GENETICS AND PLANT BREEDING

Dynamics of proteins, carbohydrates and global DNA methylation patterns during induction of nodular cluster cultures from seeds of *Vriesea reitzii*

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ABSTRACT. Tissue culture techniques have been employed for bromeliad mass propagation by means of the morphogenetic route of nodular cluster cultures (NCs). This study aimed to assess protein, carbohydrate and global DNA methylation (GDM) level dynamics during NCs induction from *Vriesea reitzii* seeds. Seeds were inoculated into Murashige and Skoog (MS) liquid medium supplemented with 4 μM α-naphthaleneacetic acid (NAA) to induce NCs and in culture medium without plant growth regulators to form normal seedlings. Samples collected at 0, 3, 7, 10, 14, and 21 days of culture were analyzed. All parameters assessed showed the same variation pattern. However, seeds inducing NCs showed significantly lower starch (6.0 mg g^{-1} FM), carbohydrate (10.7 mg g^{-1} FM) and GDM (11.0%) levels than seeds forming normal seedlings after 21 days in culture. On the other hand, the protein content (9.1 mg g^{-1} FM) was significantly higher during induction. NCs induction process through seeds is the result of gene reprogramming in the explant, which leads to morphological, biochemical and metabolic alterations. This involves dedifferentiation, high cell proliferation, high energy demand and protein synthesis, which is related to elevated metabolic activity.

Keywords: bromeliaceae; bromeliad; germination; micropropagation; seedling.

Introduction

Bromeliads are complex ecological systems that contribute to the maintenance of the forest ecosystems stability due to their high levels of specialization (Benzing, 2000). In addition to their ecological role, bromeliads have a high landscape value, which is recognized by the ornamental industry worldwide (Guerra & Dal Vesco, 2010; Negrelle, Mitchell, & Anacleto, 2012). Among the bromeliads, *Vriesea reitzii* is an epiphytic plant that occurs in southern Brazil at altitudes ranging from 750 - 1200 m (Leme & Costa, 1991). As observed in most bromeliads, the beautiful shape and colors of *Vriesea reitzii* enable its ornamental use (Negrelle et al., 2012). In recent decades, the development of tissue culture techniques has promoted new strategies for mass propagation of bromeliads (Guerra & Dal Vesco, 2010). Previous work on bromeliad micropropagation reported an *in vitro* morphogenetic pathway based on nodular cluster cultures (NCs). NCs are defined as groups or conglomerates of organogenic nodules with high regenerative efficiency (Dal Vesco & Guerra, 2010; Dal Vesco et al., 2011). Strategies for induction of NCs in bromeliads include nodal segments (Dal Vesco, Stefenon, Welter, Guerra, & Scherer, 2011), leaf segments (Alves, Dal Vesco, & Guerra, 2006; Dal Vesco & Guerra, 2010; Scherer et al., 2013; Dal Vesco, Vieira, Corredor Prado, Guerra, & Elter, 2014b) and seeds (Dal Vesco, Pescador, Corredor Prado, Welter, & Guerra, 2014a; Corredor-Prado et al., 2015). This work has established a system of NCs induction and regeneration of shoots in response to different types and combinations of plant growth regulators (PGRs). Other studies regarding NCs multiplication and differentiation to microshoots are related to GDM levels (Scherer, Fraga, Klabunde, Silva, & Guerra, 2015) and genetic analysis using amplified fragment length polymorphism (AFLP) markers (Dal Vesco, Stefenon, Welter, Steiner, & Guerra, 2012; Scherer et al., 2015). On the other hand, characterization

of the NCs induction process has been focused on morphological and anatomical alterations (Corredor-Prado et al., 2015). Biochemical and epigenetic alterations have not been evaluated.

The development of morphogenetic pathways is accompanied by changes in cellular components such as proteins and polysaccharides (Martin et al., 2000; Cangahuala-Inocente, Silveira, Caprestano, Floh, & Guerra, 2014; Morel et al., 2014). These changes require the expression of genes necessary for synthesis or mobilization of those compounds. Therefore, epigenetic mechanisms play an important role in plant development (Valledor et al., 2007). One of the epigenetic changes is the methylation of cytosine in DNA, which refers to postsynthesis methylation of deoxycytosines at the 5' position of the pyrimidine ring of cytosine, forming methyldeoxycytosine, which interferes with gene translation and expression (Finnegan, 2010). Previous findings have shown that methylation in coding or regulatory regions prevents the expression of target genes, while demethylation events are accompanied with gene activation (Meng et al., 2012; Shan et al., 2013). Furthermore, the action of sugars as signaling molecules affects the expression of genes and the activity of enzymes in many metabolic pathways (Halford, 2010).

Considering that biochemical and epigenetic changes must be accomplished in order to induce NCs, the present work aimed to evaluate the dynamic changes in proteins, carbohydrates, and global DNA methylation (GDM) levels from seeds during NCs induction.

Material and methods

Plant materials

Seeds from mature fruits of *V. reitzii* were collected from plants kept in Curitibanos, at an altitude of 990 m (Santa Catarina State, Brazil, 27º16'58"S - 50º35'04"W). Sterilization was carried out according to the procedures described by Alves et al. (2006). For NCs induction, seeds were inoculated into Murashige and Skoog (MS) liquid medium supplemented with 4 µM NAA, previously established by Dal Vesco et al. (2014a) (hereinafter termed MS-NAA). Seeds inoculated in PGR-free MS medium (hereinafter termed MS) were considered controls. All explants were inoculated over filter paper bridges, into test tubes (22 × 150 mm) containing 12 mL of culture medium. Cultures were maintained in a growth room at 25 ± 2°C and 16h photoperiod, with a light intensity of 50-60 µmol m^{-2} s^{-1}. Samples were collected at 0, 3, 7, 10, 14, and 21 days of culture.

Protein content

Each replicate (500 mg) was prepared from approximately 350 seeds. The extraction of proteins was performed following the method of Carpentier et al. (2005) with modifications. In brief, tissue collected was ground to powder with the aid of liquid nitrogen. The macerated material was homogenized with 5.0 mL of extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 1% w/v DTT, 30% w/v sucrose, and 1 mM PMSF) and 5.0 mL of buffer-saturated phenol (pH 8.0) by vortexing for 30 min. The homogenates were centrifuged for 30 min. at 10,000 × g at 4°C. The phenolic phase was recovered, homogenized with 5.0 mL of extraction buffer, vortexed, and centrifuged again. The phenol phase was collected and precipitated with 100 mM of ammonium acetate in methanol (1:5 v/v) for 12h at -20°C. After centrifugation, the pellet was washed with pure methanol and acetone. The proteins were solubilized in 0.3 mL of solubilization buffer (7 M urea, 2 M thiourea, 3% CHAPS, 2% IPG-buffer, 1.5% DTT). Protein quantification was determined by means of the copper-based method 2-D Quant Kit® (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). The data were submitted to analysis of variance (ANOVA) and presented as the means of three biological replicates.

Total soluble carbohydrates

Samples of 500 mg were ground to powder with the aid of liquid nitrogen and subsequently submitted to an 80% ethanol extraction at 70°C for 5 min. The extracts were centrifuged at 3,000 rpm at 20°C for 10 min. and filtered through fiberglass. The extraction was repeated three times and the final volume adjusted to 5 ml with ethanol (80%). The total soluble carbohydrate content was determined using the phenol-sulfuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using glucose as a standard. The absorbance was measured at 490 nm. The data were submitted to ANOVA and presented as the means of three biological replicates.

Thin layer chromatography

Analysis for the qualitative detection of carbohydrates was carried out on the extracts described previously. Chromatographic conditions: stationary phase: Silica Gel 60 TLC aluminum plate (Alugram®); mobile phase: 2-propanol: ethyl acetate: nitroethane: acetic acid: water (45:25:10:1:19); detection reagent: orcinol-sulfuric acid- ethanol reagent; volume of sample: 2 µL. Rf values were calculated for samples and compared with standards (fructose, glucose, xylose, maltose, and sucrose).

Starch content

The pellets used in the total soluble carbohydrates extraction received the addition of 1 mL of cold distilled water and 1.3 mL of 52% perchloric acid and was maintained in an ice bath with occasional agitation. Subsequently, 2.0 mL of water was added, and the material was centrifuged at 3,000 rpm for 15 min. The extraction was repeated, and the final volume adjusted to 10 mL with distilled water. The starch content was estimated by the phenol-sulfuric method (Dubois et al., 1956), using glucose as a standard, according to the method proposed by McCready, Guggolz, Siliviera, and Owen (1950). The absorbance was measured at 490 nm. The data were submitted to ANOVA and presented as the means of three biological replicates.

Global DNA methylation (GDM)

DNA isolation was based on CTAB methodology (Doyle & Doyle 1990). Digestion procedures, purification, and analysis by high-performance liquid chromatography (HPLC) were based on the method described by Fraga et al. (2012). Samples of seeds germinated containing nucleosides, including 5-methyl-2'-deoxycytidine (5mdC) and deoxycytidine (dC), were filtered (0.22 µm pore size) and injected into HPLC. A Hyperclone 5 µm C18 column (250 x 4.6 mm) (Phenomenex®, Torrance, CA, USA), a precolumn (4.0 x 3.0 mm) (Phenomenex®), and UV detector at 280 nm were used. Two eluents were used: A (0.5% v/v methanol in 10 mM KH_2PO_4 buffer, pH adjusted to 3.7 with H_3PO_4) and B (10% v/v methanol in 10 mM KH_2PO_4 buffer, pH adjusted to 3.7 with H_3PO_4). The column was eluted with 100% eluent A for 3 min., followed by a linear gradient of 0 – 100% eluent B over 17 min. and then with 100% eluent B for 5 min. Nucleoside separation was performed at flow rate of 1 mL min.$^{-1}$, with 20 µL of each sample injected. Deoxynucleotides, dNTPs (Fermentas®, Vilnius, Lithuania), were digested for 2h with alkaline phosphatase (10 U mL^{-1}) and Tris−HCl (0.5 M, pH 8.3) to form the nucleoside standards (dA, dT, dC, 5mdC, and dG) (5 – 50 mM). The identification of each nucleoside was made by comparison with the external standards according to the peak area formed. 5mdC quantification (%) was calculated according to 5mdC concentration divided by 5mdC concentration plus dC concentration and multiplied by 100. The data were submitted to ANOVA and presented as the means of three biological replicates.

Histological analysis

Samples of seeds germinated were fixed in 2.5% paraformaldehyde in 0.2 M (pH 7.2) sodium phosphate buffer overnight. The samples were then dehydrated in increasing series of ethanol aqueous solutions. After dehydration, the samples were filtrated with Historesin (Leica® Historesin, Heidelberg, Germany). Sections (5 µm) were obtained using a manual rotation microtome (Slee Technik®) and were stained with Periodic Acid-Schiff (PAS) to identify neutral polysaccharides and Coomassie Brilliant Blue (CBB) 0.4% in Clarke's solution to identify proteins. Sections were analyzed with a camera (Olympus® DP71) attached to a microscope (Olympus® BX-40).

Results and discussion

Vriesea reitzii bromeliad seeds were germinated on the 3rd day of *in vitro* culture. Subsequently, seeds on MS culture medium resulted in the development of normal seedlings (Figure 1a and c), while seeds inoculated on MS-NAA-supplemented culture medium showed enhanced cell division and proliferation of cells with meristematic characteristics (Figure 1d and f). In this context, NAA in the culture medium altered the seedling development for NCs induction. Other studies have reported the effect of auxins combined with cytokinins, which also caused NCs induction from *Vriesea* seeds but at lower percentages (Dal Vesco et al., 2014a; Corredor-Prado et al., 2015). In morphogenetic routes, such as somatic embryogenesis, several steps including dedifferentiation and somatic cells reprogramming, are necessary for the initiation and

progression of development programs (Zhang & Ogas, 2009). In this respect, auxins are important signaling molecules related to division, elongation and differentiation regulation in plants (Perrot-Rechenmann, 2010). In the present study, carbohydrate, starch, protein, and GDM levels showed significant differences between *V. reitzii* seeds on MS and those cultured on MS-NAA medium.
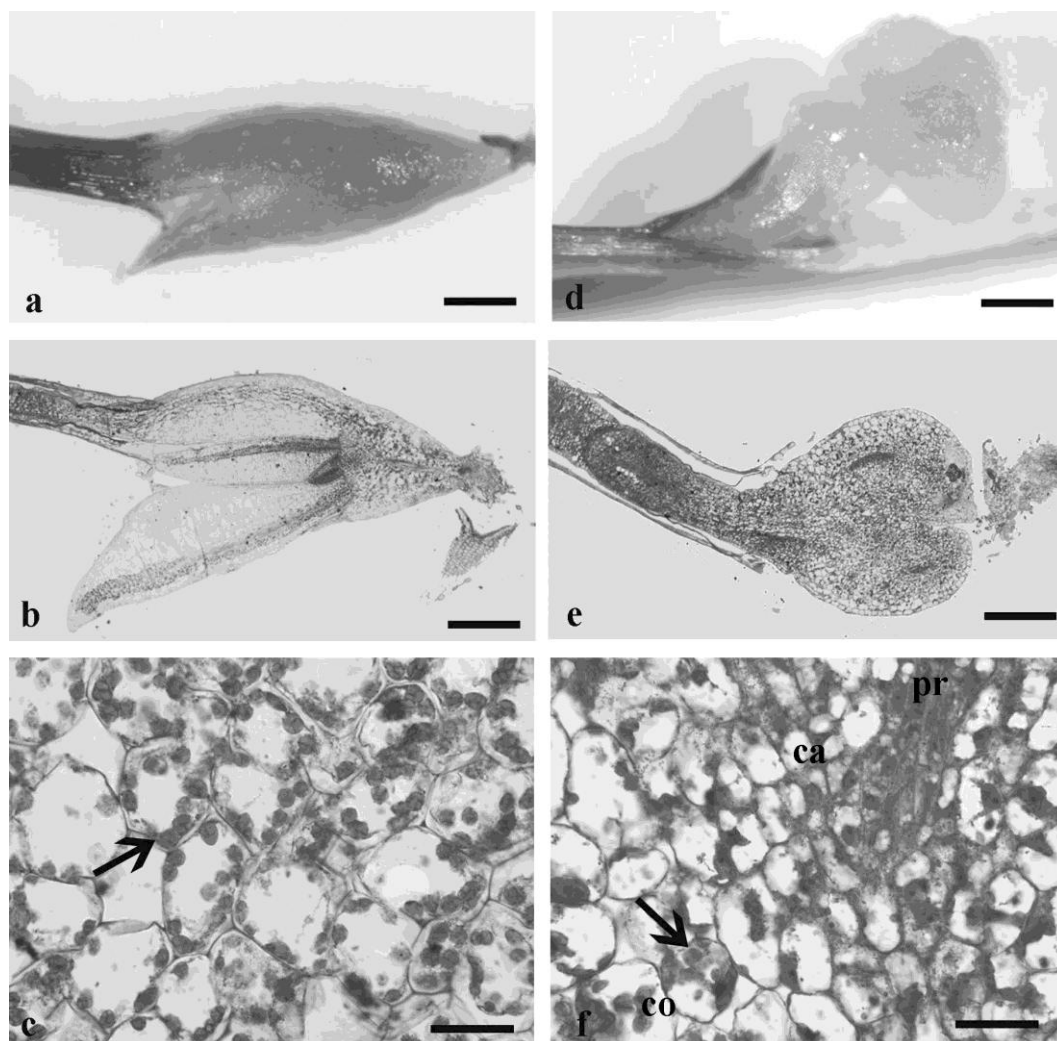
Figure 1. *Vriesea reitzii* seeds after 21 days of *in vitro* culture. Seeds on MS medium (a - c) and NCs induction medium supplemented with NAA 4 µM (d - f). Section stained with PAS-CBB (b, c, e, and f). Starch grains (arrow); cambial tissue (ca); cortex (co) provascular tissue (pr). Bars a, b, d, and e 500 µm; c and f 250 µm.

Protein and starch content

A gradual decrease in protein and starch levels was observed until the last measurement time (Figure 2a and b). Previous studies have shown that *Vriesea* genus seeds store starch and protein in the endosperm (Magalhaes & Mariath, 2012; Corredor-Prado et al., 2014). According to Bewley (1997), reserves stored in seeds are predominantly mobilized during and after radicle protrusion. Hydrolyzed amino acids release large ammonium amounts during protein degradation, which are assimilated again, synthesizing new nitrogenous molecules (Cantón, Suárez, & Cánovas, 2005), while starch degradation is related to triglyceride release and subsequent carbohydrate metabolism (Stone & Gifford, 1999).

In the present work, dry seeds (0 days of culture) with the highest protein and starch content (35.4 and 17.8 mg g^{-1} FM, respectively) showed a marked decrease on the 3rd day of culture, when seeds germinated. In barley seeds, Sreenivasulu et al. (2008) demonstrated that genes involved in reserve catabolism are expressed much earlier during seed germination, i.e., already 24 hours after imbibition. According to the authors, reserve mobilization activation is one of the first events that occur before radicle protrusion.

Reduction in the protein and starch content continued until the last days were evaluated. However, cultures on MS-NAA medium that developed NCs had higher protein (9.1 mg g^{-1} FM) and lower starch (6.0 mg g^{-1} FM) levels than seedlings after 21 days in culture (Figure 2a and b). Proteomic studies carried out in

leaves and seeds explants in the induction phase of NCs in the bromeliad *V. reitzii* showed an increase in protein content and number of spots detected in two-dimensional electrophoresis. Proteins identified were related to the regulation of effects of stress conditions in the culture medium, the adjustment of metabolism, and the increase in cell division (Corredor-Prado et al., 2016). Silveira, Floh, Handro, and Guerra (2004) also related protein level increases with high mitotic activity during exponential growth stages in *in vitro* cultures of *Pinus taeda*.

In histological sections with double staining (PAS-CBB), starch granules were observed in lower amount in NCs-inducing seeds (Figure 1f) than in seedlings (Figure 1c). This event may be related to increased metabolism of sugars, during the induction of NCs, generating a higher consumption of starch. Figure 1f, shows cells with starch, close to others with high mitotic activity, forming a cambial and provascular tissue. Thus, cortex with starch and cambial tissue surrounding the bundle would be the beginning of the NCs. Early starch presence in plant cells has been reported during *in vitro* development, acting as an energy source or as an osmotic agent that is essential for development (Martin et al., 2000). It has been suggested that starch accumulation might be a prerequisite of morphogenesis. For instance, in tobacco, starch acts as a direct cellular reserve of the energy and is rapidly converted to hexoses for the development of meristemoids (Thorpe et al., 2008).
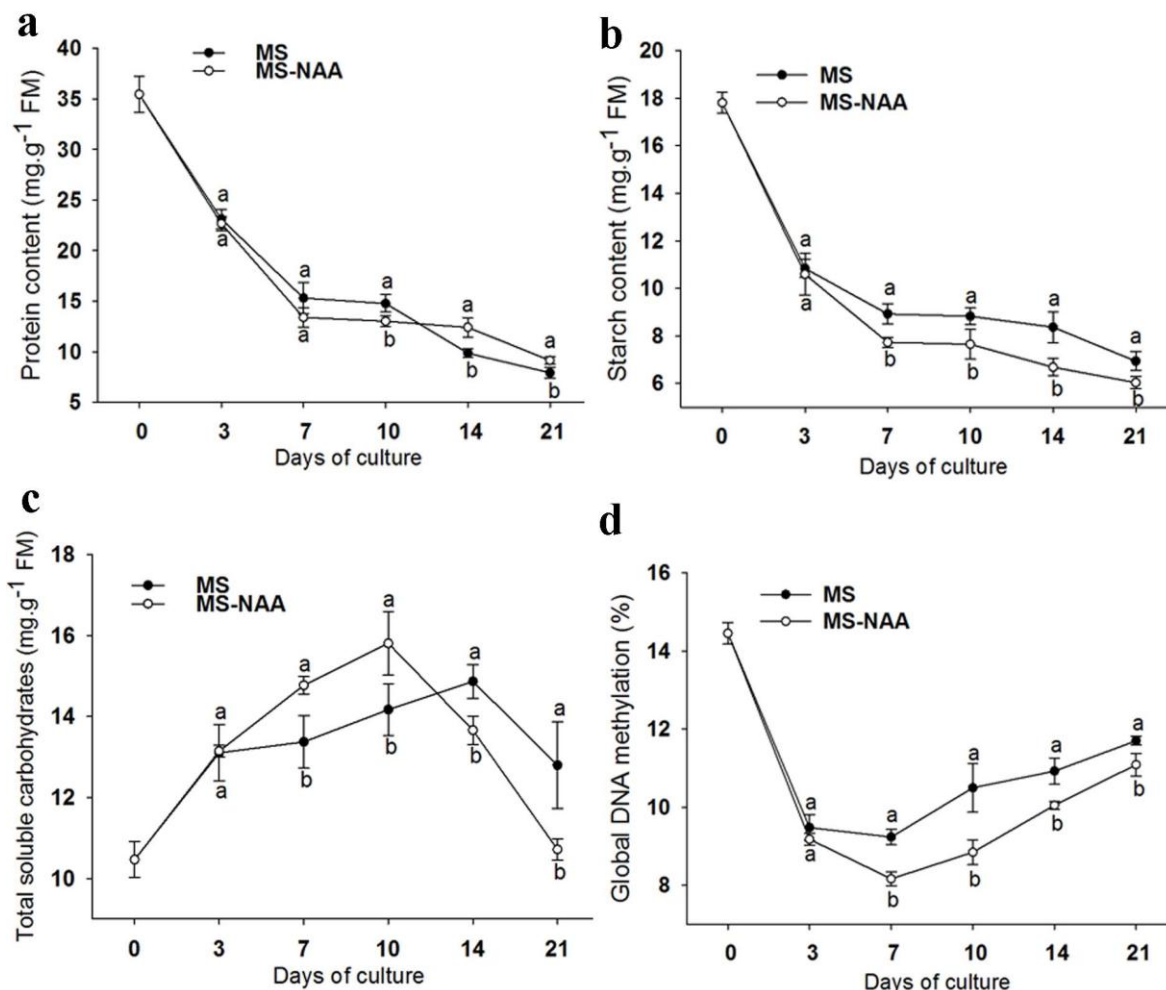

Figure 2. Content of proteins (a), starch (b), carbohydrates (c), and global DNA methylation (d) in *Vriesea reitzii* seeds cultivated on NCs induction medium (MS-NAA) and PGR-free MS medium (MS). Data are means ± standard errors.

Total soluble carbohydrates

Quantification of the carbohydrates (mono- and disaccharides), reveled the lowest value (10.4 mg g^{-1} FM) for dry seed (0 days of culture). Carbohydrates reached the highest level on the 10th day of culture in seeds inducing NCs (15.8 mg g^{-1} FM) (Figure 2c). Through qualitative thin layer chromatography, glucose monosaccharide (Rf: 0.58) and sucrose disaccharide (Rf: 0.50) were identified in seeds on MS and MS-NAA

medium (Figure 3, Table 1). Fructose, xylose, and maltose were not identified using this technique. However, at later stages of rice seed germination, Howell et al. (2009) detected several carbohydrate types, with increases primarily in fructose, glucose, and maltose. Before radicle protrusion in germination of barley seeds, activation of transcription factors for mobilization of starch reserves supplies sucrose and hexoses, which provide energy for development (Sreenivasulu et al., 2008).

Fru   Glc   Xyl   Mal   Suc   MS   MS-NAA
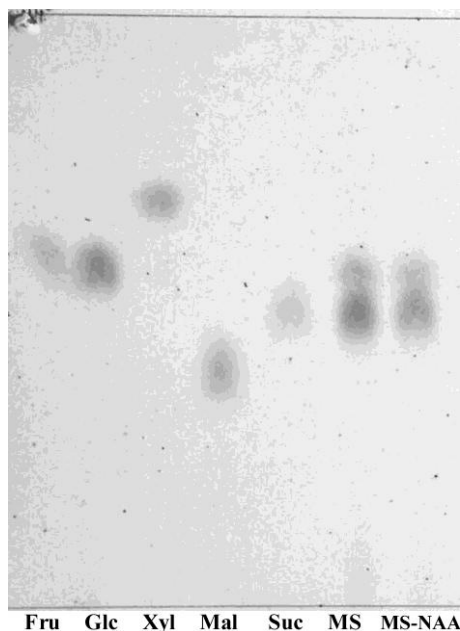
Figure 3. Thin Layer Chromatogram of *Vriesea reitzii* seeds cultivated 21 days in PGR-free medium (MS) and in NCs induction medium (MS-NAA) against standard (fructose-Fru, glucose-Glc, xylose-Xyl, maltose-Mal, and sucrose-Suc).

Table 1. Retention factor (Rf) of detected spots in thin layer chromatography (TLC) from *Vriesea reitzii* seeds. Standards used: fructose (Fru), glucose (Glc), xylose (Xyl), maltose (Mal), and sucrose (Suc). PGR-free medium (MS) and in NCs induction medium (MS-NAA).

| Days of culture | Retention factor (Rf) of detected spots | | | |
| | MS | | MS-NAA | |
	Glc (0.58)	Suc (0.50)	Glc (0.58)	Suc (0.50)
0	0.57	-	0.57	-
3	0.57	0.49	0.58	0.51
7	0.57	0.50	0.57	0.50
10	0.58	0.49	0.58	0.50
14	0.57	0.50	0.58	0.51
21	0.57	0.49	0.57	0.51

In addition to having the role of substrates for cell component synthesis, sugars also act as signaling molecules in the regulation of expression of various genes (Marsch-Martínez & Pereira, 2010). There is a strong hexose sugar increase in seeds after imbibition, followed by large changes in gene transcription (Fait et al., 2006; Howell et al., 2009). From this, a significant difference in carbohydrate content on the 10th day of culture on MS-NAA and MS medium, could be related to gene expression programming variation for NCs induction or for seedling development. According to Weber, Borisjuk, and Wobus (1997), sugars may interfere with cell cycle regulation by changing morphogenetic responses.

On the last day assessed (21 days of culture), carbohydrate content decreased in *V. reitzii* seeds. Significant differences between seeds on MS and MS-NAA medium were found (Figure 2c). Most likely, the presence of structures with different differentiation levels cause different energy levels necessary that support their metabolism. Lower values found in seeds that induced NCs (Figure 2c) indicate that this morphogenetic route has high energy demand. These results suggest that glycolytic intermediates formed by starch degradation are mainly catabolized to provide the necessary ATP for metabolism during cell proliferation that leads to NCs induction. On the other hand, higher carbohydrate content in seeds on MS medium allowed higher storage in starch (Figures 1c and 2b). In *Aechmea blanchetiana* bromeliad seedlings

cultured *in vitro* with different auxin concentrations, Chu et al. (2010) found that the highest starch and soluble carbohydrate content in plants were in the auxin-free medium. These results support the relation of auxin effect in carbohydrate metabolism.

Global DNA Methylation (GDM)

GDM levels decreased during germination until the 7th day of culture, reaching 9.2% in seeds on MS medium and 8.1% in seeds on MS-NAA medium (Figure 2d). Our results corroborate with those previously reported, in which methylation levels were higher in dry seeds than germinating seeds in *Capsicum annuum* L. (Portis, Acquadro, Comino, & Lanteri, 2004), *Brassica napus* (Lu et al., 2006), *Triticum aestivum* L. (Meng et al., 2012) and *Phelipanche ramosa* L. (Lechat et al., 2015). DNA hypomethylation during seed germination seems to be a necessary step for transcriptional activation of gene expression, contributing to the development of gene regulation (Lu et al., 2006). During wheat seed germination, Meng et al. (2012) found that the number of demethylation events was three times higher than methylation events, indicating demethylation predominance. According to Zluvova, Janousek, and Vyskot (2001), seed global hypomethylation during *Silene latifolia* germination and postgermination periods reflects transition from the metabolically quiescent seed to the actively growing and developing seedling.

On the last days evaluated (10, 14, and 21 days of culture), an increase in methylation levels was observed, reaching up to 11.7% in seeds that formed seedlings and 11.0% in seeds that induced NCs (Figure 2d). *Quercus suber* L. somatic embryo germination coincides with a significant 5mdC content decrease. However, when somatic embryos developed in seedlings with roots and initial leaves, a strong DNA methylation increase was observed (15%) (Pérez, Viejo, LaCuesta, Toorop, & Canal, 2015). Generally, differentiation coincides with increased DNA methylation, which is associated with normal ontogenic development (for review see Valledor et al., 2007).

Results on the last days also show significant GDM differences between seeds on MS and MS-NAA medium (Figure 2d). Methylation levels in seeds on MS medium could be associated with the differentiation process during normal seedling development. Therefore, the significantly lower level found in seeds that induced NCs is associated with tissue dedifferentiation. It has been reported during *in vitro* morphogenetic route induction, significant GDM decreases related to cell dedifferentiation and undifferentiated state cell maintenance (Fraga et al., 2016). According to Valledor et al. (2007), demethylation normally precedes the onset of new differentiation programs. However, even if GDM remains lower in seeds inducing NCs (associated with cellular dedifferentiation), the percentage keeps rising on the last days evaluated, which may be related to three factors, as follows: 1) some cells could be continuing the differentiation process, causing GDM level increases; 2) high cell proliferation, which is associated with high methylation levels (Wang et al., 2012); and 3) new meristematic zone formation in explants, which is in accordance with Scherer et al. (2015), who observed that high methylation levels were associated with new apical meristems formation in NCs of pineapple. Neelakandan and Wang (2012) conducted studies involving *Arabidopsis thaliana* epigenetic mutant characterization and observed that WUSCHEL (WUS) gene expression and signaling derived from auxins, which are crucial for meristem formation, are regulated, in part, by DNA methylation.

It is possible to relate methylation status with specific *in vitro* morphogenetic competence (Noceda et al., 2009). Accordingly, during somatic embryogenesis, embryogenic cells generally display lower GDM levels than nonembryogenic cells (Miguel & Marum, 2011). Similarly, lower GDM levels have been correlated with organogenic capability increases (Valledor, Meijón, Hasbún, Cañal, & Rodrigues, 2010). The results of this study suggest that low GDM levels are related to explant capacity for NCs induction. This indicates that NAA supplemented in induction medium results in alterations of gene expression. According to Zhang, Yuan, and Zhang (2012), there is a functional interplay between environmentally induced epigenetic modifications, response to PGRs, and phenotypic plasticity. Auxins used in PGR-supplemented treatment, have been frequently linked to compromise genomic stability through the promotion of DNA methylation deregulation coupled with gene expression modifications in embryogenic cultures of *Daucus carota* (LoSchiavo et al., 1989) and *Araucaria angustifolia* (Fraga et al., 2016).

Conclusion

The findings of this study suggest that NCs induction from *V. reitzii* seeds using the culture medium supplemented with NAA results in explant gene reprogramming, which leads to morphological, biochemical and metabolic changes. NCs induction is a complex process in which epigenetic control (related to GDM levels decrease) seems to play an important role in the initiation of the morphogenic response.

Concomitantly, dedifferentiation followed by cell proliferation, high energy demand given by starch degradation present in the tissues, and higher protein content is related to elevated metabolic activity.

However, more detailed studies should be conducted to determine specific relations between the different signaling networks that regulate changes associated with the acquisition of skills to develop this morphogenic route.

Acknowledgements

References

Alves, G., Dal Vesco, L. L., & Guerra, M. P. (2006). Micropropagation of the Brazilian endemic bromeliad *Vriesea reitzii* trough nodule clusters culture. *Scientia Horticulturae*, *110*(2), 204-207. DOI: 10.1016/j.scienta.2006.06.014

Benzing, D. H. (2000). *Bromeliaceae: Profile of an adaptive radiation*. New York, US: Cambridge University Press.

Bewley, J. (1997). Seed germination and dormancy. *Plant Cell*, *9*(7), 1055-1066. DOI: 10.1105/tpc.9.7.1055

Cangahuala-Inocente, G. C., Silveira, V., Caprestano, C. A., Floh, E. I. S., & Guerra, M. P. (2014). Dynamics of physiological and biochemical changes during somatic embryogenesis of *Acca sellowiana*. *In Vitro Cellular & Developmental Biology - Plant*, *50*(2), 166-175. DOI: 10.1007/s11627-013-9563-3

Cantón, F. R., Suárez, M. F., & Cánovas, F. M (2005). Molecular aspects of nitrogen mobilization and recycling in trees. *Photosynthesis Research*, *83*(2), 265-278. DOI: 10.1007/s11120-004-9366-9

Carpentier, S. C., Witters, E., Laukens, K., Deckers, P., Swennen, R., & Panis, B. (2005). Preparation of protein extracts from recalcitrant plant tissues: An evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics*, *5*(10), 2497-2507. DOI: 10.1002/pmic.200401222

Chu, E. P., Tavares, A. R., Kanashiro, S., Giampaoli, P., & Yokota, E. S. (2010). Effects of auxins on soluble carbohydrates, starch and soluble protein content in *Aechmea blanchetiana* (Bromeliaceae) cultured *in vitro*. *Scientia Horticulturae*, *125*(3), 451-455. DOI: 10.1016/j.scienta.2010.04.021

Corredor-Prado J. P., De Conti, D., Cangahuala-Inocente G., Guerra, M. P., Dal Vesco, L. L., & Pescador, R. (2016). Proteomic analysis in the induction of nodular cluster cultures in the bromeliad *Vriesea reitzii* Leme and Costa. *Acta Physiologiae Plantarum*, *38*(5), 1-10. DOI: 10.1007/s11738-016-2140-8

Corredor-Prado J. P., Schmidt, E. C., Steinmacher, D., Guerra, M. P., Bouzon, Z. L., Dal Vesco, L. L., & Pescador, R. (2014). Seed morphology of *Vriesea friburgensis* var. *paludosa* L.B. Sm. (Bromeliaceae). *Hoehnea*, *41*(4), 553-562. DOI: 10.1590/2236-8906-08/2013

Corredor-Prado J. P., Schmidt E. C., Guerra M. P., Bouzon, Z. L., Dal Vesco, L. L., & Pescador, R. (2015). Histodifferentiation and ultrastructure of nodular cultures from seeds of *Vriesea friburgensis* Mez var. *paludosa* (L.B. Smith) L.B. Smith and leaf explants of *Vriesea reitzii* Leme & A. Costa (Bromeliaceae). *Journal of Microscopy and Ultrastructure*, *3*(4), 200-209. DOI: 10.1016/j.jmau.2015.04.001

Dal Vesco L. L., & Guerra, M. P. (2010). *In vitro* morphogenesis and adventitious shoot mass regeneration of *Vriesea reitzii* from nodular cultures. *Scientia Horticulturae*, *125*(4), 748–755. DOI: 10.1016/j.scienta.2010.05.030

Dal Vesco, L. L., Pescador, R., Corredor Prado, J. P., Welter, L. J., & Guerra, M. P. (2014a). *In vitro* propagation of *Vriesea reitzii*, a native epiphyte bromeliad from the Atlantic rainforest. *Acta Scientiarum. Biological Sciences*, *36*(3), 271-278. DOI: 10.4025/actascibiolsci.v36i3.21006

Dal Vesco, L. L., Vieira, P. M., Corredor Prado, J. P., Pescador, R., Guerra, M. P., & Elter, L. J. (2014b). Induction and development of nodular cluster cultures in *Vriesea reitzii* (Leme and Costa), an endangered bromeliad from the Brazilian Atlantic Forest. *Journal of Horticultural Science and Biotechnology*, *89*(5), 542-548. DOI: 10.1080/14620316.2014.11513118

Dal Vesco, L. L., Stefenon, V. M., Welter, L. J., Guerra, M. P., & Scherer, R. F. (2011). Induction and scale-up of *Billbergia zebrina* nodule cluster cultures: Implications for mass propagation, improvement and conservation. *Scientia Horticulturae*, *128*(4), 515-522. DOI: 10.1016/j.scienta.2011.02.018

Dal Vesco, L. L., Stefenon, V. M., Welter, L. J., Steiner, N. & Guerra, M. P. (2012). Conservation of *Billbergia zebrina* genetic resources: AFLP polymorphism of *in vitro* regenerated genotypes. *Plant Genetic Resources*, *10*(1), 20-23. DOI: 10.1017/S1479262111000918

Doyle, J., & Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. *Focus*, *12*(1), 13-15.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugar and related substances. *Analytical Chemistry*, *28*(3), 350-256.

Fait, A., Angelovici, R., Less, H., Ohad, I., Urbanczyk-Wochniak, E., Fernie, A. R., & Galili, G. (2006). Arabidopsis seed development and germination is associated with temporally distinct metabolic switches. *Plant Physiology*, *142*(3), 839-854. DOI: 10.1104/pp.106.086694

Finnegan, E. F. (2010). DNA methylation: A dynamic regulator of genome organization and gene expression in plants. In E. C. Pua, & M. Davey (Eds.), *Plant developmental biology – Biotechnological* perspectives (p. 295-323). Berlin, GE: Springer.

Fraga, H. P. F., Vieira, L. N., Caprestano, C. A., Steinmacher, D. A., Micke, G. A., Spudeit, D.,A., Pescador, R., & Guerra, M. P. (2012). 5-Azacytidine combined with 2,4-D improves somatic embryogenesis of *Acca sellowiana* (O. Berg) Burret by means of changes in global DNA methylation levels. *Plant Cell Reports*, *31*(12), 2165-2176. DOI: 10.1007/s00299-012-1327-8

Fraga, H. P. F., Vieira, L. N., Heringer, A. S., Puttkammer, C. C., Silveira, V., & Guerra, M. P. (2016). DNA methylation and proteome profiles of *Araucaria angustifolia* (Bertol.) Kuntze embryogenic cultures as affected by plant growth regulators supplementation. *Plant Cell, Tissue and Organ Culture*, *125*(2), 353-374. DOI: 10.1007/s11240-016-0956-y

Guerra, M. P., & Dal Vesco, L. L. (2010). Protocols for *in vitro* propagation of ornamental plants. *Business*, *589*(1), 47-66. DOI: 10.1007/978-1-60327-114-1

Halford, N. G. (2010). Photosynthate Partitioning. (v. 2). In E. C. Pua, & M. R.Davey (Eds.), *Plant developmental biology - Biotechnological perspectives* (p. 67–82). Berlin,GE: Springer.

Howell, K. A., Narsai, R., Carroll, A., Ivanova, A., Lohse, M., Usadel, B., Harvey Millar, A., & Whelan, J. (2009). Mapping metabolic and transcript temporal switches during germination in rice highlights specific transcription factors and the role of RNA instability in the germination process. *Plant Physiology*, *149*(2), 961-980. DOI: 10.1104/pp.108.129874

Lechat, M. M., Brun, G., Montiel, G., Véronési, C., Simier, P., Thoiron, S., Pouvreau, J. B., & Delavault, P. (2015). Seed response to strigolactone is controlled by abscisic acid-independent DNA methylation in the obligate root parasitic plant, *Phelipanche ramosa* L. Pomel. *Journal of Experimental Botany*, *66*(11), 3129-3140. DOI: 10.1093/jxb/erv119

Leme, E M. C., & Costa, A. (1991) A new species from southern Brazil: a tribute to Father Raulino. *Journal of The Bromeliad Society*, *41*(5), 195-198.

LoSchiavo F, Pitto L, Giuliano G., Torti, G., Nuti-Ronchi, V., Marazziti, D., Vergara, R., ..., Terzi, M. (1989). DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. *Theoretical and Applied Genetics*, *77*(3), 325-331. DOI: 10.1007/BF00305823

Lu, G., Wu, X., Chen, B., Gao, G., Xu, K., & Li, X. (2006). Detection of DNA methylation changes during seed germination in rapeseed (*Brassica napus*). *Chinese Science Bulletin*, *51*(2), 182-190. DOI: 10.1007/s11434-005-1191-9

Magalhães, R. I., & Mariath, J. E. A. (2012). Seed morphoanatomy and its systematic relevance to Tillandsioideae (Bromeliaceae). *Plant Systematics and Evolution*, *298*(10), 1881-1895. DOI: 10.1007/s00606-012-0688-3

Marsch-Martínez, N., & Pereira, A. (2010). Activation tagging for gain-of-function mutants (v. 2). In E. C Pua, & Davey M. (Eds.), *Plant developmental biology - Biotechnological perspectives* (p. 345-370). Berlin, GE: Springer.

Martin, A. B., Cuadrado, Y., Guerra, H., Galeggo, P., Hita, O., Martin, L., Dourado, A., & Villalobos, N. (2000). Differences in the contents of total sugars, reducing sugars, starch and sucrose in embryogenic

and non-embryogenic calli from *Medicago arborea* L. *Plant Science*, *154*(2), 143-151. DOI: 10.1016/S0168-9452(99)00251-4

McCready, R. M., Guggolz, J., Siliviera, V., & Owens, H. S. (1950). Determination of starch and amylose in vegetables. *Analytical Chemistry*, *22*(9), 1156-1158. DOI: 10.1021/ac60045a016

Meng, F. R., Li, Y. C., Yin, J., Liu, H., Chen, X. J., Ni, Z. F., & Sun, Q. X. (2012). Analysis of DNA methylation during the germination of wheat seeds. *Biologia Plantarum*, *56*(2), 269-275. DOI: 10.1007/s10535-012-0086-2

Miguel, C., & Marum, L. (2011). An epigenetic view of plant cells cultured *in vitro*: Somaclonal variation and beyond. *Journal of Experimental Botany*, *62*(11), 3713-3725. DOI: 10.1093/jxb/err155

Morel, A., Trontin, J. F., Corbineau, F., Lomenech, A. M., Beaufour, M., Reymond, I., Le Metté, C., ... Lelu-Walter, M. A. (2014). Cotyledonary somatic embryos of *Pinus pinaster* Ait. most closely resemble fresh, maturing cotyledonary zygotic embryos: biological, carbohydrate and proteomic analyses. *Planta*, *240*(5), 1075-1095. DOI: 10.1007/s00425-014-2125-z

Msogoya, T. J., Grout, B. W., & Roberts, A. (2011). Reduction in genome size and DNA methylation alters plant and fruit development in tissue culture induced off-type banana (*Musa* spp.). *Journal of Animal and Plant Sciences*, *11*(3), 1450-1456.

Neelakandan, A. K., & Wang, K. (2012). Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. *Plant Cell Report*, *31*(4), 597-620. DOI: 10.1007/s00299-011-1202-z

Negrelle, R. R. B., Mitchell, D., & Anacleto, A. (2012). Bromeliad ornamental species: conservation issues and challenges related to commercialization. *Acta Scientiarum. Biological Sciences*, *34*(1), 91-100. DOI: 10.4025/actascibiolsci.v34i1.7314

Noceda, C., Salaj, T., Pérez, M., Canal, M. J., Rodrigues, R., Viejo, M., & Salaj, J. (2009). DNA demethylation and decrease on free polyamines is associated with the embryogenic capacity of *Pinus nigra* Arn. cell culture. *Trees*, *23*(6), 1285-1293. DOI: 10.1007/s00468-009-0370-8

Pérez, M., Viejo, M., LaCuesta, M., Toorop, P., & Canal, M. J., (2015). Epigenetic and hormonal profile during maturation of *Quercus Suber* L. somatic embryos. *Journal of Plant Physiology*, *173*(1), 51-61. DOI: 10.1016/j.jplph.2014.07.028

Perrot-Rechenmann, C. (2010). Cellular responses to auxin: Division versus expansion. *Cold Spring Harbor Perspectives in Biology*, *2*(5), a001446. DOI: 10.1101/cshperspect.a001446

Portis, E., Acquadro, A., Comino, C., & Lanteri, S. (2004). Analysis of DNA methylation during germination of pepper (*Capsicum annuum* L.) seeds using methylation-sensitive amplification polymorphism (MSAP). *Plant Science*, *166*(1), 169-178. DOI: 10.1016/j.plantsci.2003.09.004

Scherer, R. F., Fraga, H. P. F., Klabunde, G, F., Silva, D. A., & Guerra, M. P. (2015). Global DNA methylation levels during the development of nodule cluster cultures and assessment of genetic fidelity of *in vitro*-regenerated pineapple plants (*Ananas comosus* var. *comosus*). *Journal of Plant Growth Regulation*, *34*(3), 677-683. DOI: 10.1007/s00344-015-9493-x

Scherer, R. F., Garcia, A. C., Fraga, H. P. D. F., Dal Vesco, L. L., Steinmacher, & Guerra, M. P. (2013). Nodule cluster cultures and temporary immersion bioreactors as a high performance micropropagation strategy in pineapple (*Ananas comosus* var. *comosus*). *Scientia Horticulturae*, *151*(1), 38-45. DOI: 10.1016/j.scienta.2012.11.027

Shan, X., Wang, X., Yang, G., Wu, Y., Su, S., Li, S., Liu, H., & Yuan, Y. (2013). Analysis of the DNA methylation of maize (*Zea mays* L.) in response to cold stress based on methylation-sensitive amplified polymorphisms. *Journal of Plant Biology*, *56*(1), 32-38. DOI: 10.1007/s12374-012-0251-3

Silveira, V., Floh, S., Handro, W., & Guerra, M. P. (2004). Effect of plant growth regulators on the cellular growth and levels of intracellular protein, starch and polyamines in suspension cultures of *Pinus taeda*. *Plant Cell, Tissue and Organ Culture*, *76*(1), 53-60.

Sreenivasulu, N., Usadel, B., Winter, A., Radchuk, V., Scholz, U., Stein, N., Weschke, W., ... Wobus, U. (2008). Barley grain maturation and germination: Metabolic pathway and regulatory network commonalities and differences highlighted by new MapMan/PageMan profiling tools. *Plant Physiology*, *146*(4), 1738-1758. DOI: 10.1104/pp.107.111781

Stone, S. L., & Gifford, D. J. (1999). Structural and biochemical changes in loblolly pine (*Pinus taeda* L.) seeds during germination and early-seedling growth. II. Storage triacylglycerols and carbohydrates. *International Journal of Plant Science*s, *160*(4), 663-671.

Thorpe, T., Stasolla, C., Yeung, E. C., Klerk, G. J.; Roberts, A. & George, E. F. (2008). The components of plant tissue culture media. II: Organic additions, osmotic and pH effects, and support systems. (3rd ed.). In E. F. George, M. A. Hall, & G. J. De Klerk (Eds.), *Plant propagation by tissue culture* (p. 115–174). Dordrecht, GE: Springer.

Valledor L, Hasbún R, Meijón M, Rodrigues, J. L., Santamaría, E., Viejo, M., Berdasco, M., ... Rodrigues, R. (2007). Involvement of DNA methylation in tree development and micropropagation. *Plant Cell, Tissue and Organ Culture*, *91*(2), 75-86. DOI: 10.1007/s11240-007-9262-z

Valledor, L., Meijón, M., Hasbún, R., Cañal, M. J., & Rodrigues R. (2010). Variations in DNA methylation, acetylated histone H4, and methylated histone H3 during *Pinus radiata* needle maturation in relation to the loss of *in vitro* organogenic capability. *Journal of Plant Physiology*, *167*(5), 351-357. DOI: 10.1016/j.jplph.2009.09.018

Wang. Q. M., Wang, Y. Z., Sun, L. L., Gao, F. Z., Sun, W., He, J., Gao, X., & Wang, L. (2012). Direct and indirect organogenesis of *Clivia miniata* and assessment of DNA methylation changes in various regenerated plantlets. *Plant Cell Reports*, *31*(7), 1283-1296. DOI: 10.1007/s00299-012-1248-6

Weber, H., Borisjuk, L., & Wobus, U. (1997). Sugar import and metabolism during seed development. *Trends in Plant Science*, *2*(5), 169-174. DOI: 10.1016/S1360-1385(97)01030-3

Zhang, H., & Ogas, J. (2009). An epigenetic perspective on developmental regulation of seed genes. *Molecular Plant*, *2*(4), 610-627. DOI: 10.1093/mp/ssp027

Zhang, C. C., Yuan, W. Y., & Zhang, Q. F (2012). RPL1, a gene involved in epigenetic processes regulates phenotypic plasticity in rice. *Molecular Plant*, *5*(2), 482-493. DOI: 10.1093/mp/ssr091

Zluvova, J., Janousek, B., & Vyskot, B. (2001). Immunohistochemical study of DNA methylation dynamics during plant development. *Journal of Experimental Botany*, *52*(365), 2265-2273.