JOÃO PAULO RODRIGUES MARTINS

ANATOMICAL AND PHYSIOLOGICAL RESPONSES OF *Billbergia zebrina* (Bromeliaceae) UNDER DIFFERENT *IN VITRO* CONDITIONS

LAVRAS- MG

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ABSTRACT

The thesis was divided in four articles, in which three are related to *in vitro* propagation and how the microenvironmental conditions play on physiology and anatomy of *B. zebrina*. The last article is related to anatomical and physiological changes of *B. zebrina* under copper (Cu) excess stress. For all studies, *B. zebrina* plants were previously *in vitro*-established in MS medium. Plants were transferred to media at concentrations of 0%, 50%, 100%, 150% or 200% of the original salt concentration of MS 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 different sucrose concentrations. Soluble carbohydrates contents were assessed after the rooting. 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 on *in vitro* shoots. From these results, a second experiment was designed. *B. zebrina* side shoots were transferred to culture media containing 0.0, 15.0, 30.0, 45.0 or 60.0 g L^{-1} sucrose. Two different culture container sealing systems were tested: lids with a filter and a filter covered with PVC. At 45 days *in vitro* growth, *B. zebrina* plants were transplanted onto suitable soil mix and evaluated at 80 days growth in greenhouse. At 45 days *in vitro* and 80 days of acclimatization in the greenhouse, the biomass of plants was evaluated. Anatomical and physiological analysis were also performed on plants grown *in vitro*. Limited air exchange resulted in plantlets with anatomical and physiological disorders at the end of the *in vitro* period. The highest growth rate in the greenhouse was observed in plants previously propagated in unlimited gas exchange system and sugar-free medium. An environmental approach was proposed in the last study, in which copper was used. Anatomical and growth analysis were measured. Plants did not show any visible disturb, like necrosis on the leaves and all plants survived. Plants grown under 200 µM Cu showed anatomical changes that can help tolerating this metal, like high stomatal index and thicker cell wall in exodermis. Cu affected the leaf and root anatomy as well as on growth. *B. zebrina* tolerates high amounts of Cu. From the results it was possible to verify that microenvironmental conditions can change the growth, physiology and anatomy of *B. zebrina* during *in vitro* culture. *In vitro* technique showed a great potential on plant propagation of *B. zebrina* as well as it also presented an important tool for studies on plant physiology and anatomy.

Keywords: Bromeliad. *In vitro* culture. Photoautotrophic growth. Plant anatomy. Plant physiology.

RESUMO

A tese foi dividida em quatro artigos, nos quais três estão relacionados a propagação *in vitro* e como as condições microambientas afetam a fisiologia e anatomia de *B. zebrina*. O último artigo está relacionado as modificações fisiologicas e anatômicas de *B. zebrina* sob estresse ao excesso de cobre (Cu). Para todos os estudos, plantas de *B. zebrina* foram previamente estabelecidas *in vitro* em meio MS. Plantas foram transferidas para meios nas concentrações de 0%, 50%, 100%, 150% e 200% da concentração original dos sais do meio MS. Os meios foram preparados em duas concentrações diferentes, líquido estacionário e com 6 g L^{-1} de ágar. Para os estudos de enraizamento *in vitro*, brotos foram cultivados em meios suplementado com diferentes concentrações de sacarose. Carboidratos solúveis foram analisados após o enraizamento. A multiplicação *in vitro* de *B. zebrina* é melhor com o uso de 200% dos sais MS e em meio líquido. O uso de 15 g L^{-1} de sacarose aumentou os estoques de carboidratos endógenos e induziram uma boa formação do sistema radicular dos brotos *in vitro*. A partir desses resultados, um segundo experimento foi delineado. Brotos laterais de *B. zebrina* foram transferidos para meios de cultura contendo 0,0; 15,0; 30,0; 45,0 ou 60,0 g L^{-1} de sacarose. Dois diferentes tipos de vedação dos frascos foram testados: tampas com um filtro e filtro coberto com plástico PVC. Aos 45 dias de cultivo *in vitro*, as plantas de *B. zebrina* foram transplantadas para uma mistura de solo e avaliadas aos 80 dias de cultivo em casa de vegetação. Aos 45 dias *in vitro* e 80 dias de aclimatização em casa de vegetação, a biomassa das plantas foi avaliada. Análises fisiológicas e anatômicas foram feitas nas plantas cultivadas *in vitro*. A troca de ar limitada resultou em plantas com disordens anatômicas e fisiológicas ao final do período *in vitro*. A maior taxa de crescimento na casa de vegetação foi observado em plantas previamente propagadas no sistema que permitia troca gasosa e sem açucar no meio. Uma abordagem ambiental foi proposta no último capítulo, no qual o cobre (Cu) foi usado. Análises anatômicas e crescimento foram mensuradas. As plantas não apresentaram qualquer distúrbio, como necrose nas folhas e todas as plantas sobreviveram. Plantas cultivadas com 200 µM de Cu apresentaram modificações anatômicas que ajudam na tolerânica desse metal, como alto índice estomático e parede celular mais espessa na exoderme. Cu afetou a anatomia foliar e radicular, bem como o crescimento. *B. zebrina* tolera altas quantidade de Cu. A partir dos resultados obtidos foi possível verificar que as condições microambientais podem modificar o crescimento, fisiologia e anatomia de *B. zebrina* durante o cultivo *in vitro*. As técnicas *in vitro* mostraram um bom potencial na propagação vegetal de *B. zebrina* bem como também apresentou ser uma importante ferramenta para estudos sobre fisiologia e anatomia.

Palavras-chave: Bromélia. Cultivo *in vitro.* Cultivo fotoautotrófico. Anatomia vegetal. Fisiologia vegetal.

SUMMARY

1 INTRODUCTION

Many bromeliad species have been reduced in number or even eradicated due to habitat destruction as a result of anthropic action, such as increasing deforesting, and the occurrence of selective extraction (ROCHA et al., 2004). The attributes of colourful bracts and flowers which can last for several months, and leaves of high visual appeal, confer elements of a highly appreciated value to bromeliads as an ornamental plant (PEDROSO et al., 2010). Among them is *Billbergia zebrina* (Herbert) Lindley, an epiphytic tank bromeliad native to Atlantic Rainforest. As an effect of habitat loss and species exploitation, this species is considered a vulnerable species in the Rio Grande do Sul State, in Southern Brazil (VESCO et al., 2011).

In vitro propagation techniques have been widely used for rapid multiplication of several economically important or endangered plant species, such as those in the Bromeliaceae family (GUERRA; VESCO, 2010). Several studies related to *in vitro* culture of bromeliads have been published, such as Chu et al. (2010), Huang et al. (2011), Martins et al. (2013), Martins et al. (2014), Martins et al. (2015) and Silva et al. (2012).

Plant tissue culture techniques have been extensively used for the rapid multiplication of many plant species. During *in vitro* culture many factors can interfere on the *in vitro* morphogenetic responses. Studies have already reported the use of plant growth regulators (CHU et al., 2010; MARTINS et al., 2013; MARTINS et al., 2014), mineral composition of the culture medium (KURITA; TAMAKI, 2014) and carbon source (MARTINS et al., 2015; MENGESHA; AYENEW; TADESSE, 2013) as modulators of *in vitro* morphogenetic responses of bromeliads.

Mineral compounds in the culture medium play an important role during *in vitro* propagation of bromeliads. Mineral concentration in the medium may be related to growth and multiplication rate or even to physiological disorders (ARANHA-PERES et al., 2009; GIAMPAOLI et al., 2012; KANASHIRO et al., 2009; KURITA et al., 2012; KURITA; TAMAKI, 2014; MARTINS et al., 2015).

Carbon source (e.g. sucrose) as well as gas exchange also play a role on *in vitro* morphogenesis and they may induce plants with limited photosynthetic ability, physiological and anatomical disorders (IAREMA et al., 2012; MOHAMED; ALSADON, 2010; SHIN; PARK; PAEK, 2013) and it might interfere on plant survival and growth rate during later acclimatization (MOHAMED; ALSADON, 2010; SHIN; PARK; PAEK, 2014). Nevertheless, sugars in the medium act positively on carbohydrate stocks of *in vitro* propagated plants (FERREIRA et al., 2011; MARTINS et al., 2015).

In vitro techniques can also be used in plant physiological analysis as already verified by (GIAMPAOLI et al., 2012; KHATUN et al., 2008). This technique is advantageous because it allows to isolate a certain effect on the metabolism of plants from the effects of other stress types (GIAMPAOLI et al., 2012).

The aim was to verify the effects of the microenvironmental conditions under growth, physiology and anatomy of *B. zebrina* during the *in vitro* propagation.

1 BACKGROUND

2.1 General botanical aspects of bromeliads

Bromeliaceae is among the greatest in diversity and richness plant family in the Brazilian Atlantic Forest, comprising 30 genera and 904 species (FORZZA et al., 2013; RIBEIRO et al., 2009). Atlantic Forest is one of the three most threatened regions on the planet (MYERS et al., 2000) due to its species richness, extremely high levels of endemism and the small fraction of the original forest remaining (RIBEIRO et al., 2009).

Bromeliaceae family is traditionally divided in threes subfamilies: Pitcairnioideae with terrestrial species and winged seeds adapted for wind dispersal; Tillandsioideae with predominantly epiphytic species with plumose seeds, dispersed by wind; and Bromelioideae terrestrial and epiphytic species, with fleshy fruits and seeds dispersed by animals (BENZING, 2000). Molecular analyses have already confirmed Bromelioideae and Tillandsioideae as monophyletic groups, but Pitcairnioideae may be a polyphyletic group (BARFUSS et al., 2005; HORRES et al., 2000). Currently, the phylogenetic relationships and circumscriptions of the subfamilies are clearer, with eight subfamilies now established: Brocchinioideae, Lindmanioideae, Hechtioideae, Navioideae, Tillandsioideae, Pitcairnioideae, Puyoideae and Bromelioideae (GIVNISH et al., 2007; GIVNISH et al., 2011)

Bromeliads in their general structure consists of a usually short stem covered with leaves arranged spirally around forming a rosette. The leaves present trichomes in both sides of epidermis. This structure is important for being responsible for water and nutrients absorption, besides contributing to the light excess reflection, which can cause photoinhibition (BENZING,

2000; MARTIN; RUX; HERPPICH, 2013). The trichomes are connected to the epidermis by one or more cells placed in a uniseries structure (MANTOVANI et al., 2012). In bromeliad's leaves there is a water-storage parenchyma (hydrenchyma) formed by non-chlorophyll cells with thin walls (MARTINS et al., 2014). This parenchyma is responsible for the hydric maintenance of bromeliads and it protects the chlorophyll region from intense light, besides favouring the photosynthetic process (BRIGHIGNA; CECCHI-FIORDI; PALANDRI, 1984).

Approximately half of all bromeliads are epiphytes, and the success of this family in the epiphytic niche is frequently associated with the development of strategies to intercept, absorb (trichomes) and store rainwater more efficiently (BENZING, 2000). In the tank-forming bromeliads, e.g., the rainwater accumulates in external tanks formed by the overlapping of the leaf bases, allowing the plant to store water for periods of drought (SCHMIDT; ZOTZ, 2001).

In addition to morphological and anatomical specializations, a large number of epiphytic bromeliads display Crassulacean Acid Metabolism (CAM), which is a specialized photosynthetic pathway that minimizes water loss demand by opening its stomata mainly throughout the night when atmospheric vapour pressure deficits are lower (LÜTTGE, 2004). Besides water-use efficiency, another key feature is the modulation of CAM pathway, depending on the level of water supply provided by this mode of photosynthesis, which can perform either C_3 or CAM depending on the environmental conditions (PEREIRA; PURGATTO; MERCIER, 2013).

Many bromeliad species have commercial value as an ornamental plant, 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 aiming income supplement. It is threatening some species with extinction (NEGRELLE; MITCHELL; ANACLETO, 2012).

Therefore, the mass multiplication of bromeliads rises as an economical and ecological viable option. Bromeliads are frequently propagated by seeds (sexual propagation) or side shoots (asexual propagation). Nevertheless, these propagation techniques are not efficient to mass propagation required to ornamental plant species (CARNEIRO et al., 1999). Asexual propagation of bromeliads may be too low, since low number of side shoots are produced per plant (PAIVA et al., 2006). In the sexual propagation, disadvantages are related to limited production of plantlets (DAQUITA et al., 1999), because the germination rate of bromeliad seeds is low (MERCIER; KERBAUY, 1995).

In vitro propagation techniques are used to rapid multiplication of plants and it has been widely employed in bromeliads species (GUERRA; VESCO, 2010) as already verified by Huang et al. (2011) with *Guzmania* 'Hilda' and Martins et al. (2014) with *Neoregelia concentrica* (Vellozo) L.B. Smith.

2.2 General botanical aspects of *Billbergia zebrina*

Billbergia Thunb. is an ornamental genus consisting of about 64 species and 26 varieties (LUTHER, 2008) belonging to the subfamily Bromelioideae. It is distributed from Central America to southern South America (SMITH; DOWNS, 1979) and Atlantic Rain Forest is the diversity centre of this genus (BARROS; COSTA, 2008).

B. zebrina is an epiphytic species with tubular rosette (Figure 1). The flowering ranges from 50 to 80 cm. The floral bracts are flabby, pink or white, with persistent coloured with the base partially surrounding the

escape. The inflorescence consisting in 22-40 flowers and the fruits are rigid berries (BARROS; COSTA, 2008).

Figure 1 *B. zebrina* plants with pink (A) and white (B) flowering. Plants collected in Lavras-MG, Brazil. João Martins' pictures.

The distribution of *B. zebrina* occurs in the states of Bahia, Minas Gerais, Rio de Janeiro, Sao Paulo, Parana, Santa Catarina and Rio Grande do Sul, in Atlantic Rain Forest, Restinga forests, Cerrado and rock fields, up to 1.100m altitude. Its distribution is internalized to the south to Paraguay and Argentina (FONTOURA; COSTA; WENDT, 1991; MOURA et al., 2007; SMITH; DOWNS, 1979; VERSIEUX; WENDT, 2006).

B. zebrina has an important commercial value as ornamental plant, due to the beauty of its leaves and flowers. As an effect of habitat loss and species exploitation, it is considered a vulnerable species in the Rio Grande do Sul State, in Southern Brazil (VESCO et al., 2011).

This bromeliad has already been *in vitro* propagated by Vesco et al. (2011). These authors propagated *B. zebrina* by induction of nodular cultures. However, physiological and anatomical analyses to verify the quality of micropropagated plants have not been done so far, as well as in others *in vitro* propagated bromeliad species.

2.3 Plant tissue culture

Plant tissue culture may be defined as the aseptic culture of cells, tissues, organs or even whole plants under controlled nutritional and environmental conditions (THORPE, 2007). The first reports regarding plant tissue culture date back to the beginning of the 20th century (GARCÍA-GONZÁLES et al., 2010). Currently, this technique is a well-established technology, like many other technologies. It is known as mass clonal plant propagation system or just micropropagation (AKIN-IDOWU; IBITOYE; ADEMOYEGUN, 2009).

Many factors can modulate the *in vitro* morphogenetic responses, for instance plant growth regulators (CHU et al., 2010; MARTINS et al., 2013; 2014), mineral composition of the culture medium (KURITA; TAMAKI, 2014) and carbon source (MARTINS et al., 2015; MENGESHA; AYENEW; TADESSE, 2013).

Plant growth regulators are synthetic compounds added exogenously to the medium. These compounds are classified in different groups: auxins, cytokinins, gibberellins, abscisic acid and ethylene and they may interfere on *in vitro* morphogenetic responses (JIMÉNEZ, 2005). The *in vitro* multiplication may occur via side-shoots induction with aid of synthetic cytokinins and the organogenic responses may be influenced by cytokinin type added to the medium (MARTINS et al., 2014). Auxins are frequently

used to adventitious rooting induction (MARTINS et al., 2013) and ethylene may be produced by tissue, callus and plantlets. It influences on *in vitro* morphogenesis like inducing chlorophyll breakdown leading to senescence and leaf abscission (HAZARIKA, 2006).

The massive propagation of plants has traditionally been carried out in solid medium (DEBNATH, 2007). Nevertheless during the last few years, cultures in liquid medium with the objective of massive plant propagation have appeared as an alternative (AKIN-IDOWU; IBITOYE; ADEMOYEGUN, 2009), but it may induce hyperhydricity in *in vitro* plants (CASANOVA et al., 2008).

Mineral composition of the culture medium also plays an important role during *in vitro* propagation. The culture medium nutrient requirements may vary and function differently based on the species or the used technique. The mineral concentration may interfere on growth or even physiological disorders (ARANHA-PERES et al., 2009; GIAMPAOLI et al., 2012; KURITA; TAMAKI, 2014). It has led to modifications to existing media or even new formulations (GREENWAY et al., 2012).

Conventional *in vitro* propagation is carried out using closed containers and sucrose added to the medium as carbon source (XIAO; NIU; KOZAI, 2011). Sucrose supplementation in culture medium meets the energy demands for growth and physiological function (HAZARIKA, 2003). However, sugars added in the medium may induce plants with limited photosynthetic ability (SHIN; PARK; PAEK, 2013), moreover plants grown *in vitro* may show specific characteristics such as low differentiation of leaf tissues due to high humidity and low gas exchange with external environment (MAJADA et al., 2000).

Anatomical features such as reduced development mesophyll, especially chlorenchyma and vascular bundles may occur (GONÇALVES et al., 2000; ROMANO; MARTINS-LOUÇÃO, 2003). In addition, a little or limited functionality of the stomata of plants cultivated *in vitro* has already been reported (MARTINS et al., 2014) and this leads to the downregulation to water loss after transfer to *ex vitro* conditions (LAMHAMEDI et al., 2003).

The knowledge of the anatomical changes that occur during the *in vitro* culture may help on *in vitro* propagation, producing plants with a high quality and maximizing the establishment of these plants to *ex vitro* conditions (APOSTOLO; LLORENTE, 2000; CALVETE et al., 2002).

Anatomical changes during *in vitro* culture have been reported in some bromeliads species, such as *Ananas erectifolius* (PEREIRA et al., 2007), *A. comosus* (BARBOZA et al., 2006) and *Neoregelia concentrica* (MARTINS et al., 2014). However, these authors have not checked how the *in vitro* conditions may influence on survivel and growth rate of bromeliads after transfer to greenhouse conditions.

2.4 Plant tissue culture applied to an environmental approach

Plant tissue culture is an important tool for some agricultural sectors, such as ornamental plant propagation (CARNEIRO et al., 1999). Nevertheless, the applications of this technique go well beyond agriculture and horticulture (AKIN-IDOWU; IBITOYE; ADEMOYEGUN, 2009). Some researchers have used this technique to check the changes in the plant physiology and plant cell biochemistry in response to different stress conditions (GIAMPAOLI et al., 2012; RIBEIRO et al., 2014), e.g. copper (Cu) excess conditions (AHMAD et al., 2015; GIAMPAOLI et al., 2012;

KHATUN et al., 2008). According to Giampaoli et al. (2012), the *in vitro* culture technique is advantageous because it allows isolation of the effects of certain metal on plant metabolism from the effects of other stress types.

Essential metals, such as Cu, play biochemical and physiological functions in plants and animals. Cu plays an essential role in signaling of transcription and protein trafficking machinery, oxidative phosphorylation and iron mobilization. Thus, plants require Cu as an essential micronutrient for normal growth and development (YRUELA, 2005). However, at high concentrations, this metal can become extremely toxic causing symptoms such as chlorosis and necrosis, stunting, leaf discoloration and inhibition of root growth (VAN ASSCHE; CLIJSTERS, 1990).

Cu is a non-degradable heavy metal and it can accumulate in soil or leach into water sources. Its accumulation in topsoil has impacted micro and macro organisms (MACKIE; MÜLLER; KANDELER, 2012). The environmental pollution may lead this element reach toxic or poisonous concentrations, causing severe damage to living beings (NAGAJYOTI et al., 2010).

Currently, polluted environments are observed in large urban and industrial centers as well as in remote locations as a result of intense agricultural activities, mining, volcanic emissions, among others (DRAGUNSKI et al., 2009). Incineration of municipal waste has generated significant concentrations of many kinds of heavy metals, like Cu (NAGAJYOTI et al., 2010). In agricultural areas, fungicidal spraying has contributed in the Cu accumulation in enviroment (MACKIE; MÜLLER; KANDELER, 2012). This heavy metal may also be associated with dust particles and thus be carried by the wind (FIGUEIREDO et al., 2007).

An alternative way to promote the monitoring of pollution is the use of species that assimilate nutrients dispersed in the atmosphere or soil (FIGUEIREDO et al., 2007). This methodology is quite attractive because it has some advantages over conventional techniques such as low cost, the possibility of interaction with pollutants over long time intervals and simultaneous monitoring of multiple locations (DRAGUNSKI et al., 2009).

The use of bromeliad species in biomonitoring or bioindication of polluted environmentals is a viable alternative, in tropical conditions, in order to maintain life quality of the organisms (LUOMA; RAINBOW, 2005). Some bromeliads, like *Tillandsia*, have been used with this approch (AMADO et al., 2002; FIGUEIREDO et al., 2007). However, how the heavy metals, like Cu, interfer on plant growth, physiology and anatomy of bromeliads is not well clear.

3 GERAL CONSIDERATION

In vitro technique showed a great potential on plant propagation of *B. zebrina* as well as it also presented an important tool for studies on plant physiology and anatomy.

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ARTICLE 1

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

(Prepared in accordance with *Australian Journal of Crop Science*'s standards)

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 analyse the effects of MS-salts concentration and sucrose on direct organogenesis and *in vitro* rooting in *B. zebrina* plants.

Material 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.

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).

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).

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) $\hat{y} = 0.3143 + 0.0282x - 0.0001x^2$, $R^2 = 0.519$

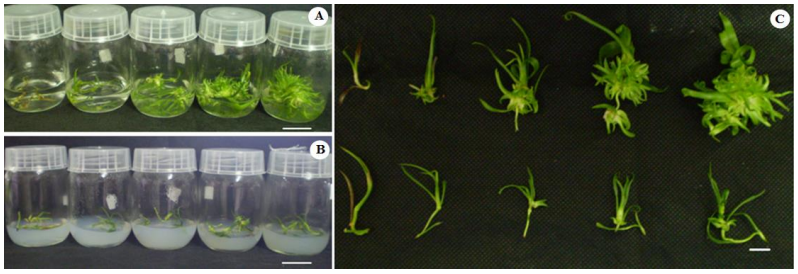

Fig 1. Direct organogenesis in *Billbergia zebrina* explants in stationary liquid medium (A) and medium solidified with agar (B). (C) Morphological aspects of explants due to MS-salt concentrations (0%; 50%; 100%; 150% and 200%, from the left to the right). Barr = 3 cm (A and B); 1 cm (C).

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).

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) $\hat{y} = 0.0029 + 0.0006x$, $R^2 = 0.988$.

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).

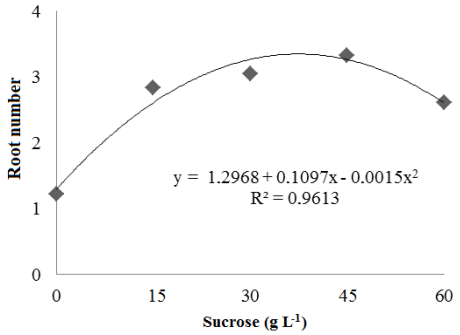

Fig 2. Root number of *Billbergia zebrina* shoots due to sucrose concentration n the medium.

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).

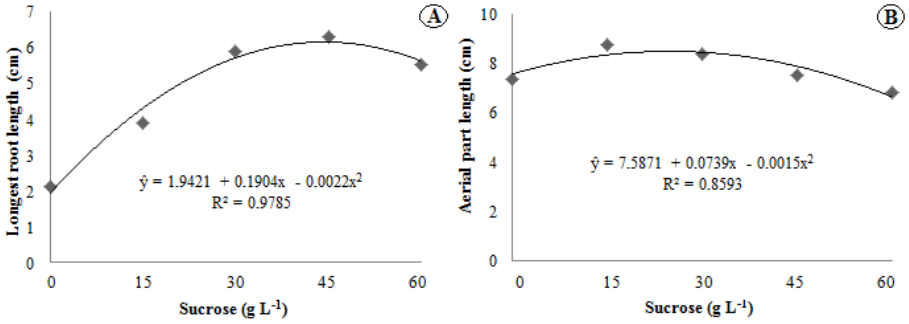

Fig 3. Longest root length and aerial part length of *B. zebrina* shoots due to sucrose concentration in the medium.

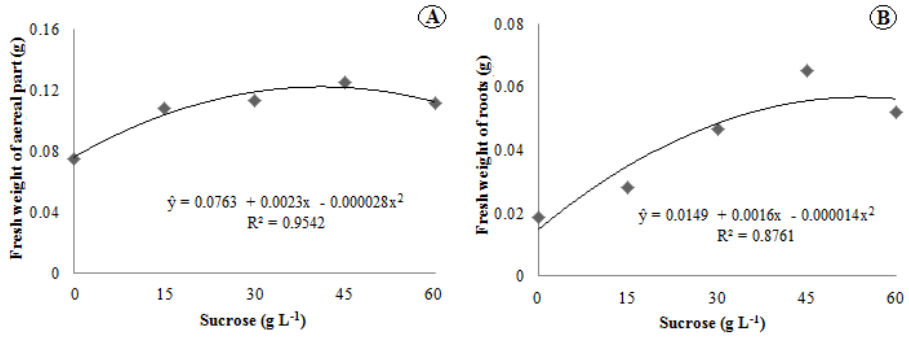

Fig 4. Fresh weight of aerial parts and roots of *B. zebrina* shoots due to sucrose concentration in the medium.

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).

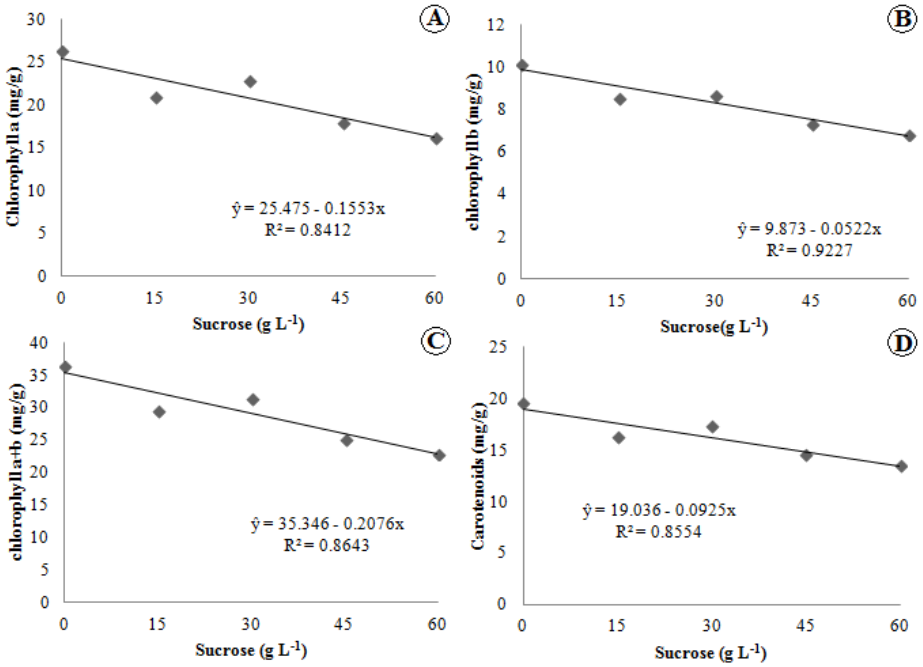

Fig 5. Photosynthetic pigment content of *B. zebrina* shoots due to sucrose concentration in the medium. (A) Chlrophlly a; (B) Chlorophlly b; (C) Chlorophlly a+b; (D) carotenoids.

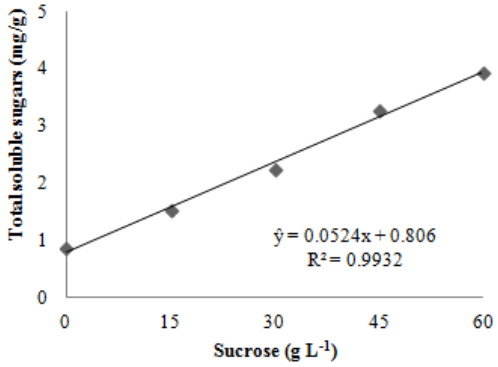

Fig 6. Total soluble sugars content of *B. zebrina* shoots due to sucrose concentration in the medium.

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 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 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.

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.

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ARTICLE 2

Impacts of photoautotrophic and photomixotrophic condition on *in vitro* propagated *Billbergia zebrina* (Bromeliaceae)

(Prepared in accordance with *Plant Cell, Tissue and Organ Culture*'s standards)

Abstract
Micro-propagation techniques contribute to the multiplication of several bromeliad species. However, micropropagated plantlets often present low survival rate due to anatomical and physiological disorders induced by *in vitro* conditions. This study aimed to evaluate the sucrose and gas exchange impact on *in vitro* propagated *Billbergia zebrina* plants and to check if there is any residual effect of the *in vitro* conditions on micropropagated plants after acclimatization. Previously *in vitro*-established *B. zebrina* plants were transferred to culture media containing 0.0, 15.0, 30.0, 45.0 or 60.0 g L^{-1} sucrose. Two different culture container sealing systems were tested: lids with a filter (permitting an excellent gas exchange) and lids with no filter (blocking fluent gas exchange). At 45 days *in vitro* growth, *B. zebrina* plantlets were transplanted onto suitable soil mix and evaluated at 80 days growth in greenhouse. At 45 days *in vitro* and 80 days of acclimatization in the greenhouse, the plants were evaluated. High sucrose levels in the *in vitro* media resulted in reduced growth. Plantlets exposed to aerated containers presented better rooting, being the sugar-free medium the best *in vitro* condition (photoautotrophic condition). Limited air exchange resulted in plantlets with anatomical and physiological disorders at the end of the *in vitro* period. The highest growth rate in the greenhouse was observed in plants previously propagated in unlimited gas exchange system and sugar-free medium. The use of photoautotrophic conditions induces *B. zebrina* plantlets without anatomical and physiological disorders and it interfere positively on *ex vitro* growth.

Keywords: bromeliad, *in vitro* plant, plant anatomy, photoautotrophic growth, tissue culture

Introduction

In vitro propagation techniques have been widely used for rapid multiplication of various economically important or endangered plant species, such as many species from the Bromeliaceae family (Guerra and Vesco 2010). Micro-propagation procedures for bromeliads have frequently been published (Martins et al. 2013, 2014; Mengesha et al. 2013; Kurita and Tamaki 2014). Most of these studies are related to the use of plant growth regulators, temperature, carbon source or mineral composition of the culture medium being the main modulators of *in vitro* morphogenetic responses.

Conventional *in vitro* propagation techniques are carried out using closed containers and sugars as carbon source added to the medium (Xiao et al. 2011). The internal atmosphere of these conventional *in vitro* containers consists of high relative humidity, variable CO_2 concentrations, and potential accumulation of ethylene and other gases (De Proft et al. 1985; Kozai 2010). Moreover, depending on the culture condition in the growth room, low irradiance, variable air and temperature may occur.

Conventional *in vitro* conditions may influence on anatomical, physiological and morphological plant characteristics, and cause great losses during later acclimatization treatment (Kitaya et al. 2005). Plants cultivated *in vitro* often present peculiar characteristics, such as poorly developed shoots, tissues with low mechanical strength, higher water content, non-functional stomata and small, thin leaves with fewer trichomes and low photoautotrophic activity (Kozai and Kubota 2001; Cha-um et al. 2011; Xiao et al. 2011). These anomalies become negative features during transfer of *in vitro* plants into the greenhouse or field conditions, causing slow establishment and low survival rate (Pospíšilová et al. 2000; Hazarika 2006; Saez et al. 2012). Only few works have been documented about anatomical

disorders on *in vitro* propagated bromeliad species (Carvalho et al. 2014; Martins et al. 2014), but how it might interfere on survival and growth rate of bromeliads after transfer to greenhouse is still unclear.

According to Fuentes et al. (2005b) the use of sucrose during *in vitro* propagation induces some physiological disorders in micropropagated plants, but sucrose increases the survival rate after greenhouse transfer. Carbohydrates are required by plant cells as carbon resources, act as energy supporting growth and biosynthetic processes, and influence in a positive way *in vitro* rooting (Ferreira et al. 2011).

Supplemental sugar in the *in vitro* medium assists in water conservation and in maintaining the osmotic potential of cells (Hazarika 2003). Sucrose is closely related to stomatal density and chlorophyll content, as well as morphogenetic behavior in some plant tissues, such as vascular and support tissues (Mohamed and Alsadon 2010; Iarema et al. 2012). The exogenous sucrose supply increases the endogenous content of carbohydrate stocks (starch, sucrose, fructose and glucose) in micropropagated plantlets. It favors acclimatization and accelerates physiological adaptations (Jo et al. 2009). However, high sucrose concentrations in the medium potentially decrease photosynthetic ability of *in vitro* plants (Fuentes et al. 2005a, b).

Container closure types ensuring improved ventilation act positively on quality of *in vitro* plantlets. Strong changes of CO_2 and ethylene during the *in vitro* magnolia cultivation have been noticed by De Proft et al. (1985) causing losses of plants when gas exchange was prohibited. The presence of sugar in the medium and accumulated ethylene in sealed vessels may let to the development of abnormal stomata which subsequently reduced the survival rate upon transfer to *ex vitro* conditions (Zobayed 2005). Permeable closures help *in vitro* plantlets to grow photomixotrophically or

photoautotrophically resulting in higher plant functionality and less propagule loss during acclimatization process (Zobayed 2000; Iarema et al. 2012).

Studies indicate that the photoautotrophic growth *in vitro* of many plant species can be significantly promoted by increasing the CO_2 concentration in the culture vessel and reducing relative humidity (Xiao et al. 2011). Photoautotrophic propagation has recently been successfully used for some plant species (Cha-um et al. 2011; Iarema et al. 2012; Shin et al. 2013; Saldanha et al. 2014).

The aim of this study was to analyze the impact of culture container gas exchange and sucrose concentration in the culture media on *in vitro* propagated *B. zebrina* plantlets and if there is any residual effect of these *in vitro* conditions on micropropagated plants after acclimatization.

Material and Methods

Plant material and culture conditions

Billbergia zebrina plantlets were previously established *in vitro* from seeds and cultivated for 60 days. The obtained plantlets were transferred to 250.0 mL glass containers offering 50.0 mL stationary liquid MS medium (Murashige and Skoog 1962), supplemented with 30.0 g L^{-1} sucrose and 13.0 µM 6-benzylaminopurine (BAP) and set at pH 5.8 (Martins et al. 2015). After 30 days of growth, side shoots were subcultivated, for 45 days, in 250.0 mL glass containers containing 50.0 mL stationary liquid MS medium with no plant growth regulator and supplemented with 30.0 g L^{-1} sucrose. Media were autoclaved at 120ºC for 20 minutes. The multiplication procedures were carried out three times on obtained side shoots in order to obtain the necessary number of side shoots with a similar morphology. *In*

vitro plant material was kept in a growth room, at 26 ± 2ºC and a 16-hour photoperiod (8:00 to 00:00 h), under fluorescent tube lamps Philips Master TL5 HO, 49W/840 providing 90 μmol m^{-2} s^{-1} of PAR light.

Sucrose and gas exchange during the *in vitro* propagation

B. zebrina side shoots with approximately 4.0 cm in length (obtained from previous *in vitro* subculture – third generation of induced side shoots), were individualized (5-8 side shoots per seedling) with the aid of a scalpel, after mixing of the explants five side shoots were taken and transferred to 280.0 mL polypropylene containers (ECO2 NV®, Geraardsbergen, Belgium) containing 50.0 mL MS medium solidified with 7.0 g L^{-1} agar (Difco - Agar Nº 1 - MC002) and 0, 15.0, 30.0, 45.0 or 60.0 g L^{-1} sucrose. This phase was carried out with five shoots per container. pH was adjusted to 5.8, before autoclaving at 120ºC, for 20 minutes. Two different sealing systems were tested: lids with a XXL filter (permitted gas exchange system - 62.83 gas replacements per day) and lids with a XXL filter covered with two layers of polyvinylchloride (PVC) transparent film (blocking fluent gas exchange – 4.19 gas replacements per day). After sterile inoculation the material was kept, for 45 days, in a growth room, at 26 ± 2ºC and a 16-hour photoperiod (8:00 to 00:00 h), under fluorescent lamps Philips Master TL5 HO, 49W/840 providing 230 μmol m^{-2} s^{-1} of PAR light.

Growth parameters of *in vitro* plantlets

To evaluate growth, twenty plantlets from each treatment were sampled randomly at 45-day growth and they were divided into four parcels. The aerial part and roots were separated to determine fresh and dry weight.

CO_2 content in the *in vitro* containers

The CO_2 content was analyzed at 45-day of *in vitro* culture at four times over a 24 hour period (6:30, 15:30, 00:30 and 6:30 h) using gas chromatography (Compact GC, Interscience, Louvain-La-Neuve, Belgium, equipped with a thermal conductivity detector). The measurements were performed on three containers per treatment each time.

Anatomical analysis

Anatomical characterization was performed on three plantlets from each treatment. Samples were randomly collected at 45-day growth and fixed in FAA (formaldehyde, acetic acid and ethanol 50%, 0.5/0.5/9, v/v) for 48 hours, followed by storage in 70% ethanol (Johansen 1940).

Paradermal and cross sections were performed in the median region of the first completely expanded leaf in the rosette central region with aid of a double edge razor. Sections were cleared with sodium hypochlorite 10% (v/v) followed by staining with safranin. Finally glycerin 50% was used to assemble the slides.

The sections were investigated using a light microscope (OLYMPUS) coupled to a digital camera. The photomicrographs were used to measure the anatomy characteristics using the software UTHSCSA-Imagetool® calibrated with a microscopy ruler.

The thickness of adaxial and abaxial epidermis (µm), hydrenchyma (µm) and chlorenchyma (µm), sclerenchyma area (μm^2) and xylem diameter (µm) were determined.

Acclimatization of the *in vitro* plantlets

At 45 days *in vitro* culture using different sucrose concentrations and sealing systems, plantlets of *B. zebrina* were transplanted to plastic pots containing peat as a potting medium and kept covered with transparent polyethylene plastic lids in order to maintain a high relative humidity for one week. After this period lids were removed. The substrate remained at a constant moisture level at full field capacity. The plant material was kept, for 80 days, in a greenhouse, at 18ºC (minimal night temperature) and a 16-hour photoperiod.

Growth of acclimatized plants

To evaluate growth, fifteen plants from each treatment were sampled randomly and they were divided into five parcels. The dry weight was measured at 80 days of *ex vitro* culture.

Statistical analysis

The experiment was performed in ten containers per treatment and a completely randomized design in a factorial arrangement (five sucrose concentrations x two different sealing systems) was adopted. The obtained data were submitted to analysis of variance (ANOVA), the averages of the factor sealing systems were compared using Tukey's test and the sucrose concentrations were subjected to regression analysis.

Results

Growth and development of *in vitro* plantlets

The gas exchange rate of the *in vitro* containers and the sucrose concentrations of the media directly influenced the morphologic responses of

B. zebrina plantlets (Fig. 1). Fresh and dry weights of the explants' aerial part showed significant interactions between the two factors examined. Fresh and dry weight of plantlets cultivated in containers with filter (aerated containers permitting unrestricted gas exchange) presented a negative linear relationships with increasing sucrose concentrations (Fig. 2A-B). When restricting the gas exchange the plantlets' dry weight showed positive relationships with increasing sucrose concentrations. Sucrose levels in the media bring up a kind of optimum around 30 g L^{-1} for the plantlets fresh weight. *B. zebrina* plantlets cultivated on sugar-free medium in aerated *in vitro* container had the highest fresh and dry weight build up (Fig. 2A-B), resulting in the most developed plantlets (Fig. 1A).

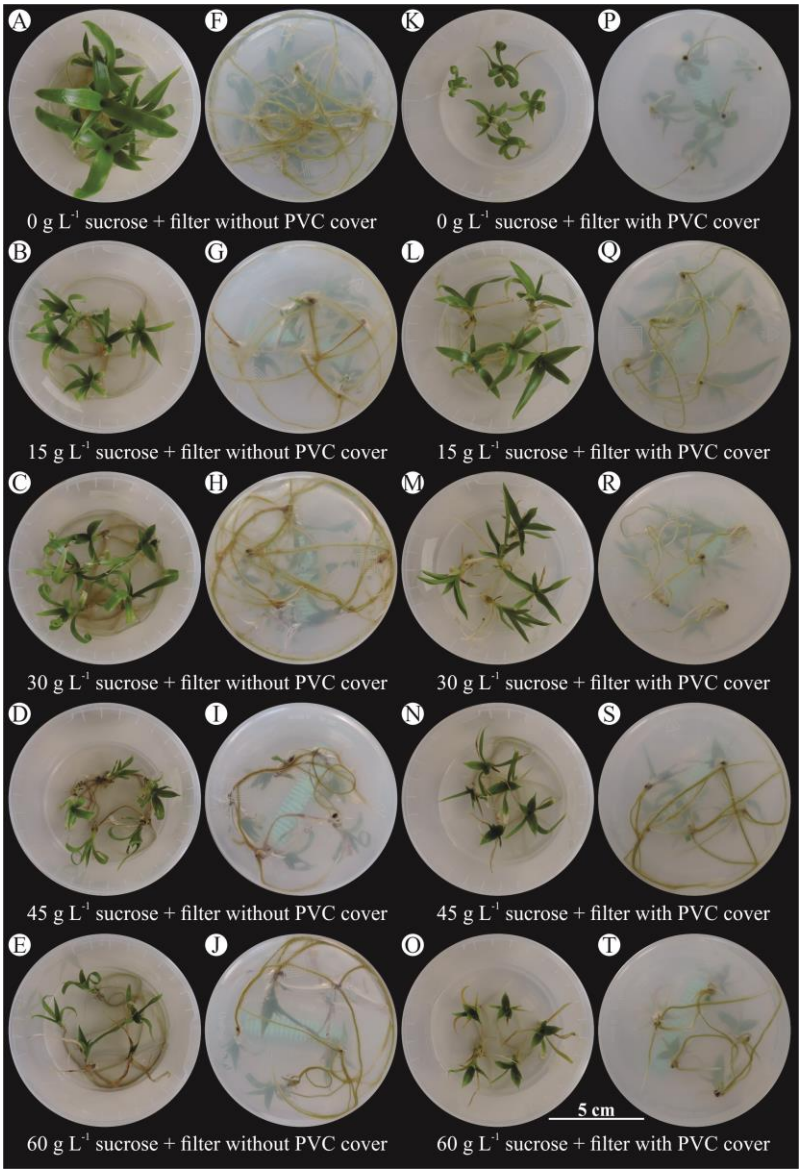

Fig. 1 *B. zebrina* plantlets at 45 days of *in vitro* growth, exposed to different sucrose concentrations in the medium and sealing systems of the containers. Aerial part and roots of plantlets grown in filter (A-J) and no filter containers (K-T) in function of sucrose concentration (0.0, 15.0, 30.0, 45.0, 60 g L^{-1} sucrose, always from top to bottom).

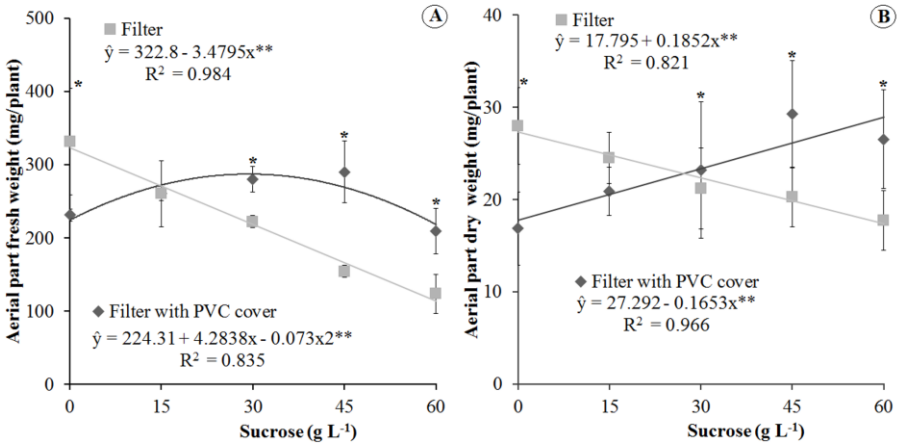

Fig. 2 Fresh (A) and dry (B) weight (mg/plant) of *B. zebrina* plantlets (aerial parts) as a function of sucrose (g L^{-1}) in the *in vitro* medium and sealing system of the culture containers. For each sucrose concentration (g L^{-1}) analyzed, averages followed by asterisk differ from each other according to the Tukey's test, at 5%.

Rooting occurred in all treatments studied (Fig.1). The roots fresh and dry weights were similar for all plantlets grown with a limited gas exchange (filter covered with PVC) (average of 60 mg/plant). When explants were cultivated with unlimited gas exchange (filter) the roots fresh and dry weight presented a negative linear correlation with increasing sucrose concentrations. Accumulation of root dry matter was lower in limited gas exchange containers (Fig. 3A-B).

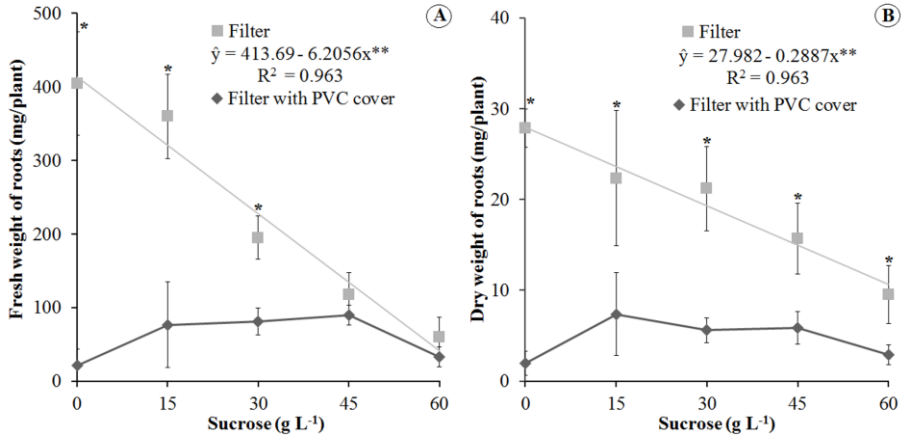

Fig. 3 Fresh (A) and dry (B) weight (mg/plant) roots from *B. zebrina* plantlets as a function of sucrose (g L^{-1}) in the medium and gas sealing of the culture containers. For each sucrose concentration (g L^{-1}) analyzed, averages followed by asterisk differ each other according to the Tukey's test, at 5%.

CO_2 concentration of the containers

The CO_2 level inside the *in vitro* culture containers was clearly influenced by the gas exchange capacity. With unrestricted gas exchange, CO_2 levels were balancing around 250 ppm (dark and light phase). Restricting the gas exchange resulted in an increased CO_2 level. The highest CO_2 values were reached at the end of the dark period (6:30h) getting up to 600 ppm. During the light period (15:30h) lower CO_2 levels were detected (3 times lower than during the dark period) (Fig. 4).

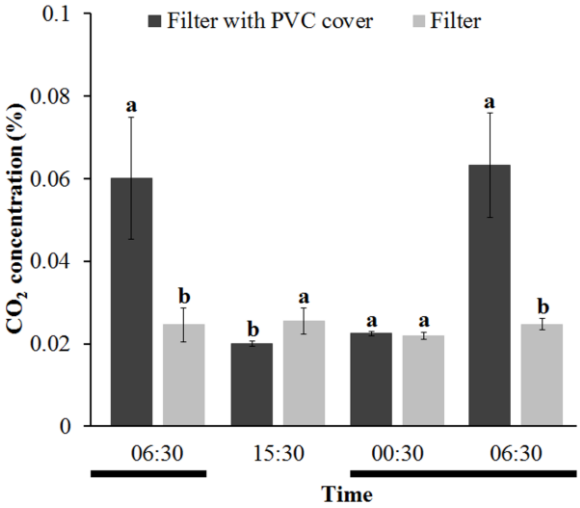

Fig. 4 CO_2 concentration (%) at different times during the day in *in vitro* containers holding *B. zebrina* plantlets. For each time analyzed, averages followed by different letters differ from each other according to Tukey's test, at 5%. The dark period is indicated by the black bar at the bottom of the figure.

Anatomical analysis

In *B. zebrina* plantlets, the surface of leaves shows tetracytic stomata with hypostomatic distribution. Transversal leaf sections present a one cell layer epidermis both sides of the leaf. The mesophyll has a dorsiventral organization. At the adaxial leaf side there is the water-storage parenchyma (hydrenchyma) containing non-chlorophyll with thin walled cells. Chlorophyll parenchyma (chlorenchyma) with isodiametric cells is located at abaxial leaf side. The vascular bundles are collateral, usually larger bundles alternating with smaller bundles and surrounded by sclerenchyma fibers (Fig. 5). This leaf structure is frequently observed and described in many bromeliads (Mantovani et al. 2012; Pereira et al. 2013; Martins et al. 2014).

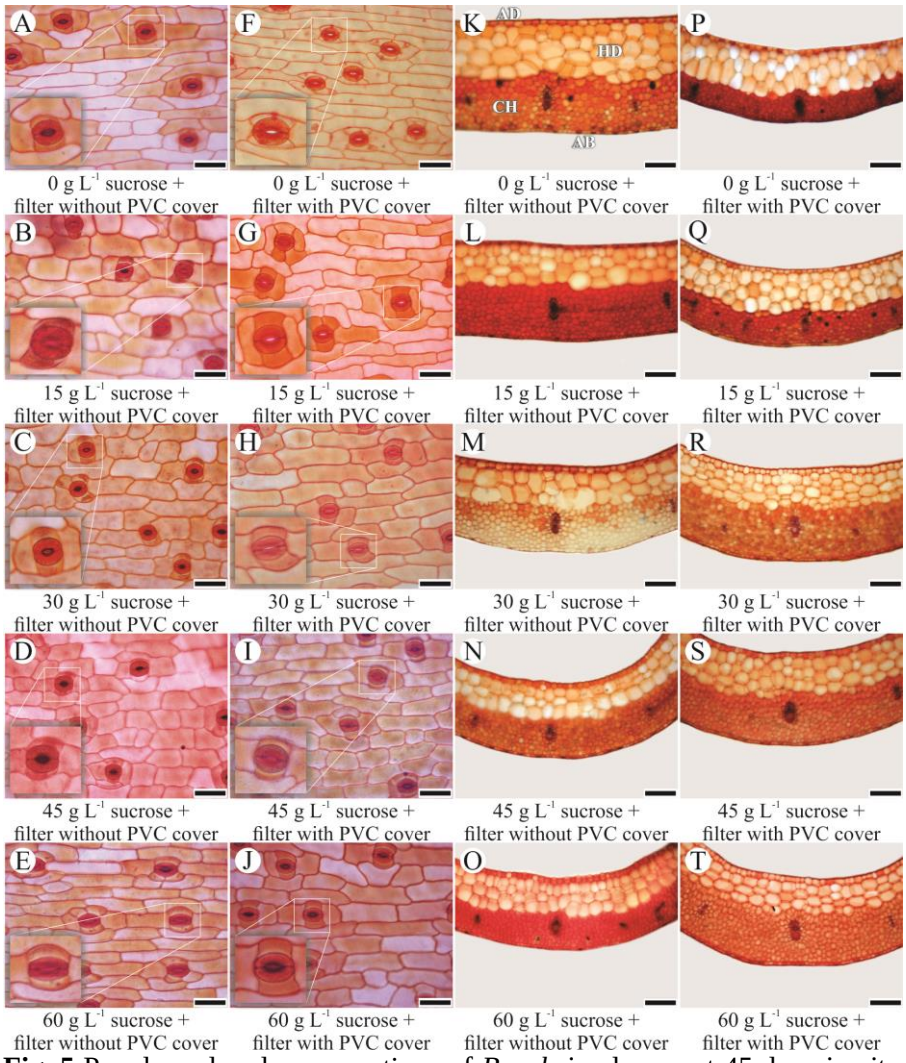

Fig. 5 Paradermal and cross sections of *B. zebrina* leaves at 45 days *in vitro* cultivation on media containing different sucrose concentrations and sealing system of the containers. Leaf paradermal and cross sections of plantlets grown with an unlimited air exchange (A-E and K-O) and with a limited air exchange containers (F-J and P-T) in combination with different sucrose concentrations (0.0, 15.0, 30.0, 45.0, 60 g L^{-1} sucrose, from top to bottom). Adaxial epidermis (AD), abaxial epidermis (AB), hydrenchyma (HD) chlorenchyma (CH). Bar = 50 µm (A-J); Bar = 200 µm (K-T).

Sucrose concentrations of the culture media influenced the stomatal shape. A larger stomatal porous was noted in treatments with limited gas exchange and low sucrose levels. Increasing sucrose in the media induced more elliptical shaped stomata and this is directly correlated to higher stomatal functionality (Fig. 5).

Adaxial epidermis thickness increased with increasing sucrose concentrations in containers with limited air exchange. Nevertheless, when the plantlets were cultivated without air exchange restriction, the adaxial epidermis did not show any difference between sucrose treatments, being as thick as those grown in high sucrose concentration and limited air exchange (Fig. 6A). Among the treatments, only the sucrose concentrations influenced the abaxial epidermis thickness regardless the sealing type of the containers. The lower epidermis, being thinner than the upper one, presented an increasing linearity as the sucrose concentrations raised (Fig. 6B).

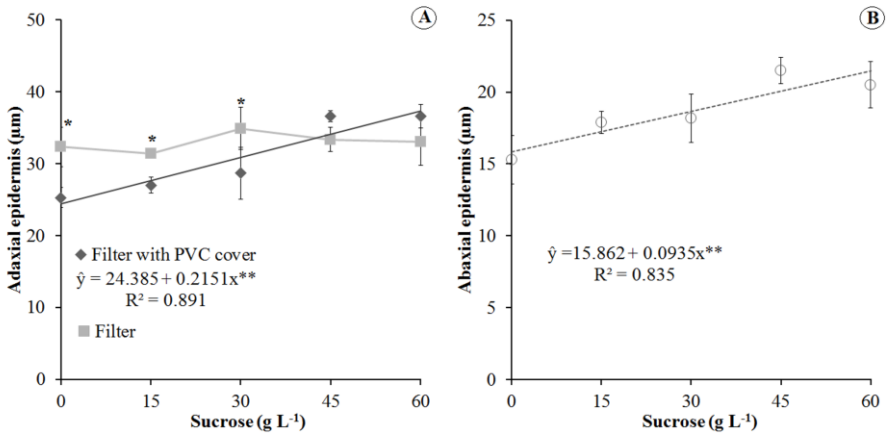

Fig. 6 Adaxial (A) and abaxial (B) epidermis thickness (µm) of *B. zebrina* leaves influenced by sucrose (g L^{-1}) in the *in vitro* medium and sealing type of the *in vitro* container at 45 days in *in vitro* culture. For each sucrose

concentration (g L^{-1}) analyzed, averages followed by asterisk differ from each other according to the Tukey's test, at 5%.

The thickest hydrenchyma was observed in plantlets grown in sugar-free medium and a free gas exchanging container. Increasing sucrose concentrations (60 g L^{-1}) resulted in a 50 % decrease of this hydrenchyma layer thickness. No differences between the sucrose treatments were noted in containers with restricted air exchange (Table 1).

The chlorochyma thickness presented a linear relation with increasing sucrose concentrations in both container sealing systems. However thickening was observed as plantlets grown under limited air exchange and sucrose levels induced a negative relationship when compared in those plantlets grown in more aerated containers (Table 1).

The sclerenchyma area and xylem vessel diameter were analyzed in central vascular bundles of *B. zebrina* leaves (Fig. 7). High sucrose concentrations had a positive effect on the sclerenchyma area size. Ventilated containers act in a positive way on sclerenchyma increment (Table 1). On the other hand, sucrose and non-ventilated containers had a negative effect on xylem vessel diameter making these water conducting vessels smaller in size (Table 1).

Table 1 Anatomical structures of *B. zebrina* leaves as a function of sucrose levels (g L^{-1}) in the *in vitro* media and container sealing type at 45 days culture

Sucrose (g L^{-1})	Hydrenchyma (µm)		Chlorenchyma (µm)		Sclerenchyma area (µm²)		**X**ylem vessel diameter (µm)	
	Filter without PVC cover	Filter with PVC cover	Filter without PVC cover	Filter with PVC cover	Filter without PVC cover	Filter with PVC cover	Filter without PVC cover	Filter with PVC cover
0.0	345 ±32^{a} (1)	220 ±29^{b}	338 ±17^{a} (2)	140 ±11^{b} (3)	1641 ±293^{a} (4)	40 ±3^{b} (5)	10.7 ±0.8^{a} (6)	6.0 0.5^{b}
15.0	280 ±18^{a}	253 ±27^{a}	293 ±5^{a}	188 ±11^{b}	1393 ±127^{a}	367 ±16^{b}	9.4 ±1.1^{a}	6.5 ±0.2^{b}
30.0	201 ±8^{b}	248 ±27^{a}	241 ±19^{b}	326 ±32^{a}	1001 ±341^{b}	1588 ±67^{a}	7.7 ±0.1^{a}	6.8 ±0.3^{a}
45.0	213 ±9^{a}	243 ±18^{a}	247 ±33^{a}	283 ±17^{a}	1176 ±363^{a}	1468 ±373^{a}	8.7 ±0.7^{a}	6.9 ±0.5^{b}
60.0	176 ±41^{b}	243 ±14^{a}	239 ±18^{b}	342 ±37^{a}	1718 ±478^{b}	2307 ±451^{a}	6.3 ±0.7^{a}	6.7 ±0.1^{a}

Averages (±SD) followed by the same letter in the row, in each anatomical struture, do not differ according to the Tukey‟s test at 5% probability. (1) $\hat{y} = 324.07 - 2.6878x^{**}$, $R^2 = 0.86$; (2) $\hat{y} = 320.4 - 1.6164x^{**}$, $R^2 = 0.79$; (3) $\hat{y} = 156.67 + 3.317x^{**}$, $R^2 = 0.80$; (4) $\hat{y} = 1705.4 - 41.3x + 0.6815x^{2**}$, $R^2 = 0.89$; (5) $\hat{y} = 27.269 + 37.572x^{**}$, $R^2 = 0.913$; (6) $\hat{y} = 10.501 - 0.0634x^{**}$, $R^2 = 0.80$.

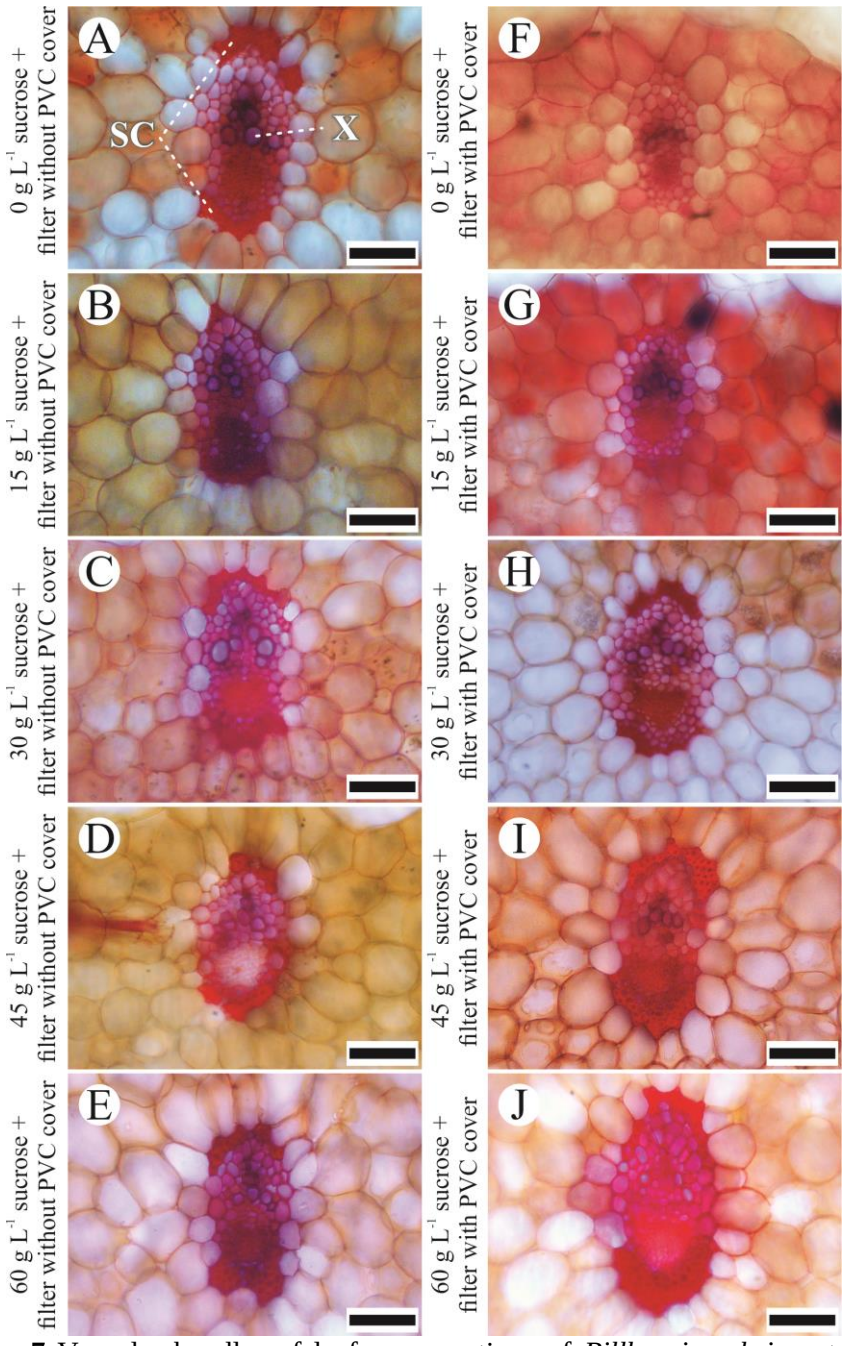

Fig. 7 Vascular bundles of leaf cross-sections of *Billbergia zebrina* at 45 days *in vitro* cultivation on media with different sucrose concentrations and sealing type systems. Leaf cross-sections of plantlets grown in aerated (A-E) and no aerated containers (F-J) in function sucrose concentration (0.0, 15.0, 30.0, 45.0, 60 g L^{-1} sucrose, always from top to bottom). Sclerenchyma (SC), Xylem vessel (X). Bar = 50 µm (A-J).

Growth and development of acclimatized plants

At 80 days *ex vitro* conditions, *B. zebrina* plants presented a survival rate of 100% in all previously examined *in vitro* treatments, but these plants showed different growth rates. Plantlets cultivated in photoautotrophic conditions *in vitro* (sugar-free in the medium and aerated containers) had aerial parts with the highest dry weight. Aerial part's dry weight of plantlets previously grown with limited air exchange showed a generally stable level with increasing sucrose concentrations after the transfer to greenhouse (Fig. 8).

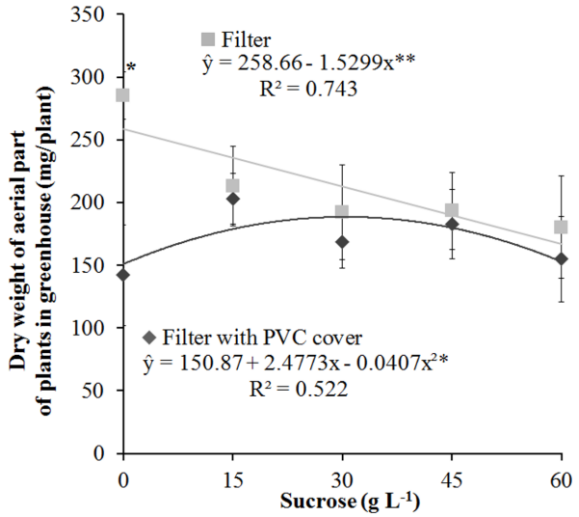

Fig. 8 Aerial part dry weight (mg/plant) of acclimatized *B. zebrina* plants in relation to the sucrose concentrations (g L^{-1}) in the medium and container sealing type during *in vitro* culture. For each sucrose concentration (g L^{-1}) analyzed, averages followed by asterisk differ from each other according to the Tukey's test, at 5%.

Discussion

This research reports for the first time the impact of sucrose and gas exchange effects on bromeliads grown under tissue culture conditions. Under photoautotrophic conditions (sugar-free media and no gas exchange restrictions) a higher growth rate and no anatomical disorders were found compared to bromeliad plantlets kept under conventional *in vitro* culture

conditions. These conditions may determine the explant survival rate after greenhouse transfer (Park et al. 2011).

B. zebrina plantlets cultivated in conventional *in vitro* containers respond clearly on the supply of sucrose (Fig. 1, 2). Plantlets cultivated in containers with filter covered with PVC, require exogenous sucrose for growth and rooting (Iarema et al. 2012; Eckstein et al. 2012). Sucrose contributes to the induction and growth of roots by acting on cell expansion and proliferation (Wang and Ruan 2013). Nevertheless, the supplementation of sucrose was not a limiting factor for root induction in *B. zebrina*. Plantlets cultivated in ventilated containers with no sucrose in de medium performed excellent root formation (Fig. 1, 3). The positive effect of container aeration is mainly due to the improved CO_2 exchange with the open air and the prevention of ethylene accumulation (Trevisan and Mendes 2005). Using gas-permeable container types allow a continuous supply of CO_2 during the light period and lower relative humidity (Xiao et al. 2011). CO_2 increase in photoautotrophic culture conditions may play a key role as carbon and energy support for root initiation and development (Hazarika 2003; Chan-um et al. 2011). However, the improved ventilation of containers enhances evapotranspiration which may alter the physical properties of culture media attributed to water loss (Ivanova and Staden 2010). Evaporation of artificial media decreases the osmotic potential due to an increased concentration of the dissolved compounds resulting into osmotic stress of the *in vitro* plants (Cui et al. 2010).

When subjected to environmental stress, plants actively reduce their vegetative growth to conserve and redistribute resources and increasing their chance of survival if stress continuous (Skirycz and Inzé 2010). This was verified on *B. zebrina* plantlets grown on media with sucrose in ventilated containers (Fig. 1, 2). Media in these aerated containers had substantial water loss (around 34% of water loss) at the same time reduced plant growth was noticed probably due to low osmotic potential of the media in combination with a reduced air humidity.

B. zebrina plantlets grown in aerated *in vitro* containers demonstrated a CO_2 level equal during the light and dark period (Fig. 4). This CO_2 level equals the level of the ambient atmosphere. The low CO_2 level (>0.02%) in the *in vitro* containers during the light period does not make use of the full photosynthesis capacity and it may result in a net negative balance of CO_2 uptake per day. *In vitro* plants compensate this unbalance by using sucrose supplied trough the medium (Hazarika 2006). This was confirmed by plantlets grown in sugar-free and no gas exchange conditions. They had the lowest growth rate due to the absence of a compensatory carbon source in the medium (Fig. 1, 2).

Adequate CO_2 levels are required for *in vitro* propagation with limited or no sucrose supply. CO_2 addition into sealed containers or containers having a gas delivery system may stimulate growth of *in vitro* plantlets (Xiao et al. 2011; Iarema et al. 2012).

Substantial differences were revealed on leaf anatomy of *B. zebrina* plantlets, which are of importance for the growing success after they are transferred to *ex vitro* conditions (Fig. 5-7 and Table 1). The main adaptation problem is developing an accurate transpiration control by the micropropagated plants, more specifically by stomatal functionality (Dias et al. 2014b). The stomatal functioning is an important feature to avoid water deficiency under *ex vitro* environment. Most of the time plantlets grown *in vitro* have dysfunctional stomata (Dias et al. 2014a). Plantlets of *B. zebrina* from containers with no air exchange kept stomata open in plantlets cultivated on sugar-free and 15 g L^{-1} sucrose, expressing a lower functionally (Fig. 5F-G). Immediately after *ex vitro* transfer, *in vitro* plants dehydrate quickly, becoming wilted due to a deficient functionality of the stomata (Dias et al. 2013). Plantlets from aerated containers contain functional stomata, since they had the closed ostioles, especially in plants cultivated on 60 g L^{-1} sucrose (Fig. 5A-E). This enhanced stomatal functioning prevents excessive desiccation of the micropropagated plants after transplanting,

increasing the chances of survival in *ex vitro* environment (Deccetti et al. 2008).

Well-developed dermal tissue is crucial for checking undue water loss under low availability of water, particularly under osmotic stress (Hameed et al. 2013). This supports the observed increase of epidermis thickness with increasing sucrose concentrations (Fig. 5H-Q and Fig. 6A-B). In addition, ventilated container also acts on epidermis thickness (Mohamed and Alsadon 2010), as verified on adaxial epidermis of *B. zebrina* plantlets. Larger epidermal cells developed in plantlets grown in ventilated containers might support the mechanisms preventing water loss, favouring the acclimatization during adaptation *ex vitro*.

Besides avoiding water loss, the hydric maintenance is essential for the *ex vitro* growth after transfer from *in vitro*. Hydrenchyma cells are specialized in storing water. Increased volume of this tissue results in a higher water storage capacity (Pedroso et al. 2010; Martins et al. 2014). This water-storage parenchyma has a water buffering action, avoiding dehydration of *in vitro* propagated plantlets during the first days after transplantation, contributing to a higher survival rate (Barboza et al. 2006). *B. zebrina* plantlets cultivated in sugar-free media in a ventilated container do have a thicker hydrenchyma (Fig. 5 and Table 1), bringing up a 100% survival of the *in vitro* plantlets during the acclimatization.

Possessing a good photosynthesis rate is very relevant for plant growth during acclimatization. Higher photosynthetic capacity may be correlated with a larger mesophyll volume (Freschi et al. 2010). Thus, stimulation of the photoautotrophic behaviour of plantlets grown *in vitro* is potential to improve quality of the plants. *B. zebrina* plantlets cultivated under these conditions had the highest chlorophyll containing parenchyma thickness (Fig. 5 and Table 1). Moderate hydric stress may increase the mesophyll thickness, as already demonstrated by Aragón et al. (2012) for pineapple plants. Leaf plasticity is important to maintain the control of water use in plants and it may reduce leaf area (Shao et al. 2008), as verified in *B.*

zebrina plantlets cultivated in high sucrose. Plantlets cultivated under high air humidity (filter covered with PVC) and osmotic stress due to high sucrose concentrations had smaller and thicker leaves (Fig. 1, 5 and Table 1).

Proper development of sclerenchyma cells is important for plant shape and functioning. Sclerenchyma containing tissue provides critical mechanical support for plants. Being part of vascular bundles sclerenchyma containing tissues support transport of water, nutrients and signalling molecules throughout the plants (Bao et al. 2012). However, plants grown *in vitro* have considerably less sclerenchyma than plants grown in open air (Hazarika 2006). Sclerenchyma in leaves provide rigidity and offers protection of specific structures like vascular bundles (Ola et al. 2012). *B. zebrina* plantlets demonstrate good development of sclerenchyma tissue under sucrose concentration higher than 30 g L^{-1} (Fig. 5, 7 and Table 1). This may be a consequence of the osmotic stress caused by the *in vitro* medium. Plants under osmotic stress present smaller leaves and well-developed sclerenchyma in these leaves (Salih et al. 1999), supporting our observations. Ventilation of the culture container also resulted in an increasing sclerenchyma area around the vascular bundles. Plantlets cultivated under photoautotrophic conditions showed clearly more sclerenchyma (Fig. 7 and Table 1).

The xylem vessel diameter was larger in plantlets grown in aerated containers. The vascular system of leaves under the ventilated conditions offered a good development xylem as demonstrated in potato plants by Mohamed and Alsadon (2010). The largest xylem vessel diameter was observed in plantlets cultivated in sugar-free and ventilated containers. (Fig. 7 and Table 1). Larger xylem vessels are related to a higher hydraulic conductance (Rodríguez-Gamir et al. 2010). Efficient xylem is characterized by wide conduits and long vessels (Bresta et al. 2011).

The *in vitro* culture history had a high impact on plant growth of *B. zebrina* still at 80 days greenhouse growth. Plants cultivated in photoautotrophic conditions (sugar-free and aerated containers) presented

the best growth (Fig. 8). This was supported by leaf anatomic features, which provide a good functionality of the physiologic apparatus. *In vitro* culture conditions may contribute to a plant phenotype that cannot survive in *ex vitro* environmental conditions (Hazarika 2006). A low survival rate side by side with a slow growth rate of tissue culture plants are critical problems facing the success of tissue culture as a commercial technique (Mohamed 2008).

Conclusion

Culture container gas exchange and sucrose concentration of the media during *in vitro* growth have an influence on *in vitro* and *ex vitro* plant growth, anatomical and physiological functioning. Photoautotrophic conditions induce *B. zebrina* plantlets to grow and develop without anatomical and physiological disorders. This induced a behaviour that interfered positively on *ex vitro* growth. Conventional *in vitro* culture of plantlets showed a slower growth after transfer to *ex vitro* greenhouse conditions.

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ARTICLE 3

Physiological responses of *Billbergia zebrina* (Bromeliaceae) in controlled microenvironmental conditions

(Prepared in accordance with *Biologia Plantarum*'s standards)

Abstract
Microenvironmental conditions may act on growth of *in vitro* plants. Sucrose, being the most common used carbon source in conventional *in vitro* culture, and limited air exchange by the culture containers are conditions acting on growth and development of tissue cultured plants. However, it may induce physiological disorders and decrease survival rate of these plants after transfer to *ex vitro* conditions. The aim was to analyze the effects of gas exchange and sucrose concentration on *B. zebrina* plantlets during *in vitro* propagation. Previously *in vitro*-established *B. zebrina* plants were transferred to culture media containing 0, 15, 30, 45 or 60 g dm^{-3} 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) contents were analysed in *B. zebrina* plantlets at 45-d of culture. Added sucrose in the medium reduced the Chl a+b content of the plantlets. On the other hand, supplied sucrose acted positively on carbohydrate stock formation of *in vitro* propagated *B. zebrina* plantlets. Photoautotrophic conditions (air exchange and sugar-free) induced plantlets with normal levels of photoassimilates. It became clear that a photoautotrophic system offers good prospects for the *in vitro* propagation of *B. zebrina* without creating physiological disorders.

Additional key words: bromeliad, *in vitro* plant, photoautotrophic growth, physiological disorders, sucrose, tissue culture

Abbreviations: Chl a+b - chlorophyll a+b; MS - Murashige and Skoog medium.

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Introduction

Micropropagation is by far the most used method for large-scale cloning of several high value crops, such as bulbous plants, fruit trees and ornamentals. Bromeliads being grown as flowering potted ornamentals with high commercial value represent a large part of the flower industry worldwide (Zhang *et al.* 2012). *Billbergia zebrina* (Herbert) Lindley, is an epiphytic tank bromeliad native to Atlantic Rainforest of Brazil. This plant has commercial value as an ornamental due to the beauty of its leaves and inflorescence (Vesco *et al.* 2011).

Application of plant tissue culture techniques for the *in vitro* propagation of bromeliads has already been reported (Chu *et al.* 2010, Huang *et al.* 2011, Martins *et al.* 2013, Martins *et al.* 2014). Most of these studies are related to the use of plant growth regulators as major modulator of *in vitro* morphogenesis. *In vitro* culture conditions are considered to be stressful for plants (Desjardins *et al.* 2009), as such, influencing plant development (Shi *et al.* 2013). Conventional *in vitro* propagation has been proven to induce anatomical and physiological disorders on plantlets (Mohamed and Alsadon 2010, Iarema *et al.* 2012) and may interfere on growth and survival rates of plants after transfer to *ex vitro* conditions (Fuentes *et al*. 2005, Shi *et al.* 2014).

Disorders observed on *in vitro* plants are directly or indirectly related to the heterotrophic conditions in a conventional micropropagation system (Hazarika 2006). Conventional *in vitro* propagation is mostly carried out using small closed glass culture containers and composed nutrient media containing sucrose as the major carbon source (Xiao *et al.* 2011). This external sugar supply is adequate for growth and organogenesis support (Jo *et al.* 2009). In the case of bromeliads 3% sucrose in the medium is recommended (Chu *et al.* 2010, Martins *et al.* 2013). Sucrose effects on the physiology of *in vitro* plants has been documented (Mohamed and Alsadon 2010, Iarema *et al.* 2012, Shi *et al.* 2013, Shi *et al.* 2014). Reduced photosynthetic ability (Shi *et al.* 2013) and plant survival and growth rate

during later acclimatization arise from previous sucrose treatments (Shi *et al.* 2014). However, supplementing sugars acts positively on carbohydrate stock formation of *in vitro* propagated plants (Ferreira *et al.* 2011). A high carbohydrate stock improves plant performance during the acclimatization phase (Fuentes *et al.* 2005, Chu *et al.* 2010).

Limited gas exchange, caused by the type of culture containers used, acts on the development of *in vitro* grown plants. *In vitro* environment is characterized by high relative humidity, a potential of ethylene build up, stagnant air, and fluctuating CO_2 concentration caused by day and night cycles (Fujiwara and Kozai 1995). CO_2 concentrations have a high impact on photosynthesis and growth of *in vitro* plants and it affects the metabolism of these plants (Iarema *et al.* 2012, Shi *et al.* 2013). Gas exchange improves in ventilated culture containers helping *in vitro* plantlets to growth photomixotrophically or even photoautotrophically (sugar-free and gas exchange potentials) resulting in higher plant quality and less propagule losses during the acclimatization process (Zobayed *et al.* 2000, Shi *et al.* 2014). The aim of this study was to analyze how the gas exchange and sucrose concentration can act on the physiology of *B. zebrina* plantlets during the *in vitro* propagation.

Material and Methods

Plant material and culture conditions

Billbergia zebrina plantlets previously established *in vitro* through seeds were transferred to 250 mL glass containers containing 50 mL stationary liquid Murashige and Skoog medium (1962; MS), supplemented with 30 g dm^{-3} sucrose and 13 µM 6-benzylaminopurine. After 30-d of growth, side shoots were subcultivated, for 45-d, in 250 mL glass containers containing 50 mL stationary liquid MS medium with no plant growth regulator and supplemented with 30 g dm^{-3} sucrose. Medium pH was set at 5.8, before autoclaving at 120ºC for 20 min. After sterile inoculation , plant cultures were kept in a growth room, at 26 ± 2ºC and a 16-h photoperiod

(8:00 to 00:00 h), under fluorescent lamps Philips Master TL5 HO, 49W/840 providing 90 μmol m^{-2} s^{-1} of PAR light.

Sucrose and gas exchange during the in vitro propagation

B. zebrina side shoots approximately 4.0 cm in length, from previous *in vitro* stage, were individualized 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 dm^{-3} agar and supplemented with 0, 15, 30, 45 or 60 g dm^{-3} sucrose. Each container received five shoots. The pH was adjusted to 5.8, before autoclaving at 120ºC, for 20 min. Two different sealing systems were used : container lids with a XXL filter (permitted gas exchange system – minimal 63 air exchanges per day) and the same container lids covered with 2 layers of polyvinyl chloride (PVC) transparent film (blocking gas exchange ofthe XXL filter). After sterile inoculation the cultured plants were kept, for 45-d, in a growth room, at 26 ± 2ºC and a 16-h photoperiod (8:00 to 00:00 h), under fluorescent lamps Philips Master TL5 HO, 49W/840 providing 230 μmol m^{-2} s^{-1} of PAR light.

Extraction and analyses of chlorophyll

For chlorophyll analysis, the aerial part of five plants per treatment were used and divided into five parcels. After weighing, chlorophyll was extracted from the plant material using dimethyl-formamide by a 72 hours dark incubation. The absorbance at 647 and 663 nm was measured by a spectrophotometer (UV-1800, Shimadzu). The final determination of the chlorophyll content was done based on the work of Wellburn (1994).

Extraction and analyses of metabolites

To determine plant metabolites, fifteen plants per treatment were collected at 6:00 h, after mixing the plants three parcels were taken. Leaves and roots were collected and individualized with the aid of a scalpel. All

samples were immediately frozen in liquid nitrogen. Plant material was lyophilized and crushed before taking a known amount of plant material on which metabolite analyses were carried out. For determination of malic acid, glucose, fructose and sucrose contents we used the ENZYTEC system (Enzytec, Scil Diagnostics GmbH, Germany) using a spectrophotometer set at 340 nm (DU-65; Beckman, Fullerton, CA) according to the protocol described by Ceusters at al. (2008). Starch content was determined as glucose equivalents following digestion with amyloglucosidase according the instructions of Enzytec (Enzytec, Scil Diagnostics GmbH, Germany). Analyses were performed on three independent biological samples. To quantify glucose, fructose and sucrose contents in the culture media, three independent samples per treatment were collected in different containers and performed as mentioned above.

Water loss of containers

To evaluate water loss of the containers used in this study, five containers from each treatment were sampled randomly. They were weighed at 0 and 45-day and the differences of weights were used to determine water loss (%).

Statistical analysis

The experiments were performed in a completely randomized design in a factorial arrangement (five sucrose concentrations x two different sealing systems). The obtained data were submitted to analysis of variance (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 with increasing sucrose concentrations, independent of the sealing system (Fig. 1*A*). However, when

air exchange was possible Chl a+b content (517.79 µg gfw^{-1}) was higher than those plants grown in containers without filter (433.65 µg gfw^{-1}).

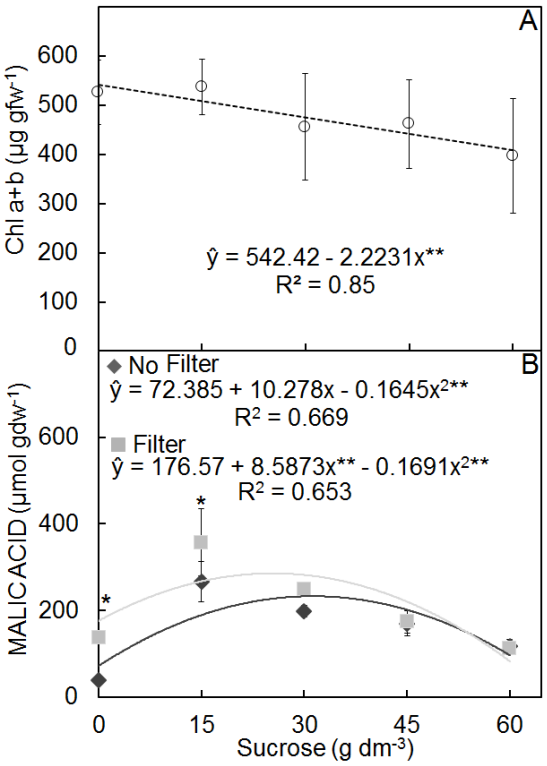

Fig. 1. Chlorophyll a+b (A) and malic acid (B) content of leaves from *B. zebrina* plantlets exposed to different sucrose concentrations in the medium and culture containers with a sealed system. For each sucrose concentration analyzed, averages followed by asterisk differ from each other according to the Tukey's test, at 5%.

The initial level of different metabolites in *B. zebrina* shoots at the start of the treatments is shown in Table 1. These shoots contained high monosaccharide (glucose and fructose) contents, but sucrose was not detectable. Starch, expressed in glucose equivalents, was present in comparable amounts as the monosaccharides. Since *B. zebrina* is a CAM plant, malic acid was also detected in the leaves.

Table 1. Carbon metabolite contents (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. *not detectable.

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

Among the findings, malic acid content presented a positive quadratic relationship with increasing sucrose concentrations in both container sealing systems after 45-d of growth. Plantlets cultivated on sugar-free or 15 g dm^{-3} sucrose in the medium and filter containers had higher malic acid levels than plantlets kept in the same sucrose concentrations and no filter containers (Fig. 1*B*).

Glucose and fructose content of the leaves presented positive linear and negative quadratic models with increasing sucrose concentrations when they were cultivated in no filter and filter containers, respectively. Plants grown on medium with 60 g dm^{-3} sucrose and no aeration of the container had the highest glucose content (Fig. 2*A*). Conversely, the fructose content was the highest in plants cultivated on sugar-free medium and in an aerated container (Fig. 2*B*). The monosaccharide contents were in general lower in roots than in leaves. Glucose content in roots showed a positive linear model independent of the container sealing system (Fig. 2*E*). However, fructose content was higher in plants grown in aerated containers and there was an increment of the carbohydrate content relationships with increasing sucrose concentrations (Fig. 2*F*).

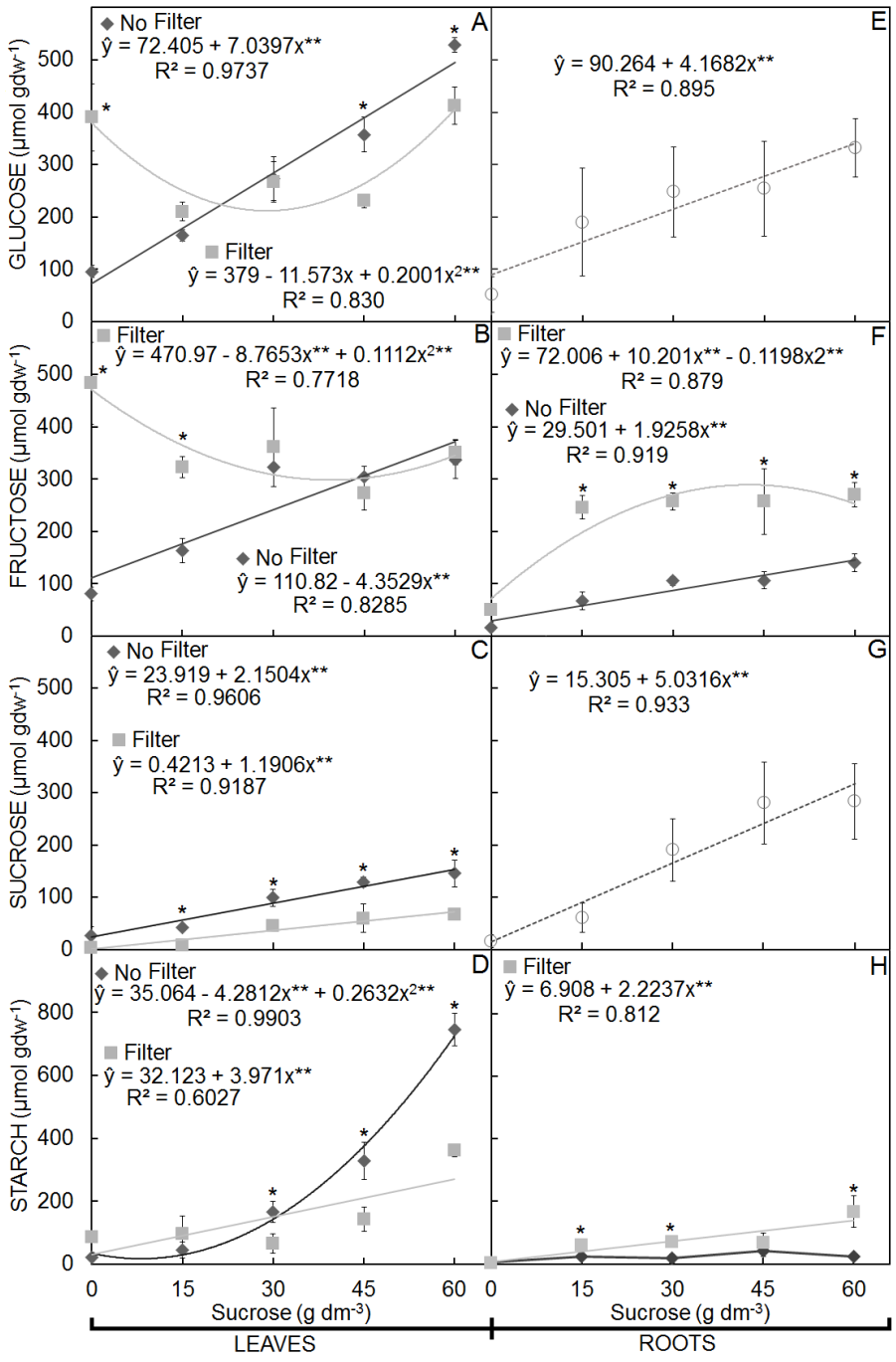

Fig. 2. Carbohydrate content in leaves (A-D) and roots (E-H) determined at the end of the dark period (6:00am) of *B. zebrina* plants in relation with the sucrose concentrations in the medium and container sealing system. For each sucrose concentration analyzed, averages followed by asterisk differ from each other according to the Tukey's test, at 5%.

Sucrose content was higher in roots than in leaves of plants cultivated on different sucrose levels in the *in vitro* media. Sucrose levels for both plant organs presented linear relationships with increasing sucrose concentration in the medium (Fig. 2*C, G*). Starch contents were higher in leaves than roots at 45-d of *in vitro* growth. The increment of starch was positively related with increasing of sucrose concentration in the medium. The highest starch content was verified in leaves of plants grown in containers with limited air exchange and a high sucrose content of the medium. However, the content of starch in roots was rather low for all plants grown in containers with no aeration (Fig. 2*D, H*).

The carbohydrate content of all nutrient media was analysed. When compared the two sealing systems, a higher glucose and fructose content was verified in the media with prior addition of 15 and 30 g dm^{-3} sucrose and filter (Table 2). In containers without gas exchange, supplemented sucrose was consumed by the plants or converted into glucose or fructose. A slight increasing of carbohydrate accumulation in the media with previous addition of 45 and 60 g dm^{-3} sucrose when the air exchange was observed (Table 2).

All treatments were performed in containers without plants and the carbohydrate contents verified at 45-d in the same growth conditions. As shown in the Table 2, there were no differences in the sucrose contents between container sealing systems, as expected (Table 2).

Table 2. Carbohydrate contents in the culture media at 45-days in containers with and without plants. Averages followed by the same letter in the row for each carbohydrate content, do not differ according to the Tukey's test, at 5%.

Sucrose (g dm^{-3})	Carbohydrate contents in the culture media with plants					
	Glucose (µmol gfw^{-1})		Fructose (µmol gfw^{-1})		Sucrose (µmol gfw^{-1})	
	No Filter	Filter	No Filter	Filter	No Filter	Filter
0	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
15	0.18 ± 0.07b	0.33 ± 0.09a	0.18 ± 0.07b	0.33 ± 0.10a	0.27 ± 0.12a	0.49 ± 0.20a
30	0.16 ± 0.03b	0.34 ± 0.16a	0.16 ± 0.03b	0.34 ± 0.15a	1.06 ± 0.11a	0.89 ± 0.11a
45	0.23 ± 0.02a	0.23 ± 0.02a	0.22 ± 0.02a	0.23 ± 0.02a	1.68 ± 0.10b	2.38 ± 0.29a
60	0.25 ± 0.07a	0.29 ± 0.05a	0.29 ± 0.16a	0.30 ± 0.05a	2.46 ± 0.08b	3.42 ± 0.62a
Sucrose (g dm^{-3})	Carbohydrate contents in the culture media without plants					
	Glucose (µmol gfw^{-1})		Fructose (µmol gfw^{-1})		Sucrose (µmol gfw^{-1})	
	No Filter	Filter	No Filter	Filter	No Filter	Filter
0	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
15	0.01 ± 0.00a	0.02 ± 0.00a	0.01 ± 0.00a	0.02 ± 0.00a	0.83 ± 0.13a	0.79 ± 0.21a
30	0.02 ± 0.01b	0.04 ± 0.01a	0.02 ± 0.01b	0.04 ± 0.01a	1.36 ± 0.13a	1.77 ± 0.30a
45	0.02 ± 0.00b	0.05 ± 0.01a	0.03 ± 0.00b	0.05 ± 0.01a	2.41 ± 0.23a	2.27 ± 0.61a
60	0.06 ± 0.00a	0.07 ± 0.01a	0.06 ± 0.00a	0.07 ± 0.01a	2.78 ± 0.04a	2.95 ± 0.54a

Water loss was observed in both sealing sytem, independent of sucrose concentration added in the medium. However, a higher dehydration of medium was verified in containers with filter (Fig. 3).

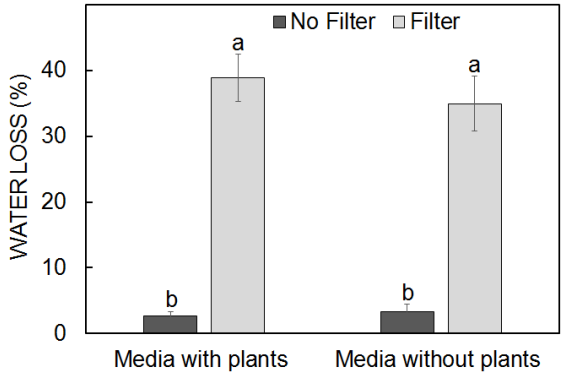

Fig. 3. Water loss at 45 days in containers with plants and without plants due to sealing system. For each plant culture system in the containers analyzed, averages followed by different letters differ from each other according to Tukey's test, at 5%.

Discussion

B. zebrina plants show different physiologic responses due to sealing systems and sucrose concentrations. *In vitro* plantlets may present poor chlorophyll content, as verified in *B. zebrina* plants (Fig. 1*A*). This is because of the exogenous supply of sucrose, which does not necessitate the normal development of photosynthetic apparatus. Although such plantlets may appear normal, their photosynthetic apparatus may be not actively functional (Hazarika 2006). Reduction of this pigment results in a limitation of 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 the photosynthetic potential and apparatus status (Alvarez *et al.* 2012, Sáez *et al.* 2012). Less energy input correlated well to biochemical processes related to both 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 an increased plant susceptibility to feedback inhibition, which is possibly associated with an excessive accumulation of hexoses and starch (Le *et al.* 2001). This agrees with was observed in *B. zebrina*, presenting low chlorophyll content (Fig. 1*A*), but high carbohydrate content (Fig. 2).

B. zebrina plants presented malic acid in all treatments, including shoots cultivated in liquid medium (Fig. 1*B* and Table 1). Modulation of the CAM pathway occurs in the bromeliad *Guzmania monostachia*, depending on the level of water supply (Pereira *et al.* 2013). The decrease of malic acid content observed in plants cultivated in sucrose concentrations higher than 30 g dm^{-3} probably was due to osmotic stress created by the *in vitro* media. Sucrose concentrations higher than 30 g dm^{-3} in the medium may induce osmotic stress on *in vitro* plants (Cui *et al.* 2010). Photosynthesis is one of the most sensitive processes for any kind of stress (Walters 2005). The malic acid content decreases in the leaves under a long period of water stress, as proven for in the CAM plant *Aechmea* 'Maya' by Ceusters *et al.* (2009). These authors mentioned that possibly roots are involved in the metabolic response to water limitation. On the other hand, lowest malic acid content (39.35 µmol gdw^{-1}) in plants grown in sugar-free medium (no osmotic stress) may be related to insufficient availability of CO_2. An increased CO_2 concentration increases the rate of malic acid formation by PEPc and its concentration in the cytoplasm (Zobayed *et al.* 2000). The increment in malic acid content in *Doritaenopsis* occurred with additional CO_2 during the *in vitro* photoautotrophic culture (Shin *et al.* 2013). During CAM action, glycolytic breakdown of storage polysaccharides (e.g., starch) or soluble sugars (e.g., glucose, fructose and sucrose) previously formed are also fundamental as substrates for the production of the CO_2 acceptor phosphoenolpyruvate (PEP) in the dark period (Ceusters and Borland 2011). It supports the results verified in plants grown in sugar-free medium and no filter containers, since those plants did not have enough carbohydrate stock to regenerate PEPc enzyme.

Sucrose concentrations in the *in vitro* media created higher monosaccharide and sucrose stocks in leaves and roots (Fig. 2). Sucrose supplement in plant tissue culture decrease the water potential of media and increase glucose, fructose, sucrose and starch contents in leaf tissue in a dose dependent manner. Monosaccharides are effective osmotic agents in plants. Osmotic stress in roots may increase monosaccharide accumulation (Xie *et al.* 2009). Sugars, especially glucose, fructose and sucrose, play an important role on stress alleviation through regulation of plant osmotic potential (Tuteja and Sopory 2008, Polanco *et al.* 2014). In photoautotrophic conditions, the plants showed high monosaccharide contents in the leaves (Fig. 2) and accumulation of different carbohydrates, evidencing good performance of photosynthetic apparatus. The presence of organic reserves and a functional photosynthetic apparatus in the *in vitro* plants should improve acclimatization performance (Shi *et al.* 2013, Shi *et al.* 2014).

Sugars are crucial for building substances for plants as well as a key source of energy necessary for inciting biochemical processes (Piotrowska *et al.* 2010). Plants cultivated in sugar-free medium and no ventilated containers showed the lowest carbohydrate stocks (Fig. 2) due to limited photosynthesis rate. Carbohydrates are required by plant cells as carbon resources, act as energy for growth and biosynthetic processes (Ferreira et al. 2011). Increasing sucrose in the media induced plants with higher starch content (Fig. 2*D*, *H*). Plants cultured in high sucrose concentrations during *in vitro* growth may present bigger and higher number of starch granules in the 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, the synthesis of starch begins in the chloroplasts during the light period (Dennis and Blakely 2000).

The use of containers with unlimited air exchange induced plants with lower starch content (Fig. 2*D*). High osmotic stress induced by dissolved sugars in the media can limit the starch formation. In general,

under osmotic and drought stress soluble sugars tend to increase, while starch content decreases (Chaves 1991). Starch does not play directly as an osmoprotectant, since it is not soluble in water. *B. zebrina* plants decreased the water osmotic potential of roots by increasing the soluble sugars content. Lower starch content and higher soluble sugars content could facilitated water flux from the medium into the plants.

At 45 days of growth, *B. zebrina* plants did not have consumed all added sucrose in the media, even in those without air exchange (Table 2). Some plant species have also been presented a low consume in sugar reserves in the culture medium (Palonen and Junttila 1999). After transfer of plants to the culture medium, they may hydrolyse sucrose into glucose and fructose by the activity of invertase released to the culture medium (Karhu 1997). This extracellular enzymatic system associated with the sucrose hydrolysis is initiated by presence of plant tissues and keeping activated even without plants, increasing fructose and glucose contents in the medium (Tremblay and Tremblay 1995). Sucrose hydrolysed to glucose and fructose, nearly doubling the osmolality of the medium (Bishnoi *et al.* 2000). In our study, sucrose hydrolysis increased the osmolality of the medium. This effect was probably even higher when combined with water loss (Fig. 3) from the media in containers with filter, increasing the effects by low solvent content.

Plants under environmental stress, e.g. osmotic stress, may present a membrane lipid peroxidation, which induces increase of cell membrane permeability and extravasation of cell-soluble substances (Zeng *et al*. 2006). It agrees with our results, since plants grown under ventilated conditions and in medium with 45 and 60 g dm^{-3} sucrose showed a slight increment in sugars into the medium. It was probably due to extravasation of cell content that includes glucose, fructose and sucrose. Under those conditions the plants did not growth (data did not show), showing toxic effect of the osmotic stress, as chlorosis and necrosis.

Conclusion

In vitro culture condition influences the physiology of *B. zebrina* plantlets during micropropagation time. Sucrose in the culture medium leads to reduced chlorophyll content and increased starch concentrations. Supplemented sucrose acts as an osmotic stress factor on *B. zebrina.* Ventilating the culture containers increases these stress responses evoking water loss and increasing sugar content in the media. Plantlets grown in photoautotrophic conditions (air exchange and sugar-free) did not show any physiological disorders and showed a normal production of photoassimilates.

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ARTICLE 4

Anatomical and physiological responses of *Billbergia zebrina* (Bromeliaceae) under copper excess

(Prepared in accordance with *Environmental and Experimental Botany*'s standards)

Abstract
Copper (Cu) plays biochemical and physiological functions in plants. However, at high concentrations, Cu can become extremely toxic, inducing several physiological changes and symptoms of toxicity such as necrosis. The aim was to analyze anatomical and physiological changes of *Billbergia zebrina* induced by Cu excess in controlled conditions. Previously *in vitro*-established *B. zebrina* plants were transferred to culture media containing 0.0, 2.0, 20.0 or 200.0 µM Cu. Growth (number of leaves and roots) and enzyme activity (superoxide dismutase and ascorbate peroxidase) analysis were measured at 20, 40, 60 and 80 days *in vitro* growth. To verify the biological activity of roots grown under Cu concentrations, a Biospeckle laser was used at 20 and 80 days. Biomass accumulation and anatomical analyses were performed at 80 days. Plants did not show any visible disturb, as necrosis on the leaves, and all plants survived. However, anatomical and physiological changes were verified. Plants grown under 200 µM Cu showed anatomical changes that may help tolerating the metal, like high stomatal index and thicker cell wall in exodermis. High biological activity was verified only at 20 days, when the analysed root area was still young. This activity was linked to high increment in cell wall at exodermis. Enzymatic antioxidant system was functional in all treatments. Cu affected the leaf and root anatomy as well as its growth, but this metal induced low changes on enzyme activity. *B. zebrina* tolerates high amounts of Cu.

Keywords: biological activity, bromeliad, *in vitro* culture, plant anatomy, plant physiology

Introduction

Essential metals, such as copper (Cu), play biochemical and physiological functions in plants and animals. Cu is found naturally in the environment in low concentrations and it is essential for the metabolism of many plant species (Nagajyoti et al. 2010). This metal plays an important role in signaling of transcription and protein trafficking machinery, oxidative phosphorylation and iron mobilization. Cu is also necessary in others physiological processes, including photosynthesis, respiration, antioxidant activity and cell wall lignification metabolism (Himelblau and Amasino 2000, Pilon et al. 2006, Burkhead et al. 2009, Sharma et al. 2012).

At high concentrations, Cu can become extremely toxic causing symptoms such as chlorosis and necrosis, stunting, leaf discoloration and inhibition of root growth (Van Assche and Clijsters 1990). Excess Cu may also induce nutritional deficiencies in plants. It accumulates in plant tissues and induces many physiological problems and biochemical changes, reducing growth, especially after chronic exposure (Wannaz et al. 2003, Khatun et al. 2008).

Cu is a non-degradable heavy metal and it can accumulate in soil or leach into water sources. Its accumulation in topsoil has impacted micro and macro organisms (Mackie et al. 2012). The environmental pollution may lead Cu reach toxic or poisonous concentrations, causing severe damage to living beings. Incineration of municipal waste has generated significant concentrations of many kinds of heavy metals, like Cu (Nagajyoti et al. 2010). In agricultural areas, fungicidal spraying has contributed in the Cu accumulation in enviroment (Mackie et al. 2012). In addition, this heavy metal may also be associated with dust particles and thus be carried by the wind (Figueiredo et al. 2007).

The pollution monitoring may be performed by plant species that assimilate nutrients dispersed in the environment. These plants can be called bioindicators and they are defined as organisms which are able to provide information on the quality of their environment (Figueiredo et al. 2007, Elis

et al. 2008). This methodology is quite attractive because it has some advantages over conventional techniques such as low cost, the possibility of interaction with pollutants over a long period and simultaneously monitoring of multiple locations. The use of bromeliads as a pollution indicator has been published (Figueiredo et al. 2007, Alves et al. 2008, Bermudez and Pignata 2011). However, most of these studies are related to the use of *Tillandsia* species.

Anatomical changes of plants may indicate the quality of the environment. Phenotypic plasticity is the key to adaptive process of plants to environmental conditions, being each species having distinctive characteristics (Gomes et al. 2012). Studies on anatomical changes of bromeliads under heavy metal exposure have been focused only on leaves, especially about changes on trichomes and stomata (Amando-Filho et al. 2002, Alves et al. 2008, Kováčik et al. 2012). Although the trichomes on leaves are responsible for absorbing water and nutrients, bromeliads may also absorb nutrients from the roots (Proença and Sajo 2008). Absorbent tank roots contribute in mineral uptake and they constitute a highly specialized root system that penetrates the spaces between of the basal parts of tank-forming leaves (Lüttge 2010). The toxic effect of metals on the root system is of particular importance for the whole plant. Since the root is an entry site for water and nutrients into the plant, any defect or malformation of the root may create problems for plant growth and development (Panou-Filotheou et al. 2001).

Physiological mechanisms are also involved to tolerate heavy metal stress. Plants have been stimulated antioxidant system to combat the oxidative injury induced by the heavy metals (Zabalza et al. 2008). These include several reactive oxygen species (ROS) removing enzymes such as superoxide dismutase (SOD), catalases (CAT), ascorbate peroxidase (APX), among others. ROS are damaging to essential cellular components and it may lead to oxidative stress affecting plant growth (Giampaoli et al. 2012).

Effects of Cu on physiology of *Aechmea blanchetiana* (Bromeliaceae) have been studied by Giampaoli et al. (2012). Nevertheless, this study was realized on *in vitro* conditions. Physiological changes under Cu exposure in controlled microenvironment conditions have also been verified by Khatun et al. (2008) and Son et al. (2014). *In vitro* technique is advantageous because it enables to isolate the effects of a certain metal on plant metabolism from the effects from other stresses (Giampaoli et al. 2012).

The aim of this work was analyse anatomical and physiological changes induced by Cu in controlled microenviroment conditions of *Billbergia zebrina* (Herbert) Lindley, a bromeliad native to Brazil and considered an endangered species.

Material and Methods

Plant material and culture conditions

Billbergia zebrina fruits were collected from adult plants grown in a greenhouse and the seeds were used as initial explant. *B. zebrina* plantlets previously established *in vitro* through seeds were transferred to 250.0 mL glass containers containing 50.0 mL stationary liquid MS medium (Murashige and Skoog 1962), supplemented with 30.0 g L^{-1} sucrose and 13.0 µM 6-benzylaminopurine (Martins et al. 2015). After 30 days growth, side shoots were subcultivated, for 45 days, in 250.0 mL glass containers with 50.0 mL stationary liquid MS medium with no plant growth regulator and supplemented with 30.0 g L^{-1} sucrose. Medium pH was set at 5.8, before autoclaving at 120ºC for 20 minutes. After inoculation in a laminar flow cabinet, plant cultures were kept in a growth room, at 27 ± 2ºC and 16-hour photoperiod under fluorescent lamps providing 25.2 µmol m^{-2} s^{-1} of photosynthetic photon flux.

Copper during the *in vitro* growth

B. zebrina side shoots with approximately 4.0 cm in length, from previous *in vitro* stage, were individualized with the aid of a scalpel and transferred to test tubes containing 10.0 mL MS medium solidified with 6.0 g L^{-1} agar and supplemented with 0, 2, 20 or 200 μM Cu. Each test tube received one side shoot. Medium pH was adjusted to 5.8, before autoclaving at 120ºC, for 20 minutes. After sterile inoculation the cultured plants were kept in a growth room for until 80 days, at 27 ± 2ºC and 16-hour photoperiod under fluorescent lamps providing 25.2 μmol m^{-2} s^{-1} of photosynthetic photon flux.

Plant anatomy analysis

Leaf and root anatomy characterization was carried out on three plants from each treatment. Samples were randomly collected at 80-day growth and fixed in FAA (formaldehyde, acetic acid and ethanol 50%, 0.5/0.5/9, v/v) for 72 hours, followed by storage in 50% ethanol (Johansen 1940).

Paradermal and cross sections were performed in the median region of the first completely expanded leaf in the rosette central region with aid of a double edge razor. Cross sections were also performed at the root basis (0.5 cm from shoot) with aid of a double edge razor. Sections were cleared with sodium hypochlorite 10% (v/v) followed by staining with safranin and astra-blue solution. Finally glycerin 50% was used to assemble the slides. The sections were investigated using a light microscope (Zeiss MicroImaging GmbH Scope.A1, Göttingen, Germany) coupled to a digital camera (Canon A630). The photomicrographs were used to measure the anatomy characteristics using the software UTHSCSA-Imagetool®.

To determine the lignin distribution in root tissues, the second staining procedure on root cross-sections was carried out with berberine hemi-sulphate solution 0.1% (w/v) for 1 hour and aniline blue 0.5% (w/v) for 30 min. Glycerin 50% (v/v) containing 0.1% (w/v) of $FeC1_3$ was used

assemble the slides. This entire procedure was performed according to Brundrett et al. (1988). The sections were observed using a fluorescence microscope Olympus BX60 equipped with cooled monochrome camera (Olympus Optical CO., LTD., Tokyo, Japan). Images were captured at ultraviolet wavelengths with excitation/emission waves of 358-461 nm (Brundrett et al. 1988).

The stomatal density (cm^2) and index (%), thickness of adaxial and abaxial epidermis (µm), hydrenchyma (µm) and chlorenchyma (µm) and xylem diameter (µm) were determined in leaves.

The total area of roots (mm^2) and central cylinder (mm^2), thickness of cell walls of cortex (µm), endodermis (µm), diameter of xylem vessel (µm) and number of xylem vessels were determine in roots.

Biological activity of roots

Biological activity of *B. zebrina* roots (between 0.5 to 1.0 cm from shoot) was measured at 20 and 80 days of growth with the aid of Biospeckle laser (or Dynamic Laser Speckle). Four test tubes from each treatment containing one plant were illuminated by a coherent light provided by a diode laser (635 nm, pumped by a source offering 66 mA). The experimental configuration was the back-scattering with a 90^o angle between the expanded laser and the camera (Ribeiro et al. 2014). The camera was a digitalmini-microscope (Dino-Lite brand AM 413zt). The interference patterns formed by the interaction of the light with the material studied was collected by the portable digital microscope, assembling a collection of 128 images in gray levels, with a resolution of 1,280 x 1,024 pixels and 0.08 s of interval between frames. The analysis and interpretation of the data were performed using graphical and numerical analysis, Standard Deviation (Nothdurft and Yao 2005) and Absolute Value of the Differences (Braga et al. 2011).

Enzyme activity analysis

To determine the antioxidant enzyme activities, plants were collected at 20, 40, 60 and 80 days growth. Each time, nine plants per treatment were collected and after mixing the plants three parcels were taken. All samples were immediately frozen in liquid nitrogen. Plant material was stored in freezer -80 ^{o}C until the analysis. The whole plant was used as plant material. The samples were crushed before taking a known amount of plant material on which extractions were carried out with a mix of 100 mM potassium phosphate buffer (pH = 7.8), 0.1 mM EDTA and 10 mM ascorbate acid. The samples were centrifuged at 13,000 g for 10 min at 4 °C and the supernatants were used for the assay of enzyme activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX).

SOD activity was assayed as described by Giannopolitis and Ries (1977) by determining its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). APX activity was analyzed according to Nakano and Asada (1981) by monitoring the decrease of ascorbate at 290 nm for 1 min. Enzyme activity was expressed as Unit mg^{-1} fw (SOD) and µM H_2O_2 min^{-1} mg^{-1} fw (APX).

Growth trait analysis

To evaluate growth traits, thirty plantlets from each treatment were randomly collected at 20, 40, 60 and 80-day growth and they were divided over three parcels. The number of leaves and roots, root length (cm) and fresh weight were determined.

Statistical analysis

For the analysis of the anatomical structures, a completely randomized design was adopted. For the analysis of the biological activity of roots (two time of analysis x four Cu concentrations), enzyme activity and growth traits (four times of analysis x four Cu concentrations) a completely randomized design in a factorial arrangement was performed. The obtained

data were submitted to analysis of variance (ANOVA), the averages were compared using Tukey's test or regression analysis at 5% probability.

Results

Leaf anatomy analysis

In *B. zebrina* plants, the surface of leaves showed tetracytic stomata with hypostomatic distribution. Transversal leaf sections presented one cell layer epidermis both sides of the leaf, water-storage parenchyma (hydrenchyma) containing non-chlorophyll with thin walled cells at the adaxial leaf side, chlorophyll parenchyma (chlorenchyma) with isodiametric cells, collateral vascular bundles, usually larger bundles alternating with smaller bundles and surrounded by sclerenchyma fibers. The mesophyll has a dorsiventral organization (Figure 1). This leaf structuring is frequently observed and described in many bromeliads (Pereira et al. 2013, Martins et al. 2014).

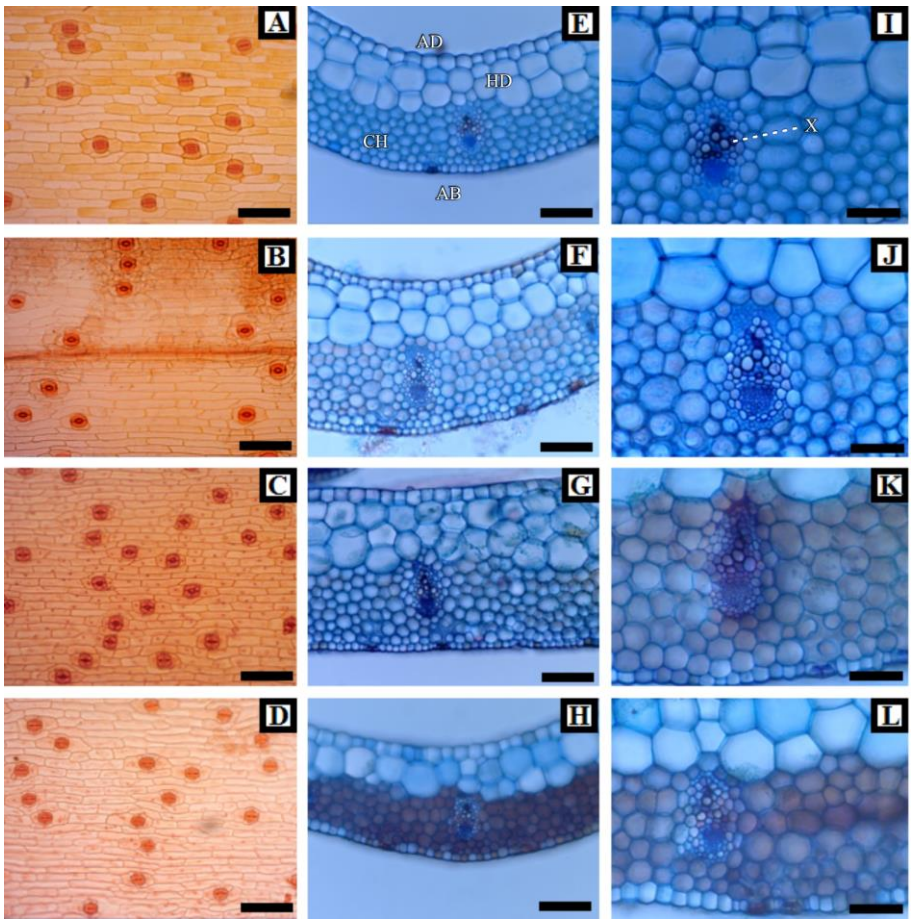

Figure 1. Paradermal and cross sections of *B. zebrina* leaves at 80 days *in vitro* cultivation on media containing different copper (Cu) concentrations (0, 2, 20 or 200 µM, from top to bottom). Adaxial epidermis (AD), abaxial epidermis (AB), hydrenchyma (HD), chlorenchyma (CH), xylem (X). Bar = 50 µm (A-H); Bar = 100 µm (I-L).

B. zebrina leaves showed an increase of stomatal index and density in function of the Cu supplementation in the medium. The lowest stomatal index and density was verified on leaves of plants grown in medium without Cu (0 µM). They presented values between 1.38 and 1.5 times lower (stomatal index and density respectively) when compared with leaves of plants grown in 200 µM Cu (Figure 2).

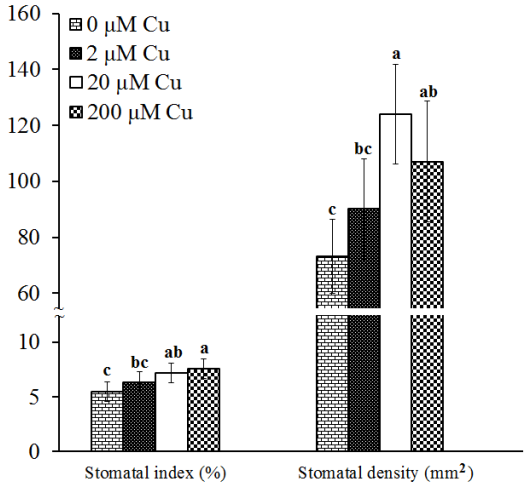

Figure 2. Stomatal index (%) and density (mm^2) of *B. zebrina* leaves as a function of Cu (µM). For each anatomical trait, averages followed by the same letter do not differ according to Tukey's test at 5% probability.

The epidermis thickness did not show any difference throughout all treatments. The averages of adaxial and abaxial side of the epidermis were 24.67 µm and 15.25 µm respectively (Figure 3). Nevertheless, the other plant tissues showed significant differences between the Cu concentrations. Plants grown on media with 2 and 20 µM Cu presented the thickest hydrenchyma and chlorenchyma as well as the largest xylem diameters. On the other hand, plants cultured on medium with 200 µM showed the lowest thickness of those leaf tissues, besides a reduced xylem diameter (Figure 3).

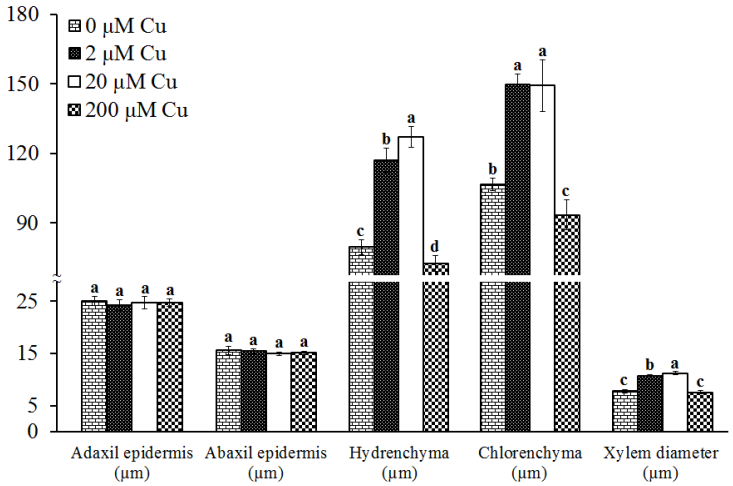

Figure 3. Anatomical structures (µm) of *B. zebrina* leaves as a function of Cu (µM). For each anatomical trait, averages followed by the same letter do not differ according toTukey's test at 5% probability.

Root anatomy analysis

Transversal root sections of *B. zebrina* present a uniseriate epidermis layer with unicellular hairs, velamen with multiseriate cell layers and an outer and inner cortex. The outer cortex (exodermis) is parenchymatous with multiseriate cell layers and the cell walls are thickened. Internal cortex shows thin walled cells, and its inner layers, close to the endodermis, there are air gap formation. The endodermis is recognized by the casparian strips and the central cylinder is delimited by a one-layered pericycle formed by thin-walled cells (Figure 4). These root anatomical structures of bromeliads are are according to those have already been documented (Segecin and Scatena 2004, Proença and Sajo 2008, Silva and Scatena 2011).

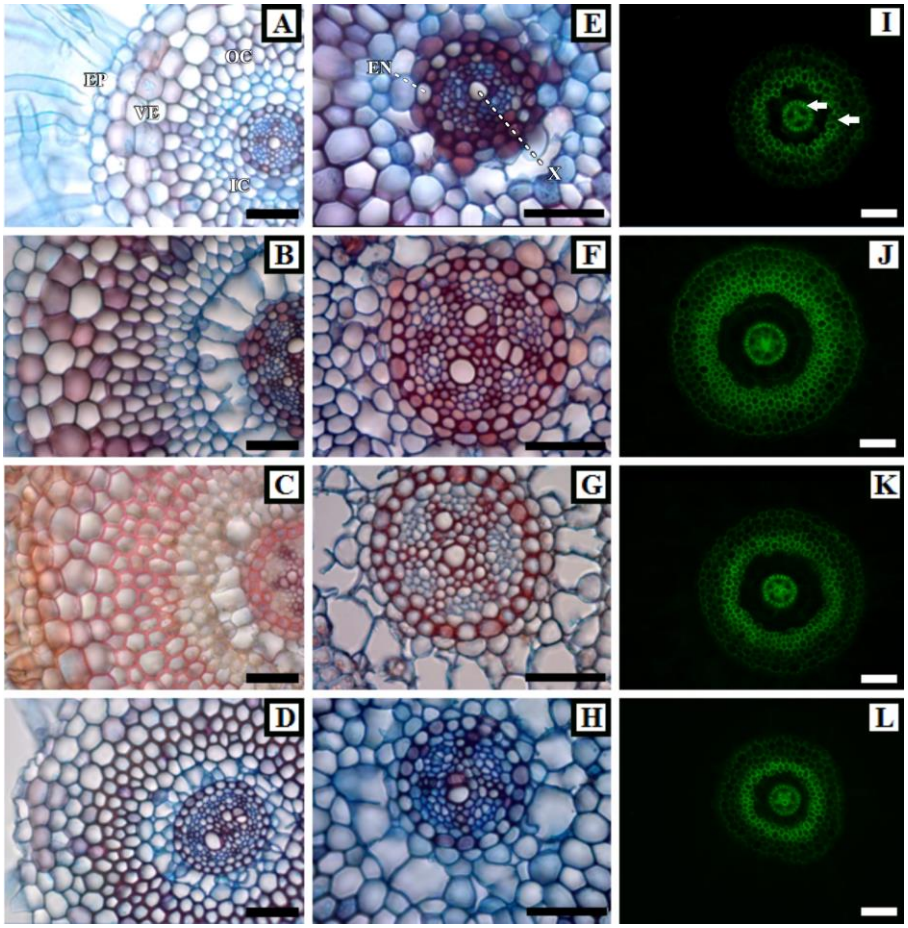

Figure 4. Cross sections of *B. zebrina* roots at 80 days *in vitro* cultivation in media containing different copper (Cu) concentrations (0, 2, 20 or 200 µM, from top to bottom). Epidermis (EP), velamen (VE), outer cortex (OC), inner cortex (IC), endodermis (EN), xylem (X). Arrows show outer cortex (exodermis) and endodermis stained by berberine hemi-sulphate and aniline blue solution. Bar = 50 µm (A-H); Bar = 100 µm (I-L).

Cu concentrations induced modifications in the *B. zebrina* root's area. A significant decrease was evident in the total root area and central cylinder area on absence of metal exposure, as well as those roots grown in concentrations higher than 2 µM Cu. The lowest areas were verified in roots grown in medium without Cu (Figure 4-5).

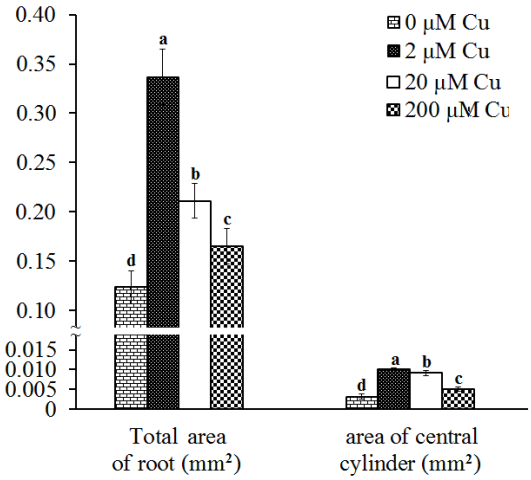

Figure 5. Root total area (mm^2) and central cylinder area (mm^2) of *B. zebrina* roots as a function of Cu (µM). For each anatomical trait, averages followed by the same letter do not differ according to Tukey's test at 5% probability.

The walls of outer cortex cells (exodermis) showed a lignin thickening in all treatments. The roots exposed to Cu presented a thicker cell wall. This thickening rose with increasing metal concentrations (Figure 4 and 6). However, endodermis thickness did not show any difference between the treatments. Xylem diameter was different only in roots grown in medium without Cu. This was lower than those cultivated with Cu. Nevertheless, the number of xylem vessels decreased with increasing Cu concentration (Figure 4 and 6).

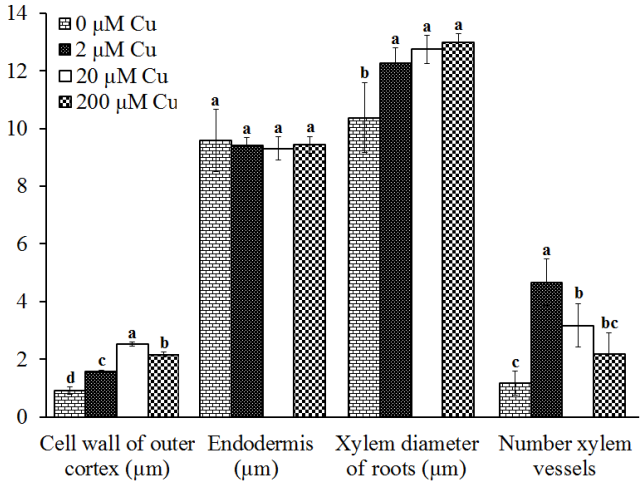

Figure 6. Anatomical structures (µm) of *B. zebrina* roots as a function of Cu (µM). For each anatomical trait, averages followed by the same letter do not differ according to Tukey's test at 5% probability.

Root biological activity

Biospeckle outcomes identified differences on biological activity between the treatments (Figure 7). A higher root biological activity at 20 days growth was observed when compared with roots at 80 days. The outcomes were presented by the Co-occurrence Matrix (COM) (Braga et al. 2011), where the dispersion of the values far from the main diagonal represents higher activities, and by the SD method, where the pseudo-colours represent the level of activity, i.e. from blue to red the activities can be classified from low to high respectively. At 20 days, roots grown in medium with 200 µM Cu showed the highest biological activity. At 80 days all treatments presented similar biological activity (Figure 7-8). At Fig 8, the biological activities represented by the AVD (Braga et al. 2011) expressed the differences between 20 and 80 days, as well as the changing of activity within 20 days linked to the Cu concentration.

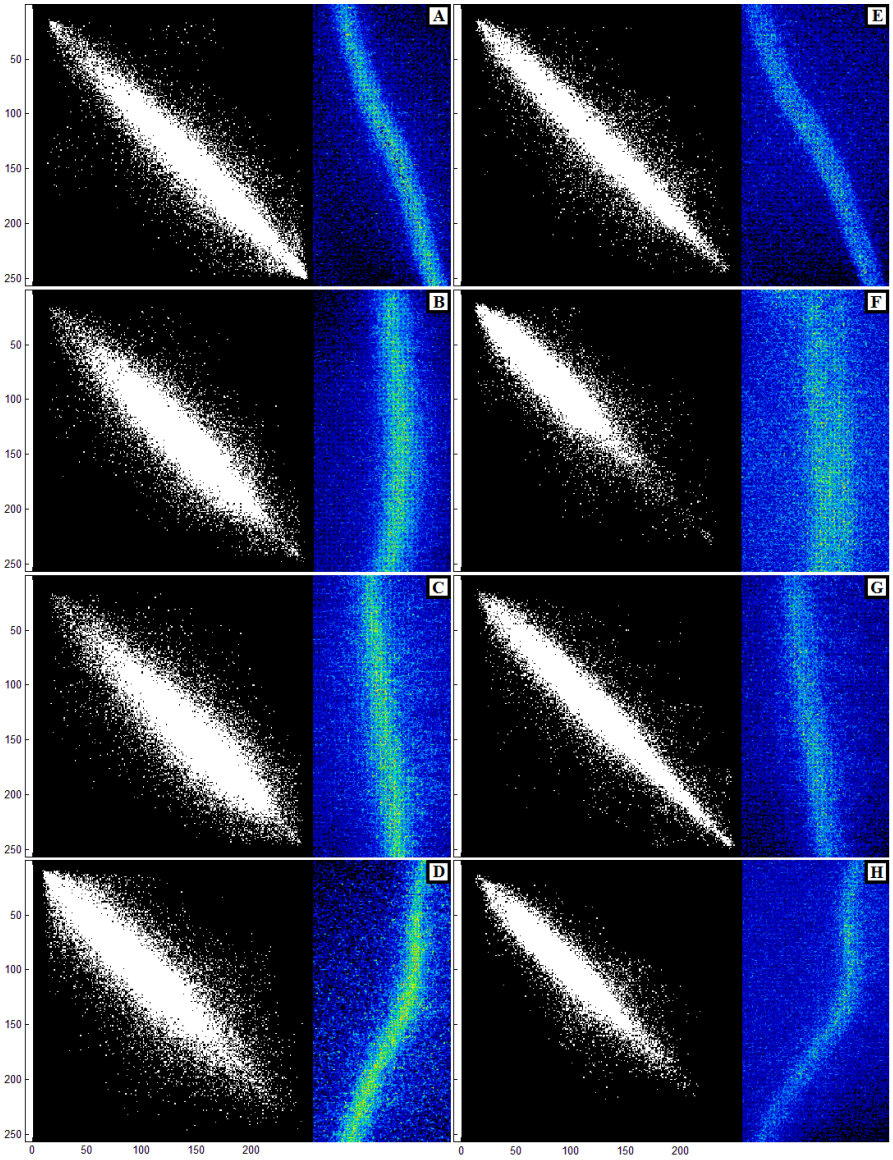

Figure 7. Biological activity by COM and SD analysis of *B. zebrina* roots as a function of Cu (0, 2, 20 or 200 µM, from top to bottom) at 20 (A-D) and 80 (E-H) days of growth.

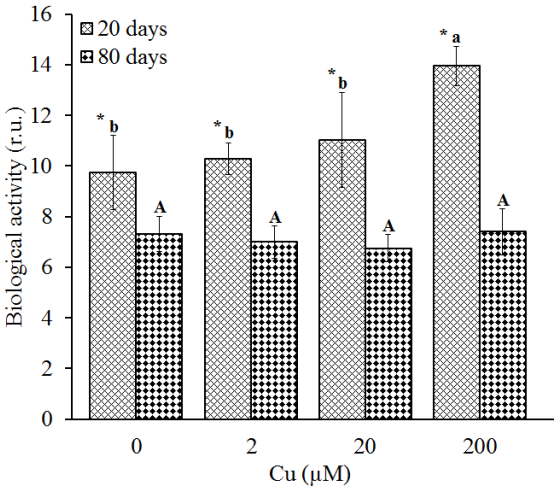

Figure 8. Biological activity by the AVD of *B. zebrina* roots as a function of Cu (µM) at 20 and 80 days of *in vitro* growth. For each time, averages followed by the same letter do not differ according to Tukey's test at 5% probability. For each Cu concentration, averages followed by asterisk differ according to Tukey's test at 5% probability.

Enzyme activity

SOD activity was influenced by concentrations and growth time, but these factors acted separately. SOD presented the highest activity when the *B. zebrina* plants were grown in medium with 200 µM independent of growth time. Nevertheless, SOD activity increased over time, in general (Figure 9). APX activity was constant and it did not show any difference between the treatments (Table 1).

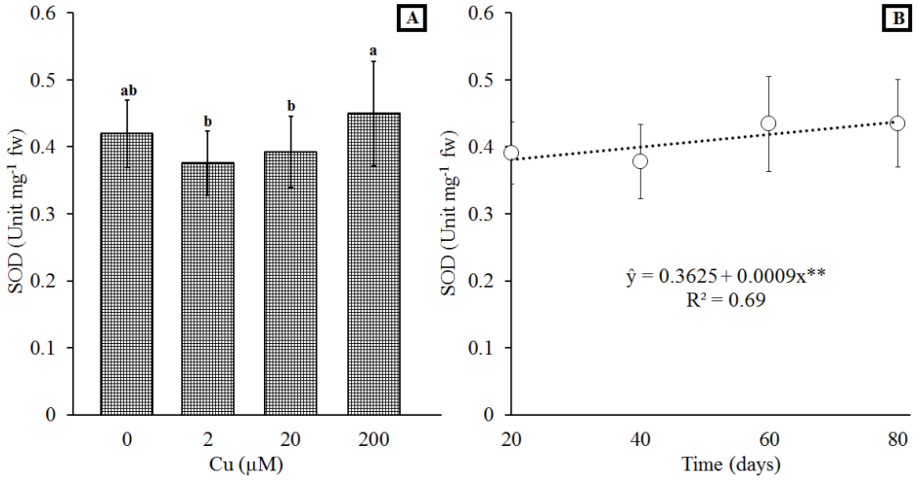

Figure 9. SOD activity of *B. zebrina* plants as a function of Cu (µM) (A) and time of *in vitro* growth (B). For each time, averages followed by the same letter do not differ according to Tukey's test at 5% probability.

Table 1. APX activity of *B. zebrina* plants as a function of Cu (µM)

Cu (µM)	APX (µM H_2O_2 min^{-1} mg^{-1} fw)
0	1.45 ± 0.69 a
2	1.35 ± 0.35 a
20	1.37 ± 0.45 a
200	1.81 ± 0.73 a

Averages (±SD) followed by the same letter in the column are not differ according to Tukey's test at 5% probability.

Growth traits

During *in vitro* culture the plants did not show any visible disturbance, like necrosis on the leaves and all plants survived. The number of leaves was not influenced by Cu concentrations at each time of analysis. However, the number of leaves presented a positive linear relation with increasing cultivation time (Figure 10a), showing that Cu concentrations did not induce a lower growth of aerial part in any treatment. Nevertheless, the size of leaves presented smaller as a function of Cu. The whole rooting process occurred after transfer of shoots to the Cu treatments and it was observed 100% of rooting in all treatments. The adventitious rooting began before 20 days growth in treatments with Cu. The plants did not present a

significant increase on number of roots over time (until 80 days of culture). Plants grown under 200 µM Cu showed higher number of roots than the others treatments (Figure 10b).

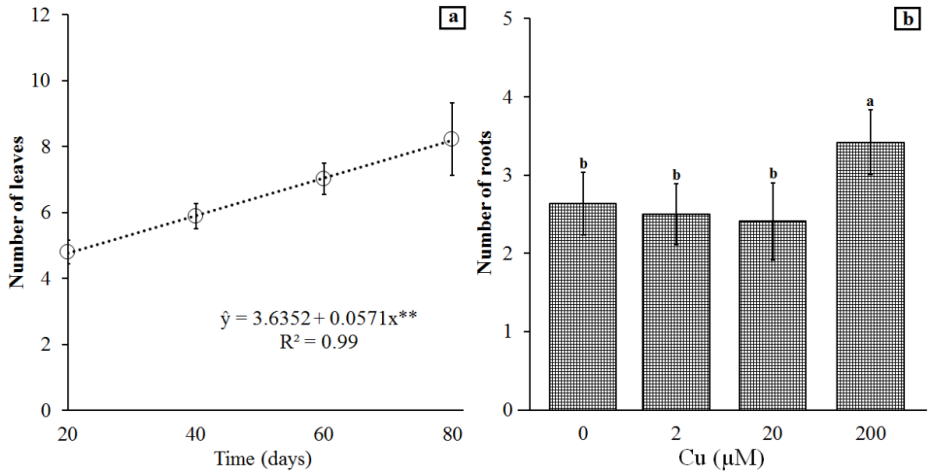

Figure 10. Number of leaves (a) at different times during the *in vitro* growth and number of roots (b) as a function of Cu (µM) in the culture medium of *B. zebrina* plants. Averages followed by the same letter are not differ according to Tukey's test at 5% probability.

The root length of *B. zebrina* plants was similar in all treatments at 20 and 40 days *in vitro* growth. Nevertheless, from 60 days the plants grown in medium with 200 µM Cu presented the shortest length of roots. The longest roots were verified in plants cultured in 2 µM Cu at 80 days. When root length was verified in each treatment over time, an increase was observed. All treatments showed a positive linear relationships with increasing growth time (Table 2).

Table 2. Root length (cm) of *B. zebrina* as a function of Cu (µM) in the medium and time of *in vitro* growth

Cu (µM)	Root length (cm)			
	20 days	40 days	60 days	80 days
0	2.40 ± 0.19 a[(1)]	4.05 ± 0.32 a	6.16 ± 2.56 a	8.09 ± 0.72 b
2	2.12 ± 0.22 a[(2)]	4.66 ± 0.52 a	6.46 ± 0.97 a	10.36 ± 0.21 a
20	2.07 ± 0.12 a[(3)]	4.09 ± 0.18 a	5.19 ± 0.30 a	8.67 ± 1.05 ab
200	1.56 ± 0.13 a[(4)]	3.55 ± 0.64 a	2.60 ± 0.56 b	7.38 ± 0.87 b

Averages (±SD) followed by the same letter in the column are not differ according to Tukey's test at 5% probability. [(1)] $\hat{y} = 0.3806 + 0.0959x^{**}$, $R^2 = 0.997$; [(2)] $\hat{y} = -0.735 + 0.1327x^{**}$, $R^2 = 0.976$; [(3)] $\hat{y} = -0.2167 + 0.1045x^{**}$, $R^2 = 0.953$; [(4)] $\hat{y} = -0.3483 + 0.0825x^{**}$, $R^2 = 0.704$.

The Cu supplementation influenced on fresh weight of *B. zebrina* plants. The lowest values were verified in plants cultured without Cu, but the addition of Cu higher than 2 µM also showed a negative effect on growth of *B. zebrina* (Figure 11).

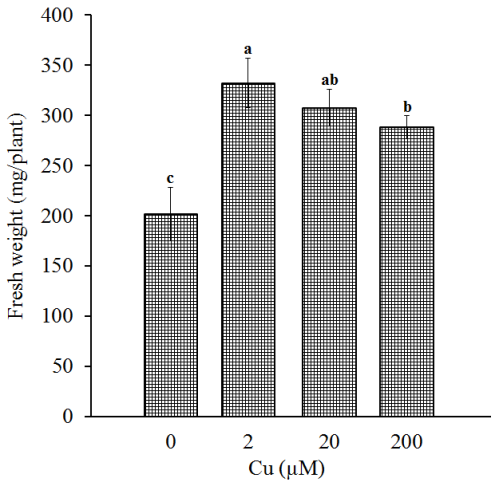

Figure 11. Fresh weight (mg/plant) as a function of Cu (µM) in the culture medium of *B. zebrina* plants. Averages followed by the same letter do not differ according to Tukey's test at 5% probability.

Discussion

This research reports for the first time the impact of Cu excess on leaf and root anatomy as well as on physiology of a bromeliad. *B. zebrina* plants showed different anatomical and physiological responses due to Cu concentrations. However, under imposed conditions, plants did not present mortality rate or any visible physiological disturb, like chlorosis, necrosis, and leaf discoloration.

On leaf surface, the stomatal density is one of the most important factors in plants under stress. Increasing stomatal density is an often response to decreasing leaf surface area to ensure surface gas exchange and, consequently, adequate carbon influx (Abbruzzese et al. 2009). An increased stomatal density may represent anatomical leaf tolerance mechanisms for controlling transpiration processes (Gomes et al. 2012). This increasing in stomata number may not be due to an increased stomatal formation but rather due to a reduction in epidermal cell size caused by water deficit caused by heavy metals, like Cu (Wainwright and Woolhouse 1977). However, from the stomatal index it was possible to verify a higher investment in stomatal formation per area with an increment in Cu concentration (Figure 1-2). An increased stomatal index with raised heavy metal concentration has also been verified in other plant species, like *Vigna radiata* (Gupta and Bhatnagar 2015). The increase in stomatal frequency is supposed to be an adaptive response to sustain adequate rate of gaseous exchange necessary for growth and development (Neelu et al. 2000).

Leaf epidermis thickness of *B. zebrina* did not change as a function of Cu concentration (Figure 1, 3). It has also been verified in *Myriophyllum alterniflorum* plants by Delmail et al. (2011). However, the chlorenchyma

and hydrenchyma thickness were related to Cu concentration (Figure 1, 3). Chlorenchyma thickness presents a great plasticity and this tissue is sensitive to heavy metals (Shi and Cai 2009). Exposure to heavy metals may lead to a reduction in the mesophyll cell size (Zhao et al. 2000, Srighar et al. 2005). Nevertheless, in our study, even though we did not measure cell size, the reduction of the leaf blade seems to be due to lower number of hydrenchyma and chlorenchyma cell layers in the treatments exposed to 200 µM Cu (Figure 1, 3). Cu excess can inhibit cell elongation and cell division of plant tissues (Panou-Filotheou and Bosabalidis 2004).

A reduction of xylem diameter was observed on leaves of *B. zebrina* grown in medium with 200 µM Cu (Figure 1, 3). This reduction is one of the factors affecting the capacity as translocation conduits (Poschenrieder and Barceló 1999). A larger xylem vessel is related to a higher hydraulic conductance (Rodríguez-Gamir et al. 2010). Thus, a smaller diameter of xylem vessels may be related as a protection factor, since it is expected a lower translocation of Cu through the plant, reducing the toxic effects of Cu excess in the aerial part.

Cu concentration also presented an impact on anatomy at analyzed root area (Figure 4-6). In young roots, four areas are traditionally recognized in root cap, zone of active cell division, zone of cell elongation, and zone of maturation. However, roots grown under heavy metal conditions may change the position of cell division/maturation of some tissues from the root apex (Lux et al. 2011, Vaculík et al. 2012). For this reason, in our study, we focused the anatomical analysis in the basis of the roots. An excess of Cu may increase abnormalities at root tip mitosis (Li and Xiong, 2004). Cu affects the cell integrity in the transition zone, region where cells coming out

of the meristem prepare for fast expansion (Madejón et al. 2009). Thus, abnormalities on rooting process may interfere on formation of root tissues and this change might be clear in mature root areas.

The root cortex of bromeliads is differentiated outer and inner cortex. The outer cortex is a multilayered tissue formed by sclerified cells probably helps to hinder the water evaporation from the inner root cortex (Proença and Sajo 2008). The inner cortex has thin walls that may collapse at maturity and form air gaps, as verified in *B. zebrina*'s roots (Figure 4). The formed intercellular spaces are related to transport water by capillary action through these spaces (Segecin and Scatena 2004).

Outer cortex is also called exodermis (Silva and Scatena 2011) and its lignification of cell walls is natural processes, as verified in all treatments by berberine hemi-sulphate and aniline blue solution (Figure 4I-L). However, the cell wall thickness was influenced by Cu concentration (Figure 4, 6). Cu contributes to cell wall metabolism among several physiological processes in plants (Kabata-Pendias 2010). The functional adaptation of the exodermis has been related to an external sealing tissue of the root that is in direct contact with the surrounding environment and so serves as the first guard preventing excessive biotic and abiotic toxins entering the root, like heavy metals (Deng et al. 2009, Cheng et al. 2010, Cheng et al. 2012). Exodermis tend to decrease the capacity for metal uptake by roots, coinciding with increased lignification (Cheng et al. 2012).

The endodermis also acts as a barrier for diffusion of metals into the vascular system (Eapen and D'Souza 2005). In our study thickness of this tissue did not change as a function of Cu concentration (Figure 4, 6). A thicker endodermis may not act as an improved apoplastic barrier against

heavy metals (Geldner 2013). Moreover, an increased thickness of the outer cortex could have acted as a first apoplastic barrier, thereby reducing the metal toxicity. The evidence that thicker cell walls are, in fact, effective barriers to apoplastic ion movement is widespread and convincing (Enstone et al. 2003).

A decrease in the size and/or number of xylem vessels in roots is a common symptom of heavy metal toxicity that strongly diminishes the uptake and transport of water by the plants (Gong et al. 2005). It agrees with what we observed in *B. zebrina*, which presented lower number of xylem vessels under Cu excess (Figure 4, 6). The root's diameter of vessels of xylem did not decrease under high Cu concentration and it, combined with low number of vessels, could be a risk to the plant. Since any damage in a vessel would interfere negatively on water and nutrients transport. However, these plants have invested in a higher number of roots (Figure 10b), which could potentially compensate a loss (due to eventual damage in a vessel) of solute transport and explore a larger medium area.

Root growth involves cell division and enzyme activity and these activities are differenced along the root (Schreibera et al. 2005). During the root growth of *B. zebrina* plants, the biological activity showed differences between the treatments at 20 days of growth (Figure 7-8). This may be related to cell wall formation in exodermis (Figure 4, 6) as a reaction to the metal concentration. Highest rates of biosynthesis of substances linked to thickening of the cell walls are located in younger root zones (Schreibera et al. 2005). At 20 days the root areas analysed by Biospeckle were probably still immature, since roots were still short (Table 2). Those roots were longer at 80 days and the analyzed root areas were probably mature. The similar

biological activity at 80 days may also be an indication that the anatomical changes, i.e. thicker cell walls at exodermis, ensured the establishment of plants at non-favorable conditions. Cheng et al. (2012) have also verified changes in root anatomy as a strategy to avoid Cu entry into the plant.

Plant establishment under heavy metal conditions involves physiological changes, e.g. on enzymatic and non-enzymatic system activity. Several published works have discussed the involvement of SOD and APX against reactive oxygen species (ROS) in plants under heavy metal stress (Khatun et al. 2008, Madejón et al. 2009, Bermudez and Pignata 2011, Giampaoli et al. 2012). The detoxification of excess ROS is achieved by action of antioxidant enzymes. However, ROS are produced as an unavoidable consequence of plant cellular metabolism (Sharma et al. 2012). SOD is the first line of defence against ROS. SOD is a metalloenzyme that dismutates superoxide radical to H_2O_2 and oxygen. Then, APX detoxify H_2O_2 using ascorbate for reduction (Thounaojam et al. 2012). Increase in SOD activities in stressed plants was indicative of enhanced O_2^- production and oxidative stress tolerance. It has been proposed as an important enzyme for plant stress tolerance (Sharma et al. 2012, Thounaojam et al. 2012). APX is regarded as one of the most widely distributed antioxidant enzymes in plant cells, and it has high affinity to H_2O_2, making APX efficient scavengers of H_2O_2 under stressful conditions (Wang et al. 1999). In our study, a suitable increase in SOD activity was detected in plants grown under high Cu concentration (Figure 9). However, the increased H_2O_2 production probably was not high enough to induce a higher increment in APX activity in *B. zebrina* (Table 1). Antioxidant system probably was in good condition and functional, since both a high concentration of ROS and low enzyme

activity of SOD and APX might induce serious physiological disturbs that could be visualized as necrosis or chlorosis.

B. zebrina plants did not stop growing under any Cu treatment, showing that those changes were effective in the establishment of *B. zebrina* under imposed conditions (Figure 10 and Table 2). Nevertheless, plants grown without Cu showed lower biomass accumulation (Figure 11). Cu is an essential micronutrient and plays important roles in metal homeostasis and normal metabolism in plants. It is involved in a wide range of biological processes (Zhang et al. 2008). In our study, it was possible verify that some biological processes related to Cu are also involved in leaf and root tissue formation (Figure 1-6). However, Cu was toxic to plants at higher concentrations. It may cause changes in nitrogen metabolism with a reduction of total nitrogen (Llorens et al. 2000). A common effect of heavy metals is a reduced growth (Mishra et al. 2008). This decline in growth, especially on biomass production, can be explain, in part, due to inhibition of cell division by copper dependent concentration. It agrees with the verified on leaf and root cross-sections (Figure 3-8).

Conclusion

Cu affected the leaf and root anatomy of *B. zebrina* as well as its growth, but this metal induced low changes in antioxidant enzyme activity. *B. zebrina* tolerates high amounts of Cu and this species may be indicated for biomonitoring use.

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