CHAPTER 3

Materials and Methods

MATERIAL AND METHODS

The chapter describes the materials and methods used for tissue culture studies in two tree species ie. *Oroxylum indicum* and *Stereospermum suaveolens* belonging to family Bignoniaceae. The methodology was divided into three sections ie. Seed germination, Regeneration and Synthetic seed studies.

3.1 Plant material and explant source

Certified seeds of both the tree species ie. *O.indicum* and *S.suaveolens* were procured from Rajpipla Forest division and used to raise seedlings explants.

Seeds were also used for raising *O. indicum* plants (2-3 months) as a source of nodal explants (Fig.6a). *S. suaveolens* plants of (1-2 year old) were procured from Rajpipla Forest division for nodal explants (Fig.6b).

3.2 Glassware

All the borosil glasswares were procured from local suppliers (Durga Scientific Ltd) and utilised for respective experiments. These are given below:

- The Erlenmeyer flasks of 50,100,150,250,500, 1000, 2000 ml capacity were used.
- Culture tubes (25x150mm) were utilised for culturing the explants and they were plugged with cotton bunks or plastic caps.
- Measuring cylinders of 25,50,100,1000 ml capacity were used.
- Beakers of 50,100,150,250,500 ml used.
- Petridishes 100 x15 mm size.

Washing of glasswares: The glasswares were initially dipped in chromic solution (Potassium dichromate and saturated solution prepared in conc.sulphuric acid) overnight and next day washed with liquid detergent followed by rinsing with tap water. A final wash was done with distill water and dried in hot air oven at 60ºC.

3.3 Chemicals

All chemicals, PGRs used were of high purity (AR grade) and were procured from various companies.

Fig. 6: *O.indicum* and *S.suaveolens* plants

a. *O. indicum* plants (2-3 months old) grown in garden pots

b. *S. suaveolens* (1-2 year old) plants

Different chemicals used are as follows:

- The media constituents of MS and WPM medium were procured from Sisco Research Laboratory (SRL) Mumbai, India.
- Carbon source ie. Sucrose pure was of SRL, Mumbai
- Gelling agents ie.Agar(SRL, Mumbai)
- Sodium alginate was of Sd fine chemicals, Mumbai and calcium chloride(SRL, Mumbai).
- The different PGRs (BAP extrapure AR, Kinetin extrapure AR, IAA, NAA, IBA and GA_3) used were of SRL Mumbai, India and TDZ of Sigma Life Science(USA).
- Additives like Casein hydrolysate (Himedia, Mumbai), Silver nitrate (Oum chemicals,Ahmedabad), Calcium pantothenate (SRL,Mumbai).
- Others chemicals like Labolene was from Fisher Scientific,Mumbai.
- Ascorbic acid, Citric acid, PVP, Streptomycin sulphate were of SRL,Mumbai and $HgCl_2$ from Qualigen Chemicals,Mumbai.

3.4 Instruments and Equipments

Major and minor instruments and equipment for carrying out tissue culture work were:

- Glass bead steriliser (Model BS-1000,Dent-Eq)
- Horizontal Autoclave –.
    - 400 x 600 mm (MAC company, Delhi)
    - 510 x 910 mm (Mediquip company,Vadodara)
- pH meter (Analab scientific, Vadodara)
- Single pan electronic balance (Sartorious, Mumbai)

Minor equipments :

- Distillation unit (Borosil)
- Hot Plate(Jay Scientific Instruments,Baroda)
- Hot air oven(Scientific industries, Mumbai)
- Micropipette:(20-100µl);(100-1000µl);(1000-5000µl)(Fischer scientific,Mumbai) .

Other things include: Parafilm (4 IN.x125 FT. paper backing produced by Bemis NA, based in Neenah,WI (United States) was used for sealing petridishes. Chromatographic paper sheets were utilised for making filter paper bridge for rooting.

3.5 Media composition

In the present studies Murashige and Skoog's ,1962 (MS) and Llyod and McCown, 1980 (WPM) media were used for establishing cultures.The media contains various macronutrients, micronutrients, vitamins etc in different concentrations. The composition of MS and WPM media is given in Table 3.

Table 3. Composition of MS and WPM medium

Constituents	MS	WPM
	(mg/1)	
Macronutrients (1x)		
$MgSO_4.7H_2O$	370	370
KNO_3	1900	-
NH_4NO_3	1650	400
$CaCl_2 2H_2O$	440	96
KH_2PO_4	170	170
$Ca(NO_3)_2 4H_2O$	-	556
Micronutrients(1X)		
H_3BO_3	6.2	6.2
$MnSO_4.4H_2O$	22.3	22.3
$ZnSO_4 7H_2O$	8.6	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4 5H_2O$	0.025	0.25
$CoCl_2.6H_2O$	0.025	-
KI	0.83	-
Na_2EDTA	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8
Vitamins and other supplements		
Thiamine HCl	0.5	1
Pyridoxine HCI	0.5	0.5
Nicotinic acid	0.5	0.5
Myo-Inositol	100	100
Glycine	2	-
Sucrose	3%	3%

3.6 Preparation of MS and WPM stocks

The different constituents of MS and WPM medium were prepared in the form of four different stock solutions. MS medium consist of stock A as major salts, stock B as minor salts, stock F as $FeSO_4$ and EDTA and Stock G as Vitamins.

The WPM medium differs only in macrosalts, so stock A of WPM was prepared separately and the other stocks B, G ,F were the same as of MS medium. All the stocks were prepared in distill water and the chemicals were dissolved one by one and finally the required volume was made up with distill water. The stocks were stored in amber coloured bottles at 4ºC and checked for contamination before use (Table.4).

Table 4. MS and WPM medium stocks

Stock	**MS medium**			**WPM medium**		
	Stock Concentration (X)	**Volume of Stock (ml)**	**Volume per litre (ml)**	**Stock concentration (X)**	**Volume of stock (ml)**	**Volume per litre (ml)**
Major salts (A)	10	500	50	5	500	100
Minor salts (B)	200	400	2	200	400	2
Vitamin (G)	200	400	2	200	400	2
$FeSO_4$ and EDTA (F)	100	200	2	100	200	2

3.7 Preparation of PGR Stocks

The media in addition to the nutrients are fortified with necessary plant growth regulators like auxins, cytokinins or gibberellins, to support good growth of tissues and organs. The requirement of these growth regulators is in µM concentration, and varies with the type of organ or tissue. In present work, the stocks of plant growth regulator (1 mM) were prepared by dissolving either 1N NaOH or 1N HCl, (Table.5)made to their respective volume with distill water and stored at 4 ºC. The PGRs were added to the medium in µM concentration and their conversion from mM to µM is given below.

$N_1V_1 = N_2 V_2$

Where,

N_1- Concentration of stock

V_1-Volume of stock

N_2-Concentration of medium

V_2-Volume of medium

Table 5. Preparation of 1mM PGR stocks

Growth Regulator	Molecular weight(g)	Disssolving Solvent
Cytokinins		
BAP	225.25	1N HCl
Kn	215.22	1N HCl
TDZ	220.2	1N NaOH
Auxins		
IAA	186.21	1N NaOH
IBA	346.37	EtOH
NAA	175.19	1N NaOH
Gibberllic Acid		
*GA_3	203.24	1N NaOH

*** GA_3 (Gibberllic acid) –** This hormone is thermolabile and loose its activity during autoclave and therefore it is prepared freshly during inoculation and added in require quantity in medium (The medium is boiled before inoculation and after adding GA_3 it is cooled).

3.8 Medium Preparation (MS/WPM)

Both the media were prepared by adding all the stocks, KH_2PO_4, Myo-inositol, sucrose and agar as follows:

Preparation of 1 litre medium

MS stock A (50ml) / WPM stock A (100ml)

↓

Add stock B (2ml)

↓

Add stock G (2ml)

↓

Add stock F (2ml)

↓

Dissolve KH_2PO_4 (170 mg)

↓

Then add Myo-inositol (100 mg)

↓

Add sucrose (3%)

↓

Add PGR as per requirement and then make up to the required volume

↓

Adjust medium to pH (5.8)

↓

Add agar (0.8%) and boil till it dissolves

↓

Dispense in culture tubes (20 ml) and flasks (50ml)

↓

Sterilize in autoclave for 25 min at 15 psi and 121ºC

↓

Keep medium in culture room overnight

In both species the work was divided into three sections:

I. **Seed germination studies**
II. **Regeneration studies**
III. **Encapsulation studies**

3.9 Section I: Seed germination studies *in Oroxylum indicum* and *Stereospermum suaveolens*

Seed germination studies in both the species were carried out as follows:

- Seeds were germinated in different substrates in both the species for calculating % germination.
- In *O. indicum*, seedlings growth was assessed by studying seedling length, collar diameter and biomass (fresh weight and dry weight).
- In *S. Suaveolens*, germination was very less compared to *O. indicum* therefore different germination parameters were evaluated to study the germination rate of seeds.

3.9.1 Seed treatment of *O.indicum and S.suaveolens* prior to germination

- The winged pericarp was removed from the seeds and then soaked overnight in distill water before placing in the different substrates.
- Next day seeds were kept in running water for 1 hour followed by washing with labolene.
- Then they were treated with bavistin for 2 min and surface sterilised with $HgCl_2$ for 2 min and rinsed with distill water three times after each treatment.
- Thereafter the seeds were placed for germination in different substrates

Germination of seeds was tried in the following substrate for selection of a suitable substrate (which could generate large number of seedlings):

- Cocopeat
- Soil
- Sand
- Cocopeat:Soil (1:1)
- Cocopeat:Sand (1:1)
- Filter paper
- Medium(MS and WPM)

3.9.2 Germination of *O.indicum* and *S.suaveolens* seeds in different substrates

❖ **Germination under natural conditions**

The seed germination in following substrates were carried out in Botanical garden.

- **Garden soil** was filled in pots and the seeds (five per pot) were placed in it.
- **Sand** was filled in polyethylene bags and one seed was placed in each bag.

❖ **Germination under Lab conditions**

➢ **Preparation of cocopeat substrate**

Dry cocopeat (100g) was soaked overnight in distill water (volume of water was four times that of dry weight).

- **Cocopeat, Cocopeat:Soil, Cocopeat:Sand**

Moist cocopeat was combined with soil and sand in 1:1 ratio and then kept in an aluminium tray which was covered with aluminium foil. These substrates were sterilised in autoclave for 45-50 min. After removing from autoclave the next day the substrate/s were filled in plastic root trainers (98 wells) in laminar air flow cabinet. After 30 minutes of irradiation under UV light the treated seeds were placed singly in each well.

After placing all the seeds bavistin solution was sprayed in each well to avoid fungal contamination and all the root trainers were kept in culture room at 25 ± 2 °C with 16 hr photoperiod.

- **Petridish with Filter paper:** The filter paper was also used as substrate for germination of seeds. It was placed in petridish and sterilised. 5-10 seeds were kept in each petridish and 5-10 ml distil water was poured in it.
- **Medium:** The **MS and WPM static** basal medium with sucrose 3% was prepared and dispended in 150 ml flask after sterilisation and 5 seeds were placed in each flask.

The above substrates were sterilised at 121°C and 15 psi for 25 min. All the Petridishes and flasks were kept in culture room at 25 ± 2 °C.

% germination was recorded after 4 weeks as follows in all the above substrates and were compared together.

% Germination = Number of seeds germinated/ Total number of seeds inoculated

3.9.3 Growth parameters in *O. indicum*

Following methodology was used for studying growth parameters:

- Seeds were soaked in distill water overnight by removing the wings of the seed and were germinated next day in sterilised cocopeat, cocopeat: soil (1:1),cocopeat: sand (1:1) substrates. Seeds were inoculated aseptically in each well of the root trainers containing respective substrates. At the same time approximately 5 ml of freshly prepared bavistin solution (200mg/l) was added to the wells to reduce contamination.
- For each planting substrate, the seed emergence was regularly checked and bavistin was sprayed three times in a week on the substrates and irrigated with distil water every alternate day. The entire root trainer was covered by a glass case to maintain humidity.

  Observations for different growth parameters like collar diameter, biomass (fresh weight and dry weight), and shoot as well as root length were recorded at weekly interval for each type of substrate by selecting five seedlings randomly. The data for all the parameters were taken after four weeks.

3.9.4 Germination parameters in *S.suaveolens*

The following different germination parameters were studied in both the species.

1. Mean daily germination (MDG) were calculated as follows (Panwar and Bhardwaj 2005):

   FG = n / N x 100

   MDG = FG / D,

   where *n* is the number of germinated seeds, *N* is the total number of seeds and *D* is the number of days to final germination.

2. The germination rate (GR) as well as germination index (GI) were calculated as follows (Panwar and Bhardwaj 2005):

$$GR = \sum_{i=1}^{n} n_i / t_i$$

$$GI = \sum_{i=1}^{n} n_i t_i / N$$

   where ni is the number of germinated seeds in day ti.

3.10 Section II: Regeneration studies in *O. indicum* and *S. suaveolens*

In regeneration studies the cultures were established on MS and WPM medium fortified with different PGRs utilising suitable explants.

3.10.1 Explant treatment

In both the tree species, cocopeat was suitable substrate for germinating seeds and therefore was used for generating seedlings explants for the regeneration studies.

The 20 days old seedlings of *O. indicum* (Fig.7a) and *S.suaveolens* (Fig.7b) as well as nodal explants of *O.indicum* (Fig.7c)and *S.suaveolens* (Fig.7d)were harvested and given the following treatments:

- The seedlings were placed in running water for 1 hour and nodal explants for 2 hours in both the species.
- These were washed with 1-2 drops of labolene for 5-10 min.
- Seedling were treated with PVP (100 mg/l) for 3 min, bavistin (200 mg/l) for 3 min and then with $HgCl_2$ (0.1 %) for 3 min.
- Nodal explants were treated with PVP (100 mg/l) for 3 min, bavistin (500 mg/l) and streptomycin (500 mg/l) for 5 min and then with $HgCl_2$ (0.1 %) for 5 min.
- Both the explants were rinsed with sterile water for three times after each treatment
- The following explants were cut in appropriate sizes and used to establish cultures:
    - Cotyledonary leaf (1×1 cm)
    - Cotyledonary node (1.5 cm)
    - Hypocotyl (1 cm)
    - Nodal explants (1.5 cm)

1.10.2 Establishment of cultures

Cultures were established in MS and WPM basal medium, and media fortified with different concentrations of individual cytokinin, combination of cytokinins and combination of cytokinin and auxin which are given below and the methodology for regeneration in both the species has been given in Fig.8.

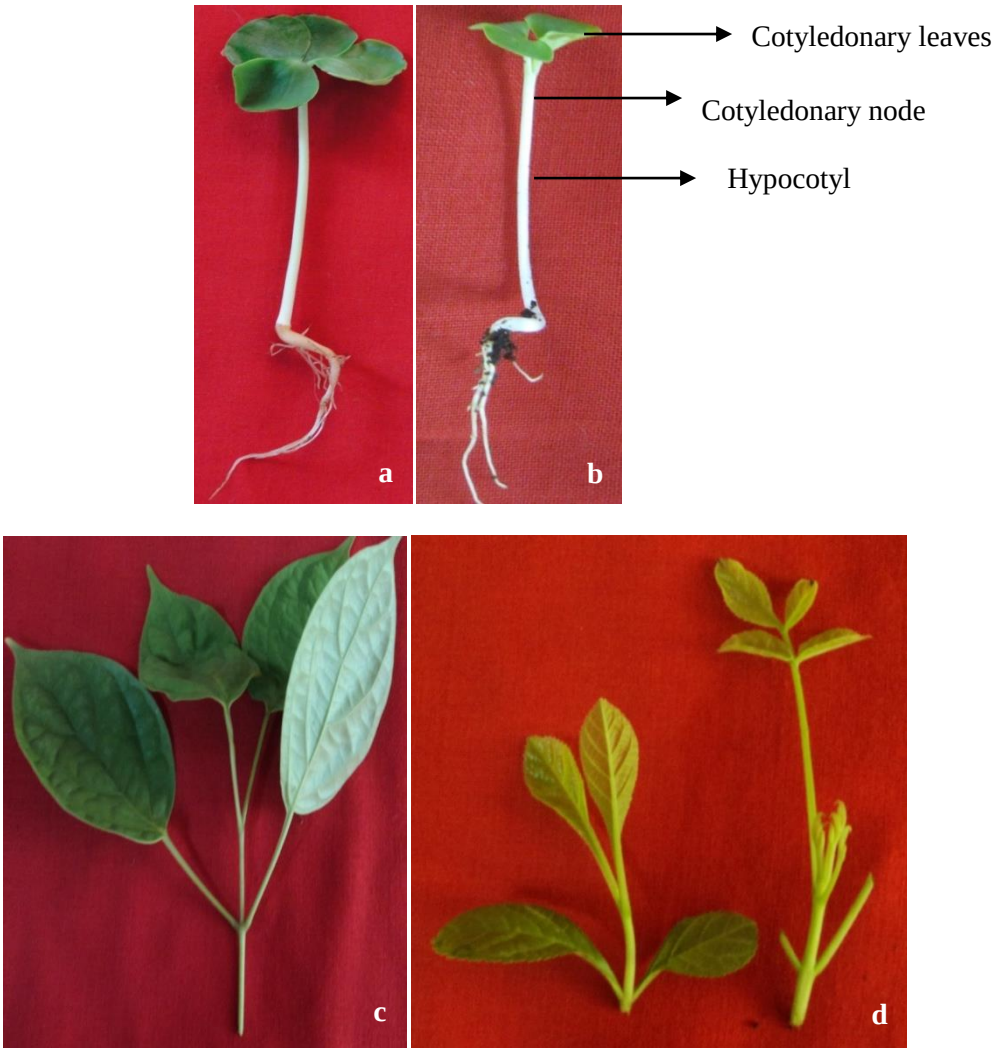

Fig.7 Explants of *O.indicum* and *S.suaveolens*

a. Single seedling of *O. indicum* with cotyledonary node, cotyledonary leaf and hypocotyl

b. Single seedling of *S.suaveolens* with cotyledonary node, cotyledonary leaf and hypocotyl

c. Nodal explants of *O. indicum*

d. Nodal explants of *S.suaveolens*

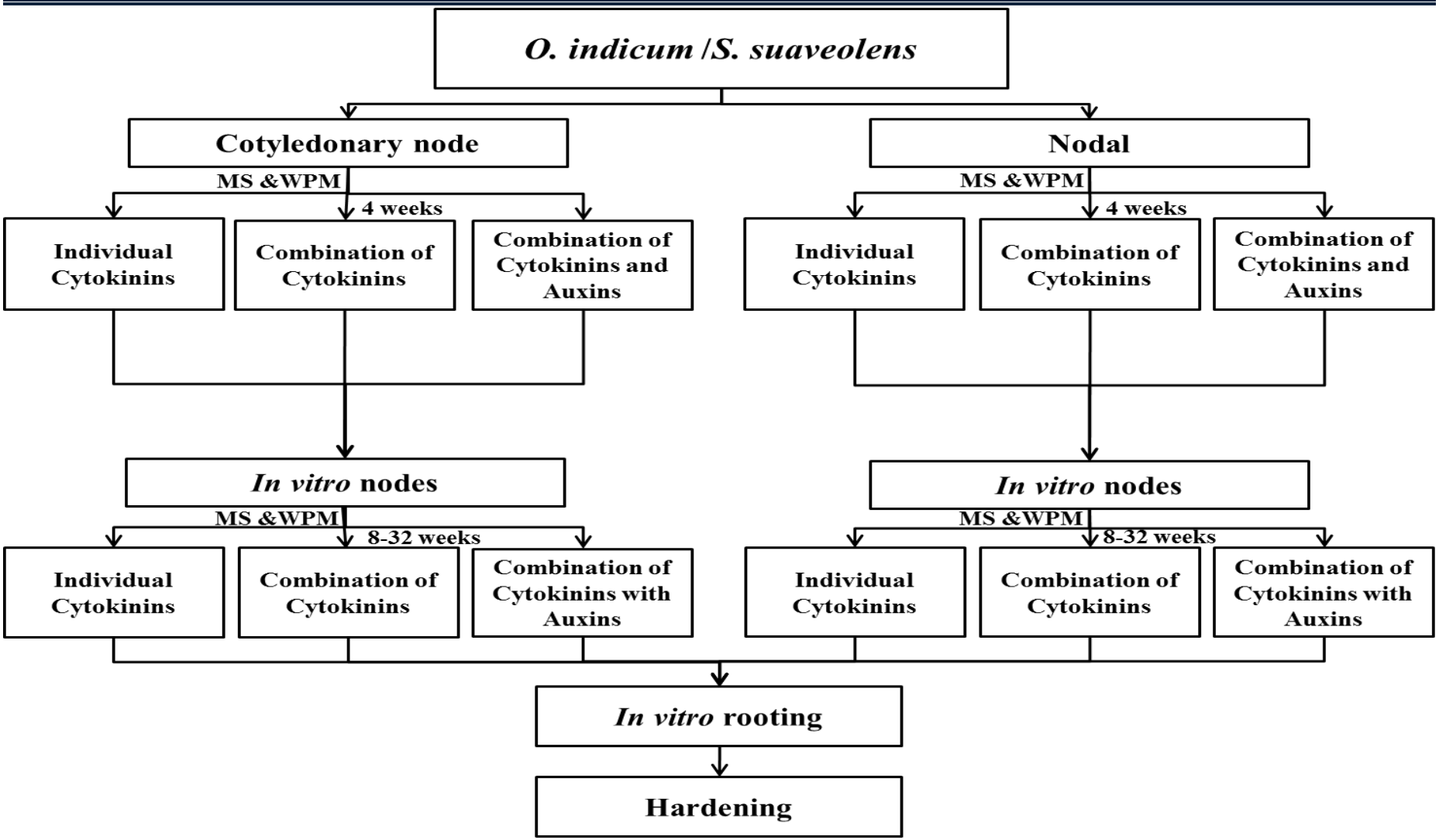

Fig. 8: Methodology for regeneration studies in *O.indicum* and *S.suaveolens*

- **Individual cytokinins:** In *O.indicum* and *S.suaveolens* the seedling explants (cotyledonary leaf ,cotyledonary node , hypocotyl (below the cotyledonary axis) and nodal explants were placed in media fortified with individual cytokinins:
  - MS/WPM + S (3%) + BAP (2-30µM)
  - MS/WPM + S (3%) + Kn (2-30µM)
  - MS/WPM + S (3%) + TDZ (0.1-2µM)
- **Combination of cytokinins:** The cotyledonary node and nodal explants were placed in the following combination of cytokinins:

O.indicum

  - MS/WPM + S (3%) +BAP(8,16 and 20µM) with Kn (2,4,8,16µM),
  - MS/WPM + S (3%) +BAP(8,16 and 20µM) with TDZ(0.1,0.2,0.25,0.5µM)
  - MS/WPM + S (3%) + Kn (2,4 and 8µM) with TDZ(0.1,0.2,0.25,0.5µM)

S.suaveolens

A) Cotyledonary node explants

  - MS + S (3%) +BAP(8,16,20µM) + Kn (2,4,8µM)
  - MS+ S (3%) +BAP(8,16,20µM)+TDZ(0.1,0.2,0.25µM) and
  - MS + S (3%) + Kn(2,4,8µM) + TDZ(0.1,0.2,0.25µM)
  - WPM+ S (3%) +BAP(2,4,8µM) + Kn(2,4,8µM)
  - WPM+ S (3%) +BAP(2,4,8µM)+TDZ(0.1,0.2,0.25µM)
  - WPM+ S (3%) + Kn(2,4,8µM) + TDZ(0.1,0.2,0.25µM)

B) Nodal explants

  - MS/WPM+ S (3%) +BAP(2,4,8µM) + Kn(2,4,8µM)
  - MS/WPM+ S (3%) +BAP(2,4,8µM)+TDZ(0.1,0.2,0.25µM) and
  - MS/WPM+ S (3%) + Kn(2,4,8µM) + TDZ(0.1,0.2,0.25µM)

- **Combination of cytokinins and auxins:** The cotyledonary node and nodal explants of both the species were placed in MS and WPM medium fortified with combinations of cytokinins and auxins:

O. indicum

A) Cotyledonary node explants

  - MS+ S (3%) +BAP(20 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)
  - MS+ S (3%) + Kn(8 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)
  - MS+ S (3%) +TDZ(0.1 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)

- WPM + S (3%)+ BAP(16 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)
- WPM+ S (3%)+ Kn(8 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)
- WPM+ S (3%)+ TDZ(0.1 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)

B) Nodal explants

- MS + S (3%) +BAP(20 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)
- MS+ S (3%)+ Kn(8 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)
- MS+ S (3%) +TDZ(0.1 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)
- WPM + (3%) +BAP(16 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)
- WPM+ S (3%)+ Kn(8 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)
- WPM+ S (3%) +TDZ(0.5 µM) with IAA/IBA/NAA(0.1,0.5,1 µM)

S.suaveolens

A) Cotyledonary node explants

- MS + S (3%) +BAP(20 µM) with IAA/NAA(0.1,0.5,1 µM)
- MS+ S (3%) + Kn(8 µM) with IAA/NAA(0.1,0.5,1 µM)
- MS+ S (3%) +TDZ(0.2 µM) with IAA/NAA(0.1,0.5,1 µM)
- WPM + S (3%) +BAP(8 µM) with IAA/NAA(0.1,0.5,1 µM)
- WPM + S (3%) +Kn(2 µM) with IAA/NAA(0.1,0.5,1 µM)
- WPM + S (3%) +TDZ(0.1 µM) with IAA/NAA(0.1,0.5,1 µM)

B) Nodal explants

- MS + S (3%) + BAP(2 µM) with IAA/NAA(0.1,0.5,1 µM)
- MS+ S (3%)+ Kn(2 µM) with IAA/NAA(0.1,0.5,1 µM)
- MS+S (3%) + TDZ(1 µM) with IAA/NAA(0.1,0.5,1 µM)
- WPM + S (3%) +BAP(8 µM) with IAA/NAA(0.1,0.5,1 µM)
- WPM+ S (3%) +Kn(2 µM) with IAA/NAA(0.1,0.5,1 µM)
- WPM+ S (3%) +TDZ(0.2 µM) with IAA/NAA(0.1,0.5,1 µM)

Observations were recorded after 4 weeks for percent response, shoot number per node and number of *in vitro* nodes per shoot which were counted in each individual combinations as follows:

- % Response = $\frac{\text{No. of explants responded}}{\text{No. of replicates}} \times 100$
- Average number of shoots = $\frac{\text{Total number of shoots developed}}{\text{No. of replicates}}$
- Average number of in vitro nodes = $\frac{\text{Total number of } \textit{in vitro} \text{ nodes in each shoot}}{\text{No. of replicates}}$

3.10.3 Multiplication of shoots

In both the species those combinations which induced axillary bud proliferation and developed into a shoot were selected for further transfer and the combinations which failed to respond within 4 weeks were not carried forward.

To enhance the number of shoots in *O.indicum* and *S.suaveolens*, certain additives like, silver nitrate(20mg/l) casein hydrolysate(1g/l) , calcium pantothenate (0.5mg/l) were also added in some medium to observe its effect on multiplication of shoots. For elongation of shoots GA_3 (1µM) and coconut water (10%) was added in medium wherever required.

3.10.3.1 Multiplication through *in vitro* nodes

In both the species the *in vitro* nodes of shoots which developed after 4 weeks from cotyledonary node and nodal explants in MS and WPM media fortified with respective individual cytokinins (BAP/Kn/TDZ), combinations of cytokinins (BAP+Kn,BAP+TDZ and Kn+TDZ) and cytokinin (BAP/Kn/TDZ) with auxins (IAA/NAA/IBA) were subcultured as follows:

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In vitro shoots (developed from cotyledonary node and nodal explants)were taken

↓

Excision of *in vitro* nodes (2-5) from these shoots and placed in respective medium

↓

After 4 weeks *in vitro* nodes were again excised from these shoots and transferred to a fresh medium with same composition till 5 passages ie.(8,12,16,24,32) weeks

↓

The % response and number of shoots developed were recorded after 8,12,16,24,32 weeks

3.10.3.2 Differentiaton through morphogenic callus(Indirect organogenesis)

When cotyledonary node and nodal explants were culture in MS and WPM medium fortified with different concentrations of PGRs in some combination there was differentiation of morphogenic callus from the base of explants. This callus was excised and placed on respective media for further differentiation of shoot buds and growth of shoots. Observations for formation of shoots was recorded after 8, 12,16,24,32 weeks in respective combinations.

3.10.4 Rooting of microshoots

The microshoots of both the species were placed for rooting in half and full strength MS and WPM (liquid and static) media as follows:

- The *in vitro* elongated shoots (4-6 cm) were aseptically harvested and the leaves of the lower two nodes were excised.
- After that they were washed with distil water for one minute to remove traces of agar attached to it.
- Then the shoots were placed in bavistin (100mg/l) for one minute to avoid fungal contamination.
- These shoots were placed vertically in liquid (MS and WPM)media (without agar) with the support of filter paper bridge and in static(MS and WPM) media(with agar) fortified with different concentrations of IBA and NAA as follows:

O. indicum

- ½ MS + S (1%)+ IBA/NAA(0, 2,2.5,5,10 µM)
- MS + S (1%)+ IBA/ NAA(0, 2,2.5,5,10 µM)
- ½ WPM + S (1%) + IBA/NAA(0, 2,2.5,5,10 µM)
- WPM + S (1%)+ IBA/ NAA(0, 2,2.5,5,10 µM)

S. suaveolens

- ½ MS + S (1%) + IBA/NAA(0,1,2,2.5,5 µM)
- MS + S (1%) + IBA/NAA(0,1,2,2.5,5 µM)
- ½ WPM + S (1 %) + IBA/ NAA(0,1,2,2.5,5µM)
- WPM + S (1%)+ IBA/NAA(0,1,2,2.5,5 µM)

Observations for % response, root number and root length were recorded after 4 weeks.

- % Response = $\frac{\text{No. of shoots forming roots}}{\text{No. of replicates}} \times 100$
- Average root number = $\frac{\text{Total number of roots developed}}{\text{No. of replicates}}$
- Average root length = $\frac{\text{Total root length in each shoot}}{\text{No. of replicates}}$

3.10.5 Hardening of plantlets

The hardening of both the plants were carried out under lab conditions and under greenhouse conditions as follows:

Under Lab conditions: The plantlets of both the species were placed initially under lab conditions in sterilised substrates like cocopeat, cocopeat:soil (1:1) and cocopeat:sand(1:1) and then transfered to greenhouse.

The *in vitro* plantlets were removed and washed with water to remove traces of medium.

- The thermocol cups were filled with substrates (80g) and then the plantlets were placed in it and covered with perforated polythene bags.
- Regular watering was done in cups and observations were recorded for growth of plants after one month.

Under Greenhouse conditions: The plantlets of both the species were directly transferred to greenhouse conditions and were hardened in different planting substrates like:

  - Cocopeat
  - Sand
  - Soil
  - Cocopeat:Soil(1:1)
  - Cocopeat:Sand(1:1)
  - Cocopeat:Sand:Soil(1:1:1)
  - Sand:Soil(1:1)

- All the substrates were filled (80g) in thermocol cups. *In vitro* plantlets with well developed roots (from liquid and static medium) were washed with water.
- Prior to transfer to substrates the initial data of *in vitro* plants were recorded for following parameters:
  - Initial shoot length
  - Initial root length
  - Initial plant height
- Then they were placed in thermocol cups and were covered with perforated polythene bags. The plantlets were watered every alternate day till one month.
- Observations for following growth parameters of plants were recorded after one month (after 4 weeks).

- Final shoot length
- Final root length
- Final plant height
- Number of new leaves and leaf length

- The plants from the respective substrate were transfered to plastic pots for their further growth and development. The pots were initially covered with polythene bags to maintain humidity and after 5 days polythene was removed. The plants were irrigated and were monitored for their growth.
- Again the same parameters (ie. shoot length, root length,plant height)were recorded after one month(after 8 weeks) before transferring them to garden pots filled with soil for their normal growth. The plants after one or two months of transfer to garden pot in greenhouse were transfered to open sunlight for their growth under natural conditions.The increase in plant height and girth above soil were recorded in survived plants in garden.

3.11 Section III: Synthetic seed studies in *O.indicum* and *S.suaveolens*

The last section was to carry out studies on preparation and germination of synthetic seeds.

3.11.1 Explant source: *In vitro* nodes were selected as explants for encapsulation in *O.indicum* and *S.suaveolens*.

3.11.2 Synthetic seed preparation: The synthetic seeds were prepared through encapsulation technique given below:

In vitro nodes (3mm) were excised

↓

Aqueous sodium alginate (3 %) was taken in blunt pipette of 0.7cm diameter

↓

In vitro nodes were inserted with help of forcep and placed in aqueous sodium alginate

↓

Then dropped in $CaCl_2$ (75 mM) solution

↓

Incubated for 30 minutes for complexation

↓

The solution was drained by a sieve to retain the synseeds

↓

Synseeds were washed 3 times with distil water

↓

Synseeds were placed in respective substrate, stored at 4ºC in refrigerator and then transferred to regenerative media at regular time intervals to observe their viability.

3.11.3 Substrate for storage:

In both the species synseeds were stored in petridish with filter paper were filled with liquid medium of the same strength as follows:

Filter paper substrate : In *O.indicum* half/full strength liquid WPM basal medium was poured (15ml) in petridish and in *S.suaveolens* half/ full strength liquid MS basal medium was poured (15ml) in petridish and then *in vitro* nodes were encapsulated in the respective strength matrix were kept in it for storage at 4 ºC by sealing the petridish with parafilm.

The synseeds prepared in various matrices stored in filter paper substrate were transferred to different regenerative media and from these the matrix and regenerative medium were selected (with optimum synseed germination) and placed on agar substrate.

Agar substrate: In this static medium (15ml) was poured in petridish and allowed to cool. In *O.indicum* WPM medium of respective strength was poured in petridishes and in *S.suaveolens* MS medium with respective strength was poured in petridishes and the *in vitro* nodes encapsulated in respective strength matrix were kept in it for storage by sealing the petridish with parafilm.

3.11.4 Storage period: The encapsulated beads were stored at 4ºC for 0,7,15,30 days.

3.11.5 Encapsulation Matrices and Regenerative media:

O.indicum

The *in vitro* nodes were encapsulated in different matrix prepared in WPM medium with sodium alginate(3%) fortified without or with PGRs which were stored in filter paper substrate and transferred at regular time interval to following static regenerative media in *O.indicum* as follows(Table 6):

Table 6. Different encapsulation matrices and regenerative media in *O.indicum*

Matrix medium	Regenerative media
Half strength	
½ WPM	• ½ WPM+ S(1%) • ½ WPM + (1%)+BAP(16µM) +IAA(1µM) • ½ WPM+ S(1%)+GA_3(10µM) • ½ WPM+S(1%)+ CW(10%)
½ WPM+BAP(16µM)	• ½ WPM+ S(1%) • ½WPM+ S(1%)+BAP(16µM) +Kn(4µM) • ½ WPM +S(1%)+ NAA(5µM)
½WPM+BAP(16µM)+IBA(0.1µM)	• ½ WPM + S(1%) • ½ WPM+ S(1%)+ NAA(5µM)
Full strength	
WPM	• WPM+ S(3%) • WPM + S(3%)+BAP(16µM) +IAA(1µM) • WPM+ S(3%)+GA_3(10µM) • WPM+ S(3%)+ CW(10%)
WPM+BAP(16µM)	• WPM+ S(3%) • WPM+ S(3%)+BAP(16µM) +Kn(4µM) • WPM+ S(3%)+NAA(5µM)
WPM+BAP(16µM)+IBA(0.1µM)	• WPM+ S (3%) • WPM+ S(3%) +NAA(5µM)

The following matrix and regenerative medium were tried for the synseeds stored in agar substrate.

- Matrix: ½ WPM+BAP(16µM) +IBA(0.1µM): RM: ½ WPM +NAA(5µM)

S.suaveolens

The *in vitro* nodes were encapsulated in different matrix prepared in MS medium with sodium alginate (3%) fortified without or with PGRs and transferred at regular time interval to following regenerative liquid and static media(Table 7).

Table 7. Different encapsulation matrices and regenerative media in *S.suaveolens*

Matrix	**Regenerative medium** (Liquid and Static)
	Half strength
½ MS	• ½ MS+ S(1%) • ½ MS+ S(1%)+IBA(2µM) • ½ MS+ S(1%)+NAA(2µM) • ½ MS+ S(1%)+GA_3 (10µM) • ½ MS+ S(1%)+CW(10%)
½ MS+BAP(20µM)	• ½ MS+ S(1%) • ½ MS+ S(1%)+IBA(2µM) • ½ MS+ S(1%)+NAA(2µM) • ½ MS+ S(1%)+Kn(8µM)
½ MS+Kn(8µM)	• ½ MS+ S(1%) • ½ MS+ S(1%)+IBA(2µM) • ½ MS+ S(1%)+NAA(2µM) • ½ MS+ S(1%)+BAP(4µM)+Kn(8µM)
½ MS+TDZ(0.2µM)	• ½ MS+ S(1%) • ½ MS+ S(1%)+IBA(2µM) • ½ MS+ S(1%)+Kn(8µM) +TDZ(0.2µM)
½ MS+BAP(8µM) +TDZ(0.2µM)	• ½ MS+ S(1%) • ½ MS+ S(1%)+IBA(2µM) • ½ MS+ S(1%)+NAA(2µM)
	Full strength
MS	• MS+S(3%) • MS+ S(3%)+IBA(2µM) • MS+ S(3%)+NAA(2µM) • MS+ S(3%)+GA_3 (10µM) • MS+ S(3%)+CW(10%)
MS+BAP(20µM)	• MS+ S(3%) • MS+ S(3%)+ IBA(2µM) • MS+ S(3%)+NAA(2µM) • MS+ S(3%)+Kn(8µM)
MS+Kn(8µM)	• MS+ S(3%) • MS+ S(3%)+IBA(2µM) • MS+ S(3%)+NAA(2µM) • MS+ S(3%)+BAP(4µM)+Kn(8µM)

MS+TDZ(0.2µM)	• MS+ S(3%) • MS+ S(3%)+IBA(2µM) • MS+ S(3%)+Kn(8µM)+TDZ(0.2µM)
MS+BAP(8µM) +TDZ(0.2µM)	• MS+ S(3%) • MS+ S(3%)+IBA(2µM) • MS+ S(3%)+NAA(2µM)

The synseeds stored in agar substrate were tried for germination in following matrix and regenerative medium:

- Matrix : MS+Kn(8µM) RM: MS+NAA(2µM)

Observations were recorded after 4 weeks for the following parameters:

- % shoot emergence = $\frac{\text{No. of synseeds emerging into shoots}}{\text{Total no. of synseeds}} \times 100$
- % shoot and root emergence = $\frac{\text{No. of synseeds emerged into shoots and root}}{\text{Total no. of synseeds}} \times 100$
- Average number of shoots = $\frac{\text{Total number of shoots emergence from synseeds}}{\text{Total no. of synseeds}}$
- Average number of roots= $\frac{\text{Total number of roots emerged from synseeds}}{\text{Total no. of synseeds}}$

3.12 Culture conditions

All the cultures were incubated in light in culture room at 25 ± 2 ºC with 16 hour photoperiod and 8 hours dark period.

3.13 Statistical Analysis

Statistical methods were used for comparison of treatment means during optimization of the parameters for *in vitro* regeneration using both seedling and mature explants. All the experiments were conducted with six replicates per treatment and repeated once or twice. Each treatment represents one explant per culture vial. All the data are expressed as the mean ± S.E and were analyzed statistically through one-way ANOVA using Microsoft Excel 2007. The significance of difference among treatment means were tested using Duncan multiple range test (DMRT) at a 5% probability level ($P \leq 0.05$).