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ORIGINAL PAPER

Agrobacterium tumefaciens mediated genetic transformation of selected elite clone(s) of *Eucalyptus tereticornis*

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Abstract Procedure for the *Agrobacterium tumefaciens* mediated T-DNA delivery into the elite clone(s) of *Eucalyptus tereticornis* using leaf explants from microshoots has been developed. Amongst two strains of *A. tumefaciens* namely, EHA105 and LBA4404 (harbouring pBI121 plasmid), strain EHA105 was found to be more efficient. Pre-culturing of tissue (2 days) on medium supplemented with 100 μM acetosyringone, before bacterial infection significantly increased transient expression of reporter gene (GUS). Co-cultivation period of 2 days and a bacterial density of 0.8 OD_{600} resulted in higher transient GUS expression. Method of injury to tissue, presence of acetosyringone in co-cultivation medium and photoperiod during co-cultivation also influenced the expression of transient GUS activity. Amongst the three clones tested, maximum transient GUS activity was recorded in clone 'CE2' followed by clone 'T1'. Regeneration of transformed shoots was achieved on modified Murashige and Skoog medium (potassium nitrate was replaced with 990 mg/l potassium sulphate and ammonium nitrate with 392 mg/l ammonium sulphate, and mesoinositol concentration was increased to 200 mg/l). Stable transformation was confirmed on the basis of GUS activity and PCR amplification of DNA fragments specific to *uidA* and *nptII* genes. The absence of bacteria in the stable transformed tissues was confirmed by PCR amplification of fragment specific to 16S rRNA of bacteria.

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	Benzyladenine
CaMV 35S	35S promoter of the cauliflower mosaic virus
GUS	*β*-Glucuronidase
MS	Murashige and Skoog (1962)
NAA	α-Naphthaleneacetic acid
Nos	Nopaline synthase
nptII	Neomycin phosphotransferase
OD_{600}	Optical density at 600 nm
PCR	Polymerase chain reaction
X-Gluc	5-bromo-4-chloro-3-indolyl-*β*-D-glucuronic acid

Introduction

Plantation forestry, with optimized and increased productivity is the major source of wood products for many purposes (Turnbull 1999). The increase in forest productivity and refinement of wood quality through genetic manipulations is becoming increasingly important (Grattapaglia and Kirst 2008). Improvement of tree species by conventional breeding is constrained by their long reproductive cycles and complex genetic characteristics, including self-incompatibility and high degree of heterozygosity. Plant genetic transformation offers an attractive alternative to conventional breeding, because it provides the potential of incorporating novel trait specific gene(s) into selected genotypes without affecting their desirable genetic combination. In addition, some major

bottlenecks in tree breeding, such as long juvenile phase and segregate evaluations, could be avoided (Peña and Séguin 2001).

Plantations of *Eucalyptus* are the source of wood biomass for several industrial applications including pulp and paper (Turnbull 1999). The genus *Eucalyptus* comprises of more than 700 species (Brooker 2000) and *E. tereticornis* Sm. is most widely cultivated in India. Significant progress has been made towards the development of regeneration protocol for some of *Eucalyptus* species via organogenesis (Subbaiah and Minocha 1990; Mullins et al. 1997; Barrueto Cid et al. 1999; Nugent et al. 2001; Dibax et al. 2005; Pinto et al. 2008; Aggarwal et al. 2010) and somatic embryogenesis (Nugent et al. 2001; Pinto et al. 2008). However, a few studies are available on the development of genetic transformation protocol of *Eucalyptus* using *Agrobacterium* (Ho et al. 1998; Tournier et al. 2003; Prakash and Gurumurthi 2009). These studies mainly focused on the development of genetic transformation protocol using juvenile tissues of seed origin (Ho et al. 1998; Tournier et al. 2003; Prakash and Gurumurthi 2009). Only one report on *A. tumefaciens* mediated genetic transformation of *E. tereticornis* using hypocotyls taken from zygotic embryos is available (Prakash and Gurumurthi 2009). A close survey of literature could not reveal any report on development of genetic transformation protocol for the selected elite clone(s) of *E. tereticornis.* Further, *Agrobacterium* mediated genetic transformation offers several advantages, such as defined integration of transgenes, low copy number and preferential integration into transcriptionally active regions of the chromosomes and hence is the most powerful tool for plant genetic transformations (Gelvin 2005).

Therefore, this study was aimed at to develop an efficient protocol for *Agrobacterium tumefaciens* mediated T-DNA delivery into the tissues taken from selected *E. tereticornis* elite clones and subsequent regeneration of transformed shoots.

Materials and methods

Plant material, chemicals, glassware and culture establishment

Plants (about 12 years old) of three selected elite clones of *E. tereticornis* Sm. namely 'T1', 'CE2' and 'Y8' growing at Thapar University Campus were selected for this study. All routinely used chemicals were purchased from HiMedia Laboratories (Mumbai, India), growth regulators and antibiotics were purchased from Sigma Chemical Co. (St Louis, MO, USA). Unless otherwise mentioned all experiments were conducted in 300 ml glass culture bottles (Kasablanka, Mumbai) containing 50 ml of medium. The pH of medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. Cultures were established following the procedure mentioned earlier (Aggarwal et al. 2010) and incubated at 25 ± 1°C under cool white fluorescent lamps (Philips India Ltd, Mumbai) with the light intensity of 42 μmol m^{-2} s^{-1} inside the culture vessel in 16-h light/8-h dark cycle. The actively growing shoot cultures were maintained on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 2.5 μM benzyladenine (BA) and 0.5 μM α-naphthaleneacetic acid (NAA). The shoot elongation and leaf expansion were achieved in these actively proliferating shoots on MS medium supplemented with 0.1 μM BA and 0.5 μM NAA. The fully expanded leaves (14–20 days old) from these elongated microshoots were used as explants for the transformation experiments.

Binary vector and *Agrobacterium* strain

The binary vector pBI121 having *uidA* gene (*β*-glucuronidase) (GUS) and selection marker gene *nptII* (neomycin phosphotransferase II) was used for genetic transformation. These genes were on the T-DNA under the control of *CaMV 35S* and *nos* promoters, respectively (Jefferson et al. 1987). The plasmid was introduced into *Agrobacterium tumefaciens* disarmed strains EHA105 (Hood et al. 1993) and LBA4404 (Hoekema et al. 1983) by the freeze-thaw method (Holsters et al. 1978). The presence of pBI121 plasmid was confirmed in the antibiotic resistant bacterial colonies by PCR using *nptII* gene specific primers (Sun and Meng 2010). The transformed *A. tumefaciens* strains were maintained at 28°C on yeast extract peptone (YEP) agar medium (10 g/l bacto peptone, 10 g/l yeast extract, 0.5 g/l NaCl and 1.5 g/l agar pH-7) containing 15 μg/ml rifampicin and 50 μg/ml kanamycin and used for the genetic transformation experiments.

Determination of antibiotic sensitivity

Tolerance limits (sensitivity) of the leaf segments for kanamycin was determined by culturing these on shoot induction medium (SIM, Table 1) supplemented with different concentrations of kanamycin (0, 10, 20, 30, 40, 50, 70 and 100 μg/ml). Kanamycin was filter sterilized and added to the medium after autoclaving when the temperature has come down to 40°C.

Transformation

Co-cultivation and infection

A single bacterial colony was inoculated in 10 ml liquid YEP medium supplemented with 50 μg/ml kanamycin and 15 μg/ml rifampicin and grown overnight at 28°C on a

Table 1 Different media combinations used for *Agrobacterium*-mediated genetic transformation of *E. tereticornis*

Culture medium	Composition
Shoot induction medium (SIM)	MS medium + 5.0 μM BA + 1 μM 2,4-D + 0.7% agar + 30 g/l sucrose, pH 5.8
Pre culture medium (PCM)	MS medium + 5.0 μM BA +1 μM 2,4-D + 0.7% agar + 30 g/l sucrose + 100 μM Acetosyringone, pH 5.8
Co-cultivation medium(CCM)	MS medium + 0.7% agar + 30 g/l sucrose + 100 μM Acetosyringone, pH 5.2
Selection medium-I (SM-I)	MS medium + 5.0 μM BA + 1 μM 2,4-D + 0.7% agar + 30 g/l sucrose + 50 mg/l kanamycin + 500 mg/l cefotaxime, pH 5.8
Selection medium-II (SM-II)	MS medium—KNO_3 + NH_4NO_3 = 391.8 mg/l + K_2SO_4 = 990 mg/l + NH_4SO_4 = 323 mg/l + Inositol = 200 mg/l + 5.0 μM BA +1 μM 2,4-D + 0.7% agar + 30 g/l sucrose + 50 mg/l kanamycin + 500 mg/l cefotaxime, pH 5.8

gyratory shaker (250 rpm). From the overnight grown culture, 0.5 ml was freshly inoculated to 50 ml of YEP medium supplemented with 50 μg/ml kanamycin and 15 μg/ml rifampicin and these bacterial cultures were grown for 24 h. Bacterial cells were pelleted by centrifugation (4000×*g*, 2 min) and suspended in YEP medium supplemented with 100 μM acetosyringone (Sigma Chemical Co., St Louis, MO) to attain the desired OD_{600}. Leaf explants that were cultured on pre-culture medium (PCM, Table 1) for 0–5 days were injured following different procedures namely, pricking with hypodermic needle, cutting with surgical blade, rubbing with carborundum paper or glass beads. Then these were infected with above mentioned suspension of *A. tumefaciens* for different time periods (10 ml, 0–30 min) in Petri plates. After infection, tissues were blotted with sterile filter paper to remove the excess of bacterial cells and medium**.** These were then cultured (1–5 days) on antibiotic-free co-cultivation medium (CCM, Table 1). Cultures were sealed with cling film and incubated under different photoperiods viz continuous light, 16-h light/8-h dark or continues dark.

Selection of transformed tissue

Following co-cultivation, leaf explants were washed 4–5 times with sterile distilled water containing 500 μg/ml cefotaxime, blotted on sterile filter paper and transferred to culture bottles containing MS medium supplemented with 5.0 μM BA, 1.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 50 μg/ml kanamycin and 500 μg/ml cefotaxime (selection-*cum*-shoot induction medium: SM-I; Table 1). The cultures were sub-cultured on same medium at every 20 days interval.

Regeneration of transformed shoots

Following genetic transformation of tissue, shoot organogenesis was not observed with the protocol reported earlier (Aggarwal et al. 2010). Therefore, certain changes were made in the basal MS medium to achieve shoot organogenesis. The composition of medium used to achieve shoot organogenesis (SM–II) is mentioned in Table 1.

Histochemical GUS assay

GUS assay was carried out using regenerated kanamycin resistant shoots and freshly infected explants after 2 days of incubation on SM-I for scoring transient expression following the method of Jefferson et al. (1987). For transient GUS assay, in total of 90 explants were scored for each treatment. Tissues were incubated overnight at 37°C in 100 mM sodium phosphate buffer (pH 7.0), containing 1 mM X-Gluc, 0.5 mM potassium ferrocyanide and 0.1% (v/v) Triton X-100. Following incubation, tissue was cleared to remove chlorophyll by washing several times with 70% ethanol and then slowly increased to absolute ethanol. The tissue showing blue colour was scored.

Molecular analysis

DNA was isolated from tissue following CTAB method (Doyle and Doyle 1990). The PCR amplification of DNA fragments specific to *nptll* and *uidA* genes was carried out using genomic DNA isolated from the leaves of GUS positive putative transgenic shoots and untransformed plants. The PCR reaction mixture consisted of 20 ng of genomic DNA, 1.0 U of *Taq* DNA polymerase (Larova, Teltow, Germany), 100 μmol $dNTP_S$ mixture, 2.0 μl reaction buffer (10X), 10 nmol each primer and sterile Milli-Q water (Millipore India, Bangalore) was added to make up the volume to 20 μl. Amplification conditions were as follows: initial denaturation 94°C for 5 min followed by 31 cycles of 94°C for 1 min, 58°C for 45 s and 72°C for 1.5 min a with final extension at 72°C for 5 min. A fragment of about 1,500 bp specific to *uidA* gene was amplified using primer pairs (forward primer 5-GGTGGGAAAGCGCGTTACAAG-3 and reverse primer 5-GTTTACGCGTTGCTTCCGCCA-3) and a fragment of about 760 bp specific to *nptII* was amplified using primer

pair (forward primer 5-GAGGCTATTCGGCTATGAC TC-3 and reverse primer 5-ATCGGGAGAGGCGATA CCGTA-3). Plasmid DNA of pBI121 was used as a positive control and leaves from untransformed shoots of *E. tereticornis* were the source of DNA for negative control.

PCR amplification of 16S rRNA fragment of about 1,500 bp was carried out using DNA extracted from putative transformed and untransformed shoots using primer pair 5-AGAGTTTGATCCTGGCTCAG-3 and 5-ACGGG CGGTGTGTTC-3 (Weisburg et al. 1991) specific to bacterial 16S rRNA. Bacterial genomic DNA was used as positive control. Amplification conditions were same as mentioned above. The amplified products were separated on a 1.0% (w/v) agarose gel and viewed using UV transilluminator (BioRad, CA, USA) following ethidium bromide staining.

Statistical analysis

Experiments were conducted in triplicates and repeated three times. The data were analyzed by analysis of variance and the means were compared using Student-Newman–Keuls-test ($P < 0.05$). All the analyses were performed using GraphPad Prism 4 software (GraphPad, San Diego, CA).

Results and discussion

In this investigation, factors influencing efficiency of T-DNA delivery into the selected elite clones of *E. tereticornis* using *A. tumefaciens* was studied. This is the first report investigating factors influencing the genetic transformation of selected elite clones of *E. tereticornis*. Subsequently, shoot organogenesis was achieved by modifying earlier reported protocol (Aggarwal et al. 2010).

Experimental results investigating the sensitivity of the leaf explants showed that the presence of kanamycin in the medium caused considerable toxicity to explant and resulted in drastic decline in shoot regeneration potential compared to those cultured on kanamycin-free medium. On medium lacking kanamycin, about 14% of explants resulted in shoot organogenesis, whereas incorporation of kanamycin inhibited shoot organogenesis and all the explants died on media containing >50 μg/ml kanamycin (Fig. 1). Therefore, the concentration of kanamycin was kept at 50 μg/ml in all experiments (unless otherwise mentioned). These results are in line with the earlier reports on *Eucalyptus* spp. (Ho et al. 1998; Tournier et al. 2003; Prakash and Gurumurthi 2009), *Leucaena leucocephala* (Jube and Borthakur 2009) and *Quercus suber* (Alvarez and Ordas 2007).

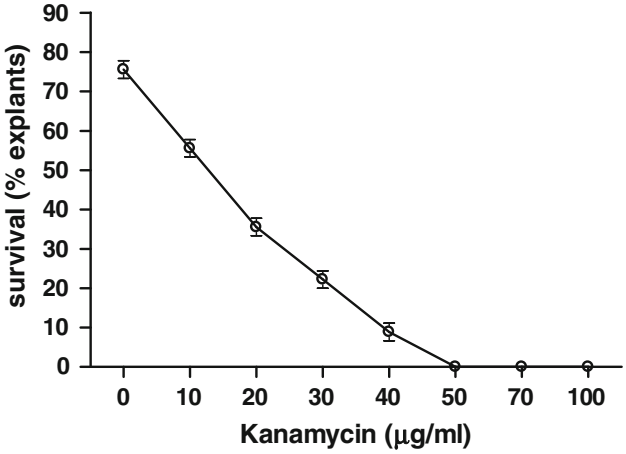

Fig. 1 The effect of kanamycin on the survival of leaf explants taken from microshoots of *E. tereticornis*. Data were recorded after 6 weeks of culture. Values are the means of three experiments consisting of three replicates each (ten explants in each replicate)

The efficiency of two strains of *A. tumefaciens* namely, EHA105 and LBA4404 were tested for genetic transformation. Strain EHA105 induced significantly higher transient GUS activity (54.4% explants) as compared to strain LBA4404 (46.5% explants). Therefore, strain EHA105 was used in all subsequent experiments. Strain EHA105 is derived from super virulent strain A 281 (Hood et al. 1993), while strain LBA4404 is derived from the less virulent strain Ach 5 (Hoekema et al. 1983). The strain EHA105 has been reported to be more efficient than other strains for transformation of *Punica granatum* (Terakami et al. 2007) and *Saccharum* spp. (Manickavasagam et al. 2004). However, strain LBA4404 has also been used successfully for transformation of plants including *Eucalyptus* (Prakash and Gurumurthi 2009). It is reported that the elimination of strain LBA4404 from plant tissues is relatively easier using lower concentration of antibiotics (Maheswaran et al. 1992) and it is difficult to eliminate strain EHA105 (Terakami et al. 2007). Therefore, in this investigation concentration of cefotaxime was kept 500 μg/ml in subsequent subculture cycles to eliminate the bacteria. Further, it was reported that cefotaxime enhanced shoot organogenic potential of leaf explants of *E. tereticornis* (Aggarwal et al. 2010).

The various factors namely pre-culture, bacterial density, mode of injury, incubation conditions, acetosyringone etc., influenced transformation efficiency of leaf explants (Table 2). Culture medium and incubation conditions of the explants, prior to *Agrobacterium* infection have been reported to enhance T-DNA delivery in some plant species (Ho et al. 1998; Padmanabhan and Sahi 2009; Yevtushenko and Misra 2010). In this study, leaves pre-cultured on PCM medium (Table 1) containing 50 μM acetosyringone for 2 days and incubated under 16 h light cycle showed

Table 2 The effect of different parameters on transient GUS expression after two days of co-cultivation with *Agrobacterium tumefaciens* strain EHA105 harbouring binary vector pBI121

Factors	Variable	% *GUS* expression
Infection time	5 min	44.4 bc
	10 min	52.2 a
	15 min	50.0 ab
	20 min	43.3 bc
	30 min	38.8 c
pH of co-cultivation medium	5.2	54.4 a
	5.5	50.0 a
	5.8	45.5 a
Pre-culture	0 day	38.8 d
	1 day	47.7 bc
	2 days	59.0 a
	3 days	52.2 b
	4 days	45.5 c
	5 days	40.0 d
Co-cultivation period	1 day	45.5 bc
	2 days	56.6 a
	3 days	48.8 b
	4 days	43.3 bc
	5 days	41.1 c
O.D value	0.2	35.5 d
	0.4	44.4 c
	0.6	54.3 ab
	0.8	60.0 a
	1.0	48.8 bc
Method of injury	Intact	35.5 c
	With hypodermic needle	57.7 a
	With surgical blade	46.6 b
	With carborundum	42.2 bc
	With glass beads	38.8 c
Acetosyringone (μM)	0	41.1 c
	50	59.4 a
	100	62.2 a
	200	47.7 b
Photoperiod	24 h light	43.3 b
	24 h dark	47.7 b
	16 h light/8 h dark	55.5 a

The effect of acetosyringone was evaluated by incorporating it in CCM medium. Values are the means of three experiments consisting of three replicates each (ten explants in each replicate). All the explants were examined for gus activity. Mean values within column (each factor analyzed separately) followed by same letter are not significantly different according to Student-Newman–Keuls-test ($P < 0.05$)

maximum transient GUS activity (59%; Table 2). The higher GUS activity is probably due to the presence of cytokinin resulting in increased cell division (Sangwan et al. 1992) and/or presence of acetosyringone that is known to induce *vir* genes and enhance T-DNA transfer (Stachel et al. 1985). Earlier, pre-culturing of explants on a particular medium prior to infection with *Agrobacterium* has been reported to enhance transformation efficiency in many plant species (Ho et al. 1998; Padmanabhan and Sahi 2009; Yasmeen 2009) including *E. camaldulensis* (Ho et al. 1998).

Incorporation of 100 μM acetosyringone in the co-cultivation medium increased transient GUS activity from 41.1% (on medium lacking acetosyringone) to 62% (Table 2). Earlier acetosyringone has been shown to enhance *Agrobacterium* mediated genetic transformation efficiency in *Eucalyptus* spp. (Tournier et al. 2003) and *Camellia sinensis* (Lopez et al. 2004).

Method of injury to the tissue prior to bacterial infection was also observed to play an important role in T-DNA delivery. Pricking of tissue using hypodermic needle enhanced transient GUS activity from 35.5% (in intact explants) to 57.7% explants (Table 2). Wounding of tissue before infection could allow bacterial penetration deep into the tissue facilitating the accessibility of plant cells to *Agrobacterium* on one hand and may stimulate the induction of *vir* genes as a result of phenolics secretion on other (Stachel et al. 1986), which could be the main reasons for enhanced bacterial efficiency for T-DNA delivery (Binns and Thomashow 1988). Method of injury to plant tissue before co-cultivation has also been shown to influence transformation frequency in *Vitis vinifera* (Dutt et al. 2007) and *Vigna radiata* (Sonia et al. 2007).

The pH of medium during co-cultivation also influenced the efficiency of T-DNA delivery. Higher frequency of explants showed transient GUS activity when cultured on medium with pH of 5.2 (54.4%) as compared to pH of 5.8 (45.5%), however, these differences were statistically non significant (Table 2). Low pH during co-cultivation was reported to be beneficial for *Agrobacterium* mediated transformation across the species by Godwin et al. (1991).

The density of bacterial suspension used for infection of the explant also influenced transient GUS activity (Table 2). Maximum transient GUS activity was obtained in explants that were infected with the bacterial suspension having an OD_{600} of 0.8. At higher bacterial density, the decrease in transient GUS activity was observed (Table 2). This could be due to increased production of toxic compounds due to bacterial overgrowth (Sonia et al. 2007) resulting in the necrosis of the tissue.

A co-cultivation period following *Agrobacterium* infection influenced the expression of transient GUS activity (Table 2). A maximum of 56.6% explants showed transient GUS activity when these were co-cultivated for 2 days. It is known that co-cultivation of explants with *A. tumefaciens* for an appropriate duration improves the

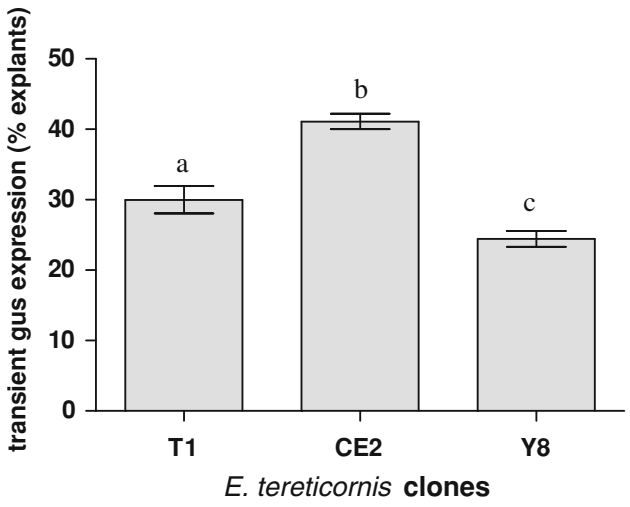

Fig. 2 Transformation efficiencies of different clones of *E. tereticornis*. Values are the means of three experiments consisting of three replicates each (ten explants in each replicate). Values sharing common letter are not significant at $P \leq 0.05$

transformation efficiency, but prolonged co-cultivation was reported to result in death of explants, resulting from overgrowth of bacteria (James et al. 1993). In this study, co–cultivation period of more than 3 days caused excessive bacterial growth leading to necrosis of explants. Although, an increased transformation frequency has been positively correlated with longer co-cultivation periods (2–5 days), yet it has been emphasized that for higher transformation efficiency, an optimum co-cultivation period is required (Niu et al. 2000).

Photoperiod during co-cultivation also had an impact on the transient GUS activity (Table 2). A photoperiod of 16-h was found to induce transient GUS activity in maximum explants (55.5%). Prakash and Gurumurthi (2009) successfully used 16-h photoperiod for the transformation of hypocotyls segments of *E. tereticornis*. Beneficial effect of light on *Agrobacterium* mediated T-DNA transfer to *Phaseolus acutifolius* and *Arabidopsis thaliana* has been reported (Zambre et al. 2003). Photoperiod during co-cultivation was also reported to influence the efficiency of transient expression of reporter gene in *Dianthus caryophyllus* (Zuker et al. 1999).

The transformation efficiency also significantly varied from clone to clone, and amongst the three clones tested namely, 'T1', 'CE2' and 'Y8', the percent explants showing transient GUS expression was 30.0, 41.1 and 24.4%, respectively (Fig. 2). Mullins et al. (1997) also reported clonal variations with respect to *Agrobacterium* mediated genetic transformation in *Eucalyptus* spp. These clonal variations are likely to be due to differential virulence of *Agrobacterium* towards these clones.

The overall transformation efficiency was also influenced by the regeneration potential of transformants. These

Table 3 Frequency of transformed shoots in different clones of *E. tereticornis* regenerated on selection medium

Clone	No. of kanamycin resistant calli	No. of kanamycin resistant shoots	Frequency of transformed shoots (%)
CE-2	75	14	2.33 ± 0.95
Y-8	62	9	1.50 ± 0.73
T-1	54	7	1.16 ± 0.85

Transformation frequency based on surviving shoots on selection medium expressing the reporter gene and tested positive by PCR. Mean value in each experiment is average of ten sets of experiment with 60 leaf explants in each set

transformation and regeneration parameters did not appear to be correlated since the clones showing better regeneration potential did not show better transient expression. Earlier, Aggarwal et al. (2010) reported that the clone T1 showed higher regeneration potential. It is now observed that the transient expression was higher in clone CE2 (Fig. 2).

Although, most of the parameters were found to influence T-DNA transfer, yet a lower transformation efficiency was observed in this study (Table 3). This could be due to the use of explants taken from the mature plants of selected elite clones, which is essential in retaining the superior genetic makeup of the existing genotype. It has been reported earlier that tissue taken from selected clones results in lower transformation and organogenesis as compared to juvenile tissues taken from seedlings (Tournier et al. 2003; Aggarwal et al. 2010). Further, in *E. globulus,* regeneration following transformation was lower, and it was difficult to recover transgenic shoots even if stable transformation was achieved (Serrano et al. 1996). In this study, it took 35–40 weeks to recover transgenic shoots on selection medium containing kanamycin.

Since problems were faced in the recovery of transgenic shoots following transformation using protocol reported earlier, certain changes were made in the composition of basal medium initially used for selection of transgenic plants (SM-I). The composition of new medium (SM-II) used to achieve shoot organogenesis is mentioned in table 1. Potassium nitrate was replaced with 990 mg/l potassium sulphate and ammonium nitrate with 392 mg/l ammonium sulphate. It has been documented that a proper NO_3^-/NH_4^+ ratio influences morphogenesis (Ramage and Williams 2002) but the range of optimal ratios depends on the species (Tazawa and Reinert 1969; Ivanova and Van Staden 2009). Recently, Ivanova and Van Staden (2009) also highlighted the role of nitrogen sources and optimal ratio of NO_3^-/NH_4^+ for successful shoot organogenesis in *Aloe polyphylla*. These changes resulted in successful shoot organogenesis from transformed tissues (Fig. 3a). Further,

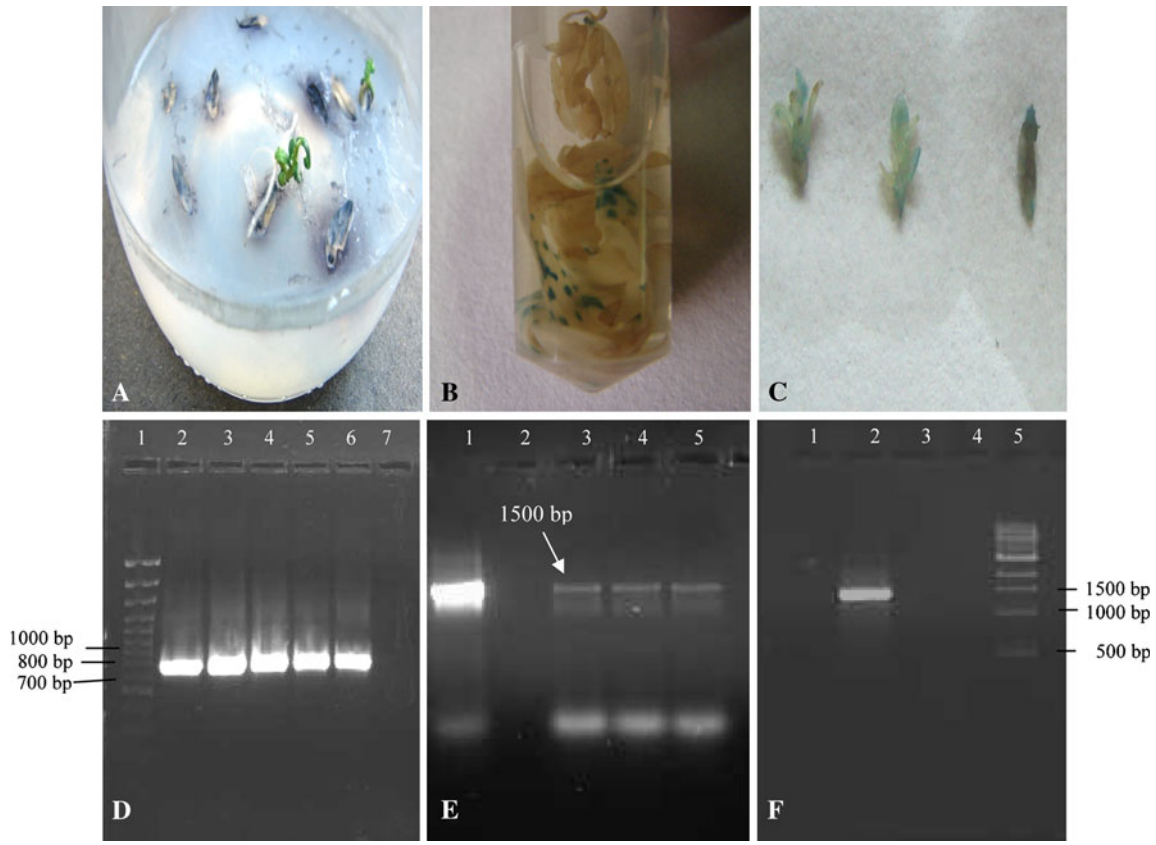

Fig. 3 **a** Explants of *E. tereticornis* clone T1 showing shoot organogenesis on selection medium containing kanamycin following transformation. **b** Leaf explants showing transient GUS activity following 2 days of co-cultivation. **c** Transformed *E. tereticornis* shoots showing stable GUS expression after 8 cycles of subculture. **d** Amplification of *nptll* gene (~760 bp) from genomic DNA, *Lane-1* 100 bp DNA ladder, *Lane-2* positive control (amplification from pBI121), *Lanes-3–6* amplification from DNA of transformed shoots, *Lane-7* negative control (untransformed tissue). **e** Amplification of DNA fragment specific to *uidA* gene (~1500 bp) from genomic DNA, *Lane-1* positive control (amplification from pBI121), *Lane-2* negative control (untransformed tissue), *Lane-3* amplification from DNA of transformed callus, *Lane-4/5* amplification from DNA of transformed shoots. **f** Amplification of 16S rDNA fragment (~1,500 bp), *Lane-1* negative control (untransformed tissue), *Lane-2* positive control (amplification from bacterial genomic DNA), *Lane-3/4* amplification from DNA of transformed shoots, *Lane-5* 1 kb ladder

increasing meso-inositol concentration improved shoots organogenesis from explants after co-cultivation with *Agrobacterium*. Earlier, the use of higher concentration of mesoinositol has been reported to be beneficial for shoot organogenesis from transformed tissue of hybrid *Eucalyptus* (Tournier et al. 2003).

Although the kanamycin resistance shoots was indicative of expression of the *nptII* gene, yet GUS activity was also examined to further confirm the expression of these newly incorporated genes. The kanamycin resistant shoots showed positive GUS activity (Fig. 3b, c). These results were confirmed by PCR amplification of DNA fragments of 750 bp specific to *nptII* gene (Fig. 3d) and 1,500 bp specific to *uidA* gene (Fig. 3e) from transgenic shoots. Amplification of DNA fragment specific to 16S rRNA locus of DNA isolated from transgenic shoots was not observed indicating the complete elimination of bacteria from these tissues (Fig. 3f). Earlier, such analysis has been successfully used for the detection of bacterial contamination in the plant tissues (Thomas et al. 2007; Luna et al. 2008).

In summary, the protocol for delivery of T-DNA using *A. tumefaciens* has been developed and subsequent regeneration of transformed shoots has been achieved in selected clones of *E. tereticornis*. This seems to be first report of successful transformation of elite clones of *E. tereticornis* using *A. tumefaciens*. This protocol has the potential to facilitate work on genetic modification of these clones incorporating genes of important traits.

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