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PHYSICAL AND ULTRASTRUCTURAL BASIS OF BLUE LEAF IRIDESCENCE IN TWO NEOTROPICAL FERNS[1]

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Iridescent blue leaf coloration in two neotropical ferns, *Danaea nodosa* (L.) Sm. (Marattiaceae) and *Trichomanes elegans* L. C. Rich. (Hymenophyllaceae), is caused by thin film constructive interference. The ultrastructural basis for the film in *D. nodosa* is multiple layers of cellulose microfibrils in the adaxial cell walls of the adaxial epidermis. The apparent helicoidal arrangement of the fibrils is analogous to similar color production in arthropods. In *T. elegans* the blue-green coloration is caused by the remarkably uniform thickness and arrangement of grana in specialized chloroplasts adjacent to the adaxial wall of the adaxial epidermis. The selective advantage of this color production, if any, is unknown but apparently different from that previously studied in *Selaginella.*

Iridescent blue leaf coloration occurs in a few plants growing in the extreme shade of tropical rain forests (Richards, 1952; Lee, 1986). In the plants examined no blue pigment is extractable by solvents, and the basis for this coloration appears to be physical (Lee, 1977). Research on the physical and ultrastructural basis, and ecological function, of such coloration has been limited to old world *Selaginella* species (Lee and Lowry, 1975; Hébant and Lee, 1984). Iridescent blue color is also produced by leaves of a few neotropical rain forest species, particularly ferns (Lee and Graham, 1986).

Iridescent coloration frequently occurs in animals, particularly insects and birds (Fox, 1976). This color is produced by three physical mechanisms: thin film interference, diffraction, and Tyndall scattering. These mechanisms differ in the purity of colors produced at different angles of incidence and in the peak wavelengths of reflectance. Diffraction produces different colors at different angles, and Tyndall scattering increases the intensity of color production at shorter wavelengths and requires a dark pigmented background. All previous research on iridescent blue leaves suggests that the basis of color production is thin film interference (Lee, 1977). More critical testing of such a hypothesis for a particular plant requires ultrastructural examination of the film apparatus by transmission electron microscopy (TEM), since the film must be similar in thickness to the wavelengths reflected (constructively interfered). Knowledge of the peak wavelength of reflectance and the approximate refractive index of the biological material allows the prediction of the film thickness using the basic Newtonian equation (Jenkins and White, 1957):

$$\text{thickness} = \frac{\lambda}{4\mu\cos\partial}$$

Where ∂ = angle of incidence, λ = wavelength of peak reflectance, and μ = refractive index, If μ of the filter is smaller than the surrounding medium, a phase shift occurs requiring twice the thickness for the same wavelength of constructive interference.

This paper describes the physical and ultrastructural basis of blue leaf iridescence in two neotropical ferns, *Danaea nodosa* (L.) Sm. (Marattiaceae) and *Trichomanes elegans* L. C. Rich. (Hymenophyllaceae), revealing the sources of interference as remarkable cellular structures in each taxon.

MATERIALS AND METHODS

Both species occur in Central American and Amazonian rain forests. Plants were observed and collected at the Finca La Selva Research Station, operated by the Organization of Tropical Studies in Heredia Province, Costa Rica. Both taxa occur in extremely moist and shady microhabitats in the forest. Leaves were collected, kept humid, and fixed or optically measured within 2 hr.

Leaf reflectance was measured in an integrating sphere attached to a Li-1800 spectroradiometer (Li-Cor Instruments, Lincoln, NE 68505) at 2-nm intervals. Five mature and healthy leaves were sampled from *T. elegans* and five from juvenile and adult plants of *D. nodosa.* The sphere geometry provided a 0° angle of incidence for the filter calculations.

Samples of six leaves from each taxon were prepared for TEM. For light microscopy samples were fixed in 3.5% gluteraldehyde in 0.024 M phosphate buffer at pH 7.0, dehydrated in an ethanol series, and embedded in JB-4 resin (Ladd Research Industries, Burlington, VT 05402). Sections, 0.5–1.0 μm thick, were stained with 0.05% toluidine blue. Samples for TEM were initially fixed in 3% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) at 20 C for 2 hr, then postfixed in 1% aqueous osmium tetroxide for 2 hr. After dehydration in an ethanol series the samples were infiltrated with Spurr's resin (Polysciences Inc., Warrington, PA 18976). Transverse sections approximately 70 nm thick were cut with a diamond knife on a Sorvall Porter Blum MT-2 ultramicrotome (Sorvall Instruments, Newtown, CT 06470), and were stained with uranyl acetate and lead citrate. Specimens were examined and pho-

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tographed in a Phillips 200 transmission electron microscope at 60 kV, previously checked for magnification with a calibration grid. Measurements of prints and negatives were performed with a dial caliper accurate to 0.05 mm.

RESULTS AND DISCUSSION

Danaea nodosa**—**Location of the filter in this taxon is simplified by the comparison of iridescent blue leaves in small juvenile plants with green leaves of larger adults. The difference in diffuse reflectances of the two leaf types (Fig. 1) shows the distinct reflectance peak at 480 ± standard error (SE) of 0 nm for the blue leaves, in addition to the normal green reflectance at 550 nm for both. Surface reflectance observed with a microscope indicated the blue color originating from the adaxial epidermal cell wall. Solving the equation for constructive interference predicted a filter thickness of approximately 83 nm. We used a value for μ of 1.45, for semihydrated cellulose (Woolley, 1975).

Viewed by light microscopy the anatomy of juvenile and adult leaves was typical of extreme shade-adapted plants (Björkman, 1981; Roth, 1984; Lee et al., 1990). Leaves contained approximately seven cell layers, the juveniles 160 ± SE 3 μm thick, with little differentiation between palisade and spongy mesophyll cell shape (Fig. 2). In TEM photographs all leaf cells of both stages contained chloroplasts with the thick grana stacks typical of extreme shade-adapted plants (Fig. 3). The striking difference between the two leaf types was the presence of alternating electron-opaque and translucent layers on the abaxial side of the adaxial epidermal cell wall (Figs. 3, 4). These layers were absent in two of the five adult leaves examined (Fig. 5), and few and variable in thickness in the other three. In the juveniles, 18–30 of light and dark bands of even thickness occurred in all leaves examined. Layers in the middle of this region in three cell walls of each leaf were measured, 15 measurements per leaf. The electron-translucent layer (84 ± SE 3 nm), compared to the electron-opaque layer (49 ± SE 10 nm), is of the thickness predicted from reflectance measurements and appears to be the layer responsible for constructive interference.

Chloroplasts in mesophyll cells surrounding vascular bundles (Fig. 6) and in the abaxial epidermis (Fig. 7) were similar to those in the adaxial epidermis (Fig. 3) but with more plastoglobuli, more and larger starch grains, and thicker grana stacks.

Trichomanes elegans**—**All healthy individuals of this filmy fern are an iridescent blue-green, bearing a striking resemblance to artificial plastic plants. It grows in frequently inundated creek beds by steep slopes in some of the shadiest forest environments at La Selva (Lee, 1987). Diffuse reflectance measurements of leaves include the typical peak at 550 nm supplemented by a shoulder at 530 ± SE 0 nm (Fig. 8). Examination of the leaf surface with incident light in a microscope revealed numerous flecks of blue-green color produced from within the epidermal cells, just beneath the adaxial cell walls. The leaf anatomy of this filmy fern is unusual: thin (135 ± SE 12 nm), two to three cells thick, no cuticle or stomata, and elongated chloroplasts adjacent to the adaxial cell wall

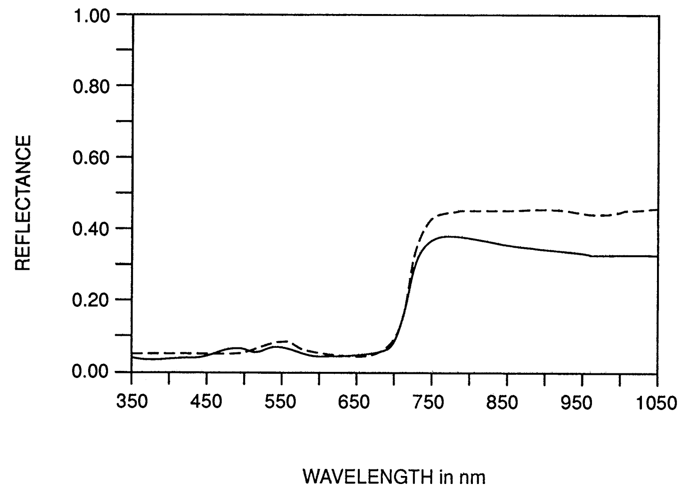

Fig. 1. Mean diffuse reflectance of five juvenile (solid line) and adult leaves (dashed line) of *Danaea nodosa*. Peak at 480 nm documents blue iridescence.

(Fig. 9). The pattern of iridescence and the location of the chloroplasts suggested that these organelles were the source of the constructive interference. Thorne, Duniec, and Lee (1980) estimated the refractive indices of chloroplasts as $\mu = 1.35$ for the stroma and $\mu = 1.47$ for dark acclimated grana. Using the latter value for these shade-adapted plants, we calculated a filter thickness of 90 nm.

TEM photographs revealed the remarkable chloroplast ultrastructure that is the probable basis for constructive interference in these plants. In transverse section the chloroplasts are filled with thin parallel grana stacks, normally five closely appressed thylakoids, interspersed with uniform and electron-translucent stromal spaces (Figs. 10, 11). The thickness of these grana, with five thylakoids, and the intervening stroma were measured in a minimum of ten grana in the middle of two chloroplasts in each of six leaves. The grana (91 ± SE 4 nm) were close the the thickness predicted for the thin film. The stroma (86 ± SE 4 nm), as regions of lower μ, would cause a phase shift and could only account for interference of much shorter wavelengths. The lower surfaces of these leaves were not iridescent. The chloroplasts present in the abaxial epidermal cells were more typical of extreme shade chloroplasts, with thick grana stacks (Fig. 12). Chloroplasts in paraveinal cells were similar to those in the abaxial epidermis, but with much greater accumulations of starch grains and plastoglobuli (Fig. 13). Thus, the striking chloroplast ultrastructure is associated with iridescent blue-green leaf coloration in *Trichomanes elegans*.

Iridescent blue coloration in both ferns is produced by constructive interference, but by different cell structures. Both taxa produce multiple ultrastructural layers that should intensify iridescent color production (Fox, 1976; Lee, 1991).

The banding described here for *D. nodosa* has been reported in the cell walls of other plants (Neville and Levy, 1985). The dense horizontal banding of cellulose microfibrils alternating with apparently vertically curved microfibrils has been termed helicoidal (Bouligand, 1965). The best explanation for this pattern appears to be the layering of fibrils produced at slightly different angles from

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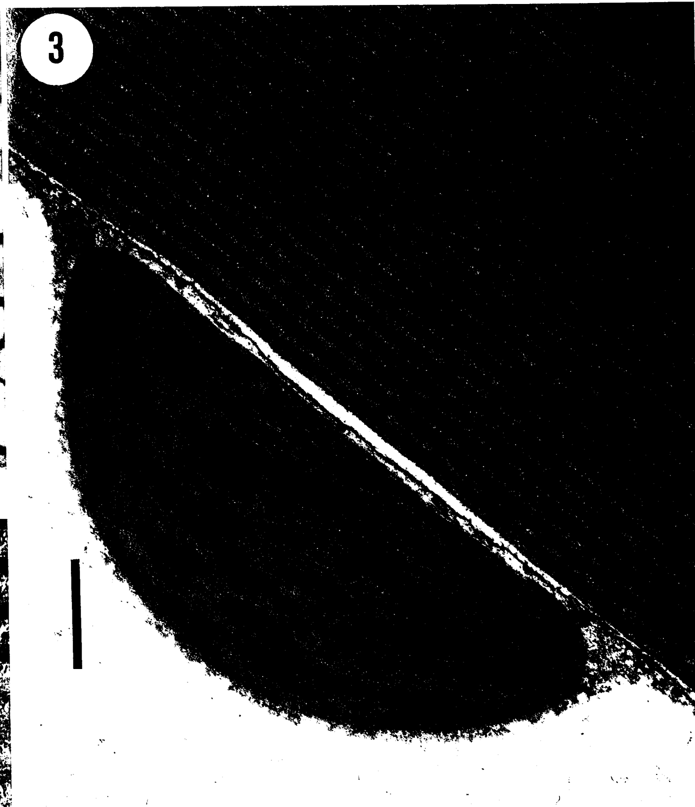
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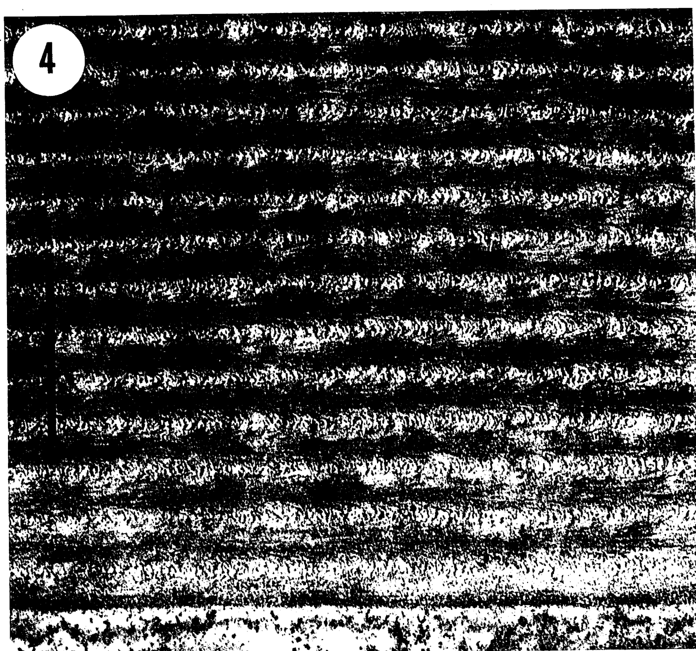
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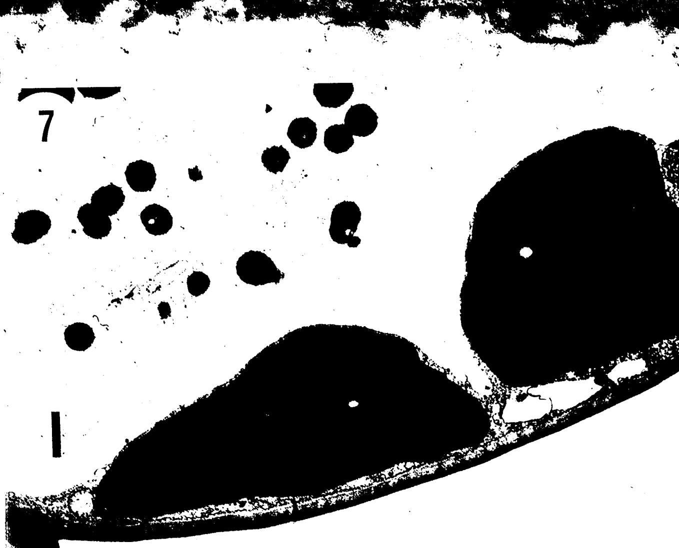
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each other. The helicoidal effect may be due to the appearance of angles more perpendicular to the two-dimensional plane, observable at slight angles from the perpendicular. The critical test for this arrangement is TEM analysis with a goniometric stage, not available to us. Very similar helicoidal arrangements have been described in insect cuticles (Neville and Caveney, 1969) and account for the metallic coloration of scarabaeid beetles. At incident angles greater than zero, the differing angles of cellulose fibrils could cause slight shifts in μ, and set up conditions for interference.

Helicoidal arrangements of cellulose microfibrils apparently do not produce constructive interference in other terrestrial plants that have been investigated for cell wall structure (Neville and Levy, 1984, 1985). Blue iridescence in *Selaginella* leaves is associated with electron-opaque bands in the adaxial epidermal walls (Hébant and Lee, 1984). In the strikingly iridescent blue fruits of *Elaeocarpus angustifolius* extracellular and cellulose containing iridosomes are the basis for color production (Lee, 1991). A helicoidal pattern of microfibril arrangement was not observed in these examples. Iridescence in marine algae, particularly the Rhodophyta, is caused by cuticular layers or cellular inclusions (Feldmann, 1970; Gerwick and Lang, 1977; Pederson, Roomans, and Hofsten, 1980).

The remarkable grana arrangement in the chloroplasts of *Trichomanes elegans* is, to our knowledge, unique among all photosynthetic eukaryotes. The greatest similarities are seen in the grana arrangement of certain marine algae (Murakami and Packer, 1970) and the eyespots of some motile unicellular algae (Foster and Smyth, 1980). In *Ulva* and *Porphyra* the grana are long and contain two to three thylakoids. Algal eyespots contain a pigmented region of alternating electron-opaque and translucent bands, superficially like those seen in *T. elegans.* Resemblance between chloroplasts in *T. elegans* and *Rhodella* (Rhodophyta) is due to the arrangement of phycobilosomes on the thylakoid membrane of the latter (Patrone, Broadwater, and Scott, 1991). In typical chloroplasts the subtle selective scattering effects that have been used to study chloroplast biochemistry are probably due to interference, although the ultrastructural basis is not precisely known (Bialek et al., 1977; Duniec and Thorne, 1977).

In both of the taxa there is a variation in chloroplast ultrastructure in different cell layers in the leaf, similar to observations of Nasrulhaq-Boyce and Duckett (1991). In contrast to the adaxial epidermal chloroplasts of *T. elegans,* chloroplasts of the abaxial epidermis and cells surrounding vascular bundles are more typical of those acclimated or adapted to shade, with thick grana stacks (Figs. 12, 13; Boardman, 1977; Chow et al., 1988). In *D. nodosa* chloroplasts of the adaxial epidermis, mesophyll and abaxial epidermis all contain thick grana stacks (Figs. 3, 6, 7). However, there is a clear association between chloroplast location and the presence of starch grains and plastoglobuli in both taxa.

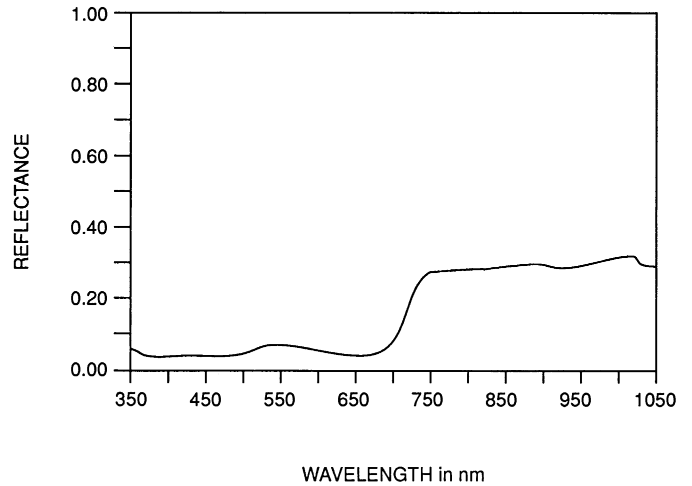

Fig. 8. Mean diffuse reflectance of five leaves of *Trichomanes elegans.* Broad peak 520–560 nm documents blue-green iridescence and chlorophyll reflectance.

Adaxial chloroplasts were conspicuous for their absence of starch bodies, these being most prominent in the mesophyll chloroplasts. A similar trend was seen for the plastoglobuli. Nasrulhaq-Boyce and Duckett (1991) speculated that these differences may reflect a functional specialization among the chloroplasts, with an export of sugars to chloroplasts adjacent to the vascular bundles, where starch accumulates. Perhaps there is selection against starch accumulation in the more light-exposed chloroplasts of the adaxial epidermis because of a correspondingly increased light scatter and reduction in absorption.

The mechanisms of constructive interference described in this paper complicate our previous understanding of the probable selective advantage of blue leaf iridescence. In *Selaginella,* interference occurs at the leaf surface, blue coloration temporarily disappearing when the leaf is placed in water (Fox and Wells, 1971). Lee and Lowry (1975) hypothesized that this superficial iridescence functions as an antireflection coating, reflecting short wavelengths and enhancing absorption of longer wavelengths through destructive interference. Differences in leaf optical properties between green and blue iridescent leaf forms were consistent with this hypothesis (Lee, 1986). However, leaf color of *D. nodosa* and *T. elegans* is not affected by contact with water, and absorption by *D. nodosa* was not increased at higher wavelengths in the iridescent leaves (Lee and Graham, 1986; Lee, unpublished results). It is possible that leaf iridescence in these two taxa is a by-product of ultrastructure and has no selective advantage, but the strong association of blue leaf iridescence with the extreme shade of humid tropical forests suggests otherwise. Iridescence may alter internal light environments in these shade-adapted leaves in a way that is advantageous to the

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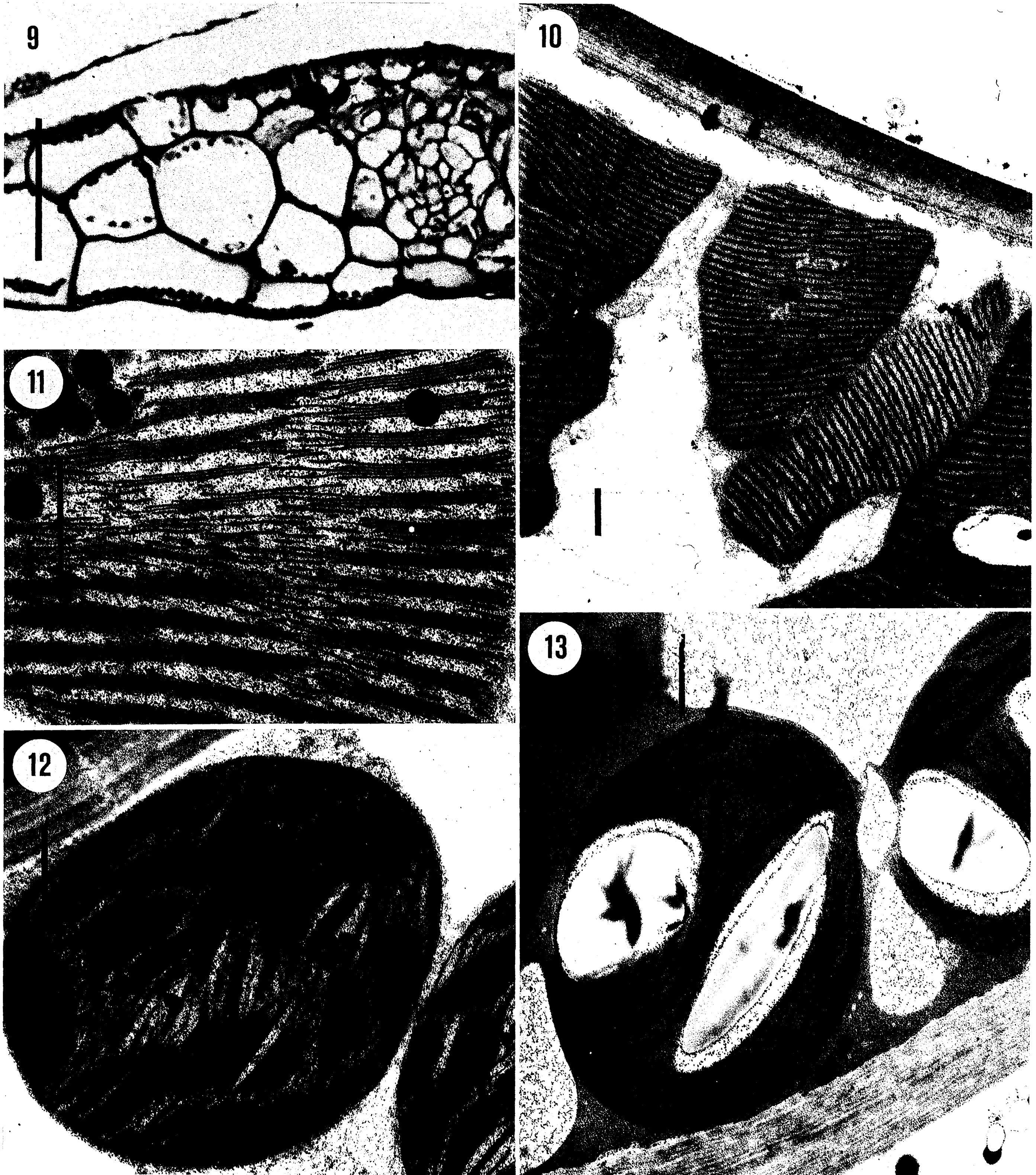
Figs. 2–7. Anatomy (2) and ultrastructure (3–7) of leaves of *Danaea nodosa.* **2.** Juvenile leaf transverse section, ×312. **3.** Adaxial epidermal wall with associated chloroplast, from juvenile leaf. **4.** Portion of adaxial epidermal wall of juvenile leaf. **5.** Adaxial wall of epidermal cell from adult leaf. **6.** Chloroplast from mesophyll cell adjacent to vascular bundle of juvenile leaf. **7.** Chloroplast from abaxial epidermal cell of juvenile leaf. Bars = 100 μm in Fig. 2 and 1 μm in Figs. 3–7.



Figs. 9–13. Anatomy (9) and ultrastructure (10–13) of leaves of *Trichomanes elegans*. **9.** Leaf transverse section, ×312. **10.** Adaxial epidermis with chloroplasts appressed to adaxial wall. **11.** Stromal and granal thylakoids of epidermal chloroplast. **12.** Chloroplast from abaxial epidermis. **13.** Chloroplast from mesophyll cell adjacent to vascular bundle. Bars = 100 μm in Fig. 9 and 1 μm in Figs. 10–13.

plants, but further research in its effect on the physiology and biochemistry of photosynthesis is needed.

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