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OPTIMIZATION OF *AGROBACTERIUM TUMEFACIENS* MEDIATED TRANSFORMATION IN *EUCALYPTUS CAMALDULENSIS*

AMMARA AHAD, ASMA MAQBOOL AND KAUSER ABDULLA MALIK*

Department of Biological Sciences
Forman Christian College, Lahore 54600, Pakistan
**Corresponding author:kauser45@gmail.com*

Abstract

This study was conducted to optimize *Agrobacterium tumefaciens* mediated transformation for *Eucalyptus camaldulensis.* Transformation was done by using LBA4404 containing binary plasmid pGA482 with *uidA (Gus)* gene under *CamV35S* promoter and *nptII* gene under *nos* promoter. For optimization, different explants (Cotyledonary leaves, plantlet leaves and hypocotyls of young *In vitro* plants and calli) with and without preculture were infected with a range of optical densities ($O.D._{600nm}$=0.3-0.6). Effect of different concentrations of Acetosyringone, infection time and co-cultivation time on transformation efficiency was evaluated. Confirmation of transformation was done through GUS histochemical staining & through PCR. Callogenesis and regeneration was found fast on MS medium containing 0.5 mg/L NAA and 1.5 mg/L BAP. Highest transformation efficiency was obtained with bacterial suspension of $O.D._{600nm}$ = 0.5 for non-precultured explants and $O.D._{600nm}$=0.3 for precultured explants.

Introduction

Eucalyptus camaldulensis belongs to family Myrtaceae, known as Mallee is native to Australia (Bown, 1995), has become frequently planted tree in the world (Turnbull, 1991; Grattapaglia, 2008).The fast growth rates (to over 100 m^3/ha per year) and short rotations (5-7 years in most cases) of Eucalyptus species are two of the major reasons for the extensive use of Eucalyptus in commercial reforestation in many parts of the world (Ho *et al.,* 1998). *Eucalyptus camaldulensis* is superior to other species in wood production on infertile dry lands (Horng *et al*., 1991). Large scale plantation of *E. camaldulensis*, *E. citriodora*, *E. melanopholia*, *E. microtheca and E. robusta* has been done in Pakistan (Qadri, 1966). Eucalyptus species are recognized for the production of industrial wood, fuel wood and essential oils (Mullins *et al.,* 1997).

Development of efficient transformation protocol is the major key for introduction of desirable characteristics in *E. camaldulensis*. Data for genetic transformation of Eucalyptus spp. has been published by Alcantara *et al.,* (2011); Matsunaga *et al.,* (2012); Prakash & Gurumurthi (2009) and Harcourt *et al.*, (2000). Lainé & David in 1994 reported whole plant regeneration through either organogenesis or somatic embryogenesis in *E. grandis*. However, using Agrobacterium-mediated genetic transformation, these systems normally lose their ability to regenerate plants from transformed tissues. Especially, the inadequacy of well developed rooting systems to initiate roots from transformed shoots shows the major hurdle to the success of regenerating whole transgenic plants. *Agrobacterium rhizogenes* inoculation has been suggested as a key to success in this context and well developed rooting system had been developed for *E. grandis*, *E. gunnii*, and *E. nitens* (Macrae & Van Staden, 1993). Creation of transgenic plant by Agrobacterium mediated transformation for these species is still problematic and needs to be significantly improved. Other approaches for genetic transformation like direct DNA transfer through chemical stimulation, electroporation and microprojectile bombardment have been used to acquire transient gene expression in protoplasts of *E. gunnii* and *E. citriodora* and in *E. globulus* embryos (Teulieres *et al*., 1991, Manders *et al*., 1992; Rochange *et al*., 1995).

The present study was carried out to develop excellent and efficient transformation system for *Eucalyptus camaldulensis* through *Agrobacterium tumefaciens* LBA4404 containing binary plasmid pGA482 with *uidA (Gus)* gene under *CamV35S* promoter and *nptII* gene under *nos* promoter. Furthermore the optimization of different parameters i.e. optical density of bacterial culture, infection and co-cultivation time, concentration of Acetosyringone and effect of preculture treatment was carried out.

Materials and Methods

Plant material & bacterial strain: Seeds were taken from Ayub Agricultural Research Institute, Faisalabad. Cotyledonary and plantlet leaves (0.5-0.8cm in size), hypocotyls and calli with and without preculturing were used in transformation experiments. For callogenesis, young leaves of field grown plants were also used as explants. *Agrobacterium tumefaciens* strain LBA4404 containing binary plasmid pGA482 with *Gus* (*uidA*) gene and *nptII* gene was used in transformation experiments.

Sterilization & germination of seeds: Sterilization of seeds is an important step towards *In vitro* growth of seedlings. For sterilization different approaches were used. In first case (C1), seeds were immersed in 70% ethanol for 2 minutes followed by 1-2 washings with autoclaved distilled water and treatment with 0.1% $HgCl_2$ solution for 10 minutes. Final washing was performed with autoclaved distilled water for 3-4 times. Seeds were put on sterile filter paper for 10-15 minutes. In second case (C2), procedure was modified and a few drops of Tween 20 were added along with 0.1% $HgCl_2$ and the rest of the procedure was same as mentioned in above case. In third case (C3), 100% Clorox for 20 minutes was used instead of 0.1% $HgCl_2$,

followed by an additional washing with 70% ethanol. Remaining procedure was same as in case one. However, in fourth case (C4), 30% sodium hypochlorite was also found to be effective.

Seeds were germinated on full and half strength MS basal medium (Murashige & Skoog, 1962) having 3% sucrose and 4% Gelzan (*PhytoTechnology* Laboratories ®). Plates were incubated at 25°C, under light conditions.

Preparation of explants: Explants were prepared from cotyledonary leaves, plantlet leaves and hypocotyls. Calli were also used as explants and were prepared using MS basal medium, with different combinations of plant growth hormones of varying concentrations (Table 1).

Transformation: *Agrobacterium tumefaciens* was grown overnight at 28°C in LB medium supplemented with Rifampicin and Kanamycin at concentrations of 50mg/L each. Bacterial suspensions of different $O.D._{600nm}$=0.3-0.6 were used for precultured and non precultered explants. While for calli $O.D._{600nm}$=0.2-0.8 was used. Effect of different infection times (1 hour and 2 hours), co-cultivation time (1day and 2 days) and different concentrations of Acetosyringone (0 µM, 200 µM and 400 µM) in co-cultivation media were studied to enhance transformation efficiency. After 2 days of co-cultivation, a few explants were selected for GUS staining and incubated in X-Gluc solution (1M Na_2HPO_4, 1M NaH_2PO_4, 0.5M EDTA, 1% Triton X-100, X-Gluc (dissolved in dimethyl sulfoxide / dimethyl foramide), methanol and ultrapure water), at 37°C for 2 days, then random explants were examined for Gus histochemical reaction. Remaining explants were shifted on MS regeneration medium. Regenerated shoots were transferred on selection medium CIM1 supplemented with Kanamycin (25mg/L). Three rounds of selection were given to the regenerated shoots. Plants were subcultured after twenty days.

Genomic DNA isolation and polymerase chain reaction (PCR) analysis: Plant genomic DNA was extracted from 0.5g of shoots by using CTAB Method (Murray & Thompson, 1980). The confirmation of putative transformants was conducted by amplification of a fragment of *uidA* gene by using gene specific forward primer 5′-TTC GCG TCG GCA TCC GCT CAG TGG CA- 3′ and Reverse primer 5′-GCG GAC GGG TAT CCG GTT CGT TGG CA- 3′, described by Prakash & Gurumurthi, 2009. PCR analysis of genomic DNA was carried out in a 25 µL reaction volume, by using 50-100ng of isolated DNA. The PCR amplification was performed by initial denaturation at 95°C (4 min) followed by 40 cycles at 95°C (1min), annealing at 64°C (1 min), elongation at 72°C (30 sec) and final elongation at 72°C (10 min). The PCR products were run on 1.5% (w/v) agarose gel in 1x TAE buffer. The gels were visualized by GelDoc-It® Imager.

Results and Discussion

Seed sterilization is an important step in order to get *In vitro* grown seedlings. The sterilization of explants taken from field grown mature plants has proved to be more problematic. Same results have been reported for sterilization of Eucalyptus species explants (McComb & Bennet, 1986; Ikemori, 1987 & Warrag *et al*., 1990). In present study, seeds and explants were sterilized by using different sterilization methods (C1, C2, C3 and C4). 100% sterilization was obtained by using C3 method, while minimum sterilization efficiency was shown by C1 method (Table 2).

Survival rate of explants sterilized by C3 was also good, when cultured on media. These results were supported by Dibax *et al*., (2010) and Nugent *et al*., (2001) who reported that different concentrations of NaOCl were effective for sterilization of Eucalyptus explants. $HgCl_2$ has been also reported as effective sterilization agent for Eucalyptus explants by Rahim *et al*., (2003) and Mamaghani *et al*., (2010).

For germination of seeds full and half strength of MS medium was used. Fast germination rate was observed on half strength MS while on full strength slow germination was observed (Table 3). Germination on half and full strength MS medium was also done by Dibax *et al*., (2005). MS medium is widely used as basal medium for germination in tissue culture studies (Prakash & Gurumurthi, 2009; Ho *et al.,* 1998).

Table 1. Combinations of different plant growth hormones used for callogenesis and shoot regeneration of *E. Camaldulensis* and preculturing of explants.

Name of media	Concentrations of plant growth hormones (in MS basal medium)
CIM1	1.5mg/L BAP + 0.5mg/L NAA
CIM2	2mg/L Kn + 3mg/L 2,4-D
CIM3	1mg/L BAP + 0.5mg/L Kn
CIM4	2mg/L BAP + 1mg/L 2,4-D
CIM5	2mg/L BAP
SIM1	1mg/L BAP
SIM2	0.2mg/L BAP + 1mg/L Zn
Preculturing media	0.5mg/L BAP + 0.1mg/L NAA

CIM = Callus induction medium, SIM = Shoot induction medium

Table 2. Sterilization efficiency for seeds and explants.

Method	Chemical agents	Explants sterilized	Explants survived	Sterilization efficiency
C1	$HgCl_2$	23	7	30.43%
C2	$HgCl_2$ & Tween20	27	21	77.78%
C3	Clorox	60	60	100%
C4	NaOCl	30	27	90%

Table 3. Germination rate.

Media	Germination time	Seeds inoculated per plate	Seeds germinated per plate	Germination rate
½ MS	5-7 days	13	7	53.84%
Full MS	14-16 days	13	3	23.07%

MS medium with different concentration of hormones was employed for callogenesis and organogenesis (Table 1). CIM3 was proved to be the best medium for callogenesis and embryonic calli were also observed on this medium (Table 4, Fig. 1c). On CIM1, CIM2 and CIM4, embryonic as well as hard brown calli were formed (in case of *In vitro* grown explants), while green proliferating calli were formed only on CIM5 when young leaves of field grown plants were used as explants (Table 1, Fig. 1a, b, d, and e). Compact hard and brown somatic calli were observed on CIM1, CIM2 and CIM4 when young leaves of field grown plants were used. It means age of explants used may have an effect on callogenesis. Different concentrations of BAP and NAA were found to be effective for callogenesis in Eucalyptus species (Bandyopadhyay & Hamill, 2000).

Bacterial culture (*Agrobacterium tumefaciens*) with optical densities from 0.3-0.6 at 600nm was used to transform cotyledonary leaves, plantlet leaves and hypocotyls with and without preculture treatment. While for calli transformation, bacterial culture having $O.D._{600nm}$=0.2-0.8 was used, but none of the calli was found positive in Gus histochemical staining. In case of precultured explants, maximum transformation efficiency (100%) was observed at $O.D._{600nm}$= 0.3. While in case of non-precultured explants, maximum efficiency (66.67%) was at $O.D._{600nm=}$ 0.5 (Table 5). These results were supported by Matsunaga *et al.,* (2012), who transformed hypocotyls of *Eucalyptus globules* using *Agrobacterium tumefaciens* strain EHA105 at $O.D._{600nm}$ = 0.5. Transformation in young leaves of micropropagated hybrid (*E. grandis x E. urophylla)* was reported by Alcantara *et al.,* (2011) using *Agrobacterium tumefaciens* strain AGL1 at $O.D_{600nm}$=0.5. Furthermore, Spokevicius *et al.,* (2005) transformed wood producing apical stem segments of *Eucalyptus globules* by using Agrobacterium strains (AGL1 and LBA4404) with optical densities from 0.4-0.6. The positive result at different optical density of bacterial culture was possible due to different genetic makeup of the plant species and Agrobacterium strain. Average transformation efficiency was found to be higher in case of two days precultured explants, as compared to non-precultured plants (Table 5, Fig. 2b, c and d). Preculture treatment was found to influence the *uidA* gene expression, as reported by Tzfira & Citovsky (2006). The mechanism for the influence of preculture treatment has not been understood but some scientists indicated that preculturing of explants enhanced the binding of *Agrobacterium tumefaciens*. Moralejo *et al.,* (1998) observed that four to six days of preculture treatment enhanced transformation efficiency in cotyledons and hypocotyls of *E. globules* while in our case preculturing of explants for two days seems to be effective.

Infection time of 1hour was seemed to be optimum. When infection time was increased from 1 hour to 2 hour, it resulted in overgrowth of *Agrobacterium* and made explants prone to desiccation (Table 6).

Acetosyringone is an alcoholic compound that enhances the *Agrobacterium* infection and T-DNA delivery system. Different concentrations (0 µM, 200 µM and 400µM) of Acetosyringone were used at the time of co-cultivation and a concentration of 200 µM (1 day co-cultivation time) was found to be optimum. Acetosyringone in 400µM concentration enhanced the growth *of Agrobacterium*. Results were supported by McCormack *et al*., (1998) and Ke *et al*., (2002) who reported that increased concentration of Acetosyringone (100 µM) in wheat and barley increased the efficiency of transformation. Difference in optimized concentration of Acetosyringone could be due to difference of monocot and dicot plants.

The effect of co-cultivation time (1 day and 2 days) on transformation efficiency was also studied. 1 day co-cultivation showed good results. However, co-cultivation period more than one day resulted in the *Agrobacterium tumefaciens* overgrowth. Different days of co-cultivation have been reported previously in *Eucalyptus* spp. Ho *et al.,* (1998) reported two days of co-cultivation time in *E. camaldulensis,* in our case this is one day. The difference may be due to using different strains of Agrobacterium for infection. Spokevicius *et al.,* (2005) reported 2-7 days of co-cultivation time in apical segments of *E. globules*.

Different shoot regeneration media were used (Table 1). Callus induction media (CIM1) was also found to be effective for shoot regeneration. Shoot regeneration was observed after 20 days of callogenesis (Fig. 2a). No regeneration was observed on SIM1 and SIM2. Same results were given by Chang & Yang (1995) who studied micropropagation of *E. camaldulensis* and found the same harmone combination effective for callogenesis as well as for regeneration. However, the basal medium used by Chang & Yang (1995) was B5 instead of MS basal medium.

Kanamycin (25mg/L) selection was given to 50 regenerated shoots for 2 months, out of which 35 shoots survived. A fragment of predicted size (461 bp) was amplified from putative transgenic shoots, by using *uidA* gene specific primers. No amplification was observed in non transgenic control. This indicated the successful integration of T-DNA into the Eucalyptus genome. Overall transformation efficiency based on PCR amplification was found to be 14% (Fig. 3).

Table 4. Results of callogenesis on different CIM.

Media	Number of days	
	Swelling of explants	Callus formation
CIM1	19	28
CIM2	17	30
CIM3	14	20
CIM4	21	40
CIM5	18	30

CIM = Callus induction media

Table 5. Transformation efficiency for precultured & non-precultured explants at different optical densities.

Optical density	0.3	0.4	0.5
Preculture explants			
Infected explants	15	20	12
Positive for gus staining	15	06	04
Transformation efficiency	100%	30%	33.33%
Non-precultured explants			
Infected explants	21	24	30
Positive for gus staining	07	06	20
Transformation efficiency	33.33%	25%	66.67%

Table 6. Effect of Co-cultivation time and infection time on overgrowth of *Agrobacterium tumefaciens*.

1 Day co-cultivation time		2 Days co-cultivation time	
Infection time	*Agrobacterium tumefaciens* overgrowth	Infection time	*Agrobacterium tumefaciens* overgrowth
1 hour	Absent	1 hour	Absent
2 hours	Present	2 hours	Present

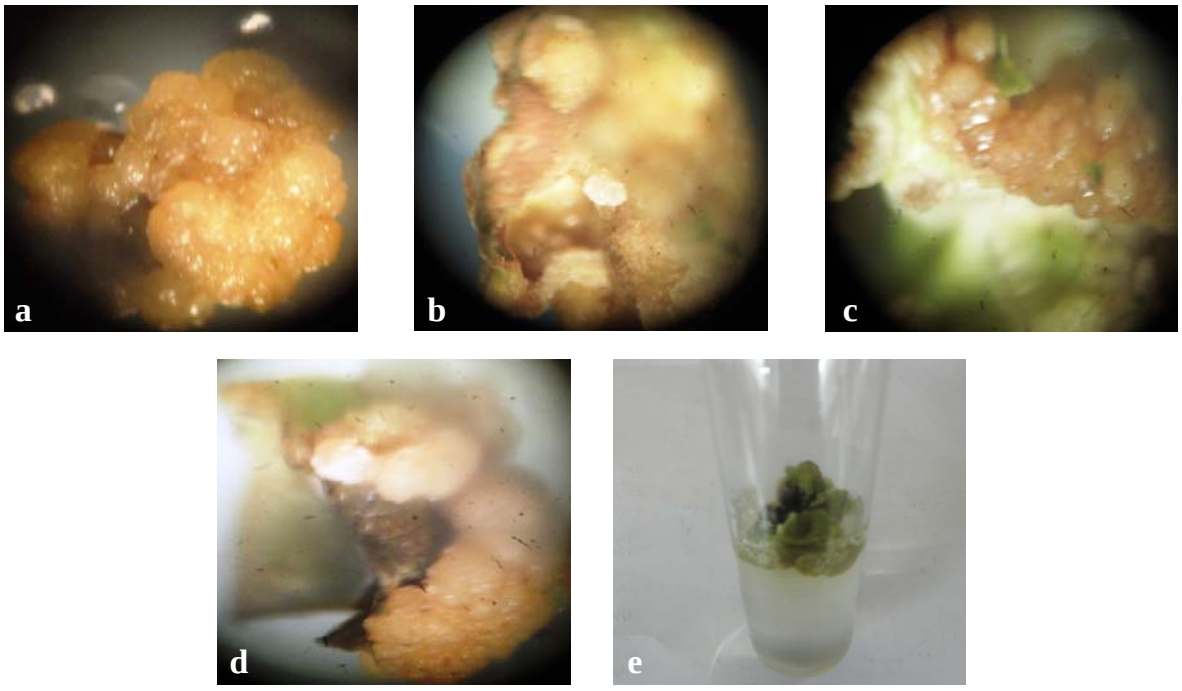

Fig. 1. Callogenesis on different plant growth hormonal combinations.
A: Callus on CIM1, B: Callus on CIM2, C: Callus on CIM3, D: Callus on CIM4, E: Callus on CIM5

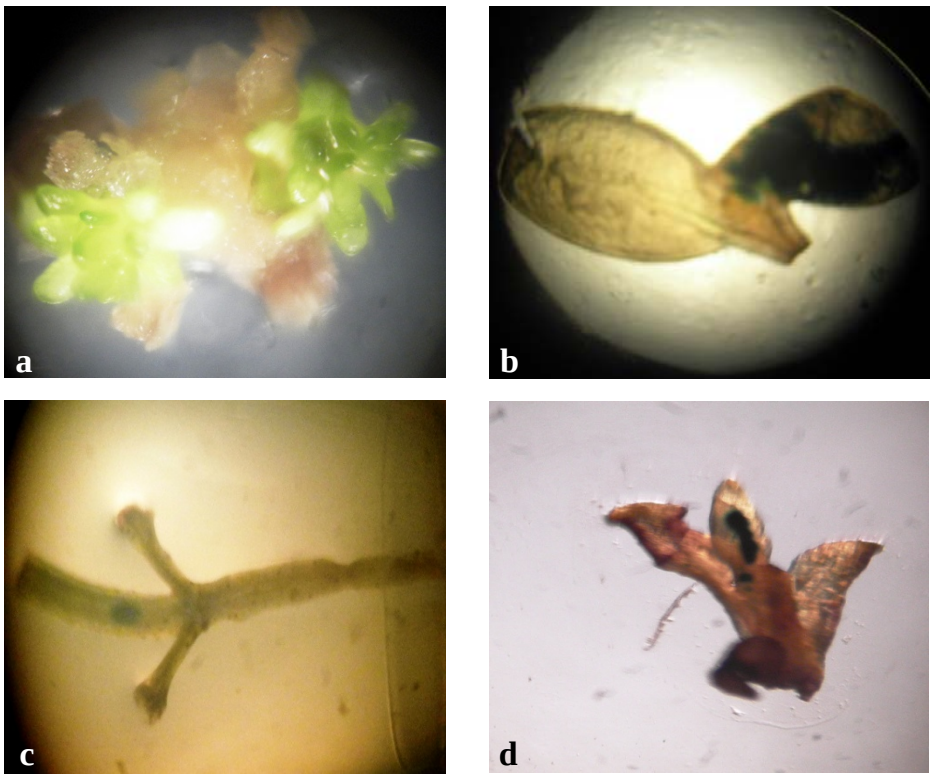

Fig. 2. Photographic presentation of *Agrobacterium tumefaciens* mediated transformation and shoot regeneration of *Eucalyptus camaldulensis*.
a. Shoot regeneration on MS basal medium with 1.5 BAP + 0.5 NAA + 400mg/L Timentin
b. GUS expression of nonprecultured hypocotyls at $O.D_{600nm}$=0.5
c. GUS expression of nonprecultured plantlet leaf at $O.D_{600nm}$=0.5
d. GUS expression of precultured plantlet leaf at $O.D_{600nm}$=0.3

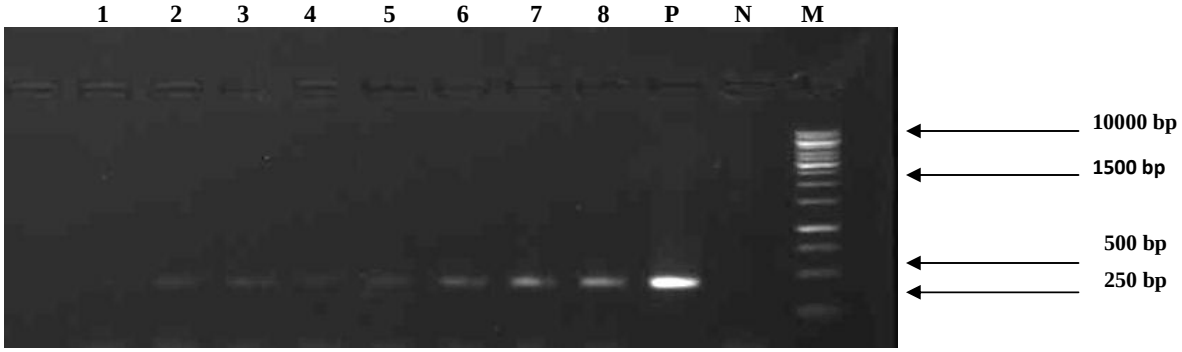

Fig. 3. PCR Result of putative transgenic plants showing integration of *uidA* gene.
1= Non transgenic, 2-8= Transformants, P= pGR1 (Positive control), N= Negative control, M = Marker of 1 kb

Conclusion

The protocol developed in this study provides a simple and efficient system for introduction of desirable characters in *Eucalyptus camaldulensis*. It also suggests that *In vitro* seedlings are good target tissues for producing transgenic plants.

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References

Alcantara, G.B., J.C. Bespalhok-Filho and M. Quoirin. 2011. Organogenesis and transient genetic transformation of the hybrid *Eucalyptus grandis* × *Eucalyptus urophylla* .*Scientia Agricola*, 68(2): 246-251.

Bandyopadhyay, S. and J.D. Hamill. 2000. Ultrastructural studies of somatic embryos of *Eucalyptus nitens* and comparisons with zygotic embryos found in mature seeds. *Annals of Botany*, 86: 237-244.

Bown, D. 1995. *Encyclopedia of Herb and Their Uses*. DK Publishing Inc, New York, pp. 280.

Chang, S.H. and J.C. Yang. 1995. Callus culture and regeneration of *Eucalyptus camaldulensis* (in Chinese). *Bull Taiwan for Res Inst New Ser*, 10: 15-24.

Dibax R., R.C. Quisen, C. Bona and M. Quoirin. 2010. Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis* dehn and histological study of organogenesis *In vitro*. *Brazilian Archives of Biology and Technology,* 53(2): 311-318.

Dibax, R., C.L. Eisfeld, F.L. Cuquel, H. Koehler and M. Quoirin. 2005. Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis*. *Scientia Agricola (Piracicaba, Brazil)*, 62: 406-412.

Grattapaglia, D. 2008. Genomics of Eucalyptus, a global tree for energy, paper and wood. In: *Genomics of Tropical Crop Plants Volume 1*, Springer Publisher, New York, pp. 259.

Harcourt, R.L., J. Kyozuka, R.B. Floyd, K.S. Bateman, H. Tanaka, V. Decroocq, D.J. Llewellyn, X. Zhu, W.J. Peacock and E.S. Dennis. 2000. Insect- and herbicide-resistant transgenic eucalypts. *Molecular Breeding*, 6(3): 307-315.

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

Horng, W., T.A. Shen, C.F. Chen and J.C. Yang. 1991. Studies on management of fast growing and high quality fiber producing tree species. In: *Proceeding of the COA symposium for 14 Current Advances On Research Projects of Forest Management & Economics*, Taipei, pp. 143-151.

Ikemori, Y.K. 1987. Epicormic shoots from the branches of *Eucalyptus grandis* as an explant source for *In vitro* culture. *Commonwealth Forestry Review,* 66: 351-356.

Ke, Xia-Yi., A.C. McCormac, A. Harvey, D. Lonsdale Chen Dong-Fang and M.C. Elliot. 2002. Manipulation of discriminatory T-DNA delivery by *Agrobacterium* into cells of immature embryos of barley and wheat. *J. Euphytica*, 126: 333-343.

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

Macrae, S. and J. Van Staden. 1993. *Agrobacterium rhizogenes*-mediated transformation to improve rooting ability of eucalypts. *Tree Physiol*., 12: 411-418.

Mamaghani, M.S., T.R. Shojaei, M. Matinizadeh and M. Forootan. 2010. Microsatellite loci and peroxidase alleles correlation in somaclonal variation of *Eucalyptus microtheca* F. Muell. *African Journal of Biotechnology*, 9(29): 4521-4528.

Manders, G., A.V.P dos. Santos, F.B. d'Utra Vaz, M.R. Davey and J.B. Power. 1992. Transient gene expression in electroporated protoplasts of *Eucalyptus citriodora* Hook. *Plant Cell Tissue Organ Cult*., 30: 69-75.

Matsunaga, E., K. Nanto, M. Oishi, H. Ebinuma, Y. Morishita, N. Sakurai, H. Suzuki, D. Shibata and T. Shimada. 2012. *Agrobacterium-mediated* transformation of *Eucalyptus globules* using explants with shoot apex with introduction of bacterial choline oxidase gene to enhance salt tolerance. *Plant Cell Rep*., DOI 10.1007/ s00299-011-1159-y.

McComb, J.A. and I.J. Bennett. 1986. Eucalyptus (*Eucalyptus* spp.). In: *Biotechnology in agriculture and forestry, trees I.* (Ed.): Y.P.S. Bajaj. Springer, Berlin Heidelberg New York, pp. 340-362.

McCormac, A.C., H. Wu, M. Bao, Y. Wang, R. Xu, M.C. Elliott and Chen Dong-Fang. 1998. The use of visual marker genes as cell specific reporters of *Agrobacterium*-mediated TDNA delivery to wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). *J. Euphytica*, 99: 17-25.

Moralejo, M., F. Rochange, A.M. Boudet and C. Teulières. 1998. Generation of transgenic *Eucalyptus globulus* plantlets through *Agrobacterium tumefaciens*-mediated transformation. *Australian Journal of Plant Physiology*, 25: 207-212.

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

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

Murray, M.G. and W.F. Thompson. 1980. Rapid isolation of high molecular-weight plant DNA. *Nucleic Acids Res*., 8: 4321-4325.

Nugent, G., S.F. Chandler, P. Whiteman and T.W. Stevenson. 2001. Adventitious bud induction in *Eucalyptus globulus* Labill. *In Vitro Cellular and Development Biology-Plant*, 37: 338-391.

Prakash, M.G. and K. Gurumurthi. 2009. Genetic transformation and regeneration of transgenic plants from precultured cotyledon and hypocotyl explants of *Eucalyptus tereticornis* Sm. using *Agrobacterium tumefaciens*. *In Vitro Cell Dev. Biol. Plant.*, 45: 429-434.

Qadri, S.M.A. 1966. Selection of Eucalyptus species for in afforstation in West Pakistan, in Proceedings of Second Pakistan Silvicultural Conference, pp. 145.

Rahim, F., M. Jabeen and I. Ilahi. 2003. Mass propagation in *Eucalyptus camaldulensis* Dehn. *Asian Journal of Plant Sciences*, 2(2): 184-187.

Rochange, F., L. Serrano, C. Marque, C. Teulieres and A.-M. Boudet. 1995. DNA delivery into *Eucalyptus globulus* zygotic embryos through biolistics: optimization of the biological and physical parameters of bombardment for two different particle guns. *Plant Cell Rep*., 14: 674-678.

Spokevicius, A.V., K.V. Beveren, M.A. Leitch and G. Bossinger. 2005. *Agrobacterium*-mediated *In vitro* transformation of wood producing stem segments in eucalypts. *Plant Cell Reports*, 23: 617-624.

Teulieres, C., J. Grima-Pettenati, C. Curie, J. Teissie and A.M. Boudet. 1991. Transient foreign gene expression in polyethylene/ glycol treated or electropulsated *Eucalyptus gunnii* protoplasts. *Plant Cell Tissue Organ Cult*., 25: 125-132.

Turnbull, J.W. 1991. Future use of eucalypts: opportunities and problems. In: *IUFRO Symp Intensive For – The role of eucalypts*. (Ed.): A.P.G. Schonau. Southern African Institute of Forestry, Pretoria, pp. 2-27.

Tzfira, T. and V. Citovsky. 2006. *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Current Opinion in Biotechnology*, 17: 147-154.

Warrag, E.L., M.S. Lesney and D.L. Rockwood. 1990. Micropropagation of field-tested superior *Eucalyptus grandis* hybrids. *New Forests*, 4(2): 67-79.

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