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A N I N T E R N A T I O N A L J O U R N A L

Effect of Carbon Source on Morphology and Histodifferentiation of *Araucaria angustifolia* Embryogenic Cultures

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ABSTRACT

The aim of the present work was to establish in vitro *conditions for the induction, stabilization and proliferation of embryogenic cultures of* A. angustifolia. *Pre-cotyledonary staged zygotic embryos inoculated BM medium supplemented with 5 µM 2,4- D, 2 µM BAP and Kin, and 3% maltose or sucrose resulted in 66.7% induction rate. The rate of induction of embryogenic cultures was affected by the carbon source, as well the multiplication and morphology of the embryogenic cultures. Embryogenic cultures maintained in BM medium with maltose presented bipolar morphology. Globular somatic embryos were obtained BM medium with 9% (PEG) and (9%) maltose. These results could establish an* in vitro *regenerative protocol towards the conservation and improvement of this important species.*

Key words: Cell competence, somatic embryogenesis, carbohydrate, conifers

INTRODUCTION

In conifers, remarkable advances were recently established in the protocols of somatic embryogenesis as is the case of *Pinus strobus* (Klimaszewska et al., 2000) and *Pinus pinaster* (Ramarosandratana et al., 2001), as well as for conifers threatened with extinction as *Cedrus libani* in Lebanon (Khuri et al., 2000) and *Araucaria angustifolia* (Bert) O. Kuntze (Silveira et al., 2002). This last species, native to Southern Brazil was severely logged during the last century. It presents a long life cycle their seeds are highly recalcitrant and the conventional methods for vegetative propagation are difficult to establish (Guerra et al., 2000).

Conifers present zygotic polyembryogenesis restricted to the early stages of embryo development. This remarkable adaptive and evolutionary mechanism may occur in response to the fertilization of the four ovules and/or by the cleavage of a single pro-embryo resulting in multiple embryos (Gifford and Foster, 1989). The reconstitution of polyembryos *in vitro* may occur by means of the somatic polyembryogenesis (Gupta and Grob, 1995).

Somatic embryogenesis relies in a sequence of steps including induction, proliferation,

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maturation, germination and finally *ex vitrum* acclimatization (Bozhkov et al., 2002). During embryogenesis, along with differentiation of apical and basal regions (morphogenesis), which establishes a polar axis, primary meristematic tissues and apical meristem are differentiated (histogenesis). For most coniferous species including *A. angustifolia*, the mechanism and proper regulation of somatic embryo formation has not been completely explained. The study of *in vitro* morphogenetic events is essential for the elucidation of control points associated with the induction and control of somatic embryogenesis in plants. In this study, we investigated some aspects associated with the induction, proliferation, development, and morphogenesis of embryogenic cultures of *Araucaria angustifolia*, with emphasis on the effect of carbohydrate source in the development of globular somatic embryos as fundamental steps towards a somatic embryogenesis protocol. The present work is part of a study involving the use of biotechnological tools for the conservation and improvement of this important and endangered Brazilian conifer.

MATERIAL AND METHODS

Plant material

From November 2002 to February 2003, immature female cones of *A. angustifolia* were collected from three plants of a natural population located at Bom Retiro County, Santa Catarina State, Southern Brazil. The cones were stored at 4ºC until has processing. The seeds were then isolated from the cones, immersed in alcohol (70%) 2 min; in sodium hypochlorite (2%) for 20 min and then washed three times with sterile water. In aseptic chamber, the immature zygotic embryos were isolated under stereomicroscope.

Induction of embryogenic cultures

The developmental stage of zygotic embryos used as explants was assessed at every collection time by staining with acetocamine (1%) and Evan's blue (0.5%) according to (Durzan, 1988). These explants were inoculated BM medium (Gupta and Pullman, 1991) supplemented with casein hydrolysate (0.5 g L^{-1}), L-glutamine (1 g L^{-1}), myo-inositol (1 g L^{-1}), 2,4-D (5μM), BAP (2 and 4μM) and Kin (2μM) and sucrose (3%) or maltose (3%). These compounds were filter-sterilized and added to the culture medium after autoclaving at 121 ºC for 15 min. The pH of the culture medium was adjusted to 5.8 before adding phytagel® (0.25%). Explants were inoculated in Petri dishes (90 x 10 mm) containing 25 mL of culture medium. The cultures were incubated in the dark at 25 ± 2ºC.

Proliferation of embryogenic cultures

After 30 days, the proliferating embryogenic cultures were subcultured to the same culture medium with the concentration of plant growth regulators reduced to half, subcultured three times every 20 days and then considered stabilized. The quality of cultures was evaluated by cytochemical procedures under light microscope based on acetocarmine and Evan's blue staining.

Somatic embryo development

Samples of 500 mg of embryogenic cultures originated the culture medium supplemented with sucrose (3%), or maltose (3%) and with 2,4-D (2μM), BAP and Kin (0.5 μM each) were subcultured Petri dishes containing 25 ml of BM culture medium supplemented with Kin (1μM), PEG (9%), maltose (9%) and sucrose (3%). The cultures were incubated in the dark at 25 ± 2ºC and subcultured every 20 days. Before submitted to maturation treatment, the cultures were evaluated according their morphological features based on von Arnold et al. (1996) and on cytochemical evaluations with acetocarmine and Evan's blue.

Experimental design and statistical analysis

All experiments were established in a completely randomized blocks design. The treatments were performed in triplicate and the data were submitted to the analysis of variance. Student-Newman-Keuls mean separation test was used to indicate significant differences (5%).

RESULTS AND DISCUSSION

Somatic embryogenesis initiation and proliferation responses

In this study, the relationship between collecting time and developmental stage of zygotic embryo was difficult to establish. For the same collecting date, embryos used as explants from the three genotypes reached different developmental stages, i.e. while proembryos were found in genotype A,

globular and torpedo embryos were present in genotype B and C, respectively (Table 1). Different maturation times of cones could explain there differences because *A. angustifolia* was a native non-domesticated species, possibly with two or more botanical varieties cohabiting in the same region (Reitz, 1966).

Differences in zygotic embryo maturation had direct effect in embryogenic induction rate (Table 2). In conifers, *in vitro* embryogenic induction rate was associated with several factors including culture environment, choice of explants, genotype and plant growth regulators (Zoglauer, 2003; Stasolla et al., 2003).

Table 1 - Developmental stages of *Araucaria angustifolia* zygotic embryos from three different genotype in different colleting times.

Genotype	Collecting time		
	15/12/02	**04/01/03**	**26/01/03**
A	Proembryo	Globular	Torpedo
B	Torpedo	Pre-cotyledonary	Cotyledonary
C	Globular	Torpedo	Pre-cotyledonary

The embryogenic induction in *A. angustifolia* was observed after 30 days culture. This process was characterized by the extrusion of a group of friable and translucent cells starting from the embryonal apex of the zygotic embryo with a subsequent enhanced cell proliferation. In all the induction tested, the embryogenic competence was influenced by the developmental stage of the explants (Table 2), the highest rate being observed in pre-cotyledonary zygotic embryos. Explants from proembryos and globular embryos showed induction rates of 13.3 and 31.3%, respectively. Zygotic embryos in cotyledonary stage resulted in low induction rates and these cultures did not show embryogenic competence (data not show). The embryogenic induction rates observed in the present work were higher than those observed in other conifer species such as *Pinus taeda* that presented a mean induction rate of 17.9% (Pullman et al., 2003).

The data obtained in the present work confirmed previous results with *A. angustifolia* (Guerra et al., 2000; Santos et al., 2002, Silveira et al., 2002) in which the pre-cotyledonary stage of developmental showed enhanced competence for embryogenic induction. These authors concluded that the competence for the induction of embryogenic culture was lost after the development of cotyledons. The strict requirement of juvenile explants, often of embryonic origin, indicated that re-direction of developmental programs in conifers was difficult to achieve in *in vitro* cultures (Stasolla et al., 2003).

The mechanism of embryogenic cultures induction is not fully understood. According to Zoglauer (2003), during the induction phase, cells cease their program of specialization and start a new developmental program. The specialization program is negatively affected by the methylation of DNA for which the auxins may play a fundamental role (Lo Schiavo et al., 1989). The carbon source and plant growth regulators supplemented to the culture medium significantly affected the embryogenic induction rate. Sucrose was superior to maltose (Table 3). Culture media supplemented with sucrose also showed an enhanced cell proliferation, specially in combination with 2,4-D (5μM), BAP and Kin (2μM each). However, the supplementation of the culture medium with maltose (3%) plus 2,4-D (5μM) and BAP and Kin (2μM each), even resulting in a reduced cell proliferation, favored the further development of somatic pro-embryos with bipolar morphology which had their cells reactive to acetocarmine (Figure 1d). Most of the pro-embryos developed in the culture medium supplemented with sucrose were less organized showing disturbed polarity and a diffuse morphology (Figure 1c). Similar patterns were observed in *Picea abies* embryogenic cultures (Filonova et al., 2000).

According to Blanc (2002), the effect of maltose in the morphology and histodifferentiation of embryogenic cultures could be ascribed to the low supply of hexoses. This suggested that the slow hydrolysis of maltose was the biochemical signal conducting to the formation of somatic embryos. On the contrary, rapid hydrolysis of sucrose could increase the content of hexoses and storage compounds, directing the cells to a fast proliferation rates (Blanc, 2002), which was consistent with the results observed in the present

work. In embryogenic cultures of *A. angustifolia*, the supplementation of culture medium with sucrose was an event associated with the unorganized cell proliferation, while maltose was possibly the key factor to start the organized embryonic development.

Table 2 - Percentage of embryogenic cultures of *Araucaria angustifolia* induced from zygotic embryos in different developmental stages in culture medium BM supplemented with maltose or sucrose.

Developmental Stage	Sample Size	Induction Rate (%)[1]
Pro-embryo	180	13.3 c
Globular	360	31.3 b
Torpedo	540	52.8 a
Pre-cotyledonary	360	66.7 a
Mean		41.09
CV (%)		32.9[2]

[1] Means followed by same letters are not statistically different according to the SNK test (5%). 2- Data transformed to log (x+2).

Table 3 - Effect of carbon source in the induction of *Araucaria angustifolia* embryogenic cultures.

Carbon Source	Sample Size	Induction Rate (%)[1]
Sucrose	720	56.6 a
Maltose	720	35.4 b
Mean		46.0
CV (%)		33.4[2]

[1] Means followed by same letters are not statistically different according to the SNK test (5%). 2- Data transformed to log (x+2).

Cell morphology and embryogenic competence

Embryogenic cultures of *A. angustifolia* presented mainly two cell types: elongated cells, which were vacuolated (Figure 1b), and isodiametric cells, which were smaller and with a dense cytoplasm (Figure 1a). These cells were clustered into aggregates of proliferating embryogenic cultures, showing variations in size and morphology and revealing different affinity to the staining procedures. Small cells were reactive to acetocarmine and stained bright red (Figure 1a) and elongated cells were permeable to Evan's blue (Figure 1b). The positive reaction to acetocarmine (Durzan, 1988) and the formation of somatic pro-embryos with bipolar morphology (von Arnold et al., 1996) are features associated with the cell competence to develop complete somatic embryos after maturation treatments.

In several *in vitro* conifer embryogenic systems the cell morphology may be associated to the embryogenic competence (Egertsdotter and von Arnold, 1998) and the differences in the cell morphology may be influenced by the explant source and culture medium composition (Fehér et al., 2003).

In the present work, the carbon source significantly affected the induction rates and morphological organization of the embryogenic cultures. The requirement of a certain degree of morphological organization within the embryogenic culture prior to exposure to maturation treatment was also proposed in previous investigations (Jalonen and von Arnold, 1991; Bozhkov et al., 2002; Stasolla et al., 2003).

In *Picea abies*, embryogenic cell lines were classified in two groups based on the cell morphology (Mo et al., 1996). Cells of the group A, contained large embryogenic heads, formed complete somatic embryos in response to the maturation treatments, and cells of the group B, smaller and structurally disorganized embryogenic heads resulted in abnormal or few somatic embryos. In accordance with this classification, in the present work, two distinct cellular groups based on morphology as well as on developmental pattern were observed. Embryogenic cultures of *A. angustifolia* induced and maintained in BM medium supplemented with maltose presented bipolar morphology (type A), in that the pro-embryo are formed by aggregation of embryogenic

cells attached to suspensors cells (Figure 1d). Cultures maintained in medium with sucrose presented diffused morphology (type B), in which the polarity was disturbed and the pro-embryo consisted in an embryogenic cells aggregates surrounded by suspensors cells (Figure 1c).

The developmental transition from pro-embryonic cultures to globular somatic embryos seemed to be a critical point in *A. angustifolia* somatic embryogenesis and the presence of maltose in the culture medium was a determinant factor for this transition leading to the histodifferentiation of subsequent developmental stages of somatic embryos.

Somatic embryo development

Embryogenic cultures of *A. angustifolia* originated in the inductive culture medium supplemented with sucrose or with maltose and plant growth regulators were responsive to the maturation treatments. Globular somatic embryos developed in culture medium supplemented with PEG (9%) and maltose (9%). It has been shown that the early stages of somatic embryogenesis are crucial for successful completion of the overall process. In *Picea alba*, even though similarities in transition process from embryogenic culture with different degree of morphological organization to complete developed somatic embryos were observed, this morphological organization limited the yield of somatic embryos (Filonova et al., 2000). Cytochemical analysis of *A. angustifolia* globular somatic embryos revealed that the suspensor cells were degraded and early somatic embryos appeared structurally organized (Figure 2c). Further developmental stages were not observed in response to the maturation treatments. Maltose and PEG were also effective in the promotion of development of late embryogenic stages of *Pinus taeda* (Li et al., 1998) and *Pinus pinaster* (Ramarosandratana et al., 2001). It was postulated that PEG generated a osmotic stress in the cells with a consequent alteration of phase change (Svobodová et al., 1999). This compound was also associated with lipid, starch and protein accumulation in somatic embryos (Misra et al., 1995). The PEG-stimulatory effects are important in order to allow the organization of developing embryos (Stasolla et al., 2003). In the present work, the presence of PEG in culture medium, although necessary for embryo development, was not sufficient for the completion of the maturation program.

Also, it has been proposed that the carbohydrates supplemented to the culture medium may play multiple roles, including the histodifferentiation of somatic embryos by means of the direct regulation of gene expression (Lipavska et al., 2000). Some authors (Finkelstein and Gibson, 2001; Leo and Shee, 2003) suggested that maltose could activate a common metabolic route with ABA. It was not clear that by which mechanism maltose promoted the development of globular somatic embryos in *A. angustifolia*. In the present work it was observed that the supplementation of the culture medium with maltose was fundamental for the morphological re-organization and histodifferentiation of the embryogenic competent cells leading to the developmental stage.

The transition from pro-embryogenic cultures to somatic embryo is a critical developmental event affecting the final yield of somatic embryos produced in culture. Thus, a proper understanding of the factors regulating these developmental processes would be of great value for the improvement of the overall embryogenic process.

In conclusion, in the present work we were successful in establishing *in vitro* conditions for the induction, stabilization and proliferation of embryogenic cultures of *A. angustifolia* as well as in describing patterns of morphogenesis and hystodifferentiation of embryogenic cultures and somatic embryos of *A. angustifolia* as affected by the carbohydrate source supplemented to the culture medium. The development of a complete regenerative protocol based on this morphogenetic route could be envisaged from the results obtained. The final goal is the development of biotechnological tools for the conservation and improvement of this important and endangered Brazilian species. The comparatively high rate of embryogenic induction and cell proliferation observed represent an additional advantage of this *in vitro* morphogenetic system for advanced studies in developmental and cell biology in plants starting from undifferentiated tissues.

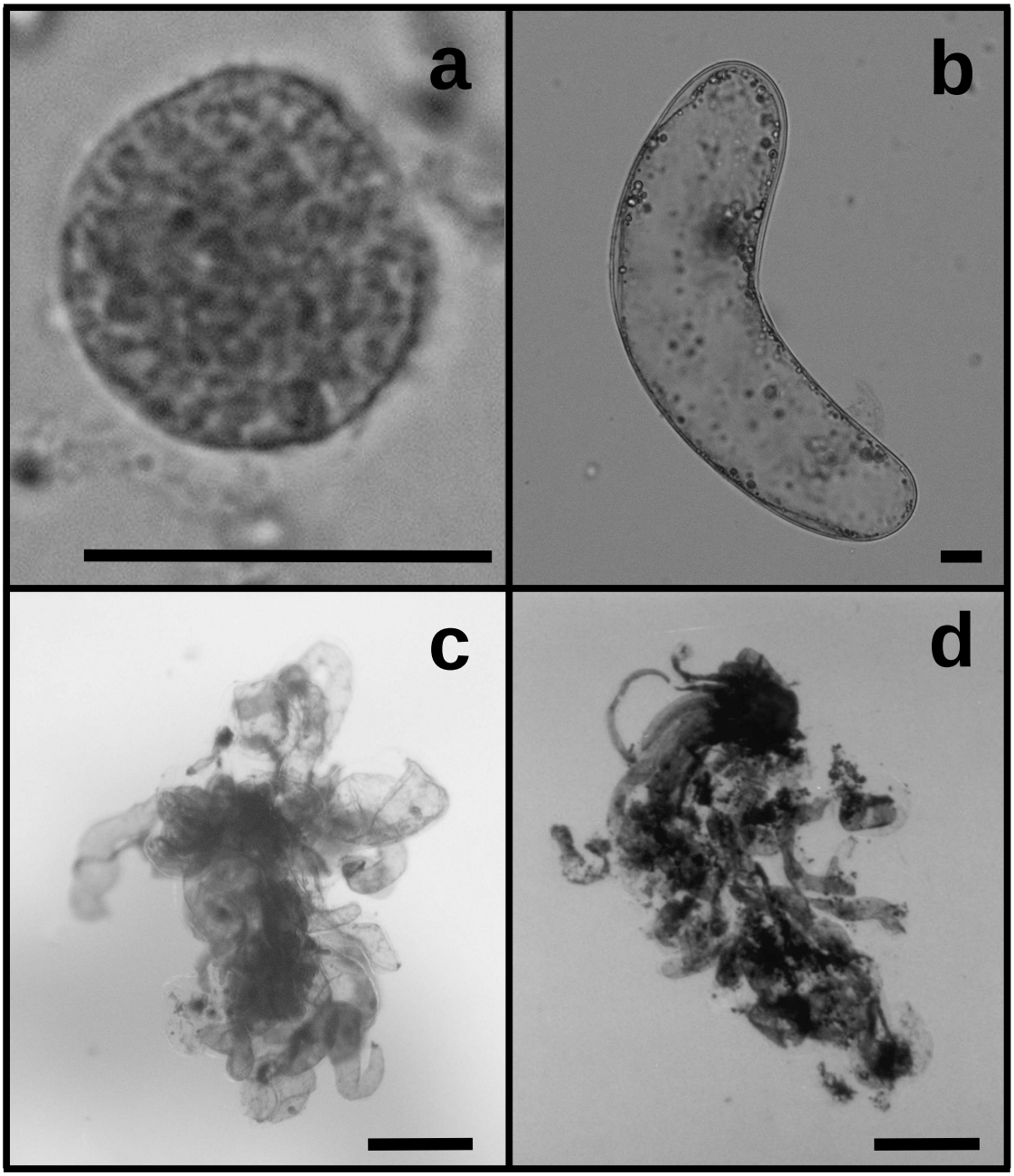

Figure 1 - Embryogenic culture of *A. angustifolia* stained with acetocarmine and Evan's blue. a) small isodiametric cells reactive to acetocarmine, b) elongated and vacuolated cells permeable to Evan's blue, c) Cultures maintained in BM medium with sucrose presented diffuse morphology, in that the polarity is disturbed and the pro-embryo consists in an aggregate of embryogenic cells surrounded by suspensors cells, d) embryogenic cultures induced and maintained in BM medium supplemented with maltose with bipolar morphology. The pro-embryo is formed by aggregates of embryogenic cells attached to suspensors cells (Bars a,b = 15μm and b,c = 250μm).

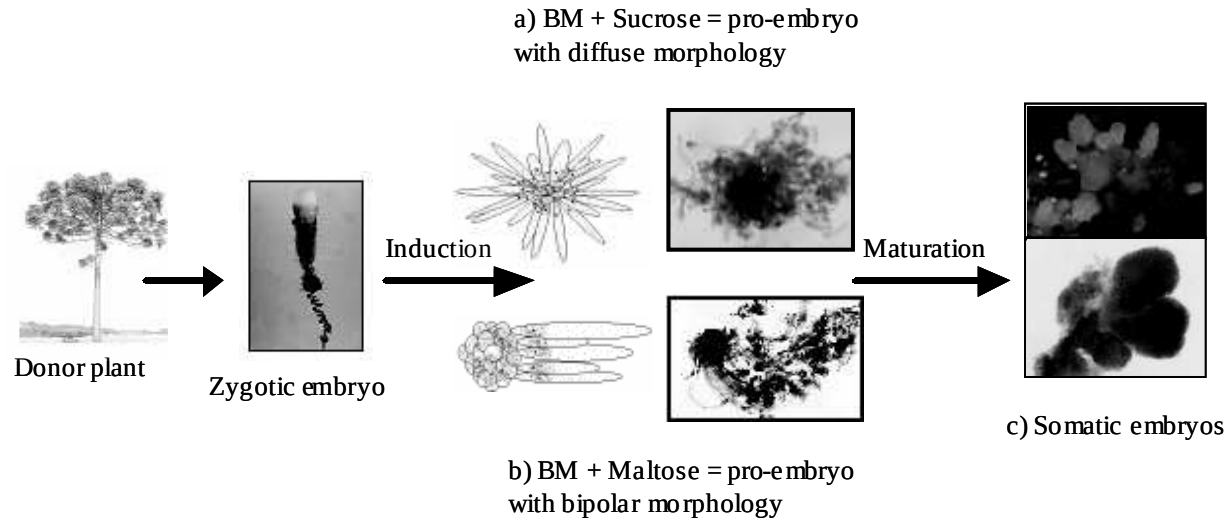

Figure 2 - Diagram of the morphogenetic events associated to the somatic embryogenesis protocol in *A. angustifolia*. a,b) Effect of carbon source in the morphology of embryogenic cultures and hystodifferentiation of somatic embryos, c) Globular somatic embryos obtained on BM medium culture supplemented with 9% (PEG) and (9%) maltose**.**

RESUMO

No presente trabalho foram investigadas as condições para a indução, estabilização e proliferação de culturas embriogênicas de *A. angustifolia*. Foram também descritos os padrões de morfogênese e histodiferenciação de culturas embriogênicas e embriões somáticos desta espécie em resposta a diferentes fontes e concentrações de carboidratos. Embriões zigóticos no estágio pré-cotiledonar, inoculados em meio de cultura BM, suplementados com 5 µM de 2,4-D, 2 µM de BAP e Kin e 3% de maltose ou sacarose resultaram em uma taxa de indução de 66,7%. A fonte de carbono afetou a taxa de indução, a multiplicação e a morfologia das culturas. Culturas embriogênicas mantidas em meio de cultura BM suplementado com maltose apresentaram morfologia bipolar. Embriões somáticos globulares foram obtidos em meio de cultura BM suplementado com PEG e maltose. Os resultados obtidos permitirão avançar na direção de um protocolo regenerativo *in vitro* visando à conservação e o melhoramento genético desta espécie.

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