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Influence of Phytoregulators on Production of Gymnemic acid in the Callus Cultures from the Leaves of *Gymnema sylvestre* R.br.,

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ABSTRACT

Gymnema sylvestre (R.Br.) is an important medicinal plant distributed throughout the world, constitutes bioactive compound called gymnemic acid, a group of oleanane saponins known for its anti-diabetic activity. The present research was designed to investigate the influence of phytoregulators on growth, biomass yield and gymnemic acid production in the callus cultures developed from the leaves of *G. Sylvestre*. Callus was induced in all the tested concentrations of phytoregulators, but short duration for initiation (12 days), optimal induction (88%), maximum biomass yield (Dry weight 114 mg culture^{-1}) and highest production of gymnemic acid (1.69 mg/dry weight culture^{-1}) were achieved using 2.0mg/L of 2,4-dichlorophenoxy acetic acid (2,4-D) in combination with 1.0mg/L of kinetin (Kn). Growth curve analysis reveals that biomass yield and gymnemic acid production were significantly growth related. Highest biomass yield and gymnemic acid production were observed after 5 weeks of inoculation. The present study reveals that auxins alone were poor in inducing callus while auxins, particularly 2,4-D in combination with cytokinins particularly kinetin was very effective in inducing the callus in terms of callus growth, biomass yield and gymnemic acid content.

Keywords: Gymnemic acid, Gymnemagenin, Growth curve, Biomass yield, High performance liquid chromatography

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INTRODUCTION

Gymnema sylvestre R. br. is a wild plant commonly known as gurmar classified in the Asclepiadaceae family and is widely distributed in Southern India, tropical Africa and Australia. *G. sylvestre* is widely used as a health food in Western India, Tropical Africa, Vietnam, Malaysia, Srilanka, Germany and the USA [1]. The leaves of this plant are used for inhibiting the taste of sweetness and are used in the control of diabetes [2]. The leaves also possesses anti hyperlipidemic [3], anti-hypercholesterolemic [4], hepatoprotective [5], anti-allergic [6], anti inflammatory [7], free radical scavenging [8], antimicrobial [9], antiviral [10] and anti larvicidal [11] activities. The major phytoconstituents of *Gymnema sylvestre* are triterpene saponins belonging to oleanane and dammarene classes. The total oleanane type saponins [1] fraction of the leaves commonly known as "gymnemic acid", has an anti-sweetening effect. Besides this the other plant constituents are flavones, anthraquinones, phytins, resins, d-quercitol, tartaric acid, butyric acid, lupeol, β-amyrin related glycosides and stigmasterol and alkaloids. Among these bioactive compounds, gymnemic acids have anti-diabetic, anti-saccharine and anti-inflammatory activities [12,13]. The antidiabetic array of molecules has been identified as a group of closely related gymnemic acids.

The conventional propagation of *Gymnema sylvestre* has been hampered because of poor seed viability, low germination rate, and poor rooting ability of vegetative cuttings. As a result, its commercial exploitation could not be achieved. Therefore, the use of in vitro propagation methods would accelerate large scale plant multiplication and conservation. *In vitro* techniques are very useful in ensuring sustainable, optimized sources of plant-derived natural products. The ability of plants to produce certain bioactive substances is greatly influenced by the physical and chemical environments in which they grow. In the past few decades, secondary metabolite production from plant tissue culture has been identified as a tremendous resource for new drug development and clinical research in the fields of pharmacology and medicine. Plant cell culture extracts have also been used widely in the form of fractions and isolated compounds as potential bioactive molecules [14]. In vitro developed callus tends to produce various active compounds, including gymnemic acid and gymnemagenin [15]. HPLC methods have already been reported for the estimation of gymnemic acid in *G.sylvestre* [16,17]. There were reports on the establishment of callus culture, effect of plant growth hormones on the growth of callus, fresh weight and dry weight, however this is the first systematic approach to study the effect of different phytoregurators on the callus induction duration, growth rate of callus, fresh weight, dry weight of callus and gymnemic acid content in the callus derived from leaf explants.

MATERIALS AND METHODS

Chemicals

Standard Gymnemagenin and Phytohormones were purchased from Sigma Aldrich chemicals, USA. The medium components were of tissue culture grade and purchased from Hi-

Media chemicals, Hyderabad, India. The solvents used for chromatographic purposes were of HPLC grade and other solvents were of reagent grade.

Plant material

Gymnema Sylvestre plants were collected from the Tirumala hills, and confirmed with taxonomist, Department of Botany and maintained in medicinal plants garden of the Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

Explant sterilization

The young leaves were excised and washed thoroughly under running tap water for 10 min. There after leaves were soaked in 1% teepol solution for about 5 min and washed thoroughly with double distilled water. Then leaves were transferred to laminar air flow (LAF) chamber. Under LAF, leaves were disinfected with 10% sodium hypochlorite (NaOCl) solution for 60 sec followed by sterile double distilled water. Then leaves were surface sterilized with 0.1% $HgCl_2$ for 30 sec followed by a final rinse (3-5 times) with sterile double distilled water.

Callus induction

The sterilized leaves were dissected into 2 - 3 equal parts and inoculated on MS media [18] supplemented with different phytohormones viz., i). MS + 2.0mg/L 2,4-dichlorophenoxyaceticacid (2,4-D), ii). MS+2.0mg/L α-napthaleneaceticacid (NAA), iii). MS + 2.0mg/L 2,4-D + 1.0mg/L Kinetin (Kn), iv). MS + 2.0mg/L 2,4-D + 1.0mg/L 6-benzylaminopurine (BAP), v). MS+2.0mg/L NAA+1.0mg/L Kn vi). MS + 2.0mg/L NAA + 1.0mg/L BAP. The cultures were maintained at 23 ± 2 °C and 60-70 % relative humidity under photoperiod of 16-h light/8-h dark with light intensity 40–45 µ mol/m^2/s. The friable and white colored globular callus (3 - 5 mm in diameter) was sub cultured at every 2 weeks interval.

Callus Growth measurement and Biomass yield

Callus initiation time (in days) and the percentage of callus induction was recorded. Morphological changes of the callus was observed. For biomass yield, the proliferated fresh callus was harvested and dried in an oven at 40°C for 24 h and the dry weight (DW) was recorded. Dry weight was determined at 15, 22, 29, 36, 43, 50 days. Callus growth curve was drawn on dry weight basis and analyzed for suitable callus harvesting time.

Gymnemic acid extraction by acid-base hydrolysis

500 mg dried powder of *in vitro* callus of *Gymnema sylvestre* was taken into a 500 mL round bottom flask and 50 mL of extraction solvent (1:1 volume of methanol and water) and 10 mL of 12% potassium hydroxide solution was added. This mixture was refluxed for an hour and then 10 mL of 4N HCl was added and refluxed again for 1 h. The mixture was cooled to room temperature, the extract was filtered through 0.45µm nylon filter (Millipore, USA) and the

volume was made up to 100 ml with extraction solvent and the clear supernatant was used for HPLC analysis.

Chromatographic separation of Gymnemic acid

Armed with CXTH-3000 software program, the HPLC system consisted of a P3000-M HPLC Pump (isocratic) with a 10 ml Pump head, the UV3000-M variable-wavelength ultraviolet/visible detector. A Rheodyne model 7725 sample injection valve equipped with a 20 µL sample loop. Reverse phase separations were carried out using kromasil RP C18 Column (250 x 4.6mm i.d; particle size 5.0 µm). The chromatographic separation was performed with C18 (5.0 µm) Column using mobile phase, acetonitrile:water (80:20) with the flow rate of 1.0 mL/min, and the column temperature was maintained between 26-27^{o}C. Gymnemic acid was detected by UV absorption at 220nm. Each injection volume was 20 µL. Validation of quantitative method was done with samples consisting of three injections of 20 µL each.

The quantity of gymnemagenin was calculated by comparing the peak of the desired analyte with that of standard peak and using the following equation

$$\gamma = \alpha \times c \times v/\beta$$

where γ = concentration of desired product (ug/g) ; α = respective peak area of sample aliquot ; β = peak area of the respective standard; c = concentration of standard solution (ug/ mL); v = volume made for sample extract.

The conversion of gymnemagenin to gymnemic acid was done using the following equation.

$$C = X\ (809.0/\ 506.7)$$

where, C is the content of gymnemic acid in the sample; X is the content of gymnemagenin present in the sample, 506.7 is the molecular weight of gymnemagenin ($C_{30}H_{50}O_6$) and 809.0 is the molecular weight of gymnemic acid ($C_{43}H_{68}O_{14}$).

Statistical analysis

All the experiments were set up in a completely randomized way and the significance of differences among means was calculated utilizing Duncan's multiple-range test [19] ($P \leq 0.05$).

RESULTS

Callus induction

Callus was initiated in all the tested concentrations of phytohormones between 12 days to 23 days after inoculation. Short duration for initiation (12 days) and maximum induction (88%) were observed in the MS media supplemented with 2.0 mg/L 2,4-D and 1.0 mg/L Kn

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while long initiation period (23 days) and lowest induction (41%) were observed in the MS media supplemented with 2.0 mg/L NAA alone (Table. 1). The auxin 2.0mg/L 2,4-D alone was very poor in inducing the callus (56%) and the initiation time was 18 days. The combination of phytohormones viz., 2.0 mg/L 2,4-D and 1.0 mg/L BAP (78%) 2.0 mg/L NAA and 1.0 mg/L kinetin (75%) were also found to be good for the initiation of the callus. MS media supplemented with 2.0 mg/L NAA and 1.0 mg/L BAP was not effective in inducing the callus where the callus induction was about 66% (Table. 1).

Table 1: Growth of callus derived from *G.sylvestre* leaves under different combinations of phytohormones [mg L^{-1}]. Growth was determined at 15, 22, 29, 36, 43, 50 days of inoculation.

Medium		Percentage of callus induction	Time taken for callus initiation	Callus score	Callus morphology
MS + 2,4-D (2.0mg/l)		56%	18days	15= - 22=+ 29=++ 36=+++ 43=+++ 50=+++	_ light green whitish and loose yellowish and loose yellowish and loose yellowish and loose
MS +NAA (2.0mg/l)		41%	23 days	15= - 22= - 29=+ 36=++ 43=+++ 50=+++	_ _ light yellow yellowish and loose yellowish and loose yellowish and loose
MS +2,4-D (2.0mg/l) + Kn(1.0mg/l)		88%	12 days	15= + 22=++ 29=+++ 36=++++ 43=++++ 50=++++	light green green whitish and compact whitish and friable yellowish and friable yellowish and loose
MS +2,4-D (2.0mg/l) + BAP (1.0mg/l)		78%	14 days	15= + 22=++ 29=+++ 36=+++ 43=++++ 50=++++	light yellowish whitish whitish and compact whitish and friable yellowish and loose yellowish and loose
MS + NAA (2.0mg/l) + Kn(1.0mg/l)		75%	13 days	15= + 22=++ 29=+++ 36=++++ 43=++++ 50=++++	light Green whitish whitish and compact whitish and friable yellowish and loose yellowish and loose
MS + NAA (2.0 mg/l) + BAP (1.0mg/l)		66%	17 days	15= - 22=+ 29=++ 36=+++ 43=+++ 50=+++	_ light yellowish whitish and friable whitish and loose yellowish and loose yellowish and loose

- = No callus; +=Low amount; ++=Good;+++=High;++++=Intense or very high

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Callus morphology

Leaf explants of *G.sylvestre* inoculated in medium supplemented with various growth regulators exhibited different morphological changes in their colour and texture such as, light green, green, light yellow and white (Table. 1). The texture was ranged from compact, friable to loose. The prolong incubation resulted in brown and compact callus For combinations such as 2.0 mg/L 2,4-D and 1.0 mg/L kinetin, 2.0 mg/L 2,4-D and 1.0 mg/L BA the morphological changes from 2nd week to 6th week were almost similar (Figure. 1a-d). Whitish and friable callus after two weeks of subculturing was shown in Figure. 1e.

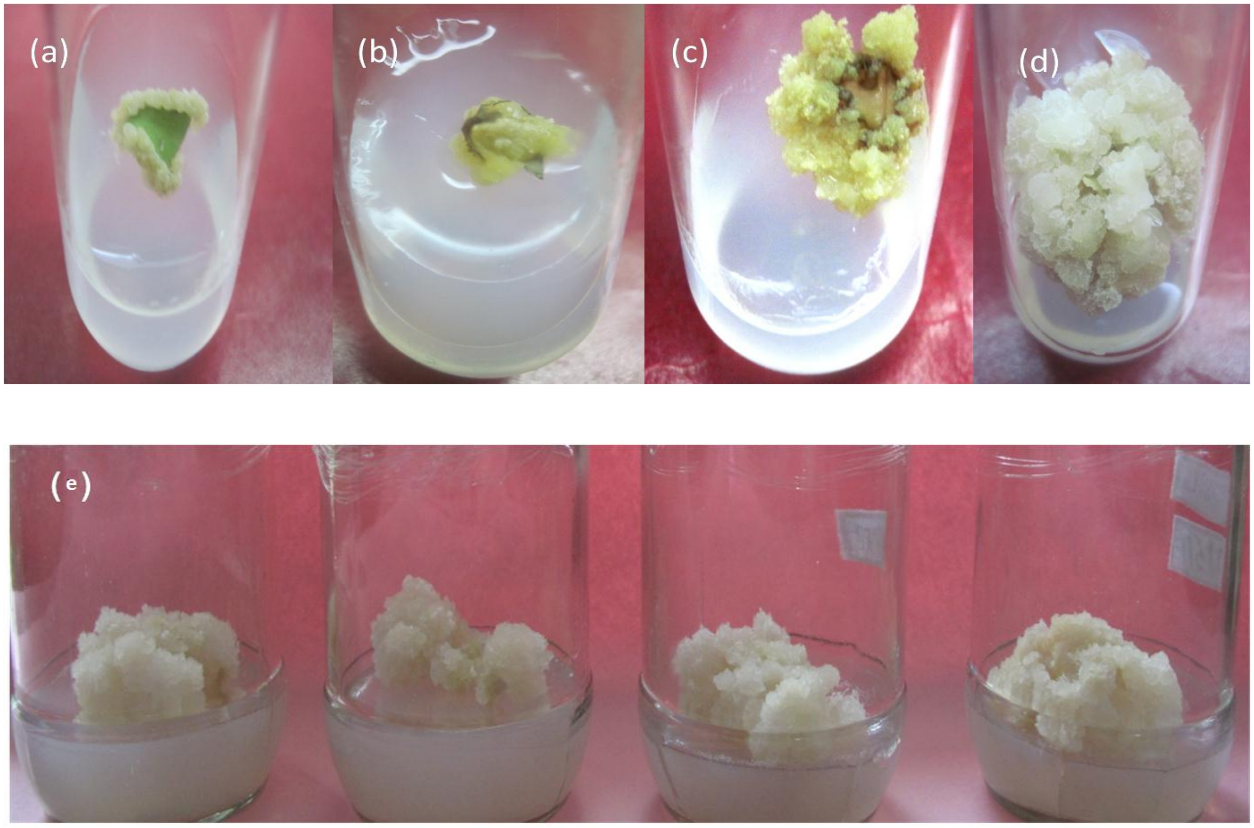

Figure 1: Callus derived from the leaves of *G. sylvestre* on MS media supplemented with 2,4-D (2.0mg/L) and Kn (1.0mg/L) (a) after 12 days (b) light green callus after 15 days (c) greenish callus after 29 days (d) white and compact callus after 36 days of inoculation. (e) whitish and friable callus after two weeks of sub culturing on MS medium containing 2,4-D (2.0mg/L) and kinetin (1.0mg/L).

Callus Biomass yield

The dry weight biomass yield was determined at 15,22,29,36,43,50 days of inoculation. Optimal biomass yield was obtained after 5 weeks of inoculation in all the tested

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concentrations. Highest biomass yield was obtained by the use of 2,4-D (2.0 mg/L) with kinetin (1.0mg/L) (DW-114 mg) on 36th day. However MS media with 2,4-D (2.0mg/L) and BAP (1.0mg/L), MS media with NAA (2.0mg/L) and Kn (1.0mg/L), MS media with NAA (2.0mg/L) and BAP(1.0mg/L), MS media with 2,4-D (2.0mg/L) resulted in less biomass yield and the optimal values are 89.08mg, 78.02mg, 67.68mg and 65mg respectively. Callus cultures containg MS media supplemented with NAA (2.0mg/L) was very poor in inducing the callus and the optimal value was 56.01mg only.

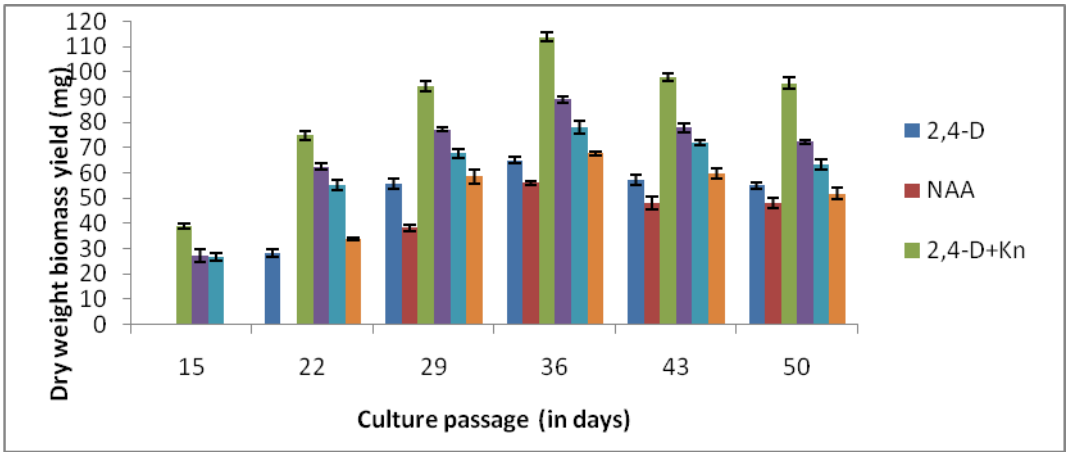

Figure 2: Biomass yield (mg) on the dry weight (DW) basis in the *G.sylvestre* callus cultures induced by the application of different phytohormones on MS media during culture passage with 7days interval. Data represents mean values±SE of three replicates; each experiment was repeated twice.

Gymnemic acid production

The chromatogram of the desired analyte was compared with respective standard (Figure. 3a-b) and the concentration of the desired product was calculated. The maximum accumulation of biomass was recorded by using 2,4-D (2.0mg/L) with kinetin (1.0mg/L). Gymnemic acid accumulation in the callus cultures directly related with biomass accumulation and the highest gymnemic acid yield from callus (1.69 mg/dry weight culture^{-1}) was obtained on the MS medium containing 2.0 mg/L 2, 4- D with 1.0 mg/L kinetin. The good accumulation of gymnemic acid was also recorded in the callus cultures containing MS media supplemented with 2.0 mg/L 2,4-D and 1.0 mg/L BAP(Gymnemic acid content; 1.12 mg/dry weight culture^{-1}); MS media supplemented with 2.0 mg/L NAA and 1.0 mg/L Kn (Gymnemic acid content; 1.03 mg/dry weight culture^{-1}); MS media supplemented with 2.0 mg/L NAA and 1.0 mg/L BAP (Gymnemic acid content; 0.99 mg/dry weight culture^{-1}). MS media supplemented with 2.0mg/L 2,4-D and MS media supplemented with 2.0mg/L alone were not suitable for gymnemic acid production and the values are 0.97mg and 0.84 mg respectively (Figure. 4).

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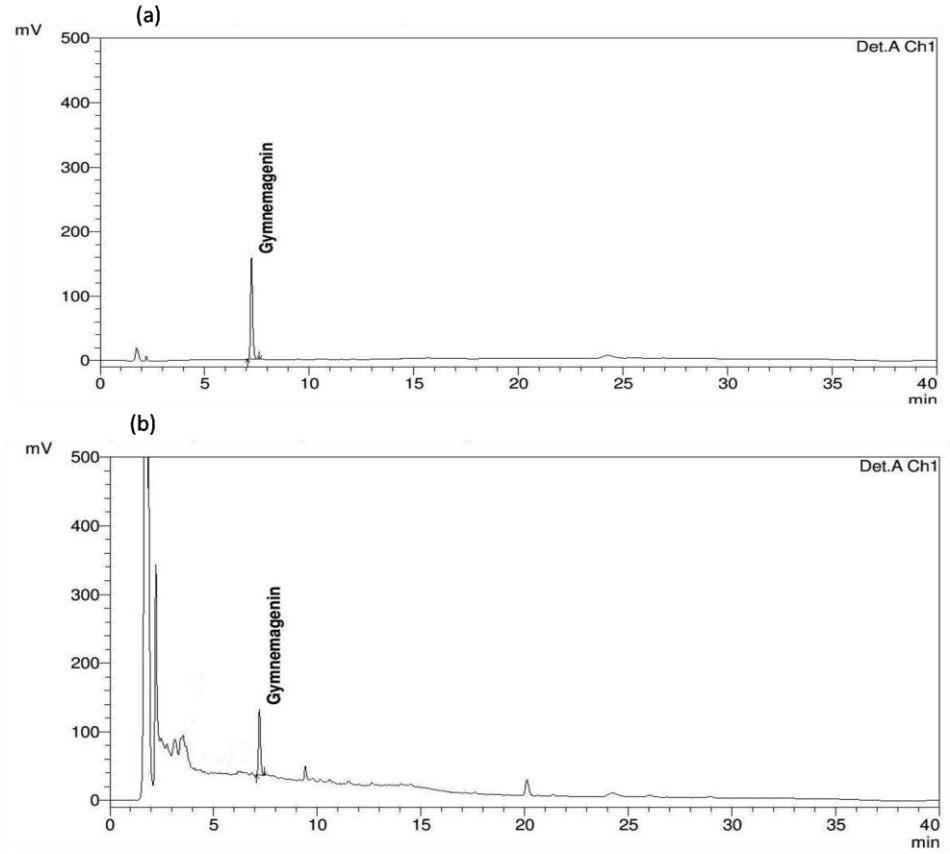

Figure 3: Chromatogram of standard Gymnemagenin (a) Gymnemagenin content (b) in the callus cultures containing MS media supplemented with 2,4-D(2.0mg/L) and Kn(1.0mg/L) harvested on 36th day of inoculation.

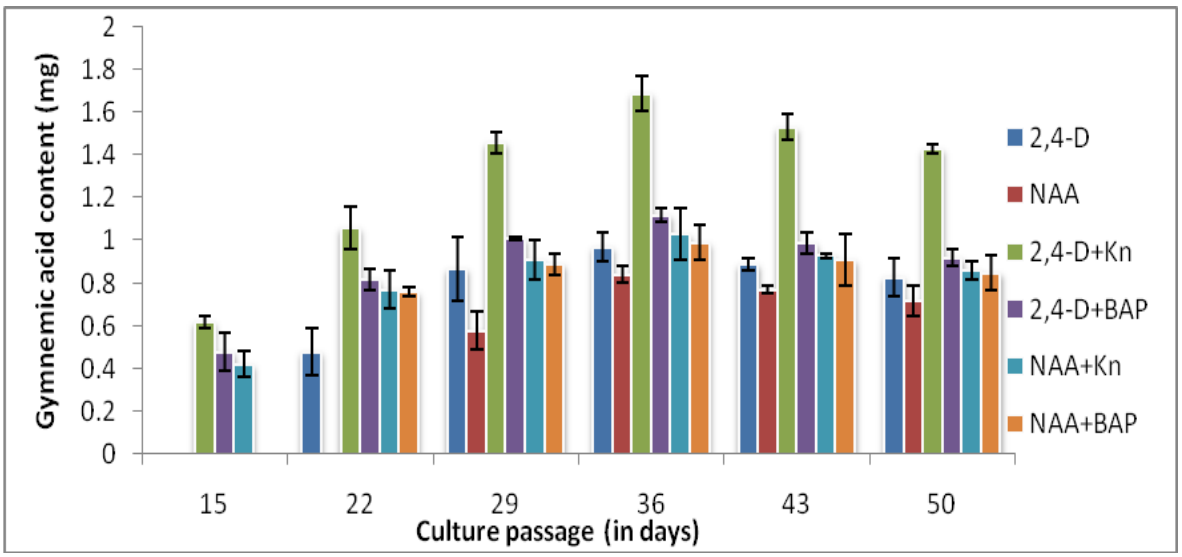

Figure 4: Gymnemic acid (mg/DWculture^{-1}) content in the *G.sylvestre* callus cultures induced by the application of different phytohormones on MS media during culture passage with 7days interval. Data represents mean values±SE of three replicates; each experiment was repeated twice.

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Callus Growth curve

Biomass yield and gymnemic acid production were determined with 7day interval from 15 days to 50 days. Callus growth curve was drawn based on biomass yield and gymnemic acid production. Callus growth curve analysis revealed the suitable callus harvesting time point with highest biomass yield and gymnemic acid production (Figure. 5) Successful production of gymnemic acid was observed between 29 days to 43 days of inoculation. But the optimal biomass yield and gymnemic acid content was observed on day 36 or after 5 weeks of inoculation. Following these time courses it was clear that the biomass yield and gymnemic acid accumulation are growth related.

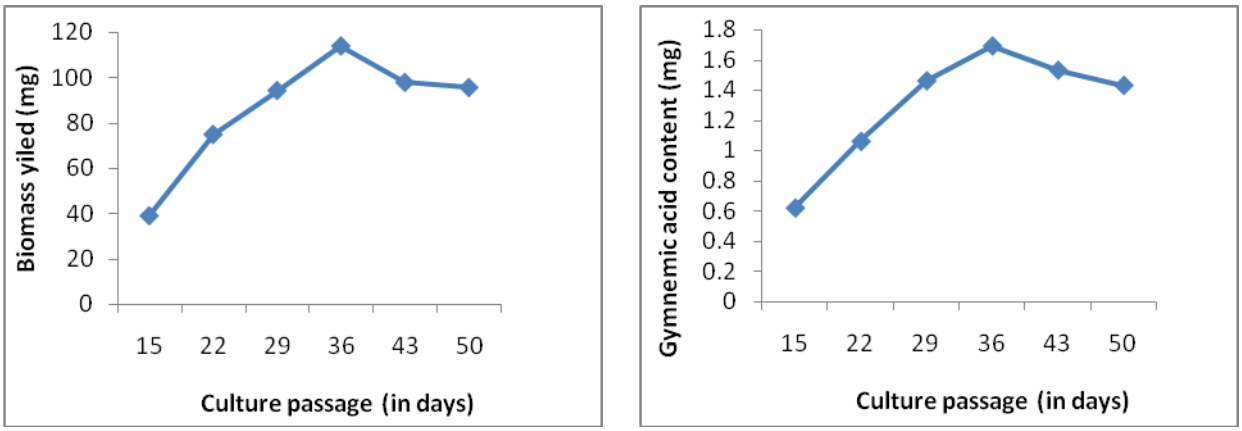

Figure 5: Growth curve of callus based on the biomass yield and gymnemic acid content during culture growth at different intervals in the milligram proliferation medium (MS + 2.0mg/L 2,4-D + 1.0mg/L kn).

DISCUSSION

Gymnema sylvestre (R.Br.) is an important medicinal plant which yields pharmaceutically active compounds called gymnemic acid (GA). Gymnemic acid is the main phytoconstituent of *G.sylvestre* and possesses different pharmacological activities. Considering medicinal properties and over-exploitation of *G. sylvestre* the requirement for a tissue culture technique as an alternative production system was crucial. In this study we reported the influence of auxins alone as well as combination of auxins with cytokinins on in vitro callus cultures of *G.sylvestre* in terms of callus induction, callus morphology, biomass yield and gymnemic acid content. The auxins 2,4-D and NAA alone were found to be very poor in inducing callus from the leaves of *G.sylvestre* and produced the callus response 56% and 41% only. The callus was loose in texture, white in color and resulted in low biomass yield and gymnemic acid accumulation.

Further investigation on callus induction was carried out using combinations of auxins with cytokinins. Highly proliferating whitish green calli was observed in the medium supplemented with 2.0 mg/L 2,4-D with 1.0 mg/L Kn producing an 88% callus response with in short duration and resulted in high biomass yield and gymnemic acid content. A further screening of suitable growth regulators for *G.sylvestre* callus induction was conducted through culturing of young leaf explants on the MS medium supplemented with the combination of 2.0

mg/L NAA and with Kn, 2.0 mg/L NAA and with BAP. The calli were found initiated with in short duration, but percentage of callusing was low and also resulted in less biomass production and gymnemic acid content. Although the combination of NAA with BA and kinetin is not suitable for callus induction but produced better results when compared to auxins alone. Based on the callus growth curve analysis, it was clear that biomass yield and gymnemic acid production are growth related and highest biomass yield and gymnemic acid content were obtained after 5 weeks of inoculation.

In conclusion, the present report on callus cultures of *G. sylvestre* has shown that both biomass yield and gymnemic acid production were influenced by phytoregulators. The optimal production of biomass and highest production of gymnemic acid content was recorded in the cultures supplemented with 2.0mg/L 2,4-D and 1.0 mg/L Kn and between 29-43 days of inoculation. These results are very useful for large scale production of gymnemic acid in the callus cultures of *G. sylvestre.*

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CONFLICT OF INTEREST

All the authors declare that there is no conflict of interest.

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