MATERIALS AND METHODS

General *in vitro* technique was followed for the cultivation of selected plants under study:

3.2.1 Sterilization of glass wares and other accessories

All the materials like glassware, instruments, medium, explants etc to be used in culture work must be sterilized to remove microbes using several techniques. The conventional method of sterilizing laboratory glass wares is to soak every glassware for 4 hours in sodium dichromate and then wash with detergent under tap water. After washing with detergent double distilled water was used to wash the glassware again. Then the glassware kept in hot air oven at 100°C for about 1-2 hours. For reused the glassware, the culture bottles or flask with unwanted media were autoclaved and same process was repeated.

Table 3.1s Sterilization technique used in plant Tissue Culture

Technique	Materials sterilized
Steam sterilization/ Autoclaving	Nutrient media, culture vessels, glassware and plasticwares.
Dry heat	Instruments, (scalpel, forceps, needles etc.), glassware, pipettes, tips and other plasticwares.
Flame sterilization	Instruments (scalpel, forceps, needles etc), mouth of culture vessels.
Filter sterilization (membrane filter made of cellulose acetate of 0.45-0.22µm pore size)	Thermolabile substances like growth factors, amino acids, vitamins and enzymes.
Alcohol sterilization	Worker's hands, laminar flow cabinet.
Surface sterilization (sodium hypochlorite, hydrogen peroxide, mercuric chloride etc)	Explants.

Other than glassware, other accessories like forceps, scalpels cleaned and wiped with absolute alcohol and after that wrapped with aluminium foil and sterilized by autoclaving (at 15lb/inch^2 pressure for 20 minutes). Filter paper, cotton, blotting paper, pipettes, beakers, petriplates, conical flask etc which were used during inoculation was also sterilized by autoclaving in same pressure and time after treated with dry heat.

3.2.2 Culture Media

The type and composition of culture media very strongly govern the growth and morphogenesis of plant tissues. The selection of tissue culture medium largely depends upon the species to be cultured. The present study was done by using standard Murashige and Skoog (MS) medium(1962). Other medium like Gamborg's $B_{5,}$ Knudson C medium was tried but satisfactory growth of selected plant species was observed in MS medium.

3.2.2.1 Chemicals used in Murashige and Skoog (1962) medium with its quantities

Micro-nutrients	**Concentration (mg/L)**
Potassium nitrate (KNO_3)	1900
Ammonium nitrate (NH_4NO_3)	1650
Calcium chloride ($CaCl_2.2H_2O$)	440
Magnesium sulphate ($MgSO_4.7H_2O$)	370
Potassium dihydrogen phosphate(KH_2PO_4)	170
Micro-nutrients	
Manganese sulphate ($MnSO_4.4H_2O$)	22.3
Zinc sulphate ($ZnSO_4.7H_2O$)	8.6
Boric acid (H_3BO_3)	6.2
Sodium molybdate ($NaMoO_4.2H_2O$)	0.25
Copper sulphate ($CuSO_4.5H_2O$)	0.025
Cobalt chloride ($CoCl_2.6H_2O$)	0.025
Vitamins	
Thiamine HCl (Vit.B_1)	0.1
Pyridoxine HCl (Vit.B_6)	0.5
Nicotinic acid	0.5
Mesoinositol	100
Iron Source	
Ferrous sulphate ($FeSO_4$)	27.8
Sodium salt of Ethylene diamine Tetra acetic acid($Na_2EDTA.2H_2O$)	37.3
Potassium Iodied (KI)	0.83

Amino Acid

Glycine	2.0

Carbon Source (g/L)

Sucrose	30

Gelling Agent

Agar	8

pH

pH of the medium is adjusted to 5.8 with 1N HCl or 1N NaOH.

3.2.2.2 Pant growth regulators (Phytohormones) used

Auxins

I. Indole-3-acetic acid (IAA)

II. α-napthalene acetic acid (NAA)

Cytokinins

I. Kinetin (KIN)

II. 6- benzylaminopurine (BAP)

3.2.2.3 Preparation of stock solution

Stock solution of micro-salts and macro-salts, vitamins, iron(EDTA), mesoinositol, potassium iodied and glycine were prepared. All these are when mixed together in appropriate quantities basal medium was formed. All stock solutions were stored in reagent bottles at low temperature in the refrigerator for further use. It is necessary to shake well the bottles of stock solution before use. Stock solution were discarded when the solution appear cloudy.

Macro-nutrient stock solution was prepared ten times (10X) of the actual concentration. Micro- nutrient, vitamins (except mesoinositol) and iron EDTAstock solution were prepared hundred times (100X) of their actual concentration. Dark coloured bottles were used to store iron stalk solution.

A. **Macro-salts (10x)**

Macro elements are classified as those elements which are required in concentration greater than 0.5mM/L and it includes nitrogen, potassioum, phosphorous, calcium, magnesium and sulphur in form of salt in media.

Table 3.2. **Preparation of stock solution of macrosalts (10x)**

Chemical constituent	**Amount (mg/L) present in original need**	**Amount (g/L)for stock solution**	**Final volume of stock solution (mL)**
KNO_3	1900	19.00	
NH_4NO_3	1650	16.50	
$CaCl_2.2H_2O$	440	4.40	500
$MgSO_4.7H_2O$	370	3.70	
KH_2PO_4	170	1.70	

Each component was separately dissolved double distilled water and then mixed with others. The final volume was made 500mLwith double distilled water in volumetric flask.

B. Micro-salts

Micro-salts are those elements which are required at a concentration less than 0.05mM/L and include iron, manganese, zinc, boron, copper and molybdenum.

Each component dissolved in double distilled water separately and then mixed with other solution. The final volume was made 100mL in volumetric flask with double distilled water.

Table 3.3 Preparation of stock solution of MS microsalts (100x)

Chemical constituent	Amount(mg/L) present in original need	Amount (g/L) for stock solution	Final volume of stock solution(mL)
$MnSO_4.4H_2O$	22.3	2.23	
$ZnSO_4.7H_2O$	8.6	0.86	
H_3BO_3	6.2	0.62	100
$NaMoO_4.2H_2O$	0.25	0.025	
$CuSO_4.5H_2O$	0.025	0.0025	
$CoCl_2.6H_2O$	0.025	0.0025	

C. Vitamins

Vitamins are the organic substances required for metabolic processes. For optimum growth of the cultured plants medium should be supplemented with vitamins.

Table 3.4. Preparation of stock solution of vitamins* (100x)

Chemical constituent	**Amount(mg/L) present in original need**	**Amount (g/L) for stock solution**	**Final volume of stock solution(mL)**
Thiamine HCl	0.1	0.01	100
Pyridoxine HCl	0.5	0.05	100
Nicotinic acid	0.5	0.005	100

*** Note:** Plant endogenously synthesizes vitamins which used in various metabolic processes but when plant grown *in vitro* media must be supplemented with some essential vitamins to record the optimum growth of the cultured plants.

D. Stock solution of mesoinositol (100X)

Mesoinositol prepared fresh for use in culture medium.1.0g mesoinositol was dissolved in 20mL double distilled water.

E. Stock solution of iron (20X)

In little amount of double distilled water 0.745g of Na_2EDTA and 0.557g of $FeSO_4.7H_2O$ were dissolved. The solution was heated, shaken and cooled and final volume was made upto 100mL in a volumetric flask.

F. Stock solution of potassium iodied (100x)

0.083g potassium iodied was dissolved in 100mL of double distilled water.

G. Stock solution of Glycine (10x)

20 mg of glycine was dissolved in 10 mL of double distilled water.

3.2.2.4 Preparation of Plant growth regulators

Plant growth regulators (Auxins and cytokinins) Himedia make were used in this investigation. Solution of auxins and cytokinin were prepared by dissolving them in any one of these – Ethanol, 1N NaOH, 1N HCl and making the final volume (100 mL) with double distilled water, stored in deep freezer for future use.

Table 3.5 Preparation of stock solution of growth regulators

Plant Growth Regulators	Procedure
1. IAA (indole acetic acid) 2. NAA (α naphthalene acetic acid) 3. KIN (Kinetin) 4. BAP (benzylamino purine)	100 mg of each growth hormone were dissolved separately in a few drops of 1N HCl or 1N NaOH or Ethanol and by adding double distilled water final volume was made upto 100 mL.

3.2.2.5 Preparation of Culture Media

Culture media was prepared from the above stock solution. Different amount of stock solution was pipetted out in a volumetric flask to make the 1Litre of MS basal medium. The following volumes of stock solutions were taken to prepare 1Litre of MS basal medium.

Amount of different stock solutions taken to prepare 1Litre MS basal media

Stock solution of macrosalts	50 mL
Stock solution of microsalts	1mL
Stock solution of vitamins	1mL
Stock solution of mesoinositol (fresh)	2mL
Stock solution of Na_2EDTA	5mL
Stock solution of Potassium iodied	1mL
Stock solution of Glycine	1mL

30g of sucrose was dissolved in small amount of double distilled water and added to the above mixture. For different growth regulators, proportionate amount of stock solution was taken in 100mL volumetric flask and volume was adjusted with double distilled water upto the mark. The pH was adjusted to 5.6-5.8 with 1N HCl or 1N NaOH. 8g of agar was added to the media and boiled properly to dissolve the agar after the adjustment of pH. Solidifying capacity of agar may lose if it is boiled for longer period. Culture media was distributed in culture tubes and culture bottles. Culture tubes were sealed with non-absorbent cotton plugs and wrapped with aluminium foils and sterilized in an autoclave.

3.2.3 Sterilization of Culture Medium

Culture tubes and bottles with media were autoclaved under 15 lb pressures and 121°C temperature for 15-20 minutes (from the time of media reached the required temperature). Volume of the medium to be sterilized decides the time period required for sterilization. The pressure should be maintained at 15 lb

because higher pressure may cause degeneration of sucrose and other components of the media.

After autoclaving, the culture tubes and bottles were kept in the culture room (plate 1) and allowed to cool. Culture tubes were kept in slating position (45°C) or kept upright in test tube stand.

Table 3.6. Minimum time necessary for autoclaving nutrient media

Volume (mL) of media per container	Minimum sterilization time (minutes) at 121°C 15lb pressure.
20-25	15
75	20
250-500	25
1000	30
1500	35
2000	40

3.2.4 Sterilization of double distilled water

Conical flask with double distilled water was sealed and autoclaved to sterilize along with the culture medium.

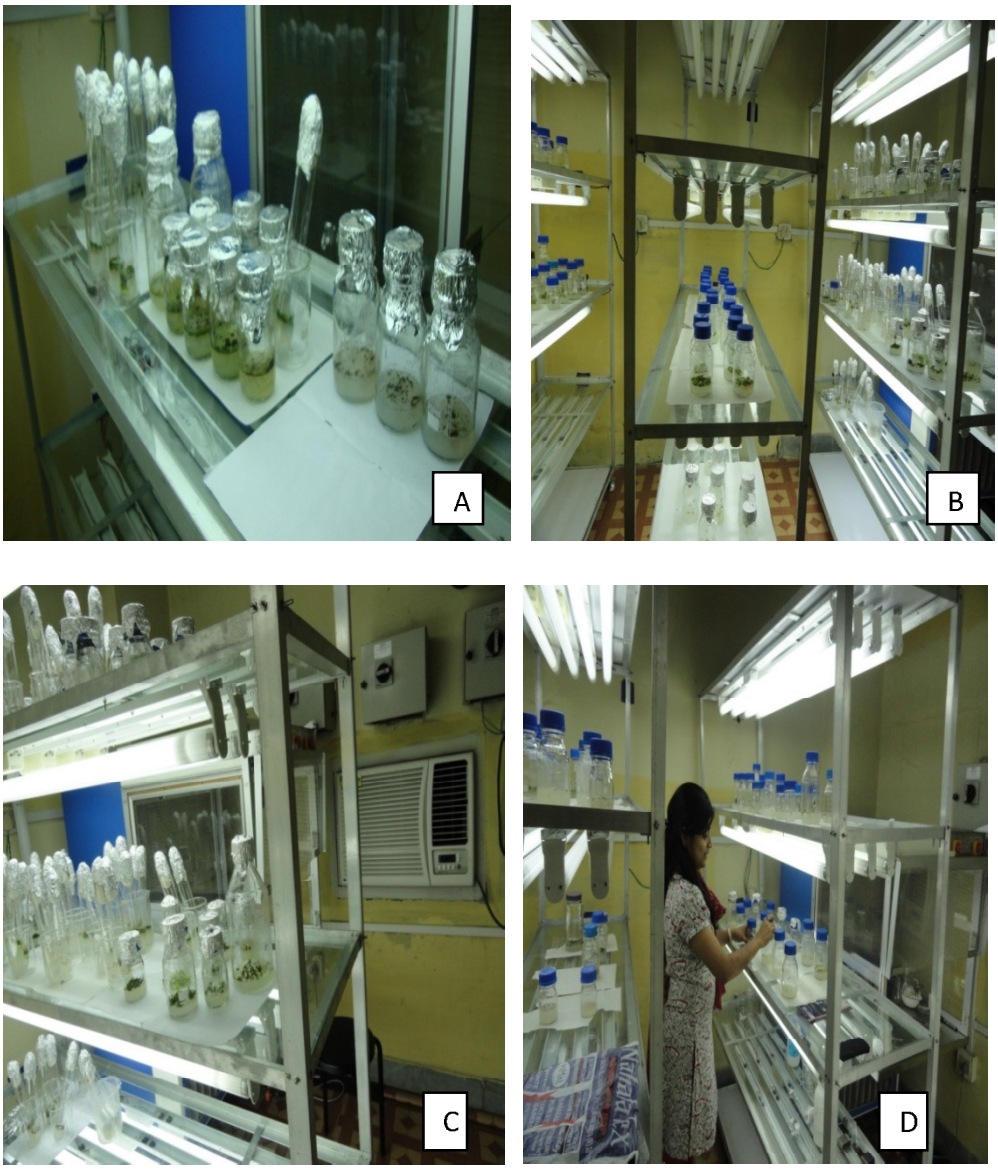

Plate1. Plants under study in culture room (*in vitro* condition)

[A] [B] [C] [D]

Materials and Methods used for *Cyathea gigantea* Wall ex. Hook

Explants Used

Spores of *Cyathea gigantea* was used as explants for present investigation.

Collection of spore :

Sporophylls of *Cyathea gigantea* (Wall. ex. Hook.) were collected from sporophytes growing in natural habitat at Churaibari area of Karimgang District. The spores were allowed to separate from sporangia and then these were stored in sterilized glass vessels at 10°c.

Surface sterilization of spores:

Explants used for *in vitro* culture must be sterilized properly to avoid contaminations which disturb the growth of culture plants within the culture vessels. The procedure and type of sterilants used largely depends on the source and type of tissue of the explants which determines the contamination percentage and tolerance to the sterilizing agent.

The procedures for surface sterilization of spores given below:

- Surface sterilization of spores were done with 30% (w/v) solution of sodium hypochlorite (4% active chlorine) for 20 minutes.
- The spores were then filtered through autoclaved filter paper after washing several times with sterile double distilled water.
- The spores were then inoculated in medium with wet condition.

Further sterilization was carried out under Laminar air flow cabinet. To avoid contamination hands were wiped with 70% alcohol. The platform of Laminar air flow cabinet was wiped with cotton soaked in absolute alcohol and all sterile equipments, media, glassware,cotton and culture bottles with media were placed into the laminar air flow cabinet. The UV light was switched on for 45 minutes under hood cover.

Procedure for inoculation

Surface sterilized spores were inoculated under laminar air flow cabinet. During the inoculation blower motor was switched on. Precaution was taken to avoid contamination and prevent the entry of microorganism. The inner side and platform of the laminar air flow cabinet and hands again wiped with absolute alcohol.

The surface sterilized spores were taken on a sterilized filter paper in a sterile petridish in front of burning spirit lamp. The forceps, scalpels and blade holders were again sterilized by using flame. The explants were then inoculated in the medium.

The transferring process was also carried out under the flame. The neck portion of the culture bottles was flamed and then removed the cap. Sterile forceps was used to place the explants on the nutrient media. Before sealing the culture bottle caps were again flamed. The whole process was carried out very carefully and quickly to maintain the aseptic condition.

Incubation of culture

The cultured spores were incubated in Growth Chamber of the Tissue culture Laboratory at 25°C ± 1°C, under 16 hours photoperiod at 2500 – 3000 Lux. High temperature may hampered the cultured tissues and at very low temperature also not supportive for tissue growth.

Illumination was provided by cool white fluorescent light. The culture bottles were incubated with 16:8hours of light: dark period (Bertland *et al*., 1999; Fernandez *et al*.,1999).

The cultures were checked periodically at an interval of 7 days and sub cultured after 15 days in the same composition with same supplementation. It was necessary to discard and remove the contaminated culture bottles from the culture room.

Germination Test

For the germination test about 5mg of sterilized spores were sown in 20 ml of liquid medium. The flasks were plugged with sterile cotton plugs wrapped with aluminium foil. All procedure was carried out in Laminar Air Flow Cabinet.

Viability Test

Spores were taken on a slide and mounted with glycerine solution and observed under microscope. Fertile spores were round and dark brown in colour.

Effect of different Plant Growth Regulators

Different concentration of IAA (0.5mg/L, 0.6mg/L,0.7mg/L,0.8mg/Land 0.9mg/L) and Kinetin (1mg/L, 2mg/L,3mg/L, 4mg/L, 5mg/L) was used to observe its effect on growth of the plant.

Morphological characters studied:

a. Prothallus structure (gametophyte)
b. Sporophyte length
c. Fresh weight of gametophyte
d. Dry weight of the gametophyte
e. Germination percentage

Germination percentage (%)=Total germinating spores in each microscopic field/ Total spore x 100

Standardization of Medium

Two media namely Murashige & Skoog (1962) medium and Fern propagation medium with and without sugar were selected for culture of spores. Growth measured in terms of fresh weight of prothallus. As very negligible growth was observed in Fern propagation medium so it is discarded for further work.

Statistical Analysis

In the present experiment, all the data are means of 3 replicates. MS excel software (Microsoft office Excel 2007. Ink) was used to determine the growth of the sporophytes.

Acclimatization of cultured plantlets

Pots were filled with brick bats, leaf mold, charcoal, dried moss in 1: 1: 1:1 proportion.

Sterilization of the potting mixture

The earthen pots containing the aforesaid potting mixture were autoclaved at 20lbs/sq inch, pressure for 30 minutes. After sterilization the pots were allowed to cool down for 48 hours and then kept in room temperature in a well ventilated room.

Transplantation

Plants were taken out from culture bottles and washed thoroughly with autoclaved distilled water to remove culture medium sticking to the plants. After that plants were dipped in 2% Diethane 45 (fungicide) for 30 seconds and transplanted in pots containing sterile mixture (vermiculite) and covered with polythene bags. The pots were kept in tissue culture laboratory. The seedlings were regularly sprayed with MS solution up to 15days of transfer and during the next 15 days these were sprayed with half strength MS solution.

After one month the seedlings were transplanted to earthen pots containing mixture of brick bats, charcoal, dried moss and leaf mold in 1:1:1:1 ratio.

Materials and Methods for *Dioscorea alata* L.

Explant Source

Field grown plants of *Dioscorea alata* L. propagated from a wild tuber were used as source of explants for *in vitro* culture. Plants were collected from Badarpur area of Karimganj District. Nodal segments were used as explant.

Surface Sterilization of nodal segment

Explants used for *in vitro* culture must be sterilized properly to avoid contaminations which disturb the growth of culture plants within the culture vessels. The procedure and type of sterilants used largely depends on the source and type of tissue of the explants which determines the contamination percentage and tolerance to the sterilizing agent.

The procedures for surface sterilization of nodal segments given below:

- Surface sterilization of nodal segments was done with 0.1% (w/v) solution mercuric chloride for 7-8 minutes.
- The nodal segments were then washed several times with sterile double distilled water.
- The surface sterilized segments were then sized to 0.5-1.0cm length containing one node with sterilized blade.
- Explants were then inoculated in medium with wet condition.

Further sterilization was carried out under Laminar air flow cabinet. To avoid contamination hands were wiped with 70% alcohol. The platform of Laminar air flow cabinet was wiped with cotton soaked in absolute alcohol and all sterile equipments, media, glassware, cotton and culture bottles with media were placed into the laminar air flow cabinet. The UV light was switched on for 45 minutes under hood cover.

Procedure for inoculation

Surface sterilized explants were inoculated under laminar air flow cabinet. During the inoculation blower motor was switched on. Precaution was taken to

avoid contamination and prevent the entry of microorganism. The inner side and platform of the laminar air flow cabinet and hands again wiped with absolute alcohol.

The surface sterilized nodal segments were taken on a sterilized filter paper in a sterile petridish in front of burning spirit lamp. The forceps, scalpels and blade holders were again sterilized by using flame. The explants were then inoculated in the medium.

The transferring process was also carried out under the flame. The neck portion of the culture bottles was flamed and then removed the cap. Sterile forceps was used to place the explants on the nutrient media. Before sealing the culture bottle caps were again flamed. The whole process was carried out very carefully and quickly to maintain the aseptic condition.

Culture Medium and Incubation Condition

MS media was selected for *in vitro* culture of explants, the pH of the medium was adjusted with 0.1N HCl and 1N NaOH solution between 5.7- 5.8. After adjusting the pH agar powder was mixed with the medium and boiled for sometime to obtain clear solution. After that 40ml of medium was taken in each 100ml screw capped bottle autoclaved at 121°C at 15lbs/sq inch for 20 min.

The screw capped bottles containing the medium were then allowed to cool for 24 hours in the culture laboratory. The surface sterilized explants were placed vertically on the MS media with and without growth regulators. Various concentration of indole acetic acid acid (0.5,1.0, 1.5, 2.0 and 2.5mg/L) and kinetin (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) was used to observe its effect on axillary bud proliferation, shoot initiation, root initiation.

The cultures were maintained at 25°c±1°c under 16 hour photoperiod at 2500-3000 Lux provided by white fluorescent tubes. After every two week the culture materials were transferred into new medium for better growth of plants.

Statistical Analysis

Ten explants were used per treatment on each multiplication and rooting medium. Experiments were repeated thrice. MS excel software (Microsoft office Excel 2007. Ink) was used to determine the growth of the plants.

Sterilization of the potting mixture

The earthen pots containing the potting mixture were autoclaved at 20lbs/sq inch, pressure for 30 minutes. After sterilization the pots were allowed to cool down for 48 hours and then kept in room temperature in a well ventilated room.

Transplantation

Plants were taken out from culture bottles and washed thoroughly with water to remove culture medium sticking to the plants. After that plants were dipped in 2% Diethane 45 (fungicide) for 30 seconds and transplanted in pots containing sterile mixture (vermiculite) and covered with polythene bags. The pots were kept in tissue culture laboratory. The seedlings were regularly sprayed with half strength MS solution up to 15days of transfer and during the next 15 days these were sprayed with liquid MS (half strength) solution without agar and sugar.

After one month the plants were transferred to acclimatization media and medium comprised of pre sterilized Brick bats + charcoal + dried moss + leaf molds + soil (1:1:1:1:1).

Parameters Studied

1. Shoot length
2. Shoot number per explants
3. Root length
4. Root number
5. Percentage of explants response
6. Days required to root initiation
7. Days required to axillary bud proliferation

Materials and Methods for *Arundina graminifolia* (D. Don) Hochr.

Explant Source

Field grown plants of *Arundina graminifolia* (D. Don) Hochr. was used as source of explants for *in vitro* culture. The plants were collected from Jiribam in the month of February, 2012. Nodal segments of *Arundina graminifolia* used as explants.

Surface Sterilization of Explant

Explants used for *in vitro* culture must be sterilized properly to avoid contaminations which disturb the growth of culture plants within the culture vessels. The procedure and type of sterilants used largely depends on the source and type of tissue of the explants which determines the contamination percentage and tolerance to the sterilizing agent.

The procedures for surface sterilization of nodal segments given below:

- Surface sterilization of nodal segments was done with 0.1% (w/v) solution mercuric chloride for 5 minutes.
- The nodal segments were then washed several times with sterile double distilled water.
- Sterilized segments were then sized to 0.5-1.0cm length containing one node with sterilized blade.
- Explants were then inoculated in medium with wet condition.

Further sterilization was carried out under Laminar air flow cabinet. To avoid contamination hands were wiped with 70% alcohol. The platform of Laminar air flow cabinet was wiped with cotton soaked in absolute alcohol and all sterile equipments, media, glassware, cotton and culture bottles with media were placed into the laminar air flow cabinet. The UV light was switched on for 45 minutes under hood cover.

Procedure for inoculation

Surface sterilized explants were inoculated under laminar air flow cabinet. During the inoculation blower motor was switched on. Precaution was taken to avoid contamination and prevent the entry of microorganism. The inner side and platform of the laminar air flow cabinet and hands again wiped with absolute alcohol.The surface sterilized nodal segments were taken on a sterilized filter paper in a sterile petridish in front of burning spirit lamp. The forceps, scalpels and blade holders were again sterilized by using flame. The explants were then inoculated in the medium.

The transferring process was also carried out under the flame. The neck portion of the culture bottles was flamed and then removed the cap. Sterile forceps was used to place the explants on the nutrient media. Before sealing the culture bottle caps were again flamed. The whole process was carried out very carefully and quickly to maintain the aseptic condition.

Culture Medium and Incubation Condition

MS medium was selected for *in vitro* culture of explants, the pH of the medium was adjusted with 1N HCl and 1N NaOH solution between 5.7- 5.8. After adjusting the pH agar powder was mixed with the medium and boiled for sometime to obtain clear solution. After that 40ml of medium was taken in each 100ml screw capped bottle autoclaved at 121°C at 15lbs/sq inch for 20 min.

The screw capped bottles containing the medium were then allowed to cool for 24 hours in the culture laboratory. The surface sterilized explants were placed vertically on the MS media with and without growth regulators. Various concentration of α napthalene acetic acid acid (0.5,1.0, 1.5, 2.0 and 2.5mg/L) and kinetin (0.5mg/L, 1.0mg/L, 1.5mg/L, 2.0mg/L and 2.5 mg/L) was used to observe its effect on axillary bud proliferation, shoot initiation, root initiation.

The cultures were maintained at 25°c±1°c under 16 hour photoperiod at 2500-3000 Lux provided by white fluorescent tubes. After every two week the

culture materials were transferred into new medium for better growth of plants.

Sterilization of the potting mixture

The earthen pots containing the potting mixture containing brick bats, charcoal, dried moss, leaf molds, sand were autoclaved at 20lbs/sq inch, pressure for 30 minutes. After sterilization the pots were allowed to cool down for 48 hours and then kept in room temperature in a well ventilated room.

Transplantation

Plants were taken out from culture bottles and washed thoroughly with water to remove culture medium sticking to the plants. After that plants were dipped in 2% Diethane 45 (fungicide) for 30 seconds and transplanted in pots containing sterile mixture and covered with polythene bags. The pots were kept in tissue culture laboratory. The seedlings were regularly sprayed with MS solution up to 15days of transfer and during the next 15 days these were sprayed with liquid MS solution (half strength) without agar and sugar.

After one month the plants were transferred to acclimatization media and medium comprised of pre sterilized Brick bats + charcoal + dried moss + leaf molds + soil (1:1:1:1:1).

Statistical Analysis

Ten explants were used per treatment on each multiplication and rooting medium. Experiments were repeated thrice. MS excel software (Microsoft office Excel 2007. Ink) was used to determine the growth of the plant.

Parameters Studied

1. Days required to axillary bud proliferation
2. Shoot number per explants
3. Shoot length
4. Days required to root initiation
5. Root length
6. Root number per explant.

Materials and Methods for *Kaempferia parviflora* Wall Ex. Baker

Explant Source

Field grown plants of *Kaempferia parviflora* Wall. EX. Baker was used as source of explants for *in vitro* culture. The plants were collected from Hailakandi in the month of July, 2012. Rhizome bud was used as explant.

Surface Sterilization of Expant

Explants used for *in vitro* culture must be sterilized properly to avoid contaminations which disturb the growth of culture plants within the culture vessels. The procedure and type of sterilants used largely depends on the source and type of tissue of the explants which determines the contamination percentage and tolerance to the sterilizing agent.

The procedures for surface sterilization of rhizome bud given below:

- Surface sterilization of rhizome bud was done with 0.1% (w/v) mercuric chloride for 9 minutes.
- The rhizome buds were then washed several times with autoclaved double distilled water.
- Explants were then inoculated in medium with wet condition.

Further sterilization was carried out under Laminar air flow cabinet. To avoid contamination hands were wiped with 70% alcohol. The platform of Laminar air flow cabinet was wiped with cotton soaked in absolute alcohol and all sterile equipments, media, glassware, cotton and culture bottles with media were placed into the laminar air flow cabinet. The UV light was switched on for 45 minutes under hood cover.

Procedure for inoculation

Surface sterilized explants (rhizome buds) were inoculated under laminar air flow cabinet. During the inoculation blower motor was switched on. Precaution was taken to avoid contamination and prevent the entry of microorganism. The inner side and platform of the laminar air flow cabinet and hands again wiped with absolute alcohol.

The surface sterilized rhizome buds were taken on a sterilized filter paper in a sterile petridish in front of burning spirit lamp. The forceps, scalpels and blade holders were again sterilized by using flame. The explants were then inoculated in the medium.

The transferring process was also carried out under the flame. The neck portion of the culture bottles was flamed and then removed the cap. Sterile forceps was used to place the explants on the nutrient media. Before sealing the culture bottle caps were again flamed. The whole process was carried out very carefully and quickly to maintain the aseptic condition.

Culture Medium and Incubation Condition

MS medium was selected for *in vitro* culture of explants, the pH of the medium was adjusted with 1N HCl and 1N NaOH solution between 5.7- 5.8. After adjusting the pH agar powder was mixed with the medium and boiled for sometime to obtain clear solution. After that 40ml of medium was taken in each 100ml screw capped bottle autoclaved at 121°C at 15lbs/sq inch for 20 min.

The screw capped bottles containing the medium were then allowed to cool for 24 hours in the culture laboratory. The surface sterilized explants were placed vertically on the MS media with and without growth regulators. Various concentrations of α napthalene acetic acid acid (1.0, 1.5, 2.0, 2.5, 3.0, 3.5mg/L) kinetin and BAP (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mg/L) was used to observe its effect on axillary bud proliferation, shoot initiation, root initiation. The cultures were maintained at 25°C±1°C under 16 hour photoperiod at 2500-3000 Lux provided by white fluorescent tubes. After every two week the culture materials were transferred into new medium for better growth of plants.

Sterilization of the potting mixture

The earthen pots containing the potting mixture containing brick bats, charcoal, dried moss, leaf molds, sand were autoclaved at 20lbs/sq inch,

pressure for 30 minutes. After sterilization the pots were allowed to cool down for 48 hours and then kept in room temperature in a well ventilated room.

Transplantation

Plants were taken out from culture bottles and washed thoroughly with water to remove culture medium sticking to the plants. After that plants were dipped in 2% Diethane 45 (fungicide) for 30 seconds and transplanted in pots containing sterile mixture (vermiculite) and covered with polythene bags. The pots were kept in tissue culture laboratory. The seedlings were regularly sprayed with half strength MS solution up to 15days of transfer and during the next 15 days these were sprayed with liquid MS solution without agar and sugar.

After one month the plants were transferred to acclimatization media and medium comprised of pre sterilized leaf molds + soil (1:1).

Statistical Analysis

Ten explants were used per treatment on each multiplication and rooting medium. Experiments were repeated thrice. MS excel software (Microsoft office Excel 2007. Ink) was used to determine the growth of the plant.

Parameters Studied

1. Shoot number per explants

2. Shoot length

3. Root number per explants

4. Root length

5. Percentage of explants response

6. Days required for rooting