

LEAFLET ANATOMY OF *ZAMIA DISODON* D.W. STEV. & SABATO AND *Z. RESTREPOI* (D.W. STEV.) A. LINDSTR.
Anatomía de los folíolos de *Zamia disodon* D.W. Stev. & Sabato y *Z. restrepoi*

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ABSTRACT

The genus *Zamia* is morphologically and ecologically the most diverse of the Cycadales. Colombia is the country where most living species of *Zamia* have been recorded. Even though in the past this genus extended beyond the Neotropical region, nowadays it is almost entirely restricted to that biogeographical realm. As with the remaining Cycadales, this genus shows several unusual anatomical traits in the leaflets. The objective of this research was to study and compare the leaflet anatomy of *Zamia disodon* and *Z. restrepoi* and establish possible relationships between the anatomical traits and the habitats of these species. We found that both species share several unusual traits with each other and with other species of the genus, such as the parenchyma morphology, the distribution of tissues between the veins and the stomata morphology. The main differences between these species were seen in their fiber clusters and in the distribution and abundance of the photosynthetic tissue near the vein areas. The presence of stomata both adaxially and abaxially could be the result of the very wet habitats where both species are native, while the presence of fiber clusters just at the vein areas could account for the longevity of the leaves of *Zamia*. On the other hand the unique traits of *Zamia restrepoi* are related to the presence of a midvein, a trait not found in any other species of *Zamia*.

Key words. Colombia, Leaflet anatomy, Scanning electron microscopy, *Zamia disodon*, *Zamia restrepoi*.

RESUMEN

Zamia es morfológica y ecológicamente el género más diverso de Cycadales. Colombia es el país con más especies vivientes, descritas de *Zamia*. Aunque en el pasado este género tuvo una distribución más amplia, en la actualidad está básicamente restringido a la región neotropical. Como con otras Cycadales, *Zamia* presenta varias características anatómicas inusuales en sus folíolos. El objetivo de este trabajo consiste en comparar la anatomía de los folíolos de *Zamia disodon* y *Z. restrepoi* y establecer posibles relaciones entre sus características anatómicas y los ambientes que estas especies habitan. Encontramos que ambas especies comparten varias características en común, como la morfología del parénquima, la distribución de los tejidos en las áreas entre venas y la morfología de los estomas. Las diferencias más notables entre especies se vieron a nivel de sus paquetes de fibras y en la

distribución y abundancia de los tejidos en las regiones cerca de las venas. La presencia de estomas en las superficies adaxial y abaxial puede deberse a la elevada humedad de los hábitats en los que ambas especies son nativas, mientras que la distribución de los paquetes de fibras puede permitir la longevidad de las hojas de *Zamia*. Por otra parte muchas de las características únicas de *Zamia restrepoi* son resultado de la presencia de una vena media muy conspicua, que es única de esta especie dentro del género.

Palabras clave. Colombia, Anatomía de los folíolos, *Zamia disodon*, *Zamia restrepoi*.

INTRODUCTION

Zamia L. is the second largest genus of the order Cycadales (ca. 70 described species) and the most speciose of its family (Osborne *et al.* 2012). It can be distinguished from other genera of Zamiaceae by the combination of the following characters: articulated leaflets, absence of terminal leaflets both in seedlings and adults, leaflets with no prominent mid-vein (except in *Z. restrepoi* (D.W. Stev.) A. Lindstr.), regularly branched trichomes, leaves and cataphylls with vestigial stipules (at least as seedlings), peltate megasporophylls without spine- or horn-like projections and foveolate pollen (Stevenson 1992, Jones 2002). Nowadays this genus is restricted to the Neotropical region (except *Z. integrifolia* L.f. which extends into southeastern USA) from northwestern Mexico, to Bolivia, the Guianas and Amazonian Brazil (Stevenson 2001, Jones 2002). The genus has two geographical diversity centers: One in Southeastern Mexico into Northern Central America (Nicolade-Morejón *et al.* 2009) and the other in Panama and Colombia. In fact, Colombia is the country with most species of *Zamia*, with 18 described species, while Panama is the most diverse country of Cycadales per unit area (Taylor *et al.* 2008).

Fossil evidence indicates that in the past the distribution of this taxon was even more extensive, as there are fossils as far north as the central USA and to the south as far as Argentinean Patagonia (Artabe & Stevenson

2002). However there's no evidence that this genus ever extended outside the Americas since the Paleocene (Jones 2002). This surprisingly broad distribution, for an almost exclusively tropical genus, was probably a consequence of the warmer world climate during most of the Tertiary (Taylor *et al.* 2008, Graham 2010). The oldest fossils are from North America, pointing to a Laurasian center of origin, along with *Ceratozamia* Brongn. and *Microcycas* Miq. (Norstog & Nicholls 1998).

As a genus, *Zamia*, exhibits the widest spectra of morphological, ecological and karyological diversities of the order (Norstog 1981, Vovides 1983, Caputo *et al.* 1996, Stevenson 2001, Jones 2002). Within this taxon there are species which show adaptations that are unique among gymnosperms like the Panamanian *Zamia pseudoparasitica* which is epiphytic (Jones 2002). A few species tolerate saline water such as *Z. roezlii* Linden in the Colombian Pacific mangroves (Stevenson 2001), and *Z. nesophila* A.S. Taylor, J.L. Haynes & Holzman in western Panama beaches (Taylor *et al.* 2008). Also, *Zamia* is the only cycadalean genus in which there is karyotypic variation, both within and between the species (Norstog 1981, Vovides 1983, Caputo *et al.* 1996).

Cycadalean leaves show clear adaptations for a xerophytic environment, although these could be inherited from an ancestor from a dry and strongly irradiated environment,

back in the Mesozoic (Norstog & Nicholls 1998): a thick cuticle, a strongly developed hypodermis with thick walls and sunken stomata usually present only on the abaxial surface of the leaflets. All species of *Zamia* have pinnate leaves (Jones 2002) but the number of leaflets per leaf varies considerably between species. The main axis of the leaf consists of the petiole, which can be prickly or smooth, and the rachis which supports the leaflets. The young leaves show erect vernation in aerial stemmed species, and inflexed vernation in those with subterranean stems (Stevenson 1981), probably to protect the fragile new leaves as they emerge from the soil. The internal anatomy of the petiole-rachis axis is characterized by an abundance of parenchyma along with mucilage channels and relatively few and small discrete vascular strands, which show an omega (Ω) shape, if seen in cross section (Norstog & Nicholls 1998). The leaflets are variable between species in regards to shape, length, width, margin and texture, and do not show a distinct main midvein (except in one species, *Z. restrepoi*). Internally, in some species at least, such as *Zamia integrifolia*, the palisade parenchyma is indistinct, unlike in *Cycas* (Norstog & Nicholls 1998). However, there is a subepidermal compact parenchyma under the adaxial surface in some tropical species. Also, idioblasts (Norstog & Nicholls 1998, Jones 2002) and occasionally isolated cells (Acuña-Castillo & Marín-Méndez 2012) with thickened secondary walls can be found in the mesophyll.

Zamia foliar anatomy, both internal and external, has been studied in previous works. Probably the most relevant one is that by Greguss (1968), which considered most cycad species known at the time, and was centered mostly on characters useful in paleobotany, such as epidermal anatomy. More recent research includes the works by Newell (1985, 1989) which emphasized inter- and intra-population

variation of external leaflet morphology of several West Indian species. Stevenson (1981, 1990), Stevenson *et al.* (1996) and Norstog & Nicholls (1998) provided detailed descriptions about the general foliar anatomy and the differences between the genera of the order. Zuo *et al.* (2004) have even analyzed how the ultrastructure and function of the chloroplasts change relative to carbon dioxide concentrations. The scarcity of recent literature on cycadalean anatomy shows the need for more research, especially in species that are virtually unstudied, as is the case for most Central and South American species of *Zamia*. Anatomical traits can be helpful in trying to establish the relationships of the species within the genus, as has happened with *Cycas*, a genus in which the series and species groups have similar foliar anatomy (Hill 1996). Previous studies of *Zamia fairchildiana* L.D. Gómez and *Z. neurophyllidia* D.W. Stev. from Costa Rica confirmed that even though the general anatomical features within the genus follow a common general pattern, there are many details which have potential taxonomic applications, and also demonstrated many interesting features that could allow these species to survive in their natural wet forest undergrowth environments (Acuña-Castillo y Marín-Méndez 2012).

The objective of this research was to study and compare the leaflet anatomy of *Zamia disodon* D.W. Stev. & Sabato and *Z. restrepoi*, both native to Colombia, and establish possible relationships between the anatomical traits and the habitats of these species.

MATERIALS AND METHODS

We worked with *Zamia disodon* and *Z. restrepoi*. Both species inhabit wet forests to rain forests in the lowlands and mid elevations of northwestern Colombia. *Zamia disodon* is known only from Antioquia (and

apparently Huánuco in Perú, but whether this population is indeed *Z. disodon* is not yet clear), at elevations between 400 and 800 m, while *Z. restrepoi* is endemic to southern Cordoba, at elevations around 500 m. Both have very restricted natural ranges, and their natural populations are critically endangered, and so even slight habitat alterations could result in their disappearance.

Leaflet material was collected from fully developed, mature leaves from plants cultivated in the Nong Nooch Tropical Botanical Garden, Thailand. All samples for microscopic observation were about 0.5 cm², mostly from the center of the leaflet. The samples were first fixed in Karnovsky solution (glutaraldehyde 2.5% / paraformaldehyde 2% / sodium phosphate buffer 0.1M, pH. 7.4), for 24 hours at 4 °C. The material was then washed in sodium phosphate buffer, and postfixed using 1% Osmium tetroxide (OsO₄). The samples were further washed in distilled water and were then processed for scanning electron microscopy or light microscopy.

For scanning electron microscopy, the samples were dehydrated through an ascending series of ethanol solutions and then the material was left in tert-butanol for 24 hours at 4°C, to be sublimed thereafter in a vacuum sublimizer Eiko ID-2. Then the material was mounted on aluminum bases and covered with a 30 nm thick gold-palladium layer. Samples were observed using a Hitachi S-570 scanning electron microscope, with an acceleration voltage of 15 KV. For light microscopy, the material was dehydrated through an ascending series of propanone (acetone) solutions, then embedded in Spurr's epoxy resin. The embedded samples were sectioned at 500 nm thick with a Power Tome PC (RMC Products) Ultramicrotome, dyed with Touluidin Blue and observed with an Olympus IX-51 inverted light microscope.

RESULTS

Zamia disodon has membranaceous, sometimes even translucent, leaflets. These are elliptical with a doubly serrate margin towards the tip. The venation is more evident on the adaxial side of the leaflet as opposed to many plicate leaved *Zamias*, which have venation most evident on the abaxial side. The leaves have divergent veins that branch dichotomously. In contrast, *Z. restrepoi* has symmetric, lanceolate to rarely linear lanceolate leaflets (the latter were formerly considered a different species: *Chigua bernalii* D.W. Stev.) with conspicuously dentate margins. They show an obvious venation in which the midrib can be clearly distinguished and from which secondary veins branch at oblique angles. This species is the only *Zamia* known to have a conspicuous midrib.

The leaflets in both species are amphistomatic, although the stomata on the abaxial surface of the leaflet are more abundant (Figs. 1a, 2a). The stomata are sunken and syndetocheilic, in which the subsidiary cells surround guard cells and are above them. The main axis of the epidermal cells is parallel to the leaflet's main axis and the cells over the veins are narrower and more elongated than those between the veins. The latter tend to be more sinuous, especially those abaxially located, possibly because of the abundance of stomata. The structure of the mesophyll in the area between veins is similar for both species, as they have a relatively compact, adaxially located parenchyma composed of small cells. Below this tissue there is an extensive spongy parenchyma with large air chambers (Fig. 1b). Even though the size of the chambers differs between the species, the main axis of such chambers is always perpendicular to the main axis of the leaflet, while the chamber's walls are one cell thick and these cells are tubular, curved and branched (Figs. 1b, 2a).

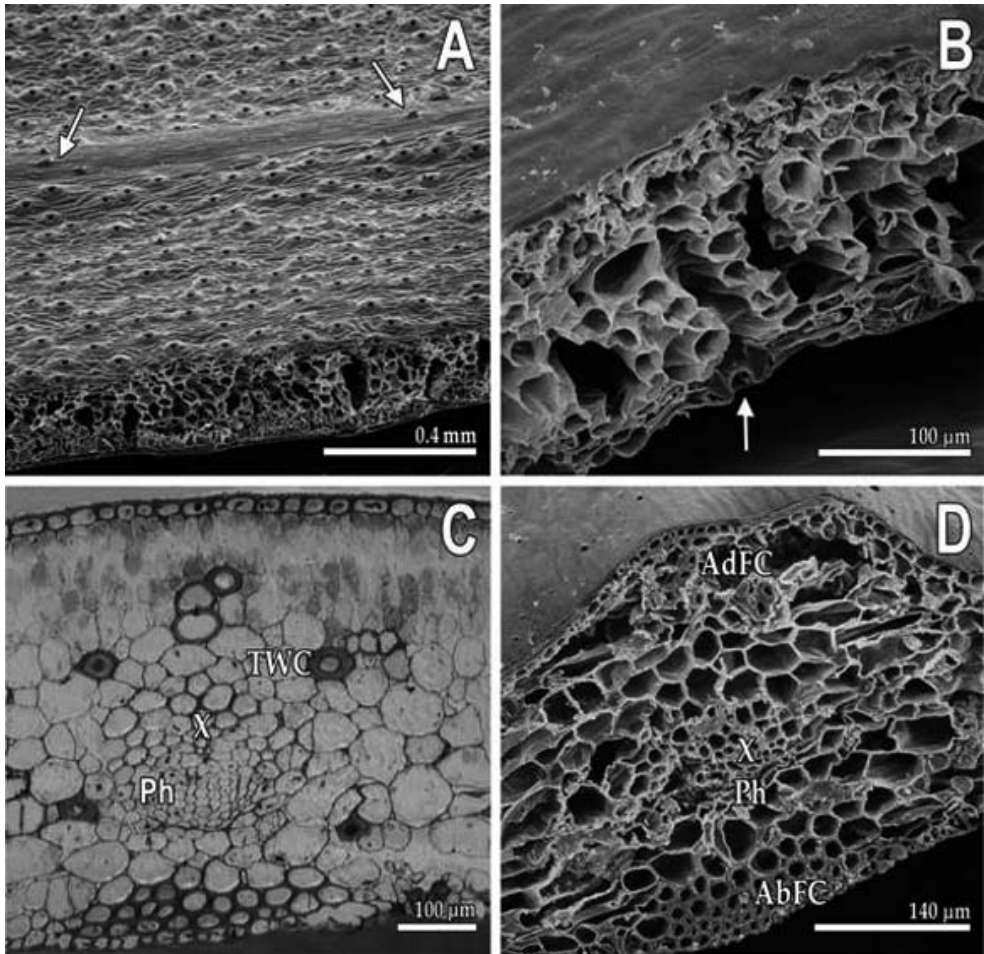


Figure 1. *Zamia disodon*.

A. SEM panoramic view of the abaxial surface of leaflet showing abundant stomata, the surface of the vein, trichome-like structures (arrows) and a longitudinal section of the blade showing tissue distribution. B. SEM detail of the longitudinal section of the blade, showing the air chambers associated with abaxial stomata (arrow). C. Light microscopy cross section of the leaflet at the vein area. D. SEM cross section of the vein. Acronyms: Abaxial fiber cluster (AbFC), adaxial fiber cluster (AdFC), phloem (Ph), scanning electron microscopy (SEM), thick walled cells (TWC), xylem (X).

The veins of *Zamia disodon* are raised adaxially. On the abaxial surface, the flat veins are clearly distinguished from the area between the veins due to differences in cell shape (Fig. 1c). Also on the abaxial side of the vein, some conic or truncated structures with a trichome-like appearance can be seen. These structures are almost entirely restricted to the epidermis over the veins, although a few of them can be observed in areas between the veins (Fig. 1a).

In the vein area and under the epidermal cells there are clusters of fibers, both abaxially and adaxially (Figs. 1d, 1c). These fiber clusters are restricted to the veins, vertically aligned with the vascular tissues, and no similar structures were seen between the veins. The abaxial fiber cluster is trapezoid shaped and is notoriously more conspicuous than the adaxial cluster, which is composed of rather few cells. Between the fiber cluster and the

vascular tissue there is parenchyma with cells of variable diameter and relatively small intercellular spaces. Occasionally, scarce thick walled cells can be seen. In the area between veins it is possible to distinguish air chambers (Fig. 1b).

The midrib of *Zamia restrepoi* is raised both adaxially and abaxially, although it is more prominent on the adaxial surface (Fig. 2b).

Even though the stomata are more frequent in the areas between the veins, some can be found on the midrib edges. On the midrib it is possible to see trichome-like projections over the surface. These structures are conical, truncated or cylindrical (Fig. 2c). As in *Z. disodon*, there are also similar structures in the areas between veins, but in such areas they are very rare.

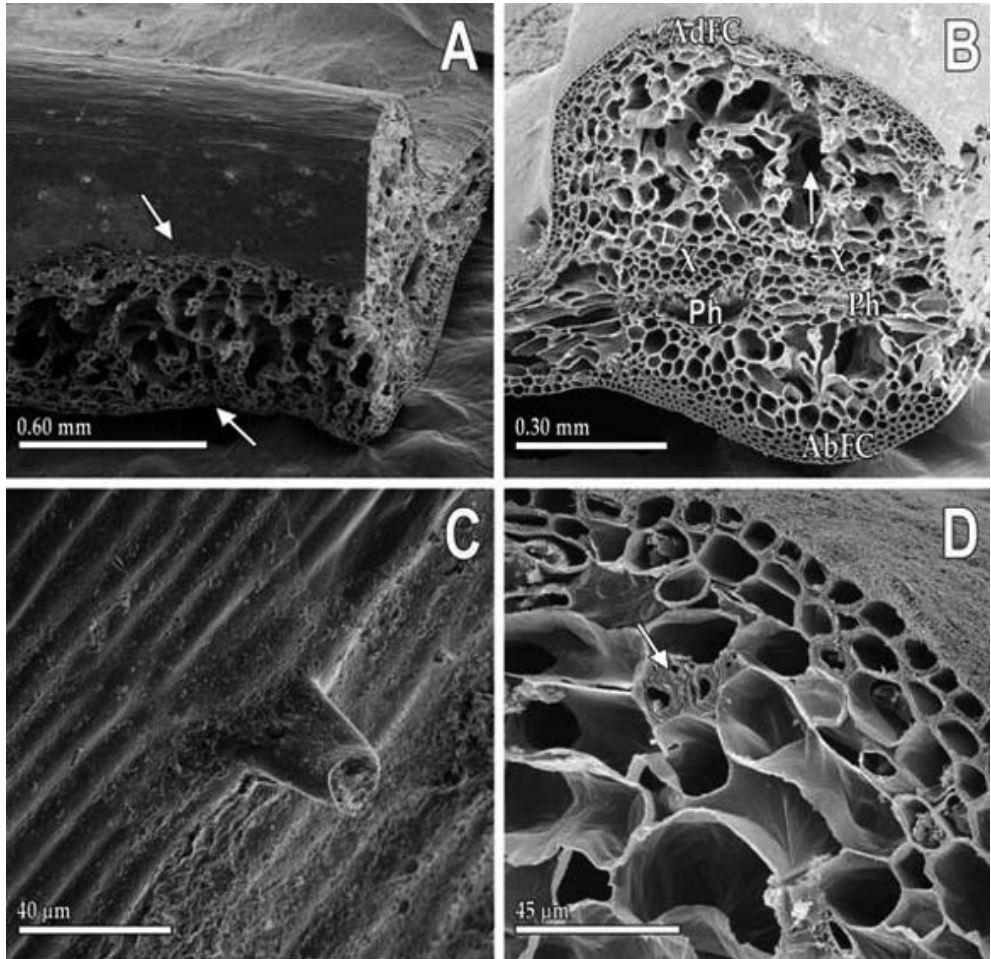


Figure 2. *Zamia restrepoi*.

A. SEM panoramic view and longitudinal section of the area near the midrib, showing spongy parenchyma, air chambers and stomata on both epidermis surfaces (arrows). B. SEM cross section of the main vein. Spongy parenchyma and air chambers are evident towards the adaxial surface. (arrow) C. SEM detail of a trichome-like structure on the vein abaxial surface. D. SEM cross section near the midrib showing thick-walled cells (arrow), close to the epidermis. Acronyms: Abaxial fiber cluster (AbFC), adaxial fiber cluster (AdFC), phloem (Ph), scanning electron microscopy (SEM), xylem (X).

Under the adaxial epidermis of the midrib there are 2-4 layers of fibers. Under these fibers there is spongy parenchyma, similar to that observed in the area between the veins (Fig. 2b), formed by tubular cells, with rather large air chambers that are connected to the spongy areas between the veins (Fig. 2a). Under the spongy parenchyma there is a relatively compact parenchyma surrounding the vascular tissue. There are 2-5 collateral vascular strands within the midrib and they are relatively small, with little xylem and crushed protophloem. The parenchyma towards the abaxial side, next to the vascular tissue, is more compact than that under the adaxial surface, and with cells of larger diameters. Under it there are 3-5 layers of abaxial, subepidermic fibers, even more developed than those located adaxially. The fiber clusters are entirely restricted to the veins, as in *Zamia disodon*. Rarely, there are thick-walled cells dispersed in the parenchyma (Fig. 2d).

DISCUSSION

Most cycad species are hypostomatic (Greguss 1968), but in a few species, and occasionally in some individuals of some species, it is possible to find amphistomatic leaflets; however, the stomatal density on the adaxial surface of the leaflet is usually much lower than that of the abaxial surface. This was noticed in the samples of *Zamia disodon*, in which the abaxial density of stomata seemed higher compared to the upper surface. It is likely that the density of the stomata and their presence on just the abaxial surface of the leaflet, or on both surfaces, could be related to the atmospheric humidity and the climate, especially because amphistomatic species are chiefly from very humid environments such as the undergrowth of tropical wet forests (Greguss 1968). The structure of the stomata and their subsidiary cells in the studied species is the same as that described by Greguss (1968)

and Acuña-Catillo & Marín-Méndez (2012) for other species of the genus: sunken guard cells and raised subsidiary cells that form the walls of the epistomatic chamber. As pointed out by Norstog & Nicholls (1998), this is an adaptation for xeric environments, and in mesic habitat species, this could be an evolutionary remnant that was present in the ancestors of the living species. As was reported for *Zamia fairchildiana* and *Z. neurophyllidia* by Acuña-Catillo & Marín-Méndez (2012), *Z. disodon* and *Z. restrepoi* have trichome-like structures on their leaflets, especially on the veins. These seem to be the basal cells of the branched trichomes, which are very common in young leaves of *Zamia* (Stevenson 1981), although these are no longer present on mature leaves. It appears that all that remains of the trichome is the basal cell, after the branched apical cell has been shed. This suggests that these trichomes are very fragile.

In *Zamia disodon*, a very uncommon leaf feature was observed: the leaflet veins are raised on the adaxial surface, but flat at the abaxial side of the leaflet. This is in contrast to the general pattern, in which the leaflets are either flat or the veins are raised abaxially (Jones 2002). However, the general structure of the leaflet is very similar to that of other species of *Zamia*, the main differences being the texture of the leaflet and the size of the fiber clusters above and below the vascular strands. These fiber clusters could indeed have taxonomic importance. However, we are not aware of their use in this way, even though in *Cycas* the location and abundance of foliar tissues have been applied in taxonomic studies (Hill 1993).

The conspicuous midrib seen in all leaflets of *Zamia restrepoi* was the main trait used to separate this species in the genus *Chigua* D.W. Stev. (Stevenson 1990), although the most recent genetic evidence suggests that what was considered *Chigua* is nested

within a clade of mostly South American *Zamia*. Nonetheless, the foliar anatomy of *Zamia restrepoi* shows several unusual traits, mostly related to the midrib. As was pointed out by Stevenson *et al.* (1996), the midrib in this species is very similar to that seen in the South African endemic, *Stangeria eriopus* (Kunze) Baill., as it is composed mostly of parenchyma and has several discrete vascular strands. However, in the samples analyzed we found air chambers in the adaxial side of the midrib. This is a rare feature in leaf anatomy and to our knowledge this is the first report of this characteristic in Cycadales. We think that either the presence or size of such air chambers is variable, and probably related to relatively higher transpiration rates. This hypothesis remains to be tested. The spongy parenchyma of the vein is connected to the spongy parenchyma of the blade. Besides the particularities associated with such a midvein, *Z. restrepoi* has several traits in common with other species of *Zamia*. The main cluster of fibers is located on the abaxial side of the leaflet, and even though those on the adaxial surface are abundant, they have thinner walls than the abaxial fibers. We suspect that fiber location and development could be related to structural factors adding resistance and longevity to the leaflets, because even though there is no data on the longevity of leaves in either of the studied species, some other species of the genus, such as *Zamia 'skinneri'* Warsz. (*Z. neurophyllidia* in the most current listings) in Costa Rica, have leaves that can stay attached to the plant for periods of five years or even more (Clark & Clark 1986, Clark *et al.* 1992)

The abundance and size of the air chambers in both species could be related to the very humid environment of the wet forest undergrowth, where these species are native (Stevenson 2001, Jones 2002), similar characteristics were noticed in *Zamia fairchildiana* and *Z. neurophyllidia* (Acuña-Castillo & Marín-Méndez 2012). These chambers perhaps

allow the leaflets to increase gas exchange in order to eliminate excess water and fix carbon. In contrast to *Cycas* there is no evident transfusion tissue in *Zamia* (Norstog & Nicholls 1998), although in this genus the elongated tubular spongy parenchyma cells could be involved in horizontal transfer of substances and are therefore probably fulfilling the role of a transfusion tissue.

ACKNOWLEDGEMENTS

We are indebted to Anders Lindström, from the Nong Nooch Tropical Botanical Garden, Thailand, for providing the material for this study. To the staff of Centro de investigación en Estructuras Microscópicas at the University of Costa Rica for their valuable help during this research, especially to Ethel Sánchez-Chacón and Alexander Rodríguez for their help with the electron microscopy study and the image processing. We are also thankful to Paul Hanson for reviewing the manuscript. Financial support for this research was provided by Vicerrectoría de Investigación, Universidad de Costa Rica through grant No 810-B0-042.

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Recibido: 15/12/2011

Aceptado: 28/01/2013