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PLANT TISSUE CULTURE METHODS

A LABORATORY MANUAL

Compiled by
Sunitha Siriwardana, M.S.

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CONTRIBUTORS

O.L. Gamborg (Ph.D.), TCCP, Colorado State University

A.S. Kumar (Ph.D.), TCCP, Colorado State University

H. Hughes (Ph.D.), Department of Horticulture /TCCP, Colorado State University

C. Livingston (Ph.D.), Department of Plant Pathology and Weed Science, Colorado State University

C. MacKinnon (Ph.D.) (Formerly of TCCP, Colorado State University

S. Siriwardana (M.S.), TCCP, Colorado State University

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LABORATORY 1 STOCK SOLUTION PREPARATION

Sunitha Siriwardana

INTRODUCTION

Several plant tissue culture media are available in pre-packaged powdered forms. These are useful for small scale media preparations. For large scale media preparations it is convenient to have the ingredients as stock solutions made with double distilled or deionized water. Stock solutions eliminate the need to weigh out chemicals before each media preparation.

PURPOSE

The purpose of this exercise is to help the student learn proper methods ofpreparation and storage of stock solutions in plant tissue culture.

MATERIALS

chemicals, preferably of the highest grade available
milligram balance
deionized or double distilled water
glass or plastic containers for storage of solutions
refrigerator or freezer to store chemicals

PROCEDURES

1. Media Composition

   For composition of commonly used media, refer to Appendix 1. A basic culture medium consists of inorganic salts, sugars, organic supplements and vitamins.

2. Chemical Storage

   The inorganic salts used as major and trace elements should be stored in a chemical cabinet in the preparatory area. The organic supplements, e.g., vitamins and EDTA, as well a growth regulators, are generally stored in freezer. Sugar, salt (NaCl) and agar can be stored in the preparatory area in clean dry containers.

3. Weighing

   Balances should not be moved at any time. It is essential to learn proper use of the balance: clean the balance after each use by brushing off chemicals fallen on it; Spatulas used for weighing chemicals should be rinsed thoroughly with deionized/distilled water and dried after use; Do not lay spatulas directly on counter tops.

4. Stock Solution Preparation

   a. Water - Use double distilled (in glass) or deionized water.

   b. Concentration of stocks - Generally 10 x or 100 x of the desired strength.

c. Mineral salts and organic components - Use highest grade available. Major and trace elements are usually completely soluble in water. In the case of trace elements and vitamins, where stock solutions are a mixture of different chemicals, dissolve one at a time. Each major stock solution is stored in plastic or glass containers separately for convenient use. Stocks of vitamins and organic supplements (FeEDTA, amino acids etc) are to be stored in the refrigerator. Trace elements need not be refrigerated.

d. Growth regulators/hormone (see Appendix 2). All cytokinins may be autoclaved for dissolution, or dissolved in acid.
   Auxins 2,4-D - heat dissolution
   IAA - minimal volume of KOH (store in dark bottles)
   NAA - minimal volume of ethanol

APPENDIX 1. COMPOSITION OF COMMONLY USED PLANT TISSUE CULTURE MEDIA

	*MS		**LS		***B 5	
Major Nutrients (Store each solution separately)	mg/l	STOCK CONCENTRATION g/l(x10)	mg/l	STOCK CONCENTRATION g/l(x10)	mg/l	STOCK CONCENTRATION g/l(x10)
NH_4NO_3	1650	16.5			-	-
KNO_3	1900	19.0			2500	25
$CaCl_2 \cdot 2H_2O$	440	4.4	Same as MS		150	1.5
$MgSO_4 \cdot 7H_2O$	370	3.7			250	2.5
KH_2PO_4	170	1.7			-	-
$(NH_4)_2 SO_4$	-	-			134	1.3
$NaH_2PO_4H_2O$	-	-			150	1.5
Micronutrients (store in one bottle)		g/l(x100)		g/l(x100)		g/l(x100)
KI	0.83	0.083			0.75	0.075
H_3BO_3	6.2	0.620			3.0	0.3
$MnSO_4.H_2O$	15.6	1.56			10.0	1.0
$ZnSO_4.7H_2O$	8.6	0.86			2.0	0.2
$NaMoO_4 2H_2O$	0.25	0.025			0.25	0.025
$CuSO_4.5H_2O$	0.025	0.0025	Same as MS		0.025	0.0025
$CoCl_2.6H_2O$	0.025	0.0025			0.025	0.0025
Iron as:						
NaEDTA	37.3	3.73			37.3	3.73
$FeSO_4.7H_2O$	27.8	2.78			27.8	2.78
-OR-						
FeEDTA	50	5.0			50	5.0
Vitamins (store in one bottle in refrigerator)	mg/l	g/l(x100)	mg/l	g/l(x100)	mg/l	g/l(x100)
Myo-Inositol	100	10	100	10	100	10
Nicotinic Acid	0.5	0.05			1	0.1
Pyridoxine HCl	0.5	0.05			100	0.1
Thiamine HCl	0.1	0.01	0.4		10	1
Sucrose	3%		4%		2%	
pH	5.7		5.5		5.5	

* Murashige, T and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant 15:473-497

** Linsmaier E. M. and F. Skoog (1965). Organic growth factor requirements of tobacco tissue culture. Physiol. Plant 18:100.

*** Gamborg, O. L., R. A. Miller and K. Ojima. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158.

Appendix 2. Preparation of Growth Hormone Stock Solutions*

COMPOUND	ABBREV.	M.W.	1mM mg/50 ml.	DISSOLVE IN
CYTOKININS:				
Benzyladenine	BA	225.2	11.25	2-5 ml of 0.5 N HCl. Heat slightly. Make to volume Adjust to pH 5
Isopentenyl Adenine	2iP	203.2	10.15	
Kinetin	K	215	10.25	
Zeatin	Z	219	10.95	
AUXINS:				
2,4-Dichlorophenoxyacetic acid	2,4-D	221	11.05	2-5 ml of Ethanol. Gradually add water. Heat slightly. Adjust to pH 5 Make to volume Adjust to pH 5 Or in minimal volume of 1N KOH.
2,4,5-Trichlorophenoxyacetic acid	2,4,5-T	255.5	12.75	
Naphtaleneacetic Acid	NAA	186.2	9.3	
Indolebutyric Acid*	IBA	203.2	10.65	
Indoleacetic Acid*	IAA	175.2	8.25	
MISCELLANEOUS:				
Gibberellic Acid**	GA	346.4	17.3	
Picloram	P	241.2	12.0	2-5 ml 0.2 MKOH. Adjust to pH 5.0
Abscisic Acid**	ABA	264	13.2	

* Stored in refrigerator.
** Filter sterilize.

EXERCISE

Prepare 1. 10 liters each of the stock macronutrients
2. 1 liter of stock micronutrients
3. 1 liter of stock Fe EDTA
4. 1 liter of vitamins (LS/MS)

DISCUSSION/COMMENTS

REFERENCES

George E.F and P.D. Sherrington (1984). Tissue culture media In: Plant Propagation by Tissue Culture; Handbook and directory of commercial laboratories, pp 184-283 Eastern Press, Reading, Berks.

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LABORATORY 2 MEDIA PREPARATION

Sunitha Siriwardana

INTRODUCTION

Isolated tissues and organs in culture have different nutritive requirements in comparison to whole plants. An understanding of these requirements is hence important in plant tissue culture. The basis of all plant tissue culture media commonly used have mineral salts, a source of carbon, usually sucrose, vitamins and various growth regulators as the essential component. Organic nitrogen compounds and undefined complex substances such as coconut milk etc make up the optional component. A basal medium is generally referred to as one without any growth regulator. Modified media contain growth regulators at various concentrations, determined mainly by the requirements of the explants.

PURPOSE

This exercise is to gain experience in making different media in tissue culture.

MATERIALS (For MS media)

Salts of major and micro nutrients
Sugar (sucrose)
Vitamins
Growth regulators
Milligram balance
Graduated cylinders, pipettes, beakers
pH meter
Culture vessels
Pressure cooker/Autoclave
Hotplate/Microwave
Deionized/Double distilled water

PROCEDURES

1. Formulation of a recipe for media making

   a. Major stocks - use 10 ml of stock of 10x stock per 100 ml of medium.
   b. Micro/Trace stocks- use 1 ml of 100 x stock per 100 ml of medium
   c. Vitamins - use 1 ml of 100 x stock per 100 ml of medium
   d. Fe EDTA - use 1 ml of 100 x stock per 100 ml of medium
   e. Hormones - as required
   f. Adjust to required volume with water.
   g. Sugar - 2-4 gm/100 ml of medium
   h. Adjust pH to 5.5
   i. Agar 0.8-1 gm per 100 ml of media (omit for liquid media)

2. Label the culture vessels before dispensing the media.

3. Heat media until agar is melted and dispense in to appropriate containers.
4. Autoclave at 121°C for 18 minutes (liquid cycle).

EXERCISE

1. Prepare 100 vials containing 10 mls each of MS media.

2. Prepare 80 vials containing 10 ml each of MS media supplemented with 2 mg/l 2,4-D and 0.5 mg/l BA.

DISCUSSION/COMMENTS

REFERENCES

Gamborg O.L and L.R. Wetter eds (1982). Plant Tissue Culture Methods, pp 1-9. National Research Council of Canada, Saskatoon, Saskachewan.

George E.F and P.D.Sherrington (1984). Tissue Culture Media In Plant Propagation By Tissue Culture. Hand book and Directory of Commercial laboratories, pp 184-283. Eastern Press Reading, Berks.

LABORATORY 3 ASEPTIC TECHNIQUE

Sunitha Siriwardana

INTRODUCTION

The rigorous exclusion of contaminating microorganisms is an absolute necessity in tissue culture. Nutrient media, culture vessels and instruments used in manipulating the tissue and the plant material itself must be sterile. Cleanliness, efficient organization and routine sterilization of all material reduces the risks of contamination.

PURPOSE

The students will learn different sterilization techniques used in plant tissue culture in this laboratory.

MATERIALS

1. Ethanol
2. Chlorox (Household bleach), or 5-6% sodium hypochlorite
3. Tween^{R}-20 (wetting agent)
4. Sterile double distilled or deionozed water
5. Explant material - Rice seeds, tobacco seeds, tobacco leaves and stems from green house
6. Culture vessels, sterile strainer, sieve, forceps, scalpel, petr dishes, magnetic stir plate, beakers and alcohol lamp
7. Laminar flow hood or sterile transfer facility
8. LS media

PROCEDURE

I. Preparation of sterile tissue for culture

A. From seeds

1. Rinse seeds in 70% ethanol for 1-2 minutes, decant.

2. Immerse in 20% chlorox solution containing 1 drop of Tween-20 in a clean stoppered jar. To ensure thorough sterilization, stir solution on magnetic stirrer for 15-20 minutes.

3. For larger grains (rice, wheat etc.): In the laminar flow hood, decant the floating seeds and sterilant, rinse with sterile water at least 5 times.

4. For extra small seeds (tobacco, carrot etc.):
In the laminar hood,
   a. Presterilize a fine mesh sieve in 20% clorox for 30 minutes, or by autoclaving sieve wrapped up in aluminum foil.
   b. Filter through sieve; wash several times with sterile water, through sieve.
   c. Invert sieve on a sterile petri dish, and pour a few mls of sterile water over the sieve; cover petri dish immediately.

B. Chlorine gas sterilization for certain types of explant materials: seeds, tubers.

1. Place material in a suitable container, uncovered in a

dessicator in a fume hood. Place a beaker containing about a 100 ml of clorox (bleach). Add 3 ml of 10 N HCl to the clorox, and place cover of derssicator immediately. Leave for 1-2 hours, depending on sensitivity of explant.

2. Open dessicator lid, cover the explant (eg. If the explant is in a petri dish, with petri lid.

C. From plants grown in the green house or growth chamber:

1. Bud, leaf and stem sections: Immerse for 1-3 minutes in 70% ethanol, followed by 2-3 rinses in sterile deionized or double distilled water, in the laminar flow hood.

2. If explant is known to be highly contaminated, stir and rinse in 20% clorox for 10-15 minutes.

3. Tubers, bulbs, rhizomes, roots and fruits: Immerse in 70% ethanol for 3-5 minutes followed by immersion in 20% commercial bleach solution. Rinse several times in sterile water.

NOTE: A specific sterilization procedure has to be developed for each individual explant type, if the general procedures given above are not suitable. This is done by trial and error, mostly.

II. Plating/culture of sterilized explant material

1. Place sterilized explant material in a covered sterile petri dish.

2. Dip the forceps in ethanol, (We use autoclaved instruments) flame over an alcohol lamp or bunsen burner and let cool. Using this sterile instruments transfer explant onto previously autoclaved culture vessels containing suitable nutrient media.

3. If the material requires further dissection before culturing, place on a sterile surface and dissect rapidly with sterile instruments.

III. Culture conditions

1. Incubate at constant temperature (25°C) in constant light intensity of about 500 ft.candles, or in the dark depending on the requirement of the explant.

2. Check periodically for contamination. Minor contamination can be removed by a sterilized instrument under aseptic conditions.

IV. Use of laminar flow hood

1. Switch on light and air flow. Leave on air flow for about 15 minutes before using the hood.

2. Spray work surface and the materials (culture vessels instruments etc.) placed in the hood with 70% ethanol. DO NOT SPRAY balances or the microscopes with alcohol.

3. Spray hands with alcohol and let dry.

4. Alcohol lamps (if used) are lit only after ensuring that the hood is dry.

5. Manipulate the explant material as necessary.

LAB EXERCISE

1. Sterilize the given explant material using appropriate procedures and transfer onto media.
   Compare sterilization with:
   a. 50%, 70% and ethanol (20% clorox for 20 min., Tween-20)
   b. 10, 20, and clorox for 20 min. each (70% ethanol, Tween-20).
   c. Different sterilization times, 20 minutes etc. (70% ethanol, 20% clorox, Tween-20)
   d. Tween-20 and without Tween-20 (70% ethanol, 20% clorox for 20 min.)

2. Testing sterility of laminar hood vs laboratory bench

   1. Uncover sterile petri plates containing previously autoclaved media, for 2-5 minutes, a. In the laminar hood
      b. In the general laboratory area.
      Compare % contamination after a few days.

   2. Transfer one set of explant material in a laminar hood vs the counter top in the laboratory, covered with wet paper towels. Compare the % of contamination after 2-3 days.

DISCUSSION/COMMENTS

REFERENCES

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LABORATORY 4 CALLUS CULTURE

Sunitha Siriwardana

INTRODUCTION

Natural causes such as wounding, pests and other stresses induce plants to form undifferentiated tissue called callus. When explants are placed in suitable culture media under sterile conditions, callus is initiated. During culture explant tissue is said to undergo dedifferentiation. Tissues from a range of organs and a great number of plants show potential to divide and proliferate in the presence of exogenous hormones. However some tissues are more predisposed to active division than others. Initiation of callus tissue is affected by many factors such as the explant itself as well as its environment.

PURPOSE

A. Callus induction - To become familiar with plant tissue culture techniques for establishing callus cultures using different explant sources. In vitro culture responses of tobacco pith, (stem cuttings), shoot cultures and rice seeds will be investigated.

MATERIALS

Tobacco pith, stem cuttings (green house), sterily grown tobacco shoots
Rice seeds.
Culture jars, LS media

PROCEDURES:

Trainees will work in pairs. Demonstration material as well as those prepared by trainees, will be used. The basal culture is L&S with 4% sucrose, 1% agar and various concentrations of growth regulators.

1. Secure tobacco stems from green house grown plants, and sterile leaf tissue from tobacco shoot (consult Training Coordinator). Count out thirty seeds of rice from stock container.

2. Sterilize leaves and seeds separately following the steps outlined in the demonstrations.

3. Under sterile conditions (consult procedures presented in the demonstrations), cut stem into 5-8 mm thick sections, and place in sterile water. Using forceps, plant leaf sections in jars (one explant piece per jar). In addition, plant one sterilized seed of rice per jar. The culture medium used for all explants is L&S salts and vitamins,plus 2 mg/l 2,4-D and 0.5 BA. Glass jars measure 35mm x 75mm, and have screw cap lids. Repeat procedures with materials provided from the demonstrations. For each set of of materials, 20 jars should be planted with each explant type.

4. Incubate tobacco sections in the dark; rice seeds are incubated in light. The temperature of the culture room should be maintained at 25-28 °C.

5. After 3-4 weeks (= passage), the callus should grow to a size 5-10 times that of the explant, and can be subcultured onto fresh medium.

B. Identification of cell and callus types - To observe and recognise different morphological callus types, and cell types of different calli.

PROCEDURES:

1. Secure cultures of rice, tobacco, and sorghum.

2. Under sterile conditions in a transfer hood, examine cultures (using a dissecting microscope) for the following morphological characteristics (a sterile scalpel and probe are useful for this):

   a. Friability (nonembryogenic)
   b. Compactness (embryogenic; pre-embryogenic)
   c. Differentiated structures(s) (roots, embryoids, leaves/shoot)
   d. Color(s) (brown, creamy, green)
   e. Size
      Any one callus mass may be composed of differing morpholo--gical callus types.

3. Dissect and separate different callus types. Each morphological type may be subcultured separately as an additional exercise.

4. At the lab bench, dissect away a small piece of callus (less than 1 mm^3). Place in drop of water on a microscope slide, and macerate with a scalpel. Place a coverslip over the tissue, and then tap or press gently over the coverslip. Observe under the microscope. Repeat procedure for each of the callus types.

C. To investigate the different effects of auxins on callus induction and differentiation.

PROCEDURES

1. Prepare media of the following formulations:
   use LS media with 4% sucrose and 0.9% agar

   a. 2 mg/l IAA
   b. 0.5 mg/l IAA & 0.2 mg/l BA
   c. 0.5 mg/l 2,4-D
   d. 2 mg/l 2,4-D
   e. 1 mg/l 2,4-D + 100 mg/l Trp + 0.5 mg/l K
   f. 1 mg/l 2,4-D + 50 mg/l Trp + 0.5 mg/l K
   g. 1 mg/l IAA + 0.5 mg/l BAP
   h. 0.1 mg/l IAA + 0.5 mg/l BAP (2% sucrose)

2. Secure callus cultures of rice, sorghum and wheat. Under sterile conditions, separate embryogenic callus from the rest of the callus mass. Subculture embryogenic callus on appropriate media given below.

   i. For IR 36 - b from Section 1
   ii. For Calrose - h from Section 1
   iii. For sorghum - g from Section 1
   iv. For wheat - h from Section 1

3. Sterilize rice seeds as instructed. Under sterile conditions, plant one seed per jar/vial containing the following media: a, c, d, e and f from Section 1 (15 vials of each type).

   Incubate rice seeds in the dark at =27°C ± 1.

4. Observation and report - After 2-3 days, examine all cultures for contamiation, remove and autoclave contaminated vials/jars right away.

   At 1 week intervals, check for callus formation or differentiation. Correlate influence of explant source, culture medium on callus response.

DISCUSSION/COMMENTS

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LABORATORY 5 SUSPENSION CULTURE

Oluf L. Gamborg

INTRODUCTION

Suspension cultures consist of a mixture of single cells and cell aggregates. They are usually grown in flasks on a shaker. The agitation is essential to permit aeration. A culture can be initiated from callus produced on agar or directly from a tissue explant. When a cell culture is being initiated it is important to start with a ratio of cell mass or tissue to liquid medium of 10 mg: 1 ml or less.

In the initial stages of culturing it is necessary to remove a portion of the spent medium at 10 - 14 day intervals by pipetting and replacing the volume with fresh medium. A cell suspension should form within 4 - 6 weeks. Subculturing can then be done at the dilution rate of 1:4 to 1:10 of inoculum to fresh medium.

Eventually the cell culture should attain a doubling time of 24 - 36 hours and consist of single cells and small multicellular clusters.

PURPOSE

To enable the student to gain experience in establishing and maintaining cell suspension cultures.

MATERIALS

Previously cultured callus, or cell suspension culture for inoculum, sterile spatulas, forceps, sterile erlenmeyer flasks (125 ml), 5 and 10 ml wide bore pipettes with cotton plugs, media supplemented with 1 mg/l 2,4-D.

MS and B5 media.

PROCEDURES

I. <u>Initiate a culture</u>

1. Transfer the callus, which should be friable, to 10 ml of medium in a 50 ml erlenmeyer. Keep the callus mass to liquid medium to about 10:1. Set up 4 - 6 flasks.

2. Cover the flasks with a stopper or aluminum foil and place them on a shaker (150 rpm) at 26 °C.

3. After 10 - 14 days, remove about 25% of the medium from each flask and replace with an equal volume fresh medium.

4. When a cell suspension has been established subculture every 2 weeks or less into fresh medium. Begin with the ratio of 1 ml of inoculum to 4 ml of medium (5x dilution).

5. Observe the formations of cells by taking small samples at intervals and transfer to petri dishes. Observe the formation of cell types and evidence of dividing and embryogenic cells.

II. Subculture of Cell Suspension cultures. Details to be given at the time of the lab.

1. Subculture 10 ml of the inoculum into a flask containing 90 mls of sterile MS media supplemented with 1 mg/l.

2. Observe for 2-4 days for contamination by observing a small sample of the suspension culture under sterile conditions.

DISCUSSION / COMMENTS

REFERENCES

Ammirato P.V. (1984). Induction, maintenance, and manipulation of development in embryogenic cell suspension cultures. In: I.K Vasil (ed) Cell Culture and Somatic Cell Genetics of Plants Vol 1., pp. 139-149. Academic Press Inc., New York.

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LABORATORY 6 MERISTEM CULTURE

Harrison Hughes

INTRODUCTION

The technique involves the excision of the apical meristematic dome and the associated leaf primordia. Although technically the term should be used for the dome alone, common usage has employed the term meristem-tip and just meristem for from 2-4 leaf primordia and the apical dome.

Meristem culture was first employed in the elimination of fungal and bacterial contaminants in plants and shortly thereafter in production of virus free plants. Best results for virus free plant production was most often obtained in association with heat therapy for prolonged periods.

Significant differences in the percentages of virus-free plants are observed with the size of the meristem tip excised. In general, the fewer leaf primordia excised in association with the dome, the higher the percentage of virus-free plants produced.

Meristem culture has more recently become an important tool of rapid propagation of some plant species. Although the numbers of plants produced are somewhat fewer than other tissue culture techniques, there are advantages. The greatest advantage lies in the observation of fewer variants with meristem culture. This is particularly important in the clonal production of food crops.

The required conditions for each plant species vary. The conditions requiring experimental determination in order of importance are growth hormone ratios, choice of medium and culture conditions for meristems.

PURPOSE

The purpose of this laboratory is to provide experience in meristem culture techniques. More specifically, the students will observe the influence of different hormones and hormone ratios on various plant species.

MATERIALS

Shoots of carnation and strawberry
Razor blades, scalpels and forceps
Chlorox, ethanol
Dissecting microscope

PROCEDURE

Each group will excise 5 shoot-apices for each of two species, carnation (*Dianthus carophyllis*) and strawberry (*Fragaria x ananassa*).

1. Preparation of sterile tissue

   a. Remove the shoot apices of young sprouts 3-5 cm long from the parent plant with a sharp razor blade.
   b. Remove the leaves and rinse apices thoroughly in 70% ethanol.

c. Soak in 7% solution of sodium hypochlorite or in 20 % bleach for 3-5 minutes. Tween 20 or 80 (0.1%) may be added to the disinfectant to increase its wetting power.
d. Rinse several times in sterile deionizes water.
e. After the final rinse, dip explant in 1% ascorbic acid solution until ready for dissection.

2. Dissection of meristematic domes.
These operations are to be performed aseptically, preferably in a laminar flow cabinet. They can also be conducted under semi-sterile conditions since the meristematic domes are enclosed in many whorls of leaves and are usually free of contaminants.

1. Sterilize the instruments (knives, needles and forceps) by immersing in 85% ethanol and flame.
Note: This should be done before each operation and as frequently as possible.

2. Wipe the microscope and the dissection stage with 70% ethanol. Avoid wetting the lenses with ethanol, as the glue which holds them is water soluble.

3. Hold the disinfected shoot apex with a sterile pair of forceps with one hand under a workable magnification (10-50x) of the microscope.

4. Remove the outer sterile leaves with sharp sterile knives until the meristematic dome is reached. It will appear glossy under the microscope.

5. By manipulating the knives make four cuts at right angles to each other, gently remove the dome and plant it immediately on the agar medium.
NOTE: The meristematic dome should contain portions of procambial tissue.

6. Incubate the tubes containing the meristems in the growth cabinet.

7. When plantlets are formed, generally after 4-6 weeks, remove them from tubes, wash the agar from the root system in running tap water and plant them into pots containing either vermiculite or perlite. The plants should be kept covered with either glass beakers or plastic bags until they are established. Water with Hoaglands nutrient solution weekly.

TISSUE CULTURE MEDIUM FOR STRAWBERRIES

	MERISTEMS(I)	BUDDING(II)	ROOTING(III)
MACRONUTRIENTS	Knop/MS	Knop/MS	Knop/MS
MICRONUTRIENTS	MS	MS	MS
VITAMIN MIXTURE	MS	MS	MS
HORMONES (mg/l)	BA 0.1	1.0	0
	IBA 1.0	1.0	1.0
	GA 0.1	0.1	0
GLUCOSE (g/l)	40.0	40.0	40.0
AGAR (g/l)	8.0	8.0	8.0
pH	5.6-5.8	5.6-5.8	5.6-5.8

TISSUE CULTURE MEDIUM FOR CARNATION

MS Medium supplemented with 1 ppm NAA

DISCUSSION /COMMENTS

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LABORATORY 7 *IN VITRO* FERTILIZATION

Harrison Hughes

INTRODUCTION

Self-pollination is limited in nature since most angiosperms are out crossers; further, interspecific and intergeneric hybridization is also rare. Hence, selfing and hybridization are two important aspects of achieving desirable genotypes in plant breeding. The fertilization of the stigmatic heads of the pistils, placental pollination and ovule pollination has been successfully used to obtain mature seeds *in vitro*. Its potential use lies in the production of interspecific and intergeneric crosses as well as a means of overcoming self-incompatibility.

PURPOSE

The purpose of this laboratory is to demonstrate the technique of *in vitro* pollination of *Petunia hybrida*.

MATERIALS

Semi open flowers of *Petunia hybrida*
Sterile forceps, needles and knives
20% clorox solution
Culture medium and culture vessels

PROCEDURE

1. The following culture medium will be used.

   Amount per liter of medium
   500 mg $Ca(NO_3)_2 \cdot 4H_2O$
   125 mg KNO_3
   125 mg KH_2PO_4
   125 mg $MgSO_4 \cdot 7H_2O$

   MS trace elements and vitamins (full strength; see page 3)

   0.25 mg calcium Pantothenate
   500 mg casein Hydrolysate
   50 mg FeEDTA
   7.5 g glycine
   40 g sucrose
   8 g agar

2. Aseptic excision of anthers

   A. Surface sterilize appropriate flowers of *P. hybrida* for 2-3 minutes in 20% bleach with a wetting agent.

   B. Rinse flowers in sterile water prior to removal of anthers.

   C. Excise the anthers from the flowers and transfer to sterile petri dishes.

   D. Leave anthers for 12-24 hours to dehisce.

3. Aseptic excision of pistils, ovary and ovules

A Surface sterilize appropriate flowers of P. hybrida for 2-3 minutes in 20% bleach with a wetting agent. Rinse in sterile water.

B. Remove the sepals, petals and anthers in a sterile hood.

C. Shorten the pedicel and then plate the pistil onto media.

E. Repeat as in A but excise ovary and split prior to plating onto media.

F. Repeat as in A, but excise ovules and plate onto media with pollination the next day.

Observe cultures every 2 days for changes in size of ovules or ovary.
Mature seed should be observed within 24 days of pollination.
Remove and germinate mature seeds.

DISCUSSION/COMMENTS

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LABORATORY 8 EMBRYO CULTURE

Harrison Hughes

INTRODUCTION

The development of embryos in vivo is influenced by many factors. For example, in nature manyembryos are subject to long dormancy periods and hybrid embryos do not survive often, due to factors inhibiting embryo development.Isolation and culture of such embryos circumvent such developmental barriers.

The culture of plant embryos dates back to the early 1900's. Most of the early work was done with well-developed embryos. As early as 1942 small embryos (less than 200 um) were successfully cultured using the addition of coconut milk to the media. Numerous studies since have indicated that the less differentiated embryos require greater media complexity.

PURPOSE

This exercise demonstrates the culture of tomato embryos using White's media supplemented with casein hydrolysate and certain organics.

MATERIALS

Tomato fruits of different sizes
70% ethanol, 20% bleach solution
Scalpels, forceps
Culture vessels
Modified White's media

PROCEDURE

A. Excision of tomato embryos
   1. Select flowers which are fully open and therefore recently pollinated.

   2. Select fruits which are approximately a. 0.5cm
      b. 1-2 cm in diameter.

B. Surface sterilize the fruit by a quick dip in alcohol followed by 3 minutes in 20% bleach.

C. Exciseseeds(embryos) from each of the fruit and noterelative size of each. Plate at least 2 embryos from each fruit.

D. Observe at 2 day intervals and note relative success ofcultures and growth rate.

DISCUSSION/COMMENTS

REFERENCES

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LABORATORY 9 MORPHOGENESIS

Harrison Hughes

INTRODUCTION

Tobacco is a model system for in vitro studies of morphogenesis in plants. Procedures for morphogenesis from plants, callus production followed by morphogenesisandembryogenesisfromsuspensionculturesshave allbeen performed routinely.

PURPOSE

Tobacco, Nicotiana tabacum will be used to demonstrate direct morphogenesis from leaf tissue and callus induction followed by morphogenesis. The initial results obtained from tissue explant, direct morphogenesis or callus is mediated by hormones used.

MATERIALS

Sterile seedlings of Tobacco/ green house grown tobacco leaves
70% ethanol, 20% bleac solution

PROCEDURES

I. Explant source

A. Sterile seedlings germinated in petri dishes as follows:

1. Seeds transferred in to 70% ethanol in a flask and shaken for two minutes.

2. Alcoholdecantedand 20% commercial bleach(containing5% hypochlorite or 1-2% sodium hypochlorite) added. Flask is shaken for 15-20 minutes and solution decanted along with floating seeds. Sunken seeds retained.

3. Seeds are then rinsed in sterile distilled water.

4. Seedsare then placed in a double layer ofpre-sterilized filterpaperin petri dishes. Sterile water is addedand dishes are sealed with parafilm.

B. Plants grown under normal greenhouse conditions but must be used prior to flowering. The youngest fully expanded leaves are collectedfrom tobacco plants either 1-2 hours after sunrise orfromdark pretreated tobacco plants.

1. Leaves are washed with mild detergent in water and rinsed first with 70% EtOH, and then distilled water.

2. Leaves are sterilized in 20% commercial hypochlorite solution for 5 minutes. It is necessary to expose all leaf surfaces to the sterilizing solution. This is often done by placing leaves in a small beaker on a gyratory shaker.

3. Leaves are rinsed in sterile distilled water three times and air dried for approximately 5 minutes in sterile petri dishes.

II. Culture Initiation

A. Direct morphogenesis

1. Leaf sections 1 cm x 1 cm (including a vein) are cut and transferred into culture medium. Do at least 5 culture vessels from each explant source.

2. An MS medium with 5 uM 6BA will be used. Shoots should appear in 3-4 weeks.

3. Culture vessels should be held at 22-28°C with light.

4. Shootscanbe transferred to a rooting media ofhormone-free full or half-strength MS.

5. Rooted plantlets may then be transplanted in to Jiffy 7 pots and placed under high humidity for several days.

B. Callus production followed by morphogenesis.

1. Place 1x1 cm leaf sections into culture medium (MS) with 4.5 uM 2,4-D and 1.5 g/l of casein hydrolysate. Do at least 5 jars per explant source.

2. Callus formation will be visible in about 4 weeks.

3. Transfer callus to MS medium supplemented with 5 uM 6BA.

4. Culture conditions and rooting as described in A.

DISCUSSION/COMMENTS

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LABORATORY 10 SOMATIC EMBRYOGENESIS

Harrison Hughes

INTRODUCTION

Somatic embryogenesis is the initiation of embryos from cells which are not products of sexual fusion. Observations of embryo development from somatic tissues were recognized and reported as early as 1958. Carrot, Daucus carota, is the classic example of somatic embryogenesis in plant tissue cultures. Somatic embryogenesis has been observed in vivo in many taxa.It has also been induced in vitro in many species of plants. The ability to initiate embryos in somatic tissues in culture offers an important pathway of plant regeneration from tissue cultures.

PURPOSE

This lab demonstrates the procedures for production of somatic embryos in Daucus carota.

MATERIALS

Carrot seeds or sterile carrot seedlings
MS media supplemented with 2,4-D and ABA as appropriate (see procedure)
Culture vessels
Gyratory shaker

PROCEDURE

I. Explant source and callus production

A. Sterile seedlings grown as per previous laboratory will be used as an explant source.

1. 0.5-1 cm petiole and leaf sections will be used as the explant fortissue culture.

2. An MS agar medium plus 4.5 uM 2,4-D will be used for culturing. Do at least 5 culture vessels.

B. Culture conditions may be 22-28°C either in light (1000 lux for 12-24 hours) or dark.

II. Subculturing callus and embryo production.

A. Enough callus should be produced after 4-5 weeks for subculturing.

1. Approximately 2.0-3.0 gms of callus should be added to 15-20 ml of MS liquid medium with 4.5 uM 2,4-D in a 50 ml erlenmeyer flask.

2. Flasks are placed on a gyratory shaker (125-160 rpm) at 25°C with or without light. Do 5 flasks.

3. Subculture at approximately 14-18 day intervals.

B. Initiation of embryo development.

1. Decant medium after 14-18 days of culture and resuspend into MS with no hormones.

2. A more uniform embryo population may be obtained by passing through a series of stainless steel mesh sieves.

   a. Start at 200 u and proceed to finer sieves.

   b. For carrot the 74 u sieve produces a fairly dense suspension of single and multiple clumps.

   c. Sieving with a 43 u sieve produces a very fine suspension at a low density.

3. The sieved suspension should then be transferred to 50 ml of MS liquid medium with no hormones in a 250 ml Erlenmeyer flask.

   a. A more normal embryo development, along with inhibition of precocious germination, may be obtained by adding 0.1-1 uM ABA to the media.

   b. ABA is dissolved slowly in distilled water at ambient temperature in complete darkness and should be filter-sterilized prior to its addition.

4. Cultures should be grown in complete darkness for more normal development. Embryos should appear in 8 days and reach mature size in 10-15 days.

5. Embryos then may be placed on an agar medium devoid of 2,4-D for plantlet development.

DISCUSSION/COMMENTS

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LABORATORY 11 IN VITRO SELECTION FOR STRESS

Christy MacKinnon and Sunitha Siriwardana

INTRODUCTION

Large areas of the world are unsuitable for agriculture because of the limitations imposed by many environmental stresses. Tissue culture techniques are being recognized as a useful tool for developing plants which are better adapted to stresses. Many features of plant tissue culture enable its application to in vitro selection of variant or mutant cells. By administering suitable selective agents which allow preferential growth of variant or mutant cells, in vitro selection techniques allows the screening of large numbers of cells in less space and time.

PURPOSE

To study in vitro selection schemes applied under different stress conditions.

MATERIALS

Rice seeds
Sodium chloride, Aluminum chloride, Polyethylene glycol
LS media supplemented with hormones (see procedures)

PROCEDURE

A. Salt selection

1. Prepare L&S media for rice, IR-36 (1 mg/l 2,4-D plus 0.5 mg/l kinetin, and 50 mg/l Tryptophan, 4% sucrose, 1% agar) in the following way:

   a. Mix together stock solution and water for 4 liters of media, and pH.

   b. Pour 1 liter of medium in 4 separate containers. (250 ml in each)

   c. Add NaCl as follows:
      Container #1. 0 NaCl
      Container #2. 6 gm NaCl
      Container #3. 9 gm Nacl
      Container #4. 11 gm NaCl

   d. Add agar to each container, microwave and dispense into vials and autoclave.

2. Transfer about 0.1 g of embryogenic callus (E) to medium containing salt, and incubate in light for 2 to 3 passages (each 4 weeks long).

3. Store each vial at the end of a passage for
   a) presence of E callus
   b) increase in weight
   c) survival of callus

4. At the end of stress period, transfer any remaining E callus to regeneration medium. (LS medium + 0.5 mg/l IAA + 0.2 mg/l BAP for IR-36; LS medium + 0.5 mg/l BAP for Calrose 76).

B. Drought selection with PEG on solid media.

1. Prepare L&S media for rice as follws:

   a. Mix stock solutions for 1 liter of medium, pH and add agar. Pour into 1 liter flask. DO NOT ADD REQUIRED AMOUNT OF WATER AT THIS POINT.

   b. Preparation of PEG "concentrations"

gm PEG/100 ml	
1	= PEG 10
2	= PEG 20
2.5	= PEG 25
5.0	= PEG 50
7.5	= PEG 75
10.0	= PEG 100

   c. In a separate 1 liter flask, mix 25 gm of 5000 MW PEG and water. Add the required amount of water .

   d. Autoclave flasks. Cool slightly. Autoclave also empty flasks to mix up media later.

   e. In a sterile 2 liter container (see c.), in the transfer hood, mix together the contents of each flask. The resulting mixture will be thick.

   f. Dispense mixed media as directed.

2. Inoculate each vial 0.1 g embryogenic callus. Incubate in light for 4 weeks. Score each vial for presence of embryogenic callus. Repeat for 2 to 3 passages.

3. Proceed with steps 2 - 4 in salt selection.

C. Drought selection with PEG on liquid medium

1. Prepare media as for B but without agar. Use filter, paper folded in the shape of an "M" for support of callus.

2. Repeat all other steps in B.

D. *In vitro* selection for aluminum tolerance

1. Prepare L&S media for rice as follows:

   a. Mix together stocks, water for 1 liter of medium; pH and add agar.

b. Prepare stock solutions of $AlCl_3$:

Dissolve $AlCl_3$ in water.

Stock(gm/100ml)	Final concentration(ppm)
2.0	200
2.5	250
5.0	500
7.5	750
10.0	1000
15.0	1500

Injection of 0.1 ml per 10 ml of stock solution yields final ppm

d. Autoclave all stocks separately first and then inject 0.1 ml per stock in to vials in a sterile hood.

e. Follow steps 2 - 4 in salt selection.

DISCUSSION/COMMENTS

REFERENCES

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LABORATORY 12. ISOLATION AND CULTURE OF PLANT PROTOPLASTS

Oluf L. Gamborg and Suresh Kumar

INTRODUCTION

Protoplasts can be isolated from tissues or cultured cells by enzymatic digestion of the cell walls. The enzymes used for this purpose are preparations which are commercially available. The success of protoplast isolation depends especially on the tissue and the combination of enzymes.

There is no standard method for the isolation and culture of protoplasts. However, the procedure follows a general pattern. The individual cell or tissue source may required special conditions for isolation or for culture. Protoplasts from cell suspension cultures generally are the most readily obtained and usually regenerate into dividing cells at a reasonable frequency.

For the experiments a suspension culture of carrot (*Daucus carota*) cv. will be used. The cells are grown in Murashige-Skoog (MS) medium with 0.5 mg/l of 2,4-D. Subculturing is performed weekly.

The other source of protoplasts are leaf tissue of tobacco (*Nicotiana tabacum*). The leaves are obtained from shoot cultures grown in MS medium with no hormone. The best materials are newly expanded leaves.
The procedure described below outlines the steps for isolation and culture of protoplasts.

MATERIALS

A. Plant materials

1. Cell suspension cultures of carrot (*Daucus carota*) started 1985. Cultured in MS medium with 0.5 mg/l 2,4-D.
2. Leaf tissue of tobacco (*Nicotiana tabacum*) from shoot cultures grown on MS medium with no hormone.

B. Enzymes

Cellulase: ONOZUKA RS

Hemicellulase: Sigma

Pectolyase: Sigma

C. Solutions and Media

Use demineralized or distilled water.

I. Culture Medium

Stock Solutions:

Major salts, (B5)	g/1000 ml (10 x conc)
KNO_3	25.00
$(NH_4)_2SO_4$	1.34
$MgSO_4.7H_2O$	2.50
$NaH2PO_4.7H_2$	1.50
$CaCl_2.2H_2O$	8.85

Use 100 ml per liter

Micronutrients (B5)	mg/1000 ml (100 x)
$MnSO_4.H_2O$	1000
H_3BO_3	300
$ZnSO_4.7H_2O$	200
$Na_2\ MoO_4.2H_2O$	25
$CuSO_4.5H_2O$	2.5
$CoCl_2.6H_2O$	2.5
5 KI	8.3

Use 10 ml per liter

Vitamins	mg/100 ml (100 x)
Nicotinic Acid	100
Thiamine HCl	1000
Pyridoxine HCl	100
Myo-inositol	10000
Ascorbic Acid	100
P. Aminobenzoic Acid	1
Riboflavin	10
Calcium panthothenate	50
Biotin	1

Store frozen - use 1 ml per liter

Fe-EDTA	mg/100 ml (10 x)
Na_2EDTA	372.5
$FeSO_4.7H_2O$	279.0

Store in refrigerator - use 10 ml per liter

1. Protoplast Culture Medium (PCM)

	Amt/1000 ml (1x)
Major salts (stock)	100 ml
Micronutrients (stock)	10.0 ml
Fe-EDTA	10.0 ml
Vitamins	1.0 ml
Casein hydrolyzate	250 mg
Ribose (if available)	125 mg
Sucrose	150 mg
L - glutamine	300 mg
Na - citrate (or citric acid)	40 mg
Malic acid	40 mg
Sorbitol 0.2 M (final)	36 g
Glucose 0.2 M (final)	36 g
2,4-D (make stock)	1.0 mg
BA (make stock)	1.0 mg

Adjust pH to 5.6 and filter sterilize

II. Protoplast Isolation Medium (IS-1)

Stock Solution for Isolation Medium

	Amount/100 ml (1x)
Major salts (stock solution)	10.0 ml
Micronutrients (stock solution)	1.0 ml
Fe-EDTA (stock solution)	1.0 ml
Vitamins (stock solution)	0.1 ml
Sorbitol (0.3 M)	5.4 gm
Glucose (0.2 M)	3.6 gm
MES Buffer	120 mg

Adjust pH to 5.6.

Filter sterilize. Distribute in four flasks.

Washing Medium (WS-1)

Similar to that of protoplast culture medium + 0.05 M Sorbitol and 0.05 M Glucose. For 100 ml of WS-1 medium, dissolve 900 mg of sorbitol and 900 mg of glucose.

III. Enzyme Solutions:

Dissolve the following enzymes:

A. (E-1) in 10 ml of IS-1 stock solution for carrot

Cellulase:	Onozuka RS	200 mg
Hemicellulase:	Sigma	100 mg
Pectolyase:	Sigma	12.5 mg

Check pH at 5.6

B. (E-2) in 10 ml of IS-1 stock solution (for tobacco leaves)

Cellulysin	200 mg
Hemicellulase	100 mg
Macerase	50 mg

Check pH at 5.6

Purification with Percoll[R]

To determine the concentration for the best separation and highest purity.

Desired % Percoll	Mix the following (ml)	
	Percoll	PCM
19	3.8	16.2
20	4.0	16.0

Protoplast Isolation Procedure

The enzyme solution is made up immediately prior to incubation with the plant materials and filter sterilized.

I. Isolation from Leaf Tissues

1. The leaves are excised from shoot cultures grown on 0 - MS agar media. Select newly expanded leaves.
2. Place leaves in a small amount of IS-1 medium in a petri dish. Cut

the leaves into fine strips, parallel to the veins.

3. Transfer a portion of the cut leaf material by forceps to 5-10 ml of enzyme solution of the 1:1 mixture of E-1 media and protoplast culture medium in a 50 x 9 mm petri dish with tight lid.
4. Seal the dish and incubate at 22-25°C. Agitate the petri dish by hand at 30 min. intervals.
5. Observe the release of protoplasts at intervals on the stage of an inverted microscope.

Proceed to Section III.

II. Isolation from Cells of Suspension Cultures

1. Centrifuge 5-10 ml of cell suspension culture. Remove supernatant.
2. Mix 5-10 ml of enzyme solution (E-1) with the cells in the centrifuge tube.
3. Transfer to 50 x 9 mm sterile petri dish.
4. Seal the dish and incubate at about 25°C. Agitate the mixture gently by hand at 30 min. intervals and observe the release of protoplasts.

III. Filter, Wash, Purification

1. Transfer the isolation mixture from the petri dish to a 50-100 um stainless steel screen or nylon filter and funnel into a sterile centrifuge tube.
2. Rinse by transferring 5 ml of WS-1 through the screen.
3. Transfer the filtrate to a 30-44 um screen and funnel into a sterile centrifuge tube to remove fine debris. Wash with 2 to 2.5 ml WS-1.
4. Centrifuge at 150 g for 5 minutes.
5. Remove the supernatant.
6. Resuspend the protoplast pellet in 10 ml of PCM.
7. Carefully pipette 10 ml of the 19% Percoll solution to the bottom of

the centrifuge tube. The protoplast suspension should be floating on the Percoll solution. Note: The Percoll may not be the correct concentration.

8. Centrifuge at 150 x g for 5 minutes.
9. Carefully pipette the band at the interface between the two layers and transfer to a centrifuge tube. Add an equal volume of culture medium (PCM).
10. Centrifuge at 150 x g for 15 minutes.
11. Remove the supernatant and resuspend the protoplast pellet in 1 to 2 ml of culture medium. The density should be about 10^5/ml.
12. Count the protoplasts in a haemocytometer. Count 64 fields, 0.25mm x 0.25 mm. See Diagram.
13. Calculate the average number of protoplasts per field and per ml. One field = 0.25mm x 0.25mm x 0.1 mm. - 10 = 6.25×10^{-6} ml. (See Diagram).
14. Adjust density if necessary to 10^5/ml by adding culture medium.

Protoplast Culture

1. Distribute the protoplast suspension in 6-8 small drops (ca 100 ul) per petri dish.
2. Seal and place the petri dishes in a plastic box humidified with paper towel wetted with 1% copper sulphate. Cover the box and incubate in the dark or diffused light at 28°C.
3. Observe the drops daily and note the frequency of cell wall regeneration (change from spherical to oval).
4. After 36 hrs. note evidence of cell division.
5. Fresh medium at 1/2 the original osmolarity should be added after 7-10 days to double the volume of the droplet.

II. Protoplast fusion

All operations should be perforemd aseptically in a laminar air flow cabinet.

1. Place a 3 ul drop of Silicone 200 fluid (autoclaved) in a 60 x 15 mm petri dish using a 1 ml plastic syringe.
2. Place a 22 x 22 mm cover slip on the drop.
3. Pipette ca 150 ul of protoplast suspension onto the cover slip with a pasteur pipette. (The protoplast suspension is a mixture of the two sources).
4. Allow the protoplasts to settle and from a thin layer.
5. Slowly add dropwise 450 ul of PEG solution to the protoplast solution. Observe the protoplast adhesion on the stage of an inverted microscope.
6. Incubate in the PEG solution for 5 to 20 minutes.
7. Begin elution by gently adding 0.5 ml eluting solution. Repeat after 10 minutes. After another 10 minutes, add ca 1 ml of protoplast culture medium.
8. Wash the proplasts 5 times by adding 1 to 2 ml of cutlure medium at ca 5 minute intervals. NOTE: Leave a thin layer of medium on the cover slip each time before adding fresh medium.
9. Leave ca 500 ul cutlrue medium on the cover slip (fused and unfused protoplasts) and put 0.5-1.0 ml of medium in droplets in the petri dish to maintain humidity.
10. Seal the petri dish with parafilm^R and incubate at ca 25°C in a 60-100 lux light in a plastic box humidified with blotting paper moistened with 1% $CuSO_4$ placed in a beaker.

Addenda: Fusion procedure - timing

Solution (a) PEG (PEG)

(b) Elution Solution (ES)

(c) Culture Medium (CM)

Starting: Protoplast mixture on coverslip

150 ml (about 2 drops)

1. Add 6 drops (ca 450 ml) PEG and leave 10-20 minutes.
2. Add 8-10 drops of ES, leave 10 minutes.
3. Add 8-10 drops of ES, leave 10 minutes.
4. Gently remove liquid and add 16-20 drops of CM. Leave ca. 5 min.
5. Remove liquid. Add 120 drops CM. Leave 5 minutes.
6. Repeat as in 5.

Assignments

A. Observe wall digestion and regeneration on protoplasts stained with Calcofluor in fluorescence microscope.

B. Determine the frequency of surviving protoplasts with Evans blue.

C. Determine the frequency of multinucleated protoplasts. Fix the protoplasts before staining them with carbol-fuchsin.

1. Fix samples of cell culture for 30 min. (Fix samples of protoplast preparation for 30 min).
2. Stain fixed samples of cell and protoplast preparations with carbol-fuchsin.
3. Determine the frequency of multinucleated protoplasts.
4. Determine the mitotic index of cell culture (protoplast source) and protoplasts during and after isolation.

D. Determine mitotic index

1. Transfer a sample of fixed cells onto a slide and add an equivalent amount of stain.

2. Wait for a few minutes, cover preparation with a cover slip and heat the slide until small bubbles appear.

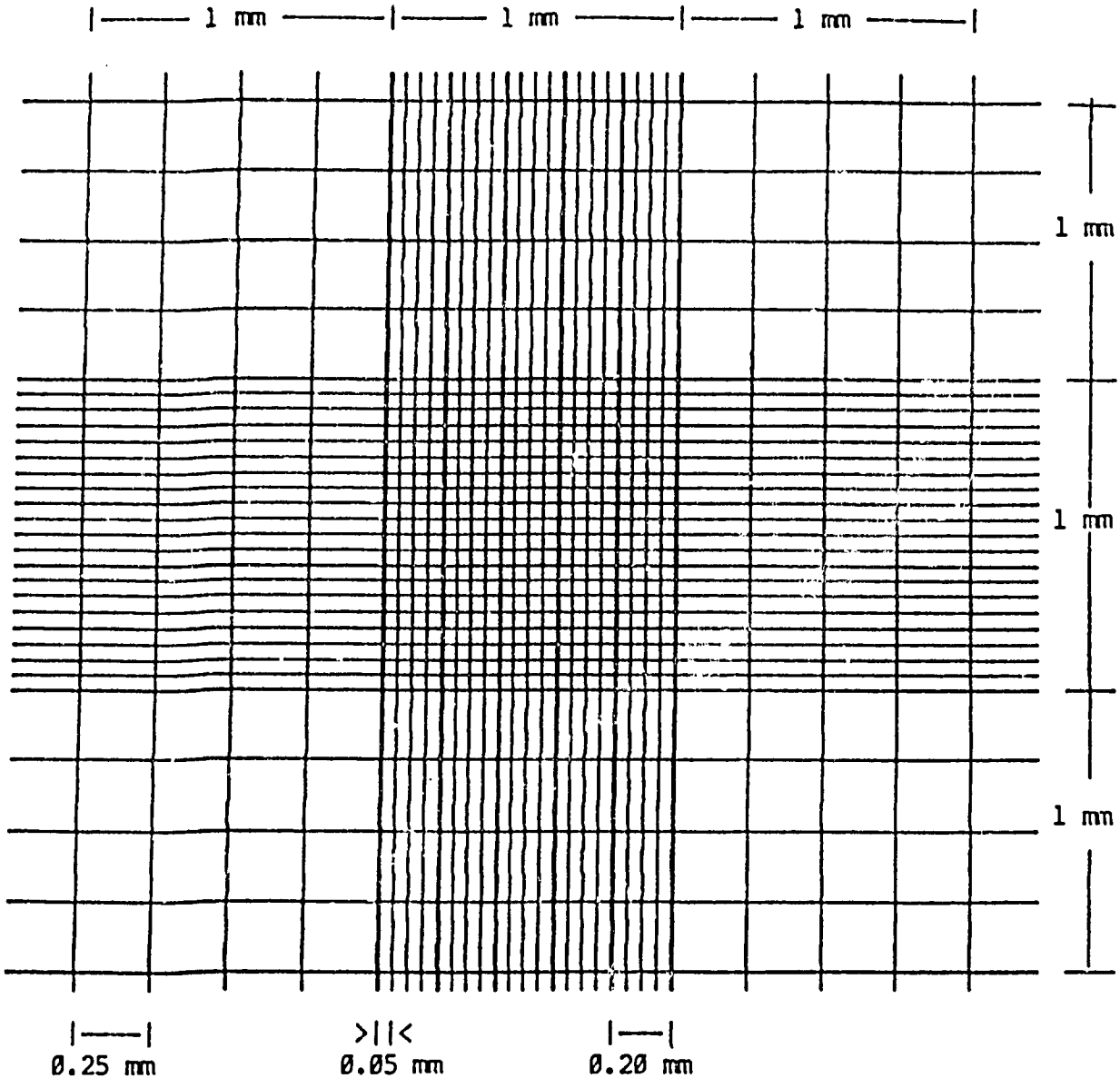
3. Cover slide with paper tissue and squash the preparation gently.

4. Count 1000 nuclei and determine the number of nuclei in mitosis.

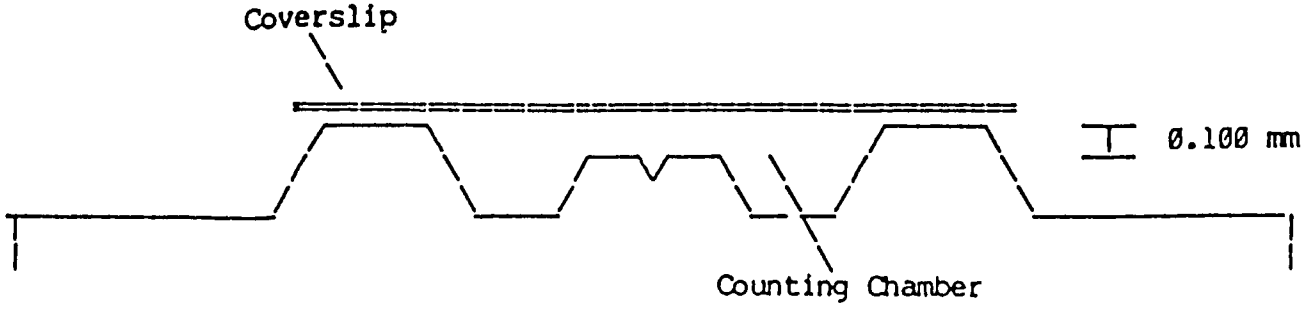
$$MI = \frac{\text{number of nuclei in mitosis}}{\text{total number of nuclei observed}}$$

RESULTS/DISCUSSION

GRADUATIONS FOR 'AO BRIGHT-LINE' HAEMACYTOMETER COUNTING CHAMBER



EDGE VIEW OF COUNTING CHAMBER WITH COVERSLIP



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LABORATORY 13 PLANT VIROLOGY

C.H. Livingston

INTRODUCTION

It has been reported that over 40 percent of the world's most devastating plant diseases are caused by viruses. This estimate does not take into account those virus-like diseases caused by mycoplasms, viroids,preons and various toxemias and physiological disorders which induce virus-like symptoms in plants.

Viruses and other sub-organism level pathogens are not visable in the light microscope, cannot be readily cultured, if at all, and exhibit little or no evidence of a life cycle as found among the plant pathogens that you have studied.

However, viruses do have a definable genetic constitution. Viruses redirect the metabolism of host plant cells to produce more virus particles at the expense of the host cell economy. This results in disease. This host-pathogen relationship is generally regarded to be the highest form of parasitism as it relies upon the host cell metabolism for virus replicaiton rather than parasitism for food as with other pathogens.

Well! How does this all fit into the scheme of Koch's postulates? Transmission is the true test! Viruses are transmitted while toxemias and physiologic disorders are not.

Transmission to appropriate susceptible host plants which allow for virus replication and diagnostic symptom expression is the first essential step in identification. This is also the simplest technique available as it does not require sophisticated equipment or special training on the part of the investigator. A third step is the examination of suspected plant sap by electron microscopy to detect virus particles. It is unlikely that most investigators would access to a scope of this kind. A fourth step is the use of serology. Virus particles present in plant sap have a strong affinity for specific antibodies which ahve been produced by the reaction of warm blooded animals to injection with a series of purified virus aliquots which immunizes the animal. The serum fraction of the blood contains the specific antibodies which can be used to do extensive testing on many suspect plants at a minimal cost. Antisera is commercially available for many common plant viruses.

Transmission and serology are the most readily available and easily assay methods available to investigators such as agricultural consultants and Peace Corp people in remote areas.

PURPOSE

The purpose of this laboratory exercise is to allow the student to actually use both indicator host and serological testing methods for several plant viruses.

Each student will:

1. Make a virus transmission test to appropriate indicator plants
2. Serologically test for virus by radial double-diffusion in agar
3. Learn how to make the proper calculations for the construction of buffer solutions essential for the contraction of virus from plant tissues

MATERIALS

1. Virus infected host plants
2. Indicator plant (Gomphrena globosa)
3. Mortar and pestle
4. Carborundum
5. Phosphate buffer (pH 7.5)
6. Microslide coated with 0.9% agar

PROCEDURES

The laboratory exercise will be conducted in concert with the students working together in groups of three. Each member of the group will work with a virus preparation which is different from the other two. The results will then be observed, recorded and comparisons made as a group. Conclusions will be drawn from the experiment following a group discussion one week later.

A. Your group will be provided with a set of three virus infected host plants which are expressing diagnostic symptoms for each of the three viruses--Potato virus X (PVX), Tobacco mosaic virus (TMV), and turnip yellows mosaic virus (TYMV). Do not touch the plants at this time as you will risk contamination of subsequent experiments. Make a sketch on your data sheet of a single leaf of the three plants and develop a short description of the symptoms to accompany your symptom sketch.

B. Remove one leaf from your own plant and place it in the mortar which contains a phosphate buffer solution (pH 7.5). Using the pestle grind the leaf to a pulp in the mortar. The small narrow leafed plant which is in front of you is a local lesion indicator plant (Gomphrena globosa) which your instructor has sprayed with a suspension of 600 mesh carborundum. Carborundum serves as an abrasive to penetrate the cuticle of the leaf when rubbed with inoculum. This allows virus particles to penetrate and infect the host plant. All plant viruses are wound pathogens and can only infect plants at sites of mechanical wounds or through injection by specific vectors. Gently apply inoculum with a circular motion of the pestle to the upper surface of several of the indicator plant leaves while supporting the leaf with your fingers. Write your name and the virus vernacular on the pot label. Place your inoculated plant in a greenhouse tray and wah your hands thoroughly with soap and water for subsequent experiments. (Why this?)

C. Each member of your group is provided a microslide coated with 0.9% lonagar in phosphate buffer pH 7.5. The cooled agar layer has been punched and cores removed for a double diffusion serological assay. Your instructor has charged the center well of each pair of grids on your slide with antiserum to you virus. Using capillary tubes, charge the wells according to the scheme at the bottom of your data sheet with yours and each of your partners' virus preparations. Your instructor will demonstrate the method or technique you should use for this step. Avoid contamination. Return your cover to the petri dish incubator with the moistened paper which maintain humidity during the incubation period. Write your name on the petri dish cover with a wax pencil and stack your dish with your partners to keep them as a group of three for your observation after a 24 hour incubation. Complete your data sheet by drawing in the diffusion line patterns in each of your groups plates. One week after inoculating your indicator plant, examine the inoculated leaves and sketch the symptom pattern on you data sheet. When your data sheet is complete, confer with your group members and draw conclusons, and check with one of the instructors.

DATA SHEET

A. Sketch a description of symptoms in systemically infected host plants

PVX infected -

TMV infected -

TYMV infected -

B. Sketch indicator-host plant reactions - symptoms

Descriptions:

3. Charge wells on the microscopic slide with anitigen and antiserum according to the following diagrams:

Charge center wells with ANTISERUM to:

```
1. PVX                    2. TMV                    3. TYMV
 ____________________      ____________________      ____________________
|                    |    |                    |    |                    |
|   0 0      0 0     |    |   0 0      0 0     |    |   0 0      0 0     |
|  0 0 0    0 0 0    |    |  0 0 0    0 0 0    |    |  0 0 0    0 0 0    |
|   0 0      0 0     |    |   0 0      0 0     |    |   0 0      0 0     |
|____________________|    |____________________|    |____________________|
```

Peripheral walls charged with ANTIGEN according to the following diagram:

```
 ____________________________________________________
|                                                    |
|          C                          .              |
|     C         A               C         A          |
|         As                          As             |
|     B         A               .         .          |
|          B                          B              |
|____________________________________________________|
```

Code:
A = PVX
B = TMV
C = TYMV
1 = PUX antioserum
2 = TMV antiserum
3 = TYMV antiserum
As = Antiserum

QUESTIONS

1. What is a buffer?

2. Why is the use of a buffer important in the exercise?

3. What is pH?

4. Why is a local lesion indicator plant used?

5. How have you attempted to apply Koch's postulates?

6. What is a virus? Define.

7. How do viruses cause plant diseases?

8. What are the chemical constituents of a virus particle?

9. Do viruses exibit a genetic constitution?

10. Are the viruses in your group different with regard to particle morphology?

11. Are the three viruses in your group serologically related?

12. How would you go about obtaining seed for indicator plants?

13. Explain the reaction of antibody to antigen in your serological reaction.

14. Discuss controls or lack thereof in these experiments?

15. Forty percent of the world's most devastating plant diseases are caused by viruses. True or false?

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LABORATORY 14 ANTHER CULTURE

Harrison Hughes

INTRODUCTION

The use of anthers to produce haploid plants has been attempted in many species since it's first successful report by Guha and Maheswari in 1966. Tobacco is one of those species which has been most studied relative to this procedure.

PURPOSE

The purpose of this laboratory is to demonstrate the above technique using tobacco. It will also show how you might correlate morphological traits of the flower (size) with the meiotic stage in pollen development.

MATERIALS

Tobacco flower buds of different growth stages.
70% ethanol, Tween^{R}-80, chlorox
Forceps, dissecting needle, glass rod/spatula
Carnoy's solution
Aceto-carmine or aceto-orcein
Microscope slides, cover slips
Culture medium

PROCEDURE

A. Composition of the anther culture medium (Nitsch 1972)

Component	mg/l
KNO_3	950
NH_4NO_3	720
$MgSO_4.7H_2O$	185
$CaCl_2.2H_2O$	166
KH_2PO_4	68
$MnSO_4.4H_2O$	25
H_3BO_3	10
$ZnSO_4.7H_2O$	10
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
FeEDTA	50
Sucrose	20,000
IAA	0.1
Adjust pH to 5.5	

B. Anther culture of tobacco

1. Select plants of flowers of varying length from those showing no exposed petals (sepals enclosing the petals) to those with fully elongated petals not yet open.

2. Measure each flower and record general morphological traits prior to culture.

3. Surface sterilize the flower buds with a quick dip in alcohol followed by 10 min. in 20% clorox solution plus a wetting agent.

4. Rinse the buds several times in sterile water.

5. Using forceps and a dissecting needle, carefully tease open the buds and remove the anthers.

6. Plate all but one anther onto the culture medium.

7. Take one anther for determination of stage of pollen development as according to B.

C. Determination of meiotic stage related to flower size.

Anther smears are used for the study of meiosis in pollen mother cells (PMC) and for microspore division. For chromosome counts, the late prophase of the first division of the PMC is the desirable stage. Proper bud size must be determined by experiment. Usually, if the anthers are yellow, pollen has already formed.

1. Fix intact anthers for 15 minutes at 60° C in Carnoy's solution.

2. Remove anthers from Carnoy's solution and place a small drop of aceto-carmine or aceto-orcein on top of them on a clean slide.

3. Squash the anther with a tip of a glass rod or spatula until the contents have been forced out. Remove any remaining piece of anther wall.

4. Pick up a clean cover slip and add a tiny bit of stain to the lower edge and lower the slip carefully over the PMC's avoiding air bubbles.

5. Warm the slide over an alcohol flame several times (Do not boil).

6. Now place the slide within the fold of a paper towel, and with the thumb placed exactly in the center of the slide, press down rather hard, being careful to keep the edges of the cover glass parallel with the edges of the slide itself.

7. Record the following:

Floral Stage:	No. anthers cultured	% anthers produced plantlets	No. plantlets per anther	Range in No. plant-lets per anther	Stage of Meiosis
1. Anthesis (prior to)					
2. Petals 2 x's longer than sepal					
3. Petals slightly longer than sepals					
4. Petals same length as sepals					
5. Small bud- corolla not visible					

DISCUSSION/COMMENTS

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