Botanical Sciences 91 (3): 251-260, 2013

The chloroplasts and unlignified parenchyma of two tropical pioneer forest tree species (Urticaceae)

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Abstract: The influence of light on chloroplast density and formation of unlignified parenchyma was determined in stems of *Myriocarpa longipes* and *Urera glabriuscula*, two woody pioneer species from the Mexican tropics, growing either under the shade or under sunny conditions. The goals of the present study were: (1) to quantify and characterize chloroplasts in this tissue and (2) to determine the influence of two light conditions in chloroplast number and unlignified parenchyma formation. Sunshine increases the presence of chloroplasts and the area of unlignified parenchyma in *M. longipes* stems, whereas in *U. glabriuscula* it occurs the opposite. Differences in the presence of some features in the pioneer species studied, such as the abundance of mucilaginous ducts (*M. longipes*), type of cystolith (*U. glabriuscula*), and the area occupied by the pith in stem cross sections, occur as a response to canopy gaps in this tropical forest.
Keywords: canopy gaps, chloroplasts formation, nonfoliar photosynthesis, unlignified parenchyma.

Resumen: Se determinó la influencia de la luz sobre la densidad de cloroplastos y la formación de parénquima no lignificado en tallos de *Myriocarpa longipes* y *Urera glabriuscula*, dos especies pioneras del trópico mexicano que pueden crecer bajo condiciones de sombra o de sol. Los objetivos del presente estudio fueron: (1) cuantificar y caracterizar los cloroplastos en este tejido y (2) determinar la influencia de dos niveles de iluminación sobre el número de cloroplastos y la formación de parénquima no lignificado. La luz del sol induce un aumento en la presencia de cloroplastos en el área de parénquima no lignificado en *M. longipes*, pero en *U. glabriuscula* ocurre lo contrario. Se observaron diferencias en la presencia de algunas características en estas especies pioneras, tales como la abundancia de ductos mucilaginosos (*M. longipes*), tipo de cistolito (*U. glabriuscula*) y el área transversal del tallo ocupada por la médula, que pudieran estar determinadas por claros en el dosel de la selva tropical.
Palabras clave: abertura del dosel, formación de cloroplastos, fotosíntesis no foliar, parénquima no lignificado.

Chloroplasts are not restricted to leaves, but rather can also be present in other parts of the plant including stems, branches, roots, flowers, fruit, and cotyledons as an additional strategy for carbon gain (Aschan and Pfanz, 2003; Dima *et al*., 2006). Among stem tissues with chloroplasts there is unlignified parenchyma (ULP), a tissue that functions as a repository for secondary metabolites and water, and which commonly forms tangential bands or clusters in the secondary xylem of a dozen plant families (Angeles *et al*., 2004; InsideWood, 2004).

The chloroplasts found inside trunks and branches can be functional and have the ability to actively assimilate reductive CO_2 in the presence of nutrients, water and carbon dioxide (Pfanz *et al*., 2002). The stems of woody plants have structural modifications that allow light to pass to the interior and reach the light-harvesting complex in the thylakoid membrane of the chloroplasts (Sun *et al*., 2003). Inside the stem, the parenchyma cells of the bark, xylem rays, and the pith actively consume oxygen and produce carbon dioxide as a result of mitochondrial activity. The different layers of bark have a high degree of resistance to gas diffusion, resulting in a barrier to atmospheric O_2 (Pfanz and Aschan, 2001). As a consequence, CO_2 accumulates within the stem, eventually leading to protoplasm acidification in the inner tissues and nonfoliar photosynthesis causes the refixation of endogenously produced CO_2 inside the stem (Pfanz *et al*., 2002). Some authors have shown that the parenchyma cells surrounding the vessels in the xylem are able to assimilate

soluble carbon dioxide from the sap, since their chloroplasts have an effective morphology, enzymatic machinery, and photosynthesis (Fineran, 1995; Aschan *et al*., 2001; Aschan and Pfanz, 2003; Berveiller *et al*., 2007).

During plant growth and development, light is one of the most important environmental factors that influence metabolic activities via photomorphogenesis. Studies have shown that ca. 730 nm light, conducted axially by vascular tissues, is important for photomorphogenesis when radiation leaks into the surrounding living tissue, such as ULP (Vogelmann, 1993; Sun *et al*., 2003). As a response to changes in light from the environment, ULP may undergo modifications in the number, size, shape, and arrangement of cells and pigment concentration (Givnish, 1988). In tropical forests, for example, canopy openings can suddenly increase light incidence in the understory from 1% to 100% (Poulson and Platt, 1989), promoting the rapid growth of pioneer species. These sudden changes in the light environment could cause rapid changes in whole plant architecture but also in the foliar and nonfoliar chloroplasts of plants growing in the understory (Pearcy, 1990; Bai *et al*., 2008; Martínez-Sánchez *et al*., 2008).

Previous research on the physiology of woody tissue has focused mostly on carbon gain (Teskey *et al*., 2008; Saveyn *et al*., 2010), the presence of the enzymes involved in photosynthesis, such as RuBisCO (Buns *et al*., 1993), corticular photosynthesis (Aschan *et al*., 2001; Aschan and Pfanz, 2003), the ultrastructure and arrangement of chloroplasts (Larcher *et al*., 1988; Fineran, 1995), and chlorophyll (van Cleve *et al*., 1993; Dima *et al*., 2006). However, the ultrastructure of nonfoliar chloroplasts growing under different light levels has not yet been compared. Pfanz *et al*. (2002) compared chloroplasts in the chlorenchyma of the woody stems of several groups of plants. They estimated the density, height, and width of grana stacks in the stem layers as well as among life stages.

Thus, for this study we selected two tropical pioneer tree species with stems that have ULP to determine the density and distribution of chloroplasts in this tissue, and to find out whether these differ under sunny and shady conditions. We chose *Myriocarpa longipes* Liebm. and *Urera glabriuscula* V.W.Steinm. in the Urticaceae family because they form both LP (lignified parenchyma) and ULP in their stems, and because they are pioneer trees in the tropical rain forest reserve at Los Tuxtlas, Veracruz, Mexico, where the dynamics of tree replacement after a gap opens has been well documented (Martínez-Ramos *et al*., 1988; Martínez-Ramos and García-Orth, 2007; Sánchez-Gallen *et al*., 2010). Secondary or pioneer trees require large openings (> 100 m^2) to regenerate and sprout (González *et al*., 1997; Ibarra-Manríquez *et al*., 1997; Howe *et al*., 2010). In this forest solar radiation reaching the forest understory through the canopy varies from 0.1% to 2%, whereas in canopy gaps radiation is 100% (Orozco-Segovia *et al*., 1987; González *et al*., 1997; Figueroa and Vazquez-Yanes, 2002).

In summary, the objectives of the present study were: (1) to quantify and characterize the chloroplasts of ULP, and (2) to determine the influence of two light intensities on the number of chloroplasts and ULP formation.

Materials and methods

Plant material. In the spring of 2011, samples from eight *Urera glabriuscula* and eight *Myriocarpa longipes* trees that were approximately ten years old, and with a dbh 10-15 cm were collected in the Los Tuxtlas Tropical Biology Field Station in Veracruz, Mexico (95° 03' 49'' W, 18° 29' 44'' N, 443 m a.s.l.). Although *U. glabriuscula* and *M. longipes* are pioneer species, mature individuals can be found growing in openings or in the understory.

Light intensity. Two different light environments were chosen: (1) sunny, where the trees grew at the edge of roads or clearings and (2) shady, where trees grew under the forest canopy. Four trees each of *Myriocarpa longipes* and *Urera glabriuscula* were selected for each environment to evaluate the effect of solar radiation on the amount of ULP and the number of chloroplasts. Photonic flux density (PFD) was measured on every stem surface; data were recorded every hour from 12:00-16:00 h with a Datalogger (LI-COR 1000, LI-COR Lincoln, Nebraska, EUA). Microclimate information collected with the Datalogger was compared with information gathered from the meteorology station located in the Los Tuxtlas Biological Field Station.

Chloroplast arrangement. To determine the number of chloroplasts per area in ULP, each stem sample was handsectioned for immediate observation. Fresh slices were examined with a Nikon Eclipse E600 microscope, using fluorescence optics (mercury lamp UV 50 W) equipped with a 450-490 nm exciting filter (chromatic transmission divisor DM500) and a 515 nm barrier filter. Images were taken with a Nikon CoolPix 950 digital camera. To acquire semiquantitative data, samples were also examined with an Olympus FV1000 confocal laser microscope. Two stain-excitement signals were used at 488 and 543 nm; for fluorescence, signal emission detection used two long-step filters of 644-692 nm and 500-530 nm. Pictures in X/Y were taken with a resolution of 800 x 800 pixels. Fourteen images were taken in Z, each 4.5 μm, and these were then combined into a single X/Y image.

Each image was processed with Image-J version 1.45e (National Institutes of Health, United States, http://rsb.info.nih.gov) to minimize noise and sharpen the contrast between red autofluorescence chloroplasts and the background. A macro (text file) was developed to optimize the image processing (Appendix). First, the color information (R, G, and B) of the images was divided, creating new red information images. Then, the background was removed from each

image to create a new dark monochromatic background while keeping the chloroplasts red in color. Subsequently, the images were converted to an 8-bit format, thus creating a bichromatic image. The threshold option in the Intermodes algorithm (Prewitt and Mendelsohn, 1966) was used to obtain an image with a light background and the chloroplasts in black. The *Close*, *Open* and *Fill Holes* options were used to avoid black dots and extra black pixels in the image, and 1 mm^2 area was drawn on the image with the chloroplasts located in the ULP, allowing us to measure the total area occupied by chloroplasts. The *Analyze Particles* function was used to calculate the area occupied by the chloroplasts. The average area of an individual chloroplast for the two tree species was 0.0002 mm^2.

Chloroplast ultrastructure. To observe the ultrastructure of chloroplasts in ULP cells, transverse ultra-thin sections were examined with a transmission electron microscope (TEM). Blocks of wood measuring 5 × 2 mm and containing ULP were obtained from stems and then fixed in 2% glutaraldehyde in 25 mM phosphate buffer PBS ($NaHPO_4 \cdot 7H_2O$ 100 mM and $NaH_2PO_4 \cdot H_2O$ pH 7) at 4 °C (Chaffey, 2002) for 48 h; samples were washed in four PBS changes, for 15 min each time. Postfixation was done with 1% OsO_4 in phosphate buffer for two hours at 4 °C. Samples were dehydrated in a series of graded ethanol concentrations for 15 min each at 4 °C: 40%, 50%, 60%, 70%, 80%, 90%, and 100% ethanol, and then two more changes with 100% ethanol, each one lasting 15 min. In addition, two changes with 100% propylene oxide (PPO) for 15 min each were done. Samples were left in PPO-Epoxy resin (Epon 812) (1:1) in a desiccator for 48 h, at room temperature. One change of 100% Epon 812 was done in a rotating device for 4 h. The embedded material was polymerized after 48 h at 60 °C (Ruzin, 1999). Sections were made (90 nm) with an ultramicrotome (Reichert-Jung) and contrasted with 2% uranyl acetate for 30 min, followed by 2% lead citrate for 10 min. Finally, the samples were examined in a JEM-1200 EXII (JEOL) transmission electron microscope equipped with a digital camera (Ditabis, Pforzheim, Germany) to record selected images.

Measuring the area of ULP. Stem samples from each individual were hand-sectioned in transverse slices approximately 100 μm thick and then analyzed with a Leica MZ8 stereomicroscope using incident light from a Leica CLS150X lamp.

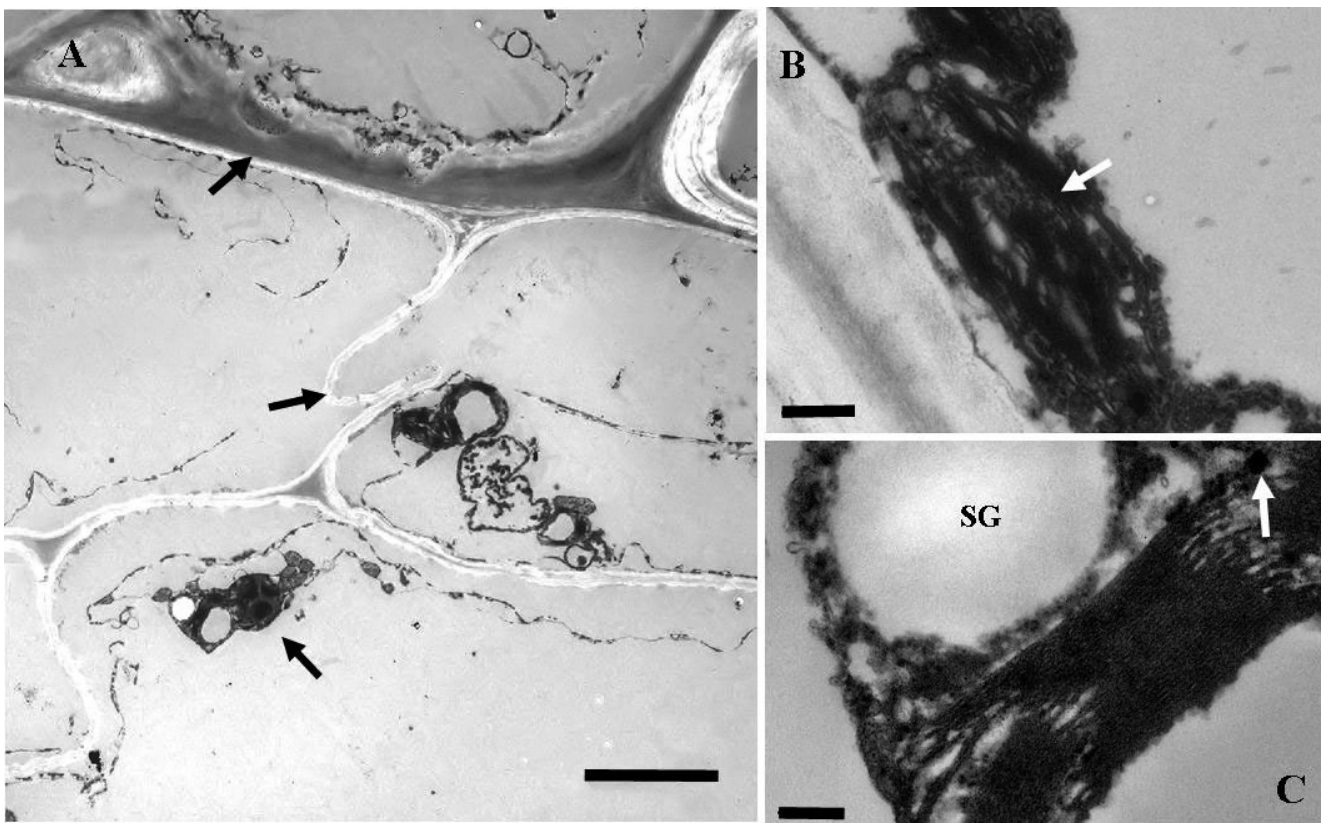

Figure 1. Cross section of secondary xylem from sunny environments (TEM). (A) Intermediate zone with lignified parenchyma cells (top black arrow) and ULP cells (middle black arrow) with ovoid chloroplasts (bottom black arrow) within cells in *Myriocarpa longipes*. (B-C) Chloroplast with no plastoglobuli and stacked thylakoid sheets (white arrows) in *Urera glabriuscula*. Scale of bars: A = 5 μm; B = 200 nm; C = 0.5 μm.

Pictures were taken with a Nikon CoolPix 950 digital camera. The captured images were used to calculate the area of ULP using the software Image-J version 1.45e (National Institutes of Health, U.S.A.; http://rsb.info.nih.gov). Each image was modified to minimize noise and sharpen the contrast between the lignified and unlignified tissue. First, the original light background was removed from each image, creating a new monochromatic background while keeping the color of the stem tissue. Next, the image was inverted so the background was dark and the lignified tissue was white. At this point the ULP appeared as a dark object because the software recognizes black pixels for the purpose of counting. The images were then converted to an 8-bit format, creating a bichromatic image. Using the threshold adjustments, the contrast was modified under the red-scale setting in order to delineate each cluster of ULP more clearly. Using the drawing tools, some particles were adjusted manually to avoid black dots in the image. Then, the *Analyze Particles* function was used to measure the area covered by ULP, but first, it was necessary to obtain the smallest and the largest values of the cluster size. This step excluded objects that were too small or too large, and allowed us to measure just the area occupied by ULP. In an 8-bit image format, each individual ULP cluster for the two species studied had an area of 0.02 to 6 mm^2. These values were consistent with the average particle size calculated as part of the *Analyze Particles* output. A total of 50 images were processed. In order to determine the accuracy and consistency of the Image-J measurements, the measurements produced by the *Wand tool* in each cluster were compared with those obtained using the *Analyze Particles* tool. ULP area was expressed as a ratio that this tissue occupies in the whole stem area.

Statistical analysis. Comparisons between chloroplasts number and ULP area were made with a T-test, data were logarithm base 10 transformed, but untransformed data are reported in text and figures.

Results

Structure and number of chloroplasts. ULP cells of the secondary xylem contain chloroplasts everywhere in the transverse stem sections (Figure 1A), although the number of chloroplasts decreased as stem diameter increased. Chloroplasts in both species had the same ultrastructure but differences were observed between the two environments. Chloroplasts are mostly ovoid (Figure 1A) to elongated (Figure 2A, B) in shape, the diameter to length ratio is approximately 1:1.5-2.5. The chloroplasts of plants from sunny places have few plastoglobuli (Figure 1C) and their grana thylakoids have stacked thylakoid sheets (Figure 1B), with few grana that are two times longer than their diameter. Occasionally, within the stroma matrix one to three small ovoid starch grains per chloroplast were visible as an electron-transparent zone in both species in both light conditions (Figure 1C). In plants from shady places, the grana consisted of stacks of closely packed thylakoids with dark stained portions (Figure 2C), and an assortment of parallel overlapping lamellae eventually forming rudimentary grana. The distance between individual grana was constant; the chloroplasts from the shade were more irregular in shape and had more abundant membranes than the chloroplasts of sunny environments (Figure 2D, Table 1).

Chloroplast density was not statistically different between species and environments (Table 2). The laser scanning confocal and fluorescence microscopy revealed the chlorophyll in the inner layers of *Myriocarpa longipes* and *Urera glabriuscula*, and also in the ULP, characterized by a strong red autofluorescence (Figure 3A). The intensity of the fluorescence in the inner stem tissues was greater in young stems than in older ones. Fluorescence was more intense in the cortex and less intense in the cambial zone. Fluorescence was seen throughout the secondary xylem, mainly in the paratracheal and radial parenchyma and also in the pith zone (Figure 3B, C).

ULP area. The secondary xylem of *Myriocarpa longipes* and *Urera glabriuscula* is divided into sections: lignified and ULP (Figure 3D, E). In *U. glabriuscula* ULP cells occur in discontinuous bands (Figure 3D), while in *M. longipes* they are in clusters (Figure 3E). These two types of arrangements measured up to 200 μm^2 in the transverse section. ULP tissue is composed of living, turgid, vacuolated, and polyhedral cells, their walls are primary unlignified with uniform thickness. Although the ULP is variable in size and shape, it is more conspicuous in the intermediate section of secondary xylem. Discontinuous bands and clusters are smaller in the internal (closer to pith zone) than in external sections (close to cambial zone). In *M. longipes* and *U. glabriuscula* the ULP occupies up to 33.2 % of the stem area (Figure 3D, E). The statistical analysis revealed differences in the ULP ratio between environments in *U. glabriuscula* ($P < 0.05$), in *M. longipes* also differences were detected ($P < 0.05$) between environments (Table 2).

Discussion

Structure and number of chloroplasts. As previously reported for the stems of other vascular plants, the chloroplasts of *Myriocarpa longipes* and *Urera glabriuscula* contained plastoglobuli and few starch grains, these resembling the chloroplasts of shade-type leaves (Anderson *et al*., 1973; Givnish, 1988). Our results found grana stacks (length-width) in a ratio 1:2 in shady and 1:1.5 in sunny environments, coinciding with previous reports such as that for the grana stacks of *Korthalsella salicornoides* (Fineran, 1995).

Our observations detected high quantities and larger chloroplasts in *Myriocarpa longipes* in sunny environments.

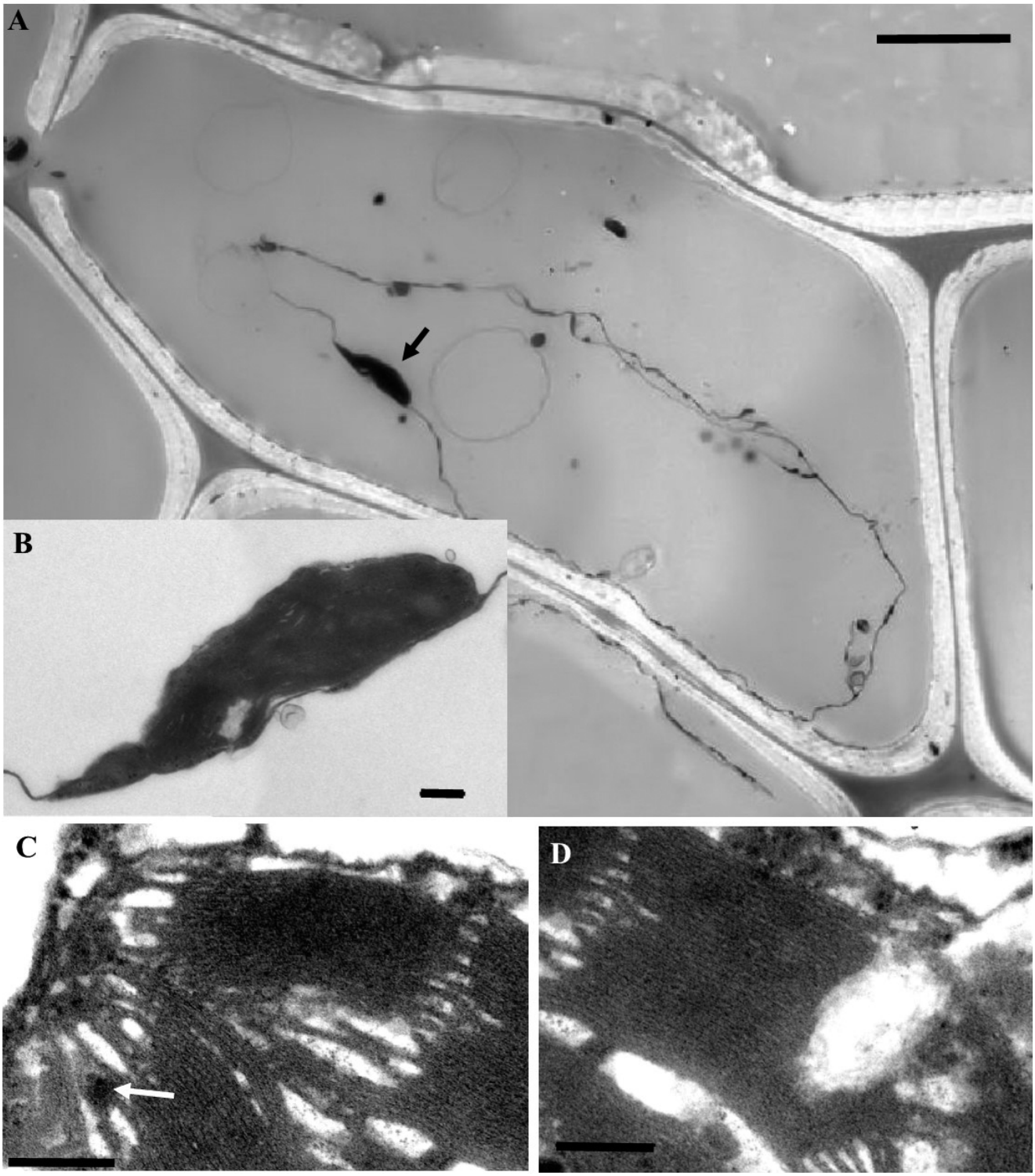

Figure 2. Cross section of secondary xylem from shady environments (TEM). (A) *Urera glabriuscula* ULP cell with an elongated chloroplast (black arrow) in the middle. (B) Detail of the chloroplast shown in A with extensive grana stacks. (C) Dense stacks of thylakoids and electron-dense plastoglobuli (white arrow) in *U. glabriuscula*. (D) Electron-transparent ovoid starch grain in *Myriocarpa longipes*. Scale of bars: A, B = 5 μm; C, D = 200 nm.

Table 1. Qualitative and quantitative differences in the chloroplasts of ULP under two different light levels. Abundance: + = 0-5%: ++ = 6-10%; +++ = 11-50%.

Feature	*Urera glabriuscula*		*Myriocarpa longipes*	
	Sunny	Shady	Sunny	Shady
Chloroplast shape	ovoid	elongated	ovoid	elongated
Chloroplast diameter to length ratio	1 : 15		1 : 2.5	
Plastoglobuli	+	++	+	++
Thylakoid sheets	+	++	+	+
Thylakoid layers	++	+++	+	+++
Grana length to diameter ratio	1 : 1.5		1 : 2	
Grana shape	normal	irregular	normal	irregular
Starch grains	1 - 2	~ 1	1 - 3	~ 1
Membranes	+	++	+	++

The density of chloroplasts in stems of *M. longipes* coincided with chloroplast density from the leaves of sun-adapted plants (Givnish, 1988). In general, chloroplasts from sun-adapted leaves are larger, but less abundant than those of shade-adapted leaves (Boardman, 1977). In *M. longipes* we found a similar pattern: stems growing at the edges of forest openings, and exposed to elevated light radiation that can penetrate deep into their tissues formed larger chloroplasts, although not as many as those of the stems growing under the canopy. This effect responds to an adaptation to avoid photoinhibition, by reducing the exposed area per unit mass of chloroplast. The reduction in the size and number of chloroplasts inside the stems of *M. longipes* could also be attributed to the optical characteristics of the stem interior. Like other parenchymatic cells, ULP cells possess multiple facets, dispersing and reflecting light in different directions within the stem (Sun *et al.*, 2003). In a shady environment, the limited incident radiation does not favor light conduction within the stems down to the regions in the plant close to the forest soil. In the absence of light moving down in the stems, the enzymes remain underexpressed and chloroplasts do not form.

The use of epifluorescence microscopy to detect chlorophyll by autofluorescence allowed us to determine whether the chloroplasts are functional (chlorophyll is identified by its red color) as has been demonstrated in a $^{14}CO_2$ labeling experiment (Langenfeld-Heyser, 1989). Chloroplasts have been detected by these methods in the cortex, xylem rays, perimedular, and pith cells for several plant groups (Dima *et al.*, 2006; Pilarski and Tokarz, 2006). Our observations using epifluorescence microscopy detected chlorophyll in large quantities in rays, in wood parenchyma cells, and in pith cells, but mainly in ULP cells in the two Urticaceae species studied. The location of chlorophyll in those tissues suggest that they play a part in stem carbon assimilation by using CO_2 dissolved in the sap stream, as reported in herbaceous species (Hibberd and Quick, 2002).

Unlignified parenchyma. The results of the t-test indicate differences in the area of ULP formed in stems of the two species. In the stems of *Myriocarpa longipes* growing under sunny conditions, we found larger areas of ULP than in shady environments. In sunny environments, there is an increase in the availability of water, light, and nutrients for plant growth and development (Martínez-Ramos, 1985). In the stems of *Urera glabriuscula* more ULP was formed under shady conditions. The allocation of these resources in a plant is driven by intrinsic photoreceptors (phytochromes) located in living cells, like ULP, which react to specific wavelengths with photomorphogenic response inside the stems (Briggs

Table 2. Summary of chloroplast density, ULP ratio in stem, and environmental conditions where the plants were growing. Values are mean ± standard error. Differences in ULP are indicated by (a) and (b) with significant differences at $P < 0.05$.

	Chloroplast density (per mm^2)	ULP area (mm^2)	Radiation ratio	Temperature Ratio
Urera glabriuscula				
Sunny	13.27 ± 2.58	8.42 ± 1.94 a	0.09 ± 0.02	0.910 ± 0.01
Shady	19.6 ± 3.86	15.59 ± 2.28 b	0.06 ± 0.02	0.88 ± 0.01
Myriocarpa longipes				
Sunny	23.27 ± 5.21	14.51 ± 6.64 b	0.166 ± 0.09	0.93 ± 0.01
Shady	14.6 ± 3.76	6.46 ± 3.10 a	0.02 ± 0.03	0.9 ± 0.02

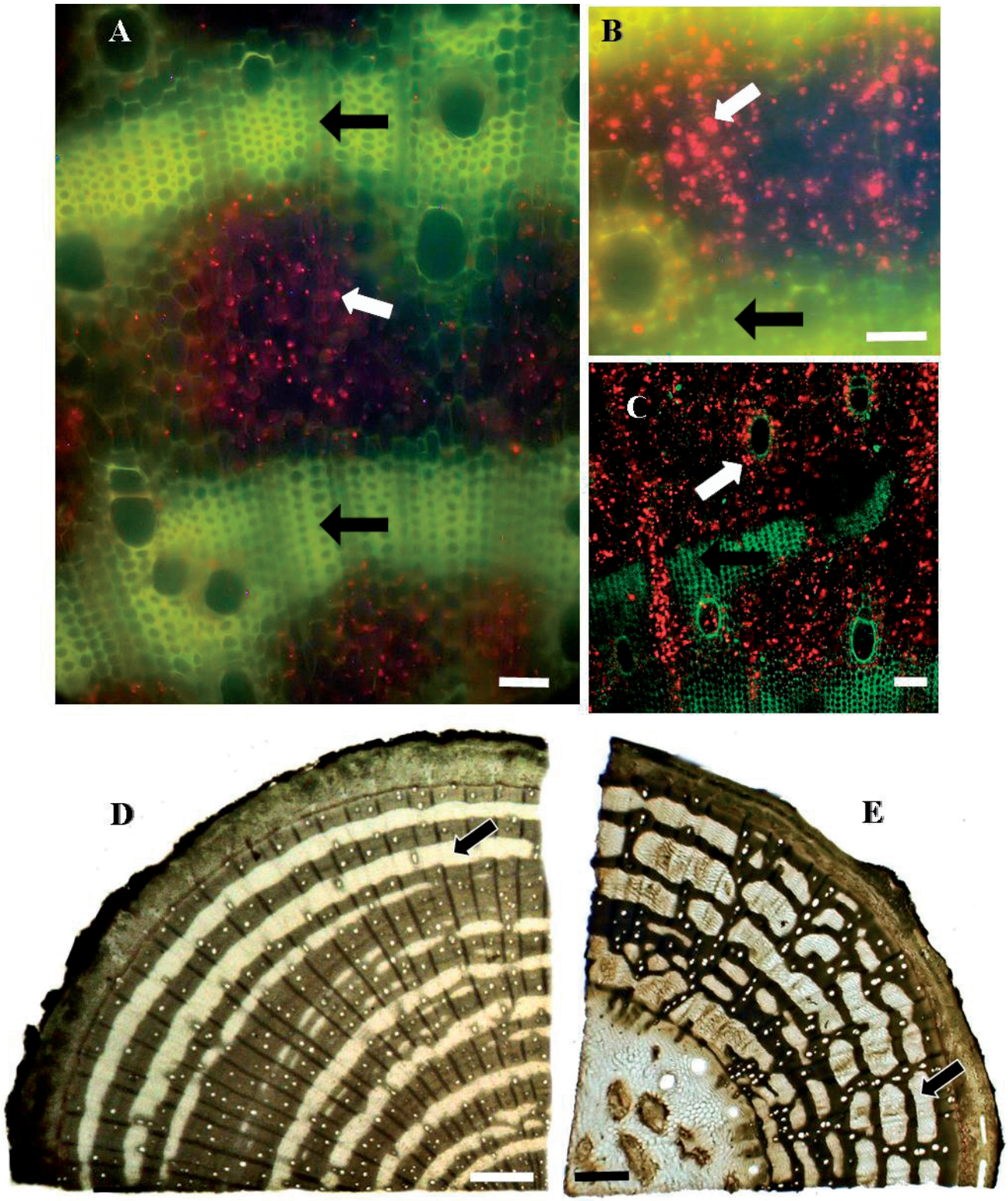

Figure 3. Photomicrographs of ULP. Chloroplasts mainly occur in the ULP of *Urera glabriuscula* as revealed by the red autofluorescence of chlorophyll (A), but it is common to find chloroplasts in the parenchyma surrounding the vessels in (B) *U. glabriuscula* (fluorescence microscopy) and (C) *Myriocarpa longipes* (confocal laser scanning microscope). (D) ULP in tangential bands (arrow) in a transverse section of *U. glabriuscula* (optical microscope). (E) Clustered ULP of *Myriocarpa longipes* (optical microscope). White arrows show the chloroplasts presence; dark arrows indicate the ULP. Scale of bars: A, B, C = 100 μm; D, E = 20 mm.

and Olney, 2001). Determination of light effects in other parts of the plant, such as the leaves, have shown that they become thicker and have increased chlorophyll content in relation to leaf area, altering their chlorophyll content while keeping a constant thickness (Vogelmann, 1993).

Conclusion

Differences were found in number of chloroplasts under the two light levels in *Myriocarpa longipes* and *Urera glabriuscula*, as well as in the allocation of ULP. Although chloroplast structure was similar in both species, major differences were observed between light levels. In sunny conditions, chloroplasts had fewer plastoglobuli but more starch grains per chloroplast than in shady environments. Under shade thylakoids and membranes were more abundant than in the open. The presence of these features requires the coordinated expression of chloroplast genes, in combination with the right environmental signals. The area covered by ULP in *M. longipes* stems was larger in the sunny than in the shady environment, while the opposite was found for ULP area in *U. glabriuscula*. This is the first report of the presence of chloroplasts in the ULP of stems of these two Urticaceae. Our results did not show clear differences for ULP or chloroplast formation under sunny and shady environments, perhaps because the difference in solar radiation in the two environments we used was not sufficient to induce visible morphogenic effects. These findings, however, do open the door to further research on a phenomenon that has received little attention until now: the formation and function of ULP in woody stems.

Acknowledgments

We are particularly grateful to Victor Steinmann, Laura Yáñez, and Guillermo Laguna for their careful review of previous versions of this manuscript; to Armando Martinez and Roger Guevara for their assistance in statistical analysis. We thank Arith Pérez and the personnel at the Los Tuxtlas Tropical Biology Field Station (UNAM) for their assistance in the lab and field, respectively. A scholarship awarded to EC by CONACyT (336218) is gratefully acknowledged. The Instituto de Ecología A. C. (Secretaria de Posgrado) provided additional funds. This study constitutes E. Cocoletzi's Master of Science thesis at the Instituto de Ecología, A. C. Two anonymous reviewers provided very thoughtful comments to our original manuscript.

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Received: September 2nd, 2012
Accepted: December 27th, 2012

Appendix. Script for the macro that was run to measure the area of ULP and the number of chloroplasts in *Urera glabriuscula* and *Myriocarpa longipes* on the transverse sections.

```
run("Duplicate...", "title =1.JPG");
run("Merge Channels...", "red = [1.JPG] green = *None* blue = *None* gray = *None*");
run("Subtract Background...", "rolling = 50");
run("8-bit");
//run("Threshold...");
setAutoThreshold("Intermodes dark");
run("Convert to Mask");
run("Close-");
run("Open");
run("Fill Holes");
run("Set Measurements...", "area mean standard min display redirect = RGB decimal = 9");
run("Analyze Particles...", "size = 0.002-200 circularity = 0.00-1.00 show = Outlines display exclude in_situ");
String.copyResults();
```