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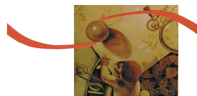

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Morphoanatomy and physiology of *Pouteria gardneriana* Radlk plantlets grown *in vitro* at varied photosynthetic photon flux densities

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ABSTRACT. Micropropagation is an important tool for the multiplication of native Cerrado species. However, understanding the responses of these species under *in vitro* culture conditions is still incomplete. Thus, the present study aimed to analyze the growth, anatomical behavior and physiology of *Pouteria gardneriana* cultivated *in vitro* under photoautotrophic conditions. Nodal segments were cultured at photosynthetic photon flux densities (PPFD) of 75, 100 and 150 μmol m^{-2} s^{-1} in culture medium MS 50% solidified with 3.5 g L^{-1} of agar and 2.0 g L^{-1} of activated charcoal, in the absence and presence of 30 g L^{-1} of sucrose. After 60 days of *in vitro* culture, the *P. gardneriana* plantlets only regenerated when sucrose was present in the culture medium. Higher fresh and dry weights, higher palisade parenchyma thickness and larger stomatal polar and equatorial diameters were observed in the plantlets cultured at PPFD 150 μmol m^{-2} s^{-1}. The PPFD difference used in the present study was sufficient to provide additional understanding of the behavior of this species *in vitro*.

Keywords: Cerrado, photoautotrophism, Sapotaceae.

Morfoanatomia e fisiologia de plântulas de *Pouteria gardneriana* Radlk, cultivadas *in vitro* sob diferentes densidades de fluxo de fótons fotossintéticos

RESUMO. A micropropagação constitui ferramenta importante para multiplicação de espécies nativas do Cerrado. No entanto, o conhecimento sobre as respostas destas espécies sob condições de cultivo *in vitro* ainda é incipiente. Assim, objetivou-se com este trabalho, analisar o crescimento, comportamento anatômico e fisiológico de *Pouteria gardneriana* cultivadas *in vitro* em condições fotoautotróficas. Os segmentos nodais foram cultivados nas densidades de fluxo de fótons fotossintéticos de 75, 100 e 150 μmol m^{-2} s^{-1}, e meio de cultivo MS 50% solidificado com 3.5 g L^{-1} de ágar e 2.0 g L^{-1} de carvão ativado, na ausência e presença de 30 g L^{-1} de sacarose. Após 60 dias de cultivo *in vitro* observou-se regeneração de plântulas de *P. gardneriana* apenas quando a sacarose estava presente no meio de cultivo. Maior massa fresca e seca, maior espessura do parênquima paliçádico e diâmetro polar e equatorial foram observadas nas plântulas cultivadas no PPFD de 150 μmol m^{-2} s^{-1}. As diferentes PPFD utilizadas neste estudo foram suficientes para compreender o comportamento desta espécie *in vitro*.

Palavras-chave: Cerrado, fotoautotrofismo, Sapotaceae.

Introduction

Guapeva (*Pouteria gardneriana* Radlk) is a native Cerrado tree species belonging to the family Sapotaceae, which is known in Brazil as 'pêssego-do-campo' and 'cabo-de-machado'. These plants have economic importance, as the fruits either are used raw or are processed by the population for the production of desserts, juices, jellies and liqueurs (Vieira, Agostini-Costa, Silva, Ferreira, & Sano, 2006; Rocha et al., 2011). *P. gardneriana* seeds have been described as recalcitrant, a characteristic that limits their storage (Cabral, Sales, Silva, Branquinho, & Oliveira, 2013) and compromises species conservation. Given these factors, plant tissue culture is a viable tool to mass produce plantlets, benefiting reforestation programs or commercial cultivation.

Traditional *in vitro* culture comprises plant management in a room at with low photosynthetic photon flux densities (PPFDs) and low gas exchange, with sucrose as the metabolic energy source for the explants to sustain their growth (Jesus, Villa, Lara, & Pasqual, 2011; Zhang, Zhao, Ma, Li, & Xiao, 2009). Plantlets cultured in this system demonstrate heterotrophic character, with low capacity to perform photosynthesis and consequently low survival during the acclimatization process (Brondani, de Wit Ondas, Baccarin, Gonçalves, & de Almeida, 2012; Fernandes, Azevedo, Costa, & Brondani, 2013; Greenway, Phillips, Lloyd, Hubstenberger, & Phillips, 2012). Thus, photoautotrophic micropropagation has been evaluated to obtain plants with anatomical and physiological characteristics that enable them to survive *ex vitro* conditions like higher chlorophyll content and active photosynthetic apparatus (Xiao, Niu, & Kozai, 2011, Zhang et al., 2009).

Photoautotrophic techniques, including greater gas exchange between the environment and the inside of the culture flasks (Iarema et al., 2012; Saldanha et al., 2012), that enrich the atmosphere with CO_2 combined with or without more porous support materials (Saldanha et al., 2014), increase PPFD (Shin, Park, & Paek, 2013; Sáez, Bravo, Latsague, Sánchez, & Ríos, 2012) and decrease or eliminate sucrose from the culture medium (Xiao & Kozai, 2006) have been investigated. Studies that seek to improve the environmental conditions for the *in vitro* culture of native species help in understanding the optimal environmental factors to obtain plantlets with better performance during the acclimatization process.

Light is one of the most important requirements for plant growth and development. Plants cultured at different PPFD demonstrate morphological, photosynthetic and metabolic differences (Dai et al., 2009). Overall, plants that grow in an environment with optimal light conditions undergo normal development compared to plants that grow in environments with low or high irradiance. Plants grown at low PPFD have low photosynthetic capacity and a low CO_2 assimilation rate. In contrast, an environment with high irradiance can negatively affect photosystem II (PSII) and photosynthesis declines (Guo, Guo, Zhou, Hu, & Shen, 2006).

Although native Cerrado fruit trees are important ecologically and economically, little is known regarding the growth conditions of this species. Studies seeking to understand the optimal PPFD for the anatomical and physiological development of *P. gardneriana* plantlets cultured *in vitro* are extremely relevant and can aid in obtaining high-quality plants, favoring their successful acclimatization and large-scale plantlet production.

Studies focused on adjusting the culture environment, such as PPFD, and determining the sucrose requirement by plants in the culture medium are essential. Thus, the present study aimed to test how PPFD can interfere with the growth, anatomy and physiology of *P. gardneriana* plantlets during *in vitro* culture.

Material and methods

Source of the plant material

Mature fruits of *P. gardneriana* Radlk were collected between November 2014 and January 2015 at the Goiano Federal Institute – Rio Verde Campus (latitude 17º48'202"S, longitude 50º54'397"W and elevation of 749 m) and at the Água Amarela farm in Ouroana municipality in Goiás State, Brazil (latitude 18º11'824"S, longitude 50º34'180"W and elevation of 656 m).

Mucilage attached to the seeds was removed by immersing them in 5% sodium hydroxide solution for 5 minutes. One hundred seeds were germinated in plastic trays (53 x 37 x 8 cm) in the presence of washed and sieved coarse sand as substrate and kept in a growth room with a mean temperature of 25 ± 3°C and a 16-hour photoperiod until seedlings were obtained. Pest control of the seedlings consisted of spraying a commercial product (0.2% Derosal® systemic fungicide solution) 24 hours before inoculation. The seedlings were watered every 15 days with nutrient solution consisting of 50% MS medium salts (Murashige & Skoog, 1962).

In vitro establishment and experimental conditions

Healthy and homogenous *P. gardneriana* seedlings were selected and 2.0 cm long nodal segments with one axillary bud were used for the *in vitro* establishment. The segments were covered by gauze and washed under running water for 15 minutes. Next, the disinfection methods were conducted under a laminar flow hood. The explants were dipped in 70% alcohol for one minute and in 20% sodium hypochlorite - NaOCl solution (commercial bleach 2.0 – 2.5% active chlorine) for 20 minutes and were then rinsed three times with sterile water.

After disinfection, the explants were cultured in test tubes (25 x 150 mm) containing 20 mL of MS medium with 50% salts, with 2 g L^{-1} of activated charcoal and solidified with 3.5 g L^{-1} of agar for 30 days. The explants were kept in a growth room with an average temperature 25 ± 3ºC, 16-hour photoperiod, and active photosynthetic radiation of 45-55 μmol m^{-2} s^{-1}, generated using white fluorescent bulbs. After this period, these explants were transferred into flasks containing 50 mL of MS 50% medium, with 2 g L^{-1} of activated charcoal and solidified with 3.5 g L^{-1} of agar. The pH of the culture medium was adjusted to 5.7 ± 0.03 and the medium was then autoclaved at 121°C for 20 minutes. PVC (polyvinylchloride) film was used to seal the flasks after inoculation.

After 30 days *in vitro* establishment, the nodal segments were cultured in the absence and presence of 30 g L^{-1} of sucrose. The flasks were placed in acclimatized chamber (Fitotron®) and three photosynthetic photon flux densities (PPFD) were evaluated (75, 100, and 150 μmol m^2 s^{-1}) using white fluorescent bulbs. These light intensities were adjusted using a photosynthetically active radiation sensor, QSO-S model (Decagon Devices, Pullman, WA, USA). All treatments were kept at 25 ± 2°C with 60% air relative humidity in the climatized chamber (Fitotron®).

Biometric analyses

The biometric evaluations were conducted after 60 days of *in vitro* culture using the following characteristics: fresh weight (g), dry weight (g), leaf area (cm^2), shoot percentage (%), leaf number and

seedling length (cm). Leaf area was obtained from image integration in ImageJ® *software* (Rasband, W. S.; U. S. ImageJ. Bethesda, MD, USA). The length measurements were obtained using a millimeter ruler. After drying the material in an air-circulation oven at 65ºC for 72 hours until obtaining constant weights, the fresh and dry weights were determined using a digital analytical balance.

Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence analysis was conducted to obtain the maximum quantum yield (Fv/Fm), photochemical quenching (qP), effective quantum yield (ΔF/Fm'), relative electron transport rate (ETR) (Bilger, Schreiber, & Bock, 1995) and non-photochemical quenching (NPQ) (Bilger & Bjorkman, 1990).

Chlorophyll *a* fluorescence was measured using a mini-PAM modulated fluorometer (Walz, Effeltrich, Germany). The analyses were conducted using the methods by Bilger et al. (1995) and Rascher, Liebig, and Lüttge (2000), which were adapted for *in vitro* plants according to Costa et al. (2014). First, the leaves were dark-acclimated for 30 minutes and were then exposed to a pulse of low-intensity red light (0.03 μmol $m^{-2} s^{-1}$) to measure the initial fluorescence (Fo). Next, the leaves were exposed to a pulse of saturating actinic light (> 6.000 μmol $m^{-2} s^{-1}$) for 0.8 s to measure maximum fluorescence (Fm). Finally, the same plantlets were placed in a climatic chamber (Fitotron®) for 20 minutes at the same aforementioned growth irradiance to measure the light fluorescence parameters.

Determination of the chlorophyll content

The chlorophyll *a*, chlorophyll *b*, carotenoid and total chlorophyll pigment levels and pheophytinization index were determined according to Costa et al. (2014) with adaptations to the method. Three leaf disks (5 mm wide) were incubated in closed flasks wrapped in aluminum foil containing 5 mL of dimethyl sulfoxide (DMSO) saturated with calcium carbonate - $CaCO_3$ (50 g L^{-1}) for a 24-hour period at 50ºC in a water bath. Next, the absorbance of the extract was determined using an Evolution 60S UV-VIS spectrophotometer (Thermo Fischer Scientific, Madison – USA). The wavelengths, equations and calculations to determine the pigment levels were based on the study by Wellburn (1994).

Anatomical characterization

The anatomical analyses of the *P. gardneriana* leaves were performed using two methods. The first was the diaphanization process, which analyzes the tissue surface and the second was fixation, in which the leaf tissue was embedded in resins to obtain cross-sections.

During diaphanization, the leaves were immersed in 5% sodium hydroxide for 24 hours, clarified with chloral hydrate 1:6:1 (p/v) for 24 more hours and stained with 1% safranin in 50% ethanol (Arnott, 1959). Upon studying the leaf surface, the *P. gardneriana* leaves were classified regarding stomatal location, stomatal morphology, stomatal density and stomatal polar and equatorial diameters.

The leaves were fixed in Karnovsky solution (Karnovsky, 1965) for 48 hours. Next, the leaves were dehydrated in an ascending ethanol series and pre-infiltrated and infiltrated with historesin (Historesin Leica, Erviegas Ltda: São Paulo, São Paulo State, Brazil) to obtain historesin blocks with included plant material. After drying the blocks in silica gel, the material was transversely sectioned 5 μm thick in a rotary microtome (RM 2155 model, Leica). The obtained cuts were stained with 0.05% toluidine blue, pH 4.0 (O'Brien, Feder, & McCully, 1964) to evaluate palisade parenchyma thickness, adaxial and abaxial epidermis thickness and mesophyll thickness.

The images were captured using an optical microscope (BX61 model, Olympus Corporation – Tokyo city, Japan) with the U-photo system. The micromorphometric measurements were obtained using image integration in image analysis software (ImageJ®).

Experimental design and statistical analysis

The experiment was conducted in a completely randomized design (CRD) in factorial arrangement (2 x 3) in the absence and presence of 30 g L^{-1} of sucrose and at three PPFD levels (75, 100, and 150 μmol $m^{-2} s^{-1}$), with eight replicates and three explants per flask. The data were subjected to analysis of variance (ANOVA), applying the F test and the means were compared using the Tukey test (5% probability). The percentage data were arc-sine $\sqrt{x/100}$ transformed and the number count was $\sqrt{x+0.5}$ transformed. SISVAR software (Ferreira, 2011) was used for the data analysis.

Results

Plant growth and development at varied in photosynthetic photon flux densities

After 60 days of culture, different growth and development profiles were observed in *P. gardneriana* plantlets cultured at different PPFDs in the presence and absence of sucrose (Figure 1). Some species have the capacity to grow *in vitro* in the absence of sucrose under light and gas exchange conditions sufficient for them to undergo photosynthesis. However, in the present study, the increase in environmental PPFD did not suppress the requirement for sucrose by the shoots in the culture medium, and plantlet growth did not occur (Figure 1D, E and F).

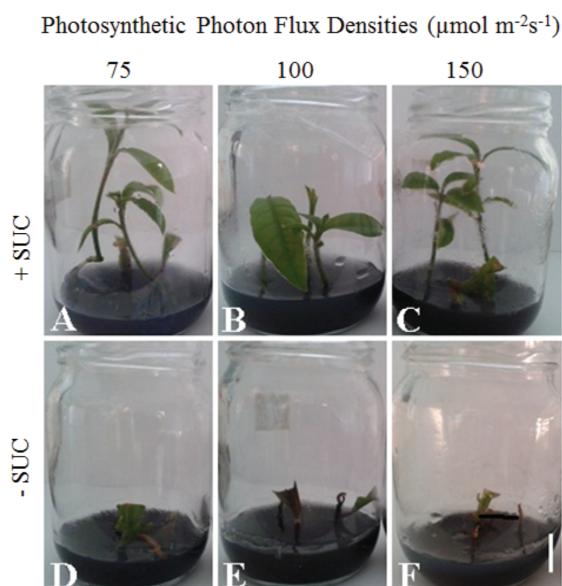

Figure 1. *P. gardneriana* Radlk plantlets growth in MS 50% culture medium supplemented with 30 g L^{-1} of sucrose (A - C) (+ SUC) and in sucrose-free medium (D – F) (- SUC) at PPFD of 75, 100 and 150 μmol m^{-2} s^{-1}, respectively. Scale bar: 1 cm.

A PPFD of 150 µmol m^{-2} s^{-1} positively affected plant biomass. Higher fresh and dry weights were obtained at an irradiance of 150 µmol m^{-2} s^{-1}, with means of 0.55 and 0.28 g, respectively, consistent with the observed leaf area value (22.7 cm^2). There were no differences between the irradiances for the shoot percentage, leaf number and plantlets length characteristics (means of 46.96%, 1.56 and 4.66 cm, respectively) (Table 1).

Parameters and photosynthetic pigments

Photosynthetic pigment levels (Cl.*a*, Cl.*b*, carotenoids and total chlorophyll) and pheophytinization index did not vary in relation to variation in PPFD (Table 2). The observed means for these pigments were 18.38, 7.78, 4.50, 26.17 μg cm^{-2} and 1.32, respectively.

The effect of PPFD on chlorophyll *a* fluorescence in *P. gardneriana* plantlets is shown in Table 3. The highest Fv/Fm value was obtained at 75 µmol m^{-2} s^{-1}, with a mean of 0.73; however, the Fv/Fm did not differ from the 100 µmol m^{-2} s^{-1}, with a mean of 0.70. The lowest Fv/Fm value (0.67) was obtained at 150 µmol m^{-2} s^{-1}. PPFD did not affect qP, NPQ, ΔF/Fm' and ETR, with means of 0.39, 0.87, 0.22, and 38.36, respectively.

Anatomical plasticity

PPFD affected the evaluated anatomical characteristics, especially the palisade parenchyma thickness and stomatal polar and equatorial diameters. This information supports understanding of the physiological responses observed in the *P. gardneriana* plantlets (Table 4).

Greater palisade parenchyma and stomatal polar and equatorial diameters were observed at 150 µmol m^{-2} s^{-1}, with means of 19.3, 20.0 and 15.9 µm, respectively. There were no differences in the adaxial and abaxial epidermis thickness, mesophyll thickness and stomatal density characteristics at different PPFDs, with means of 7.26, 7.5, 67.6 µm and 388.2 stomata mm^{-2}, respectively (Table 4).

Table 1. Fresh weight (g), dry weight (g), leaf area (cm^2), shoot percentage (%), leaf number and shoot length (cm) of *Pouteria gardneriana* Radlk cultured in MS 50% for 60 days in medium supplemented with 30 g L^{-1} in sucrose at PPFD of 75, 100 and 150 µmol m^{-2} s^{-1}.

PPFD	Biometric characteristics					
(µmol m^{-2} s^{-1})	Fresh weight (g)	Dry weight (g)	Leaf area (cm^2)	Shoot	Leaf number	Plantlets Length (cm)
75	0.47±0.02*b[z]	0.23±0.01b	20.30±3.47ab	47.40±0.81a	1.60±0.08a	4.96±0.22a
100	0.43±0.02b	0.19±0.01b	14.60±4.76b	48.21±0.01a	1.41±0.13a	4.00±0.32a
150	0.55±0.02a	0.28±0.01a	22.70±5.09a	45.28±2.15a	1.67±0.25a	5.04±0.69a

[Z]Means followed by the same letter do not differ statistically using the Tukey test at 5% probability. *Standard error of the mean.

Table 2. Chlorophyll *a*, chlorophyll *b*, carotenoids, total chlorophyll and pheophytinization index (P.I.) of *Pouteria gardneriana* Radlk plantlets cultured in MS 50% for 60 days in medium supplemented with 30 g L^{-1} sucrose at PPFD of 75, 100 and 150 µmol m^{-2} s^{-1}.

PPFD	Physiological characteristics				
(µmol m^{-2} s^{-1})	Chlorophyll *a* (μg cm^{-2})	Chlorophyll *b* (μg cm^{-2})	Carotenoids (μg cm^{-2})	Total Chlorophyll (μg cm^{-2})	P.I (A_{435}/A_{415})
75	18.11±1.31*a[z]	7.27±0.61a	4.20±0.25a	25.39±1.83a	1.30±0.02a
100	18.16±1.41a	7.64±0.33a	4.53±0.27a	25.80±1.72a	1.35±0.02a
150	18.88±1.64a	8.44±0.47a	4.78±0.24a	27.33±1.88a	1.31±0.03a

[Z]Means followed by the same letter do not differ statistically using the Tukey test at 5% probability. *Standard error of the mean.

Table 3. Maximum quantum yield (Fv/Fm), photochemical quenching (qP), non-photochemical fluorescence quenching (NPQ), effective quantum yield (ΔF/Fm') and electron transport rate (ETR) in the leaves of *Pouteria gardneriana* Radlk plantlets cultured in MS 50% for 60 days in medium supplemented with 30 g L^{-1} sucrose at PPFD of 75, 100 and 150 µmol m^{-2} s^{-1}.

PPFD	Physiological characteristics				
(µmol m^{-2} s^{-1})	Fv/Fm	qP	NPQ	ΔF/Fm'	ETR (µmol m^{-2} s^{-1})
75	0.73±0.01*a[z]	0.37±0.04a	0.45±0.10a	0.24±0.02a	41.8±4.30a
100	0.70±0.02ab	0.38±0.03a	0.93±0.17a	0.21±0.03a	36.2±4.69a
150	0.67±0.01b	0.44±0.03a	1.24±0.31a	0.22±0.03a	37.1±5.96a

[Z]Means followed by the same letter do not differ statistically using the Tukey test at 5% probability. *Standard error of the mean.

Table 4. Palisade parenchyma (P.P.), polar diameter (P.D.), equatorial diameter (Eq.D.), adaxial (Ad.Ep.T.) and abaxial epidermis thickness (Ab.Ep.T), mesophyll thickness (Me) (μm) and stomatal density (S.D.) in the leaves from *Pouteria gardneriana* Radlk plantlets cultured in MS 50% in for 60 days in medium supplemented with 30 g L^{-1} sucrose at PPFDs of 75, 100 and 150 μmol m^{-2} s^{-1}.

PPFD	Anatomical characteristics						
(μmol $m^{-2}s^{-1}$)	P.P. (μm)	P.D. (μm)	Eq.D. (μm)	Ad. Ep. T. (μm)	Ab.Ep.T. (μm)	Me (μm)	S.D. (Stomata/mm^2)
75	16.9±0.4*b[z]	15.6±0.2b	13.7±0.4b	7.7±0.4a	7.8±0.1a	68.6±0.9a	425.5±37.4a
100	15.9±0.1b	15.7±0.2b	14.0±0.1ab	7.4±0.3a	7.4±0.4a	67.3±4.6a	399.7±58.8a
150	19.3±0.5a	20.0±0.1a	15.9±0.7a	6.7±0.6a	7.3±0.5a	66.9±4.6a	339.5±33.2a

[Z]Means followed by the same letter do not differ statistically using the Tukey test at 5% probability. *Standard error of the mean.

The epidermis of both faces of the *P. gardneriana* leaves was uniseriate, consisting of rectangular cells with flat or slightly convex periclinals, external walls. The organization of the mesophyll was dorsoventrally heterogeneous, with palisade parenchyma consisting of juxtaposed columnar cells and stratified spongy parenchyma with irregular-shaped cells (Figure 2a, b, c).

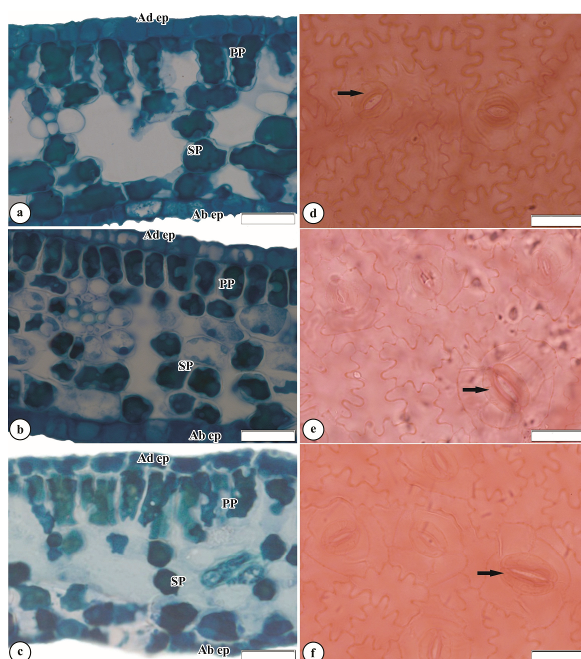

Figure 2. Electron photomicrographs of cross-sectional regions of the leaf (a - c) and the abaxial face surface (d – f) from *Pouteria gardneriana* Radlk cultured in medium supplemented with 30 g L^{-1} of sucrose at PPFD of 75 μmol m^{-2} s^{-1} (a and d), 100 μmol m^{-2} s^{-1} (b and e), and 150 μmol m^{-2} s^{-1} (c and f). Adaxial epidermis (Ad ep), abaxial epidermis (Ab ep), palisade parenchyma (PP) and spongy parenchyma (SP). Arrows = stomata. Scale bar: 50 μm.

The presence of stomata was identified only on the abaxial face, characterizing the leaf as hypostomatic. Based on the organization of the subsidiary cells, the stomata were classified as anisocytic. These characteristics were observed in all *P. gardneriana* plantlets, a genetic trait was minimally affected by the variation in PPFD. According to the ratio between the polar and equatorial stomata diameters, an increasingly ellipsoid shape was observed with increased PPFD (Figure 2d, e, f).

Discussion

Sucrose requirement by *P. gardneriana* plantlets in the culture medium was not suppressed by increased environmental photosynthetic photon flux densities

Under light and gas exchange conditions sufficient for the plants to undergo photosynthesis *in vitro,* many species have the capacity to grow in the absence of sucrose in the culture medium, as observed in *Pfaffia glomerata* (Spreng) Pedersen (Iarema et al., 2012; Saldanha et al., 2014) and hybrids of orchid *Doritaenopsis* (Shin et al., 2013). For *Mouriri elliptica* (Mart.), a native Cerrado species, increased PPFD 150 μmol m^{-2} s^{-1}) decreases the requirement for sucrose in the culture medium and led to plantlets growth (Assis et al., 2016). However, in the present study, shoot formation of the *P. gardneriana* was dependent on sucrose addition in the culture medium.

A sucrose requirement as the metabolic energy source for the growth and development of seedlings *in vitro* has been observed in several species, including the herbaceous plant *Pfaffia tuberosa* (Spreng.) (Flores, Uliana, Pimentel, & Garlet, 2013) and the tree species *Acrocomia aculeata* (Jacq.) (Bandeira, Xavier, Lani, & Otoni, 2013). According to Jesus et al. (2011), sucrose allows viable or normal plants to be obtained in most species, as observed for the plantlets under study.

Physiological profile of the *P. gardneriana* plantlets at varied photosynthetic photon flux densities

In the present study, the photosynthetic capacity of *P. gardneriana* seedlings cultured at PPFD of 75, 100, and 150 μmol m^{-2} s^{-1} was evaluates. Chlorophyll content is an important parameter that determines the photosynthetic pattern and regulates plant growth. In this study, there were no changes in the pigment levels of the plantlets in response to the variations in light. These results indicate that the different PPFDs used in the study do not influence the pigment levels or structural responses (Table 2).

Data regarding the effective quantum yield of photosystem II (ΔF/Fm), non-photochemical quenching (NPQ), electron transport rate (ETR) and photochemical quenching (qP) did not differ between the plantlets cultured at different PPFDs. However, the Fv/Fm values varied ranging from 0.67 to 0.73. A lower Fv/Fm value (0.67) was observed at 150 μmol m^{-2} s^{-1}, corroborating the results described by Shin et al. (2013); however, the difference in this parameter may be a characteristic of the species used.

The capacity to maintain high Fv/Fm ratios can be indicative of use radiation efficiency by photochemistry and consequently carbon assimilation (Tester & Bacic, 2005). Fan et al. (2013) considered excess light to reduce photosynthetic efficiency due to the inability of the photosynthetic apparatus to dissipate this excess; thus, photoinhibition and damage in the photosystem reaction centers can occur. Although a lower Fv/Fm ratio was observed in the plantlets cultured at 150 μmol m^{-2} s^{-1} they did not demonstrate photoinhibition and the biomass accumulation was higher. Photoinhibition was observed in apple plants cultured *in vitro* (Zanandrea, Bacarin, Falqueto, Braga, & Peters, 2007) and in young 'jatobá-do-cerrado' (*Hymenaea stigonocarpa*) plants grown in a greenhouse (Costa et al., 2015), when they were exposed to PPFDs higher than the ideal range for photosynthesis.

Low anatomical plasticity was observed in leaves from *P. gardneriana* cultured *in vitro* at varied photosynthetic photon flux densities

The *P. gardneriana* plants cultivated at 150 μmol m^{-2} s^{-1} showed structural alterations in the expansion of the palisade parenchyma, suggesting variation in anatomical adaptability. In the study conducted by Fan et al. (2013) when a tomato culture was subjected to PPFDs of 300 and 450 μmol m^{-2} s^{-1}, the mesophyll was thicker and the number of imperfections in the palisade parenchyma was higher. In *Mouriri elliptica* (Mart.), the morphoanatomic variations observed in the leaves were attributed to the plasticity and adaptive characteristics of the species, which are important factors for seedling survival when they are subjected to *ex vitro* culture conditions (Assis et al., 2016).

Stomatal density is another anatomical characteristic that correlates well with the photosynthetic capacity of plants; the higher the number of stomata mm^{-2}, the lower the resistance to leaf gas diffusionis (Lima Jr., Alvarenga, & Castro, 2006). According to Chirinéa, Pasqual, Araújo, Pereira, and Castro (2012), stomatal density in the leaves varies with the species and culture conditions. None of the PPFD used in the present study led to elevation of this variable in the *P. gardneriana* leaves.

The stomata in the *P. gardneriana* leaves were formed by cells with increased kidney shape and ellipsoid characteristics with increase of PPFD, showing their functionality. Consistent with these results, many studies demonstrate that the structural characteristics of the stomata are related to their functionality (Hazarika, 2006). *Castanea sativa* demonstrate ellipsoid characteristic of stomata when grown in PPFD of 150 μmol m^{-2} s^{-1} (Sáez et al., 2012).

The structural alterations of the palisade parenchyma of plantlets cultivated at 150 μmol m^{-2} s^{-1} do not reduce their photosynthetic capacity because it does not differ from other parameters, like effective quantum yield from photosystem II (ΔF/Fm') and increased CO_2 assimilation, consistent with the dry matter content observed in these plantlets.

The variation PPFD used in the present study was sufficient to understand the behavior of this species *in vitro* and to inform future studies seeking more and better quality plantlet production. Although the species' sucrose requirement was observed in the culture medium, the development of new studies evaluating concentrations lower than 30 g L^{-1} of this supplement combined with higher PPFD, atmosphere CO_2 enrichment and alternative substrates will be both interesting and important.

Conclusion

Shoot formation of *P. gardneriana* plants *in vitro* was dependent on sucrose addition to the culture medium. The organization of leaf tissue was a characteristic phenotypic minimally influenced at the PPFD values used in this study. However, 150 μmol m^{-2} s^{-1} PPFD led to higher biomass accumulation and certain anatomical adaptability with expansion of the leaf palisade parenchyma.

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