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Determination of endogenous IAA and carbohydrates during the induction and development of protocorm-like bodies of *Cattleya tigrina* A. Richard

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ABSTRACT. Tissue culture techniques have been employed for orchid mass propagation by means of the morphogenetic route of protocorm like-bodies (PLBs). This study aimed to analyze and compare Indole-acetic acid (IAA) and sugar endogenous levels in protocorm-like bodies (PLBs) induction and development in *Cattleya tigrina*, in order to better understand this process and to optimize micropropagation procedure protocols. Leaves grown on MS (Murashige and Skoog) culture medium, suplemented with 9 µM TDZ for PLBs induction and development were collected after 0, 2, 7, 14, 30, 60 and 100 days of cultivation, for further analysis. Increase of IAA and reduction of sugar levels are strongly related to morphogenetic response, that is, PLBs formation over the preexisting ones and leaf primordia formation. Sucrose, fructose and glucose presence in this study is related to cell signaling. Thus, hormonal signals and carbohydrates alter metabolism, triggering PLBs initiation and development in *C. tigrina*.

Keywords: orchid; hormones; sugars; morphogenesis.

Determinação de AIA e carboidratos endógenos durante a indução e desenvolvimento de estruturas semelhantes a protocormos de *Cattleya tigrina* A. Richard

RESUMO. Técnicas de cultura de tecidos têm sido empregadas para a propagação em massa de orquídeas por meio da via morfogenética de estruturas semelhantes a protocormos (ESPs). O estudo teve como objetivo analisar e comparar os níveis endógenos de ácido indol-3-acético (AIA) e de açúcares na indução e no desenvolvimento de estruturas semelhantes a protocormos (ESPs) em *Cattleya tigrina*, visando à melhor compreensão deste processo e à otimização de protocolos de micropropagação. Folhas cultivadas em meio de cultura MS (Murashige & Skoog), suplementadas com 9 µM de TDZ para indução e desenvolvimento de ESPs foram coletadas após 0, 2, 7, 14, 30, 60 e 100 dias de cultivo, para posterior análise. O aumento dos níveis de AIA e a redução dos níveis de açúcar estão fortemente relacionados à resposta morfogenética, ou seja, à formação de ESPs sobre os pré-existentes e à formação de primórdios foliares. A presença de sacarose, frutose e glicose neste estudo está relacionada à sinalização celular. Assim, sinais hormonais e carboidratos alteram o metabolismo, desencadeando a iniciação e o desenvolvimento de ESPs em *C. tigrina*.

Palavras-chave: orquídeas; hormônios; açúcares; morfogênese.

Introduction

The family Orchidaceae, with about 20,000 species distributed in 850 genera, is one of the largest and most specialized in the plant kingdom, found on almost all continents, especially in tropical and subtropical regions (Hossain et al., 2013). Due to ornamental and landscape features, orchids are inordinately taken from their natural habitats, leading many species to extinction and significantly threatening biodiversity maintenance (Swarts & Dixon, 2009).

Plant tissue culture technique has been widely used with success in the propagation of different land and epiphyte orchid species (Díaz & Álvarez, 2009), allowing rapid multiplication. Plant micropropagation can be used in the preservation and propagation of endangered species and as a model to study metabolism, differentiation and cell morphogenesis, providing information on physiological and biochemical events leading to *in vitro* morphogenesis modulation.

During *in vitro* establishment, a morphogenic response pattern defined as protocorm-like bodies (PLBs) has been proposed for several orchid species, such as the species under study (Almeida, Fraga,

Navarro, Guerra, & Pescador, 2017). PLBs are globular structures named after their similarity with zygotic origin embryos (protocorms) (Arditti, 2008). These structures can be directly induced from meristematic shoots (Mondal, Aditya, & Banderjee, 2013), flower stalks (Chen & Chang, 2000), leaf segments (Khoddamzadeh et al., 2011; Deb & Pongener, 2013) or indirectly, with an intermediate callus stage (Jainol & Gansau, 2017).

Many authors state that PLBs are somatic embryos (Hossain et al., 2013; Lee, Hsu, & Yeung, 2013) or that somatic embryogenesis is the initial step on the PLBs formation (Zhao, Wu, Feng, & Wang, 2008).

With histological and histochemical studies, it was possible to verify that during the early stages of PLB formation, the cells presented cytological characteristics and cell wall markers similar for the development of the zygote embryo (Liau et al., 2003). Further studies on the development of PLBs in other orchid species are essential for advancing research in this area.

Besides morphological changes, PLBs formation comprises several biochemical and molecular changes, including hormonal changes and sugars. Both endogenous and exogenous plant hormones are closely related to *in vitro* cell differentiation (Silva, 2009; Sun & Hong, 2010). They are considered key factors in triggering plant morphogenesis, including somatic embryogenesis and organogenesis (Huang, Lee, & Chen, 2012)

In the last decade, several studies investigated the association between endogenous hormone levels and plant morphogenesis, with special focus on somatic embryogenesis routes (Steiner, Santa-Catarina, Silveira, Floh, & Guerra, 2007; Pescador et al., 2012; Leljak-Levani et al., 2016). In contrast, only few reports mention endogenous hormonal changes associated with other *in vitro* morphogenesis routes. Among growth regulators, stands out auxin, which has a strong influence on a wide variety of development responses (Sairanen et al., 2012). Indole-3-acetic acid (IAA), the primary auxin in plants, is associated with histodifferentiation embryogenic pattern regulation, and is an integral regulator in cell expansion, division and differentiation (Sairanen et al., 2012).

Carbohydrates are also involved in physiological processes related to growth and development in plants, acting as energy sources for cells and carbon sources for biosynthetic processes (Zhang, Fu, & Hu, 2012; Kubeš, Drážná, Konrádová, & Lipavská, 2014). They also act as osmotic agents, helping maintaining plasma membrane integrity, besides being important signaling molecules that modulate several processes in plant development (Kubeš et al., 2014; Lastdrager, Hanson, & Smeekens, 2014). The molecular networks driving cell division and expansion largely rely on the availability of carbohydrates to provide energy and biomass (Lastdrager et al., 2014). There are several mechanisms that coordinate hormonal processes, so that they are energetically compatible with the plant carbon state. These mechanisms may act modulating hormone synthesis, transport and signaling, so that hormonal responses that promote growth are inhibited under limited carbon conditions (Eveland & Jackson, 2012; Ljung, Nemhauser, & Perata, 2015).

Thus, the aim of this study was to analyze and compare IAA and sugar endogenous levels in *C. tigrina* PLBs induction and development, in order to better understand this process and to optimize micropropagation procedure protocols.

Material and methods

Plant material

Leaf explants (±1 cm) were obtained from young plants, micropropagated and maintained *in vitro* at the Physiology Laboratory of Plant Development and Genetics (LFDGV), Agricultural Science Center (CCA), *Universidade Federal de Santa Catarina* (UFSC), Florianópolis, Santa Catarina State, Brazil. The leaf explants were detached and inoculated in MS/2 medium (Murashige & Skoog, 1962), supplemented with 9 μM TDZ (Thidiazuron) for PLBs induction.

After inoculation of the explants, they were kept in growth room with an average temperature of 25 ± 2°C, 16 hours light, with a light intensity of 50 μmol m^{-2} s^{-1}. At zero (which corresponded to the beginning of the experiment, being the starting explant), 2nd, 7, 14, 30, 60 and 100th days of cultivation, plant materials were collected for histological, hormonal and sugar analysis. For histological studies, the five explants of each culture time were collected. For hormone analysis, about 50 mg lyophilized samples were processed in triplicate for each cultivation time. With regard to sugar analysis, three biological replicates were collected for each cultivation time, each replicate was formed by 500 mg fresh pasta starting from a pool of plant material, corresponding basically to 30 leaf explants and stored at -80°C for later analysis.

Histological analysis

The material was fixed in 2.5% paraformaldehyde in 0.2 M (pH 7.2) phosphate buffer overnight. Subsequently, samples were dehydrated in increasing series of ethanol aqueous solutions (Schmidt, Scariot, Rover, & Bouzon, 2009). After dehydration, samples were infiltrated with Historesin (Leica Historesin, Heidelberg, Germany). Sections (5 μm) were obtained using a manual rotary microtome (Slee Technik®) and were double-stained with Periodic Acid-Schiff (PAS) to identify neutral polysaccharides (Gahan, 1984) and 0.4% Coomassie Brilliant Blue (CBB) in Clarke's solution to identify proteins (Gahan, 1984). Sections were analyzed with a camera (Olympus® DP71) attached to a microscope (Olympus® BX-40).

IAA determination

IAA content was determined according to Ludwig-Müller, Georgiev, and Bley (2008) with modifications. The lyophilized samples were extracted with a mixture of 1 mL isopropanol and acetic acid (95:5 v v^{-1}). Exactly 0.5 µg [13C6]-IAA (Cambridge Isotopes, Inc.) was added to each sample as internal standard. The extracts were then incubated with continuous shaking for 40 min at 4°C. Samples were centrifuged for 10 min at 25,000 g at 4°C, the supernatant was removed and evaporated to the aqueous phase under SpeedVac until only 50 μL. The aqueous phase was then extracted with ethyl acetate and water, the organic fraction was removed and the sample was taken up in methanol. The sample was evaporated to the aqueous phase using SpeedVac as described above. Purified samples were evaporated, resuspended in 50 μL pyridine followed by a 60 min derivatization at 92°C using 50 μL N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide. The analysis was performed on a gas chromatograph coupled to a mass spectrometer (model GCMS-QP2010 SE, Shimadzu) in selective ion monitoring mode. The samples were injected in the splitless mode; linear velocity 33.9 cm s^{-1}. Column DB-5 MS (30 m, ID 0.25 mm, 0.25 μm thick internal film). Column flow: 0.83 mL min. The temperature program for metabolite analysis was: 2 min at 100°C, followed by ramp of 10°C min^{-1} to 140°C, 25°C min^{-1} to 160°C, 35°C min^{-1} to 250°C, 20°C min^{-1} to 270°C and 30°C min^{-1} to 300°C. The injector temperature was 250°C, and the following MS operating parameters were used: ion source temperature 230°C; and interface temperature, 260°C. Solvent cut 5 min – mode SIM (0.30 s). Ions with a mass ratio/charge (m z^{-1}) of 244, 202 and 130 (corresponding to endogenous IAA), 250, 208 and 136 (corresponding to [13C6]-IAA) were monitored. Endogenous IAA concentrations were calculated based on extracted chromatograms at m z^{-1} 244 and 250. Data were tested by ANOVA and presented as means of three biological replicates.

Total soluble carbohydrate content

Samples of 500 mg were ground to powder with the aid of liquid nitrogen and subsequently subjected to 80% ethanol extraction at 70°C for 5 min. The extracts were centrifuged at 3,000 rpm, 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 were determined using phenol-sulfuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), using glucose as standard. The absorbance was measured at 490 nm. Data were tested by ANOVA and presented as means of three biological replicates.

Thin-layer Chromatography (TLC)

Qualitative analysis of sugars was carried out on the extracts described previously by TLC on aluminum plates coated with silica G60 (Alugram®). Mobile phase consisted of 2-Propanol/Ethyl acetate/Nitroethane /Acetyl hydroxide/water (45:25:10:1:19). Detection of carbohydrates was performed by spraying solution of orcinol, sulfuric acid and ethanol followed by heating the plate. They were used as markers fructose, glucose, xylose, maltose, and sucrose.

Starch content

The pellets used in the total soluble carbohydrates extraction received the addition of 1 mL cold distilled water and 1.3 mL 52% perchloric acid and was maintained in ice bath with occasional agitation. Subsequently, 2.0 mL 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 Owens (1950). The absorbance was measured at 490 nm. Data were tested by ANOVA and presented as means of three biological replicates.

Results and discussion

Induction and development of PLBs

Inoculated leaf explants cultured on induction medium allowed PLBs proliferation. Through

anatomical analysis, it was observed that after zero and two days of cultivation, the basal region of the leaf segment was characterized by protrusion absence (Figure 1A-F). After PAS and CBB double staining, starch granules associated with the protein material (Figure1C and F), in both cultivation times, was observed. PLBs histodifferentiation began on the 7th day of cultivation (Figure 1G-I). However, on the 14th day of cultivation, PLBs formation was observed, in which elongated cells in the epidermis were observed (Figure 1J-L). Starch grains and protein material were present on the 7 and 14th days of cultivation, with gradual increase through the analyzed times (Figure 1I and L). Significant predominance of starch and protein material reserves was observed on the 30th day of cultivation, when, by histochemical analysis, the formation of large PLB amounts was verified, which resulted from new PLB formation over the preexisting ones (Figure 1M-O). Leaf primordia formation began on the 60th day of cultivation, and the complete primordia formation was concluded on the 100th day of cultivation (Figure 1Q and T). In the 60 and 100th days of cultivation, there was a gradual decrease in relation to neutral polysaccharides and increased protein material, mainly on the 100th day of cultivation (Figure 1R and U).

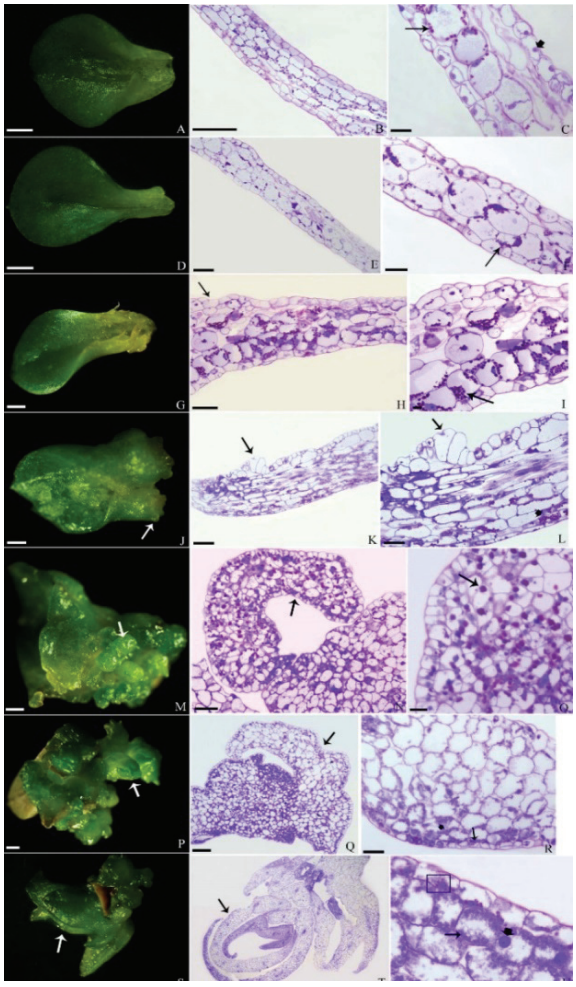

Figure 1. Histological (double-stained: PAS/CBB) aspects of protocorm-like bodies (PLBs) from leaf explants of *C. tigrina* A. Rich. (A) Overview of the adaxial surface of the initial leaf. (B) Cross-section of the initial explant. (C) Longitudinal section. Blue-stained prominent nuclei (broad arrow) and red-stained starch granules (arrow). (D) After two days of culture, overview of the adaxial surface of the leaf. (E) Cross-section of leaf induced after two days of culture. (F) Red-stained starch granules (arrow). (G) Overview of the adaxial surface of induced leaf after seven days of culture. (H) Cross-section of the leaf induced after seven days of culture (arrow). (I) Longitudinal section. Red-stained starch granules (arrow). (J) On the 14th day of culture, the early formation of PLBs (arrow). (K) Cross-section of the leaf induced on the 14th day of culture, with evidence of mitotic activity in the epidermis (arrow). (L) Longitudinal section. Elongated cells in the epidermis (arrow) and red-stained starch granules (broad arrow). (M) After 30 days of culture, with many PLBs (arrow) concentrated at the leaf base. (N) Longitudinal section of a PLB (O) Red-stained starch granules (arrow). (P) Overview of leaf inoculated at 60 days of culture, yellowish, showing deterioration, but loads of PLBs (arrow) present at the leaf base. (Q) Longitudinal section showing the beginning of the formation of leaf (arrow). (R) Blue-stained prominent proteins (broad arrow) and red-stained starch granules (arrow). (S) Overview of leaf inoculated at 100 days of culture with a developed leaf (arrow). (T) PLBs longitudinal sections showing developed leaves (arrow). (U) Blue-stained prominent nuclei (broad arrow), proteins decorated with blue (arrow) and red-stained starch granules (square). Bars: (A, D, T): 1 mm; (G, J, M, P, S): 2 mm; (C, F, I, O, R, U): 50 μm; (E, N): 100 μm; (B, H, K, L, Q): 200 μm.

Endogenous hormone

The endogenous levels of IAA is clearly altered during the induction and development of PLBs (Figure 2A). On zero, 7 and 100th days of cultivation, there was a progressive and substantial IAA accumulation in relation to the other cultivation times. However, in the other cultivation times, a sharp IAA decrease began, ranging within relatively low levels, except on the 7 and 60th days of cultivation, where there was IAA concentration increase.

Sugar contents

Total soluble carbohydrate content varied during PLBs induction and development (Figure 2B). Larger soluble sugar amounts were found on the 2nd, 7, 14th, 30 and 60th days of cultivation after induction, with no significant difference from each other. The only difference occurred on the 100th day of cultivation, when there was a significant concentration decline (8.21 mg g^{-1} FM). Regarding the initial explant (day zero), there was a slight decrease in total sugar amount, with significant differences in relation to the 2nd and 100th days of cultivation.

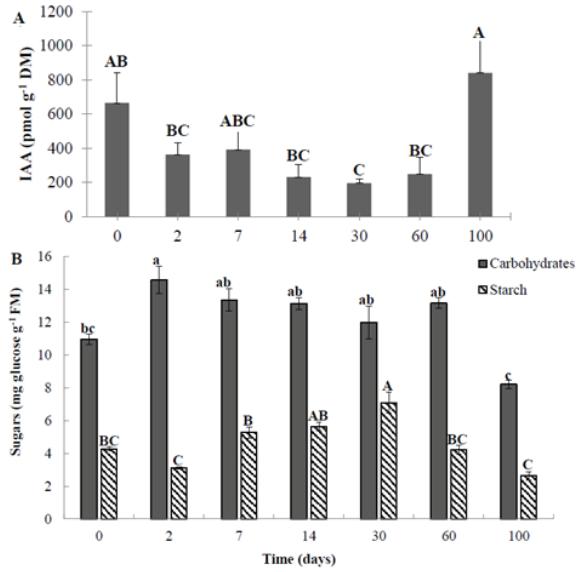

Figure 2. Changes in endogenous indole-3-acetic acid (IAA) content (A) and total soluble carbohydrate and starch content (B), during the induction and development of PLBs in *C. tigrina* after explant inoculation. Vertical bars represent the standard deviation for each measure. Means followed by different letters are significantly different according to Tukey's test at 5%.

As for starch contents, it was observed that the highest starch concentration occurred on the 30th day (7.1 mg g^{-1} FM), statistically different from the other cultivation times, with the exception of the 14th day after starting induction. On the zero, 7, 14 and 60th days of cultivation, there were no statistical differences. On the 2nd and 100 days of cultivation, starch levels were lower, with concentrations of 3.11 and 2.63 mg g^{-1} FM, respectively (Figure 2B).

Composition analysis of soluble sugars in the different cultivation times, during PLB induction and development, showed the presence of sugar alcohols, especially glucose, fructose and sucrose (Figure 3), in all culture times.

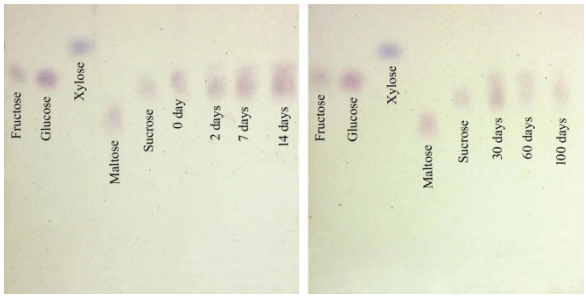

Figure 3. Qualification of sugars by Thin-layer Chromatography during the induction and development of PLBs in *C. tigrina*. Fructose, glucose, xylose, maltose and sucrose were used as markers.

Regeneration from organogenesis or somatic embryogenesis is well known, and it is known that regeneration is strongly correlated with the concentration of endogenous hormones or hormones externally applied to the culture medium (Grzyb, Kalandyk, Waligórski, & Mikuła, 2017). IAA, the main natural representative of the auxin group, is responsible for regulating plant growth and development, besides being related to a wide variety of plant responses (Sairanen et al., 2012).

IAA levels on the 2nd, 7, 14 and 30th days of cultivation are strongly related to cell division and differentiation. During these cultivation times, *C. tigrina* leaves showed evidence of PLBs development and formation, whose epidermal cells began to undergo mitotic division, initiating cell differentiation and formation of PLBs component tissues. Significant IAA level increase on the 100th day of cultivation is strongly related to morphogenetic response. This peak occurred in the period coinciding with the moment in which cells are becoming irreversibly affected by a specific morphogenic response (leaf primordia formation, in this case). Furthermore, on the 100th day of cultivation, there was PLBs formation over the preexisting ones, which is a cyclic regeneration. According to Sairanen et al. (2012), auxins perform functions in cell elongation, cell division beginning, organ definition and vascular system differentiation processes. High auxin concentrations, such as the IAA, indicate their importance for zygotic embryo development, especially with respect to cell elongation and division (Taiz, Zeiger, Møller, & Murphy, 2017).

Regarding soluble sugar amounts, the sharp increase on the 2nd day of cultivation may be due to sucrose presence on the culture medium. Similar data were found in *Medicago sativa* somatic embryo development (Horbowicz, Obendorf, McKersie, & Viands, 1995) and in *Cedrela fissilis* organogenesis (Aragão et al., 2016), in which high sugar content was associated with high carbon concentrations on the culture medium. According to Gemas and Bessa (2006), in *Anacardium occidentale* nodal explants, carbohydrate absorption from the culture medium in the beginning of cultivation is important to start sprout induction and promote their growth. Similar results were observed in sprouts obtained through *Digitalis lanata* callus (Fatima, Mujib, Fatima, Arshi, & Umar, 2009).

The decrease in total sugars on the 100th day of cultivation is related to increased energy demand for the metabolic processes occurring there. Cangahuala-Inocente, Silveira, Caprestano, Floh, and Guerra, (2014) while working with *Acca sellowiana* somatic embryogenesis (SE), observed that total soluble sugar contents differed between various

stages of embryo development, with similar values in cordiform and torpedo stages, followed by decrease in the pre-cotyledon stage. Soluble sugars influenced plant growth in two ways, as follows: serving as carbon sources, from which energy is derived for glycolysis and respiration, and acting as signaling molecules through receptor kinases action (Wind, Smeekens, & Hanson, 2010). Both ways lead to cell induction and differentiation (Lulsdorf, Tautorus, Kikcio, & Dunstan, 1992) in the present study, during PLB formation.

The same pattern is observed for starch content, and its increase on the 14 and 30th days of cultivation may serve as carbon temporary storage reserves, which can be used later for biosynthesis of other storage materials. He et al. (2011), while working with SE in soybean, also found starch content increase, followed by a sharp decrease. According to the authors, starch may serve as a carbon temporary storage reserve, which can be later used for the biosynthesis of other seed storage materials (Scofield et al., 2009). On the 60th day of cultivation, there was a significant starch content decline, and this apparent catabolism may be associated with cell proliferation and differentiation, observed in this cultivation period. Starch contents remained constant during the first 30 days in *Acca sellowiana* somatic embryo development, although these values significantly decreased compared to the initial inoculum (Cangahuala-Inocente et al., 2014). Starch content decrease may be related to its degradation to produce glucose as an energy source for various metabolic reactions that occur during cell vital metabolism growth and maintenance (Oliveira et al., 2017). In this context, the reduction in starch granules observed in dividing cells at the developmental stages of PLBs in *C. tigrina* suggests that these were required for the development of these structures.

In this study, glucose, fructose and sucrose were observed in all cultivation times, demonstrating their importance in the induction and development of PLBs. According to Aragão et al. (2016), glucose and fructose are the first molecules to act in cell signaling, while the role of sucrose may be initially to provide hexoses. Sucrose is considered the main sugar form used by cells as carbon skeleton and energy source during growth and development (Lastdrager et al., 2014). Besides, it plays a central role in plant development as a possible signaling molecule, regulating a number of genes (Barbier et al., 2015). Glucose also has a major role in metabolism, as it may serve as a bridge between carbohydrate and phytohormone signaling (Hartig & Beck, 2006). In addition, glucose has been highlighted as a signaling molecule in various processes associated with growth and development, such as germination, hypocotyl elongation, cotyledon expansion and leaf development (Lembrechts, Ceusters, Proft, & Ceusters, 2017). Hexoses and sucrose are generally associated with different seed development stages. The authors suggested that sucrose regulates cell differentiation and reserve substances storage, while hexoses control cell growth and metabolism (Wang & Ruan, 2013). According to Wang and Ruan (2013), there is strong evidence that sucrose and hexoses can indeed modulate expression of genes encoding enzymes involved in carbohydrate metabolism and in the biosynthesis of hormones implicated in seed development.

Recent studies showed that auxins and sugars (glucose and sucrose) act together, and auxin metabolism is regulated by free sugars availability (Sairanen et al., 2012; Ljung et al., 2015). Biosynthesis regulation and auxin degradation (mainly IAA) by sugars requires changes in the expression of multiple genes and metabolites associated with various IAA biosynthesis routes (Sairanen et al., 2012; Ljung et al., 2015). In order to study soluble sugar effects in auxin homeostasis, wild *Arabidopsis* seedlings were incubated with various glucose concentrations after 10 days of germination. Then, it was possible to verify the induction of the expression of several genes encoding enzymes in auxin biosynthesis from tryptophan, including YUCCA8 and YUCCA9 (Sairanen et al., 2012). Previous studies reported that a YUCCA corn putative gene was strongly induced by glucose (LeClere, Schmelz, & Chourey, 2010).

Therefore, it was observed that plant growth and development plasticity is exemplified by the complex sugar and hormone signaling interaction (Ljung et al., 2015).

Conclusion

The results of this study show the dynamics of physiological and biochemical changes that occur during PLBs induction and development in *C. tigrina*. Endogenous IAA and sugar biochemical profiles indicate that these biomolecules play an important role in cell events. IAA content increase and sugar content decrease are strongly related to morphogenetic response, that is, PLBs formation over the preexisting ones and leaf primordia formation. This study is the first to report the relationship between endogenous levels of IAA and carbohydrates during PLB induction and development.

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