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Chloroplast dimorphism in leaves of *Cabomba caroliniana* (Cabombaceae)

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ABSTRACT

Cabomba Aublet is useful as a genetic model of angiosperm evolution. Previous anatomical studies and preliminary light microscopic observations on *C. caroliniana* A Gray revealed the presence of dimorphic chloroplasts in both types of leaves, floating and submerged leaves. Leaf anatomy and chloroplasts ultrastructure were analyzed and compared. The chloroplast ultrastructure of the mesophyll and epidermal cells is different. Mesophyll chloroplasts have several plastoglobuli, large starch grains and grana formed by an average of 29 thylakoids. Epidermal chloroplasts are smaller, have grana formed by an average of 9 thylakoids and starch grains are occasional and smaller. A statistical analysis (*t*-test) was made to determine if differences between chloroplasts are significant. The ultrastructure of the epidermis chloroplast is similar to the one of sun leaves chloroplasts and the ultrastructure of the mesophyll chloroplast is similar to the one of shade chloroplast observed in other species.

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1. Introduction

Molecular phylogenetic studies indicate that the orders Amborellales, Nymphaeales and Austrobaileyales (ANA grade) diverged as separate lineages from a remaining angiosperm clade at a very early stage in flowering plant evolution. *Cabomba* Aublet (Nymphaeales) shows features that make it potentially useful as a genetic model of angiosperm evolution (Vialette-Guiraud et al., 2011). It is a New World genus of aquatic plants with most species restricted to tropical or subtropical regions (Orgaard, 1991).

Aquatic angiosperms generally have anatomical features that differ from terrestrial angiosperms (Sculthorpe, 1985). A marked foliar dimorphism is present in *Cabomba* (Moseley et al., 1984). Heterophylly is considered a result of heteroblastic development (Briggs and Walters, 1984; Kerstetter and Poeting, 1998) or of plastic development (Titus and Sullivan, 2001). *C. caroliniana* A. Gray presents floating peltate leaves with entire lamina and submerged leaves with trichotomously dissected lamina (Galati, 1981). The floating leaves are produced by flowering shoots (Fig. 1A and B). Previous anatomical studies on *Cabomba* (Galati, 1981) and preliminary light microscopic observations revealed dimorphic chloroplasts in both types of leaves of *C. caroliniana*. The aim of this research is to describe the leaf anatomy and to compare the ultrastructure of the different chloroplasts present in both types of leaves.

2. Materials and methods

Leaves of three different populations of *C. caroliniana* were collected: one population was cultivated in an outdoor pond of the Botanical Garden of the Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina; another population was from one natural population of INTA Delta (Latitude 59°20′ S. Longitude 34°40′ O) and the third population was from an indoor fishbowl culture. The reference material is deposited in the Herbarium Gaspar Xuarez (BAA). Material was pre-fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 2 h and post-fixed in 1.5% OsO_4 at 2 °C in the same buffer for 3 h. Leaves were dehydrated in ascending acetone series and embedded in Spurr's resin. Fine sections were made on a Reichert-Jung ultramicrotome and stained with toluidine blue, observed and photographed with a Motic digital light microscope. Ultrathin sections were stained with

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Fig. 1. Live photograph of *Cabomba caroliniana*. (A) Submerged leaves (sl). (B) Detail of (A) where floating leaves (fl) can be easily distinguished while accompanying the flower.

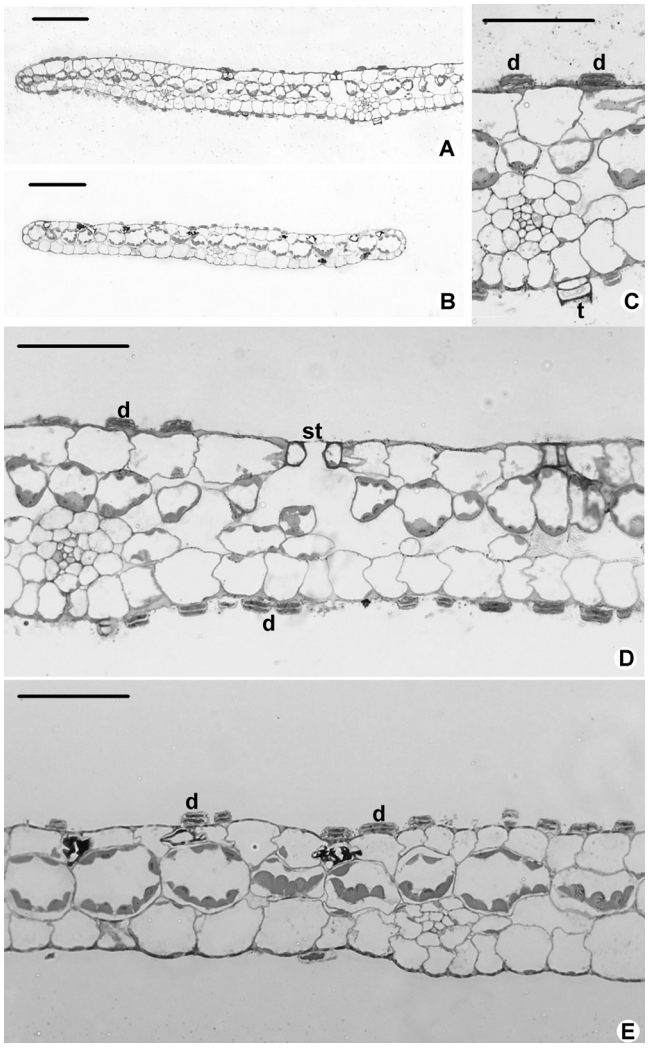

Fig. 2. Transverse sections of submerged and floating leaves observed with Light Microscope. (A) Floating leaf. (B) Submerged leaf. (C) Detail of A. d: diatoms; t: trichome. (D) Detail of a floating leaf showing a stoma (st). (E) Detail of submerged leaf. Scale bars (A) and (B) 100 μm, (C), (D) and (E) 50 μm.

uranyl acetate and lead citrate (Zarlavsky, 2014), observed and photographed in a JEOL 1200 EX II.

Free hand sections of fresh leaves were made and observed and photographed with a fluorescence microscope Zeiss Axioplan, in order to compare chlorophyll auto-fluorescence (wavelength 395 nm).

For statistical analysis, the following variables were measured in the epidermis and mesophyll chloroplasts: number of thylakoids per granum, wide of the chloroplast, and length of the chloroplast. Number of measurements for each of the variables studied is specified in Table 1. A parametric test (nominal significance level $\alpha = 0.05$) was performed after homogeneity of variance was verified. A *t*-test was used since it allows testing the hypothesis on the expectation of the random variable defined as a difference of sample means. It is assumed to have two independent samples: mesophyll and epidermis chloroplasts. The test is a tool for comparison of means in two samples. All calculations were performed using the statistical software Infostat (Di Rienzo et al., 2009).

3. Results

The anatomy of floating and submerged leaves is similar (Fig. 1A and B). The mesophyll consists of a single layer of cylindrical cells with conspicuous and numerous chloroplasts (Fig. 2A–E). Floating leaves have larger intercellular spaces in this tissue. Small chloroplasts are observed in the epidermis of both submerged and floating leaves. Stomata are present in the adaxial epidermis and tetracellular trichomes on the abaxial face of the floating leaves (Fig. 2A, C and D).

Epidermal chloroplasts are smaller and fewer than the ones present in the mesophyll (Fig. 3A). Chlorophyll observed in mesophyll chloroplasts has a brighter fluorescence than chloroplasts in the epidermal cells (Fig. 3B).

The epidermal and mesophyll chloroplasts have the same ultrastructural differences in both types of leaves. Mesophyll chloroplasts are, on average, 9.4 μm long and 4.5 μm wide. They have between 12 and 26 plastoglobuli and one or two starch grains of 6.1 μm long and 2.5 μm wide on average (Fig. 4A and B). Grana are formed by an average of 29 thylakoids. Abundant intergrana thylakoids are present in these chloroplasts (Fig. 4C). Many mitochondria are observed around the chloroplasts of mesophyll cells (Fig. 4B).

Ribosomes and endoplasmic reticulum surround the epidermal chloroplasts that are smaller than the ones of mesophyll cells (Fig. 5A–C). They are 4.4 μm long and 1.1 μm high on average. Between six and seven plastoglobuli are present in these chloroplasts and starch grains are occasional and smaller (0.65 μm long and 0.35 μm wide on average) (Fig. 5B and C). Grana have an average of 9 thylakoids and intergrana thylakoids are scarce (Fig. 5 D).

The parametric test results showed significant differences ($p < 0.0001$) in the analyzed variables (number of thylakoids per

Table 1
t-Test for independent samples. Comparative table of mean for number of thylakoids per granum, length of chloroplasts, and diameter of chloroplasts analyzed between mesophyll and epidermis chloroplasts; gl: degree of freedom; t: *t*-test.

Characteristic	Epidermis			Mesophyll			gl	t	*p*-Value
	n	Median	Variance	*n*	Median	Variance			
Number of thylakoids	32	9.47	24.26	32	29.41	184.25	39	−7.81	<0.0001
Length of the chloroplast	15	1.11	0.11	15	4.58	1.93	34	−13.51	<0.0001
Wide of the chloroplast	15	4.47	0.88	15	9.4	17.16	35	−6.5	<0.0001

granum, wide of the chloroplast, and length of the chloroplast) of the mesophyll chloroplasts with respect to the epidermis chloroplasts (Table 1). Therefore, the number of thylakoids is higher in the mesophyll chloroplasts than in the epidermis chloroplasts, and mesophyll chloroplasts are wider and longer than the epidermis chloroplasts.

4. Discussion

The presence of chloroplasts in the leaf epidermis is common in aquatic plants and some terrestrial plants, especially shade adapted species (Esau, 1965; Mickel, 1972; Dickison, 2000). In *Cabomba*, chloroplasts of the epidermis differ in size and ultrastructure from those present in the mesophyll.

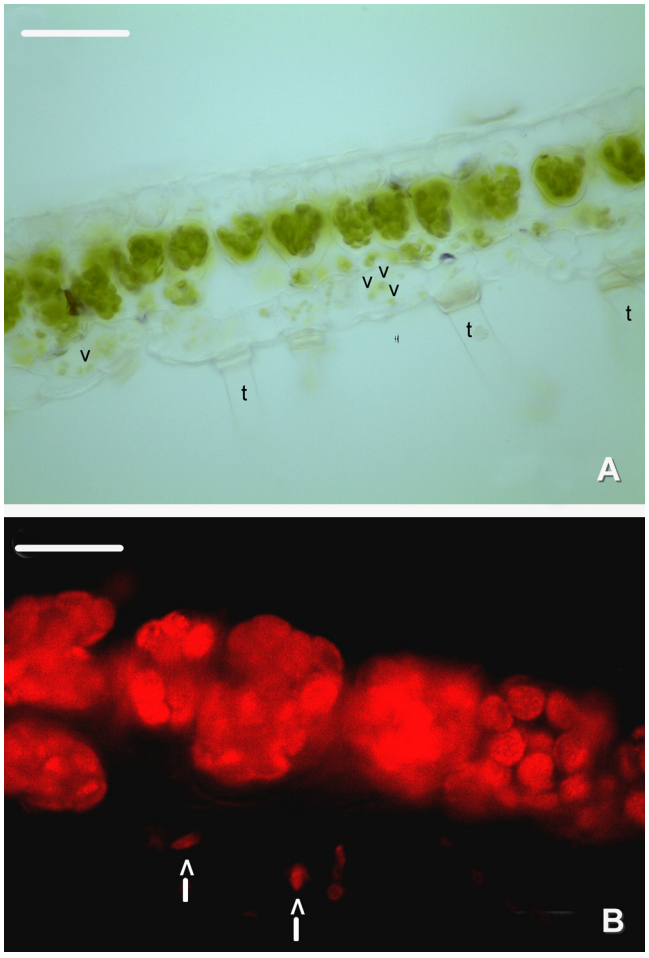

Fig. 3. Hand slide section of floating leaves. (A) Chloroplasts of the epidermis and mesophyll can be observed with light microscopy. Arrow heads indicate the epidermal chloroplasts. Trichomes (t) in abaxial epidermis. (B) Fluorescence of chloroplasts, arrows showing epidermal chloroplasts. Scale bar (A) 50 μm, (B) 25 μm.

Differences in size and ultrastructure of chloroplast in mesophyll and bundle sheath cells are common in C_4 grasses and dicots (Laetsch and Price, 1969; Johnson and Brown, 1973; Fisher and Evert, 1982; Sage and Monson, 1999). *Cabomba* presents dimorphism of chloroplasts similar to many C4 species. However, the C_4 pathway first evolved 24–30 million years ago (Sage, 2004) and basal angiosperms have existed for about 130 million years (Vialette-Guiraud et al., 2011) suggesting that *Cabomba* should be a C_3 plant.

There are some reports of chloroplasts polymorphism in some C_3 species. Dimorphic chloroplasts were described in the dorsal root epidermis and leaf epidermis of Podostemoideae by Fujinami et al. (2011). These authors observed small and large chloroplasts located separately in each epidermal cell along its upper and inner tangential walls respectively. This position of chloroplasts is similar to that observed in *C. caroliniana*, but in this last species these dimorphic organelles are located in different cells of the leaves, the smaller chloroplasts in the epidermal cells, and the larger ones more internally, in the mesophyll cells. Moreover, the larger chloroplasts of Podostemoideae (Fujinami et al., 2011) have well developed starch grains coinciding with those observed in *C. caroliniana*. According to Fujinami et al. (2011) Podostemoids could use HCO_3 and therefore the small chloroplasts lying on the outer tangential wall of the epidermal cell may supply energy to this uptake process.

Kordyum and Klimenko (2013) found differences in the ultrastructure of chloroplasts in floating, aerial and submerged leaves of *Nuphar lutea*. The floating and aerial leaves have chloroplasts with numerous plastoglobuli and grana consisting of 2–5 thylakoids while the submerged leaves have chloroplasts with scarce plastoglobuli and grana formed by 3–41 thylakoids. Coincidentally, in *C. caroliniana* the epidermal chloroplasts, which would be more exposed to light as the floating and aerial leaves of *Nuphar*, have grana formed by fewer thylakoids than the mesophyll chloroplasts that are less exposed to light as the submerged leaves of *Nuphar*. This chloroplast dimorphism was corroborated by a statistical analysis.

Light assimilation depends on the anatomical structure of leaves and is associated with the number and distribution of chloroplasts in each cell and the volume of chlorophyll (Vogelmann and Martin, 1993). Shade-type chloroplasts present more thylakoids per granum and smaller stroma compared to sun-type chloroplasts (Terashima et al., 1986). According to Lambers et al. (2008) such differences are also found between sun and shade leaves on a single plant and in different faces of a relatively thick leaf where the adaxial regions have chloroplast with sun leaves ultrastructure and shade like chloroplasts in the abaxial zone. In the same way, chloroplasts of the epidermis of *Cabomba* are similar to those found on sun leaves and chloroplasts of the mesophyll may be acting as

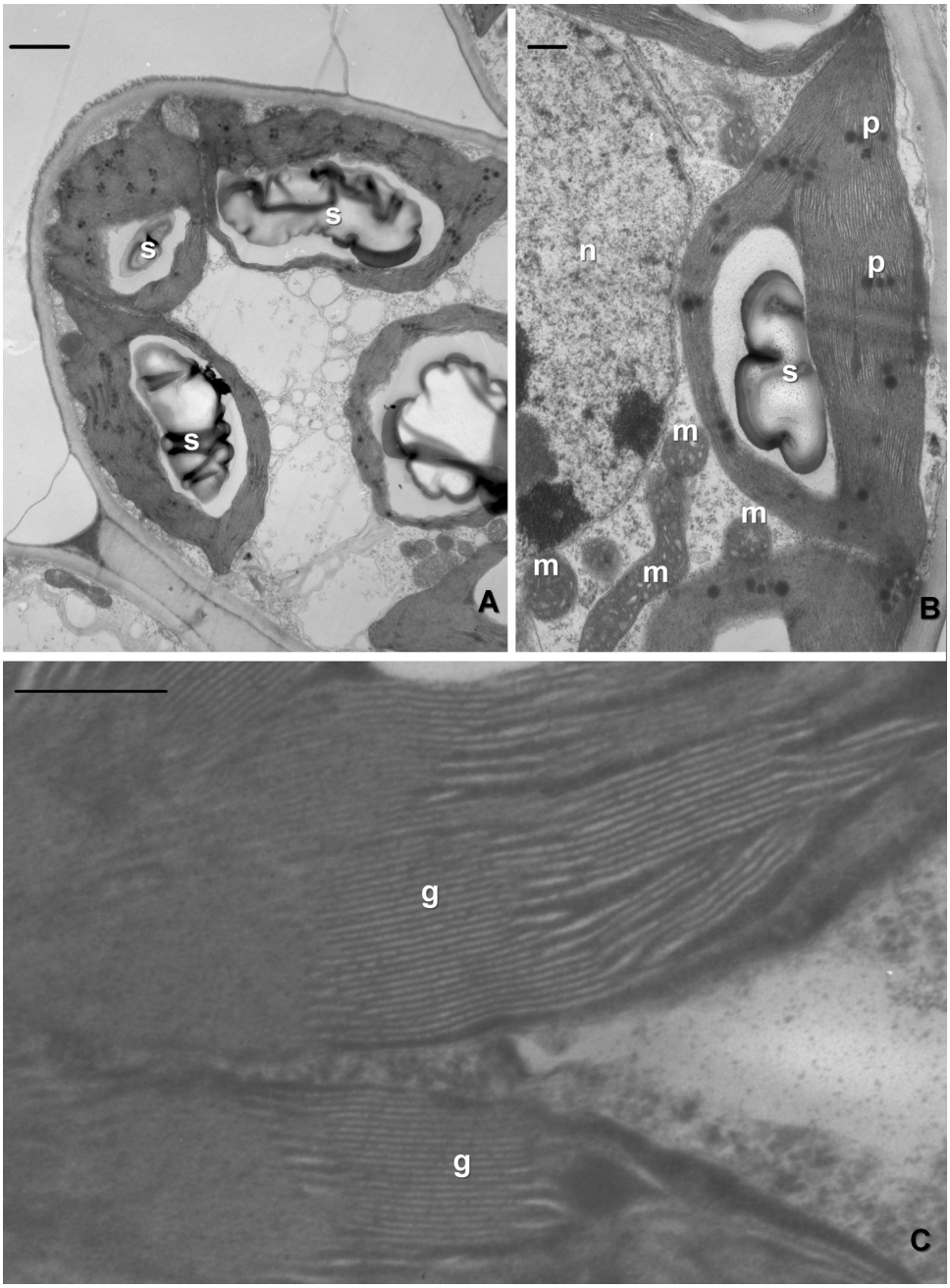

Fig. 4. Ultrastructure of the mesophyll chloroplasts (TEM). (A) Mesophyll cell of submerged leave showing chloroplasts with large starch grains (s); (B) detail of a mesophyll cell of floating leaf with nucleus (n), mitochondria (m), chloroplast with starch grain (s), and plastoglobuli (p). (C) Detail of grana (g) showing numerous thylakoids of submerged leaf. Scale bars (A) 2 μm, (B) 0.5 μm, (C) 0.5 μm.

shade adapted. On the other hand, the chlorophyll fluorescence is more intense in chloroplasts of mesophyll cells. This may be due to the presence of larger volume of chlorophyll *a* since these organelles have more grana where PSII is located (Lambers et al., 2008).

In *C. caroliniana* the epidermal chloroplasts have scarce intergranal thylakoids while the mesophyll chloroplasts have abundant intergranal thylakoids. This characteristic has not been described for other species with dimorphism of chloroplasts. Since the photosystem I is located in intergranal thylakoids and photosystem II in grana (Lambers et al., 2008), the ultrastructural differences of both chloroplasts could be related to the differential presence of the two photosystems (I and II). These observations could lay the foundations for further physiological studies.

Studies of chloroplast DNA evolution were carried out (Graham et al., 2000) but there are no registers about the chloroplast ultrastructure evolution in the basal Angiosperms. Therefore, the detailed description of chloroplast ultrastructure here given lays the foundation to compare them with the chloroplast characteristics of other representatives of the ANA grade. Further discussion of the early evolution of angiosperm chloroplast considering phylogenetic analyses should be done.

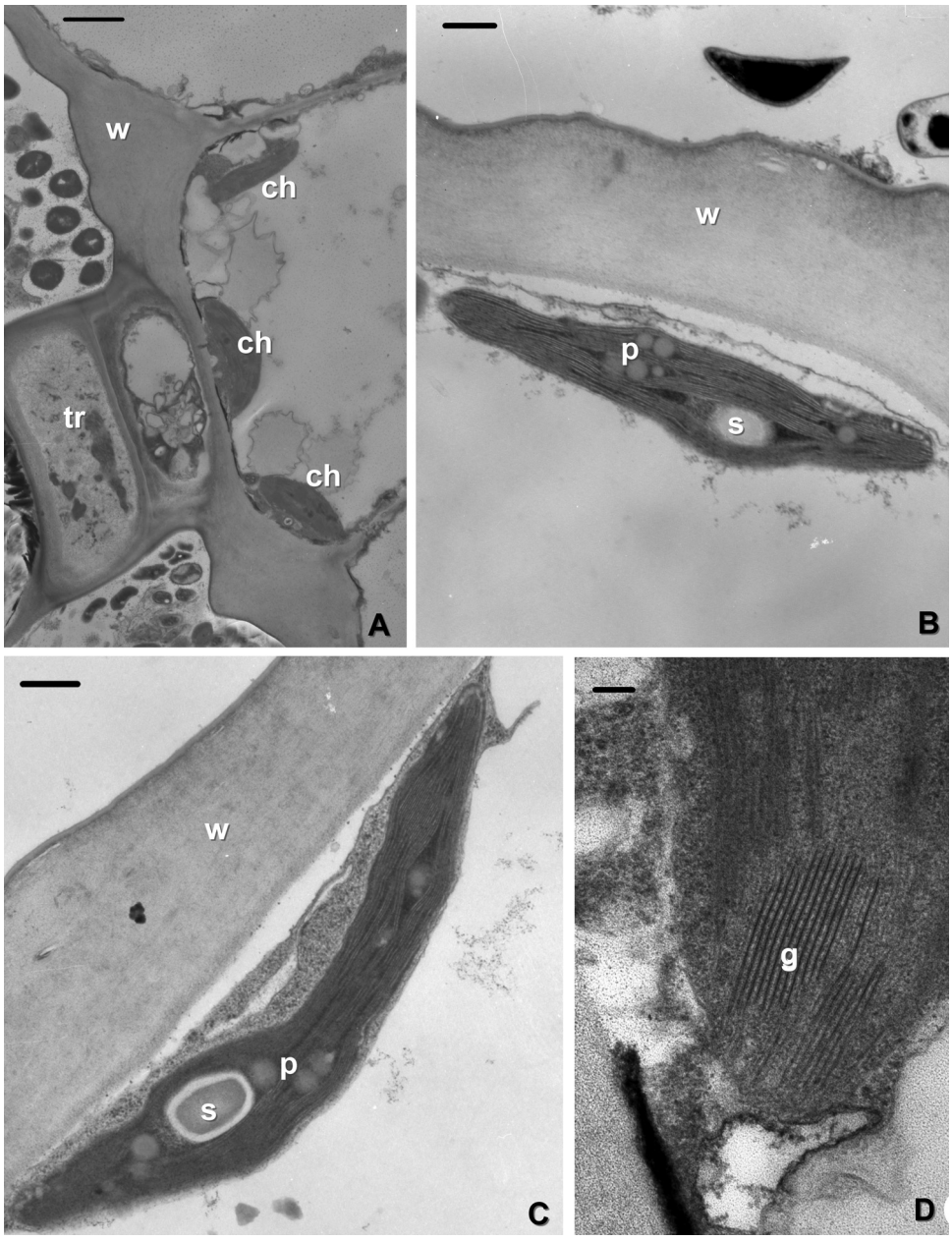

Fig. 5. Ultrastructure of the epidermal chloroplasts (TEM). (A) Basal cell of a trichome (t) with chloroplasts (ch), thick outer tangential wall (w) of epidermal cells is observed in a floating leaf; (B) and (C) Detail of outer tangential wall (w) of epidermal cell and a chloroplast with a small starch grain (s) and few plastoglobuli (p) of a submerged leaf; (D) detail of grana showing thylakoids in a submerged leaf. Scale bars (A) 2 μm, (B) 0.5 μm, (C) 0.5 μm, (D) 0.1 μm.

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