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Callus induction and Cytology of Vulnerable Tree Species *Diospyros candolleana* Wight

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ABSTRACT

In recent years over exploitation, habitat loss, encroachment, illegal felling and other human activities had made a drastic changes and decline of biodiversity which has led to loss of valuable plant species. It is our prime responsibility to give priority for the conservation of species and to keep the Biodiversity under balance. In the present study a vulnerable tree species, *Diospyros candolleana* of the family Ebenaceae has been selected for the conservation purpose through tissue culture techniques. A protocol for callus induction from leaf explant has been standardized with the reduced the rate of contamination. MS medium supplemented with individual and combinations of plant growth regulators were used to study the effects on callus induction *viz.,* 2, 4-D, Zeatin, Kinetin, BAP, NAA and Indole acetic acid. For callus proliferation it took approximately 2 months and callus were subcultured frequently. 2, 4-D, Zeatin, NAA showed callus induction; while combination of Zeatin and BAP with 2, 4-D showed profuse callus. Among these the rate of callus induction is comparatively more in media supplemented with 1 mg/l 2, 4-D (individual growth regulator) and the combination of 2, 4-D + Zeatin and BAP.

Key words : *Diospyros candolleana, Callus proliferation, Growth regulators, 2, 4-D • Zeatin*

Introduction

Every species of biodiversity, are interdependent for food, shelter and for various activities to lead their normal life process. If any disturbance in that, such as climate change, destruction of habitats, hunting, deforestation, pollution, overexploitation etc, causes sequence of changes or damages for the biodiversity leads to loss of species. One or the other way floral diversity is indispensable for all the species. Due to extensive human activities shrinkage in floral diversity cause loss of valuable medicinal plants. To conserve these medicinal plants many strategies are there. In the current study, one of the medicinal and vulnerable tree species, *Diospyros candolleana* of the family Ebenaceae, has been opted to conserve through tissue culture technique. Decoction of *D. candolleana*root bark is used to cure joint pain and swelling and it has anthelmintic and antiprotozoal properties (Dev *et al*., 2012).

Diospyros is a large genus belongs to the family Ebenaceae and the species of this genushas more economic values because fruits of few species are used as economic crops. To improve, to produce specific traits, to preserve the cultivar etc., these species are propagated by tissue culture techniques (Sugiura, 2008; Kochanova *et al*. 2011).Various explants such as leaf, shoot tips, dormant buds, adventitious bud, fruits etc., were used for callus culture and regeneration The purpose of this study is to conserve *D. candolleana species* using tissue culture techniques.

Materials and Methods Collection of plant material

Diospyros candolleana leaf explants was collected from Agumbe, Tirthahalli, Shivamogga, Karnataka, India, (Latitude-13.5692N, Longitude- 75.1338E[f] and Altitude- 664.33 meter) (Fig. 1). The species was authenticated by Dr. Sampathkumara K. K. The herbarium was submitted to the Department of Botany, University of Mysore, Mysuru. The specimen vocher number is UOMBOT22DC33.

Fig. 1.*Diospyros candolleana Habit*
A. Tree (habit), B-Young leaves

Preparation of culture media

For the inoculation of leaf explants, Murashige and Skoog (1962) (MS) media with different growth regulators (GR) in different concentrations such as., 2, 4-Dichlorophenoxyacetic acid (2, 4-D), Zeatin (Zn), Kinetin (Kn), Benzyl amino purine (BAP), Naphthalene acetic acid (NAA), Indole acetic acid (IAA) each individually supplemented with culture media and also with different combinations of 2 4-D with BAP, Zn and NAA. Outline of MS medium composition is represented in Table 1.

Methods of explants preparation and sterilization before inoculation

The young leaves were excised from the mother plant and thoroughly washed with running tap water for 5-10min, cut into small pieces and washed again in sterile distilled water for 5 min. This was followed by treating with cween 20 (10 drops in 100 mL of sterile distilled water for 8-9 min), 3 % sodium hypochlorite (8-9 min), 70% alcohol (1 mim) and 0.1 % mercuric chloride (1 min). The explants were washed 3 times in sterile distilled water. Explants were dried completely with sterile blotter discs and inoculated aseptically to the previously prepared culture media.

Table 1. Chemical composition of Murashige and Skoog media

Name of thestock	Name of the compound	Molecular formula of compound	Concentration of compound (gL^{-1})
Nitrate stock	Ammonium nitrates	NH_4NO_3	165.0
	Potassium nitrate	KNO_3	190.0
Sulphate stock	Magnesium Sulphate	$MgSO_4.7H_2O$	37.0
	Manganous Sulphate	$MnSO_4.H_2O$	1.69
	Zinc Sulphate	$ZnSO_4.7H_20$	0.86
	Cupric Sulphate	$CuSO_4.5H_2O$	0.0025
Halide stock	Calcium chloride anhydrous	$CaCl2.2H_2O$	44.0
	Potassium iodide	KI	0.083
	Cobalt chloride	CoCl233.6H20	0.0025
Phosphate stock	Potassium phosphate	KH_2PO_4	17.0
	Boric acid	H_3BO_3	0.620
	Sodium molybdate	$Na2MoO_4.2H2O$	0.025
Iron stock	Ferrous Sulphate	$FeSO_2.7H_2O$	2.784
	Ethylenediamineteraacetic acid, disodium salt	Na2EDTA	3.724
Vitamin stock	Myo-inositol		0.1
	Glycine		0.002
	Nicotinic acid		0.0005
	Pyridoxine HCL		0.0005
	Thiamine HCL		0.0001
Carbon source	Sucrose		30 g
Gelling agent	Agar		8 g

(Murashige and Skoog 1962; Smith 2006)

Three replicates of ten numbers of each concentration media having single explant and Inoculated cultures were kept in incubation chamber providing 16 hr/day light using white fluorescent tubes (3000 lux intensity) at 21± 1ºC. The data regarding response of explants, nature of callus and percentage of callus parameters were recorded from the day of inoculation and every 60 days of intervals, callus was sub cultured. Percentage of callus induction was calculated using following formula

$$\text{Percent callus induction} = \frac{\text{No. of explants produced the callus}}{\text{Total number of explants culture}} \times 100$$

Cytological studies of *D. candolleana* leaf derived callus

Callus of different stages were collected aseptically for cytological study according to the procedure given by Johansen (1940). Fresh callus were pretreated for three and half hrs with 8- hydroxyquinoline (0.02 M) and washed with distilled water and fixed in carnoy's fixative containing absolute alcohol and Glacial acetic acid mixture in the ratio 3:1 for one day. The callus were washed with distilled water and stored in 70% alcohol until further use. Small bits of callus were treated with 0.5 % mordant for 5 min, and stained with 2 % hematoxylin stain for 10 min 45% propionic acid was used to squash the callus, and the prepared slide was observed under the microscopic.

Results

The leaf, stem, flower and axillary buds of *D. candolleana* were used as explants and inoculated on MS and woody plant media with different concentration of growth regulators individually and in combinations viz., 2, 4-Dichlorophenoxyacetic acid (2, 4-D), Zeatin (Zn), Kinetin (Kn), Benzyl amino purine (BAP), Naphthalene acetic acid (NAA), Indole acetic acid (IAA) and 2,4- D+Zn, 2,4-D+NAA, 2,4-D+BAP. Except leaf explant, all other explants showed high rate of contamination and the Woody plant media showed very less and slow response. Hence further work was continued with only leaf explants using MS media with different combinations of growth regulators.

Leaf explants response to the MS media with different growth regulators

The leaf explants showed best response to the MS media with different growth regulators except IAA, BAP and Kn. The day after the inoculation, in all individual and combination of growth regulators, explants showed curling and bulging. The leaf explants curled outwards, and the edges of the explants attached to the media (Fig. 2A, B and C). Callus was initiated on the midrib, leaf edges and on the surface of the leaf explants (Fig. 2D and E). The region where the callus initiated, browning of media was observed and slowly it became darker as callus matured because of polyphenol like substances released from the callus (Fig. 2F). Proliferation of callus was slow after the callus initiation, it took nearly two months.

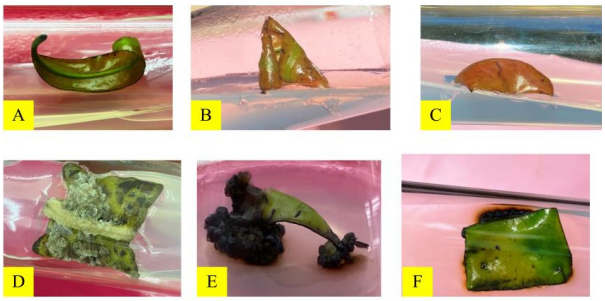

Fig. 2. Response of *D. candolleana* leaf explants to MS media
A and **B**- Curling of Leaf outward, **C**-Bulging of Leaf, **D**-callus proliferation on leaf midrib and surface, **E**-Callus proliferation from leaf edges, **F**-Black coloured callus with browning of proliferated region.

The young staged callus appeared as white or yellowish white or light brownish white coloured callus. As the callus matured it appeared dark black in colour and glossy in nature. On the matured black coloured callus, new callus cells growth appeared as white to yellowish white callus. In matured callus, three different nature of callus were produced irrespective of growth regulators and concentrations such as soft or hard callus they appeared as thick arrangement of globular like structures (Fig. 4A and B), hard callus with irregular shape (Fig. 4c) and loosely arranged small bits of soft callus with irregular shape (Fig. 4D).

Response of callus induction to different growth regulators

The callus induction from *D. candolleana* leaf explants was observed in 2,4-D, Zn and NAA growth regulators. Among these 1 mg/L 2,4-D showed best results so this concentration of growth regulator was

further used in combination with Zn, NAA and BAP. Explants not responded to, only BAP containing culture media but BAP with 2,4-D showed best amount of callus induction. This results depict that 2,4-D was the high responsive growth regulator for the callus induction in *D. candolleana* species and Zn also showed high response of the callus induction. The amount of callus produced was more in Zn (1 mg/l) +2,4-D (1 mg/l) compared to 1 mg/l 2, 4-D. combination. The amount of callus proliferation was more in Zn+2, 4-D > 2,4- D > BAP+2,4-D >Zn. In 2, 4-D+zeatin combinations callus proliferation decreases with increase in Zn concentration (Fig. 3A-E) similarly in 2, 4-D combinations. Compare to all growth regulators, NAA showed very less amount of callus proliferation.

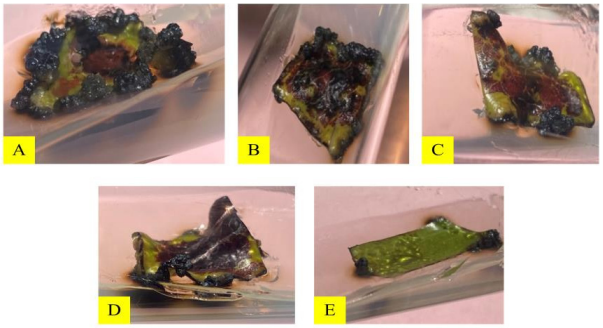

Fig. 3. Response of *D. candolleana* leaf explants to the combination of 2, 4-D and Zeatin growth regulators. Proliferation of callus decreased with increase in Zn concentraion with constant concentration of 2, 4-D (1 mg/l). A-1 mg/l, B- 2 mg/l, C-3 mg/l, D-4 mg/l, E- 5 mg/l (all zeatin growth regulator)

Response of percent callus induction

The Percentage of callus induction was more in 1 mg/ml of 2,4-D (95.23%) and in the combination of 1 mg/ml of 2,4-D + 1 mg/ml of Zn (95.23%) followed by 4 mg/l of 2,4-D (individual GR), 1 mg/ml of 2,4-D + 2 mg/mL of Zn (combination of GR) and 1 mg/ml of 2,4- D + 4 mg/ml of BAP (combination of GR). The percentage of callus induction was less in 2 mg/l NAA (57.14 %) is represented in the Table 2.

Experiment repeated thrice (n= 10 & 10 bits of explants used). Values represented Mean ± SE followed by the same letters within the column are not significantly different at ($p<0.05$) according to Duncan's Multiple Range Test (DMRT)

Results of cytological studies

The cytological study of *D. candolleana* leaf derived

Table 2. Effect of different concentration of growth regulators of media on *D. candolleana*

Plant Growth Regulators (mg/l)		% of Callus Induction
2, 4-D	1	95.2381 ± 4.7619^{a}
	2	85.7143 ± 8.2479^{abc}
	3	71.4286 ± 8.2479^{abcd}
	4	90.4762 ± 4.7619^{ab}
	5	80.9524 ± 9.5238^{abcd}
Zeatin	1	66.6667 ± 4.7619^{bcd}
	2	80.9524 ± 9.5238^{abcd}
	3	76.1905 ± 4.7619^{abcd}
	4	85.7143 ± 8.2479^{abc}
	5	80.9524 ± 4.7619^{abcd}
NAA	1	61.9048 ± 4.7619 ^{cd}
	2	57.1429 ± 0 ^{d}
	3	61.9048 ± 4.7619 ^{cd}
	4	66.6666 ± 12.5988^{bcd}
	5	71.4286 ± 0^{abcd}
2, 4-D + Zeatin	1 + 1	95.2381 ± 4.7619^{a}
	1 + 2	90.4762 ± 4.7619^{ab}
	1 + 3	76.1905 ± 4.7619^{abcd}
	1 + 4	80.9524 ± 9.5238^{abcd}
	1 + 5	76.1905 ± 4.7619^{abcd}
2, 4-D + NAA	1 + 1	61.9048 ± 4.7619 ^{cd}
	1 + 2	71.4286 ± 8.2479^{abcd}
	1 + 3	85.7143 ± 0^{abc}
	1 + 4	76.1905 ± 4.7619^{abcd}
	1 + 5	80.9524 ± 12.5988^{abcd}
2, 4-D + BAP	1 + 1	85.7143 ± 8.2479^{abc}
	1 + 2	85.7143 ± 8.2479^{abc}
	1 + 3	76.1905 ± 4.7619^{abcd}
	1 + 4	90.4762 ± 9.5238^{ab}
	1 + 5	76.1905 ± 4.7619^{abcd}

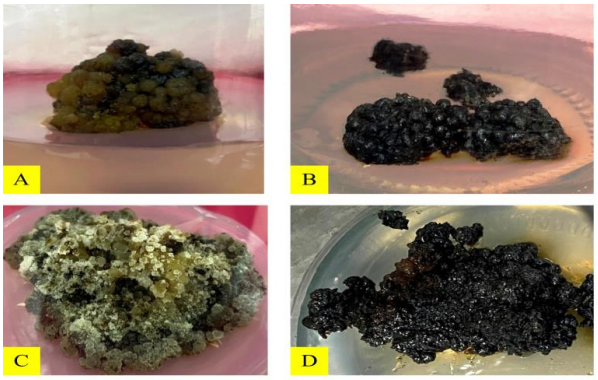

Fig. 4. Different nature of callus produced from D. candolleana leaf explants
A and **B** - Yellowish white and Black coloured globular callus, **C**-Hard irregular shaped callus, **D**- Soft callus made up of small bits.

callus showed prominent nucleus and nucleolous with irregular shaped mass of cells and some cells were enucleated (Fig. 5 A and B). Different shapes of xylary elements were seen that showed xylogenisis, it indicates that cells have the capability of organogenesis (Fig. 5 C and D).

Discussion

The economic crops of genus *Diospyros* are interesting for researchers to get clonal rootstocks through micropropagation using tissue culture techniques to overcome ancient poor characteristics having rootstocks obtained from seed propagation (Giordani *et al*., 2002). Sugiura (2008) reported that85%-100% of callus was induced 4° after 2 weeks, from the 2, 4-D+BAP (3 µM +10 µM) combination in *D. kaki*. Tao and Sugiura, (1992) reported that, hard globular calli proliferated from leaf primordial of *D. kaki*. In the earlier stage callus showing yellowish white colour later it became brown to black, similarly leaf explants also turns to brown or black colour gradually after inoculation because of the reason of exudation of polyphenol like substances was reported by Sugiura, 2008; Choi *et al*., 2001; Sarathchandra and Burch, 1991.

Conclusion

In the present study an efficient protocol for callus induction from leaf explants of *D. candolleana* using MS media supplemented with 2, 4-D and Zeatin has been standardized and this is the first report for this species.

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References

Choi, J.Y., Kim, H.J., Lee, C.H., Bae, J. M., Chung, Y. S., Shin, J. S. and Hyung, N. I. 2001. Efficient and simple plant regeneration via organogenesis from leaf segment cultures of persimmon (*Diospyros kaki* thunb.).

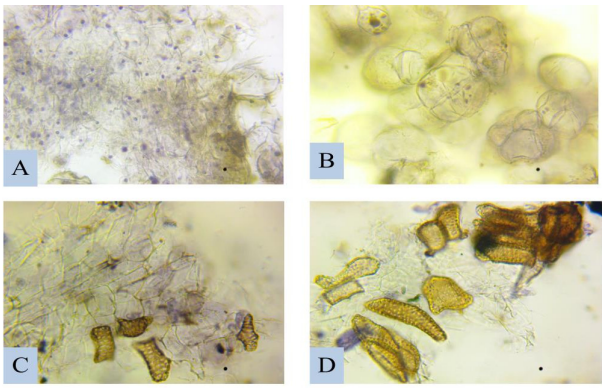

Fig. 5. Cytological studies of D. candolleana leaf derived callus
A and **B** - Nuclcatc and cnucleated cells, **C**-and **D**-Different shapes of xylary elements

In Vitro Cellular and Developmental Biology. Plant. 37: 274–279.

Dev, M. J. A., Rajarajeshwari, N., Ganapaty, S., Parixit, B. and Brun, R. 2012. Antiprotozoal and anthelmintic naphthoquinones from three unexplored species of Diospyros. *Journal of Natural Remedies*. 12(2): 129-134.

Giordani, E., Perria, R. and Bellini, E. 2002. Tissue culture of European accessions of persimmon: callusing and proliferation. *Options Méditerranéennes. Série A: Séminaires Méditerranéens (CIHEAM)*. 51: 93-96.

Johansen, D. A. 1940. Plant Microtechnique. McGraw-hill Book Co., New York, and meristematic tissue. *Staining Technology*. 9: 91-2. London.

Kochanova, Z., Onus, N. and Brindza, J. 2011. Adventitious shoot regeneration from dormant buds of persimmon (*Diospyros kaki* Thunb.) cv. Hachiya. *Journal of Agrobiology*. 28(2): 113-118.

Sarathchandra, S. U. and Burch, G. 1991. Micropropagation of Japanese persimmon (*Diospyros kaki* Thun.) cv. 'Hiratanenashi'. *New Zealand Journal of Crop and Horticultural Science.* 19: 113-120.

Smith, R. H. 2006. *Plant Tissue Culture Techniues and Experiments.* Third edition. United States of America: All Academic Press publications.

Sugiura, A. 2008. Somatic Embryogenesis and plant regeneration from Immature Persimmon (*Diospyros kaki* Thunb.) *Embryos. Hort Science.* 43(1): 211-214.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*. **15:** 473-497.

Tao, R. and Sugiura, A. 1992. Adventitious Bud Formation from Callus Cultures of Japanese Persimmon. *Hortscience*. 27(3): 259-261.