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In vitro multiple shoot and gymnemic acid production in *Gymnema sylvestre* (Retz.) R. Br. Ex. Sm.

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Gymnema sylvestre (Retz.) R. Br. Ex Sm. is a vulnerable, slow growing, perennial, woody climber belonging to family Apocynaceae, commonly used in indigenous system of medicine to control diabetes mellitus. Optimized 6 benzyl amino purine (BAP) and Kinetin (KIN) concentration (4.44 + 2.32 µM) was supplemented with naphthalene acetic acid (NAA) concentrations (0.54, 2.69, 5.37 µM) for enhancing multiple shoot production. Maximum multiple shoots (11.18/culture) were obtained on BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM). Maximum percentage of shoots rooted (77.7%) was observed on half-strength Murashinge & Skoog (MS) medium supplemented with indole 3 butyric acid (IBA) + indole 3 acetic acid (IAA) (4.92 + 5.70 µM). The cultures were harvested every week upto eight weeks to assess biomass (FW, DW) and gymnemic acid content (mg/g DW). Highest biomass (FW 0.51 g, DW 0.139 g) was obtained on BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM). Maximum gymnemic acid content (mg/g, DW) was obtained for multiple shoots cultured on BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM) followed by BAP + KIN (4.44 + 2.32 µM), BAP (4.44 µM), KIN (2.32 µM) and control i.e. 10.37, 9.36, 9.21, 9.03 and 6.86 mg/g (DW) respectively. Successful establishment of multiple shoots through nodal cultures and quantification of gymnemic acid from these multiple shoots for 8 weeks has been carried out. To the best of our knowledge, this is the first report on gymnemic acid production from multiple shoots cultures.

Keywords: *Gymnema sylvestre*, multiple shoots, nodal culture, HPTLC, gymnemic acid

Introduction

Gymnema sylvestre (Retz.) R. Br. Ex Sm. is a vulnerable, slow growing, perennial, woody climber[1] belonging to family Apocynaceae[2] (previously Asclepiadaceae). It is found in India and southwest region of China[3]. Leaves are commonly used in indigenous system of medicine to control diabetes mellitus[4]. *G. sylvestre* has been used as remedy for snake bite[5] and it has anti-bacterial, anti-viral, anti-sweetener and anti-inflammatory properties[3,6-8]. It has-hypoglycemic activity[4] and also used as a stimulant[9], laxative and diuretic[3]. *G. sylvestre* shows poor seed viability, low rate of germination, poor rooting ability of vegetative cutting and lacks proper cultivation practices[10,11]. Indiscriminate collection of *G. sylvestre* from wild has classified the plant as endangered[12]. *In vitro* propagation can play a key role in making available mass production for commercial use without disturbing the natural resource. The medicinal properties of *G. sylvestre* are mainly due to the presence of gymnemic acid, an oleanane type of triterpenoid saponin[13]. Production of gymnemic acid from *in vitro* cultures of *G. sylvestre* was limited to cell suspension cultures[5,10,13-16] which is not genetically stable as organ cultures. Plant growth regulator such as 2, 4-dichlorophenoxyacetic acid (2,4-D) is usually used for raising callus and cell suspension cultures and influences endopolyploidy, metabolic activity[17] and causes metabolic variations with the course of time[18].

To overcome the limitation with cell suspension cultures, the present investigation aims to study effect of different plant growth regulation (PGRs) on *G. sylvestre* for multiple shoot production, biomass (FW, DW) and assessing its gymnemic acid content in the obtained *in vitro* shoot cultures.

Materials and Methodology

Collection and Sterilization of Explants

Nodal explants were collected from Botanical Garden, S. P. Pune University Pune Maharashtra. Explants were kept under running water for 30 min. with 2-3 drops of Tween-80, followed by quick wash of 70% alcohol for 10 sec. For further sterilization of explants 1% bavistin and 0.1% $HgCl_2$ were used for 30 min, and 3 min, respectively.

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Medium used and Inoculation of Nodal Explants

Surface sterilized explants were inoculated on MS medium[19] supplemented with different PGRs. The media was also supplemented with citric acid (100 mg/l) and $AgNO_3$ (5 mg/l) prior to autoclaving. Medium was autoclaved at 121°C at 15 lbs for 20 min. Nodal explants were inoculated with basal cut surface down on MS medium supplemented with different concentrations of BAP, KIN and NAA (µM) alone and in combinations. After every four weeks cultures were subcultured on fresh medium with same combinations.

Rooting of *In Vitro* Obtained Multiple Shoots

Multiple shoots (3-4 cm long) obtained from nodal sectors were separated and transferred on full- and half-strength MS medium supplemented with indole-3-butyric acid (IBA) (2.46, 4.92, 7.38, 9.84, 12.30, 14.76 µM) and indole-3-acetic acid (IAA) (2.85, 5.70, 8.56, 11.41, 14.27, 17.12 µM) in combinations. These were maintained for 4 weeks duration.

Culture Conditions

The cultures were incubated at 25 ± 2°C under photoperiod 16/8 h (light/dark). The light source used was cool white florescent tubes providing an illumination of 2000 lux/m^2/s.

Biomass Production in *In Vitro* Multiple shoots

Four different media which exhibited maximum number of multiple shoots were used viz., BAP (4.44 µM), KIN (2.32 µM), BAP + KIN (4.44 + 2.32 µM), BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM). Growth was assessed in terms of fresh weight (FW) and dry weight (DW) (g) at the end of each week and was recorded upto 8 weeks consecutively.

Production of Gymnemic Acid (mg/g DW) in *In Vitro* Multiple Shoots

Oven dried (60°C) plant samples (multiple shoots) were powdered and aliquots (1 g) were cold macerated in 50 ml of mixture of 95% ethanol : water (1:1 v/v) for 48 h. The obtained solution was filtered and concentrated under vacuum, redissolved in methanol (25 ml) in a water bath at 55°C and the final volume was made upto 50 ml. This extract was used for high performance thin layer chromatography (HPTLC)[1].

Chemicals and Reagents

As the gymnemic acid (glycon) is commercially not available the quantification of gymnemic acid was done by using gymnemagenin (aglycon)[1]. Gymnemagenin gave rise to gymnemic acids and derivatives through glycosylation mechanism by glycosyltransferases. Standard gymnemagenin was procured from Natural Remedies, Bangalore. All other chemicals used were of analytical grade (Merck, India).

HPTLC Instrumentation and Experimental Conditions

Sample solutions were applied onto the TLC plates (10 × 10 cm) with automated TLC sampler Linomat V (Camag, Muttenz, Switzerland) and plates were developed using mobile phase chloroform : methanol (8 : 2) v/v. After development, chromatographic plates were dipped into modified vanillin-sulphuric acid derivatization reagent and further plates were heated at 110°C for 15 min in a pre-heated oven. The bluish-black colored spot corresponding to gymnemagenin was observed. The plates were scanned at 610 nm.

Conversion of Gymnemagenin to Gymnemic acid

The conversion of gymnemagenin to gymnemic acid was done using the equation as follows 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, and 809.0 is the molecular weight of gymnemic acid[1].

Statistical Analysis

All the experiments were carried out in triplicates and the data were expressed as means ± standard deviation. One way analysis of variance (ANOVA) analysis followed by the Duncan's multiple range test (DMRT) was used to determine significant ($p \geq 0.05$) differences.

Results and Discussion

Percent Response, Cultures with Multiple Shoots and Multiple Shoot Production

In the present investigation maximum (75.55%) and minimum (39.99 %) percent response was obtained for BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM) and BAP (2.22 µM), respectively. Control showed higher percent response (42.22%) as compared to the response observed in BAP (2.22 µM). Maximum percent of multiple shoots (64.44%) were recorded in MS media supplemented with BAP + KIN (4.44 + 2.32 µM) whereas minimum was in control (0.00%) (Table 1). Our results showed concurrence with Komalavalli & Rao (2000)[11] who indicated that for sprouting and multiple shoot production in *G. sylvestre*, MS media should be supplemented with cytokinins. We observed MS media supplemented with BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM) produced

Table 1 — Effect of different plant growth regulators on multiple shoots induction of *G. sylvestre* for eight week study

MS with PGR (µM)	% Response	Cultures with MS (%)	No. of multiple shoots/culture
BAP 2.22	39.99 ± 6.66 de	38.72 ± 4.88 de	2.88 ± 0.53 ef
BAP 4.44	44.44 ± 3.84 cde	49.99 ± 7.14 bc	4.83 ± 0.44 d
BAP 6.66	42.22 ± 3.84 cde	31.74 ± 2.74 e	2.00 ± 0.50 g
KIN 2.32	46.66 ± 6.66 cd	47.61 ± 4.12 bc	3.30 ± 0.04 e
KIN 4.65	46.66 ± 6.66 cd	37.89 ± 4.77 de	2.22 ± 0.19 fg
KIN 6.97	44.44 ± 3.84 cde	45.23 ± 4.12 cd	1.55 ± 0.19 g
BAP + KIN (4.44 + 0.46)	51.10 ± 3.85 c	56.54 ± 6.27 ab	3.12 ± 0.28 e
BAP + KIN (4.44 + 2.32)	62.22 ± 3.84 b	64.44 ± 3.84 a	6.27 ± 0.50 c
BAP + KIN (4.44 + 6.97)	35.55 ± 3.85 e	62.22 ± 3.84 a	1.52 ± 0.23 g
BAP + KIN + NAA (4.44 + 2.32 + 0.54)	62.22 ± 3.84 b	49.99 ± 5.55 bc	6.65 ± 0.80 c
BAP + KIN + NAA (4.44 + 2.32 + 2.69)	75.55 ± 3.85 a	56.05 ± 6.94 ab	11.18 ± 0.41 a
BAP + KIN + NAA(4.44 + 2.32 + 5.37)	66.66 ± 6.66 b	49.66 ± 5.05 bc	7.63 ± 0.77 b
Control	42.22 ± 3.84 cde	0.00 ± 0.00 f	0.00 ± 0.00 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

maximum number of multiple shoots (11.18/culture) followed by BAP + KIN + NAA (4.44 + 2.32 + 5.37 µM), BAP + KIN (4.44 + 2.32 µM), BAP (4.44 µM) and KIN (2.32 µM) (Table 1, Fig.1).

We observed that BAP (4.44 µM) produced more number of multiple shoots/culture (4.83/culture) as compared to that of KIN (2.32, 4.65, and 6.97 µM). Our findings showed similarity with that of Reddy *et al*[20], Devi and Srinivasan[10] Komalavalli and Rao[11] who indicated that MS media, supplemented with BAP was more effective for production of multiple shoots in *G. sylvestre* than KIN when used alone. When MS media was supplemented with BAP alone and KIN alone, maximum multiple shoots (4.83/culture and 3.30/culture) were recorded in BAP (4.44 µM) and KIN (2.32 µM), respectively. Further, these concentrations were used in combination which enhanced the number of multiple shoots to 6.27 multiple shoots/culture. It is reported that supplementation of NAA along with BAP and KIN produces multiple shoots[11]. An attempt was made by supplementing NAA (0.54, 2.69 and 5.37 µM) along with the optimized BAP and KIN concentration for enhancing number of multiple shoots. Maximum multiple shoots (11.18/culture) were recorded in BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM) (Table 1, Fig. 1). Komalavalli and Rao[11] supplemented MS media with BAP + KIN + NAA, malt extract, citric acid (100 mg/ l) and Devi and Srinivasan[10] used 2, 4-D + KIN along with vitamin B_2 and various antioxidants (citric acid, activated charcoal and ascorbic acid). Variation in the results as compared to those of previous findings could be due to use of different plant growth regulators and enhancers used for multiple shoots. Also, Komalavalli and Rao[11] and Devi and Srinivasan[10] have used *in vitro* grown seedlings for their experiments whereas we have used *in vivo* grown plant material. Propagation rate and morphogenetic responses can respond variably according to the explant type used[11,21]. Supplementation of NAA enhanced multiple shoot production upto two subcultures i.e. upto 8 weeks and further remained constant which showed agreement with Komalavalli and Rao[11]. Phenolic leaching and leaf drop was observed and similar observations are also reported[11], to overcome this problem we used citric acid (100 mg/l) and $AgNO_3$ (5 mg/l). Silver ions play an important role in blocking of ethylene pathway and has effect on abscission, senescence of leaves, morphogenesis and shoot formation[22].

Fig. 1 — Culture response of *G. sylvestre* nodal section on MS media supplemented with BAP + Kin + NAA (4.44 + 2.32 + 2.69 µM) producing 11.18 multiple shoots/culture.

Rooting of *In Vitro* Obtained Shoots

In vitro grown multiple shoots (3-4 cm length) were separated from nodal sector and were inoculated on full- and half-strength MS medium supplemented with different concentrations and combinations of IBA and IAA (Table 2). Maximum percentage of rooting was observed on shoots inoculated on half-MS as compared to full-MS. Maximum percent shoots rooted (77.7%) was recorded on half-strength MS medium supplemented with IBA + IAA (4.92 + 5.70 µM). As the combination of IBA + IAA concentrations increased, percentage of shoots rooting increased gradually upto a concentration of 4.92 + 5.70 µM, and later declined from there onwards (Table 2, Fig 2). Full-strength MS medium supplemented with different concentration and combinations of IBA and IAA also produced rooting. Full-strength MS medium showed maximum percentage rooting of shoots (31.1%) on IBA + IAA (7.38 + 8.56 µM) (Table 2).

The maximum percentage of shoots rooted on half-strength MS medium supplemented with IBA + IAA (4.92 + 5.70 µM) is higher as compared to previous reports[10,11,20] which can be due to various combinations and concentrations of PGRs used. Variation in strengths of MS medium also affects on rooting[20] and our findings are in agreement. Also, along with the variation in MS medium strength, effect of auxin concentrations on rooting of plantlets also plays an important role[10,11,23].

Table 2 — Effect of MS medium strength (full and half) and different concentrations of IBA, IAA (µM) in combination on rooting of *G. sylvestre*

MS medium Strength	IBA (µM)	IAA (µM)	% shoots rooted
Full	---	---	0 ± 0 j
Full	2.46	2.85	15.5 ± 3.8 i
Full	4.92	5.70	24.4 ± 3.8 fg
Full	7.38	8.56	31.1 ± 3.8 e
Full	9.84	11.41	22.2 ± 3.8 gh
Full	12.30	14.27	0 ± 0 j
Full	14.76	17.12	0 ± 0 j
Half	---	---	44.4 ± 3.8 b
Half	2.46	2.85	62.2 ± 3.5 d
Half	4.92	5.70	77.7 ± 3.8 a
Half	7.38	8.56	55.5 ± 3.8 c
Half	9.84	11.41	31.1 ± 3.8 e
Half	12.30	14.27	28.8 ± 3.8 ef
Half	14.76	17.12	17.7 ± 3.8 hi

Data represents mean values ± SD of three replicates mean with same letters are not significantly different at 0.05% probability level according to DMRT

Biomass and Gymnemic Acid Production in *In Vitro* Multiple Shoots

For analysis of biomass and gymnemic acid content multiple shoots grown on BAP (4.44 µM), KIN (2.32 µM) alone, BAP + KIN (4.44 + 2.32 µM) and BAP + KIN in combination with NAA (2.69 µM) were used. The cultures were harvested every week upto eight weeks to assess biomass and gymnemic acid content. Maximum FW (0.51 g) and DW (0.139 g) was obtained on BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM) (Fig. 3 and 4). Maximum gymnemic acid content was obtained for multiple shoots cultured on BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM) followed by BAP + KIN (4.44 + 2.32 µM), BAP (4.44 µM), KIN (2.32 µM) and control (10.37, 9.36, 9.21, 9.03 and 6.86 mg/g DW) respectively (Fig. 5). In the present study, we have quantified gymnemic acid content in the multiple shoots grown at different durations. Also a correlation between enhanced multiple shoots, use of different concentrations and combinations of PGRs and gymnemic acid content of obtained multiple shoots was studied. BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM) which had maximum multiple shoots/culture showed maximum gymnemic acid content (Table 1, Fig. 5). *In vitro* biomass production using cell and shoot cultures can be enhanced using different plant growth regulators. Further, this biomass can be used as a source for secondary metabolites. Our results supported the findings of other workers who have used PGRs for cell cultures of *G. sylvestre*[5], *Withania somnifera*[24] for enhanced biomass; and for shoot cultures of different families such as *Mentha piperita*[25], *Ophiorrhiza rugosa*[26], *Catharanthus roseus*[27], *Panax ginseng*[28], *Aconitum violaceum*[29], *Leptadenia reticulat*[30],

Fig. 2 — Effect on MS media strength (full and half) and different concentrations of IBA and IAA on rooting of *G. sylvestre.*

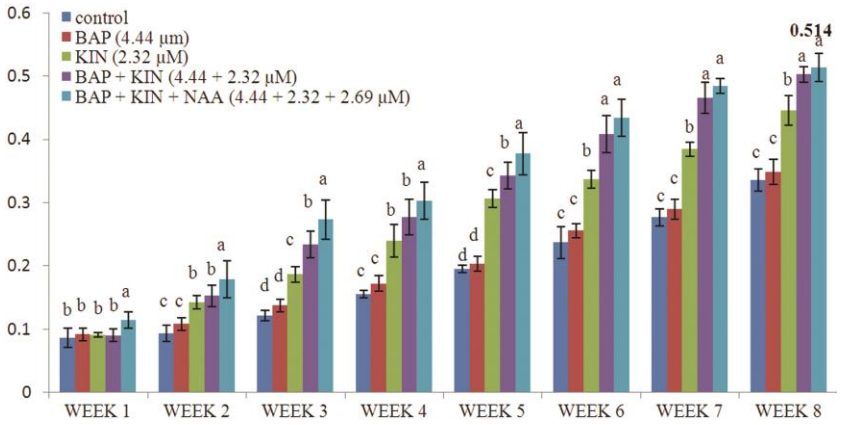

Fig. 3 — Growth analysis of *G. sylvestre* multiple shoots cultures using fresh weight (g FW) for 8 weeks study.

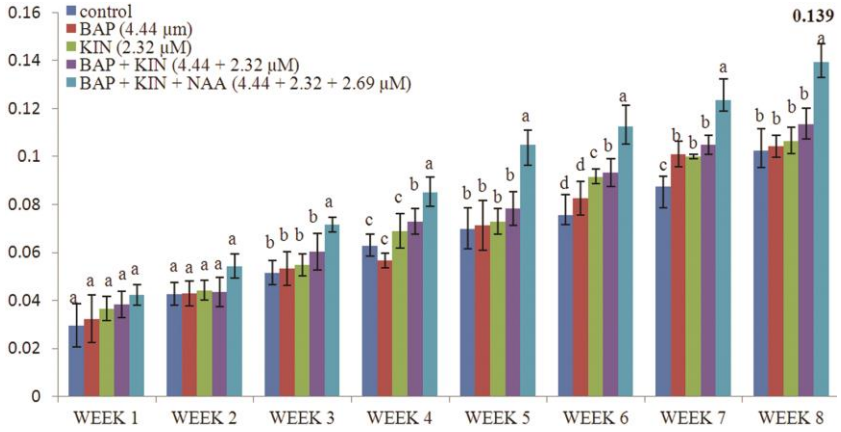

Fig. 4 — Growth analysis of *G. sylvestre* multiple shoots cultures using dry weight (g DW) for 8 weeks study.

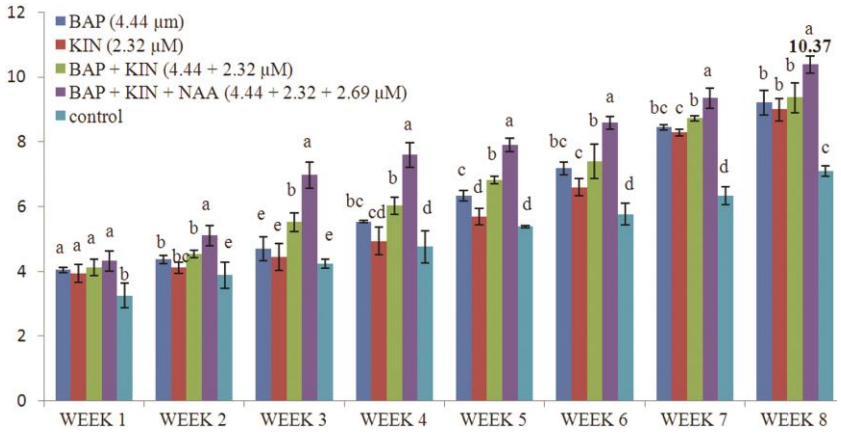

Fig. 5 — Effect of different media compositions on gymnemic acid concentration (mg/g DW) in multiple shoots cultures of *G. sylvestre* for 8 weeks study.

Hypericum perforatum[31], *Eryngium maritimum*[32]. To the best of our knowledge, this is the first report on gymnemic acid production from multiple shoots cultures.

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