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REGULAR ARTICLE

COST EFFECTIVE APPROACH FOR *IN VITRO* PROPAGATION OF (*LEPTADENIA RETICULATA* WIGHT & ARN.) - A THREATENED PLANT OF MEDICINAL IMPORTANCE

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SUMMARY

The present study deals with the development of an efficient and simple protocol for high frequency *in vitro* regeneration of *Leptadenia reticulata*, a threatened medicinal plant. A range of cytokinins with different concentration and affect of various media have been investigated for multiple shoot induction using nodes, internodes, meristem as explants. The best response for multiplication was obtained in MS media supplemented with 0.25mg/l BA and 0.25mg/l Kn. B5 media efficiently proliferated callus. Among the all the explants tested only those of old axillary nodes showed positive morphogenetic response and readily developed healthy multiple shoots, where as the other explants such as young axillary nodes, apical meristem and internodes did not respond satisfactorily. Maximum numbers of roots were observed when the *in vitro* grown shoots were maintained on full strength MS media containing 2mg/l IBA followed by 200 mg/l activated charcoal. The cost of the media was reduced by using tap water and table sugar in the media. About 93% of the plants were successfully acclimatized in the field.

Key words: Multiple shoot induction, Axillary nodes, *In vitro* regeneration, Asclepiadaceae

Abbreviations: BA: 6-benzyladenine, IAA: Indole-3-aceticacid, IBA: Indole-3-butyric acid, Kn: Kinetin, NAA: α-naphthalene acetic acid, MS: Murashige&Skoog's medium, SE.: Standard error

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1. Introduction

Leptadenia reticulata (Retz) wight & Arn. belonging to family Asclepiadaceae is an important medicinal plant with synonyms in Indian language as Jivanti, Dori, Swarn Jivanti etc. This plant species is distributed in tropical and subtropical parts of Asia, Africa, Burma, Srilanka, Philippines and Madagascar. In India it is found in Gujarat, Punjab, Himalayan ranges, konkon, Nilgiris and Southern part of India. The true center of origin of this plant is not known, but the oldest description in Athrava Veda suggests of Indian origin. Use of this plant for medicinal purpose dates back to abut 4500 to 1600 BC as mentioned in Atharva Veda. The Atharva Veda mentioned its use as a life and strength giver, propagator of milk and its use in many other ailments while Charka (one of the Samhitas) described it as an important rasayan drug, capable of maintaing youthful vigor and strength. The various reports on its multiple uses in curing several diseases such as hematopoesis, emaciation, cough, dyspnoea, fever, burning sensation, night blindness draws the attention for utilization of this plant for drugs. It is regarded as good cure for tuberculosis and effectively used for several ear and nose problems. 50% methanolic extract of this plant is having antibacterial activity and is used for the treatment of skin infection and wounds [1]. Anjaria in 1967 [2] tried Leptaden tablets (a formulation from *Leptadenia reticulata*) on some clinical cases and reported its beneficial use as galactogogue for increasing milk

yielding capacity in cattle and egg lying capacity in poultry.

This plant is also a rich source of biologically active cardiac and pregnane glycosides which are known to possess anti tumor and anti cancer activity. The chemical analysis of alcoholic extract of this plant revealed the presence of leptadenol, N-tricontane, cetyl alcohol, β- cytosterol, β-amyrin acetate, lupanol-3-o-diglucoside and alkaloids.

The natural strand of this species is first disappearing due to its restricted distribution and indiscriminate exploitation for medicinal use by pharmaceutical industry. As a result, it is now listed as an endangered species by the international union for conservation of nature and natural resources. Commercial exploitation for production and conventional propagation is hampered due to its poor seed viability, low rate of germination and seasonal availability. To date, there are only few reports on the micro propagation of *Leptadenia reticulata* [3], [4], [5]. The increasing demand for this plant material and loss of habitat will put this medicinal species under more pressure which may endanger human health. This in turn may lead to loss of consumer confidence in herbal medicines. Therefore, the need of development of rapid multiplication of this important herb has become imperative in order to reduce the existing pressure on natural population and supply of constant plant material in need.

In view of the overwhelming interest in this plant, it becomes very necessary to device a low cost approach for mass propagation by employing tissue culture techniques. The present paper describes an efficient, cost effective approach for micropropagation of *Leptadenia reticulata* using suitable explants, tap water and table sugar.

2. Materials and Methods

Healthy and young shoot cuttings of *Leptadenia reticulata* bearing 6 to 8 nodes were collected from mature plants growing in experimental garden of Rishi Herbal technology, Bangalore, Karnataka. After removing the leaves, the nodal segments (1-1.5cm) were swabbed with 80% (v/v) ethanol and were thoroughly washed under running tap water (20 min) followed by treatment with bavistin (45 mins). Under aseptic condition, the explants were surface sterilized with 0.1% of mercuric chloride for 6 minutes and finally washed six times with sterile water. The surface sterilized explant were cultured on various media such as M.S [6], L.S. and B5 [7] supplemented with BAP (0.25mg/l). Then MS media fortified with various concentrations (mg/ml) of cytokinins alone or in combination (BAP and Kn: 0.10mg/l - 2.0mg/l) were investigated to optimize the hormonal requirement for bud sprouting and multiple shoot induction. The morphogenetic response of different explant types (axillary nodes, shoot tips, leaf, inter nodal segments) were also investigated for efficient and reproducible multiplication. The pH of the media were adjusted to 5.8 with 1N NaOH or HCl and supplemented with 0.8% (w/v) agar, 2% (w/v) sucrose before molten media were dispensed in to culture tubes. The media were autoclaved at 121^0C at 15 psi ($1.04 kgcm^2$) pressure for 20 min. Explants were placed vertically in culture tubes containing 20 ml of culture media and capped tightly.

Cultures were maintained at 25 ± 2^0C under 16 hour photo period provided by cool white florescent tubes. *In vitro* derived shoots from the explants were excised after 4 weeks and sub cultured on to a fresh medium with the same concentrations of growth regulators. For rooting, 6 -8 cm long regenerated shoots were excised and cultured on half strength or full strength MS medium supplemented with different concentrations of activated charcoal (100 and 200 mg/l), IAA and IBA (0.5, 0.1 and 2.0 mg/l) for rooting. Plantlets with well developed root and shoot system were removed and transferred to plastic cups containing sterilized sand and soil (1:2) and acclimatized in the green house.

The cost of the medium per liter was worked out with modifications in its components. The conventional carbon source i.e., sucrose was replaced by table sugar and distilled water was replaced by tap autoclaved tap water. The prevailing costs of

carbon sources (sucrose and market sugar), water, activated charcoal and other components of medium (MS salts, vitamins, growth regulators and ascorbic acid) at the time of conducting the experiment were taken to calculate cost of each medium. The cost was expressed in Indian rupees.

Statistical analysis

The effect of various treatment on multiple shoot induction was compared to detect the significance of differences between the treatment mean using nested design (ANOVA) at 5% probability level according to Gomez and Gomez (1984) [8]. The experiment had three replicates; each replicate consisted of 20 culture tubes. The number of shoot buds was recorded after 4 weeks. Result of experiments was expressed in term of mean± standard error.

3. Result and Discussion

Optimization of micropropagation protocol depends upon various factors viz., media, explants, growth factors and cultural conditions. Hence in the present various stages of micropropagation were standardized by screening different factors.

Maintenance and selection of mother plants

Healthy cuttings with 3 nodes were procured from plants growing at Rishi Herbal garden and planted in polybags with 1:1:1 ratio of sand, soil and manure mixture. Only 20% of rooting was observed. These rooted cuttings were transferred to field and maintained as mother plants. Some plants were also maintained under greenhouse conditions.

Influence of the explants

The plant which was maintained in the green house provided a good source of explant throughout the year as collection of explant from wild variety is season dependent. Also the explant, obtained from the green house found to be easy to surface sterilize than field grown plants in term of the major problem of contamination. Hard and strong shoot segments proved to be better explant in terms of providing response to bud break and axillary branching. The apical shoots showed necrosis while the leaf explants did not respond at all.

Influence of Medium

Axillary buds remained green and fresh but failed to sprout on any media without cytokinins. Among the different media tested, MS basal medium was found be the best for shoots sprouting and multiplication. Though the shoot buds sprouted on B5 medium showed only limited development even if they were maintained for longer period and resulted in callus formation. *In vitro* propagation of plants belonging to the family *Asclepiadaceae* has also been shown to have optimum growth in MS medium [9], [10], [11]. Thus the degree of growth and differentiation varied considerably with the constitution of the medium [12], [13]. The need of MS salts for shoots sprouting and proliferation indicated the high salts requirements for growth of *Leptadenia reticulata*.

Influence of plant growth regulators

Shoot initiation occurred from axillary buds within 10 days of culture (Fig. 1A and B). The combination treatment (0.25 mg/l BA along with 0.25 mg/l Kn) was found to exhibit highest frequency of shoot multiplication (90%) (Fig.1C). The highest mean number of shoot (5.70 ± 0.23) and mean shoot length (5.33 ± 0.65 cm) was also evidenced in the same treatment (Table 1).

Figure 1: Various stages of *in vitro* propagation of *Leptadenia reticulata*: (A, B) - Initiation of axillary shoot from nodal explants on MS medium supplemented with 0.25mg/l BA. (C) - Multiple shoots from nodal explant on MS medium supplemented with 0.25mg/l BA and 0.25mg/l Kn. (D - Formation of callus at the base with increase in the concentration of BA and Kn beyond 0.25mg/l. (E) - Induction of roots from shoots on MS medium supplemented with 2mg/l IBA. (F)- *In vitro* raised plantlets grown normally in the soil

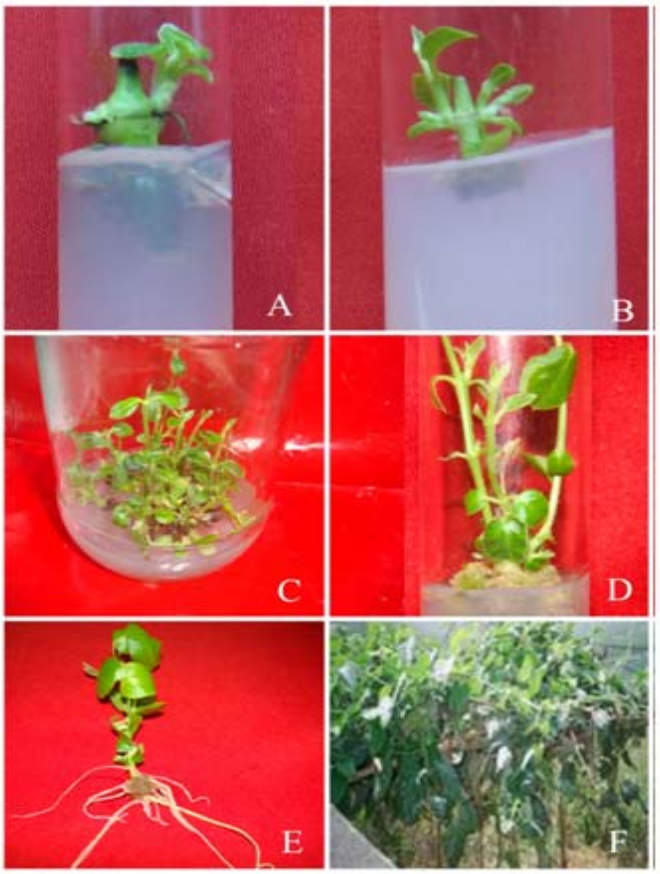
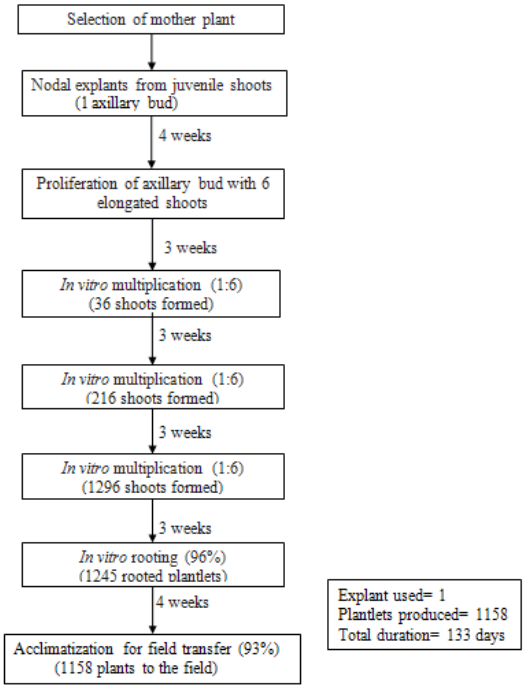


Figure 2. Flow-diagram illustrating entire protocol for multiplication of *Leptadenia reticulata* by axillary branching technique



The percentage of shoot induction decreased and formation of callus increased with increase in the concentration of BA (Fig. 1D). Though the nodal segments on MS medium with BA or Kn alone responded moderately, the shoot number, shoot length and percentage of shoot induction was very less when compared to BA & Kn in combination. Superior effect of the combination of BA and KN may be due to the synergy of cytokinins as reported in *Pogostemon cablin*, *Rollinia mucosa* and *Solanum surrattense* [14], [15], [16].

Table 1. Influence of different concentration of growth regulators added to MS medium on multiple shoot induction from axillary meristem of *Leptadenia reticulate.*

Growth Regulators (Mg l^{-1})	Mean No. of shoot per explant (±SE)	Mean Shoot length(±SE)	Mean No. of Shoot with basal callus(±SE)	Mean No. of Callus (±SE)
BA				
0.10	1.98±0.12	2.34±0.28	0.46±0.12	0.00±0.14
0.25	4.67±0.57	4.68±0.70	1.67±0.58	0.00±0.55
0.5	3.33±0.35	4.67±0.60	9.00±0.91	2.33±0.76
1.0	2.67±0.24	5.00±0.07	10.00±1.43	4.00±0.78
1.5	2.00±0.12	4.62±0.31	8.00±0.32	10.00±0.82
2.0	1.67±0.44	4.60±0.31	4.00±0.88	15.00±3.50
Kinetin				
0.10	2.96±0.26	3.21±0.23	3.44±0.28	0.54±0.12
0.25	3.67±0.57	3.67±0.31	4.33±1.18	0.78±0.32
0.5	2.33±0.44	2.33±0.27	5.00±0.37	0.67±0.46
1.0	1.33±0.35	2.00±0.07	7.00±0.32	0.67±0.50
1.5	1.00±0.12	1.67±0.31	9.33±0.91	8.00±0.26
2.0	1.33±0.24	1.33±0.37	8.00±0.37	160.71
BAP+ Kinetin				
0.5+0.5	3.00±0.53	2.00±0.51	8.00±0.34	11.00±0.38
0.25+0.25	5.70±0.23	5.33±0.65	1.52±0.24	0.00±0.19
0.25+0.1	3.80±0.18	2.67±0.69	2.00±0.3	0.00±0.19
0.1+0.1	3.20±0.11	2.83±0.53	1.32±0.12	0.00±0.19
0.1+0.25	2.40±0.08	2.17±0.20	1.84±0.21	0.00±0.19
0.5+0.5	1.40±0.17	1.33±0.36	10.00±0.62	10.00±0.96
0.1+0.05	2.00±0.34	1.67±0.32	1.24±0.22	0.00±0.19
0.2+0.05	2.13±0.11	2.67±0.71	1.52±0.28	0.00±0.19
0.2+0.1	1.70±0.21	2.170.20	2.12±0.34	0.0±0.19

Computed F value 59.38

$P<0.05$

Each mean is based on three replicates, each of which consist of 20 individual culture tubes

Data are recorded after 4 weeks of culture initiation.

Rooting of *in vitro* grown shoots and establishment of plantlets

For induction of roots, *in vitro* grown shoots of size (6- 8cm) were transferred to half and full strength MS medium along with different concentration of IAA, IBA and activated charcoal. The best rooting was evidenced with the use of full strength MS medium compared to half strength MS medium. Of the different concentration hormone tested, best response was obtained with 2.0 mg/l IBA (Fig. 1E). Where as IAA failed to produce satisfactory result (Table 2). Though the roots were observed in case of 0.25mg/l IBA but are short and weak as compare to the roots developed in case of 2.0mg/l IBA. This supports the finding of Hariharan et al [4], who established the root culture with IBA. Effect of IBA in root induction was also reported in many plants like *Tridex procumbens* [17], *Calotropis gigentea* [18] etc. The use of activated charcoal for rooting produced the better results (Table 2), so the same can be used further as the substitute for hormone and also to minimize the cost of media. Similar results are also been reported in *Pogostemon cablin* [14].

Table 2: Influence of MS salt strength, different concentration of auxins and activated charcoal on In vitro root induction of *L.reticulata* after four weeks of culture.

Medium (strength) + Auxin (mg/l)	Root induction (%)	Mean number of roots/explant (cm) ± SE	Mean root length (cm) ±SE
Half strength			
control	12	1.6±0.24	1.20±0.69
IAA			
0.5	28	1.2±0.8	2.18±0.32
1.0	42	2.3±0.5	2.16±0.52
2.0	34	1.9±0.8	2.21±0.50
IBA			
0.5	46	2.3±0.8	2.62±0.68
1.0	52	2.6±1.3	2.28±0.27
2.00	65	3.2±0.5	2.56±0.29
Activated charcoal			
100	52	2.6±0.24	2.20±0.23
200	68	3.00±0.28	2.66±0.56
Full strength			
Control	24	2.2±0.78	1.82±0.24
IAA			
0.5	36	1.3±0.20	2.20±0.33
1.0	50	2.4±0.26	2.28±0.68
2.0	28	2.2±0.88	2.23±.42
IBA			
0.5	58	3.8±0.22	3.22±0.62
1.0	68	5.2±0.89	3.36±0.52
2.00	87	7.6±0.56	5.12±0.46
Activated charcoal			
100	72	5.4±0.66	3.89±0.28
200	85	7.4±1.2	5.09±0.56

Computed F value 24.52

$P<0.05$

Each mean is based on three replicates, each of which consist of 20 individual culture tubes Data are recorded after 4 weeks of culture initiation.

Plantlets with well-developed shoot and root system were transferred to cups containing mixture of soil and sand (2:1) and then sequential hardening process was carried in the field. High relative humidity was maintained by covering the plants with transparent polythene bags under controlled condition. After 4 weeks, the plants were transferred to the soil and 93% survival was recorded (Fig. 1F).

Cost analysis of the medium

The composition of the culture media used for shoot proliferation and rooting has tremendous influence on production costs. Sucrose, agar and distilled water add significantly to the media cost. Many laboratories have reported the use of table sugar in plant propagation medium. In the present study, the use of table sugar and tap water instead of sucrose and distilled water did not show any significant difference in shoot multiplication rate (Table 3). Zapata [19] has successfully reduced the cost of banana tissue culture by 90% by replacing the tissue culture sucrose grade with a commercial sugar. Distilled water produced through electrical distillation is expensive. In some cases, alternative water sources can be

used to lower the cost of the medium [20]Prakas. If tap water is free from heavy metals and contaminants, it can be substituted for distilled water. Tap water has been used for *in vitro* propagation of banana [21] and ginger, *Zingiber officinale* [22]. The low cost medium identified in the present study holds great promise for rapid multiplication of *Leptadenia reticulata*. This is the first report of its kind in *Letadenia reticulata*.

4. Conclusion

In conclusion, the induction of multiple shoots through axillary branching is now recognized as a useful technique for propagation and *in vitro* conservation of threatened plants. Our present investigation offers a potential and cost effective production system for conservation and mass propagation of *Leptadenia reticulata* from nodal explants. Using this protocol it is possible to produce 1158 plants from a single explant within 133 days (Fig 2).

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