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Research Article

# ENHANCED PRODUCTION OF GYMNEMIC ACID USING HR BIOELICITOR EXTRACTED FROM *XANTHOMONAS SPP.*

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**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 have 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 Murashige and Skoog salts supplemented with IAA 1.5mg/l and BA 0.5mg/l gave maximum percentage of callus formation compared to other treatments evaluated. The growth rate and gymnemic acid accumulation in the callus suspension culture was determined. The HR protein from *Xanthomonas spp.* was used as an elicitor for the production of gymnemic acid. When compared to non-elicited cultures, two fold increase of gymnemic acids yield in elicited cultures was observed. The quantification of gymnemic acid was done using HPLC. The total gynemagenin after 21st day of incubation was 30.2389mg/100ml. This method can be used economically in pilot scale studies.
**Keywords**: Bioelicitation, HR elicitor, *Gymnema sylvestre*, plant cell culture

## INTRODUCTION

The past studies were basically on the plant and its phytochemical analysis which mainly lead to the extinction of the plants. As the medicinal plants are getting extinct day by day due to it's over exploitation for commercial and pharmaceutical purpose[1]. Plant tissue culture techniques are the best method to conserve these plants from extinction by increasing its number in the habitat. Callus culture and suspension culture by taking plant leaves as explants can be used for regeneration of these plants. The whole plant can also be derived from a single callus varying its hormonal concentration.[2-3-4] These callus culture techniques can also be used for the production of secondary metabolites which are bio active compounds. Several major strategies have been explored for up regulation of metabolism including precursor feeding, hormonal signaling, elicitation, immobilization of the plant cells and nutrient stress.[5] The process of enhancing or inducing secondary metabolite production by the plants to maintain their survival, persistence and competitiveness is referred as elicitation.[6] Elicitors are of two types: abiotic and biotic elicitor. In abiotic elicitor various types of metals are used for inducing the stress.[7] Various types of microorganism such as algae, fungi as well as bacteria[8], which are mainly plant pathogen in nature, can be used. The hypersensitivity reaction which is caused in the plant cells in presence of microorganism leads to the production of the secondary metabolite as a defense mechanism is the main principle behind this stress.[9] This method is cost effective as a single colony of microorganism can produce large amount of elicitor proteins and purification is also very cheap in pilot scale. The microorganism does not affect humans as they are mainly plant pathogens. The aim of the present research is mainly based on the *Gymnema sylvestre* suspension culture and enhanced the production of the secondary metabolite responsible for the antidiabetic activity.

The novelty of this work lies in using bacterial plant pathogen, *Xanthomonas* spp as bio elicitor for increasing the yield of the secondary metabolite production of *Gymnema sylvestre*.

## MATERIALS AND METHODS

### Collection of plant material

Leaves of *Gymnema sylvestre* were collected from one single plant grown in the natural habitat of Porur forest located in Javadhu hills located 45-60 kilometers from Vellore, Tamil Nadu, India and it was identified by the taxonomist from Botanical Survey of India, Coimbatore, Tamil Nadu.

### Callus culture

The surface sterilized auxiliary nodal explants of 12 cm height were cultured on Murashige and Skoog medium supplemented with 3% sucrose and solidified with 0.8% agar with 1.5 mg/l of IAA and 0.5 mg/l of BA and the pH of the medium was maintained at 5.8.[4] Samples were grown at a photoperiod of 16 h light and 8 h darkness at 25 ± 1°C with the light intensity of 1000-2000 lux provided by cool white fluorescent lamps during the photoperiod. Subcultures were carried out for every 15 days.

### Suspension culture

After 7-8 subcultures, calli were inoculated in 250 ml conical flask containing 100 ml of the MS medium with BA 0.5 $mgl^{-1}$ and IAA 1.5 $mgl^{-1}$, without agar and maintained on a rotary shaker at 120 rpm. After 7 days in liquid culture, the cells released from calli were transferred to fresh liquid medium.[4] The calli which turned to black color in the liquid medium were discarded and the healthy callus aggregates were repeatedly selected and transferred into another flask containing fresh liquid medium for every 6 days. After

several rounds of selection, the callus suspension culture was established. The subculture calli were maintained by replacing the liquid medium for every 10 days.

**Preparation of bacterial elicitor**

*Xanthomonas spp.* was isolated from citrus canker of lemon leaves and was identified by standard microbiological methods. *Xanthomonas* spp. was grown on plates containing YEPG media. Seed cultures were inoculated from these plates and grown in 50% LB at approximately 27° C (room temperature), until an optical density (620 nm) of 0.5 to 0.8 was achieved. The seed cultures were then used to inoculate minimal media cultures in a manner such that no LB was introduced into the minimal media. A 1: 10 ratio of seed culture to minimal media was used to inoculate into the minimal media cultures (i. e. the cell pellet from a 50 ml seed culture was used to inoculate 500 ml of minimal media). In the minimal media containing $K_2HPO_4$ of 39.2g/l, $KH_2PO_4$ of 71.5g/l, $(NH_4)_2.SO_4$ of 10.0g/l, $MgCl_2$ of 3.5g/l, NaCl of 1.0g/l, sucrose of 34.23g/l served to induce the bacteria to produce significant amount of the HR elicitor. The minimal media culture was grown at approximately 27° C (room temperature) until an OD of 1.7 to 2.0 was achieved at 620nm.Once the desired OD was reached, the minimal media culture was centrifuged. The resulting cell pellet was resuspended at a 1: 10, weight to volume ratio in lysis buffer (20mM NaCI and 20mM Tris-HCl at pH 8.0). The resuspended cells were ruptured by sonication. The resulting sonicate was maintained on ice until the heat-treatment. The sonicate was placed on a preheated stir plate, and brought to a rolling boil. The boil was maintained for 5 minutes. The solution was then placed in an ice water bath and cooled to approximately 10º C. Once cooled, the sonicate was brought back to its original volume with double distilled water (replacing the volume lost to evaporation during boiling). The solution was then centrifuged at 15000 rpm for 30 minutes. The centrifugation was repeated for 3 times. The resulting supernatants were combined and frozen at -80° C.[10]

1 g of fresh healthy callus was transferred to 100 ml MS liquid medium with 1.5mg/l of IAA and 0.5mg/ l of BA. One flask was introduced with 3 ml of HR bio elicitor and the other was kept as control (without elicitor). The flasks were incubated at 25±2˚C for 21 days in 16 h of light and 8 h of dark. Both the flasks were kept in a shaker at 120 rpm.

**Extraction of gymnemic acids**

Release of gymnemic acids into the medium was extracted by two methods. In the first method[11] the callus in the suspension culture was filtered, and the medium was centrifuged at 10,000 rpm for 10 minutes 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 mare was carried out by maceration for 2 days in each solvent at room temperature (25 ± 2°C). The aqueous extract of *Gymnema* callus was filtered, pH of the solvent extracted material is reduced to 2 by adding 10 per cent 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 second method[4], dried samples of *in vitro Gymnema sylvestre* cultures were extracted with ethanol in an 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 minutes 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, and the precipitate was centrifuged and washed with water and dried.

**Identification and quantification of gymnemic acid**

The major bioactive compound of *Gymnema sylvestre* is a group of 'gymnemic acid". Non- availability of the different reference standards make the job more difficult, therefore the estimation of the different gymnemic acid was performed by hydrolyzing the extract first with alkali and then with acid. The '*Gymnema*genin' thus obtained was estimated by HPLC [12] and the total gymnemic acid was calculated by applying the molecular weight.

**Estimation of gymnemic acids in *gymnema sylvestre* extract**

**Preparation of test solution**

The sample of 0.75g was weighed accurately and dissolved it in 50 per cent ethanol to make 50 ml. To 10 ml of this solution, 2 ml of 12.0 % KOH was added and heated on a boiling water bath for 1h. After cooling, 5.5 ml of 4 N HCl was added and heated on a boiling water bath for 1h. After cooling 12.0 % KOH was added to make pH between 7.5 and 8.5 and 50% ethanol is added to make 100 ml. Then it was filtered through Whatmann No.1 filter paper and subjected to 50 per cent ethanol to make 100 ml. Then it was filtered and subjected to HPLC.

Preparation of a standard solution, Gynemagenin of 0.010g was weighed accurately and dissolved in 50 % ethanol to make 100 ml.

Testing procedure: Test solution and standard solution were subjected to HPLC separately.[15] HPLC operating conditions are Wakosil II 5C18 P 4.6 mm x 250 mm column with Acetonitrile – Water (80: 20) and $KH_2PO_4$ – Water (0.1 – 100) from 0 to 20 minutes with flow rate of 1.5 ml / minute. The amount of sample injected was 20 µl with UV detector 210 nm λ with column temperature of 40°C .[4]

**RESULT AND DISCUSSIONS**

**Callus Induction**

The growth and frequency of callus induction was observed till 30$^{th}$ day from the day of incubation. The callus induction was observed on 20$^{th}$ day in IAA+BA combination, while in NAA+BA there was no callus induction till 30$^{th}$ day. As the frequency of the callus induction was more in IAA+BA[4], this is unlike many reported literature for micropropogation of *Gymnema sylvestre* and other medicinal plants [3-4-14], where there is callus induction with NAA+BA. From the above observation IAA+BA was observed to be the better combination for the callus induction of *Gymnema sylvestre*. The color of the callus was cream to pale yellow. The callus was increased after 9 days of its incubation. The color of the callus was pale green(Fig. 1).

**Suspension culture**

Initially the growth rate of the callus was very slow and then gradually increased after 7 to 10 days. The calli which turned black in the liquid medium was removed. The gymnemic acid was more accumulated in the callus itself; the release of gymnemic acid into the suspension was facilitated using shaker with 120±2rpm. The fragile callus gave a ball like appearance with cream in color in the suspension medium.

The fragile callus gets dissociated into smaller calli while kept in shaker to produce sand like calli at the bottom of the conical flasks. The production of secondary metabolites in the suspension is detected by color change of the media to yellow (Fig 1). When the shaking was continued for more than 21 days the callus turned dark green color, which indicates the organogenesis of the callus (Fig. 1). This also resulted in decrease in production of gymnemic acid. The highest production of the gymnemic acid was on 18th day. Unlike the other suspension cultures reported previously the gymnemic acid was accumulated inside the callus other than releasing out in the media was detected by phytochemical analysis and HPLC [4-14]. The darkening of callus occurred after 30th day from the day of inoculation when kept continuously in shaker in the same media. The callus was more fragile in the combination with IAA+BA than 2, 4-D +NAA+BA. For increasing the growth and fragility of the callus for the further experiments the subculturing of the callus to the fresh media was done after every 10 days. The growth of the callus in the suspension culture was significantly higher with IAA+BA combination than 2,4D+NAA+BA combination which was reported previously.1[4] Different kinds and concentrations of auxins were tested for cell culture of *Gymnema sylvestre*. Cell growth was greatest at 5.0 mg $L^{-1}$ NAA, while significantly inhibited by IBA treatment.[15] So the hormone combination of IAA+BA was used for the further experiments as it was suitable for the *Gymnema* callus growth.

**Bioelicitation using *xanthomonas spp.***

Hypersensitive Response (HR) elicitor from *Xanthomonas* spp was added into the medium. After 21 days the biomass content in the elicited suspension culture was significantly higher than the non-elicited culture. The color of the callus changed from pale colored to dirty pink in colored due to the hypersensitivity response of the elicitor (Fig 1). The amount of initial inoculum added was 1g/100ml. After 21 days of incubation, the biomass in the elicited callus suspension was found to be 22884 mg/100ml and in the non-elicited it was 15478 mg/100ml. The gymnemic acid production was also high in elicited than in non-elicited suspension. The detection and quantification of g*ymnema*genin was done using HPLC analysis.

**HPLC analysis**

The samples extracted from callus were subjected to HPLC analysis. The total content of 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. Fig. 2, 3, 4 shows the HPLC profile of gymnemagenin. The retention time of gymnemic acid was 0.90. Fig.2 shows the typical separation profile of gymnemagenin standard. Fig.3 and Fig.4 shows the separation profile of the test samples. The gymnemagenin content of each sample was measured from the corresponding peak and the quantity calibrated with that of the external standards. The content of gymnema saponin in cell suspension culture of *Gymnema sylvestre* before and after treatment with biotic elicitor is given in the table 1. When compared to non-elicited cultures very high yield was achieved in elicited cultures. The maximum gymnemagenin yield of elicited cultures was found to be 30.2389 mg/100ml. The increase in *Gymnema*genin yield was approximately twofold in the elicited callus culture than the control suspension. The mechanism of action of this elicitor is still not known properly, but the metabolic pathways of secondary metabolite production are helpful in understanding the microbial interactions between the plants cells and the microbes. It has been previously reported that various elicitors like MJ, yeast extract, pectin and chitin treatment could be effective for enhancing gymnemic acid production in cell suspension cultures.[16] Aspergillus *niger* cell extract was also used as a bio-elicitor [17], the productivity increase was considerably greater than that obtained with non-elicited cultures. 8-9 fold increase in gymnemic acid was observed in fermentor and two fold in shake flask.[17] *In vitro* salt stress also induced the production of gymnemic acid in callus cultures of *Gymnema sylvestre* R.Br. The gymnemic acid content increased with increasing concentration of 2,4-D along with NaCl.[18] 2,4-D (2.0 mg/L) and Kinetin (0.1 mg/L), and 3% w/v sucrose was found best for the accumulation of biomass and gymnemic acid content (9.95 mg/g dry weight) [19]

In *vitro* production of gymnemic acid through callus culture under abiotic stress was also reported.[20] In abiotic elicitor various types of metals are used for inducing the stress. For the laboratory scale studies of the abiotic elicitor it is cost effective but when used in pilot scale production of the bioactive compound it becomes highly economic due to the cost of the metal as well as the purification steps involved in the downstream processing. The improper purification of these metals may also lead to lethal effects for humans. These problems can be overcome by using biotic elicitor such as microorganism for giving stress to the callus. The hypersensitivity reaction which is caused in the plant cells in presence of microorganism leads to the production of the secondary metabolite as a defense mechanism is the main principle behind this stress. [9]

In conclusion addition of HR elicitor to cell suspension cultures of *G. sylvestre* enhanced the production of gymnemic acid.

**Table 1: The content of *Gymnema*genin in cell suspension cultures of Gymnema sylvestre before and after treatment with HR bio elicitor.**

| Cell suspension | Metabolite content mg/100ml (21st day) |
|---|---|
| Control | 14.97±1.35 |
| Elicitated by HR elicitor of *Xanthomonas* spp | 30.89±1.02 |

Values are mean ± SE of 10 replicates

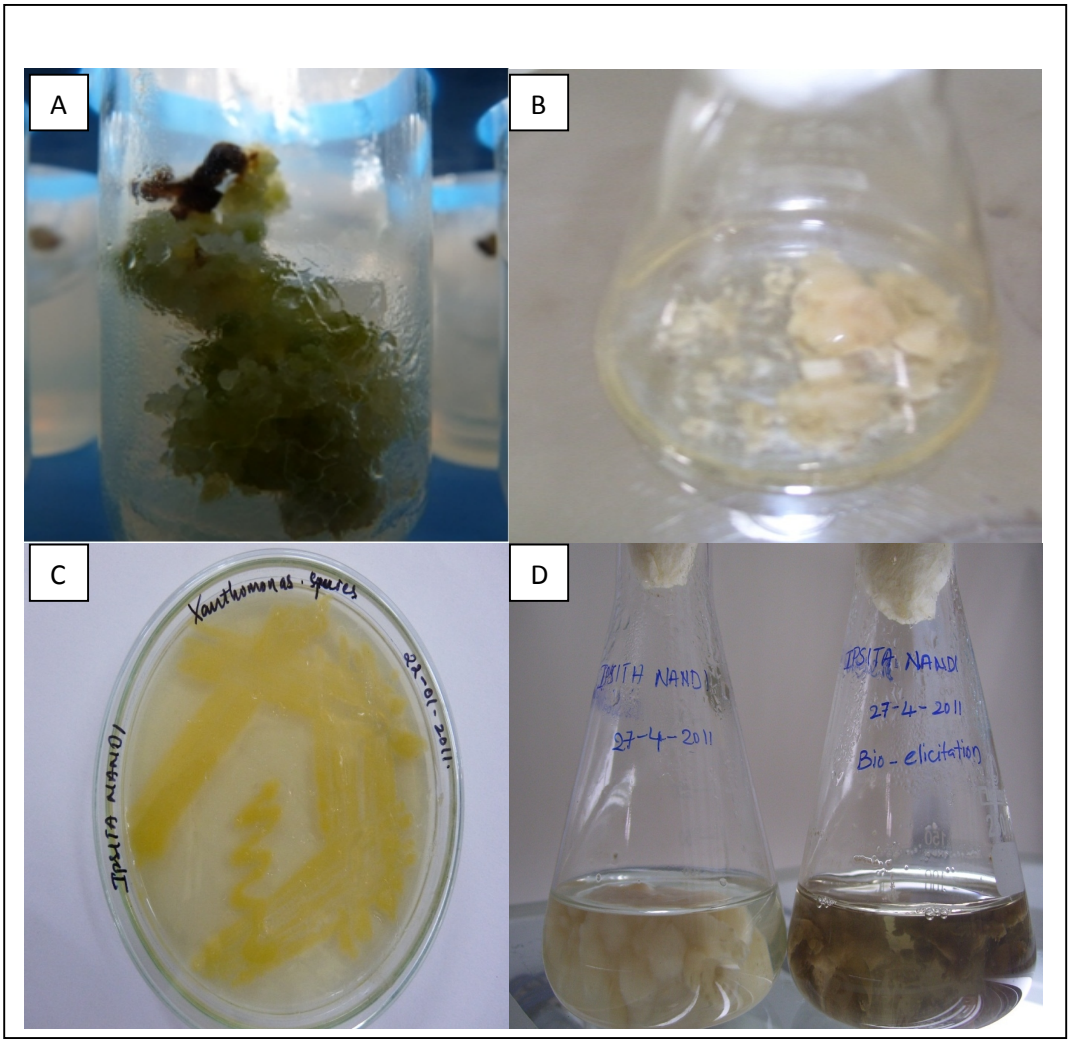
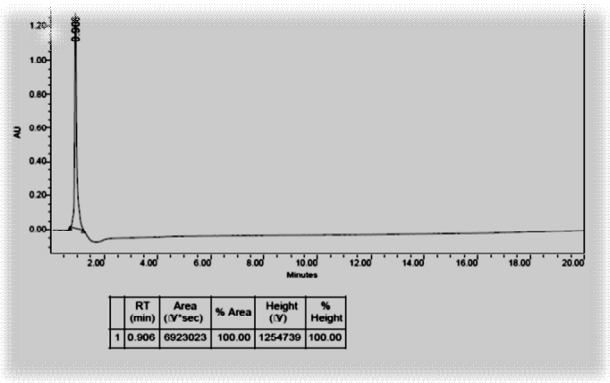
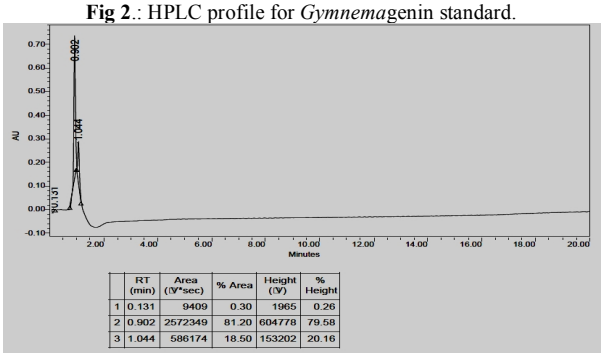



**Fig 1: A**- Callus of *Gymnema sylvestre* on solid media; B- Callus in suspension culture; C- Pure culture of *Xanthomonas species* on Yeast extract peptone glucose agar; D- Suspension cultures of *Gymnema sylvestre* treated with HR elicitor of *Xanthomonas species.*



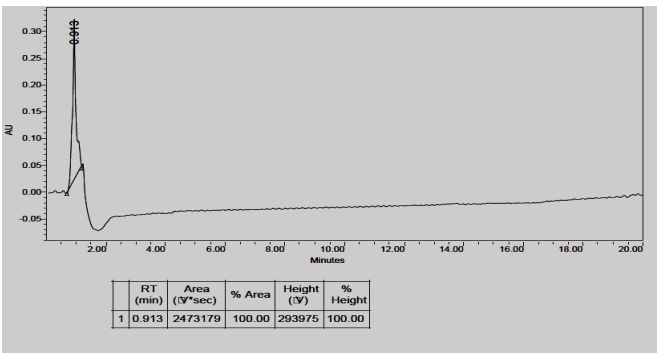

| | RT (min) | Area (µV*sec) | % Area | Height (µV) | % Height |
|---|---|---|---|---|---|
| 1 | 0.906 | 6923023 | 100.00 | 1254739 | 100.00 |

**Fig 2**.: HPLC profile for *Gymnema*genin standard.




| | RT (min) | Area (µV*sec) | % Area | Height (µV) | % Height |
|---|---|---|---|---|---|
| 1 | 0.131 | 9409 | 0.30 | 1965 | 0.26 |
| 2 | 0.902 | 2572349 | 81.20 | 604778 | 79.58 |
| 3 | 1.044 | 586174 | 18.50 | 153202 | 20.16 |

**Fig 3**.: HPLC profile for bioelicitated callus




| | RT (min) | Area (µV*sec) | % Area | Height (µV) | % Height |
|---|---|---|---|---|---|
| 1 | 0.913 | 2473179 | 100.00 | 293975 | 100.00 |

**Fig 4:** HPLC profile for control

## CONCLUSION

*In vitro* propagation of *Gymnema sylvestre* with enriched bioactive principle and cell culture methodologies for selective metabolite production is found to be highly useful for commercial production of gymnemic acid. Bioelicitors could be used as enhancers of plant secondary metabolite synthesis and could play an important role in biosynthetic pathways to enhanced production of commercially important compounds. The increased production, through elicitation, of the secondary metabolites from plant cell cultures has opened up a new area of research, which could have important

economical benefits for bio industry. Elicitation studies have shown promise in increasing yields and cutting production costs.

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