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In-vitro callus regeneration and rhizogenesis in *Diploknema butyracea*: a valuable tree borne oilseed

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ABSTRACT

In-vitro callus regeneration and rhizogenesis is achieved from leaf segments of mature tree of *Diploknema butyracea* (Roxb.) Lam. Explants sterilization was standardized using 0.1% $HgCl_2$ for 3 minutes and NaOCl (40%) for 5 minutes. High frequency callus was regenerated in MS medium with 4.44 µM BAP + 2.26 µM 2, 4-D. Maximum rhizogenesis was recorded in MS medium with 4.44 µM BAP + 1.14 µM IAA + 0.58 µM GA3. Callus culture failed to produce shoot in all tried combination of PGRs in MS medium.

1. INTRODUCTION

Diploknema butyracea (roxb.) Lam. Syn. *Aesandra butyracea* commonly called as 'Indian butter tree' is a large tree of family Sapotaceae which distributed naturally throughout the forest of lower Himalayas between an altitude ranges of 300 – 1500 m[1]. Tree attains a height of 15 m and girth 1.8 m, fruits are berries, 1-3 seeded and contain about 2.0 cm long almond shaped kernel[2]. The tree provides oil seeds, saponin, latex, tannin, leaf fodder and reported to have theurapeutical usage. Seed oil yield is 42 – 47 % of the seeds weight or 60 – 66 % of the kernel weight and called as cheura or cheuri ghee. It is an important tree borne oilseed (TBO) of Nepal, where they have developed traditional technology to extract cheura ghee from seeds of this species[3]. This may become an important TBO in India as well but there is need of concentrated efforts for its domestication, genetic improvement and standardized propagation techniques along with post-harvest technology and extraction of oil.

Tree produces enough seeds but due to demand for oil extraction its seeds are removed from forest floor, further seeds are being recalcitrant for storage (20 days viability) also hampers natural regeneration[1,4]. This species is not amenable to macropropagation[5], therefore tissue culture techniques may be an appropriate tool for rapid multiplication. Micropropagation through axillary bud proliferation is fairly slow because of strong apical dominance and consequent poor lateral growth[6]. Through callus, plant regeneration is possible on faster rate through somatic organogenesis and embryogenesis. This research was conducted to standardize *in vitro* callus induction through leaf segments of *D. butyracea* and somatic organogenesis.

2. MATERIALS AND METHODS

Leaves explant were collected from 30 years old mature tree of *D. butyracea* growing at Arboretum of Forest Research Institute, Dehradun. Leaves (1–5 cm^2) segments were cut and soaked and gently agitated for in liquid disinfectant (Cetramide 5- 6 drops/100 ml) for 3 minutes along with gentle agitations, subsequently washed with distilled water. Further leaf segments were treated with an aqueous solution of fungicide 0.1 % bavistin® (Carbendazim WP; 1 % w/v) for 3 minutes and were re-washed with distilled water. Explants were subjected to surface sterilization in a laminar air flow with 0.05 %, 0.1 % and 0.15 % w/v of mercuric chloride ($HgCl_2$) and 20 %, 40 % and 60 % v/v dilution of sodium hypochlorite (NaOCl; 5%) both for 3, 5 and 7 minutes (Table 1). Leaf segments were finally washed with sterilized distilled water for 4-5 times. The sterilized explants were inoculated on appropriate culture medium for callus induction and somatic embryogenesis. All media were supplemented with required amount of plant growth regulators (PGRs), agar (0.7 %) or phytagel (0.2 %) were added for solidifying the medium and sucrose as a carbon source at pH 5.8. Standard tissue culture conditions were provided for culture growth.

Plant growth regulators (PGRs) viz., BAP (4.44 µM) and 2, 4-D (1.13 µM, 2.26 µM, 3.39 µM and 4.53

µM) in various combinations were investigated for callus induction through leaf explants of *D. butyracea* (Table 2). Murashige and Skoog's (MS) medium without PGRs served as control. Induced callus was maintained on MS media with BAP (4.44 µM). For indirect regeneration, leaf callus were transferred under different sets of PGRs combinations of BAP (4.44 µM and 8.88 µM) and GA_3 (0.29 µM, 0.58 µM and 0.87 µM) individually as well as BAP (4.44 µM) and GA_3 (0.29 µM, 0.58 µM and 0.87 µM) in combination with IAA (0.57 µM, 1.14 µM, 1.71 µM). Experiments were laid in completely randomized design (CRD) with 7 replicates per treatment. The data was analyzed using analysis of variance (ANOVA) for CRD in Genstats ® 5 edition 3.2 for PC/Windows NT (Copyright 1995, LAWES Agricultural Trust) and treatments' mean were compared with least significance difference (LSD), where $p \leq 0.05$.

3. RESULTS AND DISCUSSION

Establishment of aseptic culture is first and important step in tissue culture. Contamination with microorganisms is considered to be the single most important reason for losses during *in-vitro* culture of plants[8]. Out of 18 sterilization treatments tried, for contamination free leaf explants, best result i.e., 100 % was observed in case of two sterilization treatments; $HgCl_2$ (0.1 %) for 7 minutes and HgCl2 (0.15 %) for 7 minutes but resulted in comparatively low survival, 42.9 % and 14.3 % respectively. On the other hand, treatment with HgCl2 (0.1 %) for 5 minutes which yielded 71.4 % contamination free cultures from leaf explants and all of them survived. Treatments of explants with $HgCl_2$ (0.1 %) for 5 and 7 minutes and NaOCl (40 %) for 5 minutes were significantly at par in terms of survival of explants (Table1). It seems that there is a trade-off between contamination free cultures and survival of explants for applied quantities of $HgCl_2$. $HgCl_2$ and NaOCl are most used microbicides in tissue culture. $HgCl_2$ is at one hand very effective but also it is dangerous to handle. Treatment of NaOCl (40 %) for 5 minutes is also significantly at par with the best treatment in terms of survival of explants to establish aseptic culture and can be an alternative to $HgCl_2$.

A significant variation for PGRs combination and their concentration was discernible with respect to callus induction. Maximum 85.7 % callus induction was observed in MS medium supplemented with 4.44 µM BAP + 2.26 µM 2, 4- D in leaf explants. Only doses of 2, 4- D higher than 3.39 µM in combination with 4.44 µM BAP were significantly low induction than best treatment for callus induction (Table 2). Callus induction occurred over the entire surface of leaf explants (Figure.1) and callus clumps developed within 4 weeks of culture based on the auxin and cytokinin concentrations in the culture medium (Figure.2). The combination of a cytokinin with an auxin has been reported to strongly enhance callus induction in dicots[7,8,9].

Callus culture *in-vitro* may develop shoots or roots depending on the morphogenic potentiality of the cells. During the study, large numbers of combinations with cytokinins (BAP and Kn) as well as auxins (NAA), were tried for shoot differentiation from callus cultures (data not shown). But, no shoots were regenerated from leaf callus of *D. butyracea*. However, frequent rhizogenesis was noticed from the callus cultures (Figure.3). Often hard-to-root species only express their rooting capacity in vitro under suitable conditions through direct or indirect rhizogenesis10. The present study showed that callus rhizogenesis depends on different concentrations and type of PGRs used. Here, maximum rhizogenesis (57.1 %), root number (2.29) and root length (1.24 cm) was recorded in MS with 4.44 µM BAP + 1.14 µM IAA + 0.58 µM GA3 within four weeks in culture. However 0.58 µM GA3 alone resulted in 42.9 % rhizogenesis (Table 3). Similar observation was recorded in *Erythrina variegates*[11].

These roots were subcultured on MS medium supplemented with auxins and cytokinins either alone or in combinations but after four weeks of culture, friable callus developed which ultimately turned brown (data not given). The brown callus with roots was subcultured again on MS medium containing auxins and cytokinins for shoot differentiation, but no shoots were observed. This is the first report of success of callus initiation and rhizogenesis in *D. butyracea*. So, two stages of organogenesis have been achieved in this study and third stage that is shoot organogenesis has to be standardized.

Table.1.Effect of sterilization treatment (different sterilizing agents with different concentrations and duration of application) on leaf explants for contamination free leaf explants (%) and survival of leaf explants (%) of *D. butyracea*. Data recorded after 4 weeks

Treatment Number	Sterilizing agents	Concen-tration	Treatment Duration (min.)	Contamination Free Leaf Explants (%)	Survival of Leaf Explants (%)
1	$HgCl_2$	0.05%	3	0.00	0.00
2			5	0.00	0.00
3			7	14.3	14.3
4		0.1%	3	28.6	28.6
5			5	71.4	71.4
6			7	100	42.9

7		0.15%	3	28.6	28.6
8			5	42.9	28.6
9			7	100	14.3
10	**NaOCl**	20%	3	0.00	0.00
11			5	0.00	0.00
12			7	0.00	0.00
13		40%	3	0.00	0.00
14			5	57.1	42.9
15			7	57.1	28.6
16		60%	3	28.6	28.6
17			5	42.9	28.6
18			7	57.1	14.3
			LSD at p<0.05	39.67	40.78

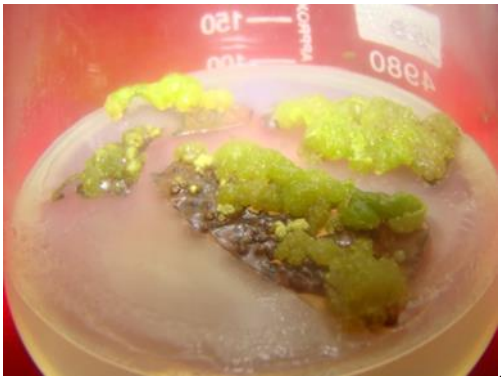
Table.2.Effect of PGRs (BAP + 2, 4-D) in MS medium on callus induction (%) from leaf segments of *D. butyracea*. Data recorded after 4 weeks

PGRs Concentration (µM)	PGR free MS medium (Control)	4.44 BAP +1.13 2, 4-D	4.44 BAP + 2.26 2, 4-D	4.44 BAP + 3.39 2, 4-D	4.44 BAP + 4.53 2, 4-D	LSD at p<0.05
Callus Induction (%)	0.0	57.1	85.7	42.9	28.6	4.6

Table.3.Effect of different PGRs (BAP, IAA and GA3) concentration in MS media on in vitro rhizogenesis of *D. butyracea*. Data recorded after 4 weeks

Hormone (µM)	**Mean Root Number**	**Mean Root Length (cm)**
PGR free MS medium	0.00	0.00
4.44 BAP	0.00	0.00
8.88 BAP	0.00	0.00
0.29 GA_3	0.71	0.31
0.58 GA_3	1.14	0.57
0.87 GA_3	0.29	0.17
4.44 BAP + 0.57 IAA +	0.00	0.00
4.44 BAP + 0.57 IAA +	0.43	0.17
4.44 BAP + 0.57 IAA +	0.00	0.00
4.44 BAP + 1.14 IAA +	1.00	0.36
4.44 BAP + 1.14 IAA +	2.29	1.24
4.44 BAP + 1.14 IAA +	0.43	0.19
4.44 BAP + 1.71 IAA +	0.29	0.17
4.44 BAP + 1.71 IAA +	0.86	0.41
4.44 BAP + 1.71 IAA +	0.14	0.19
LS D at p<0.05	1.25	0.60

Figure.1.*In-vitro* callus regeneration of *Diploknema butyracea*. Callus induced from cut ends of leaves on MS medium supplemented with 4.44 µM BAP + 2.26 µM 2, 4-D


A


B

Figure.2.Callus multiplication in *Diploknema butyracea*. (A) After one month of culture (B) After two months of culture

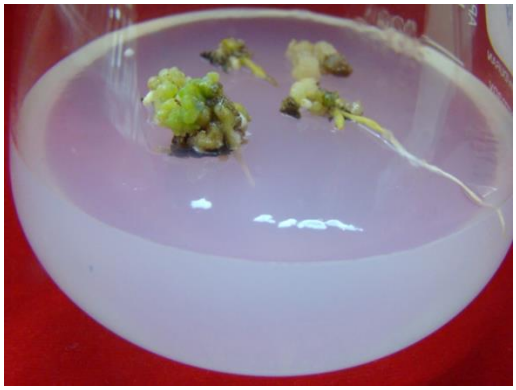


Figure.3.Rhizogenesis in *Diploknema butyracea*. Formation of roots on MS medium supplemented with 4.44 µM BAP + 1.14 µM IAA + 0.58 µM GA3

4. CONCLUSION

In-vitro callus callus was regenerated from leaf segments of mature tree of *Diploknema butyracea* (Roxb.) in MS medium with 4.44 µM BAP + 2.26 µM 2, 4-D. Maximum rhizogenesis is achieved in MS medium with 4.44 µM BAP + 1.14 µM IAA + 0.58 µM GA3.

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