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Potential mevalonate pathway precursors for enhanced production of gymnemic acid

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Gymnema sylvestre, locally called *Gurmar* (meaning sugar destroyer), is well known in Indian medicine for treatment of diabetes which is attributed to its triterpenoid saponin content gymnemic acid. Quantity of the gymnemic acid in is influenced by geographical and seasonal variations. Use of precursors enhances production of gymnemic acid *in vitro*. Here, we explored three different precursors, namely isopentenyl pyrophosphate (IPP), squalene, and 2,3-oxidosqualene for their effect on gymnemic acid production in *in vitro* cultures of *Gymnema sylvestre*. Gymnemic acid production in shoot cultures treated with 2,3-oxidosqualene (23.31 mg g^{-1} DW) was highest among all three precursors studied, followed by squalene (18.12 mg g^{-1} DW) and IPP (15.94 mg g^{-1} DW). Addition of these precursors might enhance the activity of respective enzymes, such as IPP isomerase, squalene synthase (SS), squalene epoxidase (SE), and β-amyrin synthase (β-AS) which are the key enzymes in terpenoid production. Higher concentration and higher harvest duration of all three precursors reduced the production of gymnemic acid. Results have shown that addition of these precursors is effective in enhancement of gymnemic acid production and can be successfully utilized for commercial application.

Keywords: Australian cowplant, *Gurmar*, *Gymnema*, Isopentenyl pyrophosphate, MVA pathway, 2,3-Oxidosqualene, Squalene

Diabetes is an endocrine and metabolic, multifunctional disease associated with the dysfunction and failure of vital organs[1,2]. The chronic diabetic complications are broadly divided into microvascular (neuropathy, nephropathy, and retinopathy) and macrovascular (cardiovascular disease, stroke, and peripheral artery disease)[3]. It is an enormously growing clinical and public health issue around the world and according to the International Diabetes Federation (IDF) 415 million adults were suffering from diabetes and this number will be raised to 642 million by 2040[4]. Though diabetes is incurable it can be administered with insulin, diet, oral hypoglycemic drugs[5,6] and also naturopathy[7]. Currently, various synthetic hypoglycemic drugs are available, which are expensive and cause severe side effects[8]. Hence, more attention has been paid to herbal medicines and medicinal plants which have hypoglycemic activity[9,10].

Gymnema sylvestre (Retz.) R. Br. ex Sm. Fam. Apocynaceae, commonly called Australian cowplant, locally, *gurmar* (sugar destroyer) is used in the indigenous system of medicine to control diabetes mellitus[11]. It is a good source of various secondary metabolites such as tannins, alkaloids, saponins[12]. The medicinal property of *G. sylvestre* is mainly due to gymnemic acid, which is an oleanane type of triterpenoid saponin[13]. Leaves of *G. sylvestre* have antidiabetic potential which helps in the rejuvenation of pancreatic β-cells and improves the secretion of insulin, to overcome allied diabetic complications[14,15]. Climatic and environmental gradients cause variability in the chemical constituents of plants[16]. The influence of geographical regions on gymnemic acid content has been studied from different states of India[17-20]. To make available continuous supply of gymnemic acid, *in vitro* cultures can be a promising choice[21]. For enhancement of secondary metabolites in *in vitro* production, different strategies are practiced and one of them is precursor feeding[22] and exogenously supplied initial or intermediate precursors might increase the quantity of end products[16].

In the present investigation, we explored three different precursors for their effect on gymnemic acid production in shoot cultures of *Gymnema sylvestre*.

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Materials and Methods

Collection of plant material and authentication

Plant material (*Gymnema sylvestre* shoots) for tissue culture experiments was collected from Botanical garden, Department of Botany, S. P. Pune University. Collected plant material was authenticated and the herbarium was submitted to the Botanical Survey of India, Western circle, Pune, India (BSI/WRC/Cert./2015).

Initiation of shoot cultures

Collected nodal explants were surface sterilized under running water for 30 min. with 2-3 drops of Tween-80, followed by a quick wash of 70% alcohol for 10 s. Thereafter, explants treated with 1% bavistin and 0.1% mercury chloride for 30 and 3 min, respectively. Surface sterilized nodal explants (1.0 cm) were inoculated on MS medium[23] with 6-benzylaminopurine (BAP) + kinetin (KIN) + naphthalene acetic acid (NAA) (4.44 + 2.32 + 5.37 µM)[24]. Four weeks old *in vitro* grown shoots were used for the present investigation.

Precursor feeding experiment

Three different precursors of the mevalonic acid pathway (MVA) *viz*., isopentenyl pyrophosphate (IPP), squalene, and 2,3-oxidosqualene were purchased from Sigma, USA. About 100 mM stock of IPP, squalene, and 2,3-oxidosqualene was prepared in DW, acetone and chloroform, respectively, which was stored at 4°C till next use. Optimized shoot cultures were treated with different concentrations [0 (control), 2.5, 5.0, 7.5 and 10.0 mM] of these precursors and were harvested at different time intervals such as 2, 24, 36, and 48 h.

Extraction of gymnemagenin and HPTLC analysis

The extraction of gymnemagenin and its HPTLC analysis was done by the method described earlier[25]. The conversion of gymnemagenin to gymnemic acid was done using the equation C = X (809.0/506.7) where, C = the content of gymnemic acid in the sample; X = the content of gymnemagenin present in the sample; 506.7 = mol. wt. of gymnemagenin; 809.0 = mol. wt. of gymnemic acid.

Statistical analysis

All the experiments were carried out in triplicates and the data were expressed as mean ± SD. One-way ANOVA analysis followed by Duncan's multiple range test was used to determine significant ($P \leq 0.05$) differences.

Results and Discussion

Proposed pathway for gymnemic acid production

The mechanism of gymnemic acid (oleanane type of triterpenoids saponin) production in *G. sylvestre* is not fully known and it is difficult to predict the pathway for biosynthesis of gymnemic acid[26]. The pathway for triterpenoid saponin production is well studied in *Panax ginseng* for the production and enhancement of ginsenoside[27,28]. In plants, terpenoids are produced by two pathways namely as mevalonic acid (MVA) pathway and methylerythritol phosphate (MEP) pathway[29,30]. This MVA pathway leads to the production of the triterpenoid pathway via IPP, squalene, and 2,3-oxidosqualene[31]. Three different precursors like isopentenyl pyrophosphate (IPP), squalene, and 2,3-oxidosqualene have been used for the production of gymnemic acid in *G. sylvestre* (Fig. 1).

Effect of precursor feeding on gymnemic acid production

In the present study, shoot cultures treated with IPP showed maximum (15.94 mg g^{-1} DW) content of gymnemic acid, for 10.0 mM concentration at 24 h harvest (Fig. 2A). As compared to the control IPP treated shoot cultures showed 66.56% increase in

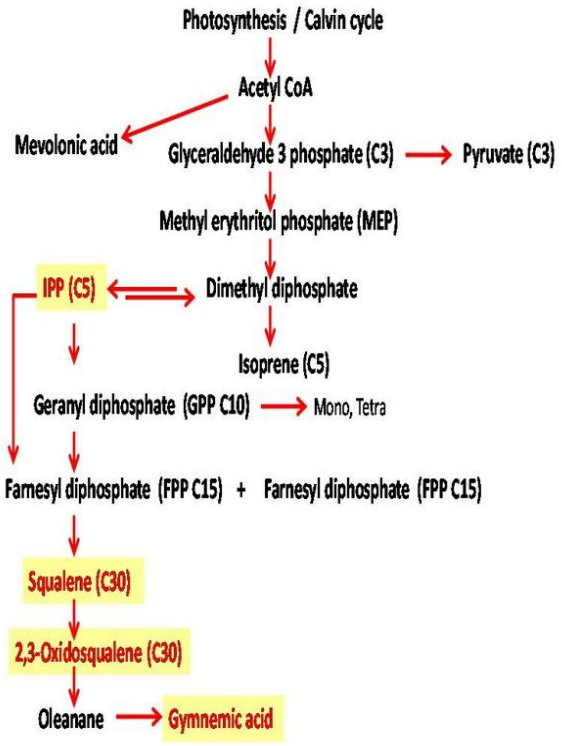

Fig. 1 — Proposed pathway for gymnemic acid production in *Gymnema sylvestre*

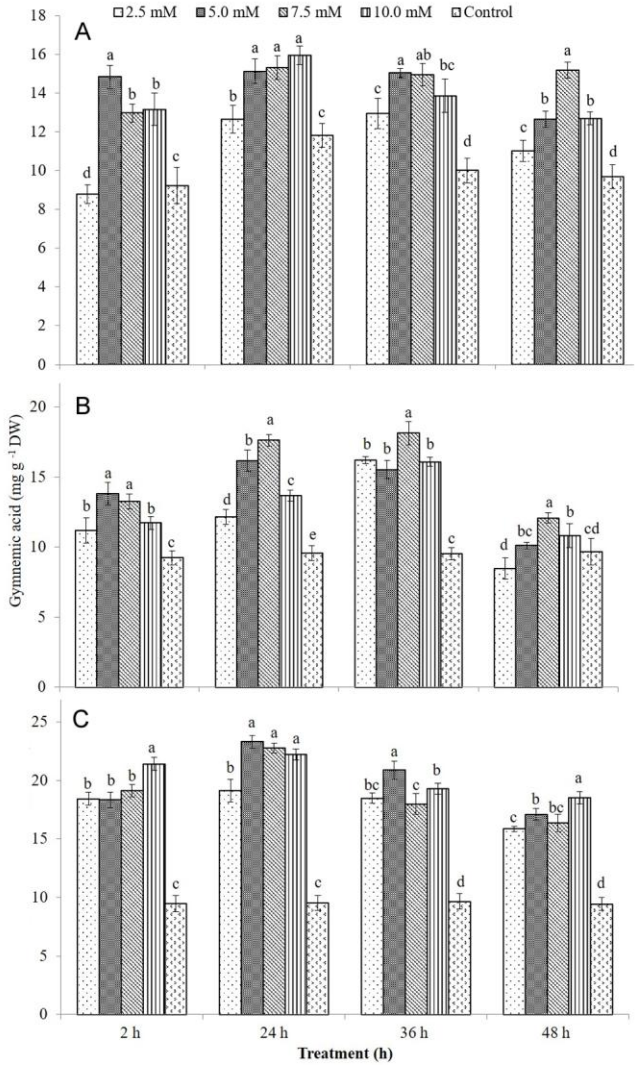

Fig. 2 — Effect of (A) IPP; (B) squalene; and (C) 2,3-oxidosqualene on gymnemic acid production (mg g^{-1} DW) in shoot cultures of *G. sylvestre* at different harvest duration (2, 24, 36 & 48 h). [Data represents mean values ± SD of three replicates. Mean with same letters are not significantly different at 0.05% probability level according to DMRT]

gymnemic acid content. Among the three precursors used, IPP showed the lowest gymnemic acid production in all the concentrations used at different harvest durations. Since IPP is a central intermediate in the isoprenoid pathway, it produces triterpene by MVA pathway and monoterpenes, diterpenes, sesquiterpenes, tetraterpenes by MEP pathway[29,32,33]. Being a central intermediate the exchange of exogenously supplemented IPP can be transported between compartments (cytosol and plastid)[34] which is the reason for low production of gymnemic acid. Higher content of gymnemic acid (18.12 mg g^{-1} DW) was produced in shoot cultures treated with squalene at a concentration of 7.5 mM at 36 h harvest (Fig. 2B) and showed 90.18% increase as compared to control.

Squalene plays an important role in the triterpenoid production which is close to the endpoint of the triterpene biosynthetic pathway and gives rise and gets converted into final product easily[35]. The higher concentration of squalene at increased harvest duration decreased the gymnemic acid production which could be due to the conversion of squalene into some other terpenoids rather than gymnemic acid, and compartmenting of pathways that allow plant cells to separate enzymes from their substrates and end products[35]. The enzymatic cyclization of squalene and 2,3-oxidosqualene is the most remarkable step in the biosynthesis of triterpenoids. Squalene and 2,3-oxido-squalene are converted into triterpenes by various enzymes, such as squalene cyclase and oxidosqualene cyclase (OSC)[36,37]. Squalene epoxidase converts squalene into 2,3-oxidosqualene which later converts into β-amyrin by β-amyrin synthase (β-AS) and finally, β-amyrin leads to the production of oleanane type of triterpenoids[38] such as gymnemic acid. In the present investigation, highest gymnemic acid (23.31 mg g^{-1} DW) was produced in the shoot cultures supplemented with a concentration of 5 mM 2,3-oxido-squalene at 24 h (Fig. 2C) among all the three precursors used which showed. As compared to the control 2,3-oxidosqualene showed 146.43% increment in gymnemic acid. Precursor 2,3-oxido-squalene is important for triterpenoid biosynthesis and cyclization of 2,3-oxidosqualene by OSC followed hydroxylation and glycosylation which produce triterpenoid saponin[39,40]. The OSC genes *viz*. *β-AS* has been reported to produce triterpenoid saponins such as gymnemic acid by cytochrome P450 monooxygenase (CYP450s) and UDP glycosyltransferases (UGTs) in *Panax*[41].

The last precursor in the MVA pathway is 2,3-oxidosqualene, and its supplementation to the cultures can be resulting in conversion into the final product easily and rapidly. Since 2,3-oxidosqualene is one of the important branch-point of terpenes and sterol biosynthesis[41], it has chances of getting converted to sterol instead of terpenes. The addition of IPP, squalene, and 2,3-oxidosqualene precursors might enhance the activity of respective enzymes, such as IPP isomerase, squalene synthase (SS), squalene epoxidase (SE), and β-AS which are the key enzymes

in terpenoid production[42,43]. In the present study, the enhanced enzyme activity could be attributed for gymnemic acid production.

Conclusion

Shoot cultures treated with IPP, squalene, and 2,3-oxidosqualene showed 66.56, 90.18, and 146.43 % increase in gymnemic acid respectively as compared to the control. The highest gymnemic acid enhancement was recorded in the shoot cultures of *G. sylvestre* treated with 2,3-oxidosqualene. The results have shown that the concentration of the precursor, harvest duration and the precursor in the pathway are critical factors for production of the secondary metabolites. Precursor feeding is an advantage over natural random plant material collection for gymnemic acid, because it is independent of climatic and environmental gradients. To the best of our knowledge this is the first report of precursor feeding and its effects on gymnemic acid production in shoot cultures of *G. sylvestre*. Findings of the present investigation have the potential to be used for continuous supply of gymnemic acid.

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Conflict of interest

Authors declare no conflict of interests.

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