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ORIGINAL ARTICLE

Improved gymnemic acid production in the suspension cultures of *Gymnema sylvestre* through biotic elicitation

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Abstract Enhancement of secondary metabolite accumulation in cultured plant cells through biotic and abiotic elicitation has been recognised as an important biotechnological strategy. *Gymnema sylvestre* is a rich source of triterpenoid saponins—gymnemic acids used mainly in the treatment of diabetes I and II. The cell suspension cultures initiated from the leaves and stalks of in vitro-grown plantlets have shown to accumulate large amounts of gymnemic acid. The cell-free extracts of *Aspergillus niger*, *Saccharomyces cerevisiae*, *Agrobacterium rhizogenes*, *Bacillus subtilis* and *Escherichia coli* were employed as sources of biotic elicitors to study the effect on secondary metabolite production. All the elicitors have shown a positive response in terms of gymnemic acid, with the highest response induced by *A. niger* [98.65 ± 0.93 mg/gram dry cell weight (gDCW)], 11.2-fold, and the lowest by *E. coli* (33.25 ± 1.38 mg/gDCW), 3.8-fold, in comparison to the untreated cultures (8.79 ± 0.82 mg/gDCW). The suspension cultures of *G. sylvestre* can serve as a continuous source of gymnemic acids throughout the year, irrespective of the climatic and geographical barriers.

Keywords *Gymnema sylvestre* · Suspensions · Saponins · Gymnemic acid · Biotic elicitors · *Aspergillus niger*

Introduction

Plant secondary metabolites occurring in low concentration may not be directly involved in plant growth and development but play a very important role in protecting the plant under abiotic stress conditions as well as from herbivore and pathogen attacks (Wu et al. 2005). Saponins, a diverse group of secondary metabolites, are responsible for potential pharmacological activities such as: anti-platelet, hypocholesterolemic, anti-tumoral (Yan et al. 2006; Ma et al. 2007; Kuo et al. 2009), anti-HIV (Kuo et al. 2009), immunoadjuvant (Fleck et al. 2006; Sun et al. 2004), anti-inflammatory (Kupeli et al. 2007; Ponou et al. 2008) and antimicrobial (Sparg et al. 2004).

Gymnema sylvestre, native to central and western India, tropical Africa, and Australia, is a perennial woody climber rich in triterpenoid saponins belonging to the oleanane (gymnemic acids) and dammarene (gymnemasides) classes (Parijat et al. 2007). The gymnemic acids are a group of closely related molecules isolated from the leaves of *G. sylvestre* (Liu et al. 1992; Manni and Sinsheimer 1965). The anti-diabetic, anti-sweet, and anti-inflammatory activities of *G. sylvestre* have been attributed to the presence of gymnemic acids; the other phytoconstituents include flavones, anthraquinones, hentriacontane, resins, d-quercitol, lupeol, β-amyrin-related glycosides, and stigmasterol. The accumulation of gymnemic acids in higher amounts in the in vitro-grown callus cultures of *G. sylvestre* has been reported (Gopi and Vatsala 2006; Kanetkar et al. 2006; Bhuvaneswari et al. 2012). The complex nature of triterpenoid saponins makes the chemical synthesis economically uncompetitive for large-scale production. Although gymnemic acid production has been achieved in the suspension cultures of *G. Sylvestre* through elicitor treatments (Rani et al. 2010; Bhuvaneswari et al. 2012; Bakrudeen Ali Ahmed et al. 2012; Subathra Devi et al. 2012), the present study is an attempt towards achieving higher levels of gymnemic acid using varied microbial sources.

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Materials and methods

Materials

The following were used: Murashige and Skoog (MS) medium, nutrient and potato dextrose agar (PDA) media, Benzyladenine (BA), 2,4–Dichlorophenoxyacetic acid (2,4–D) (Hi Media, India); HPLC grade chemicals (Merck, India); and bacterial strains, i.e. *B. subtilis* and *E. coli* (Global Hospitals, Hyderabad), *A. rhizogenes* MTCC 532, *A. niger* MTCC 2538 (IMTECH, Chandigarh) and *Saccharomyces cerevisiae* (baker's yeast). The gymnemagenin standard was procured from Plantex Laboratories, Vijayawada, India. The in vitro cultures were cultured on MS media supplemented with different phytohormones and maintained under standard culture conditions [25 ± 2 °C; 16/8 h (light/dark) regime with 40–50 μmol m^{-2} S^{-1} light].

Gymnemic acid production in suspension cultures

Callus cultures were initially established by culturing leaves and stalk portions of *G. sylvestre* shoots in MS media supplemented with different combinations of phytohormones (BA and 2,4–D). *G. sylvestre* suspensions were initiated in MS liquid media supplemented with a combination of 2,4–D (2 mg/l) and BA (0.5 mg/l) (Bhuvaneswari et al. 2012). The suspensions were incubated in an orbital shaker at 120 rpm and 25 ± 2 °C under 16/8 h light/dark cycle. The gymnemic acid content in the samples was analysed in concurrence with the growth pattern using HPLC (High Performance Liquid Chromatography) (Shirugumbi et al. 2009).

Gymnemic acid quantification

Dried callus (500 mg) was macerated in 50 ml of 50 % ethanol. After obtaining a fine suspension, 10 ml of 12 % potassium hydroxide (KOH) was added and boiled for 1 h in a water bath. HCl (4 N, 11 ml) was added on cooling and the mixture was boiled again for 1 h, followed by pH adjustment between 7.5 and 8.5 using 12 % KOH. The final volume was adjusted to 100 ml by adding 50 % ethanol. The extracts were filtered through 0.22-μm filters before subjecting them to HPLC analysis. HPLC was performed on a Shimadzu-LC-10 AT VP series using a Supelco column (250 × 4.6 mm, C 18 ODS with particle size of 5 μm). The mobile phase was prepared with acetonitrile: water (80:20). The flow rate was adjusted to 1 ml/min. Gymnemagenin was detected at 210 nm with a UV detector. Finally, the amount of gymnemic acid was obtained based on the gymnemagenin present in the samples (Bhuvaneswari et al. 2012).

Elicitor preparation

E. coli, B. subtilis, S. cerevisiae, A. rhizogenes MTCC (Microbial Type Culture Collection) 532 and *A. niger* MTCC 2538 strains were used in the study. Cultures, 48 and 72 h old, of bacteria and fungi, respectively, were activated in nutrient broth and sonicated before addition to suspensions. The microbial cultures were filtered and the cell-free filtrate was used as the source of biotic elicitor. The fungal cultures were autoclaved before use.

Elicitor treatment

Different concentrations in the range of 0.5–2.0 % (v/v) were added to the suspensions of *G. sylvestre* and the optimum concentration was determined based on the gymnemic acid content in terms of gymnemagenin. Since the maximum production of gymnemic acid in suspension cultures was observed from the 12th to the 16th day based on the previous study conducted by Bhuvaneswari et al. (2012), the elicitor addition was carried out on the 11th day, i.e. onset of stationary phase. The gymnemic acid content was monitored at 24-h intervals for 3 days.

Cell viability test

The viability of *G. sylvestre* cell suspensions preceding the addition of elicitors was assessed by selective labelling of live cells with 75 μg/ml fluorescein diacetate and expressed as gram fresh weight (gFW). (Darzynkiewicz et al. 1994).

Statistical analysis

All the experiments were performed in triplicate ($n = 3$) and the data were expressed as means ± SD. One-way ANOVA analysis followed by the Duncan's test was used to determine significant ($P \geq 0.05$) differences.

Results and discussion

Plants have many protective mechanisms against attack by physical, chemical, or biological factors. Subsequently, the defence signal transduction pathways are triggered, leading to increased secondary metabolite production by means of phytoalexins—low molecular weight substances induced upon stress situations (Evans 2002; Zhao et al. 2005). *G. sylvestre* is known to be a rich source of saponins, associated with many pharmacological properties. Saponins are characterized as phytoprotectants, which are either produced upon a stimulus conveyed by the pathogen or produced in a developmentally controlled fashion.

The leaves of *G. sylvestre* are known to contain high gymnemic acid content in comparison to the other plant parts (Komalavalli and Rao 2000). Leaves of *G. sylvestre* have been used in India for 2,000 years for the treatment of diabetes (Nadkarni 1992). Cell cultures obtained from the leaf and stalk portions of *Gymnema* are known to accumulate high gymnemic acid content in suspensions. Abiotic elicitation of *G. sylvestre* was successful in increasing the content of gymnemic acid in the cell suspension cultures in the previous study (Bhuvaneswari et al. 2012). Hence, this study was taken up to see the effect of biotic elicitors on accumulation of gymnemic acid in the suspensions. Bacterial and fungal cell wall components are also known to enhance the secondary metabolite production in plant cells (Komaraiah et al. 2002; Jeong et al. 2005; Savitha et al. 2006).

The biomass and gymnemic acid production kinetics of *G. sylvestre* suspensions were optimized in batch cultures in our previous study using abiotic elicitors (Bhuvaneswari et al. 2012). The increase in biomass reached a maximum of 21.46 gFW on day 16 of culture, while the gymnemic acid content reached its peak on day 12 (8.79 ± 0.82 mg/gDCW). Gymnemic acid production in suspensions was maintained at a constant rate from the end of exponential log phase to the middle of the stationary phase, i.e. days 12–16. But, after day 16, there was a drastic fall in gymnemic acid accumulation (Bhuvaneswari et al. 2012). The 48-h-old *S. cerevisiae* and bacterial cultures were sonicated for 10 min and the cell-free extracts were added to the suspensions on day 11, as the maximum gymnemic acid production was observed on day 12. In case of fungal elicitors, the 72-h-old cultures were autoclaved before sonication, to avoid contamination caused by the surviving fungal spores. Sonication was also carried out in addition to sterilization to help release the cellular components. These cultures were filtered by passing them through the 0.22-µm syringe-driven filters and the obtained cell-free extracts were used for elicitation experiments.

The effect of all the microbial cell-free extracts were observed at different concentrations in the range of 0.5–2.5 %. Though lower concentrations were tried, they could not evoke a noticeable response, and 0.5 % concentration was the minimum required for evoking a positive response from the suspensions. After elicitor addition, the suspensions were analysed for gymnemic acid production at 24-h intervals for 3 consecutive days (days 12, 13, and 14). The response of the suspensions varied with respect to each of the elicitors. The maximum production was observed with *A. niger* after 72 h, while the least was seen with *E. coli* after 48 h. After the optimum time period, there was a drastic fall in the secondary metabolite content (Figs. 1, 2 and 3).

Gymnemic acid production in suspension cultures (untreated) reached its maximum on day 12 after inoculation. Hence, the elicitors were added on day 11, i.e. as the culture entered the stationary phase. In some studies, the elicitors were added on the initial day of inoculation

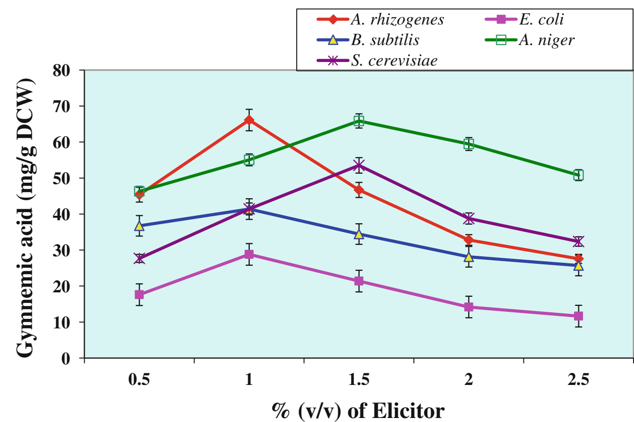

Fig. 1 Gymnemic acid content after 24 h treatment with different elicitors

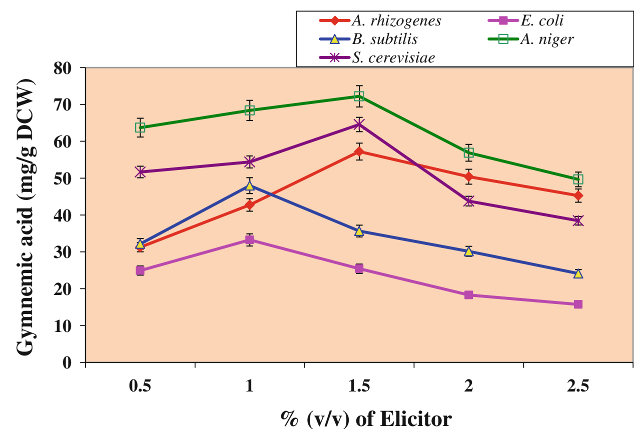

Fig. 2 Accumulation of gymnemic acid in the suspensions after 48 h elicitation treatment

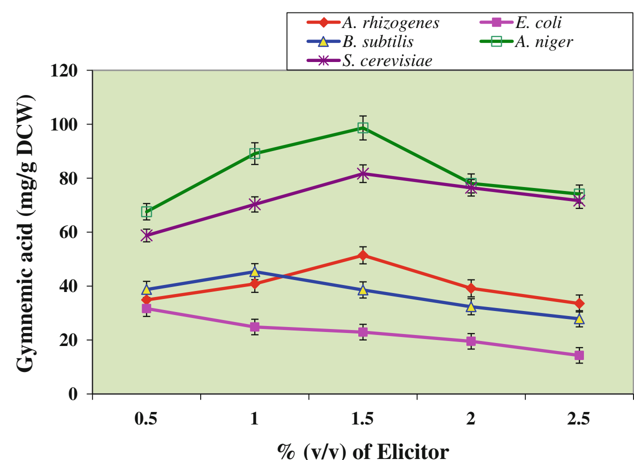

Fig. 3 Gymnemic acid content after 72 h elicitation treatment

resulting in toxicity and hence a drastic reduction in the cell growth (Veerashree et al. 2012; Hu et al. 2011; Kim et al. 2009). But, when studies were carried out to determine the optimum age of culture, it was established that the cultures when challenged during the end of the growth phase or the onset of the stationary phase gave the maximum result (Eliert 1987; Komaraiah et al. 2002; Shilpa et al. 2010). Also, when the elicitors were added during later stages of growth, the reasons for reduced accumulation of gymnemic acid cannot be differentiated between stress elicitation caused due to depletion of nutrients or the toxicity levels of the elicitor.

Elicitors of biological origin like chitin, chitosan, and glucans from the fungal cell wall and polysaccharides, peptidoglycans, and flagellin from the bacteria can be utilised for enhancement of secondary metabolites in the cell culture systems (Jurgen and Axel 1998; Shilpa et al. 2010). The gymnemic acid accumulation in the treated cultures was in the order of *A. niger* > *S. cerevisiae* > *A. rhizogenes* > *B. subtilis* > *E. coli*. Though the fungal elicitor has shown maximum accumulation, the response was obtained after a prolonged exposure time (72 h). This could be due to the fact that the heat labile fungal cellular components will be destroyed during the process of sterilisation (autoclave), whereas, in the case of *S. cerevisiae* and bacterial elicitors (24 and 48 h), only sonication and filtration were followed, hence intact cellular components were achieved evoking faster response from the suspensions.

(I) *Aspergillus niger:* Elicitation of cell cultures with fungal cell wall components for the synthesis of low molecular weight compounds has been studied in various plants, i.e. *Dioscorea zingiberensis* (Zhang et al. 2009; Zhao et al. 2011), *Plumbago rosea* (Komaraiah et al. 2002) and *Azadirachta indica* (Satdive et al. 2007). The fungal cell wall works as a polysaccharide elicitor, which induces calcium concentration in the cell and activates various defense responsive pathways leading to the accumulation of phytoalexins and low molecular weight antimicrobial compounds (Cordell 1997). The cell wall acts as a chemical messenger with specific regulatory properties. The increase in amount of gymnemic acid in the present study was found to be 11.2-fold (98.65 ± 0.93 mg/gDCW) as against the finding of 8- to 9-fold (974.79 ± 1.28 mg/l) reported by Subathra Devi and Mohana srinivasan 2011) using *A. niger*. The optimum concentration of the cell free extracts of *A. niger* towards the cell cultures was found to be 1.5 % concentration for 72 h.

(II) *Saccharomyces cerevisiae*: When *S. cerevisiae* was used, there was 9.3-fold (81.67 ± 0.89 mg/gDCW) increase in accumulation of gymnemic acid in the suspension cultures of *G. sylvestre* at 1.5 % elicitor concentration after a period of 72 h. The secondary metabolite production observed was the second highest, next to the fungal elicitor *A. niger*. There were enhanced amounts of secondary metabolites in various tissue and cell cultures of plants like *Scutellaria baicalensis* (Yoon et al. 2000), *Panax ginseng* (Lu et al. 2001), *Centella asiatica* (Kim et al. 2004), *Angelica gigas* (Rhee et al. 2010), *Pueraria candollei* (Korsangruang et al. 2010) and *Drosera burmannii* (Waraporn et al. 2010). In a study conducted by Veerashree et al. (2012), the *S. cerevisiae*-treated cultures showed a maximum accumulation of 10.64 mg/gDCW as against the present study of 81.67 ± 0.89 mg/gDCW.

(III) *Agrobacterium rhizogenes: Agrobacterium* species are soil-borne phytopathogens responsible for a variety of neoplastic diseases, i.e. crown gall (*A. tumefaciens* and *A. vitis*), hairy root (*A. rhizogenes*) and cane gall (*A. rubi*). The cell-free extracts of *A. rhizogenes* were used for eliciting gymnemic acid from the suspension cultures of *G. sylvestre*, which gave a maximum accumulation of 66.12 ± 1.76 mg/gDCW after 24 h, i.e. 7.5-fold higher than the control cultures. *Agrobacterium* being a Gram-negative bacterium acts as a source of lipo-polysaccharides, peptido-glycans, and various other cell wall components that help in the process of elicitation. Among all the bacterial elicitors, *A. rhizogenes* extracts have shown maximum enhancement of gymnemic acid production at an elicitor dose of 1 % (v/v) after 24 h.

(IV) *Bacillus subtilis: B. subtilis* was able to induce a positive response in the cell suspensions of *G. sylvestre*. But the maximum production was seen at 1 % elicitor concentration after a 48 h exposure time. There was no significant difference ($P > 0.05$) in the accumulation of gymnemic acid after 48 h (47.97 ± 1.66 mg/gDCW) and 72 h (45.35 ± 0.85 mg/gDCW) treatment.

(V) *Escherichia coli:* In case of *E. coli*, the maximum production was seen only at 1 % elicitor dose for 48 h exposure time. The amount of gymnemic acid accumulated was 33.25 ± 1.38 mg/gDCW, which was the least among all the elicitors.

The culture filtrates of non-pathogenic bacteria, *E. coli* and *B. subtilis*, were also studied for enhanced accumulation of gymnemic acid in the suspensions. Although the response obtained with both the cultures was minimal, there was a significant difference ($P < 0.05$) in the accumulation of gymnemic acid by 3.8- and 5.4-fold, respectively. This finding is similar to the report given by

Komaraiah et al. (2002) using *B. subtilis* and *P. aeruginosa*. A higher percentage of the elicitor and more exposure time was required for fungal and *S. cerevisiae* extracts to elicit the response from suspensions. The shortest exposure time was required in the case of *A. rhizogenes* (Table 1). But, after attaining the optimum gymnemic acid content, further increase in elicitor dosage and exposure time resulted in a drastic fall. This could be due to the toxicity of elicitor towards the plant cells. As the elicitor was added at the end of the log phase, there was no visible effect on cell growth, which means the cells reached their full growth potential before the suspension entered the stationary phase. Once the culture reaches stationary phase, there will not be any increase in cell density and the elicitor can only affect the production of secondary metabolites. However, until the 16th day, there was only a marginal difference in cell viability with all the elicitors, which is inversely linked to the metabolite production evident from Fig. 4. This effect of elicitors on cell growth and viability has been suggested to be caused by the diversion of metabolic flux, i.e. the activation of secondary metabolism over primary metabolism (Larronde et al. 2003; Sivakumar and Paek 2005).

Plants respond to a wide array of MAMPs (microbe-associated molecular patterns) from both pathogenic and non-pathogenic microbes including bacterial flagellin, lipopolysaccharide (LPS), elongation factors, etc. (He et al. 2007). The microbial contact with the plants would lead to the triggering of a rapid and localised defence response—the reactive oxygen species (ROS) burst (Allen and Tresini 2000). The action of ROS is thought to be mediated through changes in the cytosolic Ca^{2+} levels and generation of lipid peroxides. Several studies have shown that Ca^{2+} plays an important role in defence signalling. Ca^{2+} channel blockers have been shown to inhibit ion fluxes as well as defence responses induced by fungal and bacterial elicitors (Garcia-Brugger et al. 2006; Mc-Anish and Schroeder 2009).

Elicitor response towards plant systems varies with type and concentration, along with the treatment duration. The abiotic elicitation of *G. sylvestre* with $CdCl_2$ in the previous study (Bhuvaneswari et al. 2012) gave maximum production of 59.97 mg/gDCW of gymnemic acid, i.e. a 6.8-fold increase in comparison to the untreated cultures. In the present study, however, the maximum accumulation was observed with *A. niger*, i.e. 11.2-fold

Table 1 Effect of different biotic elicitors on gymnemic acid production

Sample no.	Organism	Amt of cell-free extract in (%v/v)	Optimum time period for elicitation (h)	Fold increase in gymnemic acid production (mg/g DCW)
1	Control	–	–	0 (8.79 ± 0.82)
2	*A. rhizogenes*	1	24	7.5 (66.12 ± 1.76)
3	*E. coli*	1	48	3.8 (33.25 ± 1.38)
4	*B. subtilis*	1	48	5.4 (47.97 ± 1.66)
5	*A. niger*	1.5	72	11.2 (98.65 ± 0.93)
6	*S. cerevisiae*	1.5	72	9.3 (81.67 ± 0.89)

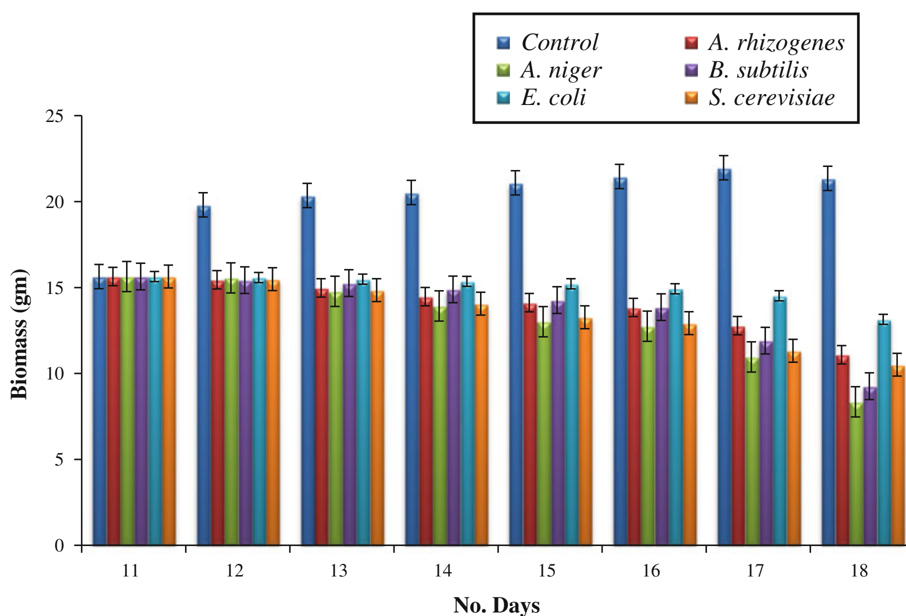

Fig. 4 Cell viability of *G. sylvestre* after elicitor addition to the suspensions in comparison to the untreated (control) cultures

(98.65 ± 0.93 mg/gDCW). This is 1.6-fold higher than the abiotic elicitor $CdCl_2$. Previous investigations on the enhancement of gymnemic acid have shown constraints and less yield in comparison to the present study: e.g. 5.79 g/l in a bioreactor (Rani et al. 2010); 4.56-fold in 45 days (Bakrudeen Ali Ahmed et al. 2012); and 8- to 9-fold in 18 days (Subathra Devi et al. 2012). Hence, we can conclude that, under the experimental conditions tested, biotic elicitors were stronger stimulators compared to the abiotic elicitors (metal salts) from the earlier study.

Gymnema sylvestre is a known commercial source of diabetic supplement. Presently, the phytochemicals are extracted from wild- or field-grown plants, which is a cumbersome process and leads to loss of genetic diversity. Also, isolation of compounds from cell cultures is easy and can be carried out continuously irrespective of the climatic and geographical barriers. The content of gymnemic acid can be further enhanced by treating the suspensions with biotic and abiotic elicitors or a combination of both. From the present study, it can be concluded that, when compared to abiotic metal salts, biotic elicitors are more successful in enhancing the accumulation of gymnemic acids in the suspension cultures of *G. sylvestre*.

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