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RESEARCH ARTICLE

AN EFFICIENT PROTOCOL DEVISED FOR RAPID CALLUS INDUCTION FROM LEAF EXPLANTS OF *SPERMADICTYONSUAVEOLENS* ROXB

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ABSTRACT

Spermadictyon suaveolens Roxb.is an ancient Indian herb used as a various medicinal products. In present investigation the callus induction of *Spermadictyon suaveolens* Roxb. were carried out by using Murashige and Skoog (MS) medium with different concentration of hormones. The explants, collected from the Matwan gaon, Taluka Dapoli, District Ratanagiri (Maharashtra) and grown on the garden of Department of Botany, University of Pune. The leaf explant respond well for callus and cell culture biomass by using MS with 1.2 mg/l TDZ.

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INTRODUCTION

Plants valued with medicine are of great interest to the researchers in the field of biotechnology. Most of the drug industries depend on the plants for the production of pharmaceutical compounds (Chand *et al*., 1997). *Spermadictyon suaveolens* Roxb. (=*Hamiltonia suaveolens* Roxb.) belongs to family Rubiaceae. The family Rubiaceae is widespread and found to be distributed in all major regions of the world except Antarctica.According to Davis *et al.*, 2009 it is the fourth largest Angiosperm family comprises of 13,143 species and 611 genera. Rubiaceae family with 10,700 species yielded the most widely used human stimulant, caffeine (Mongrand *et al*., 2005 and Ashihara *et al*., 2008). Family Rubiaceae is having many age old medicinally important plants like *Cinchona officinalis* Linn., *Coffea arabica* Linn.; *Rubia tintoria* Linn.; *Adina cordifolia* Hook.f,; *Gardenia jasminoides* Ellis.; *Morinda citrifolia* Linn.; *Anthocephalus cadamba* Miq.; *Mitragyna parviflora* Roxb.; *Spermadictyon suaveolens* Roxb. (Gupta, 1981). *S. suaveolens* Roxb. is commonly known as Forest champa. It is a branched shrub, growing up to 1-2 m tall. The leaves are oppositely arranged and elliptic-lance like about 10-20 cm in length and finely velvety in surface. Leaf stalks are 1-2 cm long.

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Flowers occur in many-flowered spherical heads which are arrange in panicles at the end of branches. The species name *suaveolens* means sweet scented, and refers to the fragrant flowers. There are manyethno-botanical survey done on this plant which helps in finding out the use of this plant as an antidiabetic.Hence, this plant is considered as antidiabetic plant in amchi system of medicine by local bush doctors as well as in indigenous system of medicines (Naik, 2010). The stem of this plant were used to cure disease Herpes zoster which is commonly known as Nagin. It led to form bands of blisters usually on just one side of the body which are full of liquid, pus, etc.The roots are also used in treatment of diabetes and rheumatoid arthritis (Sonar, 1968), in diarrhoea and for treating ulcers and wounds (Jain, 1991). Antidiabetic effect of a root extract from *S. suaveolens* also reported by (Sonar, 1968; Farnsworth and Segelman, 1971). The barks of this plant are rubbed on the body when suffering from fever (Anonymous, 1948-1976). It is potent antidiabetic plant and used in folk, Ayurvedic and homeopathic systems of medicine (Kapoor, 1997; Ravi *et al*., 1995 and Mitra, 1985). The stem bark of this plant is boiled in water and vapors are allowed over the body of a person suffering from fever and also in case of anemic persons. Active principles, medicinal properties and uses of these plants have been listed by (Kumar *etal*., 2009; Mahmoodreza, 2010; Modupe, 2010; Zhu *et al.*, 2010; Sirisha *et al.*, 2011). The chemical constituents isolated from *S. suaveolens* by using GC-MS analysis is Azulene, Tetratetracontane, 9-Nonadecane, n-hexadecanoic acid

(Palmitic acid), Phenol, 2-methoxy-4-(1-propenyl)-, (E), Tritetracontane and Ergost-5-en-3-ol, 22, 23-dimethyl-, acetate, (3β). The chemicals is used against skin irritation, anti-inflammatory effects and antibacterial properties, anti-ulcergeni and antifungal properties (Kulkarni and Sathe, 2013). One of the important chemical which we studied in the present investigation is hexadecanoic acid. It is a major component of the oil from palm trees (palm oil, palm kernel oil, and coconut oil) but can also be found in meat, cheese, butter and dairy products. Palmitate is a term for the salts and esters of palmitic acid. It was observed that rats fed on a diet of 20% palmitic acid and 80% carbohydrate showed alterations in central nervous system control of insulin secretion and suppression of the body's natural appetite-suppressing signals from leptin and insulin (Hadadare and Salunkhe, 2013). The chemical n-hexadecanoic acid (Palmitic acid) is used against anti-bacterial and antifungal properties (Modupe, 2010).

It is a live hedge around orchard of *Anacardium occidentale* Linn. Efforts were made to collect the plant material in flowering and fruiting condition and authenticated from Botanical Survey of India, Regional office, Western circle-Pune, 411001 (Specimen voucher Number-BSI/WRC/IDEN.CER./2016/240). The plant was collected and perpetuated by method of stem cuttings in the garden of Department of Botany, Savitribai Phule Pune University.

Media and culture conditions

Murashige and Skoog (1962) medium supplemented with different concentration of growth regulators used for the study. The collected leaf explant from the botanical garden were washed with the running tap water. Then the explants were cut and kept in Tween 20 for 10 minutes then thoroughly washed under running tap water.

Table 1. Influence of auxins and cytokinins alone on the growth of callus biomass by using leaf explant of spermadictyon suaveolens roxb

MS + PGRs (mg/l)					Callus			
Auxins		Cytokinins			Callus culture		Cell suspension	
2,4-D	NAA	TDZ	BA	Kin	Moisture (%)	D.W (gm)	Moisture %	D.W(gm)
Control								
0.6					60.3± 2.4^{b}	0.85 ± 0.05^{c}	63.1 ± 3.03^{c}	0.82 ± 0.11^{c}
0.8					60.9± 4.3^{b}	1.02 ± 0.08^{b}	69.3 ± 0.97^{c}	0.93 ± 0.04^{d}
1.0					69.8 ± 1.41^{b}	1.29 ± 0.32^{b}	72.4 ± 0.98^{b}	1.20 ± 0.18^{c}
1.2					75.7± 0.51^{a}	1.48 ± 0.005^{a}	80.5 ± 0.06^{a}	1.59 ± 0.003^{a}
	0.6				61.2 ± 0.35^{c}	1.08 ± 0.017^{c}	73.8 ± 0.20^{b}	1.43± 0.015^{ab}
	0.8				72.4 ± 0.41^{a}	1.43 ± 0.006^{a}	73.4 ± 2.9^{b}	1.31 ± 0.11^{b}
	1.0				78.9± 0.12^{a}	1.50 ± 0.01^{a}	80.9 ± 0.25^{a}	1.59 ± 0.046^{a}
	1.2				72.4 ± 0.26^{a}	1.43 ± 0.003^{a}	79.3 ± 0.23^{a}	1.47 ± 0.008^{b}
		0.6			80.3 ± 0.42^{c}	2.19 ± 0.46^{b}	75.2 ± 0.10^{d}	1.58 ± 0.028^{b}
		0.8			79.6 ± 0.32^{c}	2.06 ± 0.008^{b}	78.4 ± 00^{c}	1.7 ± 0.012^{b}
		1.0			83.1 ± 0.12^{b}	2.37 ± 0.24^{b}	82.7 ± 0.95^{b}	2.20 ± 0.037^{b}
		1.2			96.6 ± 0.89^{a}	8.21 ± 1.03^{a}	97.0 ± 0.61^{a}	11.26 ± 2.41^{a}
			0.6		66.7 ± 0.29^{d}	1.32 ± 0.008^{c}	70.2 ± 1.27^{c}	1.33 ± 0.076^{b}
			0.8		69.4 ± 0.33^{c}	1.30 ± 0.008^{c}	72.6 ± 0.95^{c}	1.47 ± 0.017^{b}
			1.0		75.1 ± 0.22^{b}	1.77 ± 0.015^{b}	75.7 ± 0.80^{b}	1.67 ± 0.02^{b}
			1.2		90.5 ± 0.23^{a}	2.59 ± 0.015^{a}	95.2 ± 0.64^{a}	7.78 ± 0.88^{a}
				0.6	69.9 ± 0.92^{c}	1.23 ± 0.058^{c}	76.0 ± 2.3^{b}	1.07 ± 0.055^{c}
				0.8	80.4 ± 0.64^{b}	1.15 ± 0.024^{c}	78.0 ± 0.75^{b}	1.35 ± 0.044^{b}
				1.0	83.9 ± 0.15^{a}	1.38 ± 0.018^{b}	80.3 ± 0.78^{ab}	1.47± 0.027^{ab}
				1.2	83.6 ± 0.78^{a}	1.53 ± 0.055^{a}	84.4 ± 0.24^{a}	1.54 ± 0.029^{a}

Data recorded after 4 weeks of culture incubation.The value represent the mean ± SE calculated on three independent experiments, each based on minimum of 15 replicates. Values followed by the same letter were not significantly different at 5% level (DMRT).

These varied uses have increased utilization and exploitation of *S. suaveolens* for medicinal purposes. The plant grows wild in forests and among other areas. But in the comparison of the other propagation method the *in-vitro* culture shows the results faster and the techniques produced large amount of the active constituents. Zhao *et al*., 2001 reported that callus culture could provide alternative supply of active metabolites for use in medicine and stimulating the production of novel compounds not found *in-vivo*. Therefore, an effort has been undertaken to develop a reliable protocol for callus biomass production of *S. suaveolens* by using tissue culture in present investigation.

MATERIAL AND METHODS

Collection and Identification of Plant Material

The plant of *Spermadictyon suaveolens* Roxb. was collected from Matwan gaon, Taluka Dapoli, District Ratanagiri.

Again the explants is placed in savlon (1%) for 5 min and washed with running tap water. Then the explants are soaked in the antioxidant solution containing PVP (100mg/l), Ascorbic acid (100-500mg/l) and citric acid (50-100mg/l) for 30 minutes. This was washed with the sterile distilled water in laminar air flow for 4-5 times.

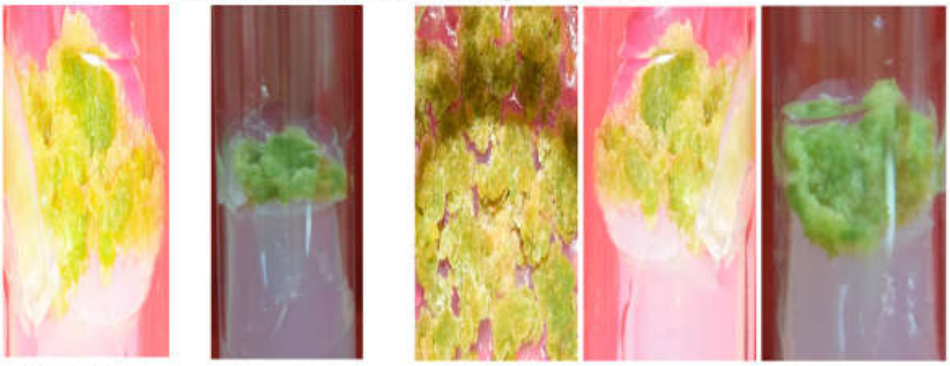
Then the explants are kept in 0.1% $HgCl_2$ for 2 min. Shaking was done during this period to get through sterilization. It was then rinsed with sterilized distilled water for 4-5 times to remove the unwanted materials from explants. The surface sterilized explants were then inoculated immediately after surface sterilization in the prepared test tubes containing media. The inoculated explants in different concentration of MS media were exposed to 16 hours light and 8 hours dark cycle at 25 ± 2º C.

Table 2. Influence of cytokinins in combination with auxing on the growth of callus biomass bu using leaf explant of spermadictyon suaveolen roxb

MS + PGR				Callus			
Cytokinins (mg/l)		Auxins (mg/l)		Callus culture		Cell suspension	
BA	TDZ	NAA	2-4,D	Moisture (%)	D.W (gm)	Moisture (%)	D.W (gm)
Control							
1.2		0.6		75.0 ± 0.25^{d}	1.54 ± 0.012^{d}	78.56 ± 2.08^{c}	1.94 ± 0.22^{c}
		0.8		82.7 ± 0.49^{b}	2.18 ± 0.018b^{c}	83.8 ± 0.48^{b}	2.31 ± 0.03^{b}
		1.0		81.7 ± 0.27^{b}	2.15 ± 0.012b^{c}	83.0 ± 0.47^{b}	2.29 ± 0.028^{b}
		1.2		89.4 ± 0.06^{a}	2.67 ± 0.003^{a}	89.4 ± 0.00^{a}	2.67 ± 0.003^{a}
			0.6	57.1 ± 0.26^{d}	0.79 ± 0.006^{d}	56.8 ± 0.23^{d}	0.77 ± 0.007^{d}
			0.8	62.5 ± 0.12^{c}	1.06 ± 0.003^{c}	70.5 ± 2.56^{b}	1.27 ± 0.85^{b}
			1.0	68.0 ± 0.12^{b}	1.21 ± 0.003^{b}	80.9 ± 1.19^{a}	1.51 ± 0.027^{a}
			1.2	69.4 ± 0.06^{b}	1.31 ± 0.003^{b}	80.9 ± 0.120^{a}	1.6 ± 0.015^{a}
	1.2	0.6		81.4 ± 0.17^{de}	2.33 ± 0.006^{d}	80.8 ± 0.55^{e}	2.32 ± 0.006^{d}
		0.8		82.9 ± 0.24^{c}	2.64 ± 0.046^{c}	82.1 ± 0.15^{cd}	2.62 ± 0.003^{c}
		1.0		80.4 ± 0.29^{e}	2.26 ± 0.008^{d}	84.1 ± 0.62^{b}	2.29 ± 0.29^{d}
		1.2		94.2 ± 0.088^{a}	7.82 ± 0.008^{a}	94.9 ± 0.35^{a}	7.72 ± 0.027^{b}
			0.6	60.2 ± 0.41^{f}	0.89 ± 0.006^{e}	62.9 ± 0.15^{e}	0.96 ± 0.003^{d}
			0.8	63.1 ± 0.43^{e}	0.99 ± 0.012^{d}	70.4 ± 0.29^{c}	1.16 ± 0.016^{c}
			1.0	68.5 ± 0.82^{d}	1.19 ± 0.015^{c}	74.4 ± 0.92^{b}	1.36 ± 0.015^{b}
			1.2	71.2 ± 0.15^{c}	1.39 ± 0.008^{b}	77.1 ± 0.80^{a}	1.53 ± 0.017^{a}

Effects of auxins and cytokinins alone on the growth of callus biomass by using leaf explant of *S. suaveolens* Roxb.

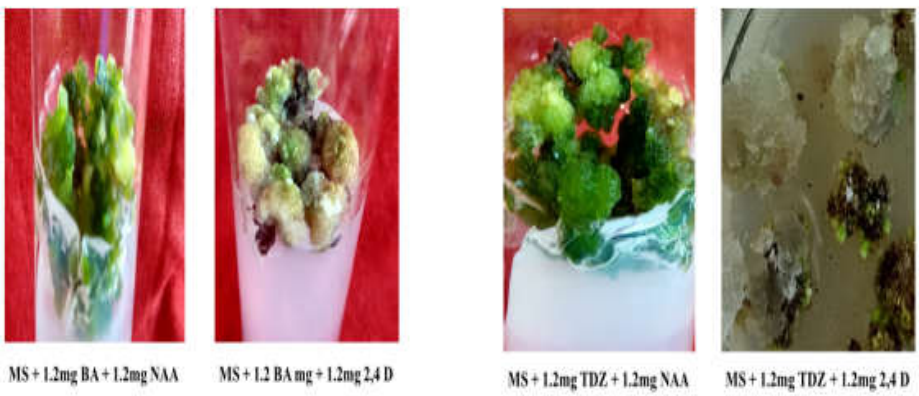
Callus induction from leaf explant cultured on:



MS + 1.2mg 2,4, D | MS + 1.0mg NAA | MS + 1.2mg TDZ | MS + 1.2mg BA | MS + 1.2mg Kin

Effects of cytokinins in combination with auxin on the growth of callus biomass by using leaf explant of *S. suaveolens* Roxb

Callus induction from leaf explant cultured on:



MS + 1.2mg BA + 1.2mg NAA | MS + 1.2 BA mg + 1.2mg 2,4 D | MS + 1.2mg TDZ + 1.2mg NAA | MS + 1.2mg TDZ + 1.2mg 2,4 D

Callus and Cell culture

The explants were cultured on MS (Murashige & Skoog) basal medium supplemented with different concentrations of auxins and cytokinins alone and in combinations with each other. Considering the quantity and quality of callus and cell culture for better response best explants were selected. About 30 day, old callus was collected and sub cultured on fresh medium with same growth regulator combinations and repeated twice with two week time interval. All the cultures were incubated at 24±2° C under 16 h photoperiod provided by cool white florescent lights. Leaf explants were excised aseptically and cultured on MS medium supplemented with different concentrations of auxins and cytokinins alone and in combinations.

Experimental design, data collection and statistical analysis

All the experiments were repeated thrice with 15 replicates for each treatment. The results are expressed as mean ± SD of three experiments.

RESULTS AND DISCUSSION

Callus culture studies

In the present investigation, callus cultures were initiated from leaf explants of *Spermadictyonsuaveolens* Roxb.on MS medium supplemented with auxins and cytokinins alone or in combinations. The explants failed to induce the callus on MS medium without growth regulators. The different auxins used were 2, 4-D and NAA and cytokinins used were BA, Kin and TDZ. Data were analyzed after four weeks of first subculture and the results showed the variation in days for callus initiation, percentages of explants respond for callus,callus color& texture (Table 1)., among all the growth regulators used, TDZ and BA resulted in extensive proliferation of callus by using leaf explants of *Spermadictyonsuaveolens*Roxb. while on other plants growth hormones the callus proliferation was notedless.

It was observed and noted that high concentration of cytokinins TDZ and BA (1.2mg/l)shows maximum callus induction in both callus culture and cell culture i.e. 8.21 ± 1.03;2.59 ± 0.01 and 11.26 ± 2.41;7.78 ± 0.88 respectively.The callus produced on the media alone with TDZ and BA is greenish white in colour and compact and fragile in nature. Both the cytokinins used showed the highest moisture % in both callus culture (96.6 ± 0.089 and 90.5 ± 0.23) and cell culture (97.0 ± 0.61 and 95.2 ± 0.64) respectively. It was recorded that Kinetin used alone shows very low response to the callus induction in both culture. The result on Table 1 recorded that out of two different auxins used alone for callus induction NAA (1.0 mg/l) showed maximum moisture % and dry weight per gram on both callus culture (78.9 ±0.12%; 1.50 ± 0.01 g) and cell culture (80.9 ± 0.25%; 1.59 ± 0.046 g).On the other side 2,4-D with concentration of (1.2 mg/l) shows maximum percent of moisture content and dry weight per gram i.e. 75.7 ± 0.51and 1.48 ± 0.005 (callus culture) and 80.5 ± 0.06 and 1.59 ± 0.003 (cell culture). The callus produced on media with 2,4- D alone shows cream colour, soft and compact nature and on NAA shows green colour, soft and fragile type of callus. The results for cytokinin in combination with auxins interpreted in Table 2. The media containing cytokinins with 1.2 mg/l TDZ and BA were incorporated with different concentration of NAA and 2, 4-D.The data recorded after 4 weeks and tabulated on Table 10, Plate X. It was observed that the TDZ (1.2 mg/l) with the same concentration (1.2 mg/l) of NAA shows maximum moisture % and dry weight of callus culture (94.2 ± 0.08%; 7.82 ± 0.008) and cell culture (94.9 ± 0.35%; 7.72 ± 0.027). On the other side BA 1.2mg/l in combination with 1.2 mg/l NAA shows the maximum callus induction in callus culture (89.4 ± 0.06%; 2.67 ± 0.003) and cell culture (89.4 ± 0; 2.67 ± 0.003).The result indicated that the high concentration of the growth hormones shows good response for callus induction in both callus culture and cell culture in compare to low concentration. On combination of TDZ and BA with auxin 2,4- D shows less amount of callus dry weight per gram. Out of both the cytokinins 1.2 mg/l TDZ with 1.2 mg/l 2,4-D shows maximum percent of moisture and callus per gram dry weight on callus culture (71.2 ± 0.15%; 1.39 ± 0.008). Nature of callus were recorded on the different combination. BA with NAA shows greenish white, compact and fragile and BA with 2-4 D shows cream colour soft and compact. On the same side TDZ with NAA shows green colour, soft and compact and TDZ with 2, 4-D shows cream colour, soft and compact.

Conclusion

The outcome of the present investigation will be helpful for Pharmaceutical industries as over increasing demand this plant. In addition, it is also helpful for the traditional practioners and researchers.

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