**BIOTECHNOLOGY STANDARD OPERATING PROCEDURES**

**Standard Operation Procedures for DNA Extraction**

1. **DNA extraction**

**1. Title:** Standard Operating Procedure for plant DNA extraction for genetic analysis using molecular marker techniques.

**2. Scope:** This procedure is applicable to plant species under genetic analysis using molecular markers

**3. Purpose:** The purpose of this procedure is to extract pure DNA from plant for genetics analysis of trees.

**4. Terms, Acronyms and Definitions:**

1. DNA: Deoxyribonucleic acid is a nucleic acid containing the genetic instructions.
2. IB: Isolation buffer
3. CIA: Chloroform Isoamyl Alcohol
4. CTAB: Cetyl trimethyl ammonium bromide
5. NaOAc: Sodium acetate
6. EDTA: Ethylenediaminetetraacetic acid, is an aminopolycarboxylic acid and a colourless, water-soluble solid.
7. RNAse: Ribonuclease is a type of nuclease that catalyzes the degradation of RNA into smaller components. An enzyme that promotes the breakdown of RNA into oligonucleotides and smaller molecules.
8. RNA: ribonucleic acid, a nucleic acid present in all living cells. Its principal role is to act as a messenger carrying instructions from DNA for controlling the synthesis of proteins.
9. DNAse: an enzyme that catalyzes the hydrolysis of DNA into oligonucleotides and smaller molecules

**5. Apparatus/equipment**

1. Ball mill grinder
2. Zarconian balls
3. Refrigerated centrifuge
4. Vortex
5. Fume hood
6. Water bath
7. Electrophoresis system
8. Thermocycler
9. Digital camera
10. UV illumination table
11. Computer
12. Eppendorf tubes
13. Pippette tips (10μl- 1000μl)
14. Micro pipettes (10μl- 1000μl)
15. Oven
16. Microwave
17. Leave samples

**6. Reagents and chemicals**

1. CTAB
2. Tris base
3. Sodium Chloride (NaCl)
4. Sodium Acetate (NaOAc)
5. Disodium EDTA
6. Chloroform
7. Isoamyl alcohol
8. Isopropanol
9. Ethanol
10. Agarose
11. RNAse
12. SYBR safe/ green
13. β-mercaptoethanol
14. Sorbitol

**7. Procedure**

* 1. Grind dried plant tissue (0.1 – 0.2 g) in ball mill grinder to a fine powder in a 2ml tube.
  2. Add 500 μl IB (10% polyethylene glycol MW 6,000, 0.35 M sorbitol, 1 M Tris-HCl,0.5% β-mercaptoethanol (add just before extraction)) and mix by vortexing.
  3. Centrifuge at 10,000 rpm at 4°C for 2–3 minutes.
  4. Remove supernatant IB and add 800 μl IB. Then remix by vortexing.
  5. Repeat steps 7.3 and 7.4 until supernatant IB becomes less viscous.
  6. Remove supernatant IB and add 800 μl CTAB solution (1% cetyltrimethylammonium bromide, 0.05 M Tris-HCl, 0.7 M NaCl, 0.5% β-mercaptoethanol (add just before extraction), RNase).
  7. Incubate at 65°C for 30 – 60 minutes, then incubate at 37°C for 30 – 60 minutes.
  8. Add an equal volume (normally 800 μl) of CIA (chloroform : isoamyl alcohol = 24) and mix by inversion for 10 minutes.
  9. Centrifuge at 14,000 rpm at room temperature for 10 minutes.
  10. Transfer only the upper aqueous phase to a new 2.0 ml microfuge tube.
  11. Add an equal volume of CIA and mix by inversion for 10 minutes.
  12. Centrifuge at 14,000 rpm at room temperature for 10 minutes.
  13. Transfer only the upper aqueous phase to a new 1.5ml microfuge tube.
  14. Add 1/10 volume of 3 M NaOAc.
  15. Add an equal volume of 99.5% isopropanol or 2.5 times the volume of 99.5% ethanol, and mix the solution by inversion.
  16. Centrifuge at 15,000 rpm at 4°C for 5 minutes.
  17. Discard all the supernatants, then add 800 μl ,70% ethanol and flip the tube to wash the DNA.
  18. Centrifuge at 15,000 rpm at 4°C for 5 minutes.
  19. Discard all the supernatant and dry the DNA pellet.
  20. Add 100 μl DNase-free water and dissolve the DNA.

**8. References:**

* 1. Milligan B.G. (1992) Plant DNA isolation. In: Hoelzel A.R. (ed.). Molecular Genetic Analysis of Populations: A Practical Approach. IRL Press, Oxford, pp 59–88.
  2. Wagner D.B. et al. (1987) Chloroplast DNA polymorphism in lodgepole and jack pines and their hybrids. Proc. Natl. Acad. Sci. USA 84: 2097–2100.

**Standard Operating Procedures for Polymerase Chain Reaction (PCR)**

**1. Title** Standard Operating Procedure for Polymerase Chain Reaction (PCR) in plant and microbial genetic analysis.

**2. Scope:** This procedure covers all activities of plant and microbial genetic analysis using molecular marker techniques.

**3. Purpose:** The purpose of this procedure is to amplify copies of fragments of DNA synthesised by the markers used.

**4. Terms, Acronyms and Definitions:**

1. PCR: Polymerase Chain Reaction: Is a technique that amplifies specific DNA regions.
2. DNA: Deoxyribonucleic acid (DNA) is a nucleic acid containing the genetic instructions.
3. dNTPs: Deoxynucleotide triphosphates, the building blocks from which the DNA polymerase synthesizes a new DNA strand
4. Primers: short DNA fragments containing of known sequence.

**5. Apparatus/equipment**

1. Vortex
2. Water bath
3. Electrophoresis system
4. Thermocycler
5. Digital camera
6. UV illumination table
7. Computer
8. Eppendorf tubes
9. Pippette tips (10μl- 1000μl)
10. Micro pipettes (10μl- 1000μl)
11. Microwave
12. Ice maker

**6. Reagents and chemicals**

PCR Reagents (Master Mix)

1. 10×PCR buffer (MgCl2)
2. 10mM dNTP mixture
3. 50mM MgCl2
4. Primer Mix (10μM each)
5. Template DNA (10-50ng / μl)
6. Taq DNA Polymerase (5U /μl)
7. Sterilized water to 100 μl

**7. Procedure**

7.1 Prepare the PCR master mix as follows

1. 10 μl 10×PCR buffer (-MgCl2) (final 1×)
2. 2 μl 10mM dNTP mixture (final 0.2mM each)
3. 3 μl 50mM MgCl2 (final 1.5mM)
4. 5 μl Primer Mix (10μM each) (final 0.5μM each)
5. 1 μl Template DNA (10-50ng / μl)
6. 0.2-0.5 μl Taq DNA Polymerase (5U /μl) (1.0 - 2.5 unit) to 100 μl sterlized water
   1. The reaction mix is prepared on ice.
   2. Place your reaction mix in the thermocycler with the programmed PCR temperature profile as shown below.

Stage 1:

95 °C for 3 minutes

Stage 2:   
95 °C for 45 seconds  
55 °C for 30 seconds(the annealing temperature depends with the primer used and varies between 37°C-57°C)  
72 °C for 90 seconds  
\*25 – 35 cycles

Stage 3:   
72 °C for 10 minutes

7.4 The PCR products are then analyzed through electrophoresis

1. **References:**
2. Milligan B.G. (1992) Plant DNA isolation. In: Hoelzel A.R. (ed.). Molecular Genetic Analysis of Populations: A Practical Approach. IRL Press, Oxford, pp 59–88.
3. Wagner D.B. et al. (1987) Chloroplast DNA polymorphism in lodgepole and jack pines and their hybrids. Proc. Natl. Acad. Sci. USA 84: 2097–2100.

**Standard Operating Procedures for Gel Electrophoresis**

**1. Title:** Standard Operating Procedure to separate macromolecules (DNA, RNA and proteins) based on their size and charge.

**2. Scope:** This procedure covers all types of plant and microbial DNA molecules under genetic analysis studies.

**3. Purpose:** The purpose of this procedure is to separate DNA molecules fragments so as to detect the presence or absence of a fragment in genetics analysis.

**4. Terms, Acronyms and Definitions:**

1. RNA; Ribo Nucleic Acid – a single stranded DNA

**5. Apparatus/equipment**

1. Vortex
2. Electrophoresis system
3. Digital camera
4. UV illumination table
5. Computer
6. Eppendorf tubes
7. Pippette tips (10μl- 1000μl)
8. Micro pipettes (10μl- 1000μl)
9. Microwave

**6. Reagents and chemicals**

1. Agarose
2. Tris base
3. Disodium EDTA
4. Double distilled water
5. SYBR safe/green

**6.1 Preparation reagents**

1. TAE preparation reagents

1. 50× TAE2 M Tris1 M acetic acid0.5 M EDTA

SYBR Green

Agarose

1. 1× TAE (with SYBR Green I) (1 L)20 ml 50× TAE960 ml Water   
   50 μl SYBR Green I
2. Loading buffer (10 ml)  
   25 mg bromophenol blue (final 0.25% w/v)  
   25 mg xylene cyanol (final 0.25% w/v)  
   500 mM EDTA (final 5 mM)  
   3 ml glycerol (final 30% v/v)

2. Agarose gel

1. Set up the gel cassette and comb.

2. Measure the appropriate volume of TAE buffer (including SYBR Green or Safe and pour into a conical flask.

3. Add agarose (the concentration is determined by the DNA fragment size to be analyzed 1-2.5%) and heat using a microwave or other means until agarose is completely dissolved in the buffer.

4. Cool to about 60°C and then add the appropriate SYBR Green or safe and mix.

5. Pour the agarose solution into the gel cassette and allow toset to a semi solid gel

6. Place the cassette into the electrophoresis tank with enough TAE to cover the gel.

**7. Procedure**

1. Add 2 μl of loading buffer to 8 μl of PCR products.

2. Load the stained DNA into the wells of the gel

3. Apply the size marker (0.2 – 0.5μg) into the first and last well of the gel.

4. Connect the terminals to the outlet of electric supply and separate the DNA at 80-100 Volts

5. Turn on the electrophoresis system, and wait for 20–60 minutes (depending on molecular weight).

6. Switch off the electrophoresis system, and remove the gel.

7. Place the gel on a UV transilluminator table.

8. Take photos of the gel using a digital camera.

**8. References:**

1. Milligan B.G. (1992) Plant DNA isolation. In: Hoelzel A.R. (ed.). Molecular Genetic Analysis of Populations: A Practical Approach. IRL Press, Oxford, pp 59–88.
2. Wagner D.B. et al. (1987) Chloroplast DNA polymorphism in lodgepole and jack pines and their hybrids. Proc. Natl. Acad. Sci. USA 84: 2097–2100.

**Standard Operating Procedure for preparation of Bio-fertilizer**

1. **Preparation of rhizobia culture media**

**1. Title:** Standard Operating Procedure for preparation of bio-fertilizer inoculants.

**2. Scope:** This procedure applies to all rhizobia from all leguminous plant/tree species

**3. Purpose:** The purpose of this procedure is to produce a bio-fertilizer based on rhizobia (bacteria) for leguminous plants/trees species to enhance soil fertility.

**4. Terms, Acronyms and Definitions:**

1. Biofertilizer:is a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant.
2. Rhizobia:are soil parasitic bacteria that fix nitrogen (diazotrophs) inside root nodules of legumes (Fabaceae).

**5. Apparatus/equipment**

1. Weighing balance
2. Autoclave
3. Incubator
4. Petri dishes
5. Bunsen burner
6. LPG gas
7. Wire Loop
8. Glass rod
9. Air laminar flow cabinet
10. Oven
11. Pipette 10µl- 1000µl, 1 ml, 5 ml,
12. Pipette tips
13. High density polythene paper
14. pH meter
15. Conical flasks 500 ml- 1000 mls

**6. Reagents and chemicals**

1. Constituents of yeast Mannitol Agar (YMA)
   * 1. Mannitol -
     2. Potassium Hydrogen Phosphate (K2HPO4) -
     3. Magnesium sulphate hydrated (MgSO4.7H2O)-
     4. Sodium Chloride (NaCl ) -
     5. Yeast Extract
     6. Agar
     7. Distilled Water
2. Yeast Mannitol Broth 1 litre is YMA without agar
3. Filter mud
4. Ethanol
5. Nodules from leguminous plant roots

**7. Procedure**

7.1 Weigh the above chemicals as per recipe for Yeast Extract Mannitol Agar (YMA).

7.2 Dissolve in distilled water except agar.

7.3 Adjust media pH to 6.8 using 1 M HCL or 1M NaOH depending on the alkalinity or acidity of the media.

7.4 Dispense the media in a conical flask or appropriate containers depending on the volume of the media.

7.5 Add 1.5 % agar to the media prepared in 6.4 above.

7.6 Sterilize the media by autoclaving at a temperature of 121 °C for 15 min.

7.7 Allow the media to cool to approximately 60 °C and dispense into sterile Petri dishes under aseptic conditions to solidify. The media prepared is used in Rhizobia culture after isolation in procedure B below.

**8. References:**

1. P. Somasegaran and H. J. Hoben; May, 1985 , Methods In Legume-Rhizobium Technology

**B. Rhizobium isolation**

**1. Title:** Isolation of rhizobia bacteria from nodules of legumes trees/plants.

**2. Scope:** The procedureis applicable to all rhizobia strains of leguminous plants/trees.

**3. Purpose:** The purpose of the procedure is to obtain pure rhizobia bacteria to prepare bio-fertilizer.

**4. Terms, Acronyms and Definitions:**

1. YMA: Yeast Mannitol Agar

**5. Apparatus/equipment**

1. Weighing balance
2. Autoclave
3. Incubator
4. Petri dishes
5. Bunsen burner
6. LPG gas
7. Wire Loop
8. Glass rod
9. Air laminar flow cabinet
10. Oven
11. Pipette 10µl- 1000µl, 1 ml, 5 ml,
12. Pipette tips
13. High density polythene paper
14. pH meter
15. Conical flasks 500 ml- 1000 mls

**6. Reagents and chemicals**

1. Constituents of yeast Mannitol Agar (YMA)
   * 1. Mannitol -
     2. Potassium Hydrogen Phosphate (K2HPO4) -
     3. Magnesium sulphate hydrated (MgSO4.7H2O)-
     4. Sodium Chloride (NaCl ) -
     5. Yeast Extract
     6. Agar
     7. Distilled Water
2. Yeast Mannitol Broth 1 litre is YMA without agar
3. Filter mud
4. Nodules from leguminous plant roots
5. 95% Ethanol for sterilization
6. Sodium hypochlorite (NaOCl)

**7. Procedure**

7.1Collect nodules from the roots of leguminous plants and wash them thoroughly clean

7.2 Immerse the nodules in 95 % ethanol for 5 seconds.

7.3 Transfer nodules to 3 % sodium hypochlorite (NaOCl) and immerse for 1 minute.

7.4 Rinse the nodules in six changes of sterile distilled water.

7.5 Crush each nodule in a drop of sterile distilled water.

7.6 Pick a loopful of nodule suspension and streak on YMA media on a Petri dish.

7.7 Incubate the inoculated media in the dark at a temperature of 28 °C for 3-10 days depending on growth rate.

7.8 The isolated Rhizobia bacteria is used to make the bio-fertilizer

*Notes:*

1. *Work in a positive-flow clean bench.*
2. *Wipe benches using 75 % ethanol.*

**C. Preparation of filter mud-based** inoculants ( 250 x 100 g packets)

**1. Title:** Procedure for preparation of rhizobia inoculants (Bio-fertilizer) using filter mud as a carrier.

**2. Scope:** This procedure applies to all rhizobia bacteria from all legumes.

**3. Purpose:** The purpose of this procedure is to incorporate rhizobia bacteria into the filter mud.

**4. Terms, Acronyms and Definitions:**

1. Filter mud: Industrial waste residue of the filtration of sugarcane juice mostly used as soil conditioner or soil fertilizer.
2. Broth: it is media without agar

**5. Apparatus/equipment**

1. Grinder
2. Syringe
3. High density polythene bags
4. Sieve
5. Polythene sealing machine
6. Autoclave
7. Air laminar flow cabinet
8. Incubator
9. Mechanical shaker
10. Sterilized petri dishes
11. Conical flasks
12. Beakers
13. Micro pipettes
14. Loop wire

**6. Reagents and chemicals**

1. Filter mud
2. Rhizobia

**7. Procedure:**

7.1 Make and sterilize 1000 mL broth at 121 °C for 15 min in a flask.

7.2 Inoculate the broth with selected superior rhizobial strain(s).

7.3 Aerate by bubbling sterile air through the broth until turbid.

7.4 Add 10 mL tap water to 50 g finely grinded filter mud contained in a High Density poly pack and sterilize at 121 °C for 1 hour.

7.5 Seal the packet containing sterile filter mud.

7.6 Using a sterile syringe inject 40 mL turbid broth into the filter mud. The injection hole is made at one corner end of the packet.

7.7 Seal the injection hole using a adhesive sticker.

7.8 Incubate the inoculant for 3 days.

7.9 Double pack the inoculant using branded paper with point of use instruction.

7.10 Transfer the inoculant packets to 4 °C for long term storage or store at room temperature for 6 months.

7.11 An example of a packaging label of final product of bio-fertilizer inoculant.

***Notes:***

1. *Perform quality control for populations and contamination checks on sampled packets of every batch prepared.*
2. *Perform quality control through authentication on host legume.*

**8. References:**

1. P. Somasegaran and H. J. Hoben; May, 1985 , Methods In Legume-Rhizobium Technology
2. Philpotts, H. 1976. Filter mud as a carrier for *Rhizobium* inoculants. J. appl. Bacteriol. 41:277-281.

## **KEFRIFIX**

Rhizobium Legume Inoculant

# Environmental friendly nitrogen source

Net weight 100g

Seed inoculation

1. Clean the seeds by washing to remove detergents, fungicides, herbicides, insecticides and excessive acid or alkaline fertilizers
2. Prepare 15 % sucrose solution/slurry (i.e. 15 g of ordinary sugar in 100 ml of water). Sucrose can be replaced by Methylcellulose or Gum arabic.
3. Immerse seed (up to 1.5 kg) in the slurry mix and then drain off the slurry
4. Pour 100 gm of the inoculant into the seeds and mix thoroughly to uniformly coat each seed
5. Leave seeds to air-dry briefly to eliminate any stickiness of the seed and sow the seeds directly in wet soil. Avoid direct sunlight.

Inoculating seedlings

1. For 1000 seedlings add 100g-inoculant filtermud to 20 litres of water.
2. The mixer should be stirred regularly to avoid filtermud settling.
3. Water on to the base of the seedlings. It is desirable to follow inoculation with a second watering to wash the bacteria down on to the root system.

For long-term use store unopened pack at 4oC

Store this pack of inoculum in a cool place.

Do not store open packs of inoculum

MFD: June 2012

Best before: March 2013

Unit price: KShs 200

### **BIOFERTILIZER**

Legume species*: Calliandra*

**Standard Operation Procedures for Plant Tissue Culture**

1. **Title:**Standard operating procedure for plant tissue culture.

**2. Scope:** The procedure applies to all forest tree species.

**3. Purpose:**The purpose of the procedure is to mass propagate plants *in-vitro* to enhance provision of planting materials

**4. Terms, Acronyms and Definitions:**

1. Plant tissue culture:- Techniques used to initiate and maintain growth of plant, tissues,organs and even cells under sterile conditions in a formulated nutrient medium supplemented with plant growth regulators.
2. *In-vitro*: -A process performed or taking place in a test tube, culture dish, or elsewhere outsidea living organism.
3. Auxins:- A plant hormone which causes the elongation of cells in shoots and is involved in regulating plant growth.
4. Cytokinins:- A plant hormone that promote cell division, or cytokinesis, in plant roots and shoots.
5. Direct organogenesis: – in vitro spontaneous regeneration ofwhole plants from inoculated explants
6. Callus induction: – in vitro proliferation of a mass of undifferentiated cells.
7. Shoot proliferation: – in vitro mass regene**Error! Bookmark not defined.**ration of shoots
8. Rhizogenesis: - in vitro regeneration of roots
9. Cell culture: - in vitro proliferation of single cells
10. Embryogenesis: - in vitro induction of somatic embryos
11. Embryo maturation: - in vitro maturation of somatic embryos

**5. Apparatus/equipment**

1. Weighing balance
2. Conical Flasks(50-500ml)
3. Measuring cylinders
4. Beakers(50-500ml )
5. pHmeter
6. Autoclave
7. Oven
8. Growth incubator
9. Air lamina flow cabinet
10. Bunsen burner
11. LPG gas
12. Forceps
13. Surgical blades
14. Hot plate magnetic stirrer
15. Fridge
16. Polyethelyne containers(50-1000ml)

**6. Reagents and chemicals**

|  |
| --- |
| 1. Hydrated Cobolt chloride( CoCl2.6H2O) |
| 1. Hydrated Copper sulphate (CuSO4.5H2O) |
| 1. Ferrous sodium EDTA (FeNaEDTA) |
| 1. Boric acid (H3BO3) |
| 1. Potassium iodide (KI) |
| 1. Hydrated Manganese sulphate (MnSO4.H2O) |
| 1. Sodium molybdate (Na2MoO4.2H2O) |
| 1. Calcium chloride (CaCl2) |
| 1. Potassium di hydrogen phosphate (KH2PO4) |
| 1. Potassium Nitrate (KNO3) |
| 1. MagnesiumSulphate (MgSO4) |
| 1. Glycine |
| 1. myo-inositol |
| 1. Nicotinic acid |
| 1. Pyridoxine HCl 2. Benzyl Amino Purine (BAP) 3. Indole Acetic Acid (IAA) 4. Napthalene Acetic Acid (NAA) 5. Indole Butylic Acid (IBA) 6. Zeatin 7. Gibberilic Acid (GA) 8. Tween ®20 9. 70% Ethanol 10. 6% Sodium hypochlorite 11. 1 molar HCL 12. 1 Molar Sodium hydroxide |

**7. Procedure**

* 1. **Media preparation**

1. Various formulations of the basic media exist and one has a choice based on the type of target species. However, in order to induce *in vitro* multiplication and growth the media is supplemented with varying concentrations of growth hormones to achieve the following;*Direct organogenesis, Callus induction, Shoot proliferation, Rhizogenesis, Cell culture, Embryogenesis and Embryo maturation.*
2. Nutrient media can be prepared as shown in the tables 1,2, 3,4 and 5
3. The media is then steam sterilized by autoclaving at 121oC for at least 15 Minutes.
4. After cooling the media it is dispensed into culture vessels depending on the experimental design.
   1. **Media formulation**

A suitable medium is better formulated by testing the individual addition of a series of concentrations of a given compound to a universal basal medium such as Murashige & Skoog Medium (MS), McCOWN Woody Plant Medium (WPM) or Gamborg B5 Medium (B5).

# **7.3 Hormone treatments**

Treatments are varied from a hypothetical low to a hypothetical high based on literature or other past experiment. The optimal concentration necessary lies between these limits. Table 4 shows the commonly used plant growth hormones and their relative function in tissue culture while table 5 gives a range of hormone concentration necessary for induction and proliferation of plant tissues.

# **7.4 Dispensing the media**

All media can be made either as liquid or solid with the addition of a gelling agent, usually purified agar at between 1- 2 %. Once media has been sterilized and cooled, it is dispensed off into treatment vials/bottles/kilner jars.

Table 1: Murashige & Skoog Medium

| **Element** | **Concentration (mg/l)** | **Concentration (M)** |
| --- | --- | --- |
| ***Micro elements*** |  |  |
| CoCl2.6H2O | 0.025 | 0.11µM |
| CuSO4.5H2O | 0.025 | 0.10µM |
| FeNaEDTA**Error! Bookmark not defined.** | 36.70 | 0.10mM |
| H3BO3 | 6.20 | 0.10 mM |
| KI | 0.83 | 5.00µM |
| MnSO4.H2O | 16.90 | 0.10 mM |
| Na2MoO4.2H2O | 0.25 | 1.03µM |
| ZnSO4.7H2O | 8.60 | 29.91µM |
| ***Macro elements*** |  |  |
| CaCl2 | 332.02 | 2.99 mM |
| KH2PO4 | 170.00 | 1.25 mM |
| KNO3 | 1900.00 | 18.79 mM |
| MgSO4 | 180.54 | 1.50 mM |
| NH4NO3 | 1650.00 | 20.61 mM |
| Total | 4302.09 |  |
| ***Vitamins*** |  |  |
| Glycine | 2.00 | 26.64µM |
| myo-inositol | 100.00 | 0.56 mM |
| Nicotinic acid | 0.50 | 4.06µM |
| Pyridoxine HCl | 0.50 | 2.43µM |
| Thiamine HCl | 0.10 | 0.30µM |
| Total | 4405.19 |  |

Table 2: GAMBORG B5 MEDIUM.

| **Element** | **Concentration mg/l** | **Concentration M** |
| --- | --- | --- |
| ***Micro elements*** |  |  |
| CoCl2.6H2O | 0.025 | 0.11µM |
| CuSO4.5H2O | 0.025 | 0.10µM |
| FeNaEDTA | 36.70 | 0.10 mM |
| H3BO3 | 3.00 | 48.52µM |
| KI | 0.75 | 4.52µM |
| MnSO4.H2O | 10.00 | 59.16µM |
| Na2MoO4.2H2O | 0.25 | 1.03µM |
| ZnSO4.7H2O | 2.00 | 6.96µM |
| ***Macro elements*** |  |  |
| CaCl2 | 113.23 | 1.02 mM |
| KNO3 | 2500.00 | 24.73 mM |
| MgSO4 | 121.56 | 1.01 mM |
| NaH2PO4 | 130.44 | 1.09 mM |
| (NH4)2SO4 | 134.00 | 1.01 mM |
| Total | 3051.98 |  |
| ***Vitamins*** |  |  |
| myo-inositol | 100.00 | 0.56 mM |
| Nicotinic acid | 1.00 | 8.12µM |
| Pyridoxine HCl | 1.00 | 4.86µM |
| Thiamine HCl | 10.00 | 29.65µM |
| Total | 3163.98 |  |

Table 3. McCOWN Woody Plant Medium (WPM)

| **Element** | **Concentration mg/l** | **Concentration M** |
| --- | --- | --- |
| ***Micro elements*** |  |  |
| CuSO4.5H2O | 0.25 | 1.00µM |
| FeNaEDTA**Error! Bookmark not defined.** | 36.70 | 0.10 mM |
| H3BO3 | 6.20 | 0.10 mM |
| MnSO4.H2O | 22.30 | 0.13mM |
| Na2MoO4.2H2O | 0.25 | 1.03µM |
| ZnSO4.7H2O | 8.60 | 29.91µM |
| ***Macro elements*** |  |  |
| CaCl2 | 72.50 | 0.65 mM |
| Ca(NO3)2 | 386.80 | 2.35 mM |
| KH2PO4 | 170.00 | 1.25 mM |
| K2SO4 | 990.00 | 5.68 mM |
| MgSO4 | 180.54 | 1.50 mM |
| NH4NO3 | 400.00 | 5.00 mM |
| Total | 2274.14 |  |
| ***Vitamins*** |  |  |
| Glycine | 2.00 | 26.64µM |
| myo-inositol | 100.00 | 0.56 mM |
| Nicotinic acid | 0.50 | 4.06µM |
| Pyridoxine HCl | 0.50 | 2.43µM |
| Thiamine HCl | 1.00 | 2.96µM |
| Total | 2378.14 |  |

Table 4. Commonly used plant growth hormones in tissue culture.

| **PGR** | **Hormone treament** | **Effect in plant tissue culture** |
| --- | --- | --- |
| Auxins | Indole-3-Acetic Acid  Indole-3-Butyric Acid  Indole-3-Butyric Acid, K-Salt  α-Naphthaleneacetic Acid  α-Naphthaleneacetic Acid, K-Salt  2,4-D (Solutions)  ρ-Chlorophenoxyacetic acid  Picloram  Dicamba | • Adventitious root formation (high concentration)  • Adventitious shoot formation (low concentration)  • Induction of somatic embryos  • Cell Division  • Callus formation and growth  • Inhibition of axillary buds  • Inhibition of root elongation |
| Cytokinins | 6-Benzylaminopurine  6-(γ,γ-Dimethylallylamino)purine (2iP)  2iP-2HCl  Kinetin  Thidiazuron (TDZ)  N-(2-Chloro-4-pyridyl)-N-phenylurea  Zeatin  Zeatin Riboside | • Adventitious shoot formation  • Inhibition of root formation  • Promotes cell division  • Modulates callus initiation and growth  • Stimulation of axillary bud breaking and growth  • Inhibition of shoot elongation  • Inhibition of leaf senescence |
| Gibberellins | Gibberellic Acid (GA3)  GA4/7 | • Stimulates shoot elongation  • Release seeds, embryos, and apical buds from dormancy,  • Inhibits adventitious root formation |
| Abscisic Acid | Abscisic Acid | • Stimulates bulb and tuber formation  • Stimulates the maturation of embryos  • Promotes the start of dormancy, leaf abscission |
| Polyamines | Putrescine  Spermidine | • Promotes adventitious root formation  • Promotes somatic embryogenesis  • Promotes shoot formation |

Table 5. An example of a range of hormone concentration necessary for induction and proliferation of plant tissues

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Hormone** | **Direct organogenesis** | **Shoot proliferation** | **Root induction** | **Callus induction** | **Somatic embryogenesis** |
| Cytokinins | Cytokinins/Auxins | 0.1-10.0 mg/L. | Inhibits |  |  |
| Auxins | Cytokinins/Auxins | Inhibits | 0.01-10.0 mg/L. | 0.01-10.0 mg/L. | 0 - 10µM (2,4-D) 0-10µg/L (TDZ) |
| Polyamines: |  | 10-1000 mM. | 10-1000 mM. |  |  |
| Gibberellins |  | 1 - 10µM |  |  |  |

Other ingredients of the media may include antibiotics (for control of contamination), polyethylene glycol (PEG**Error! Bookmark not defined.**) and Activated Charcoal (for the control of polyphenols production). Addition of thermolabile organic constituents such as vitamins, growth regulators and amino acids is done through filtration through microporus filters (0.22- 0.45) after media sterilization.

**7.5 Choice of explant material**

The best explant material is obtained from young, healthy and actively growing tissues such as meristematic tissues e.g. apical meristems, axillary buds, leave discs, and roots.

**7.6 Harvesting of explant material**

Collection of explants material varies from collecting the tissues from standing plants or harvesting from in vitro plantlets.

**7.7 Sterilization**

Explants are thoroughly washed in detergent such as Tween ®20 and thereafter surface sterilized. The common sterilizing agent is sodium or calcium hypochlorite (Common bleach). Explants sterilization is key for the elimination of in vitro contaminants. Metallic and glassware instruments are steam sterilized in an autoclave.

**7.8 Inoculation**

Explants inoculation only carried out in the clean bench. Inoculated tissues are immediately transferred into growth chambers.

**7.9 Incubation room**

Plant tissues inoculated in vitro are immediately incubated under controlled light and temperature conditions. The optimal conditions are determined by the physiology of the target plant species. Most tropical plants are incubated at around 250C at 16-hour photoperiod.

**7.10 Assessment**

Most common parameters that are assessed in tissues culture include; Fungal contamination, Bacterial Contamination, Tissue necrosis, Media browning, Callus formation, Shoot formation, Root formation and Vitrification

**7.11****Sub-culturing**

Proliferating in vitro cultures are constantly sub cultured into fresh media for various reasons but most commonly for multiplication, root initiation, embryogenesis and extension of longevity.

**7.12 Habituation**

A controlled (acclimatization) room for optimized habituation of tissue-cultured plants is necessary to adapt the plantlets in harsher environment *ex vitro* and ensure mass production.

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