

Review Article

IN VITRO RESPONSE OF GYMNEMA SYLVESTRE: A REVIEW

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ABSTRACT

Gymnema sylvestre has acquired worldwide attention as a most popular antidiabetic and antiobesity plant in recent years. A number of companies are involved in the trade of *Gymnema*, which supply the material either as a whole plant, leaves or leaf extract. Overexploitation of plant necessitates an effective protocol for clonal propagation of plants, otherwise plant will be at risk of extinction. Hence, in vitro techniques are applied on this plant not only for large-scale propagation of plant for germplasm conservation and making it available to the scientists and patients, but also for large-scale production of gymnemic acid, the principal antidiabetic constituent, in suspension culture in bioreactors. In vitro response of *Gymnema* has been investigated variously by a number of scientists. Induction of callus, maintenance of suspension culture, micropropagation through axillary bud enhancement, somatic embryogenesis and multiplication of in vitro raised seedlings has been successfully achieved. Recently there has been great advancement in the research of gymnemic acid production in suspension culture. Optimization of cultural conditions, use of biotic and abiotic elicitors have been resulted in enhanced production of biomass and hence the gymnemic acid. Application of bioreactors has further accelerated the process. This review focuses on both aspects of tissue culture to provide consolidated information to the scientists working in this field. So that these methods can be adopted successfully by industries at economic level.

Keywords: *In vitro, Micropropagation, Gymnemic Acid, Antidiabetic, Elicitors*

INTRODUCTION

Medicinal plants are again a point of focus due to the awareness of the side effects of synthetic drugs as well as advancements in bio techniques, which led to the extraction of novel compounds from various plants with enormous medicinal values. Plants have been used for curing a number of diseases all over the world since ancient time and were valuable component of the traditional health care system. *Gymnema sylvestre* R.Br. has got an important place among these medicinal herbs. *G. sylvestre* belonging to the family Asclepiadaceae is a perennial woody climber. It is well known to the Asian people since ancient days as a source of anti-diabetic drugs. Its common name is periploca of the wood in English and gurmar in Hindi. Leaves of *Gymnema* suppress the taste of sugar upon chewing, hence these are also used to lower the intense desire of consuming sugary products (Saneja *et al.*, , 2010; Rani *et al.*, , 2012). The chemical components of the plant are: resin, saponins; gymnemasine, gymnemaside, gymnestrogenine and gymnemagenine, stigmasterol, quercitol. Leaves are reported to contain acidic glycosides, anthroquinones and their derivatives. The medicinal activities of *Gymnema* are attributed to a group of oleanane type triterpenoidsaponins known as gymnemic acids and dammarenesaponins called gymnemasides. Besides these, other therapeutic biomolecules are flavones, α and β - chlorophylls, phytin, formic acid, butyric acid, tartaric acid, hentri-acontane, lupeol, β -amylin related glycosides, pentatriacontane and stigmasterol (Hong-Min *et al.*, 1992; Nadkarni 1993; Wen-Cai *et al.*, 2000; Porchezian and Dobriyal, 2003; Kokate *et al.*, 2006; Khamrov *et al.*, 2008; Sangeetha and Jegadeesan, 2012). According to the Indian system of traditional medicine; Ayurveda, it is anthelmintic, bitter cardio tonic, digestive, diuretic, Anti-arthritis, antipyretic, anticancer, anti-inflammatory, emetic, alexipharmac, expectorant, laxative, acrid, stimulant, stomachic, astringent, anodyne, uterine tonic. The plant is also useful in asthma, cardiopathy, amenorrhea, conjunctivitis, bronchitis, constipation, dyspepsia, hemorrhoids, cough, hepatosplenomegaly, jaundice, inflammations, intermittent fever, hypercholesterolemia and Leucoderma. It removes cough by inducing vomiting and paste is used as an ointment in case of insect bite (Malik *et al.*, 2008; Khanna and Kannabiran, 2009; Malik *et al.*, 2010; Osman *et al.*, 2010; Tiwari *et al.*, 2014).

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Persaud *et al.*, (1999) carried out silica gel chromatography of plant extract and obtained gymnemic acid concentrated fractions on the basis of size exclusion. The fraction was tested in vivo on rat and in vitro on several pancreatic beta-cell lines and increase in insulin secretion was observed in both cases. The extract has been used in such Ayurvedic preparations which are used to control blood sugar and cholesterol levels in diabetic animals and humans (Porchezian and Dobriyal, 2003). The principal antidiabetic constituent of the plant is gymnemic acid, which is used to cure diabetes and obesity (Kanetkar *et al.*, 2007). This property is attributed to increases in insulin secretion by extract as noticed by Al-Romaiyan *et al.*, (2010), in human pancreatic islets in vivo and in vitro. Gymnemic acid, depresses appetite and cause weight reduction, it restores pancreas function, and has anti-tooth decaying effect. Gymnemic acid possesses different therapeutic activities like suppression of taste buds activity for sweetness, inhibition of intestinal glucose absorption and lowers the plasma glucose levels (Patel *et al.*, 2012; Solanki *et al.*, 2013). The broad range pharmacological activities of *Gymnema* are due to a wide spectrum of triterpenoids found in different parts of the plant (Fabio *et al.*, 2014).

In nature *Gymnema* is propagated by seeds, however, their germination rates are very poor in natural conditions due to short viability of seeds and less endosperm. Efforts have been made to propagate this plant by conventional means. Factors which can improve seed germination, vegetative propagation through stem cutting and rooting of stem cuttings have been optimized to grow plant in field conditions and in vitro conditions (Arunakumara and Subasinghe, 2004; Singh *et al.*, 2005; Arunakumara *et al.*, 2013). Yet there are problems associated with conventional propagation methods, like depending on natural climatic conditions, season etc. It has now become essential to propagate this valuable medicinal vine by tissue culture methods not only for its conservation, but for extraction of gymnemic acid at commercial level. The foregoing review focuses on in vitro regeneration potential of *Gymnema* as well as on the in vitro production of gymnemic acid, the principal antidiabetic component. Gymnemic acid is found in plant only in trace amounts. A commercially viable production system is a major necessity to exploit its antidiabetic potential as a drug. In vitro regeneration of callus and maintenance of cell suspension culture allows the opportunity to not only produce gymnemic acid in large quantity, but also maximize its production at the commercial level by manipulating cultural conditions (Lee *et al.*, 2006).

Micropropagation

Micropropagation is a well-established process for mass-scale production of plants with clonal stability. In vitro multiplication of *Gymnema* was first of all attempted by Reddy *et al.*, (1998). Maximum number of shoots were achieved on MS medium containing BAP (5 mg l^{-1}) and NAA (0.2 mg l^{-1}). Multiplied shoots were brought to rooting on $\frac{1}{2}$ strength MS (Murashige and Skoog, 1962) medium without adding any plant growth regulator. Komalavalli and Rao (2000) investigated the effect of various factors which remarkably affected in vitro regeneration of *Gymnema* that were; seedling age, the nature of the explant, basal medium, plant hormones, antioxidants (activated charcoal, ascorbic acid, citric acid and poly vinyl pyrrolidone) and undefined supplements (coconut milk, yeast extract, casein hydrolysate and malt extract). A maximum of 57.2 shoots were induced from 30 day old seedling axillary node explants incubated on MS medium supplemented with 0.1 mg l^{-1} NAA, 1.0 mg l^{-1} BA, 0.5 mg l^{-1} Kn, 100 mg l^{-1} citric acid and 100 mg l^{-1} malt extract. Best root regeneration was observed on shoots derived from axillary nodal explant (50%) on $\frac{1}{2}$ MS medium supplemented with 3.0 mg l^{-1} IBA. The rooted shoots were subjected to hardening in soil and successfully acclimatized to natural conditions. Karthic and Seshadri (2009) developed a cost effective mass multiplication of *G. sylvestre* in hydroponic system. Effect of media and moisture on rooting of *Gymnema sylvestre* stem cuttings was also studied by Arunakumara *et al.*, (2013). Subathra and Srinivasan (2008) concluded that the requirement of MS mediums for shoot bud activation and propagation confirms the requirement of rich salts for the regeneration of *Gymnema sylvestre*. Influence of various growth hormones like; 2, 4-D, IAA, BAP, and Kinetin on the breaking of axillary bud dormancy was studied and synergistic action of vitamin B2 in relation to these plant growth regulators was also worked out. To minimize phenolic release by explants different antioxidants; activated charcoal, ascorbic acid and citric acid were also added in culture medium. Citric acid at a concentration of 100 mg l^{-1} prevented blackening of medium and enhanced the number of

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healthy micropropagated shoots in *Gymnema*. Both qualitative and quantitative improvement on rooting was obtained on $\frac{1}{2}$ strength MS medium, where 53 % shoots were induced to root within 45 days. MS medium supplemented with 1.0 mg l^{-1} BA+ 0.5 mg l^{-1} IAA+ 100 mg l^{-1} vitamin B2+ 100 mg l^{-1} citric acid was best for shoot proliferation and $\frac{1}{2}$ strength MS medium with 3.0 mg l^{-1} IBA was best for root induction. Sharma and Bansal (2010) achieved in vitro shoot regeneration using shoot tips as explants. A number of concentrations of Kinetin and BAP were tested in MS medium for getting effective shoot regeneration protocol. Best results regarding the number of shoots were regenerated on MS medium containing Kn ($4.64 \mu\text{M}$) and BAP ($4.44 \mu\text{M}$) with 3.0 % sucrose. Shoots were rooted using $\frac{1}{2}$ strength MS media supplemented with IAA. A total of 85 percent of rooted shoots survived transfer field. Amarasinghe *et al.*, (2011) also carried out in vitro propagation of *Gymnema*. Multiple shoot regeneration has also been achieved by multiplying in vitro germinated seedlings. Firstly the conditions for in vitro germination of seeds were standardized. The maximum percentage of seed germination was found on $\frac{1}{2}$ MS medium, incubated in a dark period for 120 hours from immature green seeds. The best seedling growth and multiple shoot regeneration were obtained on MS medium containing BAP 1.0 mg l^{-1} + NAA 0.1 mg l^{-1} + KI 0.5 mg l^{-1} within 45 days of culture (Jaybhaye and Deokule, 2010; Gupta *et al.*, 2012). Nodal explants have been proved as best material for in vitro propagation of *Gymnema* (Manonmani and Francisca 2012). Solanki and Gupta (2013a) reported highly reproducible plant regeneration protocol from young shoots of mature plant. Highest multiple shoots (80%) were observed on MS medium supplemented with 5.0 mg l^{-1} BAP with Mean shoot length of $2.57 \pm 1.91 \text{ cm}$. Shoots were not induced on any level of 2,4-D/IBA supplemented in MS or B5 (Gamborg *et al.*, 1968) medium. Factors affecting rooting were also studied. Highest 80 percent rooting was obtained on $\frac{1}{2}$ MS medium without any Growth hormone.

Somatic Embryogenesis

Somatic embryos are induced in somatic tissue of plants by giving proper stimulus of a particular plant growth regulator in vitro. Applications of this technique include: large-scale production of clones, virus elimination, providing source tissue for gene transfer, production of synthetic seeds. Somatic embryos can be obtained on explant either directly or by the intervention of callus phase. It depends on the presence of competent cells in explant. A proper stimulus by a specific growth regulator induces cells of explant to form an embryo. Whole plant regeneration by somatic embryo formation has been achieved by callus obtained from hypocotyl, cotyledon and leaf explants excised from in vitro raised seedlings of *Gymnema*. MS medium containing ($0.5\text{--}5.0 \mu\text{M}$) 2,4-D + ($0.5\text{--}2.0 \mu\text{M}$) BA and 2.0 percent (w/v) sucrose, induced embryogenic callus within 6–8 weeks after initiation of culture. On this medium globular and heart shape stage embryos were obtained, which further developed into a torpedo and cotyledonary stage in a medium supplemented with MS salts, B5 vitamins, $0.5 \mu\text{M}$ BA and 2.0% sucrose. Subculturing on the same medium resulted in embryo germination and formation of plantlets, which were successfully adapted to greenhouse conditions (Kumar *et al.*, 2002). Ahmed *et al.*, (2009a) standardized a protocol for the formation of somatic embryos by suspension culture of *Gymnema*. Callus cultures were induced on MS medium with growth regulators 0.5 mg l^{-1} 2,4-D (or) 1.0 mg l^{-1} NAA and 10 percent coconut water. They were transferred into an MS liquid medium containing 1.0 mg l^{-1} NAA, 1.0 mg l^{-1} BA, 3.0 percent sucrose (w/v), 10 percent coconut water, citric acid 1.0 mg l^{-1} and glutamine 10 mg l^{-1} for induction of somatic embryos from callus. Various stages of somatic embryo development like; globular, heart, torpedo and cotyledonary were identified in suspension cultures within 8 weeks. The maturation of embryos was found to be considerably influenced by plant growth regulators and length of light and dark cycles. Plantlets were germinated from 5-7 % of embryos induced on semisolid MS salts with B5 vitamins, 3.0 percent sucrose and 0.8 percent agar (w/v). After transferring in field plantlets have shown similar traits as that of source plant. The various factors affecting callus production in *Gymnema* have been investigated in detail, including the type and age of explant, media, carbon source and antioxidants. Leaves and stem cuttings from young plant were tested for their regeneration potential by inoculating on various concentrations of different combinations of auxins and cytokinins added in MS and B5 medium. Callus induction was shown by 100% explants on all the levels of 2,4-D ($0.5\text{--}5.0 \text{ mg l}^{-1}$) within 3-4 weeks. Callus obtained on 2,4-D was pale yellow and friable but on NAA compact callus was formed. On

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combinations of BAP and 2,4-D only leaf explants responded to form compact yellow, green callus within 25-30 days. Among the different media tried callus was obtained only on MS medium (Solanki and Gupta 2013b). Among different types of antioxidants (adenine sulfate, ascorbic acid and citric acid) and carbon source (glucose, maltose and sucrose) tested, citric acid at 30 mg^{-1} concentration and sucrose at 3% concentration produced the highest amount of light green, compact callus from leaves in 9-10 weeks after inoculation. (Solanki and Gupta 2013c).

In vitro Production of Gymnemic Acid

Production of secondary metabolites in culture is a very efficient system nowadays. A great variety of phytochemicals are being produced commercially by several industries globally. Several strategies are being applied to optimize production of secondary metabolites in culture which include media manipulation, use of elicitors, use of bioreactors, biotransformation etc. (Kanetkar *et al.*, 2006; Subathra *et al.*, 2006a; Ahmed *et al.*, 2009b; Aneesa *et al.*, 2010; Chodisetti *et al.*, 2013). Before attempting on secondary metabolite production it is necessary to optimize biomass production. Lee *et al.*, (2006), investigated the influence of sucrose concentration, inoculum density and auxins in batch cultures of *Gymnema* and the effect of aeration in bioreactor culture. Optimum results were obtained on 3% sucrose and 5 mg l^{-1} NAA with 60 g (wet wt) l^{-1} inoculum density in flask culture. In bioreactor culture aeration of 0.40 vvm was proved to be optimum up to only 15 days of culture. Gopi and Vatsala (2006) obtained callus from leaves and nodal cuttings of *Gymnema* on MS medium with 3% sucrose supplemented with various concentrations of auxins and cytokinins. Regeneration of callus was achieved on 0.5 mg l^{-1} 2,4-D containing medium for both explants. Bioactive components were then extracted from callus. These extracts were subjected to HPLC analysis, which confirmed the presence of gymnemic acid and gymnemagenin as major constituents. Subathra *et al.*, 2006b obtained callus on combination of IAA and BA from leaves. Elicitation by the HR protein of *Xanthomonas* species in suspension culture enhanced gymnemic acid production. Mandal *et al.*, (2009) had evolved an effective and eco-friendly microwave assisted extraction (MAE) technique for efficient and extensive extraction of gymnemagenin from *Gymnema* cultures. By this technique a yield of 4.3% w/w gymnemagenin was produced in 6 min while extraction by heat reflux took 6 hrs and maceration and stirring consume 24 h in complete extraction. Treatment of blue light as an elicitor had not only induced production of gymnemic acid in *Gymnema* cultures but biomass production also. In a medium containing 1.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} Kn, maximum amount of green compact callus was developed in blue light in 45 days, which was confirmed by growth curve analysis. HPLC, HPTLC and TLC analysis of extracts confirmed that methanolic extracts contained sufficient amounts of gymnemic acid. The research reported that these extracts significantly increased the weight of the liver, pancreas and whole body. It also enhanced liver glycogen in alloxan dosed diabetic rats (Wistar rats). These extracts increased the regeneration of β -cells in pancreas of testing rats, as compared to normal diabetic rats. Hence it may be employed as a potential pharmaceutical drug for insulin-dependent diabetes mellitus (Ahmed *et al.*, 2010).

Upendra *et al.*, (2010) have tested a number of plant growth regulators in the medium for callus culture of *Gymnema* and the effect of salt stress on induction of gymnemic acid formation in culture. The maximum quantity of biomass was produced in the medium having various concentrations of 2,4-D. The gymnemic acid production was found to be increased on increasing 2,4-D concentration along with sodium chloride. Subathra and Srinivasan (2011) made use of *Aspergillusniger* crude culture extract as a biotic elicitor to increase quantity of gymnemic acid. Comparatively 9 fold increase in gymnemic acid yield was found in fungal extract treated cultures. Chodisetti *et al.*, (2011) gave elicitation of various metallic salts to *Gymnema* cultures for enhancing production of gymnemic acid. The compounds applied were cadmium chloride, mercuric chloride, silver nitrate, cupric chloride, cobaltous chloride and calcium chloride. Among all of them CdCl₂ gave a maximum response when added 12 d of culture, at 2mM concentration. Induction occurred after 24 h of treatment. Nagella *et al.*, (2011) investigated the effect of inoculum densities, the strength of the MS medium, carbon source (sucrose, glucose, fructose, maltose), and the concentration of the sucrose to study their influence on callus formation and yield of gymnemic acid. The optimum results were found with 10 gl^{-1} of inoculum density, full-strength MS medium supplemented

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with 2,4-D (2.0 mg l⁻¹) and Kinetin (0.1 mg l⁻¹), and 3% w/v sucrose for the increase in biomass and gymnemic acid yield. Praveen *et al.*, (2011), achieved improvement in biomass regeneration and gymnemic acid yield by manipulating the concentration of macro elements of MS medium and nitrogen source in liquid cultures of *Gymnema* cells.

Veerashree *et al.*, (2012) elicited suspension culture of *Gymnema* by four complex compounds; chitin, methyl jasmonate, pectin and yeast extract. The highest gymnemic acid was obtained in cultures elicited by 0.5 g l⁻¹ yeast extract after 20 days of treatment. It was 5.25 fold greater than control amounting about 52 mg l⁻¹ dry weight basis. Abiotic elicitors have also been used to provide stress to culture in various studies. Gymnemic acid production was found to be influenced by other factors also like; culture medium, type of explants, PGRs, colour of light, temperature, photoperiod, and sucrose sources.

Callus of *Gymnema* was incubated under different light colours (blue, white, red and green). A significant increase (4.4x) in gymnemic acid yield was obtained with blue light as compared to cultures incubated in white fluorescent light. HPLC, HPTLC and gravimetric methods were standardized for the recognition and measurement of gymnemic acid in methanolic extracts of *Gymnema* (Ahmed *et al.*, 2012; Bakrudeen *et al.*, 2012; Ahmed *et al.*, 2013).

Vats and Kamal (2013) evaluated phytosterols in leaves and callus of *Gymnema*. The highest amount of callus was obtained on MS medium with 0.5 mg l⁻¹ of 2,4-D. Extracts of callus and leaves were analyzed by chromatography and spectroscopy for pointing out phytosterols. The results have shown the presence of beta-sitosterol, stigmasterol and campesterol in both types of extracts, while lanosterol was identified only in callus culture.

Hairy root cultures are established to maximize production of secondary metabolites by genetic transformation of plant cells with *Agrobacterium rhizogenes*. Nagella *et al.*, (2013), established hairy root cultures of *Gymnema* and obtained 9.4 times increase in biomass with 4.7 fold increase in the gymnemic acid amount as compared to non-transformed cultures. Hairy root cultures of *G. sylvestre* were given elicitation after 15 days of culture by oleic and linolenic acid at different concentrations and roots were harvested after 20 days. An increase of 7.78 fold in gymnemic acid yield was obtained with 5 µM linolenic acid as compared to the non-elicited cultures (Praveen *et al.*, 2014).

CONCLUSION

Gymnema sylvestre is an herb belonging to the Asclepiadaceae family. It has been utilized to cure diabetes in folk, Ayurvedic and Homeopathic systems of medicine. *Gymnema* has been used by traditional medical practitioners of India and other Asian countries for several centuries. Indiscriminate collection and over exploitation has resulted in fast disappearing of natural strands of *Gymnema* from natural resources for commercial purposes to fulfil the needs of the pharmaceutical industry. Due to the poor natural regeneration of plant, tissue culture methods represent important potential for its propagation over conventional methods for conservation and large-scale plantation.

This review summarizes the available literature on various in vitro micropropagation techniques including axillary bud activation, somatic embryogenesis and multiple shoot production from in vitro germinated seedlings. Pharmaceutical properties of *Gymnema* are attributed to triterpenoidicsaponins, mainly gymnemic acid which maintains blood glucose level through increased insulin secretion provided by repair or regeneration of pancreatic cells. In vitro production of secondary metabolites is an effective application of plant biotechnology. Recently extensive work on the optimization of strategies of gymnemic acid production in culture is being carried out all over the world. In present review there is a collection of all of these important contributions, so that these studies can be used in the development of a commercially viable system for production of gymnemic acid.

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