Australian Journal of Crop Science AJCS

AJCS 9(1):85-91 (2015) ISSN:1835-2707

Effects of salts and sucrose concentrations on *in vitro* propagation of *Billbergia zebrina* (Herbert) Lindley (Bromeliaceae)

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Abstract

Tissue culture can contribute to the multiplication of several species with commercial interest, such as bromeliads. This study aimed to evaluate the effects of MS-salts (Murashige and Skoog) and sucrose concentrations on the *in vitro* multiplication and rooting of *Billbergia zebrina,* respectively. The *in vitro*-established *B. zebrina* plants were inoculated on MS-salts at concentrations of 0%, 50%, 100%, 150% or 200% of the original salt concentration of the medium. The media were prepared in two different consistencies, stationary liquid and 6 g L^{-1} agar. For *in vitro* rooting studies, the shoots grew in a medium supplemented with 0, 15, 30, 45 or 60 g L^{-1} sucrose. Soluble carbohydrates and photosynthetic pigment contents were assessed after the rooting. Significant differences were verified observed in the evaluated characteristics due to the treatments. The use of liquid medium and the 200% concentration of MS-salts induced the highest shoot number per explant (23.94 shoots) and 100% budding. The explants cultivated in liquid and solid medium without MS-salts did not display shoot formation. High sucrose concentrations (45 and 60 g L^{-1}) induced greater root numbers and higher carbohydrate stocks, but shorter plants with a reduction of photosynthetic pigments content compared to plants grown on medium without sucrose. The *in vitro* multiplication of *B. zebrina* shoots is enhanced by using 200% of MS-salts concentration and liquid medium. The use of 15 g L^{-1} sucrose increased endogenous carbohydrate stocks and induced a good formation of the root systems in *in vitro* shoots.

Keywords: bromeliad; ornamental plant; plant propagation; *in vitro* rooting; tissue culture.
Abbreviations: ANOVA_ analysis of variance; BA_ 6-benzilaminopurine; MS_ Murashige and Skoog medium.

Introduction

The Bromeliaceae family is highly diverse, with terrestrial, rock and epiphytic species (Benzing, 2000). Many bromeliads have commercial value as ornamental plants, due to the beauty of their leaves and flowers (Vesco et al., 2011). For this reason, illegal gathering has been carried out in natural environments for the purpose of supplementing income. Illegal gathering is threatening some species with extinction (Negrelle et al., 2012). Among them is *Billbergia zebrina* (Herbert) Lindley, which is classified as endangered according to the list of threatened species (Martinelli et al., 2008; Vesco et al., 2011). *In vitro* propagation techniques have been widely used for rapid multiplication of various economically important plant species or endangered species, such as those in the Bromeliaceae family (Guerra and Vesco, 2010). Several studies related to the *in vitro* culture of bromeliads have been published, such as Chu et al. (2010), Huang et al. (2011), Silva et al. (2012) and Martins et al. (2013). However, most of these previous studies relate to the use of plant growth regulator as the main modulator of *in vitro* morphogenetic responses of bromeliads. Mineral compounds in the culture medium play an important role in regeneration processes (Ramage and Williams, 2002). The culture medium nutrient requirements may vary and function differently based on the species or the technique used. These differences have led to modifications to existing media or even new formulations (Greenway et al., 2012). The formulation created by Murashige and Skoog (1962) is used most commonly for *in vitro* culture of bromeliads. It has already been employed during the direct organogenesis of *Neoglaziovia variegata* (Arr. Cam.) Mez (Silveira et al., 2009), *Neoregelia cruenta* (R.Graham) L.B.Sm., *Tillandsia stricta* Sol., *Vriesea gigantea* Gaudich., *V. guttata* Linden & André, *V. incurvata* Gaudich. (Mengarda et al., 2009), *Nidularium procerum* Lindm., *N. innocentii* Lem. (Silva et al., 2012), *Aechmea blanchetiana* (Baker) L.B.Sm. and *A. distichantha* Lem. (Santa-Rosa et al., 2013). The consistency of the medium may also influence the *in vitro* organogenesis of bromeliads (Mengarda et al., 2009; Silva et al., 2012). Agar is a gelling medium commonly employed in plant tissue culture and its use in low concentrations may promote vigorous shoot induction in some plant species (Mengarda et al., 2009; Suthar et al., 2011). Nevertheless, it also may induce the growth of hyperhydric plants (Casanova et al., 2008). Another essential factor for *in vitro* propagation is the inclusion of carbohydrates in the growth medium. Carbohydrates are required by plant cells as carbon resources, act as energy for growth and biosynthetic processes, and may influence *in vitro* rooting (Ferreira et al., 2011). The most common carbohydrate used is sucrose, at a concentration of 3% as recommended by Murashige and Skoog (1962). The

supplemental sugar in the *in vitro* medium may assist in water conservation and maintaining the osmotic potential of cells (Hazarika, 2003). Sucrose is also closely related to stomatal density and photosynthetic pigment content, as well as development induction in some plant tissues, such as vascular and support tissues, (Mohamed and Alsadon, 2010; Iarema et al., 2012). The exogenous sucrose supply may increase the endogenous content of carbohydrate stocks such as starch, sucrose, fructose and glucose in micropropagated plants. It may favor acclimatization and accelerate physiological adaptations (Jo et al., 2009). However, high sucrose concentrations in the medium may decrease the photosynthetic ability of *in vitro* plants (Fuentes et al., 2005a, b). The aim of this study was to analyze the effects of MS-salts concentration and sucrose on direct organogenesis and *in vitro* rooting in *B. zebrina* plants.

Results

In vitro multiplication under different MS-salt concentrations

The MS-salts concentration and medium consistency directly influenced *in vitro* morphogenic responses in *B. zebrina* shoots (Fig 1). The explants cultivated on growth medium without mineral salts did not display shoot formation, even when exogenous cytokinin was used in the medium. Explants cultivated on stationary liquid medium had higher budding percentages and shoot numbers per explant than explants cultivated on medium solidified with agar. Shoot formation was stimulated when MS-salts concentrations were raised (Table 1). The fresh and dry weights of explants showed significant interactions between the factors examined. The explants cultivated on liquid medium with salts added had higher fresh weights than those cultivated in the same saline concentration in solid medium. The dry weight was higher only in explants that were cultivated on liquid medium with 150% and 200% MS salts relative to the same MS-salts concentration in solid medium (Table 2). Among the MS-salts concentrations tested, only the explants cultivated on liquid medium showed differences in the fresh and dry weights due to the treatments. They showed a positive linear relationship with salts concentration (Table 2).

In vitro rooting under different sucrose concentration

The induction of roots occurred in all treatments, with an average rooting percentage higher than 97%. The root number was confirmed to be influenced by sucrose. Root number followed a positive quadratic relationship with sucrose concentration in the medium (Fig 2). The longest root lengths and aerial parts presented quadratic relationships with increasing sucrose concentrations (Fig 3). The root growth was best at 43.27 g L^{-1} sucrose (Fig 3A). Plants cultivated on sucrose concentrations higher than 24.63 g L^{-1} showed shorter aerial parts (Fig 3B). When the accumulation of matter in the plants was analyzed, we verified that the fresh weight accumulation of the aerial parts and roots presented a positive quadratic relationship with sucrose (Fig 4). The chlorophyll a, b and carotenoids contents were affected by sucrose addition in the medium. An increase in sucrose had a negative effect, and we verified a decreasing linear model for all of the pigments analyzed (Fig 5). Conversely, the content of total soluble sugars in *B. zebrina* shoots increased with increasing sucrose concentrations (Fig 6).

Discussion

There are several studies that consider the use of plant growth regulators as the primary direct organogenesis modulators of plant species (Silveira et al., 2009; Santa-Rosa et al., 2013; Sangeetha and Venkatachalam, 2014). In this study we examined whether the agar and minerals in the growth medium play fundamental roles in the direct organogenesis of *B. zebrina* shoots. The use of agar may interfere with organogenesis response in some plant species. It may reduce the formation of lateral shoots (Ivanova and Staden, 2011). The employment of a gelling agent in the medium may decrease water availability, mineral salts and plant growth regulators (Debergh, 1983) and may also decrease endogenous cytokinin (Ivanova et al., 2006). Mengarda et al. (2009) worked with different bromeliad species and obtained higher multiplication rates with stationary liquid medium relative to medium solidified with agar. Nevertheless, the findings of Silva et al. (2012) with *Nidularium procerum* and *N. innocentii* do not apply to all bromeliad species, which do not display differences in multiplication rate between liquid and solid media. The employment of a gelling agent is important for some plant species, and it may assist with plant formation without physiological disturbances such as hyperhydricity (Ivanova and Staden, 2011). Hyperhydricity during plant formation on liquid medium or on medium with low agar concentrations has already been noted in some *in vitro* plant species (Casanova et al., 2008). Hyperhydricity is more common in plants grown on liquid medium, most likely because the tissues are kept submerged and undergo marked oxidative stress, in addition to high concentrations of reactive oxygen species associated with changes in the activities of antioxidant enzymes (Ziv, 2005). However, hyperhydricity symptoms were not observed in *B. zebrina* shoots, which did not show morphological changes such as vitrification even when explants were kept submerged in medium for 45 days (Fig 1). Increased MS-salts concentrations stimulated shoot formation in *B. zebrina* explants (Table 1). Mineral components in the growth medium are vitally important for the *in vitro* regeneration process in plants (Williams, 1993). Some mineral compounds are related to endogenous cytokinin biosynthesis. An increase in nitrate resources may induce the expression of genes responsible for the biosynthesis of cytokinins, resulting in accumulation of these hormones in plants (Takei et al., 2004; Wang et al., 2004). Cytokinins are primarily responsible for breaking apical dominance and consequent lateral shoot induction. The breaking apical dominance is fundamental in the first cell division (Pasternak et al., 2000). However, as this study verified in *B. zebrina* explants, the addition of only a cytokinin resource in the medium could not ensure an organogenesis response, indicating that the concentration of salts is very important to ensure and enhance cell division (Table 1). Low water availability in media solidified with agar has a direct effect on fresh and dry weights, which decrease with increasing concentrations of gelling agent (Suthar et al., 2011). Agar may also adsorb some nutrients from the medium. Nutrients may become unavailable to the explants (Romberger and Tabor, 1971), restricting their growth and consequently the increase in explant mass. Fresh and dry mass accumulation relate to nutrition (Huang et al., 2010). Aranda-Peres et al. (2009) worked with mineral nutrition of different bromeliad species and have verified that calcium plays an important role during *in vitro* growth. Furthermore, it exerts a positive influence on the absorption of other nutrients in the medium. They have also verified that

Table 1. Budding (%) and shoot number under different MS-salts concentrations.

MS medium (%)	Budding (%)		Shoot number	
	Liquid	Solid	Liquid	Solid
0	0 a*	0 a	0 a [(1)]	0 a [(2)]
50	100 a	66.67 b	6.60 a	1.94 b
100	100 a	60.00 b	11.00 a	2.47 b
150	100 a	33.33 b	21.31 a	1.00 b
200	100 a	53.33 b	23.94 a	2.00 b

*For each phytotechnical characteristic analyzed, averages followed by the same letter in the line do not differ according to the Tukey test, at 5%. [(1)] $\hat{y} = 0.0511 + 0.1252x$, $R^2 = 0.975$; [(2)] $\hat{y} = 0.3143 + 0.0282x - 0.0001x^2$, $R^2 = 0.519$

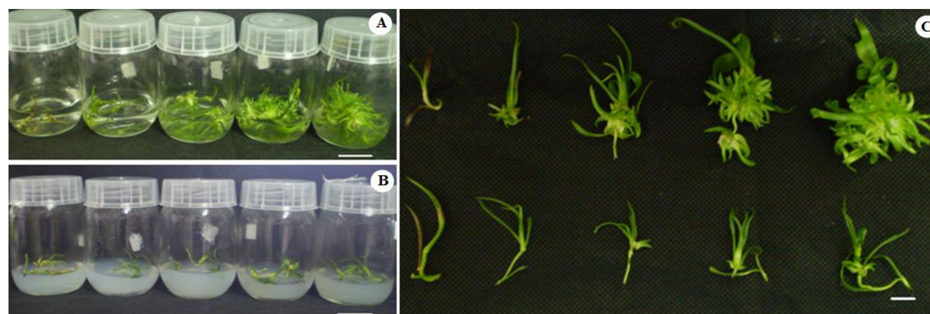

Fig 1. Direct organogenesis in *Billbergia zebrina* explants in stationary liquid medium (A) and medium solidified with agar (B). (C) Morphological aspects of explants due to MS-salt concentrations (0%; 50%; 100%; 150% and 200%, from the left to the right). Barr = 3 cm (A and B); 1 cm (C).

Table 2. Fresh and dry weight due to MS-salts concentrations in the medium

MS medium (%)	Fresh weight (g)		Dry weight (g)	
	Liquid	Solid	Liquid	Solid
0	0.083 a* [(1)]	0.059 a	0.0073 a [(2)]	0.0050 a
50	0.382 a	0.103 b	0.0276 a	0.0089 a
100	1.048 a	0.103 b	0.0552 a	0.0078 a
150	2.060 a	0.093 b	0.0948 a	0.0082 b
200	2.575 a	0.110 b	0.1176 a	0.0091 b

*For each phytotechnical characteristic analyzed, averages followed by the same letter in the line do not differ according to the Tukey test, at 5%. [(1)] $\hat{y} = -0.1026 + 0.0133x$, $R^2 = 0.972$; [(2)] $\hat{y} = 0.0029 + 0.0006x$, $R^2 = 0.988$.

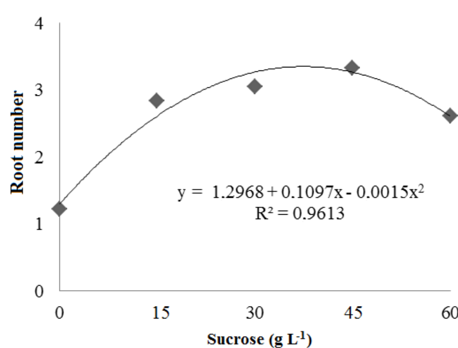

Fig 2. Root number of *Billbergia zebrina* shoots due to sucrose concentration of the medium.

increasing calcium concentration is a promising method for increasing fresh and dry weights of *in vitro* bromeliad explants. The explants of *B. zebrina* showed high rooting frequencies. The same behavior has already been verified in other plant species, in which rooting occurred in sugar-free medium (Cha-um et al., 2011; Iarema et al., 2012). Adventitious root induction is more related to the concentration and endogenous balance of plant hormones (Dong et al., 2012). Auxins are the major plant hormones responsible for initiation of adventitious roots. The most abundant endogenous auxin in plants is 3-indoleacetic acid (IAA) (Li et al., 2009). IAA concentration in shoots may ensure the *in vitro* rhizogenic response (Martins et al., 2013). It may also explain the *in vitro* rooting rate of *B. zebrina* shoots on medium without sucrose and exogenous auxin. The rhizogenic process requires available energy in the explant, given that the root number typically responds to sucrose (Jo et al., 2009; Rocha et al., 2013). Kumar et al. (1999) reported that carbohydrates used as carbon sources in the medium act on root frequency and quality, determining plant success after the transfer to an *ex vitro* environment. Carbohydrates are transported to meristematic cells, regulating rhizogenesis by modulating gene expression and enzyme activity (Pawlicki et al., 1995). The carbohydrate source is also related to the

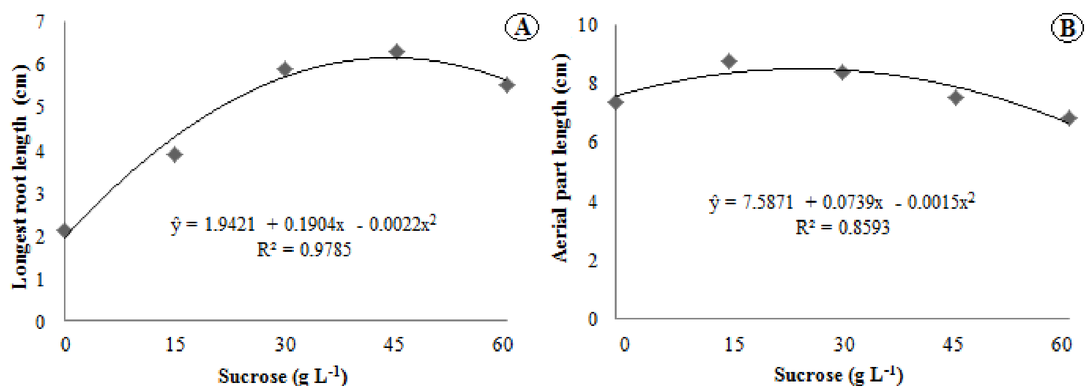

Fig 3. Longest root length and aerial part length of *B. zebrina* shoots due to sucrose concentration in the medium.

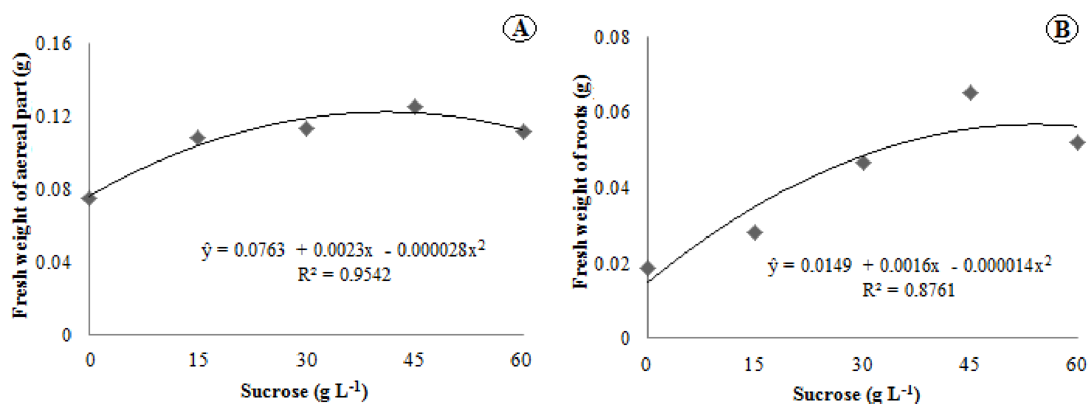

Fig 4. Fresh weight of aerial parts and roots of *B. zebrina* shoots due to sucrose concentration in the medium.

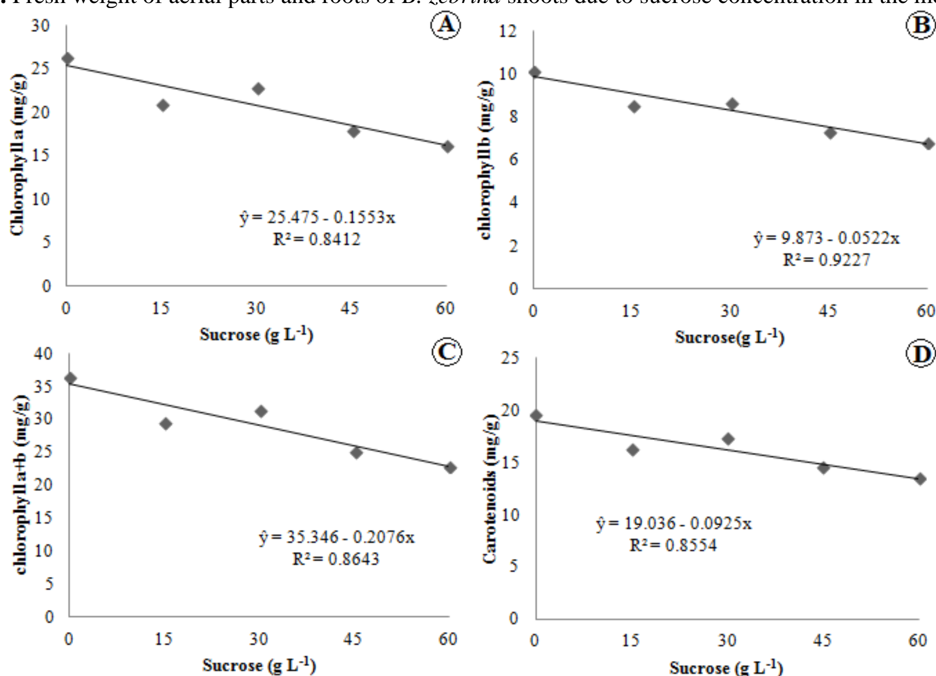

Fig 5. Photosynthetic pigment content of *B. zebrina* shoots due to sucrose concentration in the medium. (A) Chlrophlly a; (B) Chlorophlly b; (C) Chlorophlly a+b; (D) carotenoids.

growth hormone production (auxins) involved in the rooting process (Rolland et al., 2002). Sucrose contributes to the growth of roots because it acts on cell expansion and proliferation (Wang and Ruan, 2013). Nevertheless, high sugar concentrations can inhibit the growth of aerial plant parts (Al-Khateeb, 2008), as verified on *B. zebrina* shoots. Inhibition is due to osmotic stress in the medium with a high sucrose concentration (Nowak et al., 2004; Jo et al., 2009). Osmotic potential may interfere with nutrient abortion by the cells, which is essential to growth and cell division in the aerial parts (Siwach et al., 2011). Sucrose cleavage in the medium results in glucose and fructose production. It may accelerate cell division and consequently increase the explant weight and volume (Gurel and Gulsen, 1998). However, high sucrose concentrations may limit growth, due to an increase in the osmotic potential in the medium caused by sucrose (Rejšková et al., 2007). This supports our observations on *B. zebrina* shoots, which have reduced weights with sucrose concentrations higher than 41 g L^{-1} (Fig 4A). The reduction in chlorophyll content in *in vitro* plants may reduce photosynthetic ability by decreasing light absorption (Sivanesan et al., 2008). The decrease in photosynthetic pigments due to carbohydrate addition in the medium has already noted in previous literature, such as Mohamed and Alsadon (2010) and Swamy et al. (2010). These authors concluded that lower sucrose concentrations may stimulate the chlorophyll production in *in vitro* plants. A higher total soluble sugars content in *B. zebrina* shoots on media with high sucrose concentrations was verified (Fig 6). Similar results have been reported in *Dendrobium* Second Love shoots. The soluble carbohydrate increased concomitantly with growth medium sucrose concentrations in shoots of this orchid (Ferreira et al., 2011). High stock-carbohydrate concentrations in *in vitro*-formed plant tissues may improve the performance of plants during the acclimatization phase (Chu et al., 2010). Plants cultivated on medium without sugar may have better photosynthetic rates *in vitro* when they are compared with plants grown on medium with sucrose. Nevertheless, the absence of stock substances may result in low survival rates after transfer to *ex vitro* conditions, and carbon source additions to the medium in low concentrations are recommended (Fuentes et al., 2005b). This supports the use of 15 g L^{-1} sucrose during the rooting phase. Since *B. zebrina* shoots did not have a high decreasing photosynthetic pigments content and increased carbohydrate stocks in the shoots.

Materials and Methods

In vitro establishment

Billbergia zebrina fruits were collected from adult plants grown in a greenhouse (voucher specimen 27.329 – ESAL herbarium). They were submitted to disinfestation in 70% ethanol for one minute and sodium hypochlorite (30% commercial solution and 2.5% activated chlorine) for 20 minutes. Subsequently, the seeds were washed tree times in autoclaved distilled water to remove excess disinfesting solution and were then inoculated in test tubes containing 10 mL of MS medium (Murashige and Skoog, 1962) at half the original concentration, supplemented with 30 g L^{-1} of sucrose and solidified with 7 g L^{-1} of agar. The medium pH was adjusted to 5.8 before autoclaving at 120ºC for 20 minutes. After inoculation in a horizontal laminar flow cabinet, the plant material was kept in a growth room at 27±2 ºC with a 16-hour photoperiod under fluorescent lamps providing 25.2 µmol m^{-2} s^{-1} of photosynthetic photon flux.

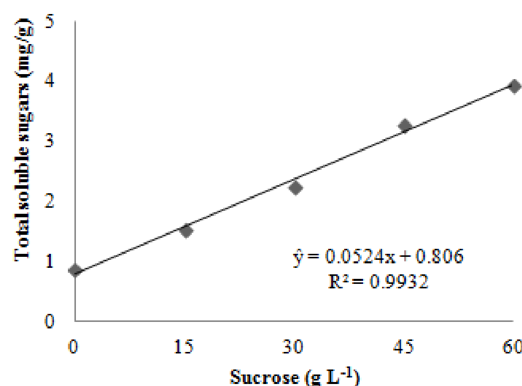

Fig 6. Total soluble sugars content of *B. zebrina* shoots due to sucrose concentration in the medium.

MS-salt concentrations effects in vitro multiplication

Plants that were 60 days old were inoculated in glass vessels containing 50 mL MS medium (Murashige and Skoog, 1962) at 0%, 50%, 100%, 150% or 200% of the original saline concentration, 30 g L^{-1} sucrose and 15 µM 6-benzilaminopurine (BA). The media were prepared in two different consistencies, stationary liquid (no gelling agent) and 6 g L^{-1} agar. The pH was adjusted to 5.8 before autoclaving at 120ºC for 20 minutes. After inoculation in a horizontal laminar flow cabinet, the plant material was kept in a growth room at 27±2 ºC with a 16-hour photoperiod under fluorescent lamps providing 25.2 µmol m^{-2} s^{-1} of photosynthetic photon flux. The evaluation was performed after 45 days of cultivation, with 15 plants per treatment divided into five parcels. The analyzed phytotechnical features were as follows: budding (%), shoot number, and fresh- and dry-weight (g) of the buds. To obtain dry mass, the plant material was kept in a forced ventilation oven at 65 ºC until stabilization.

Sucrose concentrations in vitro rooting and growth

B. zebrina shoots were maintained in MS liquid medium with 100% of the original saline concentration, 15 µM BAP, 30 g L^{-1} sucrose and no gelling agent for 45 days for shoot induction. The newly formed shoots were transferred to 250 mL vessels with 50 mL of MS stationary liquid medium without plant growth regulators until they were 30 days old. Then, the shoots were identified with the aid of a scalpel and inoculated in 50 mL vessels with MS medium at 0, 15, 30, 45 or 60 g L^{-1} sucrose and solidified with 7 g L^{-1} agar. The pH was adjusted to 5.8 before autoclaving at 120ºC for 20 minutes. After inoculation in a horizontal laminar flow cabinet, the plant material was kept in a growth room at 27±2 ºC with a 16-hour photoperiod under fluorescent lamps providing 25.2 µmol m^{-2} s^{-1} of photosynthetic photon flux. The evaluation was performed after 45 days of cultivation, with 18 plants per treatment divided into six parcels. The analyzed phytotechnical features were as follows: rooting (%), number of roots, longest root length (cm), total fresh weight (g), and fresh weight of aerial parts (g) and roots (g).

Photosynthetic pigment content analysis

For pigment content analysis, the aerial parts of 15 plants per treatment were used and were divided into three parcels. The plant material was weighed and macerated in liquid nitrogen and placed in 80% acetone. The material was then centrifuged at 8,000 g for 15 minutes, the supernatant was collected and diluted to 25 ml. The instrument estimates the pigment content on the basis of the absorbance at 470(A470), 647 (A647) and 663 nm (A663), for chlorophyll a, chlorophyll b (Engel and Poggiane, 1991) and carotenoids (Higby, 1962), respectively. The equations used were according to Li et al. (2013).

Total soluble carbohydrates analysis

To analyze the total soluble carbohydrates, 600 mg of dry material from aerial parts in each treatment were used and were divided into three parcels. The total soluble carbohydrates were extracted and determined using the anthrone method (Dische, 1962).

Statistical analysis

For the analysis of *in vitro* multiplication, a completely randomized design in a factorial arrangement (five MS concentrations x two medium consistencies) was adopted. The obtained data were submitted to analysis of variance (ANOVA), the averages of the factor medium consistencies were compared using Tukey's test at 5% probability, and the MS-salts concentrations were subjected to regression analysis. For the analysis of *in vitro* rooting, a completely randomized design was adopted. The data were subjected to analysis of variance and regression analyses. All statistical analyses were performed using the Genes software (Cruz, 2013).

Conclusion

The concentrations of MS-salts and sucrose have direct effects on morphological responses in *B. zebrina* shoots during *in vitro* growth. The *in vitro* multiplication of *B. zebrina* shoots is enhanced by doubling the original concentration of MS-salts (200%) and liquid media. While the use of agar during the multiplication phase limits the organogenesis response in *B. zebrina* shoots. Sucrose does not determine root induction in *B. zebrina* shoots but it induces better growth of roots. The use of 15 g L^{-1} sucrose could be an interesting method to increase the endogenous carbohydrate stock with few negative effects on photosynthetic pigments and maintaining a good growth.

Acknowledgements

The authors would like to thank the CAPES, CNPq and FAPEMIG (Brazil) for their financial support.

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