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Effect of Growth Hormones on Biomass Production and Glycyrrhetinic Acid content in Callus and Cell Culture of *Glycyrrhiza glabra* Linn

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The present investigation was aimed at the production of medicinally important glycyrrhetinic acid from callus and cell suspension culture of *Glycyrrhiza glabra* using various phytohormones. Supplementation of MS medium with various combinations and concentrations of phytohormones enhanced biomass production as well as glycyrrhetinic acid level in stem, leaf and root derived calli and cell suspension culture of *G. glabra*.

Among the secondary plant metabolites, glycosides play a very important and dominating role as a source of medicinal agents and hence are one of the most extensively investigated classes of compounds. Extensively explored *G. glabra* is used as an expectorant, antitussive, antiinflammatory and antiallergic agent[1-5]. Revers[6-8] and Landerlan[9] reported antiulcerative property which is due to β-glycyrrhetinic acid and its glycoside, glycyrrhizin. These two compounds have also been found effective in rheumatoid arthritis, hepatitis and in Addison's disease[10,11]. The antimicrobial activity of *G. glabra* is due to the presence of a flavone, licoflavone, which has been found effective in preventing cytotoxicity against *E. coli*[12-14]. Hatano *et al.*[15] isolated an antiHIV phenolic constituent, licopyranocoumarin from roots and stolons of *Glycyrrhiza spp.*

In the recent past, static and suspension cultures of this plant have been examined for biomass production, however, not much work has been reported on the regulatory aspect of glycyrrhetinic acid production. Therefore, in this investigation attempts have been made to study the effects of phytohormones, naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxy acetic acid (2,4-D) and benzyl amino purine (BAP) on the glycyrrhetinic acid production in callus and cell suspension culture of *G. glabra*.

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MATERIALS AND METHODS

Murashige and Skoog's medium[16] was used for the present studies. Various manipulations in MS medium were made using different combinations and concentration of IAA, NAA, 2,4-D and BAP and the media were sterilized (120°, 15 lb/sq. in. for 30 min).

After proper surface sterilization nearly 5 mm long stem, leaf and root explants of *G. glabra* were transferred separately on to culture slants and culture tubes were stored in dark for one week to initiate callus proliferation, after which the culture tubes were exposed to fluorescent light (1600-2000 Lux, 16 h) at 26±1°. After five weeks the calli were withdrawn from culture tubes and growth index [(final dry wt.-initial dry wt.)/initial dry wt.] determined. The callus mass was dried at 60° for 48 h and the dried mass so obtained was estimated for glycyrrhetinic acid content.

About 4 m old stem derived callus (grown on MS medium containing 2 mg/l each of NAA and BAP), leaf derived callus (grown on MS medium containing 2 mg/l of NAA and 2.5 mg/l BAP) and root derived callus (grown on MS medium containing 0.5 mg/l of IAA, 0.5 mg/l of 2,4-D and 0.1 mg/l of BAP) were used for establishing

suspension cultures as these media produced the callus of fairly good friability with a better growth index. Suspension cultures were initiated by transfer of these friable calli (about 2.0 g) to Erlenmeyer flasks (250 ml) each containing about 50 ml of medium without agar. These flasks were then shaken on incubator cum shaker at 100-110 rpm at 25±2° temperature. After 7 d the cells were filtered through sieve (No. 250) and transferred to new flask containing fresh medium. After 7 d the same procedure was repeated but taking only ¼ th of cells as the inoculum. After 4-5 transfer at an interval of 7 d each, the cultures contain only suspension culture of single cells along with some cell aggregates. These suspension cultures were used for subculturing by transferring about 10 ml of suspension using sterile pipette. Subsequently the cell growth (cell dry weight/100 ml of culture medium) after 1st, 2nd and 3rd week of incubation were taken and the effect of different phytohormones in various combinations and concentrations were studied on cell biomass and glycyrrhetinic acid production.

Estimation of glycyrrhetinic acid (GA):

Several methods for the estimation of glycyrrhetinic acid in plants have been reported. Killacky and Turner[17] determined GA content in *Glycyrrhiza* by HPLC. Many methods of GA estimation include gravimetric[18], colorimetry[19], polarography[20], paper chromatography[21], TLC[22], GLC[23] and TLC densitometer[24]. The procedure for GA estimation prescribed in British Pharmacopoeia[25] was followed in the present investigations. Briefly, the estimation procedure involved hydrolysis of one gram callus powder with 1M HCl and 1,4-dioxan for 2 h. After filtering, the filter was dried at 105° and extracted with chloroform. The chloroform extract was evaporated to dryness and the residue was dissolved in 10 ml of mixture containing equal volumes of chloroform and methanol. Measured

TABLE 1: EFFECT OF GROWTH HORMONES ON GI* AND GA** IN CALLUS CULTURE OF *G. GLABRA*

S. No	Growth hormones (mg/l)	STEM		LEAF		ROOT	
		GI±SD*	%GA±SD**	GI±SD	%GA±SD	GI±SD	%GA±SD
	2,4-D:BAP						
1	1:0.1	4.12±0.12	1.08±0.06	4.84±0.12	1.12±0.05	3.46±0.12	2.34±0.07
2	2:0.1	5.62±0.13	1.86±0.04	5.96±0.14	1.64±0.04	4.38±0.11	0.92±0.06
3	3:0.1	5.18±0.13	1.22±0.07	3.68±0.11	0.98±0.03	3.52±0.10	2.66±0.07
4	4:0.1	4.22±0.14	0.94±0.05	3.22±0.10	0.92±0.04	3.90±0.12	2.52±0.05
5	2:0.2	3.68±0.16	0.90±0.05	4.62±0.14	1.22±0.04	3.48±0.14	3.22±0.06
6	2:0.3	3.86±0.15	0.88±0.05	3.46±0.13	1.12±0.05	3.04±0.14	3.10±0.05
	NAA:BAP						
7	2:1.0	3.86±0.11	1.66±0.05	4.48±0.10	1.52±0.04	3.02±0.12	2.32±0.04
8	2:1.5	4.32±0.10	1.98±0.04	5.92±0.12	1.82±0.05	3.42±0.10	2.60±0.05
9	2:2.0	5.86±0.12	2.14±0.06	6.22±0.13	1.96±0.04	3.11±0.13	2.96±0.07
10	2:2.5	5.12±0.13	2.02±0.05	6.12±0.16	2.04±0.05	2.98±0.10	2.50±0.05
11	1:1.0	3.66±0.14	1.52±0.04	3.98±0.13	1.46±0.07	2.88±0.13	1.90±0.04
12	1:2.0	3.92±0.12	1.64±0.05	4.32±0.12	1.42±0.06	3.08±0.12	1.88±0.06
	IAA:2,4-D:BAP						
13	1.0:1.0:0.1	4.50±0.13	1.18±0.03	5.02±0.11	1.12±0.04	3.20±0.12	3.02±0.06
14	0.5:0.5:0.1	5.03±0.11	1.62±0.05	5.33±0.12	1.48±0.03	3.96±0.12	3.90±0.08
15	0.5:1.0:0.1	4.68±0.14	1.40±0.04	4.92±0.11	1.23±0.04	3.83±0.12	3.12±0.04
16	0.5:2.0:0.1	4.00±0.12	1.10±0.05	4.60±0.13	1.02±0.03	3.63±0.11	2.98±0.06
17	control	1.84±0.08	0.56±0.04	1.78±0.11	0.48±0.02	—	—

Culture age is 5 w. *Represents growth index as calculated using the formula, final day weight - initial dry weight/initial drug weight and ** represents glycyrrhetinic acid (GA) content.

TABLE 2: EFFECT OF GROWTH HORMONES ON GROWTH AND GA** IN SUSPENSION CULTURE OF G. GLABRA

S. No	Growth hormones (mg/1)	STEM		LEAF		ROOT	
		GI±SD*	%GA±SD**	GI±SD	%GA±SD	GI±SD	%GA±SD
	2,4-D:BAP						
1	1:0.1	0.26±0.02	1.68±0.04	0.18±0.02	1.54±0.01	0.28±0.04	0.64±0.01
2	2:0.1	0.30±0.02	1.82±0.05	0.24±0.02	1.72±0.01	0.30±0.02	2.85±0.02
3	3:0.1	0.38±0.02	1.96±0.03	0.30±0.03	1.85±0.02	0.33±0.04	2.75±0.01
4	4:0.1	0.35±0.03	1.85±0.02	0.21±0.03	1.55±0.01	0.26±0.04	2.53±0.01
	NAA:BAP						
5	1:0.1	0.34±0.03	2.08±0.02	0.19±0.03	1.66±0.01	0.34±0.03	2.18±0.04
6	3:0.1	0.48±0.04	2.12±0.02	0.30±0.04	1.82±0.01	0.42±0.04	2.42±0.04
7	5:0.1	0.53±0.03	2.20±0.03	0.42±0.04	1.94±0.01	0.58±0.03	3.16±0.04
8	7:0.1	0.42±0.04	2.02±0.03	0.25±0.03	1.74±0.01	0.51±0.03	2.28±0.03
	NAA:2,4D:BAP						
9	1.0:2.0:0.1	0.22±0.03	1.60±0.02	0.16±0.02	1.58±0.01	0.38±0.01	2.55±0.01
10	2.0:2.0:0.1	0.28±0.02	1.71±0.03	0.20±0.02	1.80±0.01	0.46±0.02	2.78±0.02
11	3.0:2.0:0.1	0.32±0.02	1.84±0.03	0.28±0.03	1.72±0.02	0.52±0.01	3.03±0.02
12	4.0:2.1:0.1	0.30±0.02	1.72±0.02	0.19±0.02	1.64±0.01	0.47±0.01	2.41±0.01

Culture age is 3 w. *Represents cell dry weight (gl 100 ml of culture medium) and ** glycyrrhetinic acid (GA) content

quantity of this solution was applied on to chromatoplates of silica gel G and placed in TLC chamber containing ethyl acetate, ammonia (1 M) and absolute ethanol (60:27:13). The developed plates were dried and examined in UV light, the area corresponding to glycyrrhetinic acid was scrapped off, treated with 10 ml of absolute ethanol and filtered through sintered glass filter. The GA content was determined spectrophotometrically by measuring absorbance at 250 nm.

RESULTS AND DISCUSSION

Various combinations of phytohormones in all used concentrations favored the callus growth in stem, leaf and root segments of *G. glabra* as compared to phytohormone free (control) medium. In stem and leaf segments the best growth, however, was obtained with the medium containing 2 mg/l each of NAA and BAP that exhibited maximum GI of 5.86 (control: 1.84) in stem and 6.22 (control: 1.78) in leaf derived callus. In case of root segment, the highest GI of 4.38 was obtained with the medium containing 2 mg/l of 2,4-D and 0.1 mg/l of BAP while phytohormone free MS medium could not induce callusing even in five weeks time.

Enhanced production of glycyrrhetinic acid was exhibited when the MS medium was supplemented with various combinations of phytohormones. In stem derived callus the best result however, was obtained with medium containing 2 mg/l each of NAA and BAP which produced maximum GA of 2.14% w/w (control 0.56 w/w). In leaf derived callus maximum amount of GA 2.04% w/w (control 0.48% w/w) was obtained with medium containing 2 mg/l of NAA and 2.5 mg/l of BAP while in case of root derived callus maximum GA of 3.90% w/w was obtained with the medium containing 0.5 mg/l of IAA, 0.5 mg/l of 2,4-D and 0.1 mg/l of BAP.

Observations indicate that various combinations of phytohormones enhance cell growth (cell dry weight/100 ml of culture medium) and glycyrrhetinic acid content in stem, leaf and root derived suspension culture of *G. glabra*. However, it was 5 mg/l of NAA in combination with 0.1 mg/l of BAP that exhibited the best results as the cell growth and glycyrrhetinic acid production was

maximum as compared to other used concentrations of phytohormones. The combination of NAA and BAP (5.0:0.1 mg/l) produced maximum cell dry weight 0.53 g/100 ml with stem, 0.42 g/100 ml with leaf and 0.58 g/100 ml with root derived suspension culture. The same combination produced maximum glycyrrhetinic acid 2.20% w/w stem, 1.94% w/w with leaf and 3.16% w/w with root derived suspension culture of *G. glabra* in three weeks time.

The demand for *G. glabra* and its important bioactive constituents glycyrrhizin and glycyrrhetinic acid is increasing because of its high therapeutic value but due to non-conducive climatic conditions the drug does not grow well. Keeping this in mind, biotechnological approach was adopted for the production of medicinally important glycyrrhetinic acid from the callus and cell suspension culture of *G. glabra* using various phytohormones. Present investigations reveal that MS medium containing different combinations and concentrations of phytohormones favors the growth and glycyrrhetinic acid production in the callus and cell suspension culture derived from stem, leaf and root explants of *G. glabra*.

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