BRIEF COMMUNICATION

Somatic embryogenesis in *Araucaria angustifolia*

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Abstract

Immature and mature zygotic embryos were used as source of explants for induction of somatic embryogenesis in *Araucaria angustifolia*. Embryogenic cultures (EC) were only obtained from immature zygotic embryos. Basic medium, carbon source, and genotype showed a significant influence on the formation of stage I somatic embryos (SE). When EC were submitted to maturation conditions, SE continued their individual development until stage II, but mature embryos were not obtained. Proteins secreted by embryogenic cultures were, to a certain degree, genotype specific and included an extracellular class IV chitinase and β-1-3-glucanase.

Additional key words: *Araucariaceae*, cell suspension cultures, extracellular proteins.

A somatic embryogenesis protocol has been developed for *Araucaria angustifolia*, a threatened subtropical conifer species from South Brazil. However, the use of somatic embryogenesis for clonal propagation and *ex situ* conservation of *A. angustifolia* has been hampered by the low number of bipolar somatic embryos (SE). Optimization of the culture conditions early in the process and the characterization of proteins directly involved in SE development can increase both number and quality of embryos produced in culture (Stasolla and Yeung 2003, Lippert *et al.* 2005).

The objective of the present experiments were: *1*) to test mature zygotic embryos as a source of explants for the induction of EC in *A. angustifolia*, *2*) to compare different basic media and carbon sources, *3*) to describe the pathway of development of early SE in consideration of different genotypes, *4*) to analyze the pattern of extracellular proteins with emphasis on the pathogenesis related proteins β-1,3-glucanase and class IV chitinase in suspension cell cultures, and *5*) to test the capacity of established EC for maturation of SE.

Immature cones and mature cones of *Araucaria angustifolia* (Bert) O. Ktze were collected, from open-pollinated trees grown in South of Brazil. Embryogenic cultures (EC) initiated from immature zygotic embryos were cultivated on BM basic medium (Gupta and Pullman 1991) containing 5 μM 2,4-dichlorophenoxy-acetic acid (2,4-D), 2 μM 6-benzylaminopurine (BA) and 2 μM kinetin (Kin) in the dark at 25 ± 2° C as described by Santos *et al*. (2002). Mature zygotic embryos were inoculated in 25 cm^3 of MSG basic medium (Becwar *et al*. 1989) supplemented with 1.46 g dm^{-3} L-glutamine, 30 g dm^{-3} sucrose, 3 g dm^{-3} *Gelrite*® (*Carl Roth GmbH & Co*., Karlsruhe, Germany) and different concentrations of 2,4-D (5, 10, 20 μM) and BA (5, 10 μM). Petri dishes containing explants were incubated in the dark at 25 ± 2 ° C. Twelve repetitions of each treatment were employed, each repetition consisting of a Petri dish inoculated with three explants. After 30 d, Petri dishes were evaluated for induction of SE.

In order to improve conditions for the establishment of EC and development of stage I SE (see Hakman and Von Arnold 1988), the basic Murashige and Skoog (1962; MS) medium supplemented with 0.75 g dm^{-3}

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Abbreviations: ABA - abscisic acid; BA - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; Kin - kinetin.
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L-glutamine, MSG supplemented with 1.46 g dm^{-3} L-glutamine, and LM medium (Litvay *et al.* 1981) and carbon sources (30 g dm^{-3} of sucrose, lactose or maltose) were tested. After 14 d of culture the number of EC containing stage I SE on the culture surface was counted. Cell suspension cultures (Ar1, Ar5, and Ar6) were established according to Rahmat (2002) with minor modifications. Cell suspensions were subcultured on culture medium MSG supplemented with 1 μM 2,4-D and 0.5 μM BA (MSG-1) in intervals of 12 d in the dark at 25 ± 2° C, rotated at 120 rpm in orbital shakers. The development of early stage SE was monitored by light microscopy using aliquots of 1 cm^3 suspension cells stained with 1 % (m/v) acetocarmine and 0.05 % (m/v) Evans blue (Gupta and Durzan 1987).

For analyses of extracellular proteins the liquid medium from cell suspension cultures Ar1, Ar5, and Ar6 was harvested. Aliquots of 2 cm^3 from filtered medium were desalted on prepacked *Sephadex G-25* columns (*PD-10*, *Amersham Bioscience*, Uppsala, Sweden). Eluates were lyophilized and resuspended in water. Protein concentration was determined by the method of Bradford (1976). SDS-PAGE of extracellular proteins was performed according to Laemmli (1970). Immunoblotting was realized according to Wiweger *et al.* (2003). Polyclonal antibody against class IV chitinase (Mikkelsen *et al.* 1992) and polyclonal antibody against β-1,3-glu-canase were used as primary antibody.

Established clones Ar1, Ar5, and Ar6 were screened for their capacity to develop later stages of SE. As a maturation medium, the following combinations were investigated: *a*) ½ BLG (Verhagen and Wann 1989) supplemented with 0.05 g dm^{-3} L-asparagin, 0.75 g dm^{-3} L-glutamine, 7.5 % (m/v) polyethylene glycol (PEG) 4000, 30 g dm^{-3} maltose, 3 g dm^{-3} *Gelrite*®, and different concentrations of abscisic acid (ABA; 0, 5, 15, 40, 80, 120, 240 μM) *b*) MSG supplemented with 1.46 g dm^{-3} L-glutamine, 6 % (m/v) PEG 3350, 60 g dm^{-3} maltose, 30 g dm^{-3} sucrose, 3 g dm^{-3} *Gelrite*®, and ABA (0, 15, 40, 80 μM). EC were maintained in the dark at 25 ± 2 °C and subcultured every 8 weeks. After 16 weeks, the number of SE, their developmental stage and morphology were evaluated. Data were subjected to analysis of variance (*ANOVA*), followed by the SNK test, both at a significance level of $\alpha = 0.05$.

When pre-cotyledonar zygotic embryos of *A. angustifolia* were cultured on medium supplemented with auxin (2,4-D) and cytokinin (BA and Kin), a white translucent mucilaginous cell mass developed after 3 weeks (Astatita and Guerra 1998). Although in some conifers, somatic embryogenesis can be achieved from mature zygotic embryos (Harry and Thorpe 1991, Zoglauer *et al.* 2003) it was impossible to obtain induction of SE using mature zygotic embryos as source of explants in *A. angustifolia*. However, for the first time in *A. angustifolia*, it was possible to obtain the development of apical buds from the embryonal shoot meristem in 52.3 % of explants on medium free of growth regulators (MSG-0). However, under the conditions tested root formation did not occur and germination was not completed.

Of the proliferation media tested, the formation of stage I SE was observed only in EC maintained on MSG basic medium. The development of at least one stage I SE was registered in eight (66.6 %) out of twelve repetitions. In conifers, the capacity of EC to develop stage I SE during the proliferation phase is considered important for predicting yield and quality of SE during maturation trials (Bozhkov *et al.* 2002). So far, a similar degree of development was obtained in *A. angustifolia* only after inclusion of osmotic agents in culture medium but independent on application of ABA (Santos *et al.* 2002, Silveira *et al.* 2002).

After 14 d of culture, the use of different carbon sources and genotypes resulted in significant differences concerning formation of stage I SE (Table 1). The highest percentage of stage I embryos occurred on media supplemented with sucrose. Considering the influence of genotype, clone Ar5 was superior to clones Ar1 and Ar6 for development of SE.

Table 1. Effect of carbon source (sucrose, maltose and lactose) and genotype on the formation of stage I somatic embryo (SE) in embryogenic cultures of *A. angustifolia*. The sugars were applied to the culture medium MSG at 30 g dm^{-3}. The average number of stage I embryos were recorded after 2 weeks. Experiments were repeated twice. Means of thirteen replications followed by different letters in the column are significant at the 0.05 level according to SNK test.

Sugar	Number of SE [culture $dish^{-1}$]	Genotype	Number of SE [culture $dish^{-1}$]
Sucrose	1.37a	Ar1	0.47b
Maltose	0.23b	Ar5	0.87a
Lactose	0.03b	Ar6	0.30b

During proliferation of EC of *A. angustifolia*, sucrose is rapidly hydrolyzed to glucose and fructose, suggesting high invertase activity (Astarita and Guerra 2000). In *Picea mariana* and *Picea glauca*, invertase activity was normally higher during early stages of SE development compared to late stage embryos. In both species, a high cellular hexose/sucrose ratio seemed to be necessary during periods of intensive growth and cell division as observed during maintenance of EC (Iraqui and Tremblay 2001).

The development of early SE in *A. angustifolia* started from bipolar structures composed of irregular cell clumps containing cells with densely cytoplasm that react positively to acetocarmine stain (embryogenic cells), connected with a single or a few elongated and vacuolated cell showing reactivity to Evan's blue (suspensor-like cells) (Fig. 1*A*). These structures resemble the description of proembryogenic masses given by Filanova *et al.* (2000) in *P. abies* and for early SE in *Pinus nigra* (Jasik *et al.* 1995). In *A. angustifolia*

cultures, these structures developed new, vacuolated cells (secondary suspensor, Fig. 1*B*) until the formation of stage I SE (Fig. 1*C*). When cultured on semi-solid medium, early SE were visible on the culture surface (Fig. 1*D*).

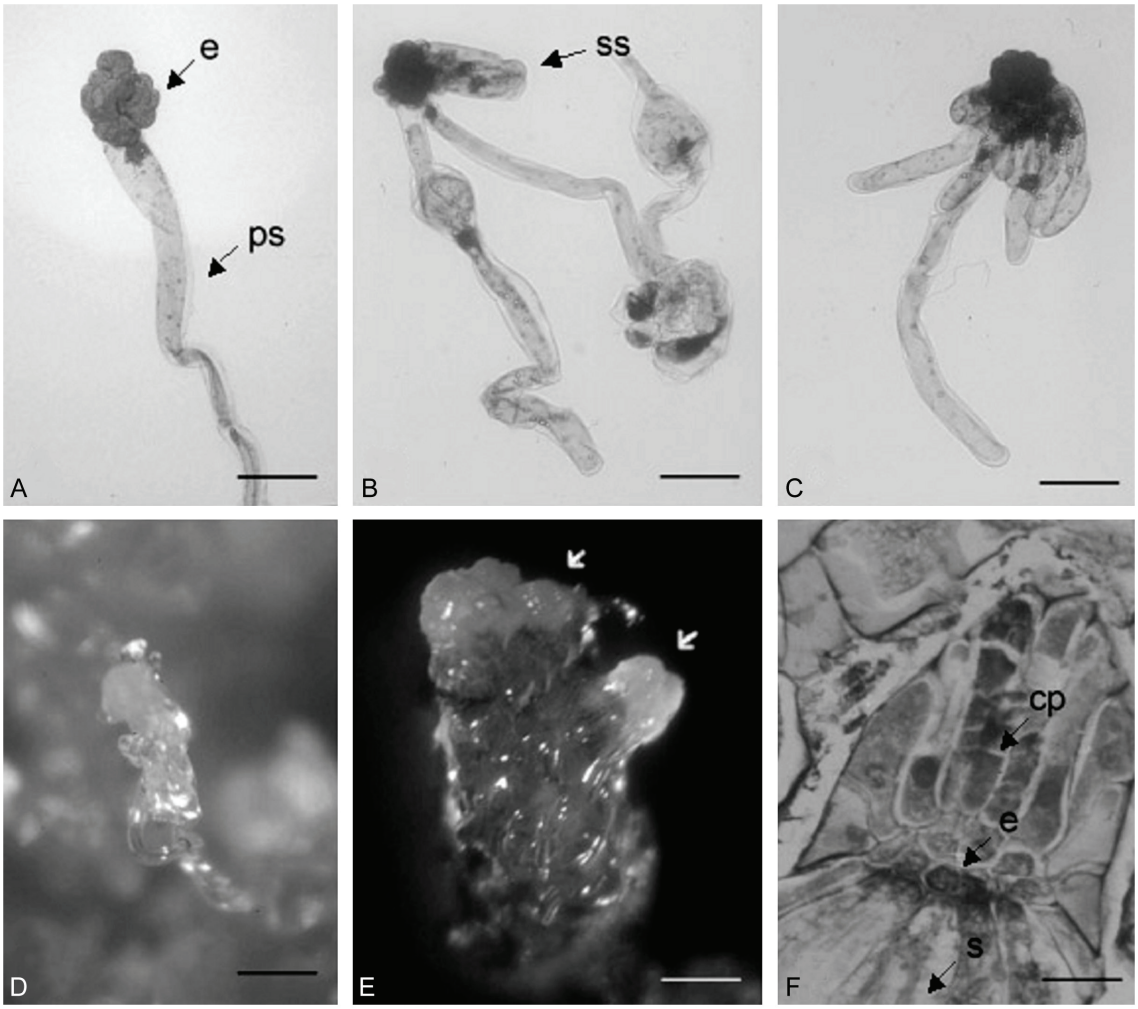

Fig. 1. Developmental pathway of early somatic embryos and zygotic embryo structure during early embryogenesis in *A. angustifolia*. *A* - early somatic embryo showing a clump of embryogenic cells (e) adjacent to a suspensor like cell (ps); *B* - development of secondary suspensor cells (ss) in early somatic embryos; *C* - stage I somatic embryo isolated from cell suspension cultivated in MSG-0 liquid medium; *D* - stage I somatic embryo anchored to the embryogenic culture by its secondary suspensor; *E* - early somatic embryos showing cleavage polyembryony (*arrow*). *F* - zygotic embryo formed by cap cells (cp), embryo cells (e) and suspensor cells (s); (*bar*: 140 μm in *A* and *C*, 180 μm in *B*, 0.3 mm in *D* and E, 107.5 μm in *F*).

During culture we observed cleavage polyembryony in early SE (Fig. 2*E*). In *Araucariaceae*, only simple polyembryony has been reported (Haines and Prakash 1980). In early embryogeny of *A. angustifolia* the upper peripheral proembryonal cells form the suspensor, the lower cells give rise to the cap, and the central cells take part in forming the embryo (Fig. 2*F*). The presence of cap cells, an exclusive early embryogenic feature present in the *Araucariaceae* family, is supposed to prevent the formation of new embryos by cleavage during early seed development (Haines and Prakash 1980). However, cap cells were never observed *in vitro* during early SE *A. angustifolia.* Similarly, no analogy between the earliest stages of SE development and the corresponding stage in zygotic embryo development was seen in *Larix deciduas* (Korlach and Zoglauer 1995) and *P. abies* (Filanova *et al.* 2000).

Despite recent reports (Salajová and Salaj 2005, Vooková and Kormuťák, 2006) stressing the importance of the degree of SE organization before the exposure to ABA, well organized EC of *A. angustifolia* did not develop fully mature SE when exposed to different ABA concentrations (5 - 240 μM). In medium containing 3 % sucrose, 6 % maltose, and 6 % PEG 3350, the development and frequency of stage II SE was improved. However, only Ar5 developed stage II embryos with

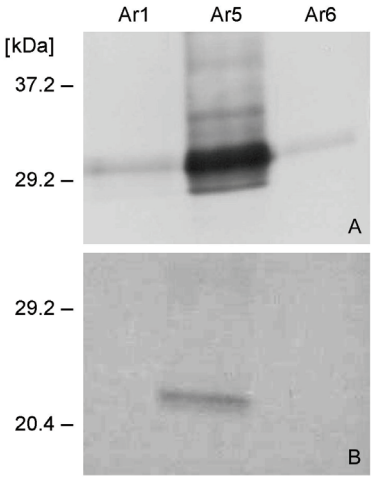

Fig. 2. Detection of extracellular proteins in conditioned medium of cell suspensions of the clones Ar1, Ar5 and Ar6 in *A. angustifolia*. *A* - immunoblot of an SDS PAGE gel showing cross reactivity between extracellular proteins and polyclonal antibody raised against class IV chitinase; *B* - immunoblot of an SDS PAGE gel showing cross reactivity between extracellular proteins and polyclonal antibody raised against β-1,3-glucanase.

smooth outer surface indicative of the development of a protodermal layer, exhibiting morphology similar to its zygotic embryo counterpart.

In EC Ar5, a *ca*. 30 kDa band strongly cross reacted with the antibody specific for class IV chitinase (Fig. 2*A*). For EC Ar1 and Ar6, the same band was detected at low intensity after prolonged exposure of the membrane to alkaline phosphatase staining. Using the β-1,3 glucanase antibody, a 21 kDa band was observed only in the medium of EC Ar5 (Fig. 2*B*).

It has been suggested that during *in vitro* SE, extracellular chitinases (*i.e*. specially of class IV) are able to cleave glycosidic bond of glucosamine and N-acetyl-glucosamine residues present in the sugar moiety of arabinogalactan proteins (Van Hengel *et al.* 2001) generating oligossacharide signal molecules essential for early stages of plant embryogenesis (Malinowski and Filipecki 2002). In embryogenic cultures of *Cichorium*, the presence of a β-1,3 glucanase (37.7 kDa) positively correlated with the formation of SE (Helleboid *et al.* 1998). The presence of extracellular chitinases and β-1,3-glucanases might be considered an indicator of a genotypic superiority of clone Ar5 for the development of SE.

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