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Research Article

Optimizing Growth Regulators for Micropropagation of Industrially Adaptable *Eucalyptus* Hybrids

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Abstract

Eucalyptus is one of the highly economic tree species in the developing countries like India. In the present investigation, experiments on optimizing plant growth regulators in the micropropagation of promising inter specific *Eucalyptus* hybrid clones namely TNPL 191(*E. camaldulensis* × *E. teriticornis*), TNPL 192 (*E. camaldulensis* × *E. pellita*) and intra specific hybrid clone TNPL 193 (*E. camaldulensis* × *E. camaldulensis*) were conducted, following standard protocols developed for *Eucalyptus*. The results showed that the BAP concentration of 0.50 mg L^{-1} for bud induction, IAA concentration of 3.0 mg L^{-1} for shoot elongation and IBA concentration of 1.0 mg L^{-1} for rooting of all these clones were found optimal. However, these three hybrid clones responded differently to the concentration of BAP at shoot proliferation stage. While the hybrid TNPL 191 showed maximum shoot proliferation rate at the concentration of 0.2 mg L^{-1} of BAP, the hybrids TNPL 192 and TNPL 193 showed highest response at 0.15 mg L^{-1}. All the *in vitro* rooted plantlets were acclimatized successfully to the prevailing natural environment. Thus, the protocols developed with respect to optimizing the plant growth regulators can be adapted in large scale micro propagation of inter and intra specific *Eucalyptus* hybrid clones.

Keywords

Eucalyptus Hybrid, Micropropagation, BAP, IAA, IBA

1. Introduction

The genus *Eucalyptus* (Family: Myrtaceae; 2n= 22), reportedly having about 900 different species is native to Australia and commercially cultivated in developing countries like India, owing to its high productivity, augmented demand and multi end uses like fiber and hardwood [1]. As an industrial point of view, the *Eucalyptus* species is mainly exploited as pulpwood by pulp and paper industries and feedstock for renewable bio-energy [2]. In India, *E. camaldulensis* and *E. teriticornis* are the most widely cultivated species due to their adaptability in arid and semi- arid conditions with relatively lower productivity. The lack of genetic variability is one of the main reasons for low productivity of commercial eucalyptus plantations in India as compared to other countries [3]. In recent times, genetic improvement programs on *Eucalyptus* species were initiated with introduction of germplasms from a wide range of natural provenances from Australia and elsewhere from the world. As the demand for wood stocks growing, inter and intra- hybridization using promising *Eucalyptus* accessions became priority in genetic improvement research.

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Planting stock with better genetic quality is the accepted mean for increasing the productivity of plantations, better farm income and sustainable supply to the wood based industries [4]. The conventional regeneration programs involve large scale seedling production with the challenges of persisting genetic load and relatively long generation time [1]. Poor rooting ability of stem cuttings and graft incompatibility are some of the reported constraints experienced with vegetative propagation of *Eucalyptus* hybrids. The biotechnological tools such as plant tissue culture along with traditional breeding program can be considered as viable solution for meeting the growing demand of forest products by industrial sectors.

In recent times, considerable attention on micropropagation of *Eucalyptus* has been given to produce large scale *Eucalyptus* clonal plants for reforestation and raising industrial plantations owing to the advantages of producing disease free and genetically identical plantlets. Well established *in vitro* propagation protocols for various *Eucalyptus* species were reported by some researchers [5-8]. But the process of somatic embryogenesis/ organogenesis and rooting differs within species and among hybrids and therefore, evolving specific protocols for each species/ hybrids need to be developed further. The cost- effective *in vitro* propagation protocols play key role in the development of low cost tissue culture technologies in the developing countries like India [9]).

Growth regulators play crucial role in promoting shoot proliferation and rooting of targeted species, especially *Eucalyptus* and their hybrids. For example, the type and concentration of cytokinins and auxins in the culture medium can affect the quality of shoot formation. The concentrations of plant growth regulators added to media needs careful consideration as an excess will result in antagonistic effect on the explants. Optimizing the hormones in micropropagation protocols can maximize the efficiency of shoot proliferation and production of healthy, true- to-type plantletes.

Therefore, this study was focused on optimizing growth regulators in micropropagation of three *Eucalyptus* hybrids developed for industrial plantations namely TNPL 191 (Interspecific hybrid of *E. camaldulensis* × *E. teriticornis*), TNPL 192 (Inter- specific hybrid of *E. camaldulensis* × *E. pellita*) and TNPL 193 (Intra- specific hybrid of *E. camaldulensis* × *E. camaldulensis*) for improvement in adventitious rooting and productivity under large scale plantlets production.

2. Materials and Methods

2.1. Eucalyptus Ex Situ Conservation and Plant Materials

Nodal buds from active coppice shoots of the *Eucalyptus* clones, grown in sand beds were used as explants. This method is considered to be effective in *ex situ* conservation of these clones [10]. Shoot intermediate nodes of about 1- 1.5 cm bearing nodal segments with auxiliary buds were detached and washed with liquid detergent initially, then under running tap water for 60 min to clear the microbial load and dust particles. The cleaned explants were washed with deionized water for 3- 5 times followed with the fungicide carbendazim suspension (1: 100 w/v) for 1 h [11]. Finally, the explants were washed with deionized water for 2- 3 times and stored for further culturing.

2.2. Disinfection and Initial Establishment of in Vitro Plant Material

The explants were sterilized with the previously standardized protocol at this laboratory [12]: initial treatment with 70% ethanol for 1 min, followed by treatment with3% sodium hypochlorite (NaOCl) with immersion duration of 12 minutes and finally with 0.1% mercuric chloride ($HgCl_2$) for 1 min. These explants were finally rinsed 4-5 times with sterilized deionized water and placed individually in borosilicate glass test tubes containing Murashige- Skoog (MS) medium [13]. The cultures in all the following experiments were maintained in a plant growth room having 16h/ 8h light/ dark photoperiod under cool white fluorescent lamps (light intensity of 40 µmol $m^{-2}s^{-1}$) and with day/ night temperature of25 ± 2 ℃.

2.3. In Vitro Shoot Initiation

The *in vitro* shoot initiation experiments were conducted using shoot- tip explants derived from the *in vitro* cultures as explained above. The explants were transferred into borosilicate glass bottles of 300 mL capacity and containing 100 mL of MS culture media, fortified with 0.00, 0.25, 0.50, 0.75 and 1.00 mg L^{-1} of 6-Benzylaminopurine (BAP). The percent shoot induction and shoot length were measured at 30 days after culture initiation. Each treatment consist 15 explants distributed in 3 culture bottles (each containing 5 explants) and each culture bottles were considered as one replication.

2.4. In Vitro Shoot Proliferation

The shoot proliferation experiments were conducted using adventitious shoot buds obtained from the shoot initiation experiment. The non- contaminated and well initiated buds were transferred into culture bottles containing MS media supplemented with five levels of BAP (0, 0.10, 0.15, 0.20 and 0.25 mg L^{-1}). The number of shoots formed per explant clump and shoot length was measured at 30 days after culturing.

2.5. In Vitro Shoot Elongation

In vitro shoot elongation experiment was conducted using shoot- tip explants derived from in vitro cultures as stated above. The shoot- tips were transferred to the culture bottle containing MS culture medium enriched with different doses of Indole 3-acetic acid (IAA)viz. 0, 2.0, 2.5, 3.0 and 3.5 mg L^{-1}. At 30 days after explants transfer, the number of new

shoots formed per clump and length of the shoots were measured. The non- hyperhydric shoots of at least 0.5 cm length were considered for measurement.

2.6. In Vitro Rooting

The *in vitro* rooting experiments were conducted using the shoot- tip explants obtained from the shoot proliferation above. The explants were transferred to culture bottles containing half strength MS culture medium supplemented with0, 0.5, 1.0, 1.5 and 2.0 mg $L^{-1}$of Indole butyric acid (IBA). The rooting percent and number of roots per explant were enumerated at after 30 days of culture inoculation.

2.7. Ex Vitro Acclimatization

Ex vitro acclimatization experiment was carried out using rooted explants from the *in vitro* rooting experiments above. After washing with tap water to remove the adhering medium, the rooted explants were planted in root trainers filled with composted coir pith as rooting medium. The root trainers were initially placed in the mist chambers (80-90% RH, 35-40 ℃/ 20-25 ℃ day/ night air temperature). After 15 days, the plants were shifted to 50%shade house (50-55% RH and 30-35 ℃/ 20-25 ℃ day/ night air temperature) for 15 days and then to open nursery having environment of 30-35 ℃ and 45-50% RH. The percent acclimatization in each of the *Eucalyptus* hybrids was assessed at 30 days after planting of the rooted explants.

2.8. Statistical Analysis

The data for each *Eucalyptus* hybrid consisting three replicates were processed with descriptive statistics and analyzed for one way analysis of varience (ANOVA). Least Significant Difference (LSD) and the post hoc test of Duncan's multiple range tests ($P < 0.05$) were performed to compare the means between various doses of PGRs at each experiments when ANOVA results indicated significant differences at $P = \leq 0.05$. Data analyses were performed using SPSS statistics for Windows (IBM SPSS statistics version 29.0).

3. Results and Discussion

3.1. Shoot Initiation

The shoot initiation rate and corresponding shoot length of three *Eucalyptus* hybrid cultures are shown in Table 1. The different concentrations of BAP were found significantly influencing the shoot induction and shoot length ($P< 0.05$). The concentration of 0.75 mg L^{-1} recorded highest shoot initiation in all the clones (Figure 1). However, the BAP concentration of 50 mg L^{-1} was found statistically on par with the best treatment in the hybrids TNPL 192 and TNPL 193. The influence of BAP was extended to increasing shoot length of eucalyptus clones as the dose of 0.5 mg L^{-1} was found best for all these hybrids. This was because of the explants apparently derives nutrients and hormones from the medium that favors the development of organogenesis [14]. Further, BAP is most efficient synthetic cytokinin which promotes induction of adventitious buds at the base of explants by stimulating cell division [15, 16]. The effective response of BAP on induction of adventitious bud in *Eucalyptus* sp. has been reported previously by Baccrin*et al*. [17]. Therefore, considering the above biometrics, the BAP dose of 0.50 mg L^{-1} was found optimum for the conversion of excised buds in to shoots, besides increasing the shoot length in all the three hybrid clones.

Table 1. *Effect of BAP on shoot initiation on eucalyptus hybrids.*

Shoot initiation						
BAP(mg L^{-1})	**Shoot initiation rate (%)**			**Shoot length (cm)**		
	TNPL 191	**TNPL 192**	**TNPL193**	**TNPL 191**	**TNPL 192**	**TNPL 193**
0.25	47.07 ±0.64^{c}	58.78 ±2.89^{c}	44.40 ±2.34^{c}	2.30 ±0.40^{b}	2.03 ±0.25^{c}	1.53 ±0.35^{b}
0.50	58.79 ±2.48a^{b}	76.78 ±2.70^{ab}	55.36 ±2.70^{a}	3.53 ±0.45^{a}	3.70 ±0.20^{a}	2.73 ±0.25^{a}
0.75	59.67 ±1.81^{a}	70.43 ±2.40^{a}	52.27 ±1.53^{ab}	2.70 ±0.60^{ab}	3.03 ±0.45^{b}	2.03 ±0.25^{b}
1.00	54.57 ±2.45^{b}	58.37 ±2.63^{b}	50.41 ±4.84^{ab}	2.53 ±0.55^{b}	2.13 ±0.15^{c}	1.73 ±0.35^{b}
Control	12.37 ±1.64^{d}	19.30 ±1.09^{d}	9.71 ±0.79^{d}	0.73 ±0.25^{c}	0.57 ±0.31^{d}	0.57 ±0.31^{c}
LSD (P< 0.05)	4.245	8.809	23.96	0.218	0.085	0.093

Note: BAP= 6- Benzylamino purine, LSD= Least Significant Difference, The mean values ±standard deviation (SD) represented in the corresponding column followed by different superscript alphabet are significantly different at $P < 0.05$ as analyzed by one way ANOVA and by DMRT.

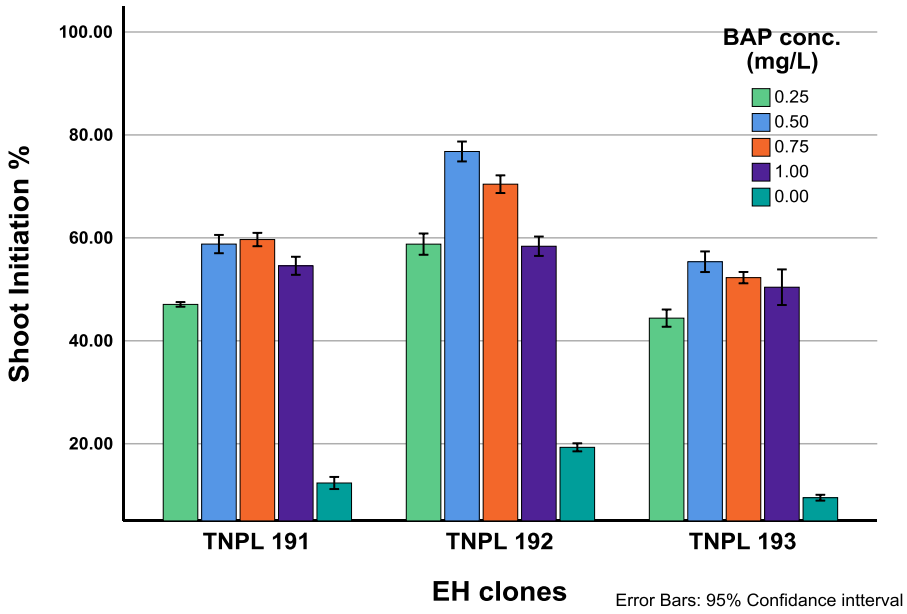

Figure 1. *Effect of various concentrations of BAP on the shoot initiation of Eucalyptus hybrid clones.*

3.2. Shoot Multiplication

The different doses of BAP significantly persuade the shoot multiplication processes in all the *Eucalyptus* hybrid clones ($P< 0.05$; Table 2). MS medium solidified with 0.2 mg L^{-1} of BAP recorded the highest number of shoots per clump and highest shoot length for the culture TNPL 191. But at 0.15 mg L^{-1} BAP, the shoots of TNPL 192 and TNPL 193 responded well by registering significantly highest number of shoots and shoot length. The exogenous application of cytokinins needs to be optimized as excess quantity may lead to lesser response in the proliferation of shoots [18-20] as observed with clones TNPL 192 and TNPL 193in our present study. Thus it was noted that the culture medium containing 0.2 mg L^{-1} of BAP was optimal for the culture TNPL 191 (*E. camaldulensis* × *E. teriticornis*) and 0.15 mg L^{-1} BAP for the cultures TNPL 192 (*E. camaldulensis* × *E. pellita*) and TNPL 193 (*E. camaldulensis* × *E. camaldulensis*) respectively for shoot proliferation.

Table 2. *Effect of BAP on shoot multiplication of eucalyptus hybrids.*

Shoot multiplication						
BAP(mg L^{-1})	**Number of shoots per clump**			**Shoot length (cm)**		
	TNPL 191	**TNPL 192**	**TNPL193**	**TNPL 191**	**TNPL 192**	**TNPL 193**
0.10	10.00 ±4.58^{c}	25.00 ±5.00^{b}	20.33 ±2.08^{c}	0.30 ±0.05^{cd}	0.70 ±0.07^{c}	0.50 ±0.04^{c}
0.15	16.67 ±3.51^{b}	36.33 ±4.51^{a}	32.33 ±3.51^{a}	0.50 ±0.03^{c}	1.30 ±0.05^{a}	1.00 ±0.09^{a}
0.20	24.33 ±5.51^{a}	28.67 ±4.04^{b}	26.33 ±5.51^{b}	1.01 ±0.04^{a}	0.90 ±0.04^{a}	0.70 ±0.05^{b}
0.25	21.00 ±4.00^{a}	23.00 ±2.00^{bc}	25.33 ±3.51^{bc}	0.80 ±0.04^{b}	0.75 ±0.05^{c}	0.50 ±0.04^{c}
Control	4.33 ±0.58^{d}	7.67 ±2.52^{c}	7.00 ±2.65^{d}	0.49 ±0.06^{d}	0.49 ±0.04^{d}	0.50 ±0.03^{c}
LSD (P< 0.05)	4.25	8.81	4.77	0.218	0.085	0.093

Note: BAP= 6- Benzylamino purine, LSD= Least Significant Difference, The mean values ±standard deviation (SD) represented in the corresponding column followed by different superscript alphabet are significantly different at $P < 0.05$ as analyzed by one way ANOVA and by DMRT.

3.3. Shoot Elongation

In this experiment, the transplanted shoots showed difference in the shoot induction and shoot length with various doses of IAA (Table 3). The MS medium with 3.0 mg $L^{-1}$of IAA was found superior in shoot induction and shoot length in all the three hybrid clones. The concentration of 3.5 mg $L^{-1}$was found statistically equal in inducing shoots (TNPL 191 and TNPL 192) and increasing shoot length (TNPL 191 and TNPL 193). The analyzed data implies that concentration of 3.0 mg L^{-1} IAA was optimal for better shoot induction and increasing the shoot length in all the three clones. Shoot growth was stimulated by the effect of optimal IAA dose, as it stimulates shoot elongation under *in situ* conditions [21]. It appears that the applied IAA was quickly conjugated and metabolized within *Eucalyptus* tissues which resulted more number of shoots and better shoots length of the inoculated explants. A similar result of increased shoot elongation response in *Eucalyptus* sp. with IAA was reported by some researchers [2, 22, 23].

Table 3. *Effect of IAA on shoot elongation of eucalyptus hybrids.*

IAA(mg L^{-1})	Shoot elongation					
	Number of shoots per clump			Shoot length (cm)		
	TNPL 191	TNPL 192	TNPL193	TNPL 191	TNPL 192	TNPL 193
2.0	3.00 ±1.00^{bc}	4.33 ±0.58^{c}	3.33 ±1.15^{bc}	2.33 ±0.58^{b}	2.52 ±0.38^{c}	2.00 ±0.75^{b}
2.5	4.67 ±2.08a^{bc}	6.67 ±1.53^{ab}	5.33 ±0.58^{ab}	4.33 ±1.53^{a}	5.25 ±1.15^{b}	4.42 ±1.13^{a}
3.0	7.33 ±2.52^{a}	9.33 ±2.52^{a}	7.33 ±0.58^{a}	6.33 ±1.36^{a}	7.42 ±1.91^{a}	5.92 ±1.38^{a}
3.5	5.33 ±1.53^{ab}	9.33 ±4.51^{a}	5.00 ±2.00^{b}	4.67 ±0.58^{a}	6.58 ±0.63^{ab}	5.08 ±0.63^{a}
Control	1.67 ±0.58^{d}	3.67 ±0.58^{c}	2.67 ±0.58^{d}	1.33 ±0.45^{b}	1.33 ±0.29^{c}	0.97 ±0.10^{b}
LSD (P< 0.05)	2.87	5.93	1.27	1.13	1.12	

Note: IAA= Indole 3-acetic acid, LSD= Least Significant Difference, The mean values ± standard deviation (SD) represented in the corresponding column followed by different superscript alphabet are significantly different at $P < 0.05$ as analyzed by one way ANOVA and by DMRT.

3.4. Rooting

The per cent rooting and number of roots per shoot were significantly influenced by different doses of IBA (0 to 2 mg L^{-1}; Table 4). The control treatment did not root in any of the cultured clones. Among the various doses, ½ strength MS + 1.0 mg L^{-1} or ½ strength MS + 1.5 mg L^{-1}showed higher rooting percent in all the hybrid clones implying that these two doses were equally efficient in inducing the rooting. However, the number of roots per shoot was found significantly highest with ½ strength MS + 1.0 mg $L^{-1}$of IBA in all the clones (P< 0.05). Thus, considering the rooting ability and increasing number of roots in these cultured clones, the media½ strength MS + 1.0 mg $L^{-1}$of IBA was found to be optimal for these three hybrid clones. No callus formation was noticed with any of the cultured clones in the rooting experiment.

Table 4. *Effect of IBA on rooting of eucalyptus hybrids.*

IBA(mg L^{-1})	Rooting					
	Rooting (%)			Number of roots per shoot		
	TNPL 191	TNPL 192	TNPL193	TNPL 191	TNPL 192	TNPL 193
0.5	18.56 ±1.22^{c}	25.41 ±2.17^{c}	23.46 ±2.35^{c}	2.33 ±0.64^{b}	3.67 ±0.68^{c}	4.67 ±0.58^{bc}

IBA(mg L^{-1})	Rooting					
	Rooting (%)			Number of roots per shoot		
	TNPL 191	TNPL 192	TNPL193	TNPL 191	TNPL 192	TNPL 193
1.0	86.33 ±2.25[a]	94.25 ±2.49[a]	87.29 ±2.13[a]	5.33 ±1.53[a]	8.33 ±0.77[a]	8.00 ±1.00[a]
1.5	87.31 ±1.94[a]	94.16 ±2.65[a]	85.18 ±1.69[a]	5.33 ±0.58[a]	6.67 ±0.58[b]	5.33 ±0.86[b]
2.0	65.24 ±1.72[b]	87.12 ±1.91[b]	73.15 ±2.66[b]	3.33 ±0.64[b]	4.33 ±0.56[c]	3.67 ±0.58[c]
Control	0.00 ±0.00[d]	0.00 ±0.00[d]	0.00 ±0.00[d]	0.00 ±0.00[c]	0.00 ±0.00[d]	0.00 ±0.00[d]
LSD (P< 0.05)	5.69	5.38	3.11	0.67	0.27	0.40

Note: IBA= Indole butyric acid, LSD= Least Significant Difference, The mean values ± standard deviation (SD) represented in the corresponding column followed by different superscript alphabet are significantly different at $P < 0.05$ as analyzed by one way ANOVA and by DMRT.

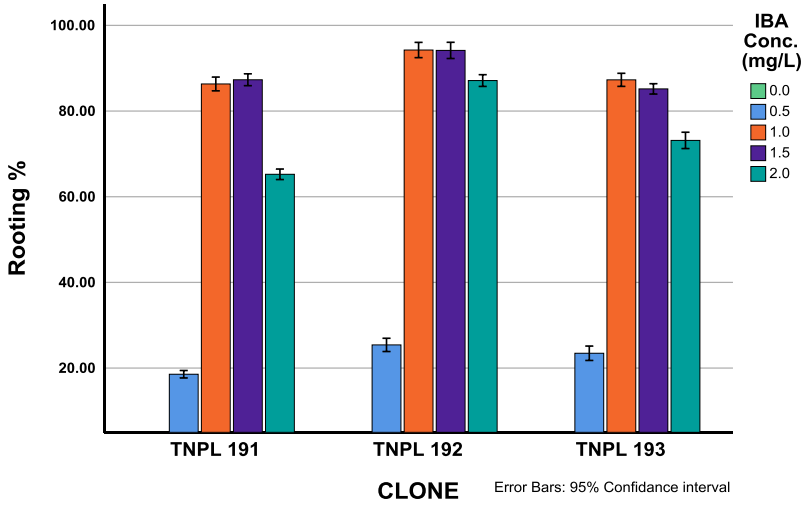

Figure 2. *Effect of different concentrations of IBA on the rooting% of Eucalyptus hybrid clones.*

The addition of different doses of plant growth regulators, especially IBA to the media is one of the most important factors that influences on cellular competence, triggering embryogenic re-differentiation, dedifferentiation and organs formation [24]. In addition, the prolonged exposure of the culture to higher dose of IBA could negatively impact the rooting ability of the *in vitro* cultures [25]. Therefore, significant differences in rooting percent and number of roots were found among different doses of IBA used in the present study. Similar result of enhanced rooting in *Eucalyptus camaldulensis* by using different concentrations of IBA was reported earlier by Shanthi et al. [2]. The results of the optimization experiment showed that ½ strength MS + 1.0 mg $L^{-1}$of IBA can be recommended for increasing the rooting percent and number of roots in the *Eucalyptus* hybrid clones.

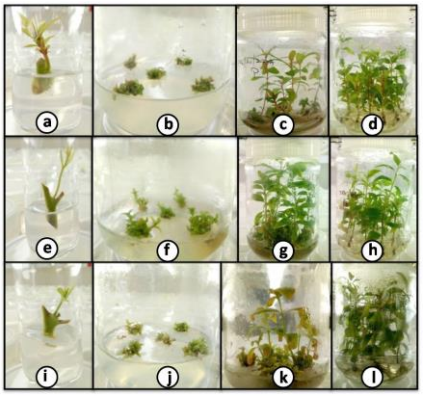

Figure 3. *In vitro propagation of Eucalyptus hybrid clones; a–d: TNPL 191 (a - initiation; b - multiplication, c - elongation & d – rooting); e-h: TNPL 192 (e - initiation; f - multiplication, g - elongation & h- rooting); i-l: TNPL 193 (i - initiation; j- multiplication, k - elongation & l – rooting).*

3.5. Ex Vitro Acclimatization

The rooted shoots were implanted into the root trainers as mentioned in section 2.7. After 30 days from the implantation, the survived plantlets were enumerated under normal prevailed environment. All the *in vitro* raised plantlets survived in the open nursery and formation of new roots and offsets were observed (Figure 4) indicating 100% successful acclimatization of the *in vitro* plantlets.

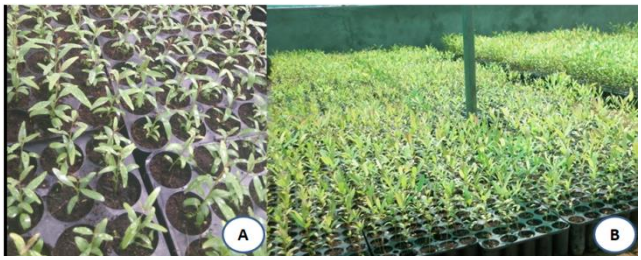

Figure 4. *Ex vitro acclimatization of Eucalyptus hybrid clones; A- in the mist chamber; B- in the shade house.*

4. Conclusion

The micropropagation of new generation *Eucalyptus* hybrids were successfully carried out by optimizing the growth regulators at shoot initiation (BAP), shoot proliferation (BAP), shoot elongation (IAA) and rooting (IBA) stages. To sum up, the BAP concentration of 0.50 mg L^{-1} for bud induction, IAA concentration of 3.0 mg L^{-1} for shoot elongation and IBA concentration of 1.0 mg L^{-1} for rooting of *Eucalyptus* hybrid clones were found optimal. However, for shoot proliferation, the optimum concentration of BAP for these clones differed. The culture medium containing 0.2 mg L^{-1} of BAP was optimal for the clone TNPL 191 and the BAP strength of 0.15 mg $L^{-1}$was found best for the clones TNPL 192 and TNPL 193. The results of this study can be applied in large scale micropropagation of inter and intra specific *Eucalyptus* hybrid clones. Future study is to be focused on successful establishment of these *in vitro Eucalyptus* hybrid plantlets in the field conditions for developing large scale plantations as a raw material inventory for pulp and paper industries.

Abbreviations

PGR	Plant Growth Regulators
IAA	Indole 3-acetic Acid
IBA	Indole Butyric Acid
BAP	6-Benzylaminopurine
MS	Murashige- Skoog
RH	Relative Humidity
ANOVA	Analysis of Variance
LSD	Least Significant Difference
DMRT	Duncan's Multiple Range Test

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Author Contributions

Malaimuthu Chinnama Naickar: Conceptualization, Methodology, Writing – review & editing

Chezhian Palanisamy: Conceptualization, Writing – review & editing

Prasath Vazram: Methodology, Writing – original draft

Jayakumar Kuppusamy: Project administration, Supervision

Stalin Thangavel: Methodology

Rajesh Ramasamy: Methodology

Conflicts of Interest

The authors declare no conflicts of interest.

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