

Clonal propagation of *Santalum album* to get high and uniformly yielding plants

FINAL
REPORT

NSF Grant
RG/09/BT/02

WTPSK SENARATH

Department of Botany

University of Sri Jayewardenepura

Nugegoda

(2010 -2013)



FINAL REPORT

1. SECTION 1

- i. **GRANT NUMBER:**
RG/2009/BT/02
- ii. **TITLE OF THE PROJECT:**
Clonal propagation of *Santalum album* to get high and uniformly yielding plants
- iii. **PRINCIPAL INVESTIGATOR:**
Prof. WTPSK Senarath
- iv. **CO-INVESTIGATORS:**
None
- v. **INSTITUTION WHERE THE RESEARCH WAS BEING CARRIED OUT:**
University of Sri Jayewardenepura, Nugegoda
- vi. **DATE OF AWARD:**
26.01.2009, Date of initiation of the project (if different from above) - 17.06.2010
- vii. **DATE OF COMPLETION OF PROJECT:**
31.12.2013
- viii. **TOTAL ALLOCATION OF FUNDS:**
Rs. 1,691,750.00
- ix. **TOTAL SPENT (Rs.):**
Rs. 1,439,608.95
- x. **NUMBER OF RESEARCH STUDENTS EMPLOYED:**
04 (All the RA's employed got permanent jobs and left the project, two as SLAS officers, one as a teacher)
- xi. **POSTGRADUATE DEGREE COMPLETED WITH DATES:**
None
- xii. **NUMBER OF TECHNICAL ASSISTANTS AND/ OR LABOURERS EMPLOYED AND PERIOD OF SERVICE:**
None on permanent basis however a labourer was hired when ever needed
- xiii. **PUBLICATIONS/COMMUNICATIONS ARISING FROM THE PROJECT DURING THE REPORTING PERIOD:**
One manuscript has been submitted to the Journal of National Science Foundation

2. SECTION 2: EXCLUSIVE SUMMARY OF THE PROJECT:

Santalum album (Sandalwood) is a valuable tropical plant belongs to family Satalaceae. Because of its fragrant heartwood and oil along with its medicinal qualities this species has been threaten by over exploitation. Therefore it is important to establish a protocol for clonal propagation through tissue culture using elite trees for establishment of Sandalwood plantations.

Nodal segments, cotyledonary parts from *in vitro* germinated mature and immature seeds were tested for callus induction and it was observed that MS medium supplemented with 1.0 mg/L 2,4-D found to be the best medium for induction of callus from cotyledonary parts. Single node explants found to be better over double node explants for shoot induction. Best shoot induction medium was MS medium supplemented with 0.5 mg/L BA while decreasing the level of BA to 0.25 mg/L enhances the multiple shoot induction. Although *in vitro* rooting was less successful *ex vitro* root induction was successful. However the percentage rooting was low. Plantlets regenerated survived well in the potting mixture (Sand: coir dust 2: ½) during acclimatization.

The objective of the current project is to obtain plants for commercial scale plantations, thus it is necessary to consider about the cost per plant. Therefore some other alternatives options were tested such as use of low cost media for shoot induction. For shoot growth use of 18% sago as gelling agent was better over 8% commercial agar and the shoots were more healthy and greener in the presence of sago. Albert solution: Maxicrop at the ratio of 2: 1 could be an alternative to MS basal medium for induction of shoots of *S. Album*.

As root induction was less successful possibility of obtaining plantlets through somatic embryogenesis has been tested. Nodal segments and mature seeds were the best explants sources for embryonic callus induction. Embryonic callus induction was successful in MS medium supplemented with 2.5 mg/L 2,4 D and 3.0 mg/L kin after 4 weeks of incubation. MS medium supplemented with 0.5 mg/L BAP, 1.0 mg/L IAA and 0.5 mg/L kin induced somatic embryos from embryonic calli. While MS medium supplemented with 2.0 mg/L GA₃ found to be the best medium for somatic embryo germination. Plantlet regeneration was observed in MS medium supplemented with 0.4 mg/L BAP and 0.2 mg/L IAA

Establishment of cell and hairy root cultures was tested and when callus was placed in liquid medium in 100 rpm on a shaker it was observed that this speed is not suitable, however increasing the speed to 250 rpm is better for cell culture establishment. Hairy root cultures could be established by transferring the callus in to MS medium supplemented with 2.5 mg/L 2,4-D, 2.0 mg/L kin and 1.0 mg/L IAA

3. SECTION 3:

REPROT IN DETAIL

i. INTRODUCTION/BACKGROUND:

Santalum album (Sandalwood) has been a part of Sri Lankan culture for hundreds of years. The heartwood yields fragrant oil, which is used mainly in the perfumery industry but also has medicinal properties. Sandalwood oil (about 92% is Santalol) has antipyretic, antiseptic, antiscabietic and diuretic properties (Rai, 1990). Thus oil is important in indigenous medicine too. Sandalwood is an evergreen tree which grows upto a height of 20 m and attains the girth of over 1.5 m. It flowers twice a year but changes according to the climatic conditions thus certain plant flower once a year and some do not flower regularly. This species is capable of growing in different kinds of soils like sand, clay and loam. Therefore it has a great interest in tree plantation projects which become a recent investment industry in Sri Lanka.

Vegetative propagation of *S. album* is by seeds where fruit has to be depulped, washed thoroughly in water and dried under shade before germination. However viability of seeds loses within 6-9 months of storage. Therefore propagation through seed germination has limited success. On the other hand, private sector which has currently established Sandalwood plantations faces another problem with seed raised plants that some of the seedlings do not give the typical fragrance with the maturity (per. Comm.). Rooting of cuttings also has been tested but the success rate is low due to hemi-parasitic nature of the species. There are records of tissue culture of *Sanalum sp.* in India (Mujab, 2005) and also in Australia where it was observed that the conditions required for mass propagation through tissue culture is different for Indian species than those in W. Australia (Rugkhla and Jones, 1998). Therefore it would be useful to establish a protocol for clonal propagation through tissue culture using elite trees for establishment of Sandalwood plantations in Sri Lanka. In Sandalwood trees, the oil content is highest in the root, next in the stem at ground level and gradually tapered off towards the tip of the stem. Therefore for harvesting oil, mature plants either have to be clear fell or damaged in order to obtain maximum yield. Use of cell and hairy root cultures is another possible way of obtaining secondary metabolites without damaging to mature plants. Establishment of cell cultures and/or hairy root cultures of Sandalwood would be an attractive method for obtaining Santalol for commercial purposes. This also will have potential in industry as Sandalwood oil is widely used in cosmetic and herbal industry of Sri Lanka.

ii. SCIENTIFIC SCOPE OF THE PROJECT (OBJECTIVES):

Determination of the factors affecting clonal propagation of *Santalum album* through tissue culture and field establishment of *in vitro* propagated plantlets

iii. MATERIALS AND METHODS:

Collection of mother stock and maintenance

Initially thirty mother plants were collected and re-potted in 1:1:1: topsoil, compost and sand mixture. Topsin (5.0 g/L) and Thiram (1.4 g/L) was sprayed alternatively to mother stock. Fungicide (Tilt, 10.0 ml/L) was sprayed 24 hours before collection of explants to reduce the contamination in cultures, Albert solution was applied (50.0 mL per plant) weekly. As *S. album* is a hemi parasitic plant, Buffalo grasses, *Desmodium sp.* (Udupiyaliya) and *Alternanthera sp.* (Mukunuwenna) were planted on pots with sandalwood seedlings. Plants were maintained under shade house condition.

Establishment of aseptic cultures

Development of surface sterilization procedure for seeds

Immature (Green color) seeds were washed for 5 min in Teepol® solution and kept 30 minutes under running tap water and then kept 30 minutes in fungicide solution (Tilt®, 1.25 mL/L) and finally thoroughly washed in sterile distilled water. After that seeds were washed in 10% Clorox for different exposure time (10, 15 and 20 min). Followed by washing in 70% EtOH for 10 mins. Then they were cultured in growth regulator free MS medium and incubated at 25±1°C in 16 h photoperiod. There were 20 replicate in each treatment. Percentage of contamination free cultures was counted after four weeks of incubation.

Surface sterilization of nodal segments and shoot tips

Young shoot tips were collected and initially washed 5 minutes in a Teepol® solution and placed under running tap water for 20 minutes. Then they were dipped 30 minutes in a fungicide solution (Topsin, 2.0 g/L) for 30 min. . Different concentrations and exposure time of Clorox was tested (Table 1) before washing in 70% ethanol for 10 min. Each treatment was followed by two successive washings in sterile distilled water. Then they were cultured in growth regulator free MS medium and incubated at 25±1°C in 16 h photoperiod. There were 20 replicate in each treatment.

Table 1: Different surface sterilization methods used for nodal segments and shoot tips

Treatment code	Soap water	Running tap water	Fungicide (1.0 g/L)	Clorox concentration	Exposure Time	70% Ethanol
T1	5 min	30 min	30 min	20%	15 min	10 min
T2	5 min	30 min	30 min	20%	10 min	10 min
T3	5 min	30 min	30 min	15%	15 min	10 min
T4	5 min	30 min	30 min	15%	20 min	10 min
T5	5 min	30 min	30 min	15%	10 min	10 min

DIRECT ORGANOGENESIS

Explants were collected in the morning and washed in soap water for 5 min and kept under running tap water for 30 min. They were dipped in a solution of Captan[™] (2.0 g/L) for 30 min. Then explants were washed in 15% Clorox for 15 min, after that in 70% ethanol for 10 min, each followed by three successive washings in sterile distilled water.

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was used as the basal medium and the pH was adjusted to 5.8. Cultures were incubated in 16 h photoperiod at 25±1 °C under otherwise stated. Completely Randomized Design (CRD) was used in all experiments with 20 replicates in each treatment.

Determination of best growth regulator combination for callus induction from cotyledonary parts

Seeds were surface sterilized and were established *in vitro* in growth regulator free MS medium. When the seeds were germinated cotyledonary parts were dissected and cultured in MS medium supplemented with different BA and 2,4-D concentrations (Table 2). Cultures were incubated in dark for callus induction. Diameter of the callus was measured in weekly intervals over a period of 16 weeks. Callus obtained was sub cultured in to both liquid and solid media and incubated in dark for further growth. And also cultures were incubated under 16 h photoperiod for shoot induction.

Table 2: Different growth regulator combinations used for callus induction from cotyledons

Treatment code	2,4-D (mg/L)	BA (mg/L)
T1 (Control)	0.0	0.0
T2	0.5	0.0
T3	1.0	0.0
T4	0.0	0.5
T5	0.5	0.5
T6	1.0	0.5
T7	0.0	1.0
T8	0.5	1.0
T9	1.0	1.0

Determination of best basal medium and the growth regulator combination for shoot induction from nodal segments

Table 3: Different media used in shoot induction from nodal segments

Treatment code	Composition	No of nodes in explant
T1	WPM + 0.5 mg/L BA	1
T2	WPM + 1.0 mg/L BA	1
T3	WPM + 1.5 mg/L BA	1
T4	WPM + 2.0 mg/L BA	1
T5	WPM + 0.5 mg/L BA	2
T6	WPM + 1.0 mg/L BA	2
T7	WPM + 1.5 mg/L BA	2
T8	WPM + 2.0 mg/L BA	2
T9	MS + 0.5 mg/L BA	1
T10	MS + 1.0 mg/L BA	1
T11	MS + 1.5 mg/L BA	1
T12	MS + 2.0 mg/L BA	1
T13	MS + 0.5 mg/L BA	2
T14	MS + 1.0 mg/L BA	2
T15	MS + 1.5 mg/L BA	2
T16	MS + 2.0 mg/L BA	2

Single nodal and double nodal shoot tips were surface sterilized and cultured in either MS or WPM medium supplemented with different concentrations of BA (0.5 – 3.0 mg/L) (Table 3). Number of shoots / axillary buds induced in each explants was counted weekly.

Determination of the best growth regulator combination for multiplication

Elongated axillary buds (with at least three nodes) obtained from both double and single nodal explants were transferred to multiplication media in order to increase the number of shoots. Combination of different concentration of BAP (0.10 – 0.35 mg/L) were used in MS medium supplemented with 0.20 mg/L 2,4-D and. Number of shoots, plant height and the diameter of callus (if any) were recorded in every 2 weeks interval.

LOW COST MEDIA FORMULATION

Sago as an alternative gelling agent in shoot induction medium

Surface sterilized single node explant were established *in vitro* in MS medium supplemented with 0.25 mg/L BA. Different concentrations of sago (Table 4) was used as the gelling agent. MS medium supplemented with 0.25 mg/L BA solidified with 8% agar was used as a control. Number of shoots / axillary buds produced was counted over a period of 4 weeks.

Table 4: Different concentrations of sago used in shoot induction media.

Treatment code	Concentration of Sago (%)
T0 (Control)	8% agar
T1	18
T2	20
T3	22
T4	24
T5	26

Albert's solution and Maxicrop combination as alternative media components for shoot induction medium

Single node explants were surface sterilized and were established in a medium containing different concentrations of Albert's solution and Maxicrop solution (Table 5). Sucrose (30.0 g/L), BA (0.5 mg/L), coconut water (15% v/v), Myo inositol (0.1g/L), vitamin B (0.18 g/L) and agar (10.0 g/L) was added as supplements. MS medium supplemented with sucrose (30.0 g/L), BA (0.5 mg/L), coconut water (15% v/v), Myo inositol (0.1g/L), vitamin B (0.18 g/L) and agar (10.0 g/L) was used as the control.

Table 5: Different concentrations of Albert's solution and Maxicrop solution used as an alternative media components

Treatment code	Albert's solution (g/L)	Maxicrop (mL/L)
T0 (control)	-	-
T1	3	1
T2	3	2
T3	3	3
T4	4	1
T5	4	2
T6	4	3

Determination of suitable concentrations of Albert's solution and Maxicrop combination as substitutes to basal salt mixtures with Sago, kurakkan (*Eleusine coracana*), rulan and jelly moss as gelling agents

Single node explants were surface sterilized and was cultured in a medium containing different concentrations of Albert's solution and Maxicrop solution (Table 6). Sucrose (30.0 g/L), BA (0.5 mg/L), coconut water (15% v/v), Myo inositol (0.1g/L), vitamin B (0.18 g/L) was added to the medium and sago (18.0 g/L), Jell moss (8.0 g/L), Kurakan, (20.0 g/L) and rualn (15.0 g/L) was used as gelling agents. Plant height, number of branches and length of the branches were measured.

Table 6: Different concentrations of Albert’s solution and Maxicrop solution used as supplement to basal salts

Albert’s solution g/L	Maxicrop mL/L
2.0	1.0
2.0	2.0
2.0	3.0
3.0	1.0
3.0	2.0
3.0	3.0
4.0	1.0
4.0	2.0
4.0	3.0

ROOT INDUCTION OF *IN VITRO* SHOOTS

In vitro rooting

In vitro produced shoots (at least 10 cm long) were transferred to different rooting media to facilitate root induction. Different concentration of growth regulators in MS medium were tested as in follow:

- 1.1 Shoots were transferred in to full and half strength MS medium supplemented with different growth regulator combinations IBA (0.5 – 2.0 mg/L) + IAA (0.5 – 2.0 mg/L), IBA (0.5 – 2.0 mg/L) + NAA (0.5 – 2.0 mg/L), and NAA (0.5 – 2.0 mg/L) + IAA (0.5 – 2.0 mg/L). Activated charcoal (2.0 g/L) was added to all the media. Cultures were incubated in 16 h light.
- 1.2 As no root induction was observed in any of the combinations used in 1.1, elongated shoots were transferred to full strength MS medium supplemented with higher concentrations of IBA (20.0, 22.0, 24.0, 26.0, 28.0 and 30.0 mg/L) and activated charcoal (2.0 g/L).

- 1.3 *In vitro* elongated shoots were inoculated into half strength MS medium supplemented with NAA (0.5 and 1.0 mg/L) + IBA (0.5 and 1.0 mg/L) together with adenine sulphate (0.1g/L) and activated charcoal (2.0g/l). Cultured were incubated in both 16 h day light and complete dark conditions.
- 1.4 *In vitro* elongated shoots were inoculated into half strength MS medium supplemented with NAA (0.25, 0.5 mg/L) & IBA (0.25, 0.5mg/L), charcoal (2.0 g/L) in the presence of adenine sulphate (0.1g/L) and without adenine sulphate. Cultures were incubated in both light and dark conditions.
- 1.5 *In vitro* elongated shoots were inoculated into half strength & quarter strength MS medium containing different concentrations of IAA, NAA & IBA, charcoal (2.0g/l), adenine sulphate (0.1g/L). Cultures were incubated in both light and dark conditions.

All the data were collected in two weeks intervals.

***Ex vitro* rooting**

- 2.1 *In vitro* elongated shoots were incubated in 25.0 mg/L IBA for 48 h as a pulse treatment and potted in different potting media (a). wet coir dust only, b). Sand: soil 1:1, c). sand: soil: coir dust 1: 1: 0.5,d). Sand: coir dust 2:1/2) and followed acclimatization process. Data were collected in three days intervals.
- 2.2 Successfully established shoots in *in vitro* conditions were potted in different potting media (a)wet coir dust only, b) sand: soil 1:1,c) sand: soil: coir dust 1: 1: 0.5, d) sand: coir dust 2:1/2) without following any growth regulator treatments and followed acclimatization process. Data were collected in three days intervals

SOMATIC EMBRYOGENESIS

Explants were collected in the morning and washed in soap water for 5 min and kept under running tap water for 30 min. They were dipped in a solution of Captan[™] (2.0 g/L) for 30 min. Then explants were washed in 15% Clorox for 15 min, after that in 70% ethanol for 10 min, each followed by three successive washings in sterile distilled water.

MS medium was used as the basal medium and the pH was adjusted to 5.8. Commercial jelly moss (8.0 g/L) was used as the solidifying agent to reduce the cost per plant. Unless otherwise stated, cultures were incubated at 25±1⁰C in 16h photoperiod. Completely Randomized Design (CRD) was used in all experiments with 20 replicates in each treatment.

Determination of best explant source for embryonic callus induction

For the selection of best explant source for callus initiation nodal segments (2.0 cm long), mature and immature seeds and leaf discs (1.0 cm²) were used. After surface sterilization they were cultured in MS medium supplemented with 2.5 mg/L 2, 4 - D and 3.0 mg/L kin. Growth regulator free MS medium was used as the control. They were incubated in dark at 25±1⁰C. Mean callus diameter and percentage of callus production were measured in two weeks intervals over a period of 8weeks.

Determination of best growth regulator combination for embryonic callus induction from nodal segments

Nodal segments were transferred to MS medium supplemented with different combinations of kin (1.00 – 3.00 mg/L) and 2,4-D (1.00 – 3.50 mg/L). Growth regulator free MS medium was used as the control. Cultures were incubated in dark at 25±1⁰C. Mean callus diameter was measured in every two weeks interval over a period of eight weeks.

Determination of best growth regulator combination for somatic embryo induction

Embryonic calli (2.0 cm) were transferred into MS medium supplemented with different combinations of kin (0.25 -1.50 mg/L), IAA (0.25 -1.00 mg/L) and BAP (0.25 -2.00 mg/L) (Table 7). Growth regulator free MS medium was used as the control. Cultures were incubated in dark at 25±1⁰C. Number of somatic embryos per callus was counted in every two week interval over a period of 8 weeks.

Table 7: Different growth regulator combinations tested for somatic embryo induction

Treatment code	BAP (mg/L)	IAA (mg/L)	kin (mg/L)
SEI1	0.25	0.25	-
SEI 2	0.25	0.25	0.25
SEI 3	0.25	0.50	-
SEI 4	0.25	0.50	0.25
SEI 5	0.50	0.25	0.25
SEI 6	0.50	0.50	0.25
SEI 7	1.00	0.50	0.50
SEI 8	1.00	1.00	1.00
SEI 9	1.50	1.00	1.00
SEI 10	1.50	1.50	1.50
SEI 11	2.00	1.00	1.00
SEI 12	2.00	1.50	1.00
SEI 13	2.00	1.50	1.50
SEI 14	2.00	1.00	1.50

Determination of best growth regulator combination for the somatic embryo germination

In vitro induced embryos were transferred to MS medium supplemented with a range of GA₃ (0.5 mg/L – 3.5 mg/L). Growth regulator free medium was used as the control. Percentage of somatic embryo germination was observed in every two weeks interval over a period of eight weeks.

Determination of best growth regulator combination for the plantlet development from germinated somatic embryos

In vitro germinated somatic embryos were transferred to the MS medium supplemented with a range of BAP (0.2– 0.6 mg/L) and IAA (0.1– 0.4 mg/L) (Table 8). Growth regulator free MS medium was used as the control. Cultures were incubated in 16 h photo period at 25±1⁰C. Number of plantlets developed was counted in two weeks interval over a period of eight weeks.

Table 8: Different growth regulator combinations tested for plantlet development from germinated somatic embryos

Treatment code	BAP (mg/L)	IAA (mg/L)
SEP 1	0.2	0.1
SEP 2	0.2	0.2
SEP 3	0.2	0.3
SEP 4	0.2	0.4
SEP 5	0.4	0.1
SEP 6	0.4	0.2
SEP 7	0.4	0.3
SEP 8	0.4	0.4
SEP 9	0.6	0.1
SEP 10	0.6	0.2
SEP 11	0.6	0.3
SEP 12	0.6	0.4

CELL AND HAIRY ROOT CULTURES

Establishment of cell cultures

Cell cultures were established by placing callus in MS liquid medium supplemented with 2.5 mg/L 2,4 D and 2.0 mg/L kin. Cultures were placed in 100 rpm on a shaker and then increased the speed to 250 rpm. Cultures were incubated at 25±1°C in dark.

Establishment of hairy root culture

Callus was placed in liquid MS medium supplemented with 2.5 mg/L 2,4 D and 2.0 mg/L kin and 1.0 mg/L IAA. Cultures were placed in 100 rpm on a shaker and then increased the speed to 250 rpm. Cultures were incubated at 25±1°C in dark.

iv. RESULTS/OUTPUTS:

Establishment of aseptic cultures

Development of surface sterilization procedure for seeds

Results are summarized in Table 9. Among all tested treatments it was observed that T3 is the best surface sterilization method that could be used in sterilization of young green seeds. Seeds exposed to 20% Clorox and the seeds exposed to 15% Clorox for longer (20 min.) showed less survival.

Table 9: Percentage of seeds without contamination

Treatment code	Teepol	Running Tap water	Fungicide (10.0 ml/L)	Clorox		70% Ethanol	Success %
				Concentration	Exposure Time		
T1	5 min	30 min	30 min	10%	10 min	10 min	40%
T2	5 min	30 min	30 min	10%	15 min	10 min	60%
T3	5 min	30 min	30 min	10%	20 min	10 min	100%

Surface sterilization of nodal segments and shoot tips

Explants washing in 15% Clorox for 15 min. Followed by 10 min. in 70% EtOH (T3) found to be the best treatment for surface sterilization of nodal segments and shoot tips. And this treatment assures the 100% survival of explants without any contamination facilitating the growth of the tissues (Table 10).

Table 10: Percentage explants survived without contamination

Treatment code	Soap water	Running tap water	Fungicide Concentration 1.0 g/L	Clorox		70% Ethanol	Percentage Success
				concentration	Time		
T1	5 min	30 min	30 min	20%	15 min	10 min	0
T2	5 min	30 min	30 min	20%	10 min	10 min	40%
T3	5 min	30 min	30 min	15%	15 min	10 min	100%
T4	5 min	30 min	30 min	15%	20 min	10 min	60%
T5	5 min	30 min	30 min	15%	10 min	10 min	40%

DIRECT ORGANOGENESIS

Determination of best growth regulator combination for callus induction from cotyledonary parts

Callus initiation was successful with cotyledon parts in all tested treatments. Callus induction was observed in MS medium supplemented with 1.0 mg/L 2,4-D and 0.5 mg/L BA (Table 11). Embryo elongation and shoot induction were also observed (Figure 1A-D).

Table 11: Different growth regulator combinations used for callus induction from cotyledons

Treatment code	2,4-D (mg/L)	BA (mg/L)	Response of cotyledon
T1 (Control)	0.0	0.0	Shoot elongation + Callus
T2	0.5	0.0	Shoot elongation + Callus
T3	1.0	0.0	Shoot elongation + Callus
T4	0.0	0.5	Shoot elongation + Callus
T5	0.5	0.5	Shoot elongation + Callus
T6	1.0	0.5	Callus
T7	0.0	1.0	Shoot elongation
T8	0.5	1.0	Shoot elongation
T9	1.0	1.0	Shoot elongation

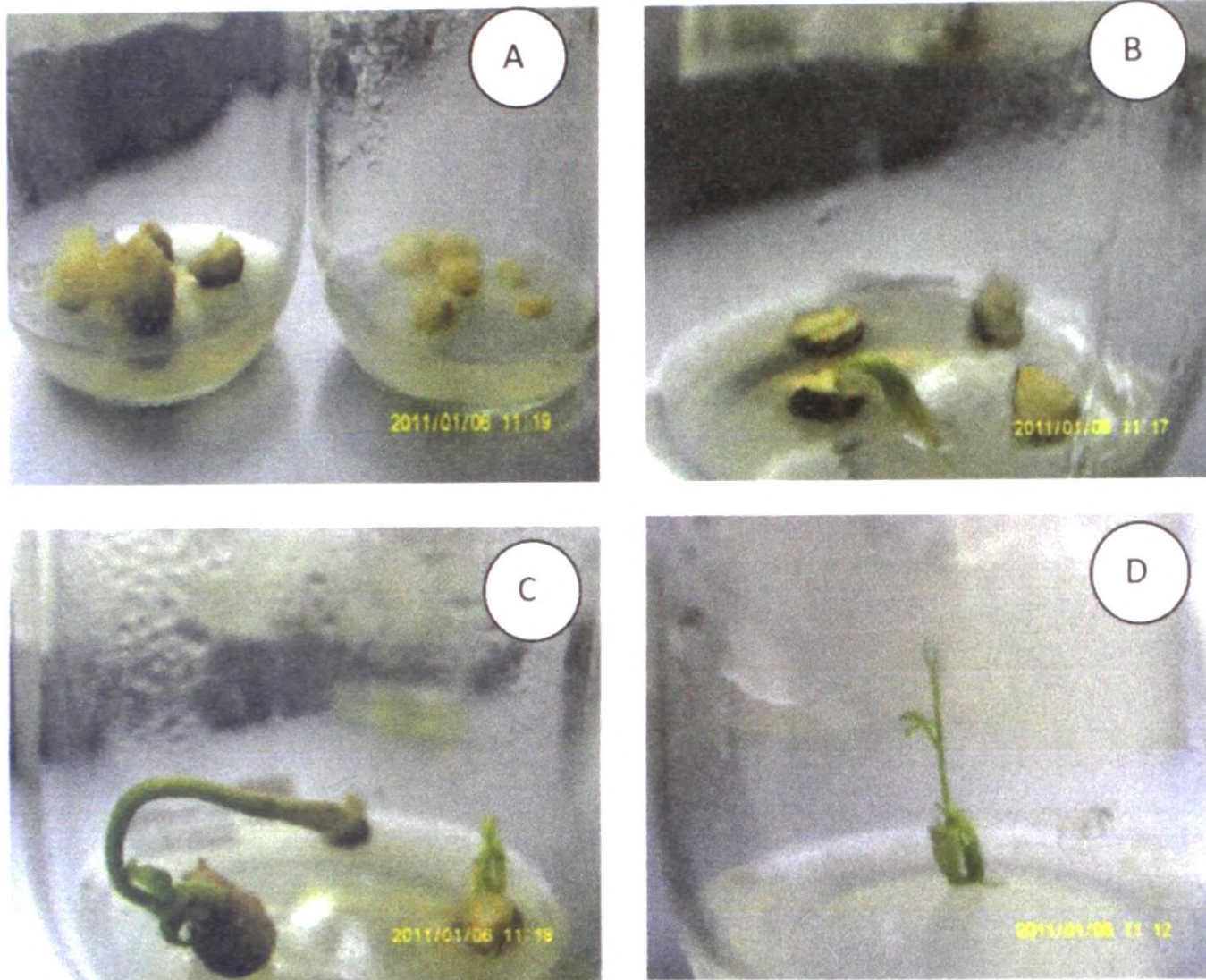


Figure 1: A) Callus induction from cotyledons, B) Initiation of shoots from seeds, C) *In vitro* seed germination, D) Elongated shoots

Determination of best basal medium and the growth regulator combination for shoot induction from nodal segments

It was observed that MS is more suitable for shoot induction from both single and double node explants. Increasing the level of BA from 0.5 to 2.0 significantly reduced the number of shoots induced (Table 12). Single node explants found to be better over double node explants and produced higher number of shoots per explant (Figure 2). MS medium supplemented with 0.5 mg/L BA (T1) found to be the best medium for shoot induction producing highest number of shoots per explants (20.3 ± 0.02).

Table 12: Number of shoots / axillary buds induced in each explants after 6 weeks of incubation

Treatment code	Composition	No. of nodes / explant	Mean number of shoots / axillary buds/explant \pm SE
T1	MS + 0.5 mg/L BA	1	20.3\pm0.02
T2	MS + 1.0 mg/L BA	1	16.4 \pm 0.01
T3	MS + 1.5 mg/L BA	1	14.2 \pm 0.02
T4	MS + 2.0 mg/L BA	1	12.2 \pm 0.01
T5	MS + 0.5 mg/L BA	2	16.3 \pm 0.02
T6	MS + 1.0 mg/L BA	2	12.4 \pm 0.01
T7	MS + 1.5 mg/L BA	2	11.2 \pm 0.02
T8	MS+ 2.0 mg/L BA	2	9.2 \pm 0.01
T9	WPM + 0.5 mg/L BA	1	11.5 \pm 0.03
T10	WPM + 1.0 mg/L BA	1	9.6 \pm 0.02
T11	WPM + 1.5 mg/L BA	1	7.2 \pm 0.03
T12	WPM + 2.0 mg/L BA	1	6.1 \pm 0.01
T13	WPM + 0.5 mg/L BA	2	8.7 \pm 0.01
T14	WPM + 1.0 mg/L BA	2	5.2 \pm 0.03
T15	WPM + 1.5 mg/L BA	2	5.2 \pm 0.02
T16	WPM + 2.0 mg/L BA	2	3.6 \pm 0.03
LSD 5%			0.02

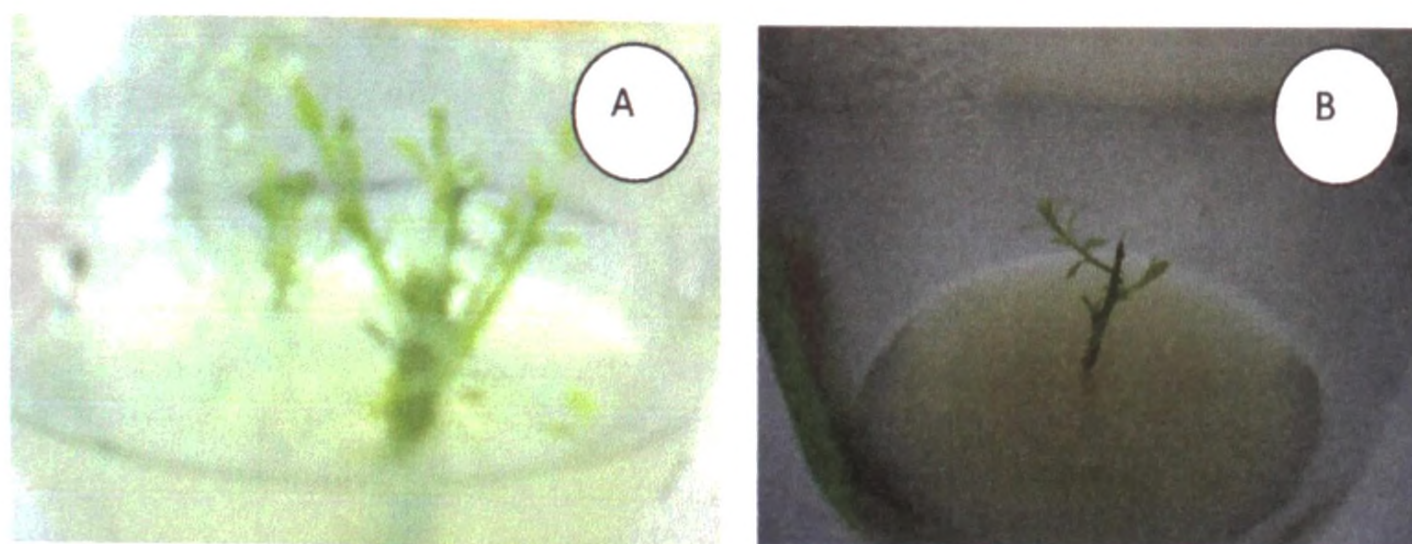


Figure 2: Multiple shoot induction from A) Single node and B) Double node explants in WPM supplemented with 0.5 mg/L BA after 3 weeks

Determination of the best growth regulator combination for multiplication

Initiation of shoots and multiplication from nodal segments in MS medium supplemented with different growth regulator combinations were observed after 14 days of incubation period in 16 h photoperiod at $25^{\circ}\text{C} \pm 1$ in all tested treatments. The results obtained are summarized in Table 13.

Table 13: The effect of BAP on shoot induction and multiplication from nodal segments of *Santalum album* after 12 weeks of incubation in 16 h photo period at $25 \pm 1^{\circ}\text{C}$

BAP (mg /L)	Plant height (cm)	Number of branches	Branch length (cm)
0.10	$0.67 \pm 0.04^{\text{a}}$	3.87 ± 0.24	0.63 ± 0.04
0.15	$0.67 \pm 0.04^{\text{a}}$	8.33 ± 0.44	1.13 ± 0.29
0.20	1.27 ± 0.09	8.60 ± 0.51	1.35 ± 0.05
0.25	1.61 ± 0.07	12.67 ± 0.39	1.61 ± 0.06
0.30	$0.86 \pm 0.04^{\text{b}}$	9.67 ± 0.05	$0.91 \pm 0.04^{\text{c}}$
0.35	$0.83 \pm 0.04^{\text{b}}$	9.00 ± 0.57	$0.90 \pm 0.04^{\text{c}}$
LSD 5%	0.01	0.02	0.01

* Measurements are the means of twenty replicates

*Means within the columns with same latter are not significantly different

Analysis of Variance showed that there was a significant difference between the mean increment of plant height of the cultured nodal segments, mean number of branches and mean branch length per shoots. MS medium supplemented with 0.25 mg/L BA showed highest shoot growth with highest mean increment of plant height (1.61 ± 0.07 cm) after 12 weeks of incubation. Highest mean number of branches (12.67 ± 0.39) and highest branch length (1.61 ± 0.06 cm) were also observed in MS medium supplemented with 0.25 mg/L BA (Figure 3). The medium supplemented with 0.20 mg /L BA showed the second highest shoot induction and multiplication with 1.27 ± 0.09 cm of mean plant height, 8.6 ± 0.51 mean number of branches and 1.35 ± 0.05 cm of mean branch length per shoot.



Figure 3: Multiplication of *in vitro* established shoot in MS medium supplemented with 0.25 mg/L BA

LOW COST MEDIA FORMULATION

Formulation of low cost culture media

Sago as an alternative gelling agent

When sago was powdered and added to the medium best solidification was observed at 18% level. Lower than 18% sago causes the sloppiness of the medium and as sago concentration increases it causes the reduction in multiple shoot induction. However it was observed adding 18% sago (Figure 4) as solidifying agent is better than adding 8% agar as it induced higher number of shoots/ explant (Table 14). And also use of sago cut down the cost of the culture medium too.

Table 14: Number of shoots/ explant after 16 weeks of incubation in Sago at different concentrations in MS medium supplemented with 0.25 mg/L BA

Treatment code	Concentration of Sago (%)	Mean number of shoots per explant±SE
T0 (Control)	8% agar	19.6±0.2
T1	18	22.8±0.1
T2	20	14.3±0.1
T3	22	14.5±0.1
T4	24	11.6±0.3
T5	26	7.1±0.2
LSD 5%		0.20



Figure 4: Growth of shoots in cultures with 18% sago as solidifying agent after 2 weeks of incubation

Albert’s solution and Maxicrop combination as alternative media components for shoot induction medium

Results are summarized in Table 15. Mixture of Albert solution and Maxicrop (2.0 :1.0) produced the highest number of shoots (16.2±0.02) and this was lower than the control (MS basal medium). However the difference was nonsignificant. All the other tested Albert solution and Maxicrop combinations produced lower number of shoots and those were significantly lower than Control and T1.

Table 15: Mean number of shoots induced when different concentrations of Albert's solution and Maxicrop solution used as an alternative media

Treatment code	Albert's solution (g/L)	Maxicrop (mL/L)	Mean number of shoots±
T1	2.0	1.0	17.62±0.01
T2	2.0	2.0	16.20±0.02
T3	2.0	3.0	1136±0.10
T4	3.0	1.0	10.41±0.02
T5	3.0	2.0	10.50±0.11
T6	3.0	3.0	10.21±0.21
LSD 5%			0.12

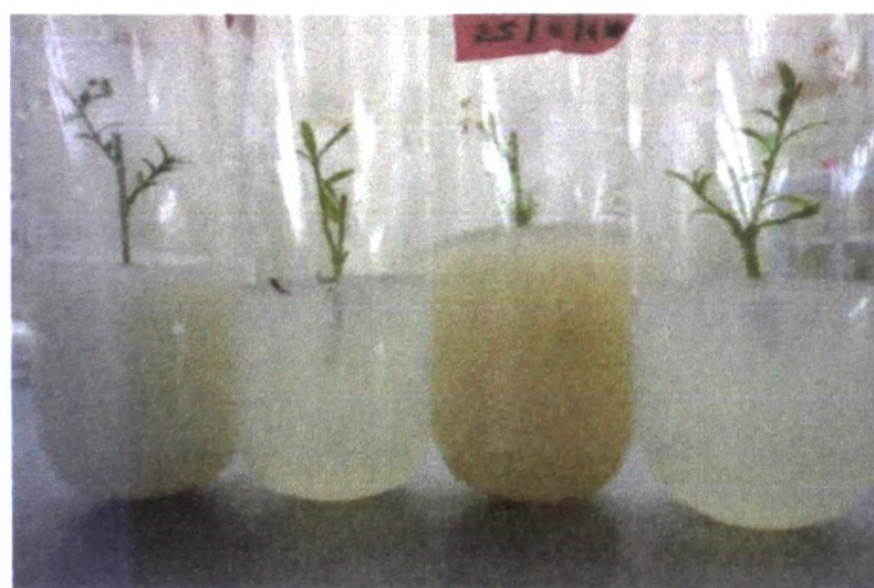


Figure 5: Growth of shoots in different low cost media

Determination of suitable concentrations of Albert's solution and Maxicrop combination as substitutes to basal salt mixtures with Sago, kurakkan (*Eleusine coracana*), rulan and jelly moss as gelling agents

Table 17-20 summarises the results of the experiment. Shoot initiation was observed in all the tested media after 2 weeks of incubation period. Analysis of Variance showed that there was a significant difference between mean plant height, mean number of branches and mean branch

length in different treatments. Highest mean plant height (1.61 ± 0.07 cm), highest mean number of branches (12.67 ± 0.39) and highest branch length (1.61 ± 0.06 cm) were observed when MS medium was used as the basal medium (Control). Media containing jelly moss as the gelling agent supplemented with 2.0 g/L Albert's solution with 1.0 mL/L Maxicrop with 0.25 mL/L BA showed the highest mean plant height of 1.37 ± 0.09 cm, highest number of branches per shoots with 9.67 ± 0.05 branches of mean number of shoots and highest branch length of 1.25 ± 0.05 cm (Table 20). In the medium solidified with 15.0% rulan supplemented with 2.0 g/L Albert's solution and 1.0 mL /L Maxicrop showed the second highest plant height (1.35 ± 0.50 cm), highest number of branches (9.25 ± 0.25) and highest branch length (1.02 ± 0.25 cm) (Table 19). When 20.0% kurakkan, was used as the solidifying agent in the medium supplemented with 2.0 g/L Albert's solution and 1.0 mL/L Maxicrop, the highest mean plant height (1.33 ± 0.02 cm), highest number of branches (8.32 ± 0.44) per shoot was observed (Table 18). The medium supplemented with 2.0 g/L Albert's solution and 1.0 mL/L Maxicrop solidified with 18% sago found to be the best low cost medium for shoot induction and showed the highest mean plant height (1.20 ± 0.07 cm).

Table 17: Shoot initiation in low cost basal media and Sago (18%) as gelling agent

Treatment code	Albert's solution g/L	Maxicrop mL/L	18% Sago		
			Plant height (cm)	Number of branches	Branch length (cm)
Tc	-	-	1.61 ± 0.07	12.67 ± 0.39	1.61 ± 0.06
TL1	2.0	1.0	1.20 ± 0.07	9.23 ± 0.01	0.98 ± 0.05
TL2	2.0	2.0	0.81 ± 0.05	2.30 ± 0.03	0.91 ± 0.03
TL3	2.0	3.0	1.05 ± 0.20	6.23 ± 0.44	0.62 ± 0.10
TL4	3.0	1.0	0.85 ± 0.07	10.11 ± 0.06	0.86 ± 0.06
TL5	3.0	2.0	0.95 ± 0.03	6.27 ± 0.15	0.89 ± 0.05
TL6	3.0	3.0	0.45 ± 0.10	4.52 ± 0.01	0.54 ± 0.11
TL7	4.0	1.0	0.67 ± 0.04	8.53 ± 0.23	0.95 ± 0.03
TL8	4.0	2.0	0.21 ± 0.03	6.12 ± 0.05^a	0.88 ± 0.21
TL9	4.0	3.0	0.27 ± 0.04	6.13 ± 0.48^a	0.50 ± 0.02

Table 18: Shoot initiation in low cost basal media and kurakkan (20%) as gelling agent

Treatment code	Albert's solution g/L	Maxicrop mL/L	20% Kurakkan		
			Plant height (cm)	Number of branches	Branch length (cm)
Tc	-	-	1.61 ± 0.07	12.67 ± 0.39	1.61 ± 0.06
TL10	2.0	1.0	0.92 ± 0.12	8.25 ± 0.08	0.63 ± 0.03 ^c
TL11	2.0	2.0	0.65 ± 0.05 ^b	4.57 ± 0.67	1.20 ± 0.06
TL12	2.0	3.0	0.54 ± 0.01	7.88 ± 0.33	0.81 ± 0.09
TL13	3.0	1.0	1.02 ± 0.03	3.54 ± 0.51	0.92 ± 0.12
TL14	3.0	2.0	1.33 ± 0.02	8.32 ± 0.44	0.55 ± 0.33
TL15	3.0	3.0	0.65 ± 0.01 ^b	6.57 ± 0.39	0.12 ± 0.45
TL16	4.0	1.0	0.67 ± 0.03	5.50 ± 0.35	0.35 ± 0.94
TL17	4.0	2.0	1.10 ± 0.12	7.23 ± 0.34	0.57 ± 0.25
TL18	4.0	3.0	0.89 ± 0.09	2.31 ± 0.12	0.63 ± 0.66 ^c

Table 19: Shoot initiation in low cost basal media and rulan (15%) as gelling agent

Treatment code	Albert's solution g/L	Maxicrop mL/L	15% Rulan		
			Plant height (cm)	Number of branches	Branch length (cm)
Tc	-	-	1.61 ± 0.07	12.67 ± 0.39	1.61 ± 0.06
TL19	2.0	1.0	1.35 ± 0.50	9.25 ± 0.25	1.02 ± 0.25
TL20	2.0	2.0	0.95 ± 0.02	6.45 ± 0.34	0.68 ± 0.33 ^d
TL21	2.0	3.0	0.85 ± 0.09	4.33 ± 0.68	0.35 ± 0.14
TL22	3.0	1.0	1.10 ± 0.05	6.89 ± 0.23	0.45 ± 0.05
TL23	3.0	2.0	0.65 ± 0.21	2.54 ± 0.44	0.68 ± 0.11 ^d
TL24	3.0	3.0	0.31 ± 0.20	8.74 ± 0.66	0.97 ± 0.65
TL25	4.0	1.0	0.10 ± 0.54	6.33 ± 0.36	0.13 ± 0.22
TL26	4.0	2.0	0.15 ± 0.33	5.78 ± 0.05	0.56 ± 0.35
TL27	4.0	3.0	0.45 ± 0.05	6.23 ± 0.48	0.24 ± 0.65

Table 20: Shoot initiation in low cost basal media and jelly moss (8%) as gelling agent

Treatment code	Albert's solution g/L	Maxicrop mL/L	8% Jelly moss		
			Plant height (cm)	Number of branches	Branch length (cm)
Tc	-	-	1.61 ± 0.07	12.67 ± 0.39	1.61 ± 0.06
TL28	2.0	1.0	1.37 ± 0.09	9.67 ± 0.05	1.25 ± 0.05
TL29	2.0	2.0	0.95 ± 0.03	6.23 ± 0.36	0.88 ± 0.01
TL30	2.0	3.0	0.57 ± 0.11 ^e	2.11 ± 0.74	0.96 ± 0.03
TL31	3.0	1.0	0.75 ± 0.35	8.89 ± 0.61	0.31 ± 0.01
TL32	3.0	2.0	0.23 ± 0.45	5.62 ± 0.33	0.48 ± 0.11
TL33	3.0	3.0	0.88 ± 0.32	7.44 ± 0.41	0.55 ± 0.04
TL34	4.0	1.0	0.36 ± 0.45	3.52 ± 0.32	0.76 ± 0.06
TL35	4.0	2.0	0.56 ± 0.06 ^e	6.54 ± 0.44	0.21 ± 0.11
TL36	4.0	3.0	0.34 ± 0.14	7.89 ± 0.27	0.44 ± 0.06

* Means within the columns with same letter are not significantly different

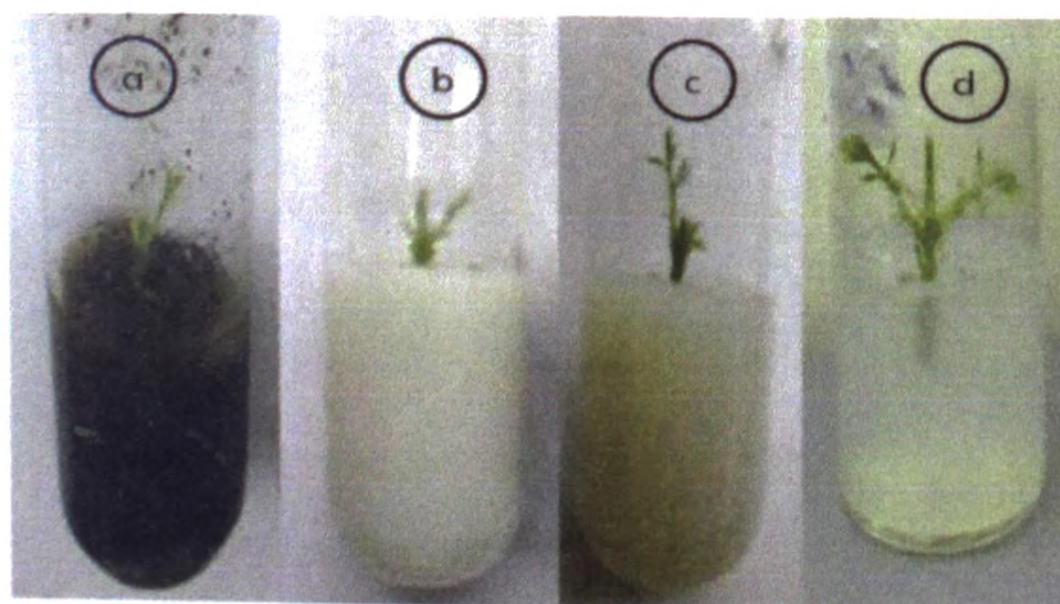


Figure 6: Single node shoot segments in Albert's (2.0 g/L) and Maxicrop (1.0 mL/L) combination medium with low cost gelling agents a) 20% kurakkan, b) 18% sago, c) 15% rulan and d) 8% jelly moss.

ROOT INDUCTION OF *IN VITRO* SHOOTS

In vitro rooting

Although number of different treatments have been used to induce *in vitro* roots, the success rate was very low. And the roots produced were weak and the number was very low (less than 3 roots per shoot).

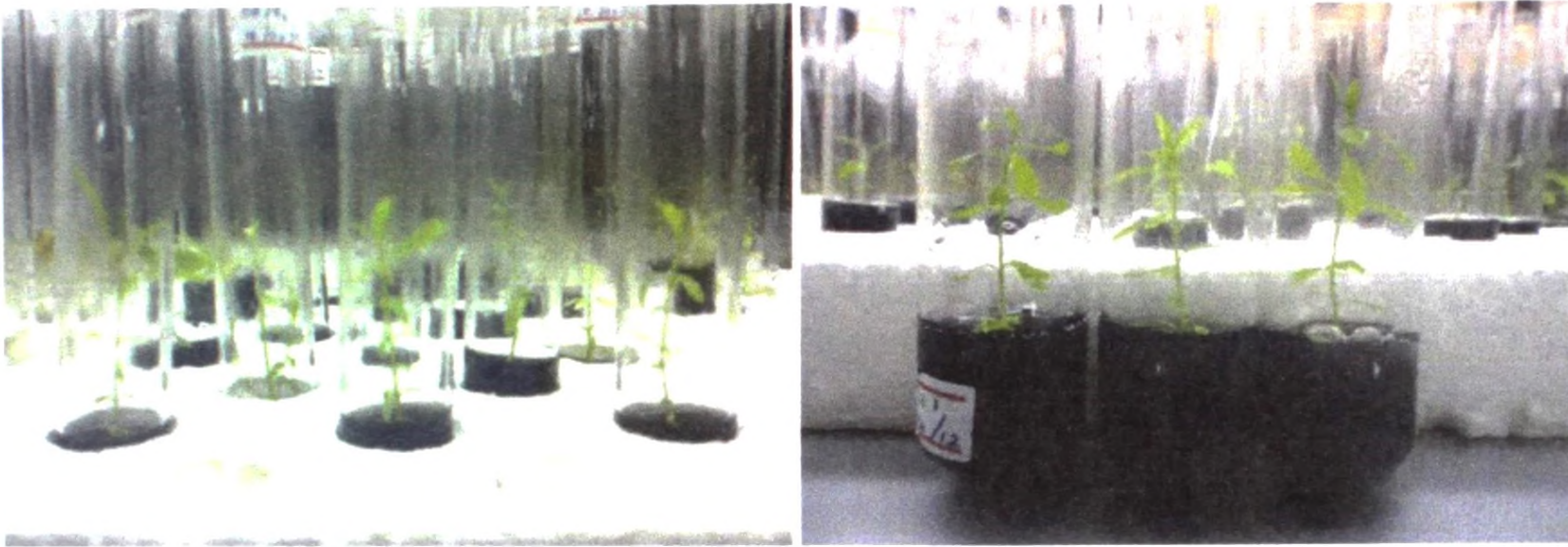


Figure 7 : Shoots in different rooting media

Ex vitro rooting

Ex vitro root induction was more successful than *in vitro* rooting (Figure 8a). Roots produced were healthier and strong and survive well in potting media during acclimatization (Figure 8 b&c).

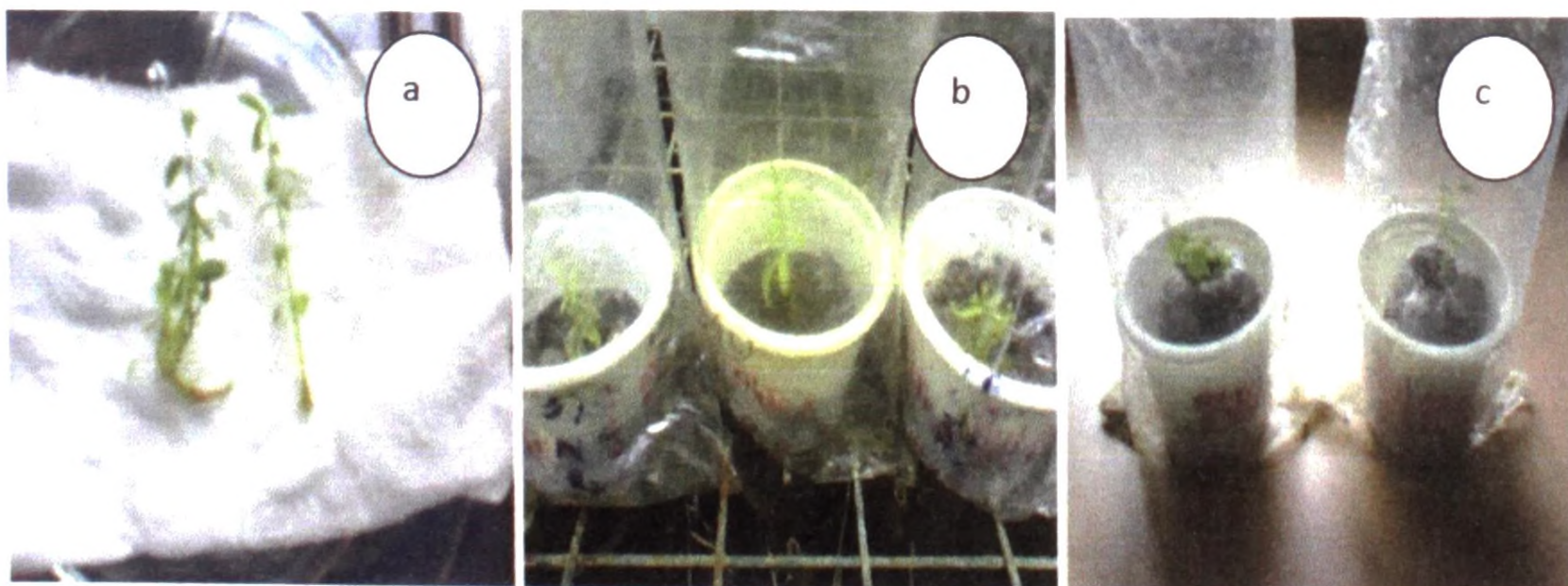


Figure 8: a) Successfully established shoots , b) & c) Plantlets in different potting media

Determination of best explant source for embryonic callus induction

Results are summarised in Table 21.

Table 21: Mean callus diameter obtained with different explant sources in MS medium supplemented with 2.5 mg /L 2, 4 –D and 3 mg /L kin after 8 weeks of incubation.

Explant type	Mean callus diameter (cm)	% of callus production
Nodal segments	2.48 ± 0.05^a	95.64 ± 0.12
Immature seeds	0.54 ± 0.07 ^b	85.52 ± 0.09 ^d
Mature seeds	0.53 ± 0.05^b	95.34 ± 0.11
Leaf discs	0.82 ± 0.10 ^c	85.32 ± 0.17 ^d
LSD	0.11	0.13

*Measurements are the means of twenty replicates ± SE

*Means within the columns with same latter are not significantly different

All the types of explant material which have been tested were able to produce callus in MS medium supplemented with 2.5 mg/L 2, 4 – D and 3.0 mg/L kin, at 25 ± 1⁰C in dark. Calli obtained were pale cream colour and had fragile texture. No callus induction was observed in growth regulator free MS medium (Control). There was a significant difference between mean callus diameters and percentages of callus induction in different treatments. After 8 weeks of incubation period the highest callus diameter (2.48 ± 0.15 cm) (Figure 7) and highest percentage of callus production (95.64 ± 0.12%) was obtained from the nodal segments and significantly higher than all tested explant types. Leaf discs cultured on the same medium showed the second highest callus diameter (0.82 ± 0.10 cm) while mature seeds showed the second highest percentage of callus production (95.34 ± 0.11). Mature seeds produced the callus with lowest diameter (0.54 ± 0.07 cm) (Figure 7a –c).

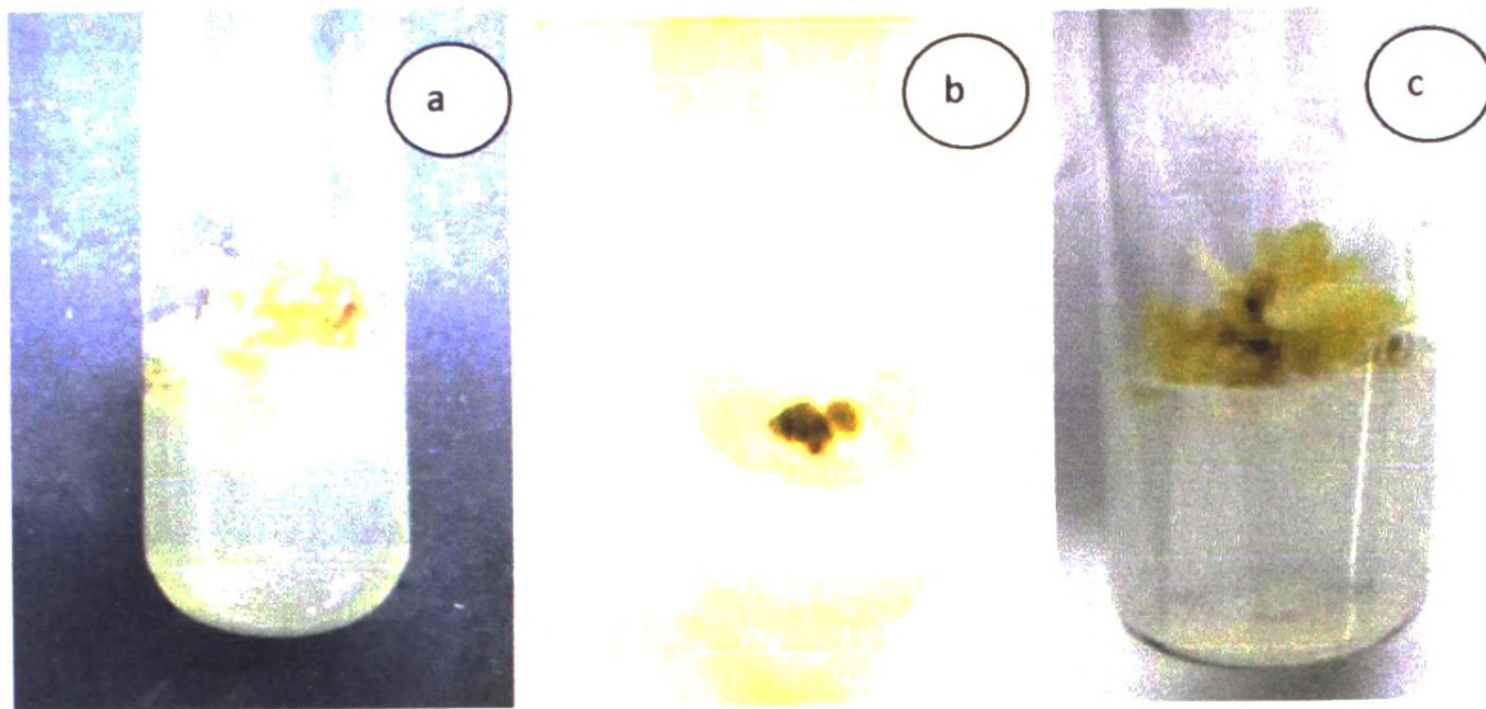


Figure 7: a) Embryonic callus derived from the nodal segments, b) immature seeds, c) mature seeds in MS medium supplemented with 2.5 mg/L 2, 4 – D and 3.0 mg/L and kin after 8 weeks of incubation

Determination of best growth regulator combination for embryonic callus induction

Increase in mean callus diameter over a period of 8 weeks in MS medium supplemented with different combinations of 2,4-D and kin is summarised in Table 22.

Table 22: Induction of embryonic callus from nodal segment explants cultured in MS medium with different concentrations of 2, 4 – D and kin.

Growth regulator combinations	Mean callus diameter \pm SE (cm)			
	Second week	Forth week	Sixth week	Eighth week
2, 4 – D : kin				
1.0 :1.0	0.28 \pm 0.04	0.40 \pm 0.05	0.52 \pm 0.06	0.63 \pm 0.07
1.0 :1.5	0.44 \pm 0.05 ^a	0.60 \pm 0.05	0.76 \pm 0.06	0.97 \pm 0.08
1.0 :2.0	0.57 \pm 0.07 ^b	0.79 \pm 0.07	1.06 \pm 0.08	1.32 \pm 0.08
1.0 :2.5	0.53 \pm 0.06	0.72 \pm 0.08	0.99 \pm 0.10	1.25 \pm 0.12
1.0 :3.0	0.57 \pm 0.08 ^b	0.75 \pm 0.09	0.94 \pm 0.10	1.17 \pm 0.12
1.0 : 3.5	0.61 \pm 0.06	0.87 \pm 0.06	1.12 \pm 0.07	1.37 \pm 0.07
1.5:1.0	0.44 \pm 0.04 ^a	0.65 \pm 0.05	0.78 \pm 0.05	0.94 \pm 0.06
1.5: 1.5	0.38 \pm 0.05	0.56 \pm 0.05	0.74 \pm 0.06	0.90 \pm 0.06

1.5 : 2.0	0.36 ± 0.04	0.52 ± 0.06	0.62 ± 0.06	0.81 ± 0.07
1.5 : 2.5	0.59 ± 0.09	0.90 ± 0.10	1.14 ± 0.11	1.35 ± 0.11
1.5 : 3.0	0.75 ± 0.09	0.96 ± 0.10	1.21 ± 0.11	1.41 ± 0.11
1.5 : 3.5	0.79 ± 0.08	1.06 ± 0.08	1.31 ± 0.09	1.60 ± 0.07
2.0 : 1.0	0.65 ± 0.09	1.03 ± 0.12	1.27 ± 0.12	1.58 ± 0.12
2.0 : 1.5	0.92 ± 0.11	1.32 ± 0.12	1.57 ± 0.13	1.83 ± 0.16
2.0 : 2.0	1.16 ± 0.13	1.52 ± 0.12 ^f	1.75 ± 0.11 ⁱ	1.97 ± 0.11
2.0 : 2.5	1.21 ± 0.14	1.51 ± 0.15 ^f	1.82 ± 0.16	2.08 ± 0.15
2.0 : 3.0	1.25 ± 0.14 ^c	1.57 ± 0.12	1.86 ± 0.11	2.20 ± 0.10
2.0 : 3.5	1.35 ± 0.13 ^d	1.74 ± 0.12	2.01 ± 0.10	2.36 ± 0.10
2.5 : 1.0	1.53 ± 0.15	1.92 ± 0.15	2.21 ± 0.12	2.49 ± 0.11
2.5 : 1.5	1.83 ± 0.12	2.11 ± 0.12 ^g	2.40 ± 0.11	2.69 ± 0.12
2.5 : 2.0	1.75 ± 0.14	2.22 ± 0.11	2.56 ± 0.10 ^k	2.89 ± 0.08
2.5 : 2.5	2.04 ± 0.11	2.31 ± 0.10	2.55 ± 0.09 ^k	2.91 ± 0.10
2.5 : 3.0	2.11 ± 0.12	2.41 ± 0.12	2.77 ± 0.11	3.22 ± 0.10
2.5 : 3.5	1.87 ± 0.11	2.13 ± 0.10 ^g	2.32 ± 0.11	2.51 ± 0.09
3.0 : 1.0	1.49 ± 0.12	1.76 ± 0.11	1.97 ± 0.11	2.12 ± 0.10 ^m
3.0 : 1.5	1.26 ± 0.13 ^c	1.44 ± 0.10	1.66 ± 0.13	1.90 ± 0.14
3.0 : 2.0	1.39 ± 0.10	1.66 ± 0.10 ^h	1.84 ± 0.10	2.06 ± 0.09
3.0 : 2.5	1.62 ± 0.10	1.97 ± 0.09	2.26 ± 0.06	2.46 ± 0.07
3.0 : 3.0	1.15 ± 0.11	1.45 ± 0.11	1.67 ± 0.11	1.88 ± 0.10
3.0 : 3.5	1.23 ± 0.10 ^e	1.51 ± 0.10 ^f	1.78 ± 0.10	2.04 ± 0.11
3.5 : 1.0	1.20 ± 0.12	1.47 ± 0.12	1.68 ± 0.11	1.91 ± 0.12
3.5 : 1.5	1.34 ± 0.07 ^d	1.58 ± 0.07	1.78 ± 0.07	1.99 ± 0.08
3.5 : 2.0	1.29 ± 0.09	1.56 ± 0.11	1.84 ± 0.11 ⁱ	2.12 ± 0.11 ^m
3.5 : 2.5	1.22 ± 0.12 ^e	1.50 ± 0.13	1.76 ± 0.12 ^j	2.02 ± 0.12

3.5 : 3.0	1.38 ± 0.09	1.64 ± 0.08 ^h	1.84 ± 0.09 ⁱ	2.00 ± 0.09
3.5 : 3.5	1.62 ± 0.08	1.87 ± 0.08	2.06 ± 0.09	2.34 ± 0.09
LSD	0.12	0.11	0.11	0.12

*Measurements are the means of twenty replicates ± SE

*Means within the columns with same letter are not significantly different

Calli initiation from nodal explants in MS medium supplemented with different growth regulator combinations were observed after 10 days of incubation period in dark, in all tested treatments. No callus induction was observed in the growth regulator free MS medium (Control). Analysis of Variance showed that there was a significant difference between mean callus diameters in different treatments. After 8 weeks of incubation period the highest mean callus diameter (3.22 ± 0.1 cm) was obtained in the medium supplemented with 2.5 mg/L 2, 4 – D and 3.0 mg/L kin and significantly higher than all tested treatments. MS medium supplemented 2.5 mg/L 2, 4 –D and 2.5 mg/L kin was the second best growth regulator combination for the induction of embryonic callus with a mean callus diameter of 2.91 ± 0.10 cm. MS medium supplemented with 1.0 mg/L 2, 4 –D and 1.0 mg/L kin showed the least mean callus diameter (0.63 ± 0.07 cm) within 8 weeks of incubation period. After twelve weeks of incubation period calli became brownish color and lost the fragile texture.

Determination of best growth regulator combination for induction of somatic embryos

Induction of somatic embryos was observed in all the tested treatments after two weeks of incubation period in dark at $25 \pm 1^{\circ}\text{C}$. The results are summarized in Table 23. No embryo induction was observed in the growth regulator free MS medium. Analysis of Variance showed that there was a significant difference between the number of somatic embryos induced per callus ($\sim 2 \text{ cm}^2$) in different treatments. MS medium supplemented with 0.5 mg /L BA , 1.0 mg/L IAA and 0.5 mg/L kin showed the highest number of somatic embryo induction (10.20 ± 0.66) and significantly higher than all tested treatments. The least number of somatic embryo inductions (6.93 ± 0.74) was observed in the medium supplemented with 0.25 mg/L BAP, 0.25 mg/L IAA and 0.25 mg/L kin after 8 weeks of incubation period at $25 \pm 1^{\circ}\text{C}$ in complete dark. According to the results the somatic embryo induction increases with the kin, BAP and IAA concentrations but started to reduce when the concentrations of BAP: IAA: kin are 0.50: 1.00: 1.00.

Table 23: The effect of growth regulator combination of BAP, IAA and kin on somatic embryo induction in MS medium after eight weeks of incubation period at $25 \pm 1^{\circ}\text{C}$ in complete dark

Growth regulator combination (mg/L)	Number of somatic embryos induced per callus \pm SE
BAP: IAA: kin	
0.00: 0.00: 0.00	0.00 \pm 0.00
0.25: 0.25: 0.25	6.93 \pm 0.76
0.25: 0.25: 0.50	7.67 \pm 0.70
0.50: 0.50: 0.25	8.87 \pm 0.58
0.50: 0.50: 0.50	8.80 \pm 0.46
0.50: 1.00: 0.25	9.13 \pm 0.91
0.50: 1.00: 0.50	10.20 \pm 0.66
0.50: 1.00: 1.00	8.07 \pm 0.73
LSD	0.50

*Measurements are means of twenty replicates \pm SE

*All the means of the column were significantly different from each other

Determination of best growth regulator combination for somatic embryo germination

Somatic embryo germination was observed in all the tested treatments after two weeks of incubation in 16 h photoperiod. The results are summarized in Table 24. No embryo germination was observed in growth regulator free MS medium. Embryo germination from embryonic callus was successful and best medium for somatic embryo germination was MS medium supplemented with 2.0 mg/L GA₃ (Table 24). Analysis of Variance showed that there was a significant difference between mean percentages of embryo germination in different treatments. In the medium supplemented with 2.0 mg/L GA₃ showed the highest mean percentage of embryo germination (58 ± 4.13) (Figure 13) and significantly higher than all other treatments. The medium supplemented with 1.5 g/L GA₃ showed secondly highest mean percentage of germination (50.67 ± 4.41). The least germination percentage of 18.00 ± 3.12 was observed in the

medium supplemented with 0.5 mg/L GA₃ (Table 24). It is significantly lesser than all other treatments.

Table 24: The effect of different combinations of GA₃ on percentage of somatic embryo germination after eight weeks of incubation period at 25 ± 1°C in 16 h photoperiod

GA ₃ (mg/L)	Mean percentage of somatic embryo germination ± SE
0.0	00.00 ± 0.00
0.5	18.00 ± 3.12
1.0	42.00 ± 3.27 ^a
1.5	50.67 ± 4.41
2.0	58.98 ± 4.13
2.5	42.00 ± 4.80 ^a
3.0	42.67 ± 4.82
3.5	44.67 ± 5.29
LSD	3.50

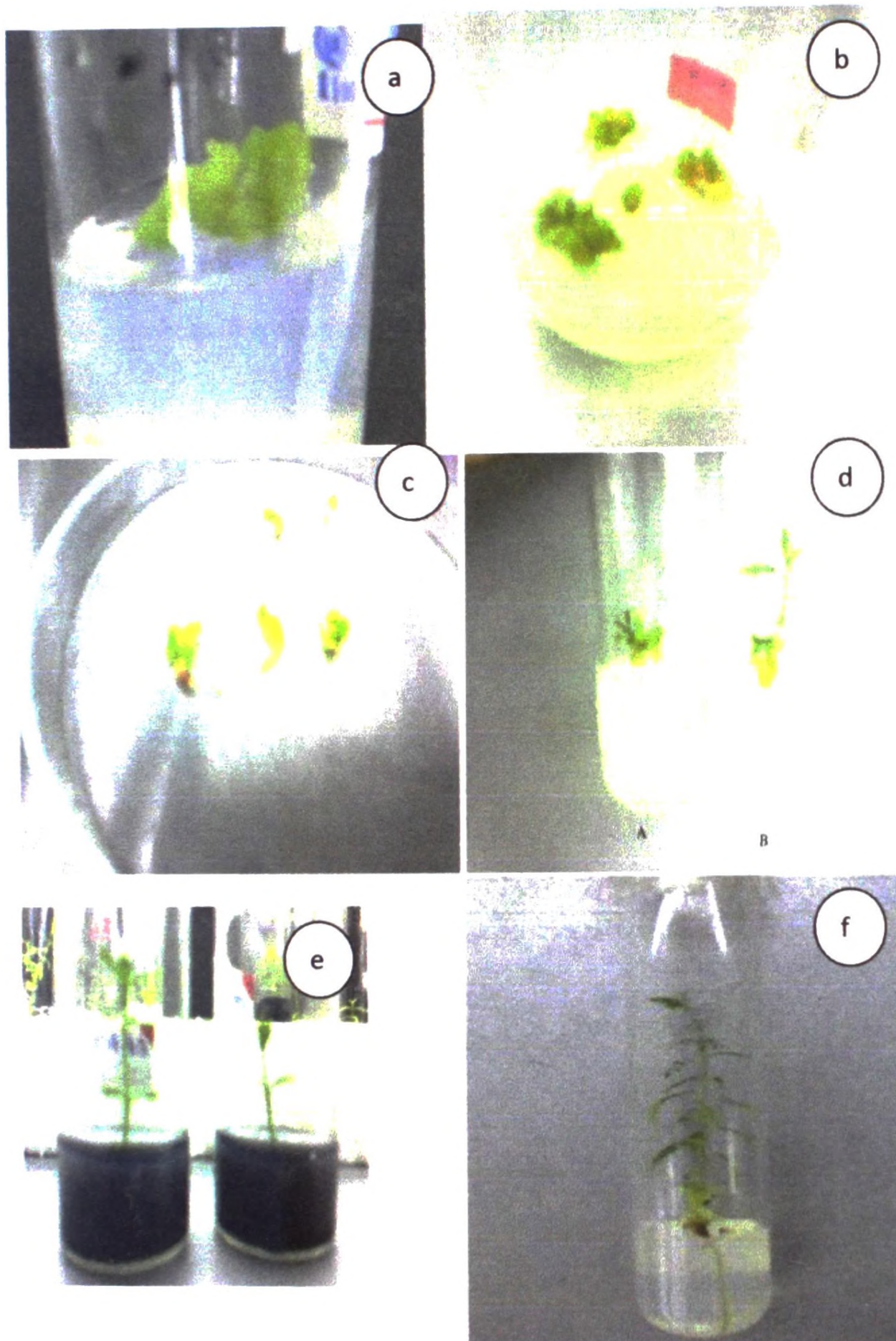


Figure 13: a). Germinating somatic embryos, b) Greening of embryonic callus, c) Germinated somatic embryos with less formed tap root, d-f). Growing plantlets with elongated roots from somatic embryos

Roots were observed in shoots produced through somatic embryogenesis (Figure 13d-f) and the plantlets survived well in acclimatization medium.

Determination of best growth regulator combination for the plantlet development from germinated somatic embryos

Results are summarized in Table 25. Plantlets development was observed in all the tested treatments within two weeks of incubation in 16 h photo period. Analysis of Variance showed that there was a significant difference between plantlets development in different treatments. Among the tested treatments, medium supplemented with 0.4 mg/L BAP and 0.2 mg/L IAA growth regulator combination was able to produce highest percentage number of plantlets (mean percentage of 76.67 ± 3.03) with healthy root and shoot part (Figure 13f). And second best plant growth regulator combination for plantlet development (mean percentage of 58.67 ± 4.01) was 0.6 mg/L BAP and 0.2 mg/L IAA. The least mean percentage of plantlet development (44.67 ± 2.56) showed in the medium supplemented with 0.2 mg/L BAP and 0.3 mg/L IAA (Table 25).

Table 25: Percentage plantlet development in the presence of different concentrations of BAP and IAA

Growth regulator combination (mg/L)	Percentage of plantlet development \pm SE
BAP: IAA	
0.0 : 0.0	00.00 \pm 0.00
0.2 : 0.1	57.33 \pm 5.73
0.2: 0.2	46.00 \pm 4.76
0.2 : 0.3	54.67 \pm 3.89
0.2 : 0.4	44.67 \pm 2.56
0.4 : 0.1	55.00 \pm 4.32
0.4 : 0.2	76.67 \pm 3.03
0.4 : 0.3	56.67 \pm 3.86 ^a
0.4 : 0.4	56.67 \pm 4.22 ^a
0.6 : 0.1	55.71 \pm 3.75
0.6 : 0.2	58.67 \pm 4.01
0.6 : 0.3	56.00 \pm 3.57
0.6 : 0.4	50.67 \pm 4.22
LSD	2.20

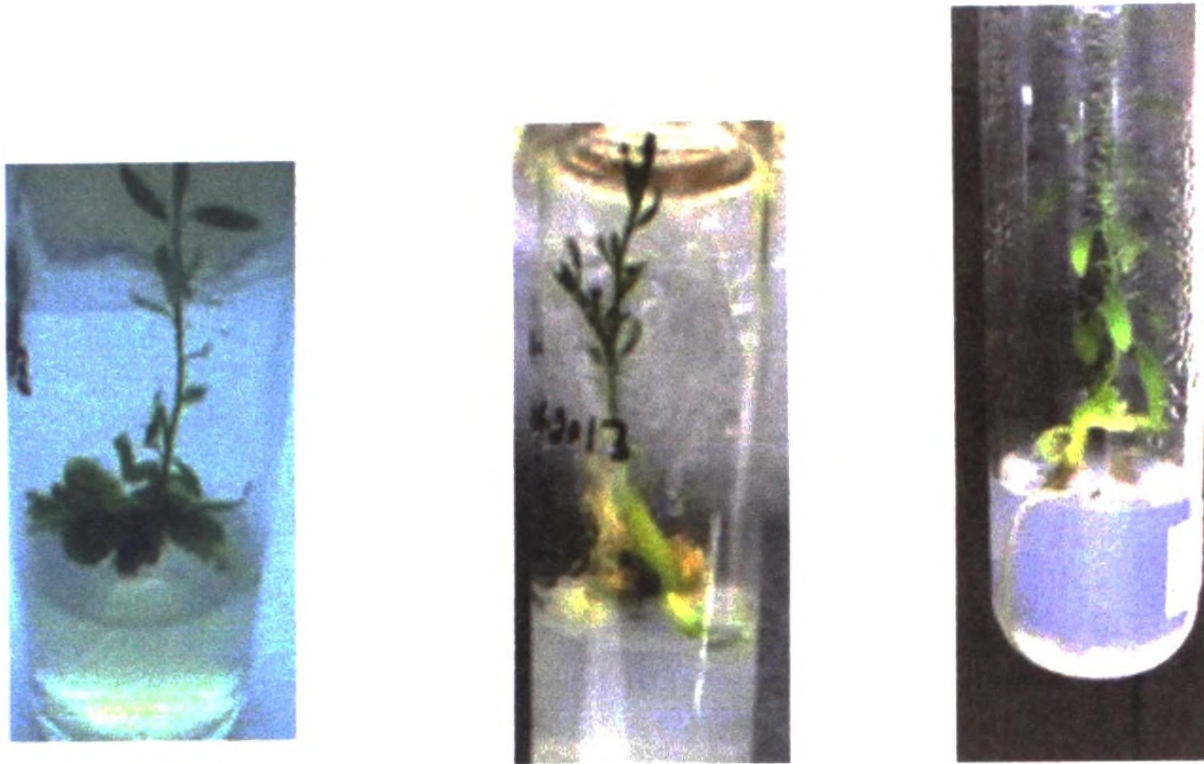


Figure 14: Plantlet developed from germinated embryos in MS medium supplemented with 0.4 mg/L BAP and 0.2 mg/L IAA

Plantlets potted in soil: sand: coir dust 1:1:1 was died and few of shoots (10%) potted in coir discs & shoots (~10%) in sand: coir dust 2: 1/2 survived successfully. They started producing new leaves.



Figure 15: Plantlets establish in coir pellets

ESTABLISHMENT OF CELL CULTURES

Cell cultures were established MS liquid medium containing 2.5 mg/L 2,4 D and 2.0 mg/L kin. When callus placed in the liquid medium it was observed that 100 rpm speed was not enough for separation of cells but 250 rpm was the optimum to obtain a homogenous cell culture.



Figure16: Cell culture established from callus

Hairy root culture

Hairy root culture was established in MS medium supplemented with 2.5 mg/L 2,4 D and 2.0 mg/L kin and 1.0 mg/L IAA.

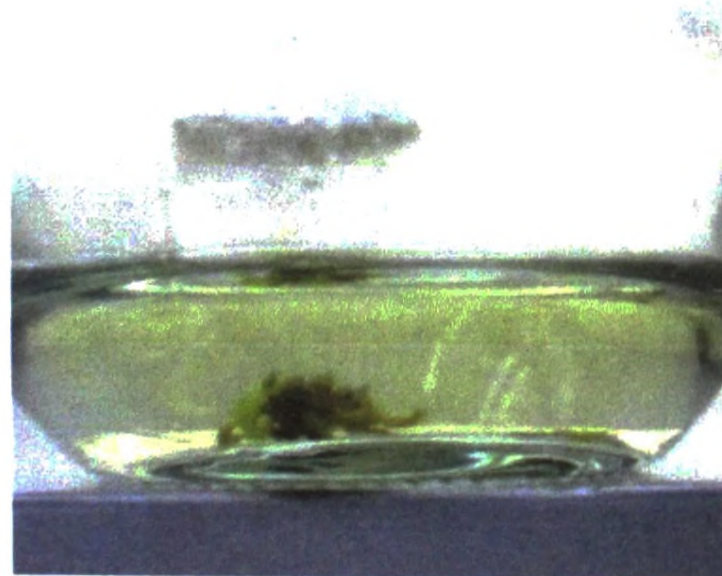


Figure 17: Hairy root cultures established

v. CONCLUSIONS

- MS medium supplemented with 1.0 mg/L 2,4-D found to be the best medium for induction of callus from cotyledonary parts.
- Shoot induction was observed in MS medium supplemented with 0.5 mg/L BA while decreasing the level of BA to 0.25 mg/L enhances the multiple shoot induction.
- For shoot growth use of 18% sago as gelling agent was better over 8% commercial agar and the shoots were more healthy and greener in the presence of sago.
- Albert solution: Maxicrop at the ratio of 2: 1 could be an alternative to MS basal medium for induction of shoots of *S. Album*.
- Albert solution: Maxicrop at the ratio of 2: 1 with 18% sago as solidifying agent found to be the best low cost medium for shoot regeneration in *S. Album*
- Although *in vitro* rooting was less successful *ex vitro* root induction was successful. However the percentage rooting was low. Plantlets regenerated survived well in the potting mixture (Sand: coir dust 2: ½) during acclimatization.
- Nodal segments and mature seeds were the best explants sources for embryonic callus induction.
- Embryonic callus induction was successful in MS medium supplemented with 2.5 mg/L 2,4 D and 3.0 mg/L kin after 4 weeks of incubation
- MS medium supplemented with 0.5 mg/L BAP, 1.0 mg/L IAA and 0.5 mg/L kin induced somatic embryos from embryonic calli
- MS medium supplemented with 2.0 mg/L GA₃ is the best medium for somatic embryo germination.
- Plantlet regeneration was observed in MS medium supplemented with 0.4 mg/L BAP and 0.2 mg/L IAA
- Sand: coir dust (2: ½) was the best acclimatization medium
- For cell culture establishment 100 rpm is not suitable while increasing the speed to 250 rpm is better for cell culture establishment.
- Hairy root cultures could be established by transferring the callus in to MS medium supplemented with 2.5 mg/L 2,4-D, 2.0 mg/L kin and 1.0 mg/L IAA

vi. PROBLEMS IF ANY, ENCOUNTERED

Changing the Research assistance regularly when the students get permanent jobs was the main problem encountered. This greatly affected the work. However it is suggested to formulate a mechanism to charge the postgraduate registration fee from students (if they utilize the NSF funds) if they leave the grant before completion of the grant/ post graduate studies.

vii. MAJOR FINDINGS AND FOLLOW UP ACTIVITIES:

- Shoot induction and multiplication was successful and the multiplication rate was very high in *S. album* however rooting was the problem and the success rate was low.
- *Ex vitro* rooting was a better alternative and the plantlets survived well in acclimatization medium
- Somatic embryogenesis is the best method for *in vitro* plantlet regeneration of *S. album* and the plantlets regenerated were healthy and survived well in potting mixtures used.
- Albert's solution: Maxicrop (2:1) with 18% sago as solidifying agent could be used as an alternative low cost medium for *in vitro* shoot induction and multiplication of *S. album*
- It is possible to establish both cell and hairy root cultures from callus
- It is needed to study rooting process in detail and the field establishment of propagated plantlets.
- Production of santalol need to be determined in cell and hairy root cultures

4. SECTION 4:

IMPACT OF RESEARCH RESULTS

i. RELEVANCE OF RESULTS ACHIEVED TO SCIENTIFIC ENHANCEMENT:

Tissue culture protocol for *S. album* for large scale plant regeneration has not been studied before. Although root induction was not successful, developing the rooting medium would facilitate the plantlet regeneration *in vitro* as the multiplication rate obtained was high.

ii. RELEVANCE OF RESULTS ACHIEVED TO NATIONAL/SOCIO-ECONOMIC DEVELOPMENT:

When the protocol is developed for rooting of *in vitro* shoots and if plantlets regenerated through somatic embryogenesis could survive well in the field this could be used for commercial scale cultivations.

iii. DISSEMINATION/APPLICATION OF RESEARCH OUTPUT:

Touchwood Investment Ltd, which initially has the interest in purchasing the protocol is now collapsed however Jiffy International is willing to continue with protocol development.

5. SECTION 5:

MISCELLANEOUS

i. LIST OF MAJOR EQUIPMENTS ACQUIRED DURING THE PROJECT PERIOD AND THEIR FUNCTIONALITY:

Magnetic stirrer with hotplate (5"x7") – Sigma Z26,212-9

Bench top shaker

Stereo microscope

ii. LIST OF PUBLICATIONS/COMMUNICATIONS ARISING FROM THE PROJECT AND/ OR PRESENTATIONS MADE AT SEMINARS, WORKSHOPS ETC.:

One manuscript has submitted to Journal of National Science Foundation and comments from reviewers have received. Another two full papers are in the process of finalising.

6. SECTION 6:

SUMMARY STATEMENT OF EXPENDITURE:

Grant Number -: RG/2009/BT/02
Statement of Funds Received & Expenditure
For the quarter ending -25.03. 2014

	Total Funds Received RS.		Total Expenditure Rs.	Balance Rs
Personnel - Research Student	934,000.00		807,932.75	126,067.25
Technical Assistant	-		-	-
Other	36,000.00		8,000.00	28,000.00
Equipment	450,000.00	12,387.20	462,387.20	-
Consumable	162,750.00	(98.20)	113,844.00	48,807.80
Lab Services	-		-	-
Travel & Subsistance	45,000.00	(12,289.00)	6,811.00	25,900.00
Miscellaneous	64,000.00		40,634.00	23,366.00
PG Registration fee	-		-	-
Calib. O f instruments	-		-	-
Other	-		-	-
Total	1,691,750.00	-	1,439,608.95	252,141.05

Unspent balance of the fund received

Fund Received	1,691,750.00
Actual Expenditure	1,439,608.95
Balance	252,141.05
Cash advance	-
Balance	252,141.05

Prepared By - *Ases 3/26*

Snr.Asst.Bursar/Accountant

Date *27.03.2014*

C.c -: Prof.S.Senarath - Dept.of Botany *[Signature]*

Senior Assistant Bursar
Faculty of Graduate Studies
University of Sri Jayawardenapura

7. SECTION 7:

i. GRATEE'S SIGNATURE:

Maruthi

ii. COMMENTS OF THE HEAD OF THE DEPARTMENT /SIGNATURE:

Recommended
[Signature]

iii. SIGNATURE OF THE VICE CHANCELLOR

Strongly recommended and forwarded

[Signature]
20/10/20

Dr. N. L. A. Karunaratne
Vice-Chancellor
University of Sri Jayewardenepura
Nugegoda, Sri Lanka.

National Digitization Project
National Science Foundation

Institute : National Science Foundation

1. Place of Scanning : Sanje (Private) Ltd, Hokandara

2. Date Scanned : 2017/04/18

3. Name of Digitizing Company : Sanje (Private) Ltd, No 435/16, Kottawa Rd,
Hokandara North, Arangala, Hokandara

4. Scanning Officer

Name : H.P.A.V. Caldera

Signature : K. Yeeb

Certification of Scanning

I hereby certify that the scanning of this document was carried out under my supervision, according to the norms and standards of digital scanning accurately, also keeping with the originality of the original document to be accepted in a court of law.

Certifying Officer

Designation : Information Officer

Name : Renuka Sugathadasa

Signature : K.P. Sugathadasa

Date :

"This document/publication was digitized under National Digitization Project of the National Science Foundation, Sri Lanka"