Effect of plant growth regulators on *in vitro* morphogenesis of *Leptadenia reticulata* (Retz.) W.&A. from nodal explants

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Multiple shoot production was successfully achieved from axillary buds of *Leptadenia reticulata* using nodes as explants, and simultaneous organogenesis via callus produced from the base of the nodes. The Murashige and Skoog basal medium was supplemented with combinations of indole-3-butyric acid (IBA) and kinetin (Kn) for multiple shoot production. Maximum of six shoots were produced per node with the combination of IBA (1 mg/l) and Kn (10 mg/l) from the axillary bud. Callus produced from the base of the nodes in combination with indole-3-acetic acid and Kn was isolated for organogenesis. Organogenesis from callus was achieved with the combination of naphthaleneacetic acid (1.5 mg/l) and Kn (10 mg/l) as well as IBA (1 and 1.5 mg/l) with Kn (2 mg/l). Microshoots were transferred for rooting in IBA-containing medium, in which maximum 13 roots were produced in IBA (1 mg/l). Through sequential hardening process, well-rooted plantlets were established in the field.

Keywords: Callus formation, mass propagation, multiple shoots, organogenesis.

LEPTADENIA reticulata (Retz.) W.&A., commonly known as 'dodi', is an Indian medicinal plant used since 4500 BC. It belongs to the family Asclepiadaceae. The whole plant ameliorates 'tridoshas' (Vatta, Pitta and Kapha), and is of great value in general debility, involuntary seminal discharge, as a stimulant[1], abortifacient, tonic, restorative, bacteriocidal, antifabrifuge, prostatitis, wound healer and in mouth ulcer[2]. Roots are used in many ayurvedic/herbal formulations[3] in diseases of the ear and nose, skin infections and general debility[4]. It is also used for increasing milk-yielding capacity in cattle[5,6] and to increase the egg-laying capacity of hen in poultry industry. Flowers are good for eyesight. The plant contains leptadenol, *n*-triacontane, cetyl alcohol, β-sitosterol, β-amyrin acetate, lupanol 3-O-diglucoside, leptidin and 0.5% alkaloids. More than 23 pharmaceutical products are available in the market containing this plant as one of the ingredients. The plant has great demand in both the local as well as the international market, at Rs 211 per kg of dry powder. Its flowers and tender leaves are used as vegetable[7] and to make bread[8]. Flowers are costly and are sold at Rs 80 per kg. The plant naturally occurs on hedges. It is now becoming a threatened/nearly extinct species[9] due to over-exploitation of vital parts – flowers. Destruction of its natural habitat is one of the reasons for poor reproduction[10]. Earlier reports are available on micropropagation[11], somatic embryogenesis from leaf explants[12] and *in vitro* adventitious root formation from leaf explants for the production of secondary metabolites[13] of this plant. The present work, however, mainly focuses on mass propagation via a high number of multiple shoot production and formation of meristematic nodules from nodal explants/calluses, which increase the number of plantlets. Propagation and distribution of this species will improve biodiversity. It will be a good step to save a nearly extinct species.

Nodes of *L. reticulata* were collected from Anoopam Mission, Anand District, Gujarat, India. Isolated nodes, used as explant, were thoroughly washed with Tween-40 in running tap water for 15 min, and then treated with the mixture of three antimicrobial agents, namely 0.5% (v/v) Savlon, 0.1% (w/v) Bavistin and 0.1% (v/v) Roger solution for 10 min. These were washed thoroughly under running tap water and finally treated with 0.5% (v/v) sodium hypochloride for 15 min and rinsed with sterile distilled water. The entire process constitutes the presterilization of the explants. Surface sterilization of the explants was done with 0.1% (w/v) mercuric chloride treatment for 5 min and repeated washing with sterile distilled water. The explants were inoculated on Murashige and Skoog (MS) basal medium[14] containing 3% (w/v) sucrose, and 0.8% (w/v) agar-agar with different hormone combinations. For rooting of microshoots, 0.6% (w/v) agar was used in the medium. The pH of the medium was adjusted to 5.8 and autoclaved at 1.1 kg/cm^2 (121°C) for 15 min. The inoculated tubes were incubated at 25 ± 2°C under 16 h photoperiod provided by a fluorescent light of about 3000 lux intensity.

The nodes were cultured on MS medium fortified with auxin indole-3-butyric acid (IBA; 1, 1.5 and 2 mg/l) and kinetin (Kn; 2, 5 and 10 mg/l) for multiple shoot induction. MS medium in combination with either indole-3-acetic acid (IAA) or naphthaleneacetic acid (NAA) 1, 1.5, 2 mg/l and/or kinetin (2, 5 and 10 mg/l) was used for callus induction. The MS basal medium along with yeast extract at 5% w/v was used without hormones for multiple shoot production. Isolated callus was subcultured in MS medium with IBA/NAA (1, 1.5 and 2 mg/l) and Kn (2, 5 and 10 mg/l) for organogenesis. Microshoots from the callus were transferred into MS basal medium for elongation. Subculturing was done into fresh solid medium at every four weeks[15]. The growth parameters were measured after two months.

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Table 1. Multiple shoot formation from node of *Leptademia reticulata* with various combinations of IBA and Kn

PGR (mg/l) IBA	Kn	Yeast extract (g/l)	Explant response (%)	Mean no. of shoots/ explant ± SE	Mean shoot length ± SE (cm)	Mean no. of meristematic nodules from top ± SE	Frequency of meristematic nodules from top (%)
0	0	0	85	1	24.6 ± 0.63	0	0
0	0	50	92	2.0 ± 0.0	5.2 ± 0.22	0	0
1	2	0	72	3.4 ± 0.36	5.2 ± 0.30	10.2 ± 0.21	50
1	5	0	61	4.0 ± 0.23	4.7 ± 0.10	9.4 ± 0.45	37
1	10	0	66	6.8 ± 0.12	3.7 ± 0.30	4.3 ± 0.64	43
1.5	2	0	56	2.1 ± 0.17	5.0 ± 0.50	8.7 ± 0.52	40
1.5	5	0	66	2.0 ± 0.20	4.3 ± 0.80	8.2 ± 0.12	33
1.5	10	0	51	3.0 ± 0.33	3.8 ± 0.20	15.3 ± 0.62	30
2	2	0	62	6.0 ± 0.20	4.5 ± 0.22	7.4 ± 0.45	36
2	5	0	51	3.6 ± 0.19	3.0 ± 0.82	8.9 ± 0.63	34
2	10	0	44	3.0 ± 0.12	2.6 ± 0.60	6.4 ± 0.42	42

PGR, Plant growth regulator; SE, Standard error.

Microshoots more than 3 cm long were transferred for rooting in MS medium with 0.25–10 mg/l concentrations of IBA, NAA or IAA. Rooted microshoots were washed carefully to remove agar from the roots and transferred to the pasteurized mixture of soil, sand and cocopit (made up from coconut coir) in equal proportions filled in plastic cups. These plantlets were subjected to hardening before field-transfer.

Multiple shoot production was successfully achieved from axillary bud, and organogenesis was achieved from callus in *L. reticulata*. A portion of the nodes was selected as explants source. Britto *et al.*[16] were successful in producing multiple shoots from the same nodal explants in 6-benzylaminopurine (BAP) and NAA in *Ceropegia bulbosa* Roxb. var. *bulbosa*. In all experiments MS basal medium was used either with or without hormones. The hormones play an important key role in plant morphogenesis. The combination of hormones tried for multiple shoot induction from the node was IBA (1, 1.5 and 2 mg/l) and Kn (2, 5 and 10 mg/l), which showed varied responses (Table 1). IAA and NAA failed to produce multiple shoots with Kn, however, both were good for callus production. Initiation of shoots from axillary bud was observed in 10 to 12 days and there was a maximum of six shoots with the combination of IBA (1 mg/l) with Kn (10 mg/l) (Figure 1 *a*) and IBA (2.0 mg/l) with Kn (2.0 mg/l). Minimum shoot initiation was two in number with combination of IBA (1.5 mg/l) and Kn (2 and 5 mg/l). Three to four shoots were produced from the node with IAA and N^6-benzyladenine in *L. reticulata*[11]. However, the present study shows that IBA and Kn are more effective for multiple shoot formation, while IAA/NAA and Kn are more effective for callus formation in *L. reticulata*. Murashige[17] concluded that Kn is less effective than N^6-benzyladenine in the formation of multiple shoot buds, particularly in woody plants, but this is not so in the case in herbacious plants. In the present study, minimum shoot length (2.6 cm) was observed with IBA (2 mg/l) and Kn (10 mg/l). This may be because of the high level of Kn. Hu and Wang[18] reported that elongation of micropropagated shoots is often inhibited by higher cytokinin level. Maximum shoot length (24.6 cm) was observed in MS basal medium, with only one shoot in two months. Shoot initiation from axillary buds in MS basal medium indicates a good amount of endogenous hormones in *L. reticulata*. Shoot initiation period was less in MS basal medium (8 days) and shoot elongated up to 24.6 cm in two months in comparison to hormone combinations. The advantage of hormone combinations has been the achievement of multiple shoots from a single node. Yeast extract at 5% w/v without hormones in nutrient medium reduces the time period of new shoot initiation up to 3–4 days as well as new shoot proliferation from axillary buds. Yeast extract has also increased the percentage of explant response to 92, the highest in all combinations. The number of shoots initiated in yeast extract without any hormone is less, only two, but this proved the best supplement for shoot initiation and explant response.

Along with multiple shoots from the node, meristematic nodules were also initiated from the top cut end of the node. The number of such meristematic nodules per explant and their frequency in culture are shown in Table 1. Maximum of 15 meristematic nodules were formed with IBA (1.5 mg/l) and Kn (10 mg/l) combination (Figure 1 *b*). Meristematic nodules formation is useful for the multiplication of this species.

Various combinations of IAA/NAA (1, 1.5 and 2 mg/l) with Kn (2, 5 and 10 mg/l) were tried to obtain the callus from the base of the nodes. Combination of either 2 mg/l IAA or NAA with 5 mg/l Kn resulted in the best callus formation. The amount of callus was more with IAA in comparison to NAA. Callus grown in combination with NAA (1.5 mg/l) and Kn (10 mg/l) appeared nodular and formed meristematic centres (Figure 1 *c*). However, shoot and root initiation was observed from the callus with the combination of IBA (1 and 1.5 mg/l) and Kn (2 mg/l; Figure 1 *d*). These shoots elongated further upon subculturing on basal medium containing yeast extract.

Table 2. Root formation from microshoots of *L. reticulata* with various concentrations of IBA and NAA after two months of culture

PGR (mg/l) IBA	Microshoots response from root (%)	Mean no. of roots/micro-shoot ± SE	Mean root length (cm) ± SE	PGR (mg/l) NAA	Microshoots response for root (%)	Mean no. of roots/ explant ± SE	Mean root length (cm) ± SE
0	35	2.5 ± 0.52	3.3 ± 0.70	0	35	2.5 ± 0.52	3.3 ± 0.70
0.25	45	8.0 ± 0.21	5.0 ± 0.52	0.25	80	2.5 ± 0.54	1.9 ± 0.23
0.5	32	1.0 ± 0.35	0.5 ± 0.10	0.5	50	4.0 ± 0.12	3.3 ± 0.64
1	85	13 ± 0.12	3.1 ± 0.31	1	33	7.0 ± 0.45	2.7 ± 0.46
1.5	40	2.0 ± 0.32	4.1 ± 0.42	1.5	30	4.0 ± 0.53	3.3 ± 0.34
2	33	1.0 ± 0.10	2.5 ± 0.10	2	42	3.1 ± 0.62	2.0 ± 0.13
2.5	66	4.0 ± 0.15	1.8 ± 0.63	2.5	64	3.4 ± 0.0	1.0 ± 0.32
5	50	7.2 ± 0.22	2.4 ± 0.52	5	82	5.7 ± 0.12	2.6 ± 0.12
10	30	2.2 ± 0.10	1.3 ± 0.42	10	36	2.6 ± 0.22	0.8 ± 0.14

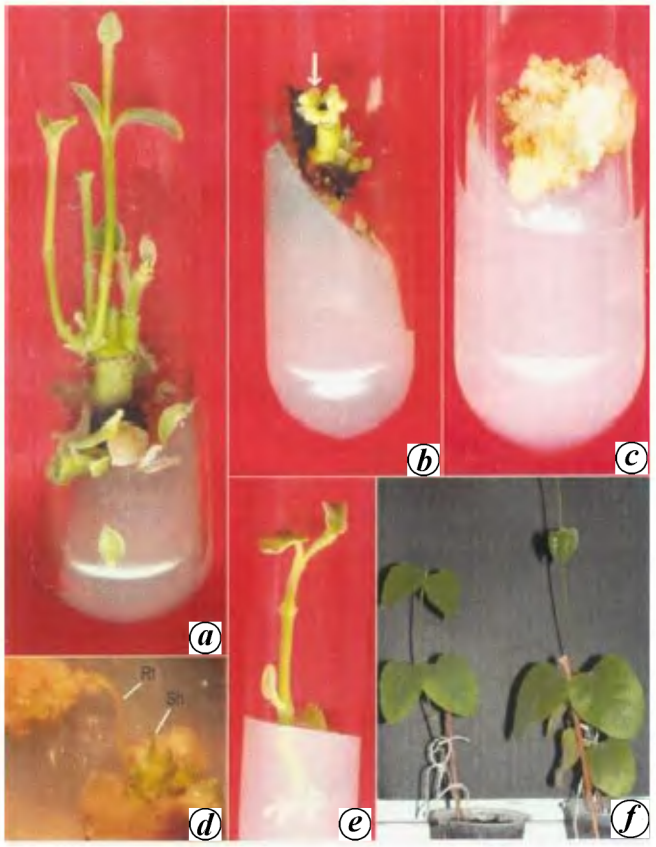

Figure 1. ***a***, Multiple shoots from axillary buds of node in the presence of IBA (1 mg/l) with Kn (10 mg/l), showing six buds per node. ***b***, Meristematic nodules from the cut top end of the node in the presence of IBA (1.5 mg/l) with Kn (10 mg/l) (arrow). ***c***, Nodular callus development in NAA (1.5 mg/l) with Kn (10 mg/l). ***d***, Initiation of shoot and root from callus in the presence of IBA (1.5 mg/l) with Kn (2 mg/l; Sh, Shoot; Rt, Root). ***e***, Root initiation from microshoots in the presence of IBA (1 mg/l). ***f***, Plants ready for field transfer after the stepwise hardening process.

Martin[12] had reported somatic embryogenesis with IBA and N^6-benzyladenine from callus produced from the shoot-tip and node. He had established plantlets on half-strength MS solid medium with gibberellic acid (GA_3) and N^6-benzyladenine. Siddique *et al.*[19] reported organogenic callus induction with NAA and Kn in *Hemidesmus indicus*. In the present investigation, organogenesis from callus was observed with NAA/IBA and Kn, which supports the contribution of NAA/IBA and Kn for organogenesis in the family Asclepiadaceae.

In vitro rooting was successfully achieved from microshoots with various concentrations of IAA, IBA and NAA (0.25–10 mg/l) as shown in Table 2. IAA failed to produce roots from microshoots (not included in Table 2), while IBA/NAA produced enough thick and branched roots. This supports the findings of Bhatt *et al.*[13], who established root culture with IBA and NAA. Initiation of roots was observed after 18–25 days. Maximum thirteen roots were observed in 1mg/l IBA (Figure 1 *e*), but maximum root length (5 cm) was achieved with 0.25 mg/l IBA in two months. The roots observed with 0.25 mg/l IBA were thinner than those observed with 1 mg/l IBA. Successful rooting was also achieved by Roy *et al.*[20] in IBA from microshoots produced from callus in *Calotropis gigentea*. In *L. reticulata*, IBA and NAA play a key role in root formation. The newly rooted microshoots were transferred to MS basal medium for fast growth and proper development of the root system. The same technique was also applied for *Rauwolfia tetraphylla*[21].

Microshoots with a proper root system were ready for field transfer. Microshoots were transferred to potting mixture filled in plastic cups (Figure 1 *f*) and then to field condition through stepwise hardening process. Initially the plantlets were covered with polythene bags to maintain high relative humidity. They were kept in a separate room under observation with ambient temperature and normal daylight photoperiod. The bags were removed periodically for gradual hardening. After 2 weeks when new leaves emerged, they were taken outside the room and kept in a shady place under normal temperature and light. After 8 weeks, when the plantlets had achieved a height of 15–20 cm, they were transplanted in the soil.

To save time and labour, direct rooting with shoot initiation from the nodes was attempted. Nodes were inoculated in auxins IBA, IAA or NAA at various concentrations (0.25, 1, 2, 5 and 10 mg/l). IAA failed to produce a root

system, while IBA and NAA produced shoots as well as roots. IBA/NAA initiated an average of two shoots per node in all concentrations used and simultaneously a root system containing five to eight roots at the base. The plantlets were ready for soil transfer within 41 days of culture and thus reduced the time period. Therefore, only IBA/NAA can be used for rooting of shoots of *L. reticulata*.

The timescale for initiation of organogenesis to the final hardening process from the callus took nearly 8 months, while the same process from the node took 3–4 months. Therefore, mass propagation from the multiple shoot production from the node was preferable instead of the organogenic pathway. Plantlets were successfully established in the soil by one month and ready for the field transfer in 3–4 months. Plantlets were transferred to the field during July to September. This period gives less mortality and high survival rate of plantlets during the hardening stage because of high moisture content of the air, which prevents quick desiccation of the plantlets.

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Direct determination of aquifer configuration using geoelectrical techniques in a piedmont zone, Himalayan foothills region, India

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Geoelectrical techniques have been used to define the geometry of aquifer systems in the Pathri-Rao watershed situated in the piedmont zone of the Himalayan foothill region, Uttarakhand, India. This has been done by integrating the results of dc resistivity and electromagnetic data recorded in the area. Two-dimensional resistivity imaging was carried out to define the horizontal and vertical geometry of the aquifer system and to infer the local groundwater flow condition. On the basis of resistivity values it was found that shallow and deep aquifers have different degrees of interaction in the area. The study demonstrates the versatility of geoelectrical techniques.

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