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ORIGINAL ARTICLE

Chloroplast ultrastructure and hormone endogenous levels are differently affected under light and dark conditions during in vitro culture of *Guadua chacoensis* (Rojas) Londoño & P. M. Peterson

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Abstract

Guadua chacoensis (Poaceae) is a woody bamboo native from the Atlantic forest biome. Morphogenetic and physiological studies are scarce in bamboos, and tissue culture-based biotechnologies tools can be used to investigate ultrastructure and physiological processes as well as to mass-propagate specific genotypes. This study evaluated the effect of light and dark conditions on chloroplast biogenesis as well as in the endogenous levels of zeatin (Z), abscisic acid (ABA), gibberellic acid (GA_4), and jasmonic acid (JA) during in vitro culture of *G. chacoensis*. An increase was observed, followed by a decrease in starch content in response to light treatment, and in contrast, in darkness, an accumulation of starch which is associated to amyloplast formation at day 30 was observed. No etioplast formation was observed even in the dark and this was associated with the presence of fully developed chloroplast at the beginning of the experiment. Z levels quantified showed distinct behavior, as in light, no difference in the levels was observed, except at day 10, and in darkness, the levels increased along the evaluation time. ABA, GA_4, and JA biosynthesis increased along the time of evaluation in light condition. In contrast, in darkness, the levels remained unaltered, excepted for an increase in JA levels at day 10, suggesting a strong relationship between light and these phytohormone levels. Possible reasons why light's presence and absence cause changes in chloroplast ultrastructure and hormone endogenous levels in in vitro cultivated culms of *G. chacoensis* are discussed.

Keywords Bamboos · Micropropagation · Transmission electron microscopy · Plant physiology · Phytohormone

Introduction

Bamboos belong to the grass family Poaceae, subfamily Bambusoideae, comprising 1641 species and 120 genera (Soreng et al. 2015). Bamboos are distributed in almost all continents, Antarctic being the only exception (Akinlabi et al. 2017), from the lowlands to more than 4000 m of altitude (Kelchner and BPG 2013). America is the second continent in richness of bamboo species and in terms of diversity, south America is the first in the New World (Bystriakova et al. 2004).

Bambuseae is the largest tribe among the three tribes of Bambusoideae, along with Arundinareae and Olyreae. Bambuseae covers the tropical and subtropical woody bamboos, with about 893 species and 68 genera (Soreng et al. 2015). The genus *Guadua* corresponds to one of five biggest genera of bamboos in Brazil (Filgueiras and Gonçalves 2004), and is the most economically important genus in tropical America (Londoño 1998). *Guadua chacoensis* (Londoño and

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Peterson 1992) is a woody native bamboo from the Atlantic forest, with natural occurrence in southern Brazil, Paraguay, Uruguay, and northeastern Argentina (Guerreiro 2014).

The flowering interval of *G. chacoensis* is estimated as 28 years (Vega and Hernández 2008), producing scarce and low-viability seeds. Vegetative propagation through offset and culm cutting is also season-dependent, with low availablity of propagules, and the conventional propagation methods are expensive and inefficient for bamboos' mass propagation. Thus, the use of biotechnology tools, such as micropropagation, is essential for large-scale bamboo propagation (Singh et al. 2013).

The physiological and genetic features of the explant are essential factors in the successful micropropagation protocol and should be investigated aiming to improve the in vitro environmental conditions. An important factor that directly affects micropropagation is light condition, which is related with in vitro growth and morphogenesis as a consequence of wavelength, time of exposure and photoperiod (George et al. 2008). Furthermore, its influence on plastid differentiation, photosynthesis and chloroplast maturation has been demonstrated (Solymosi and Aronsson 2013).

Chloroplast belongs to the diverse group of organelles known as plastids, which includes proplastids, etioplasts, gerontoplast, chromoplasts, and leucoplasts—for instance amyloplasts (Lindquist et al. 2016). These plastids diverge in their function, chemical compounds, and inner structure (Solymosi and Aronsson 2013) and reveal remarkable interconvertion plasticity (Solymosi and Keresztes 2012; Lindquist et al. 2016). Chloroplast differentiation has been designated as a light-dependent process, thus if light is reduced or either not supplied, the proplastid can differentiate into etioplast (Staehelin 2003). Etioplasts are usually seen as a temporary stage of plastids, which are formed during the dark growth, although they are rapidly converted to functional chloroplast with light supply (Wise 2007). Therefore, the investigation of the plastid interconversion dynamics can help in the better understanding plant's morphogenesis.

Phytohormones are essential in plant development, acting as signaling molecules in plant response during development, growth, adaptation, stress, and physiological response (Erland et al. 2017), and also involved in many plastid differentiation processes and function (Stetler and Laetsch 1965). Cytokinins (CK), such as zeatin (Z), are strongly related with chloroplast biogenesis, especially with its maturation, which has been reported in several studies (Stetler and Laetsch 1965; Polanská et al. 2006; Cortleven and Schmülling 2015). Phytohormones are also involved in the promotion of thylakoid formation; disintegration of prolamellar bodies in de-etiolation process (Cortleven and Schmülling 2015), participates in photomorphogenesis stimulus in dark growth of *Arabidopsis* (Lochmanová et al. 2008) and in promotion of chloroplast-related gene expression (Parthier 1979). On the other hand, abscisic acid (ABA) is generally associated with the repression of chloroplast-related genes (Yamburenko et al. 2013). ABA biosynthesis pathway is also related with plastids, such as the production of carotenoids, and its precursor occurs in plastids (Seo and Koshiba 2002).

Previous reports demonstrated the effect of gibberellic acid (GA) in plant morphogenesis responses (Fleet and Sun 2005; Alabadí et al. 2008; Li et al. 2015). GA is known to repress the photomorphogenesis in darkness because of the negative regulation of DELLA proteins. This regulation results in repression of PIFs (phytochrome interacting factors) that are responsible for repressing photomorphogenesis (Li et al. 2015). Another phytohormone that is related with different response in interplay with light is jasmonic acid (JA), where both JA co-receptors and components of light signaling can influence each other's response (Kazan and Manners 2011).

Studies regarding *Guadua* morphoanatomy and ultrastructure are still scarce, highlighting the importance of investigating anatomical aspects of this genera. Previous works have showed culms characterization using light microscopy in *Guadua angustifolia* (Londoño et al. 2002), as well as leaves and culms description using scanning electron microscopy in the same species (Montiel et al. 2006). In *Guadua chacoensis* studies can be found with different purposes, but always in leaves, using both morphoanatomy, by light microscopy, and ultrastructure by scanning electron microscopy (Vega et al. 2016; Panizzo et al. 2017). Though some investigation had already been published with anatomical aspects of *Guadua* species, none of them focused on chloroplast ultrastructure in in vitro culms.

Considering the importance of light and phytohormones in plant morphogenesis response and plastid biogenesis, the present study aimed to evaluate the influence of light presence and absence during in vitro culture of *G. chacoensis*. We emphasized two aspects: chloroplast biogenesis and quantification of the endogenous levels of the phytohormones Z, ABA, GA, and JA, and traced a comprehensive interrelation of these hormones and plastids dynamics. For the best of our knowledge, this is the first report of ultrastructural analysis of chloroplasts in *Guadua chacoensis* in vitro culms.

Materials and methods

Plant material

Nodal segments of *G. chacoensis* were introduced in vitro according to Ornellas et al. (2017), with modifications in the inoculation culture medium composition. The inoculation culture medium consisted of MS basal salts (Murashige

and Skoog 1962) supplemented with Morel vitamins (Morel and Wetmore 1951), 30 g L^{-1} of sucrose, 13 µM of 6-benzylaminopurine (BAP), 2 mL L^{-1} of PPM (Plant Preservative Mixture) and gelled with 2 g L^{-1} of Phytagel®. The multiplication culture medium was the same as described above, but PPM-free. The pH was adjusted to 5.8, prior to autoclaving for 20 min at 121 °C, 1.5 atm. The cultures were kept in shelves with white LED light (Green Power TLED W; PhilipsTM; 77 µmol m^{-2} s^{-1}) at 25 ± 2 °C and 16 h photoperiod. Subculturing was performed every 30 days to a fresh culture medium.

Light conditions experiment

Individualized culms (Fig. 1a) measuring 2–3 cm were cultivated in flasks (67.2 mm × 129.3 mm/350 mL) containing 30 mL of MS multiplication culture medium. The experiment had two conditions, darkness and light, and was held in a completely randomized design, with 30 flasks per treatment, each one containing five individualized culms. The flasks maintained in darkness were wrapped with aluminum foil. All flasks were kept in the same growth room at 25 ± 2 °C, with white LED light (Green Power TLED W; PhilipsTM; 77 µmol m^{-2} s^{-1}) and 16 h photoperiod. Samples of culms were collected at 0, 10, 20, and 30 days after inoculation, and stored at − 80 °C for hormone quantification analysis or fixed for transmission electron microscopy (TEM) characterization.

Transmission electron microscopy

Solid culms without leaf sheath were taken for processing TEM analysis with the aim to evaluate the chloroplast ultrastructure under light and dark conditions. For that, three samples were randomly collected from the middle region of the culms at 0, 10, 20, and 30 days of culture from both conditions. The collects were always performed after 8 h of light exposure and the culms analyzed were categorized as green and yellow–white culms, for light and darkness, respectively (Fig. 1). TEM procedures were realized according to Schmidt et al. (2012), fixing the material with 2.5% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.2) and 18 mM sucrose during 48 h.

After that, samples were washed with sodium cacodylate buffer and post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 4 h. Then, the samples were dehydrated in acetone series, and embedded in Spurr's resin (EMS Diasum, Hatfield, PA), according to the manufacturer instructions. Transversal ultrathin sections were treated with uranyl acetate followed by Reynolds' lead citrate (1963). TEM representative images were obtained with TEM JEM 1011 (JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 80 kV. After the acquisition of the images, 30 chloroplasts from each treatment were selected to calculate the average of chloroplast with the following features: (a) chloroplast without starch grains; (b) chloroplast with starch grains; (c) amyloplast presence; (d) disorganized chloroplasts; and (e) chloroplasts with peripheral reticulum.

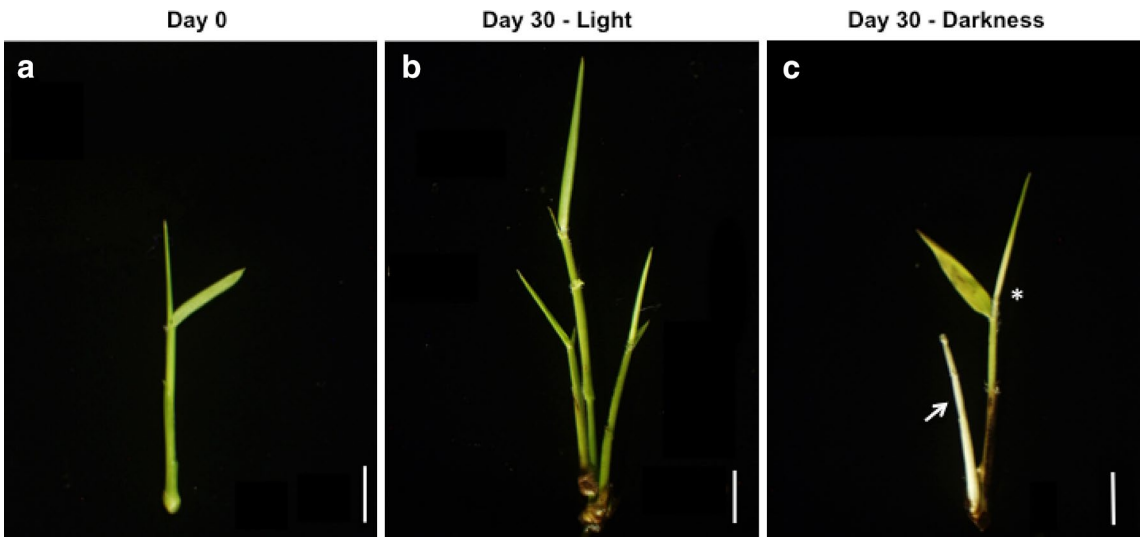

Fig. 1 *Guadua chacoensis* culms. **a** Day 0; **b** day 30 at light condition; **c** day 30 at dark condition. Bars represent 5 mm. Arrow indicates yellow–white culms. *Partially green culms

Hormone quantification

Hormone quantification (Z, GA_4, ABA and JA) was performed according to Fraga et al. (2016), with modifications. Samples of 100 mg of fresh material were ground in liquid nitrogen and transferred to a 2 mL microtube containing 1 mL of extraction buffer (methanol, water and formic acid, 75:20:5, v/v/v with 2 mM of citric acid). The tubes were mixed using a vortex mixer, incubated at − 20 °C for 3 h, followed by ultrasonic bath (40 kHz frequency) at 4 °C for 25 min and a centrifugation at 9800 g at 4 °C for 10 min, and then the supernatant was collected. The pellets were re-extracted twice, with 750 µL of extraction buffer with time of incubation of 6 h and 12 h, sonicated and centrifuged as described above. Finally, the three supernatants were combined and dried in a vacuum concentrator at 40 °C for 22,500 g and resuspended in 1 mL of mili-Q water.

The resuspended extract was purified through Oasis MCX column (150 mg Sorbent, Waters Technologies, USA), according to the manufacturer instructions. The eluent was collected, concentrated, and dried in vacuum concentrator at 40 °C for 22,500 g, resuspended in 100 µL of methanol and filtered through 0.22 µm PTFE filter.

Samples quantification was performed by LC–MS/MS consisting of an Acquity UPLC™ System (Waters, USA) quaternary pump equipped with an autosampler. The column used was Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 µm) (Waters, USA) and the mobile phase in the chromatographic separation consisted of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acetic acid in acetonitrile). Gradient used was 1% B until 1 min, followed by a linear increase up to 6 min reaching 38% B, followed by 100% B until 8.5 min, as a cleaning step, and finally changing to initial 1% B condition up to 9.5 min. The flow rate was 0.45 ml min^{-1} and column temperature of 40 °C. A Waters Xevo™ triple quadrupole mass spectrometer system (MS/MS) with an ESI interface was used in tandem MS analyses with the following conditions: capillary voltage, 2.7 kV; source temperature, 150 °C; desolvation temperature; 400 °C, desolvation gas flow, 800 L h^{-1}; cone gas flow, 50 L h^{-1}.

The parameters of MS/MS detection were optimized to each hormone and multiple reaction-monitoring (MRM) mode was applied in this analysis. Concentrations of 5, 10, 50, 100, 500, 1000, and 1500 ng mL^{-1} were prepared in three independents dilutions in methanol to obtain standard curve, and the analysis/quantification was performed in LC–MS/MS in triplicate. The quantification was achieved by the use of TargetLynz™ software (Waters, USA), with limit of detection (LOD) greater than 3, and the limit of quantification (LOQ) greater than 10. The recovery efficiency and matrix effect were determined according to Trufelli et al. (2011), with standard spikes (40 ng mL^{-1} of all hormones) during the extraction and detection steps. All variations in recovery and matrix effect were considered in the final concentration of each hormone.

After the verification of the variance homogeneity of the data sets, data quantified were subject to factorial analysis of variance (ANOVA). Student–Newman–Keuls (SNK) post hoc test ($p \leq 0.05$) was used for the separation of mean values and the statistical analysis was performed using the software Statistica®.

Chlorophyll and carotenoid quantification

At day 30 of light conditions experiment, three types of culms were selected for quantification of chlorophyll a, b, and total, and carotenoids: green culms, partially green culms (presenting some green and white regions) and yellow–white culms (Fig. 1b, c). The extraction was performed according to Hiscox and Israelstam (1979) with modifications, where 20 mg of fresh culms was added in microtube with 2 mL of dimethyl sulfoxide (DMSO). The microtubes were kept in bath water at 65 °C for 2 h and then the extracted material was filtered and pipetted in a micro plate. The quantification was performed spectrophotometrically at, 665 nm for chlorophyll a (Chl a), 649 nm for chlorophyll b (Chl b) and 480 nm for carotenoids (C x + c) (Wellburn 1994), using microplate reader SpectraMax® Paradigm® Multi-Mode. The final concentration of chlorophyll a, b, and total, and carotenoids was calculated according to Wellburn (1994).

The analysis was performed in biological and technical triplicate, and the data were subject to Student's *t* test to make pairwise comparisons ($p \leq 0.05$).

Results

Chloroplast ultrastructure under light and dark conditions

When observed under light, the chloroplasts at day 0 appeared to be large organelles, occupying a large space of plant cell (Fig. 2a) and showing mature features, i.e., the presence of double-membrane envelope (the inner and outer membranes), plastoglobuli, starch grains, and organized thylakoids stacked forming grana (Fig. 2b). The first modification observed at day 10 of culture was an increase in starch grain content in comparison with day 0. The number of chloroplasts with starch observed at day 0 was, on average, 0.46, and increased to 0.80 at day 10. These starch grains showed to be incorporated in chloroplast (Fig. 2c), which presented normal features such as thylakoids stacked in grana, presence of plastoglobuli and starch grains in large amount (Fig. 2d).

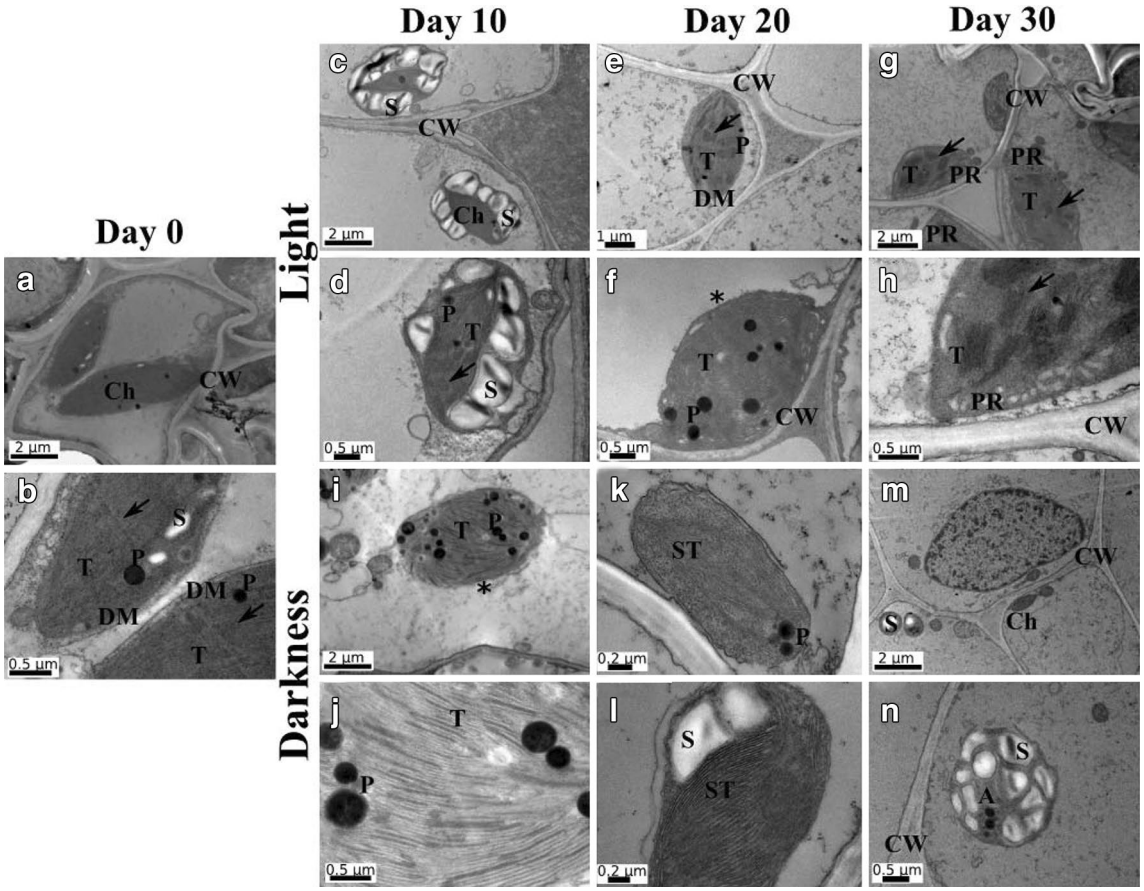

Fig. 2 Plastids' ultrastructure under light and dark conditions at 0, 10, 20, and 30 days in culture of *Guadua chacoensis* in in vitro plantlets. **a** Day 0 with the presence of two mature chloroplasts; **b** day 0, detail of mature chloroplast with double-membrane envelope, thylakoids stacked in grana (black arrows), and presence of plastoglobuli; **c** incorporation of starch grain in chloroplast at day 10 in light conditions; **d** detail of a mature chloroplast, with thylakoids stacked in grana (black arrow), plastoglobuli and incorporation of starch grains; **e** mature chloroplast with double-membrane envelope, thylakoids stacked in grana (black arrow), and presence of plastoglobuli, at day 20 in light conditions; **f** chloroplast in degradation with disorganized thylakoids, increased plastoglobuli number and rupture of the double membrane (*); **g** mature chloroplast with peripheral reticulum (PR) formed at day 30 in light conditions; **h** detail of PR formation; **i** chloroplast in degradation with disorganized thylakoids, increased plastoglobuli number and rupture of the double membrane (*), at day 10 in dark conditions; **j** detail of thylakoids derangement; **k** stroma thylakoids formed instead of stacked thylakoids at day 20 in dark conditions; **l** starch incorporation in a chloroplast with stroma thylakoids; **m** chloroplast transition to amyloplast with starch granules incorporated in chloroplast and normal chloroplast, at day 30 in dark conditions; **n** amyloplast. *Ch* chloroplast, *CW* cell wall, *DM* double-membrane envelope, *T* thylakoids, *P* plastoglobuli, *S* starch, *PR* peripheral reticulum, *ST* stroma thylakoids, *A* amyloplast, black arrows grana formation, *absence of double membrane

Mature chloroplasts were observed at day 20 in light conditions, without the presence of starch grains (Fig. 2e). Some senescent chloroplasts were observed showing the absence of double-membrane envelope, increased plastoglobuli number and disorganized thylakoids, not forming grana (Fig. 2f) The average of chloroplasts with disorganized structure was 0.46 at that time, this value being 6.5 times higher than that observed at day 0 (i.e., 0.07). At day 30 peripheral reticulum was observed attached to the inner membrane of a mature chloroplast, also lacking starch grains (Fig. 2g, h). The number of chloroplasts with peripheral reticulum observed at day 30 was, on average, 0.74. Indeed, the absence of starch grains at days 20 and 30, indicates a possible consumption of starch in light after day 20.

The chloroplast development was influenced by the darkness condition. Chloroplasts with unorganized double-membrane envelope and thylakoids, and increased plastoglobuli number were observed (Fig. 2i, j) after 10 days. The average of chloroplasts with disorganized structure, was 0.56 at day 10 and 0.60 at day 20. The presence of stroma thylakoids disposed in lines in chloroplast stroma (Fig. 2k, l) was seen after 20 days in dark conditions, as well as starch incorporation (Fig. 2l). The average of chloroplast with starch at day 0 was 0.1 and increased to 0.4 at day 20. Electron-dense plastoglobuli were also present (Fig. 2k). Starch grains,

chloroplast (Fig. 2m), and amyloplast formation (Fig. 2n) was observed at day 30 in darkness.

Hormone quantification

Zeatin (Z) quantification indicated a different behavior under light and dark conditions (Fig. 3). In light condition the levels were kept unchangeable along the time of evaluation, except at day 10, where a decrease was observed. In contrast, in darkness a statistically increased level of zeatin was observed at day 20 in comparison with days 0 and 10, and the level was maintained at day 30. Additionally, it was observed that the level of this hormone in days 10 and 20 was higher in darkness than in light conditions.

Abscisic acid (ABA) levels revealed two distinct responses under light and darkness (Fig. 4). In light condition, ABA levels remain unaltered until day 10, followed by a marked increase at day 20, which was maintained at day 30. Differently, under dark condition, ABA levels did not present considerable changes and, it was kept in lowest levels than in light conditions at day 20 and 30.

Gibberellic acid (GA_4) levels in response to light and darkness (Fig. 5) revealed different responses. In light conditions, GA_4 levels presented significant increase at day 30. In darkness, the levels of this hormone were kept unchanged along the evaluation time. Also, at day 30, the level of GA_4 was higher in light than in dark conditions.

Jasmonic acid (JA) levels presented a similar pattern under light and dark conditions. A considerable increase in its levels was observed at day 10, followed by a decrease in day 20 and maintained at day 30 (Fig. 6). Although the JA level increased significantly at day 10 in darkness, at days 20 and 30 these values returned to the same level as day 0.

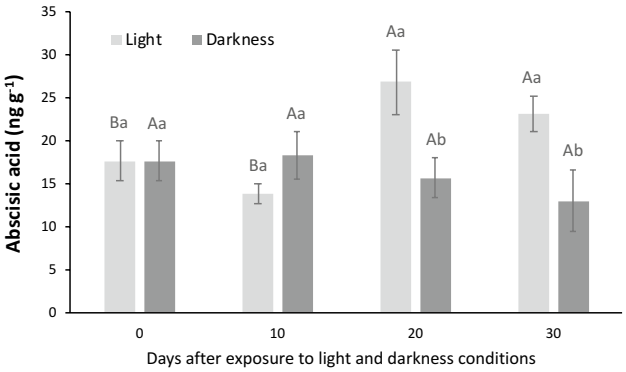

Fig. 4 Abscisic acid (ABA) endogenous levels in *Guadua chacoensis* in vitro culms at 0, 10, 20, and 30 days cultured in light and dark conditions. Mean values ± standard deviation. Different uppercase and lowercase letters indicate significant differences between treatments in light/dark conditions along the evaluation time, and between treatments at the same evaluation time, respectively, according to SNK test ($p < 0.05$). CV—light: 13.0%; 8.3%; 13.9; 8.8%; darkness: 13.0%; 15.0%; 14.8%; 27.6%

Differently, in light treatment, JA levels were higher at days 20 and 30 than day 0, demonstrating an accumulation of JA in light conditions. This accumulation was also observed when a comparison between treatments was made at days 10, 20, and 30, where the JA levels were higher in light than in dark conditions.

Chlorophyll and carotenoid quantification

Chlorophyll and carotenoid quantification demonstrated in a general view, that partially green and yellow–white culms presented lower content of these pigments than green culms

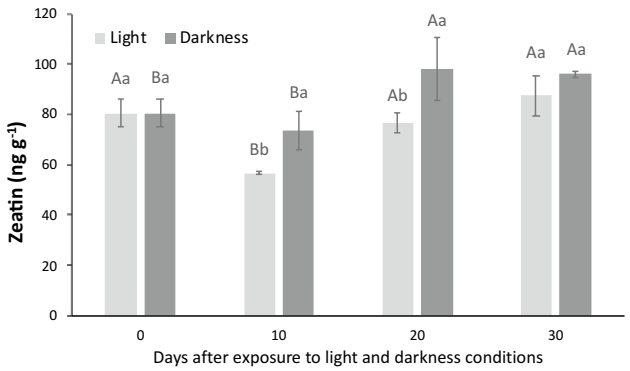

Fig. 3 Zeatin (Z) endogenous levels in *Guadua chacoensis* in vitro culms at 0, 10, 20, and 30 days cultured in light and dark conditions. Mean values ± standard deviation. Different uppercase and lowercase letters indicate significant differences between treatments in light/dark conditions along the evaluation time, and between treatments at the same evaluation time, respectively, according to SNK test ($p < 0.05$). Coefficient of variation (CV)—light: 6.7%; 0.9%; 5.1%; 8.9; darkness: 6.7%; 10.2%; 12.8%; 1.3%

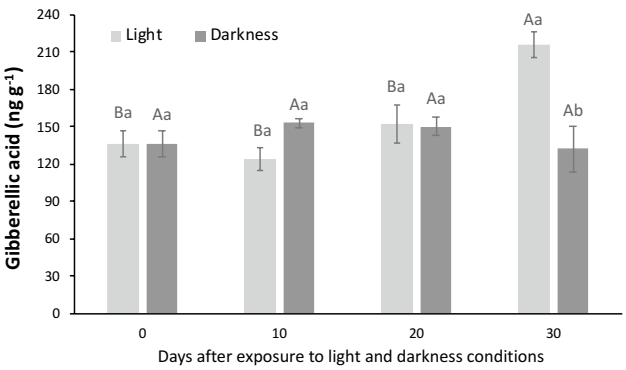

Fig. 5 Gibberellic acid (GA_4) endogenous levels in *Guadua chacoensis* in vitro culms at 0, 10, 20, and 30 days cultured in light and dark conditions. Mean values ± standard deviation. Different uppercase and lowercase letters indicate significant differences between treatments in light/dark conditions along the evaluation time, and between treatments at the same evaluation time, respectively, according to SNK test ($p < 0.05$).CV—light: 7.8%; 7.8%; 9.9%; 4.9%; darkness: 7.8%; 2.5%; 5.2%; 14.1%

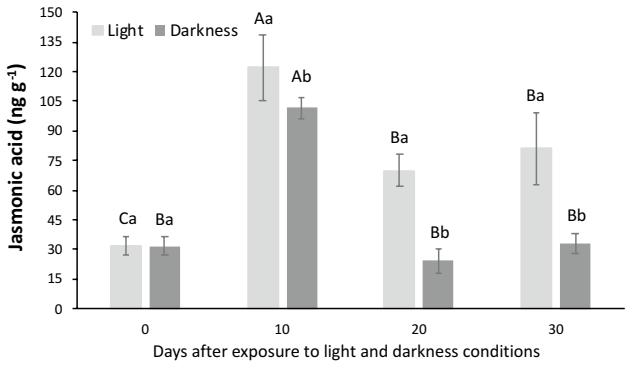

Fig. 6 Jasmonic acid (JA) endogenous levels in *Guadua chacoensis* in vitro culms at 0, 10, 20 and 30 days cultured in light and dark conditions. Mean values ± standard deviation. Different uppercase and lowercase letters indicate significant differences between treatments in light/dark conditions along the evaluation time, and between treatments at the same evaluation time, respectively, according to SNK test ($p < 0.05$). CV—light: 13.6%; 13.5%; 11.3%; 22.1%; darkness: 13.6%; 5.6%; 25.1%; 14.9%

Table 1 Levels of chlorophyll a, b, and total, and carotenoids of green, partially green and yellow–white culms of *Guadua chacoensis* at day 30 in culture

	Chl a (µg mg^{-1} FM)	Chl b (µg mg^{-1}FM)	Chl total (µg mg^{-1} FM)	C_{x+c} (µg mg^{-1} FM)
Green culms	0.133a	0.040a	0.173a	0.027a
Partially green	0.042b	0.007a	0.049b	0.013b
Yellow–white	*	*	*	*

Different lowercase letters in column indicate significant differences according to the Student's *t* test ($p < 0.05$)

*Values below the detection limit

FM fresh material, C_{x+c} carotenoids

(Table 1). However, when green and partially green culms were compared, it is observed that the levels of chlorophyll a, and total, and carotenoids were significantly lower in partially green than in green culms, but the content of chlorophyll b did not differ between them. On the other hand, yellow–white culms presented values below the limit of detection, indicating that chlorophyll and carotenoids were practically absent in these culms.

Discussion

Chloroplast ultrastructure under light and dark conditions

Chloroplast ultrastructure analysis in in vitro culture of *G. chacoensis* culms provided some evidences that light is a key factor in normal chloroplast development. In this condition, chloroplasts usually presented a mature structure, with all the typical characteristics, including double-membrane envelope, thylakoids organized in grana and plastoglobuli. In contrast, in darkness, we observed some atypical characteristics, such as the reduction in the number of thylakoids membranes.

In vitro cultures usually require carbohydrate supplementation as a carbon source and osmotic agents, being a photomixotrophic environment with low rates of gas exchange, low CO_2 concentration and poor quality of light (Sandhu et al. 2017). As a consequence of the environment conditions, in vitro cultures are not able to produce its own carbohydrate, thus, sucrose is usually supplemented to the culture medium.

Sucrose can be catabolized and stored as starch, the major form of carbohydrate storage in plants, with its synthesis occurring inside the plastids (Geigenberger 2011). In our study, the accumulation of starch granules in the early stages of chloroplast development under light at day 10 (Fig. 2c, d), and its subsequent absence after 20 and 30 days were observed (Fig. 2e–g). Previous studies (Mangat et al. 1990; Sulpice et al. 2009; Lee and Huang 2013) demonstrated the relationship between the starch granules formation in response to sucrose supplementation to different species in in vitro cultured, and in both organogenesis and somatic embryogenesis morphogenetic routes.

Mangat et al. (1990) related that during *Begonia rex* organogenesis, with 3% sucrose supplementation, starch accumulation was observed in the first days of culture, with a maximum in about 4–6 days. These starch granules decreased as the shoots were under development, thus suggesting that starch was used as energy source during in vitro morphogenesis. In addition to this, Lee and Huang (2013) showed that cultures of two rice cultivars exhibited higher levels of glucose, fructose and starch during callus induction followed by a gradual decrease in the regeneration culture medium. It has been emphasized that starch accumulation in plants can be considered the driver of growth and development because it controls carbon availability (Sulpice et al. 2009).

Our results suggest the hypothesis that starch accumulation in chloroplasts observed at day 10 in light condition could be a response to the sucrose supplementation in the culture medium. Furthermore, the absence of these starch granules in chloroplasts during latter stages (days 20 and 30) could be a consequence of the consumption of these carbohydrates as energy source to sustain plant development.

On the other hand, in dark conditions, the accumulation of starch grains was observed along the time of evaluation, with amyloplast formation at day 30. Bach et al. (2018) analyzed the importance of the light quality during the in vitro shoot organogenesis of two cultivars of *Lachenalia* sp, and one of the parameters investigated was the carbohydrate content,

including starch. These authors observed that the content of starch in the cultivar Rupert was six times higher than in Ronina under dark conditions, and additionally, they showed that the content of starch directly influences the development of shoots. In our study, we proposed that the accumulation of starch in dark conditions could be related with a reduction in plant metabolism caused by the darkness, as it is sensed as a stress that provokes changes in plant metabolism.

During the life cycle of organelles, senescence is a naturally occurring process that corresponds to the last development step before organelles' death. Chloroplast is the first organelle to show ultrastructural indicators of senescence, such as thylakoids' disorder, increased number of plastoglobuli and collapse of double-membrane envelope (Blswal and Biswal 1988). In the present study, these same signals were observed at day 10 of dark conditions (Fig. 2i, j), as well as at day 20 of light conditions (Fig. 2f).

Senescence can be seen as a natural process along plastid development or can be induced by some stress condition. Plastoglobuli number can increase in response to abiotic conditions that promote stress in photosynthetic apparatus (Austin et al. 2006). Bréhélin et al. (2007) also reported the involvement of plastoglobuli in stress response and suggested that plastoglobuli should be seen as an organelle involved with secondary metabolism compounds and stress response, rather than as a simple storage organelle. In our study, senescence chloroplasts in day 20 in the presence of light could be associated with natural degradation along plastid living. In contrast, the senescence chloroplast observed during the darkness growth, at day 10, is probably consequence of the dark itself.

Hormones' involvement in senescence process has been extensively investigated, and it well known that cytokinin, gibberellic acid, and auxins retard senescence (Jibran et al. 2013). On the other hand, abscisic acid, jasmonic acid, ethylene, and salicylic acid act as senescence promoters (Munné-Bosh and Alegre 2004). Jibran et al. (2013) emphasize the importance of hormone action in development and stress and conclude that senescence is a consequence of a complex interaction between hormones cross talking, stress conditions, and developmental age. Jan et al. (2018) also highlighted that the hormonal cross talking has direct effect in the regulation of senescence. As the senescence response is usually associated with a complex interaction of hormones and not only with the action of a specific hormone, a direct correlation between the presence of senescence chloroplast, observed in our study, and senescence promoters hormones, such as JA and ABA, could not be stated.

During chloroplast development of some species, a structure named chloroplast peripheral reticulum (PR) appears connected to the inner envelope membrane. The presence of PR was detected in our study, at day 30 of light condition (Fig. 2g, h). This observed PR could be classified as Type II, which are characterized as a system of dense and packed vesicles in the plastid periphery and are usually found in C3 and C4 species (Wise and Harris 1984). Many authors suggested that PR is an adaptive structure that facilitates the metabolite transport into and out the chloroplast, as the presence of PR increases the area of the inner envelope membrane (Laetsch 1968; Heldt and Sauer 1971; Wise 2007; Szczepanik and Sowiński 2014).

The presence of peripheral reticulum has also been seen in plastids during the process of senescence. Greening et al. (1982) observed an increased presence of chloroplast peripheral reticulum during the process of senescence and postulated that this structure functions as a facilitator of metabolic transport. Otherwise, Harris (1978) hypothesizes that peripheral reticulum presence during senescence was related with the collapse of enzymes of the plastids.

Besides, chloroplast PR is often associated with low respiration rates, where the photorespiration is probably dependent on the presence of PR, as proposed by Hilliard and West (1971). According to Szczepanik and Sowiński (2014), PR presence is also related with high light intensity, which observed PR system to be better developed under high light intensity than in low light intensity in four different grass species.

In our work, the presence of PR in chloroplasts at day 30, in light condition (Fig. 2g, h), corroborates with the possible function as facilitator of metabolic transport. At this step of culture, the content of metabolites available for plant growth is probably scarce, and PR can provide the migration of some metabolites from the outside to the inside of the chloroplast, allowing chloroplast to keep their functionality and ensure plant development. On the other hand, we cannot exclude the hypothesis that the presence of PR at day 30 in light could be a signal of a beginning of chloroplast senescence, as PR have already been correlated with senescence in plastids (Harris 1978; Greening et al. 1982).

Although etioplast formation is expected to occur during dark growth, in our study, it was not observed. The most important characteristic of etioplasts is the unique paracrystalline membrane system known as prolamellar bodies (PLB) connected to prothylakoids membranes (Solymosi and Keresztes 2012). The presence of stroma thylakoids organized in lines, without the presence of PLB, was observed in our study at day 20 in darkness (Fig. 2k, l), indicating that these plastids cannot be classified as etioplast.

Previous studies have showed that cytokinin supplementation can influence chloroplast maturation (Stetler and Laetsch 1965; Polanská et al. 2006; Cortleven and Schmülling 2015) and consequently, inhibit etioplast formation. Stetler and Laetsch (1965) observed that chloroplast maturation of tobacco culture tissues was dependent on the kinetin supplementation. In addition, plastids from cultures grown

in dark conditions presented few thylakoids and were not able to develop grana and PLB, not converting to etioplast.

In this same context, Chory et al. (1994) observed that the plastid morphology of wild-type seedling of *Arabidopsis thaliana* grown in darkness was influenced by cytokinin supplementation, where the presence of cytokinin results in plastids with similar structure of the observed in *det* (*Arabidopsis thaliana* mutants for etiolation sensing) (Chory et al. 1989; Chory and Peto 1990). The same authors also proposed three models of action where light and cytokinin act simultaneously or subsequently in the generation of light response such as gene expression, chloroplast differentiation, and leaf development, thought regulation of gene DET.

On the other hand, the conversion of chloroplast to etioplast could be repressed because initial chloroplasts were fully developed, as observed in our study at day 0. Even though, plastids are known to be easily reversible between the different types, the only exception of total reversibility is between fully developed chloroplast and etioplast (Lindquist et al. 2016).

Hormone dynamics between light and dark conditions

The signaling roles of phytohormone in many processes of plant development and stress responses (Erland et al. 2017) indicate that quantification of endogenous levels of hormones can provide important insights about the behavior of *G. chacoensis* culms under dark condition.

Our results regarding Z levels indicated that a considerable decrease was observed at day 10 in light and a considerable increase was observed at day 20 and maintained at day 30 in darkness (Fig. 3). Cytokinins are involved in sucrose transport and metabolism (Gibson 2004), and the sucrose supplementation in culture medium could lead to alterations in their endogenous levels.

It has been shown that glucose supplementation in darkness growth promotes a decrease followed by an increase in CK endogenous levels (Stirk et al. 2014). This behavior was considered a consequence of the plant metabolism adjustment to initiate CK biosynthesis, using the energy supplies provided by the glucose supplementation (Stirk et al. 2014). Similar results were also observed in dark-induced treatment of *Catasetum fimbriatum* (Orchidaceae) by Suzuki et al. (2010). According to these authors, an increase in CK endogenous level could be understood as a signal of nutrient translocation to guarantee etiolation. In our study we could observe that the level of Z in days 20 and 30 in darkness was higher than at the day 0 in the same treatment. This behavior could be a result of nutrient translocation as suggested by Suzuki et al. (2010), but we also postulate that the accumulation of zeatin in *G. chacoensis* culms at days 20 and 30 in darkness could be a consequence of the BAP supplementation in the culture medium.

It is widely accepted that CKs are associated with chloroplast maturation (Stetler and Laetsch 1965; Polanská et al. 2006; Cortleven and Schmülling 2015), thylakoid formation (Cortleven and Schmülling 2015) and expression of chloroplast-related genes (Parthier 1979). The roles of CKs in such process could be reinforced with the results observed in the present work. At day 10 in light, it is possible to observe a decrease in the Z levels (Fig. 3), and at the same time well-developed chloroplast was observed in ultrastructure analysis (Fig. 2c, d). We suggest that Z decreasing at day 10 in light could be associated with the use of this hormone in chloroplast development, including maturation, thylakoid formation and gene expression regulation.

ABA corresponds to another plant hormone directly interconnected with chloroplast features and it has been associated with the repression of gene expression of some important chloroplast-related genes (Kusnetsov et al. 1994; Yamburenko et al. 2013). In this work ABA levels did not present considerable changes in darkness along the 30 days of evaluation, and it was kept in lowest levels than in light conditions at days 20 and 30 (Fig. 4). This behavior suggests a possible reduction in the ABA biosynthesis in darkness, in comparison with light after day 20, which can be related with the reduction of carotenoids (ABA precursors) endogenous content (Seo and Koshiba 2002).

Lower carotenoid endogenous content as well as lower levels of chlorophyll a, and total in yellow–white and partially green culms was observed in our work at day 30 (Table 1). It is possible to assume that ABA biosynthesis in dark condition was inhibited as a consequence of the reduction in carotenoid content. In fact, a reduction on ABA endogenous levels in etiolated explants of *Cyathea delgadii*, as compared with light-cultured explants, reinforces our findings (Grzyb et al. 2017). Additionally, the reduction of ABA endogenous levels affected by the absence of light can be associated with reduction in thylakoid number since most of carotenoids are found in thylakoid membranes (Sun et al. 2017). This could be perceived especially at days 20 and 30 in dark conditions, where a drastic reduction in the presence of thylakoids membranes was observed (Fig. 2k, m).

GA influencing plant morphogenesis has been shown in previous studies (Fleet and Sun 2005; Alabadí et al. 2008; Li et al. 2015). *G. chacoensis* culms showed higher GA_4 endogenous levels at day 30 in light than in dark conditions, and likewise, their levels on darkness growth were almost unchangeable (Fig. 5). The reduction in the production of GA_4 in darkness, in comparison with light treatment is probably correlated with the repression of photomorphogenesis, driven by PIFs. Photomorphogenesis response can also be repressed indirectly by the action of DELLA proteins that provoke repression of PIFs (Li et al. 2015). In addition,

DELLA proteins are already characterized as negative regulator of GA response (Sun 2008).

JA response to biotic stress, such as pathogen attack, is further best characterized than its response to abiotic stress. However, it is well known that plant response to biotic and abiotic stress is accomplished by changes in endogenous levels of hormones (Du et al. 2013). Accumulation of JA endogenous levels in *G. chacoensis* culms subjected to light compared with dark conditions and its increase and posterior decline in darkness proposes a negative response in JA biosynthesis under stress caused by absence of light (Fig. 6).

A significant relationship between JA biosynthesis and light has been demonstrated. A variety of JA co-receptors can influence light response and light-signaling compounds, such as phytochrome can influence JA gene expression and response (Kazan and Manners 2011). Therefore, the higher JA endogenous levels in light treatment are probably associated with the active JA biosynthesis when culms are exposed to light. As light absence is sensed by the plant as an abiotic stress, the lowest levels of JA observed in darkness compared to light conditions among the time of evaluation—days 10, 20, and 30, demonstrated that this treatment evokes a reduction in JA endogenous levels.

Our results regarding JA decreased levels in darkness are in agreement with those reported by Du et al. (2013). These authors observed that the heat stress in rice reduced JA endogenous levels and the gene expression of some JA biosynthesis genes. Despite of this, it is important to emphasize that knowledge about changes in JA levels in response to abiotic stress is still reduced, and different responses can be observed to different abiotic stresses. Du et al. (2013) also showed that cold and drought treatments caused opposite responses, with increased JA levels as well as upregulation in gene expression. Many questions about JA response to abiotic stress are still open; however, our study provides new insights about plant response under darkness growth.

Concluding remarks

Taken together, our results indicated different starch accumulation patterns in chloroplasts in light or dark conditions. In light treatment, a decreased number of starch grains was observed during in vitro culture, probably associated with energy providing for plant initiation and posterior development. Differently, in dark conditions, starch accumulation and amyloplast formation were observed, possibly as a consequence of plant metabolism reduction. Additionally, etioplast formation was not observed in the dark, suggesting that the conversion was inhibited by the presence of fully developed chloroplast at the beginning of the experiment.

Variable responses in endogenous levels of phytohormones, as a consequence of light or dark conditions, were observed in *G. chacoensis* culms in an unprecedented way. Z levels increased in darkness along the time of evaluation, indicating an accumulation of these hormones, possible associated with BAP supplementation or nutrient translocation. ABA, GA_4 and JA biosynthesis were unchangeable in darkness, except for an increase in JA levels at day 10, and, on the other hand, their levels increased along the time of evaluation in light, suggesting a strong relationship between light and these phytohormone levels. Furthermore, a reduction in the ABA biosynthesis observed in darkness is possibly related with the reduction of carotenoids' (ABA precursors) endogenous content. These results can broaden our understanding of these phytohormones' metabolism in response to different light and dark conditions.

Author contribution statement Conceived and designed the experiments: LGP, RP, HPFF, and LNV; in vitro introduction and multiplication: LGP and TSO; implementation of etiolation experiment: LGP and ASH; transmission electron microscopy analysis: LGP, LNV, and HPFF. Hormone analysis: LGP and HPS; data analysis: LGP, HPFF, LNV, ASH; manuscript writing: LGP, HPFF, LNV, and RP. Manuscript review: HPFF, LNV, ASH, MPG, RP, TSO, and HPS. Contributed with material, reagents and analysis tools: MPG, RP, and HPS.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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