International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 7 (5): 653-658, 2011
ISSN 1811-7775 / DOI: 10.3923/ijp.2011.653.658

Establishment of Callus Tissue and Effect of Growth Regulators on Enhanced Sterol Production in *Cissus quadrangularis* L.

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Abstract: The present investigation was carried out for isolation, identification and enhancement of phytosterol from *in vitro* tissue of *Cissus quadrangularis* L. Aim of this investigation was to develop an efficient protocol for enhancement of phytosterol from *in vitro* cultured callus tissue of *Cissus quadrangularis* (L.), an important bone healing plant. The production of callus tissue was achieved from mature nodal segment by culturing on MS medium supplemented with auxins and cytokinins. Identification of the steroidal substances from these tissues was done by various chromatographic techniques such as-Colour reaction, TLC, comparing IR and HPLC chromatogram. Enhancement of the steroidal substances from these tissues was achieved by the addition of various concentrations of plant growth regulators (IAA, NAA and 2, 4-D). Best results for callus induction were achieved on MS medium supplemented with 3% sucrose, NAA (2.0 mg L^{-1}) and BAP (0.5 mg L^{-1}). β-sitosterol and stigmasterol were identified by above mentioned techniques and enhanced production of these sterols up to 10-12 fold was achieved with IAA (5 ppm) and 6-8 fold with 2, 4-D (5 ppm) in callus tissue. Hence, these tissue culture and enhanced steroidal content can be used after scale-up procedure, on a large scale for commercial production of valuable steroidal compounds.

Key words: Callus, *Cissus quadrangularis*, sterols, growth regulator, bone healer

INTRODUCTION

Cissus quadrangularis L. (Vitaceae) commonly known as Hadjod is an important medicinal plant of great economic value (Panthong *et al*., 2007). The species of Vitaceae found in India are edible, ornamental and have great medicinal properties including anthelmintic, alterative, dyspeptic, antiascorbic, stomatic, antioxidant and antimicrobial (Meher *et al*., 2010; Robert *et al*., 2001; Murthy *et al*., 2003; Kar and Borthakur, 2008). Nodal stem segment of *Cissus quadrangularis* was found best for the culture of callus tissue. The effect of combination of auxins and cytokinins (NAA/IAA/2,4-D and BAP/Kn) for callus production in various plants were studied by several workers (Siwach *et al*., 2011; Satyavani *et al*., 2011). Most importantly, the stem and root powder are used in the treatment of cuts and wounds and bone fracture (Potu *et al*., 2009). The leaves are also useful in colonopathy, scurvy, asthma, burns and wounds. Ayurveda has identified many medicinal properties of this plant and it is effectively used against disease related to bone system and as laxative, wormicide and disinfectant, remove piles and eye diseases. *Cissus quadrangularis* contains various phytochemicals like steroids most importantly sitosterols, terpenoids, calcium oxalate, carotene, ascorbic acid etc. (Joseph and Raj, 2011; Sanayal *et al*., 2005; Sharma and Patni, 2006).

The most common of the plant sterols are sitosterol, stigmasterol and campesterol; they are produced by a bifurcated sterol biosynthetic pathway involving a common precursor (Noguchi *et al*., 2000). These anabolic steroidal principles from the plant show a marked influence on the rate of fracture healing by influencing early regeneration and quicker mineralization of bone callus. Every part of this plant is used in ayurvedic medicine for solving problems of fractured patients. Due to extensive utilization of this plant it is being threatened. In the present investigation, a protocol has been developed for callus establishment and enhancement in production of sterols by addition of growth regulators.

MATERIALS AND METHODS

Plant material: Healthy plants of *Cissus quadrangularis* were collected from the Kulish Smriti Van, Jaipur and from the University of Rajasthan. The nodal, internodal segments and leaves were used as explants for callus initiation. These explants were cut 8-10 mm and washed under running tap water for half an hour, subsequently followed by 1% labolene (Qualigens, India) for 5 min and then washed with sterile distilled water. They were then

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surface sterilised with 0.1% $HgCl_2$ solution for 3-5 min followed by several rinses in sterile distilled water. The sterile explants were then transferred on MS medium. The medium was supplemented with various combinations of auxins and cytokinins for callus induction. The media were congealed with agar (0.8%) and sucrose (3%) was used as a source of carbohydrate. pH of the medium was adjusted to 5.8 using 0.1 N NaOH/0.1N HCl solution before being autoclaved at 15 psi for 20 min. All the cultures were incubated at 30±2°C under 16 h photoperiod, illuminated by fluorescent light of about 2500-3000 lux intensity. Five replicates for each treatment were taken and all experiments were repeated thrice and the callus was subcultured after 4-5 weeks.

Callus culture and sterol production: Nodal segment was found to be the best explant for callus induction. Best combination and concentration of growth regulators for callus induction was NAA (2.5 mg L^{-1}) and BAP (0.5 mg L^{-1}). The callus was found to be green and fast growing. It was tested for shoot regeneration potential on MS medium supplemented with various concentrations of BAP/Kn alone or in combination with auxins. The best response was found with BAP (3 mg L^{-1}) and KN (1.0 mg L^{-1}). Growth Index (GI) of callus tissue was calculated at the end of every week up to six weeks and it was calculated by the formula giving below. GI of six week old culture (6.22) served as control for β-sitosterol, Stigmasterol and total sterol.

$$GI = \frac{\text{Final dry weight of tissue-Initial dry weight of tissue}}{\text{Initial dry weight of tissue}}$$

Sterol production from callus of nodal segment was studied with their respective growth indices. Unorganized callus tissue of *Cissus quadrangularis* was maintained on MS medium supplemented with various concentrations (1-5 ppm) of IAA, NAA, 2, 4-D. These tissue samples were harvested at their maximum growth age of 6 weeks, dried, weighed, growth indices was calculated separately and 18 months old callus tissue was used to study the effect on growth and production of sterols.

Sterol extraction from callus: The tissue samples grown at different time intervals were collected separately. This tissue was dried at 105°C for 15 min to inactivate enzymes and then at 60°C for 8 h until a constant weight was achieved; Sample was powdered in a mortar and then refluxed in 30% Hydrochloric acid (v/v) for 4 h. Each hydrolyzed sample was washed with cold water and filtered till the pH of the filtrate was 7. Finally the powdered and hydrolyzed callus was extracted in benzene using soxhlet apparatus for TLC. The best solvent for the development of chromatogram on silica gel G was found to be n-hexane: acetone (8:2) (Fazali and Hardman, 1968). The steroid content of the tissue sample was calculated (mg g^{-1} dw) at different intervals and compared with their respective growth indices. Quantification and Identification of sterols was carried out colorimetrically by TLC, IR studies and HPLC (Sharma and Patni, 2007; Ganesh and Vennila, 2011).

RESULTS AND DISCUSSION

Callus culture: Callus induction was observed within three to four weeks from each explant cultured on MS medium supplemented with varying concentrations (0.5-10 mg L^{-1}) of growth regulators viz NAA, IAA and 2,4-D (Table 1, Fig. 1). Callusing was initiated after 10-15 days of inoculation. Callus induction was always preceded by swelling of explants. Callus produced from leaf and internodal explants was slow growing but nodal explants produced vigorously growing callus. NAA was found to be the best for producing green, compact and fast growing callus, optimal concentration being 2.5 mg L^{-1}. Callus produced with 2, 4-D was yellowish pale green in colour which later turned brown and was incapable of differentiation. IAA induced less compact and less green callus which turned brown and showed limited growth. NAA (2.5 mg L^{-1}) was further tried with various concentrations of BAP/Kn (0.5-3.0 mg L^{-1}) to obtain better response. It was observed that the combination of NAA (2.5 mg L^{-1}) and BAP (0.5 mg L^{-1}) (Table 2) was best for callus induction (Fig. 1e, f).

Table 1: Effect of different auxins on callus induction

Medium	:	MS+Sucrose(3.0%)+Auxins viz. NAA, 2,4-D, IAA+ Additive [NH_4NO_3(20 mg L^{-1})].
Explant	:	Mature nodal stem explant
Incubation	:	At 30±2°C in 16 hr photoperiod (2500-3000 lux) upto 4 weeks

Auxins (mg L^{-1})	Response	Remarks
Control : (Without auxin)	-	No callusing
NAA		
0.5	CR^{+}	Callus showed rhizogenic response
1.0	CR^{+}	
1.5	CR^{++}	Callus yellowish green in colour,
2.0	CR^{++}	fast growing and friable
2.5	C^{++++}	
3.0 to 5.0	C^{++++}	Very low regenerative potential
IAA		
0.5	-	
1.0	C^{+}	Callus produced was yellowish
2.0	C^{++}	green in colour, very compact and
2.5	C^{++}	slow growing
3.0 to 5.0	C^{++}	
2,4-D		
0.5	-	
1.0	-	
1.5	C^{+}	Compact callus showing poor growth
2.0	C^{+}	
2.5	C^{+}	
3.0 to 5.0	C^{++}	

Compact callus showing poor growth. CR: Rhizogenic callus, C-Callus, +: Slight regenerative, ++: Moderate, ++++: Profuse

Table 2: Effect of NAA in combination with cytokinins on callus induction in *Cissus quadrangularis*

Medium : MS + Sucrose (3.0%) + NAA (2.5 mg L^{-1}) + BAP/Kn (0.5-3.0 mg L^{-1}) + Additive [NH_4NO_3 (20 mg L^{-1})].
Inoculum : Mature nodal stem explant
Incubation : At 30±2°C in 16 hr photoperiod (2500-3000 lux) up to 4 weeks

Cytokinin (mg L^{-1})		Response	Remarks
Control : MS+NAA (2.5 mg L^{-1})+additive [NH_4NO_3(20 mg L^{-1})].		C^{++++}	-
BAP	**Kn**		
0.5	-	C^{++++}	Callus green in colour, fast growing with high regenerative potential
1.0	-	C^{++}	
2.0	-	C^{+}	
3.0	-	C^{+}	
-	0.5	C^{++}	Callus greenish in colour and slow growing
-	1.0	C^{++}	
-	2.0	-	
-	3.0	-	

C: Callus, +: Slight regenerative, ++: Moderate, ++++: Profuse

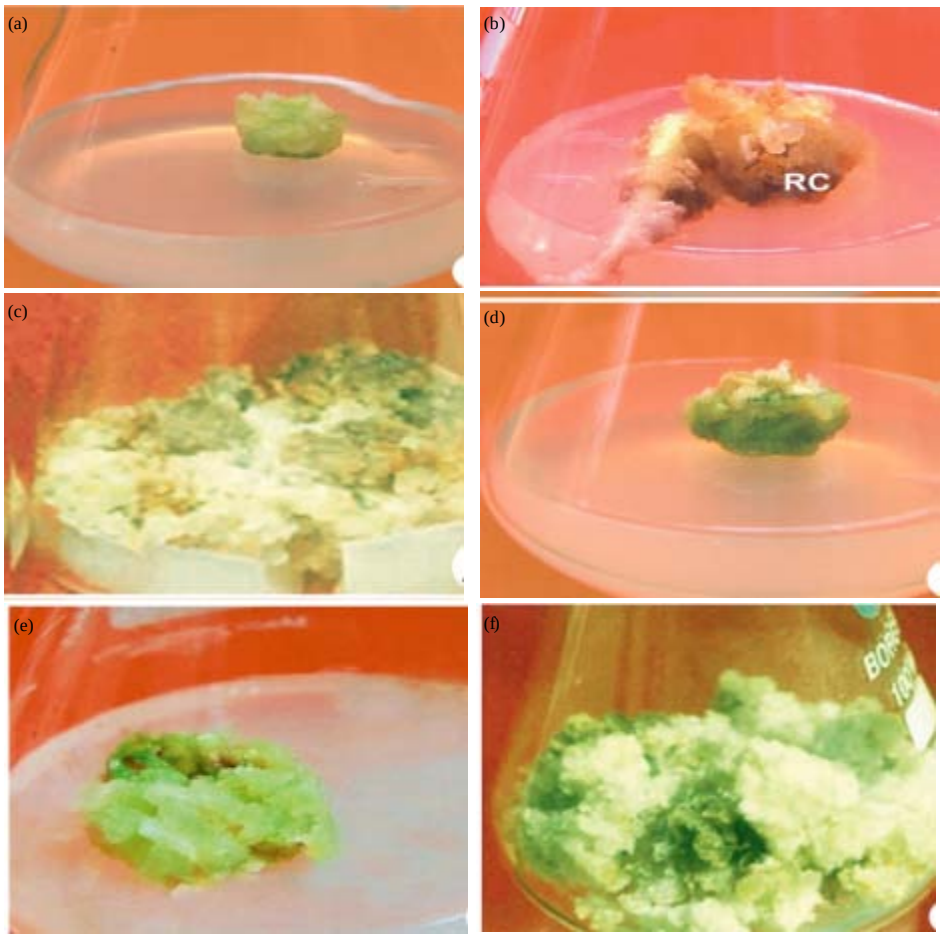

Fig. 1 (a-f): Callus induction in *Cissus quadrangularis* from nodal explants: (a-d) Callus initiation on MS+NAA (0.5 -2.0 mg L^{-1}, 4 weeks old), IAA (2.5 mg L^{-1}, 4 weeks old), NAA (2.5 mg L^{-1}, 4 weeks old), 2,4-D (2.5 mg L^{-1}, 4 weeks old), (e-f) Callus induction with combination on MS+BAP (0.5 mg L^{-1})+NAA (2.5 mg L^{-1}), 2 and 4 weeks old

Similar results were obtained in *Pluchea lanceolata*, *Allium chinense* and *Stevia rebaudiana* with auxins and cytokinin (Arya *et al*., 2008; Yan *et al*., 2009; Patel and Shah, 2009). The callus so produced was compact and green. These results are supported by earlier investigations carried out for callus induction in *Ipomoea obscura* (L.), *Dentrobium fimbriatum* and *Actinidia deliciosa* using node and leaf as explants (Roy and Banerjee, 2003; Akbas *et al*., 2009). *Cissus quadrangularis* cultured on MS medium supplemented with 3% sucrose, NAA (2.5 mg L^{-1}) and BAP (0.5 mg L^{-1}) was the best medium for callus induction (Table 2).

Effect of growth regulators on sterol production: The sample for the study of sterol production and effect of growth regulators was of 18 months old callus tissues of *Cissus quadrangularis*. This tissue was transferred to fresh MS-medium supplemented with various concentrations (1-5 ppm) of IAA, NAA and 2, 4-D and was harvested at their maximum growth age of 6 weeks, dried, weighed and growth indices was calculated separately.

Maximum growth was recorded in the tissue fed with 5 ppm of NAA (GI-9.5) as compared to control (GI-6.22) followed by 2, 4-D (GI-8.6) and IAA (GI-7.8) at 1 ppm (Table 3). Only two sterols, β-sitosterol and stigmasterol were confirmed by TLC in the entire tissue sample tested. However, content of sterols increased considerably in the tissue fed with 5 ppm of IAA followed by 2, 4-D and NAA at 5 ppm and 3 ppm, respectively (Table 3). Hence, IAA and 2, 4-D enhanced the production of sterols where as NAA did not, although its incorporation in the medium increased the growth of tissue. Since, NAA (2.5 mg L^{-1}) and BAP (0.5 mg L^{-1}) produced maximum amount of callus, simultaneously, we observed that the addition of IAA (5 ppm) increased (Table 3) the production of various phytosterols present in callus tissue. The production of phytosterols were enhanced up to 10-12 fold by the addition of IAA, up to 6-8 fold by the addition of 2,4-D but NAA showed negligible results in the enhancement of sterol production. Similar results were obtained in callus of *Euphorbia tirucalli* (Biesboer and Mahlberg, 1979). The callus culture of *Centella asiatica* produced highest amount of triterpenes in third week by addition of squalene which was identified by HPLC (Kiong *et al*., 2005). Enhance production of Sterols from *in vitro* culture of *Panax ginseng* were identified and determined quantitatively by Lee *et al*., 2004, variation in phytosterol composition during the ripening of *Carthamus tinctiris* L. seed was studied by Hamroum-Sellami *et al*. (2007).

β-sitosterol and stigmasterol from tissue samples of *Cissus quadrangularis* were confirmed by Co-TLC (β-sitosterol, Rf 0.95, Purple; Stigmasterol, Rf 0.89, Grey); m.p. (β-sitosterol 139-140°C, Stigmasterol, 142-144°C); by superimposable IR spectra (Fig. 2) of the isolated and the authentic samples of each of the sterols and by HPLC studies. In HPLC studies, *in vitro* callus tissue of *Cissus quadrangularis* showed chromatogram with peaks at 4.582 and 5.817 min as Retention Time (Rt), which was compared with standard Beta sitosterol (Rt = 4.551 min) and standard Stigmasterol (Rt = 5.821 min). Cholesterol and lanosterol could not be detected in *in vitro* tissue cultures of *Cissus quadrangularis*. Total amount of sterols (0.439 mg g^{-1}dw) was found to be higher in six weeks old callus, β-sitosterol (0.272 mg g^{-1} dw) and stigmasterol (0.167 mg g^{-1} dw) was also found higher in six week old tissue of *Cissus quadrangularis* (Table 4).

Similar results were obtained in African potato (*Hypoxis hemerocallidea*). Some time the addition of elicitor/precursor may suppresses the production of phytosterol (Haudenschild and Hartmann, 1995; Bonfill *et al*., 2010).

Table 3: Effect of various concentrations of growth regulators (auxins) on growth and production of sterols in tissue cultures of *Cissus quadrangularis* (Age of tissue-6 weeks)

MS-medium+Auxin	Conc. (ppm)	GI	β-sitosterol (%)	Stigma sterol (%)	Total sterol (%)
Control	-	6.22	0.0272	0.0167	0.0439
IAA	1	7.8	0.0772	0.0667	0.1439
	2	7.6	0.0771	0.0660	0.1431
	3	7.0	0.1772	0.1667	0.3439
	4	7.0	0.1770	0.1660	0.3430
	5	7.1	0.2472	0.2367	0.4839
2,4-D	1	8.6	0.0772	0.0667	0.1439
	2	8.5	0.0772	0.0661	0.1433
	3	7.0	0.1072	0.0967	0.2039
	4	6.9	0.1070	0.0966	0.2036
	5	7.4	0.1172	0.1067	0.2239
NAA	1	8.0	0.0172	0.0047	0.0219
	2	7.9	0.0171	0.0048	0.0219
	3	8.1	0.0177	0.0067	0.0244
	4	7.9	0.0171	0.0051	0.0222
	5	9.5	0.0170	0.0052	0.0222

Table 4: Sterols from static tissue culture of *Cissus quadrangularis*

		β-sitosterol		Stigma sterol		Total sterol	
Age of tissue week	GI	mg/g dw	%	mg/g dw	%	mg/g dw	%
2	1.55	0.257	0.0257	0.150	0.0150	0.407	0.0407
4	3.27	0.262	0.0262	0.157	0.0157	0.419	0.0419
6	6.22	0.272	0.0272	0.167	0.0167	0.439	0.0439
8	4.44	0.267	0.0267	0.162	0.0162	0.429	0.0429

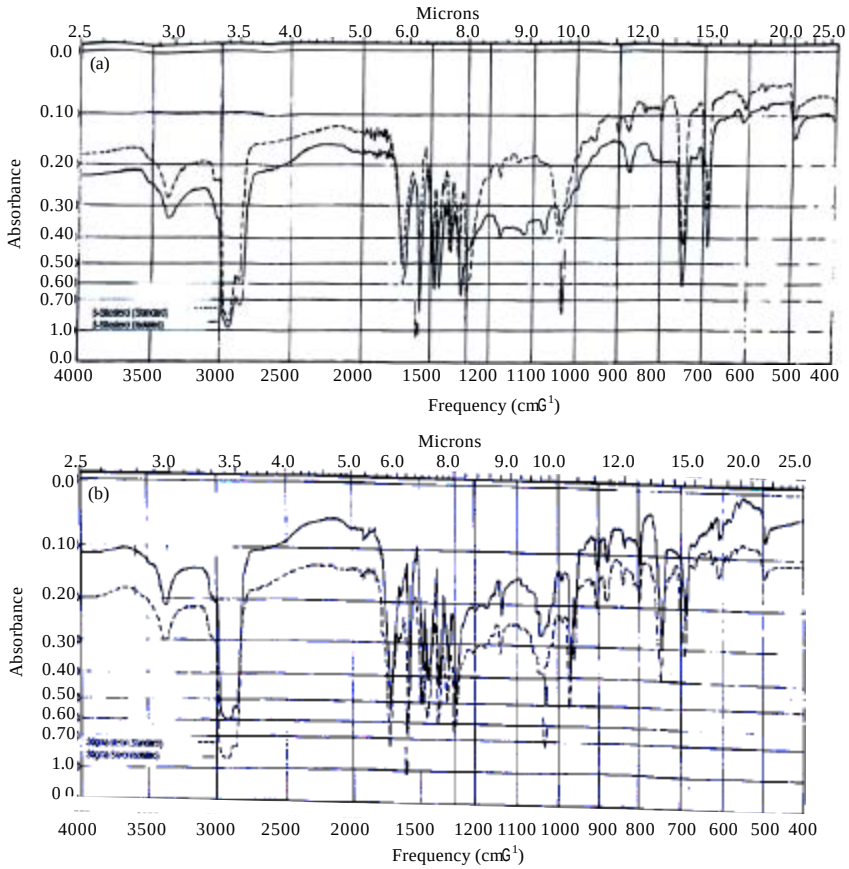

Fig. 2: Superimposed IR spectra of (a) β-sitosterol and (b) Stigmasterol

CONCLUSION

Based on findings of this study, it could be concluded that MS medium supplemented with 3% sucrose, NAA (2.5 mg L^{-1}) and BAP (0.5 mg L^{-1}) was the best medium for callus induction. Total and individual amount of callus production was found to be higher in six weeks old callus. The present study also revealed that the addition of auxins regulate the synthesis of sterols and IAA and 2,4-D enhance the production of sterols up to 12 fold. Hence, the protocol may be used on a large scale for enhancement, isolation, production and quantification of important steroidal content from callus culture of *Cissus quadrangularis,* the potent bone healer.

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