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Effect of age of cultures and hormones on the synthesis of secondary metabolites from callus of *salvia santolinifolia* (boiss), a medicinal herb

[1] Tour Jan, [2] Beena Naqvi, [3] Raiha Qadri, [1] Muhammad Nisar

[1] Department of Botany, University of Malakand, Dir Lower, Chakdara, KP, Pakistan.

[2] PCSIR Laboratories Complex, Karachi, Karachi-75270, Pakistan.

[3] Department of Botany, University of Karachi, Karachi-75270, Pakistan.

Abstract
Expression of natural products in callus culture established from nodal part of *Salvia santolinifolia* was investigated and the effect of age of culture and plant growth regulators was determined. Callus initiation started after 5-6 days of inoculation on MS1 medium and further proliferated of calli were obtained on MS2 & MS3 media. Subcultures of calli were regularly performed after 27-28 days of inoculation for nine months for the synthesis of secondary metabolites. With the age of callus maximum secondary metabolites accumulated after eight month of culture on both media (MS2 & MS3). A comparison of plant growth regulators on the bases of dry weight of callus showed that maximum synthesis of natural product occurred in MS3 medium. The phytochemical analysis of methanolic extract of callus tissues of *Salvia santolinifolia* reported stigmasterol for the first time. The structure of the isolated compound was determined by using mass spectrometry and 1D-& 2D-NMR techniques.

Keywords: Age of callus, hormones, dry weight of callus, secondary metabolites, *Salvia santolinifolia*.

Introduction

The drugs used in modern medicine are either directly isolated from plants or synthetically modified from compounds of natural origin. It was estimated that 20,000 plant species are used for medicinal purposes [11]. About 25% of all our medicines come from plants. It was estimated that the value of those drugs containing compounds from plant species is about 10 billion in the United States alone [3] but now their extraction from plants has been replaced by organic synthesis, however, plants are still a source of compounds, which are too complex or expensive to produce in any other way. In the endeavours of increasing their quantity technique of plant cell and tissue culture is used. Utilization of cultured plant materials for the production of secondary metabolites for the extraction of drugs is relatively a new field and has been recognized since the early 1950s. It has been reported low concentration of rosmaranic acid and lithospermic acid B from fresh leaves of *Salvia miltiorrhiza* whereas a high concentration was found in the leaves of 15 week old plants regenerated *in vitro* [7]. The bufadienolides content was studied *in vitro* produced material of *Drimia rotbusta* bark. Bufadienolides were present in calli and in three, six and ten month old ex-vitro plants. Quantitatively the tissue culture derived plants contained the same bufadienolides as did the mother plant [6]. Plant aging is an important factor for the biosynthesis and accumulation of ecteoside in *Osmanthus fragrances* both *in vivo* and in cultured shoots. Field-grown plants contained large amount of ecteoside in stem portion (4.72% estimated on dry weight basis) while young field-grown plants and culture shoots contained small amount of ecteoside (1% as estimated on dry weight basis) [10]. In the callus culture of *Picrasma quassioids* was detected mainly in calli grown in the presence of 2, 4-D but the highest values were found in the presence of IBA and zeatin riboside, no quassin was detected in the early culture but on subculturing quassin was detected [12]. Latex from *Papaver somniferum* (opium poppy) is a commercial source of the analgesics, morphine and codeine. Callus and suspension cultures of *Papaver somniferum* are being investigated as an alternative means for production of these compounds. Production of morphine and codeine in morphologically undifferentiated cultures has been reported [14].

The aim of the present investigation was to determine the effect of hormones on the growth of callus culture and production of secondary metabolites. The effects of age of callus were also investigated to determine the month in which the accumulation of secondary metabolites is high.

Materials and Methods

Axillary branches (7-9 cm long) were excised from the selected healthy plants. They were first defoliated and then sterilized with 0.05% Mercuric chloride (HgCl2) containing few drops of Tween-20, for 10-13 minutes followed by rinsed 3-4 time with sterile distil water. Murashige and Skoog (MS) (1962) medium were used. Plant growth regulators were added before autoclaving. pH of the medium was adjusted to 5.5 to 5.55. Agar (agar-agar Mikrobiologie, Merck, U.S.A.) (0.6%) was used as solidifying agent. Cultures were maintained in a growth chamber at 26±2 C˚ under a light regime of 16 hours day and 8 hours night. In the growth chamber light was provided from white fluorescent tubes. For callus induction the following medium was used;

MS1= NAA (0.5 mg/l)
Further multiplication of callus was obtained on;
MS2= NAA+BA (0.5+1.5 mg/l)
MS3= NAA+2iP (0.5+1.0 mg/l)

Subcultures of callus were made after 30 days intervals. Actively growing calli (0.9 gram) were subculture on callus multiplication media (MS2 & MS3). Calli were propagated till 9th subcultures. The calli of 1st and 2nd subcultures not saved for extraction because they were used in sub-culturing.

Calli of 3rd, 4th, 5th, 6th, 7th, 8th and 9th subculture which was produced on callus multiplication media (MS2&MS3) were soaked separately in methanol for a period of 5 days. TLC of each extract was performed (Fig. 1) using 0.5% methanol in chloroform (5:95). Spots on TLC were marked under UV light, and/ or were sprayed with ceric sulphate and then dried with heat gun, so that the compounds become visible. Compounds from extract of each subculture were looked similar on TLC (Figure 1). Dry weight of callus was performed for the determination of secondary metabolites accumulation with the age of callus. For this purpose 2.0 gram callus was separated randomly from a vial and dried at 60 C° in an oven for 3 days and data (dry weight) of each day were recorded.

Results

Initiation of callus

Callus was initiated on MS1 medium containing NAA (0.5 mg/l) from nodal explants (Table-1) after 3-6 days of inoculation. Callus induction first began from the cut end of the explants which was touching the medium and then spread toward the whole segments. Morphologically the calli appeared on nodal explants in the presence of NAA was yellowish-white, wet and friable.

Table 1: The effect of NAA on the induction of callus from nodal explants

Explants	Growth regulators (gm/l)	% Response	Mean fresh weight of callus (g) ±SE
Node	NAA 0.5	75.23	4.47±1.64

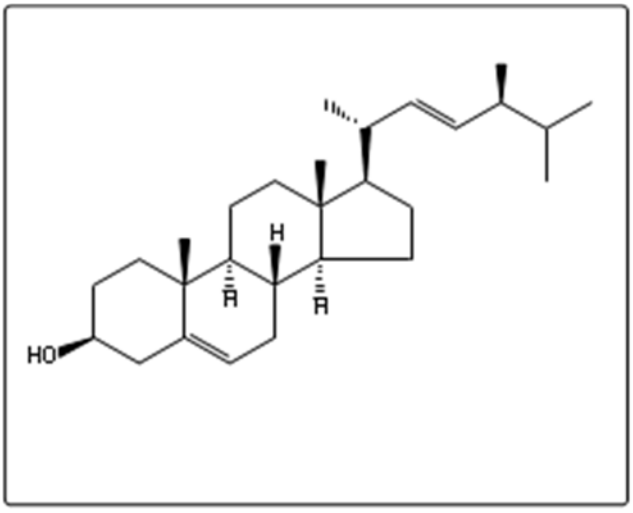
Symbol; SE: Standard Error

Effect of age of callus on the synthesis of secondary metabolites: The induced calli of MS1 medium were multiplied and maintained for 9-passages (subcultures) on MS2 & MS3 media. Calli of each subculture of the two media (MS2 & MS3) were extracted separately with methanol. Spots of compounds from extract of 3rd-8th subcultures of the two media looked similar on TLC (Figure-1) whereas, from the extract of 9th subcultures of the two media extra bands of compounds were appeared on TLC (Figure-1).

Effect of hormones on the synthesis of secondary metabolites: Callus was induced on MS1 medium and further multiply on MS2 and MS3 media, up to 9-subculture. Callus of each subcultures (3rd-9th) of the two media (MS2 & MS3) were separately extracted with methanol. The extracts of calli of each subculture of the two media (MS2 and MS3) were viewed and analysed on TLC. Comparison of compounds (base on TLC spots) extracted from each subculture of calli of two media (MS2 &MS3), indicated that under different hormonal condition the production of secondary metabolites were not affected. Because similar bands of compounds appeared from extract of each subculture (3rd to 9th) of the two media (MS2 &MS3) on TLC (Fig.-1). The dry weight of callus produced in two combinations of hormones used in MS2 & MS3 media increased progressively in subsequent subculture (3rd-9th). Maximum dry weight (0.067 g) of callus was obtained from 9th subculture while minimum dry weight (0.038 g) from 3rd subculture from callus produced on MS3 medium (Table-2). Similarly, the calli multiplied on MS2 medium, maximum dry weight of callus obtained was (0.064 g) from 9th subculture and minimum (0.034 g) from 3rd subculture (Table-2). A comparison of dry weight of callus produced on MS2 and MS3 indicated that maximum accumulation of secondary metabolites in MS3 medium (Table 2).

Extraction from callus: All calli produced during 9 subcultures was extracted with methanol and total 27.43 gram crud extract was obtained. The chromatographic analysis of methanolic extract of callus revealed the presence of 4 compounds out of which three were new to science (data not shown) and one was known (Stigmasterol).

Structure characterization of Stigmasterol: The fraction eluted with 60% chloroform in hexane yielded compound Stigmasterol. This was purified as a white solid with 80% chloroform in hexane.



Spectral data of Stimasterol: FDMS: *m/z* 412.
HREI-MS: *m/z* 412.3709 (calcd. 412.3704 for $C_{29}H_{48}O$,).
^{1}H-NMR ($CDCl_3$, 300 Mhz): □ 5.32 (1h, br. s, H-6), 5.11 (1H, dd, *J* = 15.5, 8.5 Hz, H-22), 4.98 (1H, dd, *J* = 15.5, 8.5 Hz, H-23), 1.00 (3H, d, *J* = 6.5Hz, H-21), 0.98 (3H, s, H-19), 0.82 (3H, d, *J* = 6.0 Hz, H-26), 0.78 (3H, t, *J* = 7.5 Hz, H-29), 0.77 (3H, d, *J* = 6.0 Hz, H-27) and 0.68 (3H, s, H-18).

Discussion: In this study the evaluation of composition of the accumulated product through methanolic extracts of the harvested calli reveals some difference in the composition of natural product with the age of cultures. The extracts of 3rd, 4th, 5th, 6th, 7th, 8th and 9th subcultures of the two media (MS2 & MS3) were analysed on TLC. When comparison among compounds of these extracts were made, similar bands of compounds were found from the extract of 3rd to 8th subcultures of the two media (MS2 & MS3) but from 9th subculture of the two media extra bands of compounds appeared (Fig.1). The appearance of these extra bands of compounds in 9th subcultures of the two media shows the possible accumulation of extra compounds in the callus tissues. The correlation between age of culture and accumulation of natural product are further supported by the dry weight of callus. Because maximum dry weight of callus was obtained from 9th subculture of the two media and minimum from 3rd subcultures of the two media (Table 2). During the experimental work growths of calli slow down in 8th subculture and thereafter show declined in 9th subculture. It may be possible that secondary metabolites accumulated an *in vitro* in the later stage of the growth cycle when growths of callus slow down. These secondary metabolites may show variation in yield with the age of the callus as has been reported for the accumulation of cryptotanshinone in *Salvia multiorrhza* [13]. In *Morinda citrifolia* chemical constituents accumulate in later stage of growth cycle when growth slow down or reached plateau [5]. Researchers have reported several variations with age of culture, maximum amount of flavonoids content was observed in 6 week old callus (2.90 mg/g dry weight) and minimum was in 2 week old callus cultures (1.83 mg/g dry weight) [1]. From the callus cultures of *Cephalotaxus harringtonia* 2-6 time greater amount of alkaloid was obtained [2].
In certain cases the plant growth regulators effected the production of natural products in several plants species, for example, berberin in *Coptic japonica* [9]. In some cases, however, chemical constituent's accumulation seems to be effected by phytohormones independently from their effect on growth. For example, 5 combinations of hormones were tested for their effect on growth and phenylpropanoid metabolism in cell culture of *Vanilla planifolia* [4]. In the presence of 2, 4-D or NAA, along or in combination with Kinetin or BA, growth was not effected whereas the formation of extractable phenolics was. In the case of present study with *Salvia santolinifolia* on the bases of TLC spots, the plant growth regulators used in the two media (MS2 & MS3) has no different effect on the synthesis of secondary metabolites. Because similar bands of compounds appeared on TLC from all extracts of the calli of 3rd-9th-subcultures of the two media (Fig. 1). Whereas on dry weight bases calli of MS3 media accumulated maximum secondary metabolites compared to MS2 media.

Table 2: Dry weight of callus produced on medium supplemented with NAA+BA and NAA+2iP

Subcultures	Media	Growth Regulators (mg/l)	Fresh weight of callus (g)	Dry weight of callus (g)
		NAA+BA		
3rd	MS2	0.5+1.5	2.0	0.034
5th	MS2	0.3+1.5	2.0	0.043
7th	MS2	0.3+1.5	2.0	0.045
9th	MS2	0.3+1.5	2.0	0.064
		NAA+2iP		
3rd	MS3	0.5+1.0	2.0	0.038
5th	MS3	0.3+1.0	2.0	0.046
7th	MS3	0.3+1.0	2.0	0.059
9th	MS3	0.3+1.0	2.0	0.067

Table 3: ^{13}C-NMR ($CDCl_3$, 75 MHz) data of 4.

C. No.	Multiplicity (DEPT)	^{13}C-NMR (δ)	C. No.	Multiplicity (DEPT)	^{13}C-NMR (δ)
1	CH_2	37.3	16	CH_2	26.1
2	CH_2	28.2	17	CH	56.0
3	CH	71.8	18	CH_3	12.1
4	CH_2	40.3	19	CH_3	19.4
5	C	140.8	20	CH	36.1
6	CH	121.7	21	CH_3	19.0
7	CH_2	31.7*	22	CH	138.3
8	CH	31.9*	23	CH	129.3
9	CH	51.2	24	CH	50.2
10	C	36.5	25	CH	29.2
11	CH_2	21.12**	26	CH_3	21.2
12	CH_2	39.7	27	CH_3	21.14**
13	C	42.2	28	CH_2	23.1
14	CH	56.9	29	CH_3	12.2
15	CH_2	24.4			

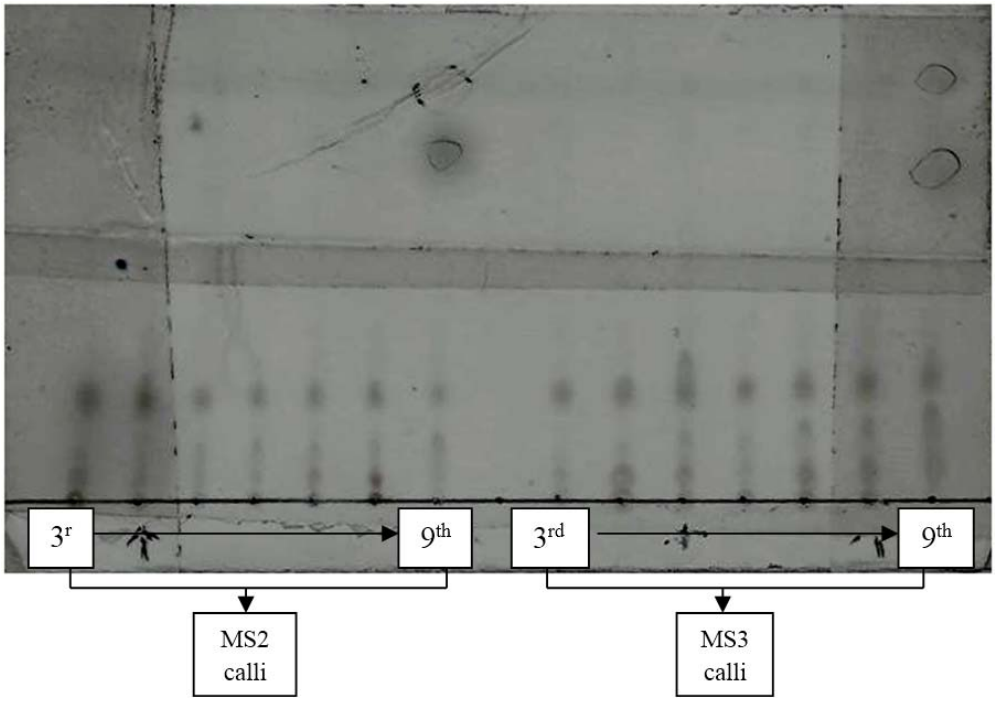


Fig 1: TLC analysis of crud extract of calli produced during 3rd-9th passages. TLC was developed in chloroform: methanol (95:5). MS2 calli produced under the influence of: NAA+BA (0.3+1.5 mg/l) and MS3 calli produced under the influence of: NAA+2iP (0.3+1.0 mg/l).

References

1. Agarwal M., Kamal R. Studies on flavonoid production using in vitro cultures of Momordica charantia L. Indian J. of Biotech. 2007; 6:277-279.
2. Delfel NE, Rothfus JA. Antitumor alkaloids in callus cultures of Cephalotaxus harringtonia. Phytochemistry. 1977; 16:1595-1598.
3. Duke JA. Hand Book of Medicinal Herbs, CRC Press, Boca Rofa florida, 1985, 420-421.
4. Funk C, Brodelius P. Influence of growth regulator and an elicitor on phenylpropanoid metabolism in suspension culture of Vanilla planifolia. Phytochemistry. 1990; 29:845-848.
5. Hagendoorn MJM, Jamar DCL, Meykamp B, Plas LHW. Cell division versus secondary metabolites production in Morinda citrifolia cell suspension culture. J Pl Physio. 1997; 150:325-330.
6. Luyt RP, Jager AK, Staden JV. Bufadienolides an in vitro derived Drimia robusta plants. S. Afr. J Bot. 1999; 65:443-445.
7. Morimoto S, Goto Y, Shoyama Y. Production of lithospermic acid and rosmarinic acid in callus tissue and regeneration plantlets of Salvia miltiorrhiza. J Nat Prod. 1994; 5:817-823.
8. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physio. Pl., 1962; 15:473-497.
9. Nakagawa K, Fukui H, Tabata M. Hormonal regulation of berberin from suspension culture of cell of Torreya nucifera var. radicans. Phytochemistry, 1986; 58:385-389.
10. Nishikawa K, Matsuura E, Ishimaru K. Tissue culture and acteoside production of osmanthus fragrans. Jpn. J Food Chem. 1998; 5:116-119.
11. Penos G. Index plantarum medicinalium totius mundi eorumque synonymorum (EMPLED). Org. Ed. Med. Fram. Nfilano, 1983, 1026.
12. Scragg AH, Allan EJ. Picrasma quassioides Bennet (Japanes quassia tree): In vitro culture and production of quassin. In: Biotechnology in agricultureand forestry 21: Medicinal and aromatic plants IV, (Ed.) Y. S. P. Bajaj). Springer-varlag Berlin Hedelberg, 1993, 264.
13. Tsay HS, Agrawal DC. Tissue culture technology of Chinese medicinal plant resources in Taiwan and their sustainable utilization. Inter. J Appl Sci and Engi. 2005; 3:215-223.
14. Yoshikawa T, Furuya T. Morphinan alkaloid production by tissue differentiated from cultured cells of Papavera somnifera. Pl. Med., 1985; 2:110-113.