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ELICITOR OPTIMIZATION FOR GYMNEMIC ACID PRODUCTION IN CELL SUSPENSION CULTURES OF *GYMNEMA SYLVESTRE* R.Br.

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ABSTRACT

Gymnema sylvestre R.Br. belongs to asclepiadaceae family, used for the treatment of diabetes. Gymnemic acid, a group of related triterpenoid compounds present in leaf are the active molecules responsible for the biological properties of *G. sylvestre*. The present study was carried out to investigate the influence of different doses of elicitors such asketoconazole, chitosan, methyl jasmonate (MeJA) and salicylic acid (SA) on cell growth and gymnemic acid production profiles in cell suspension cultures of *G. sylvestre*. Among the elicitors tested, SA at 100µM significantly increased the cell growth (172±5.7%) versus control (144±3%). All the elicitors even at lower concentrations improved gymnemic acid content than their higher dose counter parts. Based on HPLC analysis, higher elicitation efficiency in the form of gymnemic acid production was observed with MeJA at 50µM and is 8 folds higher (84.6±3.2mg/g DW) compared with control. Results suggest that, elicitor induced cell stress is imperative for the enhanced gymnemic acid production. Overall, MeJA at 50µMcould be used as suitable elicitor dose for effective growth and gymnemic acid production. The results showed that, cell suspension cultures combined with optimised elicitor doses has potential to improve the gymnemic acid production at large scale in a cost-effective manner.

Keywords: Gymnemic acid, Elicitors, Methyl jasmonate, HPLC, Cell growth

INTRODUCTION

Medicinal plants are essential source of secondary metabolites that are used for improving the longevity and life span of human population. Therapeutic properties of *Gymnema sylvestre* R. Br. have been addressed for the treatment of antidiabetic, antimicrobial, anti-hypercholesteraemic, hepatoprotective and anti-saccharine activities **[1]**. *G. sylvestre* possesses several phytochemicals such as alkaloids, tannins, terpenoids and other secondary metabolites. It has been suggested and demonstrated that, gymnemic acid, a group of related oleanane and dammarane type of triterpenoid saponin compounds are the active molecules that are responsible for the medicinal properties of *G. sylvestre* **[2]**. The potent antidiabetic activity was exhibited by stimulating insulin release possibly through pancreatic regeneration or repair and stimulation of enzymes responsible for glucose uptake and utilization **[3]**.

Normal filed grown plants are not suitable to meet the commercial demands of plant secondary metabolites because of the various constrains like geographical variation, stunted growth, prolonged cultivation period. This necessitates the alternative method for the enhanced production of gymnemic acid. Among them, elicitation is the most effective and well accepted method for the enhanced secondary metabolite production in cell and tissue cultures. Elicitors are the biological or non-biological substances that recognize cell surface receptors and show mimicking effect like pathogens. Elicitors binding lead to altering in signal transduction events that stimulates the expression of genes involved in secondary metabolism. Numerous reports also validated the elicitors effect on enhanced secondary metabolite production **[4]**. Among the elicitors, methyl jasmonate (MeJA), salicylic acid (SA), ketoconazole and chitosan have received much attention for enhanced secondary metabolite production. Several studies have shown the potential effect of MeJA and SA on secondary metabolite accumulation in cell cultures of *Hybanthus enneaspermus* **[6]**, *Psoralea corylifolia* Linn. **[7]**, chitosan mediated elicitation also studied in *Decalepis salicifolia* **[8]**. Previous studies have been conducted for the gymnemic acid production in cell and organ cultures **[9]**.

Elicitor optimisation is one of the pre-requisites for investigating their influence on suspension culture systems. The present study was aimed to demonstrate the influence and effectiveness of different doses of elicitors on growth and production

of active principles in cell suspension cultures of *G.sylvestre*.

MATERIALS AND METHODS

Plant material

Leaf (meristems) explants were collected from three years old *G. sylvestre* plants grownunder greenhouse conditions in Davangere University, Shivagangothri campus, Davangere, Karnataka, India.

Callus initiation and maintenance

Explants were surface sterilized by rinsing with a 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 then with mercuric chloride (0.1%) for 3 minutes followed by 3-4 rinses with sterile double distilled water. Further these explants were sliced along edges with sterile blade and were directly inoculated onto Murashige and Skoog **[10]** medium solidified with 0.8 % agar, fortified with 3 % sucrose, 2 mg L^{-1} BAP, 2 mg L^{-1} NAA, 0.5 mg L^{-1}, picloram and 100 mg L^{-1} casein for callus induction. Medium pH was adjusted to5.8 ± 0.1 prior to autoclaving. Cultures were maintained at 16 h light and 8 h dark photoperiod (50 µmoL $m^{-2}s^{-2}$, 24 ± 1 ºC) for 20 days or until the callus growth was established. The initiated callus was transferred aseptically to fresh culture vessels containing similar growth regulators and medium composition. Among them the best grown (pale green colour) callus was taken and sub-cultured for every 2 weeks to maintain stable cell lines.

Suspension culture establishment

One month old friable callus (500 mg) was 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 at 110 rpm in a gyratory rotatory shaker incubator at 25 ±1 ºC under dark condition. Medium pH was adjusted to 5.8 ± 0.1, autoclaved for 15 min at 121 ºC or 15 lb pressure, cooled to room temperature. The cell biomass obtained was utilized for evaluating elicitors' effect at different concentrations on growth index and gymnemic acid content.

Elicitation

Elicitors' treatments comprising of ketoconazole at 4, 8, 12 and 16 nM; chitosan at 25, 50, 75 and 100mgL^{-1}; MeJA at 25, 50, 75 and 100 µM and SA at 25, 50, 75 and 100 µM. Appropriate dilutions were done with sterile double distilled water, filter-sterilized and supplemented to the suspension culture medium under aseptic conditions on the day of callus inoculation. Sterile distilled water was used as control. All the cultures were harvested at 6days of post-elicitation and used for studying growth index and gymnemic acid production.

Determination of cell growth index

After 6days of culture period, the fresh cell mass was separated from the liquid media by passing through filter paper, rinsed with distilled water and excess surface water was blotted. The cell mass was dried in an oven at 45±2 ºC till it attains constant weight. Growth index was determined by using the formula,

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

Where, Wt final is weight of callus at the end of 6 days of culture period,
Wt initial is weight of callus at the day of inoculation (day 0).

Extraction of gymnemic acid

Cell filtrates of respective suspension cultures (100 mg DCW) was mixed with 50 mL of extraction solvent (50% methanol) and 10 mL of 12% KOH (w/v) and refluxed for one hour.Further, by adding 11 mL of 4N HCl refluxed for 1 more hour. The extract-mixture was cooled to room temperature, pH was adjusted to 7.0 ±0.5 and filtered through 0.22 µ nylon membrane filters (Hi-media, India). The clear supernatant was used for the quantitative analysis by HPLC (high performance liquid chromatography).

Quantification of gymnemic acid by HPLC

Gymnemic acid was quantified as its aglycone gymnemagenin by HPLC (Agilent 1260, quaternary pump and diode array detector VL- G1315D operated at 210 nm). Separations were performed in a ZORBAX Eclipse plus C18 analytical column (4.6 × 250 mm, 5 µm) using acetonitrile and water (80:20) as an eluent at a flow rate of 1 mL min^{-1} at 26 ºC column temperature with 20 µL of injection volume. Gymnemagenin was used as standard (Natural Remedies, Bangalore, India).

Statistical analysis

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

RESULTS AND DISCUSSION

Elicitor effect on growth index

Elicitation is one of the most effective strategy for the enhanced secondary metabolite production in cell, tissue and organ cultures **[11]**. In the present investigation, four elicitors at four different doses were tested **(Table 1)** for cell growth and gymnemic acid production were evaluated. Elicitors used in the present study showed significant effect on cell growth index on cell suspension cultures of *G. sylvestre*. However, their effect differed in terms of dosage and cell growth. Among the elicitors used, SA showed a gradual increase in cell growth at all tested concentration, and maximum effect was observed at 100µM (172±5.7%) which corresponds to an increase of about 1.2

folds higher growth compared with control (144±3%). Similar results ofSA induced cell growth promotion was also observed in *Centella asiatica* **[12]**. Ketoconazole showed maximum cell growth at 16nM (141±5.28%) and is slightly inferior to SA induced cell growth **(Figure 1)**. While chitosan treatment showed drastic decrease in cell growth even at low doses (25mgL^{-1}), it is 20% lesser even to control. Previous reports also substantiated the detrimental effect of chitosan in cell cultures of *Perovskia abrotanoides* **[13]**. Among the different doses of MeJA used, maximum cell growth was achieved with 25 µM (131±2.6%) followed by this slight decrease was observed. Overall, the elicitors used in the present study showed dose dependent effect on cell growth and is more rigorous at higher doses with the exception of SA and ketoconazole. Similar findings were also reported in cell suspension cultures of *Leucas aspera* **[14]**. Most often, elicitors at higher concentrations exert hypersensitive response that lead tosevere cellular damage and often to cell death. This becomes indispensable to elicitor-induced enhancement of secondary metabolite production. The results of the present study inferred that SA is an effective elicitor for cell growth promotion followed by ketoconazole, MeJA and chitosan.

Elicitor effect on Gymnemic acid production

Elicitors modulate gene expression responsible for the production of secondary metabolites by changing the signal transduction cascades. In the present study, all the elicitor treatments enhanced gymnemic acid production compared with control set of cultures, and accumulation patterns were appeared to be dosage dependent **(Figure 2a, b)**. Among the elicitors tested, MeJA at 50 µM showed copious amounts of gymnemic acid and is 8 folds higher (84.6±3.2mg/g DW) to untreated control (10.4±1.3 mg g^{-1} DW) **(Figure 3)** followed by 75µM (80.3±3.2mgg^{-1}DW). Previous reports also revealed that, MeJA proved to be a better choice of elicitor for the enhanced production of triterpenoids in cell cultures of *Leucas aspera* **[14]** and *Centella asiatica* **[15]**. However, during the present study with subsequent rise in MeJA concentration decline in gymnemic acid content was noticed and finally reaching to 71.3±3.4 mg g^{-1}DW. Decreased secondary metabolite production at higher MeJA higher concentration also seen in root cultures of *Papaver bracteatum* Lindl **[16]**. As shown in Figure 3, SA at 50µM concentration was found to be optimum and producing 6 folds higher gymnemic acid (61.3±3.1 mgg^{-1} DW) compared to control. Potentiation of SA for enhanced secondary metabolite

production also observed in cell suspension cultures of *Psoralea corylifolea* **[6]**.

Recent studies have demonstrated the role of SA in inducing defence response through local and systemic acquired response (SAR) **[17]**. Gradual increase in gymnemic acid was content observed with chitosan at 75mgL^{-1} and reaching to 7 folds higher levels (74.3±4.9mgg^{-1} DW) compared with control. It has been suggested that, most often, elevated secondary metabolism compromises cell growth **[18]**. In the present study increase in gymnemic acid production was correlated with decrease in cell growth in presence of chitosan treatment. Similar results were also observed in adventitious root cultures of *Polygonum multiflorum* **[19]**. The elicitor mimicking activity of chitosan was facilitated through mediation of pathogenesis-related proteins, defence-related enzymes, and secondary metabolites accumulation, as well as the complex signal transduction network **[20]**. Treatment with ketoconazole at 12nM contributed to the production of 51±1.9 mgg^{-1} DW which was about 5 folds higher compared with control. Cell suspension cultures of *G. sylvestre* showed different patterns of cell growth and gymnemic acid accumulation patterns in response to the different types of elicitor used at varied doses. However, elicitor effect depends on type of the elicitor and dosage, plant species, growth stage and culture type, co-culture with elicitor and metabolite under investigation **[21].** Selection of optimized elicitor dosage depends on the biomass accumulation as well as production of active principles. In this perspective, the present investigation speculated the utilization of MeJA at 50 µM was found to be effective elicitor concentration for the enhanced gymnemic acid production with least compromising cell growth.

Table 1: Elicitors and their concentrations used in present study

Elicitors	Units	Concentration			
		C1	C2	C3	C4
Ketoconazole	nM	4	8	12	16
Chitosan	Mg L^{-1}	25	50	75	100
SA	µM	25	50	75	100
MeJA	µM	25	50	75	100

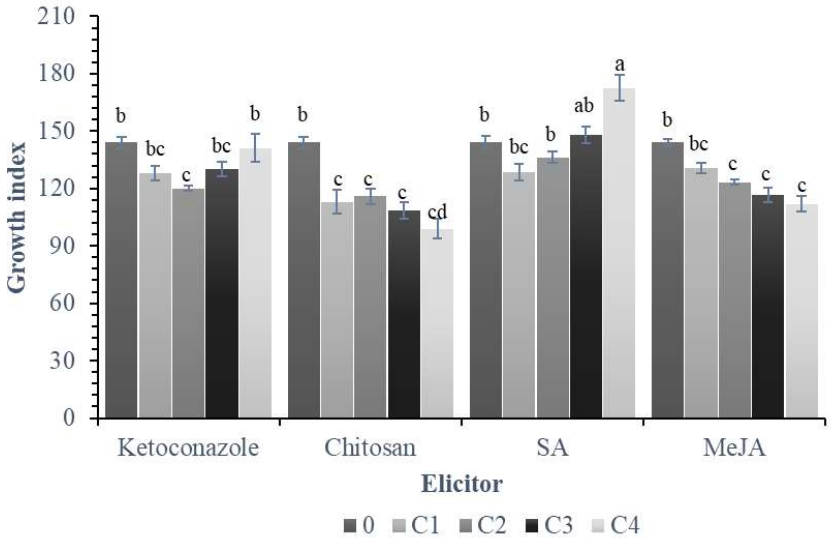

Figure 1: Elicitor effect on cell growth in cell suspension cultures of *G. sylvestre* for 6 days of culture period (as shown in Table 1 elicitors were added to the culture medium at four different concentrations C1, C2, C3 and C4).

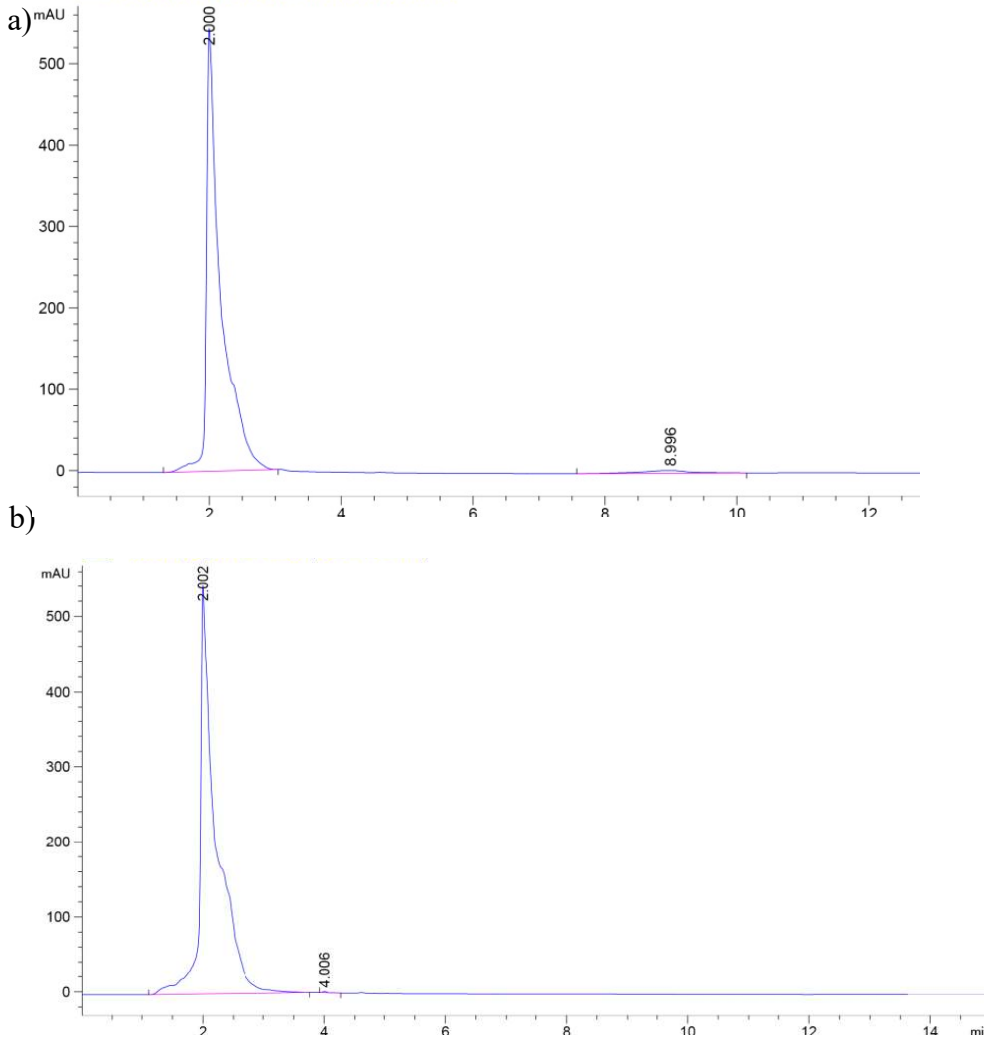

Figure 2: a) HPLC chromatogram of gymnemic acid standard; b) HPLC chromatogram of cell suspension treated with MeJA 50µM concentration.

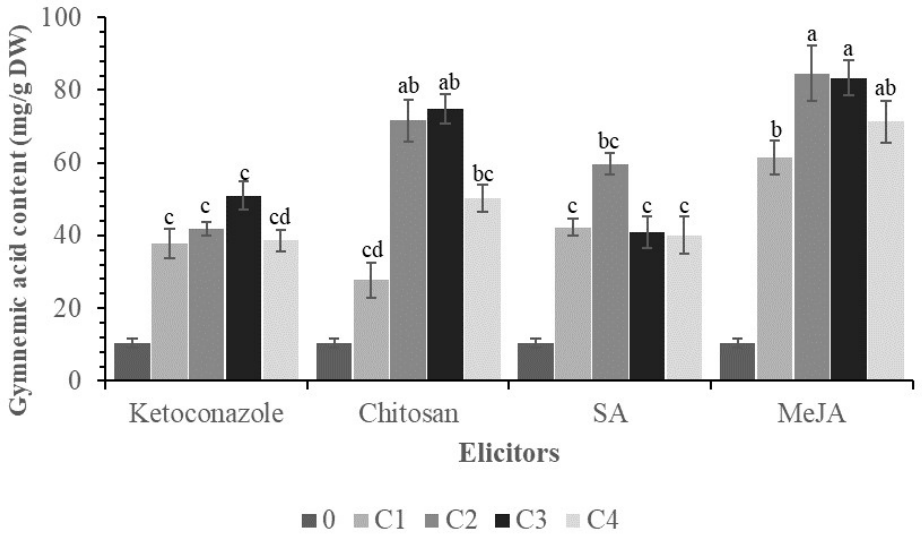

Figure 3: Elicitor effect on gymnemic acid production in cell suspension cultures of *Gymnema sylvestre* for 6 days of culture period
(as shown in Table 1 elicitors were added to the culture medium at four different concentrations C1, C2, C3 and C4)

CONCLUSION

All the elicitors used in the present study influence the gymnemic acid content and cell growth at varied tested concentrations. Even at low doses all the elicitors showed significant improvement in gymnemic acid production. Treatment with MeJA at 50µMfound to be better elicitor for the enhanced gymnemic acid production (8.5 folds to control) with least compromising cell growth (10% decrease). Whereas cellular growth was strongly influenced by SA 100µMtreatment than other elicitors used (17% higher to control). Least significant differences were observed in cell growth between MeJA, ketoconazole treatments. Results of the present study showed an alternative approach for the commercial production of gymnemic acidusing cell suspension cultures of *G. sylvestre*.

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