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The anthocerote chloroplast: a review

By KEVIN C. VAUGHN[1], ROBERTO LIGRONE2, HEATHER A. OWEN3, JIRO HASEGAWA[4], ELLA 0. CAMPBELL[5], KAREN S. RENZAGLIA[6] and JULIAN MONGE-NAJERA[7]

1. *Southern Weed Science Laboratory, USDA/ARS, P.O. Box 350, Stoneville, MS 38776, USA*
2. *Dipartimento di Biologia vegetate, via Foria 223, 1-80139 Napoti, Italy*
3. *Zoology Department, Miami University, Oxford, OH 45056, USA*
4. *Laboratory of Applied Biology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan*
5. *Botany and Zoology Department, Massey University, Palmerston North, New Zealand*
6. *BiologyDepartment, E. Tennessee State University, Johnson City, TN 37614, USA*
7. *Biologia Tropical, Universidad de Costa Rica, Ciudad Universitaria 'Rodgigo Facio', Costa Rica*

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CONTENTS

SUMMARY

This review covers previous data, together with new information from our laboratories, on the subject of the anthocerote chioroplast. Unlike all other archegoniates, most species of anthocerote have pyrenoids in their chloroplasts. The pyrenoid is the site of accumulation of the first enzyme in the C3 photosynthetic cycle, ribulose bisphosphate carboxylase/oxygenase. Unlike most algae, the hornwort pyrenoid is composed of distinct subunits, numbering up to several hundred. Pyrenoid morphology is quite variable among the genera in shape, fine structure, and distribution of inclusions. Another unique feature of the anthocerote chloroplast is the presence of thylakoids that connect adjacent granal stacks at right angles to the long axis of the granum (so-called channel thylakoids), resulting in a 'spongy' arrangement of the thylakoid system. The granal stacks of anthocerotes are like the 'pseudograna' of green algae because they lack the highly-curved end membranes typical of all other embryophytes. The channel thylakoids are enriched in photosystem (PS) I and the grana are enriched in PS II. The chloroplast envelope is a double membrane structure with regions of appression, much like that of other green plants. The apical cell of the gametophyte contains chloroplasts similar to the mature chloroplasts of the thallus, although certain gametophytic tissues may contain underdeveloped plastids. Chloroplasts in cells around *Nostoc* colonies and in cells invaded by mycorrhizal fungi have thylakoids mainly in pairs, and small or absent pyrenoids. A number of similarly reduced plastids are noted in the placental cells at the sporophybe/gametophyte junction and in developing spores. The greatest reduction is observed in spermatid cell plastids, which at maturity consist of only a small starch grain surrounded by the envelope. Chromoplast-like organelles are found in the cells of the antheridial jacket in some genera; these contain numerous osmiophilic globules that are probably pigment aggregations. Colourless bead-like plastids occur in the rhizoids; these seem to develop by fragmentation of the single chioroplast in the rhizoid initials, concomitant with the loss of chlorophyll. Chloroplast division is a tightly controlled process and, in uniplastidic species, always occurs just before nuclear division, with the participation of a unique system of chioroplast-associated microtubules. The number of chloroplasts per cell is quite variable in some genera, although most species have but a single chloroplast in each cell of the gametophyte. Chioroplast shape is also variable from ellipsoidal, dumbbell-shaped, to irregular. These data indicate that the anthocerote chloroplast is unique among the embrvophvtes and are in line with the notion of an isolated position in the plant kingdom. Certain features of chioroplast morphology appear to be typical of certain genera and might prove useful in taxonomic decisions at the generic level.

Key words: Anthocerote, Anthocerotophyta, chloroplast, Bryophyta, electron microscopy, pyrenoid.

I. INTRODUCTION

The anthocerotes are a small group of land plants characterized by having non-meristematic uniplastidic cells and pyrenoids in the chloroplasts. Although this makes the anthocerotes a very well defined group, in an isolated position within the embryophvtes, their taxonomy is extremely controversial, because of the lack of clear-cut diagnostic features.

Several hundred species have been described so far, mostly from tropical and subtropical areas, but actually the group is likely to comprise no more than a hundred species, with many of them reported in the literature under synonyms (Schuster, 1984; Hasegawa, 1988). Six genera are commonly recognized, i.e. Anthoceros, Phaeoceros, Notothylas, Dendroceros, Folioceros and Megaceros (Schofield, 1984). Folioceros is considered to be a subgenus of Anthoceros by Hasegawa (1988), whereas Häsel de Menendez (1988) recognizes two additional genera, .Leiosporoceros [treated as a subgenus of Phaeoceros by Hasegawa (1988)) and Sphaerosporoceros (considered under Anthoceros by other taxonomists).

Inter- and infrageneric differences in plastid number and morphology have been known for a long time (Campbell, 1907; McAllister, 1914,1917; Kaja, 1954), but this topic has so far been addressed at the ultrastructural level in only a limited range of species (Burr, 1970; Valentine, Campbell & Hopcroft, 1986).

This paper reports a study of the chloroplast in a number of anthocerote species (see Table 1), including representatives of Folioceros and Sphaerosporoceros, genera that have not yet been investigated. Variations in plastid structure in different tissues of both the gametophyte and sporophyte are also reported. The data are integrated with information from the literature on hornwort plastids, to evaluate the evolutionary and taxonomical significance of plastids in the anthocerotes.

II. THE CHLOROPLAST OF THE MATURE GAMETOPHYTE

(a) General considerations

nthocerote chloroplasts contain all of the elements used in defining a chloroplast (Fig. 1 A). The chloroplast is bound by a double-membraned envelope that varies in the closeness of the two membranes from well separated to completely appressed. The hylakoids are distributed throughout the interior of the chloroplast and are surrounded by stroma. In the case of species with pyrenoids, these areas are more electron-opaque than the surrounding stroma. In many species, plastoglobuli, spherical aggregations of lipid, are found throughout the stroma, often clustered in small groups.

uccessful fixation and embedding of anthocerote chloroplasts are not easy tasks. Because of this, previous analyses of these chloroplasts are incomplete or inaccurate. Vaughn et at. (1990a) report that a fixation in 6 0 (v/v) glutaraldehyde in 005 ii PIPES (pH 74), washing in cacodylate buffer, and post- fixation in 200 (v/v) 0s04 in cacodylate buffer are satisfactory for gametophytic tissue in many species. Addition of caffeine (for some Megaceros spp.) and tannic acid (for some Notothytas spp.) are useful in providing optimal resolution, by binding phenolics (caffeine) or enhancing membrane binding of osmium (tannic acid). Sporophytic tissue is fixed well with this same protocol and generally does not require the additives for successful fixation. All bryophytes are difficult to embed in epoxy resins so a slow infiltration scheme (exchanges over several days), with agitation or rotation of the sample in 10000 resin, is most effective for successful embedding (Duckett, Renzaglia & Smith, 1988). Spurr's or other low viscosity resins are the most often-utilized. Of the non-epoxy resins, L.R. White resin is the most commonly used for immunocytochemistry. Lowicryl resin, although of very low viscosity, apparently does not penetrate the tissue easily so that a very long infiltration protocol (4—6 d at 0—4 °C) is required for successful embedding.

Table 1. *List of anthocerote species that have been investigated ultrastructurally*

Species	Ref.*
Anthoceros	
punctatus L.	1, 2, 6, 9
laminiferus Steph.	1
formosae Steph.	1
lametlatus Steph.	1
fusiformis Aust.	1
ecklonii Steph.	8
Dendroceros	
granulatus IVIitt.	1, 3
tubercularis Hatt.	1, 4
japonicus Steph.	1
ualidus Steph.	1, 3
javanicus Nees	1
cavernosus Haseg.	1
Folioceros	
fuciformis Bharadw.	1
appendicutatus Haseg.	1
Megaceros	
longispirus Steph.	1, 3
leptohvmenius Steph.	1, 3, ?5
flageltaris Steph.	1, 3, 5
sp. from North Carolina, USA	1, 6
giganteus Steph.	3, 5
vincentianus Camp.	1, 5
denticulatus Steph.	1, 3
Notothytas	
orbicularis Sull.	1, 5, 6
temperata Haseg.	1
breutetii Gottsche	1
Phaeoceros	
himalayensis Prosk.	5
laevis (and carolinianus) Prosk.	1
coriaceus Steph.	1
microsporus Hässel	1
miyakeanus Hatt.	7
Sphaerosporoceros	
adscendens Hässel	1

*References: 1 This report and/or Vaughn et al. (1990 a); 2 Chauhan & Schraudholf (1986); 3 Valentine et at. (1986); 4 Ligrone & Renzaglia (1990); 5 Burr (1970); 6 Duckett & Renzaglia (1988); 7 Toyama (1974); 8 Wilsenach (1963); 9 Ajiri & Ueda (1986).

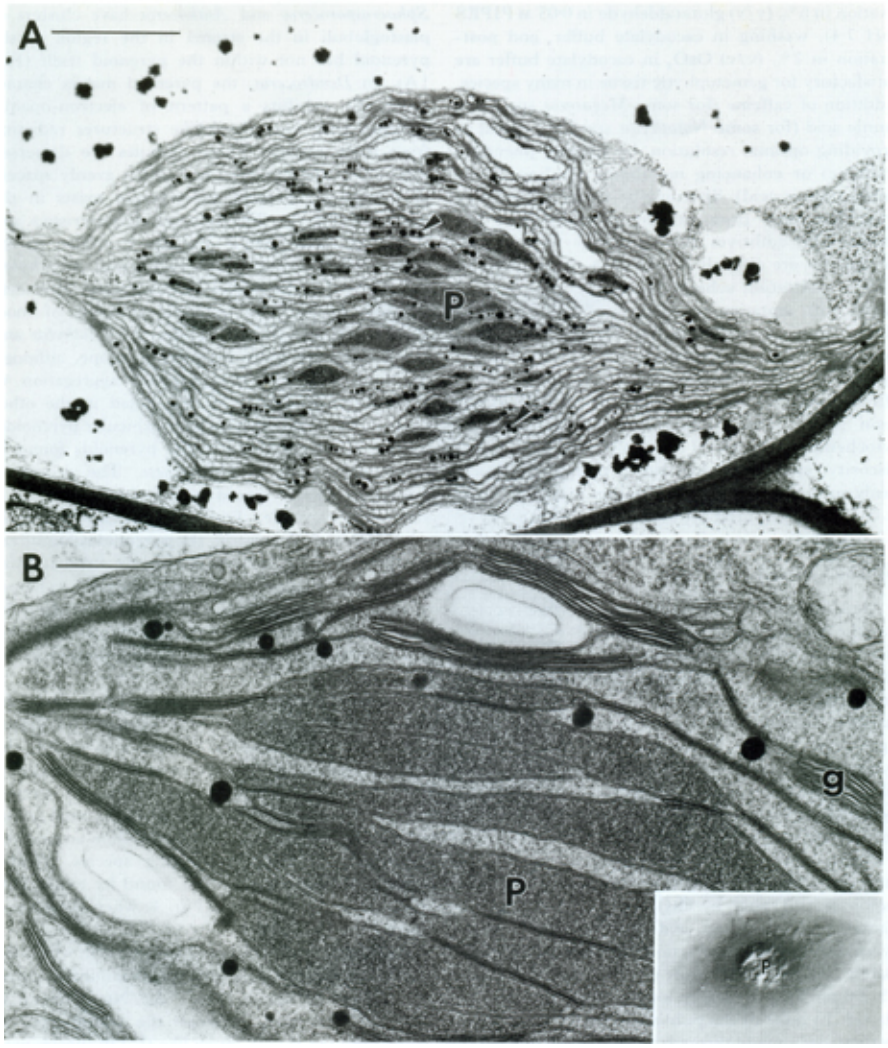

Figure 1. (A) Electron micrograph of a chloroplast of Sphaerosporoceros adscendens, a rather 'typical' anthocerote chloroplast. Subunits of the multiple pyrenoid (P) are more electron-opaque than the surrounding stroma. Channel thylakoids connect grana stacks together end-to-end. Numerous plastoglobuli (arrows) are electron-opaque spheres found in the stroma. S, starch; bar, 50 sum. (B) Electron micrograph of the pyrenoid region of Phaeoceros laevis. The pyrenoid subunits (P) are more electron-opaque than the surrounding stroma. The pyrenoid is dissected by stroma as well as thylakoids organized into both grana (g) and single (stroma) lamellae. Bar, 05 sum. Inset: Nomarski differential interference micrograph of an Anthoceros punctatus chloroplast with a prominent multiple pyrenoid (P), x 200.

(b) The pyrenoid

McAllister (1914, 1927), using light microscopy, was the first to describe the pyrenoid of Phaeoceros laevis as a multiple pyrenoid; that is, a pyrenoid consisting of up to 200 small disc-like structures aggregated in the centre of the chloroplast. This makes it distinct from the 'unit' pyrenoids of most algae (e.g. McKay & Gibbs, 1989). Anthocerote pyrenoids are especially striking using Nomarski differential interference microscopy so that the pyrenoid subunits, or pyrenosomes are seen in positive relief (Fig. 1 B inset).

Electron microscopic investigations (Fig. 1 B) of these structures in P. laevis reveal that the pyrenoid region is traversed by thylakoids, generally both stacked and unstacked, and also by areas of nonpyrenoid stroma. The pyrenoid subunits are approximately 05 ,um wide and on the average about 15—20 pm long in median longisections through the plastid. In none of the published studies have serial sections been made through the pyrenoid regions to determine if the units are discrete or are one highly dissected structure, intercalated by thylakoids and non-pyrenoid stroma. Generally, the pyrenoid occupies the central area of the chloroplast and is surrounded by starch granules, especially in older tissue.

here are numerous variations in this pyrenoid structure. In pyrenoids of Notothylas and Fotioceros, pyrenoglobuli occur at the periphery of the pyrenoid subunits (Fig. 2). Although the composition of these pyrenoglobuli is unknown, it is probable that they are lipid-containing, like the plastoglobuli of higher plants (Steinmüller & Tevini, 1985). Chloroplasts of *Sphaerosporoceros* and *Anthoceros* have clusters of plastoglobuli in the stroma in the region of the pyrenoid but not within the pyrenoid itself (Fig. 1 A). In *Dendroceros,* the pyrenoid matrix characteristically exhibits a pattern of electron-opaque globules with finer threadlike structures radiating from their surface. These globules are dispersed throughout the pyrenoid and rather evenly spaced, as though a repeating substructure exists in the pyrenoid (Fig. 3). The staining characteristics and shapes of these opaque globules is similar to the pyrenoglobuli found in *Fotioceros* and *Notothylas,* indicating that the bodies in *Dendroceros* pyrenoids are also lipids. The pyrenoid subunits of most species are disc-shaped. Those of *Notothytas* and *Dendroceros* are quite irregular in shape, although the final shape of the pyrenoid aggregation in *Dendroceros* is similar to that noted in the other genera. *Notothylas* species have orbicular pyrenoids, similar in overall shape to the pyrenoids found in green algae such as *Coleochaete.* The degree of dissection of the

pyrenoid by non-pyrenoid stroma is also quite variable. For example, in *Notothylas orbicularis* and *N. breutetii,* the pyrenoid subunits are nearly appressed, separated only by stroma thylakoids and a relatively small area of stroma, whereas in other species (including *N. temperata)* the subunits are widely separated by non-pyrenoid stroma and both stromal granal lamellae are present in the pyrenoid area.

Not all of the anthocerote species have pyrenoids. All of the *Megaceros* species investigated by Valentine *et at.* (1986) and by us lack a pyrenoid; the electron opacity of the stroma is even throughout. Burr (1970) found that in some *Megaceros* species, particular areas of the stroma had more starch grains or granules, like the starch accumulation around the pyrenoid in pyrenoid-containing species. Similar starch distributions were not found by either Valentine *et* at. (1986) or us (Fig. 4) when these same species were examined.

Anthocerosfusiformis has chloroplasts in which the starch-containing layers form a halo around a central area devoid of starch (Fig. 5A). No pyrenoid is visible in this area, although the arrangement of starch granules is very much like that in species with typical pyrenoid. This starch distribution is unlikely to be an artifact of fixation or growth condition as it was observed in specimens of this species grown under different growth and light conditions. A similar starch distribution is found in *P. coriaceus* (Fig. 5B). Moreover, observation of apical and subapical cells of both of these species with 'pseudopyrenoids' reveals a similar starch distribution to that found in the mature regions. Light microscopic stains for protein reveal an even staining throughout the plastid both in those species with anomalous starch distribution and the *Megaceros* species studied by Burr (1970), whereas pyrenoids are quite distinct from the remainder of the stroma using these same a site of accumulation of ribulose-1 ,5-bisphosphate stains. carboxylase/oxygenase (Rubisco) (e.g. Mc Kay & In algae, the biochemical and immunocyto- Gibbs, 1989). Vaughn *et at.* (1990 a) have recently chemical evidence clearly shows that the pyrenoid is examined the chloroplasts of a number of hornwort species and found that all of the Rubisco is concentrated in the pvrenoids, with only background labelling elsewhere in the non-pyrenoid stroma (Fig. 6A). Pvrenoid inclusions, such as pvrenoglobuli, are not labelled. These data indicate that the hornwort pyrenoid is homologous with the algal pyrenoids and that the Rubisco in the pyrenoid is active, because all of it is present in the pyrenoid. Chloroplasts without pyrenoids, including those with starch-free areas of stroma in the centre of the chloroplast, have an even distribution of Rubisco throughout the stroma (Fig. 6B; Vaughn *et a!.,* 1990a).

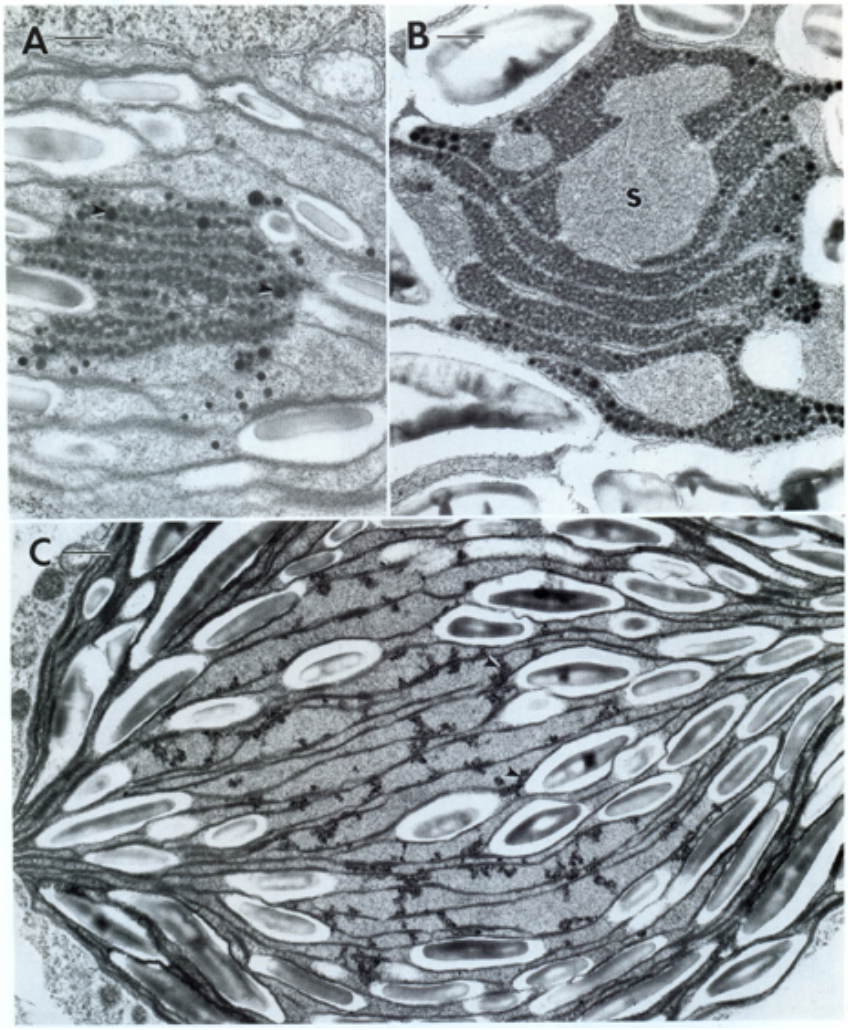

Figure 2. Electron micrographs of the pyrenoid region of (A) *Notothylas orbicularis,* (B) *N. temperata,* and (C) *Folioceros fuciformis.* In *N. orbicularis,* the pyrenoid subunits are spaced closely together with only single lamellae separating the subunits. Pvrenoglobuli border the subunits (arrows). *N. temperata* pyrenoids are more dissected by non-pyrenoid stroma (s) and lamellae occasionally occur in the grana stacks as well. The *Folioceros* pvrenoid has numerous pvrenoglobuli (arrows) as well as well-developed grana stacks that separate the subunits. Bars: (A, B) 05 /m; (C) 1 0μm.

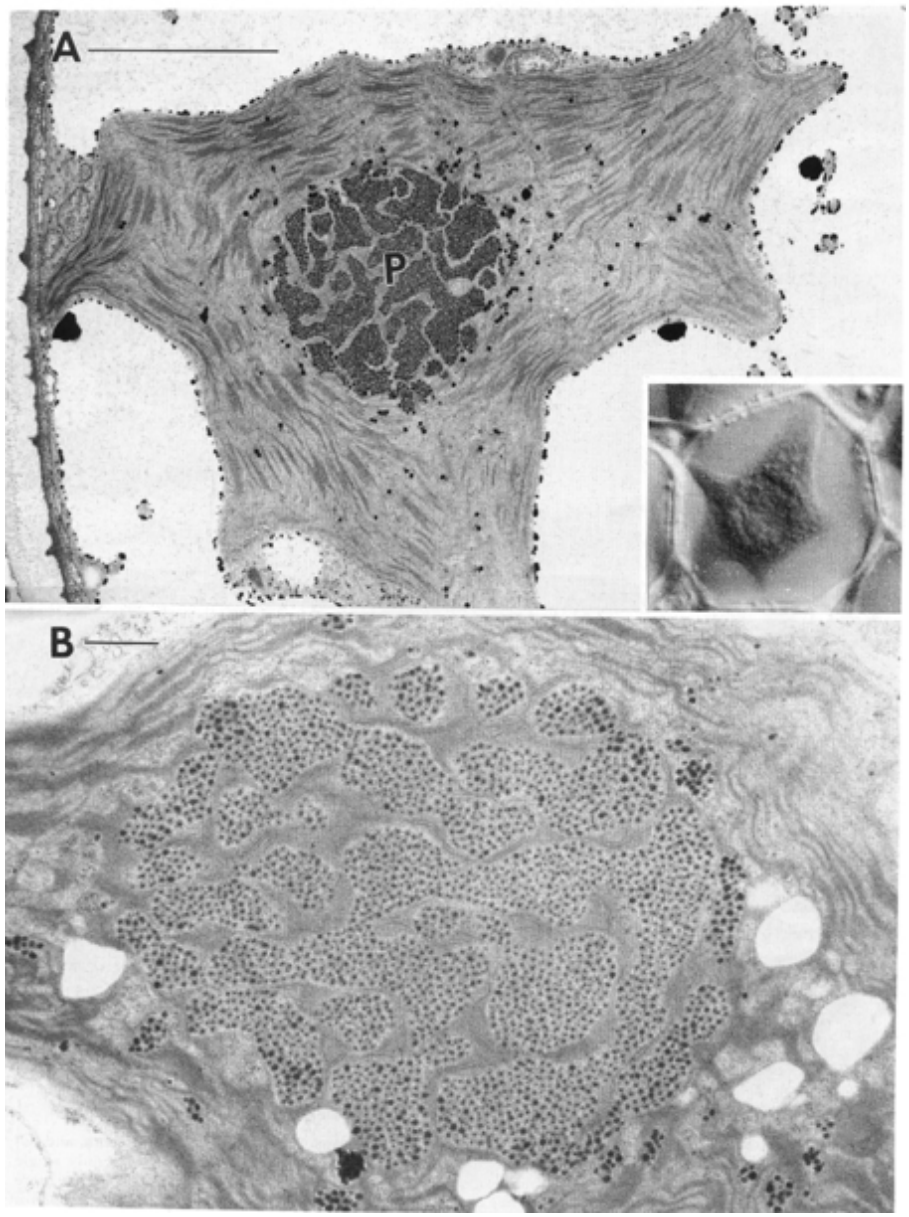

Figure 3. *Dendroceros* chloroplasts. (A) Low magnification electron micrograph of *D. tubercularis* plastid revealing the irregular chioroplast outline and the centrally localized pyrenoid (P) with irregularly shaped subunits. Bar, 50 1um. (B) Details of the pyrenoid region of *D. validus,* revealing the repeating pattern of electron-opaque pyrenoid inclusions surrounded by a spoke-like array of electron opaque lines. Bar, 05 pm. Inset is a Nomarski differential interference micrograph of the wing of the thallus of *D. tubercularis.* Note the irregular plastid shape in this profile, x 200.

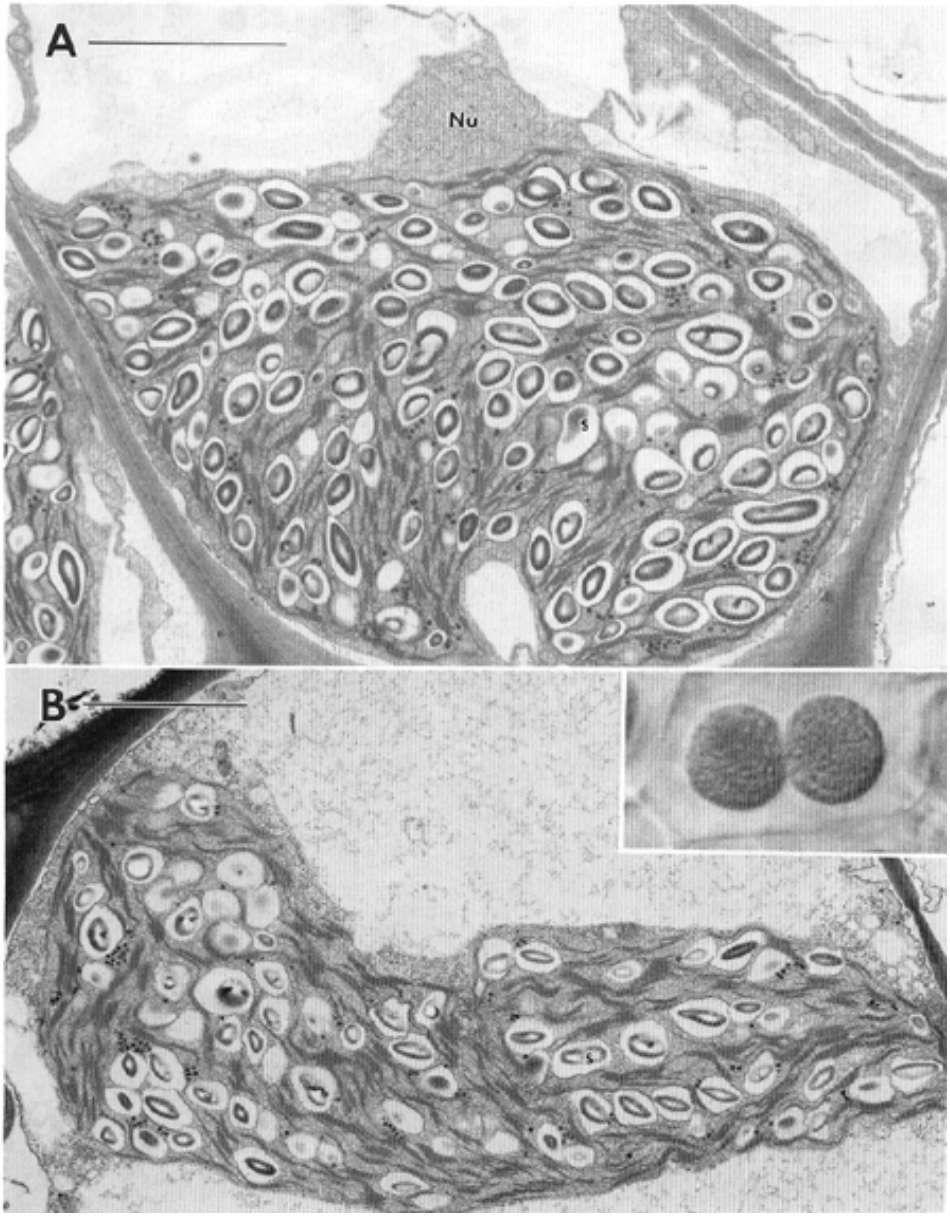

Figure 4. *Megaceros* chioroplasts. **(A)** *M. leptohymenius.* **(B)** *M. flagellaris.* No pyrenoids are found in these chloroplasts and the starch is randomly distributed. Bar, 50 pm. Nu, nucleus; s, starch. Inset is a bright-field light micrograph of an upper epidermal cell of *M. flagellaris.* Two chloroplasts are present, x 200.

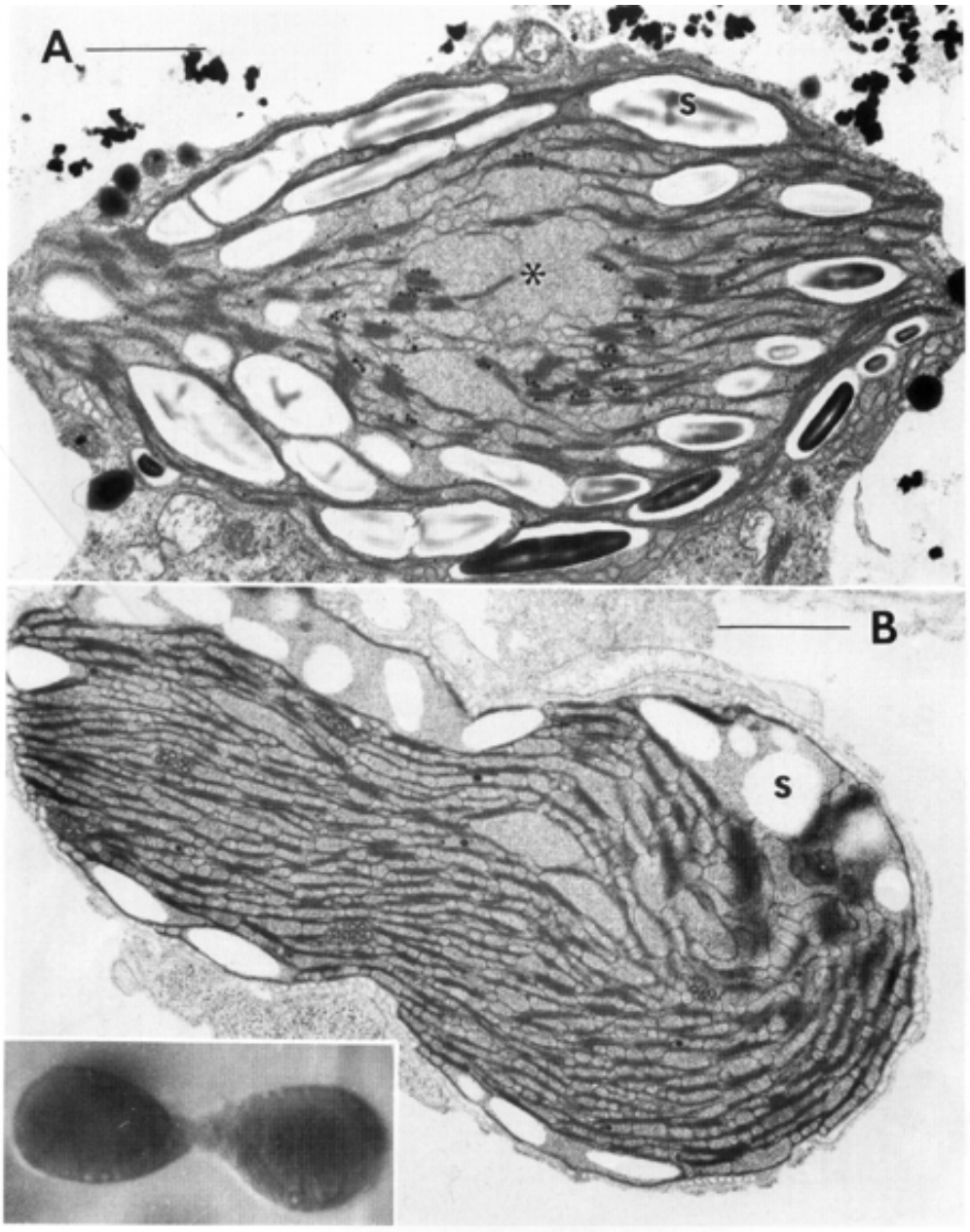

Figure 5. Chloroplasts with unusual starch distributions. (A) *Anthocerosfusiformis* with starch (s) concentration near the plastid envelope, leaving a starch-free zone (*) in the centre of the chloroplast. (B) The *Phaeoceros coriaceus* chloroplast has a concentration of starch (a) near the envelope and, in addition, has an irregular morphology. Bars, 20 /im. Inset: Nomarski differential contrast micrograph of *P. coriaceus* chloroplasts showing the dumbbell-shaped chloroplast often found in this species, x 400.

(c) Thylakoids

he presence of a pyrenoid is not the only distinguishing characteristic of the anthocerote chloroplast, as the thylakoid architecture is also unique. The granal stacks are connected to each other through the thylakoids orientated perpendicular tothe long axis of the granum (Figs 1, 6C). This gives - a 'spongy' appearance to the thylakoid system. The intergranal thylakoids were termed 'channel thylakoids' by Burr (1970) in that they dissect the stroma into channels. The granal stacks of the anthocerote chloroplast are also atypical in that they lack the highly-curved grana end membranes present in the chloroplasts of other embryophytes (Fig. 6, inset). Manton (1962) referred to anthocerote grana as 'pseudograna' to differentiate them from the true grana found in higher plants. Channel thylakoids and grana without end membranes are found in all of the species observed to date, indicating that these features may be diagnostic of hornworts (Crandall-Stotler, 1986). The channel thylakoids were misinterpreted in early electron microscopic studies (Menke, 1961; Manton, 1962). These workers assumed that the stromal space between two adjacent channel thylakoids was a swollen thylakoid lumen, i.e. the two outer membranes of adjacent channel thylakoids were assumed to bound the lumen of the thylakoid. The advent of improved fixation techniques which preserved more of the stromal matrix reconciled this misconception (Wilsenach, 1963). The immunocytochemical localization of Rubisco in this space in *Megaceros* (Vaughn *et at.,* 1990a) is further proof that it is stroma, not a swollen lumen.

ittle is known of the molecular organization of the photosynthetic apparatus in the anthocerotes. So-called 'green' gels, in which the thylakoid proteins are partially denatured, reveal similar chlorophyll proteins to those found in higher plants [the P700 chlorophyll *a* protein, the chlorophyll *a/b* light-harvesting complex, and the CP47 and CP49 polypeptides of photosystem (PS) II (Vaughn, unpublished)]. Cytochemical localization of PS I activity indicates that the channel thylakoids and the outer thylakoids of the grana stack are enriched in PS I (Fig. 7A).

he reduction of the osmiophilic tetrazolium thiocarbamyl nitroblue tetrazolium, which accepts electrons from PS II (Vaughn, 1987), occurs mainly in the partition region of the grana stacks. Thus, the distribution of photosystems in the grana stacks is similar to that in higher plants and most algae, the channel thylakoids being the equivalent of the stroma lamellae of higher plant, connecting grana and enriched in PS I.

he only other thylakoid protein investigated in the anthocerotes is polyphenol oxidase. *Phaeoceros, Notothylas,* and *Anthoceros* spp. have polyphenol oxidase activity as measured by spectrophotometric enzyme activity, gel activity stain, and cytochemistry (Sherman, Vaughn & Duke, 1991). Polyphenol oxidase is found in some charalean algae and most higher plants (Sherman *et at.,* 1991), but the function of the protein is not known (Vaughn, Lax & Duke, 1988).

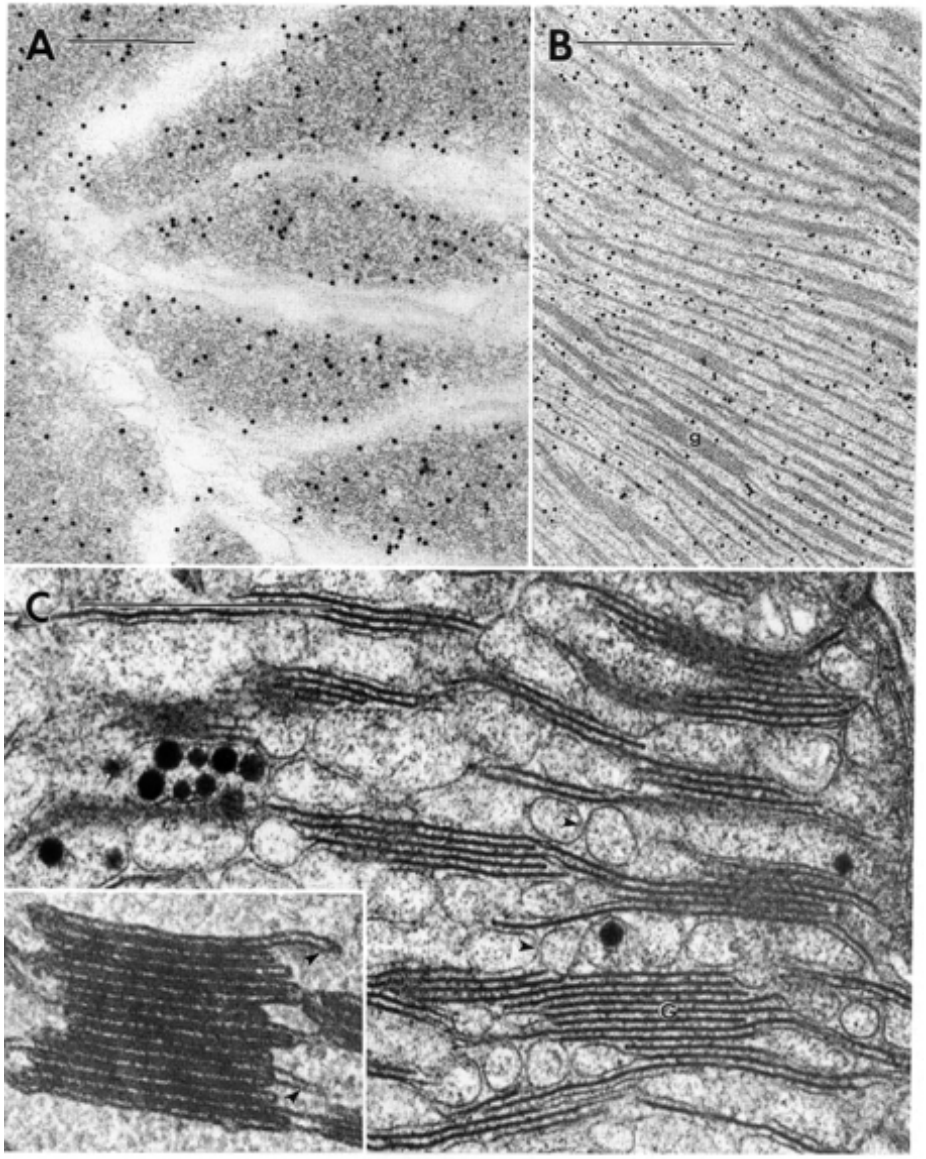

Figure 6. Immunocytochemical localization of Rubisco in (A) *Sphaerosporoceros adscendens* and (B) *Megaceros flagellaris.* In the pyrenoid-containing S. *adscendens,* the immunogold labelling is concentrated over the pyrenoid whereas the labelling is found throughout the stroma in *M. flagellaris;* g, granum. (C) Electron micrograph of thylakoid membranes of *Anthoceros punctatus* showing the characteristic channel thylakoids (arrows) that connect tops and bottoms of the grana (G). Inset: electron micrograph of thylakoid membranes of the higher plant *Vicia faba* showing the highly-curved grana end membranes (absent in anthocerotes) and stroma lamellae (arrows) connecting the grana stacks in the long axis parallel to the stack. Bars; (A, C, D) O5,im; (B) 1O,um.

(d) Starch

In most of the pyrenoid-containing anthocerotes, a layer rich in starch granules surrounds the pyrenoid (Fig. 1 A). Mature chloroplasts are often so full of starch, that the pyrenoid is difficult to discern by light microscopy. Valentine *et at.* (1986) found no starch in two species of *Dendroceros* and only small starch grains were observed in sporophyte plastids and adjacent gametophytic plastids of *D. tubercutaris* (Ligrone & Renzaglia, 1990). More abundant starch is found in sporophyte chlorenchyma in a number of *Dendroceros* species. Small starch grains are observed around the pyrenoids in chloroplasts of gametophytes of *D. tubercularis, D. japonicus,* and *D. javanicus,* but these are relatively rare, regardless of the culture of the plants or the part of the thallus examined. Valentine *et at.* (1986) speculated that the unusual lipid bodies found in the cytoplasm may represent an alternative form of storage product.

(e) Chtoroptast number, size and shape

lthough the uniplastidic condition of gametophyte cells is to be considered one of the most distinctive features of the anthocerotes, some exceptions are known. Notably, in the genus *Megaceros,* the apical cell and young epidermal cells have a single chloroplast, but mature epidermal cells generally contain 2—4 chloroplasts. The parenchyma cells are generally at least biplastidic, but occasionally they may contain up to 14 distinct chloroplasts (Burr, 1970; Campbell, 1982). Exceptions to the uniplastidic condition have also been reported in other genera, e.g. *Phaeoceros coriaceus, Phaeoceros hallii, Fotioceros fuciformis, Anthoceros fusiformis, Anthoceros formosae* (Bartlett, 1928; Bharadwaj, 1958; Campbell, 1982; Hasegawa, 1984). Gametophyte cells around the archegonia may have two chloroplasts in species with uniplastidic cells (Schuster, 1984).

As to the chloroplast number in sporophytic cells, an incorrect idea that the hornworts generally have two chloroplasts in sporophytic cells, may have been accepted without any substantiating observations. Indeed, we can see such descriptions in some textbooks (Lotsy, 1909; Campbell, 1918; Smith, 1955; Parihar, 1962). Chloroplast number in sporophytic cells varies in each species, however. For example, *Anthoceros husnotii, Anthoceros crispus, Anthoceros hawaiensis, Dendroceros tubercularis, Folioceros appendiculatus, Folioceros funciformis, Phaeoceros laevis,* and *Phaeoceros miyakeanus* have a single chloroplast in sporophytic cells (Scherrer, 1915; Bartlett, 1928; Bharadwaj, 1958; Hasegawa & Wada, unpublished), where such species as *Anthoceros formosae, Anthoceros fusiformis, Anthoceros punctatus,* and *Anthoceros subbrevis* contain two chloroplasts in their sporophytic cells (Bartlett, 1928; Bharadwaj, 1958; Hasegawa & Wada, unpublished). Moreover, Bartlett (1928) reported the four chloroplast condition in sporophytic cells of *Phaeoceros haliji* and *Phaeoceros pearsonii.* In *Megaceros* spp. where gametophyte cells contain more than two chloroplasts, the chioroplast number in sporophytic cells could be as many as twelve (Bartlett, 1928). The shape of the anthocerote chloroplast is quite variable. In the genera with multilayered thalli, notably *Phaeoceros, Anthoceros* and *Folioceros,* the upper epidermal cells contain the most well- developed chloroplasts. These are of giant sizes (over 70 pm long) and typically consist of a large central portion, containing the pyrenoid and starch, and a relatively thin peripheral area (Fig. 1 A). The peripheral area is highly pleomorphic and is responsible for the great variability in chloroplast shape. This is particularly evident in the chloroplasts of *Dendroceros* (Fig. 3, and inset). In *Dendroceros* and *Megaceros,* the chloroplasts are often very closely associated with the nucleus, that generally lies in a constriction of the dumbbell-like chloroplast (Fig. 7B). Some of the most unusual plastid shapes are found in *Phaeoceros coriaceus,* whose chloroplasts may be dumbbell-shaped, divided into two halves except by a fine thread, or oddly divided at angles (Fig. 5 B, and inset). Similar unusual plastid shapes have been observed in *Ant hoceros formosae* (Bharadwaj, 1958; Hasegawa, 1984).

Some of the variability in chloroplast structure may be due to light-dependent movements. For example, Burr (1968) found that several *Megaceros* species had very photoactive chloroplasts that were laminate under low light intensities but contracted into elliptical structures under high light intensities. The chloroplast morphology seems to be modulated by culture conditions as well (Valentine, 1984). The pleomorphism of the peripheral portions of chloroplasts in such genera as *Phaeoceros, Anthoceros* and *Folioceros* may be related to the absence of starch and pyrenoid in these areas. Whereas the stroma and thvlakoids may respond readily, the starch and pyrenoid, being less malleable, will resist movements. Campbell (1971) speculated that photo- activity may be one reason why *Megaceros* chloroplasts have lost pyrenoids.

Figure 7. For legend see opposite.

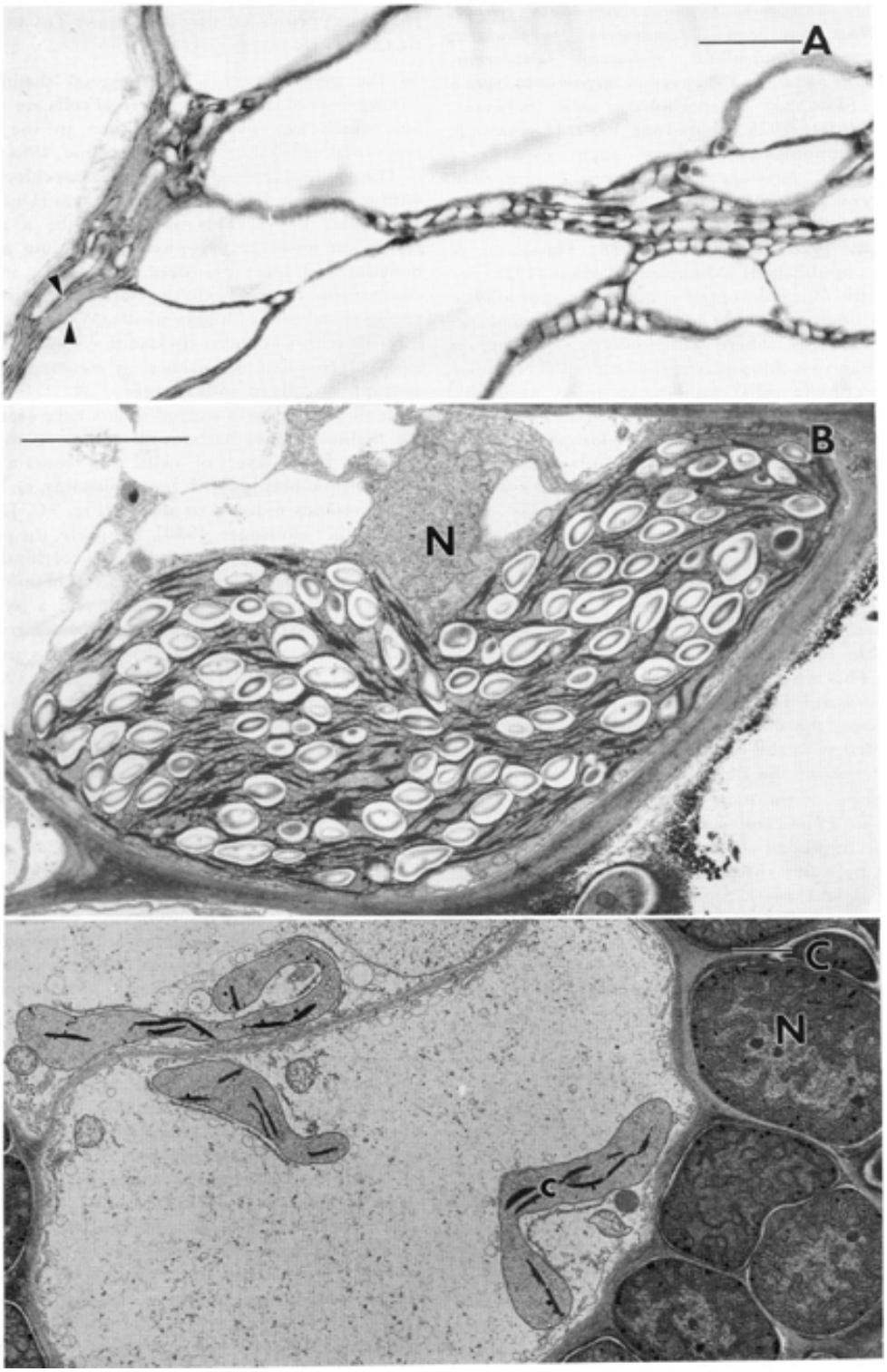

Figure 7. Cytochemical detection of photosystem I activity in *Phaeoceros laeuis.* Reaction is present on the channel thvlakoids and the tops and bottom of the grana stacks (arrows). **(B) Chloroplast of the** *Megaceros* species from North Carolina in which the nucleus (N) is associated with a constriction in the centre of the chloroplast. (C) Chloroplast (C) in a cell adjacent to a *Nostoc* (N) colony of *Notothylas orbicularis* are much less- developed than those found in other portions of the thallus. Thylakoids are mainly in doublets and pyrenoids are absent. Bars: (A) 05 /im; (B) 5() /im; (C) 2() pm.

III. VARIATIONS IN CHLOROPLAST (PLASTID) U L T R A STRUCTURE

In the genera with a multilayered thallus, the chloroplasts of the lower epidermal cells are smaller and much less pleomorphic than in the upper epidermal cells (Burr, 1968; Valentine, 1984).

The single apical cell of the thallus has chloroplasts with a well developed thylakoid system (Duckett & Renzaglia, 1988). This appears to be a general feature of lower embryophytes, including mosses, hepatics and most pteridophytes, and is in stark comparison to the underdeveloped proplastids of young apical cells of higher plants (Wellburn, 1987). Plastids with a reduced thylakoid system are, however, of frequent occurrence in gametophyte and sporophyte cells of anthocerotes.

In all of the genera we and others have examined, the thallus cavities harbouring *Nostoc* colonies are outlined by a layer of cells that contain small pleomorphic plastids with few thylakoids, no starch, and pyrenoids reduced or absent (Fig. 7C, Duckett *et at.,* 1977; Honegger, 1980). Similarly, the plastids in cells of *Phaeoceros* infected by mycorrhizal fungi are less developed than the chloroplasts of uninfected parenchyma cells and lack starch and a pyrenoid (Ligrone, 1988). Most likely, these changes in plastid structure reflect physiological interactions with the symbionts (Duckett *et at.,* 1977; Ligrone, 1988).

Several species of anthocerotes form tubers during conditions that are unfavourable for growth. Ligrone & Lopes (1989) examined the ultrastructural changes that occur during the formation of tubers in *Phaeoceros laevis.* Chloroplast structure is remarkably well preserved even at final stages of tuber formation, with extensive depositions of starch, perhaps as a reserve substance for regrowth when conditions become more favourable.

Rhizoids may develop from any epidermal cell (Renzaglia, 1978). As the rhizoid differentiates, the chloroplast of the epidermal cell fragments into beadlike structures that spread throughout the cytoplasm. Whether these are truly multiple plastids or a single plastid connected by thin connections is not known. Concomitantly, the chloroplast dedifferentiates, losing both the pyrenoid and the thylakoids. Starch is still present. No green colour or bright red fluorescence, indicative of chlorophyll, is found in the mature rhizoid of most species, indicating that the plastids are leucoplasts or amyloplasts at maturity or are lost entirely. Some chlorophyll is observed in rhizoids of *Dendroceros* species, especially in those close to the apex.

When angiosperms are exposed to prolonged periods of darkness, etioplasts develop. Ligrone & Fioretto (1987) kept growing sporophytes *ofF. laevis* in darkness for prolonged periods and monitored the ultrastructural effects as well as changes in the chlorophyll content over time. Unlike angiosperms, no prolamellar bodies are noted in the developing hornwort sporophytes, even after 60 days in darkness. The chloroplasts of dark-grown sporophytes have bigger granal stacks and less numerous channel thylakoids than in the light-grown controls. Paracrystalline membrane arrays, faintly reminiscent of prolamellar bodies, are found occasionally in developing chloroplasts but disappear during development.

One of the greatest changes to the anthocerote chloroplast occurs during the development of the male gametes. The plastids in sperm cells of all of the anthocerotes examined are much smaller than the chioroplasts of the thallus (Renzaglia & Carothers, 1986). At the mid-spermatid stage, the chloroplast is already very reduced, containing a small tear-shaped starch grain and a few vesicle-like thylakoids (Fig. 8A). At maturity, other than a tiny bit of stroma and the starch grain, there is no substructure to the chioroplast (Fig. 8 B). The mature spermatozoids of *Notothylas* and *Phaeoceros* lack plastid DNA (Renzaglia & Duckett, 1989). Antheridial chambers are obvious on the thallus surfaces as small patches of a bright orange colour in some of the genera (e.g. *Phaeoceros, Notothytas).* The orange colour is due to a layer of chromoplast-containing cells, the antheridial jacket, which surrounds the spermatids (Duckett, 1975). The jacket cell plastids are similar to chromoplasts found in higher plants in that they have fewer thylakoids than the chloroplasts of the adjoining gametophytic tissue and an abundance of plastoglobuli, which presumably contains the carotenoid pigments (Fig. 8C). In other genera (e.g. *Megaceros),* the antheridial jacket cells contain chioroplasts rather than chromoplasts.

There are no previous reports on the ultrastructure of the female gametangia, although there have been several light microscopic studies (e.g. Renzaglia, 1978). Ultrastructural investigations of archegonia of *Phaeoceros laevis* (Renzaglia, unpublished) reveal the presence of long, flat plastids with a rudimentary thylakoid system in the egg cells (Fig. 9A). The plastids of sterile archegonial cells, such as the neck and ventral canal cells, are similar in structure but also contain small pyrenoids and a slightly more elaborate thylakoid system than the egg cell. The presence of long, small-diametered well-developed chloroplasts in the egg cells, coupled with the absence of chloroplast DNA and the small size of the plastids in the male gametes, indicate the maternal inheritance of chloroplast DNA in the anthocerotes, as in most higher plants (Vaughn *et at.,* 1980), although dissimilar to the paternal inheritance of plastids in gymnosperms (Sears, 1980).

There is much greater variation in plastid structure in the sporophyte than in the gametophyte tissue. At the sporophyte/gametophyte junction in *Phaeoceros* (Gambardella, Ligrone & Castaldo, 1981; Gambardella & Ligrone, 1987), *Anthoceros* (Chauhan & Schraudolf, 1986) and *Fotioceros* (Vaughn & Hasegawa, unpublished), the gametophyte transfer cells contain pleomorphic plastids with a reduced thylakoid system and are generally devoid of starch (Fig. bA). The adjoining haustorial cells of the sporophyte exhibit large plastids with a rudimentary inner membrane system, a scarcely recognizable pyrenoid, abundant membranous envelope-associated structures similar to peripheral reticulum, and large starch grains of spheroidal shape (Fig. 10B).

In *Dendroceros tubercutaris* (Ligrone & Renzaglia, 1990), the gametophytic transfer cells have well-developed chloroplasts, whereas the haustorial cells have large plastids lacking thylakoids, pyrenoid starch, and containing numerous swollen vesicles. The opposite situation is found in *Notothylas,* where the haustorial cells have well-developed chloroplasts with abundant starch and a distinct pyrenoid, whereas the gametophytic transfer cells have plastids with a reduced thylakoid system and no recognizable pyrenoid (Ligrone, Renzaglia & Duckett, unpublished). These variations in plastid structure may possibly reflect differences in the physiological relationships between the two generations (Ligrone & Renzaglia, 1990). The plastids from cells of the basal meristem have a similar morphology: long and flat plastids with somewhat swollen-appearing thylakoids, generally in doublets or small grana stacks, and a distinct pyrenoid, in species with pyrenoids. Chioroplasts of both the sporophyte wall and the columella cells are similar to those of the gametophyte, except that the thylakoid system is less elaborate. At early developmental stages, spores (or spore mother cells) have relatively well-developed chloroplasts that contain both thylakoids and pyrenoids (Fig. bC), but later the plastids are converted to amyloplasts in most of the hornwort species (Fig. 1OD, see also Ajiri & Ueda, 1986). Similar plastid morphologies are noted in elater mother cells. The spores of *Dendroceros* are unique amongst anthocerotes in that they become multicellular before release from the capsule and appear green because of the development of chloroplasts (Renzaglia, 1978). Chloroplasts in multicellular *Dendroceros* spores are similar to those found in the later development of the gametophytic tissue.

Although the plastids of the sporophyte basal meristem are recognizable as chloroplasts, there is a gradient of development from the basal meristem to the more mature chloroplasts found in the columella and chlorenchyma cells. Wilsenach (1963) utilized this gradient of development to follow chloroplast ontogeny in *Anthoceros ecktonii.* His data indicate that thylakoids may develop from ingrowths of the inner chioroplast envelope, the stacks form from folding of lamellae, and channel thylakoids from fusion of lamellae between stacks. Ligrone & Fioretto (1987) were able to repeat these observations using more modern fixation protocols on *Phaeoceros laevis.* In higher plants, there has been some doubt whether the inner-envelope derived vesicles in

fact contribute to thylakoid development (Vaughn & Duke, 1987). In anthocerotes, the connections between welldeveloped thylakoids and envelope vesicles makes the role of these vesicles in thylakoid development more tenable.

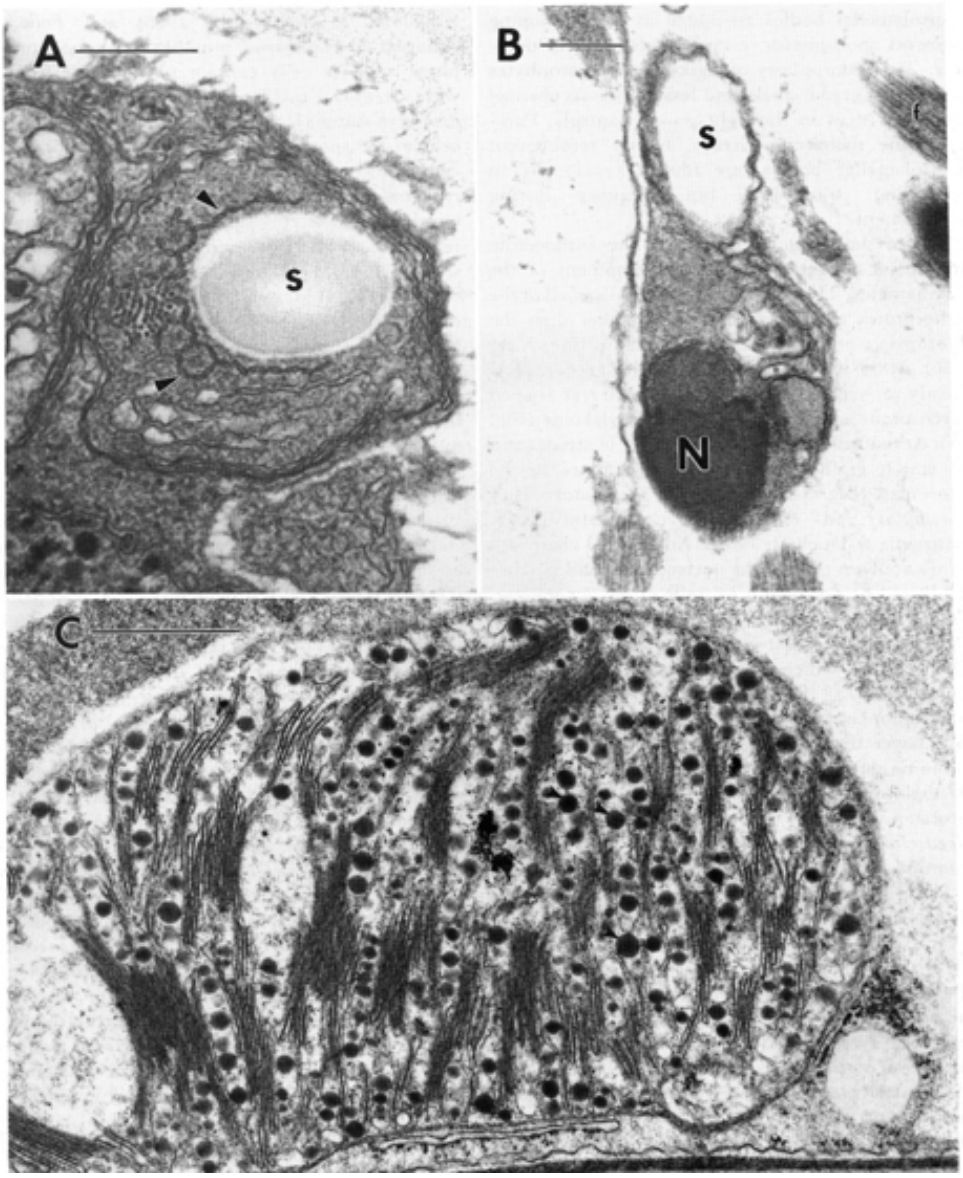

Figure 8. Ultrastructural characteristics of plastids in antheridia. (A) Plastid (P) from a mid-stage spermatid of *Megacerosflagellaris.* Only a few membranes (arrows) and a starch grain (s) are noted. (B) Mature spermatid of *Folioceros fuciformis* with plastid reduced to a single envelope-covered starch grain. N, nucleus; f, flagella. (C) Chromoplast from the antheridial jacket cell of *Folioceros fuciformis.* Although some thylakoids remain in the plastid, there are large numbers of plastoglobuli (arrows), probably the site of carotenoid accumulation. Bars; (A, B) O2pm; (C) 1Opm.

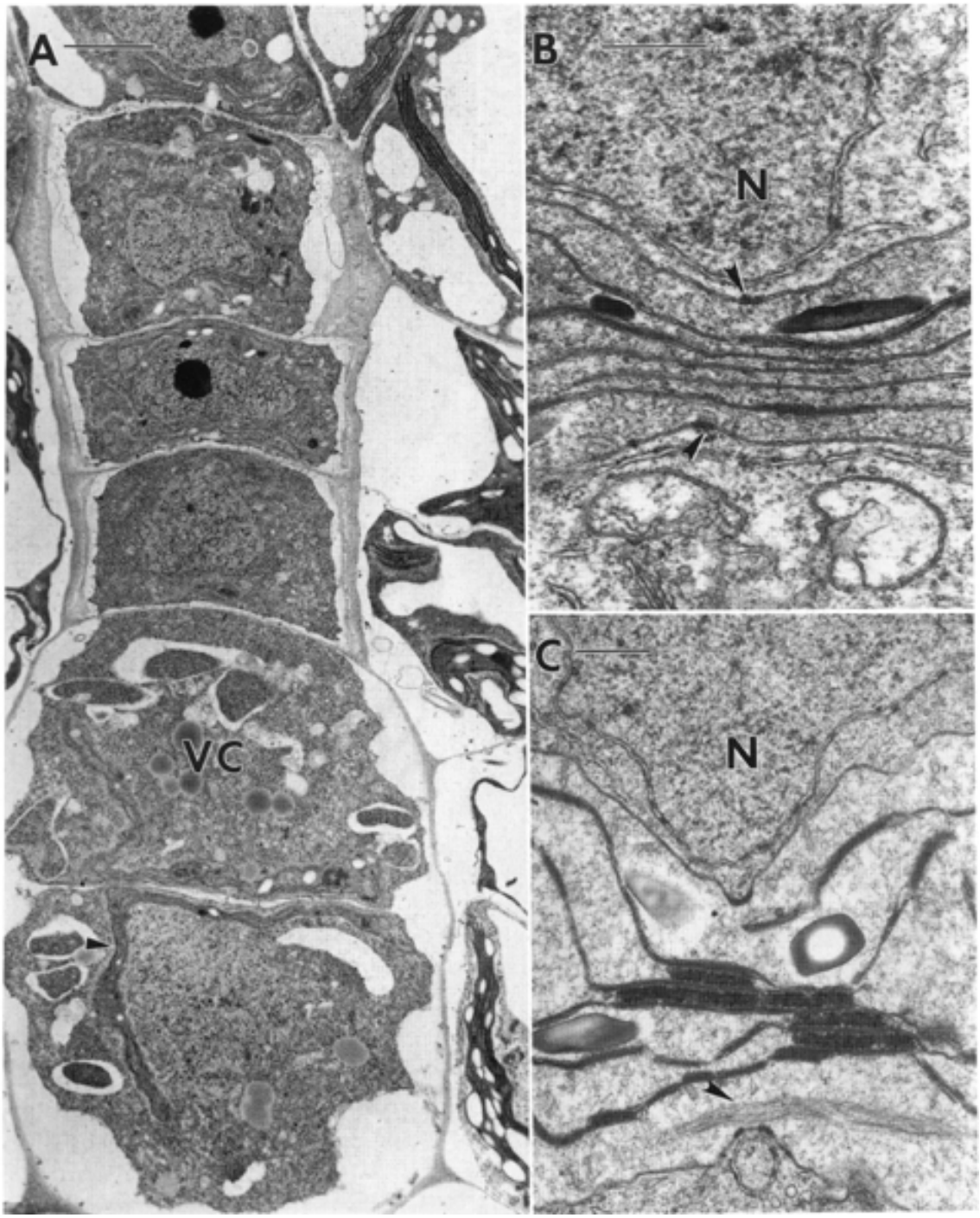

Figure 9. Archegonial plastids of *Phaeoceros laevis* are relatively long and flat structures (arrow) with a reduced membrane system. These are much more developed than the plastids found in the spermatids (Fig. 8B), however. Ventral canal cells (VC) have similar plastids to those in the archegonia. Stages of chloroplast division in the sporophyte meristem of *Folioceros fuciformis.* (B) Association of the nucleus (N) with a constriction in the chloroplast. Note the accumulation of electron-opaque material on the envelope at the site of this constriction (arrows). (C) In addition to the constriction ring, a band of filaments (arrow) is also noted in the chloroplast. Bars: (A) 20 *pm;* (B, C) 05 pm.

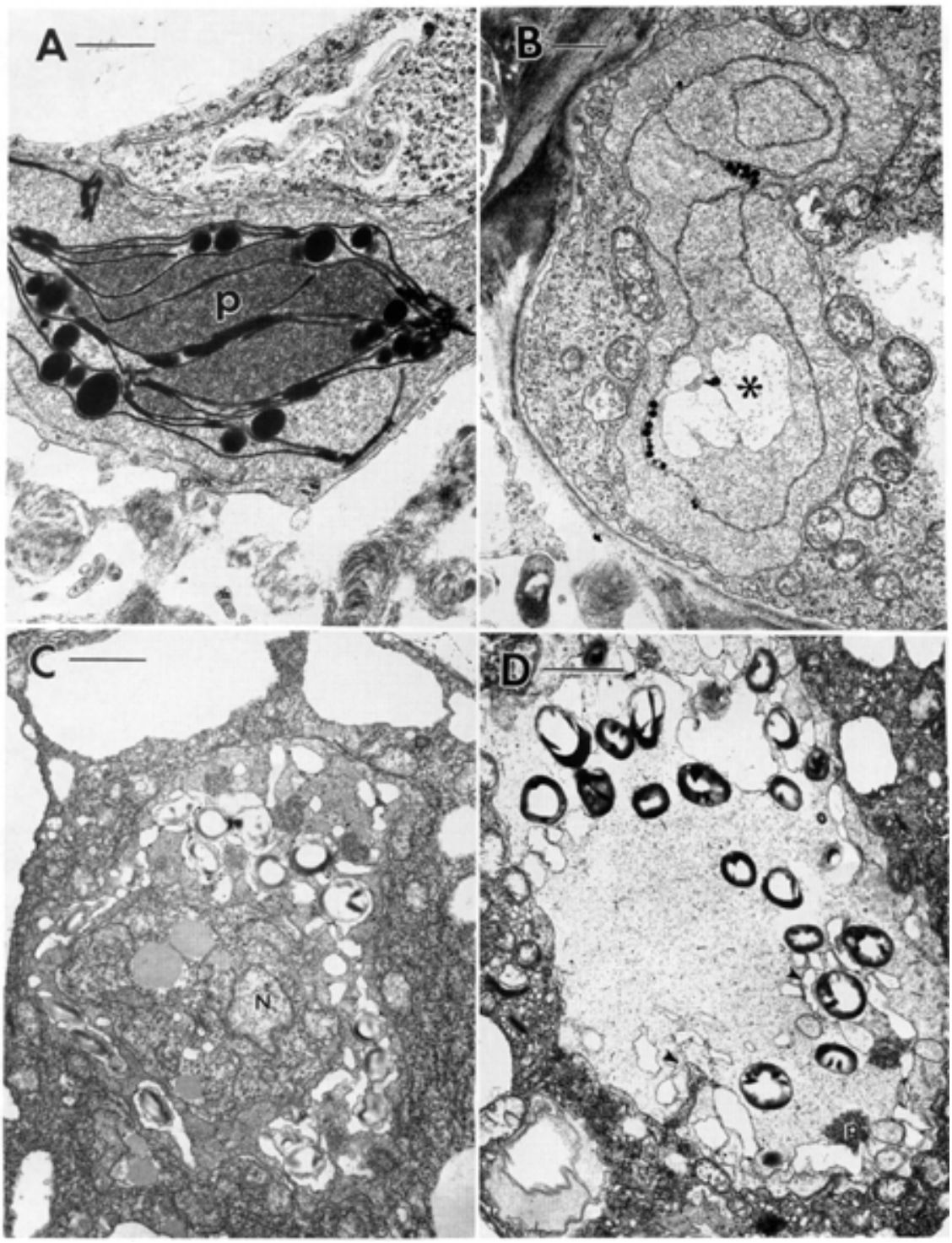

Figure 10. Ultrastructure of plastids from the placenta and sporophyte. (A) Transfer cell plastid of *Phaeoceros laevis* with a multiple pyrenoid (p) but with a greatly reduced thylakoid system. (B) Plastid from a haustorial cell of the sporophyte of *Notothylas breutelii* with very few internal membranes and a vacuole-like inclusion (*). (C) Plastid from a young spore of *Folioceros fuciformis,* still with some thylakoids. The plastid encircles the nucleus (N). (D) Plastid from a nearly mature spore of *Notothylas orbicularis* with much less electron-opaque stroma, only remnants of thylakoids (arrows) and a pyrenoid (p). Bars: (A, B) 05 /Lm; (C, D) 20 ,um.

IV. CHLOROPLAST DIVISION

Because many anthocerote species have uniplastidic cells, it is not surprising that the division of the single plastid would be tightly controlled in order to prevent the formation of apoplastidic and biplastidic cells by unequal cytokinesis. Lander (1935) described the division process from light microscopic studies of *Z\Totothylas orbicularis.* At early stages, the chloroplast is closely associated with the nucleus and is perpendicular to the future cell plate. The chloroplast then elongates and forms a central constriction with the two halves lying along opposite cell walls. Nuclear division follows and the formation of a cell plate eventually completes the chloroplast division.

Lander's light microscopic study is a surprisingly complete description, whose details have recently been substantiated and expanded with the use of immunofluorescence and electron microscopy. At the end of the chloroplast constriction, a ring of electron-opaque material accumulates (Fig. 9B). This is the first description of a constriction ring in the anthocerotes, although similar rings have been observed in chloroplast division in many plants (Oross & Possingham, 1989; Tewinkel & Volkman, 1987; Kuroiwa, 1989). It is assumed that all of these constriction rings are actin. In addition, anthocerote chloroplasts contain a bundle of fibrillar material similar in structure to actin microfilament that is oriented in the direction of division in the plastid as well (Fig. 9C). McCurdy & Williamson (1987) detected a protein that was recognized by monoclonal anti-actin in purified pea chioroplasts, but similar experiments on *Phaeoceros* plastids were negative (McCurdy, personal communication). Brown & Lemmon (1985, 1988) showed that the preprophase band of microtubules transects the plane of chloroplast division. Other microtubules, forming an axial microtubule system, are oriented parallel to the long axis of the plastid (Brown & Lemmon, 1989) and appear to have a role in plastid division. During mitosis, the nearly divided chloro plas is positioned with a mass concentrated at each pole connected by a constricted region. Spindle microtubules are organized at the tips of the chloroplast envelope, indicating that this area is a microtubule organizing centre (Fig. 12A). There fore successful chloroplast division is a requisite for nuclear division, ensuring that no apoplastidic cells are formed, as the chloroplast serves as a spindle microtubule organizing centre. As cytokinesis is completed by the formation of a cell plate, the plastid division is completed. The formation of the cell plate in hornworts is produced by a phragmoplast, like other embryophytes and some green algae (Stewart & Mattox, 1975) rather than a phycoplast like the remaining green algae (Stewart & Mattox, 1975). Peroxisomes, or perhaps a highly branched peroxi some occur at the cell plate, as is typical of both advanced algae and higher plants (Owen, 1983). In both the light microscopic studies of Lander (1935) and the electron microscopic and immunofluorescent studies of Brown & Lemmon (1985, 1988) and Owen (1983), dividing cells were observed in the basal meristem of the sporophyte. In the apical cell of the gametophyte, a similar pattern of chloroplast division is noted, although the chloroplasts are more developed than those at the sporophyte meristem (e.g. Fig. 11). Investigations of meiotic cells also reveal the involvement of plastids in meiotic divisions as spindle microtubule organizing centres (Brown & Lemmon, 1990). No examples of plastid division by methods other than median constriction (Vaughn *et al., 1990b)* have been observed in anthocerotes.

In the genus *Megaceros,* most of the thallus cells are multiplastidic, with the exception of the apical cell and some epidermal cells. Division of the chioroplast appears similar to that in uniplastidic cells, however. One of the chloroplasts associates with the nucleus and the plane of the subsequent division is set (Burr, 1968). This ensures at least one plastid in each new cell after cytokinesis even though all of the other chioroplasts may end up in a single cell. Thus, the mechanism of a coordinated nuclear and chloroplast division appears to be ubiquitous in the anthocerotes and is perhaps of taxonomic importance in defining Anthocerotophyta.

Like most bryophytes, the anthocerote gametophytes have a remarkable ability to regenerate whole plants from parts of the old. Burr (1969) investigated the changes in chloroplast number and division pattern during the regeneration process in *Megaceros flagellaris.* Surprisingly, she found that, during formation of new apical cells from the multiplastidic cells of the thallus, the chloroplast number was reduced to one. This was achieved through cell divisions without plastid division, walling off of plastids without the nucleus, and chloroplast fusion.

V. ASSOCIATIONS WITH OTHER ORGANELLES

Besides the associations with the nucleus and microtubules (above), several other associations of anthocerote chloroplasts with other organelles are worthy of note.

In higher plants, peroxisomes are often associated with the chloroplast envelope. It is believed that this close relationship is due to the presence of the important photorespiratory enzyme, glycollate oxidase, in the peroxisome (Vaughn, 1990). Similarly, peroxisomes are often found associated with the anthocerote chloroplast envelope and have been detected both in species that have and species that lack pyrenoids. Peroxisomes are also present during chioroplast and nuclear division, generally at the plastid isthmus (Owen, 1983; Brown & Lemmon, 1985).

In developing plastids of the sporophyte, arrays of both smooth and rough endoplasmic reticulum (ER) are associated with the chloroplast envelope. This plastid-associated ER was first noted by Ligrone & Fioretto (1987) in Phaeoceros laevis and has been found in all of the developing sporophytes of anthocerotes examined by us as well. Ligrone & Fioretto (1987) speculated that it may have a role in plastid development, possibly for the transport of lipids and/or proteins into the chloroplast.

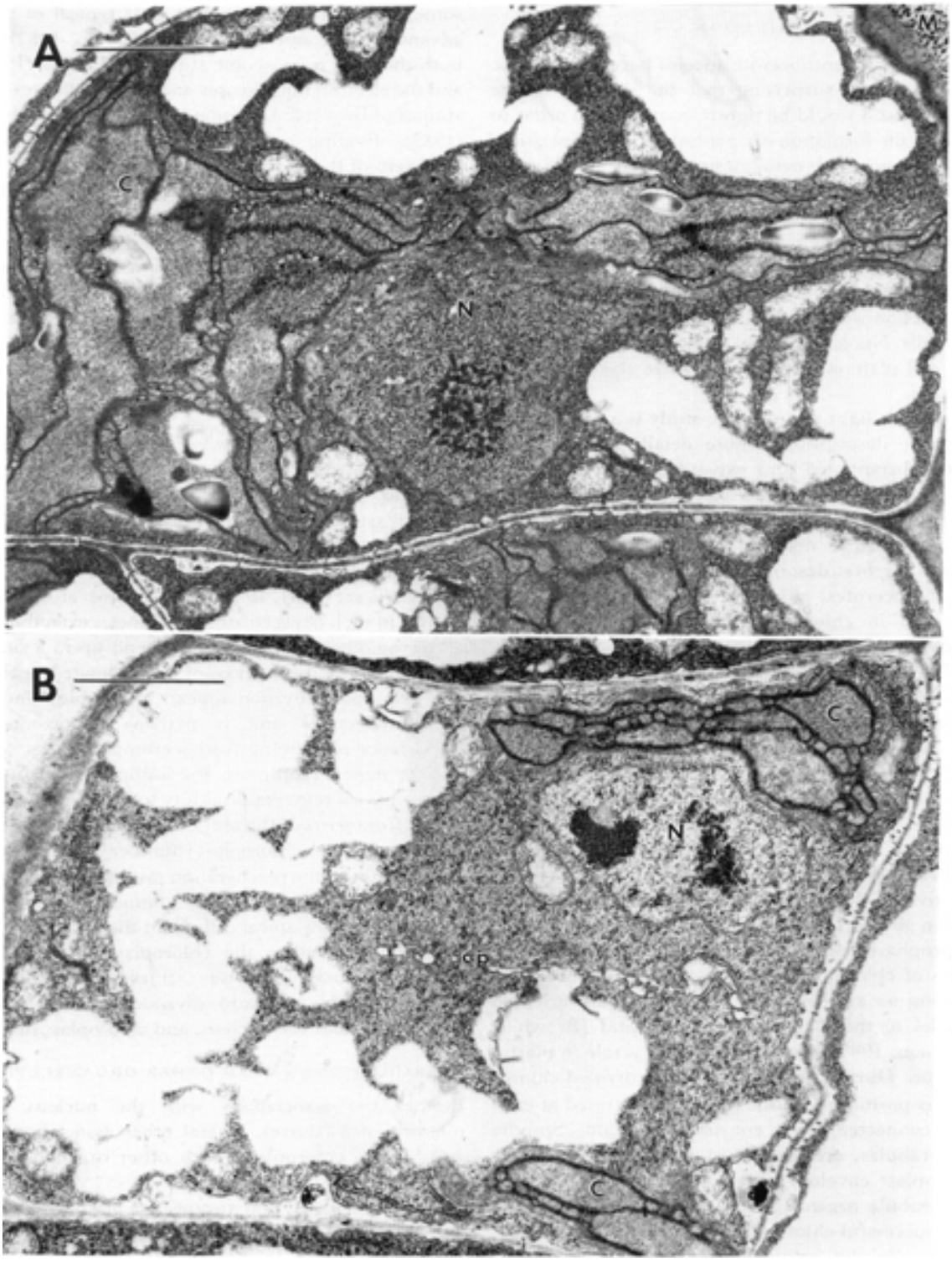

Figure 11. Early stages of plastid division (A) and telophase nuclear division (B) figures in the apical cell of a *Folioceros* sp. from Japan. N, nucleus; M, mucilage; cp, cell plate; C, chloroplast; bar, 20 1um.

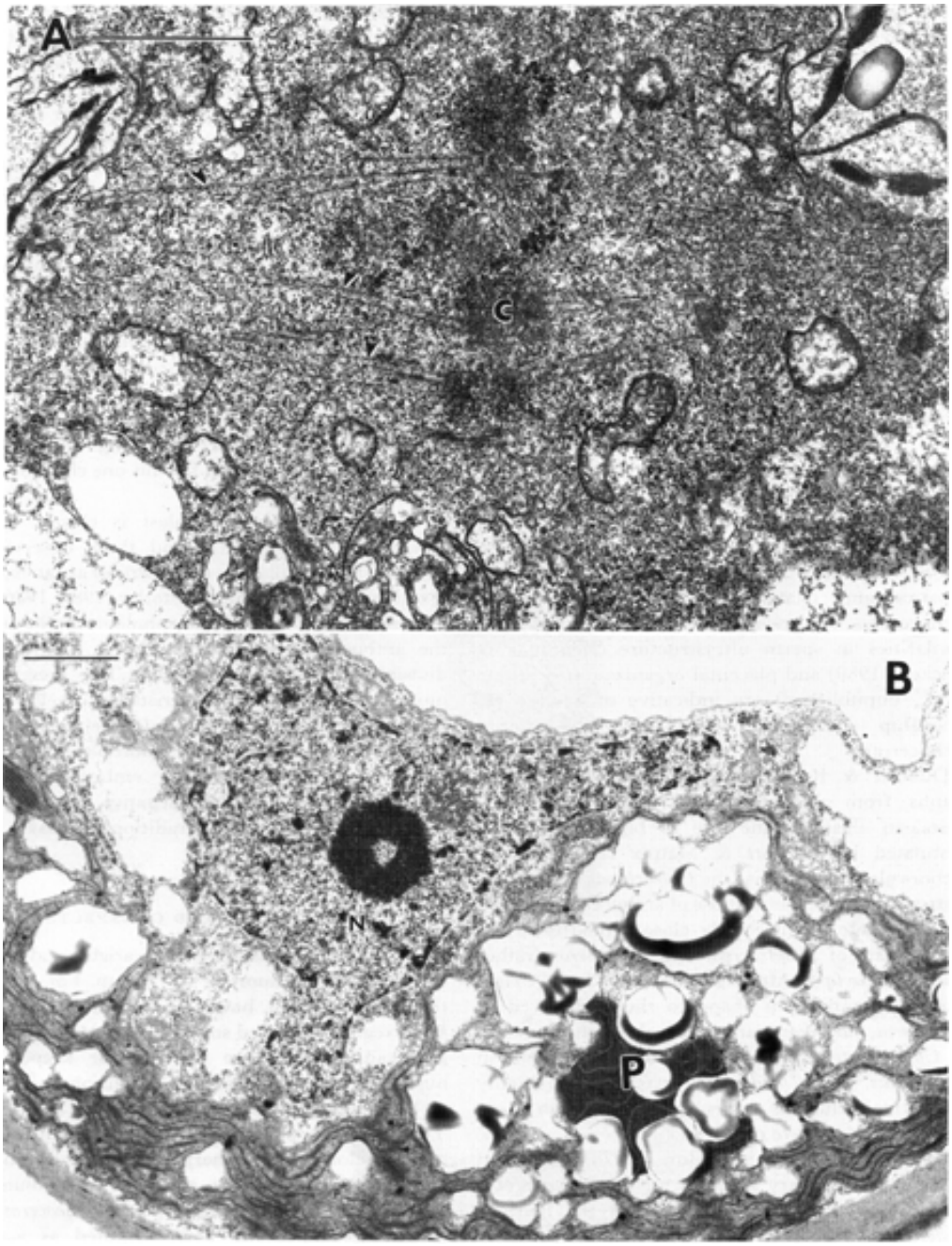

Figure 12. (A) Metaphase figure in which the termini of the microtubules is found on the chioroplast envelope (*) which appears to be a microtubule organizing centre. Arrows mark microtubules; C, chromosome. (B) Chioroplast of *Coleochaete scutata* contain a pyrenoid in which only single thylakoids cross the pyrenoid area (P). N, nucleus; bars, 2Opm.

VI. EVOLUTIONARY CONSIDERATIONS

Recently, there has been much discussion of the possibility of *Coleochaete* representing the extant green algal genus most like the progenitor of higher plants (Delwiche *et al.,* 1989). Unfortunately, from the present viewpoint, most of the investigations of these algae have centred on aspects of the biology of these plants other than the chioroplast morphology and development. *Coleochaete* chloroplasts (Fig. 12) share many characteristics with the anthocerotes, especially *Notothylas.* The granal stacks do not have end membranes in *Coleochaete,* but rather are pseudograna as is found in the anthocerotes. In addition, the thylakoids are arranged in channels, a situation which, among embryophytes, is noted only in the anthocerotes. The pyrenoid of *Coleochaete* is globular and is transected into subunits by single thylakoids. Most of these pyrenoids have very little non-pyrenoid stroma intercalating the subunits of the pyrenoid. This pyrenoid structure is very similar to that found in *Notothylas orbicularis* or *N. breutelii:* a globular structure dissected only by stroma lamellae. Although many bryologists consider that the genus *Notothylas* is advanced (i.e. its small sporophyte enclosed within the gametophyte has evolved by reduction from a more elaborate sporophyte like that found in the other anthocerote genera), the opposite situation could be true. In fact, using cladistics, Mishler & Churchill (1985) concluded that the most parsimonious cladogram is one in which *Notothylas* is separated from the anthocerotes, and set in a closer affiliation with the hepatics and charalean algae than the other anthocerotes. However, it must be noted that similarities in

sperm ultrastructure (Renzaglia & Duckett, 1989) and placental organization (Ligrone *et al.,* unpublished) are indicative of a close relationship between *Notothylas* and other anthocerotes.

Duckett & Renzaglia (1988) examined micro- graphs from published work of a number of charalean algae on the line to higher plants, as postulated by Stewart & Mattox (1975). These authors also observe that the *Coleochaete* chloroplast is structurally similar to those of anthocerote species, but they note a particularly close similarity to the chloroplasts of *Dendroceros* and *Megaceros* (rather than *Notothylas).* Although the plastids in *Coleochaete* are similar in shape to those observed in *Megaceros,* our investigations reveal that the pyrenoid of *Coleochaete* is very similar to those found in *Notothylas.* The *Dendroceros* pyrenoid has highly distinctive inclusions and is quite different in shape from the *Coleochaete* pyrenoid.

Both Kaja (1954) and Burr (1970) presented possible evolutionary schemes for the anthocerotes based upon chloroplast structure at the light or electron microscopic level, respectively. In Kaja's (1954) scheme, the *Notothylas* chioroplast is considered the most algal-like, with various other pyrenoid configurations as intermediate, and the pyrenoid-less (interpreted by Kaja as many dispersed pyrenoids) *Megaceros fiagellaris* as the most advanced. Burr (1970) examined mainly species of *Megaceros* and found variations in chioroplast number and size. However, her analysis of chloroplast ultrastructure in these species appears flawed because the areas she described as 'pyrenoids' appear to be nothing more than starch-free zones of stroma. Ultrastructural studies of Valentine *et al.* (1986) and us have revealed no pyrenoids in some of the same species referred to by Burr (1970) as having a pyrenoid actually lack a pyrenoid. In our studies, a series of variations in pyrenoid ultrastructure may be constructed similar to Kaja's scheme. This scheme draws an evolutionary sequence which is difficult to reconcile with the many other morphological characteristics that indicate that the sporophvte of *Notothylas* is derived, and not ancestral (Renzaglia, 1978). In addition, ultrastructural investigations are still restricted to a small range of species and clearly there is a large variation in plastid ultrastructure, including apparently pyrenoidless species in the genus *Notothylas* (Udar & Singh, 1981). Thus, despite the number of anthocerote species examined for chloroplast structure, drawing any kind of phylogenetic scheme even for this one character may be premature.

The anthocerote chloroplast is clearly unique among the archegoniates and these observations support the view that the anthocerotes are an isolated taxonomic group. Crandall-Stotler (1980, 1986) and Schuster (1984) argue on morphological criteria that the anthocerotes should be placed in their own division, the Anthocerotophyta. The presence of uniplastidic cells in non-meristematic cells of the gametophyte, multiple pyrenoids, pseudograna, and channel thylakoids are all chloroplast characters found nowhere else in the embryophytes and certainly not at all in the hepatics, a group with which the anthocerotes traditionally have been linked.

VII. TAXONOMIC USES AND CONSPECTUS

Pyrenoid structure is extremely variable and may be an aid in the taxonomy of this group. For example, the *Notothylas* spp. have rather rounded pyrenoids and irregularly shaped subunits. In *Dendroceros,* the pyrenoid subunits are of irregular shape, with numerous electron-opaque inclusions (pyrenoglobuli) surrounded by electron-opaque threads. The *Folioceros* pyrenoids also have pyrenoglobuli, but only along the periphery of the subunits, and the subunits are similar in shape to that found in *Anthoceros, Sphaerosporoceros,* and *Phaeoceros.* Although *Folioceros* has been disputed as a good genus by some (Schuster, 1987; Hasegawa, 1988), the presence of a concentration of pyrenoglobuli at the periphery of the pyrenoid subunits is a potential taxonomic marker for this taxon. In *Megaceros,* no pyrenoids were detected in any of the species examined. As additional taxa are examined, these generalities or generic parameters in chloroplast structure may not prove to be absolute.

Chloroplast structure and number were utilized in a recent taxonomic revision by Hasegawa (1988). *Megaceros giganteus* (formerly *Dendroceros giganteus)* has a conspicuous costa and unistratose wings, an anatomical organization that closely recalls that found in *Dendroceros* species. However, mature gametophyte cells of *M. giganteus* are multiplastidic (Hasegawa, 1988) and the chloroplasts lack a pyrenoid (Valentine *et al.,* 1986). Thus, the grouping of this species in *Megaceros* reflects the greater similarity of the chloroplasts of this species with *Megaceros* than with *Dendroceros.*

This review has centred on morphological features but this is in large part because there are almost no physiological/biochemical studies on the anthocerote chloroplast. Anthocerotes are not found abundantly in the field (except in unique localities such as the cool, wet areas of New Zealand) and, even under optimal conditions, material at the same physiological state in sufficient quantity to perform physiological/biochemical studies is difficult to obtain. The thylakoids of the hornworts represent a unique system of membranes and it would be desirable to learn how the various pigment—protein complexes are arranged in the membranes. Immuno cytochemica techniques, already used to localize Rubisco in the pyrenoid (Vaughn *et at.,* 1990 a), may be useful in determining the distribution of the complexes in the photosynthetic apparatus. Similarly, it may be helpful to determine if other carbon- fixation enzymes besides Rubisco are present in the pyrenoids of the anthocerote. Thus, immunocytochemistry offers a solution to the problem of limited tissue of a given physiological state and should be invaluable in determining many other facets of the anthocerote chloroplast.

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