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In vitro regeneration of an endangered medicinally important plant: *Lasiosiphon eriocephalus* Decne

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ABSTRACT

In-vitro multiplication of Lasiosiphon eriocephalus an endangered medicinally important species of Family Thymaleaceae is reported in the present work. Apical buds, Lateral buds, Leaves and Internodes were cultured on Murashige and Skoog's (MS) medium supplemented with 0.5, 1.0, and 2.0 mg l^{-1} 6-Benzylaminopurine (BAP), 2, 4 – dichlorophenoxyacetic acid (2,4-D), and Kinetin. Meristematic tissues (Apical and lateral buds) responded by showing multiple shooting in all the three concentration of BAP, Explants inoculated in 1 mg l^{-1} BAP responded in 7 days (swelling was observed) and shoot initiation was noticed within 15days of inoculation. Callusing was observed in the leaf explants cultured on media supplemented with 2, 4-D. Rooting was induced in the in vitro regenerated shoots by sub-culturing them to M.S. medium fortified with 0.5 mg l^{-1} IAA. Rooted cultures were then subjected to primary hardening in pro trays containing vermiculite and coco peat. Finally the plants were transferred to green house for secondary hardening.

Key words: Plant Tissue Culture, Endangered plant, *Lasiosiphon eriocephalus*, Rametha, *In-Vitro*, Multiple Shooting.

INTRODUCTION

Lasiosiphon eriocephalus Decne (syn. Gnidia glauca) is a perennial shrub or small tree belonging to the family Thymaleaceae. Herbarium of this plant is preserved in Cup-Board No. / Pigeon hole No. 26/u at Janaki Ammal Herbarium of Indian Institute of integrative Medicine, Jammu. This five to eight feet tall plant has a special place in tribal and local system of medicine. Once found all over the Western Ghats of Indian peninsula in grassy patches, because of indiscriminate collection of medicinal plants as a result of population growth urbanization and rapid agricultural development, now it has joined the list of endangered plant species and is depleting at an alarming rate. It is now seen in sparse clumps in Sagar and Sorab talukas in Shimoga district of Karnataka and also found near Kaas plateau, Mahabaleshwar and Khandala in Satara, Petkilla in Raigad and Bhimashankar, Lonavala and Talegaon in Pune district of Maharashtra.

The natural regeneration as well as conventional propagation of this plant has also been beset with several other factors, like poor fruit set, seed germination and rooting on the stem cuttings. Triphenyl tetrazolium chloride test for seed viability was performed to check the seed viability which indicated only 2% viability of the seeds. However, soaking the seeds in 100 mg l -1 Gibberellic Acid overnight helped overcome the dormancy and induced as much as 96% germination [1]. Therefore there is a need to conserve this plant and regenerate it before it hits the list of extinct plant species.

Its Medicinal uses as rubefacient have been shown by Behl et al. [2]. Later Bendre et al. [3] have shown this plant to have teeth loosening effect and recently Joshi et al. [4] could demonstrate in vitro anticoagulant activity due to *Bis*-Coumarin found in this plant. Plant owes its medicinal importance mainly to two secondary metabolites-Coumarins

(Lasiocephalin and Lasioerin) and Glycosides (Erioside, Eriocephaloside). Moreover, Bhandari et al [5] have recorde Genkwanin, β-D-Glucosides, Syringaresinol, Syringing and β-Sitosterol; and a Xanthone Glycoside was recorded by Mandal et al. [6].

MATERIALS AND METHODS

Explant Collection and Surface Sterilization: Young shoots of *Lasiosiphon eriocephalus*, with maximum of ten lateral buds were collected during the active growing season (end of monsoon) from Khandala hills of Maharashtra. To combat the inhibitory effect of phenol oxidase leaching out from cut ends, explants were immediately dipped in freshly prepared anti-oxidant solution (chilled) containing 100 mg l^{-1} ascorbic acid and 150 mg l^{-1} citric acid.

Mother plants were sprayed with 1% Bavistin to keep a check on the possible fungi that might have been lodged on the plant. Upon bringing the explants to the laboratory within 4 hrs of excision they were further cut to small pieces of about two – three centimetre and dipped in a solution of 2% Tween-20 for 10 min. These explants were then scrubbed softly with a soft paint brush and washed with demineralised water. After washing explants were again dipped in a solution containing 0.1% Bavistin + 0.25% Chloramphenicol (a bactericide from Ranbaxy India) and kept on shaker with constant stirring for 30 min. Further surface sterilization of explants was done on Laminar Air Flow bench. Explants were treated by 1% sodium hypochlorite solution for 10 min and then rinsed with autoclaved demineralised water for 10 min. They were then surface sterilized with 0.2 % mercuric chloride solution for 3 min and then explants were rinsed thrice with autoclaved demineralised water to ensure complete removal of all the sterilant. Explants were further trimmed to a length of 1 cm before inoculation.

Murashige and Skoog's (1968) medium with 3 % Sucrose and solidified with 0.8 % Agar was used as basal medium for in vitro regeneration and culture studies. Basal medium was fortified with different Plant Growth Regulators (PGRs) viz. 6-Benzyl Amino Purine (0.5, 1.0 & 2.0 mg l^{-1} BAP) or 6-Furfuryl Amino Purine (0.5, 1.0 & 2.0 mg l^{-1} Kinetin) and 2, 4-Dichloro Acetic Acid (0.5, 1.0 & 2.0 mg l^{-1} 2, 4-D). Temperature of the growth room was maintained at 23±2°C. Cultures were incubated in dark. Initiation of culture was monitored visually as well as by observing the sections of cultures under the microscope.

After a week of incubation in dark the cultures were subjected to 16 hrs photoperiod with light intensity of 3000 Lux followed by 8 hrs of dark period. Cultures were subcultured after every 22 days. A record of percentage culture responding was maintained.

For rooting, regenerated shoot clusters were excised into single plants and sub-cultured on to medium containing Indole-3-acetic acid (0.1, 0.5 & 1.0 mg l^{-1} IAA), Indole-3-butyric acid (0.1, 0.5 & 1.0 mg l^{-1} IBA) and 1-Naphthaleneacetic acid (0.01, 0.05 & 0.1 mg l^{-1} NAA). Light intensity of the growth room was reduced to 2000 Lux. Number of regenerated roots per shoot as well as percentage of shoots responding to rooting and time taken for rooting was recorded. A regular subculture at an interval of 4 weeks was done throughout the culture period.

Hardening of Rooted Plants: - Regenerated plantlets were recovered from the culture tubes and washed thoroughly with water to remove agar residue and transferred in a 50 cavity protray having 1" X 1" X 1.5" cavity size filled with steam sterilized vermiculite and coco peat. These pro trays were kept in a green house with a fan-pad cooling system and fogger to control temperature and relative humidity. The green house temperature did not exceed 29°C. The maximum day light intensity during hardening was 12,000 - 14,000 Lux. Immediately after transfer to the green house plantlets were kept at 100% relative humidity for first 3 days and then RH of 60-70% was maintained. Mortality and survival of plants during hardening was recorded.

RESULTS AND DISCUSSION

BAP induced multiple shooting in both apical and lateral meristematic explants within 7 to 18 days from all the three concentration used (Fig. 1), maximum response was noted at 1.0 mg l^{-1} BAP. The superiority of BAP over other cytokinins on shoot bud production and proliferation of shoots has been reported for several medicinal and aromatic plant species such as *Prosalea* corydifolia [7], *Eclipta alba* [8] and *Mentha viridis* [9]. Lateral buds were found to be more responsive, showing culture initiation in 86 % of the inoculated explants in 7 days than apical buds which showed only 60 % culture initiation. Further subculture to the same medium though showed very good shoot proliferation, but elongation of the shoots was very show. To increase faster shoot elongation coconut water was added to the medium.

Kinetin also showed multiple shooting in both apical bud and lateral buds, but here the cultures initiation took more than 2 weeks time and even percentage response of the cultures in Kinetin was subordinate to that of BAP [10].

Most effective concentration for both the apical and lateral bud was 1 mg l^{-1}. On increasing the concentration of BAP the response was delayed further and a reduced number of shoots were obtained [11]. Since growth on kinetin containing medium was very slow, hence efforts were concentrated towards growing the cultures in BAP fortified medium.

A microscopic examination of *In Vitro* cultured apical and lateral buds at initial stages of multiple shooting revealed that multiple shooting was due to direct organogenesis i.e. continued development of meristematic activity of axillary or lateral buds and the de novo induction of adventitious buds from various tissues [12, 13]. As it can be seen in figure 1E and 1F, the meristematic dome of both apical and lateral buds proliferated to produce shoot buds. This development exploits normal ontogeic route for branch development by lateral (pre-formed) meristem when treated with PGR. In *L. eriocephalus* both apical and lateral meristematic tissue could induce, proliferate and elongate shoots on simple medium containing only BAP. Differentiated tissues like leaf and stem could produce only short lived callus.

Both the meristematic explants i.e. apical and lateral buds did not show any growth when cultured on 2, 4-D containing medium. Whereas, leaf and internode explants started to callus within 10days of inoculation. Response to 2,4-D have been found to be very tissue specific in many plants e.g. in *Sorghum bicolor*, 2,4-D could proliferate cultures only if the leaf explants were taken from the base of young leaves, explants taken from other regions of the leaves did not [14]. Swelling was observed in leaves and internodes inoculated in 2, 4-D after eight days of inoculation. Out of the three concentrations tried 2 mg l^{-1} was best suited for callusing as 76 % of the leaf explants and 57 % of the internodes responded in that concentration. Initially callus started to appear from the mid rib but later it proliferated from the whole surface. Friable callus first appeared from the cut ends of internodes and then from whole surface. However, after 2-3 subcultures the calli from both the explants perished.

Table 1: *In Vitro* Differential response by different explants of *L. eriocephalus* to different PGRs augmented into MS basal medium.

PGRs mg l^{-1}	No of explants inoculated	Time Taken for Initiation	Shoot Apex	Lateral Buds	Young Leaf	Internode
			% response	% response	% response	% response
0.5 2,4-D	100	10	0	0	51	30
1.0 2,4-D	100	10	0	0	48	25
2.0 2,4-D	100	9	0	0	76	57
0.5 BAP	100	18	40	59	0	0
1.0 BAP	100	7	60	86	0	0
2.0 BAP	100	10	23	71	0	0
0.5 KIN	100	15	17	20	0	0
1.0 KIN	100	15	32	64	0	0
2.0 KIN	100	15	25	31	0	0

Although the concentration and type of auxins and cytokinins required for in vitro regeneration of different plants vary, but the basic approach of use of their combination has been well established and used to regenerate a wide variety of plants [15 - 18]. Though little is known about how hormones evoke a particular pattern of morphogenesis, still requirement of plant hormones are surely a must for inducing culture response in plant tissues.

Rooting - > 3 cm long regenerated shoots having at least 2 internodes and 4 - 6 leaves; were cut at the base and inoculated on rooting medium containing IAA, IBA or NAA (Table-2). Rooting occurred in IAA and NAA containing medium. Most suitable rooting medium was one fortified with IAA with a concentration of 0.5 mg/l, where each shoot showed induction of root primordia within 7 days and it took one month to have roots of approximately 2-3 cm length each.

Table 2: Rooting response by regenerated shoots of *L. eriocephalus* to different auxins. Data was recorded 30 days after subculture on to the auxin containing medium. Results are mean of 100 readings

PGRs mg l^{-1}	% Rooting	Average no of roots
IAA 0.1	20	3
IAA 0.5	55	4
IAA 1.0	10	3.5
IBA 0.1	0	0
IBA 0.5	0	0
IBA 1.0	0	0
NAA 0.01	4	3
NAA 0.05	12	3
NAA 0.1	10	2.5

Hardening - two substrates were tried for hardening the regenerated plants. Plants that were transferred to vermiculite became soggy and died. About 70% plants survived hardening on soil in poly house.

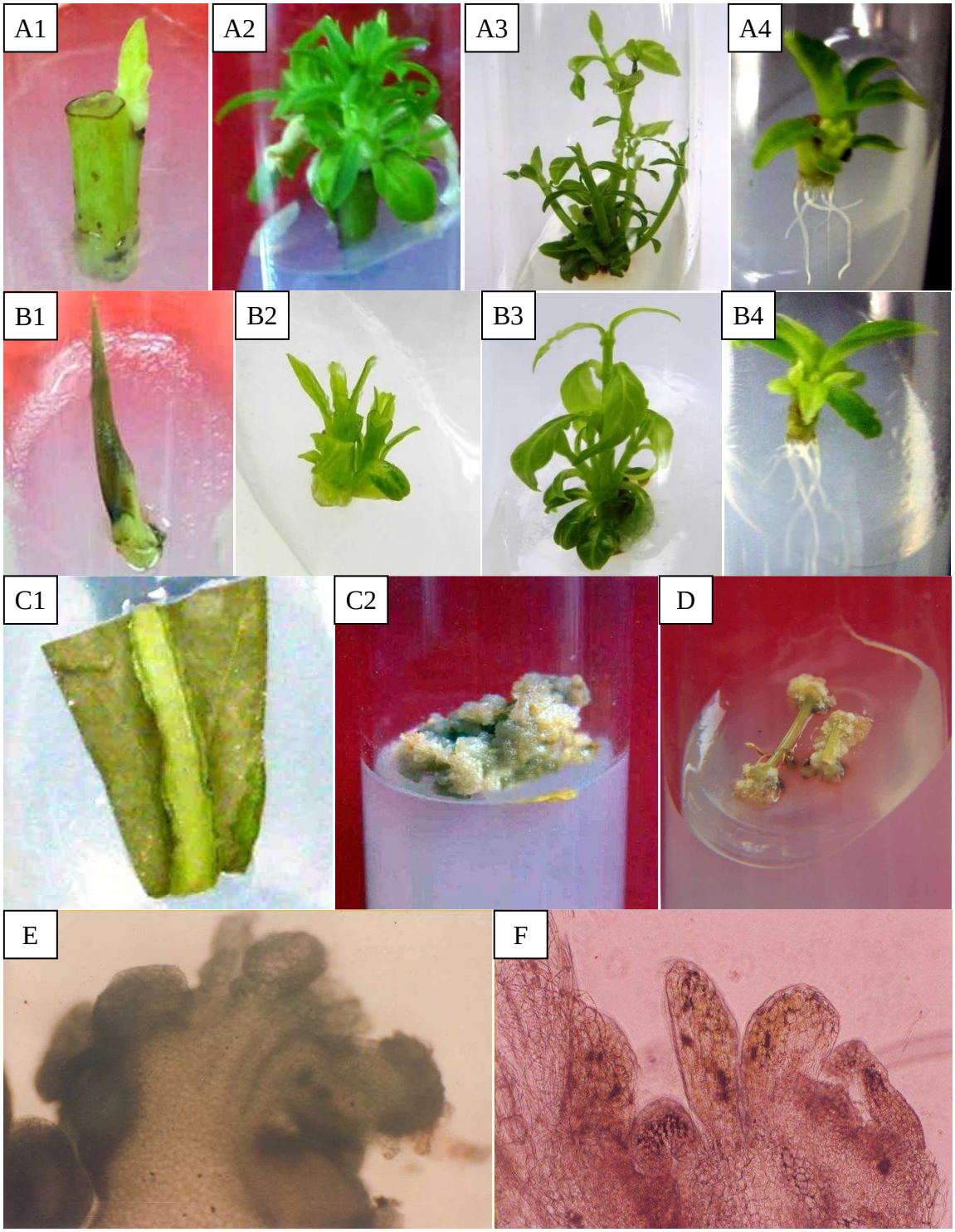

Figure. 1: *In vitro* growth of different explants of *Lasiosiphon eriocephalus Decne*

(A1-A4) Different stages of plant regeneration from Lateral Buds on MS medium supplemented with 1 mg l $^{-1}$ BAP
(B1-B4) Different stages of plant regeneration from shoot apex on MS medium supplemented with 1 mg l $^{-1}$ BAP
(C1) Friable callus from leaf mid rib and later (C2) friable callus developing from whole leaf surface on MS medium supplemented with 2, 4-D
(D) Callusing from both the cut ends as well as whole surface of stem, on MS medium supplemented with 2, 4-D and Microscopic photographs of L.S. of (E) Shoot apex and (F) Lateral bud cultured on MS medium supplemented with 1 mg l $^{-1}$ BAP; both showing multiple shoot primordia.

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