Biotechnology tolls in *Araucaria angustifolia* conservation and improvement: inductive factors affecting somatic embryogenesis

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ABSTRACT

Biotechnological techniques based on somatic embryogenesis have been employed for capturing genetic gains in forest breeding programs. The present work aimed at studying the factors associated with the induction of somatic embryogenesis in *Araucaria angustifolia*. Zygotic embryos in different developmental stages were inoculated in a LP medium supplemented with 2,4-D, BA and KIN. Embryogenic cultures were inoculated in a LP liquid medium free of plant growth regulators. ABA and/or PEG 4000 were evaluated for the development of somatic embryos. The embryogenic induction was dependent on the genotype of the mother plant, ranging from 21.9% to 55.2%. Suspension cultures showed a 5.6 times increment of the initial cell volume after 33 days in the culture medium free of plant growth regulators. It was possible to establish and multiply autonomous embryogenic cultures. The medium containing ABA (50 mM) and PEG (1%) resulted in the development of globular and torpedo somatic embryos.

KEY WORDS: Brazilian pine, improvement, conservation, somatic embryogenesis, autonomous, suspension cultures.

INTRODUCTION

Araucaria angustifolia is the only native conifer of economic importance in Brazil, representing the most exploited timber source until the 70's (Astarita and Guerra, 1998). This species is a valuable source of seeds, wood, fiber and resin. The seeds present high nutritious value being consumed by humans and the wild fauna. The wood of adult trees is employed in furniture, structural timber, and almost all other kinds of wood applications (Carvalho, 1994).

Originally, the forests of *A. angustifolia* covered an area of 20 millions hectares in the states of Rio Grande do Sul, Paraná and Santa Catarina. As the *A. angustifolia* exploitation progressed the natural reserves decreased (Ondro et al., 1995), as a result of the clear-cutting form of exploitation. Nowadays, only relicts of the natural vegetation are found, representing 1 to 2% of the original area (Guerra et al., 2000). More recently, logging was directed to the natural relicts in such intensity that this species was included in the official list of endangered Brazilian plants, under the "vulnerable" category.

The development of technologies for the conservation and genetic improvement of *A. angustifolia* is required if we intend to develop reforestation programs with this species. It has been demonstrated that, in good production sites, this species shows similar increment rates and biomass production as *Pinus taeda* or *Pinus elliottii*, the most common exotic cultivated conifers in Brazil (Guerra et al., 2000).

Vegetative propagation brings significant advantages for both breeding and mass propagation of genetically improved genotypes. Today, the use of cuttings is the most common technique used to carry out the vegetative propagation of forest trees. However, this technique has some drawbacks such as the decrease of rooting ability with the aging of the donor plant and the limited number of plantlet produced (Högberg et al., 1998). More recently, research efforts have focused on the application of biotechnological tools, mainly somatic embryogenesis (Merkle and Dean, 2000). Somatic embryogenesis has been an effective supplement to a variety of different goals in tree improvement programs (von Aderkas et al., 2001).

The applications of somatic embryogenesis include the provision of cell lines for genetic transformation, the *ex situ* conservation of rare and endangered species or populations, the research to improve our understanding of conifer genetics, and the generation of high-value clonal forestry which is the most promising application (Park et al., 1998).

Studies with *A. angustifolia* somatic embryogenesis have been done by Astarita and Guerra (1998) and Guerra et al. (2000). However, the published protocols showed limited efficiency to obtain somatic embryos of *A. angustifolia* following the proembryo stage.

The present studied investigated the effect of the mother plant genotype, the developmental stage of explants, as well as the effects of the plant growth regulators and polyethylene glycol (PEG) on the induction, growth and development of embryogenic cultures of *A. angustifolia*.

MATERIAL AND METHODS

Immature female cones of *A. angustifolia* were collected from two genotypes in Bom Retiro county, Santa Catarina State, in four dates: 11/22/97; 12/12/97; 01/04/98 and 01/22/98, representing different developmental stages. Seeds were removed from the cones, surface sterilized with 70% ethanol for 2 min and subsequently in a 40% commercial bleach solution (2-2.5% sodium hypochloride) for 10 min, and then rinsed four times with sterilized water. Immature zygotic embryos were excised from the seeds and used as primary explants (Figure 1).

The four induction treatments were based in the LP culture medium (von Arnold and Eriksson, 1981) free of plant growth regulators (D0), LP medium supplemented with 2 µM 2,4-dichlorophenoxy acetic acid (2,4-D), 0.5 µM N^6-Benzylaminopurine (BA) and 0.5 µM Kinetin (KIN) (D2), LP medium supplemented with 5 µM 2,4-D, 2 µM BA and 2 µM KIN (D5), and LP medium supplemented with 10 µM 2,4-D, 4 µM BA and 4 mM KIN (D10). Sucrose (3%) was added to culture media supplemented with 0.65% Agar (Difco). The pH of the culture media was adjusted to 5.8 prior to sterilization in autoclave (121ºC, 1.5 atm, 15 min). The solution containing the LP vitamins, 0.5 $g.L^{-1}$ casein hydrolysate, 0.45 $g.L^{-1}$ L-glutamine and 0.1 $g.L^{-1}$ inositol, was filter sterilized and added to the autoclaved basal culture medium.

Ten explants were inoculated in a 100 x 15 mm petri dish containing 25 mL inductive culture medium. The cultures were kept in the dark at 25±1ºC and the induction percentage was evaluated weekly under stereomicroscope for up to 8 weeks. Afterwards, the embryogenic cultures induced in the LP medium free of plant growth regulators were transferred to a fresh medium with the same composition. Those cultures induced in the LP medium supplemented with plant growth regulators were transferred to a fresh medium supplemented with 5 mM 2,4-D, 2 mM BA and 2 mM KIN. During the maintenance phase, the embryogenic cultures were sub- cultured to the same culture medium composition every three weeks.

The double-staining procedures with acetocarmine (2%) and Evan's blue (0.1%) described by Gupta and Durzan (1987) were used to evaluate the embryogenic cultures. The stained embryogenic cultures were observed under a BX-40 Olympus light microscope.

The treatment combinations from the three factors (explant developmental stage, genotype and culture medium) were replicated three times, being the plots completely randomized in a factorial design. The rate of induction was obtained from the number of explants that produced a translucent white and mucilaginous cell masses in relation to the total. Data were transformed to $(x+1)^{0.5}$ according to Compton (1994), and submitted to an analysis of variance. Mean separation was tested using the Student-Newman-Kuels (SNK) test at the 0.05 probability level (Steel and Torrie, 1980).

Growth dynamic of suspension cultures

Embryogenic cultures (1.5 g) maintained in LP solid medium supplemented with 5 µM 2,4-D, 2 µM BA and 2 µM KIN (LP-D5) or in LP liquid medium free of plant growth regulators (LP-D0) were inoculated in three adapted nipple flasks containing 100 ml of LP-D5 medium or LP-D0, according to the maintenance medium. The nipple flasks were incubated in the darkness on an orbital shaker at slow rotation (1 rpm). The sedimented cell volume was evaluated every three days by inverting the position of the flasks, using the graduated centrifuge of the adapted nipple flasks.

Somatic embryo development

The somatic embryo development experiment was performed by using of embryogenic cultures induced and maintained in a LP medium free of plant growth regulators. The LP culture medium supplemented with 0, 10, 20 or 50 µM of ABA and 0, 1, 3, or 7.5% of PEG 4000 was tested. The culture medium was supplemented with 3% sucrose, 0.65% agar, 0.5 $g.L^{-1}$ casein hydrolysate, 0.45 $g.L^{-1}$ L-glutamine and 0.1 $g.L^{-1}$ inositol. The pH was adjusted to 5.8 prior to sterilization. The ABA, vitamins, casein hydrolysate, L-glutamine and inositol were filter-sterilized and added to the autoclaved media. The double-staining procedures with acetocarmine (2%) and Evan's blue (0.1%) described by Gupta and Durzan (1987) and morphological observations were used to evaluate the

progression to late embryogenic developmental stages according to those described by Tautorus et al. (1991). The stained embryogenic cultures were observed under a BX-40 Olympus light microscope and the culture morphologies were observed under a SZH-10 Olympus stereomicroscope.

RESULTS

Induction of embryogenic cultures

Induction was characterized when the explants gave rise a translucent white and mucilaginous cell masses (Figures 2 and 3). After eight weeks in the inductive media, the general average of embryogenic induction was 38.5% (Table 1). The developmental stage of the explant and the genotype revealed statistically significant effect ($P<0.01$) on the induction percentages of embryogenic cultures. The addition of plant growth regulators to the culture medium did not resulted in statistically significant effects ($P<0.01$). In addition, two single interactions (developmental stage either with genotype and culture medium) as well as the triple interactions were statistically significant ($P<0.01$).

Effect of the genotype on the embryogenic induction

Induction of embryogenic cultures in *A. angustifolia* was obtained in both genotypes tested, with values ranging from 21.9% to 55.2%. Data analysis showed a significant effect ($P<0.01$) of the mother plant genotype on the embryogenic induction rates (Table 1).

Table 1. Percentage of embryogenic cultures induced from immature zygotic embryos of two genotypes of *Araucaria angustifolia.*

Genotype	Sample size	Mean [1/]
Plant 1	24	21.9 b
Plant 2	24	55.2 a
Mean		38.5
Coefficient of variation (%)		47.7

[1/] Means followed by same letters are not statistically different according to the SNK test (5%).

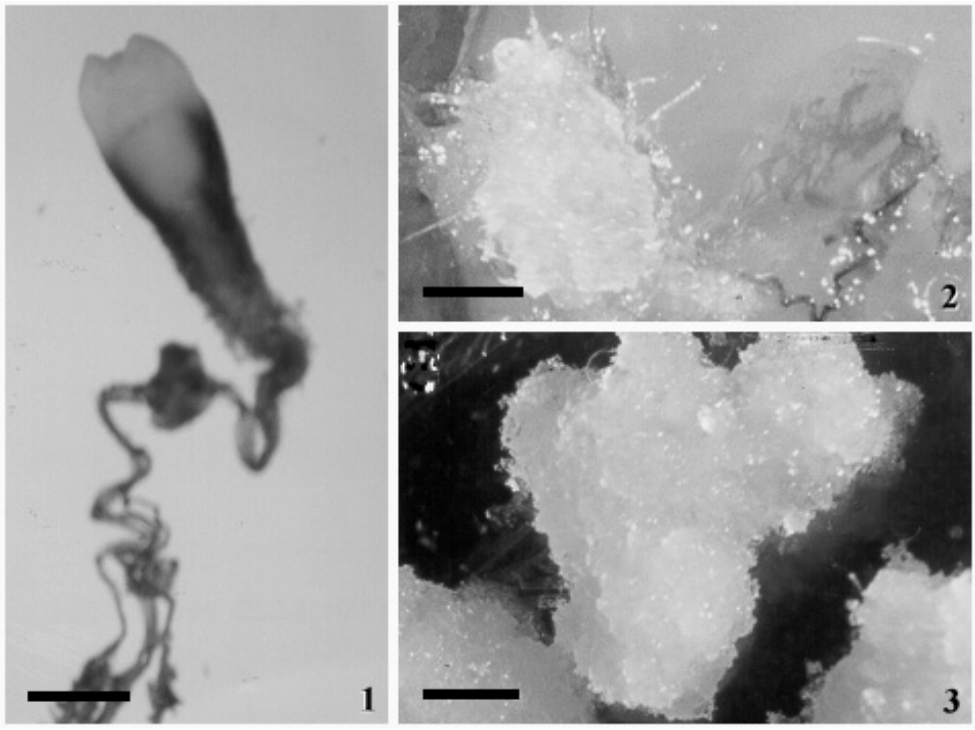

Figure 1-3. Somatic embryogenesis induction in *Araucaria angustifolia*. 1) Isolated precotyledonary zygotic embryo (bar 126 mm); 2) Induction of embryogenic culture from immature zygotic embryo on LP culture medium medium supplemented with 10 µM 2,4-D, 4 µM BA and 4 µM KIN (LP-D10) (bar 352 mm) and 3) Embryogenic culture maintenance in LP culture medium free of plant growth regulators (bar 2 mm).

Effect of developmental stage on the embryogenic induction

The developmental stages of the zygotic embryos used as primary explants ranged from proembryo in the first collecting time, to pre-cotyledonary in the last one. The highest embryogenic induction rate (52.1%) was obtained by pre-cotyledonary embryos (Figure 1) as explant, although this result was not statistically different from those obtained by globular embryos (Table 2). Embryogenic induction rates lower than 30% were obtained by proembryos and torpedo stage embryos (Table 2).

Embryogenic induction

The induction of embryogenic cultures was observed even in the LP medium free of plant growth regulators (D0), although, in this case the induction rate was the lowest (33.3%). The highest induction rate was obtained by the D10 medium, where 43.8% of the explants resulted in embryogenic cultures. However, the data analysis did not show any significant effect ($P>0.05$) of plant growth regulators on the embryogenic induction (Table 3).

Growth of suspension cultures

An expressive growth was observed in the suspension cultures of *A. angustifolia* in LP liquid culture medium free of plant growth regulators. The cell volume increased 5.6 times in 33 days, and the sedimented cell volume at the end of the growth dynamic was 22.5 mL. The growth dynamic of cell suspension cultures of *A. angustifolia* in the LP medium supplemented with 5 µM of 2,4-D, 2 µM of BA and 2 µM of KIN showed a 7.8-fold increase in its initial volume after the culture period of 54 days (Figure 4).

Somatic embryo development

The culture medium supplemented with ABA (50 µM) and PEG 4000 (1%) resulted in the development of somatic proembryos (Figures 5 and 6) that gave rise to globular and torpedo somatic embryos (Figures 7 and 8, respectively) after ninety days of culture. No further development of cotiledonary embryos was observed. The development of early staged somatic embryos was not observed under other treatments as well.

DISCUSSION

Embryogenic induction in *A. angustifolia* was characterized by the proliferation of a translucent to white and mucilaginous embryogenic cell masses (Figure 2). These features were also observed previously in other conifer species such as *Pinus pinaster* and *P. sylvestris* (Lelu et al. 1999) and *P. taeda* (Li et al., 1998).

In *A. angustifolia*, the induction rate of embryogenic culture was dependent on the genotype of the mother plant. Under the same conditions of culture medium, differences of up to 33.3% in the induction percentage of embryogenic cultures were observed. According to von Arnold et al. (1995), the quality of the seeds and the genotype may affect the somatic embryogenesis induction rates. The effect of the genotype on the embryogenic induction rate has also been observed in other conifer species such as *Picea glauca* (Högberg et al., 1998), *Abies alba* x *Abies cephalonica* and *Abies alba* x *Abies numidica* (Salajova et al., 1996), *Picea abies* (von Arnold et al., 1996), *Pinus pinaster* and *Pinus sylvestris* (Lelu et al., 1999). This genotype effect could be revealed by testing a larger number of trees and by controlling crosses between plant showing different abilities to induct embryogenic cultures (Lelu et al., 1999).

Table 2. Percentage of embryogenic cultures induced from four different developmental stages of zygotic embryos of *Araucaria angustifolia*.

Collecting time	Sample size	Developmental stage	Mean [1/]
11/22/97	24	Proembryo	29.2 b
12/12/97	24	Globular	43.8 ab
01/04/98	24	Torpedo	29.2 b
01/22/98	24	Pre-cotyledonary	52.1 a
Mean			38.5
Coeficient of variation (%)			47.7

[1/] Means followed by same letters are not statistically different according to the SNK test (5%).

Table 3. Percentage of embryogenic cultures induced from immature zygotic embryos of *Araucaria angustifolia* of four different culture media composition.

Media composition [1/]	Sample size	Mean [2/]
D0	24	33.3 a
D2	24	41.7 a
D5	24	35.4 a
D10	24	43.8 a
Mean		38.5
Coefficient of variation (%)		47.7

[1/] D0: LP medium (von Arnold and Eriksson, 1981) free of plant growth regulators; D2: LP medium plus 2 µM 2,4-D, 0.5 µM BA and 0.5 µM KIN; D5: LP medium plus 5 µM 2,4-D, 2 µM BA and 2 µM KIN and D10: LP medium plus 10 µM 2,4-D, 4 µM BA and 4 µM KIN;
[2/] Means followed by same letters are not statistically different according to the SNK test (5%).

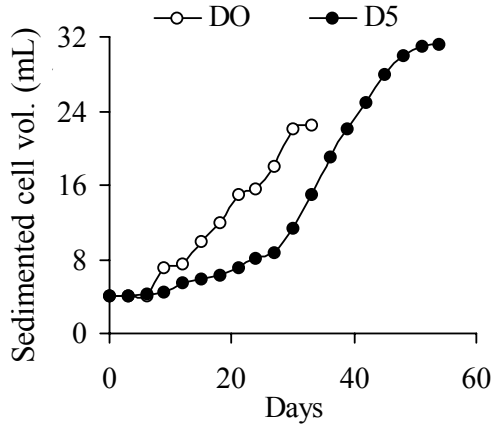

Figure 4. Growth dynamic of *A. angustifolia* embryogenic suspension cultures in LP medium (von Arnold and Eriksson 1981) free of (D0), and in LP medium supplemented with 5 µM 2,4-D, 2 µM BA and 2 µM KIN (D5).

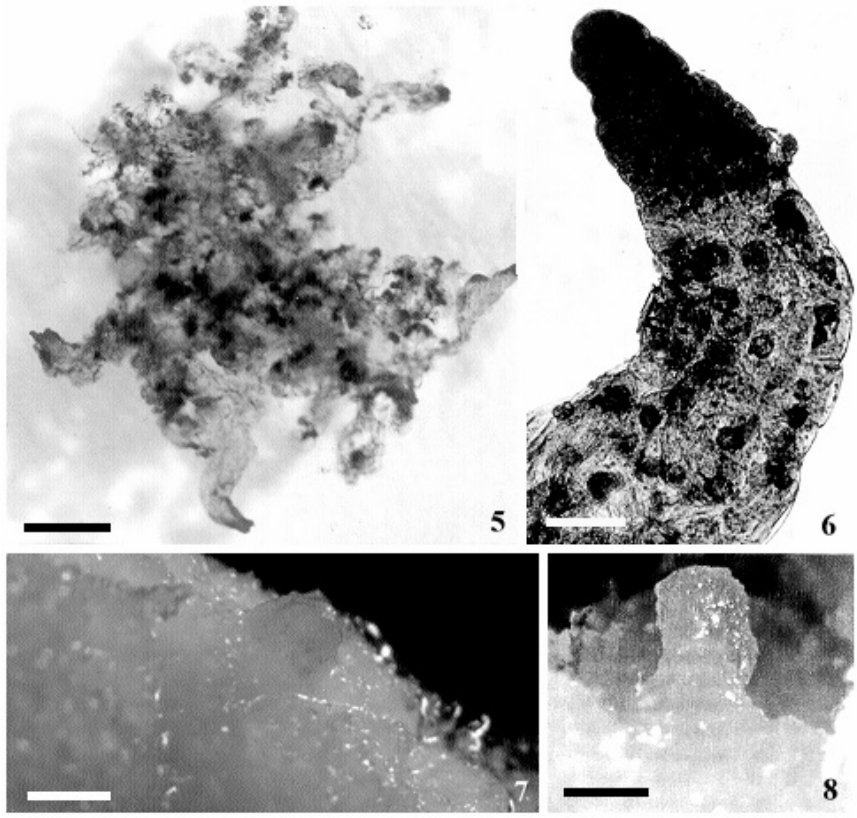

Figure 5 - 8. Somatic embryo development of *Araucaria angustifoia*. 5) Double stain with acetocarmine and Evan's blue of embryogenic culture kept on LP medium (von Arnold and Eriksson 1981) supplemented with 50 µM ABA and 1% PEG (bar 176 mm); 6) Somatic proembryo isolated from embryogenic culture described in figure 5 (bar 33.3 mm); 7) Globular stage somatic embryos on LP culture medium supplemented with 50 µM ABA and 1% PEG (bar 1.76 mm) and 8) Torpedo stage somatic embryos on LP culture medium supplemented 50 µM ABA and 1% PEG (bar 1.17 mm).

Some conifers species like *A. angustifolia* present polyzygotic polyembryony in addition to dioecy. As a result, each embryo, taken as explant, could become a specific allelic association (genotype). Thus, the high values (47.7%) of the coefficient of variation obtained in such experiments were expected.

In the somatic embryogenesis of *A. angustifolia,* the developmental stage of zygotic embryos is a critical factor in the induction of embryogenic cultures (Astarita and Guerra, 1998). In the present work, the best results were obtained from pre-cotyledonary embryos (Figure 1). In this species the embryogenic induction competence was lost when the explant was in the cotyledonary stage (Astarita and Guerra, 1998; Guerra et al., 2000). Pre-cotyledonary embryos have been used as explant source for many conifers species such as *Pinus taeda* (Becwar et al., 1991), *Pinus pinaster* (Lelu et al., 1999) and *Pinus strobus* (Klimaszewska and Smith, 1997). The critical factor in conifer somatic embryogenesis seems to be the developmental stage of the explant, which is associated with the morphological features of the female cones. In some cases, such as in *Pinus taeda,* the fertilization time is considered the reference point (Li and Huang, 1996). However, in *A. angustifolia* it is difficult to establish the fertilization time since the reproductive cycle, from the primordial carpel to the seed takes approximately four years. Thus, the best way is monitoring the female cone development in order to identify the time that the embryo would be at pre-cotyledonary stage.

In the present work the induction of *A. angustifolia* embryogenic cultures was achieved even in the absence of plant growth regulators. This occurrence has also been reported in other conifer species (Lelu et al., 1999). However, the induction rates were lower than in the present work. It is important to stress that the embryogenic induction rate obtained in the present work is the highest one reported so far for conifer species.

The use of low levels of plant growth regulators for the embryogenic induction showed to be advantageous in other conifers species, such as in *Pinus elliottii* (Jain et al., 1989) and in *Pinus taeda* (Li et al., 1998). However, the auxin and cytokinin balance in the culture medium seems to be more important than the absolute concentrations of these plant growth regulators (Guerra et al., 2000).

In the present work, the suspension culture in LP liquid medium free of plant growth regulators resulted in an increment rate of 5.6-fold in the initial sedimented cell volume after 33 days of culture. Suspension culture systems have been employed for the multiplication of embryogenic cultures in many conifer species. A large number of somatic proembryos can be obtained in suspension cultured in bioreactors (Tautorus et al., 1991), orbital or conventional shakers. The cell proliferation in culture medium free of plant growth regulators may be a precondition for the subsequent embryonic development. It has been suggested that the presence of auxins in the culture medium influences the formation of non-polar proembryos affecting the further development of somatic embryos in the maturation phase (Korlach and Zoglauer, 1995).

In the somatic embryo development experiment, the LP culture medium supplemented with ABA (50 μM) and PEG 4000 (1%) resulted in the development of torpedo stage somatic embryos (Figure 8). Although those embryos kept the morphology during the experimental period, further development of cotiledonary stage embryos was not observed. Astarita and Guerra (1998) obtained the highest number of early somatic embryos of *A. angustifolia* (103 proembryo.g^{-1}) when 1% of PEG was added to the LP medium. However, globular and torpedo somatic embryos were not observed in that work.

In general, the maturation of zygotic embryos involves physiological processes that ensure embryo dormancy, including the accumulation of abscisic acid (ABA), growth inhibition and the maintenance of quiescence (Dodeman et al., 1997). Also, the maturation of conifer somatic embryos occurs in the presence of ABA and an osmotic agent (Attree and Fowke, 1993). The presence of an osmotic agent allows the osmotic potential to be similar to the level observed during the early stages of the zygotic embryo development. Furthermore, ABA and water stress are involved reciprocally in the accumulation of storage reserves in conifer somatic embryos. Maturation is currently the least efficient stage of conifer plantlet regeneration from somatic embryos (Attree and Fowke, 1993).

In conclusion, the results of the present work showed that the induction percentage of somatic embryogenesis in *A. angustifolia* was dependent on the genotype and the developmental stage of the explant. Plant growth regulators were not essential for the induction and multiplication of embryogenic cultures. This finding is highly representative since the addition of plant growth regulators to the culture medium could reduce the maturation and conversion ability of the somatic embryos. In addition, in the maturation phase ABA and PEG supplemented to the culture medium allowed the development of torpedo

somatic embryos. Although much work on somatic embryogenesis of *A. angustifolia* has been done in our laboratory, additional work is needed to accomplish the last stage of embryo development in order to support breeding, mass propagation and conservation of this endangered conifer species.

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RESUMO

Técnicas biotecnológicas na conservação e melhoramento de *Araucaria angustifolia*: fatores indutivos que afetam a embriogênese somática

Técnicas biotecnológicas baseadas na embriogênese somática vêm sendo empregadas para a fixação de ganhos genéticos em programas de melhoramento genético florestal. Contudo, a aplicação desta técnica é dependente do estabelecimento de protocolos associados à sua indução e controle. Assim, o presente trabalho teve como objetivos identificar os fatores associados à indução, estabelecimento e desenvolvimento de culturas embriogênicas de *A. angustifolia*. Clones femininos imaturos foram coletados de dois genótipos em Bom Retiro-SC, entre novembro de 1997 e fevereiro de 1998. Os embriões zigóticos imaturos foram excisados e inoculados em quatro formulações de meio de cultura LP, suplementados com 2,4-D (0; 2; 5 e 10 μM), BA (0; 0,5; 2 and 4 μM) e KIN (0; 0,5; 2 and 4 μM). Suspensões celulares foram estabelecidas em frascos "nipple" adaptados, contendo 100 mL de meio líquido LP isento de reguladores de crescimento. No experimento para o desenvolvimento de embriões somáticos foi testado o meio de cultura LP suplementado com ABA (0; 10; 20 e 50 μM) e PEG 4000 (0; 1; 3 e 7,5%). A porcentagem de indução foi dependente do genótipo da planta, variando de 21,9% a 55,2%. A taxa de indução mais elevada foi de 43,8% em meio de cultura LP suplementado com 2,4-D (2,0 μM), BA e KIN (0,5 μM). Após 33 dias de cultura, as suspensões celulares mostraram um incremento de 5,6 vezes no volume celular sedimentado inicial, em meio de cultura isento de reguladores de crescimento, revelando que é possível estabelecer linhagens celulares embriogênicas autônomas. O meio de cultura basal, contendo ABA (50 μM) e PEG 4000 (1%), permitiu a progressão dos embriões somáticos até os estádios globular e torpedo de desenvolvimento.

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