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Full Length Research Paper

In vitro studies on effects of plant growth regulators on callus and suspension culture biomass yield from *Gymnema sylvestre R.Br*

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Callus cultures were initiated from nodal segments and leaf explants of *Gymnema sylvestre* on Murashige and Skoog (1962) medium containing basic salts and 30 g/l sucrose supplemented with different concentrations (0.10, 0.25, 0.5, 1.0, 2.0 and 5.0 mg/l) of 2,4-dichlorophenoxy acetic acid (2,4-D), α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), kinetin (KN) and 6-benzyladenine (BA). Callus induction was observed in 0.5 mg/l of 2, 4-D supplemented medium for both explants. At the initial stage, some parts of explants enlarged and gave raise to pale yellowish calli after 2-3 weeks of incubation. The harvested cell biomass was subjected to extraction of active principles. In this study, cell biomass extracts were compared with extracts from leaves of naturally growing gymnema plants. HPLC analysis of these extracts showed that the main components of the active principles namely gymnemic acids and gymnemagenin were present in sufficiently large amounts in the cultured undifferentiated cells.

Key words: *Gymnema sylvestre*, antidiabetic compounds, gymnemic acids, callus, cell culture.

INTRODUCTION

Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavours and fragrances ingredients, food additives, and pesticides (Balandrin and Klocke, 1988). It has been mentioned that natural habitats for medicinal plants are disappearing fast and together with environmental and geopolitical instabilities; it is increasingly difficult to acquire plant-derived compounds. This has prompted industries, as well as scientists to consider the possibilities of investigation into cell cultures as an alternative supply for the production of plant pharmaceuticals (Mulabagal and Tsay, 2004). In the search for alternative to production of desirable medicinal compounds form plants, biotechnological approaches, especially, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Dicosmo and Misawa, 1995; Ramanchandra Rao and Ravishankar, 2002).

Plant cell suspension culture system could be used for large scale culturing of plant cells from which secondary metabolites could be extracted. In order to obtain high yields suitable for commercial exploitation, efforts have been taken to utilise the highly valuable medicinal plant *Gymnema sylvestre* R.Br. *G. sylvestre* belongs to the family Asclepiadaceae. The plant is distributed over most parts of India and tropical Africa. The plant is popularly called as “Gru-mar” for its distinctive property of temporarily destroying the taste of sweetness and is used in the treatment of diabetes (Chopra et al., 1956; Nadkarni, 1982; Shanmugasundaram et al., 1983; Suttisri et al., 1995; Bailey and Day, 1989; Zhang and Xiao, 1993; Raman and Skett, 1998) in India. *G. sylvestre* foods in tea bags, tablets, beverages and confectionaries

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in recent years in Japan. The users of these health food often expect weight reduction or improvement of diabetes because of their ability to suppress the taste of sweetness and inhibit glucose absorption (Nakamura, 1988; Ueno, 1997). Several products, under such brand name as Body Slatto Tea®, Gymnema®, Gymnema Diet® and Sugar Off® are commercially available (Suttisri et al., 1995). The major bioactive constituents of *G. sylvestre* are a group of oleanane type triterpenoid saponins known as "gymnemic acids" (Min et al., 1992; Kuzuko, 1992).

There are a number of studies on *G. sylvestre* active principles, pharmacological activity, phytochemistry and propagation by *in vitro* technique (Reddy et al., 1998; Komalavalli and Rao, 2000; Kumar et al., 2002). Nevertheless, there are no reports available on *G. sylvestre in vitro* cell mass production and screening of biosynthetic potential using modern techniques. Hence, the present investigation was carried out with the objective of providing new vistas on the cell and tissue culture and to elucidate the effect of plant growth regulators on cell growth and production of total gymnemic acids.

MATERIAL AND METHODS

Cultivation of cuttings

Gymnema sylvestre R.Br. plants raised in pots containing soil and farmyard manure (FYM) 1:1 ratio at our Centre served as the source for experimental materials. The mother cuttings for these plants were obtained from medicinal plant conservation park (MPCP), Centre for Indian Medical Heritage (CIMH), AVP-campus, Kanjikde P.O, Palaghat Distt, Kerala.

Explant preparation

Small young twigs were collected from 4 months old healthy plants, cut into 1-1.5 cm nodal segments with axillary buds. Young leaves were also used as explants for culture. These explants were washed in the liquid detergent Tween 20 (few drops per 100 ml solution) for 3 min and then rinsed in running tap water for 10 min. The cleaned explants were surface sterilised with aqueous 0.1% mercuric chloride solution for 8 min followed by 10 rinses in sterile distilled water with one minute intervals. After trimming the cut ends, surface sterilised explants were planted on the culture medium.

Media and callus initiation

Murashige and Skoog (1962) (MS) medium supplemented with various auxins viz. 2,4-dichlorophenoxy acetic acid (2,4-D), α-naphthalene acetic acid (NAA), Indole-3 acetic acid (IAA), Indole 3-butyric acid (IBA) and cytokinins viz. 6-benzyladenine (BA) and kinetin (KN) alone or in combination at different concentration (0.10, 0.25, 0.5, 1.0, 2.0 and 5.0 mg/l) was used for callus initiation and suspension culture development. The cultures were incubated under cool fluorescent lights with 1500-2000 Lux for 16 h at a temperature of 25± 2ºC and 70±10 relative humidity. Each experiment had 25 replicates and repeated at least three times. Data were documented up to seven weeks of culture.

Callus induction

Initial study proved that young leaf and nodal explants produced callus. The callusing response from nodal explant is much higher than that of the leaf. The calli were transferred either to the same initiation medium. After 2 or 3 subcultures on the MS basal medium, the calli were placed on MS basal medium containing different concentration and combinations of 2,4-D, IAA, IBA, BA and KN and the comparative responses of these PGRs on callus development were evaluated.

Callus and cell suspension culture

Induced calli were transferred periodically to freshly prepared culture medium: MS supplemented with 0.5 mg/l 2, 4-D, 2.5 mg/l NAA, 0.5 mg/l BA, 3% sucrose and 0.85% of agar. The stock cultures were subcultured every four weeks. Suspension culture was generated as follow: approximately 500 mg of fresh weight of callus were cultured in 50 ml liquid in MS supplemented with same compositions as the callus maintenance media without agar in a 250 ml conical flask. The flasks were placed on a rotary shaker (ORBIT, India) with speed of 110 rpm in growth room.

Growth measurement

Growth of *G. sylvestre* was measured in terms of fresh and dry weight. Fresh weights of cells/callus were taken after removing the excess of moisture on the surface using blotting paper. Dry weight of cells/callus was determined by drying in a hot air oven at 60ºC for 24 h.

Extraction for active principle

The active components extractions from the callus and leaf samples were analysed as prescribed by Shimizu et al. (1997). The dried callus cells and dried leaf tissue (about 1.0 g each) were powdered and the active principles were extracted with 75% ethanol. The dried extract was mixed with n-butanol and water (2:1 ratio), and then the layer of n-butanol was evaporated under vacuum. The residue was washed with petroleum ether to remove fatty components and then treated with methanol. After filtration, the concentrated extract in methanol was used for separation by high performance liquid chromatography (HPLC).

The extract was dissolved in HPLC grade water and remaining residue was dissolved in of HPLC grade methanol separately. These extracts were filtered through Sartorius RC-membrane syringe filter (0.20 µm) and 10 µl of the filtrate was used for HPLC analysis using Shimadzu HPLC with auto sampler and standard C18 column (25 cm x 4.6 mm). The mobile phase consisting of acetonitrile : water (80:20) was used for extraction purpose. Flow rate was 1.5 ml/min was maintained with pressures at 250 psi and the column temperature was 40°C. The compounds were read at 210 nm in an UV detector. The total run time was 30 min but it was preferable if the time was extended to 40 min.

RESULTS AND DISCUSSION

Initially callus was initiated form nodal and leaf explants of *G. sylvestre* on basal MS medium supplemented with 2,4-D at different concentrations (0.10, 0.25, 0.5, 1.0, 2.5, and 5.0 mg/l). The proliferation efficiency of callus of

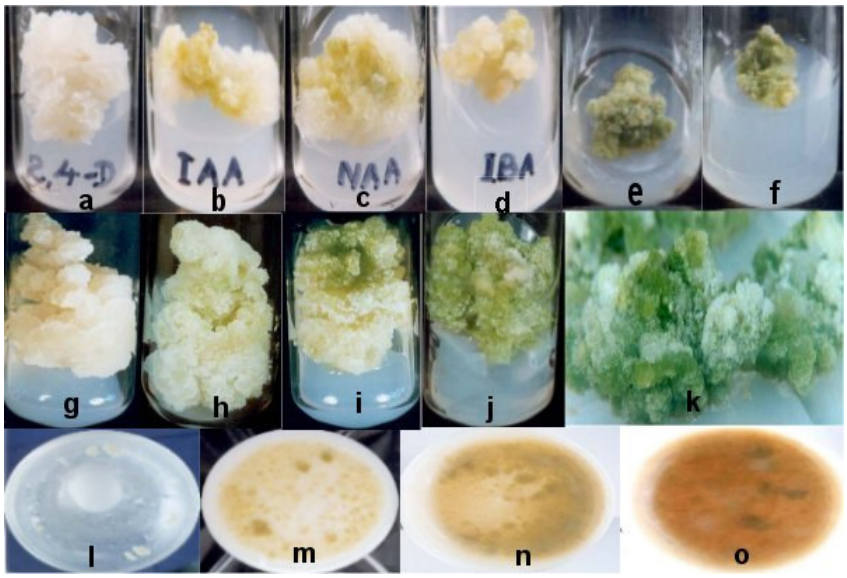

Figure 1. (a-o) Callus and suspension culture developments from *Gymnema sylvestre* a- Callus culture in MS + 2,4-D at 0.5 mg/l; b- Callus culture in MS + IAA at 2.5 mg/l ; c- Callus culture in MS + NAA at 2.5 mg/l; d- Callus culture in MS + IBA at 2.5 mg/l ; e- Callus culture in MS + BA at 2.5 mg/l; f- Callus culture in MS + KN at 2.5 mg/l; g- Callus culture in MS + 2,4-D 0.5 mg/l+NAA 1.0 mg/l; h- Callus culture in MS + 2,4-D 0.5 mg/l+NAA 2.5 mg/l; i- Callus culture in MS + 2,4-D 0.5 mg/l+NAA 2.5 mg/l+BA 0.1 mg/l; j- Callus culture in MS + 2,4-D 0.5 mg/l+NAA 2.5 mg/l+BA 0.25 mg/l; k- Callus culture in MS + 2,4-D 0.5 mg/l+NAA 2.5 mg/l+BA 0.5 mg/l; i-o Cell suspension culture in MS + 2,4-D 0.5 mg/l+NAA 2.5 mg/l+BA 0.5 mg/l; l- fifth day culture; m- tenth day culture; n-fifteenth day culture; and o- twenty day old culture.

nodal explants was significantly higher than that of leaf explant for four to five weeks incubation of culture. The remarkable callus induction was obtained in MS medium containing 0.5 mg/l 2,4-D. The callus nature was delicate with pale yellow in colour.

In order to study the effects of plant growth regulators (PGRs) on callus culture, different PGRs were tried at different concentration and various combinations. Based on the results the maximum growth of callus was obtained in MS medium individually amended PGRs such as 2,4-D at 0.5 mg/l followed by NAA, IAA, IBA at 2.5 mg/l among the auxins. The cytokinins, BA at 2.5 mg/l followed by KN at 5.0 mg/l, in MS medium were also tested. The callusing response of most of these PGRs has been studied while multiplication of *G. sylvestre* was reported by Reddy et al. (1998). The maximum callus growth was found with auxins such as 2,4-D and NAA, and also with BA among the cytokinins. Callus in 2,4-D supplemented medium was well developed, albino, spongy, and loosely arranged (Figure 1a). The moisture content of callus was high as compared to other auxins-supplemented media. In NAA supplemented medium, the callus was pale, yellowish green in colour more friable, hard and granular (Figure 1b). But in IAA and IBA supplemented media, the callus was solid in nature and pale green in colour (Figure 1c-d). Yield of fresh biomass was high on medium with 2,4-D (169 g fw/l and 9.825 g dw/l) followed by NAA (78 g fw /l and 7.0 g dw/l), IAA (41 g fw/l and 2.82 g dw/l) and IBA (38.40 g fw/l and 2.38 dw/l) (see Table 1). The nature of callus was green in colour and more compact, hard and granular in BA-supplemented medium, whereas in KN-supplemented medium, the callus was light green, less hard and compact (Figure 1e-f). In BA medium, more biomass yield was achieved than of KN.

Concentration of 2, 4-D higher than 0.5 mg/l results in increased moisture content resulting in low dry matter yield.

Based on the results of the previous experiments, the high biomass yielding concentration of 2,4-D and NAA were selected for synergistic effects of auxins on callus culture. The culture grew very fast within 15 days in all combinations tested. The callus was pale yellowish green in colour at lower concentration of NAA (1.0 mg/l), but turned into green in higher concentrations of NAA (2.5 mg/l). The tissue turns brown by the third week onwards in higher concentration of NAA (5.0 mg/l) in combination with 2,4-D (0.5 mg/l). These results reveal that higher levels of NAA (2.5 mg/l) in the medium induced maximum callus growth during 15 to 18 days with maximum yield of biomass (222 g fw/l and 13.64 g dw/l) achieved. The results of biomass yield from combination effect of auxins 2,4-D and NAA with BA are presented in Figure 2. About 2.0 g of actively growing calli were inoculated in conical flasks each containing 50 ml of solid medium. Combination of 2,4-D + NAA + BA showed more callus

Table 1. Individual effect of auxins and cytokinins on *Gymnema sylvestre* callus culture

PGR mg/l	Auxins								Cytokinins			
	2,4-D		NAA		IAA		IBA		BA		KN	
	fw	dw	fw	dw	fw	dw	fw	dw	fw	dw	fw	dw
0.10	108.57	7.53	7.01	0.62	6.24	0.50	5.96	0.39	14.00	1.05	6.80	0.53
0.25	159.85	9.52	12.00	1.10	12.60	0.60	8.00	0.86	16.00	1.20	9.00	0.68
0.50	169.18	9.82	28.30	2.92	16.90	1.06	15.00	1.42	22.50	1.30	12.90	1.00
1.00	146.12	8.02	57.40	5.12	31.50	2.10	24.80	1.63	24.30	2.32	14.00	1.08
2.50	105.57	6.01	78.00	7.00	41.50	2.82	38.40	2.38	26.50	2.51	18.80	1.45
5.00	94.64	4.28	72.02	6.56	39.10	2.77	35.10	2.29	23.50	2.38	17.60	1.33

fw: fresh weight g/l; dw: dry weight g/l; PGR: plant growth regulator

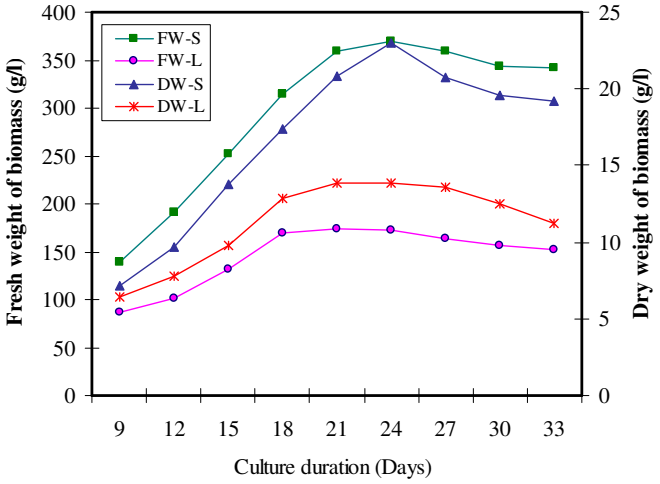

Figure 2. *Gymnema sylvestre* callus/cell biomass in solid and liquid medium culture at different age.

biomass yield than the combination auxins with KN. The nature of callus was more compact, hard and dark green in colour in BA-supplemented medium but with KN, the callus was green in colour and less compact. The yield of fresh biomass in BA-supplemented medium was about two times more than that in KN-supplemented medium. Dry biomass was about one and half times higher in BA than KN-supplemented medium. The lower concentration of BA (0.5 mg/l) produced more fresh calli. On the basis of yield of both fresh and dry biomass, BA has greater effect than KN. Based on the above results, the PGR combinations of 0.5 mg/l 2,4-D + 2.5 mg/l NAA + 0.5 mg/l BA (named as MS-G) which yielded maximum biomass 370 g fw/l and 23.12 g dw/l this combination was used for further studies (Figure 1k).

Further study was carried out to see the age influence of callus/cell growth. About 2.0 g of actively growing calli were inoculated in conical flasks containing 50 ml of MS-G solid medium. The cultures were incubated for 16 h photoperiod at 25 ± 1°C. Observations were made from the ninth day after incubation and up to the 33rd day with three days intervals, and callus biomass yield data were recorded. Average increase in fresh weight was compared. The results are presented in the Figure 2. The yield of biomass was expressed as fresh weight and dry weight (g/l). The yield of fresh biomass was high in callus cultured in solid medium. The period of maximum biomass yield (348 g fw/l) was during 21st to 27th day after that the biomass yield started to decrease. The culture remained green unto 36 days and then started browning showing symptoms of death phase. The percentage of dry weight biomass in solid medium culture yield was less compared to that in liquid medium culture. The Figure 2 showed that the liquid medium culture, the average maximum biomass yield was from 18th day to 24th day after that the biomass production decreased and the pale yellowish green colour of biomass turned into pale brownish colour (Figure lo).

In order to determine the active principles (total gymnemic acids) present, callus/cell was extracted by using both methanol and water and compared with leaf extract of *G. sylvestre* through HPLC analysis. The results of chromatogram of callus/cell samples showed most of compounds present in leaf extract chromatogram. Interestingly, additional compounds were also found in both methanol and water extract (not shown). The accumulation of active principles in cultured cells at a higher level than those in native plants through optimization of cultural conditions has been observed in *Panax ginseng* (Ushiyama, 1991). Rosmarinic acid by *Colleus blumei* (Ulbrich et al., 1983), shikonin by *Lithospermum erythrorhizon* (Takahashi and Fujita 1991), diosgenin by *Dioscorea* (Rokem et al., 1984), ubiquinone-10 by *Nicotiana tabacum* (Matsumoto et al., 1980) were accumulated in much higher levels in cultured cells than in the intact plants. Sometimes cultured plant cells often produce reduced quantities and different profiles of secondary metabolites when compared with the intact plant (Whitaker et al., 1986). This report coincides with

our where there were additional peaks in HPLC chromatogram of callus water extract sample which was not seen in leaf water extract sample (not shown).

Since the demand in pharmaceutical industries for plant based raw materials is ever increasing. The present study is a stepping stone for *in vitro* production of required active principles of *G. sylvestre*. So far, there is no known report of production of active principles of *G. sylvestre* by callus. This is the first successful attempt of production of secondary metabolites of *G. sylvestre* where, production levels can be manipulated with appropriate PGRs. Further studies will be directed towards large scale production, testing the efficacy of secondary metabolites through animal cell lines and exploring market potential.

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