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DIRECT MULTIPLE SHOOT BUDS REGENERATION FROM SHOOT APEX AND NODAL EXPLANTS FOR MASS PROPAGATION OF *SANTALUM ALBUM* LINN.

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ABSTRACT

In vitro propagation studies were initiated with *Santalum album* Linn. for rapid micropropagation using shoot apex and nodal explants. Direct regeneration of multiple shoot bud was observed from both explants in Murashige and Skoog (MS) basal medium supplemented with various concentrations and combinations of BAP (6-Benzylaminopurine), Kn (kinetin), IAA (Indole-3-acetic acid) and NAA(α-Naphthalene acetic acid). The highest shoot regeneration frequency (100%) and mean number (4.76 ± 0.33) of shoots per explant was achieved from nodal segments cultured on MS medium fortified with 2.0 mg/l BAP + 0.5 mg/l IAA within 15 days of inoculation. 2.0 mg/l BAP + 1.0 mg/l IAA found to be best for shoot elongation. In terms of rooting, *In vitro* derived well developed shoots were excised and implanted individually on half strength of MS with different concentrations of IAA, IBA and NAA. Maximum number (3.11 ± 0.12) and highest length (3.73 cm) of root was observed on half strength MS medium containing 0.5 mg/l IAA + 1.0 mg/l IBA. Well rooted plantlets transferred to plastic pots containing soil and compost mixture (1:1) and showed 91% survival when transferred to outdoor.

KEYWORDS: Explants, *In vitro*, Micropropagation, Multiplication, *Santalum album.*

INTRODUCTION

Santalum album Linn. belongs to the Santalaceae family, is a threatened [1] small tropical aromatic and medicinal tree species, commonly known as *Sandalwood* or *Chandon*. It is native to semi-arid areas of Indian sub-continent and is now planted in China, Sri Lanka, Indonesia, Malaysia, the Philippines, Northern Australia and also in Bangladesh. The heartwood containing 1.5-5% of strong specific fragrance of oil which has been used in perfumes, cosmetics, religious and medicine purposes and was predicted to have anti-melanoma compounds [2, 3]. Sandalwood oil is widely used for the treatment of coldness, fever, weakness, skin diseases, and bronchitis, hear diseases, infection of urinary system, liver and gall bladder complaints etc. [4]. Many research articles reported that this plants species also has anti-oxidant, anti-microbial, and anti-hyperglycemic potentiality [5, 6]. Due to the over exploitation of this plant species for harvesting wood to obtain santalol (sandalwood oil) and also for other purposes such as to use in wood carving industries and aboriginal medicine. These activities contribute towards the destruction of *S. album* plants in Bangladesh.

Conventionally, the plant is propagated by seed but the success rate of seed germination is very low [7]. Normally seeds remain dormant for two months and gradually viability is decreased after 9-10 months period. The other methods of propagation by cutting or by grafting are of limited use in sandal because of poor success rate. Hence, there is an urgent need to develop alternative propagation techniques to fulfill the current requirement and also to conserve this and other

valuable medicinal plant species [8]. Plant tissue culture and micropropagation would possibly be a better technique to contend with this growing problem [9-11]. Therefore, the objective of the present study was to develop a successful rapid *in vitro* protocol for mass propagation of *Santalum album* Linn. through direct organogenesis using shoot apex and nodal explants.

MATERIALS AND METHODS

Plant Materials and Explants Sterilization

Shoot apex and nodal explants were collected from two years old plants of *Santalum album* Linn. grown in the medicinal block of Bangladesh Forest Research Institute, Chittagong, Bangladesh. Both explants were thoroughly washed under running tap water for 20 minutes, treated with liquid detergent (Tween 20) for 10 minutes, followed by dipping in 5% (v/v) savlon solution for 10 minutes. The plant materials were then washed 6-7 times with distilled water. After rinsing with 70% ethanol for less than 60 seconds, they were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10 minutes and washed with sterile double distilled water 4-5 times after each surface disinfection treatment under aseptic conditions. The surface sterilized explants were cut into small pieces (0.5-1.0cm) with a sterilized surgical blade and then inoculated onto the culture media.

Media and Culture Conditions

The culture medium used for the present study was Murashige and Skoog (MS) basal medium supplemented with different concentrations and combinations of plant growth regulators (PGRs) such as BAP, Kn, NAA and IAA were used for induction of organogenesis or embryogenesis. In some cases the multiple shoot buds (MSBs) that developed from nodal explants or from shoot apex elongated on MS supplemented with different PGRs and for rooting, elongated shoots at a height of 3-4cm were rescued aseptically from the cultured on rooting medium containing half strength MS medium fortified with different concentrations and combinations of IBA, IAA and NAA. In all cases the media were solidified with 0.8% (w/v) agar and pH was adjusted to 5.8 prior to autoclaving for 30 minutes at 121^0C under 1.1kg/cm^2 pressure. All culture vessels with inoculated explants were then taken to the culture room for incubation where a cycle of 14 hours continuous light and 10 hours continuous dark phase was maintained. The room temperature was maintained at 25 ± 2^0C.

Shoot Induction and Multiplication

Shoot apex and nodal segments were placed on MS medium supplemented with various cytokinins *viz.* BAP and Kn at different concentrations (0.5-2.0mg) either alone or in combination with IAA or NAA (0.5-1.0mg) for shoot induction. The induced shoots were allowed to grow for 10-25 days. After 10-25 days of culture, the explants producing maximum number of multiple shoot buds were sub-cultured on MS basal medium fortified with different concentrations of PGRs for proliferation of more number and highest length of micro shoots.

Root Formation

The isolated multiple shoot buds (3-4cm) with two to three pairs of leaves were harvested and subsequently transferred to half strength MS medium fortified with different concentrations of IAA, IBA and NAA. Data were recorded on percentage of rooting, mean number of roots and root length after 3-4 weeks of inoculation.

Hardening and Acclimatization of Plantlets to Soil

Plantlets with well develop shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing a mixture of soil and compost (1:1) at relative humidity 80-85% with light intensity varied 2000-3000lux and temperature of 28 ± 2^0 C following successive phases of acclimatization. For the purpose, the month of the culture vessels were kept open for one day in the culture room and they were then kept outside the culture room for 6 hours in the next day. Later on those were kept outside the culture room for 12 hours. Finally the seedling were taken out of the culture vessels and rinsed with running tap water for complete removal of medium attached to the roots.

Statistical Analysis

Experiments were set up in a Randomized Block Design (RBD) and each experiment was replicated thrice. Observations were recorded on the percentage of response, number of shoots per explants and number of roots per shoot. A minimum of 10-15 explants were used for each experiment. Means and standard deviations were calculated for each treatment. The data Means ± SD of at least three different experiments were represented.

RESULTS AND DISCUSSION

In vitro regeneration of multiple shoot buds from shoot apex and nodal explants of *S. album* without callus formation was successfully developed. Proliferation of multiple shoots was observed with high frequency from nodal segment than shoot apex within 10-25 days of inoculation. Both explants underwent direct organogenesis on MS medium containing different concentrations and combinations of auxins (0.5 - 1.0 mg/l) and cytokinins (0.5 - 2.0 mg/l). It is evident that the combinations of auxins (IAA/IBA) and cytokinins (BAP/Kn) was more effective in production of multiple shoot buds compared to the only cytokinin.

The highest frequency of multiple shoot induction was obtained (100%, 4.76 ± 0.33) on the medium augmented with 2.0 mg/l BAP + 0.5 mg/l IAA (Figure1-A&B, Table-1) from nodal segments. Conversely, the lowest percentage of multiple shoots induction was found to be (06.44%, 1.06 ± 0.20) from shoot apex on the media supplemented with 0.5 mg/l BAP. The synergistic effect of cytokinin with auxin has been demonstrated in several medicinal plants, *viz. Elephantopus scaber* [12], *Cestrum nocturnum* [13], *Plectrathus amboinicus* [14], *Plumbago zeylanica* [15] and *Stevia rebaudiana* [16].

For elongation, the micro shoots that developed directly from shoot apex and nodal explants underwent elongation when individually grown on different PGRs supplemented elongation media. Data on elongation of shoot buds were recorded after 4 weeks of culture (Table 2). The efficiency of a medium was assessed in terms of enhancing multiplication and elongation of shoots. The highest length of shoots (Figure1-C&D) was (4.81cm) obtained on the MS medium having 2.0 mg/l BAP + 1.0 mg/l IAA. On the contrary, the minimum elongation (1.23 cm) of shoot buds took place in the medium fortified with 3.0 mg/l Kn + 1.0 mg/l IAA. Cytokinins have been shown to be most critical growth regulator for shoot multiplication in some medicinal plant species, like *Gentiana kurroo* [17]. So in the present study MS medium with cytokinins and auxins acted as trigger for initiating multiplication and elongation of multiple shoot buds. Similar responses were observed in many other medicinal plants such as, *Toddalia asiatica* [18], *Aegle marmelos* [19] and *Boerhaavia diffusa* [20] and *Ruta gravelens* [21].

Table 1: Effect of Different Concentrations and Combinations of Plant Growth Regulators in MS Medium on Multiple Shoot Buds Induction from Shoot apex and nodal segments of *Santalum album.*

Concentrations of PGRs(mg/l)				Nodal Segment			Shoot Apex		
BAP	Kn	IAA	NAA	Time(Days) Required For Shoot Initiation	%of Explant Giving a Response	Average* No. of Multiple Shoot Buds Per Culture (Mean ±SE)	Time(Days) Required for Shoot Initiation	% of Explants Giving a Response	Average* No. of Multiple Shoot Buds Per Culture (Mean ±SE)
0.5	-	-	-	10-25	10. 24	1.17 ± 0.16	**10-25**	**06.44**	**1.06 ± 0.20**
1.0	-	-	-	10-25	18.03	1.32 ± 0.11	10-25	10.03	1.13 ± 0.35
2.0	-	-	-	10-25	26.56	1.66 ± 0.13	10-25	16.00	1.28 ± 0.16
1.0	-	0.5	-	10-25	76.47	2.79 ± 0.25	10-25	57.20	2.16 ± 0.41
1.0	-	1.0	-	10-25	84.61	2.86 ± 0.16	10-25	60.08	2.32 ± 0.13
1.5	-	1.0	-	10-20	91.20	3.47 ± 0.64	10-20	71.21	2.66 ± 0.61
2.0	-	**0.5**	-	**10-15**	**100**	**4.76 ± 0.33**	10-20	89.21	2.96 ± 0.11
2.0	-	1.0	-	10-15	81.03	2.56 ± 0.18	10-20	72.41	2.65 ± 0.22
1.0	-	-	0.5	10-25	65.00	1.56 ± 0.30	10-25	47.10	1.14 ± 0.02
1.0	-	-	1.0	10-20	72.41	1.79 ± 0.51	10-25	50.06	1.32 ± 0.55
1.5	-	-	1.0	10-20	80.12	2.75 ± 0.24	10-25	66.09	1.62 ± 0.53
2.0	-	-	0.5	15-20	71.00	2.46 ± 0.10	15-20	60.43	1.51 ± 0.22
2.0	-	-	1.0	15-20	62.14	1.77 ± 0.27	15-20	48.00	1.46 ± 0.37
-	1.0	1.0	-	15-20	34.17	1.55 ± 0.42	15-20	20..11	1.36 ± 0.17
-	1.5	1.0	-	15-20	68.00	1.80 ± 0.03	15-20	37.55	1.50 ± 0.19
-	2.0	1.0	-	15-20	61.07	1.68 ± 0.16	15-20	48.63	1.32 ± 0.07
-	1.0	-	1.0	10-25	36.20	1.50 ± 0.54	15-20	29.00	1.27 ± 0.16
-	1.5	-	1.0	10-25	51.22	1.31 ± 0.30	15-20	40.00	1.18 ± 0.16
-	2.0	-	1.0	10-25	44.05	1.23 ± 0.12	15-20	32.14	1.12 ± 0.16

* Values are the mean of three replicates each with 15 explants

Table 2: Effect of Different Concentrations and Combinations of Cytokinins and Auxins on Elongation of Multiple Shoot Buds of *Santalum album.*

Concentrations of PGRs(mg/l)				Average* Initial Length(cm) of Individual Shoot Bud (±SE)	Average* Length(cm) of Shoot Bud After 30days of Culture (±SE)
BAP	Kn	IAA	NAA		
0.5	-	0.5	-	1.14 ± 0.06	1.68 ± 0.17
1.0	-	0.5	-	1.51 ± 0.17	2.00 ± 0.13
1.0	-	1.0	-	1.85 ± 0.23	2.14 ± 0.04
1.5	-	0.5	-	2.31 ± 0.11	3.27 ± 0.32
1.5	-	1.0	-	2.50 ± 0.25	3.88 ± 0.18
2.0	-	**1.0**	-	**3.26± 0.34**	**4.81 ± 0.10**
1.0	-	-	0.5	1.62 ± 0.12	1.87 ± 0.23
1.0	-	-	1.0	2.11 ± 0.30	2.33 ± 0.04
2.0	-	-	1.0	3.36 ± 0.10	3.65 ± 0.16
3.0	-	-	1.0	2.70 ± 0.28	3.21 ± 0.18
-	1.0	1.0	-	1.36 ± 0.15	1.75 ± 0.37
-	1.5	1.0	-	1.68 ± 0.18	2.55 ± 0.31
-	2.0	1.0	-	1.43 ± 0.32	1.14 ± 0.07
-	**3.0**	**1.0**	-	**1.06 ± 0.21**	**1.23 ± 0.15**
-	1.0	-	1.0	1.40 ± 0.18	1.71 ± 0.30
-	2.0	-	1.0	2.37 ± 0.31	2.60 ± 0.16
-	3.0	-	1.0	1.50 ± 0.10	2.08 ± 0.22

* Values are the mean of three replicates each with 15 explants

In order to produce complete plants, multiple shoot buds were individually transferred to rooting media. Half strength MS basal medium with IAA (0.5-1.5mg/l) or IBA (0.5-1.5mg/l) or in combination of IAA, IBA and NAA(0.5-1.0mg/l) were used for root induction. Data were recorded after 3 weeks of inoculation (Table 3). The best response was recorded when 0.5 mg/l IAA + 1.0 mg/l IBA was added to half strength MS medium. In this medium combination on an average highest number (3.11 ± 0.12) and maximum length (3.73 cm) of root was observed (Figure1-E). On the other hand, low frequency (56%) of root was reported in half strength MS with 0.5 mg/l IAA. The influence of IBA and IAA for induction and proliferation of root has been reported in many medicinal plant, such as, *Viola pilosa* [22], *Aquilaria malaccensis* [23], *Plumbago zeylanica* [24] and *Mentha pulegium* [25].

After 4-5 weeks the shoots with strong and stout root system were acclimatized outside chamber for 10 days then transferred to plastic cups (Figure1-F) containing mixture of soil and compost (1:1). On an average 91% plantlets finally survived in nature.

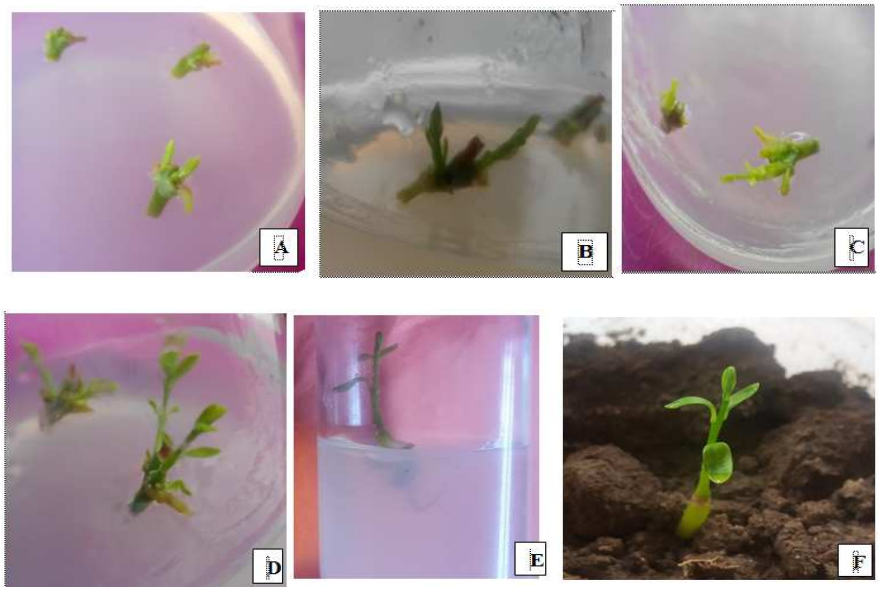

Figure 1: *In Vitro* Regeneration of Plantlets through Direct Organogenesis in *Santalum album.* A&B- Induction of Shoot Buds on MS+2.0 mg/l BAP+0.5 mg/l IAA; C&D- Elongation of Shoot Buds; E. Elongation and Rooting of Shoots on 1/2 Strength MS + 0.5mg/l IAA+1.0 mg/l IBA; F. Hardening and Acclimation of *In Vitro* Regenerated Plant.

Table 3: Effect of Different Concentrations and Combinations of IAA, IBA and NAA in Half Strength MS on Root Induction from Micro Shoots of *Santalum album*.

Concentrations of PGRs (mg/l)			Rooting %	Mean* No. of Roots per Shoot Bud(±SE)	Mean*Length of roots (cm) (±SE)
IAA	IBA	NAA			
0.5	**-**	**-**	**56**	**2.05 ± 0.13**	**2.12 ± 0.34**
1.0	-	-	68	2.62 ± 0.30	2.87 ± 0.11
1.5	-	-	76	2.75 ± 0.21	3.00 ± 0.05
-	0.5	-	65	2.66 ± 0.32	2.61 ± 0.28
-	1.0	-	73	2.72 ± 0.05	2.40 ± 0.17
-	1.5	-	82	2.81 ± 0.10	3.10 ± 0.31
0.5	0.5	-	100	2.55 ± 0.21	3.16 ± 0.28
0.5	**1.0**	**-**	**100**	**3.11 ± 0.12**	**3.73 ± 0.08**
1.0	1.0	-	100	3.02 ± 0.13	3.20 ± 0.27
0.5	-	0.5	88	2.31 ± 0.10	2.27 ± 0.15
1.0	-	1.0	93	2.65 ± 0.24	2.63 ± 0.22
1.5	-	0.5	80	2.25 ± 0.11	2.30 ± 0.20

* Values are the mean of three replicates each with 15 explants.

CONCLUTION

This research study presents the report of plant regeneration through direct organogenesis of *Santalum album* Linn. from shoot apex and nodal explants. MS basal medium containing 2.0 mg/l BAP + 0.5 mg/l IAA gave the best rate of shoot initiation. The highest length of shoot was obtained in MS fortified with 2.0 mg/l BAP and 1.0 mg/l IAA. Half strength MS basal medium with 0.5 mg/l IAA + 1.0 mg/l IBA was found to be best for the root induction. The protocol presented in this study may provide a high efficiency regeneration system for successful regeneration of adventitious shoots for *ex situ* conservation of *Santalum album* Linn. as well as genetic improvement studies for pharmaceutical, industrial uses and future research investigations.

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