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Somatic embryogenesis and plant regeneration from stem explants of *Leptadenia reticulata* (Retz.) Wight. & Arn.

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Somatic embryogenesis and plant regeneration from stem explants of *Leptadenia reticulata* (Asclepeadaceae), an endangered medicinal plant possessing galactogogue property and rich alkaloid content, have been reported. Murashige and Skoog's medium supplemented with MS + 3% sucrose + NAA (2.68 µ*M*) + BAP (4.40 µ*M*) was the best for callus formation as well as pre-embryonic mass (PEM) induction. Of the two subculture conditions tested for proper embryo induction, MS liquid medium proved superior over solid culture medium in inducing healthy embryos. Presence of NAA in the induction medium was also found to be critical for PEM formation, as the callus raised without it could not be triggered for embryogenesis in any of the subsequent subcultures. The resulting embryoids on transferring to Murashige and Skoog's basal as well as other cytokinin containing medium attained maturation with varied frequencies, of which MS basal medium triggered maximum response compared to other cytokinin containing medium. The so-developed shoots on transferring to half strength MS + IBA (4. 90 µ*M*) developed vigorous tap root system, which were later hardened on peat mass mixture with 75% survival.

Keywords: Stem explants, *Leptadenia reticulata,* somatic embryogenesis, napthalene-3-acetic acid, liquid medium

Introduction

Leptadenia reticulata (Retz.) Wight. & Arn., an important, endangered, medicinal plant, belongs to the family Asclepiadaceae. Different parts of this plant, whose medicinal uses are known since 4500 BC, offer diverse curative applications owing to the presence of copious alkaloids and other therapeutic phytochemicals[1]. Leaves and twigs of the plant are the source of hentriacontanol, α- and β-amyrin, stigmasterol and γ-sitosterol, and falvonoids, diometin and lutcolin. Pericarp and follicle contain quercetin, iso-quercitrin, ruin and hyproside, while seeds contain meso-inositol and its monomethyl ether[2]. Leaves and roots of the plants are used in the treatment of tuberculosis and eye diseases. Alcoholic (50%) extracts of roots, leaves and stem show antibacterial activity and are used as anaesthetic[3]. *L. reticulata* is also key ingredient of Ayurvedic drug Jivanti, which is used as stimulant and tonic. In Jivanti preparation, it is used along with *Dregaea volubilis*, *Sarcostemma bevistigma*, *Holostemma ada-kodien* and *Flickingeria nodosa*[2]. The plant is also reported to possess high galactogogue property, which has been clinically assed by many investigators[4-6].

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Owing to immense commercial significance for its therapeutic potential, *L. reticulata* has been subjected to overexploitation by neutraceutical companies, rendering it an endangered species. Hence, there is a need to apply non-conventional methods of propagation, in addition to *in situ* conservation, for large-scale cultivation and sustainable utilization of biodiversity of *L. reticulata*. To achieve these objectives, present study was taken up.

Materials and Methods

Explant Preparation and Culture Conditions

Young, tender twigs were collected from 1-yr-old field plants of *L. reticulata* (Fig. 1a) grown at herbal garden of Sir M Visvesvaraya Institute of Technology (SMVIT), Bangalore. The stems (internodal parts) were trimmed to 2.0 cm size and treated as explants. They were washed thoroughly in running tap water (30 min), then in 5% lab wash (Merck) and again in running tap water (10 min). The washed explants were surface-sterilized for 6 min with the mixture of 0.1% mercuric chloride + 0.1% Bavistin + 0.1% Cetrimide and rinsed five times with sterile distilled water to remove the traces of sterilants. Analytical grade chemicals obtained from Himedia Laboratories and hormones from Sigma Chemicals were used for preparing the stock solutions and subsequent media

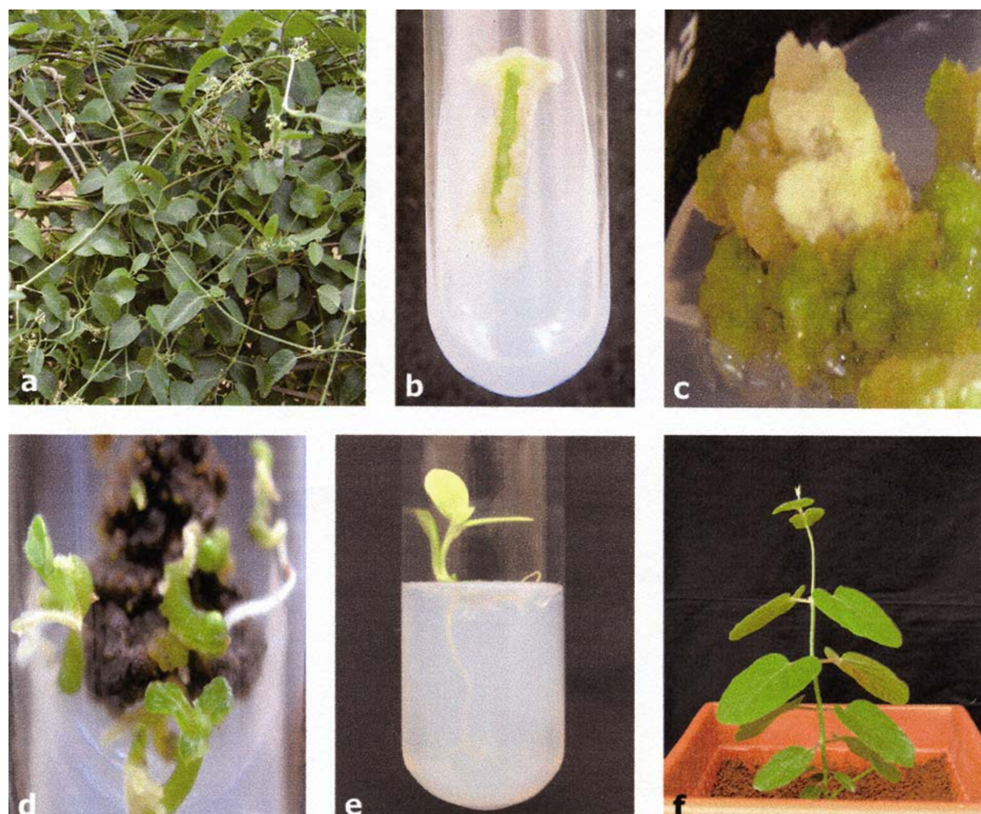

Fig. 1 (a-e)—Regeneration of plant *via* somatic embryogenesis from stem explant cultures of *L. reticulata*: a. Habit of plant maintained at SMVIT campus; b. Proliferating callus on cut-ends of stem explants cultured on MS + NAA (2.68 μ*M*) + BAP (4.40 μ*M*) after 2 wk of culture; c. Pre-embryonic mass formed from callus on MS + NAA (2.68 μ*M*) + BAP (4.40 μ*M*) after 4 wk of culture; d. Conversion of cotyledonary stage embryos into mature plantlets on MS basal medium; e. Plant regenerated *via* somatic embryogenesis rooted on half strength MS medium + IBA (2.46 μ*M*); and f. Hardened plant in pot.

preparation. Murashige & Skoog's salts[7] with 3% (w/v) sucrose was used as the basal medium. After adding the growth regulators, the *p*H of the medium was adjusted to 5.7 ± 0.1 followed by gelling with 0.8% of agar. The media were autoclaved at 121° C and 1.06 kg/cm^2 pressure for 20 min.

Somatic Embryogenesis and Plant Regeneration

The internodal explants, trimmed to 1.5 cm size were aseptically inoculated onto MS medium supplemented with different concentrations of NAA (0.53-26.85 μ*M*), 2,4-D (2.26-22.62 μ*M*) and combination of NAA (0.53 μ*M*) + BAP (0.44-22.20 μ*M*) and 2,4-D (2.26 μ*M*) + BAP (0.44-22.20 μ*M*) for callus induction. All the cultures were incubated in a growth chamber maintained at a temperature of 25 ± 2°C, relative humidity 60-80% and 16:8 h photoperiod at a photon flux density of 50 μE mol m^{-2}s^{-2} provided by day light fluorescent tubes. The calli obtained on the above medium was subcultured and maintained separately on solid as well as liquid culture medium with same hormone concentration to determine the effect of subculture condition on embryo development. The embryos developed on both the medium were then transferred onto MS basal as well as MS + BAP (0.44-22.20 μ*M*), MS + Kn (0.46-23.20 μ*M*) and MS + TDZ (0.45-22.7 μ*M*) medium to ascertain their effect on embryo maturation and development. The resultant shoots were then tested for root induction on MS + IBA (0.49-24.60 μ*M*). Well-rooted shoots were taken out and washed with sterile distilled water to remove the traces of agar. The plants were then shifted to micro-pots containing peat mass wetted with quarter strength MS salts and acclimatized in culture room for 2 wk before transferring to earthen pots containing soil, and finally to the field.

Statistical Analysis

The cultures were examined after 4 wk and all the experiments were repeated at least thrice. Data were analyzed using one-way analysis of variance

(ANOVA) and comparisons between the mean values of treatments were made by Tukey's HSD test with 5% level of probability.

Results and Discussion

Callus initiation occurred at the cut ends of the explants within 1 wk and creamy-white callus developed all over the explants within 2-3 wk of the culture on almost all the media tested (Fig. 1b). However, the degree of callus formation varied with the treatments. Two of the media tested, MS + NAA (2.68 μ*M*) + BAP (4.40 μ*M*) as well as MS + 2, 4-D (2.26 μ*M*) + BAP (2.22 μ*M*) elicited steady callus growth until 3rd wk of the culture (Table 1). However, only the callus formed on MS + NAA (2.68 μ*M*) + BAP (4.40 μ*M*) continued to proliferate until 4th wk, exhibiting embryogenic trigger, which was evident from the formation of uniform pre-embryonic mass throughout the callus at the end of 4th wk (Fig. 1c). The callus formed on 2,4-D containing medium, either alone or in combination with BAP turned into hard, nodular, sparingly embryonic mass and became non-proliferative after 3rd wk of culture (Table 1). The results clearly indicate that NAA plays a critical role in the early developmental events leading to somatic embryogenesis in *L. reticulata* cultures. Similar results have also been reported in *L. reticulata* leaf cultures[8] and in garlic[9]. While on one side, the results confirm the generalization that intermediate levels of auxins, either alone or in combination with cytokinins, support initiation and maintenance of callus[10-11], they further draw attention to the critical role of specific auxins in the early embryogenic events as also reported in *Cicer arietinum*[12] , *Prunus avium*[13] and *Hardwickia binata* [14].

The friable callus formed on MS + 3% sucrose + NAA (2.68 μ*M*) + BAP (4.40 μ*M*) was subcultured onto solid as well as liquid maintenance medium with the same hormonal concentration for determining the effect of suspension on embryo development. From this, the callus was further transferred to MS solid medium supplemented with cytokinins, like BAP, Kn and TDZ as well as MS basal medium, to achieve complete embryo development and maturation. While majority of the embryonic callus developed on the solid culture medium showed incomplete embryo development, leading to either only radicle or plumule development on subsequent cultures, the embryos obtained from suspension culture exhibited all the four sequential stages of development, such as globular, heart shaped, torpedo and cotyledonary stages (Figs 2a-c); finally attaining clear-cut radicle and plumular development (Figs 2d-f). The precise development of embryos in suspension culture may be due to effective separation of embryonic mass achieved in suspension culture compared to solid culture medium as documented in *Allium fistulosum*[15] cell cultures. Of the three cytokinins (BAP, Kn and TDZ) and basal medium tested for maximum embryo maturation, MS basal medium induced large number of embryo maturation compared to cytokinin containing medium (Table 2). The experiments were repeated even with callus formed on MS + 2,4-D (2.26 μ*M*) + BAP (2.22 μ*M*) medium and the results are described in Table 3. The efficacy of basal medium to bring about embryo maturation has been widely reported in many plant species including Kodo millet[16], *Oldenlandia umbellata*[17] and *Araujia sericifera*[18]

Table 1—Analysis of callus induction from stem explants of *L. reticulata* cultured on MS medium supplemented with various hormonal concentrations

MS + hormone (μ*M*)	Morphological response of *explant*
Basal	No Response
NAA, 0.53	Creamy white, non-proliferating callus
NAA, 5.37	Creamy white, proliferating friable, rhizoidal callus
2,4-D, 0.45	Creamy white, nodular, less proliferating, callus
2,4-D, 4.52	Creamy white, less proliferating nodular, rhizoidal callus
2,4-D, 22.62	Creamy white, nodular, rhizoidal, non-proliferating callus
NAA, 2.68 + BAP, 0.44	Creamy white, proliferating, nodular callus
NAA, 2.68 + BAP, 2.22	Creamy white, greenish, nodular, proliferating callus
NAA, 2.68 + BAP, 4.40	Creamy white, nodular, highly proliferating, callus
2,4-D, 0.45 + BAP, 2.22	Creamy white, hard, nodular callus
2,4-D, 2.26 + BAP, 2.22	Creamy white, proliferating, nodular callus

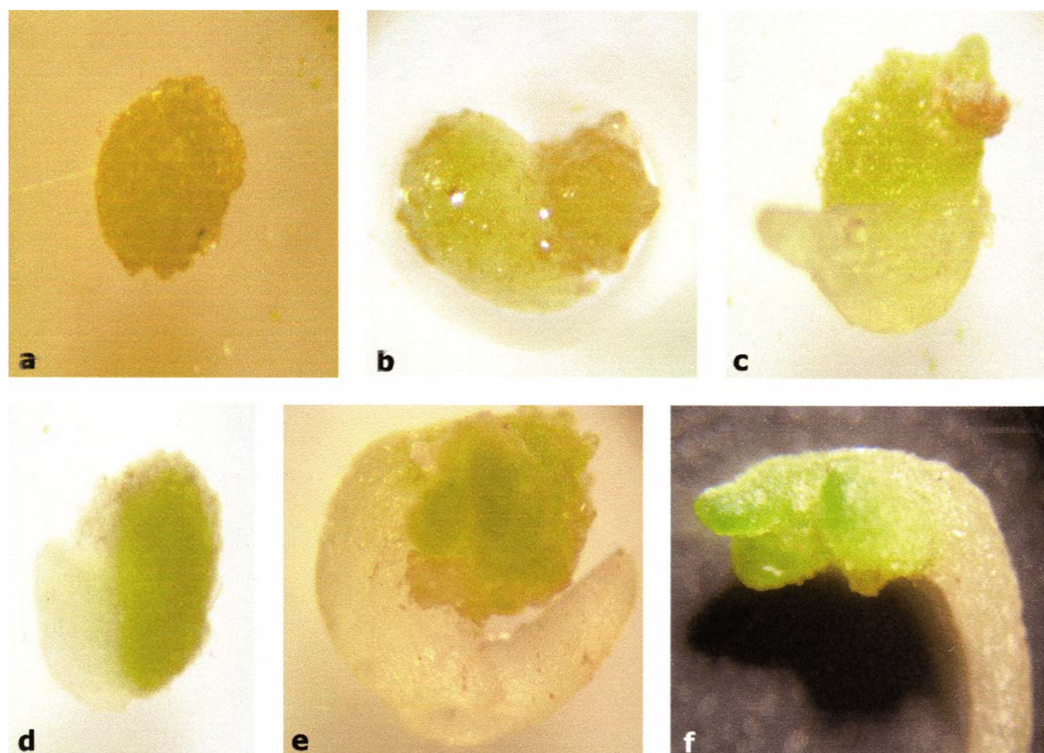

Fig. 2 (a-f)—Stages of embryo development and maturation: a. Globular embryo; b. Heart shaped embryo; c. Cotyledonary embryo showing root emergence; and d-f. Stages of embryo maturation on MS basal medium showing progressive development of radicle and plumular structures.

Table 2—Effect of various concentrations of auxins and cytokinins on embryogenesis of *L. reticulata* stem callus obtained from MS + NAA (2.68 µ*M*) + BAP (4.40 µ*M*) medium

MS + hormone (µ*M*)	*Average no. of cotyledonary embryos	% response
BAP, 0.04	3.00 ± 0.20^{b}	66
BAP, 0.22	$1.79\pm0.20b^{c}$	66
BAP, 0.44	$1.33\pm0.15b^{c}$	66
BAP, 2.22	0.33 ± 0.17^{d}	33
Kn, 2.32	$2.30\pm0.20c^{d}$	66
TDZ, 0.11	0.66 ± 0.15^{d}	33
TDZ, 0.04	0.33 ± 0.17^{d}	33
Basal	12.00 ± 0.45^{a}	99

*Values are mean± SD of three independent experiments
*Means in each column followed by different letters are significantly different according to the Tukey's HSD test at 0.05 significance level

Table 3—Effect of various concentrations of auxins and cytokinins on embryogenesis of *L. reticulata* stem callus obtained from MS + 2,4-D (2.26 µ*M*) + BAP (2.22 µ*M*) medium

MS + hormone (µ*M*)	*Average no. of cotyledonary embryos	% response
BAP, 0.04	1.00 ± 0.17^{ab}	33
BAP, 0.22	0.66 ± 0.15^{b}	33
BAP, 0.44	0.66 ± 0.15^{b}	33
BAP, 2.22	1.00 ± 0.17^{ab}	33
Kn, 4.60	0.66 ± 0.15^{b}	33
Kn, 11.62	1.66 ± 0.15^{ac}	66
TDZ, 0.11	1.33 ± 0.31^{a}	66
TDZ, 0.22	0.66 ± 0.15^{b}	33
TDZ, 0.34	0.33 ± 0.17^{c}	33

*Values are mean±SD of three independent experiments
*Means in each column followed by different letters are significantly different according to the Tukey's HSD test at 0.05 significance level

The mature plantlets developed on basal medium (Fig. 1d) were transferred to half strength MS + 3% sucrose + IBA (2.46 µ*M*) for obtaining complete plant development. Numerous roots were formed from young plantlets within 10 d of culture (Fig. 1e), exhibiting 75% survival. Effect of IBA on root induction has been reported in many plants like *Caltropis gigantea*[19], *Traidax porcumbens*[20], etc. After 4 wk of culture, well-rooted shoots were shifted to micropots containing peat mass, wetted with quarter strength of MS salts, and were acclimatized in culture room for 2 wk, recording 75% survival. After

2 wk, the plants were further hardened firstly by transferring onto pots (Fig. 1f) and finally to the field condition.

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