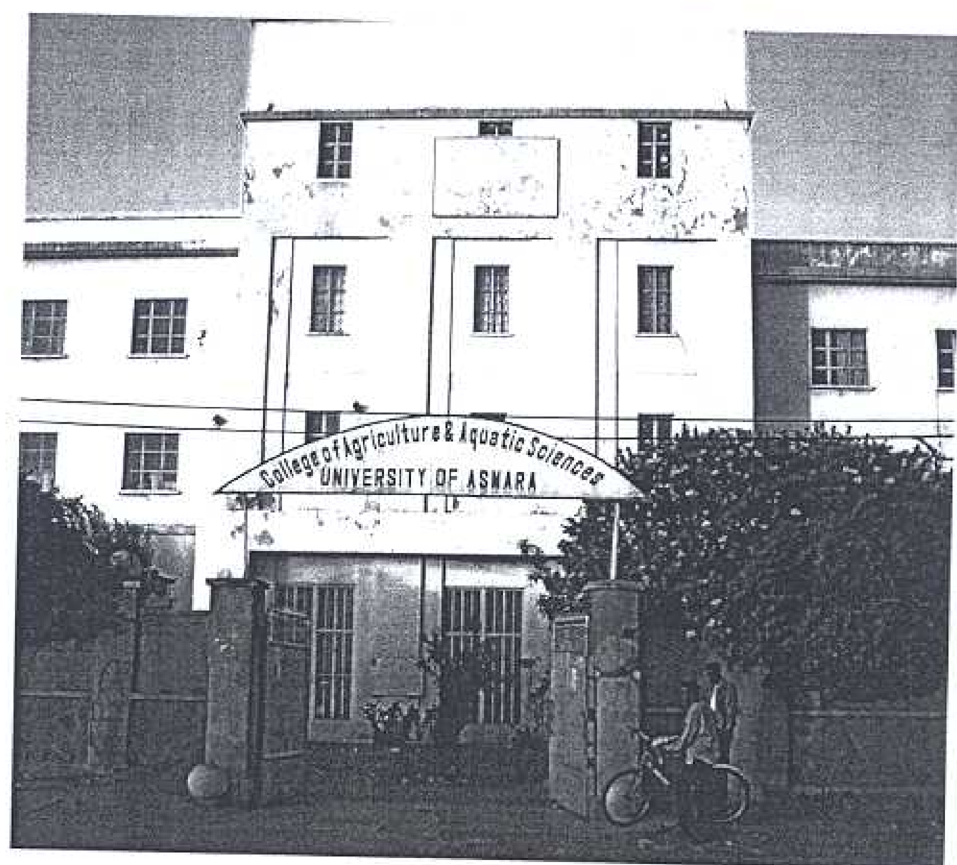
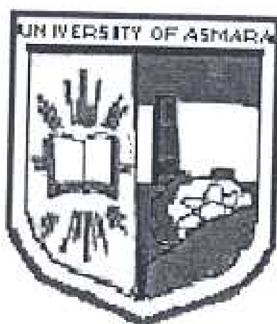


An Introduction to Plant Tissue Culture

University of Asmara
College of Agriculture

Asmara, October 2005



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RAC CHANGINS

Practice of Plant Tissue Culture

Lecture

on

IN VITRO LABORATORY TECHNIQUES

by

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Contents

Preface 4

Acknowledgements 5

I. Tissue Culture Laboratory 6

1. Basic Rule 7

2. Laboratory Organisation 7

Preparation Room 7

Storage Room 8

Sterile Room 8

Culture Room 9

3. Laboratory Management 10

Coordinator (or Manager) 10

Laboratory Technician 10

Collaborators 10

4. Laboratory Safety 11

II. Experiments 12

5. Media Preparation 13

6. Aseptic culture techniques 18

7. Micropropagation 22

8. Acclimatisation 26

III. Some useful terms 28

IV. Appendices 31

TRAINING PROGRAMME ON TISSUE CULTURE TECHNIQUES

College of Agriculture
Asmara, October 17 –21, 2005

Monday, October 17	***Tissue culture laboratory***
08.00 – 9.00	Introduction Some landmarks in Plant Tissue Culture Organisation of a Tissue Culture Laboratory Laboratory tasks
09.00 – 09.30	Break
09.30 – 10.30	Questions – Answers
10.30 – 12.30	Preparation of experimental work Chemicals, vessels, protocols
12.00 – 14.00	Lunch
14.00 – 18.00	Practical work Media preparation, sterilisation
Tuesday, October 18	***Asepsis***
08.00 – 9.00	Contamination Aseptic techniques Disinfection Modes of disinfection
09.00 – 09.30	Break
09.30 – 10.30	Questions – Answers
10.30 – 12.30	Preparation of experimental work
12.00 – 14.00	Lunch
14.00 – 18.00	Practical work Media, hemicals, instruments, plant material (potato and rose) *In vitro* Establishment (stage 1)
Wednesday, October 19	***Micropropagation*** (part I)
08.00 – 9.00	Principal techniques of micropropagation Stages of micropropagation Stage 0 : preparation of plant material Stage 1 : *in vitro* establishment Stage 2 : *in vitro* proliferation

09.00 – 09.30	Break
09.30 – 10.30	Questions – Answers
10.30 – 12.30	Preparation of experimental work Chemicals, vessels, protocols
12.00 – 14.00	Lunch
14.00 – 18.00	Practical work *In vitro* multiplication of potato
Thursday, October 20	***Micropropagation*** (part II)
08.00 – 9.00	Stage 3 : Rooting Stage 4 : Acclimatisation
09.00 – 09.30	Break
09.30 – 10.30	Questions – Answers
10.30 – 12.30	Preparation of experimental work Plant material, culture support, substrate, instruments
12.00 – 14.00	Lunch
14.00 – 18.00	Practical work Acclimatisation of potato microplants
Friday, October 21	***Recapitulation***
08.00 – 10.00	Practice of tissue culture Problems encountered in tissue culture Contamination Applications of tissue culture Example : production and conservation of high quality seed potatoes in Switzerland
10.00 – 11.00	Questions - Answers
11.00 – 12.00	Closing party
12.00 – 14.00	Lunch
14.00 – 18.00	Round – table Feed back Future works

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Preface

Tissue culture has had an important impact on the plant production and improvement during the last fifty years. Although tissue culture belongs at present to the technologies which are applied in Plant Biotechnology, it is still considered as an "Art". For the success of plant tissue culture techniques one needs a solid "know-how" as well as practical skills.

This lecture is predominantly directed to a wide range of laboratory workers (technicians, students, etc.) who are interested in tissue culture. It consists of an introductory course on the basic aseptic techniques in order to acquire the practical skills required for the applications of plant tissue culture techniques to many areas in agriculture.

Acknowledgements

I would like to acknowledge Agroscope RAC Changins for authorizing the participation in the training programme of Tissue culture in Eritrea; the Syngenta Foundation for a Sustainable Agriculture for the financial support facilitating the development and training programme of tissue culture. In particular, I am grateful to Mr. Felix Nicolier for his precious advice and important support along the setting up of the Tissue Culture laboratory at the College of Agriculture (University of Asmara). I am also grateful to Professor Woldeselassie Ogbazghi, Dean of the College of Agriculture (University of Asmara), and to Professors Bissrat Ghebru and Tadesse Mehari for their warm hospitality and for giving us the opportunity to organize the Tissue Culture course at the College of Agriculture. Finally, I am thankful to Sandra, Paul and my colleague Daniel Thomas for their help. It was a great pleasure to share with them the art of Tissue Culture.

I

TISSUE CULTURE LABORATORY

1. Basic rule

The tissue culture laboratory must be kept clean at all times. Dust and microbial spores from the environment can provoke contamination which leads to the lost of cultures. Therefore, it is important to avoid all risks of contamination by respecting some basic rules as :

- ! Change clothes and shoes (if possible) before entering the laboratory
- ! Wash correctly hands and arms with soap
- ! Discard all contaminated sources (bacteria, fungi and yeast)
- ! Cover all equipment to avoid contact with contaminants (dust, spores, etc.)
- ! Minimise the flow traffic (Move only if it is necessary!)

2. Laboratory Organisation

A laboratory devoted to Plant Tissue Culture must have appropriate areas for several activities :

Preparation Room

Tissue culture operations are generally divided into distinct areas of the laboratory. One area should be set up for media preparation and have the necessary equipment. This area will also house stock solutions. In the same area (or in an adjacent space) would be other apparatus like autoclave, oven, freezer, and a refrigerator (or a cool room). A preparation room will therefore usually contain shelves (or cupboards) for stocks of chemicals, clean glassware, containers and other supplies, etc.

This work area is a main place where culture media may be prepared with all equipments in close proximity. These are :

- Bench
- Chemicals
- Glassware
- Hotplate and Magnetic stirrer
- Balances (Analytical and precision)
- pH-meter
- Refrigerator & Freezer
- Water purification system
- Dish washing machine
- Autoclave
- etc.

Recommendations

A chemical hood should be installed for the storage of volatile and for the manipulations of dangerous (toxic) compounds.

Cleanliness of culture vessels is important for succeeding in Tissue Culture. Therefore, glassware should be washed correctly and rinsed with deionized water and finally placed in a clean area (bench, rack or oven) for drying.

Glassware dedicated to tissue culture laboratory should not be shared with other laboratory.

Contaminated cultures should be autoclaved and the media discarded before washing (by hand or in a dish washer).

Storage Room

One area in adjacent to the sterile room shoud be set up for storage of sterile vessels, sterile water and prepared media until being used. This location reduces of course the risk of contamination.

Recommendations

Glassware and disposable equipments should be kept under cover in view to protect from dust.

Stock of chemicals can also be stored in this room, but in a lockable cupboard.

Sterile Room

In this area, all operations as explant isolation and/or micropropagation with multiple transfers (or subcultures) must be conducted within a sterile environment, which will ensure that there is low (or no) risk of contamination by micro-organisms (bacteria, yeast, fungi, etc.). To this end, the most effective and convenient way to prevent contamination is to operate under sterile conditions provided by a laminar air flow cabinet.

Recommendations

The room should be designed to avoid dust and air currents. A positive air pressure system would be ideal.
Instruments (binocular microscope, surgical blades, scalpels), sterile water, chemicals (alcohol, disinfectants, etc.), sterile paper and sterilized culture media can also be stored in this section to permit to working conveniently in close proximity.

Culture Room (or Growth room)

The growth chamber (or incubation area) required for plant cultures depend greatly on the kind of work undertaken. Therefore, most experimental works done on plants with a small number of cultures could be accommodated in an incubator having light and temperature controls. For the commercial purpose, a room with multiple racks of shelving equipped with environmental control device (i.e. temperature , light, humidity and photoperiods). But, the latter is less convenient when different conditions of culture are needed.

Recommendations

This area must have temperature control, light intensity and photoperiod control and possibly humidity control.
Most of cultures can be incubated in a range of 10 to 27 °C under 16/8 hr. light/dark regime.

Concerning the light intensity, the range of light depending on the sources can be 20 to 200 μE/sec/m^2. Generally, 50 to 60 μE/sec/m^2 seems to be enough for a standard condition of culture.

Some values in foot-candles or microeinsteins (μE) per second and per square meter :

- full sun = 10'000 fc = 2'000 μE/sec/m^2
- 1μE/sec/m^2 = 6.02 x 10^{17} photons /m^2= μmol/sec/m^2= 0.215W/ m^2

3. Laboratory Management

Coordinator or Manager

The role of the coordinator or manager is to oversee the laboratory with respect to the planning of works, the personnel management and the technical equipment as well. It is necessary that this person possesses an university background in order to face difficulties arising in the field of fundamental plant biology (physiology, biochemistry, molecular biology).

Laboratory technician

The laboratory technician must be able to help the coordinator in the field of practical running of the laboratory. He (she) must know instantly the number of species cultivated, their location, their current stage. In addition, he (she) should have an inventory of all equipment and maintains them in a workable condition according to a schedule of maintenance requirements (annual, bisannual service ?). He (she) must also to purchase consumable items and keep a list of detailed chemicals (non toxic and toxic) compounds which will be stored separately in appropriate location. He (she) keeps a list of reservation for the use of laminar air flow cabinet during the week.

Recommendations

Set up an inventory containing important data on all facilities and equipment.
Use proper blanket for each specific item (see examples).
Set up a plan (semester, annual, etc.) for maintenance of equipment and apparatus.
Up-to-date record all the need of chemicals, glassware, instruments, etc. to avoid the lack of laboratory material during the run of experiments.

Collaborators

Potential users of the laboratory including scientists, students or laboratory employees must be well informed by the manager or the laboratory technician on the rules assigned to the laboratory : " Leave the laboratory in the condition you want to find it ".

4. Laboratory Safety

Every person who starts to work in the laboratory must participate in an information session. This session should give instructions on how to operate major items or equipment (water demineralizer, sterilizator, balances, pH-meter, laminar air flow cabinet, etc.). Specific recommendations will also be provided on how to dispose of different kinds of waste (used blades, broken glass, domestic waste, toxic compounds, etc.). Also, first aid kits, eye douche and portable fire extinguishers should be placed so that everyone knows where they are and how to use them.

<u>Recommendations</u> :

Never pipette by mouth.
Handling alkalis and acids with extreme caution.
Avoid handling alcohols when operating with open flames.
Switch off the electrical appliances when not in use for a long period.
Open the autoclave only when the temperature is below 100°C (i.e. pressure is falling to zero).
Toxic chemicals must be stored in a chemical hood (or ventilated cupboard).
The laboratory must not be used as a <u>Cafeteria for Eating, Drinking or Smoking.</u>

II

Experiments

5. Media preparation

The choice of the culture medium, depending on the species (or variety) and the purpose of the tissue culture technique which is to be used, is important to the success in plant tissue culture. In general, the culture medium consists of **inorganic salts, vitamins, growth regulators** and **a carbon source**. In addition, other components as **antibiotics, amino acids, natural complexes** can be added for specific purposes. A gelling agent (**Agar**) has to be added when the solid medium must be prepared.

Exercice 1:

Preparation of stock solutions (**SS**).

Procedures (overhead)

- Record data in the column "**Stock Solutions**" (see Table 1. below) using a reference preparation sheet (see appendix 6).
- For **Macronutrient salts**, weigh exact amount of each Macronutrient salt and dissolve separately in ca. 800 ml pure water (or distilled water) in four (4) recipients. Adjust to 1 litre each and label the chemical formula, the concentration and the date of preparation.
- For **Micronutrient salts**, weigh exact amount of each of Micronutrient salts and dissolve one by one in ca. 900 ml (observe that one compound is clearly dissolved completely before adding the next). Adjust to 1 litre and label the name of the solution. Example: Micro salts CMS (100X).
- **FeNaEDTA** and **KI** must be prepared and stored in amber recipients with specific labels.
- For **Vitamins** stock solution, weigh and dissolve each of vitamins in separate four (4) recipients with specific labels.
- Check :
  - ☛ The volume of all stock solutions, the label containing exact data (concentration, date of preparation and name (or initials) of the person preparing these stock solutions).
  - ☛ The closure of the recipients.
- Store stock solutions in the dark at +4°C (refrigerator).

Table 1. Compound and amount of chemicals for making CMS-Stock Solutions (CMS-SS).

Compound	Concentration		Stock Solutions	Amount to take for making 1 liter medium
			Weight	
Macro nutrients (I)	[mg/l]	[mM]	[g/l]	[ml/l]
KNO_3	1212	12.00	40.40	30
$Ca(NO_3)_2.4H_2O$	708	3.00	23.60	30
$MgSO_4.7H_2O$	370	1.50	24.65	15
$NH_4H_2PO_4$	230	2.00	11.50	20
Micro nutrients (II)	[mg/l]	[µM]	[g/l]	[ml/l]
$MnSO_4.H_2O$	8.41	50	8.41	
$ZnSO_4.7H_2O$	2.87	10	2.87	
$CuSO_4.5H_2O$	0.25	1	0.25	1
H_3BO_3	6.20	100	6.20	
$Na_2MoO_4.2H_2O$	0.25	1	0.25	
$CoCl_2.6H_2O$	0.025	0.1	0.025	
FeNaEDTA (III)	11.04	30	1.84	5
KI (IV)	0.83	5	83mg/100ml	1
Vitamins	[mg/l]	[µM]		
Myo-inositol **(V)**	100	555	5.0g/500ml	10
Thiamine-HCl **(VI)**	1.0	2.96	100mg/100ml	1

Exercice 2 :

Preparation of 1 litre CMS (Lê & Collet, 1985) medium for the culture of potato.

Procedures :

- Fill ca. 500 ml pure water (or Distilled water) in 1 litre container
- Add exact amount of each of stock solutions of **Macronutrients**, **Micronutrients**, **FeNaEDTA**, **KI** and **Vitamines** (see Table 1. above)
- Add 20g sucrose and swirl the solution.
- Make up to ca. 900 ml.
- Adjust the pH to 5.7 – 5.8 with 1N NaOH (or KOH) or HCl.
- Add 0.8% Agar (8g/l).

- Adjust the volume to 1 litre by using graduated cylinder (or plastic wash bottle).
- Heat the medium to dissolve Agar.
- Swirl gently the medium to homogenize nutrient solution.
- Dispense the Agar-medium to culture vessels (tubes, jars, etc.).
- Close the culture vessels with caps.
- Wrap up the vessels in aluminium foil.
- Label the vessels with the correct name of the medium, the date of preparation and the acronym of the person preparing the medium (see example).
- Put on top a temperature indicator strip for checking the correct sterilisation.
- Autoclave for 15 minutes at 121°C (15 p.s.i.)
- Wait until the pressure drop down to 0 bar (less than 100 °C) before bringing out the sterilised medium.
- Cool the medium in a clean area with a blanket (ex. house-hold linen) in view to avoid dust (i.e. contamination).
- Store the sterilised medium in the dark at room temperature, if possible in a cupboard, for short term storage (2 – 3 weeks). For longer period of conservation, transfer the medium in a room with a temperature ranging from 4 to10 °C.

Exercice 3 :

Preparation of 1 litre medium for the culture of Rose (cv. *Rosa ssp.*).

Procedures :

- Fill ca. 500 ml pure water (or Distilled water) in 1 litre container
- Add exact amount of each of stock solutions of **Macronutrients**, **Micronutrients**, **FeNaEDTA**, **KI** and **Vitamines** (see Table 2. above)
- Add 30g sucrose and swirl gently the solution.
- Add growth regulators (see Table 2. above)
- Swirl gently the solution.
- Make up to ca. 900 ml.
- Adjust the pH to 5.7 – 5.8 with 1N NaOH (or KOH) or HCl.
- Add 0.8% Agar (8g/l).
- Bring the solution to the final volume (1 litre) using graduated cylinder (or plastic wash bottle).
- Heat the medium to dissolve Agar.
- Swirl gently the medium to homogenize nutrient solution.

- Dispense the Agar-medium and close the culture vessels with caps.
- Wrap up the vessels in aluminium foil.
- Label the vessels with the correct name of the medium, the date of preparation and the acronym of the person preparing the medium (see example).
- Put on top a temperature indicator strip for checking the correct sterilisation.
- Autoclave for 15 minutes at 121°C (15 p.s.i.)
- Wait until the pressure drop down to 0 bar (less than 100 °C) before bringing out the sterilised medium.
- Cool the medium in a clean area with a blanket (ex. house-hold linen) in view to avoid dust (i.e. contamination).
- Store the sterilised medium in the dark at room temperature, if possible in a cupboard, for short term storage (2 – 3 weeks). For longer period of conservation, transfer the medium in a room with a temperature ranging from 4 to10 °C.

Table 2. Modified Murashige and Skoog (MS) medium (1962).

Compound	Concentration		Stock Solutions	Amount to take for making 1 liter medium
			Weight	
Macro nutrients (I)	[mg/l]	[mM]	[g/l]	[ml/l]
NH_4NO_3	1650	20.60	82.50	20
KNO_3	1900	18.80	95.00	20
$MgSO_4.7H_2O$	370	1.50	24.65	15
$CaCl_2.2H_2O$	440	3.00	17.60	25
Micro nutrients (II)	[mg/l]	[μM]	[g/l]	[ml/l]
$MnSO_4.H_2O$	16.9	100	16.9	
$ZnSO_4.7H_2O$	8.6	30	8.6	
$CuSO_4.5H_2O$	0.025	0.1	0.025	1
H_3BO_3	6.20	100	6.20	
$Na_2MoO_4.2H_2O$	0.25	1	0.25	
$CoCl_2.6H_2O$	0.025	0.1	0.025	
FeNaEDTA (III)	36.70	100	1.84	20
KI (IV)	0.83	5	83mg/100ml	1
Vitamins (V)	[mg/l]	[μM]		
Myo-inositol	100	555	5.0g/500ml	10
Thiamine-HCl	1.0	2.96	100mg/100ml	1
Pyridoxine-HCl	0.5	2.43	50mg/100ml	1
Nicotinic Acid	0.5	4.06	50mg/100ml	1
Growth regulators (VI)	[mg/l]	[μM]		
BA*	1.0	4.44	5mg/500ml	100
IBA*	0.1	0.49	10mg/100ml	1

*/ see also appendix 3

6. Aseptic culture techniques

Sterilisation

Various techniques are used for sterilising media and instruments (forceps, scalpels, needles, sieves, etc.) in plant tissue culture. These are :

- *Autoclaving*
  In most of laboratories, media and instruments are sterilised by autoclaving (steam heating under pressure). The standard conditions for conducting sterilisation are 121 °C (15 psi) for 15-20 minutes. However, the larger volumes of medium need to be sterilised for longer periods (see appendix 5).

> Recommendations :
>
> It is generally recommended that the medium requires to reach a temperature of 121 °C and be kept at this temperature at least 15 minutes for obtaining sterility.
> Preheat solutions prior to autoclaving can reduce the exposure time to reach 121 °C.
> It is important to remove all air from the internal chamber of autoclave so that the steam can properly be applied on the materials.

- *Dry heat*
  Glassware and instruments (only metal tools) are also be sterilised by using an oven maintained at 180 - 200 °C for 4 h.

- *Flaming*
  Dissecting instruments are usually sterilised by soaking the ends in 95% alcohol and flaming off.

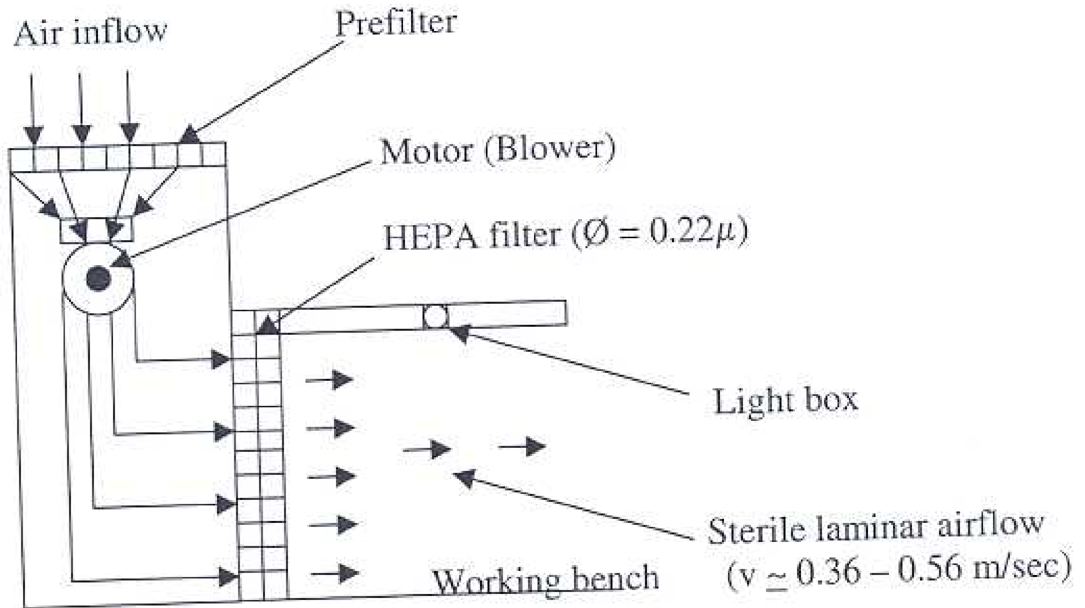
- *Glass bead steriliser*
  Ustensils such as forceps and scalpels are sterilised by heating for 15 sec. in an electric glass bead steriliser.

- *Filtration*
  Compounds highly susceptible to heat damage (thermolabile) should be sterilised by filtration and added to the autoclaved medium once its temperature is falling at ca. 40 °C.

Laminar air flow

The application of plant tissue culture requires sterile (or aseptic) conditions which are important for successful *in vitro* culture techniques. Therefore, it is important to carry out all operations in a sterile area. A laminar flow bench is in this respect necessary for any sterile work. (see figure below). The prefilter collects the larger particles from the incoming air, and any dust which gets through the prefilter is eliminated by the High-Efficiency Particulate Air (HEPA) filter.

Laminar flow bench



This should be checked regularly (i.e. every year or whenever it is moved for detecting leaks. In the same way, instruments and glassware dedicated to tissue culture should be sterilised wrapped in aluminium foil (or in a metal box) and opened at the working laminar flow bench.

Before starting to operate the laminar flow bench should be turned on at least 30 minutes and the surface of the bench should be disinfected with 70% (v/v) ethanol or isopropanol. All culture media, instruments, glassware, binocular microscope, etc. should also be installed during ventilating time in the lamina flow bench for removing contaminants (see overhead).

Recommendations

Some precautions to avoid contaminations when operating in the laminar flow bench :

- Wash correctly hands and forearms before starting to work.
- Wipe regularly the surface of the bench with 70% alcohol.
- Sterilise forceps, scalpels, etc. by flaming off or by heating in a glass-bead sterilizer and leave gently to cool.
- Alcohol is inflammable, take great care during flaming !
- Never pass hands or arms over sterile exposed plant material or open culture recipients, but passing round the sterile material.
- Disinfect hands regularly.
- Flame the mouth of culture recipient (tube, flask or jar) before opening and cover quickly with caps after transferring the plant material.
- Refrain from talking (unless it is necessary) in the laminar flow bench.
- Remove all used or unnecessary material from the air laminar flow in view to provide maximum sterile air flow in the hood.
- Roll up the sleeves of laboratory clothes.
- Cover hair (if possible).
- Keep the face away from cultures when coughing or talking.

Surface Sterilisation of Plant Tissue

All plant organs or tissues carrying on their surface micro-organisms contaminants (bacteria, fungi, yeast, etc..) must firstly be surface-sterilised using sterilisant at suitable concentration for an adequate period. To this end, various disinfectant agents have been used (see Table 3).

Table 3. Some disinfectants used in the surface-sterilisation of plant tissues.

Disinfectants	*Concentration*	*Exposure time*	*Effectiveness*
Sodium Hypochloride	0.5 to 5%	5 to 30 min.	Very good
Calcium Hypochloride	3 to 10%	5 to 30 min.	Very good
Mercuric Chloride	0.1 to 1%	1 to 15 min.	Satisfactory
Hydrogen Peroxide	3 to 10%	5 to 15 min.	Good
Bromine Water	1 to 2%	2 to 10 min.	Very good
Silver Nitrate	1%	5 to 30 min.	Good
Kohrsolin	3%	15 –20 min.	Good
Ethanol	70%	5 sec. to 5 min.	Good ?

According to the type of the plant organ or tissue, one should adjust the conditions of sterilisation for each kind of plant material (see Table 4).

Table 4. Concentration of Sodium Hypochlorite and Duration of the treatment in relation to the plant material.

NaOCl	*Concentration*	*Duration*
Thin Leaf	1.0%	12 – 15 min.
Thick Leaf	1.5%	15-20 min.
Bulbs and Tubers	1.5%	30 min.
Stems	1.5 to 2.0%	25 – 30 min.
Seeds	2.0 %	30 min.

For plant tissue taken from field culture conditions carrying a heavy load of micro-organisms, it is important to wash it correctly under running tap water during 1 hour prior to treatment with disinfectant.

Example of surface disinfection procedures (see overhead)

A/ Single treatment :

- 70% Ethanol, 5 sec.
- 1% NaOCl plus few drops of wetting agent (Teepol), 15 min.
- Three rinses in sterile distilled water, 5 min each.

B/ Combined sterilisation (double treatment):

- 70% Ethanol, 5 sec.
- 1% NaOCl plus few drops of wetting agent (Teepol), 15 min.
- One rinse in sterile distilled water, 5 min.
- 3% Kohrsolin, 15 min.
- Three rinses in sterile distilled water, 5 - 10 min. each.

C/ Combined sterilisation (triple treatment) :

- 70% Ethanol, 5 sec.
- 1% Benlate, 30 min.
- One rinse in sterile distilled water, 5 min.
- 1% NaOCl plus few drops of wetting agent (Teepol), 15 min.
- One rinse in sterile distilled water, 5 min.
- 3% Kohrsolin, 15 min.
- Three rinses in sterile distilled water, 5 – 10 min. each.

Recommendations

Mercuric chloride is toxic so that it should be used with caution.
Special care should be taken when silver nitrate is used as well.
Waste solution containing toxic compounds must be collected in a special recipient.

7. Micropropagation

See overhead

I. *In vitro* Establishment

Aim : This experiment aims at introducing practicians (laboratory technician, students, etc.) to initiate a culture free from micro-organisms (bacteria, fungi, yeast, etc.) and other contaminants.

Exercice 4 :

In vitro Establishment of commonly grown potato (var. *S. tuberosum ssp.*)

Experimental Material

- Plant material (sprouting potatoes)
- Beakers (100ml)
- Glass bottles with blue caps (250ml)
- Forceps
- Scalpels
- Knives
- Alcool 70%
- 1% NaOCl solution + few drops of wetting agent (Teepol)
- 3% Kohrsolin
- Sterile Distilled Water (SDW)
- Culture glass tubes with CMS-potato medium
- Sterile Pétri dishes (or sterilised paper)
- Towel paper
- Labels

Procedures :

1. Switch on the laminar air flow (ca. 30 min.) and wipe it clean with 70% alcohol.
2. Set out the working table according to figure 1 (see annex 1).
1. Remove sprouts from tubers and place them in a beaker.
2. Cover the beaker with a piece of gauze which is closed with a rubber band.
3. Wash thoroughly under running tap water to remove dust and surface soil.
4. Wipe them dry with a tissue paper.
5. In the laminar airflow hood cut up the sprouts into segments (explants) containing 2 to 3 nodals each.
6. Dip shortly in 70% Alcohol (3 sec.).
7. Transfer explants to 1% NaOCl and soak for 15 –20 minutes.
8. Agitate the flask by hand to ensure that the plant tissue is in close contact with the disinfectant.
9. Rinse in SDW.
10. Soak in 3% Kohrsolin for 15 minutes.
11. Wash three times in SDW for 5-7 minutes each.
12. Remove the explants after the final rinse and transfer them to a sterile Petri dish (or sterilised paper).
13. Trim and discard both ends of the explants using sterile forceps and scalpel.
14. Divide the explants into pieces containing one axillary bud each.
15. Flame the forceps and scalpel after each dividing operation.
16. Flame the neck of the culture glass tube before opening it using the little finger.
17. Transfer rapidly single node explants onto the CMS medium (place correctly the basal end in the agar solidified medium).
18. Flame again the neck of the culture tube before closing it with the cap.
19. Repeat the transfer operation (16 – 18) until all the explants have been inoculated.
20. Label the cultures (name of the plant species, culture medium , date and initials of the operator).
21. Finally, transfer the plant cultures to an incubator (or growth room) at 20°C with 14-h.photoperiod under a photon flux of 50 – 55 $\mu mol/sec/m^2$.

Recommendations

For detecting contamination of suspected materials by endophytic micro-organisms (bacteria, fungi and yeast), two media can be used during the course of culture, namely :

(i) Bacto Nutrient Agar supplemented with 1% glucose + 0.5% Yeast Extract, pH 7.
(ii) LPGA : 1% glucose + 0.5% Yeast Extract + 0.5% Bacto Peptone + 0.8 % Agar, pH 7.2.

II. *In vitro* Propagation

Aim :

The aim of this manipulation is to help laboratory workers (technicians, students, etc.) to grasp the techniques of *in vitro* micropropagation properly and efficiently.

Exercice 5 :

In vitro propagation of commonly grown potato (*S. tuberosum ssp.*)

Experimental material

- *In vitro* microplants of potato (4-5 weeks culture)
- CMS medium
- Forceps (25 cm)
- Forceps (20cm)
- Scalpels (2 X)
- N°10 Blades
- Sterile Pétri dishes (or sterilised paper)
- 95% Alcohol (for flaming instruments)
- 70% Alcohol (for surface disinfection)
- Test tube racks
- Towel paper
- Labels
- Black pencil

Procedures :

1. Before starting to operate switch on the motor of laminar airflow 20-30 minutes.
2. Wipe the air flow bench with 70% alcohol.
3. Set out the working table according to figure 2. (see annex 2).
4. Make sure (using a check-list) that all you need is ready.
5. Disinfect your hands and arms with 70% alcohol (or other disinfectant).
6. Take a test tube containing potato microplant with your left hand and flame the neck of the tube.
7. Remove the closure from a culture tube with your right hand by using the little finger and flame quickly the open end.
8. Pick up the microplant with sterile forceps and place it in a sterile Pétri dish (or sterilised paper).
9. While holding the microplant with a pair of 20 cm sterile forceps, divide it into 4-5 single nodal segments (also called explants or cuttings) using a sterile scalpel.
10. Discard the apex of the microplant and transfer single nodal segments to the CMS medium using a pair of 25 cm sterile forceps.
11. Flame the open end before closing the culture tube with cap.
12. Repeat this procedure (6 – 11) until all plant material have been used for the experiment.
13. After labelling the cultures (name of the plant, medium, date and initials of the operator) place in a rack and expose to light in an incubator (or growth room) at 20 °C with 14h-photoperiod under a photon flux of 50 – 55 $\mu mol/sec/m^2$.

8. Acclimatisation

See overhead

Aim :

This experiment aims at introducing a basic technique for succeeding the transfer of *in vitro* propagated plantlets to the external environment (green-house, field conditions, etc.).

Exercice 6 :

Acclimatisation and culture of *in vitro* potato plantlets under green-house conditions.

Experimental material

- Rooted *in vitro* microplants of potato (4-5 weeks culture)
- Plastic pots (5 cm diameter) or transplant-cell trays
- Culture substrate containing conventional potting soil, perlite or a mixture of sterilised peat:compost:sand (1:1:1)
- Plastic bag
- Nutrient solution (CMS medium without sucrose)
- Trays
- Plastic or wood labels
- Black pencil

Procedures :

1. Prepare the trays containing plastic pots filled with the culture substrate the day before transferring the rooted plantlets to soil conditions.
2. Water properly the pots and leave them to eliminate excess water.
3. Remove the plantlet from the culture tube using forceps (or by hand) and rinse them in tap water to discard excess agar from roots.
4. Make a hole in the substrate using a piece of wood (or black pencil) .
5. Place the plantlet into a pot in 2:3 depth of the stem into the substrate.
6. Fill the pot with small amount of substrate and compress the substrate around the stem using fingers.
7. Shower carefully the pot and place it in the tray.

8. Repeat this procedure (3 - 7) until all plantlets have been transferred to soil substrate.
9. Label the cultures (name of the plant species, date, initials of operator).
10. Put the tray in a plastic bag and close it in view to avoid dehydration during the first three days.
11. Place the trays in the green-house with the possibility to shade during shining days.
12. Keep the temperature in the green-house around 25 ± 1 °C and 60-70% humidity.
13. Open the plastic bag progressively in order to allow the hardening of the *in vitro* plantlets during the period under mist conditions. According to the environmental conditions (microclimate in the green-house), one should accelerate or delay the opening of the plastic bag.
14. Control regularly the growth of the acclimatised plants by providing sufficiently water and nutrient solution.
15. After ca. a month the plants can be repotted in a bigger container or transferred to the field (under insect-proof conditions) for producing tubers.

III. Some useful terms

Adventitious
Developing from unusual place (origin), such as shoots or roots from callus, leaf or stem tissue, or embryos from sources other than zygote.

Aseptic
Sterile, without infection or absence of contaminating micro-organisms.

Callus
An unorganised proliferation of plant cells on the culture medium. A wound response resulting in a tumerous growth.

Clonal propagation
Asexual reproduction or vegetative multiplication of plants which are considered to be genetically identical (uniform) and originated from a single individual or explant.

Clone
A group of plants propagated by vegetative (asexual) means from a single individual.

Culture medium
A mixture of mineral and organic nutrients used to growth plant cell, tissue and organ.

Explant
Fragment of plant organ or tissue taken from its original site and used to start a tissue culture; initial explant.

Growth regulators
Organic compounds (synthetic chemicals) other than usual nutrients that influence the growth of the plant tissue, such as Auxins, Cytokinins, Gibberellins and Ethylen.

In vitro propagation
Propagation of plants using plastic or glass recipients, aseptic techniques, appropriate medium and a controlled artificial environment.

Meristem
A group of actively dividing cells from which permanent tissue systems (root, shoot, leaf and flower) are derived.

Meristem culture
A culture of shiny, dome-like structure measuring ca. 0.1 – 0.2 mm in length which is excised from shoot apex.

Micropropagation
In vitro clonal propagation of plants from shoot tips or nodal explants, usually with an accelerated proliferation of shoots during subcultures.

Morphogenesis

The evolution of a structure from an undifferenciated to a differenciated state. The growth and development of differenciated structures.

Organ Culture
Aseptic culture of organised structures, e.g. root or shoot tip, shoot segment, etc.

Organogenesis
A process of differenciation by which plant organs are formed *de novo* or from preexisting structures.

Plant tissue culture
The growth or maintenance of plant cells, tissues and organs or whole plants *in vitro*.

Primary culture
A culture started from cells, tissues or organs excised directly from organisms.

Regeneration
A morphogenetic response to a stimulus resulting in the production of new organs, embryos or whole plants from cultured explants or calli derived from them.

Shoot tip (apex)
Terminal portion of a shoot (0.1 - 1.0 mm) comprising the meristem (0.05 – 0.1 mm) together with the primordial and developing leaves.

Stock plants
An amount of plants from which explants are taken for the use of tissue culture.

Stock solutions
Concentrated solutions (10X, 50X or 100X) from which portions are used for making culture media.

Subculture
Transfer of an explant to a fresh culture medium.

Surface disinfection
Elimination a wide range of microbial contaminants from the surfaces of plant tissue.

Sterilisation
Total elimination of all forms of microbial life.

Totipotency
Potentiality or property of normal cells to produce a whole organism.

Vegetative propagation
Reproduction of plants through a nonsexual process involving the culture of plant parts, e.g. stem cuttings, axillary buds, leaf explants.

Virus-free
Plant material certified through specified tests, e.g. ELISA-test, as being free from specified viruses.

Vitrification (or *Hyperhydricity*)
Morphological, physiological and metabolic derangements. Vitrified plants appear thick, brittle, glassy and water-soaked.

IV. Appendices

Appendix 1 :

Some basal media for plant tissue culture

Component	B5		DKW		NN
Macronutrients	[mg/l]	[mM]	[mg/l]	[mM]	[mg/l]
NH_4NO_3			1416	17.7	750
KNO_3	2500	25			950
$CaCl_2.2H_2O$	150	1.0	149	1.0	166
$Ca(NO_3)_2.4H_2O$			1968	8.3	
K_2SO_4			1559	8.9	
$MgSO_4.7H_2O$	250	1.0	740	3.0	185
KH_2PO_4			265	1.9	68
$(NH_4)_2SO_4$	134	1.0			
$Na\ H_2PO_4.\ H_2O$	150	1.1			
Micronutrients	[mg/l]	[μM]	[mg/l]	[μM]	[mg/l]
KI	0.75	4.5			
H_3BO_3	3.0	50	4.8	77.6	10
$MnSO_4.H_2O$	10	60	33.5	198.2	
$MnSO_4.4H_2O$					19
$ZnSO_4.7H_2O$	2.0	7.0			10
$Zn(NO)_2.6H_2O$			17.0	57.2	
$Na_2MoO_4.2H_2O$	0.25	1.0	0.39	1.6	0.25
$CuSO_4.5H_2O$	0.025	0.1	0.25	1.0	0.025
$CoCl_2.6H_2O$	0.025	0.1			0.025
$NiSO_4.6H_2O$			0.005	0.2	
$FeSO_4.7H_2O$*	27.8	100	33.8	121.5	27.8
Na_2EDTA*	37.3	100	45.4	121.5	37.3
Vitamins & organics	[mg/l]	[μM]	[mg/l]	[μM]	[mg/l]
Myo-inositol	100	555	100	555	100
Thiamine-HCl	10	30	2.0	5.9	0.5
Pyridoxine-HCl	1.0	5	0.5	2.5	0.5
Nicotinic Acid	1.0	8	1.0	8	5.0
Glycine			20	270	5.0
L-Glutamine			250	1710.6	
Saccharose	20 g		30 g		20 g
pH	5.5		5.5		5.5

*/ Fe-NaEDTA (36.70mg/l) is also available.

Appendix 2 :

Compounds and molecular weight

Macronutrients	*Molecular weight*
NH_4NO_3	80.09
KNO_3	101.10
$CaCl_2.2H_2O$	147.02
$Ca(NO_3)_2.4H_2O$	236.15
K_2SO_4	174.10
$MgSO_4.7H_2O$	246.50
KH_2PO_4	136.09
$(NH_4)_2SO_4$	132.14
Na H_2PO_4. H_2O	137.98
Micronutrients	
KI	166.01
H_3BO_3	61.84
$MnSO_4.H_2O$ (conv. H_2O / $4H_2O$ = 0.76)	169.10
$MnSO_4.4H_2O$ (conv. $4H_2O$ / H_2O = 1.32)	223.09
$ZnSO_4.7H_2O$	287.55
$Zn(NO)_2.6H_2O$	
$Na_2MoO_4.2H_2O$	241.95
$CuSO_4.5H_2O$	249.68
$CoCl_2.6H_2O$	237.93
$NiSO_4.6H_2O$	
$FeSO_4.7H_2O$	278.00
$Na_2EDTA.2H_2O$	372.20
Vitamins	
Myo-inositol	180.16
Thiamine-HCl	337.28
Pyridoxine-HCl	205.64
Nicotinic Acid	123.11
Growth regulators	
N^6-Benzyladenine	225.3
Kinetin	215.2
N^6-(2-isopentyl)adenine	
Zeatin	219.2
Gibberellic acid	330.0
Indole-3-acetic acid	175.2
Indole-3-butyric acid	203.2
α-Naphthaleneacetic acid	186.2
Abcissic acid	264.3
2,4-dichlorophenoxyacetic acid	221.04

Amino acids	
Alanine	89.09
Arginine	174.20
Aspartic acid	133.10
Cysteine	121.16
Glutamine	146.20
Glutamic acid	147.13
Glycine	75.10
Phenyalanine	165.19
Tyrosine	181.19
Sugars	
Fructose	180.16
Glucose	180.16
Mannitol	182.17
Ribose	150.13
Sorbitol	182.17
Sacchariose	342.30
Xylose	150.13
Antibiotics	
Carbenicillin	422.4
Rifampicin	823.0
Streptomycine	1457.4

Appendix 3 :

Preparation and conservation of plant growth regulators

Compounds	*Abbreviation*	*Solvent*	*Storage*
N^6-Benzyladenine	BA or BAP	NaOH	0 – 4 °C
Kinetin	KIN	NaOH	0 °C
N^6-(2-isopentyl)adenine	2iP	NaOH	0 °C
Zeatin	ZEA	NaOH	0 °C
Gibberellic acid	GA_3	EtOH	0 – 4 °C
Indole-3-acetic acid	IAA	EtOH/NaOH	0 °C
Indole-3-butyric acid	IBA	EtOH/NaOH	0 °C
α-Naphthaleneacetic acid	NAA	NaOH	0 – 4 °C
2,4-dichlorophenoxyacetic acid	2,4-D	EtOH/NaOH	0 – 4 °C
Abcissic acid	ABA	NaOH	0 °C

Appendix 4 :

Some notes on solutions

1 ppm (parts per million) = 1 mg per liter

1 molar (1 M or 1 mol/liter) = molecular weight in g per liter

Example : 1M KNO_3 = 101.1 g/l

1 millimolar (1 mM) = molecular weight in mg per liter

Example : 1mM KNO_3 = 101.1 mg/l

Dilution rule :

- C_1 (concentration of stock solution)
- C_2 (required concentration)
- V_1 (volume of stock required)
- V_2 (medium volume)

$$C_1 \times V_1 = C_2 \times V_2$$

$$V_1 = \frac{C_2 \times V_2}{C_1}$$

Appendix 5 :

Time required for sterilising liquid media (Burger, 1988)

Volume [ml]	Autoclave preheating time to reach 121 °C [min.]	Total sterilisation time [min.]
20 – 25	9	24
100	13.5	28.5
500	20	35
1000	25	40
2000	33	48
3000	40	55
4000	48	63

Appendix 6 :

Media N° : Purpose : Date:

Person (initials) :

Constituents	Stock Solutions (Concentration)	Amount to take for making : 1L – 2L – 4L	Final Concentration
Minerals Macro Micro			
Iron NaFeEDTA			
Sugar			
Vitamins Thiamine Pyridoxine Nicotinic Acid Myo-inositol			
Auxin IAA IBA NAA 2,4-D			
Cytokinin BA (=BAP) Kinetin 2iP Zeatin			
Gibberellin GA_3			
Organics			
pH			
Agar			
Observations			

Appendix 7 : Dilution of Alcohol (Gay-Lussac)

Amount of water to add to100 ml Alcohol.

Alcohol to dilute

Alcohol to obtain	*100*	*99*	*98*	*97*	*96*	*95*	*94*	*93*	*92*
95	6.50	5.15	3.83	2.53	1.25				
90	13.25	11.83	10.43	9.07	7.73	6.41	5.10	3.80	2.54
85	20.54	19.05	17.58	16.15	14.73	13.33	11.96	10.59	9.24
80	28.59	27.01	25.47	23.95	22.45	20.95	19.49	18.04	16.61
75	37.58	35.90	34.28	32.67	31.08	29.52	27.97	26.43	24.94
70	47.75	45.98	44.25	42.54	40.85	39.18	37.53	35.89	34.27
65	59.37	57.49	55.63	53.81	52.00	50.22	48.45	46.70	44.85
60	72.82	70.80	68.80	66.85	64.92	63.00	61.10	59.21	57.33
55	88.60	86.42	84.28	82.16	80.06	77.99	75.93	73.88	71.85
50	107.44	105.08	102.75	100.44	98.15	95.89	93.64	91.41	89.19
45	130.26	127.67	125.11	122.57	120.06	117.57	115.09	112.64	110.18
40	158.56	155.68	152.84	150.02	147.22	144.46	141.70	138.95	136.23
35	194.63	191.39	188.19	185.01	181.85	178.71	175.60	172.49	169.39
30	242.38	238.67	234.99	231.33	227.70	224.08	220.49	216.90	213.33
25	308.90	304.52	300.18	295.86	291.56	287.28	283.02	278.77	274.53
20	408.50	403.13	397.79	392.47	387.17	381.90	376.64	371.40	366.16
15	574.75	567.43	560.53	553.55	546.59	539.66	532.74	525.83	518.94
10	907.09	896.73	886.40	876.10	865.15	855.55	845.31	835.08	824.86

Alcohol to obtain	*90*	*85*	*80*	*75*	*70*	*65*	*60*	*55*	*50*
85	6.56								
80	13.79	6.83							
75	21.89	14.48	7.20						
70	31.05	23.14	15.35	7.64					
65	41.53	33.03	24.66	16.37	8.15				
60	53.65	44.48	35.44	26.47	17.58	8.76			
55	67.87	57.90	48.07	38.32	28.63	19.02	9.47		
50	84.71	73.90	63.04	52.43	41.73	31.25	20.47	10.35	
45	105.34	93.30	81.38	69.54	57.78	46.09	34.46	22.90	11.41
40	130.80	117.34	104.01	90.76	77.58	64.48	51.43	38.46	25.55
35	163.28	148.01	132.88	117.82	102.84	87.93	73.08	58.31	43.59
30	206.22	188.57	171.05	153.61	136.04	118.94	101.71	84.54	67.45
25	266.12	245.15	224.30	203.61	182.83	162.21	141.65	121.16	100.73
20	355.80	329.84	304.01	278.26	252.58	226.98	201.43	175.96	150.55
15	505.27	471.00	436.85	402.81	368.83	334.91	301.07	267.29	233.64
10	804.50	753.65	702.89	652.21	601.60	551.06	500.50	450.19	399.85