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MORFO-HISTODIFERENCIAÇÃO DE CULTURAS NODULARES DE *Billbergia alfonsi-joannis* Reitz E *B. zebrina* (Herbert) Lindley

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RESUMO

Billbergia alfonsi-joannis Reitz e *B.zebrina* (Herbert) Lindley são espécies da família Bromeliaceae, endêmicas da Mata Atlântica. A alta exploração da Mata Atlântica e o potencial ornamental e econômico destas duas espécies, levam a vulnerabilidade delas na natureza. Sendo assim, estudos envolvendo a micropropagação têm se mostrado de grande relevância para assegurar sua conservação. Uma das formas de regeneração in vitro que vem sendo utilizada com espécies de bromélias é a partir de culturas nodulares, que consistem em grupos de nódulos organogênicos globulares compostos basicamente de grupos de células meristemáticas. Entretanto, há muitas lacunas na compreensão dos eventos que envolvem o processo de indução e regeneração a partir de culturas nodulares e cada vez mais vem sendo indipensável o conhecimento estrutural e ultraestrutural destes processos. Neste contexto, este trabalho teve como objetivo geral avançar no conhecimento da morfo-histodiferenciação de culturas nodulares induzidas em *B. alfonsi-joannis* e *B. zebrina* e do processo de regeneração de brotos a partir de culturas nodulares de *B. zebrina*. A análise mofo-histológica revelou que as culturas nodulares têm origem no periciclo do caule e são constituídas por policentro, tecido cambial, parênquima cortical e tecido de revestimento. O policentro é formado por centros organizacionais dispersos em tecido parenquimático, os quais são constituídos por sistema vascular envolto por bainha de feixe. O tecido cambial envolve o policentro e é responsável pelo processo regenerativo de novos nódulos, de gemas e raízes adventícias. Observou-se também que, nódulos primários podem gerar nódulos secundários em um processo repetitivo. Além disso, as análises morfo-histológicas do processo de regeneração permitiu elucidar que células na região de formação dos brotos apresentaram características de células mitoticamente ativas com a presença de muitos plasmodesmos e microtúbulos evidentes. Os brotos formados mantiveram conexão vascular com o nódulo. Células que ligam o feixe vascular do nódulo em direção ao broto mostraram degeneração de organelas e originaram neoelementos vasculares. O posicionamento do domo em relação aos primórdios foliares variou conforme o desenvolvimento do broto, e os estômatos dos primórdios foliares apresentaram-se arredondados e geralmente mais elevados em relação à superfície epidérmica.

Palavras – chave: Bromeliaceae, histologia, micropropagação, morfologia.

ABSTRACT

Billbergia alfonsi-joannis and *B. zebrina* are species of the family Bromeliaceae and native to the Atlantic Forest. The ornamental uses of species from this family affects its preservation in nature by the economic exploitation and high anthropogenic pressure in their natural environment, the Atlantic Forest, consequently leading to its genetic erosion. Tissue culture is an important alternative for conservation of bromeliads. Specifically, the in vitro regeneration of new individuals from nodular cultures is a system that has been successfully used for Bromeliaceae species. Nodular culture consists of groups of globular organogenic nodules, composed of meristematic cell groups. Despite the existence of well-established protocols for bromeliads species, many gaps in understanding the mechanisms of this route still remain. Therefore, this work aimed to monitor the morpho-histodifferentiation of nodular clusters induced on nodal segments of *Billbergia alfonsi-joannis* and *B. zebrina* and the morpho and histodifferentiation processes of shoot regeneration from nodular cultures of *B. zebrina*. Morphological and histological analysis showed that nodular clusters originated from stem pericycles and consisted of a polycenter, cambial tissue, cortical parenchyma, and a covering tissue. The polycenter consisted of an organizational center dispersed in parenchymal tissue. Each organizational center was formed by a vascular system surrounded by a bundle sheath. A cambial tissue surrounded these polycenters, promoting the regeneration of new nodules and leading to the formation of buds and roots. Primary nodules could generate secondary nodules in a repetitive process. The analysis of the regeneration process showed that cells in the shoot formation region presented characteristics of mitotically active cells, such as the presence of numerous plasmodesmata and conspicuous microtubules. New shoots maintained vascular connection to the node. Cells binding the nodule vascular bundle toward the shoot showed degeneration of organelles, which, in turn, originated vascular neoelements. The dome presented a depressed or prominent surface, depending on shoot development. Rounded stomata were conspicuous in the leaf primordia and generally higher in number as compared to the epidermal surface.

Keywords: Bromeliaceae, histology, micropropagation, morphology.

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ABREVIAÇÕES

AAN naphthalene acetic acid
AB axillary bud
BAP benzylaminopurine
BAF Billbergia alfonsi-joannis
BD bud
BZ Billbergia zebrina
CA cambium
CC cambial cell
CL chlorenchyma
CP chloroplast
CO corpus
COC companion cell
DM donut-shaped mitochondria
DO dome
ER endoplasmic reticulum
GC golgi complex
HY hydrenchyma
LAVEG Plant Anatomy Laboratory
LCME Central Laboratory of Electron Microscopy
LE leaf
LFDGV Laboratory of Plant Development and Genetics
LP leaf primordia
LM light microscopy
LP leaf primordia
MC meristematic cell
MI mitochondria
MIS mitochondria in series
MS Murashige and Skoog (1962) basal media
NC nodular culture
NO nodule
OC organizational center
NV nodule vascularization
PA parenchyma
PL plastoglobuli
PE periderm
PV provascular cell
RO root
SEM scanning electron microscopy

SH shoot
SM stomata
SN secondary nodule
SNE Sieve neoelement
ST stem
TDZ Thidiazuron
TEM transmission electron microscopy
TN tracheal neoelement
TU tunic
VA vacuole
VB vascular bundle
VC vascular cylinder
VZ vascularization

SUMÁRIO

INTRODUÇÃO, JUSTIFICATIVA E ANTECEDENTES

A Mata Atlântica é um bioma de alta biodiversidade e com um grande número de espécies endêmicas e, por isso, é considerada um hotspot (Myers et al., 2000). Bromeliaceae é uma família com expressivo número de representantes endêmicos neste bioma e inclui cerca de 3.172 espécies, distribuídas em 58 gêneros (Luther, 2008). Espécies desta família possuem interesse ecológico, por manterem a estabilidade da floresta e servirem de abrigo para diversas espécies da fauna (Reitz, 1983) e, também econômico, por seu uso medicinal, alimentício e ornamental (Benzing, 1980). Devido ao seu potencial ornamental, as bromélias são extensivamente utilizadas pelos paisagistas em virtude de sua beleza da sua floração e folhagens, apresentando diferentes colorações como vermelha, amarela, púrpura e branca (Martinelli, 2000).

Bromeliaceae é uma família com grande diversidade ecológica e com base em estudos filogenéticos está constituída por oito subfamílias: Pitcairnioideae, Navioideae, Brocchinioideae, Lindmanioideae, Hechtioideae, Puyoideae, Bromelioideae e Tillandsoideae (Givinish et al., 2011). As espécies de Bromelioidea caracterizam-se por possuirem sementes sem apêndices, flores epígeas e folhas frequentemente com margens espinhosas (Benzing, 1980); as folhas formam tanques basais que retém água e o sistema radicular é reduzido, servindo apenas para fixação ao substrato, pois a absorção de água e nutrientes é provida por escamas que recobrem a lâmina foliar (Pittendrigh, 1948). A subfamília Bromelioidea está constituída por 30 gêneros e 425 espécies e são geralmente epifíticas (Smith & Downs, 1974; Reitz, 1983). É nesta subfamília que encontra-se o gênero *Billbergia*, representado no Brasil por 47 espécies, das quais 21 espécies são endêmicas da Mata Atlântica (Gaiotto et al., 2010). Neste gênero, duas espécies destacam-se pela beleza e semelhanças, são elas *Billbergia alfonsi-joannis* Reitz (Fig. 1a) e *B. zebrina* (Herbert) Lindley (Fig. 1b). De acordo com Gaiotto et al. (2010), elas ocorrem no mesmo tipo de ambiente e apresentam grande proximidade quanto aos caracteres morfológicos como forma e cor das pétalas e textura do escapo.

Figura 1. Aspecto geral de indivíduos férteis de espécies de *Billbergia* Wendl. a. *B. alfonsi-joannis* Reitz. b. *B. zebrina* (Herbert) Lindley. Fonte: (a) Souza, D.S. 2010 Flora RS, disponível em http://www6.ufrgs.br/fitoecologia/florars/open_sp.php?img=4616 (b) Peixoto, M. Brazil Plants disponível em http://www.brazilplants.com/bromeliaceae/ billbergia-zebrina.html.

Billbergia alfonsi-joannis é uma espécie endêmica da Mata Atlântica e está entre as espécies da flora ameaçadas de extinção segundo o Ministério do Meio Ambiente (Brasil, 2008). Distribui-se no Brasil nos estados do Paraná, Santa Catarina, Minas Gerais e São Paulo (Gaiotto et al., 2010). Conforme Reitz (1983), esta é uma bromélia epífita, de aproximadamente 90 cm de altura, com poucas folhas, as quais estão dispostas em reforçada roseta tubular, formando um enorme tubo utriculado, desde a base até a metade das folhas, estreitado para o ápice e a base; as lâminas são extremamente rijas e com fortes espinhos nas margens e a inflorescência é muito maior do que as folhas e é do tipo espiga pendente. O autor ressalta que esta é considerada uma das bromélias mais lindas.

Billbergia zebrina também é uma bromélia epífita e nativa da Mata Atlântica, que além da sua importância ecológica agrega grande interesse ornamental (Reitz, 1983), devido à beleza das suas folhas e inflorescência. Gaiotto et al. (2010) refere à ocorrência da espécie na Floresta Ombrófila Mista e na Floresta Ombrófila Densa, sendo encontrada na Argentina, Paraguai e Brasil (Minas Gerais, Rio de Janeiro, São Paulo, Paraná, Santa Catarina e Rio Grande do Sul). É uma espécie robusta, com cerca de 70 cm de altura, poucas folhas, as quais estão

dispostas em roseta tubular de coloração verde-escura e com listras transversais branco-zebradas na face inferior; a inflorescência é do tipo espiga pendente (Reitz, 1983).

Portanto, o evidente potencial ornamental destas duas espécies endêmicas da Mata Altlântica fragiliza a sua preservação na natureza, pois, como mencionado por Martinelli (2000), devido à alta exploração de espécies de interesse econômico e a pressão pelo desenvolvimento urbano ao longo da costa, a Mata Atlântica vem sofrendo devastação e os conservacionistas estimam que apenas 2 a 3% da floresta original sobreviverá. A cobertura florestal deste bioma encontra-se reduzida a cerca de 8% da área original (Brasil, 2000). Originalmente, conforme relata Oliveira Costa (1997), a Mata Atlântica cobria uma área de aproximadamente um milhão de km^2 do território brasileiro, 12% da área do país; formava, com a floresta amazônica, o conjunto das duas maiores e mais importantes florestas do continente. O autor ainda acrescenta que, apesar da devastação acentuada, a Mata Atlântica ainda contém um dos mais importantes conjuntos da diversidade biológica do Brasil, com altíssimos níveis de endemismo, o que evidencia ainda mais a importância da conservação das suas espécies nativas.

Sendo assim, torna-se cada vez mais importante a realização de estudos com espécies endêmicas deste bioma, como é o caso de *Billbergia alfonsi-joannis* (já em risco de extinção) e *B. zebrina* visando à preservação destas espécies.

Técnicas de cultura de tecidos apresentam um conjunto de estratégias que possibilitam auxiliar na propagação massal e conservação das espécies da flora nativa brasileira, reduzindo assim a pressão de coleta na natureza potencialmente com valor econômico (Guerra & Dal Vesco, 2010, Pilatti et al., 2011). Assim, procedimentos de propagação in vitro vem sendo utilizados com muitas espécies vegetais, devido à qualidade fitossanitária, com reduzido tempo para obtenção dos resultados, o que independe da época do ano e mantém a identidade genética dos indivíduos (Kozai et al., 1997; Guerra et al., 1999).

Algumas técnicas de cultura de tecidos já são conhecidas para espécies de bromélias como embriogênese somática, organogênese direta e culturas nodulares (Guerra & Dal Vesco, 2010). Esta última técnica vem sendo desenvolvida mais recentemente em bromélias porque possui um alto potencial regenerativo e consiste na regeneração de novos indivíduos a partir da formação de grupos de nódulos organogênicos globulares composto basicamente de grupos de células meristemáticas (Dal Vesco et al., 2011). Algumas espécies de bromélias já foram regeneradas a partir de culturas semelhantes às nodulares, como as observadas em *Ananas*

comusus (L.) Merr. (Teng, 1997), *Neoregelia cruenta* (Graham) L.B. Sm. (Carneiro et al., 1999), *Vriesea friburguensis* Mez (Alves e Guerra, 2001), *Vriesea reitzii* Leme & Costa, Andrea (Dal Vesco & Guerra, 2010), *Ananas comosus* (Scherer et al. 2013), *Aechmea setigera* Mart. ex Schult. & Schult. f. (Fermino Júnior et al. 2014), inclusive para espécies do gênero *Billbergia* Thunb. (Dal Vesco et al. 2011).

Apesar de protocolos de regeneração a partir de culturas nodulares já terem sido utilizadas com espécies de bromélias, há lacunas na compreensão dos aspectos com relação à gênese da morfo-histologia das estruturas. Estudos sobre a morfo-histodiferenciação podem ser ferramentas adicionais para elucidar e investigar esta questão, desde a inoculação dos explantes até a regeneração dos novos indivíduos. A análise dos eventos durante a morfo-histodiferenciação pode e tem contribuído com o entendimento dos sistemas de culturas de tecidos, pois permite evidenciar as alterações estruturais e ultraestruturais que estão ocorrendo nos explantes e desta forma fornecer informações adicionais sobre a origem de células-tecidos-órgãos e seu desenvolvimento (Yeung, 1999). A caracterização estrutural nas diferentes fases do desenvolvimento das culturas in vitro é determinante para o entendimento das rotas da morfo-histoferenciação (Dal Vesco et al., 2011). Os estudos histológicos são fundamentais não apenas para confirmar a rota morfogênica estabelecida, mas, também para determinar os tipos celulares dos quais derivam as culturas, como em casos de embriões somáticos ou brotos regenerados (Fermino Junior et al., 2009; Maciel et al., 2010).

Alguns estudos já enfocam a histodiferenciação em culturas nodulares como para plantas lenhosas (McCown et al., 1988), *Chicorium intybus* (Pierón et al., 1998), *Humulus lupulus* (Batista et al., 2000 e Fortes e Pais, 2000), *Sclerocarya birrea* (Moyo et al., 2009), *Ananas comosus* (Scherer et al., 2013), *Aechmea setigera* (Fermino Júnior et al., 2014) e *Vriesea friburgensis* (Corredor-Prado et al., 2015). Porém, apesar da importância do conhecimento morfo-histológico no processo de regeneração a partir de culturas nodulares, são poucos os estudos que agregam estas informações no desenvolvimento dos seus protocolos de regeneração. Neste sentido, investigações sobre a morfo-histogênese de culturas in vitro de espécies de Bromeliaceae micropropagadas, devido ao seu potencial ornamental, vêm somar às técnicas de cultura de tecidos, viabilizando uma maior eficiência e, por conseguinte, também uma maior eficácia nos procedimentos de multiplicação de espécies em extinção e/ou ameaçadas de extinção.

Desta forma, no presente trabalho de tese, buscou-se avançar no conhecimento da morfo-histodiferenciação de culturas nodulares induzidas em *B. alfonsi-joannis* e *B. zebrina*, e do processo de regeneração de brotos a partir de culturas nodulares de *B. zebrina*. Sendo assim, a tese foi estruturada em 2 capítulos: o primeiro capítulo trata da morfo-histodiferenciação do processo de indução de culturas nodulares a partir de segmentos nodais de *B. alfonsi-joannis* e *B. zebrina*; e o segundo capítulo trata da morfo-histodiferenciação do processo regenerativo de brotos a partir de culturas nodulares de *B. zebrina*. Ao final, as considerações finais serão apresentadas.

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CAPÍTULO 1

Este manuscrito encontra-se publicado no periódico *Protoplasma*:

Morpho-histodifferentiation of *Billbergia* Thunb. (Bromeliaceae) nodular cultures

Souza TV, Thiesen JF, Lando AP, Guerra MP, Santos M. 2016. Morpho-histodifferentiation of *Billbergia* Thunb. (Bromeliaceae) nodular cultures. Protoplasma. doi: 10.1007/s00709-016-0962-2.

Abstract

Nodule cultures are formed through an intermediate morphogenetic route that lies between organogenesis and somatic embryogenesis. Although well described in many species, different aspects of the morphological and histological development of nodules remain to be clarified. Based on their threatened status and high ornamental value, *Billbergia alfonsi-joannis* and *B. zebrina*, two epiphytic bromeliad species endemic to the South American Atlantic Forest, were studied. Nodular cultures were induced to grow from nodal segments taken from etiolated seedlings grown in vitro for 12 weeks in the dark on MS medium supplemented with 1uM TDZ. Samples were taken for analysis weekly over eight weeks of growth and analyzed under light, transmission electron, and scanning electron microscopes. Morphological and histological analysis showed that nodular clusters originated from stem pericycles and consisted of a polycenter, cambial tissue, cortical parenchyma, and a covering tissue. The polycenter consisted of an organizational center dispersed in parenchymal tissue. Each organizational center was formed by a vascular system surrounded by a bundle sheath. A cambial tissue surrounded these polycenters, promoting the regeneration of new nodules and leading to the formation of buds and roots. Primary nodules could generate secondary nodules in a repetitive process. Thus, histological analysis revealed the origin and formation of nodular cultures. These new data will support the establishment of micropropagation protocols and regeneration on a large scale for these species.

Keywords: Histology, *Billbergia zebrina*, *Billbergia alfonsi-joannis*, micropropagation, ultrastructure

Introduction

Species of *Billbergia* Thunb. (Bromeliaceae) are known for their medicinal, food, and, principally, ornamental uses (Benzing 1980). Moreover, these species have substantial ecological value by serving as cover for many animal species and, hence, sustaining forests (Reitz 1983). Two epiphytic species endemic to the South American Atlantic Forest, *Billbergia alfonsi-joannis* Reitz and *B. zebrina* (Herbert) Lindley, have attracted attention for their use as ornamental plants. However, overharvesting and overall high impact on the South American Atlantic Forest have contributed to *B. alfonsi-joannis* coming under threat of extinction, while *B. zebrina* is already considered vulnerable to extinction (Brasil 2008; Dal Vesco et al. 2011).

In vitro culture techniques have been used to optimize the propagation and conservation of endangered or threatened plant species (Fay 1992). This is especially true for bromeliads, where micropropagation is a valuable tool for germplasm conservation, clonal mass propagation of selected genotypes (Guerra and Dal Vesco 2010), and reduction of overharvesting.

Plant regeneration through induction of nodular clusters is one morphogenetic route in regenerating plantlets. Since nodular clusters consist of groups of globular organogenic nodules, with friable to light-compacted texture, they are, basically, groups of meristematic cells (Dal Vesco and Guerra 2010; Dal Vesco et al. 2011). This regeneration system is based on the formation of organogenic nodules arising directly from explant and cell suspension, which can proliferate and generate new nodules, allowing the long-term maintenance of the culture in the nodular stage (Warrag et al. 1991; Teng, 1997; Fortes and Pais 2000).

The regeneration through nodular clusters is distinct from organogenesis and somatic embryogenesis, thus the nodular cultures have been considered a third morphogenetic route (George 1993). It is possible to find studies that considered the regeneration through nodular cultures as being somatic embryogenesis, since their regenerative efficiencies are comparable (Moyo et al., 2009). However, differently from somatic embryogenesis, the regeneration through nodular cultures generates only unipolar axes, i.e. buds and roots without connection between them (McCown et al., 1988; Batista et al., 2000; Dal Vesco e Guerra, 2010). Furthermore, the nodular cultures present very particular characteristics in the organizational level, different from the other routes, such as the arrangement of organizational centers and the presence of vascularization

between the nodule and the forming buds (McCown et al., 1988; Haensch, 2004; Moyo et al., 2009).

Regeneration protocols from nodular clusters have been developed for species of several plant families (Warrag et al. 1991; Piéron et al. 1993, 1998; Fortes and Pais 2000; Batista et al. 2000; Salaj et al. 2005; Woo and Wetzstein 2008; Moyo et al. 2009; Ferreira et al. 2009), such as Bromeliaceae (Teng 1997; Carneiro et al. 1999; Alves and Guerra 2001; Alves et al. 2006; Dal Vesco and Guerra 2010; Scherer et al. 2013; Fermino Júnior et al. 2014; Corredor-Prado et al. 2015), including species of *Billbergia* (Dal Vesco et al. 2011).

Therefore, regeneration protocols based on this morphogenetic route could be considered well established; however, the origin and structure of these nodular clusters remain to be clarified. Studies have pointed to the importance of histological evaluation in the development of in vitro culture protocols. Such assessment could improve protocol efficiency and reveal new information that would allow the application of appropriate strategies for plant regeneration and, consequently, conservation (Salaj et al. 2005; Woo and Wetzstein 2008). Morpho-histodifferentiation analysis of nodular cultures could further clarify and characterize nodular clusters as distinct from somatic embryogenesis (Moyo et al. 2009; Corredor-Prado et al. 2015). Therefore, the present study aimed to study the morpho-histodifferentiation of nodular clusters induced on nodal segments of two epiphytic bromeliad species from the South American Atlantic Forest: *Billbergia alfonsi-joannis* and *B. zebrina*.

Materials and methods

Disinfection and inoculation of seeds

Seeds excised from *Billbergia alfonsi-joannis* and *B. zebrina* seed capsules were disinfected in 70% ethanol for two minutes and, subsequently, in a 30% bleach solution for 20 minutes. Seeds were inoculated in 340ml glass flasks containing 25mL of MS culture media (Murashige and Skoog 1962) with Morel and Wetmore vitamins (Morel and Wetmore 1951), 30 g L-1 sucrose, 7.5 g L-1 agar, and 1.0 g L-1 activated charcoal. Flasks were sealed with plastic lids and covered with plastic film. Medium pH was previously adjusted to 5.7 and then autoclaved for 16 minutes at 1.3 atm. The cultures were maintained in the dark at 25 °C for germination.

Nodular culture induction

Nodal segments 1.0 ± 0.3 cm in length from both species were collected from etiolated plantlets after twelve weeks of cultivation in the dark. Explants were inoculated in test tubes containing 15mL of semisolid MS media supplemented with 1.0 μM Thidiazuron (1-phenyl-3-(1,2,3-thiadiazol) 5-urea) (TDZ) to induce nodular culture, following the previously established protocol of Dal Vesco et al. (2011).

Morpho- and histodifferentiation analysis

Samples representing cultures from the two species obtained during the process of nodular cluster induction were collected weekly, starting at time zero and ending at the eighth week post-culture induction. These samples were immediately processed in accordance with the techniques described next.

Light microscopy (LM)

Samples were fixed in 2.5% glutaraldehyde in a 0.1M sodium phosphate (pH 7.2) buffer, washed in the same buffer, dehydrated in a gradual ethanol series (O´Brien and McCully 1981), and infiltrated with Jung's Historesin (Leica®, Heidelberg, Germany) in accordance with Gerrits and Smid (1983). Sections 5μm wide were obtained with a RM 2125 RT rotating microtome (Leica®, Nussloch, Germany) and stained with toluidine blue (O'Brien et al. 1964). Images were captured with a Sony P92 digital camera mounted on a MPS 30 DMLS light microscope (Leica®, Wetzlar, Germany).

Scanning Electron Microscopy (SEM)

Samples from the two species were fixed in accordance with the method described for histodifferentiation. After dehydration, the samples were submitted to critical point drying using CO2 in an EM CDP300 (Leica®, Heidelberg, Germany), following the protocol of Horridge and Tamm (1969). The dried samples were adhered to aluminum supports

with doubled-sided carbon tape and then covered with a 30nm gold-palladium film in an EM SCD 500 high vacuum sputter coater (Leica®, Vienna, Austria). Images were captured with a Jeol XL30 scanning electron microscope (Tokyo, Japan) in the Central Electronic Microscope Laboratory of the Federal University of Santa Catarina, Brazil. Samples analyzed externally were then immersed in liquid nitrogen for thirty seconds, sectioned, adhered again to aluminum supports, and metalized for internal observations.

Transmission Electron Microscopy (TEM)

Samples were fixed in 2.5% glutaraldehyde in a 0.1M sodium phosphate buffer (pH 7.2), washed in the same buffer solution, post-fixed in osmium tetroxide, dehydrated in an increasing series of acetone aqueous solutions, and infiltrated in Spurr´s resin as described in Spurr (1969). Samples were sectioned using a UC7 ultramicrotome (Leica®, Vienna, Austria), collected over copper grids, and contrasted with 5% uranyl acetate and lead citrate, following Reynolds (1963). Samples were observed and documented under a Jeol JEM-1011 transmission electron microscope (Tokyo, Japan).

Results

The initial structure of nodal segments gave no indication of mitotic activity (Fig. 1a). Initial meristematic activity on the insertion region of the leaf on the stem of *B. zebrina* nodal segments was observed after one week in culture media (Fig. 1b). This activity was observed in the peripheral region of the stem segment near the leaf axil, as shown by the clusters of dividing cells (Fig. 1b). Analysis of sequential longitudinal sections showed that the nodal region was surrounded by peripheral meristematic tissue, as observed by the band of dividing cells. Moreover, formation of provascular tissue was observed, indicating the beginning of nodule vascular tissue (Fig. 1c). After three weeks of development in nodule culture, nodule formation began in the region below leaf insertion (Fig. 1d) with vascularization originating from the peripheral region of the stem vascular cylinder (Fig. 2a). Histodifferentiation in *B. alfonsi-joannis* was similar to that of *B. zebrina* with the same origin of nodule vascularization after three weeks of culture (Fig. 2b and detailed in 2c). Ultrastructural analysis using SEM reinforced these findings in that nodules were found to develop from the nodal segment below the leaf

base (Fig. 2d). This same sample was later sectioned longitudinally in the middle of one of the nodules, as shown in Figure 2f, in order to record the vascular origin in the stem segment (Fig. 2e). In SEM imaging, the pericycle of the shoot segment, initially formed by a layer of cells, became pluristratified and faced toward the forming nodule (Fig. 2e). It is in this area that deviation of the pericycle occurred and provascular cells were found to have already incorporated into nodular masses (Fig. 2e, f).

The central area of nodules from both species presented a polycenter formed by organizational centers (OCs) dispersed in parenchymatic cells after five weeks of cultivation (Fig. 3a). These centers were formed by tracheary neoelements and still-undifferentiated meristematic cells (Fig. 3b). Each OC was surrounded by a bundle sheath (Fig. 3c). Under TEM microscopy, cellular ultrastructure showed the deposition of secondary walls in cells in OCs, confirming the formation of tracheary neoelements (Fig. 3d). In addition, other cells were observed to have ample vacuoles and cytoplasm rich in mitochondria. However, these organelles were confined to the cell periphery, characterizing the formation of sieve neoelements (Fig. 3e). The polycenter of the nodule showed delimited cambial tissue (Fig. 3a). Cells in this tissue showed flattened shape with thin walls, at times winding, and a dense protoplast (Fig. 3f). Young nodules were covered by an epidermis (Fig. 2b, c); however, this covering was replaced by a periderm in more developed nodules (Fig. 3g).

During the development of nodule culture, formed nodules gave rise to other nodules (Fig. 4a), configuring a repetitive process. The secondary nodules were derived from cambial tissue from the primary nodule and developed with the same structure, i.e., a polycenter, cambial tissue, and parenchymatic tissue (Fig. 4b).

Within one to eight weeks of cultivation in induction medium, cultures formed unipolar structures originating from the cambial tissue, resulting in the formation of adventitious buds or roots. In histological studies, induction of adventitious roots occurred after three weeks in culture (Fig. 4c). These roots were derived from meristematic cells which constituted cambial tissue surrounding a polycenter (Fig. 4c). Some adventitious roots were directed toward the exterior (Fig. 4c, d), while others crossed the cortical region of the nodule (Fig. 4c, e). These intracortical roots showed a sclerenchymatous ring with tracheal elements and alternate sieve elements (Fig. 4e).

Adventitious buds also originated from cambial tissue surrounding the polycenter. These buds were composed of meristematic tissues, tunic and body (Fig. 2c). The morphological and histological differentiation of

these buds allowed the formation of shoot tips with leaf primordia, as observed in *B. alfonsi-joannis* after eight weeks of cultivation (Fig. 4g). In more developed *B. zebrina* nodule cultures, the presence of leaves after seven weeks in culture was observed (Fig. 4f).

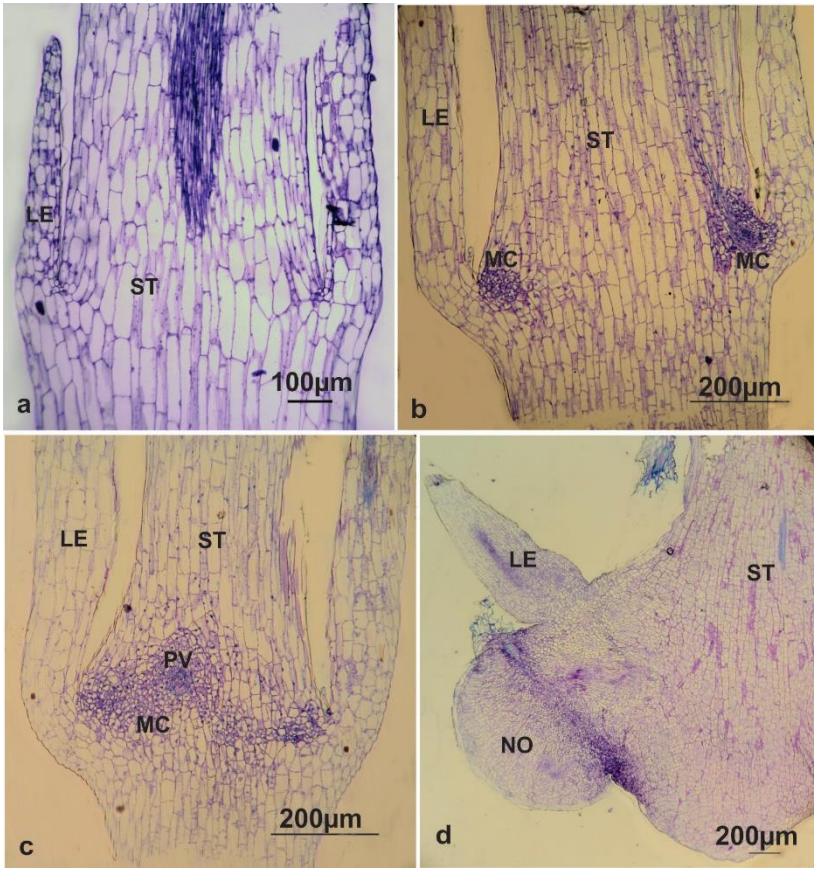

Fig. 1 Histology of nodule cultures on nodal segments of *Billbergia zebrina* from 0 to 3 weeks of culture. Longitudinal sections. a Nodal segment at an early stage (0 time – before induction). b Middle region of nodal segment after 1 week of induction. c Peripheral region of a nodal segment after 1 week of induction. d Nodal segment with a formed nodule after 3 weeks of induction.Abbreviations: LE – leaf; MC - Meristematic cell; NO - Nodule; PV - Provascular cell; ST – Stem.

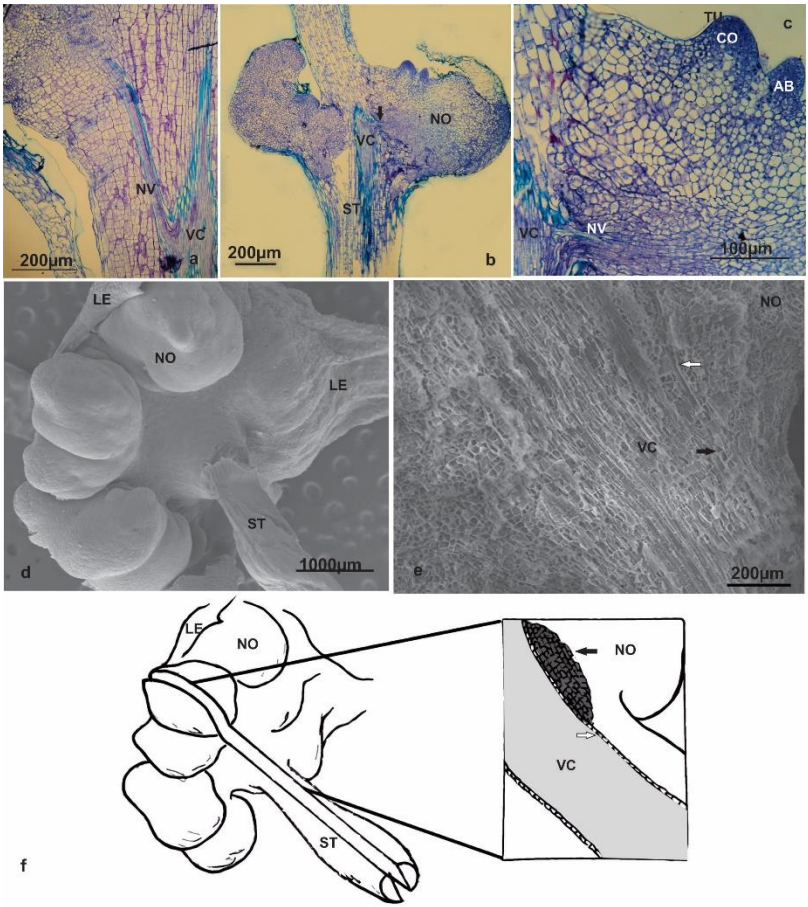

Fig. 2 Light and scanning electron microscopy of nodular cultures on nodal segments of *Billbergia alfonsi-joannis* (BAF) and *B. zebrina* (BZ) 3-5 weeks after induction. a Longitudinal section of nodal segment with early-stage BAF after 3 weeks of induction. b Longitudinal section of nodal segment with nodules of BAF after 4 weeks of induction. c Detailed image of 2B showing nodule vascularization. d Nodular culture on nodal segment of BZ after 5 weeks of induction. e Inner detail of a nodular culture (sample d sectioned), emphasizing pericycle (black arrow – unistratified pericycle and white arrow – pluristratified pericycle). f Illustrative diagram of section d and e, showing the section in the nodular culture and pluristratification of the pericycle (arrow). Abbreviations: LE

- leaf MC - Meristematic cell; NO - Nodule; NV - Nodule vascularization; PV - Provascular cell; VC - Vascular cylinder; ST – Stem

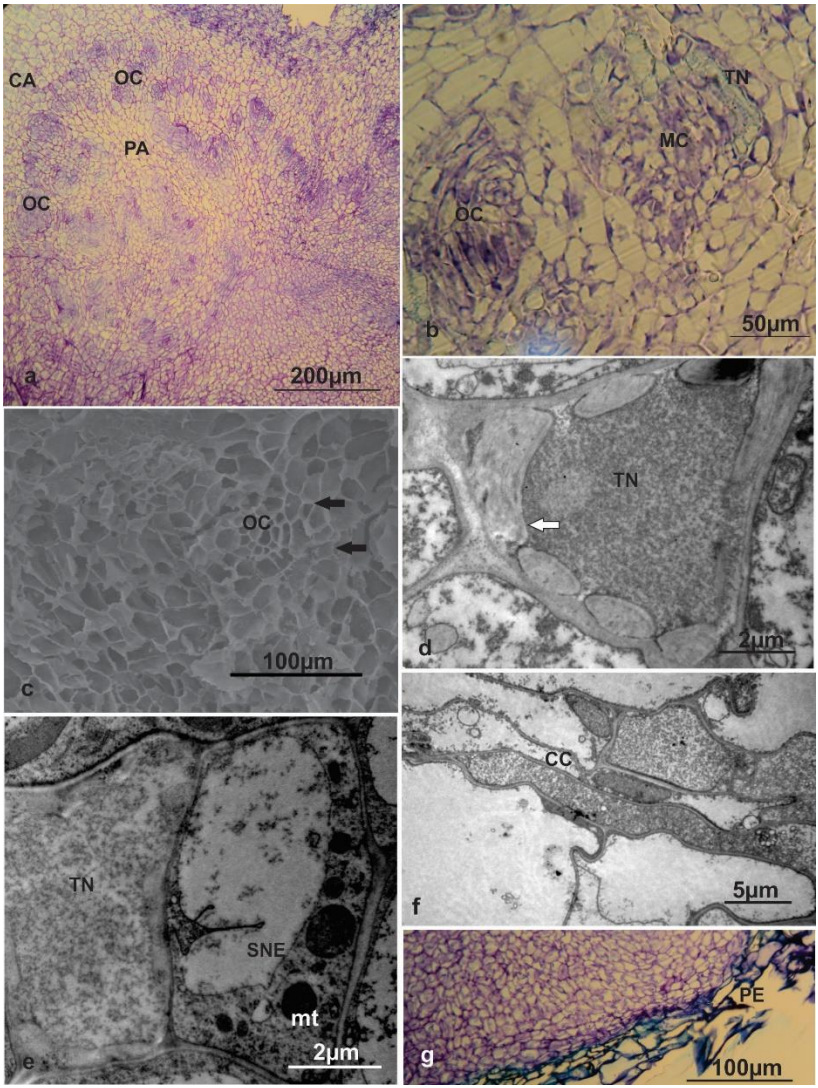

Fig. 3 Light microscopy and scanning and transmission electron microscopy of nodular cultures from nodal segments of *Billbergia alfonsi-joannis* (BAF) and *B. zebrina* (BZ) after 5 weeks of induction. a Longitudinal section of BZ nodular cultures. b Detailed organizational centers of nodular cultures of BZ. c Organizational center in BAF showing bundle sheaths (arrow). d Tracheal neoelements in the organizational center of BZ. Arrow indicates secondary wall deposition.

e Tracheal neoelements and sieve neoelements in the organizational center of BAF. f Cambial cells in the polycenter of BAF. g Periderm on a BZ nodule. Abbreviations: CA – cambium; CC - cambial cell; MC - meristematic cell ; OC - organizational center ; PA – parenchyma; PE – periderm; SNE – Sieve neoelement; TN - tracheal neoelement.

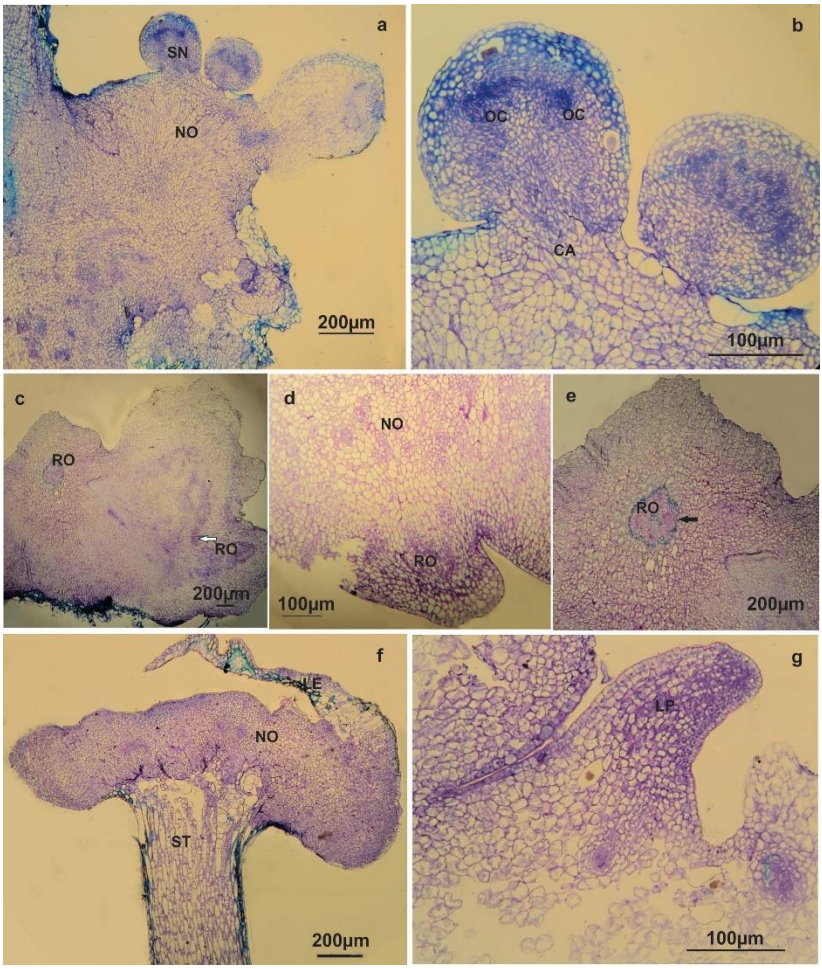

Fig. 4 Histology nodule cultures from nodal segments of *Billbergia alfonsi-joannis* (BAF) and *B. zebrina* (BZ) 5 to 8 weeks after induction. a Longitudinal section of BZ nodular culture after 6 weeks of induction. b Image detail of figure a showing secondary nodules. c Longitudinal section of BZ nodular culture with root development after 5 weeks of induction. d Detail of root formation in BAF. e Detail of root formation in BZ. Arrow indicates sclerenchymatous ring. f Longitudinal section of BAF nodular cultures with leaf formation after seven weeks of induction. g Longitudinal section of BAF leaf primordium formation after 8 weeks of induction. Abbreviations: CA – cambium; LE – nodule; LP - leaf

primordia; NO – nodule; OC - organizational center; RO – root; SN - secondary nodule; ST – stem.

Discussion

Nodular culture induction on *B. alfonsi-joannis* and *B. zebrina* occurred after one week of cultivation on MS media with 1uM TDZ. The time to obtain morphogenetic response in vitro appears to correlate with the taxonomic group. Despite the beginning of the formation of nodular cultures of *Linum usitatissimum* was reported as occurring one week after the induction (Salaj et al. 2005), most of the previous works have mentioned that two weeks are required for the formation of nodular culture of several eudicots: *Cichorium intybus* (Piéron et al. 1993), *Humulus lupulus* (Batista et al. 2000) and *Sclerocarya birrea* (Moyo et al. 2009). Nodular culture formation has been observed to occur in one week in other monocotyledonous plant, like *Digitaria exilis* (Ntui et al. 2010). Thus, the occurrence of a band of meristematic cells after one week in culture characterizes initial formation of nodular clusters of two *Billbergia* species. This strengthens the possibility that this inductive process occurs earlier in monocots than in most eudicotyledonous.

Histological analysis of origin of nodular cultures has been rarely performed. However, Ferreira et al. (2009) found that *Populus euphratica* nodule cultures originated from vascular cambium of leaf. However, the species studied here did not develop vascular cambium, and we did not find any studies reporting on nodule culture originating from monocotyledonous nodal segments. In this study, intense cellular multiplication in the nodal region was initially observed, which could be an initial indication that the beginning of nodular formation occurred from an axillary bud or activity of the intercalary meristem.

Axillary bud formation at the stem-leaf intersection is associated with adaxial leaf face (McConnell and Barton 1998), starting in the peripheral region with a bulge formation consisting of the bud tunic and body (Evert 2006). However, the formation of the nodule in the stem region below the leaf in *Billbergia* excluded the possibility of axillary bud origin.

The activity of intercalary meristem was also discarded as a point of origin based on the location of this tissue and nodules. In monocots, the intercalary meristem is located at the base of the internode and leaf sheath (Evert 2006). This would determine the formation of nodules on the adaxial face of the leaf base, as observed by Corredor-Prado et al. (2015). However, in the present study, nodules were formed under the leaf, suggesting that cells were dividing in the region of the pericycle in and below the node. This supports the idea that nodules did not arise from structures above the node, such as bud or intercalary meristem. Rather,

the nodule expanded toward the abaxial surface of the leaf, suggesting that the nodule originates from pericycle.

The pericycle is the outermost cell range of the stele, which has high meristematic potential (Krauss 1948) and may remain active throughout the plant's life (Menezes et al. 2005). This high meristematic potential, based on the capacity to form lateral roots (Menezes et al. 2005), could have contributed to the stimulation of the pericycle to initiate the formation of nodules through the action of TDZ. This plant growth regulator, TDZ has the capacity to promote cell division and differentiation and is therefore used in tissue culture systems for its powerful activity similar to that of cytokinins (Woo and Wetzstein 2008). It has also been used in studies on somatic embryogenesis, direct organogenesis (Woo and Wetzstein 2008) and nodular cultures(Dal Vesco et al. 2011).

The structure of the nodule culture has been reported by many authors (Batista et al. 2000; Fortes and Pais 2000; Ferreira et al. 2009; Guerra and Dal Vesco 2010), who describe the organizational centers as being formed from central neoformed xylematic tissue surrounded by meristematic cells. According to these authors, organizational centers in a nodule constitute polycenters or meganodules. These features were also observed in this study, adding to the evidence that not only tracheal neoelements are involved in the establishment of polycenters, but also sieve neoelements. Furthermore, a sheath of cells surrounds OCs, but with meristematic characteristics, forming what is usually called a bundle sheath. However, these cells showed meristematic characteristics. The layer of cells surrounding the vascular bundle had high meristematic potential which usually arises from endoderm (Menezes et al., 2005).

In this context, the structure of the nodular cultures could be compared to an atactostele in monocots. However, unlike a typical atactostele, where a single endoderm involves the entire stele, each vascular bundle in a nodule had its own sheath, which could divide to generate new OCs. This is reminiscent of a characteristic of more primitive phylogenetic groups. The vascular bundles of ferns, for example, are individually surrounded by endoderm, called meristeles (Ogura 1972). Each meristele can vascularize to form a new frond with differentiated and independent growth. Thus, each OC could allow vascularization of new nodules in nodular cultures.

The layer of cambial cells surrounding the polycenter, as constituted by OCs, was shown to be a highly relevant tissue for nodular culture development by their mitotic potential, essentially because evidence of an intense cell division was observed. The cambial layer has

already been described in nodular cultures of other species, such as *Humulus lupulus* (Fortes and Pais 2000). Piéron et al. (1998) observed that these cells showed ultrastructural characteristics that indicated high activity with a large number of mitochondria. In this work, the cambial layer of *Billbergia* had tortuous cell walls. This could indicate an increase in surface contact with neighboring cells, thus allowing an increase of metabolite exchange, thereby increasing the speed of onset of differentiation in these cells of cambial origin.

Similar to periderm, the presence of cells in the peripheral region has been reported in nodular cultures of other species, such as *Humulus lupulus* (Batista et al. 2000) and the bromeliad *Aechmea setigera* (Fermino Júnior et al. 2014). For *Humulus lupulus*, the formation of such cell layers appeared following the expansion of nodule diameter and that this periderm may suffer disruptions and cause division of nodular cultures (Batista et al., 2000). Young *Billbergia* nodules were shown to be delimited by the epidermis; however, this tissue was substituted by periderm after nodule expansion.

The emergence of roots from nodules, as well as adventitious buds and leaf primordia, demonstrated the differentiation of unipolar axes. Unipolarity is a characteristic of nodular cultures, unlike bipolarity, as encountered in somatic embryos (Dal Vesco and Guerra, 2010). Unipolarity during the development of nodule cultures has also been observed in studies reporting on the emergence of root in nodules of flax (Salaj et al. 2005) or on the occurrence of multiple meristems in nodules of *Vriesea reitzii* (Alves et al. 2006, Dal Vesco and Guerra 2010, Dal Vesco et al. 2011, Corredor-Prado et al. 2015). In the course of culture development, Salaj et al. (2005) commented that roots acquire a typical structure of the organ, as observed here in *Billbergia*. The roots of species of this genus even showed development of a sclerenchymatous ring. Lack of synchronicity of events in nodule cultures, e.g., roots in both initial and advanced stages in the same sample, was also reported by Woo and Wetzstein (2008).

The formation of secondary nodules from existing nodules is a characteristic of nodular cultures. Nodule multiplication can be sustained for long periods of time, as described in *Ananas comosus* (Teng 1997), *Populus euphratica* (Ferreira et al. 2009), and *Sclerocarya birrea* subsp. Caffra (Moyo et al. 2009). While other studies have already described the proliferation of nodules, the present study has provided the foundation for greater understanding of the origin of daughter nodules from cambial cells in the primary nodule. Figure 5 shows a synthesis of what has been

demonstrated by other studies and the results reported here for *Billbergia* regarding the origin, structure and regeneration of nodular cultures.

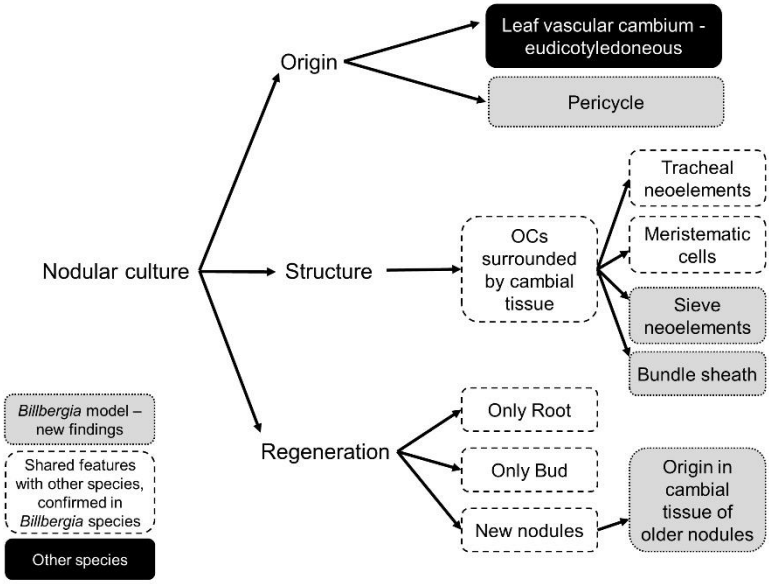

Fig. 5 Schematic diagram indicating the features of nodular culture found in other species compared with the *Billbergia* species model.

Conclusion

Morpho-histodifferentiation analysis in nodular cultures of *Billbergia* reinforced characteristics of these structures that differ from other micropropagation processes, such as the presence of polycenters, regeneration from unipolar axes during development, and development and formation of the secondary nodules from pre-existing nodules. Moreover, we present several new observations not yet described in the literature, such as the origin of nodules from the pericycle, the presence of bundle sheaths surrounding the organizational centers of the polycenter, and the origin of new nodules from cambial cells of older nodules. These results add relevant information to advance the knowledge of the regenerative in vitro route based on nodular cultures. In addition, a process of neoformation of secondary nodules, which establishes a repetitive or cyclic system responsible for the regenerative efficiency of

this model, allows, in turn, the possibility of culture multiplication for efficient plantlet production.

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CAPÍTULO 2

Este manuscrito segue as normas de formatação do periódico Plant Cell Tissue and Organ Culture e foi submetido em 11/04/2016

Morpho- and histodifferentiation of shoot regeneration of *Billbergia zebrina* (Helbert) Lindley nodular cultures

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Abstract

Regeneration of nodular cultures involves the production of new individuals from organogenic nodules. Despite the existence of well-established protocols for bromeliads species, many gaps in understanding the mechanisms of this route still remain, particularly the regeneration of new shoots, a stage little explored. Therefore, this work aimed to monitor the morpho and histodifferentiation processes of shoot regeneration from nodular cultures of *Billbergia zebrina*. To accomplish this, nodular cultures previously obtained from nodal segments of *B. zebrina* were transferred to regeneration induction medium consisting of gelled MS medium supplemented with 1.0 µM of naphthaleneacetic acid and 2.0 µM of benzylaminopurine. Cultures were maintained in a growth chamber over eight weeks, and samples were collected weekly for analysis under light, transmission electron, and scanning electron microscopy. Cells in the shoot formation region presented characteristics of mitotically active cells, such as the presence of numerous plasmodesmata and conspicuous microtubules. New shoots maintained vascular connection to the node. Cells binding the nodule vascular bundle toward the shoot showed degeneration of organelles, which, in turn, originated vascular neoelements. The dome presented a depressed or prominent surface, depending on shoot development. Rounded stomata were conspicuous in the leaf primordia and generally higher in number as compared to the epidermal surface. This study elucidated the nodular culture *in vitro* system in aspects associated to the regeneration of shoots and it is anticipated that such data will lead to the optimization of present nodular cultures based protocols with the aim of conserving endangered bromeliad species.

Keywords: morphodifferentiation, histodifferentiation, histology, micropropagation, Bromeliaceae

Introduction

Billbergia zebrina (Herbert) Lindley is a species of the family Bromeliaceae and native to the Atlantic Forest. The ornamental uses of species from this family (Reitz 1983) is from the beauty of their leaves and inflorescences. However, this same ornamental potential ironically affects its preservation in nature by the economic exploitation and high anthropogenic pressure in their natural environment, the Atlantic Forest,

consequently leading to its genetic erosion (Martinelli 2000). In some regions of Brazil, *B. zebrina* is already considered a vulnerable species (Dal Vesco et al. 2011). Thus, it is urgent to develop conservation strategies for Bromeliaceae, in general, and *B. zebrina*, in particular.

Tissue culture is an important alternative for conservation since it allows the mass micropropagation of bromeliads, and consequently reducing collection pressure on naturally growing habitats (Rech Filho et al. 2009; Guerra and Dal Vesco 2010). Specifically, the *in vitro* regeneration of new individuals from nodular cultures (NC) is a system that has been successfully used for Bromeliaceae species. NC consists of groups of globular organogenic nodules, which are greenish-yellow, friable to slightly compact in texture, and basically composed of meristematic cell groups (Dal Vesco et al. 2011). Regeneration through NC is, however, distinct from organogenesis and somatic embryogenesis, being considered a third morphogenetic pathway of micropropagation (George 1993).

Some bromeliad species have already well established regeneration NC protocols (Teng 1997; Carneiro et al. 1999; Alves and Guerra 2001; Alves et al. 2006; Dal Vesco and Guerra 2010; Scherer et al. 2013; Fermino Junior et al. 2014; Corredor-Prado et al. 2015), including the *B. zebrina* (Dal Vesco et al. 2011). Nonetheless, our understanding of this regenerative route, particularly at the structural level, is limited. Therefore, studies on morpho- and histodifferentiation may be additional tools by which it is possible to obtain a better understanding of this *in vitro* regenerative system. Indeed, the analysis of events during morpho- and histodifferentiation has already improved tissue culture systems by highlighting the structural and ultrastructural changes that occur along the morphogenesis and development (Yeung 1999).

Previous studies have shown the structural and ultrastructural features of NC systems (McCown et al. 1988; Piéron et al. 1998; Batista et al. 2000; Fortes and Pais 2000; Moyo et al. 2009; Scherer et al. 2013; Fermino Junior et al. 2014; Corredor-Prado et al. 2015). However, no studies have thus far elucidated how shoots histologically arise from NC, despite of the fact that this is a key step in obtaining plantlets. Therefore, this study aimed to investigate the morphological and histological aspects of shoot regeneration in *Billbergia zebrina* NC.

Material and Methods

Shoot regeneration

NC of *B. zebrina* were previously induced, according to the protocol established by Dal Vesco et al. (2011). Nodal segments (1.0 ± 0.3 cm in length) were taken from 12-week-old etiolated seedlings and inoculated into test tubes (25mm x 150mm) containing 15 ml of MS medium (Murashige and Skoog 1962) with Morel vitamins (Morel and Wetmore 1951), $30gL^{-1}$ sucrose, $7.5gL^{-1}$ agar, supplemented with 1.0 μM thidiazuron (TDZ) [1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea].

At 56 days, these nodule cultures were transferred to shoot regeneration medium in test tubes (25mm x 150mm) containing 15 ml of MS medium (Murashige and Skoog 1962) plus Morel vitamins (Morel and Wetmore 1951), $30gL^{-1}$ sucrose, $7.5gL^{-1}$ agar, plus 1 μM of α-naphthalene acetic acid (NAA) and 2 μM 6-benzylaminopurine (BAP). The pH of the culture medium was previously adjusted to 5.7 and autoclaved for 16 min at 1.3 atm. The tubes were covered with plastic lids, sealed with plastic film, and maintained in a growth chamber with a photoperiod of 16/8 hours (light/dark) at 25 ± 2°C.

Germination of *B. zebrina* seeds

In order to compare leaf structure between shoots regenerated from NC and seedlings *B. zebrina* seeds were germinated in covered plastic trays lined with 2 sheets of filter paper moistened with distilled water. Fifteen seeds were placed in each tray, which remained in a growth chamber with a photoperiod of 16/8 hours (light/dark) at 25 ± 2°C.

Morpho- and histodifferentiation analysis

Samples in regeneration medium were randomly collected throughout the eight weeks of culture. Meanwhile, leaf samples of conventionally germinated plantlets were collected from the first to the third week of seed germination. These samples were processed for microscopy, as follows:

Light microscopy (LM)

Samples were fixed in 2.5% glutaraldehyde in a 0.1M sodium phosphate (pH 7.2) buffer, washed in the same buffer, dehydrated in a gradual ethanol series (O´Brien and McCully 1981), and infiltrated with Jung's Historesin (Leica®, Heidelberg, Germany) in accordance with Gerrits and Smid (1983). Sections 5µm wide were obtained with a RM 2125 RT rotating microtome (Leica®, Nussloch, Germany) and stained with toluidine blue (O'Brien et al., 1964). Images were captured with an Opticam 5.1 MP digital camera attached to a MPS 30 DMLS light microscope (Leica®, Wetzlar, Germany).

Scanning Electron Microscopy (SEM)

Samples were fixed in accordance with the method described for LM. After dehydration, the samples were submitted to critical point drying using CO^2 in an EM CDP300 (Leica®, Heidelberg, Germany), following the protocol of Horridge and Tamm (1969). Dried samples were adhered to aluminium supports with doubled-sided carbon tape and then covered with a 30nm gold-palladium film in an EM SCD 500 high vacuum sputter coater (Leica®, Vienna, Austria). Images were captured with a Jeol XL30 scanning electron microscope (Tokyo, Japan) in the Central Electronic Microscope Laboratory of the Federal University of Santa Catarina (LCME-UFSC), Brazil.

Transmission Electron Microscopy (TEM)

Samples were fixed in 2.5% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.2), washed in the same buffer solution, post-fixed in osmium tetroxide, dehydrated in an increasing series of acetone aqueous solutions, and infiltrated in Spurr´s resin as described in Spurr (1969). Samples were sectioned using a UC7 ultramicrotome (Leica®, Vienna, Austria), collected over copper grids, and contrasted with 5% uranyl acetate and lead citrate, following Reynolds (1963). Samples were observed and documented under a Jeol JEM-1011 transmission electron microscope (Tokyo, Japan) at LCME-UFSC.

Results

Regeneration occurred in an unsynchronized process through time. In other words, the same NC showed shoots at different developmental stages. For example, at 14 days (Fig. 1a), the NC showed regenerated shoots with development similar to that of the same NC at 58 days (Fig.

1b). Notwithstanding this developmental characteristic, increasing cultivation time, e.g., from 14 to 58 days, did favour the regeneration process by the development of more shoots. Therefore, analyses were restricted to the developmental stage of shoots regardless of culture time.

NC also formed roots (Fig. 1c) with origin and structure independent of the shoots. Thus, the regeneration of NC resulted in the formation of only unipolar axes, i.e., either shoots or roots.

The origin of shoots was evidenced by the presence of cells showing high mitotic activity in the periphery of nodules (Fig. 1d). At the beginning of shoot development, the dome region generally showed a depressed surface by the elevated leaf primordia (Fig. 1d-e). However, as shoots developed, the dome became more prominent (Fig. 1f), and exhibited a layer of peripheral meristem cells, constituting the tunica, which consisted of a number of meristematic cells dividing in various planes, featuring the corpus. The proliferation and differentiation of cells of the corpus originated the internal tissues of the leaf primordia (Fig. 1f-g). For shoots in the early developmental stages, longitudinal sections showed leaf primordia with differences in size and degree of differentiation, indicating the arrangement of leaves in rosettes, typical of bromeliads (Fig. 1g-h). Cross sections of NC presented polycenter with branching vascularization, as leaf trace, toward the peripheral region where the shoot was starting to form (Fig. 1i). Vascular neoelements were detected in these branches (traces).

TEM analysis revealed cells with dense protoplast (Fig. 2a), forming the dome containing numerous mitochondria, evident Golgi bodies (Fig. 2b), and endoplasmic reticulum (Fig. 2c). These cells were thin-walled, but with large extracellular matrix deposition between them (Fig. 2d). Between neighbouring cells, the presence of many plasmodesmata was observed (Fig. 2e), showing a reduction in extracellular matrix deposition. Verification of cytoskeleton crossing the cell protoplast, with microtubule strands running through the periphery of the cells, gave the most significant evidence of the high mitotic potential of these cells (Fig. 2f-g).

Based on ultrastructural analysis, some nodal cells were observed to run toward the vascularization of the shoots having chloroplasts with numerous plastoglobuli (Fig. 3a). The greater size variation of plastoglobuli indicated the degradation of chloroplasts for future formation of tracheary neoelements. In addition, some chloroplasts showed highly corrugated thylakoid, forming large waves on the grana (Fig. 3b). Cells also contained large vacuoles and protoplasts restricted to the periphery (Fig. 3c), indicating the formation of sieve neoelements.

Neighbouring cells were found with reduced size and dense protoplast, featuring companion cells (Fig. 3d). Companion cells showed donut-shaped (toroidal) mitochondria (Fig. 3e) and serial mitochondria (Fig. 3f).

A comparative histological analysis was then performed between leaves of shoots regenerated from NC and those of conventionally germinated seedlings, revealing some distinct characteristics. The mesophyll of leaves from regenerated shoots (Fig. 4a) was homogeneous, with all cells containing chloroplasts, while in the leaves of seedlings (Fig. 4b), the mesophyll exhibited heterogeneity, with chlorophyllous tissue in the abaxial surface and hydrenchyma (water storing parenchyma cells, nonchlorophyllous tissue) in the adaxial surface. In leaves of seedlings, all stomata exhibited a similar position on the epidermal surface, i.e., slightly elevated and elongated in shape (Fig. 4c). In leaves of regenerated shoots, some stomata were higher and round in shape, while others were deeper on the epidermal surface (Fig. 4d).

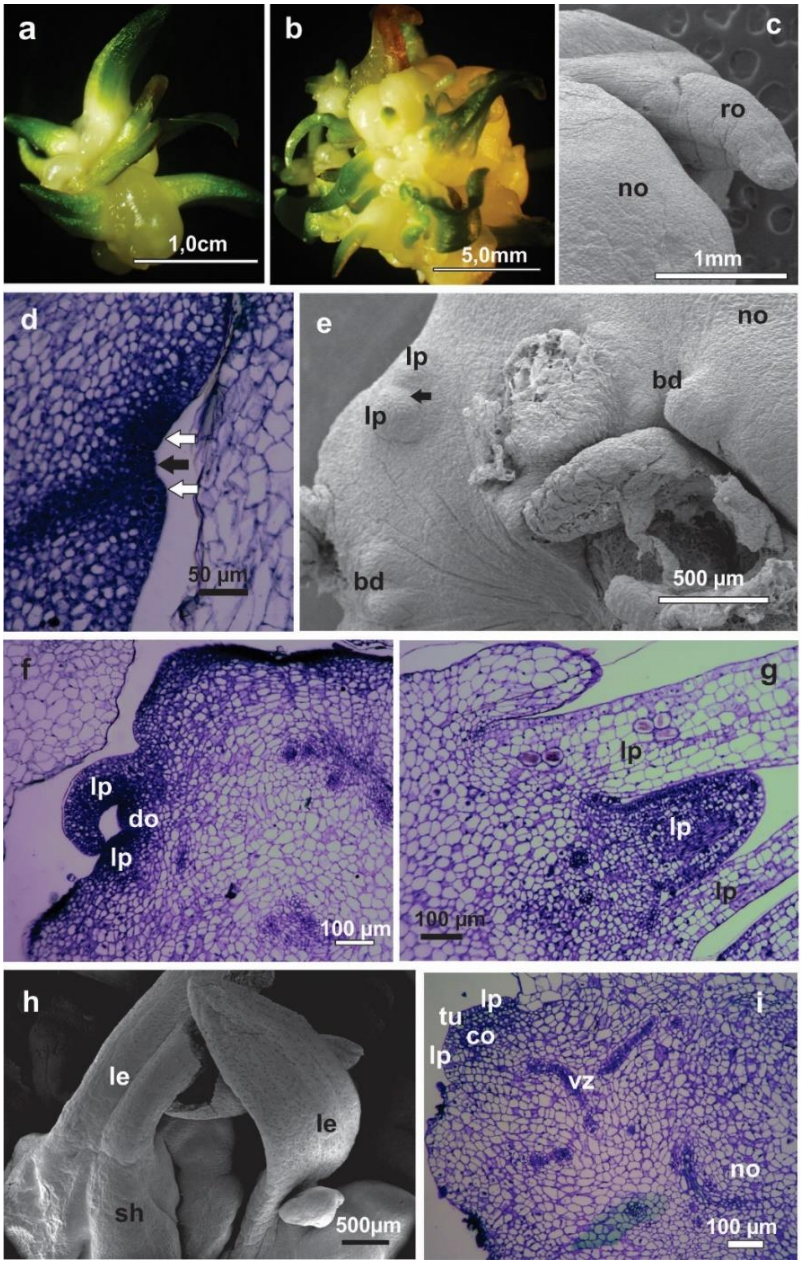

Fig.1 Light microscopy and scanning electron microscopy of the morpho- and histodifferentiation of shoot formation from *Billbergia zebrina*

nodular cultures. A. Nodular culture showing shoot formation at 14 days of cultivation B. Nodular cultures with shoot formation at 58 days. C. Root formation from nodules. D. Bud starting to form with little apparent dome. Arrow indicates dome.. E. Bud formation . F. Shoot with leaf primordia more evident. G. Leaf primordia in rosette-like formation. H. Formed shoots. I. Bud in later stage with evident dome. Abbreviations: BD – bud; CO – corpus; DO – dome; LE – leaf; LP – leaf primordia; NO – nodule; SH – shoot; TU – tunic; VZ – vascularization.

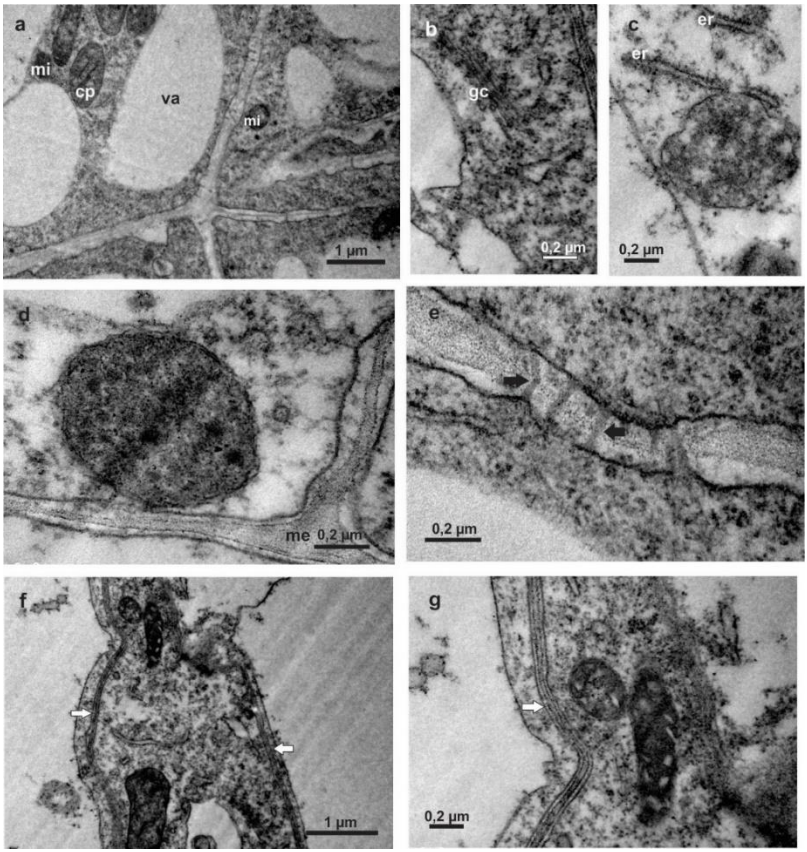

Fig. 2 Transmission electron microscopy of shoot regeneration from *B. zebrina* nodular cultures. A. Mitotically active cells of shoot formation. B. Detail of mitotically active cells, highlighting the Golgi complex. C. Detail of mitotically active cells, highlighting the endoplasmic reticulum. D. Detail highlighting the extracellular matrix. E. Detail highlighting plasmodesmata (arrows indicate plasmodemata). F. and G. Detail highlighting microtubules (arrows indicate microtubules). Abbreviations: CP – chloroplast; ER – endoplasmic reticulum; GC – golgi complex; MI – mitochondria; VA – vacuole.

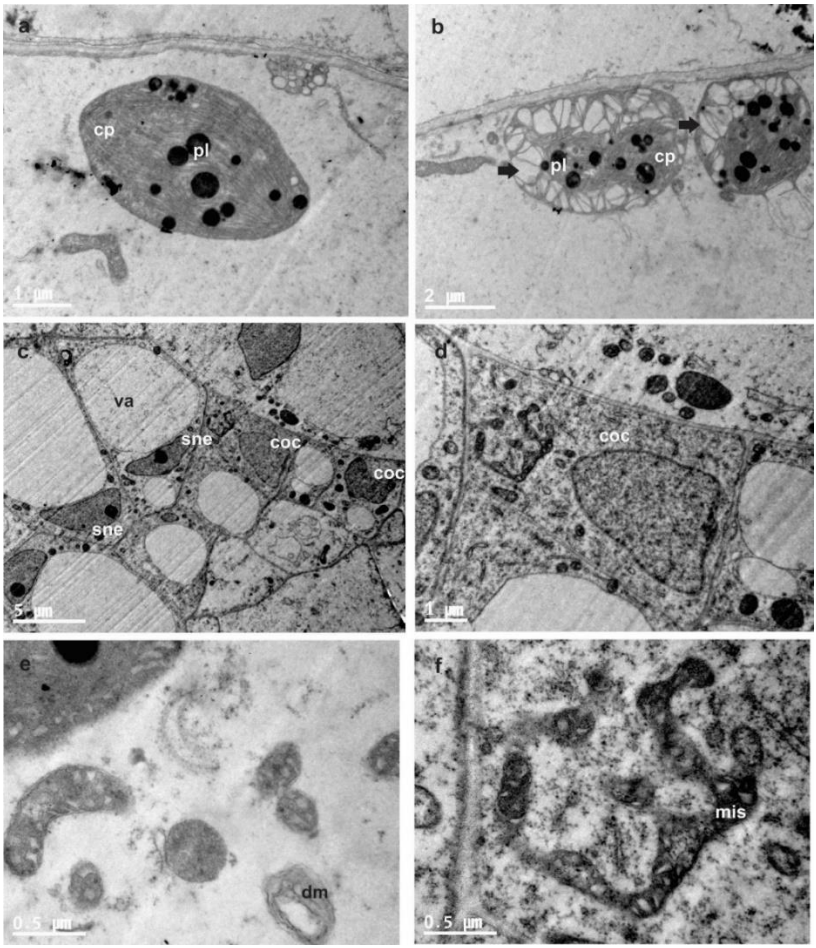

Fig. 3 Transmission electron microscopy of shoot regeneration from *B. zebrina* nodular cultures. A. and B. Cell chloroplast in neovascularization region; arrow indicates wavy crests. C. Formation of sieve neoelements. D. Companion cell formation. E. Formation of donut-shaped mitochondria. F. Formation of serial mitochondria. Abbreviations: COC – companion cell; CP – chloroplast; DM – donut-shaped mitochondria; MIS – mitochondria in series; PL – plastoglobuli; SNE – sieve neoelement.

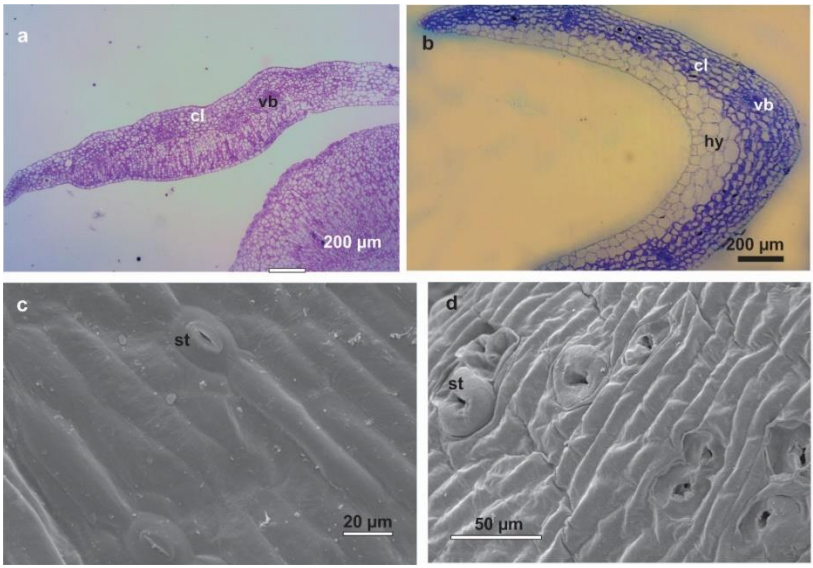

Fig. 4 Light microscopy and scanning electron microscopy comparing morpho-and histodifferentiation of leaves from shoots regenerated from nodular cultures and conventionally germinated seedlings of *B. zebrina*. A. Cross section of shoot leaf. B. Cross section of seedling leaf. C. Frontal view of seedling leaf. D. Frontal view of shoot leaf. Abbreviations: CL – chlorenchyma; HY – hydrenchyma; SM – stomata; VB – vascular bundle.

Discussion

The lack of synchronization in the shoot regeneration process from NC, as observed in the present study, was previously reported (Woo and Wetzstein 2008). In the same sample of *Elliottia racemosa*, these authors observed shoots in early formation and others already bearing several leaf primordia. Moyo et al. (2009) also found buds at different stages of development in the same sample of *Sclerocarya birrea* NC.

In addition to regenerated shoots, the regeneration of some roots from NC of *B. zebrina* was also observed. However, these shoots and roots were not connected, forming only unipolar axes of regeneration. This corroborates the difference between nodular cultures and somatic embryogenesis in that bipolar axes are not formed in regeneration cultures, only unipolar axes (McCown et al. 1988; Dal Vesco and Guerra 2010). This unipolarity had already been reported in other studies, such

as shoot regeneration in *Humulus lupulus* NC (Batista et al. 2000), multiple meristems in *Vriesea reitzii* (Alves et al. 2006) and roots in linen nodules (Salaj et al. 2005).

Dome positioning in relation to leaf primordia is variable (Evert 2008). This variation can range from virtual disappearance between the expanding leaf primordia to constant height, even with the growth of leaf primordia. Dedicová et al. (2000) in NC of *Linum usitatissimum* showed that the dome of apical meristem regenerated from nodules sometimes became wider, subsequently giving rise to other apical meristems. These changes in bud dome, as reported in the regeneration of *B. zebrina* and the work of Dedicová et al. (2000), can be compared to plastochronic changes described by Evert (2008) as morphological changes in the apex by events occurring during bud development.

The vascular connection between node and developing shoot is one of the characteristics differentiating NC regeneration from somatic embryogenesis. In somatic embryogenesis, no vascular connection is formed between the somatic embryo and the mother plant (Haensch 2004; Moyo et al. 2009). This was observed in NC of *B. zebrina* which showed increased mitotic activity and the formation of vascular connection between node and bud, as also confirmed by Fortes and Pais (2000). Studying *Humulus lupulus*, these authors also described increased mitotic activity in cells next to the nodal epidermis. In *Cichorium intybus*, Piéron et al. (1998) reported the development of neovascularization between node and leaf primordia, and they pointed out that this vascularization optimized the flow of nutrients between node and bud.

Additional evidence of nodular neovascularization toward the shoot was revealed by the presence of many plastoglobuli in the chloroplast. Senescent cells increase the number of plastoglobuli, and, thereafter, the size of these structures increases (Lichtenthaler 2013). According to Krupinska (2007) cells in senescence (in the present work represented by cells modifying for the vascularization) undergo a series of changes in chloroplasts, such as reducing the thylakoid membrane system, slackening the grana piles, swelling intrathylakoidal space, shrinking organelle size with a transition from ellipsoid to a circular shape, as well as increasing the size and number of plastoglobuli. More rounded chloroplast with loosening of its thylakoid was observed in in the present work. This loosening formed large waves in granum piles, similar to that seen in *Arapdopsis*, in which deformed chloroplasts were attributed to a loss of attraction between thylakoids and consequent rupture of membranes, changes which can be irreversible in the chloroplast (Chuartzman et al. 2008). In senescent cells the thylakoid

membranes of chloroplasts are progressively degraded and replaced by plastoglobuli (Tevini and Steinmfiller 1985). In the present work, all these factors suggest that the cells located in the connecting region between the nodule and the regenerated shoot underwent to the process of chloroplast degradation. This corresponds to the degradation process of some organelles in order to form vascular neoelements, not senescence. Thus, histodifferentiation requires transformation of cellular components with the elimination of some organelles to establish cells with suitable characteristics for proper sap conduction, such as large vacuoles for sieve neoelements and resistant cell walls for tracheal neoelements.

Cells with mitochondria in altered formats were found near the sieve neoelements. Among the types of altered mitochondria found in this study were those showing donut shape and serial arrangement. The formation of donut-shaped mitochondria begins with the autofusion of many mitochondria, and it ends with the acquisition of an annular shape (Liu and Hajnóczky 2011). This may explain the presence of mitochondria in serial arrangement, demonstrating that this form is, in fact, a step prior to the formation of donut format in these organelles.

Changes in mitochondria have already been reported, mainly in animal cells responding, for example, to oxidative stress (Jendrach et al. 2008) or cellular hypoxia (Liu and Hajnóczky 2011). For plants, the only record of mitochondrial change was in somatic embryo cells of *Araucaria angustifolia* (Fraga et al. 2015). According to Jendrach et al. (2008), mitochondria are one of the first organelles to reflect cellular response to some stressor. Interestingly, i*n vitro* culture conditions represent a combination of cellular stress factors, such as growth regulators, high or low salt concentrations, high or low light intensity and oxidative stress (Zavattieri et al. 2010). Thus, the altered mitochondria found in this study indicate a cellular response to stresses caused by *in vitro* cultivation, in addition to morphofunctional transformation. Such responses represent a structural modification to accommodate the formation of vessels.

In the shoot formation region, cells showed high mitotic activity, with the presence of organelles reflective of high metabolic activity, such as mitochondria, golgi bodies, and thin cell wall. Despite the thin cell wall, large deposits of extracellular matrix were seen between cells. According to Samaj et al. (1999), the extracellular matrix plays an important role in cell-cell adhesion and plant morphogenesis, and this matrix may be involved in the recognition of morphogenetic cells and the regulation of initial stages of morphogenesis and/or embryogenesis. Furthermore, the substances that make up the extracellular matrix may be related to the control of such mechanisms as elongation, growth and cell

differentiation (Popielarska-Konieczna et al. 2008) and therefore may be present in the bud formation region which is undergoing division processes and subsequent cell differentiation. Similar results have been reported in other *in vitro* studies, e.g., *Triticum aestivum* (Konieczny et al. 2005; Pilarska et al. 2007), *Actinidia deliciosa* (Popielarska-Konieczna et al. 2010), *Vitis vinifera* (Pereira et al. 2011) and *Oryza sativa* (Bevitore et al. 2014), but none of these studies considered regeneration processes from nodular cultures.

More evidence for high cell-cell communication was the presence of many cellular connections through plasmodesmata. Gunning (1976) explained that plasmodesmata in plants arise from a mere collection of individual cells in an interconnected community of protoplasts. Plasmodesmata were also reported to play a fundamental role in nutrient diffusion and could be associated with electrical or hormonal signals. However, not all cells are interconnected by plasmodesmata, nor is the presence and quantity of plasmodesmata necessarily related to the functional demands of cells (Ehlers and Kollmann 2001). In the case of cells with high mitotic activity, such as buds, abundant plasmodesmata are found, and their formation is a normal process during the development of these tissues (van der Schoot and Rinne 1999), as seen in the present study.

Visualization of microtubules in cells of the bud-forming region also indicates high mitotic activity. The microtubules are involved in cell elongation and cytokinesis (Cyr 1994; Nick 2000) and are therefore found in larger quantity in mitotically active cells. According to Wick et al. (1981), microtubules are considered morphogenetic tools for the production of new cells in specific areas and shapes and for modelling the progeny during their expansion and differentiation. These are two basic processes by which plants develop their characteristic configuration. Microtubules are also involved in stress response, e.g., cases of fungal or viral infection (Kobayashi and Kobayashi 2000), and, as previously reported, *in vitro* conditions create such stressful conditions as differential light conditions, salt concentrations and growth regulators. In embryogenesis studies, microtubules were observed to be involved in events leading to embryonic formation (Hause et al. 1993). However, our understanding of microtubules is limited since cytoplasmic volume negatively interferes with microscopic visualization, even under electron microscopy (Baskin et al. 1992).

In the present work, the leaf blades of regenerated shoots from NC showed differences in shape and positioning of the stomata relative to other cells of the epidermis, when compared with leaves of

conventionally germinated seedlings. The more rounded shape of the stomata, as observed in this study, has been reported for other species cultured *in vitro*, such as tobacco (Tichá et al. 1999), roses (Johansson et al. 1992) and Cymbidium (Mayer et al. 2008). Hazarika (2006) states that leaves with elevated stomata and with round-shaped guard have been found in some species cultured *in vitro*, compared with elliptical and sunken guard cells in *ex vitro* seedlings. According to Blanke and Belcher (1989), more rounded stomata always seem to remain open, but the saturated atmosphere from the culture flask generates a low moisture gradient between the intercellular space of the leaf and the environment, which causes little transpiration. The characteristic of opened stomata should receive special attention in future studies of acclimatization whenever excessive transpiration hinders adaptation.

Shoot leaves have not yet developed hydrenchyma comparable to that of seedling leaves. This absence can be explained by the high humidity of *in vitro* culture (Barboza et al. 2006) with less demand for water reserves. On the other hand, the presence of hydrenchyma in the leaves of the seedlings suggests just the opposite. In addition, these water reserve tissues are closely related to heat economy in species with crassulacean acid metabolism (Barboza et al. 2006).

Conclusion

The analysis of morpho- and histodifferentiation of shoot regeneration in *Billbergia zebrina* from NC reinforced some features of regeneration that differ from other micropropagation processes such as vascular connection between shoot and NC and the development of unipolar axes, either shoots or roots. The present study has further elucidated the NC system regarding aspects not yet explored in the regeneration phase of shoot, including, the ultrastructure of neovascularization formed between NC and shoot and the ultrastructure of the mitotically active cells for shoot formation, as well as dome and leaf primordia formation at structural and ultrastructural levels. Such morpho- and histodifferentiation analyses have not yet been described in previous studies with this *in vitro* regenerative route.

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CONSIDERAÇÕES FINAIS

Os avanços alcançados no presente trabalho permitiram a elucidação de aspectos da morfo-histodiferenciação do processo de indução de culturas nodulares e regeneração de brotos a partir destas culturas até então pouco compreendidos e explorados, principalmente em espécies de monocotiledôneas. Os resultados obtidos no capítulo 1, permitiram a confirmação e aprofundamento nos conhecimentos em relação à estruturação básica dos nódulos. Além disso, trouxe novidades estruturais dos nódulos como a presença de bainha do feixe e neoelementos crivados. Comprovou-se também o caráter cíclico das culturas nodulares, com formação de nódulos filhos, a partir de nódulos já existentes. E, somado a isto, observou-se de forma inédita a origem dos nódulos a partir do periciclo do caule.

Os estudos da morfo-histodiferenciação da regeneração de brotos a partir de culturas nodulares, apresentados no capítulo 2, permitiram um avanço substancial principalmente a nível ultraestrutural, visto que esta etapa regenerativa é raramente abordada nos poucos estudos histológicos que há em protocolos de regeneração a partir de culturas nodulares. Os resultados trouxeram detalhes da neovascularização formada entre o nódulo e o broto, e das células mitoticamente ativas que formam o broto, como a presença de muitos plasmodesmos e citoesqueleto evidente. Reforçou também a formação de somente eixos unipolares de regeneração a partir de culturas nodulares.

Estes resultados agregam informações para a otimização de protocolos de regeneração a partir de culturas nodulares, e abrem perspectivas para novos estudos. Estes novos estudos, podem seguir no acompanhamento da morfo-histodiferenciação em processos de aclimatização dos brotos regenerados. Além disso, podem dar sequência a uma investigação do surgimento de câmbio, estrutura típica de eudicotiledôneas e que surge no presente estudo na indução de culturas nodulares em espécies de monocotiledôneas. Assim como uma análise da periderme em nódulos mais desenvolvidos, para verificação do desenvolvimento de uma periderme típica originada de um felogênio ou um tecido protetor em monocotiledôneas.