In vitro Clonal Propagation of Mature Eucalyptus F_1 Hybrid (*Eucalyptus tereticornis* Sm. x *E. grandis* Hill ex. Maiden)

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Abstract

Axillary shoot bud multiplication has been achieved in Eucalyptus hybrid FRI-6 (*Eucalyptus tereticornis* Sm. x *Eucalyptus grandis* Hill ex. Maiden) by using explants from a 30-year-old, mature tree. Cultures were established on MS medium supplemented with BAP (1.0 mg/l) along with NAA (1.0 mg/l) and regular subculturing was carried out in BAP (1.0 mg/l). Proper elongation of shoots was achieved in $^1/_2$ MS medium devoid of plant growth regulators. Best rooting (75%) was observed in $^1/_2$ MS supplemented with IBA (1.0 mg/l). The method of *in vitro* clonal propagation of F_1 hybrid FRI-6 reported in this paper will be helpful in clonal multiplication of the F1 hybrids for testing them under different climatic and edaphic conditions. This interspecific hybrid is of interest as it involves two parent species viz. *E. grandis* and *E. tereticornis*, the former shows faster rate of growth, good stem form, best quality of pulp and prefers high rain fed areas while the latter is drought tolerant and it is very likely that F_1 hybrid may be suited for intermediary zones (Hybrid habitat).

Abbreviations: **MS:** Murashige and Skoog (1962); **BAP:** 6-Benzylamino purine; **NAA:** Naphthalene acetic acid; **IBA:** Indole-3-butyric acid; **GA_3:** Gibberellic acid; **KN:** Kinetin.

Key words: Eucalyptus tereticornis, Eucalyptus grandis, clonal propagation, micropropagation, tissue culture.

Introduction

Clonal forestry is gaining a lot of recognition all over the world, to bridge the gap between the growing demand and inadequate supply of wood. But there is an acute shortage of genetically improved planting stock in the country. Traditionally propagation of Eucalyptus is most commonly carried out through seeds. However, multiplication of trees through seeds is not a satisfactory means of conserving the characteristics of a desired clone (Das and Mitra, 1990) because of strong heterozygosity of trees. Vegetative propagation of elite trees/hybrids through tissue culture is considered to be economically feasible in overcoming the problem of rooting of cuttings which is most commonly faced while dealing with mature trees due to development of root inhibitors, secondary metabolites, phenolics etc. Further, this will also help in overcoming the problem of segregation in F_2 generation in case of hybrids. There are very few reports on successful propagation of Eucalyptus F_1 hybrids by using mature explants (Poissonier, 1984; Lubrano, 1988; Warrag et al. 1990; Kapoor and Chauhan, 1992; Janq-Chun et al., 1995; Bisht et al., 2000).

In the present study FRI-6 which is a controlled hybrid of *E. tereticornis* Sm. x *E. grandis* Hill ex Maiden (Venkatesh and Sharma, 1979) was selected for studies on in vitro clonal propagation by using mature explant. Since production of F_1 hybrid seeds in Eucalyptus *de novo* is a labour intensive task because of bisexual nature of flowers which require emasculation, it was thought to develop protocol for their *in vitro* clonal multiplication which will help in production of clonal material of this hybrid for testing them with regard to their adaptability under different climatic and edaphic conditions and establishment of their germplasm. This hybrid though intermediate to parental species in more than half the total number of contrasting characters studied (Venkatesh and Sharma, 1979) but is of interest because it involved *E. grandis* and *E. tereticornis* the two parent species, the former shows faster rate of growth, good stem form, provides best quality of pulp and prefers high rain fed areas while, the latter is drought tolerant and thus it is very likely that hybrids may be suited for intermediary zone (hybrid habitat). The results of micropropagation of this hybrid produced for the first time in India (Venkatesh and Sharma, 1979) are reported in this communication.

Material and Methods

Explants

Twigs were collected from a 30-year-old tree (FRI-6) of F_1 interspecific hybrid (*E. tereticornis* x *E. grandis*) growing at New Forest (Altitude 610 m, Latitude 30° N, Longitude 78° E, Annual rain fall 216 cm) the campus of Forest Research Institute, Dehradun.

Surface Sterilization

The twigs were washed thoroughly in running tap water to remove the superficial dust particles. Leaves were removed and nodal segments measuring 2–3 cm were cut. The explants were washed with dilute detergent (1–2% Teepol) solution for 15 minutes and then washed well in running tap water. The explants were subsequently taken into the laminar flow cabinet and treated with 30% $NaOCl_2$ solution for 20 minutes followed by rinsing 4–6 times with sterilized distilled water.

Culture Medium

For establishment of cultures MS basal (Murashige and Skoog, 1962) medium supplemented with 3% sucrose (as a carbohydrate source) was used. The medium was gelled with 0.7% bacteriological agar (Ranbaxy). The pH of medium was adjusted to 5.8 by using 1N NaOH or 1N HCl prior to adding of agar. The culture medium was autoclaved at 121°C and 1.0 kg/cm^2 pressure for 15 minutes.

Nodal explants were placed vertically and cultured in conical flasks (Borosil make) containing MS medium. Full strength MS medium supplemented with different concentrations of BAP (0.1–1.0 mg/l) individually and in the combination with NAA (0.1–1.0 mg/l) was used for multiple shoot induction. For studies on elongation of shoots from rosette clump of buds, clumps having 30–35 buds were transferred to one half and one fourth strength MS media with or without plant growth regulators.

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Full strength MS medium supplemented with BAP (1.0 mg/l) and different concentrations of GA_3 (0.04,0.1 and 0.4 mg/l) was also tried for elongation of *in vitro* grown shoots. One half-strength MS medium having 2.5% sucrose supplemented with auxins; IBA (0.1–1.0 mg/l) alone and in the combination with NAA (0.1–0.5 mg/l) was used for rooting of *in vitro* grown shoots. The concentration of sucrose was reduced to make the plant more autotrophic (DHAWAN and BHOJWANI, 1985). For *in vitro* hardening of plantlets, absorbent cotton soaked in $^1/_4$ strength liquid MS medium devoid of plant growth regulators was used. 24 replicates each were used for studies on shoot initiation as well as for determining the conditions for rooting. Subculturing was carried out at periodical intervals of 4 weeks.

Culture Conditions

All cultures for proliferation and rooting were maintained and incubated at 25 ± 1°C for 16 hours in light (illuminated by 40 Watt cool white fluorescent tubes, 1200 lux) and for 8 hours in dark.

Acclimatization

Plantlets after attaining a height of 2.5–3.0 cm were hardened *in vitro* by placing them on liquid $^1/_4$ strength MS medium devoid of plant growth regulators and having 2% sucrose. Absorbent cotton soaked in this liquid medium was used for supporting root system of *in vitro* raised plantlets. Plantlets were maintained in this step for 10–15 days and thereafter they were transferred to pots containing a mixture of soil, sand and manure (1:1:1) and covered with perforated polythene bags for aeration. The polythene bags were withdrawn periodically and finally removed when new pair of leaves started emerging.

Results and Discussion

Sterilization of nodal explants

One of the most critical factors in attempting to propagate genetically superior Eucalyptus trees is the successful disinfections of mature trees (WARRAG et al., 1990). During former sterilization experiments on FRI-6 it was observed that explants were very much susceptible to $HgCl_2$ and majority of them turned black and died after few days of inoculation and not even a single bud break was observed. When different concentrations of $NaOCl_2$ (10,20,30 %) were used for surface sterilization, 30% $NaOCl_2$ treatment for 20 minutes gave fruitful results and at this concentration up to 50% nodal explants remained green and uncontaminated. On the basis of these findings 30% $NaOCl_2$ for 20 minutes treatment was used for surface sterilization of nodal explants.

Shoot multiplication

Despite numerous advances and the fact that tissues from adult trees were among the first to have been introduced into culture (GAUTHERET, 1990), establishment of cultures directly from tissues derived from mature trees is still problematic. A further problem associated with the establishment of cultures is exudation of phenolic compounds from cultures that can eventually lead to necrosis and loss of cultures. The problem of phenolics was very severe in this case and after inoculation all the explants exuded phenolic compounds from the cut end irrespective of their position on the donor tree (i.e. 2nd, 3rd and so, on node). Cultures put in dark conditions did not show any favourable results. Even use of antioxidants like ascorbic acid and absorbents like charcoal failed to get desirable results. So to overcome this problem media were changed frequently at short intervals during the initial phase of establishment of cultures. Inspite of all this majority of the bud explants turned black and eventually died after few days in culture. Out of 24 bud explants inoculated only 8 which remained green from the beginning, responded to the cultural conditions. BAP alone did not facilitate any bud break. After 90 days of inoculation shoot buds started multiplying in 1.0 mg/l BAP + 1.0 mg/l NAA and after 150 days from a single bud 20–25 buds multiplied. (*Table 1, Figure B*) After this stage multiplication rate increased with every subculturing. Therefore, a clump of buds was transferred to fresh medium. As reported earlier by BISHT et al. (2000) for micropropagation of *E. tereticornis*, in this hybrid also it was observed that bud clumps were more compact and buds were rather smaller in size when subculturing was continued in auxin supplemented media. To overcome this problem buds were subcultured in MS medium supplemented with 1 mg/l BAP + 1 mg/l NAA only upto 180 days and after this regular subculturing was carried out at periodical intervals of 25–30 days in MS+1.0 mg/l BAP (*Figure C*).

Leaching of phenolics was observed even after 8th to 10th subculture and medium turned black at the base of bud clumps however, no significant effect of necrosis was observed and cultures remained healthy if subcultured regularly.

Elongation

It was observed that shoots multiplied in this way showed very poor elongation and buds were in the form of rosette clumps (*Figure B*). Although repeated subculturing showed slight increase in shoot length even then shoots required elongation prior to *in vitro* rooting. To facilitate elongation, different treatments (*Table 2*) were tried for duration of 15 days. Proper elongation was observed in $^1/_2$ strength MS medium

Table 1. – Effect of plant growth regulators on number of shoot buds per explant formed.

Sl.No.	Growth Regulators (mg/l) in MS Medium		Days after culturing	
	BAP	NAA	90 Days	150 Days
1	0.0	0.0	-	-
2	0.1	-	-	-
3	0.5	-	-	-
4	1.0	-	-	-
5	1.0	0.1	-	-
6	1.0	0.5	Red callusing on explant	6-7
7	1.0	1.0	One bud	20-25

– Denotes no bud; no growth of callus.

Table 2. – Effect of different treatments on elongation of shoots after 15 days of inoculation.

Sl.No	Media Combinations(mg/l)	Number of shoot buds per culture (approx.)	Remark
1	¼ MS	30-35	Good elongation with weak etiolated shoots
2	½ MS	30-35	Proper elongation with healthy shoots (1.5 to 2.0 cm.) This medium was found to be the best for elongation of shoots. The shoots harvested from the cultures reared on this medium gave higher percentage of rooting as compared to shoots grown on the rest of the media combinations.
3	½ MS + 0.1 BAP	30-35	Good elongation with weak shoots
4	½ MS + 0.5 BAP	30-35	Moderate elongation with healthy shoots
5	MS + 1.0 BAP	30-35	Proliferation with no elongation (Stunted growth)
6	MS + 1.0 BAP + 0.04 GA_3	30-35	Proliferation with good elongation of shoots. (2.5 to 3.0 cm)
7	MS + 1.0 BAP + 0.1 GA_3	30-35	Slight elongation with deformed (curly) narrow leaves
8	MS + 1.0 BAP + 0.4 GA_3	30-35	No elongation

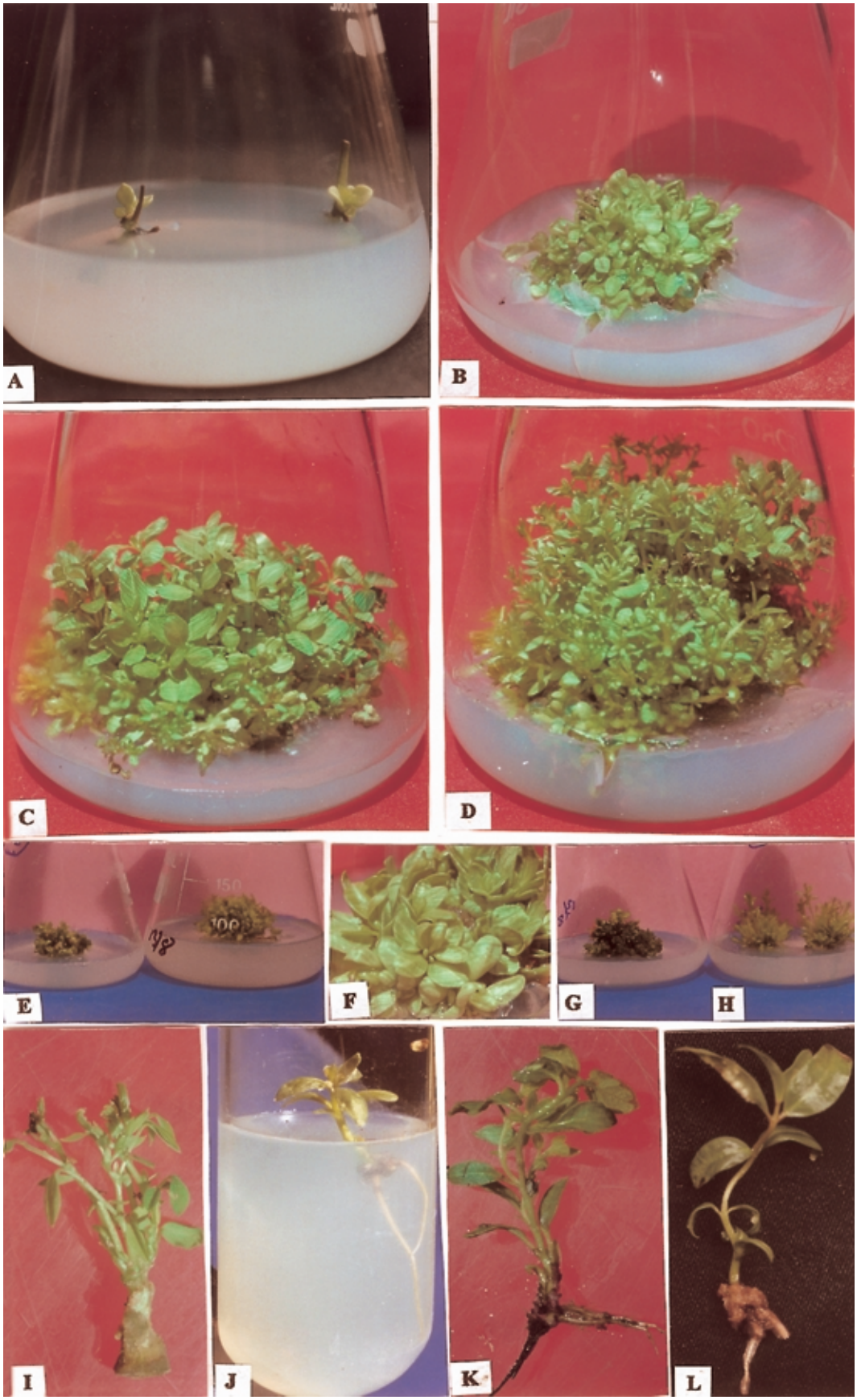

Figure (A to L). – Micropropagation of F1 hybrid of *E. tereticornis* x *E. grandis*. **A)** Bud break in nodal explants, **B)** In vitro multiple shoot formation, **C)** Multiplication of cultures, **D)** Elongation of shoots on 1/2 MS medium, **E)** Rosette clump of shoots in MS medium supplemented with BAP (1 mg/l) and GA_3 (0.4 mg/l), **F)** Close-up view of fig E, **G)** Elongation of shoots in MS supplemented with BAP (1 mg/l + 0.1 mg/l GA_3), **H)** Elongation of shoots in MS supplemented with BAP (1 mg/l) + GA_3 (0.04 mg/l), **I)** Elongated shoots prior to inoculation for rooting, **J)** In vitro rooting, **K)** In vitro rooted plantlet, **L)** In vitro rooted plantlet showing callus formation on the junction of root and collar.

Table 3. – Effect of auxins on rooting of *in vitro* grown shoots.

Sl. No.	Auxin (mg/l) in 1/2 strength MS medium		Number of shoots rooted	Rooting percentage	Number of roots per shoot (Mean ± SE)	Length of root (cm.) (Mean ± SE)
	IBA	NAA				
1	0.1	-	6	25.0	1.16 ± 0.15	5.66 ± 0.69
2	0.5	-	13	54.2	2.15 ± 0.35	5.33 ± 0.27
3	1.0	-	18	75.0	3.22 ± 0.32	2.28 ± 0.19
4	0.1	0.1	5	20.8	1.20 ± 0.17	4.40 ± 0.45
5	0.5	0.1	12	50.0	1.25 ± 0.13	3.37 ± 0.37
6	1.0	0.1	14	58.3	2.21 ± 0.24	2.30 ± 0.32
7	0.1	0.5	8	33.3	1.25 ± 0.15	4.25 ± 0.49
8	0.5	0.5	10	41.7	1.40 ± 0.16	1.60 ± 0.22
9	1.0	0.5	12	50.0	1.33 ± 0.14	1.12 ± 0.06

devoid of plant growth regulators (*Figure D*). Similar results were observed in case of F_1 hybrid of *Eucalyptus camaldulensis* x *E. tereticornis* and *Eucalyptus tereticornis* (BISHT et al., 2000a and b). However, in this hybrid the elongation of shoots was achieved within 30 days. In case of micropropagation of 23-year-old candidate plus tree of *Eucalyptus tereticornis* shoots elongation was achieved within 15 days when such rosette clump of buds were cultured on MS medium supplemented with Kn and GA_3 along with BAP.

Rooting

After elongation, individual shoots measuring 1.5–2.0 cm were transferred to half strength MS medium supplemented with IBA alone and in combination with NAA for rooting. Rooting experiments were initiated after 270 days of subculturing to get maximum material for trying different hormonal concentrations for rooting. During the course of *in vitro* rooting experiments it was observed that shoots harvested form the cultures established on medium for shoot elongation i.e. $^1/_2$ MS, gave higher percentage of rooting than shoots harvested from cultures grown on MS+ 1.0 mg/l BAP + 0.04 mg/l GA_3 (data not presented) and based on this fact this medium was found to be best (*Table 2*). Maximum rooting percentage (75%) was achieved in 1.0 mg/l IBA without intervening callus phase (*Table 3, Figure K*). An increase in concentration of auxin beyond 1.0 mg/l decreased the percentage of rooting. Furthermore, induction of callus formation was observed under higher concentration of auxin (data not presented, Figure L). So far as the number of roots per shoot is concerned highest number of roots (3.22 ± 0.32) was observed in 1mg/l IBA while the lowest number of roots (1.16 ± 0.15) in 0.1 mg/l IBA. BISHT et al. (1997) while working on juvenile hybrid of *Eucalyptus camaldulensis* DEHN x *Eucalyptus tereticornis* SM. found that higher concentration of auxins more than 1 mg/l did not yield any encouraging results. Under the present study maximum root length (5.66 ± 0.69 cm) was observed in lower concentration (0.1 mg/l) of IBA. As the concentration of auxins increased a decrease in root length was observed (*Table 3*). Minimum root length (1.12 ± 0.06 cm) was observed under combined treatment of auxins viz 1.0 mg/l IBA + 0.5 mg/l NAA. THIMANN (1977) was of opinion that root elongation is very sensitive to higher auxin concentrations.

Acclimatization

Hardening of the tissue culture derived plantlets is the most crucial step in the entire micropropagation process. The survey of literature shows numerous cases where rooting of shoots was obtained *in vitro*, but details regarding the procedures of hardening and their efficiency are often not provided. In the present study rooted shoots after attaining a height of 2.0–2.5 cm were hardened *in vitro* by placing them on liquid $^1/_4$ strength MS medium devoid of plant growth regulators and having 2% sucrose. Absorbent cotton soaked in this liquid medium was used for supporting root system of *in vitro* raised plantlets. One can skip *in vivo* hardening step in favourable conditions like in rainy season when relative humidity in the atmosphere is quite high but in the dry months this step is very much useful and gave very good results in successful hardening. After transfer of plantlets to soil in pots 90% success in survival rate was achieved.

The method for *in vitro* propagation of FRI-6 reported in this paper through axillary branching of nodal segments is ideal for rapid clonal propagation. It was observed that multiplication rate of this hybrid was fairly good as from single bud after the period of 150 days of inoculation about 20–25 shoots were produced and it increased to two folds after second subculturing and 6 fold increase was achieved after 8th subculturing.

As a result of the present study it is hoped that this method of *in vitro* propagation of promising F_1 hybrids will be helpful in their multiplication on mass scale, for testing them under different climatic and edaphic conditions and establishment of their germplasm.

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