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In-vitro production of secondary metabolites in *Gymnema sylvestre*

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ABSTRACT

Gymnemic acid (GA) is the principle constituent in G*ymnema sylvestre* , which is used in control of diabetics. Standardization of protocol for *in-vitro* culture and suspension culture to optimize the GA production was experimented. Gymnemic acid in yellowish-brown calli was 251.73 mg 100 g^{-1} dw when compared to 26.31 mg 100 g^{-1} dw in grey calli, which was mainly due to accumulation of gymnemic acid during culture. The cells were run in suspension culture at 120 rpm and sub-cultured every 20 days. The cell density, dry weight of cells, packed cell volume and viability of cells were the highest on day 15 irrespective of the treatments. The doubling time of cells was three days. The highest gymnemic acid was recorded in auxin-free medium (579.2 mg 100 ml^{-1}) followed by sucrose 60 g l^{-1} (487.96 mg 100 ml $^{-1}$) and mannitol (439.66 mg 100 ml^{-1}). The fluorescent nature of gymnemic acid was identified and photo-micrographed.

Key words: Gymnema, gymnemic acid, secondary metabolites, suspension culture.

INTRODUCTION

Gymnema sylvestre has anti-diabetic properties and the plant is used as a herbal medicine throughout the world. The plants bear a triterpenoid saponin in the cells of plant parts called gymnemic acid which is the principle constituent responsible for the control of diabetics. The inhibitory activity of gymnemic acid against glucose absorption in the intestine is the basic principle in reducing the blood glucose level (Yoshikawa and Yamahara, 17). Isolation of gymnemic acid has been carried out by many authors in Japan (Murakami *et al*., 9). Even though plants have a substantial amount of secondary metabolites which could be extracted directly, commercial production of these secondary metabolites in higher amounts could be possible only through cell suspension cultures as the yield and quality of the secondary metabolite is more consistent and the production schedule could be predicted and controlled in the laboratory. Production of secondary metabolites through cell suspension culture is more advantageous and more precise (Gopi and Vatsala, 5; Kanetkar *et al*., 7). Standardization of culture conditions to increase the gymnemic acid *in-vitro* would largely help the pharmaceutical industry to produce large quantities of these secondary metabolites independent of availability of plants, season and soil conditions.

MATERIALS AND METHODS

The experiment was conducted at the Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore. The internodal stem bits from 3rd-6th node from tip were used to initiate callus on full-strength MS medium (Murashige and Skoog, 10) supplemented with 4 mg l^{-1} 2,4-D. Culture flasks containing 50 ml of sterile medium were used for callus initiation and were maintained in a culture room at 24 ± 2°C with continuous light. Callus was sub-cultured every 45 days using the same culture conditions.

Two colours namely grey and yellowish-brown were observed. Fresh weight of the callus was measured. The callus was dried in lyophilizer and weighed (Villareal *et al*., 15). Relative Growth Rate (RGR) was calculated using the formula;

$$RGR = \frac{\text{Final fresh weight - Initial fresh weight}}{\text{Day of final FW - Day of initial FW}}$$

Gymnemic acid was quantified directly in callus. Ten gram of callus was weighed, dried in lyophilizer and extracted with 60% methanol for HPLC analysis (Villareal *et al*., 15).

One gram of callus was placed in 25 ml of liquid medium in 100 ml conical flasks, five flasks per treatment. Cultures were grown in an orbital shaker at 120 rpm in continuous light at 24 ± 2°C. Suspensions were sub- cultured every 30 days in the same treatment and culture conditions. Friable calli were used to establish suspension cultures. Following treatments were imposed in liquid MS medium. T_1 : 2,4-D 4 mg l^{-1}; T_2 : 2-4-D 6 mg l^{-1}; T_3 : Mannitol 20 g l^{-1}; T_4 : Mannitol 40 g l^{-1}; T_5 : Sucrose 60 g l^{-1}; T_6 : Sucrose 80 g l^{-1} T_7 : Sodiumazide 0.65 gl^{-1}; T_8 : Sodiumazide 3.0 gl^{-1}; T_9 : Calcium-free medium; T_{10} : Auxin free medium. Cell counts were recorded once in 5 days from zero day for 25 days and expressed as cell count × 10^4 per ml of suspension. The packed cell volume was recorded on

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zero day, continued at 5 days interval and expressed in per cent (v/v). Ten ml of the suspension cultures were subjected to the lyophilizer (Virtis Sentry 5 L) at -50°C. The dried cultures (*Villareal et al.,*15) were extracted by two successive macerations with 60% methanol, evaporated to dryness under vacuum, re-constituted in 60% HPLC methanol, filtered through acro-discs. Sample of 20 µl was injected to HPLC column ODS Lichrosfer Ò 60, 5m, 250 × 3.5 *i.d*. and UV detector 240 nm with a mobile phase of methanol : KH_2PO_4 and a flow rate of 0.5 ml min^{-1}. Retention times of gymnemic acid in samples were compared with that of the authentic gymnemic acid standard. Suitable calibration curves for quantification of the secondary metabolites in vitro were constructed.

Fresh weight was determined as filtered biomass after centrifugation and discarding the supernatant (Villareal *et al*.,15). The dry weight was recorded after drying the centrifuge tubes at 80°C for 24 hours, and allowing it to cool (Fellers *et al*., 4). Usually the cell viability was determined using the fluorescein-diacetate method. Since the cells of gymnema are naturally fluorescing in nature, the viability was measured by counting the fluorescent cells and expressed in per cent. The data was statistically analyzed by completely randomized design with two replications.

RESULTS AND DISCUSSION

The high amount of gymnemic acid was assessed and reported in this study revealed the possibility of taking up suspension culture through callus and produce secondary metabolites in large quantities even from a very small unit of a tissue of *Gymnema sylvestre*. The stem bits showed a higher response of callusing to 2,4-D 4 mg l^{-1}. Callus formation reached 90 percent after 30 days of culture (Villareal *et al*.,15).

The calli in suspension was grey in colour while few cultures showed a yellowish-brown discolouration. The occurrence of this colour change was only two percent among the grey cultures. When the yellowish-brown calli and its medium (Fig. 1) were smeared on a microscopic slide and viewed by UV fluorescence microscope (Osbourn *et al*., 13) typical yellow fluorescence was observed. Whereas, the grey calli and its medium showed little or no fluorescence (Fig. 2) under UV illumination. The fresh weight of the callus was the highest in grey coloured callus (12.10 g) on 45th day after inoculation. The yellowish-brown callus recorded 11.8 g fresh weight on the same day. Dry weight of callus was the highest in grey coloured callus (0.376 g) when compared to yellowish-brown coloured callus (0.367 g) on 45th day after inoculation. There was no significant difference between the relative growth rate of grey and yellowish-brown callus (0.230 and 0.223 RGR, respectively). The HPLC analysis of these two different coloured calli revealed a highly significant variation in the gymnemic acid content, i.e., 251.73 mg 100 g^{-1} dw in yellowish-brown and 26.31 mg 100 g^{-1} dw in grey callus (Table 1). This revealed that yellowish-brown discolouration in the calli is mainly due to the accumulation of gymnemic acid. This character would be applicable to detect and screen for higher gymnemic acid bearing cultures at the callus phase itself.

Since the occurrence of yellowish-brown callus is only 2 per cent among the grey cultures, a trial to increase the secondary metabolite in grey callus by the cell suspension culture was undertaken. In general, this study indicated that the cell density (Table 2), PCV (Table 3), dry weight (Table 4) and viability were the highest on day 15 irrespective of the treatments. The doubling time of cells was 3 days (Fellers *et al*., 4). The highest gymnemic acid yield was recorded in auxin-free medium followed by sucrose 60 g l^{-1}, mannitol 40 g l^{-1} and calcium-free medium. Treatment 4 mg l^{-1} 2,4-D also yielded higher quantity of gymnemic acid. The absence of auxin in the medium might have induced a stress in the cells of suspension culture and thus enhancing the cells to produce higher secondary metabolites as a defense mechanism (Papadapoulou *et al*., 14) (Fig. 3).

Solasodine production in *Solanum elaeagnifolium* was shown to be enhanced by increasing the sucrose concentration (Nigra *et al*., 11). In the present study the gymnemic acid yield was 487.96 mg 100 ml^{-1} with sucrose 60 g l^{-1}. However, at 80 g l^{-1} recorded a lower yield of gymnemic acid due to death of cells by high osmotic stress induced in the medium (Fellers *et al*., 4). Mannitol, a sugar which also creates an osmotic stress or shock in the culture medium would enhance the production of alkaloids to even 4-fold by (Jian *et al*. (6) in *Catharanthus roseus* cell cultures. In this study also, mannitol at 40 g l^{-1} improved the

Table 1. Growth rate and gymnemic acid of callus in *Gymnema sylvestre*.

Colour of the callus	Inoculum weight (g)	Fresh weight (g)	Dry weight (g)	RGR	Gymnemic acid (mg 100 g^{-1} dry weight)
Grey	2.0	12.10	0.376	0.230	26.31
Yellowish brown	2.0	11.92	0.367	0.223	251.73
CD (0.05)	0.0064	0.0059	NS	NS	2.044**

Table 2. Density of cells in suspension culture of *Gymnema sylvestre.*

Treatment	Day* 0 (x 10^4 ml $^{-1}$)	Day 3	Day 5	Day 10	Day 15	Day 20	Day 25
2,4-D 4 mg l^{-1}	0.6	1.3	2.2	5.5	18.5	14.2	12.0
2,4-D 6 mg l^{-1}	0.5	0.9	0.6	1.0	2.0	2.0	1.6
Mannitol 20 g l^{-1}	0.5	1.2	1.5	4.9	17.7	6.5	3.5
Mannitol 40 g l^{-1}	0.6	1.3	2.5	2.6	22.7	21.0	11.9
Sucrose 60 g l^{-1}	0.6	1.1	10.0	10.3	10.6	4.2	3.5
Sucrose 80 g l^{-1}	0.4	0.9	0.9	0.5	2.6	1.0	1.0
Sodium azide 0.65 g l^{-1}	0.2	0.5	0.5	0.5	2.5	2.2	1.1
Sodium azide 3.0 g l^{-1}	0.2	0.5	0.5	0.5	1.9	1.9	1.5
Auxin-free medium	0.5	0.8	1.5	3.8	2.8	1.6	1.2
Calcium-free medium	0.4	0.9	1.6	2.9	6.5	2.5	1.0

*2 percent inoculum

$CD_{0.05}$ Treatment = 0.34; Day = 0.28; Interaction = 0.91

Table 3. Packed cell volume in suspension culture of *Gymnema sylvestre.*

Treatment	Day* 0 (x 10^4 ml $^{-1}$)	Day 3	Day 5	Day 10	Day 15	Day 20	Day 25
2,4-D 4 mg l^{-1}	0.6	1.2	6.6	5.3	5.5	6.0	4.0
2,4-D 6 mg l^{-1}	0.6	1.3	3.3	3.3	1.3	3.3	2.6
Mannitol 20 g l^{-1}	1.3	2.6	2.0	3.3	4.6	4.6	3.3
Mannitol 40 g l^{-1}	1.3	3.0	3.3	3.3	6.0	6.0	6.0
Sucrose 60 g l^{-1}	0.6	1.5	6.0	4.0	6.0	6.0	6.0
Sucrose 80 g l^{-1}	0.6	1.5	1.3	2.0	3.3	1.3	0.6
Sodium azide 0.65 g l^{-1}	1.3	2.0	1.0	3.3	3.3	2.0	3.2
Sodium azide 3.0 g l^{-1}	1.3	2.0	1.0	2.0	1.3	1.3	0.6
Auxin-free medium	1.3	3.0	3.3	4.6	3.3	2.0	3.0
Calcium-free medium	1.3	2.5	2.6	3.16	6.0	6.0	3.3

*2 percent inoculum

$CD_{0.05}$ Treatment = 0.327; Day = 0.274; Interaction = 0.866

Table 4. Dry weight of cells (g 1^{-1}) in suspension culture of *Gymnema sylvestre.*

Treatment	Day 0	Day 7	Day 15	Day 25
2,4-D 4 mg l^{-1}	2.30	4.02	16.1	13.57
2,4-D 6 mg l^{-1}	2.39	3.19	4.95	7.28
Mannitol 20 g l^{-1}	2.15	6.62	10.13	11.28
Mannitol 40 g l^{-1}	3.07	4.66	13.57	13.72
Sucrose 60 g l^{-1}	3.99	3.86	13.36	7.15
Sucrose 80 g l^{-1}	1.90	0.74	7.15	3.80
Sodium azide 0.65 g l^{-1}	2.30	2.91	7.48	7.03
Sodium azide 3.0 g l^{-1}	2.42	2.61	4.93	5.61
Auxin-free medium	2.42	2.91	9.66	4.05
Calcium-free medium	2.15	3.96	13.11	10.46

$CD_{0.05}$ Treatment = 0.395; Day = 0.250; Interaction = 0.791

Table 5. Cell viability (%) in suspension culture of *Gymnema sylvestre.*

Treatment	Day 0	Day 7	Day 15	Day 25	Day 30
2,4-D 4 mg l^{-1}	98.0	99.0	99.0	96.0	92.5
2,4-D 6 mg l^{-1}	98.0	98.0	15.0	80.0	80.0
Mannitol 20 g l^{-1}	98.0	99.5	99.0	98.0	94.0
Mannitol 40 g l^{-1}	98.0	99.0	99.0	98.0	96.0
Sucrose 60 g l^{-1}	98.0	99.0	99.0	96.0	91.0
Sucrose 80 g l^{-1}	98.0	94.0	80.5	70.0	65.0
Sodium azide 0.65 g l^{-1}	98.0	85.5	65.0	60.0	55.0
Sodium azide 3.0 g l^{-1}	98.0	83.0	60.0	55.0	50.0
Auxin-free medium	98.0	95.0	99.0	85.0	70.0
Calcium-free medium	98.0	95.0	99.0	97.5	93.2

$CD_{0.05}$ Treatment = 0.855 ; Day = 0.604; Interaction = 1.911

production of secondary metabolites. The viability of the cells in mannitol was significantly higher (96%) even 30 days after inoculation. The higher cell density and dry weight in this treatment might be the reasons for a higher gymnemic acid yield. Earlier, Chen *et al.* (3) had reported similar results with combined effect of sucrose and mannitol in *Taxus* sp.

The 2,4-D at 4 mg l^{-1} recorded a higher gymemic acid. The higher dry weight of cells of 13.57 g l^{-1} on day 25, viability of 92.5 per cent was recorded in this treatment. A higher PCV of 5.5 per cent (w/v) was recorded on day 15, which might be due to higher cell density on day 15. This study was in accordance with Villareal *et al*. (15). Meiping (8) also reported that 2,4-D 2 mg l^{-1} along with 0.5 mg l^{-1} 6-BA as the best medium for higher saponin content in American ginseng. However, Wichers *et al*. (16) reported that 2,4-D reduced the cell growth which is contradictory in the present study. Bozhkov *et al.*, 1) recorded increased level of cell death in auxin-free medium in Norway spruce suspension cultures.

Elimination of calcium from the cell suspension culture medium promoted the production of secondary metabolites in *Digitalis thapsi* (Cacho *et al*., 2). The present study also recorded 397.77 mg 100 ml^{-1} gymnemic acid in calcium-free medium. The cell growth characters were also comparatively higher in this medium with a dry weight of 10.46 g l^{-1} on day 25. Calcium measurement studies indicated that the concentration of calcium in the cytoplasm of plant cells is maintained in the micromolar range. However, the calcium concentration in the cell wall and in organelles is in the millimolar range which accounts for intact cell wall and components (Fig. 4). Deprival of calcium in the medium would affect the cell wall and membrane integrity in due course leading to dispersion of the cellular contents in the medium (Cacho *et al*., 2).

The results showed that sodium azide even at 0.6 g l^{-1} reduced the secondary metabolite production to 55.93 mg 100 ml^{-1} when compared with other treatments. This shows that there is a possibility of inducing mutants through cell suspension culture which does not possess any secondary metabolite, which would ensure easier genetic studies for identification and isolation of genetic markers. Papadopoulou *et al.* (14) actually used sodium azide at 10 mM concentration to treat the seeds for creating saponin deficient mutant in oats. This chemical at 3 g l^{-1} reduced the cell density, PCV and viability of cells to a great extent in suspension culture. The gymnemic acid was quantified in cultures on day 40. Fluorescence microscopic study showed the cells released out their metabolites in to the medium on day 40. This is in accordance with Oncina *et al*. (12) in callus cultures of fenugreek. However, the possibility of increased accumulation of secondary metabolites in long-term culture has to be investigated. Hence, the present study indicated that yellowish-brown calli yielded high gymnemic acid and this could be selected for higher secondary metabolite production.

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