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# $CO_2$ enrichment alters morphophysiology and improves growth and acclimatization in *Etlingera Elatior* (Jack) R.M. Smith micropropagated plants

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## Abstract

Conventional in vitro propagation may affect normal morphophysiological characteristics and survival during the acclimatization of commercial plants, such as *Etlingera Elatior* (Jack) R.M. Smith. $CO_2$ enrichment, gas-permeable membranes, and reduced carbohydrate concentration in culture media can be used to mitigate these effects. Here, we aimed to determine the impact of photomixotrophic and photoautotrophic growth on morphophysiological responses of *E. elatior* and its survival during ex vitro acclimatization. In vitro-established plants were placed in flasks containing semisolid MS medium with 0.54 µM α-naphthalene acetic acid and sucrose (0 or 15 g $L^{-1}$). The flasks were sealed with lids with or without orifices covered by gas-permeable membranes and kept for 45 days under forced-air ventilation (360 or 1000 µmol$^{-1}$ $CO_2$). Growth variables, anatomical and physiological characteristics, and survival rate were evaluated. Overall, 1000 µmol $mol^{-1}$ $CO_2$, gas-permeable membranes, and 15 g $L^{-1}$ sucrose resulted in plants with higher fresh and dry mass, higher level of photosynthetic pigments, as well as more developed secondary ribs in the leaves, prominent midribs, highly organized and differentiated chlorenchyma cells (palisade and spongy parenchyma), more developed vascular bundles, and intercellular gaps were reduced, compared to conventionally in vitro-propagated plants. Moreover, these conditions promote morphophysiological responses and favored survival of *E. elatior* plants (75%) when transferred to ex vitro conditions.

**Keywords** Gas exchange · Leaf anatomy · Photomixotrophy · Photosynthetic pigments · Torch ginger

## 1 Introduction

*Etlingera elatior* (Jack) R.M. Smith (Zingiberaceae) originates from Southeast Asia and is popularly known as torch ginger (Ghasemzadeh et al. 2015; Silva et al. 2017); it has four cultivars, namely Porcelain (inflorescences with silky light pink bracts), Pink Torch (dark pink bracts), Red Torch (red bracts), and Tulip Torch Ginger (crimson tulip-shaped bracts) (Chan et al. 2011; Lins and Coelho 2003; Yunus et al. 2012). It can be employed in the manufacturing of medicines, dyes, essential oils, and cosmetics, as well as in horticulture, and in the production of food and spices (Abdelwahab et al. 2010; Yunus et al. 2012; Ghasemzadeh et al. 2015; Silva et al. 2017). Also, it can serve as a source of natural cellulosic fiber for composite materials (Quinaya and d'Almeida 2019).

To meet the demand from pharmaceutical and agrochemical industries, large volumes of Zingiberaceae plantlets must

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be produced. Plant tissue culture has been proposed as an alternative to mass clonal propagation for the production of plantlets with high genetic and phytosanitary quality. Such strategy offers an important tool for the breeding of various ornamental and medicinal plants, such as torch ginger, despite the limited information regarding in vitro propagation of this species (Yunus et al. 2012).

In addition to preventing contamination, flask sealing avoids dehydration of the tissue culture and its medium during in vitro propagation (Zobayed 2006; Kozai 2010; Pinheiro et al. 2013; Saldanha et al. 2013). However, conventional growth in sealed flasks with reduced gas exchange may result in plants with stunted shoots, reduced cuticle and epicuticular wax accumulation on the leaves, reduced supportive tissues (collenchyma and sclerenchyma), increased water content, nonfunctional stomata, and underdeveloped narrow leaves, which lead to poor photoautotrophic activity (Kozai and Kubota 2001; Xiao et al. 2011). In the conventional system, there is also a buildup of ethylene and other volatile gases inside the flasks, reductions in $CO_2$ and gas exchange, and decreased flux of photosynthetically active photons, which may limit photosynthesis as a whole (Desjardins et al. 2009; Saldanha et al. 2013; Batista et al. 2017a). As a consequence, growth and development rates decline, leading to considerable losses due to plant mortality during acclimatization (Nguyen and Kozai 2005; Zobayed 2006; Kozai 2010; Xiao et al. 2011).

Supplementation with abundant exogenous sugars promotes in vitro growth of plantlets with low photosynthetic activity. However, reducing sugar content could be advantageous in preventing the rapid proliferation of bacteria and fungi, lowering costs, and increasing plant survival during acclimatization (Kozai and Kubota 2001). Indeed, alternative approaches to enhance growth performance and photosynthetic competence include lowering or even eliminating sucrose, applying natural light, increasing the concentration of $CO_2$, and reducing $O_2$.

Ideally, in vitro propagation should provide conditions close to those found in environmental conditions, including high supplies of photosynthetically active photons, $CO_2$, and gas exchange (Xiao et al. 2011). To achieve this, gas-permeable membranes (Kozai 2010; Saldanha et al. 2012) have been shown to enable greater gas exchange, decrease ethylene accumulation into the flask, and facilitate plant acclimatization (Xiao et al. 2011). Moreover, they have a positive effect on culture establishment, improving shoot quantity and quality, while reducing the occurrence of chlorosis and leaf senescence (Rodrigues et al. 2012; Ríos-Ríos et al. 2019). Finally, they can increase plant survival during acclimatization by hardening in vitro-grown plants (Batista et al. 2017a, b).

We hypothesized that in vitro cultivation under higher gas exchange, $CO_2$ enrichment, and different sucrose concentrations could promote field-like in vitro morphogenesis and acclimatization of *E. elatior* plants. The present study aimed to: (i) identify the morphophysiological responses of *E. elatior* plants grown in vitro to photomixotrophic and photoautotrophic growth conditions accompanied by $CO_2$ enrichment; (ii) elucidate the photoautotrophic potential of *E. elatior* plantlets grown in vitro; and (iii) correlate the morphophysiological characteristics with the adaptation of plants to acclimatization.

## 2 Materials and methods

Plant material and growth conditions - Shoots of *E. elatior* cv. Porcelain were established in vitro from rhizomes at the Plant Tissue Culture Laboratory, Institute of Applied Biotechnology for Agriculture (BIOAGRO, Federal University of Viçosa, Viçosa, Brazil), and subcultured every 30 days until obtain a sufficient number of explants. The plants were cultured on MS medium (Murashige and Skoog 1962) supplemented with conventional carbohydrate concentrations (3% sucrose), 6.66 µM 6-benzyladenine, 0.54 µM $\alpha$-naphthalene acetic acid (NAA), 100 mg $L^{-1}$ myo-inositol, and 6.5 g $L^{-1}$ agar (Merck®, Darmstadt, Germany). The pH was adjusted to 5.8 before autoclaving at 121 °C and 1.5 atm for 15 min. The plant material was placed in a growth chamber at 25 ± 2 °C, with a 16-h photoperiod, and irradiance of 60 µmol $m^{-2}$ $s^{-1}$, provided by two fluorescent tubes (110 W, HO T12; Sylvania, São Paulo, Brazil).

In vitro elongation and rooting of E. elatior plants under $CO_2$ enrichment - The explants used in this study were approximately 3.0 cm long, with three or four leaves and no roots. They were then inoculated into 300-mL glass flasks, which were sealed with rigid polypropylene lids (sealed flasks) or with rigid polypropylene lids pierced whit two 10-mm holes covered by gas-permeable membranes (flasks with membranes) as proposed by Saldanha et al. (2012). Each flask was filled with 60 mL MS medium supplemented with 0.54 µM NAA, sucrose (0 or 15 g $L^{-1}$), 100 mg $L^{-1}$ myo-inositol, and 6.5 g $L^{-1}$ agar; pH was adjusted to 5.8 before autoclaving at 121 °C and 1.5 atm for 15 min. The flasks were placed in a growth chamber for 45 days in two forced-air flow boxes (360 or 1000 µmol $mol^{-1}$ $CO_2$) at 25 ± 2 °C, 16-h photoperiod, and irradiance of 121 µmol $m^{-2}$ $s^{-1}$ provided by two fluorescent tubes (110 W, HO T12; Sylvania) as proposed by Saldanha et al. (2013, 2014).

Growth analysis - The following characteristics were assessed after 45 days of in vitro cultivation: plant height (mm), oxidation rate (%; when more than 50% of the plants displayed brown coloring), number of shoots (considering the new shoots emitted from the initial plant), plant fresh, and dry weight (g), photosynthetic pigments (chlorophyll *a*,

chlorophyll *b*, total chlorophyll, and carotenoids; mg $cm^{-2}$), and micromorphometry (adaxial and abaxial epidermis, mesophyll, and leaf blade thickness; µm). Dry plant weight was determined after the samples reached a constant weight in an oven at 70 °C for 48 h.

Photosynthetic pigments analysis - The photosynthetic pigments (chlorophyll *a* and *b*, total chlorophylls, and carotenoids) were quantified based on the method proposed by Wellburn (1994). Two leaf disks (6 mm in diameter) were cut from fully expanded alternate leaves of the second and third nodes from the tip down and then incubated in 2 mL dimethyl sulfoxide saturated with calcium carbonate, and kept in the dark in test tubes for 48 h at room temperature, as proposed by Santos et al. (2008) with modifications. Sample absorbance was determined after the incubation period using a Genesys 10 UV-visible spectrophotometer (Thermo Scientific, Madison, WI, USA) with a 10-mm path cuvette. Wavelengths and equations used for calculating pigment levels were based on Wellburn (1994).

Anatomical, histochemical and micromorphometry analysis - To determine the effect of $CO_2$ enrichment on *E. elatior* growth**,** microscopy analysis was performed only in plants cultured under photoautotrophic and photomixotrophic conditions. For anatomical and histochemical analysis, sections were taken from the middle third of fully expanded leaves from the second and third nodes and then fixed in Karnovsky solution (Karnovsky 1965) modified with 2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1 M cacodylate buffer pH 7.2, and 0.001 M $CaCl_2{\cdot}2H_2O$. The samples were subsequently dehydrated in in a growing series of ethanol (from 30 to 100%) and embedded in methacrylate (Historesin; Leica Instruments, Heidelberg, Germany). To mount the blades, 5-µm-thick cross sections were removed from the middle third of fully expanded leaves using an automatic feed rotary microtome (RM2155; Leica Microsystems Inc., Deerfield, IL, USA).

For anatomical characterization, the samples were stained in toluidine blue (pH 4.0) for 10 min (O'Brien and McCully 1981). For histochemical characterization, cross sections from the middle third of fully expanded leaves were subjected to staining with Sudan black B to detect lipid bodies; periodic acid/Schiff staining (PAS) for starch, mucilage, and polysaccharide detection; combined double-staining with toluidine blue and Lugol's solution for starch; and xylidine Ponceau staining to detect total protein. The slides were mounted with Permount® medium.

For micromorphometric analysis, three images of different plants were obtained per treatment, in which epidermal thickness (adaxial and abaxial surfaces), mesophyll thickness, and total thickness of the leaf blade were measured, as proposed by Rodrigues et al. (2020) with modifications. Three measurements of each parameter per image were taken using a photomicroscope (AX70TRF; Olympus Optical, Tokyo, Japan) equipped with a U-Photo system and processed in ImageJ® software.

Plant acclimatization - After 45 days of in vitro culture, the plants were acclimatized ex vitro. They were transferred to polyethylene pots (300 mL capacity) with ~ 260 $cm^3$ Plantmax HT (Buschle & Lepper S.A., Joinville-SC, Brazil) commercial substrate (pH 5.6) containing macronutrients (N, 0.51; P, 0.41; K, 0.38; Ca, 0.90; Mg, 1.78; S, 0.27; Fe, 2.00 dag $kg^{-1}$) and micronutrients (Cu, 36.5; Zn, 45; Mn, 215; B, 13.8 mg $kg^{-1}$). The plants were left on a workbench at room temperature (28 ± 2 °C), 70 µmol $m^{-2}$ $s^{-1}$ artificial irradiance, and indirect daylight from ambient conditions. Irrigation was adjusted as required, but no other intervention regarding the maintenance of humidity was applied, to provide adverse conditions to the plants and thus certify which of them responds with superiority. After 45 days, the percentage of surviving plants was calculated.

## 2.1 Statistical analysis

The experiment was conducted following a completely randomized design consisting of six treatments (Table 1): two $CO_2$ concentrations (360 and 1000 µmol $mol^{-1}$), two sealing methods (sealed flasks and flasks with membranes), and two sucrose concentrations (0 and 15 g $L^{-1}$).

A completely randomized design was followed also for growth variables (plant height, oxidation rate, number of shoots, plant fresh and dry weight), with six treatments (Table 1), six replicates each, and the experimental unit consisting of a flask containing four plants/replicate. For the photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids), three replicates were used, and the experimental unit consisted of two leaf disks per test tube. For micromorphometric variables (adaxial and abaxial epidermis, mesophyll, and leaf blade thickness), three replicates were used, and the experimental unit consisted of three analyses of cross sections. For survival analysis, four replicates were used, each amounting to one plant per pot.

**Table 1** Treatments used during in vitro elongation and rooting of *Etlingera elatior*. Plants were maintained at different $CO_2$ concentrations (360 and 1000 µmol $mol^{-1}$) and growth conditions (sealing type with different gas exchange rates and sucrose concentrations in culture medium)

| $CO_2$ (µmol $mol^{-1)}$ | Gas-permeable membrane | Sucrose (g $L^{-1}$) |
|---|---|---|
| – | Without | 0 |
| – | Without | 15 |
| 360 | With | 0 |
| 360 | With | 15 |
| 1000 | With | 0 |
| 1000 | With | 15 |

All variables were subjected to analysis of variance, and the means were compared by the Scott–Knott test ($P \leq 0.05$), using the GENES software package (Cruz 2013). The experiments were repeated twice.

## 3 Results

Does in vitro $CO_2$ enrichment benefit *Etlingera elatior* plants when subjected to ex vitro conditions? - Analysis of variance revealed a significant difference ($P \leq 0.05$, F-test) between treatments ($CO_2$ enrichment, sealing methods, and sucrose concentrations) for the following variables: oxidation rate, plant height, fresh plant weight, dry plant weight, number of shoots, survival percentage, chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoids, adaxial and abaxial epidermis thickness, mesophyll thickness, and leaf blade thickness.

As suggested by apparent and secondary ribs on the leaves, *E. elatior* plants grew better under photomixotrophic conditions when gas-permeable membranes and a higher $CO_2$ concentration were used (Fig. 1a–f). Although plants grown in flasks with no permeable membranes did form few or rare roots (Fig. 1a), their leaves displayed less prominent ribs compared to plants grown under photomixotrophic conditions (Fig. 1d, f). In photoautotrophic conditions, with $CO_2$ enrichment but no sucrose addition, plants showed a

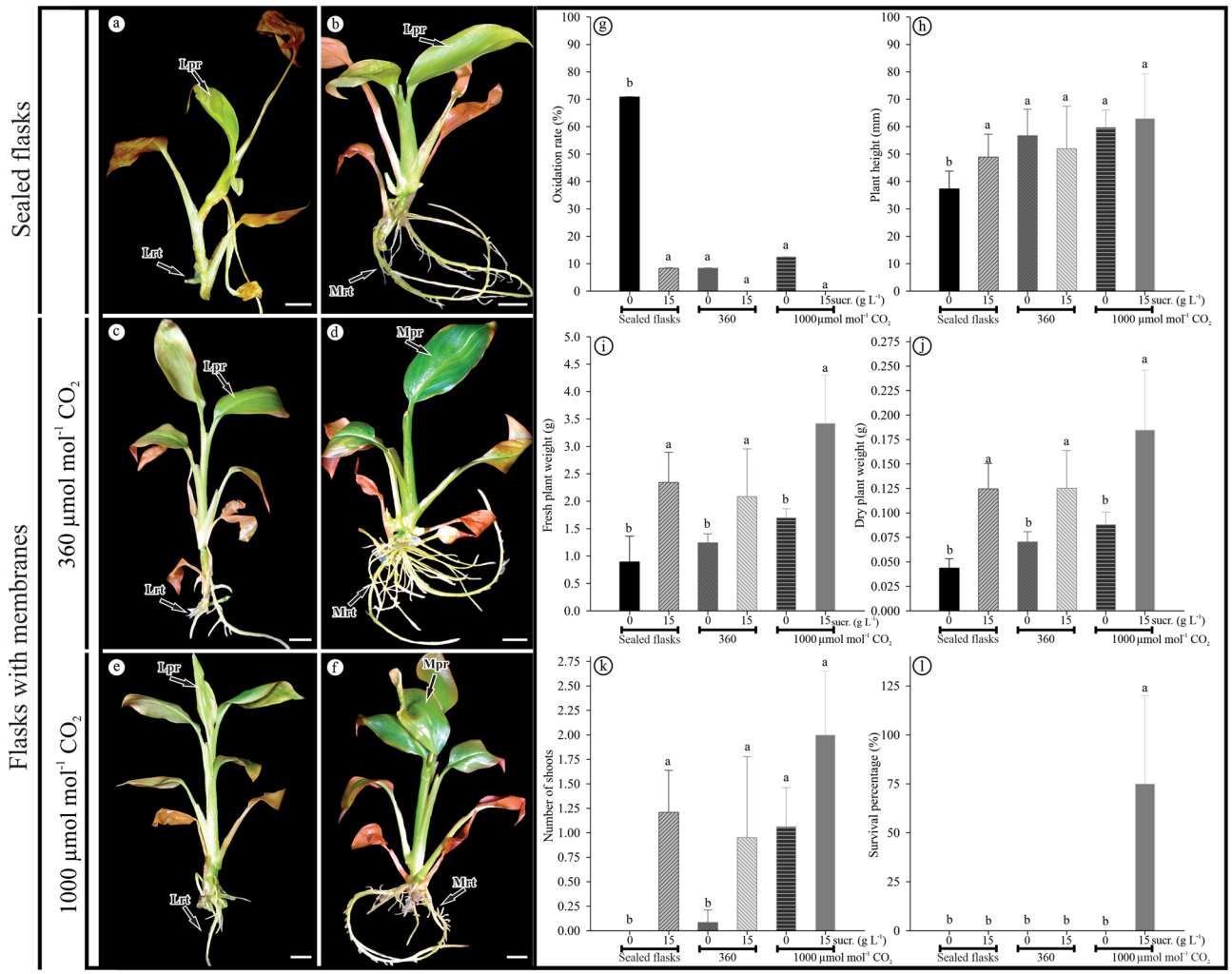


**Fig. 1** *Etlingera elatior* plants after 45 days of in vitro culture spanning elongation and rooting at different $CO_2$ concentrations (360 and 1000 µmol $mol^{-1}$) and growth conditions (sealing type with different gas exchange rates and sucrose concentration in culture medium). The specific treatments were as follows: sealed flasks without gas-permeable membranes and without **a** or with **b** sucrose; with $CO_2$ enrichment (360 µmol $mol^{-1}$), with gas-permeable membranes, and without **c** or with **d** sucrose; with $CO_2$ enrichment (1000 µmol $mol^{-1}$), with gas-permeable membranes, and without **e** or with **f** sucrose. *Abbreviations*: Lpr, less prominent ribs; Lrt, less roots; Mrt, more roots; Mpr, more prominent ribs. Bars: a–f: 10 mm. The following variables were measured: oxidation rate **g**; plant height **h**; fresh **i** and dry plant weight **j**; number of shoots **k**, and survival percentage **l**. Error bars denote the standard error. Means followed by the same letter do not differ by the Scott–Knott test ($P \leq 0.05$)

less developed rooting system (Fig. 1a, c, e), whereas under photomixotrophic conditions, with $CO_2$ enrichment and sucrose addition, plants showed a more developed rooting system (Fig. 1b, d, f) and more prominent ribs in the leaves (Fig. 1d; f).

The explants presented high oxidation rate, and consequently brown coloration of the leaves, under photoautotrophic conditions without $CO_2$ enrichment (70.8%), which prevented their use for microscopy analyses. When plants were supplemented with sucrose or subjected to conditions of photoautotrophy or photomixotrophy with gas-permeable membranes, they exhibited a common drastic reduction in oxidation rate (Fig. 1g).

Explants grown without $CO_2$ enrichment produced significantly smaller plants (37.28 mm) ($P \leq 0.05$), whereas those grown under photomixotrophic conditions and 1000 µmol $mol^{-1}$ $CO_2$ and with added sucrose were the highest (62.98 cm), albeit not significantly (Fig. 1h). Fresh and dry plant weight were significantly higher when sucrose was added, regardless of other treatments (Fig. 1i, j). In general, plants grown under photoautotrophic conditions showed lower fresh and dry weights compared to those grown in a photomixotrophic environment, irrespective of $CO_2$ enrichment.

The number of shoots was greater following addition of 15 g $L^{-1}$ of sucrose. A similar increase in shoot production was observed when the concentration of $CO_2$ was augmented to 1000 µmol $mol^{-1}$ under photoautotrophic conditions, demonstrating the importance of maintaining carbon rates in plants (Fig. 1k).

Only plants subjected to 1000 µmol $mol^{-1}$ $CO_2$, covered by a lid with gas-permeable membranes, and supplemented with 15 g $L^{-1}$ of sucrose survived after acclimatization (75%, Fig. 1l), which probably occurred due to the characteristics of these plants, i.e., the presence of amyloplasts (Am, starch grains) in the mesophyll and around the vascular bundles, and the greater development of this system that provided survival to ex vitro conditions (Fig. 1f, 3e, 4e). These conditions resulted also in higher levels of photosynthetic pigments, such as chlorophyll *a* (Fig. 2a), chlorophyll *b* (Fig. 2b), total chlorophyll (Fig. 2c), and carotenoids (Fig. 2d).

Anatomical and histochemical characteristics of *Etlingera elatior* leaves differ between treatments - The leaves of *E. elatior* cultured under 360 µmol $mol^{-1}$ $CO_2$ without or with sucrose concentration (Fig. 3b, c, respectively) and 1000 µmol $mol^{-1}$ $CO_2$ without or with sucrose concentration (Fig. 3d, e, respectively) showed the same histological organization, i.e., a uni- biseriate and hypostomatic epidermis (St) and dorsoventral mesophyll composed of one layer of palisade parenchyma cells (Pp) and four to six layers of spongy parenchyma (Sp). Presence of specialized idioblast cells (Fig. 4f–j) and vascular bundles (Vb) dispersed throughout the mesophyll in plants submitted to all treatments (Fig. 4).

Plants grown under conventional conditions in sealed flasks showed wider intercellular gaps (Ig, red square) in reduced mesophyll, a chlorenchyma with no clear differentiation between palisade and spongy parenchyma (Pp, Sp, respectively), and underdeveloped vascular bundles (Vb) (Fig. 3a). In contrast, leaves of plants exposed to $CO_2$ enrichment (1000 µmol $mol^{-1}$) showed more developed vascular bundles, differentiated chlorenchyma cells (with differences in palisade and spongy parenchyma), and reduced intercellular gaps (Fig. 3d, e). Micromorphometric analysis revealed that plants maintained at 1000 µmol $mol^{-1}$ $CO_2$ with or without sucrose supplementation had 1.5 × thinner abaxial and adaxial epidermis (Fig. 3a–g). The mesophyll and leaf blade thickness of plants kept under 1000 µmol $mol^{-1}$ $CO_2$ and supplemented with sucrose matched those recorded during the other treatments, except for plants supplied with the same amount of $CO_2$, but no sucrose (Fig. 3h, i). In general, the gain in mesophyll thickness compared to adaxial and abaxial epidermis was common in plants maintained under conventional conditions in sealed flasks (Fig. 3a) or at 360 µmol $mol^{-1}$ $CO_2$ without or with added sucrose (Fig. 3b, c). Hence, photomixotrophy, 1000 µmol $mol^{-1}$ $CO_2$, and sucrose supplementation favor plant development (Figs. 1f, 3e).

Histochemical tests revealed the presence of storage compounds in the mesophyll cells of *E. elatior* leaves. Toluidine blue and Lugol's solution highlighted the presence of starch granules in the mesophyll and especially around vascular bundles, in plants grown under 1000 µmol $mol^{-1}$ $CO_2$ and sucrose supplementation (Fig. 4e).

In all treatments, Sudan black confirmed the presence of lipid bodies in idioblast cells (Fig. 4f–j). A positive reaction to xylidine Ponceau revealed the distribution of proteins in the mesophyll, especially around vascular bundles and in palisade parenchyma (Fig. 4k–o).

PAS staining confirmed the presence of carbohydrates, reflecting the results highlighted with toluidine blue and Lugol's tests (Fig. 4p–t). Carbohydrates in the form of starch granules were detected in the mesophyll and around vascular bundles. Treatment with 1000 µmol $mol^{-1}$ $CO_2$, lid with membrane, and 15 g $L^{-1}$ sucrose caused a more intense reaction to PAS staining. In these plants, carbohydrates were also observed in palisade and spongy parenchyma cells (Fig. 4t); however, they were less noticeable without $CO_2$ enrichment (Fig. 4q, s).

## 4 Discussion

This is the first study to describe the effect of $CO_2$ enrichment on the i*n vitro* propagation of *E. elatior*. The use of different $CO_2$ concentrations, sucrose levels, and sealing

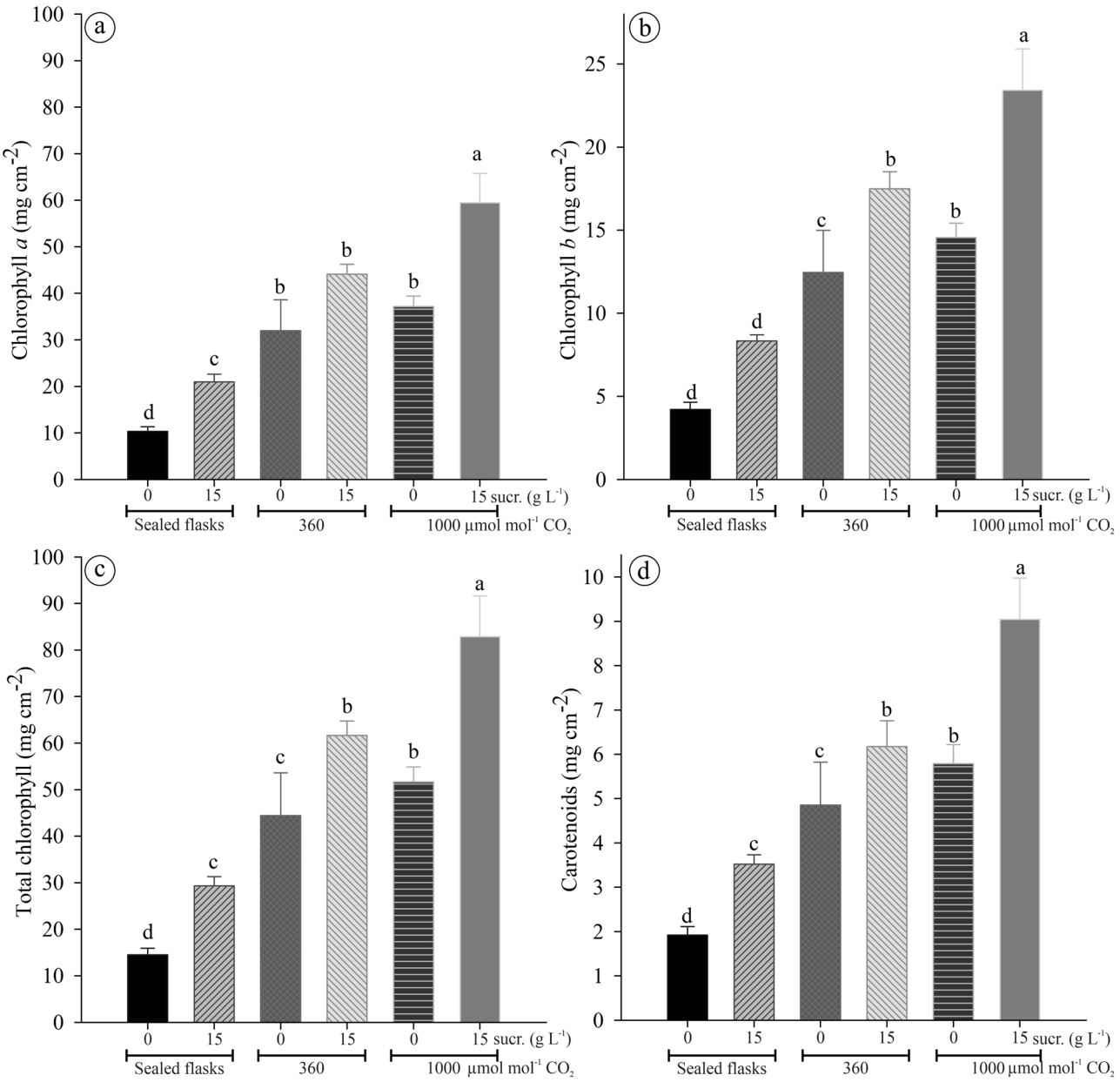


**Fig. 2** Photosynthetic pigments in leaves of *Etlingera elatior* plants maintained for 45 days through in vitro elongation and rooting at different $CO_2$ concentrations (360 and 1000 µmol $mol^{-1}$) and growth conditions (sealing type with different gas exchange rates and sucrose concentration in culture medium). **a** Chlorophyll *a* (mg $cm^{-2}$); **b** chlorophyll *b* (mg $cm^{-2}$); **c** total chlorophylls (mg $cm^{-2}$); **d** carotenoids (mg $cm^{-2}$). Error bars denote the standard error. Means followed by the same letter do not differ by the Scott–Knott test ($P \leq 0.05$)

methods led to notable differences in plant growth parameters. The use of gas-permeable membranes in association with $CO_2$ enrichment (360 or 1000 µmol $mol^{-1}$ $CO_2$) enhanced growth performance under photomixotrophic conditions, particularly when sucrose was added to the medium (Figs. 1d, 1f, 3c, 3e).

In vitro growth and development are affected by gas exchange between the flask headspace and the external ambient (Zobayed 2006; Saldanha et al. 2012, 2013) because they help maintain suitable $CO_2$ concentrations, decrease ethylene accumulation, and reduce relative humidity inside the flask. These changes are due to improved gas exchange efficiency which stimulates photosynthesis while positively influencing plant performance both when in vitro and in the ex vitro acclimatization (Kozai and Kubota 2001; Xiao et al. 2011). The use of gas-permeable membranes during the in vitro stage not only leads to more vigorous plants with improved biomass accumulation, higher growth and

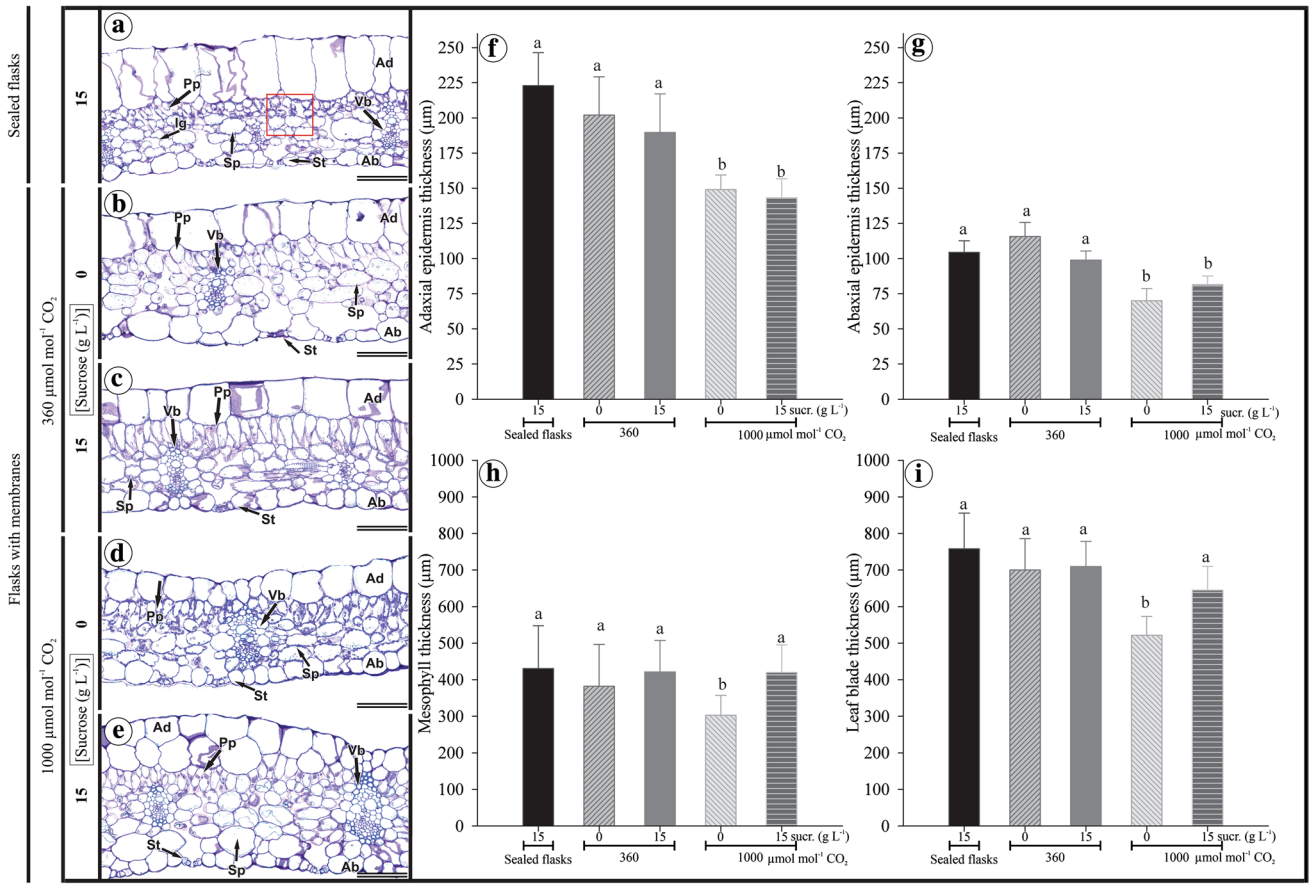


**Fig. 3** Cross sections of the middle third of fully expanded leaves of *Etlingera elatior* during in vitro elongation and rooting at different $CO_2$ concentrations (360 and 1000 µmol mol$^{-1}$) and growth conditions (sealing type with different gas exchange rates and sucrose concentration in culture medium). The specific treatments were as follows: sealed flasks without gas-permeable membranes and with **a** sucrose addition; flasks with $CO_2$ enrichment (360 µmol mol$^{-1}$), gas-permeable membranes, and without **b** or with **c** sucrose addition; with $CO_2$ enrichment (1000 µmol mol$^{-1}$), gas-permeable membranes, and without **d** or with **e** sucrose addition. *Abbreviations*: Ab, abaxial face of the epidermis; Ad, adaxial face of the epidermis; Ig, intercellular gaps; Pp, palisade parenchyma; St, stomata; Sp, spongy parenchyma; Vb, vascular system. Bars: a–e: 100 µm. Micromorphometric variables: adaxial and abaxial epidermis **f**, **g**, mesophyll **h**, and leaf blade thickness **i**. Error bars denote the standard error. Means followed by the same letter do not differ by the Scott–Knott test ($P \leq 0.05$)

photosynthetic rates, and larger, better-developed leaves and roots, but also stimulates nutrient absorption and promotes secondary metabolism (Ribeiro et al. 2009; Arigita et al. 2010; Kozai 2010; Badr et al. 2011; Iarema et al. 2012; Saldanha et al. 2013, 2014; Ríos-Ríos et al. 2019). Some of these benefits were confirmed here in plants under 1000 µmol mol$^{-1}$ $CO_2$, gas-permeable membranes, and sucrose in the culture medium.

Elevated gas exchange may induce a twofold increase in plant dry weight compared to the conventional in vitro culture system (Nguyen et al. 1999). Here, the fresh and dry weight increased when sucrose was added to the culture medium under higher gas exchange, and this supplementation was also essential for biomass and metabolite production. Without sucrose, there was little fresh and dry mass accumulation, regardless of the type of sealing or $CO_2$ concentration applied. Dry weight was greater when combining 15 g L$^{-1}$ sucrose with 1000 µmol mol$^{-1}$ $CO_2$ (Fig. 1i, j). The optimal concentration of sucrose depends on the plant species (Baque et al. 2012; Zhang et al. 2018; García-Ramírez et al. 2019), as well as gas exchange (Ríos-Ríos et al. 2019). Comparatively, shoot dry weight in *Pfaffia glomerata* (Spreng.) Pedersen (Saldanha et al. 2012) and *Piper crassinervium* Kunth (Ríos-Ríos et al. 2019) increased when gas-permeable membranes were used. The addition of sucrose to the culture medium is important as the explants depend on an energy source to enable the normal activity of physiological cell functions, such as respiration, and this dependence occurs because in the conventional in vitro propagation system the photosynthesis process does not normally occur due to the lack of $CO_2$ (Ayub et al. 2019), which can lead to high mortality rates during the acclimation stage. High addition of sucrose to the culture medium also reduces the water potential gradient and reduces water and nutrient absorption

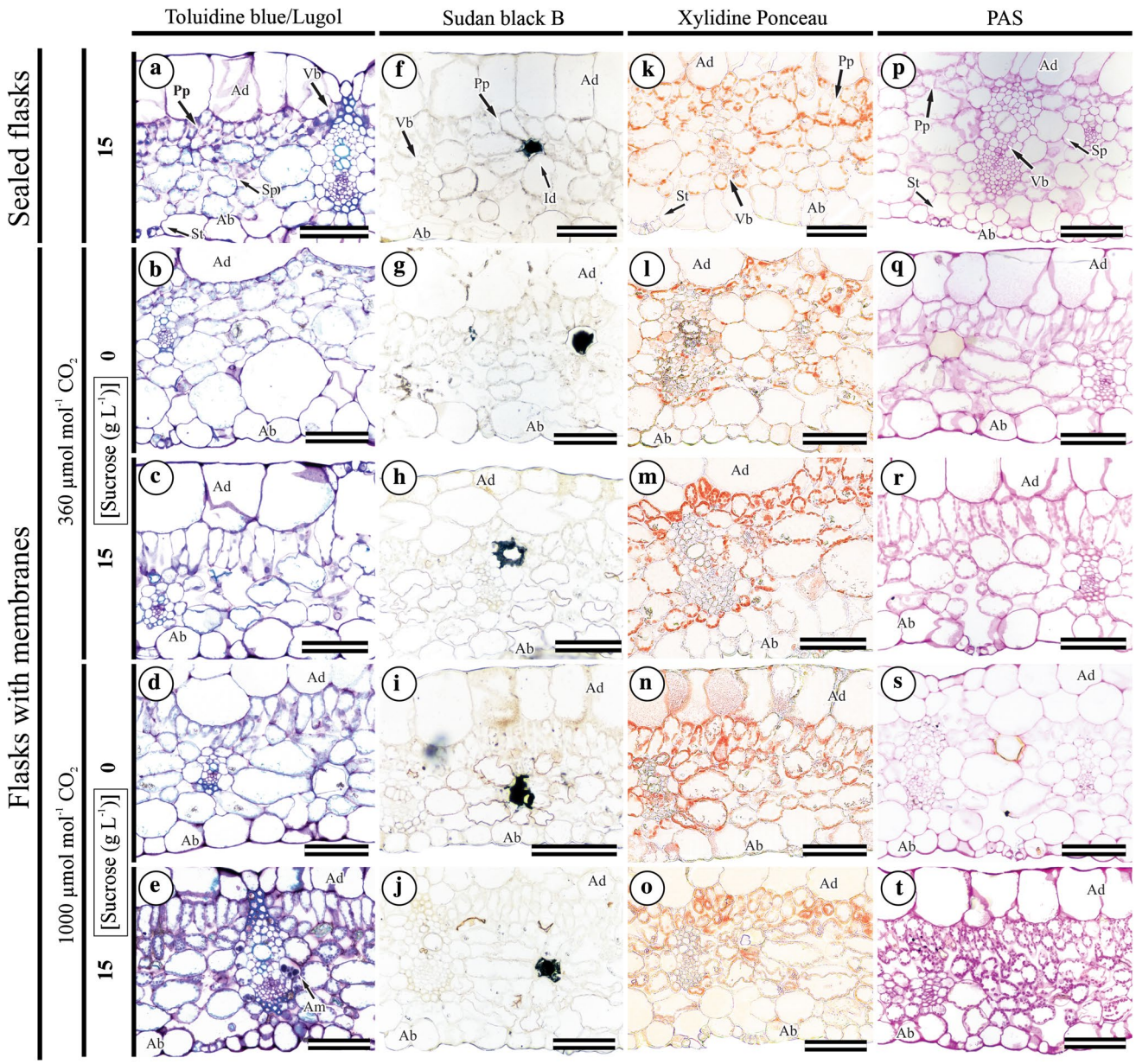


**Fig. 4** Histochemical study of the middle third of fully expanded leaves of *Etlingera elatior* during in vitro elongation and rooting at different $CO_2$ concentrations (360 and 1000 µmol $mol^{-1}$) and growth conditions (sealing type with different gas exchange rates and sucrose concentration in culture medium). The specific treatments were as follows: sealed flasks without gas-permeable membranes and with **a**, **f**, **k**, **p** sucrose addition; with $CO_2$ enrichment (360 µmol $mol^{-1}$), gas-permeable membranes, and without **b**, **g**, **l**, **q** or with **e**, **h**, **m**, **r** sucrose addition; flasks with $CO_2$ enrichment (1000 µmol $mol^{-1}$), gas-permeable membranes, and without **d, i**, **n**, **s** or with **e**, **j**, **o**, **t** sucrose addition. Cross sections of the midportion of leaves were subjected to combined double-staining with toluidine blue and Lugol's solution **a**, **b**, **c**, **d**, **e** showing positive (purple color) reaction to starch **e**, Sudan black B showing positive (dark color) reaction to lipids in idioblast cells **f**, **g**, **h**, **i**, **j**, xylidine Ponceau showing positive (red color) reaction to protein bodies **k**, **l**, **m**, **n**, **o**, and PAS showing negative reaction to carbohydrates **p, q, r, s, t**. *Abbreviations*: Ab, abaxial face of the epidermis; Ad, adaxial face of the epidermis; Am, amyloplasts (starch); Id, idioblast; Pp, palisade parenchyma; St, stomata; Sp, spongy parenchyma; Vb, vascular system. Bars: 100 µm

(Ayub et al. 2019), being one of the causes of occurrence of plant mortality (Badr et al. 2011). So far, there are no reports on changes in the concentration of sucrose associated with gas-permeable membranes in *Etlingera elatior*, and in this study gas-permeable membranes favored plant growth, under both photomixotrophic and photoautotrophic conditions, together with higher $CO_2$ concentrations.

The concentration of photosynthetic pigments is a good indicator of the functional status of the photosynthetic system (Alvarez et al. 2012), and this photosynthetic screening

of in vitro-grown plantlets is highly required before transferring plants to ex vitro conditions (Soni et al. 2021). Specifically, low photosynthetic pigment levels could affect plant performance during ex vitro acclimatization. Here, the use of 1000 µmol $mol^{-1}$ $CO_2$, gas-permeable membranes, and sucrose supplementation increased the levels of photosynthetic pigments (chlorophyll *a*, *b*, total chlorophylls, and carotenoids). The same was reported previously for *Persea americana* Miller, in which reduced sucrose levels associated with 1000 µmol $mol^{-1}$ $CO_2$ induced an increase in photosynthetic pigments and, consequently, in the photosynthetic rate (de la Viña et al. 1999). In *P. glomerata,* a higher pigment content was also associated with $CO_2$ enrichment, indicating that this condition stimulated photoautotrophic growth (Saldanha et al. 2013, 2014). Increasing gas exchange induces the biosynthesis of pigments in leaves grown in vitro (Ivanova and Staden 2010; Mohamed and Alsadon 2010; Saldanha et al. 2012). Comparatively, decreasing gas exchange inside the flask induces a decline in photosynthetic pigments, so there is less moisture loss and ethylene accumulation (Chanemougasoundharam et al. 2004; Saldanha et al. 2012; Zobayed et al. 1999), as observed in this study (Fig. 2a–d). Low photosynthetic pigment levels could, as a result, affect plant performance during the ex vitro acclimatization. That is, in vitro-grown plantlets with reduced photosynthetic potential show declined rate of survival under natural conditions (Soni et al. 2021).

Changes in the anatomy of *E. elatior* plants were observed during in vitro elongation and rooting at $CO_2$ enrichment (360 or 1000 µmol $mol^{-1}$ $CO_2$), sucrose levels, and sealing methods. Photomixotrophic conditions, including 1000 µmol $mol^{-1}$ $CO_2$, gas-permeable membranes, and sucrose addition, resulted in more vigorous plants with better-developed root and leaf systems and more developed secondary ribs in the leaves (Figs. 1f, 3e, 4e). The thickness of the epidermal faces and mesophyll varied according to treatment. Photomixotrophy associated with $CO_2$ enrichment (1000 µmol $mol^{-1}$ $CO_2$) promoted gains in mesophyll thickness and reduced adaxial and abaxial epidermis thickness (Fig. 3). Compared to conventional in vitro propagation, gas exchange yields more vigorous plants, with more differentiated anatomical structures (Ribeiro et al. 2009; Batista et al. 2017b), and, associated with sucrose reduction, increases plant survival during ex vitro acclimatization (Mohamed and Alsadon 2010).

The use of gas-permeable membranes and, consequently, efficient gas exchange results in more differentiated, organized cells and tissues (Zobayed et al. 2001a), while also promoting cell wall deposition, vascular system development (Mills et al. 2004), and differentiated palisade and spongy parenchyma cells (Zobayed et al. 2001b). These characteristics were observed in *E. elatior* plants in photomixotrophic conditions, especially in those grown under $CO_2$ enrichment (1000 µmol $mol^{-1}$ $CO_2$) and sucrose addition, where the superior morphophysiological characteristics of the plants consequently increased survival during ex vitro conditions. Therefore, anatomically well-developed leaves originating from plantlets grown in flasks with porous membranes and reduced sucrose levels increase plant survival during the ex vitro acclimatization stage (Mohamed and Alsadon 2010), due to the higher deposition of epicuticular wax on both leaf surfaces and better functioning stomata (Yoon et al. 2009).

Histochemical tests detected presence of specialized idioblast cells containing lipophilic compounds (Fig. 4f–j), a feature commonly observed in the leaves of Zingiberaceae species. These structures were also found in *Alpinia zerumbet* (Pers.) Burtt & Smith (Albuquerque and Neves 2004; Victorio et al. 2011) and in *Hedychium coronarium* J. Koenig (Martins et al. 2010) grown under field conditions. Here, starch granules were localized in the mesophyll and around vascular bundles in plants grown under photomixotrophic conditions (Fig. 4e). The observed starch distribution pattern probably reflects a boost in photosynthetic activity caused by $CO_2$ enrichment during the in vitro stage and may eventually impact survival of ex vitro-grown plants. The presence of starch during ex vitro acclimatization has been reported only in plants previously subjected to $CO_2$ enrichment (Yoon et al. 2009) and is associated with increased photosynthetic activity (Suthar et al. 2009). The accumulation of starch granules in the leaves facilitates the transfer of plants to ex vitro conditions, speeding up physiological adaptation and augmenting the success of acclimatization (Yoon et al. 2009).

*E. elatior* plants were transferred to ex vitro conditions to verify whether in vitro growth produced any morphophysiological gain that would allow them to better withstand the stress imposed by the change in growth conditions. Only plants subjected to $CO_2$ enrichment (1000 µmol $mol^{-1}$), covered with gas-permeable membranes, and supplemented with sucrose (15 g $L^{-1}$) maintained an elevated survival rate (75%). The gas exchange derived from the use of membranes and higher $CO_2$ concentration increase fresh and dry weight, ameliorate plant rusticity, facilitate the transition from in vitro to ex vitro, and improve overall plant quality (Batista et al. 2017a, b).

This study describes the use of $CO_2$ enrichment, sucrose supplementation, and different sealing methods to improve the development of *E. elatior* plantlets. The present findings reveal that *E. elatior* cv. Porcelain can be grown photomixotrophically by applying $CO_2$ enrichment (1000 µmol $mol^{-1}$), lids with gas-permeable membranes, and 15 g $L^{-1}$ sucrose in MS medium. These conditions promote better morphophysiological responses from the plants and contribute to their survival during ex vitro growth.

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## Declarations

**Conflicts of interest** The authors declare that they have no conflicts of interest.

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