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Physiological responses by *Billbergia zebrina* (Bromeliaceae) when grown under controlled microenvironmental conditions

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Sucrose, the most commonly used carbon source in conventional *in vitro* culture, and limited air exchange in the culture containers are factors that affect the growth of *in vitro*-cultured plants. They may induce physiological disorders and decrease the survival rate of plants after transfer to *ex vitro* conditions. The aim of the present study was to analyze the effects of gas exchange and sucrose concentration on *Billbergia zebrina* plantlets during *in vitro* propagation. *In vitro*-established *B. zebrina* plantlets were transferred to culture media containing 0, 15, 30, 45, or 60 g L^{-1} sucrose. Two different culture-container sealing systems were compared: lids with a filter (permitting gas exchange) and lids with no filter (blocking fluent gas exchange). Carbohydrate and chlorophyll (Chl a+b) concentrations were analyzed in plantlets at 45-days of culture. The addition of sucrose to the medium reduced the Chl a+b concentration in the plantlets. On the other hand, additional sucrose had a positive effect on the carbohydrate stock formation of the plantlets. The results showed that a photoautotrophic system (air exchange and a sugar-free medium) improves the *in vitro* propagation of *B. zebrina* without creating physiological disorders.

Key words: Bromeliad, *In vitro* plant, photoautotrophic growth, physiological disorders, sucrose.

INTRODUCTION

Plant micropropagation is the most common method used for the large-scale cloning of several horticultural crops, such as bulbous plants, fruit trees, and ornamentals. Bromeliads that are grown as flowering, potted ornamentals have a high commercial value and account for a large part of the flower industry worldwide (Zhang et al., 2012). *Billbergia zebrina* (Herbert) Lindley is an epiphytic tank bromeliad native to the Atlantic Rainforest of Brazil. This plant has commercial value as an ornamental due to the quality its leaves and

inflorescence (Vesco et al., 2011).

The application of plant tissue culture techniques to *in vitro* propagation of bromeliads has been reported previously (Huang et al., 2011; Martins et al., 2014; Resende et al., 2016; Viehmannova et al., 2016). Most of these studies are related to the use of plant growth regulators as major modulators of *in vitro* morphogenetic responses. In bromeliads, multiplication and rooting are strongly controlled by external plant growth regulators (Van Dijck et al., 1988).

In vitro culture conditions are thought to be stressful to plants (Desjardins et al., 2009) and may influence plant morphogenesis (Shin et al., 2013). Conventional *in vitro* propagation has been shown to induce plant anatomical and physiological disorders (Mohamed and Alsadon, 2010; Iarema et al., 2012), and it may interfere with the growth and survival rates of plants after transfer to *ex vitro* conditions (Fuentes et al., 2005; Shin et al., 2014).

In vitro plant disorders are directly or indirectly related to the heterotrophic conditions in a conventional micropropagation system, where sugar in the culture medium is the main cause of these disorders (Hazarika, 2006). Conventional *in vitro* propagation is mostly carried out using small, closed glass culture containers and the media most often contains sucrose as the major carbon source (Xiao et al., 2011). This external sugar supply is adequate for growth and organogenesis support (Hazarika, 2003). For bromeliads, 3% sucrose in the medium is recommended (Pérez et al., 2013; Martins et al., 2014; Resende et al., 2016). Sucrose effects on the physiology of *in vitro* plants have been previously documented (Iarema et al., 2012; Shin et al., 2014). Reduced photosynthetic ability (Shin et al., 2013), and plant survival and growth rates during later acclimatization periods are closely related to previous sucrose treatments (Mohamed and Alsadon, 2010; Shin et al., 2014). However, supplementing sugars positively affects carbohydrate stock formation by *in vitro* propagated plants (Ferreira et al., 2011) and a high carbohydrate stock improves plant performance during the acclimatization phase (Fuentes et al., 2005).

Limited gas exchange, caused by the type of culture containers used, affects the development of *in vitro* grown plants (Martins et al., 2015a). The *in vitro* environment is characterized by high relative humidity, potential ethylene build up, stagnant air, and a fluctuating CO_2 concentration caused by day and night cycles (Martins et al., 2015b). The CO_2 concentration has a considerable impact on photosynthesis and the growth of *in vitro* plants. It also affects plant metabolism (Iarema et al., 2012; Shin et al., 2013). Gas exchange improves in ventilated culture containers and this helps *in vitro* plantlets to grow photomixotrophically or even photoautotrophically, which results in improved plant quality and less propagule loss during the acclimatization process (Zobayed et al., 2000; Shin et al., 2014). Previous studies have indicated that the *in vitro*

photoautotrophic growth (sugar-free with gas exchange) of many plant species can be significantly improved by increasing the CO_2 concentration in the culture vessel and reducing the relative humidity (Xiao et al., 2011). Recently, we reported the effect of *in vitro* conditions on the growth and anatomy of *B. zebrina* plantlets (Martins et al., 2015b). We found that conventional *in vitro* culture induced anatomical plantlet leaf disorders and that these disorders had a negative effect on the acclimatization period. However, it is not clear how microenvironmental conditions influence the physiology of *in vitro* propagated bromeliads.

The aim of this study was to analyze the effects of gas exchange and sucrose concentration on the physiology of *B. zebrina* plantlets during *in vitro* propagation.

MATERIALS AND METHODS

Plant materials and culture conditions

B. zebrina plantlets, which had previously been established *in vitro* using seeds, were transferred to 250 mL glass containers containing 50 mL stationary, liquid Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 30 g L^{-1} sucrose and 3 mg L^{-1} 6-benzylaminopurine (Martins et al., 2015a). After 30 days growth, side shoots were sub-cultivated for 45 days in 250 mL glass containers containing 50 mL stationary, liquid MS medium with no plant growth regulator, but supplemented with 30 g L^{-1} sucrose. The medium pH was set at 5.8, before autoclaving at 120ºC for 20 min. After sterile inoculation, the plant cultures were kept in a growth room at 26 ± 2ºC with a 16-h photoperiod (8:00 to 00:00 h) under fluorescent lamps (Philips Master TL5 HO, 49W/840) that provided 90 µmol m^{-2} s^{-1} of PAR light.

Sucrose and gas exchange during *in vitro* propagation

B. zebrina side shoots that were approximately 4.0 cm in length were taken from the plantlets propagated in the previous *in vitro* stage. They were individualized (5 to 8 side shoots per seedling) with the aid of a scalpel and transferred to 280-mL polypropylene containers (ECO2 NV®, Geraardsbergen, Belgium) containing 50 mL MS medium solidified with 7 g L^{-1} agar and supplemented with 0, 15, 30, 45, or 60 g L^{-1} sucrose. Each container received five shoots. The pH was adjusted to 5.8 before autoclaving at 120°C for 20 min. Two different sealing systems were used: Container lids with an XXL filter (permitted gas exchange - minimum of 63 air exchanges per day) and the same container lids covered with two layers of polyvinyl chloride (PVC) transparent film (blocking gas exchange by the XXL filter exchange - 4.19 gas replacements per day). After sterile inoculation, the cultured plants were kept for 45 days in a growth room at 26 ± 2ºC with a 16-h photoperiod (8:00 to 00:00 h) and under fluorescent lamps (Philips Master TL5 HO, 49W/840) that provided 230 µmol m^{-2} s^{-1} of PAR light.

Chlorophyll extraction and analyses

The aerial parts of five plants per treatment were divided into five independent samples. After weighing, the chlorophyll was extracted from the fresh plant samples (leaf discs) by incubating them in the dark for 72-h in dimethyl-formamide. The absorbance at 647 and 663 nm was measured by a spectrophotometer (UV-1800, Shimadzu, Japan). Final determination of the chlorophyll
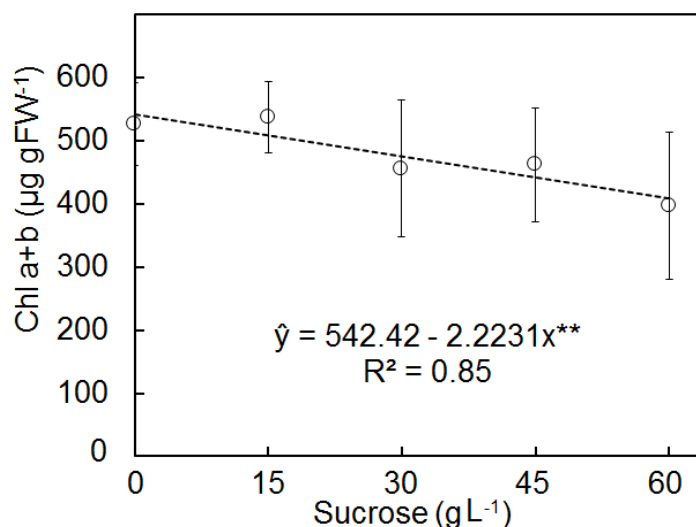
Figure 1. Chlorophyll a+b concentration of leaves taken from *B. zebrina* plantlets exposed to different sucrose concentrations.

concentration was based on Wellburn (1994) and expressed as µg gFW^{-1} (fresh weight) of tissue.

Extraction and analyses of plant metabolites

Fifteen plants per treatment were collected at 6:00 h. After mixing the plant material, three samples were taken. The leaves and roots were collected and given an identification mark with the aid of a scalpel. All the samples were immediately frozen in liquid nitrogen. The plant material (five plant mix) was lyophilized and crushed before taking a known amount of plant material for the metabolite analyses. An ENZYTEC system (Enzytec, Scil Diagnostics GmbH, Dormstadt, Germany), along with a spectrophotometer (DU-65; Beckman, Fullerton, CA, USA), set at 340 nm, were used to determine the malic acid, glucose, fructose, and sucrose concentrations in the leaves and roots. The protocol described by Ceusters et al. (2008) was followed. Starch concentration was determined as glucose equivalents after digestion with amylo-glucosidase according to the protocol described by Enzytec, Scil Diagnostics. Analyses were performed on three independent biological samples. The glucose, fructose, and sucrose concentrations in the culture media were quantified by collecting three independent samples per treatment from the different containers and performing the above mentioned analyses. The concentration of plant metabolites was expressed as µmol gDW^{-1} (dry weight) in the leaves and roots. Carbohydrate concentration in the culture media was expressed as µmol gFW^{-1} (fresh weight).

Water loss in the containers

Five containers from each treatment were sampled randomly to evaluate water loss. They were weighed at 0 and 45 days, and the differences in the weights were used to determine water loss (%).

Statistical analysis

The experiment had a completely randomized design in a factorial arrangement (five sucrose concentrations × two sealing systems). The data obtained were submitted to two-way analysis of variance

Table 1. Carbon metabolite concentrations (µmol gDW^{-1}) in *B. zebrina* shoots at the start of incubation on different sucrose concentrations and different culture container aeration Samples were taken at 6:00 am.

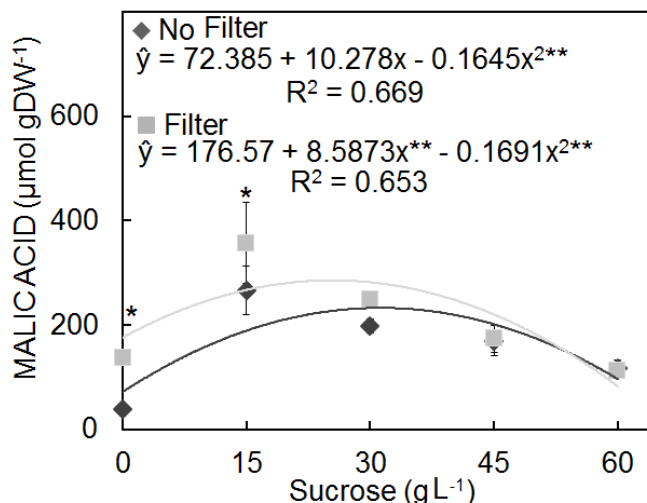| Metabolites	Concentration (µmol gDW^{-1})
Malic acid	112 ± 13
Glucose	890 ± 14
Fructose	386 ± 34
Sucrose	nd*
Starch	573 ± 223

*Not detectable.

(ANOVA), the averages of the factor sealing systems were compared using Tukey's test, and the sucrose concentrations were subjected to regression analysis.

RESULTS

Chlorophyll a+b (Chl a+b) levels decreased as the sucrose concentration rose, and this was independent of the sealing system (Figure 1). However, when air exchange was possible (filter containers), plant Chl a+b concentrations (517.79 µg gFW^{-1}) were higher than for the plants grown in containers without filters (433.65 µg gfw^{-1}).

The initial levels of the different metabolites in *B. zebrina* shoots at the start of the treatments are shown in Table 1. The monosaccharide (glucose and fructose) concentrations were high in the shoots, but sucrose was not detected. Starch, expressed in glucose equivalents, was present at similar levels as the glucose and fructose. *B. zebrina* is a CAM plant, and hence malic acid was also

◆ No Filter
$\hat{y} = 72.385 + 10.278x - 0.1645x^2**$
$R^2 = 0.669$

■ Filter
$\hat{y} = 176.57 + 8.5873x** - 0.1691x^2**$
$R^2 = 0.653$

Figure 2. Malic acid concentration of leaves taken from *B. zebrina* plantlets exposed to different sucrose concentrations and to one of two different ventilation treatments. For each sucrose concentration, means followed by an asterisk differ from each other at the $P \leq 0.05$ level according to Tukey's test.

detected in the leaves.

Malic acid concentration had a positive quadratic relationship with increasing sucrose concentrations for both container sealing systems at 45-days growth. Plants cultivated in filter containers on the sugar-free medium or with 15 g L^{-1} sucrose had higher malic acid levels than plants subjected to the same sucrose concentrations, but grown in containers without filters (Figure 2).

The glucose and fructose concentrations of the leaves had positive linear, but negative quadratic relationships with increasing sucrose concentration, respectively, when they were cultivated in no filter and filter containers. Plants grown in a container with no gas exchange and on a medium with 60 g L^{-1} sucrose had the highest glucose concentration (Figure 3A). Conversely, the fructose concentration was highest in plants cultivated on the sugar-free medium and in an aerated container (Figure 3C). The monosaccharide concentrations were generally lower in the roots than in the leaves. The glucose levels in the roots showed a positive linear relationship with sucrose levels, which was independent of the container sealing system (Figure 3B). However, the fructose concentration was higher in plants grown in aerated containers and there was an improvement in the carbohydrate concentration when the sucrose concentrations rose (Figure 3D).

The sucrose concentration was higher in the roots than in the leaves for all sucrose levels. Sucrose levels for both plant tissues had linear relationships with increasing sucrose concentration in the medium (Figure 4A and B). Starch concentrations were higher in the leaves than in the roots after 45-days *in vitro* growth. The starch increase was positively related to rises in the sucrose

concentration. The highest starch concentration was recorded in the leaves of plants grown in containers with aeration and a high sucrose concentration in the medium. However, the starch concentration in the roots was low for all plants grown in containers with no aeration (Figure 4C and D).

The carbohydrate concentrations in all the nutrient media were analyzed. Glucose and fructose presented similar course in function of the sucrose level at the start of the culture time. They presented quadratic and linear models with increasing sucrose concentrations when they were cultivated in the presence or absence of a filter, respectively (Figure 5A and B). Sucrose in the medium showed a linear relationship with sucrose added in the medium at the beginning of the culture time, showing a higher accumulation when the air exchange potential was high (Figure 5C). All sugars analyzed in the media were higher in aerated containers than the ones with no aeration. Because sucrose uptake is dependent on the sucrose level of the media more sucrose has been hydrolysed in the leaves resulting in more free glucose and fructose.

All treatments were also performed in containers without plants and the carbohydrate concentrations measured at 45-days under the same growth conditions. As shown in the Table 2, there were differences in the carbohydrate concentrations between container sealing systems. It was correlated to dehydration of medium. Water loss occurred with both sealing systems, and this was independent of sucrose concentration. However, medium dehydration was higher in the aerated containers (Figure 6).

The morphology of *B. zebrina* plants was also
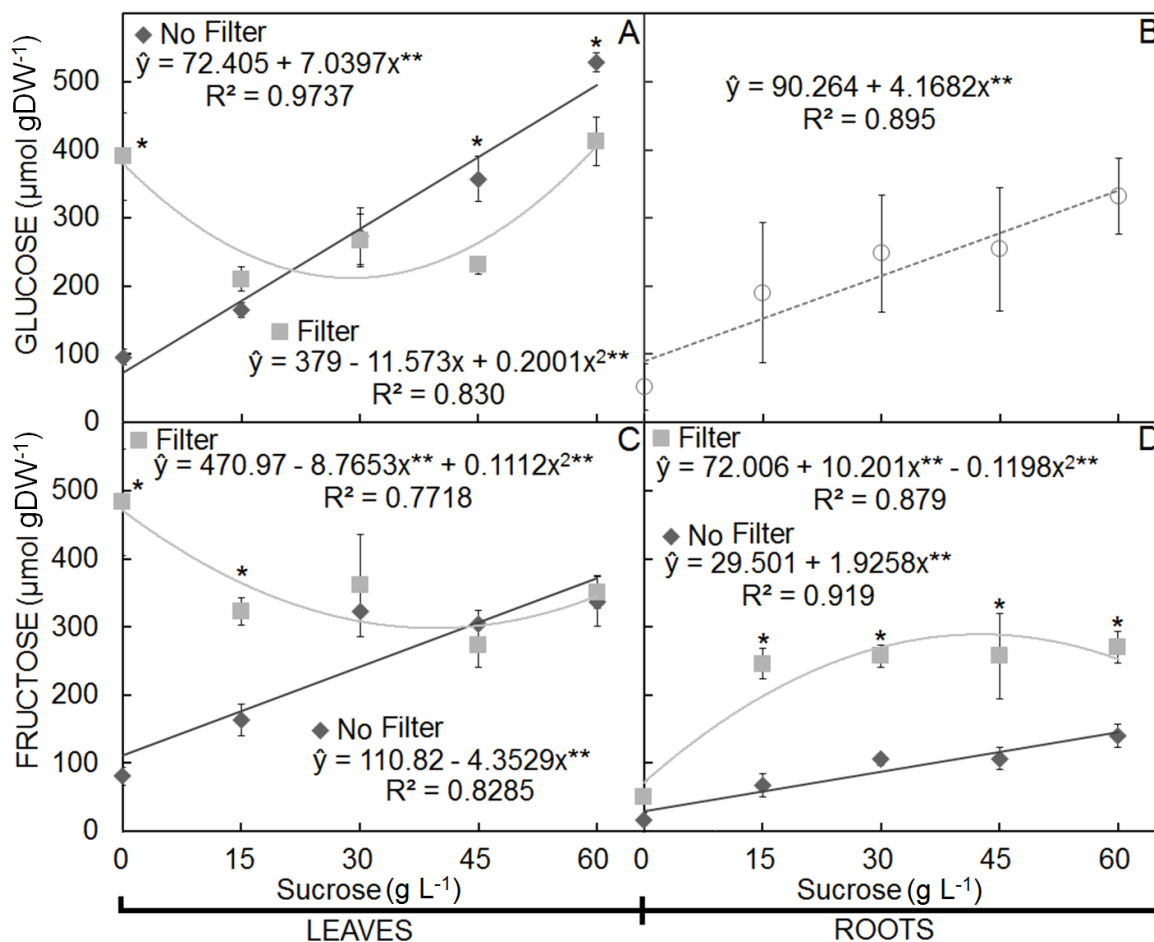
Figure 3. Glucose (A-B) and fructose (C-D) concentrations in leaves and roots at the end of the dark period (6:00am) for *B. zebrina* plants cultivated in media containing different sucrose concentrations and subjected to one of two different ventilation treatments. For each sucrose concentration, means followed by an asterisk differ from each other at the P≤ 0.05 level according to Tukey's test.

influenced by sucrose concentration and the gas exchange system. Growth was more vigorous under photoautotrophic conditions (sugar-free media and gas exchange). In contrast, plants cultivated with sucrose concentrations higher than 30 g L^{-1} had a low growth rate (data not shown) and showed leaf chlorosis, followed by necrosis (Figure 7).

DISCUSSION

B. zebrine plants showed different physiological responses to the different sealing systems and sucrose concentrations. *In vitro* plants may have poor chlorophyll concentrations, as occurred in the *B. zebrina* plants. This is because of the exogenous supply of sucrose, which does not promote the normal development of a photosynthetic apparatus. Although such plants may appear normal, their photosynthetic apparatus may not be active (Hazarika, 2006). A reduction in chlorophyll

causes a fall in light absorption and therefore provides less ATP and NADPH for the dark reactions (Sivanesan et al., 2008). In this respect, chlorophyll is a good first and easy-to-measure indicator of photosynthetic potential and apparatus status (Alvarez et al., 2012; Sáez et al., 2012). Lower energy inputs correlate well with the biochemical processes related to the quantity and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Koch, 1996). The insufficient supply of ribulose-1,5-biphosphate may also be related to increased plant susceptibility to feedback inhibition, which is possibly associated with an excessive accumulation of hexoses and starch (Le et al., 2001). This may explain the results for the *B. zebrina* plants, which had low chlorophyll levels (Figure 1), but high carbohydrate concentrations (Figures 3 and 4).

B. zebrina plants produced malic acid in all treatments, including shoots cultivated in the liquid media. Modulation of the CAM pathway occurs in the bromeliad *Guzmania monostachia*, depending on the water supply level
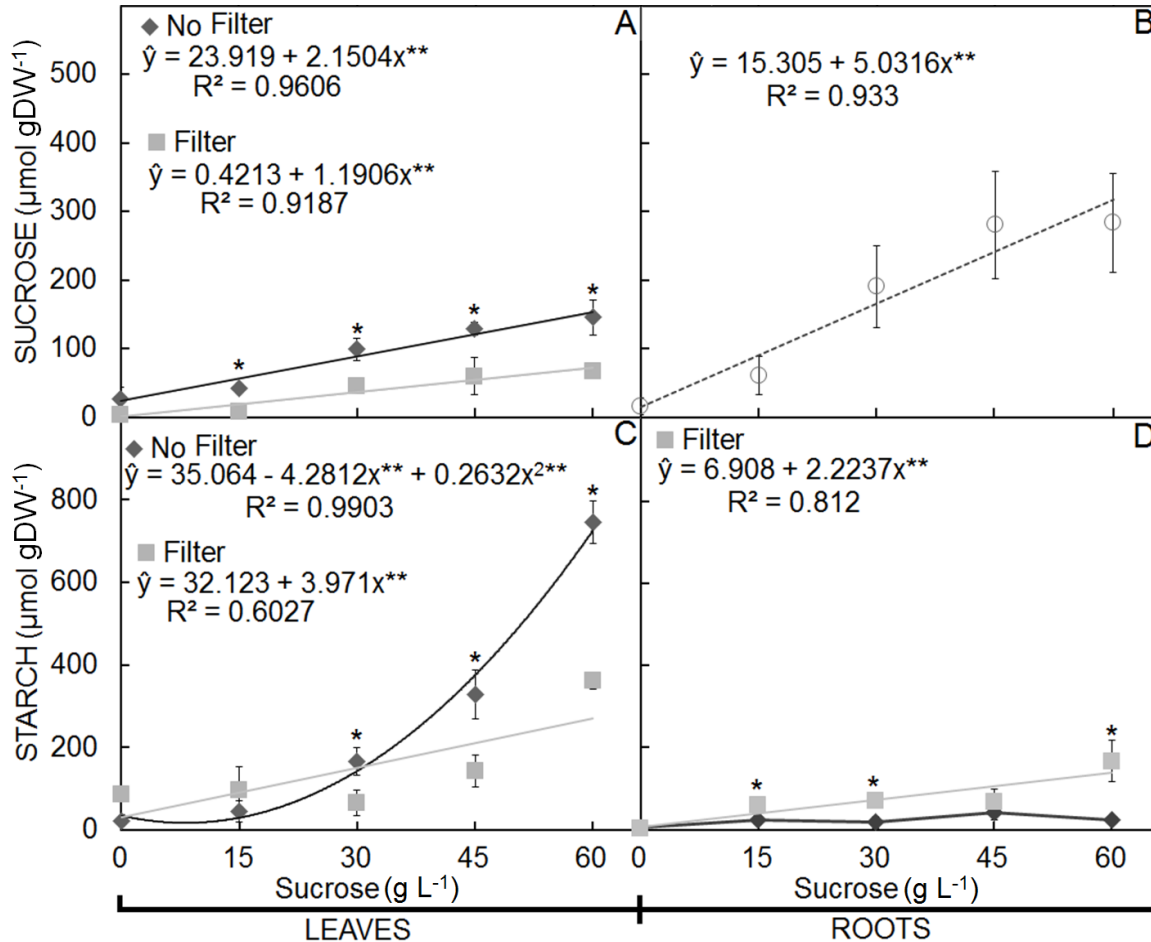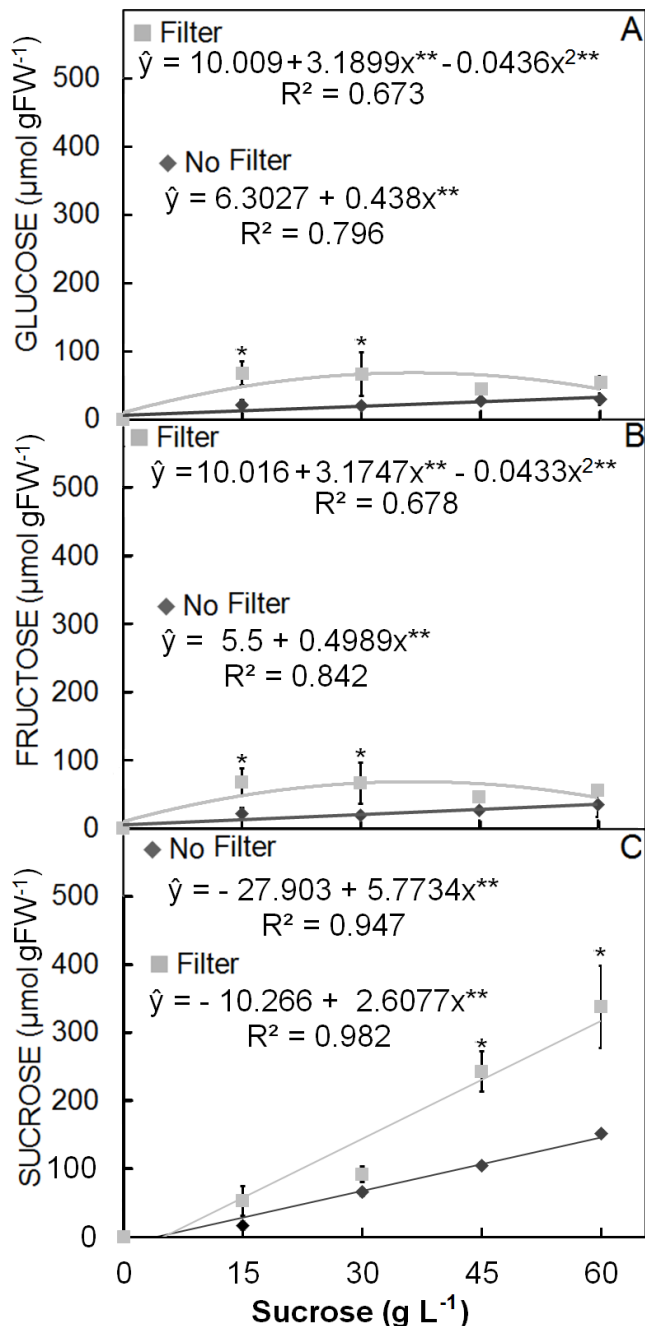
Figure 4. Sucrose (A-B) and starch (C-D) concentrations in leaves and roots at the end of the dark period (6:00am) for *B. zebrina* plants cultivated in media containing different sucrose concentrations and subjected to one of two different ventilation treatments. For each sucrose concentration, means followed by an asterisk differ from each other at the P≤ 0.05 level according to Tukey's test.

(Pereira et al., 2013). The decrease in malic acid concentration observed in plants cultivated in media where the sucrose concentrations were higher than 30 g L^{-1} was probably was due to the osmotic stress caused by the media. Sucrose concentrations higher than 30 g L^{-1} in a medium may induce osmotic stress when cultivating *in vitro* plants (Cui et al., 2010). Photosynthesis is very sensitive to stress (Walters, 2005), and malic acid concentration has been shown to fall in leaves subjected to a long period of water stress, for example in the CAM plant *Aechmea* 'Maya' (Ceusters et al., 2009). These authors suggested that the roots might be involved in the metabolic response to water limitation. In this study, the lowest malic acid concentration (39.35 µmol gDW^{-1}) was found in plants grown in the sugar-free medium (no osmotic stress), but this may be related to insufficient availability of CO_2. An increased CO_2 concentration improves the rate of malic acid formation and accumulation in the cytoplasm by PEPc (Zobayed et al., 2000). The increase in malic acid concentration in

Doritaenopsis occurred when additional CO_2 was available during *in vitro* photoautotrophic culture (Shin et al., 2013). During CAM activity, the glycolytic breakdown products of storage polysaccharides (e.g., starch) or soluble sugars (e.g., glucose, fructose, and sucrose) can be used for the production of the CO_2 acceptor phosphoenolpyruvate (PEP) during the dark period (Ceusters and Borland, 2011). This explains the results for the plants grown in the sugar-free medium and in the no ventilation containers, since those plants did not have enough carbohydrate stock to regenerate the PEPc enzyme.

Sucrose concentrations in the *in vitro* media created higher monosaccharide and sucrose stocks in the leaves and roots (Figures 3 and 4). Sucrose addition to the plant tissue cultures reduced the water potential of the media and increased the leaf tissue glucose, fructose, sucrose, and starch concentrations in a dose dependent manner. Monosaccharides are effective osmotic agents in plants because osmotic stress in roots can increase

Figure 5. Carbohydrate concentrations in the culture media at 45-days in containers with *B. zebrina* plants. For each sucrose concentration, means followed by an asterisk differ from each other at the P≤ 0.05 level according to Tukey's test.

showed that the photosynthetic apparatus was performing well. The presence of organic reserves and a functional photosynthetic apparatus in the *in vitro* plants should improve acclimatization performance (Shin et al., 2013, 2014).

Sugars are crucial for building compounds in plants and are a key source of energy that can be used to induce biochemical processes (Piotrowska et al., 2010). *B. zebrina* plants cultivated under the sugar-free and no ventilation conditions had the lowest carbohydrate stocks due to the limited photosynthesis rate. Carbohydrates are required by plant cells as carbon resources, and supply energy for growth and biosynthetic processes (Ferreira et al., 2011). Increasing the sucrose levels in the media produced plants with higher starch concentrations (Figure 4C and D). Plants cultured with high sucrose concentrations during *in vitro* growth may have larger and higher numbers of starch granules in their chloroplasts (Capellades et al., 1991). The main reserves of carbon, and therefore energy, are sucrose and starch. When the export rate of sucrose is lower than the sucrose synthesis rate, an accumulation of hexoses and triose-phosphates occurs, and as a result, starch synthesis begins in the chloroplasts during the light period (Dennis and Blakely, 2000).

The use of containers with unlimited air exchange produced plants with lower starch concentrations. High osmotic stress, induced by dissolved sugars in the media, can limit starch formation. In general, under osmotic and drought stress, soluble sugars tend to increase, while starch concentration decreases (Chaves, 1991). Starch does not directly act as an osmoprotectant because it is not soluble in water. *B. zebrina* plants decreased the water osmotic potential in their roots by increasing the soluble sugar content. Lower starch and higher soluble sugar contents could have facilitated water flux from the medium into the plants.

At 45-days growth, the *B. zebrina* plants had not consumed all the added sucrose in the media, even in those without air exchange. Other plant species have also shown a low sugar reserve consumption in the same culture medium (Palonen and Junttila, 1999). After plants are transferred to the culture medium, they may hydrolyze sucrose into glucose and fructose using invertase that has been released into the culture medium (Karhu, 1997). This extracellular enzymatic system, which is associated with sucrose hydrolysis, is initiated by the presence of plant tissues, but can remain active even when plants are not present, which increases the fructose and glucose concentrations in the medium (Tremblay and Tremblay, 1995). Sucrose was hydrolyzed to glucose and fructose, which nearly doubled the osmolality of the medium (Bishnoi et al., 2000), and in this study, sucrose hydrolysis increased the osmolality of the media containing additional sucrose. This effect was probably even higher when combined with water loss from the media in containers with a filter, which would reduce the

monosaccharide accumulation (Xie et al., 2009). Sugars, especially glucose, fructose, and sucrose, play an important role in stress alleviation through the regulation of plant osmotic potential (Polanco et al., 2014). Under photoautotrophic conditions, the plants had high monosaccharide concentrations in the leaves (Figure 3) and the accumulation of different carbohydrates

Table 2. Carbohydrate concentrations in the culture media at 45-days in containers without plants.

Sucrose (g L⁻¹)	Carbohydrate concentrations in the culture media without plants					
	Glucose (µmol/gfw)		Fructose (µmol/gfw)		Sucrose (µmol/gfw)	
	No filter	Filter	No filter	Filter	No filter	Filter
0	0.00±0.0ᵃ*	0.00±0.0ᵃ*	0.00±0.0ᵃ*	0.00±0.0ᵃ*	0.00±0.0ᵃ*	0.00±0.0ᵃ*
15	1.37±0.4ᵃ	3.12±0.8ᵃ	1.33±0.4ᵃ	2.94±0.7ᵃ	50.41±8.1ᵃ	74.72±19.9ᵃ
30	2.70±0.7ᵇ	8.37±1.9ᵃ	2.55±0.7ᵇ	7.86±1.8ᵃ	78.67±12.9ᵇ	187.57±39.6ᵃ
45	3.32±0.4ᵇ	10.74±2.8ᵃ	3.09±0.3ᵇ	10.16±2.5ᵃ	144.81±13.9ᵇ	232.55±62.7ᵃ
60	7.48±0.1ᵇ	13.32±2.6ᵃ	7.07±0.1ᵇ	12.53±2.4ᵃ	159.70±2.5ᵇ	279.57±50.7ᵃ

Averages followed by the same letter in the row for each carbohydrate concentration, do not differ according to the Tukey's test, at 5%.
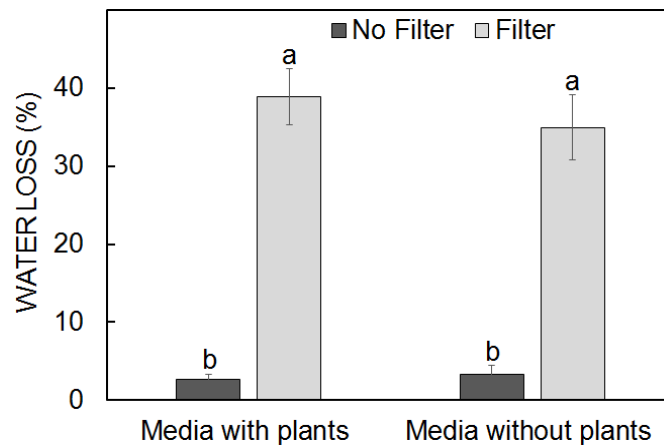
Figure 6. Water loss in containers with and without plants due to the sealing system after 45 days of growth. For each plant culture system, means followed by different letters differ from each at the P≤ 0.05 level according to Tukey's test.
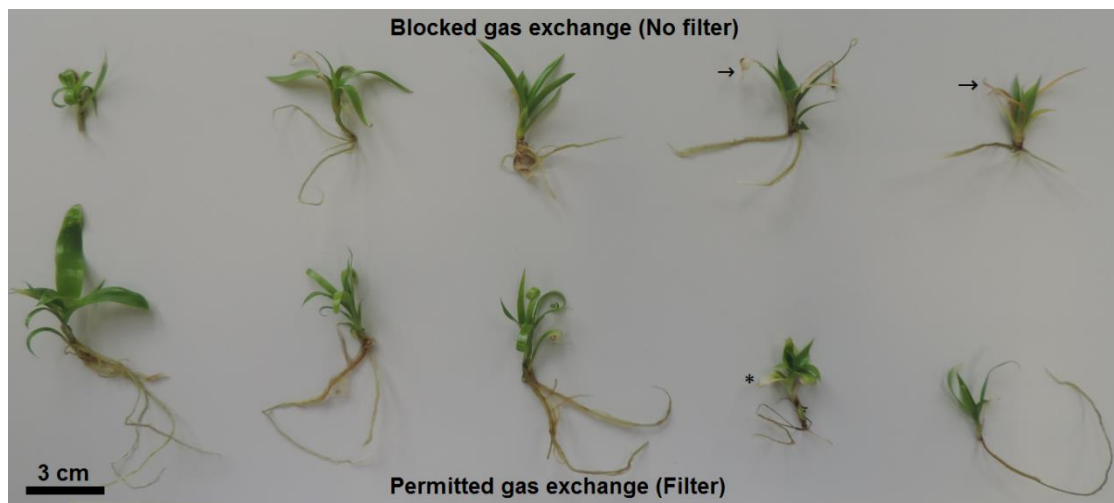
Figure 7. Visual aspects of *B. zebrina* plants after 45 days of *in vitro* growth. The plantlets had been exposed to different media sucrose concentrations (0, 15, 30, 45, or 60 g L⁻¹ sucrose, from left to right) and one of two ventilation treatments. Leaf chlorosis and necrosis are shown by an asterisk and an arrow, respectively.

solvent content.

Plants under environmental stress, e.g. osmotic stress, may show membrane lipid peroxidation, which increases cell membrane permeability and extravasation of cell-soluble substances (Zeng et al., 2006). Our results showed that plants grown under ventilated conditions and in media with 45 or 60 g L^{-1} sucrose showed leaf senescence and a reduced growth rate. The increased chlorosis and necrosis may indicate that the plants were under osmotic stress.

Conclusion

In vitro culture conditions influenced the physiology of *B. zebrina* plantlets during micropropagation. Sucrose in the culture medium led to reduced chlorophyll concentrations and increased starch concentrations. Adding sucrose osmotically stressed *B. zebrina.* Ventilating the culture containers increased the stress responses because water loss was greater and it also led to increased media sugar concentration. Plants grown under photoautotrophic conditions (air exchange and sugar-free) did not show any physiological disorders and photoassimilate production was normal.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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