

## ***In vitro* multiplication of *Gymnema sylvestre* R.Br. – An important medicinal plant**

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The objective of this study was to develop a rapid system for regenerating shoots from mature nodal explants of *Gymnema sylvestre*, an useful antidiabetic medicinal plant. Single node stem explants were inoculated on MS media containing different combinations of 6-benzylaminopurine (BAP) or kinetin with naphthaleneacetic acid (NAA). Maximum number of shoots (7 per explant) were observed on the medium containing BAP (5 mg/l) and NAA (0.2 mg/l). Regenerated shoots were rooted on MS half-strength medium without supplementing any growth regulator.

*GYMNEMA SYLVESTRE* R.Br. is a valuable medicinal plant belonging to the family Asclepiadaceae, and distributed over most parts of India and Africa. It is well recognized in traditional medicine as a remedy for diabetes mellitus, stomachic and diurea<sup>1,2</sup>. The plant is popularly known as 'gurmar' for its distinctive property of temporarily destroying the taste of sweetness<sup>3</sup>. The complex mixture of the active principles, named gymnemic acids were isolated from *Gymnema* leaves<sup>4-10</sup>. Recently, the plant has been recognized by natural products industry in North America and Europe and a number of commercial, over-the-counter herbal products are now available that contain varying amounts of *Gymnema*<sup>11</sup>. Hence, it is felt that there is a great need for cultivation of this important medicinal plant.

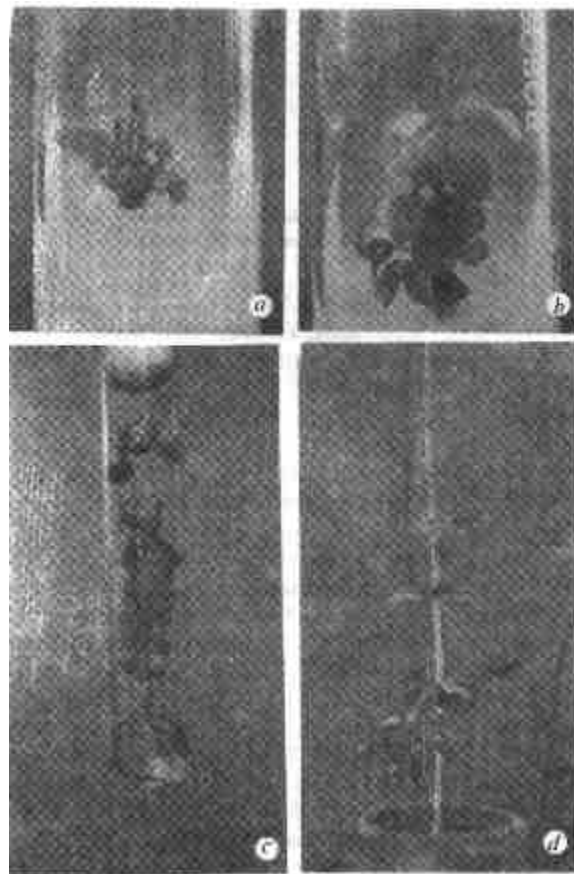
*Gymnema* is propagated by seed germination. One of the constraints in this conventional propagation is the very short span of seed viability. No alternative mode of multiplication is available to propagate and to conserve genetic stock of this useful plant. Tissue culture offers an effective alternative method for rapid multiplication of desirable clones. Relatively few studies have been published on the mass *in vitro* clonal propagation of some milk weeds like *Tylophora*<sup>12</sup>, *Hemidesmus*<sup>13</sup>, *Asclepias*<sup>14</sup> which described the use of adventitious shoot regeneration. Micropropagation by axillary bud proliferation has proved to be the most reliable method for large-scale production of many forest and medicinal plants<sup>15</sup>. The present investigation was undertaken with the aim to optimize *in vitro* conditions for mass

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multiplication of this medicinal plant having potential antidiabetic properties.

Small tender twigs were collected from 5 to 6-year old wild grown mature plants, cut into 1–1.5 cm nodal segments and used as explants for the induction of multiple shoots. Explants were washed thoroughly under running tap water for 15 min and treated with a surfactant Tween-20 (two drops per 100 ml solution). Later they were surface-sterilized with 1% fungicide (w/v) (carbendazim) for 30 min, followed by rinsing in 1% mercuric chloride (w/v) for 5 min and washed thrice with sterile distilled water. Under a laminar flow, cabinet explants were inoculated aseptically on Murashige and Skoog<sup>16</sup> (MS) medium supplemented with various concentrations of cytokinins, kinetin and 6-benzylaminopurine (BAP), and auxins indoleacetic acid (IAA) and naphthaleneacetic acid (NAA) alone or in combinations. Media were adjusted to pH 5.7–5.8 and 0.8% (w/v) agar (CDH, Mumbai) was added before autoclaving at



**Figure 1.** *a*, Formation of multiple shoots from mature nodal explant on MS medium with 5 mg/l BAP + 0.2 mg/l NAA; *b*, Elongation of multiple shoots on 0.2 mg<sup>-1</sup> BAP + 0.2 mg<sup>-1</sup> NAA; *c*, Profuse rooting of *in vitro* initiated shoot on auxin-free 1/2 strength MS basal medium; *d*, Potted *in vitro* raised plantlet after six weeks.

**Table 1.** Effect of different PGR in MS medium on induction of multiple shoots from mature nodal explants of *Gynenema sylvestre*. Observations after 10 weeks

Plant growth regulator (mg/l)			Frequency of shoot regeneration (%)	Mean number of shoots/explant	Mean length of the shoot (cm)	Basal callus
BAP	Kin	NAA				
0.2	-	0.2	83.7 <sup>a</sup>	1.0 <sup>cd</sup>	7.2 <sup>b</sup>	-
0.2	0.2	0.2	70.1 <sup>b</sup>	1.0 <sup>cd</sup>	7.0 <sup>b</sup>	+
1.0	-	-	46.4 <sup>f</sup>	1.0 <sup>cd</sup>	5.6 <sup>abc</sup>	+
1.0	-	0.2	52.4 <sup>e</sup>	1.2 <sup>cd</sup>	6.2 <sup>ab</sup>	+
1.0	0.5	0.2	55.5 <sup>d</sup>	1.0 <sup>cd</sup>	6.8 <sup>a</sup>	+++
-	0.5	0.2	30.3 <sup>g</sup>	1.0 <sup>d</sup>	3.2 <sup>d</sup>	++
-	1.0	0.2	32.1 <sup>g</sup>	1.6 <sup>cd</sup>	5.2 <sup>abc</sup>	+++
-	2.0	0.2	47.3 <sup>e</sup>	2.2 <sup>cd</sup>	5.7 <sup>ab</sup>	+++
2.0	-	-	47.1 <sup>f</sup>	2.7 <sup>c</sup>	3.8 <sup>cd</sup>	++
2.0	-	0.2	72.3 <sup>b</sup>	5.4 <sup>b</sup>	4.6 <sup>bcd</sup>	+
5.0	-	-	58.3 <sup>d</sup>	4.3 <sup>b</sup>	5.7 <sup>abc</sup>	++
5.0	-	0.2	70.5 <sup>b</sup>	7.4 <sup>a</sup>	6.2 <sup>ab</sup>	++

Number of (+) signs indicates the degree of callusing.

Values represented in the table are the mean of 20 h replicates.

Mean values followed by the same letter are not significantly different at  $P \leq 0.05$  (Student-Newman-Keul's test).

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**Table 2.** Rooting efficiency of *in vitro* raised shoots of *Gymnema sylvestre* in MS medium. Observations: After six weeks

MS medium	Frequency of root regeneration (%)	Mean no. of roots	Mean length of the root (cm)
Full-strength	46.0 ± 0.7	7.6 ± 0.3	2.3 ± 0.4
Half-strength	74.2 ± 0.4	28.5 ± 0.5	5.6 ± 0.7
Quarter-strength	52.6 ± 0.9	11.4 ± 0.6	3.5 ± 0.5

Values represented above are the mean of 20 replicates.

1.06 kg/cm<sup>2</sup> pressure for 15 min. After inoculation in test tubes (25 × 150 mm), cultures were maintained at 25 ± 2°C under 16 h daily illumination with fluorescent light (15 µE m<sup>-2</sup>s<sup>-1</sup>). In all experiments 25 replicates were used and each experiment was repeated at least three times.

Isozyme (peroxidase) analysis was performed by macerating 1 g leaf material collected from mature parent plants and 1½-year-old tissue culture raised plants, growing in the same environment. Leaf tissue was macerated in a pre-chilled mortar by adding 3 ml of 0.1M phosphate buffer (pH 7). Extract was centrifuged at 18,000 g for 15 min at 4°C. The supernatant was used as an enzyme extract<sup>17</sup>. SDS-PAGE was performed<sup>18</sup> using 12% polyacrylamide gels. 8 µg of protein was loaded in each slot. Proteins were separated by supplying a constant voltage of 200 V and 30 amp at 4°C, for 5 h. Gels were incubated in a staining solution containing benzidine (2.08 g), acetic acid (18 ml), 3% hydrogen peroxide (100 ml) and water (80 ml). When the blue-coloured bands appeared, the reaction was arrested by immersing the gel in 300 ml of 7% acetic acid solution for 10 min.

Preliminary studies proved that nodal explants on BAP with NAA combination responded better than those on BAP with IAA. Induction of multiple shoots was achieved from axillary regions with BAP and NAA, 5–6 weeks after inoculation with an average of 7 shoots per explant. Among all the concentrations tested, the best response was noticed with 5 mg/l, BAP + 0.2 mg/l NAA. Explants inoculated at higher concentrations of BAP (more than 5 mg/l) alone or in combination with NAA produced clumps of highly-reduced shoots with smaller leaves. Patnaik and Debata<sup>19</sup> found a similar response in *Hemidesmus indicus*, where such abnormal shoots were grown normally when subcultured on low concentrations of BAP. However, in the present study these shoots did not survive even after subculturing on to the medium containing low concentrations of BAP or GA<sub>3</sub>. While multiple shoots originated from leaf axil, the stem portion below the node formed callus in many treatments (Table 1). Similar observations were made in other plants<sup>20,21</sup> also. In the present study, kinetin at different concentrations did not improve the number of proliferating shoots as reported by Purohit and Dave<sup>22</sup>. Shoot length also did not improve significantly when kinetin was added to the medium. After 7–8 weeks, when regenerated shoots attained a length of more than 3 cm, they were excised and planted on full-strength and half-strength MS basal medium with and without supplementing various auxins (IAA, IBA and NAA). In cultures where the shoots were inoculated on auxin-free half-strength MS basal medium, root primordia emerged from the shoot base 15–20 days after implantation and subsequently developed into roots without basal callus (Table 2). Nevertheless when auxins were added to the medium, callus was formed from the shoot base, which did not favour root formation.

For acclimatization, plantlets were removed from rooting medium 8 weeks after root initiation, and

**Figure 2.** Banding pattern of peroxidase isozyme in leaf tissues of *G. sylvestre* (L–R) 1–3, mature parent plants; 4–6, regenerated plants.

transferred to fresh tubes containing autoclaved tap water. After 8–10 days, plantlets were subsequently transferred to plastic pots (9 × 9 cm) containing autoclaved soilrite covered with perforated polythene bags to maintain humidity and were kept under culture room conditions for about 7 days. After three weeks, polythene bags were removed and pots were transferred to the garden, and placed under shade till the new leaf appeared. Then they were planted under normal garden conditions. The transplantation success was about 75%. Isozyme (peroxidase) profile showed no variation between parent plants and *in vitro* raised plants (Figure 2).

Direct shoot multiplication is preferable for generating true-to type plants than callus regeneration, where possibilities of chromosomal variations are high. This report helps to multiply elite genotypes of this useful medicinal plant.

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