Fundamentals, advances and applications of somatic embryogenesis in selected Brazilian native species

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Abstract

Somatic embryogenesis (SE) is a complex, multifactorial model of molecular, biochemical, physiological and developmental steps of plant embryogenesis. This regenerative route arises from a single totipotent somatic cell that has dedifferentiated or redifferentiated into an embryonic state. Additionally, it comprises a high performance in vitro regenerative system for both the mass clonal propagation of an elite genotype and/or the mass propagation of endangered plant germplasm. Following coordinated subsequent cell divisions, changes in cellular contents and balances of protein, hormones, polyamines, polysaccharides, and lipids, histodifferentiation processes takes place, culminating with the full development of a mature somatic embryo, mimicking the developmental stages observed in a zygotic embryo. These dynamics are simultaneously affected by a myriad of factors, mainly associated to epigenetic reprogramming, tissue culture environment, culture media composition, and type, levels and balances of plant growth regulators. In this context, in the present review we present and discuss three SE systems of selected and typical Brazilian native woody species, representing different taxonomic groups: *Araucaria angustifolia* (*Araucariaceae*), *Acca sellowiana* (*Myrtaceae*) and *Bactris gasipaes* (*Arecaceae*). Our approach to SE wants to contribute to the better understanding of plant cell totipotentiality, as well as to address its application in capturing genetic gains from elite genotypes, and for use in conservation programs of endangered species.

Keywords: micropropagation, *Acca sellowiana*, *Araucaria angustifolia*, *Bactris gasipaes*, in vitro culture

INTRODUCTION

In 2005, the journal Science selected 25 scientific questions based on the criteria of how fundamental and comprehensive they were, and how their solutions could impact other related disciplines. One of these questions was: how can a somatic cell become a whole plant? (Vogel, 2005). Considering this and its importance to science and industry, plant cell totipotency, especially somatic embryogenesis (SE), is a priority field of study for the contemporary world.

Somatic embryogenesis is associated with a variety of applications, such as obtaining a reference model for studies on basic physiology and biochemistry, large-scale propagation and genetic transformation, somatic embryo cryopreservation integrated into breeding programs, establishment of in vitro gene banks, germplasm conservation of endangered species, and mass clonal propagation of genotypes of high commercial value (von Arnold et al., 2002; Zoglauer et al., 2003).

Under certain conditions, a somatic plant cell can dedifferentiate to a totipotent embryogenic cell (cellular competence) that has the ability to proliferate (embryogenic cell fate) and/or regenerate an embryo (Figure 1; Tautorus et al., 1991). Initiation, proliferation, maturation, and germination of somatic embryos are different stages of a complex pathway

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full of biochemical and morphological changes similar to zygotic embryogenesis (ZE) (Zimmerman, 1993; Marsoni et al., 2008).

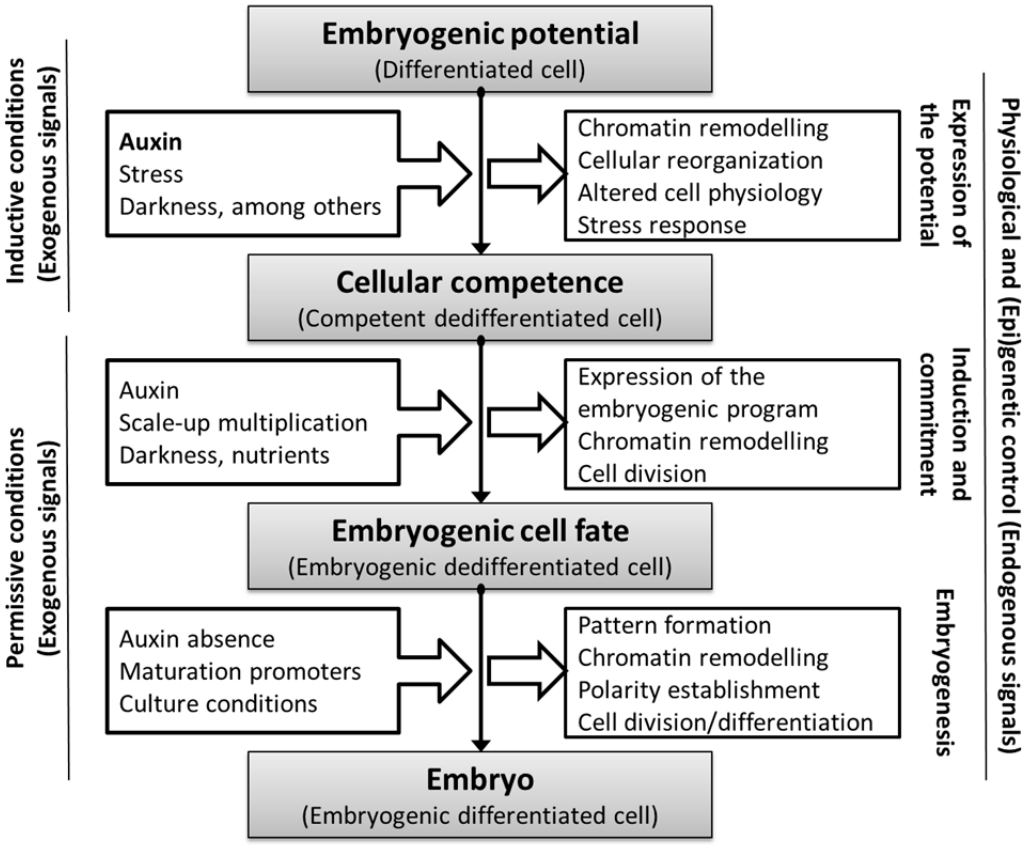

Figure 1. Hypothetical sequence of events underlying somatic embryogenesis (SE) in plants. Several signals (endogenous and exogenous), including auxins, maturation promoters, stress, evoke a wide cellular response including reorganizations at the levels of cell structure, physiology, chromatin and gene expression. The main steps of SE modulation and control involve inductive and permissive conditions, driving competent cells to an embryogenic cell fate, a period that precedes the beginning of embryogenesis per se. All these events are permeated by (epi)genetic and physiological changes, that act as modulators in the SE control (adapted from Fehér, 2006).

Morphological changes during SE are characterized by shifts in concentration of biochemical groups, such as total protein, polyamines, soluble sugars, and polysaccharides. Moreover, changes to cell morphology and biochemistry require the expression of many genes (Verdeil et al., 2007). This genetic regulation can be affected by epigenetic changes, such as chromatin remodeling and DNA methylation (Verdeil et al., 2007). Specific changes to DNA methylation in plants have been associated with embryonic developmental phases, so they are directly related to embryogenic competence (Noceda et al., 2009).

In this context, three SE models currently studied in our laboratory are discussed. We selected three native woody plant species from the Atlantic and Amazon biomes from three different taxonomic groups. *Acca sellowiana* (O. Berg) Burret (*Myrtaceae*) and *Araucaria angustifolia* (Bert.) Kuntze (*Araucariaceae*), native species of the Atlantic Forest of southern Brazil; and *Bactris gasipaes* Kunth (*Arecaceae*), a palm widely distributed in the lowland humid Amazonian region. Our objective was to understand the basis of plant cell totipotency and to apply this knowledge for capturing genetic gains from elite genotype as well as for using it in conservation programs of endangered species.

DISCUSSION

Acca sellowiana

A. sellowiana (syn. *Feijoa sellowiana*), known as feijoa or pineapple-guava, is a native *Myrtaceae* of the Atlantic Forest of southern Brazil, with secondary dispersion in northeast Uruguay (Guerra et al., 2012). In the state of Santa Catarina, Brazil, it is predominantly found in altitudes above 1000 m as a component of the sub-canopy of the Ombrophilous Mixed Forest, an ecosystem of the Atlantic Forest biome, alongside formations of *A. angustifolia*, which dominates the canopy (Finatto et al., 2011). This species is cultivated in several countries (e.g., Colombia, New Zealand, Brazil and Turkey), and its unique flavor makes it a potentially attractive fruit crop for small farmers (dos Santos et al., 2009).

Somatic embryogenesis in *A. sellowiana* was first described by Cruz et al. (1990) using the cotyledonary region of zygotic embryos cultured on MS culture medium (Murashige and Skoog, 1962) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). According to Cruz et al. (1990), small, meristematic cells containing dense cytoplasm, prominent nucleus, small starch grains, and small vacuoles were originated after two weeks. Culture medium supplemented with asparagine, arginine or glycine enhanced the induction and development of somatic embryos (Dal Vesco and Guerra, 2001).

Guerra et al. (2001) reported that pulses with high 2,4-D levels and subsequent culture in PGR-free medium resulted in early and high frequency somatic embryo induction starting from zygotic embryos as explants. Stamen filaments inoculated in LPM (von Arnold and Eriksson, 1981) culture medium supplemented with Picloram and Kinetin (Kin) have been used for SE initiation and somatic embryos able to develop plantlets have been obtained (Stefanello et al., 2005). This use of stamen filaments as explant source was the first report describing a complete SE protocol in this species using somatic tissues as explants and somatic embryo conversion into plantlets was improved in response to half-strength MS culture medium supplemented with 6-benzylaminopurine (BAP). The main steps of SE in *A. sellowiana* are summarized in Figure 2.

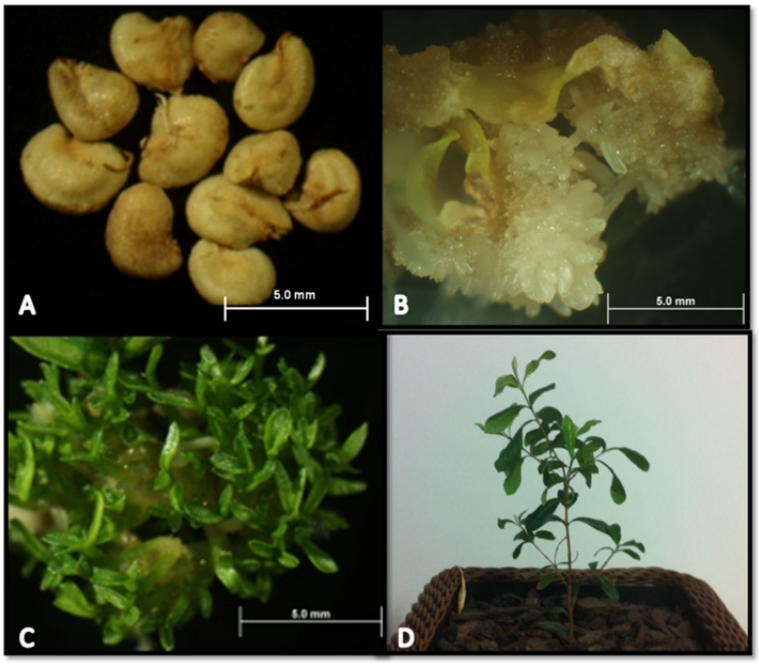

Figure 2. Steps of somatic embryogenesis protocol of *A. sellowiana*. (A) Seeds of *A. sellowiana* after disinfestation procedures; (B) Somatic embryos obtained at 45 days culture; (C) Somatic embryo conversion at 30 days culture in petri dishes; (D) Acclimatized plant.

The *A. sellowiana* SE protocol was further optimized enhancing the SE induction rate and the number of full developed somatic embryos (Cangahuala-Inocente et al., 2007). Thus, synthetic seed technology using sodium alginate resulted in increased conversion rates of encapsulated somatic embryos to plantlets. In this case the culture medium based in LP half-strength salt formulation supplemented with sucrose, BAP and gibberellic acid (GA_3) was used as a supporting medium (Cangahuala-Inocente et al., 2007).

Associated with the development of protocols for SE induction and control, several biochemical and molecular studies have been performed over the past years by our research group. Total protein levels decreased and the soluble sugars levels increase during the first 30 days culture and remained stable until day 120. Otherwise, total protein levels increased according to the progression in somatic embryos developmental stages. The levels of total sugars and starch increased in heart and cotyledonary stages and decreased in the torpedo and pre-cotyledonary stages. These findings indicate that the compounds play a central role in the somatic embryos development of *A. sellowiana* (Cangahuala-Inocente et al., 2009a).

Proteomic studies have also been performed for *A. sellowiana* SE. A high similarity in somatic embryos protein profiles was observed to be similar to zygotic embryos, suggesting that only few and specific genes are involved in the different developmental stages, and that gene expression occurs prior to morphological changes (Cangahuala-Inocente et al., 2009b). The presence of essential nitrogen metabolism proteins, such as cytosolic glutamine synthetase, was also observed (Cangahuala-Inocente et al., 2009b).

Morphological disorders in a relevant portion of emerged somatic embryos have been observed for this species, resulting in a limiting factor in the true-to-type plantlet formation. This phenomenon was also investigated by proteomic studies by our research group, as demonstrated by Fraga et al. (2013). Two proteins related to carbohydrate metabolism were only expressed in off-types after 10 days conversion, suggesting a more active respiratory pathway. A vicilin-like storage protein was only found in off-types after 20 days conversion, indicating that plantlets may present an abnormality in the mobilization of storage compounds, causing reduced vigor in the development of derived plantlets. Taking these results, the process of abnormal development of somatic plantlets seems to be driven by only a few proteins that are apparently essential for the morphogenesis of normal plantlets and their subsequent development (Fraga et al., 2013).

Pescador et al. (2012b) found decreased glutamine levels during the initial phase of somatic embryo development. They were related to protein synthesis and mobilization during embryo maturation. Absorption of glutamine in the first 2 h of culture emphasized its involvement as an important nitrogen source for somatic and zygotic embryos. The analysis of endogenous amino acid levels was also performed at different stages of development during both direct and indirect SE and during ZE (Pescador et al., 2013). In ZE, glutamine and asparagine appeared to be essential to early development of the zygotic embryos. These results suggest the involvement of amino acids in the zygotic and somatic embryos ontogenesis in *A. sellowiana*, indicating specific amino acids requirements for each development event (Pescador et al., 2013).

The study of reserve lipids revealed a high degree of similarity in the composition of total lipids between somatic and zygotic embryos. In both routes, total lipid content increased during embryo development, especially in the transition from torpedo to cotyledonary stages (Pescador et al., 2012a).

Recently, the dynamics in global methylation levels during SE development was studied (Fraga et al., 2012). In this sense, the use of the hypomethylating drug 5-Azacytidine (5-AzaC) at moderate levels combined with 2,4-D stimulated SE induction but resulted in a decreased embryo-to-plantlet conversion. This result was attributed to genome-wide deregulatory effects of 5-AzaC and 2,4-D on DNA methylation patterns changes during cellular re-differentiation. Furthermore, these studies suggested new approaches on the investigation associated to epigenetic markers of SE in future studies.

Araucaria angustifolia

Araucaria angustifolia is a dioecious perennial conifer native to South America,

occurring in south and southeastern Brazil, and small areas of northwestern Argentina and Paraguay (Guerra et al., 2000). *Araucaria* generates an ecologically-important micro-environment within the forest, allowing growth and survival of many shade-tolerant plant species, as well as many small vertebrates and invertebrates (Stefenon et al., 2009). Economically, this species is valuable due to both the production of edible seeds and its high-quality wood, which determined its historical economic importance for construction and cellulose-based products (Guerra et al., 2000).

Drastic population decline and habitat reduction culminated in inclusion of *A. angustifolia* on the IUCN international list as "critically endangered" (IUCN, 2013). However, the remaining forests of this species continue to be explored from commercial and scientific perspectives (Stefenon et al., 2009). In this context, *A. angustifolia* is a species native to Brazil with great potential for studies to support genetic conservation (Vieira et al., 2012). The application of tissue culture tools in this species, such as SE, is one of the most promising techniques for its conservation and mass propagation (Guerra et al., 2001).

Plant regeneration via SE in conifers usually includes five main steps: initiation of embryogenic cultures on a primary explant inoculated on culture medium generally supplemented with plant growth regulators (PGR), particularly auxins and cytokinins; multiplication of embryogenic cultures in liquid or on semi-solid medium culture, most often supplemented with auxins and cytokinins; pre-maturation of somatic embryos on PGR-free culture medium, in order to inhibit proliferation and promote cell differentiation; maturation of somatic embryos in culture medium supplemented with abscisic acid (ABA) and high osmotic potential; finally, plantlet development and conversion in PGR-free culture medium (von Arnold et al., 2002) (Figure 3).

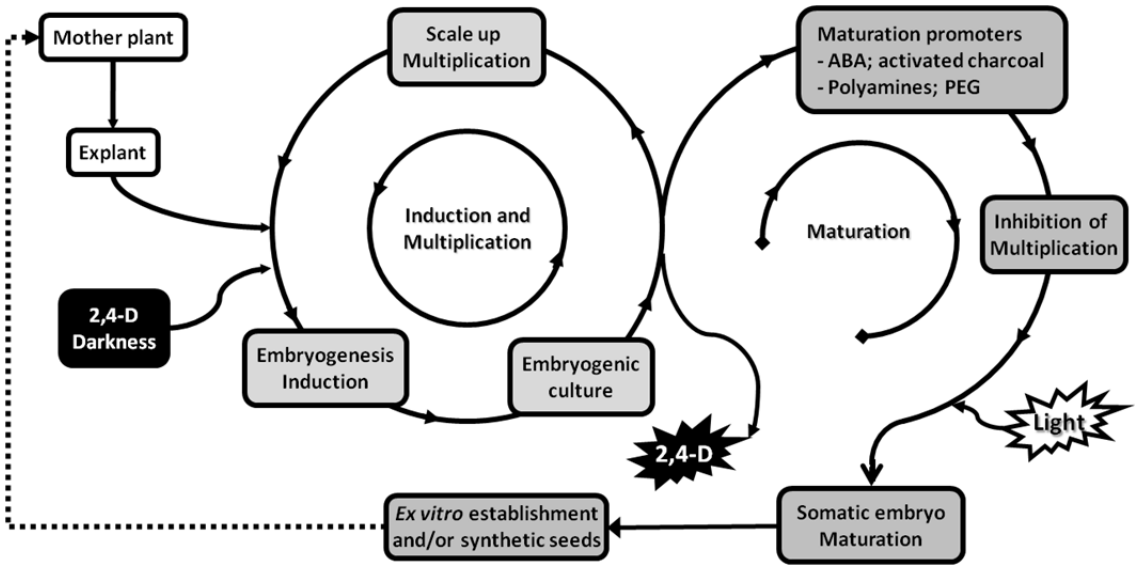

Figure 3. Schematic representation of SE modulation cycles: (1) induction and propagation cycles; (2) maturation cycle (adapted from Durzan, 1988).

More specifically, in *A. angustifolia*, SE is characterized by the development of embryogenic cultures (EC), which, in turn, are multiplied as pro-embryogenic masses (PEM) during the early stages of SE. This step is successfully achieved; however, further protocol optimization is required because an unknown factor hampers further maturation of somatic embryos from PEM (Vieira et al., 2012). However, PEM transfer to a pre-maturation PGR-free culture medium with a high osmotic potential promoted PEM transition to early somatic embryos (dos Santos et al., 2002) (Figure 4). In conifers, the development of early somatic embryos is important for predicting the yield and quality of somatic embryos in the maturation phase (Filonova et al., 2000).

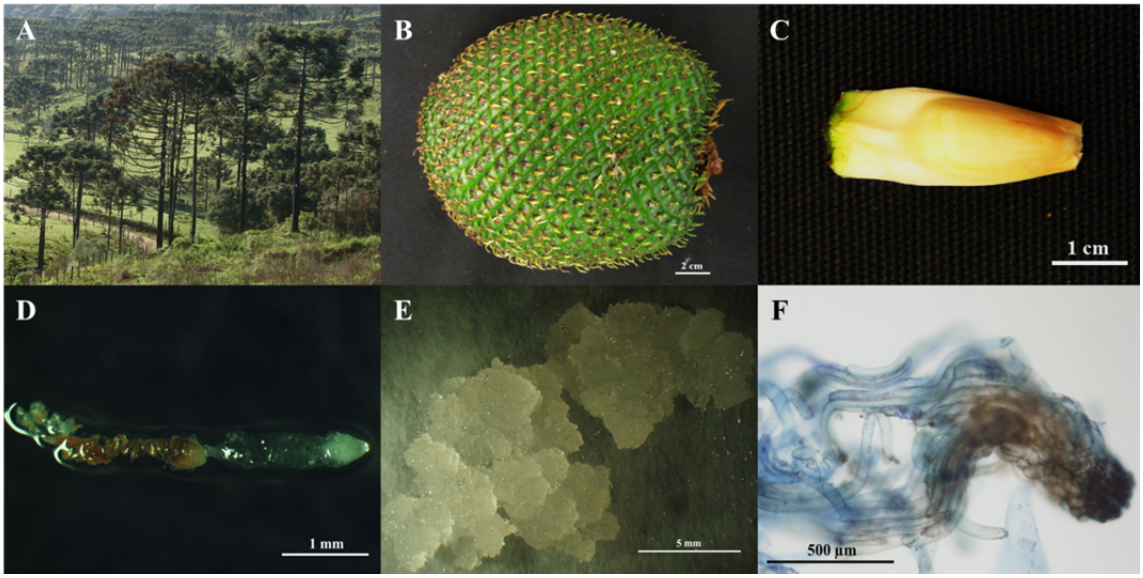

Figure 4. Steps of somatic embryogenesis (SE) induction of *A. angustifolia*. (A) Adult individuals in a natural population; (B) Immature female cone; (C) Immature seed used as explant source; (D) Globular-staged zygotic embryo excised from immature seed; (E) Embryogenic culture obtained after SE induction during multiplication phase; (F) Globular somatic embryo stained with Evans blue and acetic carmine.

Despite the lack of success in conversion of somatic embryos to plantlets, many biochemical, molecular, and histological studies performed during the initial phase of SE in *A. angustifolia* brought relevant results to better understand this morphogenetic route, generating a series of works from our laboratory: Astarita and Guerra (1998, 2000), Steiner et al. (2005, 2012), dos Santos et al. (2002, 2010), Silveira et al. (2002, 2006), Schlögl et al. (2012a, b), Vieira et al. (2012).

In order to study the genetic regulation of SE in *A. angustifolia*, homologs to genes known to regulate SE in other plants were cloned, sequenced, and analyzed by means sqRT-PCR (Schlögl et al., 2012a, b). These genes included *ARGONAUTE* (*AaAGO*), *CUP-SHAPED COTYLEDON1* (*AaCUC*), *WUSCHEL-related WOX* (*AaWOX*), *S-LOCUS LECTIN PROTEIN KINASE* (*AaLecK*), *SCARECROW-like* (*AaSCR*), *VICILIN* 7S (*AaVIC*), *LEAFY COTYLEDON* 1(*AaLEC*), and a *REVERSIBLE GLYCOSYLATED POLYPEPTIDE* (*AaRGP*). Up-regulation of *AaAGO*, *AaCUC*, *AaWOX*, *AaLecK*, and *AaVIC* was observed during transition of somatic embryos from maintenance to maturation phases. During the multiplication phase, expression of *AaAGO* and *AaSCR*, but not *AaRPG* and *AaLEC* genes was influenced by presence/absence of both auxin and cytokinin. Many of the genes analyzed could be used as embryogenesis markers, since their expression changed with progression of somatic embryo development. These findings may offer a valuable contribution to evaluation of embryogenic culture responses to changes in culture media formulation and improved somatic embryo formation in different genotypes.

We also described the cloning and characterization of the expression pattern of the *A. angustifolia* putative homolog of a *SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE* (*SERK*) gene family member (*AaSERK1*), known to be involved in early SE and ZE. The RT-PCR results showed that *AaSERK1* is preferentially expressed in embryogenic cell cultures. Additionally, in situ hybridization results showed that *AaSERK1* transcripts initially accumulate in groups of cells at the periphery of the embryogenic calli and then are restricted to the developing embryo proper (Steiner et al., 2012).

Beyond studying transcription of genes specifically associated with SE, other markers are important for elucidating the underlying regulatory mechanisms of SE. In several plant species, the influence of reduced glutathione (GSH) in cell division and differentiation was

observed to be such a marker of SE (Belmonte et al., 2005, 2006). In *Picea glauca*, by supplementing the culture medium with GSH and glutathione disulfide (GSSG), the GSH/GSSG ratio showed effects on the quantity and quality of somatic embryos obtained (Belmonte and Yeung, 2004). This same process also occurs during ZE in *Brassica napus* and *Picea glauca* (Belmonte et al., 2005; Stasolla et al., 2008). Besides a high GSH/GSSG ratio required at the beginning of embryogenesis of conifers, the presence of nitric oxide (NO) in embryogenic cells may be responsible for maintaining the polarization of pro-embryos (Silveira et al., 2006).

In our laboratory, we observed a clear relationship between the manipulation of the GSH/GSSG ratio and NO emission, and the quality and number of early somatic embryos in *A. angustifolia*. Low GSH concentrations (0.01 and 0.1 mM) were shown to be a potential NO scavenger in the culture medium. Furthermore, low GSH concentrations increased the number of early SE formed in cell suspension culture media in a few days. In gelled culture medium, high levels of GSH (5 mM) allowed the development of globular embryos presenting high NO emission on embryo apex, stressing its importance in the differentiation and cell division. These results indicate that the modification of the embryogenic cultures redox state might be an effective strategy to develop more efficient embryogenic systems for this species (Vieira et al., 2012).

Bactris gasipaes

Bactris gasipaes, a caespitose palm widely distributed in the lowland humid Amazonian Neotropics, belongs to the *Arecaceae* family, one of the most economically-important tropical plant groups (Balick, 1988). Peach palms are cultivated for its starchy or oily fruits, moderately popular in the whole region of traditional distribution, and for heart of palm, the soft edible young leaves extracted from the apex of their shoots (Clement, 2008). Brazil, the largest producer and consumer of heart of palm, also exports heart of palm to other countries (Mora-Urpí et al., 1997).

The main conservation effort for this species consists of living collections in field germplasm banks, seed banks being a non-viable strategy due to peach palm seed recalcitrance to desiccation (Mora-Urpí et al., 1997). However, considering the intrinsic risks of ex vitro collections, in vitro conservation is suggested as the most promising and cost effective technique for supporting conservation programs of peach palm (Mora-Urpí et al., 1997). Most recently, cryopreservation-based techniques have been considered more productive approaches for peach palm long-term conservation (Steinmacher et al., 2011).

Considering the importance of this palm species, several Latin American institutions have established breeding programs, where in vitro regeneration is considered an important tool. Peach palm regeneration was first observed through indirect organogenesis (Arias and Huete, 1983), later by SE (Valverde et al., 1987; Stein and Stephens, 1991), and then by direct organogenesis (Almeida and Kerbauy, 1996). Valverde et al. (1987) described a protocol for in vitro peach palm SE using 100 shoot tip explants, although only ten calli were obtained, each producing 2-8 somatic embryos per callus. Using immature inflorescences as an explant source, Almeida and Kerbauy (1996) described a protocol for in vitro organogenesis, however, a low regenerative rate was achieved. A complete protocol for SE using mature zygotic embryos as explants was published by our research group (Steinmacher et al., 2007a). Embryos were successfully induced, matured, and germinated and the regenerated plantlets were successfully acclimatized to greenhouse conditions (Steinmacher et al., 2007a) (Figure 5).

Subsequently, Steinmacher et al. (2007b) described the induction, development and conversion of somatic embryos in peach palm using inflorescences as explant source. The thin cell layer technique has also been tested using meristematic tissue, resulting in high frequency SE (Steinmacher et al., 2007c). The formation of these embryos occurred in clusters, suggesting the occurrence of secondary SE (Steinmacher et al., 2011).

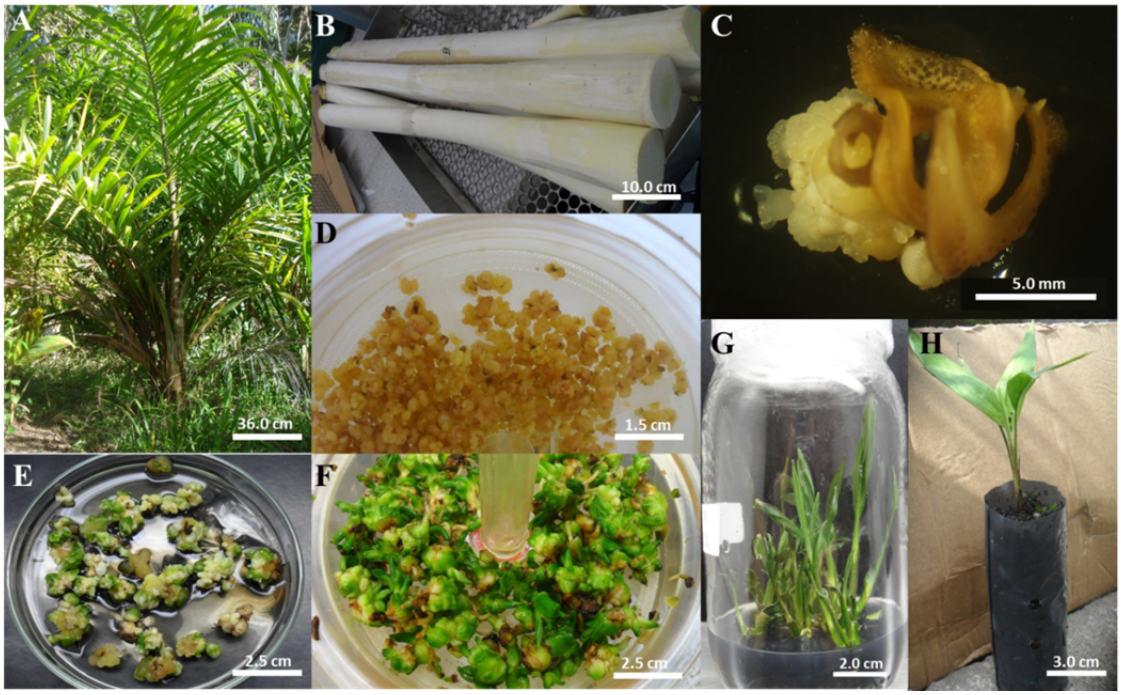

Figure 5. Steps of somatic embryogenesis (SE) protocol of *Bactris gasipaes*. (A) Adult plant; (B) Heart of palm; (C) Explant source exhibiting early somatic embryos during SE induction phase; (D) Somatic embryos during multiplication phase; (E) Somatic embryos during maturation phase; (F) Conversion phase in RITA® bioreactor; (G) Rooting and elongation phase of somatic plantlets; (H) Acclimatized plantlets derived from somatic embryogenesis.

It was also shown that temporary immersion systems (TIS) increased the regenerative capacity of embryogenic cultures. Full developed somatic embryos showed a 30% maturation rate, and none of the plantlets obtained in petri dishes reached more than 6.4 cm in height, while 51% of the plantlets obtained from TIS were greater than 6.4 cm in height.

Steinmacher et al. (2007d) established a peach palm zygotic embryo cryopreservation protocol based on the encapsulation-dehydration technique, obtaining 29% of regrowth survival. It can thus be considered an alternative conservation strategy, or serve to complement other approaches (Steinmacher et al., 2007d). Cryopreservation of somatic embryos derived from known genotypes is the ideal goal for both capturing gains from breeding programs, as well as conservation of natural genetic diversity.

Protocols for cryopreservation of *B. gasipaes* embryogenic cultures have been recently developed in our laboratory. Somatic embryo clusters (SEC) submitted to plant vitrification solution 3 (PVS3; Sakai et al., 1990) for different periods resulted in different regrowth rates. The highest regrowth rate (52.4%) was obtained in response to droplet-vitrification technique combined with 120 min incubation time (Heringer et al., 2013). Droplet-vitrification success is largely attributed due to the fast freezing and thawing features of temperature exchanges provided by foil strip properties, thus decreasing the risk of the ice crystal formation which causes cell collapse (Yi, 2012).

Parallel studies on global DNA methylation dynamics with peach palm somatic embryos regrowth after cryopreservation have been also accomplished (Heringer et al., 2013). The results showed that global DNA methylation levels were affected by both cryoprotectants and the droplet-vitrification cryopreservation procedures. Incubation of SEC in PVS3, despite being essential for SE survival after contact with LN, not only reduced the regrowth rates in a time-independent manner, but immediately increased DNA methylation levels when compared to SEC not submitted to cryopreservation. Thus, during cryopreservation of somatic embryos, changes caused by the altered state of DNA

methylation have physiological consequences with further implications in the germplasm conservation of this species. Further investigations may elucidate whether these changes can be inherited by the next generation and whether the DNA methylation changes caused during cryoprotection might influence the quality of regenerated plants from these somatic embryos.

CONCLUSIONS

In the present review we presented and discussed the main features of three selected SE model-systems of native Brazilian woody plants belonging to three different taxonomic groups. Woody plants are considered recalcitrant to SE, so we selected these species aiming to shed light in the fundamental and control points of this in vitro morphogenetic route, as well as to identify comparative and differential features of these selected systems. Additionally, these SE systems encompass valuable biotechnological tools and applications, seeking the capture and fixation of genetic gains from elite genotypes and/or the conservation of rare and endangered germplasm via mass propagation assisted by the bioreactors technology.

These SE model systems fit to both scientific advances in the field of developmental physiology and biotechnological applications. Expressive advances were generated taking into account the particularities of each SE model studied by means of several approaches including molecular, physiological, biochemical and structural techniques. Further efforts are needed to expand the knowledge on the fine-tuning modulation and of these SE models, the improvements of protocols and the effective integration of these protocols into active breeding and conservation programs.

ACKNOWLEDGEMENTS

The authors are grateful to FAPESC, CNPq, and CAPES for fellowships, grants and other financial support in several projects associated to this review.

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