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Effect of Media Strength and pH on the Growth of Hairy Roots and Production of Gymnemic Acid from *Gymnema Sylvestre*

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Abstract

Gymnema sylvestre (Madhunashini) is one of the most important medicinal plants used as a crude drug for its preventive and therapeutic properties. Among the diverse constituents of *Gymnema*, gymnemic acid is found to be a major component responsible for their biological and pharmacological actions. The present study deals with the influence of different media strength and initial medium pH on the growth of hairy roots and gymnemic acid production from *Gymnema sylvestre*. Higher strength of the media (1.5X) favoured the biomass production (114.64 g/L FW and 12.63 g/L DW) and gymnemic acid content (11.7 mg/g DW) in the tested range of 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0 X strength. Among the different hydrogen ion concentration (pH) of 4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5, initial medium pH of 6.0 favoured the biomass production (102.41 g/L FW and 11.52 g/L DW) and medium pH of 5.8 favoured the gymnemic acid production (11.30 mg/g DW).

Keywords: *Gymnema sylvestre*, Gymnemic Acid, Madhunashini, Media Strength, Hydrogen Ion Concentrations

1. Introduction

Plant secondary metabolites are the complex biologically active molecules present in a large number of plants and play a major role

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in the interaction with the environment. The productions of these molecules are often low and they greatly depend on the developmental stage and the physiological state of the plant [1]. The usage of these medicinal plants has been since ancient times, where human beings have exploited many of these medicinal plants as sources of nutraceuticals, cosmetics, drugs and food preservatives [2]. *Gymnema sylvestre* (Asclepiadaceae) is one of the important medicinal plants which has the mention in the Ayurveda. The plant is popularly known as "Gurmar" or "Madhunashini" for its distinctive property of temporarily destroying the taste of sweetness and is used in the treatment of diabetes [3]. The plant is known to possess potent anti-diabetic properties and has traditional uses in the treatment of asthma, eye complaints and snake bite. It also possesses certain pharmacological activities like anti-microbial, anti-hypercholesterolemic and hepatoprotective properties [4]. The leaves of the species contain triterpenoid saponins belonging to the oleanane (gymnemic acids) and dammarane (gymnemasides) classes [5]. The gymnemic acids are a group of closely related molecules that are isolated from the leaves of *G. sylvestre* [6]. The antidiabetic, anti-sweet and anti-inflammatory activities of *G. sylvestre* have been attributed to the presence of gymnemic acids (GA); the other phytoconstituents include flavones, anthraquinones, hentriacontane, resins, d-quercitol, lupeol,β-amyrin-related glycosides, stigmasterol and alkaloids [5].

For commercial gymnemic acid production, majorly field grown plant material is generally used but the quality of these products may be highly affected by different environmental conditions, pollutants and fungi, bacteria, viruses and insects, which can result in a heavy loss in yield and alter the medicinal content of plant. Plant cell and organ cultures are promising technologies to obtain plant specific valuable metabolites [7]. Cell and organ cultures have a higher rate of metabolism than field grown plants because the initiation of cell and organ growth in culture leads to fast proliferation of cell/organs and to a condensed biosynthetic cycle. Further, plant cell/organ cultures are not limited by environmental, ecological and climatic conditions and cells/organs thus proliferate at higher growth rates than whole plant in

cultivation [8]. In the native plant, gymnemic acid represents a very minor proportion and therefore alternative exploration of condition optimised cultures for efficient in vitro biogeneration of such gymnemic acid, which are pharmacologically promising but are severely limited in production, is important. Productions of gymnemic acid have been reported in cell suspension and hairy roots cultures [9-15]. However, optimisation of culture conditions has not been worked out in the hairy root culture. By proper manipulation of culture medium and conditions, it is possible to obtain valuable secondary metabolites in larger scale. In view of this, in the present study, the researchers have established hairy root cultures of *G. sylvestre* and examined the effect of media strength and different initial pH of the medium on biomass accumulation and gymnemic acid production.

2. Materials and methods

2.1. Hairy root culture

The hairy root cultures were initiated by culturing 500 mg of *G. sylvestre* hairy roots in 250 ml Erlenmeyer's flasks containing 50 ml of Murashige and Skoog (MS) [16] medium supplemented with 30 g/Lsucrose [10]. The initial medium pH was adjusted to 5.8 ± 0.2 before autoclaving (at121°C and 1.2 kg cm^2pressure for 15 min), and the cultures were kept under continuous agitation at 100 rpm in an orbital shaker and incubated at 25 ± 2°C with a 16 h photoperiod (40 mol $m^{-2}s^{-1}$) provided by 40 W white fluorescent lamps (Philips, Seoul, Korea Republic). The roots were sub cultured every 15 days.

2.2. Optimisation of culture conditions

Five hundred milligrams of hairy roots were inoculated in 250 mL Erlenmeyer flasks containing 50 mL of different strengths of the MS medium (0.25, 0.5, 0.75, 1.0, 1.5, and 2.0X) supplemented with 30 g/L sucrose for determining its optimum strength for promoting root growth. Various ranges of the initial medium pH (4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5) were also tested for the maximum growth of root biomass and were applied depending on the objective of the experiment. All shake flask cultures were kept in 16 h photoperiod

(40 µm mol^{-1} s^{-1}) at 25 ± 2°C and 100 rpm. After 20 days of culture, the growth of hairy roots was assessed in terms of fresh weight, dry weight, and content of gymnemic acid production was also determined.

2.3. Determination of root biomass

The roots were separated from the media by passing them through a 0.45 µm pore size stainless steel sieve (Sigma, USA). Their fresh weights (FW) were determined after they were washed with distilled water and the excess surface water was blotted away. Dry weights (DW) were recorded after the roots were dried at 60°C till constant weight is recorded.

2.4. Extraction of gymnemic acid

The extraction of gymnemic acid was done following the method of Praveen *et al*., (2014) [9]. 100 mg of sample was weighed into a 500 mL round bottom flask and 50 mL of extraction solvent (1:1 volume of methanol: water) and 10 mL of 11% potassium hydroxide solution was added. The mixture was refluxed for an hour; 9 mL of concentrated HCl was added and refluxed again for 1 h. The mixture was cooled to room temperature, the extract was filtered through 0.45 µm nylon filter (Sigma, USA) and the volume was made up to 100 mL with extraction solvent and the clear supernatant was used for HPLC analysis.

2.5. HPLC analysis of gymnemic acid

The HPLC analysis of gymnemic acid was done following the method of Praveen *et al*., (2014) [9]. The analytical HPLC experiments were performed with a High Performance Liquid Chromatography (HPLC) equipped with a variable dual wavelength detector operating at 210 nm (W2487). Separations were carried out with C18 (5 µm) column with acetonitrile: water (80:20) as an eluent at a flow rate of 1 mL/min with the injection volume of 20 µl and the column temperature was maintained at 27°C. Gymnemagenin (99% purity) standard was obtained from Natural Remedies (Bangalore, India). The identity of the compound was confirmed by comparison with retention time of standard and

the amount of gymnemic acid was obtained based on the gymnemagenin present in the samples.

2.6. Statistical analysis

All the experiments were performed in triplicates and each experiment was repeated twice. The data were expressed as means ±SE. One way ANOVA analysis followed by the Duncan's test was used to determine significant ($P \leq 0.05$) differences using SPSS version 16.

3 Results and Discussion

3.1. Effect of medium strength on biomass accumulation and gymnemic acid production

The optimum nutrient concentration is a critical determinant in controlling the growth of the cells/organs and the accumulation of secondary metabolite. In the present study, the effect of medium strength (MS medium of 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0X strength) on the biomass accumulation and gymnemic acid production from hairy root suspension cultures are represented in Figure 1. The hairy roots were cultured in MS basal medium with 3% sucrose. The highest accumulation of biomass (114.64 g/L of FW and 12.63 g/L DW) was observed in 1.5X strength MS medium (Figure 1A). Lower and higher strength of MS medium did not favour the accumulation of biomass. Lower concentration of quarter strength MS medium accumulated biomass of 78.43 g/L FW and 7.98 g/L DW whereas higher concentration of double strength MS medium accumulated biomass of 103.70 g/L FW and 11.22 g/L DW. 1.5X strength MS medium favoured the production of gymnemic acid which recoded 11.70 mg/g DW (Figure 1B). The lowest production of gymnemic acid content of 7.83 mg/g DW was recorded in the double strength MS medium whereas ¾ salt strength MS medium was optimal for the ginseng hairy root growth compared with other media. Lower salt strengths of MS medium such as ½ MS and ¾ MS were favourable for ginsenoside accumulation. Hence ¾ MS salt strength was favourable for both hairy root growth and ginsenoside production in *Panax ginseng* [17]. In contrast to our results, Yu *et al*., 2000 [18] reported that in ginseng adventitious

root cultures, half and full strength MS was suitable for biomass production whereas full strength medium was optimal for ginsenoside accumulation.

3.2. Effect of hydrogen ion concentration (pH) on biomass accumulation and gymnemic acid production

The effect of hydrogen ion concentration (pH; 4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5) on the biomass accumulation and gymnemic acid production from hairy root suspension culture is presented in Figure 2. The hairy roots were cultured in MS basal medium with 3% sucrose. The highest accumulation of biomass in terms of FW and DW was recorded at the pH of 6.0 (Figure 2A). The medium set at pH 6.0 accumulated the biomass of 102.41 g/L FW and 11.52 g/L DW. Higher level of pH 6.5 decreased in the accumulation of biomass which recorded 88.46 g/L FW and 9.51 g/L DW. The acidic pH of 4.0 had a negative effect on the biomass accumulation (70.62 g/L FW and 7.25 g/L DW). The highest production of gymnemic acid (11.30 mg/g DW) content was recorded at the pH level of 5.8, followed by pH 6.0 which produced 10.90 mg/g DW (Figure 2B). The lowest production of gymnemic acid (7.75 mg/g DW) was recorded at the pH level of 6.5. This can be explained as due to better utilisation of ammonium and nitrates as indicated by the uptake studies. However at pH 4.0 the biomass yield was low which appears to be due to poor utilisation of ammonium. The rapid changes in the pH of the medium during the culture period may be due to the nitrogen source utilised by the roots, which is also source of extra hydrogen of hydroxide ions as suggested by Dougall *et al*., (1983) [19]. Similar results were observed in the hairy root culture of *Tagetes patula* where medium pH of 5.7 was favourable for the production of thiophene. In hairy root cultures of *Trigonella foenum-graecum* L., the medium pH of 5.0, 5.5 and 5.9 did not affect the growth of the hairy roots but the diosgenin content varied. The maximum amount of diosgenin formation was recorded at pH 5.0 and it produced three fold increase when compared with the medium pH of 5.9 [20]. A 10 min exposure of pH 2.0 followed by return to standard growth medium of pH 5.5 resulted in the maximum production of betalains from hairy root cultures of *Beta vulgaris* L. [21]. Sivakumar *et al*., (2005) [17]

reported that medium pH of 6.0 and 6.5 favoured both biomass accumulation and ginsenoside production in hairy roots of *Panax ginseng*.

4. Conclusion

Culturing of hairy roots is an efficient method for producing useful bioactive molecules from medicinal plants. In the present study of flask scale system, we found that in vitro conditions strongly affected root growth and the accumulation of secondary metabolites from Gymnema hairy roots. The best performance overall was obtained in a 1.5X strength of MS medium and initial medium pH of 6.0. The above results are useful for the optimisation of the cultural conditions and for large-scale cultivation of *Gymnema sylvestre* hairy root culture for the production of gymnemic acid.

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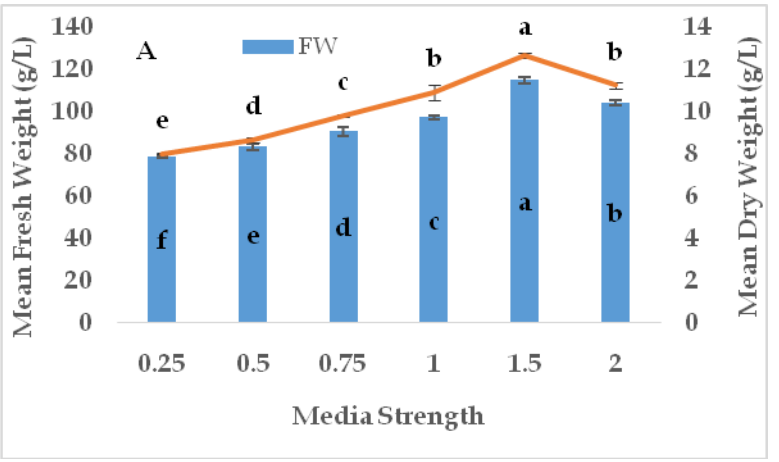

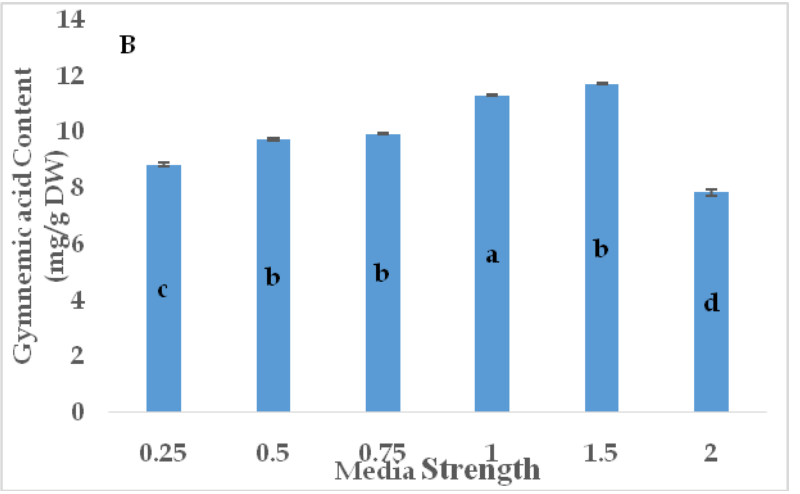

Figure 1: *Gymnema sylvestre* hairy root culture: effect of medium strength on biomass accumulation (A) and gymnemic acid production (B)[z, y].

[z]0.5 g of hairy roots was cultured in 50 ml of MS basal medium for 20 days.

[y] Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT).

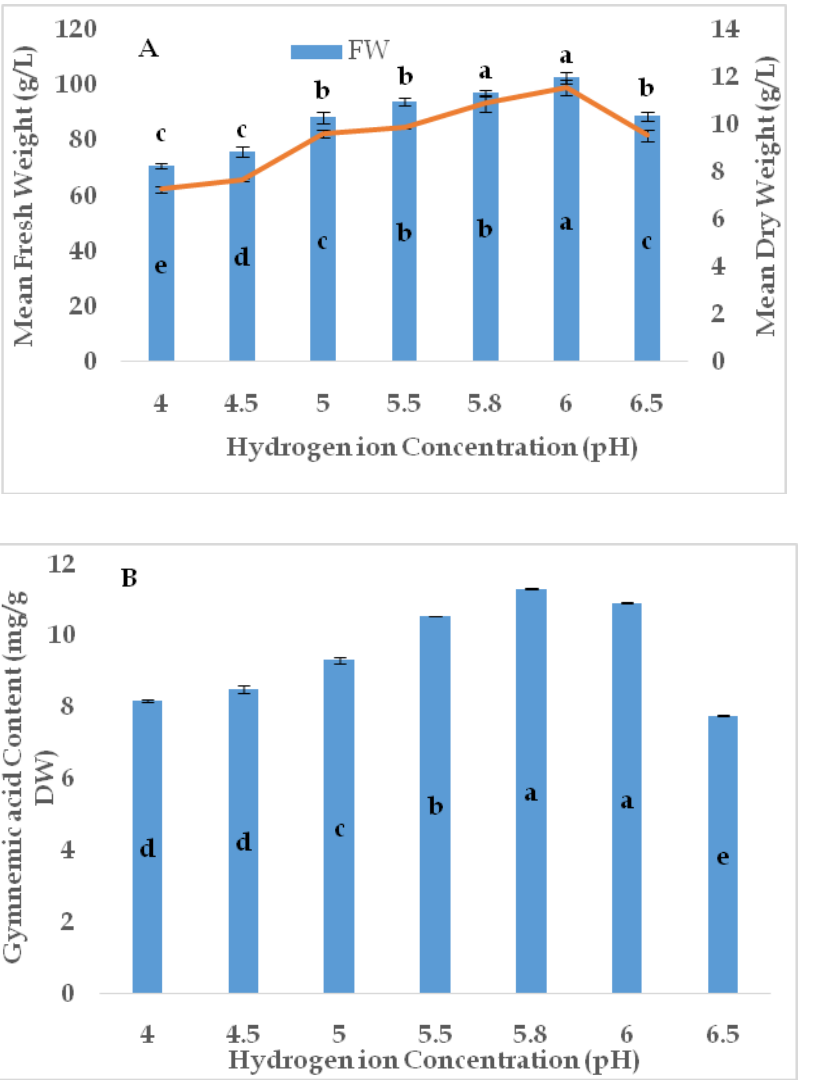

Figure 2. *Gymnema sylvestre* hairy root culture: effect of hydrogen ion concentration (pH) on biomass accumulation (A) and gymnemic acid production (B)[z, y].

[z]0.5 g of hairy roots was cultured in 50 ml of MS basal medium for 20 days.

[y]Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT).