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Somatic Embryogenesis and Plant Regeneration from Cell Suspension Culture of *Gymnema sylvestre* (Retz) R. Br. Ex Roemer & Schultes

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Abstract

We have optimized a system for the somatic embryogenesis via embryogenic suspension cultures of *Gymnema sylvestre*. Callus cultures were induced on MS medium with growth regulators (2,4 -D 0.5 mg/l (or) NAA 1.0 mg/l) and 10% coconut water. They were transferred into MS liquid medium containing NAA 1.0 mg/l, BA 1.0 mg/l, 3.0% sucrose (w/v), 10% coconut water, citric acid 1 mg/l and glutamine 10 mg/l for somatic embryogenesis from callus. Globular, heart, torpedo and cotyledonary stage of embryos were observed in suspension cultures after 8 weeks. The maturation embryos were significantly affected by growth regulators and photoperiod. Five to seven percent of embryos formed plantlets on semisolid medium containing basal MS medium with B5 vitamin, 3.0% sucrose and 0.8% agar (w/v). All plantlets established in the field exhibited morphological characters similar to those of the mother plant.

Keywords: *Gymnema sylvestre;* MS medium; embryogenic callus; cell suspension culture; somatic embryos; plant growth regulators.

1. Introduction

Plant biotechnology offers an opportunity to exploit the cell, tissue, organ or entire plant by growing them *in vitro* and to genetically manipulate them to get the desired compounds. Since the world population is increasing rapidly, there is extreme pressure on the available cultivable land to produce chemicals from plants and the available land should be used effectively. Hence, it is appropriate to develop modern technologies leading to plant improvement for better utilization of the land to meet the requirement. Equally important is the conservation of wild germplasm for posterity while maintaining genetic stability. Compared to indirect somatic embryogenesis via callus, direct somatic embryogenesis from organized tissues has shown greater genetic and cytological fidelity [1]. Plant regeneration can be achieved in two ways: through organogenesis and somatic embryogenesis. Here, we report the indirect somatic embryogenesis and plant regeneration from leaf segments of field grown plants.

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Gymnema sylvestre (Retz) R.Br. Ex Roemer & Schultes, a herb belongs to the Asclepiadaceae family commenced 100 years ago, has been employed to control diabetes by traditional medicinal practitioners of India for several centuries. *Gymnema sylvestre* is well-recognized in traditional medicine as a remedy for diabetes mellitus and used in folk, ayurvedic and homeopathic systems of medicine [2]. It brings blood glucose homeostasis through increased serum insulin levels provided by repair or regeneration of the endocrine pancreas [3]. There are only a few reports available on *in vitro* propagation of *G. sylvestre* using different explants. Micropropagation was observed in MS medium supplemented with BA (5.0 mg/l) and NAA (0.2 mg/l) which could induce 7 shoots/explant and best root induction in ½MS without growth regulators [4]. Recently reported *in vitro* callus showed that the active compounds, gymnemic acid and gymnemagenin, were present in sufficiently large amount in the cultured undifferentiated cells [5] and external phytohormone, shaking speeds, pH of the medium played important roles in growth and gymnemic acid production in suspension culture [6]. We have recently published a review of the pharmacological activities, a phytochemical investigation and *in vitro* studies of *G. sylvestre* [7]. The *G. sylvestre* leaf and callus extracts reduced blood sugar and lipid profiles such as cholesterol, triglyceride, HDL, LDL, VLDL in alloxan-induced diabetic Wistar rats [8]. Recently, we conclude that the effect of *G. sylvestre* leaf and callus extracts as strong herbal remedies is confirmed in this study, and suggest that they may be capable of fully restoring pancreatic β-cells function and thus curing type I diabetes and showing anti hyperglycaemic activity [9]. The present study attempts at optimizing somatic embryogenesis of *Gymnema sylvestre* have apparently been successful. This paper identified factors which are important for the successful standardization of somatic embryogenesis in *Gymnema sylvestre* via cell suspension cultures. Development of somatic embryogenesis system would be important in facilitating conservation and genetic engineering of this valuable medicinal plant.

2. Materials and Methods

Gymnema sylvestre plants (GS) were collected from the Pachamalai hills, Tiruchirappalli, Tamil Nadu, and maintained in the plant science garden, Bharathidasan University, Tiruchirappalli, India.

The leaf explants were collected from field grown plants of *G .sylvestre*. The collected explants were washed thoroughly in running tap water for 20 min and then washed with 2% Teepol solution (v/v) (Reckitt Benckiser, India) and continuously washed with sterile distilled water. Under aseptic condition, the washed explants were immersed in freshly prepared 70% alcohol (v/v) for 15 sec and then rinsed with the sterile distilled water. Then explants were disinfected with 3% sodium hypochlorite (v/v) for 2 min. Finally explants were treated with 0.1% mercuric chloride for 2 min then rinsed with sterile distilled water. After sterilization explants were dissected (sterile blade) into pieces of 10mm X 10mm and inoculated into the MS culture medium [10]. MS medium supplemented with sucrose (3%, w/v), agar (0.8 % w/v), BA (0.5 - 2.0 mg/l), KN (0.5 - 2.0 mg/l) in combination with 2,4-D (0.01 - 4.0 mg/l), NAA (0.01 - 4.0 mg/l), citric acid (0.5 - 2.0 mg/l), glutamine (5.0 - 20.0 mg/l) with 10% coconut water were tested in suspension culture medium for embryo maturation, plant regeneration and germination from leaf explants callus. The media pH was adjusted to 5.8 with 0.1N NaOH or HCl before autoclaving at 121°C for 15 min. For suspension culture, 100 mg of friable callus were transferred into 250 ml conical falsk which containing 50 ml of liquid medium. The suspension cultures were incubated in the dark on a rotary shaker at 90 rpm. Solid medium culture was kept in a 16h light/8h dark cycle at 25 ± 2°C.

Conversion of mature cotyledonary stage of embryo into plantlets was attempted by transferring them into MS solid medium with B5 vitamin, 3.0% sucrose, 0.8% agar (w/v) and subcultured every week unless otherwise medium changes to brown colour stated. The regenerated

plants were transferred into small plastic pots containing vermiculite and kept in a culture room for 2 weeks (25 ± 2°C). The plantlets were irrigated once in 3 days and fertilized with nutrient solution (NPK 17:17:17) at weekly intervals.

Only data which showed advantageous effect were included in the tables and presented in mean of explants per treatment and repeated three times. 30 replicates were used and repeated thrice, callus initiation, suspension culture observations were recorded on the number response of globular, heart and torpedo stages embryo induction and number of embryo germination per cotyledonary stage. All the treatments were statistically analyzed by Duncan's Multiple Range Test (DMRT) [11].

Abbrevations: BA: Benzyladenine, KN: Kinetin, CA: Citric acid, Glu: Glutamine,CW: Coconut water, NAA: α -Naphthalene acetic acid, 2,4-D: Dicholoro phenoxy acetic acid

3. Results and Discussion

3.1 Callus initiation

Leaf explants of *Gymnema sylvestre* was grown on MS medium without growth regulators formed the callus dry weight of 0.74 g/l. Callus formation on MS medium has also been reported in *Melia azedarach* [12] and *Holostemma ada-kodien* [13]. Recently, we have reported on callus production in different media such as MS, SH, WPM and B5, among which MS with auxins and cytokinins were suitable for callus production [14]. Callus was initiated on day 12 of leaf explant culture. The vigorous callus proliferation was observed after 25 days of the culture (Table 1). The maximum callus induction (98.4%) was observed on MS medium supplemented with 2,4-D (0.5 mg/l) or NAA (1.0 mg/l) and 10% coconut water. In this view of Zimmerman[15], removal of auxin from the culture medium is vital to inactivate several genes or to synthesis new gene product essential for the successful completion of embryo development. 2,4-D has proved to be useful to obtain a somatic embryogenesis response in a number of plant species [16]. In addition, 2, 4-D even at low concentration containing medium with BA significantly influenced embryogenesis [17]. Simillar data was observed in *G. Sylvestre* somatic embrogenesis experiment. All callus cultures were observed for callus initiation date, fresh weight, dry weight, biomass and the nature of callus before transferring to suspension culture (data not shown).

3.2 Phenolic excretion

The maximum somatic embryogenic callus induction was observed in *G. sylvestre* at 25th day and the phenolic excretion significantly increased. In order to control phenolic exudation from leaf callus which considerably reduced somatic embryo stage and germination, various antioxidant such as glutamine and citric acid at different concentrations (0.5 - 2.0 mg/l), glutamine (5.0 - 20.0 mg/l) were fortified in the medium containing 2,4-D or NAA with 10% coconut water. During the phenolic excretion, the embryogenic callus fresh and dry weight were significantly reduced comparing without phenolic excretion. Kawahara and Komamine[18] reported the effect of exogenous auxin on the expression of polarities in the early stages of somatic embryogenesis. Recently reported, NAA (0.2 mg/l) and 2,4-D (0.2 mg/l) to induce embryogenic callus from *Chorispora bungeana* in solid media [19].

3.3 Establishment of cell suspension cultures and embryo formation

The embryogenic friable callus was obtained on MS solid medium supplemented with 2, 4-D (0.5 mg/l) or NAA (1.0 mg/l) and 10% coconut water (Figure 1a). Suspension cultures could induce the higher number of somatic embryos than solid culture. Simillar results have been reported in *Holostemma ada-kodien* [13]. The developed globular embryo callus was transffered into MS liquid medium containing 2,4-D (0.5 mg/l) or NAA (1.0 mg/l) combination with BA (0.5 mg/l -

1.5 mg/l), 10% coconut water, glutamine (5.0 - 20.0 mg/l) and citric acid (0.5 - 2.0 mg/l) (Table 2). The efficiency of BA in the induction of somatic embryos has also been demonstrated in the other Asclepiadaceae plants such as *Tylophora indica* [20]. 2,4-D (or) NAA was belived to be the most trustworthy growth regulator for the induction of somatic embryogenesis [21]. This suspension culture showed maximum number of globular, heart, terpedo stage embryos (Figure 1b). The suspension culture of callus was determined for fresh weight, dry weight and nature of biomass callus every 10 day interval.

Initially, the suspension cultures contained large number of free single cells and cell aggregates. After 8 weeks, the cell converted into globular embryos which were either solitary or in clusters. They could be easily identified from undifferentiated one by their texture and colour. The deveopmental changes occured at more advanced stages, as globular (Figure 1-c), heart (Figure 1- d), terpedo (Figure 1- e) and cotyledonary stage of embryos (Figure 1- f) and their percentages were observed (Table 2). After the transfer into a fresh medium, the embryos were counted. When the mature embryogenic cells were kept in the same medium for long days, the embryogenic cells were dead or brown color or cell aged or cell clumping and the shape of cells were round or oblong.

3.4 Embryo germination and plant regeneration

The mature embryos, upon transfer into MS medium with B5 vitamin and without growth regulator, 3% (w/v) sucrose and 0.8% agar were used for germination (Figure 1- f). Sometimes embryos shows brown colour or black colour due to delay subculture. Kumar *et al*. [21] reported that cotyledonary embryos transferred to full strength MS medium and B5 vitamin without growth regulators facilitated low percent conversion of embryos into plantlets. Germinated plantlets were individually transferred to pots containing soil, sand and farmyard manure (1:1:1) and grown in the green house (Figure 1 - g). The survival rate was 92% and plants showed normal growth with similar phenotype of mother plants.

4. Conclusions

In conclusion, the present protocol can be used for rapid multiplication of *G. sylvestre* by somatic embryogenesis without any variation. The plants propagated by tissue culture did not show any morphological abnormality when compared to original plants.

Table 1. Embryogenic callus induction from leaf explants of *Gymnema sylvestre* on MS medium supplemented with 2,4-D and NAA with 10% coconut water, after 25 days in callus culture

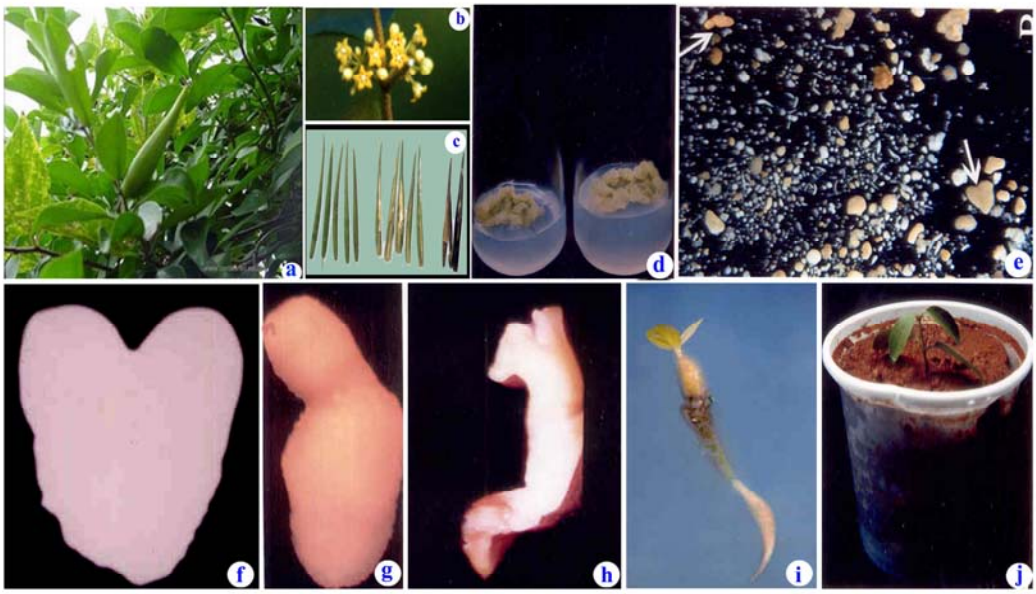
Plant growth regulators (mgl^{-1})		Embryogenic callus frequency (%)*
2,4-D	NAA	Leaf
.01	-	49.5^{gh}
0.05	-	78.2^{d}
0.1	-	87.6^{b}
0.5	-	94.5^{a}
1.0	-	83.0^{bc}
1.5	-	62.4^{e}
2.0	-	60.6^{ef}
2.5	-	52.8^{g}
3.0	-	40.5^{i}
3.5	-	37.6^{ij}
4.0	-	32.5^{k}
-	0.01	38.5^{hi}
-	0.05	45.6^{fg}
-	0.1	54.2^{de}
-	0.5	63.1^{ab}
-	1.0	65.4^{a}
-	1.5	59.5^{c}
-	2.0	56.0^{d}
-	2.5	48.5^{f}
-	3.0	39.6^{h}
-	3.5	36.5^{j}
-	4.0	30.4^{k}

*Values are mean of 30 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT.

Table 2. Somatic embryogenesis from callus induced from leaf and shoot tip explants of

Plant growth regulators (mg/l)					Globular (%)	Heart (%)	Torpedo (%)	Cotyledonary (%)*
2,4-D	NAA	Citric acid	Glutamine	BA				
0.5	-	0.5	5.0	0.5	46.6^{f}	41.3^{cd}	35.5^{b}	26.2^{b}
0.5	-	0.5	5.0	1.0	57.2b^{c}	46.4^{b}	38.8^{a}	29.6^{a}
0.5	-	0.5	5.0	1.5	49.1^{d}	39.8^{e}	28.6^{d}	21.8^{d}
-	1.0	0.5	5.0	0.5	48.5^{de}	39.6^{ef}	26.1^{f}	18.4^{f}
-	1.0	0.5	5.0	1.0	69.8^{a}	54.9^{a}	32.6^{c}	25.2^{bc}
-	1.0	0.5	5.0	1.5	61.4^{b}	41.6^{b}	28.4^{de}	20.5^{de}
0.5	-	1.0	10.0	0.5	69.7^{e}	52.3^{de}	41.2^{bc}	31.0^{bc}
0.5	-	1.0	10.0	1.0	73.2^{c}	67.6^{ab}	37.9^{d}	27.8^{d}
0.5	-	1.0	10.0	1.5	66.0^{ef}	34.1^{f}	29.5^{f}	18.2^{f}
-	1.0	1.0	10.0	0.5	72.6^{cd}	61.3^{c}	49.5^{b}	36.2^{b}
-	1.0	1.0	10.0	1.0	85.2^{a}	71.4^{a}	58.8^{a}	49.6^{a}
-	1.0	1.0	10.0	1.5	79.1^{b}	52.8^{d}	32.6^{de}	25.8^{de}
0.5	-	1.5	15.0	0.5	78.5^{b}	59.6^{bc}	46.1^{bc}	32.4^{b}
0.5	-	1.5	15.0	1.0	89.8^{a}	74.9^{a}	52.6^{a}	35.2^{a}
0.5	-	1.5	15.0	1.5	71.4^{d}	64.6^{b}	46.4^{b}	26.5^{d}
-	1.0	1.5	15.0	0.5	59.7e	42.3^{de}	31.2^{e}	22.2^{f}
-	1.0	1.5	15.0	1.0	73.2^{bc}	47.6^{d}	35.9^{d}	29.6^{c}
-	1.0	1.5	15.0	1.5	56.0^{ef}	34.1^{f}	29.5^{ef}	25.8^{de}
0.5	-	2.0	20.0	0.5	52.6^{f}	41.3^{f}	35.5^{de}	28.4^{bc}
0.5	-	2.0	20.0	1.0	65.2^{d}	48.4^{de}	38.8^{d}	35.2^{a}
0.5	-	2.0	20.0	1.5	59.1^{de}	44.8^{d}	32.6^{f}	25.5^{f}
-	1.0	2.0	20.0	0.5	78.5^{b}	59.6^{bc}	46.1^{c}	26.2^{d}
-	1.0	2.0	20.0	1.0	89.8^{a}	74.9^{a}	62.6^{a}	29.6^{b}
-	1.0	2.0	20.0	1.5	71.4^{bc}	64.6^{b}	56.4^{b}	25.8^{de}

*Values are mean of 30 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT.



Somatic embryogenesis and Plant regeneration from cell suspension culture of *Gymnema sylvestre* (Retz) R.Br. Ex Roemer & Schultes.

Figure 1a Habitat; b. Flower; c. Seed; d. Somatic embryo callus induction (leaf explants); e.Different stages somatic embryos (leaf explants); f. Heart shaped embryo; g. Torpedo stage embryo; h. Cotyledonary stage embryo; i. Embryo germination; j. Hardening

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