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Micropropagation of *Eucalyptus tereticornis* Smith.

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Abstract

Axillary shoot bud multiplication has been achieved in *Eucalyptus tereticornis* Smith. using explants from different regions of 8–10 years old elite trees, growing in the field. Results showed that addition of NAA at $0.1\,mg\,l^{-1}$ and BAP at $1.0\,mg\,l^{-1}$ to modified MS medium induced maximum number of shoot buds. For inducing axial growth in regenerated bud primordia, the hormone concentration of the medium was lowered. The addition of charcoal and gibberellic acid to the medium were beneficial. Rooting was best in Knop's medium containing $1.0\,mg\,l^{-1}$ IBA. The key factor in root induction was primarily a dark incubation for a short period. The percentage of both rooting of shoots and survival of the rooted shoots was 60–80.

Continuous trials using explants from the elite trees throughout the year showed that the period between July–September was the best season for the explant source for rapid and increased multiplication of axillary buds. Phenolic exudation was also minimum at this period. The experiments were repeated using 50 populations from different plantations. It was observed that during culture, genotypically different populations responded differently in spite of optimal growth conditions.

Introduction

Considerable work has been conducted in the last two decades on the propagation of forest trees by using tissue culture techniques [2, 3, 7]. Much work has also been done on this aspect in different species of *Eucalyptus* [4, 6, 9, 12, 14], where seedling materials [4, 12] and nodal segments [6, 9, 14] of 5–10 year old tree were used as explants. Forty to sixty per cent of in vitro clonally propagated plants of *E. globulus* [11], *E. torelliana* and *E. camaldulensis* [6] were reported to survive in field conditions. Gupta et al. [5] reported that large numbers of viable plants of *E. citriodora* could be produced from a single bud in a year with subculture every 60 days. The only report of in vitro multiplication of *E. tereticornis* [11] does not provide a full description of results.

Species of *Eucalyptus* are fast growing trees with high biomass production and usually grow well in poor soil and drought conditions. This paper reports the successful in vitro multiplication and transplantation of *E. tereticornis* with high field survival.

Materials and methods

Twigs were collected from the upper and lower bole of the main trunk of 8–10 years old elite trees of *Eucalyptus tereticornis* Smith (Mysore gum). Later coppiced shoots were also used. Plant materials used for this investigation were collected from the plantation at Satsole, Sakhisole, Sapadiha Mouza of Arabari range, Silviculture Division, Midnapore, West Bengal, India. The experiments were

carried out with the same source of materials for the last three successive years.

The twigs were washed thoroughly in running tap water to remove the superficial dust particles. Nodal segments (0.5–1.0 cm) of terminal shoots, lateral shoots from both the upper and lower bole, as well as the coppiced shoots containing axillary bud primordia were cut, washed with 5% Teepol (British Drug House) for 5 min and surface sterilized in 20% (w/v) freshly prepared commercial bleaching powder solution in water for 30 min. The explants were then washed thrice with sterile distilled water followed by their culture onto sterile media.

Nutrient media of Murashige and Skoog (MS) [13] was used with 3% sucrose and solidified with 0.7% Difco-bacto agar. However, the calcium chloride concentration of MS was reduced to 146 $mg\,l^{-1}$ and in place of normal vitamin mixture, a modified vitamin mixture consisting of thiamine-HCl 2.5 $mg\,l^{-1}$, pyridoxine-HCl 2.5 $mg\,l^{-1}$ and nicotinic acid 5.0 $mg\,l^{-1}$ was used. The media were supplemented with 6-benzylaminopurine (BAP) alone and in combination with α-naphthaleneacetic acid (NAA). Both the hormones were used at 0.0, 0.1, 0.2, 0.5, 0.7, 1.0 and 2.0 $mg\,l^{-1}$. The culture tubes containing media were sterilized in an autoclave at 1.05 $kg\,cm^{-2}$ pressure for 15 min. All the cultures were grown under a photoperiod of 16 h light from Philips white fluorescent tubes giving 30×10^8 μmoles $sec^{-1}\,m^{-2}$ and at a temperature of 25 ± 2°C. Unless otherwise stated, subcultures were carried out every 4 weeks. For rooting in shoot cultures, MS media at half-strength, White's (W) media [16] and Knop's (K) media [8] were tried. Indole-3-acetic acid (IAA) (0.5, 1.0 $mg\,l^{-1}$) plus kinetin (KIN) (0.005 $mg\,l^{-1}$) combinations and also indole-3-butyric acid (IBA) and NAA alone at 2.0 and 4.0 $mg\,l^{-1}$ were used for root induction.

Each treatment was replicated 10 times with each replicate inoculated with two explants. The experiment was repeated three times.

Results

Culture of the axillary buds taken from the terminal and basal region of the trees

Phenolic compounds exuded from the cut ends of the inoculated explants. The buds taken from the basal lateral branches of the main trunk exuded less phenolic compounds than those taken from the terminal branches and they produced more shoot buds around the basal region. The explants exuded phenolics irrespective of the cultures kept either in photoperiod or in complete darkness and in liquid or solid media. Media were changed twice or thrice at two day intervals to facilitate quick exudation of phenolics. Majority of the bud explants except a few became black after two–three days culture. Few bud explants which remained green from the beginning, responded to the cultural conditions. The difference in the behaviour of this explant namely, some remained green while others became black is not known.

To get over this disadvantage of phenolic exuda-

Table 1. Different source explants showing phenol exudation and percentage of bud induction.

	Culture of apical and axillary buds from			
	Terminal shoot of main trunk	Lateral shoot from the upper bole of main trunk	Lateral shoot from the lower bole of main trunk	Coppiced shoots from the main trunk
Exudation of phenolics	+ + +	+ +	+ ±	+
Percentage of induced buds	10	15	30	50

'+' Sign denotes amount of phenol excretion estimated visually.
+ Negligible amount
+ ± Little amount
+ + Good amount
+ + + Maximum amount where medium becomes brown within 24 h.

tion, we employed in addition to the use of upper and lower branches of the main trunk, the coppiced shoots, which were found to be the most responsive (Table 1) as the exudation of phenolics were negligible. Hence, out of different 'source explants', coppiced shoots were considered as the desirable source material for experimentation.

Culture of the axillary buds collected from the coppiced shoots of elite trees

The cultured bud explants unfolded within 10–12 days in modified MS medium containing 0.1 $mg\,l^{-1}$ NAA + 0.5 $mg\,l^{-1}$ BAP and new buds developed around their basal regions within 5–6 weeks growth period. The developing buds exhibited different morphology when cultured in different concentrations of NAA and BAP. In such a combination when NAA was increased, callus was formed at the basal cut end (Fig. 1) and when BAP was increased more buds were formed (Table 2, Fig. 2). When 1.0 $mg\,l^{-1}$ of each of NAA and BAP were used, the number of buds increased to 18–22 and the rosette bud clumps were more compact and smaller in size with little callus at the base of the regenerated buds hindering the separation of clumps into smaller units (Fig. 3a). In the best combination of NAA (0.1 $mg\,l^{-1}$) and BAP (0.5 $mg\,l^{-1}$), 14–16 buds were formed and the bud clumps were a bit loose and spreading permitting thinning of buds to smaller units (Fig. 3b). Thinned shoot buds could easily be manipulated to the next passage for further growth.

Induction of axial growth

When the small rosette bud clump units were transferred to MS medium containing NAA (0.01 $mg\,l^{-1}$), BAP (0.1 $mg\,l^{-1}$), gibberellic acid

Table 2. Effect of BAP and NAA on number of buds formed. (Observation recorded after two subcultures.)

BAP ($mg\,l^{-1}$)	NAA ($mg\,l^{-1}$)						
	0.0	0.1	0.2	0.5	0.7	1.0	2.0
0.0	–	–	–	–	–	–	–
0.1	–	–	–	Swelling of explant	Swelling of explant	±, no bud	±, no bud
0.2	–	–	–	-do-	-do-	-do-	-do-
0.5	4–8	14–16	8–14	11–12	2–4, ±	+	+ +
0.7	4–7	12–18	11–15	10–13	4–8, ±	5–7, +	+ +
1.0	5–8	18–22	12–19	12–15	15–17, ±	18–20, ±	3–7
2.0	5–7	5–9	6–8	4–7	5–6, +	2–4, +	rare, 1–2

'–' Denotes no bud, no growth of callus.
'±' Denotes slight callusing.
'+' Denotes good callusing.
'+ +' Denotes very good callusing.
Statistical analysis of the bud regeneration data are given in Table 2a.

Table 2a. Statistical data of Table 2.

BAP ($mg\,l^{-1}$)	NAA ($mg\,l^{-1}$)																				
	0.0			0.1			0.2			0.5			0.7			1.0			2.0		
	m	S.d	CV %	m	S.d	CV %	m	S.d	CV %	m	S.d	CV %	m	S.d	CV %	m	S.d	CV %	m	S.d	CV %
0.5	6	1	22	15	1	8	11	2	22	11	1	4	3	1	24	–	–	–	–	–	–
0.1	5	1	18	15	1	9	13	1	10	11	1	9	6	1	20	6	1	10	–	–	–
1.0	5	1	17	20	2	8	16	2	12	14	1	7	16	1	4	19	1	3	5	1	18
2.0	6	1	17	7	1	18	7	1	11	5	1	22	5	1	9	3	1	30	–	–	–

m = Mean   S.d. = Standard deviation   CV = Co-efficient of variation

Fig. 1. Callus formation at the base of the buds in presence of high NAA (scale 1.0 cm).

Fig. 2. Maximum multiplication of shoot buds in NAA (0.1 mg l^{-1}) plus BAP (1.0 mg l^{-1}). Culture tube in horizontal position (scale 1.0 cm).

Fig. 3. Effect of levels of growth regulators on the morphology of the bud clumps (scale 1.0 cm): (*a*) produced in NAA (1.0 mg l^{-1}) plus BAP (1.0 mg l^{-1}); (*b*) produced in NAA (0.1 mg l^{-1}) plus BAP (0.5 mg l^{-1}).

(GA, 1.0 mg l^{-1}) and activated charcoal (0.2%), the transferred buds exhibited axial growth (Table 3). However, regeneration from their basal end continued but to a lesser degree (Fig. 4). For better growth of these dominant shoots, they were excised and cultured in the same medium. Their elongation in the culture medium continued without any proliferation from the basal end. The left over proliferated buds were used as secondary bud explants for renewed multiplication.

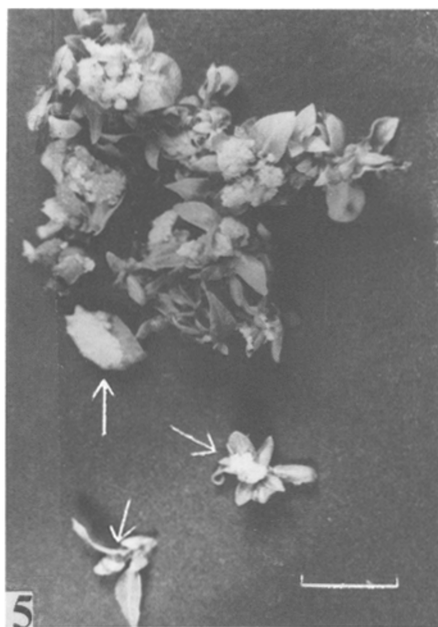
Alternatively, the bud multiples growing in MS medium containing NAA (0.1 mg l^{-1}) and BAP (0.5 mg l^{-1}) were incubated at a slightly higher temperature (30°–32°C) in the dark for 4–5 days followed by their transfer to 16 h photoperiod for the rest of the culture period. The dominant shoots appeared in the clumps, were isolated and used for root induction.

If the cultures were maintained in MS medium containing 0.1 mg l^{-1} NAA + 1.0 mg l^{-1} BAP, callus formation took place in young and old leaves as well as the shoot tips of the bud clumps (Fig. 5) and ultimately the top growth was checked. But in some cases a few buds regenerated from their basal region.

Organogenesis or embryogenesis, which initially require low concentration of auxin for morphogenetic induction, but its continued presence in subsequent cultures produce abnormalities and in such a case a balance between abscisic acid, cytokinin and GA in an appropriate ratio when added to the culture medium stops these abnormalities fostering the normal development of organs and embryos [1]. In view of this instance, abscisic acid has been used to promote organized growth in the present system by using 0.1 and 1.0 mg l^{-1}. But favourable response was not obtained.

Rooting of regenerated shoots

For rooting, a few preliminary experiments using W medium and MS medium at half-strength with 1% sucrose were tried in all the following experiments: (i) treatment with high concentration of NAA or IBA (each at 2.0 and 4.0 mg l^{-1}) for 48–96 h followed by their culture in auxin-free basal media was ineffective; (ii) treatment with high level of NAA (2.0 mg l^{-1}) followed by their culture in medium containing IAA (1.0 mg l^{-1}) and a low level of KIN (0.005 mg l^{-1}) showed induction and development of a few roots in some shoots, lacking satisfactory growth of the rooted shoots; (iii) isolated shoots were cultured continuously in media containing IAA at 0.5 or 1.0 mg l^{-1} plus KIN at



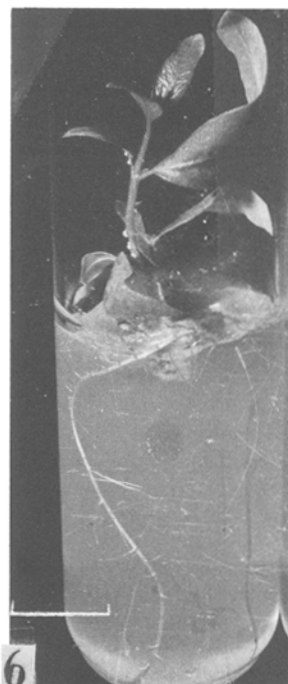


Fig. 4. Induction of axial growth in few shoot buds within the clump. The arrow indicates the formation of leader shoots (scale 1.0 cm).

Fig. 5. Formation of callus on the shoot tip and leaves (indicated by arrow) in NAA (0.1 $mg l^{-1}$) and BAP (1.0 $mg l^{-1}$) (scale 1.0 cm).

Fig. 6. Development of the rooted shoot in K-medium (scale 2.0 cm).

0.005 $mg l^{-1}$ in both solid and liquid cultures, resulting in 20% rooting within 15 days along with defoliation which could be checked by using MS medium but shoot growth was not promoted; and (iv) a modification of K-medium [8] in which macro-salts were taken at half-strength with reduced level of $Ca(NO_3)_2 4H_2O$ to one-fourth strength supplemented with half-strength of micro-salts of MS. To this basal medium, IAA (1.0 and 2.0 $mg l^{-1}$) and IBA (1.0 and 2.0 $mg l^{-1}$) alone and in combinations were tried. IBA (1.0 $mg l^{-1}$) alone was found to be the best not only for root induction but also for the

Table 3. The results of growth in length of shoots in two treatments.

Treatments	No. of buds per explant	No. of elongated shoots perexplant in the medium stated below[1]		
		1st subculture	2nd subculture	3rd subculture
1. NAA (0.1 $mg l^{-1}$) + BAP (0.5 $mg l^{-1}$)	14–16	3–4	5–8	7–10
2. NAA (0.1 $mg l^{-1}$) + BAP (1.0 $mg l^{-1}$)	18–22	-	1–3	4–6

The data are of the best explant i.e., coppiced shoot.
[1] NAA (0.01 $mg l^{-1}$) + BAP (0.1 $mg l^{-1}$) + GA (1.0 $mg l^{-1}$) + activated (charcoal 0.2%).

survival of the rooted plants in the field. Incubation of the shoots for 7–10 days in darkness was found to be an essential factor. When the cultures were transferred from darkness to photoperiod, root initials were observed after 10–15 days in 60–80% cases. After emergence of roots, the shoots were transferred to the modified K-medium without auxin and sucrose, which favoured further development of the rooted shoots (Table 4, Fig. 6). Similar introduction of dark phase in the previous experiments for rooting of shoots did not improve root induction. Thus, a total period of 20–25 days in modified K-medium (iv) is required for rooting. If culturing of rooted shoots were prolonged in the auxin medium after initiation of roots, the growth of the roots was checked and slight callusing was noticed on their leaves, nodal and internodal regions.

Table 4. Summary of the results of the rooting treatments.

Media[1] and procedure	Root formation %	Plantlet development
Treatments		
(i) NAA or IBA (each at 2.0 and 4.0 $mg l^{-1}$) for 48–96 h — Basal media	nil	-
(ii) NAA (2.0 $mg l^{-1}$) for 48–96 h — IAA (1.0 $mg l^{-1}$) + K (0.005 $mg l^{-1}$)	10	-
(iii) IAA (0.5 and 1.0 $mg l^{-1}$) + K (0.005 $mg l^{-1}$) for 15 days — Root initials — MS	20	±
(iv) IBA (1.0 $mg l^{-1}$) for 7–10 days in dark — 16 h photoperiod — root formation within 15 days — Basal media	60–80	+

[1] Basal media used for (i), (ii) and (iii) were that of White's [16] and half-strength MS [13] and for that of (iv) was modified Knop's [8] media.
'-' No growth '±' Poor growth '+' Good growth

Acclimatization of the rooted shoots

When the rooted shoots were 2.5–3.0 cm long, they were transferred to auxin-free liquid K-medium. The rooted shoots were propped up in the liquid medium with the help of filter paper strips (Fig. 7). After 15–20 days, when the plantlets attained a height of 6.0–8.0 cm, they were transferred to pots containing garden soil and sand mixture (1:1). The potted plants were enclosed inside polythene bags. The covers were periodically withdrawn to acclimatize the plants and after about one month of transplantation, the covers were permanently withdrawn (Fig. 8). Survival rate of plants in the field after transplantation was 80%. At present, there are 20 plants in the field.

Thus after standardization of all the physical and chemical milieu and starting from the explant stage to the stage of transplanted plant in the soil — the entire process took 5–6 months. At the initial stage, the process was a bit slow, but later on as the cultures were established, the multiplication rate as well as transplantation rate were increased. Thus, the final best treatments for micropropagation involve a) bud multiplication, b) shoot elongation and c) root induction which occurred, respectively in the following media: a) MS + NAA

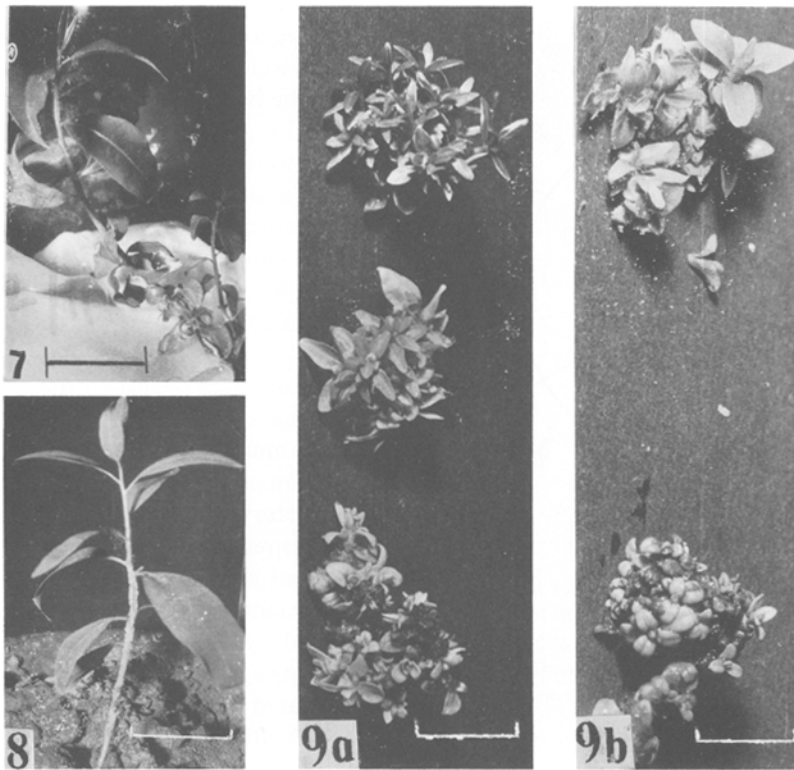


Fig. 7. Culture of the rooted shoot in liquid K-medium, supported by filter paper (scale 3.0 cm).

Fig. 8. Plantlet transplanted in the pot (3 months old) (scale 60.0 cm).

Fig. 9. Regenerated bud clumps of five different populations showing morphological differences (scale 1.0 cm).

(0.1 mg l^{-1}) + BAP (0.5 mg l^{-1}); b) MS + NAA (0.01 mg l^{-1}) + BAP (0.1 mg l^{-1}) + GA (1.0 mg l^{-1}) + activated charcoal (0.2%); c) K + IBA (1.0 mg l^{-1}).

Cytological preparations [15] of the root-tips of the regenerated shoots showed 2n = 22, the normal diploid number of the species. But as the chromosomes were in different planes it was not possible to take the photograph of the diploid plate.

Response of different populations to the same cultural conditions

In selecting the best elite tree which may respond best in cultural conditions with respect to multiplication as well as rooting and simultaneous best growth after transplantation to soil, we studied a population of 50 different trees, which were raised from seeds. Of these only 16 were found to be the suitable candidates for axillary bud multiplication after applying different media and various growth substances as described in previous pages. The different populations responded differently in spite of optimal growth conditions. So for the present plan of work the best strain was carried through. Regenerated bud clumps of each of these genotypically different populations differs in their morphology including leaf size, shape as well as in their multiplication rate and axial growth with respect to one particular treatment, namely NAA (0.1 mg l^{-1}) plus BAP (0.5 mg l^{-1}). Figures 9a, b show such bud clumps from five such populations. Within the different populations, when the rooting is induced and they begin to elongate, the differences in morphology disappear gradually and all the plantlets look alike.

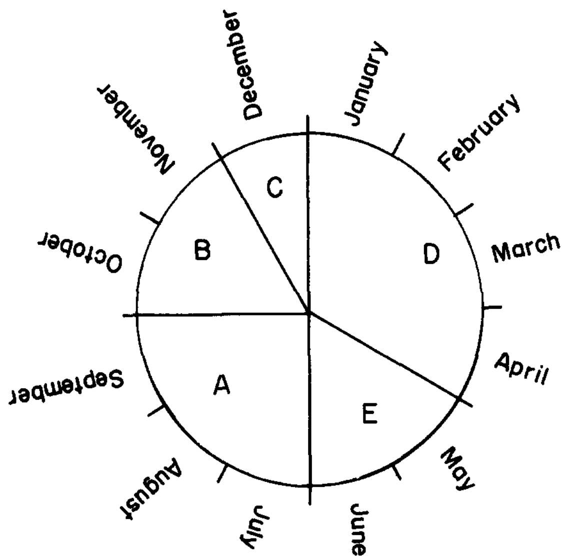


Fig. 10. Months showing regenerative capacity of the explants. A: 50–60% regeneration, phenol exudation nil. B: No regeneration, excessive phenol exudation. C: No regeneration but callus formation, phenol exudation nil. D: 10% regeneration, phenol exudation nil. E: No regeneration, excessive phenol exudation.

Effect of seasons

While repeating the experiments on the responsiveness of axillary bud explants collected in every month, it was found that explants collected during July to September were the best for in vitro studies for micropropagation. In October–November, phenol exudation was high even in the axillary bud explants from coppiced shoot. In December, callus induction was quicker from cut surface of explant but axillary bud multiplication was less and very slow. In January to April, multiplication rate of shoot buds was slow but enhanced after fourth or fifth subculture in the same medium. In May and June, this multiplication was almost negligible and associated with high amount of phenolic exudation (Fig. 10).

Discussion

In starting aseptic culture of the axillary buds for multiplication of the species, the main hazard faced was that of exudation of phenolic substances into the media. Probably because of this exudation of phenolic substances, the explants could not survive and eventually died without regenerating buds. Media were changed 3–4 times at 2–3 days interval from the beginning of the culture to overcome this exudation, but with little effect. Axillary bud explants were used from different regions, such as, the upper and lower boles of the adult tree and the coppiced shoots. Exudation of phenolics was least in the coppiced shoots which responded to bud multiplication.

It has been specified by Bonga [3] that multiplication of shoot bud explants of both tropical and temperate trees is easier if the explant is taken from germinated seedlings and their phenolic exudation is also much less than the mature tree. But multiplication of trees through seed germination is not a satisfactory means of preserving the characteristics of a desired clone. In *Fagraea* [10], shoot regeneration was achieved in explants obtained from the rooted cuttings of 10—year old trees by in vitro method, whereas explants from nodal regions of the same aged tree failed to grow and regenerate. It was stated that the new shoots developed from the rooted cuttings were juvenile. So to achieve rejuvenation, we coppiced the elite tree and the new shoots developed from the coppiced area were taken as explant source. Phenolic exudation was much less and the rate of multiplication was much better in these rejuvenated shoots (see Table 1). In subsequent cultures, multiplication potentiality of the axillary buds increased which might be due to prolonged continuous culture of the buds in the cytokinin containing media.

The second hurdle in this work was to induce axial growth in the buds of the clumps. Thinning of buds, lowering of hormone level, addition of GA and charcoal at a low level induced axial growth not in all but only in few leader shoots. Incubation for short period in darkness followed by high intensity of light also favoured growth of the shoot buds.

For root induction, minimum requirement, as present in modified K-medium was found to be the most suitable one. Again, the most essential and critical part of the rooting phase was the initial dark phase, not exceeding 7–10 days in K-medium containing IBA but extended period of darkness induced callusing in the basal region of shoots. Thus, the extent of dark period is critical for induction of rooting in the subsequent photoperiod.

The only report of multiplication of *E. tereticornis* [11] showed multiplication of shoot buds using terminal buds collected from 10–20 year old tree. For this they used MS [13] media supplemented with KIN ($0.2\,mg\,l^{-1}$) and BAP ($1.0\,mg\,l^{-1}$) followed by reduction of these growth substances in subsequent subcultures. Root induction phase as described in that reference was quite a lengthy one, which involved 3–4 subcultures and several auxins were used together for inducing roots in excised shoots. But they have not mentioned the rooting percentage and field survival was 40–60%. However, in the present investigation, these two percentages are 60–80 and 80 respectively.

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