Practical Manual

Elementary Plant Biotechnology

Course No.: ABB-255; Credit Hours: 2(1+1)

For

B.Sc. (Hons.) Horticulture 3rd Semester

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2019

College of Horticulture & Forestry

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Elementary Plant Biotechnology

Syllabus: Requirements for Plant Tissue Culture Laboratory; Techniques in Plant Tissue Culture; Media components and preparations; Sterilization techniques and Inoculation of various explants; Aseptic manipulation of various explants; Callus induction and Plant Regeneration; Micro propagation of important crops; Anther, Embryo and Endosperm culture; Hardening / Acclimatization of regenerated plants; Somatic embryogenesis and synthetic seed production; Isolation of protoplast; Demonstration of Culturing of protoplast; Demonstration of Isolation of DNA; Demonstration of Gene transfer techniques, direct methods; Demonstration of Gene transfer techniques, indirect methods; Demonstration of Confirmation of Genetic transformation; Demonstration of gel-electrophoricsis techniques. Green synthesis of nano particles and their size characterization.

Name of Student ....

Roll No. ....

Batch ....

Session ....

Semester ....

Course Name: ....

Course No.: ....

Credit ....

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No. .... as per the syllabus of B.Sc. (Hons.) Agriculture/ Horticulture/ Forestry .... semester

in the year.....................in the respective lab/field of College.

Date: Course Teacher

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Experiment No. 1

Objective: To identify the important glass wares and instruments used in the biotechnology laboratory and write their uses

Plastic and Glasswares	
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Test tube stand: ……………………………………………………………… …………………………………………………………………………………… …………………………………………………………………………………… …………………………………………………………………………………… ……………………………………………………………………………………	
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Measuring cylinder: ……………………………………………………………	
Volumetric Flask: ……………………………………………………………	
Funnel: ……………………………………………………………………	
Separatory Funnel: …………………………………………………………	
Reagent bottles: ……………………………………………………………	
Wash bottle: ………………………………………………………………	
Pasteur pipette: ……………………………………………………………	

Pestle and Mortar: …………………………………………………………… ……………………………………………………………………………………… ……………………………………………………………………………………… ……………………………………………………………………………………… ………………………………………………………………………………………	
Liquid Nitrogen cane: ……………………………………………………… ……………………………………………………………………………………… ……………………………………………………………………………………… ……………………………………………………………………………………… ………………………………………………………………………………………	
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Instruments	
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Mini-centrifuge: …………………………………… …………………………………………… …………………………………………… ……………………………………………	

Table top centrifuge:…………………………………………… …………………………………………………………………… …………………………………………………………………… …………………………………………………………………… ……………………………………………………………………	
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Multi-channel Micro-pipette: …………………….…………………………… ………………………………………………………….…….……………………….. …………………………………………………………….....…………………….. …………………………………………………………….…….……………….. …………………………………………………………..……….………………..	
Spectrophotometer: ………………………………………………………… ………………………………………………………….…….……………………….. …………………………………………………………….....…………………….. …………………………………………………………….…….……………….. …………………………………………………………..……….………………..	

Experiment No. 2

Objective: To aware about plant tissue culture laboratory organization and personnel safety

Plant Tissue Culture laboratory organization:

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Washing facilities & Drying area:

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General laboratory and Media Preparation area:

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Essential equipment and items:

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Culturing facilities:

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Transfer area:

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Green House:

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Safety aspects:

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2. ………………………………………………………………………………………………………
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5. ………………………………………………………………………………………………………

Experiment No–3

Objective: To perform the handling and standardization of plant materials under generally followed step of tissue culture techniques

Procedure: General procedure and standardization of plant materials are-

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Observation:………………………………………………………………………………………………

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Experiment No–4

Objective: To demonstrate the various sterilization techniques used in plant tissue culture

Principle:……………………………………………………………………………………………………

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(A) Steam Sterilization:

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2. ………
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3. ………
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4. ………
   ……………………………………………………………………………………………………

Observation:………………………………………………………………………………………………

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(B) Dry Sterilization:

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2. ………
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4. ………
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Observation:…………………………………………………………………………………………...................
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(C) Filter Sterilization:

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5. ………...…
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Observation:……………………………………………………………………………………………
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(D) Chemical Sterilization:

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Observation:…………………………………………………………………………………………………

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Table: Composition of effective chemical sterilants for explant

S. No.	Chemical sterilants	Working concentration	Effectiveness	Treatment time (Min)	Remarks
1.	Sodium hypochlorite				
2.	Calcium hypochlorite				
3.	Hydrogen peroxide				
4.	Bromine water				
5.	Silver nitrate				
6.	Mercuric chloride				
7.	Antibiotics				

(E): Alcohol Sterilization: …………………………………………………………………………………

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(F) Flame Sterilization:

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Observation:…………………………………………………………………………………………………

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Experiment No–5

Objective: To demonstrate the surface sterilization of the given explant samples for tissue culture purpose

Requirements:…………………………………………………………………………………………

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Procedures:

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Observation: Following observation are taken for aseptic bud culture with respect of time period are given in the Table:

Time (min) Of dipping in 70 % ethanol	Time (min) of immersion in Ca $(OCl)_2$ solution	Infiltration of Ca $(OCl)_2$ solution	Percentage of aseptic cultures sampled in the month of					
			1st	2nd	3rd	4th	5th	Mean
0.5	20	No						
0.5	20	Yes						

0.5	20	No						
0.5	20	Yes						
0.5	20	No						
0.5	20	Yes						
0.5	20	No						
0.5	20	Yes						
0.5								

Results:……………………………………………………………………………………………………....................

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Precautions:

1. ………...
2. ………
3. ………

Experiment No–6

Objective: To prepare the various nutrient media and growth regulators used in preparation of plant tissue culture

Composition of nutrient medium:

(A) Macronutrients (10x):

Required chemicals and Reagents: ………………………..

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Procedure for preparation:

1. ………………………………………………………………………………………………………..
2. ………………………………………………………………………………………………………..
3. ………………………………………………………………………………………………………..
4. ………………………………………………………………………………………………………..
5. ………………………………………………………………………………………………………..
6. ………………………………………………………………………………………………………..

(B) Micronutrients (100x):

Chemicals and Reagents required:…………………………………..………………………………………

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Procedure for preparation:

1. ……………………………………………………………………………………………………….
2. ……………………………………………………………………………………………………….
3. ……………………………………………………………………………………………………….
4. ……………………………………………………………………………………………………….
5. ………………………………………………………………………………………………………..

(C) Vitamins and growth hormones

Procedure for preparation:

1. ……………………………………………………………………………………………………….
2. ……………………………………………………………………………………………………….
3. ……………………………………………………………………………………………………….
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(D) Iron Source:

Procedure for preparation:

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(E) pH (Final amount):………………………...……………………………………………………………….…

Results:……………………………………………………………………………………………....................

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Experiment No–7

Objective: To prepare the M.S. media using micronutrient, macronutrient and growth regulators

Requirements:…………………………………………………………………………………………………

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Procedures:

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Results:……

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Experiment No–8

Objective: To demonstrate the seed germination of tomato and tobacco under controlled condition

Requirement:……………………………………………………………………………………………

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Procedure:

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Observation:……………………………………………………………………………………………

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Results:…………………………………………………………………………………………………

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Experiment No–9

Objective: To demonstrate the callus induction from tomato and tobacco explants and regeneration of plants

Requirements:……………………………………………………………………………………………………

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Procedures:

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Observation: The callus induction growth has been compared from various explants.

<table>
<tr><td rowspan="6">Growth observation</td><td>Days interval</td><td colspan="5">Callus from different explant</td></tr>
<tr><td></td><td>Shoot apex with cotyledons</td><td>Shoot apex without cotyledons</td><td>Media inserted stem base</td><td>Cotyledons</td><td>Hypocotyl section</td></tr>
<tr><td>3rd days</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>5th days</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>7th days</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>10th days</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td>15th days</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td colspan="2">Remarks</td><td colspan="5"></td></tr>
</table>

Result:………

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Experiment No–10

Objective: To determine the fresh and dry weight of callus by destructive method

Requirements:……………………………………………………………………………………………

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Procedure:

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Observation: The fresh weight, dry weight and fresh/dry weigh ration can be calculated with following formula-

Fresh weight $= [C] - [B] \times \frac{\text{Total culture volume}}{\text{Culture volume} - 2\text{ ml}}$

Dry weight $= [D] - [A] \times \frac{\text{Total culture volume}}{\text{Culture volume} - 2\text{ ml}}$

Fresh weight/dry weight ratio $= \frac{\text{Fresh weight}}{\text{Dry weight}} \times 100$

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Results:

Fresh weight =…………………mg

Dry weight = …………………..mg

Fresh/dry weight ratio**=**… : ...

Experiment No–11

Objective: To demonstrate the embryo culture techniques from the cereals (Wheat, rice, barley, maize etc.)

Requirements:……………………………………………………………………………………………

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Procedures:

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Observation: Observation will be recorded after every third day of the plantlet establishment

Week intervals	**Developmental stages**				
	I	**II**	**III**	**IV**	**V**
1st week					
2nd week					

3rd week					
4th week					
Remarks					

Results:………………………………………………………………………………………………………...................

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Experiment No–12

Objective: To demonstrate the technique of anther culture for haploid production from cereals (rice, wheat, barley, maize etc.) anther.

Requirements:……………………………………………………………………………………………

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Procedure:

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12. ……………………………………………………………………………………………………

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Observation: Observation will be recorded after every third day of the plantlet establishment

Week intervals	Developmental stages				
	I	**II**	**III**	**IV**	**V**
1st week					
2nd week					
3rd week					
4th week					
Remarks	The haploid plants can be identified at juvenile stage using PCR/molecular marker techniques				

Results:……………………………………………………………………………………………………......................

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Experiment No–13

Objective: To demonstrate the technique of protoplast isolation and culture using tomato/tobacco leaves

Requirements:…………………………………………………………………………………..………………

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Procedures:

1. ……..…
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2. ……
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8. ……
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10. ……
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11. ……
    …….
12. ……
    ……

Observation:

Cultured protoplast	**Observation**				
	I	**II**	**III**	**IV**	**V**
3rd days					
5th days					
7th days					
2nd week					
3rd week					
4th week					
Remarks:					

Results:……………………………………………………………………………………………………......................

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Experiment No–14

Objective: To prepare CTAB buffer and reagents used in plant genomic DNA isolation

Required:……

…….

Calculation for CTAB buffer (Volume 400 ml):

1. 2 % CTAB = **8 gm**
2. 100 mM Tris HCl = 157.6/1000x400x100mM/1000= **6.304 gm**
3. 1.4 M NaCl = 58.44/1000x400x1.4M=**32.72 gm**
4. 20mM EDTA=372.24/1000x400x20mM/1000=**2.97 gm**

(A) Procedure for preparation of CTAB buffer:

1. ………

   ………
2. ………

   ………
3. ………

   ………
4. ………

   ………
5. ………

   ………

(B) Procedure for preparation of ammonium acetate 7.5 M (50 ml volume):

1. ………
2. ………
3. ………
4. ………

(C) Procedure for preparation of Chloroform Isoamyl alcohol (24:1), Volume (100 ml):

1. ………
2. ………
3. ………
4. ………

(D) Procedure for preparation of 70 % ethanol (Volume 100 ml):

1. ………

   ………

2. ………

…….

3. ………

……………………………………………………………………………………………………..……

(E) Procedure for preparation of TE buffer @pH 8.0 (Volume 200 ml):

1. ………………………………………………………………………………..……………………......

2. ……………………………………………………………………………………………………........

3. ………………………………………………………………………………………………..……......

4. ……………………………………………………………………………………………………........

(F) Procedure for 50 X TAE (Tris base, acetic acid and EDTA) buffer (Volume 1000 ml)

1. …………………………………………………………………………………………………..……......

2. ………………………………………………………………………………………….........................

3. …………………………………………………………………………………………………..……......

4. ……………………………………………………………………………………………………........

Experiment No–15:

Objective: To isolate the genomic DNA from given leaf sample using CTAB buffer

Material required: ………………………………………………………………………………………..……

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Chemicals and Reagents required: ……………………..…………………………………………….………..

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Procedure:

1. ……
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11. …….
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12. …….
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Precautions:

1. ……..
2. ……..
3. ……..

Experiment No–16

Objective: To demonstrate PCR technique using thermal cycler

Requirements:……………………………………………………………………………………………………

……………………………………………………………………………………..…………………………

Procedures:

(A) Protocol (Assemble of PCR components):

1. ………
   ………
2. ………
   ………
3. ………
   ………
4. ………
   ………
5. ………
   ……..

(B) Standard PCR Reaction Mixture:

1. ……
   ………..
2. ……
   ……
3. ……
   …….

(C) **Steps of PCR**

1. **Initial Denaturation (94°C):** ……………………………………………………………………………
   ………
2. **Annealing (55 °C):** ………………………………………………………………………………………
   ……….
3. **Extension (Elongation) (72°C):** ……………..………………………………………………………
   ………

(D) Standard PCR amplification steps:

Sl. No	Steps	Temperature	Times
1.	Initial denaturation		
2.	Denaturation		
3.	Annealing		
4.	Extension		
5.	Final Extension		

Observation:…………………………………………………………………………………………………

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Result:………

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Experiment No–17

Objective: To demonstrate the agarose gel electrophoresis using plant genomic DNA

Requirement:……………………………………………………………………………………………

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Procedures:

1. ……………………………………………………………………………………………………
   ……………………………………………………………………………………………………
2. ……………………………………………………………………………………………………
   ……………………………………………………………………………………………………
3. ……………………………………………………………………………………………………
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Observation:……………………………………………………………………………………………

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Result: …………………………………………………………………………………………………

Experiment No–18

Objective: To demonstrate the non-PCR based RFLP techniques using the plant genomic DNA.

Requirements:…………………………………………………………………………………………………

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Procedures:

1. ………………………………………………………………………………………………………
2. ………………………………………………………………………………………………………
3. ………………………………………………………………………………………………………...
4. ………………………………………………………………………………………………………
5. ………………………………………………………………………………………………………
6. ……………………………………………………………………………………………………….
7. ………………………………………………………………………………………………………...
8. ……………………………………………………………………………………………………….

Observation:

DNA fragments Size	**Observed no. of DNA fragments (bp)**				
	I	II	III	IV	V
20-50 bp					
50-100 bp					
100-150 bp					
150-200 bp					
200-250 bp					
250-300 bp					

Results:…………………………………………………………………………………………..………………

GENERAL PROCEDURE AND STANDARDIZATION OF PLANT MATERIALS

1. Collection of explant materials (Pieces of seedlings, buds, stem or storage organs, leaf materials and for cereals immature embryos, mesocotyl or basal stem sections of young plants) in screw-cap bottle.
2. Sterilization of the materials by submerging in a dilute solution of the surface sterilants like calcium or sodium hypochlorite, hydrogen peroxide, bromine water, chlorine water, silver nitrate, bleaching powder etc.
3. Removal of sterilants from the surface of explant by thoroughly rinsing with several changes of the sterile distilled water.
4. Transferring the materials to a sterile Petri dish.
5. Preparation of suitable explant from the surface sterilized explants using sterilized (by dipping in 95 % alcohol and flaming and cooled) instruments (scalpels, needles, cork-borer, forceps etc.)
6. Transferring the inoculation into a suitable medium under laminar airflow.
7. Incubate at 25 to 28 ^{0}C in the dark / low light for 3 – 4 weeks for the callus development.
8. Transfer the callus into liquid culture medium under laminar airflow.
9. Incubation of the flask on a shaker at 150 rpm in continuous light at 26^{0}C for 4 -6 weeks by decanting and replacing the volume with fresh medium at two-week intervals.
10. Regeneration of plants from cell suspension culture for raising cultures to development under controlled aseptic condition.

SURFACE STERILIZATION CHART

S. No.	Disinfectants	Concentration (%)	Exposure Time (Min)	Effectiveness
1.	Calcium hypochlorite	9-10%	5-30	Very Good
2.	Sodium hypochlorite	0.5 – 1.5	5-30	Very good
3.	Hydrogen peroxide	3-12	5-15	Good
4.	Chlorine water	1-2	2-10	Very good
5.	Bromine	1-2	2-10	Good
6.	Silver nitrate	1.0	5.30	Good
7.	Mercuric chloride	0.1-1.0	2-10	Satisfactory
8.	Antibiotics	4.50 mg/l	30-60	Satisfactory

COMPOSITION OF NUTRIENT MEDIUM

(A) Macronutrients (10x):	(B) Micronutrients (100x):
NH_4NO_3	KI
KNO_3	H_3BO_3
$CaCl_2.2H_2O$	$MnSO_4.4H_2O$
$MgSO_4.7H_2O$	$ZnSO_4.7H_2O$)
KH_2PO_4	$Na_2MoO_4.2H_2O$
	$CuSO_4.5H_2O$
	$CaCl_2.6H_2O$

(C) Vitamins and growth hormones

Reagents	Solubility
Indole–3 acetic acid	1N NaOH
Indole – 3 butyric acid	1N NaOH
Kinetin	1N NaOH
Zeatin	1N NaOH
Gibberellic acid	Ethanol
Abscissic acid	1N NaOH

Vitamins and Growth Regulators
Inositol, Nicotinic acid, Pyridoxine HCl, Thiamine-HCl, IAA, Kinetin, Glycine, Trytophan, Mannitol, Sucrose,

Folic acid	1N NaOH		Agar

COMPOSITION OF MURASHIGE-SKOOG MEDIUM (MS MEDIUM) FOR PLANT TISSUE AND CELL CULTURE

Constituents	Concentration (mg/l)	Volume to be taken/liter of medium (ml)
Macro-elements (10X)		
$MgSO_4.7H_2O$	370.00	100
$CaCl_2.7H_2O$	440.00	
KNO_3	1900.00	
NH_4NO_3	1650.300	
KH_2PO_4	170.00	
$(NH_4)_2SO_4$	330.00	
Micro-element		
KI	0.83	10
H_3BO_3	6.20	
$MnSO_4.4H_2O$	22.30	
$ZnSO4.7H_2O$	8.60	
$Na_2MoO_4.2H_2O$	0.25	
$CuSO_4.5H_2O$	0.025	
$CaCl_2.6H_2O$	0.025	
Iron Source		
Fe-EDTA- Na	40.00	Added fresh
Vitamins and Hormones		
Inositol	100.00	4.00
Nicotinic acid	0.5	1.00
Pyridoxine-HCl	0.5	1.00
Thiamne-HCl	0.5	0.2
IAA	15.00	1.00
Kinetin	0.04-10	Added fresh
Sucrose	30,000.00	Added fresh
Agar	8,000.00	Added fresh
pH: Make the pH 5.7 of the final volume		

Stock solution dilution chart (Vitamins & Hormones)

Concentration of stock solutions (mg/ml)	Amount to be use (ml)	Concentration of the final solution (mg/l)				
		25 ml	500 ml	1 litre	2 litre	10 litre
0.01 mg/ml	0.1	0.004	0.002	0.0001	0.0005	0.0001
	0.5	0.02	0.01	0.005	0.0025	0.0005
	1.0	0.04	0.02	0.01	0.005	0.001
	10.0	0.4	0.2	0.1	0.05	0.01
0.1 mg/ml	0.1	0.04	0.02	0.01	0.005	0.001
	0.5	0.2	0.1	0.05	0.025	0.005
	1.0	0.4	0.2	0.1	0.05	0.01
	10.0	4.0	2.0	1.0	0.5	0.1
1.0 mg/ml	(-) 0.1	0.4	0.2	0.1	0.005	0.01
	(-) 0.5	2.0	1.0	0.5	0.25	0.05
	(-) 1.0	4.0	2.0	1.0	0.5	0.1
	(-) 10.0	40.0	20.0	10.0	5.0	1.0
10.0 mg/ml	0.1	4.0	2.0	1.0	0.5	0.1
	0.5	20.0	10.0	5.0	2.5	0.5
	1.0	4.0	20.0	10.0	5.0	1.0
	10.0	400.0	200.0	50.0	100.0	10.0

Composition of N_6 medium (Chu et al., 1978) for anther culture

Constituents	Volume (mg/l)
MgSO4.7H2O	185.00

Constituents	Volume (mg/l)
ZnSO4.7H2O	1.5

CaCl2.2H2O	166.00	H3BO3	1.6
KNO3	2830.00	EDTA disodium salt	37.3
KH2PO4	400.00	Thiamine	1.0
(NH4)2SO4	463.00	Pyridoxine	0.5
FeSO4.7H2O	27.8	Nicotinic acid	0.5
MnSO4.H2O	3.3	Sucrose	30,000.00
KI	0.8		

MEDIUM FOR CULTURING PROTOPLAST AT LOW DENSITY

Constituents	**Amount (mg/l)**	**Constituents**	**Amount (mg/l)**
1. Mineral slat			
NH_4NO_3	600.00	KI	0.75
KNO_3	1900.00	H_3BO_3	3.00
$CaCl_2.2H_2O$	600.00	$MnSO_4.H_2O$	10.00
$MgSO_4.7H_2O$	300.00	$ZnSO_4.7H_2O$	2.00
KH_2PO_4	170.00	$Na_2MoO_4.7H_2O$	0.25
KCl	300.00	$CuSO_4.5H_2O$	0.025
Sequestrene 330 Fe	28.00	$CoCl_2.6H_2O$	0.025
2. Sugars			
Glucose	68400.00	Mannose	125.00
Sucrose	125.00	Rhamnose	125.00
Fructose	125.00	Cellobiose	125.00
Ribose	125.00	Sorbitol	125.00
Xylose	125.00	Manitol	125.00
3. Organic acid (pH 5.5 with NaOH)			
Sodium pyruvate	5.00	Malic acid	10.00
Citric acid	10.00	Fumaric acid	10.00
4. Vitamins			
Inositol	100.00	Biotin	0.005
Nicotinamide	1.00	Chlorine	0.5
Pyridoxine-HCl	1.00	Riboflavin	0.1
Thiamine-HCl	1.00	Ascorbic acid	1.0
D-calcium panthothenate	0.5	Vitamin A	0.005
Folic acid	0.2	Vitamin D3	0.005
p-Aminibenzoic acid	0.1	Vitamin B12	0.01
5. Hormones			
2,4 D	1.0	Zeatin	0.1
NAA	1.0	IAA	1.0
6. Coconut water from mature fruits (Heated at 60^{0}C for 30 minutes and filter			10 ml/l

CELL PROTOPLAST WASHING MEDIUM (CPW)

Constituents	**Amount (mg/l)**
KH_2PO_4	27.2
KNO_3	101.0
$CaCl2.2H_2O$	1480.0
$MgSO_4.7H_2O$	246.0
KI	0.16
CuSO4.5H2O	0.25
Note: Adjust pH 5.8 with 0.2 N KOH or 0.2 N HCl	

PCR REACTION MIXTURE

PCR components	**Volume** (µl)
PCR Buffer, 10x	2.5
dNTPs mix	0.5
Template DNA (genomic DNA 100 ng/ **µl**)	1.0
Forward primer (250 ng/**µl**)	0.5
Reverse primes (250 ng/**µl**)	0.5

Taq DNA polymerase (3.0 u/µl)	0.5
Millique sterile water	19.5
Total reaction volume	25.0