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MICROPROPAGATION OF *RAUVOLFIA TETRAPHYLLA* L. FROM *IN VITRO* SEEDLING DERIVED EXPLANTS USING VARIOUS AMINO ACIDS

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ABSTRACT

Rauvolfia tetraphylla L. belonging to the family Apocynaceae has been used in the treatment of hypertension, snake bites and insect sting poisons since a long time. Though various attempts were reported for the regeneration of *R. tetraphylla* through explants collected from *in vivo* grown plants, this is the first attempt reporting regeneration from *in vitro* seedling derived explants. Various amino acids were also tested for their efficacy on *in vitro* regeneration of *R. tetraphylla*. The regeneration through the shoot tip and cotyledonary node explants of *in vitro* grown seedlings were achieved in MS+B_5 medium fortified with 3% sucrose and 0.8% agar. By adjusting the cytokinin (BA (Benzyl adenine), KN (Kinetin)) concentrations and various amino acids (glutamine, lysine, proline, methionine and isoleucine) in multiple shoot induction medium, the media composition was optimized for obtaining maximum results. Compared to KN, BA gave best response for multiple shoot induction. Maximum number of shoots were produced in the cotyledonary nodes than the shoot tips. Amino acids encouraged multiple shoot induction (especially glutamine and proline) response when combined with 4.44 µM BA, where 34.75 and 29.18 shoots were produced per explant with 0.51 mM glutamine and 0.66 mM proline respectively. Elongation of shoots was 100 % on medium with 0.44 µM BA and 1.156 µM GA_3 (Gibberellic acid). The rooting of elongated shoots (65%) was attained by incorporating IBA (Indole butyric acid) in the medium. The roots were thick, long, about 4.8 roots were produced per explant on medium supplied with 9.84 µM IBA. The hardening and field introduction attempts were successful with the survival rate of 78 % after 12 weeks of acclimatization.

Keywords: Cotyledonary node, amino acids, shoot tip, micropropagation.

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INTRODUCTION

Rauvolfia tetraphylla L. is an endangered, medicinally important shrub belonging to the family Apocynaceae. The plant is extensively used by Tribes of India for treating diverse ailments viz., to increase uterine contraction, for relief of muscular and rheumatism pain, to treat skin diseases and in the treatment of fever, hypertension and respiratory problems (Iqbal *et al*., 2013). *Rauvolfia* is prevalently used in Ayurveda and Unani systems of medicine (Jakaria *et al*., 2016). Various indole alkaloids present in the root of *R. tetraphylla* is responsible for the curative properties of this plant, for which the plants are uprooted and used in drug preparation. Other phytochemicals reported in this plant are phenolic compounds, saponins and resins (Gulab *et al*., 2022). The roots of *R. tetraphylla* is considered as an adulterant or alternative to the widely exploited plant R. *serpentina* in drug preparations (Mahalakshmi *et al*., 2019; Prathapa *et al*., 2020) and Sulaiman *et al*. (2020) reported that alkaloids content in *R. tetraphylla* is higher than that of R. *serpentina*. The methanolic extract of *R. tetraphylla* leaf had no toxicity effect up to 550 mg/kg bodyweight in experimental rats and exhibited toxicity effects without lethality at 2000 mg/kg body weight (Tamboli and Pandit, 2014). Extensive collection of this plant made *R. tetraphylla* as an endangered plant (Gulab *et al*., 2022), where tissue culture techniques aid its conservation. In our earlier work (Anitha and Ranjitha Kumari, 2006), we reported multiple shoot induction from *in vivo* explants, and other reports available on *in vitro* regeneration of *R. tetraphylla* through axillary buds (Patil and Jayanthi, 1997; Faisal *et al*., 2012; Mamatha *et al*., 2021) and shoot tips (Sarma *et al*., 1999) of *in vivo* derived explants.

From the available data, no research was reported for regeneration of *R. tetraphylla* from *in vitro* derived explants. Hence, the present work is carried out with an objective to standardize a reliable protocol for *in vitro* regeneration of *R. tetraphylla* from seedling derived

explants in phytohormones and amino acids supplemented medium.

MATERIALS AND METHODS

Fully ripened fruits were collected from 3-years old garden plants and the seeds were separated and soaked in water. The seeds that plunged immediately in the water were used for germination. Surface sterilization of seeds is done using 1% Teepol for 20 min. followed by 70% ethyl alcohol treatment for 1 min. Finally, mercuric chloride (0.2%) wash is given for 7 min., and the sterilizing agents were removed using sterile distilled water wash. Hard seed coat was split and the seeds were soaked in 0.001% GA_3 solution for 30 min., then sown in sterile soil mixture and on moist cotton bed (Anitha and Ranjitha Kumari, 2013). Sand, garden soil and farmyard manure at 1:2:1 ratio is used for soil mixture preparation. From 20-25 days old seedlings, explants (shoot tip and cotyledonary node) were procured and inoculated in a vertical orientation into the multiple shoot induction medium.

The medium used for the present study is MS+B_5 medium that consisted MS nutrient salts (Murashige and Skoog, 1962) and B_5 vitamins (Gamborg *et al.*, 1968), 3% sucrose, 0.8% agar and phytohormones (cytokinins and auxins). The cytokinins, BA and KN were added to the medium at 1.11-8.88 µM and 1.16-9.28 µM concentrations, respectively. Auxins such as NAA and IAA were used in the following concentrations, IAA at 0.15-1.16 µM and NAA at 0.14-1.08 µM along with BA. Various amino acids such as proline (0.22-0.88 mM), methionine (0.17-0.68 mM), glutamine (0.17-0.68 mM), lysine (0.17-0.68 mM) and isoleucine (0.19-0.76 mM) were tested individually along with BA. Phytohormones and other additives free medium was kept as control. After adjusting the pH of the medium to 5.75±0.05 by using 0.1 N NaOH or 0.1 N HCl, agar was added in the medium, melted and dispensed into boiling tubes (approximately 10 ml) and bottles (approximately 20 ml), sealed with cotton plugs and then sterilized in the autoclave for 20 min at 121ºC.

The individual shoots were separated and transferred to medium supplied with varying concentrations of BA (0.22-0.66 µM) or KN (0.464-0.928 µM) + GA_3 (0.289-1.445 µM) for shoot elongation. The shoots which reached approximately 2-2.5 cm in length were inoculated into the rooting medium augmented with auxins, IAA (0.57-8.56 µM), IBA (1.23-12.6 µM) and NAA (0.537-8.06 µM). The pH of all media was set and then sterilized in a similar way explained in the multiple shoot induction experiments. The explants inoculated in culture tubes and were incubated at 25 ± 1ºC and exposed to 16-hour photoperiod with 40 µM $m^{-2}s^{-1}$ light intensity and the relative humidity maintained was 60 - 70%. The explants were transferred to freshly prepared medium at every three weeks. Rooted Plantlets were washed gently and transferred to plastic tea cups (10 cm diameter) filled with sterilized soil mixture made up of sand, farmyard manure, garden soil at the ratio of 1:1:2 for hardening purpose. The potted plants were covered with polythene bags to maintain high humidity. Initially the cups were maintained in the culture room for 3 weeks and irrigated with tap water once a day. Afterwards, the plants were shifted to shade condition in the lab and maintained for two weeks. The polythene bags were removed and the plants were maintained under shade for another two weeks in the garden. The plants were finally transferred to normal field conditions.

Per cent response in multiple shoot induction, shoot elongation, rooting and hardening was recorded periodically. The number of shoots induced in each treatment with BA, KN and amino acids from *in vitro* derived shoot tip and cotyledonary node explants was recorded and average is presented. The length of shoot and root was measured and expressed in cm in shoot elongation and root induction experiments. In all the experiments, fifteen replicates were used in each treatment and each experiment was repeated five times. DMRT (Duncan's Multiple Range Test) at 0.05% significance level was carried out to compare the average using SPSS software version 10.0 (LEAD Technologies Inc., Chicago, USA, 1999). Different alphabets followed average are significantly different from each other at $P \leq 0.05$ according to DMRT.

RESULTS AND DISCUSSION

Emergence of shoot buds was observed after a week of culture from the explants collected from *in vitro* seedlings (Plate 1A - E). Explants showed maximum number of axillary sprouting in BA augmented medium than KN (Table 1 and 2). BA at 4.44 µM concentration induced the maximum number of shoots than the other concentrations tested, both in the cotyledonary node and the shoot tip. In the present study, cotyledonary nodes produced more shoots (16.0 shoots/ explant) than the shoot tip (6.84 shoots/ explant) explants (Table 1 and 2). In *Sesbania drummondii* regeneration protocol, the authors observed maximum number of shoots production from cotyledonary node explants than the axillary branch node at 22.2 µM BA (Cheepala *et al*., 2004). Similarly, cotyledonary nodes were reported effective for multiple shoot induction in *Commiphora wightii* (Arn.) Bhandari (Tarun *et al*., 2010). In *in-vivo* derived explants of *Rauvolfia tetraphylla*, nodal explants responded well for multiple shoot induction than the shoot tips (Hoque *et al*., 2020; Mamatha *et al*., 2021). Addition of auxins such as IAA and NAA inhibited the shoot formation response in terms of percentage of response, number of shoots per explant and length of the shoots (Table 3 and 4). In this respect, the present observation deviates from the

inferences made by other researchers in *R. tetraphylla*, using garden grown plants as a source of explant. When BA (2.5 mg/l) is used along with NAA (0.1 mg/l) and IAA (0.1 mg/l), more shoots were produced in the nodal explants (Mamatha *et al*., 2021). BA (2.2 mg/l) and NAA (0.1 mg/l) is reported for maximum shoot induction in nodal explants (Hoque *et al*., 2020) and BA (7.5 µM) with NAA (2.5 µM) is reported for maximum shoot induction response in the shoot tip explants (Faisal *et al*., 2012). But the report of Ponni and Ashalatha (2019) supports present investigation, where addition of IBA with BA reduced the shoot multiplication rate in *Ensete superbum* (Roxb.) Cheesman, whereas the other available report supports the usage of auxins with BA for multiple shoot induction in *Ensete superbum* (Mathew and Philip, 1996).

Inclusion of amino acids in multiple shoot induction medium altered the shoot production response, Glutamine exerted greater response in shoot bud induction followed by proline, and lysine (Table 5). Glutamine at 0.51 mM concentration induced 34.75 shoots per cotyledonary node explant with 100% response. Similar to present investigation, addition of glutamine enhanced the multiple shoot production in *Aquilaria malaccensis* (Papori and Jonaki, 2018). Glutamine is known to be the readily available nitrogen sources and involved in various metabolic activities, thus enhancing the regeneration potential of the explants and maintain growth rate for a longer period (Papori and Jonaki, 2018) as well biomass increase in the explants (Ponni and Ashalatha, 2019). Proline also enhanced the multiple shoot induction response, at 0.66 mM concentration, 96% response was observed in cotyledonary node explants with an average of 29.18 shoots per explant (Table 5; Plate 1F). Proline was reported to stimulate *in vitro* shoot organogenesis from cotyledon explants of C*ucumis melo* (Milazzo *et al*., 1998). According to the authors, proline activates purine biosynthesis, which enhances the synthesis of auxin and cytokinin in plants.

When BA and KN were combined with GA_3, elongation of the individual shoots was observed. BA at 0.44 µM and GA_3 at 1.156 µM concentrations induced the maximum elongation of the internodes (4.3 cm) in all the explants (100%) compared to other concentrations (Table 4). Though KN (0.696 µM) with GA_3 (0.867 µM) was superior to BA+GA_3 combinations on shoot elongation (5.0 cm), the per cent response was poor (73%) (Table 6; Plate 1G). Similarly, BAP and GA_3 combination is used for shoot elongation in the micropropagation of Tea clone Iran 100 (Reza *et al*., 2014) and for shoot elongation in apple stocks (Geng *et al*., 2016). Gibberellins are known to increase the cell number, thus increasing the length of internodes (Shan *et al*., 2021).

The rooting experiment was initially tested with IAA, IBA and NAA, whereas rooting was observed only in IBA added medium. The root initiation was observed after 15 days of culture, where IBA at 9.84 µM concentration induced the maximum rooting response (65%), and the number of roots also increased to 4.8 at this concentration (Table 7; Plate 1H). At lower concentration of IBA (1.23 µM) and in phytohormone free medium root induction was not observed. Consistent to our results, IBA was reported as best root inducing phytohormone in *R. tetraphylla* (Faisal *et al*., 2005) and in *Rauvolfia micrantha* (Patil and Jayanthi 1997). Contrarily, NAA was used for root induction in *R. micrantha* (Sudha and Seeni, 1996) and in R. *serpentina* (Roja and Heble, 1996). The combination of NAA and IBA was used for root induction in *R. tetraphylla* (Ghosh and Banerjee, 2003) and in *R. serpentina* (Ahmad *et al*., 2002). The survival rate of the regenerated plantlets gradually reduced with the exposure from culture room to field condition. When the plastic cups were kept in the tissue culture room, after two weeks, the percentage of survival was 96%. After exposure to shade conditions in the field, the survival rate reduced to 80%. Finally, 78% of plants were successfully hardened and established in the field (Figure 1; Plate 2I).

Table 1: Multiple shoot induction response in seedling derived shoot tip explants of *Rauvolfia tetraphylla* L.

Phytohormone	Phytohormone concentration (µM)	Per Cent of Shoot induction	Number of shoots/ explant (Average)	Shoot length in cm (Average)
BA	1.11	45^{d}	2.26^{d}	3.0^{b}
	2.22	60^{bc}	3.64^{bc}	2.8^{c}
	4.44	80^{a}	6.84^{a}	2.2^{ef}
	6.66	65^{b}	3.42^{c}	1.9^{gh}
	8.88	35^{e}	1.52^{e}	1.5^{i}
KN	1.16	40^{de}	1.98^{de}	2.5^{d}
	2.32	58^{c}	2.8^{cd}	3.3^{a}
	4.64	65^{b}	4.0^{b}	2.8^{c}
	6.96	60^{bc}	1.38^{ef}	2.3^{e}
	9.28	21^{f}	1.00^{f}	2.0^{g}

Data represents the observation made after 40 days of inoculation in respective treatment

Table 2: Multiple shoot induction in seedling derived cotyledonary nodal explants of *Rauvolfia tetraphylla* L.

Phytohormone	Phytohormone concentration (µM)	Per Cent of Shoot induction	Number of shoots/ explant (Average)	Shoot length in cm (Average)
BA	1.11	60^{g}	4.53^{e}	2.3^{d}
	2.22	68^{e}	9.12^{cd}	2.1^{f}
	4.44	90^{a}	16.0^{a}	1.92^{g}
	6.66	85^{b}	12.4^{b}	1.6^{h}
	8.88	79^{cd}	10.56^{c}	0.9^{i}
KN	1.16	50^{h}	2.44^{g}	3.31^{a}
	2.32	65^{ef}	3.2^{fg}	3.0^{b}
	4.64	80^{c}	4.0^{f}	2.9^{bc}
	6.96	75^{d}	4.12^{ef}	2.5^{de}
	9.28	65^{ef}	2.16^{gh}	2.0^{fg}

Data represents the observation made after 40 days of inoculation in respective treatment

Table 3: Influence of IAA and NAA with BA (4.44 µM) on multiple shoot induction in seedling derived shoot tip explants of *Rauvolfia tetraphylla* L.

Phytohormone	Phytohormone concentration (µM)	Per Cent of Shoot induction	Number of shoots/ explant (Average)	Shoot length in cm (Average)
IAA	0.15	30^{a}	1.16^{d}	1.1^{b}
	0.29	23^{b}	1.23^{bc}	0.6^{c}
	0.58	20^{c}	1.0^{c}	0.6^{c}
	0.87	10^{d}	1.0^{c}	0.3^{d}
	1.16	Nil	Nil	Nil
NAA	0.14	30^{a}	2.13^{a}	1.5^{a}
	0.27	24^{ab}	1.8^{ab}	0.7^{bc}
	0.54	21^{bc}	1.5^{b}	0.3^{d}
	0.81	12^{cd}	1.0^{c}	0.1^{e}
	1.08	0.7^{e}	1.0^{c}	0.1^{e}

Data represents the observation made after 40 days of inoculation in respective treatment

Table 4: Influence of IAA and NAA with BA (4.44 µM) on multiple shoot induction in seedling derived cotyledonary nodal explants of *Rauvolfia tetraphylla* L.

Phytohormone	Phytohormone concentration (µM)	Per Cent of Shoot induction	Number of shoots/ explant (Average)	Shoot length in cm (Average)
IAA	0.15	60^{a}	4.10^{a}	1.9^{ab}
	0.29	42^{b}	3.11^{b}	1.1^{cd}
	0.58	35^{c}	2.59^{bc}	0.6^{de}
	0.87	23^{d}	1.0^{f}	0.3^{f}
	1.16	10^{e}	1.0^{f}	0.1^{fg}
NAA	0.14	48^{ab}	3.4^{ab}	2.0^{a}
	0.27	40^{bc}	2.0^{d}	1.3^{c}
	0.54	35^{c}	1.8^{e}	0.7^{d}
	0.81	30^{cd}	1.0^{f}	0.1^{fg}
	1.08	18de	1.0^{f}	0.1^{fg}

Data represents the observation made after 40 days of inoculation in respective treatment

Table 5: Influence of amino acids along with BA on multiple shoot induction in seedling derived cotyledonary nodal explants of *Rauvolfia tetraphylla* L.

Amino acids	BA (4.44 µM) + Amino acid (mM)	Per Cent of Shoot induction	Number of shoots/ explant (Average)	Shoot length in cm (Average)
Proline	0.22	94^{c}	19.6^{ef}	2.5^{c}
	0.44	96^{b}	23.72^{d}	2.3^{e}
	0.66	96^{b}	29.18^{b}	1.8^{j}
	0.88	90^{d}	18.5^{f}	1.0^{o}
Methionine	0.17	95^{bc}	12.6^{ij}	2.6^{b}
	0.34	68^{g}	8.0^{lm}	2.7^{a}
	0.51	60^{i}	5.5^{n}	2.0^{h}
	0.68	33^{l}	2.56^{o}	1.3^{m}
Glutamine	0.17	95^{bc}	21.2^{e}	2.0^{h}
	0.34	98^{ab}	26.5^{c}	1.6^{k}
	0.51	100^{a}	34.75^{a}	1.2^{n}
	0.68	92^{cd}	15.2^{gh}	1.0^{o}
Lysine	0.17	80^{f}	16.5^{g}	2.4^{d}
	0.34	85^{e}	13.0^{i}	2.6^{b}
	0.51	82^{ef}	9.32^{l}	2.5^{c}
	0.68	65^{gh}	6.23^{mn}	2.2^{f}
Isoleucine	0.19	65^{gh}	12.5^{ij}	2.1^{g}
	0.38	55^{j}	10.0^{k}	1.9^{i}
	0.57	40^{k}	9.69^{kl}	1.4^{l}
	0.76	30^{lm}	7.8^{m}	1.0^{o}

Data represents the observation made after 40 days of inoculation in respective treatment.

Table 6: Influence of BA and KN with GA_3 on elongation of *in vitro* regenerated shoots in *Rauvolfia tetraphylla* L.

Phytohormone concentrations BA + GA_3 (µM)	Shoot elongation response (%)	Shoot length in cm (Average)	Phytohormone concentration KN + GA_3 (µM)	Shoot elongation response (%)	Shoot length in cm (Average)
0.22+0.289	78^{de}	2.1^{fg}	0.464+0.289	52^{ij}	2.8^{h}
0.22+0.578	81^{cd}	2.5^{e}	0.464+0.578	58^{g}	3.5^{ef}
0.22+0.867	74^{ef}	1.9^{gh}	0.464+0.867	62^{f}	4.0^{c}
0.22+1.156	70^{g}	1.6^{hi}	0.464+1.156	65^{d}	4.8^{ab}
0.22+1.445	65^{h}	1.2^{j}	0.464+1.445	47^{k}	3.5^{ef}
0.44+0.289	80^{d}	3.2^{d}	0.696+0.289	62^{f}	3.1^{g}
0.44+0.578	83^{c}	3.5^{c}	0.696+0.578	67^{c}	3.6^{e}
0.44+0.867	87^{b}	3.9^{b}	0.696+0.867	73^{a}	5.0^{a}
0.44+1.156	100^{a}	4.3^{a}	0.696+1.156	70^{b}	4.6^{b}
0.44+1.445	85^{bc}	3.5^{c}	0.696+1.445	64^{de}	4.0^{c}
0.66+0.289	74^{ef}	2.2^{f}	0.928+0.289	53^{i}	2.8^{h}
0.66+0.578	76^{e}	2.0^{g}	0.928+0.578	56^{h}	3.4^{f}
0.66+0.867	73^{f}	1.7^{h}	0.928+0.867	57^{gh}	3.9^{cd}
0.66+1.156	62^{i}	1.5^{i}	0.928+1.156	53^{i}	3.1^{g}
0.66+1.445	58^{j}	1.0^{jk}	0.928+1.445	45^{l}	2.0^{i}

Data represents the observation made after 30 days of inoculation in respective treatment

Table 7: Influence of IBA on rooting of *in vitro* regenerated shoots in *Rauvolfia tetraphylla* L.

IBA concentrations (µM)	Per Cent of Root induction	Number of roots/explant (Average)
-	-	-
1.23	-	-
2.46	5^{gh}	1.0^{gh}
3.69	12^{g}	1.12^{g}
4.92	22^{f}	1.9^{f}
6.15	35^{e}	2.1^{ef}
7.38	45^{cd}	2.4^{e}
8.61	57^{b}	3.2^{c}
9.84	65^{a}	4.8^{a}
11.07	60^{ab}	4.0^{b}
12.6	48^{c}	2.9^{cd}

Data represents the observation made after 40 days of inoculation in respective treatment

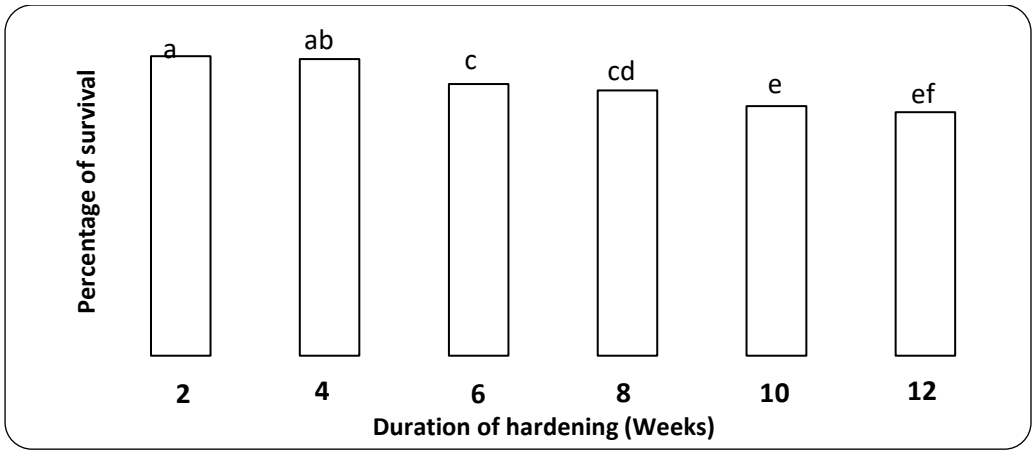

Figure 1: Hardening and survival percentage of the regenerated plantlets of *Rauvolfia tetraphylla* L. in the field

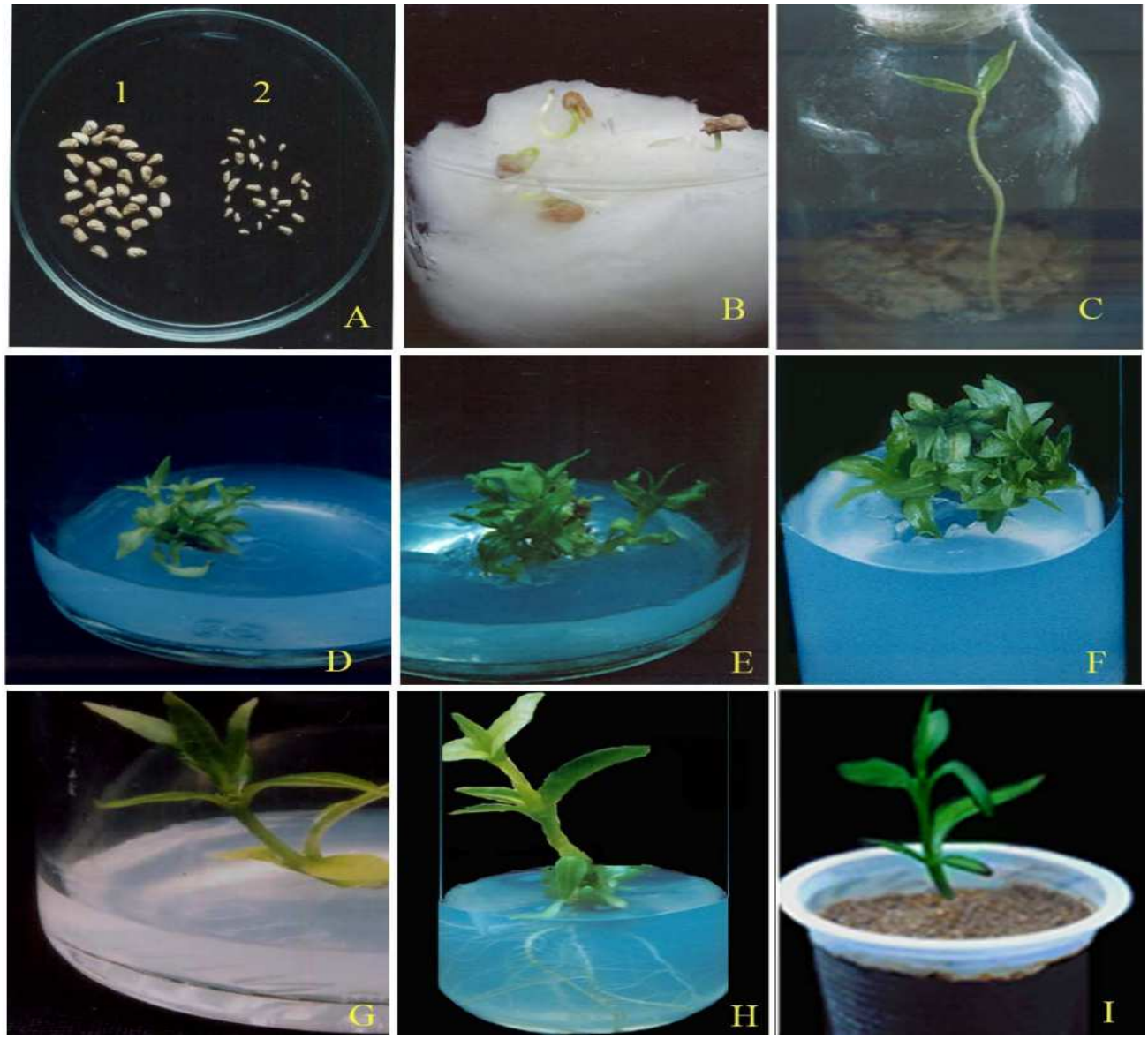

Plate 1: Micropropagation of *Rauvolfia tetraphylla* L. from shoot tip and cotyledonary node explants of the seedlings

A. Seeds collected from the field grown plants (1. Well-developed seeds, 2. Poorly developed seeds), B. Seed germination on moist cotton (after 12 days of inoculation), C. Seed germination in sterile soil mixture (after 18 days of inoculation), D. Multiple shoot induction from shoot tip explant cultured on MS+B_5 medium supplemented with 4.44 µM BA (after 40 days of culture in respective medium), E. Multiple shoot induction from cotyledonary node explant cultured on MS+B_5 medium supplemented with 4.44 µM BA (after 40 days of culture in respective medium), F. Multiple shoot induction from cotyledonary node explant cultured on MS+B_5 medium supplemented with 4.44 µM BA+0.51 mM Glutamine (after 40 days of culture in respective medium), G. Shoot elongation of *in vitro* regenerated shoot MS+B_5 medium supplemented with 0.44 µM BA+1.156 µM GA_3 (after 30 days of culture in respective medium), H. Rooting of *in vitro* regenerated shoots in MS+B_5 medium supplemented with 9.84 µM IBA (after 50 days of culture in respective medium), H. Hardening of regenerated plantlet in sterile soil mixture (at 5th week of hardening).

Conclusion: The present study provides a reliable protocol for micropropagation of *Rauvolfia tetraphylla*, which is endangered in India due to uprooting of this plant for drug preparation. Aseptic seeds were germinated in sterile cotton as well as in soil mixture for explant collection. More number of shoots production was observed in the cotyledonary nodes cultured in MS+B_5 medium added with 4.44 µM BA and 0.51 mM glutamine. Shoot elongation was achieved with BA+GA_3 combination. Rooting of the elongated shoots was attained in the medium containing IBA at 9.84 µM concentration. Hardening and field establishment was done with 12 weeks of acclimatization at 78% success rate. Age of the explants is always a key factor in the success of plant tissue culture, where young explants are the preferred source for maximum regeneration response and this work formed a simple protocol for regeneration from young explants. Further improvements in the micropropagation response may be achieved with cotyledonary node explants in *R. tetraphylla* using other phytohormones and additives in future.

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