In vitro regeneration of *Eucalyptus camaldulensis*

V. Girijashankar

Published online: 30 December 2011

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Abstract An efficient in vitro regeneration protocol enables mass multiplication, genetic modification and germplasm conservation of desired plants. In vitro plant regeneration was achieved from nodal segments of 18-months-old superior genotypes of *Eucalyptus camaldulensis* trees through direct organogenesis (DO) and direct somatic embryogenesis (DSE) pathways. Initial bud break (BB) stage occurred via DO while shoot multiplication phase followed both DO and DSE pathways. Interestingly, both BB and shoot multiplication stages were achieved on shoot induction and multiplication (SIM) media composed of Murashige and Skoog (MS) basal medium supplemented with 2 mg l⁻¹ benzyl aminopurine (BAP) and 0.1 mg l⁻¹ naphthalene acetic acid (NAA). Best shoot elongation response was observed on half strength MS fortified with 0.5 mg l⁻¹ BAP, while root induction and elongation was superior in 1/2 MS+1 mg l⁻¹ Indole butyric acid (IBA). Full strength MS fortified with cytokinins (BAP) and weak auxin (NAA) in the ratio of 20:1 favored direct regeneration pathways. Further, half strength MS supported shoot and root development. The absence of intervening callus phase in this protocol can help in minimizing the chance occurrence of somaclones. When compared to other compositions tried, hardening in 100 % coco peat resulted in maximum survival (80 %) of the in vitro raised plantlets. For mass multiplication, fortnight subculturing of a single nodal explants for eight passages on SIM medium resulted in 60–148 shoot initials. Repeated subculturing in SIM medium induced the formation of direct somatic embryos which in turn improved the

turnover capacity and enabled large scale clonal multiplication of elite and desirable trees of *E. camaldulensis*. Following this protocol, it takes a minimum time period of four-months between in vitro explant inoculation to hardening stage. In the present study, DO and DSE pathway of plant regeneration was reported occurring simultaneously in the same nodal explants of *E. camaldulensis*.

Keywords Direct organogenesis · Direct somatic embryogenesis · In vitro regeneration · Nodal stem cuttings · *Eucalyptus camaldulensis*

Abbreviations

BAP	6-Benzyle aminopurine
BB	Bud break
DO	Direct organogenesis
DSE	Direct somatic embryogenesis
DSEs	Direct somatic embryos
IBA	Indole butyric acid
MS	Murashige and Skoog medium
NAA	α -Naphthalene acetic acid
RIM	Root induction medium
SEM	Shoot elongation medium
SIM	Shoot induction and multiplication

Introduction

Eucalyptus camaldulensis Dehn. (River red gum) is one of the widely planted *Eucalyptus species* (Eldridge et al. 1994) for paper pulp, fuel wood and timber. The area under eucalyptus plantation is estimated to be around 20 million hectares worldwide (GIT Forestry 2008). This species of eucalyptus grows under rain fed conditions of semi-arid

V. Girijashankar (✉)
Plant Breeding and Genetics, ITC R&D Centre,
Hyderabad, A.P., India
e-mail: vgirija_shank@yahoo.com

tropics and is known for its drought tolerance. The widespread distribution, ability to adapt to changing rainfall (250–1600 mm) and temperature regimes makes this species economically useful and industrially important (Butcher et al. 2009). In addition to large scale clonal propagation and genetic transformation, in vitro conservation of elite germplasm of this species needs an efficient and robust in vitro regeneration protocol.

Mass multiplication of *E. camaldulensis* has been reported earlier which may follow different in vitro plant regeneration pathways such as direct organogenesis (Gupta et al. 1983; Yasodha et al. 1997; Kawaoka et al. 2006), indirect organogenesis (Diallo and Duhoux 1984; Muralidharan and Mascarenhas 1987; Mullins et al. 1997; Ho et al. 1998; Harcourt et al. 2000; Chang et al. 2000; Arezki et al. 2000; Fazal et al. 2003; Dibax et al. 2005) and indirect somatic embryogenesis (Prakash and Gurumurthi 2010). Following the above mentioned plant regeneration pathways, transgenic plants of this economically important species were generated and has been reviewed earlier (Mullins et al. 1997; Ho et al. 1998; Harcourt et al. 2000; Valerio et al. 2003; Kawaoka et al. 2006; Girijashankar 2011). These regeneration protocols at times may be laborious and difficult to follow. Further, in comparison to direct pathways, indirect regeneration protocols which involve callus formation can give rise to somaclonal variations.

It was reported that plants regenerating through somatic embryogenesis pathway mostly develop either from a single progenitor cell or through few proembryonal cells and the regenerated plants tend to be genetically uniform (Vasil 1987). Genetic transformation seldom occurs within single or few cells within the explant. In such cases, direct somatic embryogenesis (DSE) regeneration pathway enables the development of complete plants from such individually transformed cells. Further, the absence of intervening callus phase reduces the risk of developing somaclones or chimeras. So, the problems associated with in vitro regeneration methods could be minimized in DSE. Recently, Prakash and Gurumurthi (2010) reported DSE pathway of in vitro regeneration using hypocotyle explants from germinated seedlings of *E. camaldulensis*. However, they reported low frequency of somatic embryo (SE) formation i.e. only 5 among 50 hypocotyls responded to DSE. Further, they reported the occurrence of direct somatic embryos but complete plant regeneration from the same was not reported. Additionally, the use of hypocotyle explants from germinated seedlings has a hidden shortcoming. Since seed embryos are products of genetic recombination, the offspring may not be a true-to-type or clone of the desired parent. This becomes important specially when a desirable tree of this species needs to be clonally propagated or improved through genetic transformation.

Here an attempt was made to develop a simple, complete and efficient in vitro regeneration protocol for *E. camaldulensis*. The explants used in this study were nodal stem cuttings from 18 months-old trees. The results of in vitro regeneration and acclimatization are discussed, accordingly.

Materials and methods

Plant material and sterilization

Nodal cuttings of 18 months-old trees belonging to *E. camaldulensis* species were collected from five morphologically superior trees named as M1, M6, M9, M12 and M38. The explants were first surface sterilized in 70 % ethanol for 1 min following which the nodal cuttings were immersed in 1 % sodium hypochlorite solution (v/v) containing few drops of Tween-20 and were shaken for 15 min. Later, they were rinsed three times with sterile distilled water for 3 min per wash. Surface sterilization of eucalyptus explants was also carried with mercuric chloride (HgCl_2 0.1 %).

Media and culture conditions

Murashige and Skoog (1962) basal medium was gelled either with 0.8 % (w/v) bacteriological agar or 0.3 % (w/v) Gelrite. Sucrose 30 g l^{-1} was used in all experiments as carbohydrate source. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C for 20 min at 15 lbs. Filter sterilized phytohormones were added to the medium after autoclaving and transferred into 300 ml capacity culture (jam) bottles. For all experiments, cultures were maintained under photoperiod of 16 h light and 8 h dark. Light intensity of $20 \mu\text{mol m}^{-2} \text{ s}^{-1}$ was provided by white fluorescent tube lights and the room temperature was maintained at $28 \pm 2^\circ\text{C}$. MS media in combination with or without phytohormones were tried and the best combinations for in vitro regeneration are mentioned in Fig. 3.

Statistical analysis

Nodal explants from five mutated and fast growing superior individual trees were used in the study. A total of ten nodal cuttings for each clone were inoculated into six different combinations of media+phytohormones. Thus, each value in Table 1 is a mean of 50 explants. The values are expressed as mean $\pm$ standard error. Shoot elongation and rooting response in Table 2 is expressed in percentage which is calculated for 50 independent shoots inoculated for each media. Further, shoot and root lengths are expressed as range with lower and upper limits.

Table 1 Effect of different media and phytohormone combinations on bud break and shoot multiplication stages during in vitro regeneration of *E. camaldulensis* nodal stem cuttings

Medium composition	Bud break stage		Shoot multiplication stage
	Multiple shoots per explants	Shoots per nodal segment	Shoot initials developed per individual shoot
1/2 MS	4.06±2.9	2±0.4	0±0
1/2 MS+0.5 BAP	5.1±1.3	2±0.6	1.33±0.2
MS+2 BAP+0.1 NAA	24.3±2.2	4±1.4	6.3±2.1
1/2 MS+0.4 BAP+1 IBA	2.03±0.6	2±0.8	0.63±0.3
1/2 MS+1 IBA	0±0	0±0	0±0
MS+1 BAP+3 IBA	2±0.5	2±1.1	0±0

Values expressed as mean±standard error

Data was taken after 4 weeks of inoculation for each stage and nodal explants from five different trees were evaluated. A total of 10 explants per tree were inoculated for each media ± phytohormones combination tried.

Results

Surface sterilization of eucalyptus explants was carried using mercuric chloride (HgCl_2 0.1 %), 1 % sodium hypochlorite (NaOCl_2) and 70 % ethanol. Use of HgCl_2 (0.1 %) for 10 to 15 min on nodal cuttings resulted in browning, necrosis and eventually lead to death of plant tissues. After evaluating different concentrations and time of exposure, in comparison to HgCl_2 , 1 % sodium hypochlorite (NaOCl_2) for 15–17 min resulted in reduced contamination as well as minimized the injury to explants. It was noted that whenever the time of surface sterilization in NaOCl_2 was extended upto 20 min, bleaching of nodal stem cut ends was observed which in turn resulted in brownish necrotic tissue that eventually lead to death of explants. Thus, care must be taken in order to avoid over exposure of explants to NaOCl_2 .

Initial subculture immediately after 3 days after inoculation (DAI) into shoot induction and multiplication medium (SIM) composed of MS+2 mg l^{-1} BAP+0.1 mg l^{-1} NAA, improved survival rates of the nodal segments. Further,

subculturing into fresh SIM followed by trimming of the dead necrotic tips of Y shaped nodal cuttings exuding secondary metabolites not only enhanced the survival rate of explants but also improved their appearance and response towards the treatments imposed. Further, this practice of subculturing immediately 3 DAI, helped in elimination of contaminated explants at an early stage.

Bud break stage occurs via DO

Culturing of nodal explants for fortnight period in SIM media resulted in development of BB stage which occur in the axils of the explants as tiny sprouts (other than axillary buds) (Fig. 1a, b). Following 4-weeks of culturing in SIM media, multiple shoots with tiny leaflets start to proliferate and enlarge from the nodal segment (Fig. 1c, d). Various stages of DO formed during BB stage were shown in Fig. 1a–d. Interestingly, the axillary bud of the nodal segment develops necrosis and eventually gets etiolated by the end of 4th week on SIM media. Cross sectional view of BB

Table 2 Shoot elongation and rooting response in different media and phytohormone combinations

Medium composition	After 2nd wk	After 5th wk	Response @ 1 month of incubation without sub culture
1/2 MS	-	+	20 % rooting; 1–3 roots each of 2 cm length; SE varied between 1 and 2 cm.
1/2 MS+0.5 BAP	+	+	SE in 60 % explants (≥ 2 cm).
MS+2 BAP+0.1 NAA	-	-	20 % SE.
1/2 MS+0.4 BAP+1 IBA	-	+	30 % rooting; 2–3 cm long; SE slow up to 2 cm.
1/2 MS+1 IBA	+	+	60 % rooting; 2–8 roots each of 2–7 cm long; SE high 4–8 cm with 4 or more leaves.
MS+1 BAP+3 IBA	-	-	Callus formation.

Concentration of phytohormones expressed in mg l^{-1}

SE: shoot elongation

+ responded either for shoot elongation or rooting; – no response for rooting

50 independent shoots were inoculated for each media

stage revealed the occurrence of primordial shoots developing in a racemose pattern while each individual shoot had a vascular connection with the parent nodal cutting for drawing nourishment (Fig. 1e–h). Contrary to this, MS basal medium alone resulted in the formation of single shoot unlike that of multiple shoots in case of SIM medium.

Shoot multiplication occurs via DO and DSE

After 4-weeks after inoculation, the cluster of multiple shoots were separated from the parent nodal explant and placed in direct contact with SIM medium. Improving the surface contact area between the shoots and medium fostered the mass multiplication of shoot initials. This practice lead to the formation of multiple direct somatic embryos (DSEs) occurring parallel along with DO derived shoots. There is no need for separate media for somatic embryo induction and maturation as mentioned elsewhere. Various stages of DSEs formation was recorded starting from globular stage (Fig. 1i, j) to heart shaped embryos (Fig. 1k, l) which eventually lead to the development of torpedo (Fig. 1m) and cotyledonary stage embryos (Fig. 1n, o).

Initially the nodal explant followed direct organogenesis pathway of in vitro plant regeneration and produced 1–8 shoots of varied sizes in a period of 4 weeks on SIM media. Later, DSE pathway was observed especially when the shoot clusters formed on the parent nodal cuttings were separated and inoculated on SIM media for 4th subsequent sub culture which commences approximately 60 DAI into SIM medium. Interestingly, few of the shoot clusters never produced DSEs and continued in DO pathway. These DSEs unlike most other plants tend to have contact with the shoot clusters. Close examination under stereomicroscope revealed all four stages of DSE. A general observation is that it took a minimum of one-week time for conversion of globular embryo into cotyledonary stage. However, this time interval varied across (1–3 weeks) 3 of the 5 tree explants used in this study.

The differentiation between DO and DSE derived shoots was tough at cotyledonary stage. The difference between the DSEs and DO shoots (Fig. 1o & p) could be better visualized at 40× magnification under a stereomicroscope. The major difference was the number of leaflets that surround the shoot apex. It was observed that DO shoot produces numerous short leaves while DSEs produce two primordial cotyledonary leaves that cover the shoot apex.

For mass multiplication the shoot clumps were repeatedly sub cultured on SIM once every fortnight. If needed, the first formed shoot cluster can be transferred to SEM for further elongation immediately after completing their initial six-weeks of inoculation in SIM media. On the contrary, repeated fortnight subculturing of shoot clusters for eight passages on SIM medium results in the formation of 60 shoot initials

Fig. 1 Regeneration of *E. camaldulensis* through direct organogenesis and direct somatic embryogenesis in vitro. **a** bulged nodal explant 15 DAI; **b** bud break stage with shoot formation (distinct from axillary bud) after 3-weeks of inoculation in SIM; **c** multiple shoot buds originating from an individual nodal cutting via DO pathway; **d** cluster of multiple shoots elongating after their initial formation on the nodal explant after 4 weeks of inoculation; **e** Internal view of bulged axial region of bud break stage revealing the early stage of direct regeneration from the nodal explant; **f** separation of the newly formed independent egg-like primordial shoot initial from the nursing mother tissues within the bulged BB stage of the nodal explant. This is the maturation stage of the primordial shoot from the parent tissue; **g** emergence of multiple capsules of varying sizes; **h** cross section of bud break stage. Encapsulated shoot with a pair of primordial leaves. Below the embryo-like structure is a bundle of vascular tissue which draws nourishment and is connected with the conducting tissue of the parent nodal segment. Axillary globular embryo-like structure representing the early stages of axillary multiple shoots surrounded by parent tissues within the nodal explant along with vascular connection; **i** budding globular embryo emerging directly out of the nodal stem cutting; **j** cluster of globular embryos occurring in SIM media after 8 weeks of initial inoculation on SIM media; **k** early heart shaped embryo in yellow colour; **l** late heart shaped embryo showing the conversion from yellow coloured cell to green colour **m** torpedo stage; **n**, **o** early and late cotyledonary stages, respectively, with a pair of primordial leaves and shoot primordia, trying to emerge out from the embryogenic mass. It is to be noted that in any of these stages the intervening callus phase did not occur; **p** view of a DO shoot initial showing a rosette of young leaves which enables us to differentiate it from emerging cotyledonary embryo. Arrow pointing at a tiny and newly developing globular embryo near the base of DO shoot initial

(M1) while the highest observed was 148 shoots initials (Tree M6) per a single nodal explant. However, this response varied across the 5 genotypes used in the experiment. The differential response of explants to various medium compositions was given in Table 1.

Shoot elongation

Before subculturing the multiple shoots into SEM composed of half MS+0.5 mg l⁻¹ BAP, the enlarged shoot initials were separated into 3 or more clusters. This practice supported the normal growth of shoots, than otherwise. Shoots inoculated in SEM showed maximum response to shoot elongation compared to other combinations of media and phytohormones (Table 2). On an average, each shoot took 4 weeks to attain a height of 2–4 cm. Lack of proper care during shoot elongation can result in the formation of white or yellow colored callus formation on leaves and shoot (Fig. 2c).

Rooting

Different media compositions were tried among which the best response for rooting was observed in half MS basal supplemented with 1 mg l⁻¹ IBA (root induction medium, RIM) (Table 2). On the other hand, full strength MS basal medium did not favored rooting even when tried with the same concentration of IBA. The number of roots varied from 2 to 10 per shoot. Instead of fortnight subculturing,

the explants can be sub cultured to fresh RIM once in every 4 weeks interval. This process lead to maximum root growth with minimum root damage between passages. The initial response of elongated shoots inoculated into RIM is slow. Shoots obtained from long term cultures immediately responded to rooting, than otherwise. Rooting response also varied across genotypes. M1 genotype developed root initials in 15 days while others took 1 month for the same response. However, most of the explants from different clonal origin responded to root initiation by the end of 4 weeks of culturing in RIM, with few exceptions.

Elongated shoots cultured on RIM containing activated charcoal (1.5 %) showed improved rooting response especially in root induction and growth rate (data not shown). Excess concentration of IBA in RIM, greater than 1 mg l^{-1} resulted in callus formation. Subculturing of shoots on 1/2 MS basal fostered shoot and root development. Across the five genotypes studied, root formation directly improved shoot elongation and leaf development. It was observed that a well formed shoot (of height 6 cm) with a minimum of two roots each of $\geq 4 \text{ cm}$ length could easily withstand the hardening conditions.

Fig. 2 Stages of in vitro mass multiplication and acclimatization of *E. camaldulensis*. **a** Multiple shoots originated from a single nodal cutting; **b** initial shoot elongation stage; **c** cankerous growth on few elongated shoots; **d** elongated multiple shoots (≥ 2 cm) on nodal cuttings occurring within 1 month of inoculation of nodal cutting. These shoots can be directly transferred to rooting medium for immediate transfer into field or routinely sub cultured for extended periods in order to mass multiply; **e** root formation stage; **f** different types of roots observed under in vitro conditions. *Black arrow* indicates thin and

slender root formed when agar is used for gelling the media. *White arrows* indicate thick and stout roots formed when the shoot is grown on rooting medium gelled with Gelrite (0.3 %); **g** fully formed plantlet ready for hardening; **h** inverted jam bottles covering the plantlets in order to prevent evapo-transpiration losses during initial 2 weeks of hardening. This improves the survival percentage of plants during hardening; **i** plantlets hardened for ≥ 2 weeks in coco peat and ready to get transferred to polybags in nursery; **j** established plants in polythene bags under nursery shade net

Gelling agents added into the media showed marked difference on thickness of the roots and their establishment. Use of bacteriological agar (0.7 to 0.8 %) as gelling agent resulted in the formation of thin and slender roots while Gelrite (0.3 %) produced early root induction response with multiple white and stout roots (Fig. 3f). Further, when the rooted plants were sub cultured on to fresh media containing bacteriological agar, it was found that the roots could not establish properly or penetrate (roots break) into the gelled

media which eventually resulted in necrosis followed by death of roots. Contrary to this, the use of Gelrite (0.3 %) resulted in soft semisolid media which enabled easy penetration of young roots leading to easy stabilization of the plantlet and enabled normal growth. Rooted plants growing in bacteriological agar often tend to break during passages due to the sticky hard gel. Media solidified with Gelrite or Phytigel produced transparent RIM which enabled the easy separation of the plantlet with minimum damage to the roots

Fig. 3 Flow diagram of in vitro regeneration in *E. camaldulensis* with best media composition and time interval. Note: The incubation period for each stage varied with the genotypes

(Fig. 2g). We suppose that the chemical composition of Gelrite also supports plant growth. The positive effect of Gelrite was evident when the rooted plants showed higher survival percentage during hardening processes.

Acclimatization

Adaptation of plants to newer environment is acclimatization and the process of adjusting the in vitro grown plantlet to outside or surrounding climate is hardening. Different soil compositions were evaluated for successful establishment of the in vitro rooted plants (Table 3). During the first 2 weeks of hardening, defoliation of in vitro formed leaves was observed which is followed by root elongation along with induction of new leaves. Root growth was more prominent than shoot elongation under hardening conditions. Water stagnation around the root zone during hardening phase has led to death and deterioration of few plantlets. In order to enhance the initial survival of the new in vitro raised plants during hardening, covering the shoots with a polythene cover or transparent polythene to be glass container proved vital and important. This not only prevented the evapo-transpiration losses but also allowed the plants to withstand the sudden changes in the surroundings (change

from in vitro to ex vitro). A slow process of adapting the plantlets to growth room conditions was followed prior to exposure of the plants to the outside atmospheric conditions. This was initiated after 1 week of transferring the plants to the coco peat soil where the inverted jam bottles covering the plantlets were removed for an 1 hour each day. This initially resulted in wilting of plants. This process when followed for 1 week gave best results without any loss of plants and eventually withstood the complete removal of plastic covering. Thus, the in vitro grown plants were slowly acclimatized to the outside environment. Acclimatization phase was found to be one of the most crucial period because lack of proper care during this stage resulted in a drastic reduction of tissue culture turnover capacity. Later, when the plants were transferred to polybags containing sand: red soil: FYM in 1:1:1 ratio and kept under shade nursery for a minimum of 1 week period prior to their field planting resulted in maximum survival and successful field establishment.

Discussion

Various factors influencing in vitro regeneration of *E. camaldulensis* such as age, type of explant, media composition, phytohormones, antibiotics, culture conditions etc. were well documented in earlier studies (Gupta et al. 1983; Muralidharan and Mascarenhas 1987; Yasodha et al. 1997; Arya et al. 2009; Prakash and Gurumurthi 2010). Keeping this in view, we concentrated on elimination of callus phase which has few shortcomings as mentioned earlier. So for this purpose a combination of strong cytokinin and weak auxin was of our interest in the present study. We intentionally avoided the use of 2,4-D, casein hydrolysate, kinetin and zeatin because the combination of BAP and NAA was found more appropriate for direct regeneration pathway in *E. camaldulensis* hybrids (Prabha et al. 1999; Arya et al. 2009). Further, callus formation was reported in this species due to high concentration of the above mentioned auxins (Fazal et al. 2003; Prakash and Gurumurthi 2010). On the contrary, combinations of cytokinin with a weak auxin of lower concentration on eucalyptus may lead to direct regeneration pathway (Gupta et al. 1983;

Table 3 Effect of different hardening media's on plantlet establishment

Medium composition	No. of plants hardened	No. of plants successfully established in nursery	Observation
Sand+red soil+FYM (1:1:1)	25	5 (20 %)	Most of the plants died within the first week of transfer.
Coco peat+vermiculite (1:1)	25	8 (32 %)	Few plants survived.
Coco peat 100 %	25	20 (80 %)	Highest survival recorded. Roots elongated while shoot growth was medium.

Muralidharan and Mascarenhas 1987; Yasodha et al. 1997; Kawaoka et al. 2006; Prakash and Gurumurthi 2010). The nodal cuttings from a mature tree were known to have more amounts of permanent tissues and comparatively less number of meristematic cells. So, in the present study, we used young twigs of superior *E. camaldulensis* for mass multiplication and developed an in vitro regeneration protocol without intervening callus phase.

By striking an optimum balance between cytokinin: auxin (BAP 20: NAA 1) in BB and shoot multiplication stages, the plant regeneration pathway was driven towards DO and DSE. The best combinations of media and phytohormones at various stages of tissue culturing were outlined in Fig. 3. IBA at 1 mg l⁻¹ concentration was found more efficient in root induction and elongation than NAA and Indole-3-acetic acid. It is of interest to know that the first two stages (shoot induction and multiplication) need regular subculturing at every fortnight interval for better response while the remaining last two stages (shoot elongation and root induction) requires subculturing once every month.

Further, in order to induce DSEs or multiple shoots through DO, full strength MS was found to be congenial. This was in congruence with earlier eucalyptus micropropagation works (Arya et al. 2009; Prakash and Gurumurthi 2010). Gradual increase in total protein content followed by need for high carbon and nitrogen sources in the supporting media during somatic embryo's growth and maturation phases (Cangahuala-Inocente et al. 2009) explains the above responses of eucalyptus explants at early stages of in vitro regeneration. Our observations on the use of activated charcoal and 1/2MS on shoot and root development in this species are concurrent with the results of Dibax et al. (2010).

The cross section of the bud break stage clearly showed an egg like capsule within which the primordial leaves covered the shoot apex while the lower part showed a vascular connection with the parent tissues essential for drawing nourishment. This clearly demonstrates organogenesis pathway of initial regeneration. The development and morphology of shoot organogenesis in the BB stage was documented clearly. The shoot clusters, when repeatedly sub cultured on SIM medium started an alternate pathway of regeneration namely DSE. This helps in further mass multiplication of plantlets from a single nodal cutting. In eucalyptus, in vitro multiplication occurring parallel from more than one regeneration pathway was known. Bandyopadhyay et al. (1999) reported organogenesis cum somatic embryogenesis occurring via direct and indirect plant regenerations within the same explant of *E. nitens* and *E. globulus*.

Low frequency of DSEs formation from hypocotyles of *E. camaldulensis* was recently reported by Prakash and Gurumurthi (2010). Overcoming these drawbacks, here we developed a complete and high frequency direct

organogenesis cum direct somatic embryogenesis regeneration protocol for mass multiplication of *E. camaldulensis* by using nodal cuttings from 18 months old superior trees. This is a stage dependent (media composition), easy to follow and efficient regeneration protocol from which plantlets can be obtained from 4 months onwards and the number of shoots initials from a single explant can reach up to 140 plus in a period of four-months of repeated subculturing. However this response was genotype dependent. In conclusion, we report here a mixed pathway of plant regeneration in *E. camaldulensis* which can be used in genetic transformation, large scale clonal propagation and synthetic seed production from desired elite trees. Absence of intervening callus phase and use of stem segments from desired tree were the major advantages of this protocol.

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