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Dynamics in global DNA methylation and endogenous polyamine levels during protocorm-like bodies induction of *Cattleya tigrina* A. Richard

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ABSTRACT. *Cattleya tigrina* is endemic to the Atlantic forest biome and classified as vulnerable in the Red Book of Brazilian Flora. *In vitro* techniques comprise valuable tools for the conservation of endangered plant species. The aim of the present study was to evaluate the morphological features, global DNA methylation levels and free polyamines during protocorm-like bodies (PLBs) induction of *C. tigrina*. Along with that, an efficient protocol for *in vitro* propagation of this species is proposed. The first evidences of PLBs induction in *C. tigrina* occurred at seven days in culture, starting from the basal portion of the leaf abaxial surface. A hypomethylation marked the beginning of cell differentiation, followed by an increased global DNA methylation at 35 days in culture, coinciding with a subtle change in the structures morphogenetic development. During PLBs induction, putrescine exhibited higher levels as compared to spermidine and spermine, and apparently presents a major role during the PLBs induction in *C. tigrina*. Due to the apparent secondary PLBs formation, this protocol can represent a highly efficient method for *in vitro* propagation of this species.

Keywords: orchids, micropropagation, *in vitro* culture, epigenetics, PLBs.

Dinâmica da metilação do DNA global e níveis endógenos de poliaminas durante a indução de estruturas semelhantes a protocormos de *Cattleya tigrina* A. Richard

RESUMO. *Cattleya tigrina* é uma espécie endêmica do bioma Mata Atlântica e classificada como vulnerável no Livro Vermelho da Flora Brasileira. As técnicas *in vitro* compreendem ferramentas valiosas a serem empregadas na conservação de espécies de plantas ameaçadas. O objetivo do presente estudo foi avaliar as características morfológicas, os níveis globais de metilação do DNA e as poliaminas livres durante a indução de estruturas semelhantes a protocormos (ESPs). Paralelamente, um protocolo eficiente para a propagação *in vitro* desta espécie é apresentado. As primeiras evidências de indução de ESPs em *C. tigrina* foram observadas aos sete dias de cultivo, a partir da porção basal da superfície abaxial da folha. Uma hipometilação foi observada concomitante ao início da diferenciação celular, e um aumento da metilação global do DNA foi encontrada aos 35 dias de cultivo, coincidindo com uma sutil mudança no desenvolvimento morfogenético das estruturas. Durante a indução de ESPs, a putrescina exibiu níveis aumentados em comparação a espermidina e espermina e, aparentemente, apresenta um papel importante durante a indução dessas estruturas em *C. tigrina*. Devido à aparente formação secundária de ESPs, este protocolo pode representar um método altamente eficiente para a propagação *in vitro* desta espécie.

Palavras-chave: orquideas, micropropagação, cultivo *in vitro*, epigenética, ESPs.

Introduction

The Atlantic Forest biome is worldwide recognized by its high diversity and endemism of species. Considered a biodiversity hotspot, it holds about 20,000 different plant species, of which 8,000 are endemic (Myers, Mittermeier, Mittermeier, Da Fonseca, & Kent, 2000). The family Orchidaceae includes 27,801 species distributed in 899 genera (The Plant List, 2013), being one of the largest and specialized families in the plant kingdom, spread throughout almost all continents, especially in tropical and subtropical regions (Hossain et al., 2013). *Cattleya tigrina* A. Richard is an endemic orchid species to Atlantic forest biome, and classified as vulnerable in the Red Book of Brazilian Flora (Martinelli & Moraes, 2013).

In vitro micropropagation techniques have been a widely used tool for conservation of endangered plant species (Sarasan et al., 2006). The classic research approach in orchid *in vitro* conservation is the establishment of protocols for non-symbiotic germination or organogenesis. However, in the last few years, many studies aiming at the development and obtainment of *in vitro* protocorm-like bodies (PLBs) were realized (Lee, Hsu, & Yeung, 2013). These structures can be directly induced from meristematic shoots (Subramanium & Taha, 2003), flower stalks (Chen & Chang, 2000), leaf segments (Sheelavanthmath, Murthy, Hema, Hahn, & Paek, 2005), or indirectly, with an intermediate callus stage (Tokuhara & Mii, 2003).

To date, only a few studies focusing on PLBs ontogeny were performed (Lee et al., 2013). Many authors states that PLBs are somatic embryos (Hossain et al., 2013; da Silva, 2013; Lee et al., 2013), or that somatic embryogenesis is the initial step on the PLBs formation (Zhao, Wu, Feng, & Wang 2008). Through histological and histochemical studies, Lee et al. (2013) demonstrated that during the early stages of PLB formation, the cells show cytological characteristics and cell wall markers similar to zygotic embryo development. However, further studies on PLBs development in other orchid species are essential for the research advancement in this area (Liau et al., 2003).

Besides morphological changes, PLBs ontogeny comprises several biochemical and molecular changes, including those regulated by epigenetic mechanisms, such as DNA methylation. Changes in DNA methylation occur during growth and development of plants (Ramchandani, Bhattacharya, Cervoni, & Szyf, 1999). In plant tissue culture, differentiation and dedifferentiation processes, as well as cell division, are followed by tissue-specific methylation and demethylation events in genomic DNA (Msogoya, Grout, & Roberts, 2011; Fraga et al., 2016).

Polyamines (PAs) are also involved in physiological processes related to growth and development in plants, and function as regulators during seed development in both angiosperms and gymnosperms (Cohen, 1998; Stasolla & Yeung, 2003; Reis, Vale, Heringer, Santa-Catarina, & Silveira, 2016; de Oliveira et al., 2017). PAs are small polycationic, aliphatic amines capable of electrostatically interacting with macromolecules, such as nucleic acids, phospholipids, cell wall components, and proteins (Baron & Stasolla, 2008). In plants, the three major PAs (putrescine, spermidine and spermine) are synthesized from arginine and ornithine through decarboxylation process (Silveira et al., 2004). Thus, an improved understanding of the biochemical and physiological events that occur during PLBs induction and development is essential to develop more efficient micropropagation protocols.

In this sense, the aim of the present study was to evaluate the morphological features, global DNA methylation levels and free polyamines during protocorm-like bodies induction of *C. tigrina*. Along with that, an efficient protocol for *in vitro* propagation of this species is proposed.

Material and methods

Plant material

Seeds of *C. tigrina* were *in vitro* germinated and the seedlings maintained through successive subcultures (each 35 days, totaling 5 cycles) in culture medium composed by MS basal salts (Murashige & Skoog, 1962) plant growth regulators-free. Leaf sections (2.0 ± 0.5 cm) were used as the initial explant for PLBs induction. The PLBs induction culture medium consisted of MS basal salts supplemented with soybean peptone (1.0 g L^{-1}), NaH_2PO_4 (170 mg L^{-1}), Morel vitamins (Morel & Wetmore, 1951), sucrose 20 g L^{-1} and 9 µM of Thidiazuron (TDZ) (Voges, Benevenuto, Fritsche, & Guerra, 2014). The pH was adjusted to 5.8 and autoclaved at 121ºC at 1.5 atm for 20 min. Foliar segments were inoculated with the abaxial surface in contact with the culture medium in 300 mL flasks with 25 mL of volume. Sixteen leaf explants were placed in each flask, and then cultured with 16 hours photoperiod, with a low light intensity of 55 μmol m^2 s^{-1} provided by cool-White OSRAM™ fluorescent lamps, at 25 ± 2°C.

Samples from PLBs induction process were collected at the inoculation time and after 7, 14, 21, 28, and 35 days in culture for global DNA methylation analysis and free PAs quantification.

Global DNA methylation analysis

DNA extraction was performed according to Doyle and Doyle (1987) using samples of 500 mg of fresh mass (FM). Nucleic acids digestion procedures were based on the method described by Johnston et al. (2005) and Fraga et al. (2012). High performance liquid chromatography (HPLC) analysis was performed according to Johnston et al. (2005). A HyperClone™ 5 μm ODS (C18) 120 Å, LC Column 250 x 4.6 mm (Phenomenex®, Torrance, USA), guard column (4.0 × 3.0 mm) (Phenomenex®), and UV detector (280 nm) were used. The gradient program consisted of 3 min. with 100% buffer A (0.5% v/v methanol in 10 mM KH_2PO_4 adjusted to pH 3.7

with phosphoric acid, 0.22 μm filtered), followed by a linear gradient from 3 to 20 min. to 100% of buffer B (10% v/v methanol in 10 mM KH_2PO_4 adjusted to pH 3.7 with phosphoric acid, 0.22 μm filtered), and a linear gradient from 20–25 min. with 100% of buffer B. A flow rate of 1 mL min.$^{-1}$ and 20 μL of sample injection volume were applied.

The dNTPs (Fermentas, Hanover, MD, USA) used as standards (dA, dT, dC and dG) and 5mdC were digested for 2 hours at 37ºC with alkaline phosphatase (10 U mL^{-1}) and Tris-HCl (0.5 M, pH 8.3) to obtain the nucleosides. The standard nucleosides (5-50 mM) were prepared in deionized H_2O and stored at -20ºC. 5mdC quantification (%) was performed according to 5mdC concentration divided by 5mdC concentration plus dC concentration multiplied by 100. The obtained peak area was analyzed by LC Solution software (Shimadzu®, Kyoto, Japan).

Free polyamines quantification

Free polyamines were determined from samples of 200 mg FM based on the methodology described by Silveira et al. (2004) and Scherer et al. (2013). Samples were ground in 1.6 mL 5% (v/v) perchloric acid solution, and free PAs were extracted and dansylated. Free PA were derivatized by dansyl chloride and identified by HPLC, using a 5 μm reverse-phase C18 column (Shimadzu® Shim-pack CLC ODS). The gradient of absolute acetonitrile was programmed to 65% over the first 10 min., from 65 to 100% for 10 and 13 min., and 100% for 13 and 21 min., using 1 mL min.$^{-1}$ flow rate at 40ºC. The PAs concentration was determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with standard PAs: putrescine, spermidine and spermine. The 1,7-diaminoheptane (DAH) was used as internal standard.

Statistical Analysis

Different sample collection times were considered as treatments, consisting in six biological replicates. Each biological replicate was injected twice for the HPLC analysis. Data from global DNA methylation levels and free PAs were analyzed by ASSISTAT® software and submitted to analysis of variance (ANOVA), followed by Tukey post hoc test ($p < 0.05$).

Results

PLBs induction

During the first 28 days of PLBs induction, we observed the first evidences of PLBs induction in the leaf base (Figure 1A; B), followed by globular a well-defined translucent PLBs (day 14; Figure 1C), and increased PLBs clusters proliferation (day 21; Figure 1D). During the first 21 days, we also observed a continuous formation of new PLBs. After 28 days in culture, some PLBs became larger and turned to dark green color (Figure 1E).

Global DNA methylation and free endogenous polyamines

The dynamics of global DNA methylation levels of *C. tigrina* PLBs revealed significant differences between the developmental stages (Figure 2A). At the inoculation day, the explant showed 23% of global DNA methylation, followed by a decrease at 7 days in culture (19%), and reached its lowest level (16%) at 28 days in culture.

Hypomethylation coincided with the beginning of cell differentiation (Figure 1B), and was maintained during the developmental stages, that showed an increase in the number and volume of PLBs (Figure 1C, D and E). The increased global DNA methylation at 35 days in culture coincided with a subtle change in the PLBs morphogenetic development (Figure 1F).

An expressive increment in putrescine levels was observed between day zero and day 14 in culture (Figure 2A). The highest level was observed after 14 days in culture, remaining constant until day 35 in culture. The increased levels observed at day 14 in culture coincided with the beginning of PLBs formation. Spermidine and spermine levels did not show changes throughout the 35 days in culture, remaining constant (Figure 2B). However, spermidine levels were approximately 4 times higher than spermine.

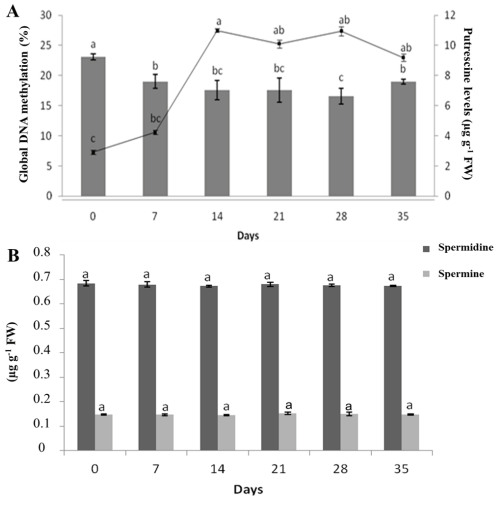

Figure 2. Global DNA methylation, putrescine, spermidine and spermine levels during protocorm like-bodies formation of *C. tigrina*. In figure A, bars represent DNA methylation levels, and line putrescine levels. Mean values followed by standard deviation (vertical bars). Means followed by different letters are significantly different according to the Tukey test ($p < 0.05$).

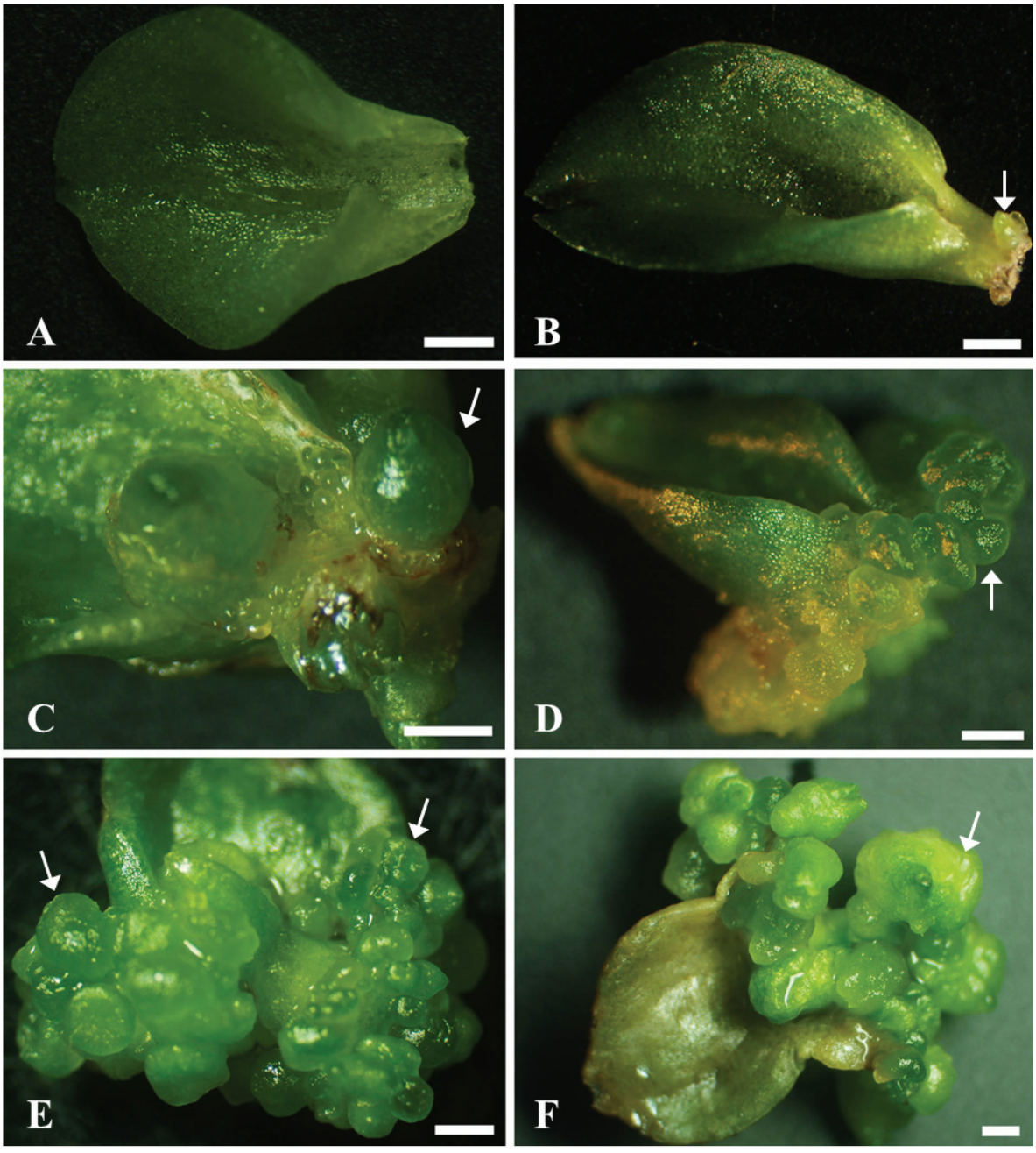

Figure 1. Protocorm-like bodies (PLBs) induction in *Cattleya tigrina*. A - Leaf explant (day zero); B - Globular and translucent PLBs beginning to emerge from leaf base after 7 days in culture (arrows); C - Larger and greenish PLBs formation at day 14 (arrows); D - Formation of PLBs clusters at day 21 in culture (arrows); E - Mixture of translucent and greenish PLBs at day 28 (arrows); F – Secondary PLBs formation from preexisting PLBs at day 35 in culture (arrows). Scale bars: 1 mm.

Discussion

PLBs induction

In the present study, PLBs formation started in the leaf base. The leaf basal region may have improved response to *in vitro* morphogenesis due to its proximity to the axillary meristem, which contain both meristematic regions and newly formed tissues with high metabolic activity (Firoozabady & Moy, 2004). In agreement to our results, the leaf basal portion of *Dendrobium* 'Chiengmai Pink' also showed a greater ability to directly form somatic embryos (Chung, Chen, & Chang, 2007).

Our results also indicated secondary PLBs induction, especially after 28 days in culture. Zhao et al. (2008) reported that the majority of *Dendrobium candidum* PLBs produced secondary PLBs originated from cells of the interior or exterior of the embryogenic callus. Chen and Chang (2006)

also observed continuous production of somatic embryos in *Phalaenopsis amabilis* through secondary embryogenesis directly from leaf explants. In this sense, due to the apparent secondary PLBs formation in *C. tigrina*, this protocol can represent an efficient method to *in vitro* propagation of this species.

Global DNA methylation levels

Transitions between different developmental stages involves changes in the cellular differentiation pattern, these changes are epigenetically regulated. DNA methylation plays an important role in plant development, being associated with control of proliferating cells development (Ruiz-García, Cervera, & Martínez-Zapater, 2005). In *Eleuterococcus senticosus*, DNA methylation levels of callus obtained from leaf explants were overall lower in the embryogenic than in the non-embryogenic callus (Chakrabarty, Yu, & Paek, 2003). These authors suggested that the differences in DNA methylation could reflect different developmental processes occurring in the plant cells, similar to animal embryos, in which a wave of demethylation following fertilization leads to an almost complete conversion of methylcytosine to cytosines (Chakrabarty et al., 2003).

In plants, as observed for *Silene latifolia*, the moderate process of global hypomethylation during seed germination and post-germination reflects the transition from a metabolically quiescent seed to the active seedlings growth and development (Zluvova, Janousek, & Vyskot, 2001). In *Pinus nigra* embryogenic cultures, the hypomethylation of specific cell populations seems to be associated with the initiation of a differentiation program, whereby embryonic structures features arise, being able to regenerate whole plants (Noceda et al., 2009).

It is also reported an inverse relationship between DNA methylation levels and embryogenic potential for *P. nigra* embryogenic cultures (Noceda et al., 2009). Higher levels of DNA methylation (30%) corresponded to cell lineages without embryogenic capacity, while levels below 18% of 5mdC enabled the formation of bipolar structures, with embryogenic potential. These authors suggested that these responses might be related to improved gene expression associated to the development of a full embryogenic program.

Similarly, in *Rosa hybrida*, cytosine demethylation events occurred at a high frequency during somatic embryogenesis and these DNA methylation patterns were transmitted to regenerated somatic plantlets (Xu, Li, & Korban, 2004). Otherwise, the observed DNA methylation pattern during shoot organogenesis of *Rosa hybrida* were not observed into regenerated shoots, leading these authors to suggest that the changes in the DNA methylation patterns are mainly associated with the embryogenic pathway.

In the present work, global DNA hypomethylation until day 28 in culture showed a clear relationship with the PLBs induction and formation. These results corroborate the idea that DNA hypomethylation plays a key role in the acquisition of the embryogenic capacity in somatic cells (Elhiti, Tahir, Gulden, Khamiss, & Stasolla, 2010).

The use of TDZ for PLBs induction in the present study may have influenced the DNA methylation levels, since this epigenetic mechanism is often induced by stress (Boyko & Kovalchuk, 2011), an effect that has been attributed to TDZ supplementation *in vitro* (Mamaghani et al., 2009). TDZ is a derivative of phenyl urea, which can act as a cytokinin-purine and present an auxin-type response (Thomas & Katterman, 1986; Visser, Qureshi, Gill, & Saxena, 1992; Jones, Cao, O'Brien, Murch, & Saxena, 2007), presumably affecting the hormone endogenous levels, and promoting the somatic embryogenesis induction in many explant sources (Murthy, Murch, & Saxena, 1995; 1998). Whereas plant growth and development are regulated by specific plant hormones, and being the modulation of DNA methylation one of the hormone modes of action in the plant (Vanyushin, Bakeeva, Zamyatnina, & Aleksandrushkina, 2004), it can be assumed that TDZ supplementation affects the global DNA methylation levels and, consequently, the expression of genes related to *C. tigrina* PLBs formation.

Thus, the results presented in this study indicated a dynamic in global DNA methylation levels during PLBs formation from leaf explants of *C. tigrina*, indicating a hypomethylation process coincident with the beginning of the PLBs formation. In addition, TDZ supplementation in culture medium is apparently essential for PLBs induction, and may be associated with factors that trigger this demethylation process.

Changes in free endogenous polyamines

During PLBs induction, putrescine exhibited higher levels as compared to spermidine and spermine (Figure 2). Similarly, embryogenic lines of *P. nigra* presented spermine as the lowest abundant PA (Noceda et al., 2009). Putrescine was also the most abundant PA found in embryogenic cultures of *Bactris gasipaes* (Nascimento-Gavioli et al., 2017) and

sugarcane callus (Silveira et al., 2013). According to Bagni and Tassoni (2001), putrescine and spermidine are generally the most abundant PAs in plants, while spermine is only present in small levels.

Saiprasad, Raghuveer, Khetarpal, and Chandra (2004) observed that putrescine was the most efficient PA for supplementation in PLBs induction culture medium of *Dendrobium* 'Sonia'. In addition, cultures exposed to spermidine or spermine presented lower PLBs induction (Saiprasad, et al., 2004). Some studies also indicate that putrescine metabolism may be involved in determining the somatic embryogenesis capacity in several plant species (Silveira et al., 2004). Gow, Chen, and Chang (2008) observed that the PAs, including putrescine, induced direct somatic embryogenesis in leaf explants of *Phalaenopsis amabilis.*

Cangahuala-Inocente, Steiner, Santos, and Guerra (2004) observed a PAs accumulation during somatic embryogenesis induction in *Acca sellowiana*, concomitantly with an intense proliferation of epidermal cells, accumulation of phenolic compounds, and improved meristem organization. In *Araucaria angustifolia,* high levels of putrescine and spermidine were observed in pre-cotyledonary somatic embryos, with subsequent decrease after the cotyledons formation (Astarita, Handro, & Floh, 2003). In *Ocotea catharinensis* improved total PA levels were observed in globular-staged somatic embryos, with decreased levels during cotyledonary stage (Santa-Catarina, Hanai, Dornellas, Viana, & Floh, 2004). Similarly, high putrescine levels were observed in *Picea rubens* pro-embryogenic cultures, while spermidine was predominant during somatic embryos development (Minocha, Minocha, & Long, 2004). Our results are in agreement with these reports, and putrescine apparently presents a major role during the PLBs induction in *C. tigrina*.

Spermidine and spermine seem to be more relevant than putrescine in later embryonic developmental stages, and are necessary for somatic and zygotic embryos histodifferentiation (Minocha, Smith, Reeves, Steele, & Minocha, 1999; Santa-Catarina, Silveira, Scherer, & Floh, 2007; Steiner, Santa-Catarina, Silveira, Floh, & Guerra, 2007). In experiments with PAs exogenous supplementation, an increased PLBs induction rate was observed in *Dendrobium huoshanense*, as well as in the PLBs conversion (Wang, Luo, Wu, & Jin, 2009). Putrescine supplementation to the culture medium also improved PLBs development of *Dendrobium officinale* (Wei, Wei, & Yang, 2010). These authors also observed that the α-DL-difluoromethylarginine supplementation, a polyamine inhibitor, reduced the frequency of PLBs conversion. In this sense, the results of the present study suggest that PAs have an important role in the *in vitro* morphogenesis process of *C. tigrina*, corroborating previous reports, and, especially putrescine, indicated high levels during PLBs induction process.

There is a possible relationship between PAs and DNA methylation, since their biosynthetic pathways share the enzyme decarboxylated S-adenosylmethionine as a common substrate (Fraga, Cañal & Rodríguez, 2002; Noceda et al., 2009). Interestingly, the low levels of spermine found in the present study could be related to DNA methylation. In addition, Hirasawa and Suzuki (1985) have shown that most of the spermine in maize chromatin is probably chromatin bound in vivo, which suggests that spermine may have a specific role in chromatin.

Conclusion

Due to the apparent secondary PLBs formation, this protocol can represent an efficient method to *in vitro* propagation of this species. Global DNA methylation levels of *C. tigrina* PLBs revealed significant differences between the structures developmental stages. A hypomethylation process coincides with the beginning of cell differentiation and remains low during the development of PLBs. These findings indicates that DNA hypomethylation plays a key role in the acquisition of the embryogenic capacity in somatic cells of *C. tigrina*.

Regarding PA analysis, the results suggested that PAs have an important role in the PLBs induction in *C. tigrina*. During PLBs induction, putrescine exhibited higher levels as compared to spermidine and spermine, and apparently presents a major role during the PLBs induction in *C. tigrina*. However, further analysis during PLBs conversion into microshoots is necessary to elucidate spermidine and spermine role in this differentiation process.

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