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Research note

In vitro propagation of *Rauwolfia micrantha*, a rare medicinal plant

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Abstract

Shoot tip and single node explants from young shoots of 1-year old flowering plants of *Rauwolfia micrantha* Hook. f. were cultured on Murashige & Skoog (MS) medium variously supplemented with 6 - benzyladenine (BA) and α - naphthaleneacetic acid (NAA). A combination of 13.2 μM BA and 2.68 μM NAA induced high frequency (77%) formation of up to 3 shoots from each node in 8 weeks. The regeneration of shoot tips from the field-grown plants and *in vitro* shoots placed horizontally differed. Repeated subculturing of the shoot tips and single nodes at 6-week intervals for over a year in combination of 4.4 μM BA and 0.27 μM NAA enabled mass multiplication of shoots without any evidence of decline. Rooting of the excised shoots on medium containing 2.6 μM NAA was preceded by callus formation. The rooted plants were removed off the callus, hardened off and 80% established in pots. Micropropagated plants displayed uniform morphological, growth, flowering, fruiting and seed germination characteristics.

Abbreviations: BA – 6-benzyladenine; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; 2-ip – 2-isopentenyladenine; MS – Murashige & Skoog (1962); NAA – α- naphthaleneacetic acid

Rauwolfia micrantha Hook.f (Apocynaceae) is a perennial woody shrub distributed at elevations up to 600m in the Tinnevelly and Travancore hills of the Western Ghats in southern India. The roots are a rich source of the antihypertensive tranquilizer alkaloids ajmalicine, reserpiline, reserpine, sarpagine and serpentine (Anonymous, 1969). *R. micrantha* is used as a substitute for *R. serpentina* to treat a variety of nervous disorders in traditional medicine (Ayurveda) especially in the State ot Kerala (Sahu, 1979).

In nature, *R. micrantha* is not over exploited. However, such factors as endemicity, restricted distribution, small populations in accessible areas, and severe anthropogenic pressure on forest lands have caused its decline in the wild. It is reported to be rare (Sahu, 1979). Conventional propagation is beset with problems of poor seed viability, low germination and scanty and delayed rooting of seedlings and vegetative cuttings. There is an urgent need to apply non-conventional propagation methods for conservation and future commercial delivery of *Rauwolfia* spp. (Upadhyay *et al.*, 1992).

In the present paper, we report on rapid propagation through axillary bud break in shoot tip and single node cultures and field establishment of uniform plants of *R. micrantha*.

Seeds collected from a 5-year old plant in the herbal garden of our Institute were chipped and soaked in 144.34 μM GA_3 solution for 20 min before sowing in 1:1 sand and soil mixture. Approximately 40% seeds germinated after 6 weeks. Top shoot cuttings having 3–4 nodes (each node with 2–3 leaves) were collected from actively growing 1-year old flowering plants and after leaf excision, were washed first in 1% (v/v) Labolene detergent (Glaxo India Pvt. Ltd., Bombay) for 15 min and then in running tap water for 10 min.

Surface decontamination of the cuttings consisted of passage through 15% (v/v) Steriliq commercial

bleach (Combi Organic Chem (P) Ltd., New Delhi) for 8 min and 0.1% (w/v) $HgCl_2$ for 10 min. After 3–4 rinses in sterile distilled water, shoot tips (0.3–0.5 cm) and single nodes (1.0–1.5 cm) were dissected out of the cuttings, rinsed once in sterile distilled water and blotted on sterile filter paper discs before planting them vertically or horizontally on solid Murashige & Skoog (1962) medium in 25×150 mm culture tubes. The medium contained 0.5% (w/v) agar (CDH India Ltd., New Delhi) and varied concentrations and combinations of BA and NAA. The pH of the medium was adjusted to 5.8 before adding agar and autoclaved at 121 °C and 108 kPa for 18 min. All the cultures were incubated at 24 ± 2 °C under 12-h photoperiod at a photon flux density of 20–50 μ mol m^{-2} s^{-1} from daylight fluorescent tubes (Philips India Ltd., Bombay). Each treatment consisted of 15–20 explants and was repeated at least once.

After 8 weeks of culture, the branched axillary shoots proliferated upon the nodal explants were separated for multiplication. Explants of axillary shoots viz. shoot tips and nodes were dissected and subcultured in vertical or horizontal orientation for 6 weeks in medium containing 4.4 μM BA and 0.27 μM NAA. Subsequently, mass propagation of shoots was achieved by repeated subculture of the shoot tips and single node explants of shoot cultures in the same medium at 6 week intervals.

Rooting of 3–5 cm shoot cuttings was obtained in medium containing half concentration MS salts, 555.06 μM myo-inositol, 3% (w/v) sucrose and varied concentrations of the auxins IAA, NAA or IBA. No attempt was made to determine possible loss of IAA activity during autoclaving. After 6 weeks, the rooted plants were removed from the culture tubes, repeatedly washed in tap water to remove the callus mass sticking to the cut ends of shoots and transplanted in a potting mixture of soil, sand and cattle manure (1:1:1) in 5×5 cm clay pots. If the callus was compact, it was surgically removed before transplantation. The potted plants were well irrigated and hardened for 2 weeks in a manually operated mist chamber maintaining 70–90% relative humidity. Established plants were maintained under greenhouse conditions for 4-weeks before transfer to field or repotting in 15 cm pots for further growth under 50% shade. Growth, flowering and fruiting characteristics of randomly selected plants were recorded after 12 to 13 months. Growth was measured in terms of total length of the shoot up to the tip and mean width of the shoot determined at basal, middle and upper first axillary branching levels of the stem using a vernier scale. Mean length of the fruits and fresh weights of the depulped seeds blotted on filter paper were also determined. Seeds collected from mature fruits were germinated by the method followed for the seeds of the field plants. Data were analysed using standard ANOVA procedures and the differences between the means were compared using the LSD multiple range test.

Bacterial infection of 20–35% of the shoot tip and single node explants was noticed 5–6 days after transfer. The remainder responded favourably, with the initiation of shoot buds in 10–15 days. Shoot tips were less effective explants, as reported in certain other systems (Hutchinson, 1982; Stapfer & Heuser, 1985). Irrespective of the vertical or horizontal positioning, all the shoot tips developed into single shoots either by longitudinal growth or by upward curvature and elongation. Solitary shoot formation, presumably due to increased apical dominance is not uncommon in woody species (Hu & Wang, 1983). Single node explants responded somewhat better with the formatian of 2–3 shoot buds in 3 weeks that developed into 2.0 – 3.5 cm shoots in 8 weeks. Examination of these explants revealed the formation of only a single shoot from each leaf axil. Although axillary shoot emergence was soon followed by callusing to various degrees in the cut and adjacent basal parts of the explants immersed in the medium, the shoot initiation sites were not affected. Phenolic exudates, frequently encountered in woody plant tissue cultures were absent.

In marked contrast to published results on the related species, *R. serpentina* (Mathur *et al.*, 1987), BA 13.2 μM induced the formation of only 1–2 shoot buds in 40–46% of the nodal explants. Both kinetin and 2-ip did not release the axillary buds. Besides, BA alone was insufficient to sustain the growth of the buds into shoots (Table 1). Influence of NAA was evident when a combination of 13.2 μM BA and 2.68 μM accelerated initiation and growth of the shoots. High frequency (76%) formation of maximum number of 3 shoots from each node in 8 weeks was recorded in this hormonal regime. Significant difference ($p \leq 0.05$) in the number of shoots formed was observed between BA and BA–NAA combinations. Each shoot so formed showed a marked tendency for enhanced axillary branching especially from the basal nodes. In most of these shoots, 1–3 axillary branches emanated from nodes overgrew while the shoots from which they were derived remained short. Combination of higher concentration (22 μM of BA and 2.68 μM NAA) was less effective with a substantial reduction in the frequency of shoot formation. Consistent with published

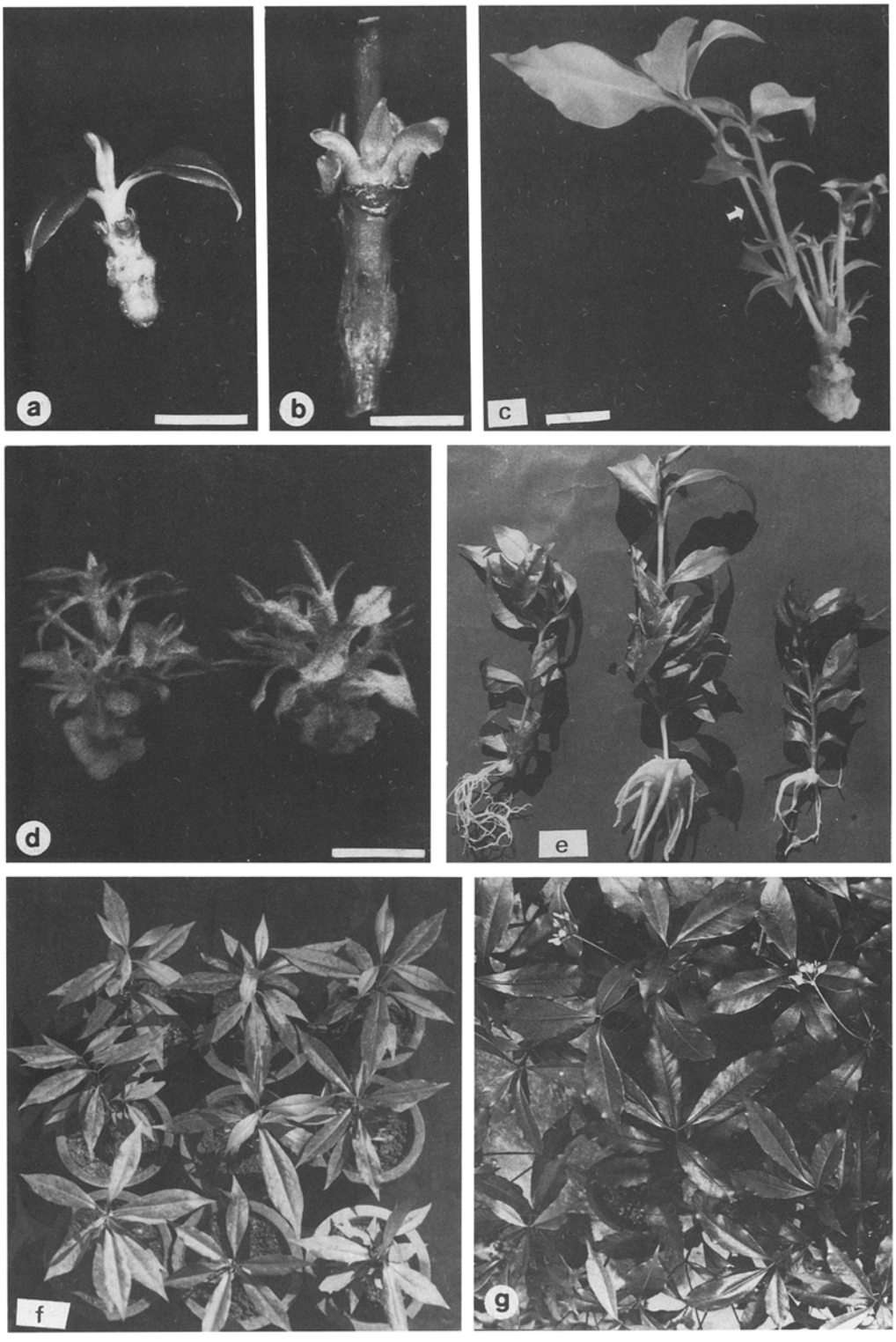

Fig. 1. In vitro propagation of *Rauwolfia micrantha*. (*a*) Single shoot developed after 8 weeks of culture from shoot tip implanted vertically in MS medium containing 16.11 μM BA and 2.68 μM NAA. (*b*) Shoot buds differentiating from nodal explant culture after 3 weeks. (*c*) Branch shoot (arrow) rapidly growing over the node-derived shoot. (*d*) Single and multiple shoot formations in vertically (L) and horizontally (R) oriented shoot tips of *in vitro* shoots after 3 weeks of culture. (*e*) Rooted plants obtained in medium containing (L to R) 2.68 μM IAA. NAA and IBA respectively (Bars represent 5 mm.)

Table 1. Axillary shoot formation in nodal segments of 1-year old flowering plants of *Rauwolfia micrantha* cultured in MS agar medium supplemented with BA and NAA. Observations were made after 8 weeks of culture.

Plant growth regulator (μM)		Frequency of shoot formation (%)	Mean number of shoots/ node	Mean number of axillary branches/shoot
BA	NAA			
5.37	0	41	1.4^{b}	0
10.74	0	40	1.6^{b}	0
16.11	0	45.5	1.9^{ab}	0
5.37	2.68	53.3	2.3^{a}	1.1^{c}
10.74	2.68	62.1	3.0^{a}	2.5^{b}
16.11	2.68	76.9	3.0^{a}	5.4^{a}
26.85	2.68	52.9	3.0^{a}	3.2^{b}

Means followed by the same letters are not significantly different ($p \leq 0.05$) using L.S.D multiple range test

Table 2. Shoot multiplication in differentially oriented shoot tip and nodal explants derived from shoot cultures. Observations were made after 4 weeks of subculture in presence of 4.4 μM and 0.27 μM NAA. Figures in parentheses indicate comparative values obtained for the explants of field plants for the same period of culture in presence of 16.11 μM BA and 2.68 μM NAA.

Explant type	Orientation	Frequency of shoot formation (%)	Mean number of shoots/explant	Mean length of shoot (cm)
Shoot tip	Horizontal	100.0(40.2)	4.00 ± 0.0(1.0 ± 00)	2.0 ± 0.1 (0.4 ± 0.8)
	Vertical	100.0(58.8)	1.8 ± 0.4(1.0 ± 0.0)	2.4 ± 0.3(0.5 ± 0.2)
Node	Horizontal	96.6(65.0)	2.6 ± 0.4(2.3 ± 0.7)	2.4 ± 0.5(1.0 ± 0.0)
	Vertical	100.0(76.9)	3.0 ± 0.0(3.0 ± 0.0)	2.8 ± 0.0(1.6 ± 0.5)

reports (Amin & Jaiswal, 1987; Maarri *et al.*, 1986; Maria *et al.*, 1990), the shoot formed in high cytokinin medium were stunted in growth with short internodes and crowded leaves.

For shoot multiplication experiments, shoot tips and single node explants of *in vitro* shoots were subcultured in a medium containing reduced levels of BA (4.4 μM) and NAA (0.27 μM). Single shoot formation, through elongation, occurred in nearly 55% of the vertically implanted shoot tips (Fig. 1d) while the rest produced 1.4 – 2.2 shoots (Table 2). All the shoot tip explants placed horizontally responded well producing more (4.0 ± 0.0) shoots than the nodal explants (2.2 – 3.0) within 4 weeks of subculture. The observation that in these explants the tip portion continued to grow by elongation even while 3 shoot buds were differentiated from the sides and the buds rapidly developed into 2.0 ± 0.1 cm shoots indicated the weak apical dominance and easy release of the axillary buds. The remarkable difference in regeneration of the shoot tip explants of field-grown plants and *in vitro* shoots could be attributed to altered endogenous concentration of hormones in the latter growing in high cytokinin medium at the time of explantation. The nodal explants of shoot cultures responded with the formation of 2.2–3.0 shoots but each shoot grew vigorously to reach a length of 4.5–5.0 cm in 6 weeks. The ability of almost all the shoot culture-derived shoot tips to produce more shoots together with the vigorous growth of the axillary shoots differentiated upon both shoot tips and nodal explants were suggestive of enhanced multiplication of shoots during subculture. Stocking of shoot cultures was thus achieved by repeated subculturing of the shoot tips and nodal explants at 6 - week intervals for over a year wherein the rate of multplication was maintained. The cultures were invariably free of callus formation at the site of shoot formation although the cut ends showed marginal callusing activity during this period. There was no evidence of axillary meristem proliferation into multiple shoots or of adventitious shoot formation.

Table 3. Effects of auxins on rooting of *in vitro* shoot cuttings of *Rauwolfia micrantha*

	Auxins (μM)	Rooted shoots (%)	Mean number of roots/shoot	Mean length of the root (cm)	Callusing	Suvrival (%)
	0.0	30.0	2.1^{e}	1.8^{d}	–	20.0
IAA	1.14	50.0	2.4^{e}	4.2^{ab}	–	45.0
	2.85	85.7	3.4^{de}	5.2^{a}	–	56.5
	5.70	63.0	5.1^{ed}	3.9^{b}	–	60.5
NAA	1.07	41.6	5.8^{c}	3.5^{bc}	–	62.7
	2.68	76.9	8.2^{b}	4.1^{b}	–	83.4
	5.37	54.5	10.2^{a}	3.1^{bc}	–	72.0
IBA	0.98	44.0	3.1^{e}	2.1^{cd}	–	32.0
	2.46	87.8	5.7^{cd}	3.2^{bc}	–	51.5
	4.92	66.6	6.1^{c}	1.3^{d}	–	43.5

Observations were made after 4–5 weeks
Means followed by the same letters are not significantly different ($p \leq 0.05$) using L.S.D. multiple range test.
Number of + signs indicates the degree of callusing: - sign indicates no response.

Table 4. Flowering, fruiting and seed characteristics of the micropropagated plants of *R. micrantha* after 12–13 months of cultivation in pots

Plant number	Shoot length (cm)	Mean width of shoot (cm)	Number of flowers/plant	Number of fruits/plant (%)	Mean length of fruit (cm)	Mean weight of seed (mg)	Seed germination after 6 weeks (%)
1	63	1.2 ± 0.4	25	18(72.0)	0.95 ± 0.1	44 ± 0.6	48
2	60	0.8 ± 0.2	20	12(60.0)	0.91 ± 0.2	39 ± 0.7	46
3	58	1.3 ± 0.3	26	17(65.4)	0.85 ± 0.5	38 ± 0.6	44
4	57	1.3 ± 0.1	25	14(56.0)	1.00 ± 0.0	40 ± 0.5	48
5	63	1.2 ± 0.2	18	12(66.7)	0.94 ± 0.3	41 ± 0.2	45
6	59	1.4 ± 0.1	16	11(68.8)	0.96 ± 0.2	39 ± 0.2	47
7	65	1.5 ± 0.3	24	15(62.5)	1.00 ± 0.2	42 ± 0.5	49
8	62	1.2 ± 0.3	20	14(70.0)	0.92 ± 0.3	44 ± 0.5	49
9	59	1.0 ± 0.3	17	17(58.8)	1.02 ± 0.0	45 ± 0.6	45
10	63	1.3 ± 0.3	20	20(50.0)	1.00 ± 0.3	43 ± 0.5	48

Figures in parentheses indicate percentage of flowers producing fruits.

Thirty per cent of the shoot cuttings transplanted into auxin - free medium produced 1 or 2 thin and short roots in 4 weeks (Table 3). Although IAA and IBA supplemented at 2.8 μM and 2.46 μM respectively induced highest frequency (85–87%) rooting devoid of callusing, the roots formed had thin laterals. The plants with such roots showed poor establishment (50–57%) after hardening. Significant differences ($p \leq 0.05$) for the number and length of the roots were observed between the auxins. The use of lower (1.14 μM and 0.98 μM) and higher (5.70 μM and 4.92 μM) concentrations of IAA and IBA respectively resulted in diminished rooting response and did not improve establishment. This problem was alleviated by the use of 2.68 μM NAA which promoted the formation of 8–10 robust roots and high percentage survival and establishment (83%) of plants. Rooting however, was preceded by the formation of friable callus, the latter being confined to the cut ends of the shoots and the roots differentiated from the sides of the shoots immediately above the site

of callusing. The shoots were maintained in this medium for 6 weeks before weaning the plants for callus removal and establishment in pots.

In spite of the prevalence of good agroclimatic conditions in the garden in the months of August, September and October 1991 more than 60% of the plants transplanted in pots and reared under greenhouse conditions were lost within 3 weeks due to shoot necrosis. Invariably the shoot apices together with the leaves got dried up leaving leafless stem or stem with 1 or 2 basal leaves. The stumps so remaining seldom produced new shoots. More than 80% of the 120 plants hardened for 2 weeks in the mist chamber established. After acclimatization under greenhouse conditions the plants were repotted in 15 cm pots and grown under 50% shade. The plants were free from growth defects as revealed by shoot length and width measurements (Table 4). After 12 months the plants flowered, the period between flower bud emergence and fruit ripening varying from 25 to 30 days. The flowering and fruiting characteristics of the randomly selected clonal plants were uniform. In all the plants approximately 65% of the flowers produced fruits, the uniform seeds collected from which were germinated at frequencies comparable to the seeds (40%) of field plants.

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