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BRIEF COMMUNICATION

Benzyladenine induced somatic embryogenesis and plant regeneration of *Leptadenia reticulata*

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Abstract

Plant regeneration through indirect somatic embryogenesis was attempted from leaf, internode, node and shoot-tip derived callus of *Leptadenia reticulata.* Somatic embryos at the highest frequency was induced on Murashige and Skoog (MS) medium supplemented with 8.87 μM benzyladenine (BA) and 2.46 μM indole-3-butyric acid (IBA). From different explants, only shoot-tip and node explant derived calli induced somatic embryos. Transfer of the embryogenic callus to suspension cultures of the same concentration of growth regulators facilitated the development of embryos. Suspension cultures with reduced concentration of BA (2.22 μM) either alone or in combination with 0.49 μM IBA fostered maturation of embryos. Half-strength MS solid medium with 1.44 μM GA_3 and BA (0.22 or 0.44 μM) facilitated conversion of embryos into plantlets at higher rate compared to that on with BA alone. About 77 plantlets were recovered from 10 mg callus. Plantlets transferred to small cups and subsequently to field survived in 80 %. All the plantlets established in the field exhibited morphological characters similar to that of the mother plant.

Additional key words: suspension culture, node callus, shoot-tip callus.

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Leptadenia reticulata (Retz.) Wt. & Arn. (*Asclepiadaceae*), a shrub distributed in tropical and subtropical parts of Asia and Africa, is a component of many drugs. Stigmasterol is the major component in the extract and it also contains β-sitosterol. The tubers contain fructosan of the inulin type and are used as vegetable (Kirtikar and Basu 1975). The stem and roots also contain many aliphatic esters. The plant is an out breeder, hence seed derived progenies are not identical genetically. Very low seed setting (< 5 %) and low germination rate of seeds (< 5 %) curb its propagation through seeds. Vegetative propagation by stem cuttings is cumbersome (< 10 %). Large-scale propagation is a prerequisite to meet the pharmaceutical needs, and also for effective conservation of this valuable medicinal plant.

Plant regeneration through indirect somatic embryogenesis has been reported in many medicinal plants of *Asclepiadaceae viz. Tylophora indica* (Manjula *et al*. 2000, Jayanthi and Mandal 2001), *Hemidesmus indicus* (Sarasan *et al*. 1994), *Araujia sericifera* (Torné *et al*. 1997), *Ceropegia* spp. (Patil 1998), and *Holostemma ada-kodien* (Martin 2003). The present investigation describes the *in vitro* propagation of *Leptadenia reticulata* through somatic embryogenesis.

Leaf, internode, node, and shoot-tip segments of *Leptadenia reticulata* were collected from the tender parts of mature plants growing in Calicut University Campus. After thorough wash under running tap water, detergent, extran and double distilled water, the segments were surface sterilized using 0.5 % (m/v) mercuric chloride solution for 10 - 14 min, washed again with sterile double distilled water and cut into appropriate sizes (leaf explant 1 cm^2; internode, shoot-tip and node explants 1 - 1.5 cm) and cultured on Murashige and Skoog (1962) medium with different growth regulators *viz*. benzyladenine (BA), kinetin (Kn), α-naphthalene-acetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D)

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Abbreviations: BA - benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; IBA - indole-3-butyric acid; Kn - kinetin; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid.
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at different concentrations either alone or in combination as specified in the text. The medium was supplemented with 3 % sucrose, and gelled with 0.8 % agar (*Merck*, Murobai, India). Suspension cultures were used without changing the medium components. The pH of the medium was adjusted to 5.8 before the addition of agar. The media were sterilized by autoclaving at a pressure of 1.06 kg cm^{-2} for 20 min. Incubation period of the initiation cultures was 60 d. Cultures were incubated at 25 ± 2 °C with 16-h photoperiod under white fluorescent tubes (irradiance of 25 μmol m^{-2} s^{-1}) unless otherwise stated. Suspension cultures were incubated on a gyratory shaker at 120 rpm in dark. Conversion of embryos into plantlets was attempted by transferring onto half strength MS agar medium fortified with different growth regulators/additives.

Plantlets transferred to small pots containing sand and soil (1:1) were kept in greenhouse for 15 d and subsequently transferred to the field. The experiments were set up in a completely randomized design. Twenty cultures were raised for each treatment, and all experiments were repeated twice. Means were compared using Duncan's (1955) multiple range test.

All types of explants of *Leptadenia reticulata* developed callus on MS growth regulator free agar medium. Callus induction on MS basal medium has also been reported in *Melia azedarach* (Handro and Floh 2001), and *Holostemma ada-kodien* (Martin 2003). According Arnholdt-Schmitt *et al.* (1995), DNA amplification during the first few hours after inoculation precedes mitotic activity. This may relate to wounding and activation of the cell cycle and/or initiation of dedifferentiation.

Auxins (NAA, IAA, 2,4-D) or cytokinins (BA, Kn) either alone or in combinations were efficient in the induction of callus from all types of explants (Table 1). Amount of the callus varied depending on the type and concentration of the growth regulators (Table 1). Callus formation at the basal cut ends of the node and shoot-tip explants of *L. reticulata* on medium enriched with BA or Kn is in concomitant with the report on *Gymnema sylvestre* (Komalavalli and Rao 2000) and *Holostemma ada-kodien* (Martin 2002). As envisaged by Marks and Simpson (1994), callus formation at the basal cut end of node and shoot-tip explants may be due the accumulation of auxin at the basal cut end, which stimulates cell proliferation especially in the presence of cytokinins.

Explant, from which the callus developed, and the type of the growth regulator were significant in the induction of somatic embryos. Friable cream coloured callus developed at the basal cut end of node and shoot-tip explants on MS medium supplemented with BA alone or in combination with auxins (NAA, IAA, IBA) at low concentration only underwent somatic embryogenesis. Calli developed from the axillary bud region, and also the callus induced by the axillary buds (in certain cases) underwent somatic embryogenesis. Friable callus developed from leaf and internode explants on MS medium containing BA alone, and also in combination with low concentrations of auxins did not induce embryos even after second subculture (*i.e.*, 140 d after establishment).

Of the callus developed from node and shoot-tip explants on MS medium with different concentrations of

Table 1. Callus induction from different explants of *L. reticulata* on MS medium fortified with different growth regulators. Culture period 40 d. Mean of 40 replicates. Means followed different letters are significantly different at 5 % level. Explant mass at inoculation: leaf 260 ± 15 mg, internode 95 ± 15 mg, node 260 ± 10 mg, shoot-tip 110 ± 15 mg. Callus fresh mass includes the explant mass.

Regulators		Callus fresh mass [mg] node	shoot-tip	leaf	internode
Basal medium	0.00	1045^{i}	1057^{i}	1051^{i}	1120^{j}
BA	2.22	1305^{f}	1305^{f}	1305^{f}	1385^{g}
	4.44	1465^{d}	1465^{d}	1465^{d}	1505^{ef}
	6.66	1520^{cd}	1520^{cd}	1520^{cd}	1586^{d}
	8.87	1575^{bc}	1575^{bc}	1575^{bc}	1645^{c}
	11.10	1475^{d}	1475^{d}	1475^{d}	1495^{f}
	13.30	1055^{i}	1055^{i}	1055^{i}	1123^{j}
Kn	2.32	1179^{h}	1179^{h}	1179^{h}	1218^{i}
	4.65	1255^{g}	1255^{g}	1255^{g}	1294^{h}
	6.97	1372^{e}	1372^{e}	1372^{e}	1379^{g}
	9.29	1245^{g}	1245^{g}	1245^{g}	1210^{i}
	13.90	1615^{b}	1615^{b}	1615^{b}	1697^{b}
NAA	2.69	1485^{d}	1485^{d}	1485^{d}	1537^{e}
	5.37	1550^{c}	1550^{c}	1550^{c}	1634^{c}
	10.80	1470^{d}	1470^{d}	1470^{d}	1490^{f}
IBA	2.46	1580^{bc}	1580^{bc}	1580^{bc}	1640^{c}
	4.90	1615^{b}	1615^{b}	1615^{b}	1697^{b}
	9.80	1735^{a}	1735^{a}	1735^{a}	1815^{a}
2,4-D	2.26	1485^{d}	1485^{d}	1485^{d}	1537^{e}
	4.52	1550^{c}	1550^{c}	1550^{c}	1634^{c}
	9.05	1470^{d}	1470^{d}	1470^{d}	1490^{f}
IAA	2.85	1580^{bc}	1580^{bc}	1580^{bc}	1640^{c}
	5.71	1487^{d}	1487^{d}	1487^{d}	1535^{e}
	11.40	1522^{cd}	1522^{cd}	1522^{cd}	1589^{d}
BA + Kn	8.87 + 0.46	1615^{b}	1615^{b}	1615^{b}	1697^{b}
	8.87 + 1.39	1735^{a}	1735^{a}	1735^{a}	1815^{a}
BA + NAA	8.87 + 0.54	1485^{d}	1485^{d}	1485^{d}	1537^{e}
	8.87 + 1.61	1550^{c}	1550^{c}	1550^{c}	1634^{c}
	8.87 + 2.69	1470^{d}	1470^{d}	1470^{d}	1490^{f}
BA + IBA	8.87 + 0.49	1580^{bc}	1580^{bc}	1580^{bc}	1640^{c}
	8.87 + 1.48	1487^{d}	1487^{d}	1487^{d}	1535^{e}
	8.87 + 2.46	1522^{cd}	1522^{cd}	1522^{cd}	1589^{d}
	8.87 + 3.94	1615^{b}	1615^{b}	1615^{b}	1697^{b}
	8.87 + 0.45	1735^{a}	1735^{a}	1735^{a}	1815^{a}
	8.87 + 1.36	1485^{d}	1485^{d}	1485^{d}	1537^{e}
	8.87 + 2.26	1550^{c}	1550^{c}	1550^{c}	1634^{c}
BA + 2,4-D	8.87 + 0.57	1470^{d}	1470^{d}	1470^{d}	1490^{f}
	8.87 + 1.71	1580^{bc}	1580^{bc}	1580^{bc}	1640^{c}
	8.87 + 2.85	1487^{d}	1487^{d}	1487^{d}	1535^{e}

Table 2. Numbers of embryos during the first subculture of embryogenic callus to solid and suspension cultures of MS medium with or without growth regulators. Culture period 40 d. Data represents the mean of 40 replicates. Means followed by different letters are significantly different at 5 % level. Embryos of all stages were counted.

Growth regulators [μM]				Number of embryos [mg^{-1} (callus)]	
BA	IBA	IAA	NAA	suspension	solid
0				1.82^{j}	0.95^{h}
2.22				4.09^{h}	1.46^{fg}
4.44				6.05^{f}	2.19^{e}
6.66				7.87^{d}	2.88^{cd}
8.87				8.54^{c}	3.64^{b}
11.1				3.48^{hi}	1.27^{gh}
13.3				1.36^{j}	0.76^{h}
8.87	0.49			9.17^{b}	3.73^{b}
8.87	1.48			10.23^{a}	4.16^{a}
8.87	2.46			8.65^{c}	2.44^{de}
8.87		0.57		7.97^{d}	2.25^{e}
8.87		1.71		9.27^{b}	3.27^{bc}
8.87		2.85		6.68^{e}	1.94^{ef}
8.87			0.54	5.94^{f}	1.76^{f}
8.87			1.61	5.15^{g}	1.78^{f}
8.87			2.69	3.88^{hi}	1.36^{g}
2.22	0.49			5.32^{g}	2.14^{e}
2.22	2.46			6.75^{e}	2.58^{d}
4.44	0.49			5.86^{f}	2.37^{e}

BA (2.22 - 13.3 μM), when used singly, BA at 8.87 μM was superior for the induction of somatic embryos. Callus initiated on 8.87 μM BA containing medium started to induce embryos after 50 d of incubation. On this medium a mean of 9.2 embryos were developed within 60 d, and the embryos were at globular and heart shaped stages. Addition of auxins along with BA differently affected the frequency of somatic embryos. Among the different auxins, addition of 2.46 μM IBA with 8.87 μM BA induced the highest number (mean of 17.3) of somatic embryos. On IBA and BA containing medium, embryos were initiated between 35 and 40 d, and some of the embryos developed up to early cotyledonary embryos. Of the activity of auxins, IAA followed IBA. BA and IAA supplemented medium initiated a mean of 12.3 embryos. Addition of lower concentration of NAA along with BA did not exhibit significant difference in the potential of embryogenesis to that on medium having BA only. Addition of 2,4-D even at low concentration to medium with BA significantly influenced embryogenesis. On BA and 2,4-D supple-mented medium, no embryos were initiated in the initiation culture. On all cases, the embryos were developed from the periphery of the callus. Subculture of the callus enhanced the number of embryos (Table 2). Suspension culture was superior for the induction of somatic embryos to semisolid medium cultures (Table 2). Suspension culture containing 8.87 μM BA and 2.46 μM IBA induced the highest number of embryos (Table 2). Associated with proliferation of callus, cultures showed various embryonic stages. Development of somatic embryos was asynchronous. However, globular embryos were more in number compared to heart and torpedo. Some of the embryos underwent maturation and conversion into plantlets. The callus produced more than 10 somatic embryos per mg (Table 2). Suspension cultures of MS medium containing reduced concentration of BA (2.22 μM) either alone or in combination with 0.49 μM IBA encouraged maturation and conversion of embryos at higher rate, but was with less number of embryos (Table 2). MS medium with 2.22 μM BA facilitated conversion of 7 - 12 embryos among the embryos developed from 10 mg callus. Embryogenic potential of node and shoot-tip explant derived callus compared to the internode and leaf callus may be due to the passage of some internal components from the pre-existing buds that are vital to evoke embryogenesis. Efficacy of BA in the induction of somatic embryos has also been demonstrated in other members of *Asclepiadaceae* such as *Araujia sericifera* (Torné *et al*. 1997) and *Tylophora indica* (Manjula *et al*. 2000). Though, 2,4-D was believed to be the most trustworthy growth regulator for the induction of somatic embryogenesis (Kumar *et al*. 2002, Vikrant and

Table 3. Conversion of embryos into plantlets upon half strength MS agar medium fortified with different growth regulators/additives. Culture period 40 d. Mean of 100 embryos (20 cultures each with 5 embryos). Means followed by different letters are significantly different at 5 % level.

Growth regulators [μM]			Additives [%]		Conversion [%]
BA	GA_3	IBA	CW	CH	
0					19.2^{hi}
0.22					34.7^{f}
0.44					51.9^{d}
0.66					16.2^{i}
	0.29				47.7^{de}
	1.44				63.2^{c}
	2.89				43.4^{e}
	4.33				36.4^{f}
			05.0		30.9^{g}
			10.0		23.1^{h}
			15.0		15.9^{i}
				0.10	18.3^{hi}
				0.15	25.2^{g}
				0.20	30.5^{g}
				0.30	19.0^{hi}
0.44		0.49			52.9^{d}
0.44		0.98			47.2^{de}
0.44		1.48			35.8^{f}
0.44	1.44				87.4^{a}
0.44	2.89				70.2^{b}
0.22	1.44				86.1^{a}
0.22	2.89				68.9^{b}

Rashid 2003) in the present study, it was inhibitory for embryogenesis. Inhibitory effect of 2,4-D to somatic embryogenesis as in the present study has also been reported in *Eucalyptus globulus* (Nugent *et al*. 2001).

Cotyledonary embryos transferred on solid full or half strength growth regulator free MS medium facilitated low percent conversion of embryos into plantlets. Half-strength MS medium fortified with 1.44 μM GA_3 and BA (0.22 or 0.44 μM) favoured the highest percentage (87.4 %) of conversion of embryos into plantlets (Table 3). Influence of GA_3 in conversion of embryos as in the present study has also been reported in *Santalum album* (Rai and McComb 2002). As per the present protocol, out of the 102 embryos per 10 mg callus, 87.4 % (89 embryos) underwent conversion. Of which, 77 (86.5 %) plantlets were recovered from per 10 mg callus.

Plantlets transferred to small pots containing sterile sand and soil (1:1) revived growth within 15 d and grew well. 92 % of plantlets survived in the field. Plantlets grew well, exhibited morphological characters similar to source plants. Established protocol enables rapid large-scale propagation of this valuable medicinal plant.

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