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In vitro Propagation of *Gymnema sylvestre*

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Abstract: The need of MS salts for shoot sprouting and proliferation shows the high salt requirement for the growth of *Gymnema sylvestre*. Influence of plant growth regulators, IAA, BA, 2,4-D and kinetin on shoot sprouting was investigated. Synergistic effect of Vitamin B_2 was studied. To overcome phenolic exudation and the effects of antioxidants, the effect of activated charcoal, citric acid and ascorbic acid were investigated. Incorporation of citric acid (100 mg L^{-1}) to the medium prevented phenolic exudation and increased the production of healthy normal shoots and shoot bud differentiation in *Gymnema sylvestre*. There was a considerable improvement in rooting as about 53% shoots could be induced to root on 1/2 MS medium within 45 days with a fairly good length and number of roots per shoot. MS medium containing 1 mg L^{-1} BA+0.5 mg L^{-1} IAA + 100 mg L^{-1} Vitamin B2+100 mg L^{-1} citric acid is best for shoot proliferation and 1/2 strength MS medium with IBA 3 mg L^{-1} is best for root induction.

Key words: Tissue culture, *Gymnema sylvestre*, *in vitro* propagation

INTRODUCTION

Use of *Gymnema sylvestre*, commonly known as periploca of the woods, has increased recently due to the pharmaceutical potential of gymnemic acids, found in its leaves. The various reports on its multiple uses attracted attention for utilization of the plant for its active principle, gymnemic acid. Gymnemic acid has been reported to effect a natural treatment for diabetes mellitus. At present 90% collection of medicinal plants is from the wild. This will not augur the sustainable use of medicinal plants and also it will not fulfill the needs of the majority of the population. *Gymnema sylvestre* natural strands are fast disappearing and are threatened with extinction due to its indiscriminate collection, over exploitation of natural resources for commercial purposes and to meet the requirements of the pharmaceutical industry. The attempt for its production and conventional propagation is hampered due to its poor seed viability, low rate of germination and poor rooting ability of vegetative cuttings. Alternative propagation methods would be beneficial in accelerating large scale multiplication, improvement and conservation of the plant.

Due to lack of proper cultivation practices, destruction of plant habitats and the illegal, indiscriminate collection of plants from these habitats, many medicinal plants are severely threatened. Advance biotechnological methods culturing plant cells and tissues should provide new means of conserving and rapid propagation of valuable, rare and endangered medicinal plants.

The present study describes the *in vitro* propagation, methods of *Gymnema sylvestre*.

MATERIALS AND METHODS

Plant material: Seeds of *Gymnema sylvestre* were collected from one single plant grown in the Kodiakarai forest, Nagapattinam district, Tamil Nadu, India, identified by the taxonomist from Botanical Survey of India, Coimbatore, Tamil Nadu.

Seed culture: The seeds were washed in running tap water for 5 min, treated with 2-3 drops of Tween 40 for 10 min and finally the seeds were washed thrice with sterile distilled water. Seeds were subsequently surface sterilized with 0.1% $HgCl_2$ for 5 min and washed thrice with sterile distilled water. The surface sterilized seeds were cultured on Murasige and Skoog medium supplemented with 2% sucrose and solidified with 0.8% agar (Himedia) (Ashok *et al*., 2002). pH of the medium was maintained at 5.8. Samples were grown at a photoperiod of 16 h light and 8 h darkness at 25±1°C with the light the light intensity of 1000 lux provided by cool white fluorescent lamps during the photoperiod. The resulting seedlings were used as explant source. Seeds and seedlings of *Gymnema sylvestre* are shown in Fig. 1.

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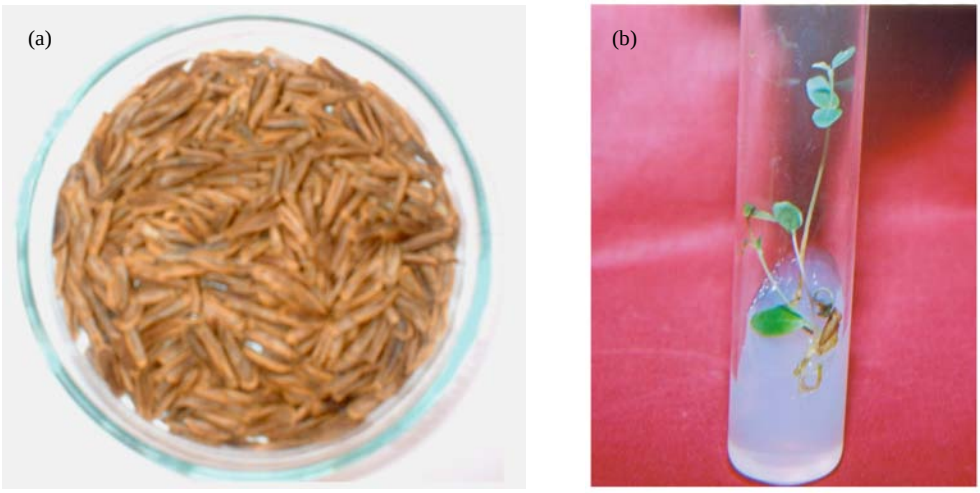


Fig. 1: (a) Seeds and (b) seedlings of *Gymnema sylvestre*

In vitro* propagation of *Gymnema sylvestre

Initiation and multiplication: Auxillary node explants were collected from a healthy plant. Explants were cultured on MS media fortified with various concentrations (mg L^{-1} w/v) of cytokinins either individually or in combination with auxins (concentration ratio was specified in the Table 3) were investigated to optimize salt and hormonal requirements for sprouting and multiple shoot induction. pH of the media was adjusted to 5.8 with 0.1 N NaOH. All culture media contained 3% sucrose (w/v) and solidified with 0.8% agar (Bacteriological grade, Himedia, India (Komalavalli and Rao, 2000), Media was sterilized. Explants were placed vertically in glass tubes (150×25 mm) containing 20 mL of culture medium and plugged tightly with non-absorbent cotton.

Rooting: Shoots (4-5 cm long) regenerated from different explants were excised and individually transferred to MS medium fortified with various concentrations of auxins (specified in the Table 6) (IAA, IBA and NAA) and onto varying strengths of MS salts (full, 3/4, 1/2, 1/4 and 1/8) for root induction. After 50 days in rooting medium, the rooted micro shoots were removed from the culture medium and the roots were washed in sterile distilled water to remove all traces of agar and transferred to soil.

RESULTS AND DISCUSSION

Influence of medium: Among the three different media tested, MS medium was found to be the best basal medium for shoot sprouting (6.8±1.0%), number (3.61±0.51) and length (2.83±0.04) of shoots with little callus formation followed by B5 and white medium (Table 1, 2). The shoot buds which sprouted on white medium showed only limited development even if they were maintained for longer period in culture as in previous report (Komalavalli and Rao, 2000). Shoots did not develop on any media in the absence of cytokinin. *In vitro* propagation of plants belonging to Asclepidiaceae have also been shown to have optimum overall growth in MS medium (Chi Won and John, 1985; Patnaik and Debata, 1996; Komalavalli and Rao, 1997). Thus the degree of growth and differentiation varied considerably with the medium constitution (Shekhawat *et al*., 1993; Das *et al*., 1996). The need of MS salts for shoot sprouting and proliferation shows the high salt requirement for the growth of *G. sylvestre*.

Table 1: Influence of various media supplemented with 0.5 mg L^{-1} BA on shoot bud induction from axillary node explants of *Gymnema sylvestre* after 30 days

Medium	Shoot sprouting frequency (%)	Shoot No./ responding explant	Shoot length (cm)
MS	53±3.2	2.3±0.32	2.24±0.01
White	1±3.4	0.3±0.12	0.10±0.03
B_5	42±2.6	1.0±0.06	1.06±0.03

Values are Mean±SE of 10 replicates

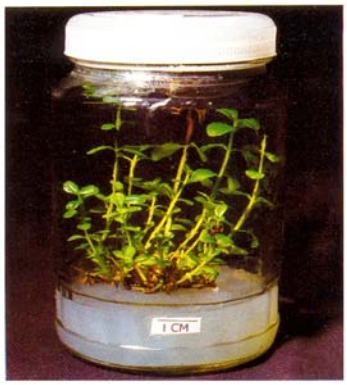
Table 2: Influence of various media supplemented with 1 mg L^{-1} BA on shoot bud induction from axillary node explants of Gymnema sylvestre after 30 days

Medium	Shoot sprouting frequency (%)	Shoot No./ responding explant	Shoot length (cm)
MS	68±1.00	3.61±0.51	2.83±0.04
White	6±2.35	0.50±0.01	0.60±0.03
B_5	53±5.25	1.83±0.32	1.56±0.06

Values are Mean±SE of 10 replicates

Influence of plant growth regulators: Axillary shoot sprouting was initiated at all concentrations of BA alone and in combinations of BA and IAA, 2, 4-D and kinetin, 2, 4-D and BA, when compared to kinetin combinations.

Shoot initiated from axillary node explant



Multiple shoots developed from
Gymnema sylvestra

Multiple shoots developed
From *Gymnema sylvestra*
(MS medium with riboflavin)

Fig. 2: Multiple shoot developed from *Gymnema sylvestre* axillary node explant, (a) Shoot initiated from

IAA and BA combinations were effective (Table 3). In the present study, combined BA (1 mg L^{-1}) and IAA (0.5 mg L^{-1}) in the culture medium promoted the shoot sprouting frequency and multiple shoot induction whereas Komalavalli and Rao (2000) reported that high concentration of IAA and IBA (above 0.1 mg L^{-1}) induced callus, occasionally root formation occurred at excised ends and prevented multiple shoot induction. In IAA and BA combinations, the shoot number was also increased (Fig. 2). Combinations of 2,4-D and BA was also tested. Shoots were sprouted in all the concentrations. When compared to IAA and BA combinations, the propagation rate was very low. Komalavalli and Rao (2000) reported auxin combined with either BA or kinetin above resulted in low propagation rate. Combination of 2,4-D and BA induced callus formation.

Reddy *et al*. (1998) reported that kinetin did not improve significantly the shoot length and the number of proliferating shoots. In the current report, combined, 2,4-D (1 mg L^{-1}) and kinetin (0.5 mg L^{-1}) in the culture medium promoted the growth of the shoots. When compared to kinetin, BA significantly improved the shoot length and the No. of proliferating shoots. MS medium containing BA was more effective than kinetin for inducing proliferation of axillary buds as in previous report (Reddy *et al*., 1998). Results of BA alone supplemented medium and combinations BA and IAA, BA and 2 ,4-D, kinetin and 2, 4-D supplemented medium were analysed. Results were shown in the Table 3. From the results it was inferred that combinations of IAA (5 mg L^{-1}) and BA (1 mg L^{-1}) increased the propagation rate, whereas Komalavalli and Rao (2000) reported that the shoot number increased when auxins (both NAA and IBA at 0.1 mg L^{-1}) combined with BA and kinetin. Superiority of BA and kinetin in combination has been found in micropropagation of other woody perenials (Das *et al*., 1996; Komalavalli and Rao, 1997). The shoots formed exhibited phenolic exudation and leaf drop, which inhibited the conversions of multiple buds into shoots as in earlier report (Komalavalli and Rao, 2000).

Table 3: Influence of plant growth regulators on multiple shoot induction from axillary node of *Gymnema sylvestre* on MS medium after 30 days

Growth regulator (mg L^{-1})	Shoot sprouting frequency (%)	Shoot No./ responding explant	Shoot length (cm)
2, 4-D kinetin			
1.0+0.5	70±1.2	5.0±0.11	2.1±0.01
2.0+0.5	62±2.01	3.2±0.02	1.7±0.03
1.5+0.5	58±1.57	4.0±0.27	1.0±0.01
1.5+0.5	42±1.06	2.0±0.38	1.1±0.06
1.0+1.5	52±1.31	4.0±0.21	2.0±0.01
2.0+1.5	48±1.06	3.1±2.23	1.0±0.05
0.5+1.5	33±2.60	1.2±0.01	1.1±0.03
1.5+1.5	45±1.30	1.5±0.11	2.0±0.01
1.0+1.0	75±3.20	70.0±0.22	3.0±0.02
2.0+1.0	38±1.63	2.0±0.33	1.8±0.01
0.5+1.0	60±1.60	3.8±0.26	2.0±0.04
1.5+1.0	42±2.60	2.1±0.31	0.5±0.01
1.0+2.0	35±1.57	1.5±0.10	2.0±0.03
2.0+2.0	22±1.21	1.0±0.01	0.4±0.01
0.5+2.0	28±3.0	1.1±0.04	0.5±0.01
1.5+2.0	38±1.01	1.5±0.08	0.5±0.02
2, 4-D+BA			
0.5+0.5	61±1.70	5.2±0.01	2.0±0.02
1.0+0.5	40±1.06	2.0±0.10	1.0±0.05
1.5+0.5	32±1.20	1.4±0.03	2.0±0.01
2.0+0.5	28±1.30	1.0±0.01	0.5±0.01
0.5+1.0	73±2.20	5.6±0.12	1.8±0.03
1.0+1.0	62±1.31	3.0±0.01	1.3±0.01
1.5+1.0	32±1.28	1.5±0.01	1.0±0.05
2.0+1.0	24±1.10	1.0±0.05	0.5±0.01
0.5+1.5	5.8±1.57	4.0±0.10	2.4±0.09
1.0+1.5	55±1.23	3.8±0.11	1.8±0.01
1.5+1.5	63±1.21	3.1±0.29	2.0±0.01
2.0+1.5	60±1.01	4.0±1.17	2.2±0.02
0.5+2.0	46±1.23	1.3±1.10	1.0±0.02
1.0+2.0	53±1.06	3.9±0.21	2.0±0.01
1.5+2.0	42±2.06	2.3±0.24	1.1±0.01
2.0+2.0	41±1.31	2.0±0.10	0.5±0.02
IAA+BA			
0.5+0.5	68±2.01	3.0±0.03	2.0±0.03
1.0+0.5	40±1.30	2.10±0.01	1.1±0.01
1.5+0.5	46±0.28	2.40±0.08	1.0±0.05
2.0+0.5	42±1.06	3.0±0.10	2.0±0.01
0.5+1.0	86±1.02	10.3±0.08	2.1±0.01
1.0+1.0	57±2.60	3.5±0.21	1.1±0.05
1.5+1.0	42±1.20	2.0±0.30	1.0±0.01
2.0+1.0	52±1.27	4.0±0.27	2.1±0.02
0.5+1.5	48±2.00	1.5±0.12	0.5±0.04
1.0+1.5	41±1.84	2.0±0.08	1.0±0.01
1.5+1.5	54±1.30	4.2±0.16	1.7±0.05
2.0+1.5	45±1.10	2.0±0.27	1.0±0.01
0.5+2.0	41±1.14	2.3±0.10	1.1±0.02
1.0+2.0	32±2.00	1.1±0.02	1.0±0.01
1.5+2.0	38±1.83	1.0±0.08	0.5±0.01
2.0+2.0	42±1.70	2.0±0.14	1.4±0.02
BA			
0.5	53±3.20	2.8±0.14	1.0±0.05
1.0	68±1.00	3.8±0.10	2.1±0.02
1.5	63±1.50	2.3±0.11	1.4±0.01
2.5	43±1.75	1.12±0.19	6.5±0.13

Values are mean±SE of 10 replicates

Influence of vitamin B_2 (Riboflavin): Synergistic effect of Vitamin B_2 was studied after determining the optimum cytokinin and auxin levels for shoot sprouting to improve the quality of the shoots. Different concentrations of Vitamin B_2 was added into the medium. Vitamin B_2 100 mg L^{-1} significantly improved the shoot sprouting frequency, shoot number and shoot length, prevented yellowing of leaves and reduced the callus formation at the cut end of the axillary node explants (Table 4). Thus Vitamin B_2 was found to be necessary for the speedy proliferation of axillary buds and to improve the quality and quantity of *Gymnema sylvestre*. Komalavalli and Rao (2000) reported the influence of complex extracts in *G. sylvestre in vitro* propagation.

Table 4: Influence of Vitamin B_2 on multiple shoot induction in 20 day old seedling from axillary node explant of *Gymnema sylvestre* on MS media fortified with 1 mg L^{-1} BA+0.5 mg L^{-1} IAA after 30 days

Concentration of vitamin B_2 (mg L^{-1})	Shoot No./ responding explant	Shoot length (cm)
100	45±0.58	5.5±0.18
75	12±0.31	2.1±0.11
50	7±0.28	1.7±0.07
25	8±0.10	1.0±0.03

Values are mean±SE of 10 replicates

Table 5: Influence of antioxidants on multiple shoot induction from 28 day old seedling axillary node of *Gymnema sylvestre* on MS medium supplemented with 1 mg L^{-1} BA+0.5 mg L^{-1} IAA+100 mg L^{-1} vitamin B_2 after 30 days

Antioxidant (mg L^{-1})	Shoot No./ explant	Shoot length (cm)
Citric acid		
50	17.80±1.20	4.3±0.021
100	28.30±0.91	5.5±0.022
200	10.12±1.03	4.9±0.015
Activated charcoal		
10	7.50±0.01	3.6±0.035
20	4.30±0.16	4.1±0.022
50	8.90±0.05	4.3±0.021
Ascorbic acid		
10	12.01±0.27	4.6±0.016
20	10.60±0.15	4.3±0.026
50	9.50±0.04	3.0±0.012

Values are Mean±SE of 10 replicates

Influence of antioxidants: To overcome phenolic exudation, the effect of activated charcoal, citric acid, ascorbic acid were investigated. Incorporation of citric acid (100 mg L^{-1}) to the medium prevented phenolic exudation, increased the production of healthy normal shoots and shoot bud differentiation in *G. sylvestre* (Table 5) as in earlier report (Komalavalli and Rao, 2000). The promotive effect of citric acid was reported by Shekhawa *et al.* (1993) in Prosopris cineraria. Activated charcoal added to the culture medium reduced the number of shoots per explant, whereas it is reported to have beneficial effects in *Eucalyptus tereticornis* (Das and Mitra, 1990) and *Sesbania sesban* (Shankar and Mohan Ram, 1999). Reduction in shoot number may be due to adsorption of essential compounds besides the inhibitory factors (Weatherhead *et al.*, 1979). Ascorbic acid added into the culture medium did not give significant results. In marked contrast, ascorbic acid (100 mg L^{-1}) proved to be better for multiple shoot

Rooting | Roots of *in vitro* derived plantlets

Roots of *in vitro* derived plantlets | Plantlets transferred to soil

Fig. 3: Rooting of *in vitro* derived *Gymnema sylvestre* plantlets

Table 6: Influence of IBA concentration and MS medium strength on rooting of *Gymnema sylvestre* derived shoots after 45 days

MS (Medium Strength)	IBA (mg L^{-1})	Percentage of shoots rooted	Root length (cm)	Shoot with basal callus (%)
1	0	0.00	0.00	0.00
1	0.5	2.50±0.01	3.9±0.13	6.30±1.23
1	1	3.00±0.03	4.8±0.20	6.80±1.12
1	2	17.00±0.10	1.3±0.22	35.00±0.51
1	3	23.10±0.14	6.1±0.31	38.00±0.89
1	5	12.30±0.20	2.8±0.11	58.00±0.52
3/4	1	10.21±0.15	4.2±0.25	31.00±0.75
3/4	3	28.26±0.30	6.3±0.27	22.00±1.60
3/4	5	20.10±0.13	5.1±0.33	20.00±0.81
1/2	0	8.90±0.11	2.1±0.13	0.00
1/2	1	28.90±0.18	6.8±0.33	0.98±0.10
1/2	3	53.00±0.30	9.2±0.38	1.60±0.02
1/2	5	20.30±0.18	5.3±0.28	8.15±0.11
1/4	1	15.00±0.20	3.8±0.10	3.81±0.04
1/4	3	27.00±0.41	6.5±0.33	2.10±0.02
1/4	5	13.20±0.15	1.2±0.21	5.15±0.02
1/8	1	12.00±0.22	1.8±0.31	2.10±0.04
1/8	3	21.20±0.35	4.6±0.33	1.90±0.02
1/8	5	10.21±0.11	3.5±0.27	4.80±0.06

Values are Mean±SE of 10 replicates

induction and to sustain overall growth in the related species of *Tylophora* and *G. elegans* (Komalavalli and Rao, 1997). Thus the culture of shoots *in vitro* in the presence of citric acid completely inhibited the explant browning by controlling the phenolic oxidation and enhanced multiple shoot induction in *G. sylvestre*.

Rooting of *in vitro* desired shoots: The shoots (4-6 cm) were cultured on various strengths of MS basal medium fortified with different concentration of IBA (Table 6). Komalavalli and Rao (2000) reported that IBA was most effective for root induction and survival in the field with minimal callus formation. IBA improved the overall growth of roots and the reduced the time duration of root induction. There was a considerable improvement in rooting as about 53% shoots could be induced to root on half strength MS medium within 45 days with a fairly good length and No. of roots per shoot. Photographs of *in vitro* derived roots and plantlets are shown in Fig. 3. Thus improvement in overall quality of roots was observed at half strength MS medium as in the earlier report (Komalavalli and Rao, 2000). In contrast, a drastic inhibitory effect on both root formation and elongation was noted at 1/4, 1/8, strength MS medium. No significant difference was observed regarding *G. elegans* and *G. sylvestre* (Komalavalli and Rao, 2000). In conclusion, the outlined procedure offers a potential system for improvement, conservation and mass propagation of *G. sylvestre* from pre-existing meristems of seedling explants. MS medium containing 1 mg L^{-1} BA+0.5 mg L^{-1} IAA+100 mg L^{-1} Riboflavin (Vitamin B_2)+100 mg L^{-1} citric acid is the best for shoot proliferation. MS basal medium supplemented with 3 mg L^{-1} IBA is the best for root

induction. The *in vitro* propagation of *G. sylvestre* is not very different from that of *G. elegans* described earlier and may be applicable for other economically important wood climbers as well. Komalavalli and Rao (2000) reported that MS medium containing 1 mg L^{-1} BA+0.5 mg L^{-1} kinetin+ 0.1 mg L^{-1} NAA+100 mg L^{-1} malt extract+100 mg L^{-1} citric acid, the best for shoot proliferation.

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