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Organogenesis and *Agrobacterium tumefaciens*-mediated transformation of *Eucalyptus saligna* with *P5CS* gene

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Abstract

The purpose of this research was *Eucalyptus saligna in vitro* regeneration and transformation with *P5CSF129A* gene, which encodes Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*), the key enzyme in proline biosynthesis. After selection of the most responsive genotype, shoot organogenesis was induced on leaf explants cultured on a callus induction medium (CI) followed by subculture on a shoot induction medium (SI). Shoots were subsequently cultured on an elongation medium (BE), then transferred to a rooting medium and finally transplanted to pots and acclimatized in a greenhouse. For genetic transformation, a binary vector carrying *P5CSF129A* and *uidA* genes, both under control of the 35SCaMV promoter, was used. Leaves were co-cultured with *Agrobacterium tumefaciens* in the dark on CI medium for 5 d. The explants were transferred to the selective callogenesis inducing medium (SCI) containing kanamycin and cefotaxime. Calli developed shoots that were cultured on an elongation medium for 14 d and finally multiplied. The presence of the transgene in the plant genome was demonstrated by PCR and confirmed by Southern blot analysis. Proline content in the leaves was four times higher in transformed than in untransformed plants while the proline content in the roots was similar in both types of plants.

Additional key words: abiotic stress, *in vitro* culture, PCR, proline, Southern blot.

Introduction

The information about *in vitro* culture of *Eucalyptus saligna* tissues is limited. Plant regeneration from nodal segments excised from field plants has been published by Fantini and Graça (1990) and Le Roux and Van Staden (1991). However, no methodology of indirect organogenesis from juvenile explants has been described so far. Recent studies concerning *Eucalyptus* genetic modification have been carried out in order to modify cellulose and hemicellulose biosynthesis and to increase biomass in a short period of time and other studies concerning biotic and abiotic stress resistance such as drought, cold and salinity, insect and herbicide resistance, acid soil tolerance and phytoremediation are currently underway for *Eucalyptus* spp. improvement (Quoirin and Quisen 2006).

The different strategies that have been used to increase cold resistance in plants include proline overproduction and accumulation in plant cells. Increased tolerance has already been obtained in some plant species [rice (Hur *et al*. 2004), *Arabidopsis* (Savouré *et al*. 1997, Nanjo *et al*. 2003), *Larix* spp. (Gleeson *et al*. 2005) and tobacco (Konstantinova *et al*. 2002)] through the introduction of Δ^1-pyrroline-5-carboxylate synthetase gene (*P5CS*) which is involved in proline synthesis.

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Abbreviations: BAP - benzylaminopurine; BE - bud elongation medium; BM - bud multiplication medium; CI - medium for callogenesis induction from leaf explants; G/MR - germination and microcutting rooting medium; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; NAA - naphthaleneacetic acid; OI - medium for organogenesis induction from cotyledonary explants; PFD - photon flux density; SCI - selective callogenesis induction medium; SE - shoot elongation medium; SI - medium for shoot induction from leaf explants; TDZ - thidiazuron.
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The objective of this study was the *Agrobacterium tumefaciens*-mediated genetic transformation of *E. saligna* with the *P5CSF129A* gene in order to increase proline biosynthesis. Studies concerning organogenesis in cotyledon and leaf explants from a selected genotype were first performed in order to establish a protocol for plant regeneration that was subsequently used in genetic transformation.

Materials and methods

***In vitro* regeneration:** Seeds of *Eucalyptus saligna* Sm., provided by EMBRAPA-*Florestas*, were composed of a mixture of seven progenies. Seeds greater than 1 mm were then immersed in 70 % (v/v) ethanol containing *Tween* 20® (5 drops in 100 cm^3) for 3 min and surface-sterilized with a 6 % sodium hypochlorite (v/v) solution for 30 min. Seeds were rinsed three times in sterile distilled water and sown in Petri dishes containing the G medium (composed of half-strength Murashige and Skoog (1962; MS) medium (mineral salts, vitamins and organic compounds, without growth regulators). Seeds were maintained in the dark for 10 d and then exposed to light for two days. Twelve days after sowing, the cotyledonary leaves were excised at the petiole base and cultured with the adaxial face in contact with the OI medium, composed of MS medium supplemented with 2.7 µM naphthaleneacetic acid (NAA) and 5.4 µM benzylaminopurine (BAP), following the procedure described by Dibax *et al*. (2005). The cultures were maintained in a growth room in the dark for 15 d and then exposed to light for 15 d. Cultures were maintained under white fluorescent tubes providing a photon flux density (PFD) of approximately 40 µmol m^{-2} s^{-1}, 16-h photoperiod and a temperature of 27 ± 2 °C. The cultures were performed in Petri dishes, containing 30 cm^3 of culture medium and sealed with PVC film, or in glass flasks containing 40 cm^3 of culture medium, sealed with rigid polypropylene caps. All media were supplemented with 3.0 % sucrose (except the germination and rooting media with 2.0 %), solidified with 0.7 % agar (*Vetec*®, Rio de Janeiro, Brazil) and the pH adjusted to 5.8 before autoclaving for 20 min at 120 °C. The explants that formed calli were transferred to the same culture medium under light. After 60 d, one clone was selected for its *in vitro* performance and cultured on BM medium, composed of MS salts with half concentrations of potassium and ammonium nitrates, full vitamins and 1.11 µM BAP.

15 d after culture on a multiplication medium, leaves were excised from shoots at the petiole base and split into two halves. The leaf explants were arranged randomly in contact with the modified MS medium containing half-strength concentrations of potassium and ammonium nitrates, 3.0 % sucrose and 0.7 % agar. The treatments were: *1*) control (without plant growth regulators), *2*) 0.1 µM NAA + 1.0 µM thidiazuron (TDZ), *3*) 0.1 µM NAA + 1.5 µM TDZ and *4*) 0.1 µM NAA + 2.0 µM TDZ. The Petri dishes were kept in the dark in the growth room. 15 d after their isolation, the explants were subcultured onto the same medium in the dark for a total period of 30 d. Then, the percentage of explants forming calli was evaluated and the browning tissues removed. The explants were subsequently transferred to a SI medium, composed of MS medium with half concentrations of potassium and ammonium nitrates, 0.67 µM NAA and 1.11 µM BAP. The cultures were covered with *Sombrite*® (50 % PFD reduction) and kept in the growth room for 15 d. After this period, the explants were transferred to fresh SI medium and cultured for 15 d under a PFD of 40 µmol m^{-2} s^{-1}. 60 d after the initial culture period, the percentage of explants regenerating shoots, the number of shoots per explant and the percentage of explants were evaluated. The experimental design was totally randomized, with 10 replicates per treatment and 10 explants per flask. Treatment effects were analysed by *ANOVA* and means compared by Tukey's multiple range test using *Statgraphics Plus 4.1* software.

Rooting and acclimatization: The explants were divided into small clumps with approximately five buds each and cultured on a bud elongation (BE) medium. For rooting, shoots were divided into 1.5 cm microcuttings with the leaf area reduced, and cultured on a MR medium, composed of half-strength MS salts, full MS vitamins and without growth regulators. The cultures were kept in the dark for 15 d and then exposed to light. After 28 d the percentage of rooted microcuttings, the number of roots and the main root length were evaluated. Acclimatization of 30 plantlets was performed on *Plantmax HT*® substrate under intermittent mist for 15 d, then without mist for 15 d, receiving manual irrigation every 3 d. Finally, the percentage of surviving plants and the average plant size were evaluated.

Genetic transformation of *E. saligna* with *P5CSF129A* gene: The *Agrobacterium tumefaciens* strain used for the genetic transformation of *E. saligna* was EHA105 (Hood *et al*. 1993) containing the binary vector pBI121-*P5CSF129A* (Hong *et al*. 2000). This vector carried the *Vigna aconitifolia P5CSF129A* mutant gene and the β-glucuronidase reporter gene (*uidA*), both under control of the CaMV35S constitutive promoter, and the neomycin phosphotransferase selection marker gene (*nptII*) under control of *nos* promoter (Fig. 1). Leaves were excised from shoots of the selected clone, transversally cut into two halves and immersed in a bacterial solution of A_{600} = 0.5 for 30 min. In this experiment four Petri dishes with 50 leaf segments were used. The control consisted of leaf explants without

bacterial inoculation and selective antibiotics, cultured on a CI medium, composed of MS medium with half concentrations of potassium and ammonium nitrates, 0.1 μM NAA and 1.0 μM TDZ. After the inoculation period, leaf segments were blotted on a sterile filter paper and co-cultured for 5 d in Petri dishes on CI medium. After this period, explants were cultured on the selective medium (CI supplemented with 50 mg dm^{-3} kanamycin and 500 mg dm^{-3} cefotaxime) for 2 periods of 28 d. They were then transferred to the same medium without kanamycin and with 250 mg dm^{-3} cefotaxime for 28 d. In all stages, the explants were kept in the dark. The calli which originated on the selective medium were isolated and transferred to SI medium and maintained under light for 28 d. Explants showing shoot formation were transferred to a SE medium composed of MS medium containing half concentrations of potassium and ammonium nitrates and 1.45 μM gibberellic acid (GA_3) for 14 d in order to facilitate the separation of shoots. Then, the shoots were transferred to flasks (6 cm diameter, 9 cm height) containing the BM medium. Several subcultures were made every 15 d until a sufficient quantity of plant material was obtained. After plant acclimatization under greenhouse conditions, four-month-old plants were used for molecular analysis, total fresh mass and proline content determination.

DNA was extracted from 100 mg of fresh young leaves and processed according to the protocol of Doyle and Doyle (1990). The analysis of the transformed plants was performed by PCR. Each reaction (0.02 cm^3) contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 100 μM of each dNTP, 1.0 U of *Taq* polymerase, 50 ng DNA and 5 μM of each oligonucleotide specific to *P5CSF129A* gene. The *P5CSF129A* primer sequences were: 5'AGCAACTCAACTCTCTCGGA-3' and 5'-CCACTCTAGACTTGTCGCCA-3'. The samples were submitted to the following amplification programme in a *PTC-100*™ thermocycler (*MJ Research*, Waltham, MA, USA): 94 °C for 4 min, followed by 32 cycles of 94 °C for 1 min, 52 °C for 45 s, 72 °C for 45 s, 72 °C for 5 min and 4 °C until insertion into the gel. The amplified fragments were subjected to electrophoresis in a 0.8 % agarose gel containing 0.5 μg ethidium bromide, and visualized under UV radiation (Brody and Kern 2004).

For the Southern blot analysis, 20 μg of genomic DNA of the transgenic event and control were digested with *Hind*III at 37 °C for 16 h. The DNA samples were then subjected to electrophoresis in agarose gel and stained with 1 % (v/v) ethidium bromide for 16 h. After electrophoresis, the DNA was transferred onto a *Hybond-N*+® membrane (*GE Healthcare*, UK) by capillarity and fixed by incubation at 80 °C for 2 h (Sambrook and Maniatis 1989). Two Southern hybridization analyses were carried out: the first with a probe of approximately 1.6 kb obtained from the plasmid pBI121-*P5CSF129A* digested with *Xba*I enzyme and the second with a probe of 519 bp obtained by PCR from an internal *uidA* gene position. The probes were labelled with ^{32}P d-CTP by random priming technique. After hybridization, membranes were washed twice at 42 °C with SSC (2 %) and SDS (0.1 %) solutions and twice with SSC (0.1 %) and SDS (0.1 %) solutions at 42 °C. Hybridization patterns were detected by exposure on a plate reader for 1 h and the images captured by a *FLA-3000* system (*Fuji* film).

Plant material (roots and shoots of 4 untransformed and 4 transformed plants, 100 mg for each sample) was collected from 4-month-old plants cultured under green-house conditions. The proline content was determined following the procedure of Bates *et al*. (1973).

Results

Indirect organogenesis from cotyledonary leaves and bud multiplication: The CI medium induced callogenesis in 85 % of the cotyledonary explants and 40 % of callus regenerated buds. Callus induction began 10 d after isolation (Fig. 2*A*). The number of buds per explant was 4.25 and mortality rate 45 %. The shoots originated predominantly from red nodular protuberances located at the petiole base after 30 d of culture. For bud

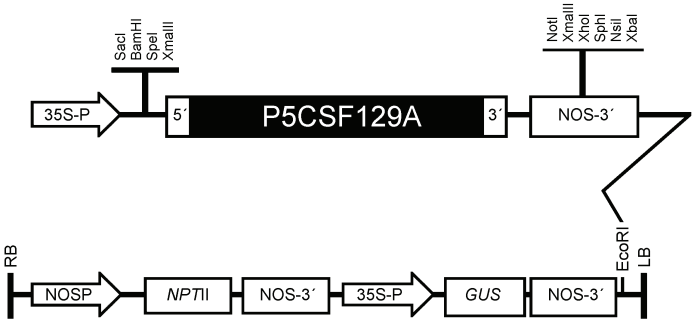

Fig. 1. T-region of the pBI121 plasmid used in *E. saligna* genetic transformation. P5CSF129A mutant gene construction (Zhang *et al.* 1995). *P5CS* - *V. aconitifolia* gene, *NPT*II - neomycin phosphotransferase gene, *GUS* - reporter gene, encodes β-glucuronidase, *NOSP* - nopaline synthase promoter gene, 35S-P - cauliflower mosaic virus (CaMV) promoter, NOS-3' - *A. tumefaciens* nopaline synthase terminator, RB - T-DNA right border, LB - T-DNA left border.

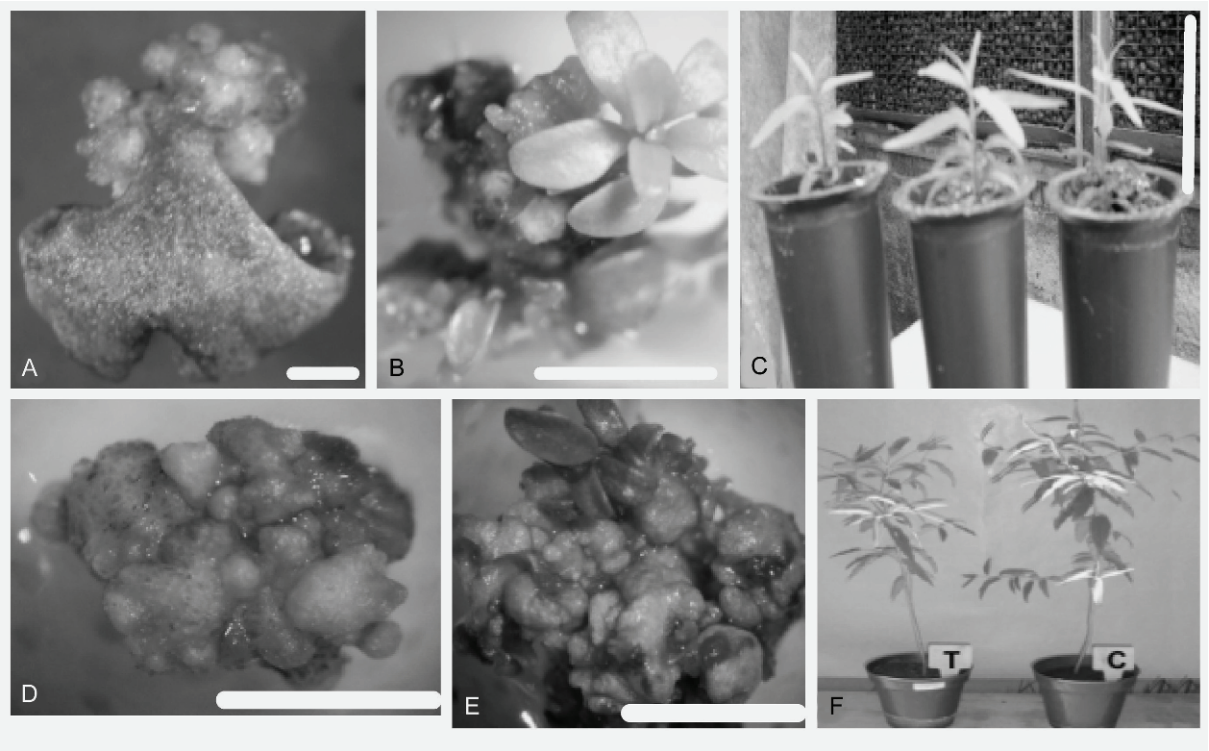

Fig. 2. *In vitro* plant regeneration and *Agrobacterium tumefaciens*-mediated genetic transformation of *Eucalyptus saligna*. *A* - Callus formation from cotyledonary leaf explants after 30 d of culture on MS medium containing 2.70 μM NAA and 4.44 μM BAP; *B* - Clump of buds covering the leaf explant after 60 d of culture on MSM medium containing 0.67 μM NAA and 1.11 μM BAP (SI medium); *C* - Acclimatization of rooted microcuttings on *Plantmax HT*® mixture after 28 d of culture in a greenhouse; *D* - Callus induced on selective MSM medium containing 0.1 μM NAA and 1.0 μM TDZ (SCI medium) 90 d after inoculation; *E* - Bud induction on MSM culture medium containing 0.67 μM NAA and 1.11 μM BAP (SI medium); *F* - transgenic (*left*) and untransformed plant (*right*) after 4 months of acclimatization under greenhouse conditions. *Bars*: *A* = 2 mm, *B* = 5 mm, *C* = 70 mm, *D* and *E* = 5 mm.

Table 1. Shoot regeneration from leaf explants of *E. saligna* on MS medium containing NAA and TDZ (CI medium) for 30 d, followed by subculture on a bud regeneration medium containing 0.67 μM NAA and 1.11 BAP (SI medium) for 30 d. Means followed by the same letter in a column do not differ according to Tukey's multiple range test ($P < 0.05$).

NAA [μM]	TDZ [μM]	Callus formation [%]	Shoot regeneration [%]	Shoot number [explant^{-1}]	Browning [%]
0.0	0.0	0c	0d	0b	-
0.1	1.0	73a	30a	13a	70b
0.1	1.5	72a	13b	9a	87a
0.1	2.0	59b	5c	11a	88a

multiplication, the BM medium provided an average number of 7 buds per explant after 28 d of culture. Hyperhydricity was not observed in any of the treatments.

Organogenesis from leaf explants: Callus formation in leaf explants occurred in all treatments except in the control. The MSM medium containing 0.1 μM NAA and 1.0 or 1.5 μM TDZ provided the best results. Callus induction began 15 d after explant isolation at the leaf cut and petiole base (Table 1, Fig. 2*B*). With regard to the percentage of explants regenerating shoots and number of shoots per explant, the best response was obtained on MSM medium supplemented with 0.1 μM NAA and 1.0 μM TDZ (Table 1) with 30 % of regenerating shoots and 13 shoots per explant 30 d after transfer to this medium. The shoot origin was the green callus formed at the petiole base and the cut region. Considering the number of buds per explant, there was no statistical difference between the three treatments (Table 1). After 60 d of culture, the lowest oxidation level (70 %) was observed in a medium with 0.1 μM NAA and 1.0 μM TDZ, while all the explants on the control treatment were oxidized.

Elongation, rooting and plant acclimatization: For bud elongation, the BE culture medium containing 2.5 % activated charcoal was efficient with 90 % of the explants presenting bud development and elongation. Mean shoot length ranged from 2.5 to 3.0 cm after 28 d of culture and the shoots showed rooting at the end of elongation phase. The percentage of rooted microcuttings was 76 and the average number of roots per plant and mean length of the main root were 2 and 1.7 cm per plant, respectively. After the acclimatization period, the plant survival rate was 80 % and the mean shoot size 7 cm, and no obvious phenotypic changes were observed (Fig. 2*C*).

Genetic transformation of *E. saligna* with *P5CSF129A* gene: After inoculation, co-culture and cultivation on selection medium, some calli were formed on leaf explants and the shoots regenerated (Table 1, Fig. 2*D*). In control explants (non inoculated), callus formation occurred after 15 d of culture, and after 30 d of initial culture period the start of shoot regeneration was observed. Adventitious buds sprouted from green calli after 60 d. In non-inoculated explants, callus induction occurred in 56 % while in the *Agrobacterium* inoculated ones, callus formation was induced in only 8 % of explants after 90 d (Fig. 2*E*). The percentage of explants with regenerating buds was 28 % for non-inoculated explants and 0.5 % for infected explants cultured on the medium with kanamycin and cefotaxime. The average number of buds per explant was 15 in control and 4 for the inoculated explants. Explant necrosis was higher after infection with *Agrobacterium* and culture on antibiotic containing media than in non-inoculated controls. The regeneration of kanamycin-resistant shoots occurred from one leaf explant that formed calli after 120 d on selective medium. The buds were elongated after 14 d on a culture medium containing 1.45 μM GA_3 and multiplied on BM medium. The histochemical assay for *GUS* activity applied 5 months after the transformation gave evidence for stable expression of the *uidA* gene. PCR amplification of the transgene sequence in the primary transformants gave the expected fragment of 598 bp on 1 % agarose gel corresponding to the expected internal region for the coding sequence of the moth bean mutated *P5CS* gene. As expected, the control plants did not show the gene fragment (Fig. 3*A*). To confirm the *P5CSF129A* gene integration and copy number, Southern blot hybridization was carried out with DNA from the PCR positive plants. Preliminary results with two different *V. aconitifolia P5CS* probes showed cross-hybridization with the endogenous *E. saligna P5CS* even at high stringency. Southern blot analysis was therefore carried out with a probe obtained from a fragment of 519 bp corresponding to internal *uidA* gene obtained by PCR to confirm T-DNA integration into the *E. saligna* genome. The Southern blot analysis revealed the presence of two insertions of the *uidA* gene (Fig. 3*B*). The proline content in leaf tissue of transgenic plants was four times greater than that in control plants. The proline content mean values were 13.39 and 3.42 μmol g^{-1}(f.m.), respectively. The proline content in roots of *E. saligna* control plants was not statistically different from the value in transgenic plants. The shoot fresh mass was 17.48 and 10.65 g in control and transgenic plants, respectively (Fig. 2*F*). The root fresh mass of control plants was 11.87 g and that of transgenic plants only 8.94 g. The roots from control plants seemed to be more lignified than those of transgenic plants.

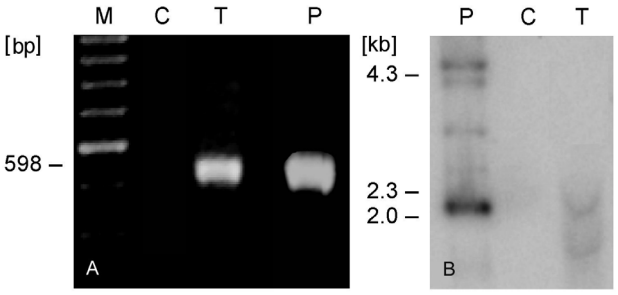

Fig. 3. *A* - PCR analysis of DNA isolated from *Eucalyptus saligna* GUS^+ plants. *Lanes*: M - molecular mass marker, C - control (non transgenic plant), T - transgenic plant 1, P - pBI121-P5CSF129A plasmid. *B* - Southern analysis with a probe for *GUS* gene. *Lanes*: P - GUS plasmid; C - untransformed plant; T - transgenic plant showing two copies of *GUS* gene insertion.

Discussion

Hervé *et al*. (2001) reported a high correlation between calli formed at the petiole base and shoot regeneration from leaf segments of *E. gunnii*. According to these authors, 8.8 % of the calli formed at the petiole leaf base developed shoots after transfer to the shoot regeneration medium. Similar shoot regeneration results were observed by Barrueto-Cid *et al*. (1999) and Alves *et al*. (2004) in seedling explants of the *E. grandis* × *E. urophylla* hybrid. These authors compared callus induction and shoot regeneration from hypocotyls, primary leaves and cotyledonary leaves. For all types of explants the SP culture medium (Barrueto-Cid *et al.* 1999) composed of half-strength MS mineral salts and quarter-strength MS FeEDTA supplemented with 2.0 μM of TDZ enabled 95 to 100 % of callus induction in the regions where the explants were cut. According to these authors, the calli kept their regeneration ability for 30 d on this medium and, after this period, no shoot regeneration was observed. Similar results were observed by Sanatombi and Sharma (2008) when leaf explants of *Capsicum* spp. derived from four-five week-old *in vitro* germinated seedlings were cultured on MS medium supplemented with 3 % (m/v) sucrose, 8.8 μM BAP and 11.4 μM IAA. In this work, the leaf explants produced

buds mainly from the petiole base. Saritha and Naidu (2008) cultured *Spilanthes acmella* on MS medium supplemented with different plant growth regulators for direct regeneration from mature leaves or leaves of *in vitro* grown plantlets. The formation of shoots was observed after 45 d either in the midrib region or basal petiole region or apical region of the leaf. Here too, the formation of callus occurred at the cut regions. Similar results were related by Sreedhar *et al*. (2008) who studied the combined effects of BAP and kinetin on leaf explants of *Stevia rebaudiana* on shoot regeneration after 7 weeks of culture. In this study, adventitious shoots emerged from either side of the midrib and especially from the petiole basis, indicating that there exists a physiological gradient of growth regulators in the explants from the proximal to the distal end. Comparing the different protocols of bud regeneration from leaf explants of *Eucalyptus* sp., it was observed that the regeneration rates obtained by Mullins *et al*. (1997) and by Lainé and David (1994) were lower than those described here. These authors obtained 60 and 38.5 % of bud regeneration from leaves of *E. camaldulensis* and *E. grandis* plants, respectively. However, due to the lack of published studies concerning organogenesis in *E. saligna* leaves, it is not possible to compare the results observed in this study with those carried out with different *Eucalyptus* species.

According to Barrueto-Cid *et al*. (1999), the biosynthesis of phenolic compounds, induced by light and high cytokinin concentrations in culture media, contribute to explant oxidation. These authors observed considerable amounts of oxidized explants during adventitious bud regeneration from cotyledonary leaves, primary leaves and hypocotyls of *E. grandis* × *E. urophylla*. For plant regeneration from *E. grandis* leaves, Lainé and David (1994) recommended the use of PVP (polyvinylpyrrolidone) or other antioxidants to minimize the deleterious effects caused by the explant browning during callus and bud induction. Shoot elongation was obtained with satisfactory results from cotyledonary leaves of *E. grandis* × *E. urophylla* when using the SPM medium (Barrueto-Cid *et al*. 1999) containing MS micronutrients at full strength, sucrose at 2 % and activated charcoal at 1 %. This result indicated the efficiency of activated charcoal on bud elongation as observed in the present case for *E. saligna*. Furthermore, Mullins *et al*. (1997) reported that buds resulting from leaf explants of *E. grandis* occasionally showed spontaneous elongation when cultured on a medium containing 2 μM BAP and 2.5 μM NAA. The rooting rate observed in the present work was similar to that reported by Ho *et al.* (1998) for *E. camaldulensis*.

Concerning the results of *E. saligna* genetic transformation, the GUS constitutive expression observed in leaves confirms the results obtained by Ho *et al*. (1998) who observed the expression in all tissues of *E. camaldulensis* transformed with *uidA* gene under the control of 35S-CaMV promoter. A similar high level of explant necrosis was registered in cotyledonary explants of *Citrullus lanatus* co-cultured with the strains LBA4404, GV3101, EHA101 of *A. tumefaciens* and a long time (6 weeks) was also required for shoot regeneration (Cho *et al*. 2008). The observed increase of proline content in tissues of *E. saligna* described here was proportionally lower than those observed for other species transformed with the *P5CS* gene grown in the absence of stress. For *Larix* spp. (Gleeson *et al*. 2005), the proline content in embryogenic cell mass expressing *P5CS* gene was approximately 30 times higher than those observed in non-transformed plants, and after plant regeneration on culture medium without kanamycin the levels remained unchanged. In tobacco plants (Kavi-Kishor *et al*. 1995) and wheat (Sawahel and Hassan 2002) expressing the *P5CS* gene, the proline contents were increased 14 and 12 times, respectively, compared to the non-transformed plants. The growth reduction observed in leaves and roots of *E. saligna* expressing the *P5CS* gene when compared with non transformed control plants may result from accumulation of free proline. Transgenic plants of *Arabidopsis thaliana* and *Nicotiana tabacum* that were engineered to overproduce osmolytes exhibit impaired growth in the absence of stress (Maggio *et al.* 2002). These authors hypothesized that plant growth reduction is associated with the additional metabolic consequences of free proline overproduction.

In conclusion, this is the first report of induction of organogenesis from cotyledonary and leaf explants in *E. saligna*. Moreover the genetic transformation of this species with the *P5CS* gene provided a transformation efficiency of 0.5 %. The information presented here may constitute the basis for optimization of the protocol for regeneration and micropropagation of other *E. saligna* genotypes and future studies of cold stress tolerance of *E. saligna* genetically transformed with *P5CS* gene.

References

Alves, E.C.S.C., Xavier, A., Otoni, W.C.: Organogênese de explante foliar de clones de *Eucalyptus grandis* × *Eucalyptus urophylla*. - Pesq. agropec. Bras. **39**: 421-430, 2004.

Barrueto-Cid, L.P., Machado, A.C.M., Carvalheira, S.R.C., Brasileiro, A.C.M.: Plant regeneration from seedling explants of *Eucalyptus grandis* × *E. urophylla*. - Plant Cell Tissue Organ Cult. **56**: 17-23, 1999.

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline for water stress studies. - Plant Soil **39**: 205-207, 1973.

Brody, J.R., Kern, S.E.: Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. - BioTechnology **36**: 214-216, 2004.

Cho, M.A., Moon C.Y., Liu J.R., Choi P.S.: *Agrobacterium*-mediated transformation in *Citrullus lanatus*. - Biol. Plant.

52: 365-369, 2008.

Dibax, R., Eisfeld, C.deL., Cuquel, F., Koehler, H., Quoirin, M.: Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis*. - Scientia Agr. **62**: 406-412, 2005.

Doyle, J.J., Doyle, J.L.: Isolation of plant DNA from fresh tissue. - Focus **12**: 13-15, 1990.

Fantini, M., Jr., Graça, M.E.C.: Propagação *in vitro* de *Eucalyptus saligna*. - In: 6 Congresso Florestal Brasileiro. Pp. 373-378. Sociedade Brasileira de Silvicultura, São Paulo 1990.

Gleeson, D., Walter, M.A.L., Parkinson, M.: Overproduction of proline in transgenic hybrid larch (*Larix leptoeuropaea* (Dengler) cultures renders them tolerant to cold, salt and frost. - Mol. Breed. **15**: 21-29, 2005.

Hervé, P., Jauneau, A., Pâques, M., Marien, J.N., Boudet, A.M., Teulières, C.A.: Procedure for shoot organogenesis *in vitro* from leaves and nodes of an elite *Eucalyptus gunnii* clone: comparative histology. - Plant Sci. **161**: 645-653, 2001.

Ho, C.K., Chang, S.H., Tsay, J.Y., Tsai, C.J., Chiang, V.L., Chen, Z.Z.: *Agrobacterium tumefaciens*-mediated transformation of *Eucalyptus camaldulensis* and production of transgenic plants. - Plant Cell Rep. **17**: 675-680, 1998.

Hong, Z., Lakkineni, K., Zhang, Z., Verma, D.P.S.: Removal of feedback inhibition of Δ^1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. - Plant Physiol. **122**: 1129-1136, 2000.

Hood, E.E., Gelvin, S.B., Melchers, L.S., Hoekema, A.: New *Agrobacterium* helper plasmids for gene transfer to plants. - Transgen. Res. **2**: 208-218, 1993.

Hur, J., Jung, K.H., Lee, C.H.: Stress-inducible *OSP5CS2* gene is essential for salt and cold tolerance in rice. - Plant Sci. **167**: 417-426, 2004.

Kavi-Kishor, P.B.K., Hong, Z., Miao, G.-H., Hu, C.A.A., Verma, D.P.S.: Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. - Plant Physiol. **108**: 1387- 1394, 1995.

Konstantinova, T., Parvanova, D., Atanassov, A., Djilianoiv, D.: Freezing tolerant tobacco, transformed to accumulate osmoprotectants. - Plant Sci. **163**: 157-164, 2002.

Lainé, E., David, A.: Regeneration of plants from leaf explants of micropropagated clonal *Eucalyptus grandis*. - Plant Cell Rep. **13**: 473-476, 1994.

Le Roux, J.J., Van Staden, J.: Micropropagation of *Eucalyptus* species. - J. hort. Sci. **26**: 199-200, 1991.

Maggio, A., Miyazaki, S., Veronese, P., Fujita, T., Ibeas, J.I., Dasm, B., Narasimhan, M.L., Hasegawa, P.M., Joly, R.J., Bressan, R.A.: Does proline accumulation play an active role in stress-induced growth reduction? - Plant J. **31**: 699-712, 2002.

Mullins, K.V., Llewellyn, D.J., Hartney, V.J., Strauss, S., Dennis, E.S.: Regeneration and transformation of *Eucalyptus camaldulensis*. - Plant Cell Rep. **16**: 787-791, 1997.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nanjo, T., Fujita, M., Seki, M., Kato, T., Tabata, S., Shinozaki, K.: Toxicity of free proline revealed in an *Arabidopsis* T-DNA-tagged mutant deficient in proline dehydrogenase. - Plant Cell Physiol. **44**: 541-548, 2003.

Quoirin, M., Quisen, R.: Advances in genetic transformation of *Eucalyptus* species. - In: Franche, C. (ed.): Molecular Biology of Tropical Plants. Pp. 41-56. Research Signpost, Kerala 2006.

Sambrook, J., Maniatis, T., Fritsch, E.F.: Molecular Cloning: a Laboratory Manual. 2nd Ed. - Cold Spring Harbor Press, New York 1989.

Sanatombi, K., Sharma, G.J.: *In vitro* plant regeneration in six cultivars of *Capsicum* spp. using different explants. - Biol. Plant. **52**: 141-145, 2008.

Saritha, K.V., Naidu, C.V.: Direct shoot regeneration from leaf explants of *Spilanthes acmella*. - Biol. Plant. **52**: 334-338, 2008.

Savouré, A., Hua, X.J., Bertauche, N., Van Montagu, M., Verbruggen, N.: Abscisic acid-independent and abscisic acid-dependent regulation of proline biosynthesis following cold and osmotic stresses in *Arabidopsis thaliana*. - Mol. Genet. Genom. **254**: 104-109, 1997.

Sawahel, W.A., Hassan, A.H.: Generation of transgenic wheat plants producing high levels of the osmoprotectant proline. - Biotech. Lett. **4**: 721-725, 2002.

Sreedhar, R.V., Venkatachalam, L., Thimmaraju, R., Bhagyalakshmi, N., Narayan, M.S., Ravishankar G.A.: Direct organogenesis from leaf explants of *Stevia rebaudiana* and cultivation in bioreactor. - Biol. Plant. **52**: 355-360, 2008.

Zhang, C.S., Lu, Q., Verma, D.S.P.: Removal of feedback inhibition of Δ^1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme catalyzing the first 2 steps of proline biosynthesis in plants. - J. biol.Chem. **270**: 20491-20496, 1995.