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A Protocol for High Frequency Regeneration Through Nodal Explant Cultures and *ex vitro* Rooting of *Plumbago rosea* L.

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Abstract: A rapid clonal multiplication scheme comprising direct multiple shoot initiation and downsizing of the node with buds proliferated upon during subculture was developed for *Plumbago rosea*. Sixty five per cent of the nodes (~2.0 cm) dissected out of young shoots from field grown plants and cultured in MS agar medium containing 3% sucrose and 15.4 μM BAP remained contamination free and responded at 95% rate with callusing at basal cut end and axillary bud break in 5 days followed by the formation of 2.41±0.14 shoots of 0.87±0.14 cm length in 3 weeks. Though differences in frequency and number of buds formed between nodes of 1-5 positions from the young shoots was negligible, the shoots emanated from the youngest node were shorter (0.92±0.19 cm) than those (2.3±0.50 cm) of the mature 5th node. Synergistic influence of BAP and auxins on caulogenesis was absent. Bud emergence in shorter (~0.5 cm) nodes was delayed up to 3 weeks and extensive callus proliferation from the cut basal end overlapped the 8.2±0.37 axillary shoots/buds formed after 7 weeks. Reduction in the size (downsized) of the 2.0 cm node with buds to 1.0 cm by dissecting out the basal internodal segment having the callus and subculture of them (~1.0 cm) with buds in contact with the medium for 3 weeks contributed to maximum multiplication of 42.1±5.40 shoot buds. Division of the shoot cluster and transfer of 2-3 shoots (0.5-1.5 cm) in a clump to MS basal liquid medium induced elongation of the shoots to 4.1±0.18 cm in 2 weeks. Shoots of 3.0-4.2 cm length were rooted within 3 weeks at 100% efficiency *in vitro* or *ex vitro* without hardening. *In vitro* rhizogenesis in presence of 0.49 μM IBA is recommended for enhanced rooting and high yield of commercially important tuberous roots during cultivation in the field.

Key words: Multiple shoot, downsized node, micropropagation

INTRODUCTION

Plumbago rosea L (Plumbaginaceae) is one of the few medicinal plant species cultivated in parts of Southern India. The tuberous roots of the plant are used as an important indigenous drug for treating a variety of symptoms including oedema, piles, intestinal worms, skin disease, common wart, rheumatism, secondary syphilis and leprae (Anonymous, 1969; Mooss, 1976; Pillai *et al*., 1981; Agarwal and Gosh, 1985). Moreover, root extract is traditionally used as abortive (Kirtikar and Basu, 1975). The medicinal properties of the roots are attributed to the presence of plumbagin, 5-hydroxy 2-methyl 1, 4-naphthoquinone (Modi, 1961). Plumbagin has many pharmacological properties of which anticancerous and antimicrobial activities are the most important (Nadkarni, 1976; Dinda *et al*., 1997; De Paiva *et al*., 2003).

Among the three species of *Plumbago* (*P. zeylanica, P. rosea* and *P. capensis*) *P. rosea* is preferred for cultivation in homesteads and in farms. Seed setting is seldom in *P. rosea* and the plant is propagated using stem cuttings and root suckers. This kind of clonal propagation is slow and the plants so raised take 18-24 months under field cultivation to produce root tubers. An intervention through micropropagation will be a desirable option for the rapid production and supply of planting materials, with the additional advantage of early harvesting of roots (Satheeshkumar and Seeni, 2003). Published reports (Kumar and Bhavanandan, 1988; Harikrishnan and Hariharan, 1996; Chetia and Handique, 2000; Das and Rout, 2002) of successful *in vitro* regeneration of *P. rosea* have suggested the need for the development of a package for production of quality planting material on sustainable basis to support the cultivation. In the present study we describe the conditions for high frequency regeneration of *P. rosea* suitably supplemented with *ex vitro* rooting and field establishment.

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MATERIALS AND METHODS

Three to four month old plants of *Plumbago rosea* from the medicinal garden of the institute served as the source of explants. Young shoots having 5-6 nodes were collected, defoliated and washed in 5% (v/v) Teepol (Glaxo India Pvt. Ltd. Mumbai) and then in running tap water for 5 min. Stem cuttings with single node were cut from the top to the bottom positions (1 to 5) and were surface sterilized by immersion in 0.1% (w/v) $HgCl_2$ for 4-7 min followed by 2-3 washes in sterile distilled water. After trimming from the cut ends, nodal explants of different lengths and positions were vertically implanted into the medium consisted of full strength inorganic and organic salts of Murashige Skoog (MS) (Murashige and Skoog, 1962) solid medium supplemented with different plant growth regulators (PGRs). For the purpose of comparison, both solid and liquid MS media were used with nodal segments of 2nd and 3rd positions. In liquid media, the explants were supported by filter paper wraps. In order to study the influence of explant size on shoot morphogenesis, two types of explants viz. node with short internodal portion (~0.5 cm) and the other with long internodal portion on either sides (2.0-3.0 cm) were prepared and inoculated onto solid medium in such a way that the nodal portion is either in immediate contact or not with the medium. In the longer explants, the nodal portion was invariably 2.0-3.0 mm above the surface of the medium while the subtending internodal base was immersed in the medium. For all the treatments, MS medium supplemented with 3% sucrose and different PGRs at varied concentrations and combinations of BAP (6-benzylaminopurine) 2.2 -22.0 μM, Kn (Kinetin) 2.35-23.5 μM, 2ip (2- isopentyladenine) 2.49-24.5 μM or permutative combinations of cytokinins and 0.57-5.7 μM IAA (indole -3-acetic acid) and pH of the medium was adjusted to 5.8 before adding agar 0.6% (w/v) (Thomas Baker, Mumbai). After dissolving the agar by boiling, the medium was dispensed in 15 mL aliquots into 20x150 mm culture tubes and autoclaved at 121°C and 1.5 kg cm^2 for 18 min. All the cultures were maintained under 12 h white fluorescent lights (30-35 μ Em^2 s^{-1}) at 26±2°C. For each treatment twenty replicates were used and repeated all the experiments at least twice.

For shoot multiplication, explant with buds, isolated buds and reducing the size of the node (downsized) by removing the internodal portion from the bottom to 1.0 cm with buds were used. In all the cases MS solid medium supplemented with 3% sucrose and different concentrations of BAP alone (2.2-22.0 μM) and combinations of BAP (2.2-22.0 μM) plus 0.54-8.1 μM α-naphthaleneacetic acid (NAA) or 2.2-22.0 μ*M* BAP plus 0.57-8.55 μM IAA were used.

After 3 weeks of subculture, clusters of shoots multiplied in media supplemented with BAP were divided and clumps of 2-3 shoots of 0.5-1.5 cm length were transferred to basal solid and liquid MS and the media supplemented with reduced level of BAP (2.2-6.6 μM) for shoot elongation.

After 2 weeks of shoot elongation, 3.0-4.2 cm long shoots were transferred to MS agar medium containing 0.49-2.46 μM indole-3- butyric acid (IBA) or combinations of IBA (0.49-2.46 μM) plus NAA (0.54-2.7μM) or IAA (0.57-2.85 μM) for rooting in 3 weeks.

Plantlets of 4-7 cm length each with 9-12 roots were deflasked, washed in running tap water and transferred to polythene bags (10×15 cm) filled with potting media consisting of sand and dried farmyard manure (3:1). No hardening procedure was followed. The plants were kept under 25% shade net house and irrigated twice a day.

For *ex vitro* rooting, shoots of 3.0-4.2 cm length were deflasked, washed thoroughly in running water and planted directly in poly bags (10×15 cm) filled with fine sand, kept under 25% shade and irrigated twice a day.

Data was subjected to analysis of variance (ANOVA) using a Completely Random Design (CRB) and means were compared by Duncan's multiple range LSD test at 5% significance level.

RESULTS AND DISCUSSION

This study was undertaken to develop a rapid micropropagation scheme for the production of planting material so as to support cultivation of the otherwise conventionally propagated *P. rosea* and possible early harvest of the plumbagin-rich tuberous roots already demonstrated in cultivated micropropagated plants (Satheeshkumar and Seeni, 2003).

Approximately 65% of the young nodal explants remained contamination free during culture initiation and efforts to reduce the percentage of contamination without affecting the viability and morphogenetic response of the explants by changing the surface sterilization procedures did not yield encouraging results. The contamination was exclusively fungal which might be due to endophytic associations (Chetia and Handique, 2000).

Shoot bud formation from nodal explants was delayed up to 3 weeks in basal medium independent of explant size and position. In media supplemented with cytokinins, 95% of the long explants (2.0-3.0 cm) readily responded with bud initiation in 5 days, followed by callusing at cut ends, while the shorter ones (~0.5 cm) responded rapidly with callus formation at the basal cut end in one week followed by bud emergence in 3 weeks. Perusal of the literature on *Cereis yunnanensis* reveals

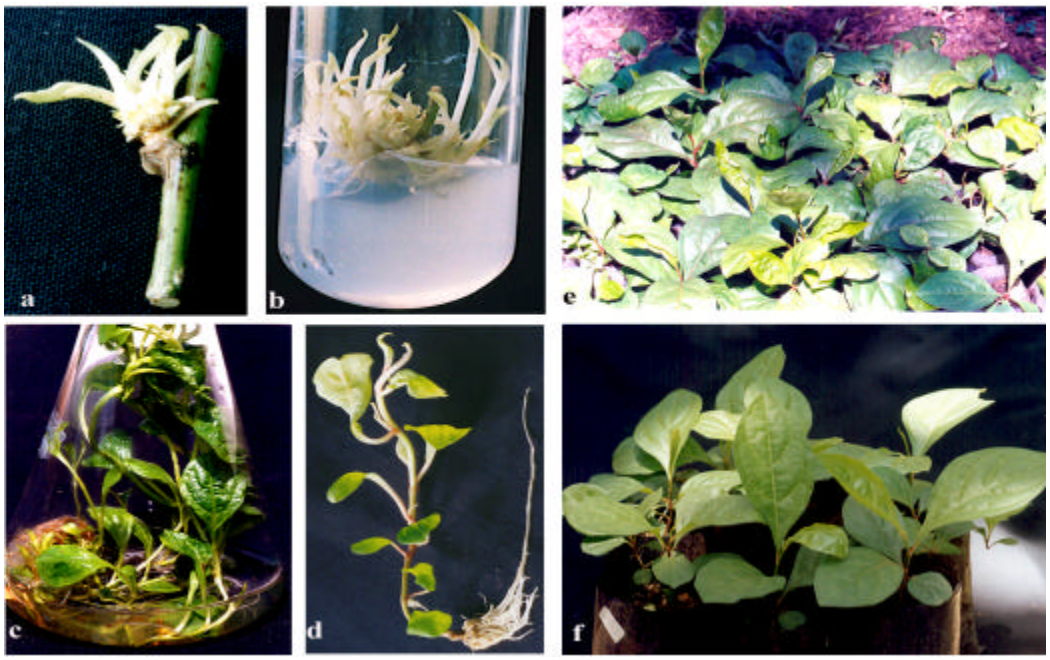

Fig. 1: a) Multiple shoots formed on nodal explants in MS medium containing 15.4 μM BAP after 3 weeks, b) Subculture of the downsized nodes produced 42.1 shoots in MS medium containing 15.4 μM BAP after 3 weeks, c) Elongated shoots in MS liquid medium after 2 weeks, d) An *in vitro* rooted plant, e) *In vitro* rooted plants established in poly bags after 5 weeks and f) *Ex vitro* rooted plants established in poly bags after 6 weeks

Table 1: Effect of growth regulators on shoot initiation in single node explant after 3 weeks of culture

Growth hormone (μM)						
Kn	2ip	BAP	IAA	No. of shoot buds	Length of shoot buds (cm)	Degree of callusing
02.35	0.00	0.00	0.00	1.00±0^{c}	5.00±0.87^{a}	-
04.7	0.00	0.00	0.00	1.42±0.20^{b}	0.81±0.33^{b}	-
09.4	0.00	0.00	0.00	1.20±0.18^{b}	0.18±0.05^{c}	+
16.4	0.00	0.00	0.00	1.00±0^{c}	1.05±0.79^{b}	+
23.5	0.00	0.00	0.00	1.6±0.4^{b}	0.90±0.49^{b}	++
0	2.49	0.00	0.00	1.00±0^{c}	1.06±0.69^{b}	-
0	4.90	0.00	0.00	1.30±0.33^{b}	0.4±0.09^{c}	-
0	9.80	0.00	0.00	1.00±0^{c}	0.72±0.22^{b}	+
0	17.10	0.00	0.00	1.00±0^{c}	0.15±0.05^{c}	++
0	24.50	0.00	0.00	1.00±0^{c}	0.13±0.03^{c}	+++
0	0.00	2.20	0.00	1.25±0.25^{b}	1.60±0.4^{b}	
0	0.00	4.40	0.00	1.66±0.33^{b}	1.40±0.44^{b}	-
0	0.00	8.80	0.00	2.00±0.58^{a}	1.30±0.34^{b}	-
0	0.00	13.20	0.00	2.20±0.49^{a}	0.79±0.25^{b}	+
0	0.00	15.40	0.00	2.41±0.14^{a}	0.87±0.14^{b}	+
0	0.00	17.60	0.00	2.33±0.66^{a}	0.94±0.36^{b}	++
0	0.00	22.00	0.00	2.25±0.48^{a}	1.08±0.32^{b}	+++
0	4.90	0.00	0.57	1.00±0^{c}	0.48±0.1^{c}	+++
0	7.40	0.00	2.15	1.00±0^{c}	0.45±0.08^{c}	+++
0	12.30	0.00	5.70	1.00±0^{c}	0.31±0.03^{c}	+++
04.7	0.00	0.00	0.57	1.25±0.25^{b}	1.00±0.06^{b}	++
07.05	0.00	0.00	2.85	1.75±0.47^{a}	0.80±0.08^{b}	+++
11.7	0.00	0.00	5.70	1.00±0.0^{c}	0.57±0.07^{b}	+++
0	0.00	4.40	0.57	1.60±0.24^{b}	1.02±0.066^{b}	++
0	0.00	6.60	2.85	2.28±0.35^{a}	0.58±0.008^{b}	+++
0	0.00	11.00	5.70	2.16±0.30^{a}	0.48±0.066^{c}	+++

±indicates degree of callusing, Mean values with same superscripts within columns are not significantly different (p = 0.05) by the LSD test

length of explant was a critical factor for *in vitro* micropropagation (Cheong and Pooler, 2003). The delayed bud breaking observed in the downsized nodes of *P. rosea* is presumably due to wounding responses leading to early and pronounced proliferation of callus at the cut end immediately affecting the emergence of the closely present otherwise dormant resident axillary meristem. In fact, the callus had attained substantial growth by the time the bud had emerged in most of the explants.

Among the individual concentrations of the cytokinins and combinations of cytokinins and IAA tested, BAP at 15.4 μM induced maximum number of shoots (2.41±0.140) with a length of 0.87±0.14 cm (Table 1) in 95% of the nodal explants in 3 weeks (Fig. 1a). Higher concentrations of BAP (>15.4 μM) induced increased callusing and relatively less number of bud formation. Though bud formation on the shorter nodes (~0.5 cm) was delayed and preceded by pronounced callusing, relatively larger number (8.2±0.37) yet smaller buds were differentiated after 7 weeks. After an initial lag, the axillary buds in the shorter nodes were activated and developed into multiple shoot buds.

The better response of *P. rosea* on BAP containing media is consistent with the findings of earlier workers (Cheong and Pooler, 2003; Raha and Chandra, 2001). However, combinations of BAP and IAA favoured more callusing than caulogenesis which is in marked contrast with the results in nodal explants of *P. rosea* (Harikrishnan and Hariharan, 1996) and on callus cultures of *P. zeylanica* (Rout *et al*., 1999). The absence of synergistic influence of the cytokinin and auxin in certain other species has been related to the endogenous auxin present in the explants (Julliard *et al*., 1992). It is also possible that the observed differences in responses of the same explant type between different workers are due to the physiological and genotypic differences in the source materials used.

Shoot morphogenesis was somewhat advanced (2.4±0.24 buds in 3 weeks) and the shoots formed were longer (1.61±0.5 cm) in liquid media than on solid media (Fig. 2). The advantage of using liquid as opposed to agar medium is sometimes related to relief from the inhibitory influence of exudates (Latha and Seeni, 1994). Since exudation was rarely encountered in the explants of *P. rosea*, freedom from possible inhibitory substances present in the agar, increased supply and absorption of nutrients by the growing shoots might account for the enhanced growth responses in liquid medium.

There was no significant difference in the number of shoot buds formed in nodes of different positions. However, the mean length of the shoots showed substantial difference with the shortest shoots

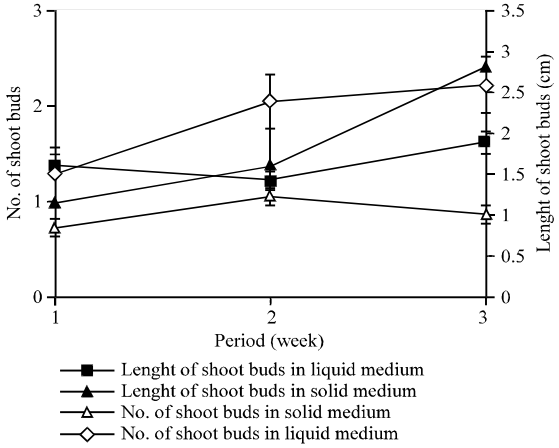

Fig. 2: Effect of MS agar and liquid media supplemented with 15.4 μM BAP on shoot initiation in single node cultures of *P. rosea*. Observations were made after 3 weeks of culture

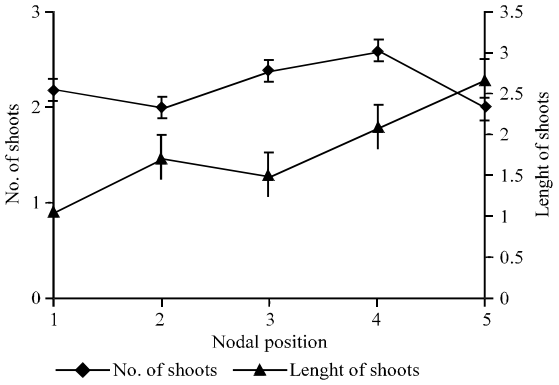

Fig. 3: Shoot initiation response on single node culture as a function of position on the growing shoot. MS agar medium supplemented with 15.4 μM BAP was used. Observations were made after 3 weeks

(0.92±0.19 cm) obtained in the 1st node and the longest (2.3±0.5 cm) in the relatively mature 5th node (Fig. 3). Earlier workers have recorded either conspicuous differences in morphogenetic competence between buds of different positions (Hirimburegama and Gamage, 1995) or no difference at all (Claire and Sharon, 2001). Number of multiple shoots formed under the influence of the cytokinins being the same in different nodes, the differences confined only in length of the bud. In a situation, where a gradient in auxin concentration exists along the entire length of the shoot, the much relieved distal buds developed into longer shoots, while the proximal ones, under the greater influence of the growing

shoot tip, produced relatively shorter shoots. Since the differences in the endogenous auxin concentration between different positions were marginal, they did not influence the number of shoots formed, particularly when optimal concentration of BAP (15.4 μM) was employed.

No attempt was made to subculture the 0.5 cm nodes having shoot buds with overlapping callus. Although shoot buds (2.41±0.14) initiated on longer (2.0-3.0 cm) explants in three weeks were a few, their multiplication was rapid and free of callusing during subculture. Rate of shoot multiplication, however, depended on the reduction in the original length of the node used for subculture. Multiplication was less (4.4±0.6) when the normal nodal explants (2.0-3.0 cm) with callus at cut ends and buds at axils were subcultured for 3 weeks in the medium containing 15.4 μM BAP. The callus at cut basal end proliferated more than shoot bud multiplication and the limited number of shoots so multiplied grew rapidly to attain a length of 2.5-3.0 cm during this period. If the shoots and buds initiated were isolated and subcultured, significant increase in multiplication occurred and an aggregate number of 7.41±2.18 shoots was harvested. Maximum multiplication of shoots was noticed when the length of the node was reduced from below and the downsized (~1.0 cm) node free of callus was subcultured with buds in contact with the medium. No further callusing was observed at cut end and obtained maximum of 42.1±5.4 shoots in three weeks (Fig. 1b).

Published works on changes needed at subculture stage are scanty and do not deal with the importance of reducing the size of the node to realize phenomenal increase in the multiplication. It should be noted that even during culture initiation a shorter node (~0.5 cm) with proliferating callus at the base produced significantly larger number of shoots than the longer node though after an initial lag. In the light of the findings, it would be worthwhile to investigate the plus effect of reduced explant size on shoot multiplication, in explants with and without callus proliferation in other species.

The influence of the PGRs tested during the subculture of the shortened nodes with buds proliferated upon supported the observations made during culture initiation (Table 2). All the combinations of the BAP and IAA promoted less number of shoot multiplication than individual concentrations of BAP that is in marked contrast to the synergistic influence of auxin addition observed in *P. zeylanica* (Rout *et al*., 1999). The antagonistic influence of IAA on caulogenesis was also observed during culture initiation could only be attributed to the threshold level of endogenous auxins present in the explants used for both culture initiation and multiplication (Okubo *et al*., 1991). Increased callus formation in response to combinations of BAP and NAA, also observed in certain other cultures (*Woodfordia fruiticosa*) was not surprising as among auxins NAA is known to induce more callusing than IAA and IBA accompanying or preceding organogenesis (Krishnan and Seeni, 1994).

Table 2: Shoot multiplication through subculture of downsized nodes (0.5 cm) devoid of callus in MS agar medium supplemented with various concentrations of BAP and combinations of BAP and auxins. Observations were made after 3 weeks of culture

Growth regulators (μM)					
BAP	IAA	NAA	No. of shoots	Length of shoots (cm)	Degree of callusing
2.2	0.00	0.00	3.60±0.50^{k}	0.92±0.10	-
4.4	0.00	0.00	4.80±0.58^{j}	0.97±0.11	-
8.8	0.00	0.00	14.00±1^{f}	0.64±0.03	-
13.2	0.00	0.00	22.25±4.9^{d}	0.34±0.02	+
15.4	0.00	0.00	42.10±5.40^{a}	0.166±0.01	++
17.6	0.00	0.00	33.60±1.85^{b}	0.298±0.01	+++
22.0	0.00	0.00	17.60±0.88^{e}	0.390±0.04	++++
2.2	0.57	0.00	2.25±0.48^{l}	1.650±0.24	-
4.4	2.15	0.00	3.00±0.40^{k}	1.070±0.19	+
6.6	2.15	0.00	5.00±0.58^{j}	0.600±0.10	+
8.8	2.15	0.00	21.60±1.20^{d}	0.440±0.02	+
11.0	5.70	0.00	26.30±1.45^{c}	0.340±0.01	+++
15.4	8.55	0.00	6.00±1.15^{j}	0.530±0.09	++++
2.2	0.00	0.54	3.00±0.57^{k}	0.790±0.08	-
4.4	0.00	2.70	5.00±0.58^{j}	0.900±0.17	++
6.6	0.00	2.70	7.50±0.5^{i}	0.500±0.07	+++
8.8	0.00	2.70	21.60±1.20^{d}	0.310±0.03	++++
11.0	0.00	5.40	10.60±2.40^{g}	0.240±0.01	++++
15.4	0.00	8.10	9.00±1.15^{h}	0.300±0.06	++++

Mean values (n = 10) with same superscript(s) within the columns are not significantly different (p = 0.05) by the LSD multiple range test

Two or three shoots (0.5-1.5 cm) in a cluster transferred to liquid and solid basal media showed growth to attain a length of 4.1±0.18 cm and 3.26±0.16 cm, respectively in 2 weeks. Supplementation of 2.2-6.6 μM BAP in the medium induced the formation of 1-2 additional shoots with marginal increase in length (1.1-1.6 cm) in the existing shoots. The results confirmed the inhibitory influence of cytokinins on shoot growth observed earlier in other systems (Bermudez *et al*., 2002) and suggested the desirability of using basal liquid media for shoot elongation, lending credence to the observations made during culture initiation. The shoots so subjected to elongation were grown to 4.0-8.0 cm long after 5 weeks (Fig. 1c).

All the shoots of 3.5-4.0 cm length transferred to basal solid media as well as media containing auxins readily formed roots with or without associated callus formation in 2 weeks. Among the auxins tested, maximum number of root (12.5±2.5) formation together with substantial callusing was induced by 1.14 μM IAA. Callus free rooting, though relatively less in number (9.8±1.2) was observed in presence of 0.49 μM IBA (Fig. 1d). Combinations of IBA and IAA as well as combination of 0.49 μM IBA and 0.54 μM NAA tested did not enhance

rhizogenesis and the later combinations induced more callusing than rooting, thereby reinforced the increasingly callogenic activity of NAA observed earlier during shoot multiplication. The formation of upto 6 roots/shoot at 100% efficiency even in the basal medium further suggested that concentration of endogenous auxin in the shoots implicated during shoot initiation and multiplication was also sufficient to induce rhizogenesis in all the shoots and exogenous addition of IBA or IAA caused only quantitative enhancement. This was further confirmed when all the shoots were successfully rooted (4.8±0.5) *ex vitro* in 4 weeks even without hardening. 100% establishment was obtained in both *in vitro* and *ex vitro* rooted plants in poly bags in the shade house under regular irrigation (Fig 1e and f). The absence of rooting in the same shoots in the basal medium during the preceding elongation phase, however, could only be related to the inhibitory concentrations of endogenous cytokinins built up during culture initiation and multiplication which got reduced sufficiently to a level to promote rooting only during the second passage through the basal medium. The bypassing of the hardening stage in the micropropagation of horticulturals may reduce the cost of propagation by 50% or more (Martin *et al*., 2003). Though *ex vitro* rooting has certain advantages, to economize plant production, in *P. rosea* it is better to opt for *in vitro* means produce significantly larger number of roots without callusing in presence of 0.49 μM IBA which contributed to the production of commercially important tuberous roots during cultivation in the field (Satheeshkumar and Seeni, 2003). Since additional formation of roots was minimal in the post-transplantation stage, a plant with initial larger number of roots was desirable to produce larger number of tuberous roots during cultivation thereby enhancing its acceptability to the farmers. Two months after establishment the plants showed uniform growth and were free from morphological abnormalities.

Based on the results obtained during culture initiation, multiplication, elongation and rooting stages, a protocol for large scale clonal multiplication of *P. rosea* was developed (Fig. 4). The method described herein together with the cultivation and production characteristics of the micropropagated plants demonstrated earlier (Satheeshkumar and Seeni, 2003) is superior to multiplication achieved through direct organogenesis reported by others (Harikrishnan and Hariharan, 1996; Chetia and Handique, 2000) and indirect organogenesis by Kumar and Bhavanandan (1988). It is complete in all respects from culture initiation up to cultivation and production and enables consistent production and supply of planting materials. However, the cost benefit ratio is yet to be worked out to ascertain the economic viability of micropropagation in relation to enhanced tuberous root harvest.

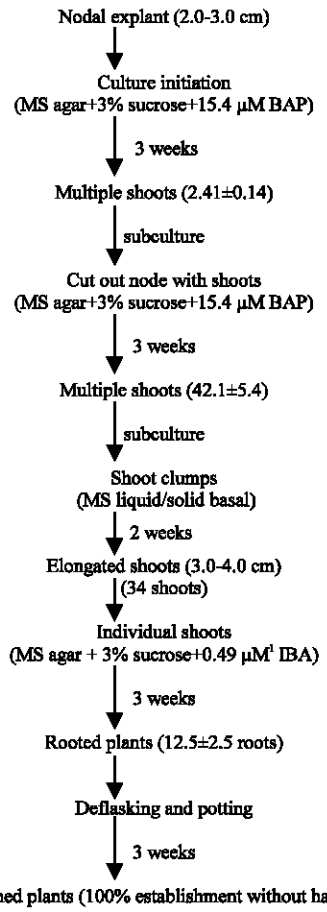

Fig. 4: Scheme for high frequency regeneration and multiplication of *Plumbago rosea* L.

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