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Establishment of suspension cell culture of *Gymnema sylvestre* R. Br.- A threatened anti-diabetic plant

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Abstract

A cell suspension culture was established from leaf explants of wild *Gymnema sylvestre* plants collected from Muniyankudisai, Tamilnadu, India. Murashige and Skoog medium supplemented with 9.0 µM l^{-1} of 2, 4- Dichlorophenoxy acetic acid and 2.1 µM l^{-1} Benzyl adenine produced yellow friable callus with green patches. The cells were subcultured conscientiously twelve times to get consistent growth of the cells in suspension. From the 10^{th} subculture onwards callus cells acclimatized to grow in suspension with aggregation and reached 168.6 g l^{-1} fw and 5.16 g l^{-1} dw of cell biomass.

Key words: *Gymnema sylvestre*, Suspension culture, Callus, Leaf explant

Introduction

Gymnema sylvestre R.Br. (Asclepiadaceae) is an anti-diabetic plant used in folk, ayurvedic, homeopathic systems and even in modern medicine (Leach 2007). The pharmacological properties of *G. sylvestre* have been attributed to a group of triterpene saponins, known as Gymnemic acids (I – XVIII, Gymnemosaponins I - V and Gymnemosides A-F). One of the 70 species collected from tropical forests in India, it has been classified as vulnerable and needs appropriate attention and immediate management focus (WWF-India 2005; Ved and Goraya 2007; Chaudhuri 2007; Pattanaik et al. 2009). Collection of *G. sylvestre* from wild is expected go up to an alarming level that might lead to extinction. This necessitates increasing the availability of this plant or developing a biotechnological process for the production of bioactive compounds under *in vitro* conditions to control indiscriminate exploitation of biodiversity.

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The capacity for plant cell culture to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology (Smetanska 2008). Though cell suspension cultures in *G. sylvestre* have been reported earlier (Gopi and Vatsala 2006; Lee et al. 2006, 2007; Subathra devi et al. 2006, Subathra devi and Mohana Srivasavan 2009; Veerashree et al. 2011) the biomass production and associated active metabolite concentration were insufficient for commercialization. Several strategies have been investigated to increase the yield of biomass and bioactive compounds in the cell cultures that includes culture type, cell aggregation, manipulation of nutrient medium, growth regulators, chemical treatment etc. Genetic potential is also one of the most important factors in enhancing the cell biomass and biochemical status of plant cell cultures. The objective of the present study is to establish suspension cell culture from *G. sylvestre* wild plants collected from dry tropical environment.

Materials and methods

Explant preparation

Leaf segments (0.5 to 1.0 cm) collected from healthy wild *G. sylvestre* plants collected from Muniyankudisai, Tamilnadu, India were raised in pots containing soil and farm yard manure (1:1 ratio) and processed for aseptic culture. Explants were surface sterilized by cleaning thoroughly under running tap water for 20 min, washed with a solution of Labolene (2-3 drops in 100 ml water) for 10 min, and again washed with sterile distilled water. The cleaned explants were then treated with 70 % ethanol for 1 min followed by $HgCl_2$ (0.1 %) treatment for 4 min under aseptic conditions and washed six times with sterile distilled water to remove traces of $HgCl_2$. Unless otherwise indicated, all the experiments were conducted using MS basal medium (MSB) (Murashige and Skoog 1962). Media pH was invariably adjusted to 5.8 before autoclaving.

Callus initiation

The surface sterilized trimmed leaf explants were inoculated onto MSB with different concentrations of 2, 4- Dichlorophenoxy acetic acid (2, 4-D) (0.0, 1.1, 2.2, 4.5, 9.0, 13.5, 18.0, and 22.6 µM l^{-1})

Table 1. Influence of various concentrations and combinations of 2, 4-D, and BA on callus induction from leaf explants *of G. sylvestre*.

Growth regulators (μM l^{-1})		Callusing response (%)	Callus fresh weight (g l^{-1})	Morphogenic response
2, 4-D	BA			
0.0	-	-	-	-
1.1	-	26.0±1.5^{g}	26.3±0.8^{h}	White, friable
2.2	-	52.0±1.5^{f}	94.0±4.3^{g}	White, friable
4.5	-	62.0±1.1^{e}	182.0±1.1^{f}	White, friable
9.0	-	84.0±0.6^{d}	321.3±4.6^{d}	Yellowish white, friable
13.5	-	88.3±0.6^{abc}	322.0±1.1^{d}	White mixed brown, wet
18.0	-	89.3±1.6^{ab}	320.6±0.6^{d}	Brown, wet
22.6	-	90.6±0.3^{a}	311.3±0.6^{e}	Brown, wet
9.0	1.1	84.3±0.6^{d}	347.6±1.4^{b}	Yellow, friable
9.0	2.2	86.6±0.6^{bcd}	355.6±2.3^{a}	Green mixed yellow, friable
9.0	4.4	86.3±0.6^{bcd}	358.6±1.6^{a}	Green, friable
9.0	8.8	86.3±1.3^{bcd}	361.0±5.0^{a}	Green, compact

Explants were cultured on MSB supplemented with 2, 4-D and BA. Data were recorded after 35 days of culture. Results represent mean of three replicated experiments. Values denoted by different letters differ significantly at $p<0.05$ level (DMRT).

alone or in combination with different concentrations of Benzyl adenine (BA) (1.1, 2.2, 4.4, and 8.8 μM l^{-1}).

Suspension culture establishment

Cell suspension cultures were developed by inoculating 2.0 ± 0.2 g of yellowish green friable callus into liquid MSB supplemented with 9.0 μM l^{-1} of 2,4-D and 2.2 μM l^{-1} of BA. This was followed by twelve continuous subcultures involving the transfer of 5 ml batches of suspension cultures to 45 ml of same medium in 250 ml Erlenmeyer flasks at an interval of 21 days.

The cultures were maintained at 22 ± 1 ℃ under 16/8 h photoperiod by cool white fluorescent tubes (60 μmol m^{-2} s^{-1}) with 55–60 % relative humidity. Three replicates were maintained for each treatment. The flasks were analyzed for cell growth and biomass. Experiments that showed some advantageous effect were repeated three times and presented in the tables as mean±SD and means were separated using Duncan's multiple range test (DMRT) ($p\leq0.05$).

Results and Discussion

Table 1 shows the influence of various concentrations and combinations of 2, 4-D, and BA on callus induction from leaf explants of *G. sylvestre.* The productive callus biomass for the establishment of suspension culture was obtained within 35 days on MSB supplemented with 2, 4-D (9.0 μM l^{-1}) in combination with BA (2.2 μM l^{-1}). The produced callus appeared friable and yellowish with green patches (Fig 2a,b). In an earlier study 2, 4-D in combination with KN was reported to produce light green compact callus and higher biomass in callus cultures of *G. sylvestre* (Ahmed et al. 2008). However, our results on using MSB supplemented with 2, 4-D in combination with BA producing productive mixed yellowish green friable callus that resulted in the establishment of suspension cell cultures corroborates with the earlier studies on *Psychotria carthagenensis, Rosa bourboniana* and *Vanilla planifolia* (Lopes et al. 2000; Janarthanam and Seshadri 2008 a, b).

Figure 1 shows influence of conscientious subculture of callus grown in MSB supplemented with 2, 4-D (9.0 μM l^{-1}) in combination with BA (2.2 μM l^{-1}) to obtain suspension cell biomass. The result shows that conscientious sub culturing process substantially improves cell growth and biomass. Initial harvest of cell mass resulted in 48.2 g l^{-1} fw and 1.8 g l^{-1} dw. From second subculture to ninth subculture the cell mass was found lesser than the initial cell mass and variations were noticed in the cell mass. During the initial to ninth subculture, cells were seen growing as free, single or joint cells of various shapes (Fig 2c). From tenth subculture onwards the cells started aggregating and cell mass started considerably increasing and the fresh weight of the cells in 10th, 11th and 12th subcultures were 126.6, 162.0 and 168.6 of g l^{-1} and the corresponding dry weights were 4.21, 5.24 and 5.16 g l^{-1} respectively. This could be attributed to frequent cell divisions at the periphery of the cell. Generally, aggregation in plant cell culture is desired because metabolite production is not always the function of a single cell but involves different cells and hence cell aggregation is a favorable property with respect to the production of secondary metabolites (Zhao et al. 2003; Fu et al. 2005).

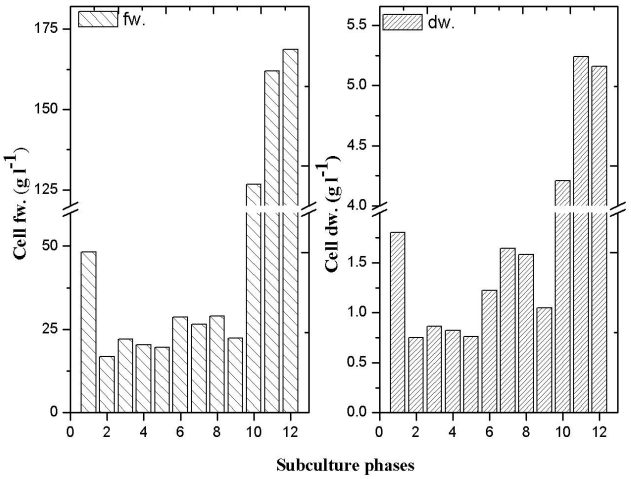

Figure 1. Influence of subculture of callus grown in MSB supplemented with 2, 4-D (9.0 μM l^{-1}) in combination with BA (2.2 μM l^{-1}).

Conclusion

The studies have established that 2, 4-D (9.0 μM l^{-1}) in combination with BA (2.2 μM l^{-1}) can be used to produce friable productive callus biomass and to establish suspension cell culture. However, refinement of the protocol along with in depth studies in bioreactors to generate enough biomass with active ingredients in shorter time would be a promising step towards preventing further loss of native plants and preservation of biodiversity.

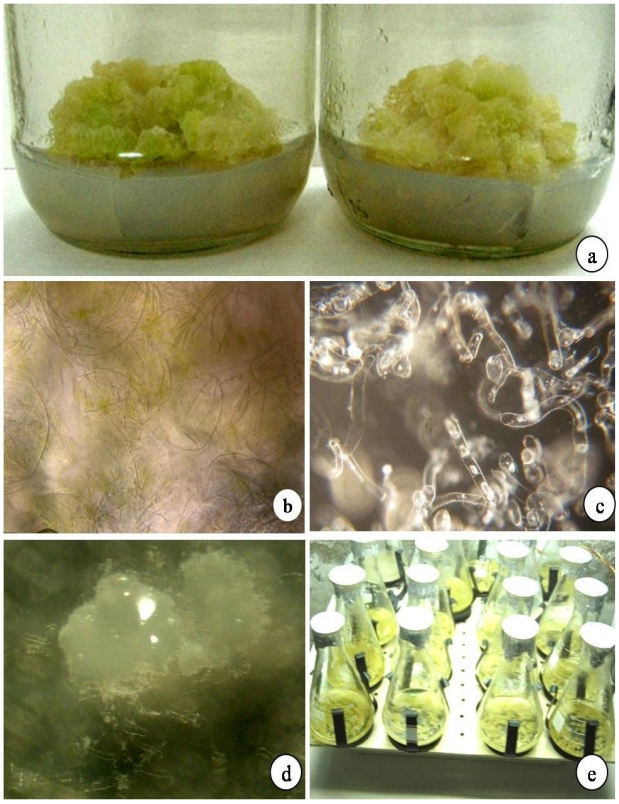

Figure 2: Callus and suspension cultures of *G. sylvestre*, a-growth of callus in on MSB supplemented with 2, 4-D (9.0 µM l^{-1}) and BA (2.2 µM l^{-1}); b-microscopic view of green mixed yellowish friable callus (40X), c-actively dividing single and joint suspension cells (10X). d-Stereo microscopic photograph of suspension cell aggregates, e- suspension cell culture growing in the flask (250ml).

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