Journal of Applied Sciences

ISSN 1812-5654

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Applied Sciences 6 (10): 2263-2268, 2006
ISSN 1812-5654

Gymnemic Acid Production in Suspension Cell Cultures of *Gymnema sylvestre*

C. Subathra Devi, S. Murugesh and V. Mohana Srinivasan
Department of Biotechnology, School of Chemical and Biotechnology,
Shanmuga Arts, Science, Technology and Research Academy,
SASTRA Deemed University, Thanjavur-613 402, Tamil Nadu, India

Abstract: Use of *Gymnema sylvestre*, commonly known as Periploca of woods an Indian medicinal woody climber has increased recently due to the pharmaceutical potential of gymnemic acids, found in its leaves. Gymnemic acids has been reported to effect a natural treatment for diabetes. This study developed a novel cell culture system for *in vitro* growth and production of this species, suggesting a possible technology for large scale production of gymnemic acids. Leaf explants grown in Muragish and Skoog salts supplemented with IAA 1.5 mg L^{-1} and BA 0.5 mg L^{-1} gave maximum percentage callus formation compared to other medium treatments evaluated. Combination of external phytohormone, shaking speeds, pH of the medium, played important roles in growth and gymnemic acid production in suspension cell cultures.

Key words: *Gymnema sylvestre*, gymnemic acid production, callus culture

INTRODUCTION

The disillusionment of consumers over modern health care has led them to seek alternatives in traditional medicine. But the lack of legislation or effective agreements on conservation of biodiversity has resulted in slaughter harvesting of medicinal plants and massive depletion of biodiversity. The increased demands for herbal medicine had renewed interest by the multinational pharmaceutical industry in bio-prospecting. The increasing awareness about side effects of drugs had made the western pharmaceutical industries to turn towards the plant based Indian and Chinese medicine. The resurgence of public interest in plant based medicine coupled with rapid expansion of pharmaceutical industries has necessitated an increased demand for medicinal plants. With deforestation, medicinal wealth is lost, such that many valuable plants are threatened with extinction. Pharmaceutical companies depend largely upon materials procured from naturally stands that are rapidly being depleted. Plant tissue culture is an alternative method of propagation and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants. Plant cell culture system represent a potential renewable source of valuable compounds, flavours, fragrances and colorants, which cannot be produced by microbial cells or chemical synthesis. Plant cell culture technology plays an even more significant role in solving world hunger by developing products that provide higher yield. The present study describes the callus and suspension culture methods that we have established in our laboratory for the production of secondary metabolite gymnemic acid from a medicinal plant *Gymnema sylvestre* (potent antidiabetic plant) and production of gymnemic acids in suspension cell cultures of *Gymnema sylvestre*. This is the first study in *Gymnema sylvestre* callus suspension culture.

MATERIALS AND METHODS

Plant material: The seeds of *Gymnema sylvestre* were collected from Kodiakarai forest, India and germinated on the solid phytohormone-free MS medium with 0.8% of agar. pH of the medium was maintained at 5.8. Samples were grown at a photoperiod of 16 h light and 8 h darkness at 25±1 °C with the light intensity of 100 lux provided by cool white fluorescent lamps during the photoperiod, the resulting seedlings were used as explant source.

Callus induction: To induce callus formation, leaf explants excised from seedlings 12 cm height were cultured on agarized 0.8% (w/v) MS medium supplemented with 2% sucrose and different concentrations of BAP, IAA and 2,4-D were added (concentration ratio was specified in the Table 1). Sub cultures were carried out every 20 days. The experiments were repeated three times and each treatment consisted of 10 replicates.

Corresponding Author: C. Subathra Devi, Department of Biotechnology, School of Chemical and Biotechnology, Shanmuga Arts, Science, Technology and Research Academy, SASTRA Deemed University, Thanjavur-613 402, Tamil Nadu, India

Table 1: Effects of various concentrations of 2,4-D, IAA in combination with BAP (0.5 mg L^{-1}) on the frequency of callus induction in *Gymnemic sylvestre*

Auxins (mg L^{-1})	Callus induction frequency (% of mean±SE)
IAA	
0.5	31.0±1.12
1.0	44.0±1.20
1.5	60.0±1.81
2.0	1.9±1.30
2,4-D	
0.5	28.0±2.65
1.0	39.0±1.0
1.5	42.0±2.31
2.0	35.0±3.62

Suspension culture: After 5-6 subculture, calli 2-3 g fresh weight were inoculated in 250 mL conical flask containing 50 mL of the MS medium with BA 0.5 mg L^{-1} and IAA 1.5 mg L^{-1}, without agar and maintained on a rotary shaker at 120 rpm. After 20 days in liquid culture, the cells released from calli were transferred to fresh liquid medium. The calli which turned a black colour in the liquid medium were discarded and the healthy callus aggregates were repeatedly selected and transferred into another flask containing fresh liquid medium every 6 days. After several rounds of selection, the callus suspension culture was established. The subculture was kept by replacing the liquid medium every 10 days.

Growth rate determination: Growth rate of each callus strain was determined by dry weight measurement. Tissues were harvested at weekly intervals, dried at 60°C in a hot oven for 48 h. The data have been presented as an average of 10 replicates in three repetitive experiments and standard error of the mean.

Extraction, quantitative measurement and identification of gymnemic acids

Extraction: Release of gymnemic acids into the medium by certain extraction methods were investigated.

In the first method, the callus in the suspension culture was filtered and the medium was centrifuged at 10,000 rpm for 10 min to remove coarse debris. The clear supernatant was taken and the pH was reduced to 2 by adding 10% HCl. At the same time, the filtered callus was immersed in petroleum ether. After that the petroleum ether extract was removed and the marc was carried out by maceration for 2 days in each solvent at room temperature (25±2°C). The aqueous extract of gymnema callus were filtered, pH of the solvent extracted material is reduced to 2 by adding 10% HCl. Then both the flask were incubated at 4°C for one day, gymnemic acid was precipitated, the precipitate was centrifuged and washed with water and dried.

In the 2nd method, dried samples of *in vitro Gymnema sylvestre* culture were extracted with ethanol in a ultrasonic bath and kept for 2 days incubation at room temperature. As a general procedure, 75 mL of the solvent was added to dried sample (1 g) and sonication carried out for 30 min with 75 mL of water (aqueous extract) and it was kept in water bath. Then the pH of the extract was reduced to 2 by adding 10% HCl. The flask was incubated at 4°C, gymnemic acid was precipitated, the precipitate was centrifuged and washed with water and dried.

HPLC analysis: Total gymnemic acids was determined by HPLC analysis as gymnemagenin which was the main sapogenin obtained on the hydrolysis of the mixture of gymnemic acids present in the extract.

Preparation of the test solution 0.75 g of the sample is weighed accurately and dissolved it in 50% ethanol to make 50 mL. To 10 mL of this solution, 2 mL of 12.0% KOH is added and heated on a boiling water bath for 1 h. After cooling, 5.5 mL of 4 N HCl is added to make pH between 7.5 and 8.5 and 50% ethanol is added to make 100 mL. Then it is filtered through Whatmann No.I filter paper and subjected to 50% ethanol to make 10 mL. Then it was passed through a filter with 0.45 μm pore size prior to HPLC analysis using a C_{18} column (Wakosil II 5C 18 P 4.6×250 mm).

HPLC operating conditions: Eluent A-Acetonitrile-water (80:20), Eluent B-KH_2PO_4-water (0.1-100), Eluation gradient% A = 25-50 from 0 to 20 min, flow rate: 1.5 mL min^{-1}, UV detector: λ 210 nm. The amount of sample injected was 20 μL and the column temperature was maintained at 40°C. The retention time of gymnemic acid was 6.5. Figure 1 shows the typical separation profile. The gymnemagenin content of each sample was measured from the corresponding peak and the quantity calibrated with that of the external standards.

The effects of culture conditions and chemical feeding on gymnemic acid yield: The callus suspension culture was supplemented with various concentrations and

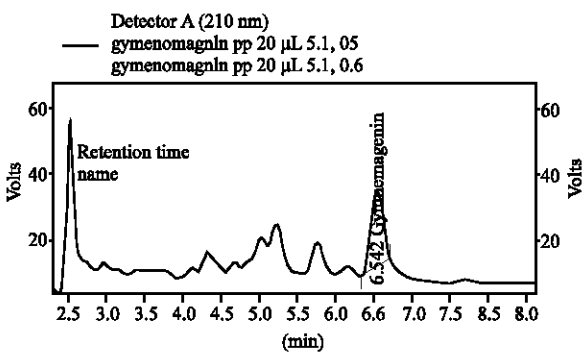

Fig. 1: HPLC Profile for gymnemagenin

combinations of external phytohormone, modified pH, different shaking speeds as specified in the result section to evaluate its effect on biomass accumulation and gymnemic acid content.

RESULTS AND DISCUSSION

Callus induction: The effects of external phytohormone on callus induction. We investigated the effects of various concentrations of IAA and 2, 4-D in combination with BAP (0.5 mg L^{-1}) on the frequency of callus induction using leaf explants of *Gymnema sylvestre*. The results were shown in Table 1. The callus was induced in all the combinations of IAA+BA and 2,4,D+BA, but the frequency of induction was more in 0.5 mg BAP + 1.5 mg IAA L^{-1}. Percentage ratio was 60±1.81(Fig. 2 and Table 1) The callus was not induced well in the medium containing 2 mg L^{-1} IAA. The effects of 2,4-D was also tested. The frequency rate was low when compared to IAA and BA combination. Among the auxins tested IAA showed better result. BA and IAA combinations induced callus formation after an inoculation period of 5-7 days. But the callus induction frequency was 35±3.62% in 2 mg L^{-1} 2,4-D concentration. Where as in IAA, it was 1.9±1.30. Growth of the callus was also high in case of IAA and BA combination. 2,4-D induced the callus formation but showed slower growth rate.

Fig. 2a: Calli developed from leaf explant of *Gymnema sylvestre*

Fig. 2b: Suspension culture of *Gymnema sylvestre*

The growth rate and gymnemic acid accumulation in the callus suspension culture: Figure 3 shows the kinetics of dry weight accumulation and gymnemic acid yield for the suspension culture over a 30 day growth period. The amount of initial inoculum inoculated was 0.345 g. The dry weight accumulation was slow during the initial 5 days after inoculation, the rate increased rapidly from day 5 to day 20 and gradually reached the stationary phase after day 25. During the early stage, the gymnemic acids were not produced. Gymnemic acids were detected on day 10. Only low level of 9.10 mg L^{-1} dry weight on day 10. The maximum gymnemic acid yield of this suspension culture was 98.63 mg gymnemic acid per liter on day 20 after the inoculation.

The optimization of gymnemic acid production in the callus suspension culture: The present study investigated the effect of altering following culturing conditions on the gymnemic acid yield; adjusting the speed, adding different external phytohormone, changing the pH of medium.

Table 2 lists the effects of external phytohormone on the gymnemic acid yield, different concentration ranges of IAA, BA and 2,4-D were tested. The highest biomass and gymnemic acid yields were obtained when the BA concentration was 1 mg L^{-1} and the IAA concentration was 0.5 mg L^{-1}. The biomass accumulation reached 1.98±0.01 g dry weight per liter and the gymnemic acid yield reached 109.71 mg L^{-1}, respectively (Fig. 2b). The combination of BA + IAA significantly stimulate the growth of the callus and gymnemic acid production. The externally added 2,4-D inhibited the dry weight accumulation of the suspension. High concentration of IAA and BA (5 and 10 mg L^{-1}) inhibited the growth and the production rate of gymnemic acid was also reduced. High concentrations of growth regulators often turned the cells black and stopped the growth. Kinetin IBA and the combinations (Kinetin, IBA and 24,4-D) were not shown significant results (data not shown).

Figure 4 shows the effects of pH, ranging from 3.7 to 6.5, on the growth and gymenmic acid content of the suspension culture. The gymnemic acid content was significantly stimulated when the pH value was 5.6, the maximum gymnemic acid yield was 98.01±0.05. The shaking speed could influence the secondary metabolism by affecting the gaseous environment of the liquid culture medium. We tested the effect of shaking speed al 100 and 150 on the 120 rpm min^{-1} gymnemic acid yield of the

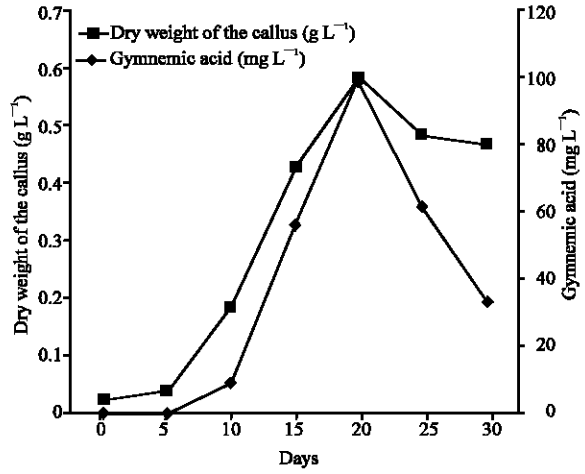

Fig. 3: The growth rate and gymnemic acid yield of callus suspension culture of *Gymnestre sylvestre*

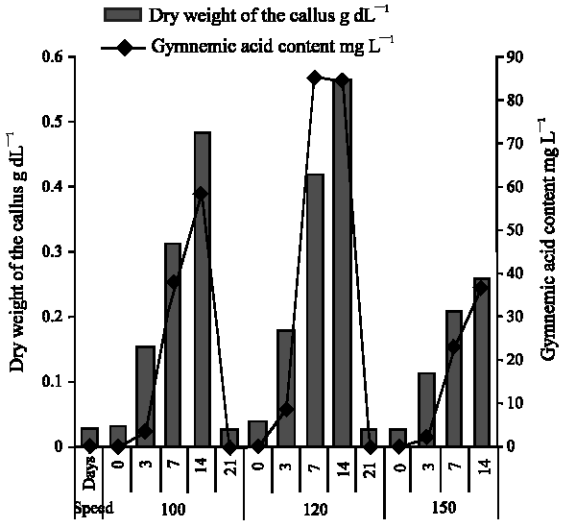

Fig. 4: Effect of pH on the growth of *Gymnema sylvestre* callus suspension culture

150 on the 120 rpm min^{-1} gymnemic acid yield of the suspension culture. The optimal shaking speed for gymnemic acid production is 120 rpm min^{-1}. The gymenmic acid yield for culture subjected to the shaking speed of 100, 120 and 150 rpm min^{-1} was 58.86±1.80, 84.68±3.30 and 37.21±1.15, respectively. Change in pH and shaking speeds were also altered the production rate of gymnemic acids. Figure 5 shows the effects of shaking speeds on the growth of *Gymnema sylvestre* suspension culture.

HPLC analysis: The samples extracted from dried callus and medium were subjected to HPLC analysis. The gymnemic acid content of each sample was measured from

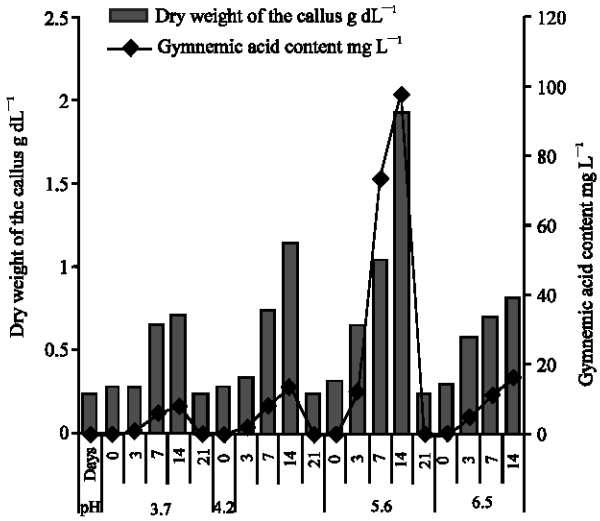

Fig. 5: The effect of shaking speeds on the growth of the *Gymnema sylvestre* callus suspension culture

the corresponding peak and compared with the standard. Dried callus samples peak were not correlated with the standard gymnemic acid. The gymnemic acids were not present in the dried callus sample. But the samples extracted from the medium contained gymnemic acids. From the results, we concluded that gymnemic acids were not accumulated there were released into the medium.

Over exploitation of natural stands has caused depletion of these plants in nature. *Gymnema sylvestre* natural strands are fast disappearing and are threatened with extinction due to its indiscriminate collection and over exploitation and natural resources for commercial purposes and to meet the requirements of the pharmaceutical industry (Choudhury, 1988). Commercial exploitation for production and conventional propagation is hampered due to its poor seed viability, low rate of germination and poor rooting ability of vegetative cuttings. Limited tissue culture work has been done on *Gymnema* species (Komalavalli and Rao, 1997; Reddy *et al.*, 1998). Komalavalli and Rao (2000) reported an efficient and rapid propagation of *Gymnema sylvestre* using various explants from *in vitro* grown seedling explants, but the multiplication rate achieved was very low. Alternative cell culture methods would be beneficial in accelerating large scale production of gymnemic acids. Cultured plant cells and tissues are widely recognised as promising alternatives for the production of valuable secondary metabolites (Lipsky, 1992; McDonald *et al.*, 1995). The successful cases are commercial production of Shikonin, berberine (Fujiita, 1988) and taxol (Blechert and Guenard, 1990). However, the low yield is still the major limitation for profitable commercialization (Shuangxiu *et al.*, 2003).

Table 2: The effects of external phytohormone on the dry weight accumulation, gymnemic acid content and yield of callus propagated in suspension culture

Concentration of phytohormone (μg L^{-1})				
IAA	BA	Biomass g dry (wt L^{-1})	Gymnemic acid content (mg g^{-1}) dry wt.	Net Gymnemic acid (mg L^{-1})
1 mg	0	1.70±0.03	5.35±0.11	9.09±0.16
	1	1.78±0.01	11.20±1.12	19.93±1.10
	2.5	1.61±0.01	6.97±0.02	11.22±0.03
	5.0	1.22±0.04	5.13±0.81	6.25±0.79
	1.10	1.10±0.01	5.01±0.05	5.51±0.03
BA	IAA			
1 mg	0	1.89±0.02	4.01±0.05	7.57±0.01
	0.5	1.98±0.01	55.41±1.67	109.71±1.55
	1.0	1.90±0.03	36.21±1.20	68.79±1.31
	1.5	1.95±0.03	10.24±1.11	19.96±0.98
	2.0	1.86±0.04	7.15±0.05	13.29±0.06
	5.0	1.92±0.02	4.18±0.13	8.02±0.08
BA + IAA	2,4-D			
2.5 + 1	0.0	1.72±0.01	9.12±2.01	15.68±2.00
	0.5	1.76±0.03	28.01±0.05	49.29±0.01
	1.0	1.83±0.05	12.31±0.31	22.52±0.28
	2.0	1.91±0.02	18.11±0.03	34.59±0.01
BA	IAA			
	0.5	1.70±0.03	15.0±1.10	25.5±1.12
	0.0	1.79±0.02	20.0±0.05	35.8±0.03
	1.0	1.85±0.02	18.0±1.21	33.3±1.18
	1.5	1.83±0.01	10.0±2.28	18.3±2.20
	2.0	1.83±0.02	6.0±1.12	10.98±1.15

The experiments were repeated three times and it consist of 10 replicates

In the present study, we established callus that can be propagated in a suspension culturing system and produced gymnemic acids under a defined culture condition. During the process, we demonstrated the profound influence of external phytohormone for the production of gymnemic acids from *Gymnema sylvestre* suspension culture. The proper combination of IAA and BA infleunce the rate of gymnemic acid production. The maximum yield of gymnemic acid is 109.71 mg L^{-1}. For the commercial production this work needs further investigation, to enhance the production rate. The synthesis of chemical compounds is often linked with the morphological and biochemical differentiation process. Chemical gradients in a differentiated tissue or callus aggregates may facilitate the synthesis of particular secondary metaoblites (Endress, 1994; Dorenburg and Knorr, 1995). Precursor feeding has been an effective method to increase the production of secondary metabolite in several culture system (Shuangxiu *et al*., 2003). The effects of precursors feeding on the product yield often indicate the distance between the precursor and product in the biosynthesis pathway. To meet the commercial demand, this plant needs future studies. Future studies involve, the addition of precursors and elicitors to increase the yield of gymnemic acids.

In vitro propagation of medicinal plants with enriched bioactive principle and cell culture methodologies for selective metabolite production is found to be highly useful for commercial production of medicinally important compounds. The increased use of plant cell culture systems in recent years is perhaps due to an improved understanding of the secondary metabolite pathway in economically important plants (Mulabhagal *et al*., 2004).

Complex and incompletely understood nature of plant cells *in vitro* cultures, case-by-case studies have been made within last few years (Mulabaghal *et al*., 2004). These new technologies will serve to extend and enhance the continued usefulness of potent anti-diabetic plant *Gymnema sylvestre*. Advances in *Gymnema sylvestre* cell culture could provide new means for the cost-effective and commercial production. We hope that a continuation and intensification efforts in this field will lead to controllable and successful biotechnological, production of specific, valuable and as yet unknown plant chemicals.

REFERENCES

Blechert, S. and D. Guenard, 1990. Taxus Alkaloids. In: The Alkaloids, Academic Press, Brossi, A. (Ed.), San Diego (Chapter 6), Vol. 39.

Choudhury, B.P., 1988. Assessment and Conservation of Medicinal Plants of Bhuvaneswar and its neighbourhood. In: Indigenous Medicinal Plants Today and Tomorrow's Printers and Publishers, New Delhi, India, pp: 211-219.

Dorenburg, H. and D. Knorr, 1995. Strategies for the improvement of secondary metabolites production in plant cell culture. Enzyme Microbiol. Technol., 17: 674-684.

Endress, R., 1994. Plant Cell Biotechnology. Springer-Verlag, Berlin, Heidelberg, pp: 121-242.

Fujita, Y., 1988. Industrial Production of Shikonin and Berberine, In: Application of Plant Cell and Tissue Culture, Bock, G. and J. Marsh (Eds.), CIBA Foundation Symposium, Wiley, Chickester, UK., 137: 228-235.

Komalavalli, N. and M.V. Rao, 1997. *In vitro* micropropagation of *Gymnema sylvestre* W and A, a rare medicinal plant. Ind. J. Exp. Biol., 35: 1088-1092.

Komalavalli, N. and M.V. Rao, 2000. *In vitro* micropropagation of *Gymnema sylvestre*-A Multipurpose medicinal plant. Plant. Cell Tissue and Organ. Culture, 61: 97-105.

Lipsky, A.K., 1992. Problems of optimization of plant cell culture processes. J. Biotechnol., 26: 83-97.

Mc Donald, K.A., A.P. Jackman, J.E. Thorup and A.M. Dandekar, 1995. Plant callus as a source of biochemicals. Applied. Biochem. Biotechnol., 54: 93-108.

Mulabagal V.C.Y., S.F. Lee, S.M. Ho, C.L. Nalawade and H.S. Tsay, 2004. Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures (Review). Bot. Bull. Acad. Sin., 45: 1-22.

Reddy, P.S., G.R. Gopal and G.L. Sita, 1998. *In vitro* multiplication of *Gymnema sylvestre* R. Br. An important medicinal plant. Curr. Sci., 75: 843-845.

Shuangxiu, W., Z. Yuanggang, W. Madeline, 2003. High yield production of salidroside in the suspension culture of *Rhodiola sachalinensis*. J. Biotechnol., 106: 33-43.