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In Vitro Mass Multiplication and Isolation of Natural Products from Callus Tissues of *Salvia santolinifolia* (Boiss)

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Abstract: Method for mass multiplication of callus of *Salvia santolinifolia* by nodal explants was investigated by optimizing the concentration and combinations of different plant growth regulators in MS medium. Nodal explants could be stimulated to form callus on MS medium supplemented with 1-Naphthalene acetic acid (NAA) (0.1, 0.5 and 1.0 mg/l). Large scale multiplication of callus was achieved on MS medium containing NAA (0.3 mg/l) plus N^6 Benzylaminopurine (BAP) (1.5 mg/l) and NAA (0.3 mg/l) plus N^6-(2-isopentyl)-adenine (2iP) (1.0 mg/l). The phytochemical investtigation of callus lead to the isolation of β-Sitosterol and Stigmasterol compounds.

Keywords: Callus, mass multiplication, *salvia*, Lamiaceae

1. Introduction

The genus *Salvia* (Lamiaceae) is represented by about 900 species in the world. Species of the genus are widely used as folk-medicinal and ornamental plants as well as for their culinary purposes. They have antibacterial, antiviral, antifungal, antimutagenic and anti-inflammatory activities. Extracts of sage species are also valued for their antioxidant properties and are widely used in cosmetics and by the food-industry (Karousou et al., 2000). Phytochemical studies have shown that this group of plants is characterized by the biosynthesis of different types of terpenoidcompounds, flavonoids and other phenolic derivatives (Ulubelen, 2000; Lu and Foo, 2002). Several species of the genus *Salvia* are used in folk medicines, for instance, *S. bucharica* is used for the treatment of liver disorders (Ahmad et al., 1999). In china, *S. miltiorrhiza* is used in

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the treatment of heart diseases, haemorrhages miscarriage and hypertension (Wan et al., 2006). *S. prionitis* is used in chinese folk medicine for the treatment of tonsillitis, pharyngitis and bacillary dysentery (Xu et al., 2006). The interesting and immense medicinal properties of the genus *Salvia* prompted us to look for its locally available species. Out of the species found naturally occurring in Pakistan, *Salvia santolinifolia* is the only species which is found in Karachi (Hedge, 1990). The objective of the present study was to establish an efficient *in vitro* method for the rapid multiplication of callus from nodal explants of *Salvia santolinifolia* and then to evaluate the secondary metabolites production of cultured tissues.

2. Materials and Methods

Branches were excised from the selected plant. For the isolation of nodes and internodes shoot apex and leaves was removed from the branches. Excised young shoots were first kept under running tap water for 10-20 min. Shoots were surface sterilized with 0.05% Mercuric Chloride containing few drops of Tween-20 for 10-15 mins. Followed by 3-4 times rinsing with sterile distilled water prior to inoculation. Murishage and Skoog (1962) (MS) medium containing 3% sugar and 0.6% agar (agar-agar Mikrobiologie, Mereck, U.S.A.) was used through out this investigation. pH was adjusted to 5.5 to 5.55. Explants were cut to desired size and inoculated on MS media supplemented with NAA (0.1, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l). The combine effect of NAA (0.5 mg/l) plus BA (0.5, 1.0 and 1.5 mg/l) and NAA (0.5 mg/l) and 2iP (0.5, 1.0 and 1.5 mg/l) were also tested in MS medium. Mass multiplication of callus was obtained on media containing NAA (0.3 mg/l) plus BA (1.5 mg/l) and NAA (0.3 mg/l) plus 2iP (1.0 mg/l). For the isolation of secondary metabolites 11.0 kg of callus was soaked in methanol for a period of 10 days. Solvent was evaporated by means of distillation at low temperature (40-45 C°) and total 27.43 gram crud extract of callus was obtained. The crude extract thus obtained was subjected to silica gel column. Column was eluted initially with hexane (pure), followed by hexane**:** chloroform (95:5) mixture (in which the chloroform concentration was gradually increased by 5% up to 95%), chloroform (pure), chloroform**:** methanol (95:5) mixture (the methanol concentration in chloroform was increased by 2% up to 15%) and finally with pure methanol as a mobile phase. Fractions were collected in fraction collectors. TLC of each fraction was performed and similar spots on TLC were combined. All fractions were re-fractionated with the above respective solvents till to the isolation of pure compounds.

3. Results

Callus Initiation on Nodal Explants:

Callus initiation began from nodal explants within 3-6 days of inoculation under the influence of NAA

(Table-1). Callus induction first taking place from the cut end of the explants and then spread toward the whole segments. NAA at 0.5 mg/l induced maximum amount (4.47±1.64 gram) of callus (fresh weight) from nodal explants while at 0.1 mg/l of NAA less amount of callus was induced (Table-1). Morphologically, the calli appeared on nodal explants yellowish-white, wet and friable.

Table 1 The effect of different concentrations of NAA on the induction of callus from leaf and nodal explants of *Salvia santolinifolia* after 30 days of culture

Explants	Growth regulators (gm/l)	% Response	Mean fresh weight of callus (g) ±SE
Nodes	NAA		
	0.1	62.82	3.14±0.32
	0.5	75.23	4.47±1.64
	1.0	62.82	4.15±1.23

Symbol; SE: Standard Error

Proliferation of Callus

Callus which had been produced on MS medium containing NAA (0.5 mg/l) was subculture on their respective level of NAA alone and in combination with different concentrations of BA and 2iP (Table-2). Results from table-2 shows that maximum amount (5.34±1.74 gram) of callus (fresh weight) was obtained on medium containing NAA (0.5 mg/l) alone but on this concentration the regeneration of roots were higher (3-13), it is therefore, NAA was not used alone in further experiments. Moreover, among the combinations of auxin (NAA) and cytokinins (BA and 2iP), maximum amount (5.2±1.32 gram) of callus (fresh weight) was obtained on medium containing NAA (0.5 mg/l) plus BA (1.5 mg/l) (Table-2) whereas on the combinations of NAA and 2iP, maximum amount (4.3±1.8 gram) of callus (fresh weight) was obtained on medium containing NAA (0.5 mg/l) plus 2iP (1.0 mg/l) (Table-2). Apart of callus production and multiplication the combinations of auxin and cytokinins completely stopped roots regeneration from callus media containing BA plus NAA while on media containing 2iP plus NAA less numbers of roots regeneration occurred (Table-2).

Table 2 The effect of cytokinins (BA, 2iP) at various concentrations in media containing auxin (NAA) during first subculture

Number of subculture	Growth regulators	Concentrations (mg/l)	% Response	Range of root formation	Mean fresh weight of callus(±SE) (g)
1st	NAA	0.5	72.44	3-13	5.34±1.74
	NAA+BA	0.5+1.0	44.23	--	4.2±2.05
		0.5+1.5	62.82	--	5.2±1.32
		0.5+2.0	62.82	--	4.6±2.5
		0.5+2.5	59.35	--	4.3±2.14
	NAA+2iP	0.5+0.5	66.21	1-3	4.1±1.23
		0.5+1.0	63.22	1-2	4.3±1.8
		0.5+1.5	63.22	1	3.9±2.03

Symbols; SE: Standard Error, --: Zero

Repeated Subculture of Callus

Copiously growing calli on media containing NAA (0.5mg/l) plus BA (1.5 mg/l) (Table 3) (Figure-1) and NAA (0.5 mg/l) plus 2iP (1.0 mg/l) (Table-4) were repeatedly subculture for 9th months. The calli produced and multiply on these two media [NAA (0.5mg/l) plus BA (1.5 mg/l) and NAA (0.5 mg/l) plus 2iP (1.0 mg/l)] were used for the extraction of secondary metabolites. Callus remained growing continuously till the end of experimental period and total 11.51 kg of callus (fresh weight) were obtained. In repeated subcultures of callus, increase in biomass declined after 8th subculture on medium containing NAA plus BA (Table-3) and after 7th subculture with NAA and 2iP (Table-4). During first subculture some roots regenerated on them (Table-2). Lowering the concentration of NAA to 0.3 mg/l from 0.5 mg/l, gradually inhibited root formation during succeeding subcultures (Table-3, 4). Complete inhibition of root formation was observed from 6th subculture on medium containing NAA plus BA (Table-3) and from 7th subculture on medium containing NAA plus 2iP (Table-4).

Table 3 Callus biomass produced from nodal segments of *Salvia santolinifolia* during repeated subculture on medium containing NAA+BA in combination

Numbers of subculture	Growth regulators (mg/)	Range of roots formation	Mean fresh weight of callus (±SE) (g)	Amount of callus harvested (g)
	NAA+BA			
2nd	0.3+1.5	9-16	4.3±2.3	0
3rd	0.3+1.5	4-21	6.33±1.32	550.40
4th	0.3+1.5	2-12	8.99±2.36	735.9
5th	0.3+1.5	1-3	13.54±4.3	620.7
6th	0.3+1.5	0	14.39±1.9	750.09
7th	0.3+1.5	0	15.84±1.5	918.34
8th	0.3+1.5	0	17.98±2.4	865.17
9th	0.3+1.5	0	17.92±1.7	988.07

Total amount of callus collected at the end of experimental period= 5428.67 g or (5.43 Kg)

Symbol; SE: Standard Error

Table 4 Callus biomass produced from nodal segments of *Salvia santolinifolia* during repeated subculture on medium containing NAA+2iP in combination

Numbers of subculture	Growth regulators (mg/l)	Range of roots formation	Mean fresh weight of callus (±SE) (g)	Amount of callus harvested (g)
	NAA+2iP			
2nd	0.3+1.0	6-19	4.1±1.3	0
3rd	0.3+1.0	2-12	6.45±1.47	771.65
4th	0.3+1.0	1-10	9.3±1.2	817.6
5th	0.3+1.0	1-7	10.55±1.67	830.3
6th	0.3+1.0	1-3	13.94±1.8	936.14
7th	0.3+1.0	0	16.31±1.94	847.94
8th	0.3+1.0	0	15.8±2.2	792.91
9th	0.3+1.0	0	15.54±1.8	1086.52

Total amount of callus collected at the end of experimental period= 6083.06 g (6.08 Kg)

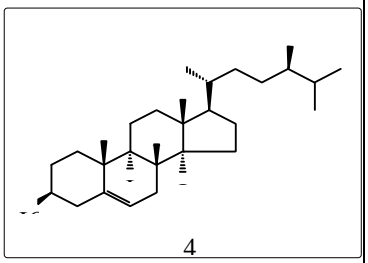
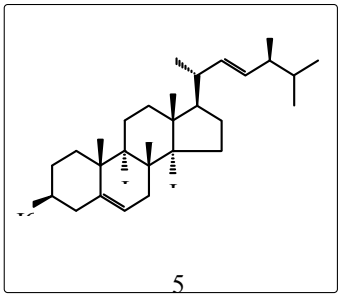
Symbol; SE: Standard Error

Figure 1: Callus induced from nodal segments on medium containing NAA (0.3 mg/l) and BA (1.5 mg/l) after 30 days of culture.

Extraction and Isolation of Natural Products from Callus Tissues

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 two sterols out of which one was β-Sitosterol and one was stigmasterol. Details structure characterizations of all these compounds are not shown.

Table 5 Identified compounds from the crude extract of callus produced in vitro from the nodal segments of *Salvia santolinifolia*

Class of compound	Compound	Structural formula	Molecular formula
Sterol	β-Sitosterol	 4	$C_{29}H_{50}O$
	Stigmasterol	 5	$C_{29}H_{48}O$

4. Discussion

The *in vitro* produced plant tissues which have been frequently used for the extraction of secondary metabolites are calli (Hegazi and El-Lamey, 2011and El-Baz et al., 2010). The nodal segments of *Salvia santolinifolia* can easily produced callus in the presence of NAA at all concentration. The callus induced at the level of 0.5 mg/l of NAA was the highest. Gostin (2008) obtained callus from nodal explants of *Salvia officinalis* on medium containing NAA. Musarurwa et al., (2010) produced callus from nodal explants of *Salvia stenophylla* with NAA and BA. NAA alone or in combination with BA was very effective in inducing callus on MS medium from stem and petioles of *Salvia canariensis* (Mederos-Molina, 2004). The callus which was induced on nodal segments of *Salvia santolinifolia* in the existence of NAA (0.5 mg/l) were further multiplied on media containing auxin (NAA) and cytokinins (BA and 2iP) for 9th passages. The use of auxins and cytokinins in combination for large scale production of callus were previously reported by several authors. For example, NAA in the presence of BA enhanced callus formation in *Salvia officinalis* (Ioja-Boldura et al., 2010), in *salvia nemrosa*, callus was formed with roots in the presence of NAA and BA (Skala and Wysokinska, 2004), the combination of NAA and BA produced best callus in *Pluchea lanceolata* (Arya et al*.,* 2008), *Citrullus colocynthis* (Meena and Patni, 2008). A high level of cytokinin with low level of auxin favoured shoots morphogenesis in tobacco callus and stem segments (Skoog and Miller, 1957) later it was found true with large number of plant species. In *Salvia santolinifolia* rapid growth of callus with the differentiation of roots occurred in this study with such combinations which may either be due to a higher level of endogenous auxin present in the explants which might have produced an additive effect with the exogenously supplied auxin leading to stimulation of profuse growth of callus and roots differentiation or it may be due to the genotype of the plant.

Calli in the present study, were grown for 9 passages (subcultures) on the medium of same composition in order to enrich callus by the continued accumulation of the synthesized chemical constituents as has been reported for the callus cultures of *Salvia multiorrhiza* (We et al., 2003); for the *in vitro* produced shoots of *Salvia officinalis* (Santos-Gomes et al., 2002), the in vitro produced shoots were cultures till 7 subcultures prior to extraction.

The chemical analysis of callus conducted in the present study revealed the presence one new compound salvialactomin, belong to class laction and four known compounds, belonging to three different classes. Out of which one flavone, 5-methylflavone, one fatty acid ester, glycerol-2-pentatriacontanoate and two sterol (β-Sitosterol) and (Stigmasterol) were identified from the methanolic extracts of callus tissue of *Salvia santolinifolia*.

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