Research article

Dose dependent effect of silver nitrate on enhanced production of gymnemic acid in cell suspension cultures of *Gymnema sylvestre* R. Br.

Jarubandi Rajashekar¹, Vadlapudi Kumar^{1*}, Dittakoppa Vijendra Poornima¹, Gajula Hari¹,
Torankumar Sannabommaji¹, Raghuramulu D.²

¹Department of Biochemistry, Davangere University, Shivagangothri, Davangere-577 007 Karnataka India

²Department of Botany, Government Degree College for Men, Kurnool-518 001, Andhra Pradesh India

(Received: January 2021

Revised: February 2021

Accepted: February 2021)

Corresponding author: **Vadlapudi Kumar**. Email: veeke2012@gmail.com

ABSTRACT

Introduction and Aim: *Gymnema sylvestre* is rich source of triterpenoid saponins and is popularly known for its anti-diabetic potential. Gymnemic acid is chiefly responsible for its therapeutic potential. In fact, gymnemic acid is a group of compounds known as gymnemagenin (oleanane group) and other related compounds are gymnemasides (dammaranes). Despite of its immense medicinal importance, very few studies were conducted for enhanced gymnemic acid production. The present study undertaken for the enhanced gymnemic acid production using silver nitrate (AgNO_3) as effective elicitor and its influence on other culture parameters.

Materials and Methods: Callus culture was initiated for cell suspension culture establishment and is used to study the elicitor effect. Different doses of AgNO_3 used at varied culture periods and its mediated effect on growth index, pH, EC and MDA content was conducted. Gymnemic acid production was estimated by HPLC.

Results: Cell growth was positively influenced by AgNO_3 with concomitant change in pH and EC of the medium. Apparent rise in MDA content in response to increased AgNO_3 dosage was observed and is positively correlated with enhanced gymnemic acid production. Dose dependent effect of AgNO_3 was observed at different culture periods used under study.

Conclusion: Substantial improvements of gymnemic acid produced in response to AgNO_3 at 100 μM dosage can be used for its enhanced production at industrial scale. It also offers opportunities for studying elicitor influence on other parameters for gymnemic acid production.

Keywords: Gymnemic acid; HPLC; elicitation

INTRODUCTION

Gymnema sylvestre R. Br. (Family, Asclepiadaceae) is a woody climber found in tropical regions of Western Ghats of India and some regions of middle Africa and Australia. It is a natural source of various secondary metabolites with ample pharmacological activities and is used for the treatment of diabetes in Indian ayurvedic and medicinal pharmacopoeia since ancient times. Numerous studies on anti-microbial, anti-cancerous, anti-hypercholesterolemic, anti-arthritis, hepatoprotective, anti-inflammatory and cardiopathic effects has been reported (1, 2). Gymnemic acid is responsible for these biological properties and is a group of oleanane and other is dammarane class (gymnemasides) of triterpenoids (3).

Medicinal plants are rich source of various phytochemicals used as therapeutic drugs, nutraceuticals, flavours, fragrances and food additives etc. These phytochemicals are known as secondary metabolites produced in response to various stress conditions to hasten the adaptation process for host plant (4). In their natural habitat, accumulation levels are very low and depends on physiological and environmental factors, moreover isolating the active

principles also time consuming and economically infeasible and require indiscriminate exploitation of whole plant. In order to compensate these limitations, plant cell and organ culture provide an attractive alternative for the production of active principles (5). Among the different strategies used, elicitation has got special attention for enhanced secondary metabolite production (6). Stress molecules or conditions that activate plant defence mechanisms for the subsequent synthesis of secondary metabolites are termed as elicitors. Even at trace amounts elicitors trigger the defence mechanism by stimulating cascade of events leading to synthesis of secondary metabolites. Many reports are in favour of elicitor effect in cell and organ cultures (7, 8). Further, understanding the elicitor induced signal transduction for enhanced secondary metabolite synthesis gains special attention for the *in vitro* culture system optimization.

Due to its immense medicinal properties and low availability in natural host, reports were attempted to enhance yield in *in vitro* culture methods (9-11) using various elicitors. Till now, no reports available for the involvement of different parameters (pH, electrical conductivity and malondialdehyde content) and their relationship with gymnemic acid production is established. In view of the above, the present study

focused on dose dependent effect of silver nitrate (AgNO_3) on enhanced gymnemic acid biosynthesis in cell suspension culture of *G. sylvestre*. In addition, to investigate the time course effect the cultures were harvested at different time intervals and production levels determined using HPLC.

MATERIALS AND METHODS

Plant material

Leaf (meristems) explants were collected from *G. sylvestre* plants grown under greenhouse conditions in Davangere University, Shivagangothri campus.

Callus initiation and maintenance

Explants were initially rinsed with running tap water for 2 minutes, then washed with solution containing 0.1% (w/v) bavistin, 0.05 gm cetrimide (0.05% (w/v) and Tween-20 (2%) in distilled water (v/v) for 4 minutes and treated with mercuric chloride (0.1%) for 3 minutes. After each treatment, explants were rinsed for 3-4 times with sterile double distilled water. The surface sterilized explants were sliced along edges with sterile blade and were directly inoculated onto Murashige and Skoog (12) medium containing 2 mg/L BAP, 2 mg/L NAA, 0.5 mg/L picloram and 100 mg/L casein for the induction of callus. Medium pH was adjusted to 5.7 ± 0.1 prior to autoclave. Cultures were maintained at 16 h light and 8 h dark photoperiod ($50 \mu\text{mol}/\text{m}^2\text{s}^2$, $24 \pm 1^\circ\text{C}$) for 20 days or until the callus establishment. The initiated callus (pale green colour) was transferred aseptically to fresh culture medium containing similar growth regulators and sub cultured for every 2 weeks to maintain stable cell lines.

Suspension culture establishment

One month old friable callus (500 mg) inoculated into 250 mL Erlenmeyer flask containing 50 mL MS liquid medium supplemented with growth regulators (as used for the callus establishment) and were maintained in a gyratory rotatory shaker incubator at 110 rpm speed, $24 \pm 1^\circ\text{C}$ temperature under dark condition. The cell biomass obtained was utilized for evaluating elicitor effect on growth index and gymnemic acid content at different doses and time courses.

Elicitation

About 0.5g (500mg) of fresh callus was inoculated into 250mL conical flask containing 50mL of MS medium. Stock solution of AgNO_3 was prepared (10mg/mL), pH adjusted to 5.8 and filter sterilised before use in concentrations ranging from 25, 50, 100 and 200 μM . Elicitor treated cultures were harvested at regular time intervals (3, 6, 9 and 12 days) and cells were collected by filtration for the determination of growth index and gymnemic acid content. Non elicited control was maintained parallelly. All the experiments were conducted in triplicates.

Determination of cell growth, medium pH and conductivity

Cell growth was assessed as growth index. After completion of successive culture periods (3, 6, 9 and 12 days), the fresh cell mass was separated from the liquid media by passing through filter paper (Whatman No. 1) two times rinsed with distilled water and excess surface water was blotted away. Dry weight was measured after the cell mass dried in an oven at $45 \pm 2^\circ\text{C}$ for 48hrs. Growth index was used to reduce the discrepancies in cell growth caused by variation in callus inoculum size. This can be determined by using the formula,

$$\text{Growth index} = \frac{\text{Wt final}}{\text{Wt initial}} \times 100$$

Where, Wt final is weight of cell mass at the harvest, Wt initial-weight of cell mass at the day of inoculation (day 0).

The pH and conductivities of the culture media were measured with pH meter (Elico LI 120, India) and conductivity meter (Systronics conductivity meter 304, India) respectively.

Lipid peroxidation assay (MDA content)

Lipid peroxidation was determined by measuring the amount of MDA (malondialdehyde) produced by thiobarbituric acid (TBA) reaction. MDA content was estimated using method developed by Heath and Packer (13). The fresh biomass was homogenised with 1.5mL of 0.5% trichloro acetic acid and centrifuged at 10,000rpm for 15min. Then 500 μL of supernatant was mixed with 2mL of TBA (0.5% w/v TBA in 20% TCA) and incubated at 95°C for 30min, rapidly cooled in ice bath, centrifuged at 5000rpm for 10min and absorbance was read at 532nm and 600nm. The amount of MDA produced measured using extinction coefficient 155mM/Cm and expressed as nM/g FW.

Extraction of gymnemic acid

Cell filtrates of respective suspension cultures (100 mg DCW) were mixed with 50 mL of extraction solvent (50% methanol) and 10 mL of 12% KOH (w/v) and refluxed for one hour. Again, refluxed for one more hour by adding 11 mL of 4N HCl. The mixture was cooled to room temperature, pH was adjusted between 7.0-7.5 and filtered through 0.22 μm nylon membrane filters (Hi-media, India). The clear supernatant was used for the quantitative analysis by HPLC.

Quantification of gymnemic acid by HPLC

Gymnemic acid was quantified as its aglycone gymnemagenin by HPLC analysis (Agilent 1260) and separations were observed at 210nm in a ZORBAX Eclipse plus C18 analytical column ($4.6 \times 250 \text{ mm}$, 5 μm) using acetonitrile and water (80:20) as an eluent at a flow rate of 1 mL/min at 26°C column temperature with 20 μL of injection volume. Presence of gymnemic acid (gymnemagenin) was confirmed by the retention time with the help of gymnemagenin standard (Natural Remedies, Bangalore, India). Total

gymnemic acid present in the sample was quantified as gymnemagenin by using the following formula with molecular weight corrections,

$$\text{Gymnemic acid content} = \frac{\text{Ht} \times \text{Ws} \times 500}{\text{Hs} \times \text{Wt} (1 - \text{Dw}/100)} \times \frac{809.0}{507.7}$$

Where, Ht peak height of test sample chromatogram, Hs peak height of standard chromatogram, Ws weight of gymnemagenin standard, Wt weight of test, Dw loss on drying test sample.

Statistical analysis

All data represented were the average of three independent determinations $\pm$ SE. Data were analysed by one-way ANOVA, and mean values of treatments were compared at $P < 0.05$ Tukey-Kramer (HSD) test.

RESULTS

Effect of AgNO₃ on cell growth

Cell growth was monitored by measuring fresh cell weight at each successive culture periods. During the present study, different doses of AgNO₃ response on

cell suspension cultures of *G. sylvestre* corresponding to cell growth was investigated at varied time courses. Significant difference in cell growth observed with increase in elicitor dosage compared to control ($p < 0.05$). Treatment with AgNO₃ at all doses has showed least significant effect during 3 days post elicitation. Then, gradual increase in cell growth observed for 9 days of culture period, later declining trend was noticed (Fig. 1). However, AgNO₃ at 50 μ M showed maximum growth promoting effect at 9 days of culture period ($221.31 \pm 5.4\%$) and the observed response is 20% higher to respective control ($184.7 \pm 4.8\%$) followed by 6 days (13% higher to control). Addition of AgNO₃ at 100 μ M also showed comparable response but is slightly inferior ($204.64 \pm 6.8\%$) to 50 μ M. Increasing culture period to 12 days, similar kinetics with respect to cell growth inhibition was observed at all tested doses. In comparison to control, AgNO₃ at 200 μ M showed largest decrease in cell growth of 22%. This implies that use of AgNO₃ at 50 μ M at 9 days post elicitation found to be effective dose.

Fig. 1: Dose dependent effect of AgNO₃ on cell growth in *G. sylvestre* cell suspension cultures at different culture periods. Each value represents mean of three independent experiments $\pm$ SE.

Effect of AgNO₃ on medium pH, EC and MDA content

To better understand AgNO₃ dose dependency on cell cultures medium pH, electrical conductivity (EC) and MDA content were investigated. Gradual decrease in

EC of the medium observed without difference between treated and control cultures. This could be ascribed to higher inorganic and mineral nutrient consumption (Fig. 2). Perhaps, AgNO₃ at 200 μ M concentration showed significant difference and is further confirmed by decrease in cell growth.

Fig. 2: Dose dependent effect of AgNO₃ on EC of the medium in *G. sylvestre* cell suspension cultures at different culture periods. Each value represents mean of three independent experiments $\pm$ SE.

As shown in Fig. 3, slight increase in medium pH observed in elicitor treatment and is in similar manner at all tested doses. However, control cultures showed significant decrease in medium pH with change in

culture period ($p < 0.05$). This variation could be nitrate absorption and thereby ammonia assimilation with consequent rise in hydrogen ion concentration.

Fig. 3: Dose dependent effect of AgNO_3 on pH of the medium in *G. sylvestre* cell suspension cultures at different culture periods. Each value represents mean of three independent experiments $\pm$ SE.

In the present study, all treated cultures showed higher MDA than control. Increase in MDA content observed with gradual increments of elicitor dosage and culture period (Fig. 4). Maximum was observed in cells

treated with 200µM at 12 days post elicitation. Strong positive correlation between MDA content to the gymnemic acid production observed.

Fig. 4: Dose dependent effect of AgNO_3 on MDA content in *G. sylvestre* cell suspension cultures at different culture periods. Each value represents mean of three independent experiments $\pm$ SE.

Effect of AgNO_3 on gymnemic acid production

In the present study, AgNO_3 tested at all the concentration ranges showed significant increase in gymnemic acid content compared to untreated control cultures. To achieve maximum yield, varied culture periods were tested with different elicitor doses. Gymnemic acid HPLC chromatograms for AgNO_3 elicited samples were illustrated in Fig. 5a and b. Among all the doses tested, application of AgNO_3 at 100µM showed greatest improvements in gymnemic acid at 12 days of culture period, which corresponds to (82.24 ± 1.78 mg/g DW) 8.5 folds higher to respective

control (Fig. 6). Steady increase in gymnemic acid accumulation levels observed over 9 days post elicitation at all doses tested and the maximum was recorded at 200 µM (72.40 ± 3.8 mg/g DW) and the production levels are 6 folds higher to respective control (Figure 6). Further, inverse correlation observed with increasing culture period (61.14 ± 4.35 mg/g DW) which represent 6.5 folds higher to control. Perhaps, AgNO_3 at 200 µM showed a rapid increase in gymnemic acid accumulation within 3 days (68.04 ± 4.6 mg/g DW) and remained higher throughout the culture period.

Fig. 5 a: HPLC chromatograms of gymnemagenin standard, b: HPLC chromatogram of AgNO₃ treated sample.

Fig. 6: Dose dependent effect of AgNO₃ on gymnemic acid content in *G. sylvestre* cell suspension cultures at different culture periods. Each value represents mean of three independent experiments $\pm$ SE.

DISCUSSION

Numerous studies have shown elicitor mediated enhanced secondary metabolite production in cell and organ culture systems (7). Elicitor specificity, culture period, dose, culture conditions (medium pH, nutrient composition, growth regulators, light conditions, shaking speed), type of cell culture (cell suspension, hairy root, adventitious roots, cambial meristems etc) and culture growth stage are the critical parameters having significant impact on elicitor mediated secondary metabolite production (6).

In this study dose dependent effect of AgNO₃ on cell growth and gymnemic acid production in cell suspension cultures of *G. sylvestre* was observed. Improved cell growth was observed with optimised elicitor dosage (50 μM) and is higher than control culture. Elicitor mediated growth promoting effect also observed in hairy root cultures of *Salvia miltiorrhiza* (14). However, cell growth retardation observed with apparent rise in elicitor dose and culture

period. This stipulates elicitor optimisation for improved cell growth. At optimised elicitor dose, linear correlation between cell growth to medium pH and EC ascribed to higher inorganic and mineral nutrient consumption with concomitant increase in culture period and the inverse is true at higher elicitor dose. Previous reports also stated the growth retardation at higher elicitor concentration (15, 16). This states that, elicitor effect dependent on elicitor dose and culture period used under study. Most often, elevated secondary metabolism showed strong negative correlation with cell growth (6). However, in the present study, higher gymnemic acid production observed with 100 μM with least growth retarding activity. This is highly preferable for obtaining notable increments of secondary metabolites in *invitro* cell culture systems.

Ethylene acts as signal molecule produced in response to various stress for the induction of defence mechanisms. At higher levels, ethylene inhibits

secondary metabolite production and the inverse is true for enhanced secondary metabolite production (17). Previously, effectiveness of ethylene inhibitors on enhanced secondary metabolite production in cell suspension cultures of *Gloriosa superba* (18) and *Hybanthus enneaspermus* (19) reported. Silver ions at higher doses strongly inhibit ethylene biosynthesis. This suggests the effectiveness of AgNO₃ as potent elicitor for the enhanced secondary metabolite production (20). In the present study, low doses of AgNO₃ inhibit ethylene mediated signalling cascade with consequent rise in gymnemic acid production. However, increase in AgNO₃ dose to 200µM decreased the cell growth and restricts metabolite production. This could be presumed to higher elicitor concentration induces hypersensitive response and leading to cell death with consequent reduction in overall productivity. Hence, optimising elicitor dose and culture period are prerequisite for increased secondary metabolite production (6). Many types of heavy metals such as Ca²⁺, Ag²⁺, Cd²⁺, Cu²⁺, Mn²⁺ and Zn²⁺ have significantly involved in enhanced secondary metabolite production (7) Ramirez Estrada). Heavy metals generate reactive oxygen species (ROS) that cause oxidative stress with subsequent membrane damage and expression of defence related genes. The produced ROS act as catalyst for enzymatic and non-enzymatic lipid peroxidation and thus Jasmonic acid (JA) biosynthesis (21, 22). The signal events mediated by JA lead to synthesis of various secondary metabolites and other related compounds (23, 24). Positive correlation between MDA content to gymnemic acid production indicate that, elicitor induced cell stress in imperative for enhanced secondary metabolite production.

It would be highly desirable for the elicitor mediated enhanced gymnemic acid production with least compromising cell growth. Considering this, the established protocol in the present study undoubtedly becomes a potential method for sustainable commercial scale production.

CONCLUSION

Results of the present study demonstrate that elicitor dosage and culture period are the critical parameters in determining the enhanced gymnemic acid production in cell suspension cultures of *G. sylvestre*. Notable increase observed with AgNO₃ at 100µM considered as most effective elicitor dose. The production level was 8.5 folds higher to respective control with least compromising cell growth. Elicitor induced cell stress is imperative for the enhanced gymnemic acid production and can be ascertained based on MDA content. Stable maintenance of pH and EC of the medium at optimised elicitor dose compared with control culture indicates that, 100 µM found to be effective elicitor dose at 12 days post elicitation. Further experiments are required to characterise the

mechanisms underlying AgNO₃ mediated response on enhanced gymnemic acid production.

CONFLICT OF INTEREST

The authors declare no conflict of interest

REFERENCES

1. Sarker, P., Rahman, M. M., Khan, F., Ming, L. C., Mohamed, I. N., Zhao, C., Rashid, M. A. Comprehensive review on phytochemicals, pharmacological and clinical potentials of *Gymnema sylvestre*. *Front. Pharmacol.* 2019; 10: 1223.
2. Tiwari, P., Ahmad, K., Hassan Baig, M. *Gymnema sylvestre* for diabetes: from traditional herb to future's therapeutic. *Cur. Pharm. Design.* 2017; 23(11): 1667-1676.
3. Ye, W. C., Zhang, Q. W., Liu, X., Che, C. T., Zhao, S. X. Oleanane saponins from *Gymnema sylvestre*. *Phytochem.* 2000; 53(8): 893-899.
4. Yang, L., Wen, K. S., Ruan, X., Zhao, Y. X., Wei, F., Wang, Q. Response of plant secondary metabolites to environmental factors. *Molecules.* 2018; 23(4): 762.
5. Namdeo, A. G. Plant cell elicitation for production of secondary metabolites: a review. *Pharmacog. Rev.* 2007; 1(1): 69-79.
6. Isah, T. Stress and defence responses in plant secondary metabolites production. *Biol. Res.* 2019; 52(1): 39.
7. Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo, D., Moyano, E., Golenioski, M., Cusidó, R. M. *et al.*, Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules.* 2016; 21(2): 182.
8. Vijendra, P. D., Jayanna, S. G., Kumar, V., Sannabommaji, T., Rajashekar, J., Gajula, H. Product enhancement of triterpenoid saponins in cell suspension cultures of *Leucas aspera* Spreng. *Ind. Crop. Prod.* 2020; 156: 112857.
9. Veerashree, V., Anuradha, C. M., Kumar, V. Elicitor-enhanced production of gymnemic acid in cell suspension cultures of *Gymnema sylvestre* R. Br. *Plant Cell. Tiss. Organ. Cult.* 2012; 108(1): 27-35.
10. Chodiseti, B., Rao, K., Gandhi, S., Giri, A. Gymnemic acid enhancement in the suspension cultures of *Gymnema sylvestre* by using the signaling molecules—methyl jasmonate and salicylic acid. *In Vitro Cell Dev. Biol.-Plant.* 2015; 51(1): 88-92.
11. Chung, I. M., Rajakumar, G., Subramanian, U., Venkidasamy, B., Thiruvengadam, M. Impact of copper oxide nanoparticles on enhancement of bioactive compounds using cell suspension cultures of *Gymnema sylvestre* (Retz.) R. Br. *Appl. Sci. Basel.* 2019; 9(10): 2165.
12. Murashige, T., Skoog, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 1962; 15(3): 473-497.
13. Heath, R.L., Packer, L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* 1968; 125(1): 189-198.
14. Xing, B., Yang, D., Guo, W., Liang, Z., Yan, X., Zhu, Y. *et al.*, Ag⁺ as a more effective elicitor for production of tanshinones than phenolic acids in *Salvia miltiorrhiza* hairy roots. *Molecules.* 2015; 20(1): 309-324.
15. Hari, G., Vadlapudi, K., Vijendra, P. D., Rajashekar, J., Sannabommaji, T., Basappa, G. A combination of elicitor and precursor enhances psoralen production in *Psoralea corylifolia* Linn. *Suspension Cultures. Ind. Crops Prod.* 2018; 15(124): 685-691.
16. Cai, Z., Kastell, A., Speiser, C., Smetanska, I. Enhanced resveratrol production in *Vitis vinifera* cell suspension cultures by heavy metals without loss of cell viability. *Appl Biochem Biotechnol.* 2013; 171: 330-340.
17. Pan, Z. W., Wang, H. Q., Zhong, J. J. Scale-up study on suspension cultures of *Taxus chinensis* cells for production of

- taxane diterpene. Enzyme. Microb. Technol. 2000; 15, 27(9): 714-723.
18. Mahendran, D., Kavikishore, P. B., Sreeramanan, S., Venkatachalam P. Enhanced biosynthesis of colchicine and thiocolchicoside contents in cell suspension cultures of *Gloriosa superba* L. exposed to ethylene inhibitor and elicitors. Ind. Crop Prod. 2018; 15,120: 123-130.
 19. Sathish, S., Vasudevan, V., Karthik, S., Pavan, G., Manickavasagam, M. Enhanced l-Dopa production from elicited cell suspension culture of *Hybanthus enneaspermus* (L.) F. Muell. Plant Biotechnol. Rep. 2019; 3(6): 613-621.
 20. Rahimi A., Hasanloo, T., Bihamta, M. R. Enhanced production of silymarin by Ag⁺ elicitor in cell suspension cultures of *Silybum marianum*. Pharm. Biol. 2010; 48: 708-715.
 21. Zhao, J., Davis, L. C., Verpoorte, R. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Adv. 2005; 23(4): 283-333.
 22. You, J., Chan Z. ROS regulation during abiotic stress responses in crop plants. Front. Plant Sci. 2015; 8,6: 1092.
 23. Ali, M., Baek, K. H. Jasmonic acid signalling pathway in response to abiotic stresses in plants. Int. J. Mol. Sci. 2020. 21(2): 621.
 24. Narayani, M., Srivastava, S. Elicitation: a stimulation of stress in in vitro plant cell/tissue cultures for enhancement of secondary metabolite production. Phytochem. Rev. 2017; 16(6): 1227-1252.