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RESEARCH ARTICLE

SOMATIC EMBRYOGENESIS AND PLANTLETS REGENERATION FROM SEEDLING EXPLANTS OF *Santalum album* L. (Santalaceae)

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ABSTRACT

India has a varietal emporium of medicinal plants and is one of the richest countries in the world with regard to the genetic resources of medicinal plants. *Santalum album* is an economically important tree harvested for heartwood and heartwood oil. Heartwood oil is useful in medicines of many diseases. A reproducible system for somatic embryogenesis and plantlet formation of sandalwood has been developed. High number of direct somatic embryo proliferation from leaf explants was observed in MS medium containing 2,4-D 13.50 µM showed 60.0 ±5.0 percentage of response. The maximum rooting response achieved on medium supplemented only with 2.46µM IBA was 70 per cent with an average of 5.3 ±0.25 roots per shoot. Rooted plantlet was transferred to paper cups containing sterile soil, sand and vermiculite.

INTRODUCTION

India is one among the twelve mega biodiversity nations and more than 15,000 plant species are used for medicine from the diverse flora. According to WHO report, 80 per cent of the world population depends on the traditional plant based medicines for their primary health care needs. In India, medicinal plants are widely used by all sections of the people as they play a very important role (Perumalsamy and Gopalakrishnakone, 2007). WHO has shown great interest in documenting the use of medicinal plants (Hasan *et al*., 2009). Medicinal plants play a vital role for the development of new drugs (Farnsworth, 1994; Srivastava *et al*., 1996). India has a varietal emporium of medicinal plants and it is one of the richest countries in the world with regard to the genetic resources of medicinal plants (Martins *et al*., 2001).

Santalum album L. belonging to the family Santalaceae, is a well known plant distributed in India, Australia, Newzealand, South America, Indonesia and other countries. In India, their presence is mostly confined to Southern States *viz.,* Tamil Nadu, Karnataka, Andhra Pradesh and Kerala (Bhattacharya and Sita, 1999; Azeez *et al*., 2009). *Santalum album* is a small to medium-sized evergreen tree, sometimes reaching up to 18m in height and 2.5m in girth. It is a root parasite and successful regeneration (both natural and artificial) requires, suitable host plants. Sandal wood oil is used as an important raw material in soap, perfume industries and other cosmetics. In addition to this heart wood oil is used to treat skin disease, leprosy, jaundice, vomiting, diabetes and inflammation (Chada, 1972; Bapat *et al*., 1985; Anandhapadmanabha, 2000). Regeneration via somatic embryogenesis of *Santalum album* can be achieved from hypocotyls, nodal and endosperm explants (Sita *et al*., 1979; Rao and Bapat, 1992). Preliminary results on somatic embryogenesis of *Santalum album* obtained following the published methods were unreliable, particularly for the two steps of inducing embryogenic callus using 2,4-D and subculturing into GA_3 (Sita *et al*., 1979; Rugkhla and Jones, 1998). Conventionally this species is propagated either by vegetative means or by seeds. In vegetative propagation success rate is always very low and time consumption is an important factor in vegetative propagation. Since sandal is cross pollinated seeds show a greater degree of heterozygosis and natural genetic uniformity is not maintained. Hence there is an urgent need to develop alternative propagation techniques to fulfill the current requirement and also to conserve this endangered species immediately (Sita, 1986; Mujib *et al.,* 1998).

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MATERIALS AND METHODS

Collection of *Santalum album*

Healthy plants of *Santalum album* seeds collected from Vandalur Forest Research Institute, Chennai, Tamil Nadu, India were raised in pots containing soil and farm yard manure

in the ratio 1:1 under green house condition in laboratory**.** The explants taken from these plants were used for all experiments.

Explants preparation

The explants (seeds) were surface sterilized following the procedures of Samantray and Upadhyaya (2010). The plant specimens were collected from healthy potted plants kept in the green house. The explants namely node, internodes, juvenile leaf and shoot tips were washed thoroughly in running tap water and then washed with 70 per cent ethanol. The explants were then immersed in 0.1 per cent (w/v) mercuric chloride for five minutes for surface sterilization and then washed repeatedly in sterile double distilled water three to five times to remove traces of mercuric chloride adhering to the explants. Therefore, the seed explants were used for culturing.

Sterilization of equipments

Media, glassware, blade holder, forceps, tweezers and scissors were washed in distilled water and steam sterilized for 15-20 minutes at 121ºC with 15 lb/inch^2 normal steam pressure in an autoclave (Purvis *et al.,* 1966).

Preparation of tissue culture media

Murashige and Skoog (1962) medium were used as basal medium. The composition of media are given in Table 1. Four individual stock solutions of macro nutrient, micro nutrient, micro-iron and vitamins were prepared and stored. Stock solutions of salts were prepared using double distilled water and the required volume made up to with standard volumetric flask. Iron stock solution was stored in amber bottle to prevent photolysis. All the stock solutions were stored in at 4°C and meso-inositol; cytokine and auxin stock solutions were freshly prepared and used every month. For preparation of medium, all four stock solutions were mixed thoroughly with required amounts of sterile distilled water. Three per cent sucrose (30 g/l), 0.01 per cent meso-inositol (100mg/l) and required amount of plant growth regulators (PGRs) were added and the pH was adjusted to 5.6 ±0.2 with 0.1N HCl or 0.1N NaOH prior to autoclaving. The gelling agent agar (Himedia Grade 301) 0.9 per cent (w/v) was added to the prepared media constituents and mixed well before dispensing into glass wares. The contents were labelled and sterilized in an autoclave at 15 lb/inch^2 steam pressure for 15-20 minutes at 121ºC. After inoculating with explants, all the culture vials were kept under 16/8h (light/dark) photoperiod at 25 ±2ºC.

Preparation of auxins

The auxins namely 2,4-D, IAA, IBA and NAA were prepared separately by dissolving 100 mg in 1ml of 0.1N NaOH and the volume was then made up to 100ml in a standard flask by adding double distilled water and stored at 4ºC.

Preparation of cytokines

The cytokines namely benzyl adenine (BA), Gibberlic acid (GA) and Kinetin (KN) were prepared separately by dissolving 100mg in 1ml of 0.1N HCl and the volume was then made up to 100ml in standard flask by adding double distilled water and stored at 4ºC.

Plant regeneration from seed explant of *Santalum album*

Explants from seeds of *Santalum album* were inoculated on MS basal medium supplemented with individual concentrations and combination of BA (1.11, 2.22, 4.44, 8.88 and 11.10 µM) and NAA (0.54 µM, 1.34, 2.69 and 5.37 µM) for germination of seeds. At the end of the experiment, percentage of entire plant developed from per seeds explants was recorded.

Direct somatic embryogenesis developed from internodal explants of *Santalum album*

Explants from internodes of *Santalum album* plant were inoculated on MS basal medium supplemented with individual concentrations and combination of 2, 4-D (1.13, 4.52, 9.04, 13.50 and 18.02 µM) for direct somatic embryo development. Mature somatic embryos were transferred onto MS medium with BA (1.11, 2.22, 4.44, 8.88 and 11.10 µM) and GA3 (0.054, 0.14, 0.27 and 0.54 µM) for shoot elongation. At the end of the experiment, percentages of shooting, shoot length were recorded.

Root proliferation

The healthy shootlets were transferred to half strength MS basal medium supplemented with individual concentrations of IBA (0.49, 0.98, 2.46, 4.92 and 12.30 µM) for root initiation. The percentage of rooting, root length and the number of roots per individual shoots were documented.

Hardening

The healthy rooted plantlets were carefully removed from the medium without disturbing the root and shoot and washed thoroughly in sterile distilled water in order to remove the medium sticking on to the roots. The plantlets were then transferred to pots (6 cm dia) containing red soil, vermiculite and farm yard manure in 1:1:1 ratio for hardening. The hardened plantlets were closed with polythene bags to prevent transpiration and to maintain relative humidity (RH) of 80 per cent.

Culture conditions

The cultures were incubated in culture chamber at 25 ºC for the light condition and the culture vials were placed on the rack at a distance of 25 cm from the light source. A 16/8 h (light / dark) photoperiod of cool white light was provided from 2000 Lux.

RESULTS AND DISCUSSION

Germination and growth of mature seeds

Surface sterilized seeds inoculated on MS medium supplemented with various concentration and combination of BA and NAA. Initiation of seed germination in most of the treatments was observed within three weeks of culture. All seeds turned green after three weeks of culture (Fig.1). Maximum seed germination (cotyledon developed) was observed in MS medium containing BAP 4.44 µM and NAA 2.69 µM showed 80 per cent response and produced seed

germination and developed cotyledons with an average length entire plant of 3.9 ±0.0 cm after 35 days. (Table 2). Whereas higher concentration of BAP (8.88 µM) combined with all concentration NAA, gradual fall in the germination seed was recorded.

Table 1. Composition of MS (Murashige and Skoog, 1962) basal medium

Stock	Ingredients	Concentration (mg/L)
Macro salts (10 folds)	NH_4NO_3	1,650.00
	KNO_3	1,900.00
	$MgSO_4.7H_2O$	370.00
	KH_2PO_4	170.00
	$CaCl_2.2H_2O$ *	440.00
Iron and EDTA 25 folds	$FeSO_4.7H_2O$	27.80
	Na_2 EDTA	33.60
Micro salts (25 folds)	$MnSO_4.4H_2O$	22.30
	$ZnSO_4.4H_2O$	8.60
	H_3BO_3	6.20
	KI	0.83
	$Na_2MoO_4.7H_2O$	0.25
	$CuSO_4.7H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
Amino acid	Glycine	2.00
Vitamins	Nicotinic acid	0.50
	Pyridoxine HCl	0.50
	Thiamine HCl	1.00
Meso-inositol	Meso-inositol *	100.00
Sucrose	Sucrose *	30.00.00
pH	-	5.6 ±0.2

* add freshly

Table 2. Effect of different concentration of cytokine (BA) and auxin (NAA) in MS medium on seed germination of *Santalum album*

Plant growth regulator		Seed germination (%)	Shoot length(cm)
BA	NAA		
1.11	0.54	25.00	2.07 ± 0.38
	1.34	36.67	1.90 ± 0.17
	2.69	40.00	3.33 ± 0.15
	5.37	25.00	1.93 ± 0.12
2.22	0.54	40.00	1.90 ±0.10
	1.34	46.67	2.93 ± 0.06
	2.69	41.67	2.00 ± 0.50
	5.37	30.00	2.03 ± 0.23
4.44	0.54	31.67	2.67 ± 0.29
	1.34	50.00	3.87 ± 0.21
	2.69	75.00	4.43 ± 0.06
	5.37	40.00	2.43 ± 0.06
11.10	0.54	25.00	1.93 ± 0.06
	1.34	26.67	2.23 ± 0.31
	2.69	35.00	1.93 ± 0.1
	5.37	30.00	1.47 ± .06

Table 3. Effect of individual concentration of auxins (2,4-D) on embryogenic response of *Santalum album*

PGR Concentration (µM)	Inter node
2,4 - D	Embryogenic response %
4.52	23.33
9.04	26.67
13.50	60.00
18.02	-

Direct somatic embryogenesis from internodal explant of *Santalum album*

Direct somatic embryogenesis was observed on inter nodal explants from *in vitro* germination seed. Inter nodal explants culture on MS medium supplemented with different individual concentrations of 2,4 –D (1.13, 4.52, 9.04, 13.50 and

Table 4. Effect of different concentration of BA and GA on MS medium on shoot elongation from direct somatic embryogenic (internodal explants)

Plant growth regulator (µM)		Inoculated direct embryogenesis from internodal explants	Shoot elongation (%)	Shoot length (cm)
BA	GA			
0.56	0.29		-	-
	0.72		-	-
	1.44		-	-
	2.89		-	-
1.11	0.29		20.0	1.0± 0.0
	0.72		21.7	1.0± 0.0
	1.44		15.0	0.5± 0.3
	2.89		10.0	0.5± 0.2
2.22	0.29		21.7	0.5± 0.2
	0.72		33.3	0.8± 0.3
	1.44		65.0	2.0 ± 0.0
	2.89		15.0	0.9± 0.2
4.44	0.29		-	-
	0.72		-	-
	1.44		-	-
	2.89		-	-

Table 5. Effect of individual concentration of auxins (IBA) on root proliferation from *invitro* shoots

Plant growth regulator (µM) IBA	Root response (%)	No. of root per shoot let	Root length
0.49	-		
0.98	35.0	20.67 ± 1.15	1.40 ± 0.10
2.46	60.0	4.00 ± 1.00	3.40 ± 0.10
4.90	41.6	1.67 ± 0.58	1.93 ± 0.12

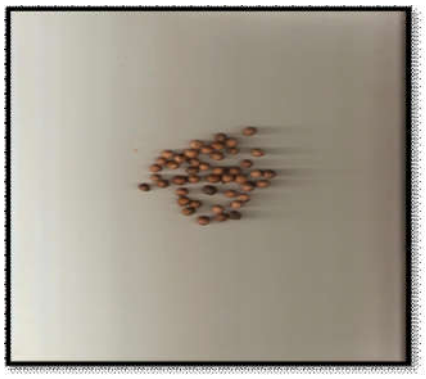

Fig. 1. Seed and germination of *Santalum album*

Fig. 2. Internodal explant of *Santalum album*

Fig. 3. Shhot elongation of *Santalum album*

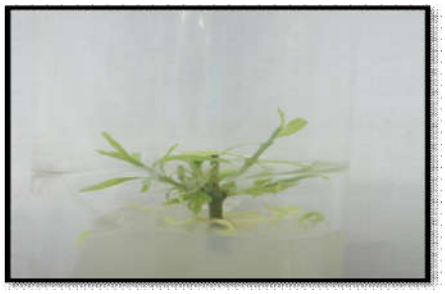

Fig. 4. Root proliferation of MS medium

Fig. 5. Hardening of *Santalum album*

18.02 µM). Direct somatic embryo in most of the treatments was observed within four weeks of culture. High number of direct embryo proliferation from leaf explants was observed in MS medium containing 2, 4-D 13.50 µM showed 60.0 ±5.0 percentage of response and produced 33.0 ±1.0 embryos per explants after six weeks of culture (Table 3, Fig. 2). Whereas lower concentration of 2,4-D (1.13, 4.52, 9.04µM) did not get somatic embryos.

Embryo germination and shoot elongation medium

The mature embryo were transferred to MS medium (elongation) supplemented with BA (1.11 - 4.44 µM) and GA_3 (0.29 -2.89 µM). The mature embryo transferred on elongation media containing 2.22 µM BA and 1.44 µM GA_3 showed better growth response and shoots elongation (mature embryo) with an average length of 2.0 ±0.0 cm after 25 days culture (Table 4, Fig.3).

Root proliferation from *in vitro* shoot (mature embryo)

Individual shoots from a multiple shoot mass were separated after 28 days transferred to half strength of MS supplemented with 2.46 µM IBA. In all media, roots appeared after one to three weeks and after 30 days, were well developed (Fig. 4). The maximum rooting response achieved on medium supplemented only with 2.46 µM IBA was 70 per cent, with an average of 5.3 ±0.25 roots per shoot (Table 5). Though lower concentration of IBA produced significantly higher rooting and root length, higher concentration of IBA did not produce results as expected.

Hardening of *in vitro* plantlets of *Santalum album*

Rooted plantlets of *Santalum album* was transferred to paper cups containing sterile soil, sand and vermiculite (1:1:1), kept at 2^oC for one week and transferred to the shade house after 35 days of normal growth (Fig. 5). In *Santalum album*, the survival rate of 70 per cent was observed on hardening in red soil, vermiculite and farmyard manure (1:1:1). However, the survival rate decreased by 10 per cent after two to three weeks of acclimatization. It was observed that gradual acclimatization of *in vitro* grown plants to external environment was most essential for *Santalum album*. More than 60 per cent of the plants transferred to pots survived and resumed growth.

Conclusion

In the present study, *Santalam album* recorded the survival rate of 70 per cent on hardening the plantlets in red soil, vermiculite and farmyard manure (1:1:1). It was observed that gradual acclimatization of *in vitro* grown plants to external environment was most essential for *Santalam album*. In spite of the decrease in the survival rate by 10 per cent after two to three weeks of acclimatization, 70 per cent of the plants of *Santalam album* transferred to pots survived.

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