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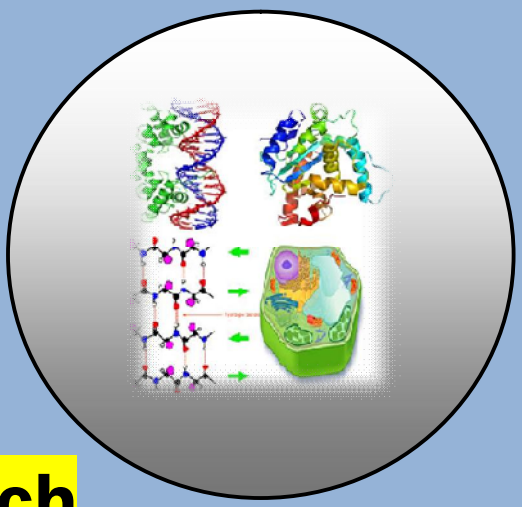

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REVIEW ARTICLE

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Principles and Techniques of Plant Tissue Culture

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ABSTRACT

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation, including:

- ***The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.***
- ***To quickly produce mature plants.***
- ***The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.***
- ***The regeneration of whole plants from plant cells that have been genetically modified.***
- ***The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.***
- ***The production of plants from seeds that otherwise have very low chances of germinating and growing, i.e.: orchids and Nepenthes.***
- ***To clean particular plants of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture***

In Addition modern plant tissue culture is performed under aseptic conditions under HEPA filtered air provided by a Laminar flow cabinet. Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization of starting material (explants) in chemical solutions (usually alcohol and sodium or calcium hypochlorite or mercuric chloride.

Key words: Laminar Flow Cabinet, Explants, Plant Tissue Culture and Micropropagation.

INTRODUCTION

Plant cells can be grown in isolation from intact plants in tissue culture systems. The cells have the characteristics of callus cells, rather than other plant cell types. These are the cells that appear on cut surfaces when a plant is wounded and which gradually cover and seal the damaged area Sathyanarayana (2007).

Pieces of plant tissue will slowly divide and grow into a colorless mass of cells if they are kept in special conditions. These are:

- initiated from the most appropriate plant tissue for the particular plant variety
- presence of a high concentration of auxin and cytokinin growth regulators in the growth media
- a growth medium containing organic and inorganic compounds to sustain the cells
- aseptic conditions during culture to exclude competition from microorganisms

The plant cells can grow on a solid surface as friable, pale-brown lumps (called callus), or as individual or small clusters of cells in a liquid medium called a suspension culture. These cells can be maintained indefinitely provided they are sub-cultured regularly into fresh growth medium.

Tissue culture cells generally lack the distinctive features of most plant cells. They have a small vacuole, lack chloroplasts and photosynthetic pathways and the structural or chemical features that distinguish so many cell types within the intact plant are absent. They are most similar to the undifferentiated cells found in meristematic regions which become fated to develop into each cell type as the plant grows. Tissue cultured cells can also be induced to re-differentiate into whole plants by alterations to the growth media.

Plant tissue cultures can be initiated from almost any part of a plant. The physiological state of the plant does have an influence on its response to attempts to initiate tissue culture. The parent plant must be healthy and free from obvious signs of disease or decay. The source, termed explants, may be dictated by the reason for carrying out the tissue culture. Younger tissue contains a higher proportion of actively dividing cells and is more responsive to a callus initiation program. The plants themselves must be actively growing, and not about to enter a period of dormancy Bhojwani and Razdan. (1996).

The exact conditions required to initiate and sustain plant cells in culture, or to regenerate intact plants from cultured cells, are different for each plant species. Each variety of a species will often have a particular set of cultural requirements. Despite all the knowledge that has been obtained about plant tissue culture during the twentieth century, these conditions have to be identified for each variety through experimentation Indra et al. (2013).

OBJECTIVES

1. To know the basics of plant tissue culturing.
2. To know the production of callus from carrot.
3. Measure the efficacy of root and shoot.

Using somaclonal variation to select for disease resistance

Plant tissue cultures isolated from even a single cell can show variation after repeated subculture.

Distinct lines can be selected with their own particular morphology and physiology. It suggests that the tissue culture contains a population of genotypes whose proportion can be altered by imposing an appropriate selection pressure. This variation can be transmitted to plants regenerated from the tissue cultures, and is called somaclonal variation. It provides an additional source of novel variation for exploitation by plant breeders.

Fig 1. Carrot family lines regenerated from tissue-culture. Both have been grown for 12 weeks in a glasshouse after 10 weeks vernalisation. Family 16 (LHS) are flowering abundantly, while Family 17 (RHS) have not flowered.

The carrot cultivar Fancy was used in our laboratory to generate a series of 197 regenerant progeny lines. These plants showed considerable morphological variation. They were tested for resistance to the leaf spot pathogen *Alternaria dauci*, which can cause total necrosis of mature leaves. They had a greater degree of variation in response than the parental cultivar, including some more resistant lines.

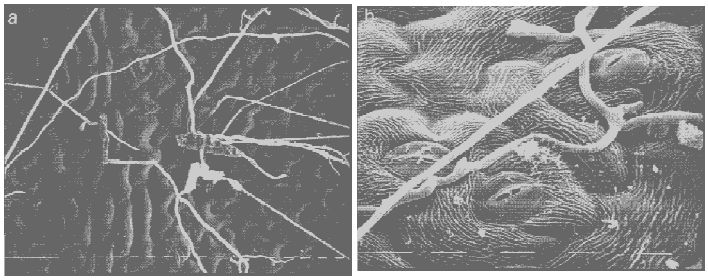

Fig 2. Scanning electron micrographs of surface of carrot leaf 3 days after inoculation with *A. dauci.* (LHS) germination from multiseptate conidiospore (RHS) penetration of hyphae through epidermal surface rather than through stomata. Scale bar = 10 micrometres

One symptom of the disease is loss of chlorophyll and total soluble polyphenol compounds. These reduce to a low level 6 days after inoculation of excised leaves with *A. dauci* spores when compared with uninoculated leaves. Regenerant progeny with high chlorophyll levels maintained higher chlorophyll levels after challenge with *A. dauci.*

After self-pollinating selected high- and low-chlorophyll regenerant plants, this characteristic was inherited by their progeny, suggesting that the capacity to resist this infection is inherited.

Utilising anther culture to select for cold hardiness

Crosses between distantly related species can bring together novel gene combinations. However, the hybrid offspring can be few in number, genetically unstable and require years of further selection and screening before any advantageous characteristics can be brought near to commercial use.

Anther culture (androgenesis), to generate haploid plants from pollen microspores, is one way to shorten this process. It allows novel allele combinations, particularly ones involving recessive characters, to be assessed in intact plants. Useful individuals can then be developed into homozygous and fertile plants through chromosome doubling techniques, and brought into a breeding program.

We have recently been involved in a collaborative project with the Institute of Grassland and Environmental Research (IGER) to use this approach to improve cold-tolerance and fodder quality in grazing grasses. Crosses between *Lolium multiflorum* (Italian ryegrass) and *Festuca arundinacea* (tall fescue) should offer valuable combinations of characteristics. The *Lolium* species should provide good growth characteristics, while the *Festuca* provides stress-tolerance. One hybrid individual (*Festulolium*) resulting from such a cross had already shown drought-tolerance characteristics. However, the out-breeding nature of these grass species, along with the hexaploid genome of *F. arundinacea* and autotetraploid *L. multiflorum* indicated that a lengthy breeding program might be necessary.

The research project therefore aimed to produce androgenic plants from the existing pentaploid *Festulolium* plant and assess them for cold tolerance. This exploited the expertise in tissue culture at the University of Liverpool together with experience in breeding for stress-tolerance at IGER.

Anthers containing immature pollen (microspores) are the starting material for androgenesis. Flowers have to be selected at the correct developmental stage, which varies from species to species. In addition, some individual genotypes may not be amenable to anther culture, or require specific pretreatments. Careful microscopy and testing of successful pre-treatments of related species are therefore necessary when dealing with a new species. For the Graminae, microspores must be at the mononucleate stage and no pre-treatment is necessary.

The cut flowers were surface sterilized and opened in sterile conditions under a binocular microscope. Anthers were dissected and transferred to a solid nutrient medium. Large numbers could be placed on each petri dish. Callus developed, which was transferred to a different medium to initiate embryos and so generate haploid plants.

Over 200 androgenic plants were produced at Liverpool, each originating from a different microspore. Each therefore represented a genetically different individual. Testing for phosphoglucosisomerase, where a different isozyme was contributed by each of the five chromosome groups within the *Festulolium* plant, indicated that the pollen-derived plants had a wide variety of chromosome combinations from each of the parents of the hybrid.

The freezing-tolerance of these plants varied considerably, with three individuals able to survive the extreme cold of -14 degrees Celsius. When the chromosome complement of two of these plants was examined using genomic *in situ* hybridisation (GISH), they carried virtually the whole genome of *F. pratensis*, a parent of *F. arundinacea* noted for its freezing-tolerance.

Unfortunately, the fertility of these two plants was not restored by chromosome doubling, so that they could not be used for further breeding. However, they demonstrated the potential of androgenesis for rapid assessment of the genetic potential available from a difficult breeding combination, indicating that this type of wide cross revealed characters of cold and drought tolerance that were worth pursuing.

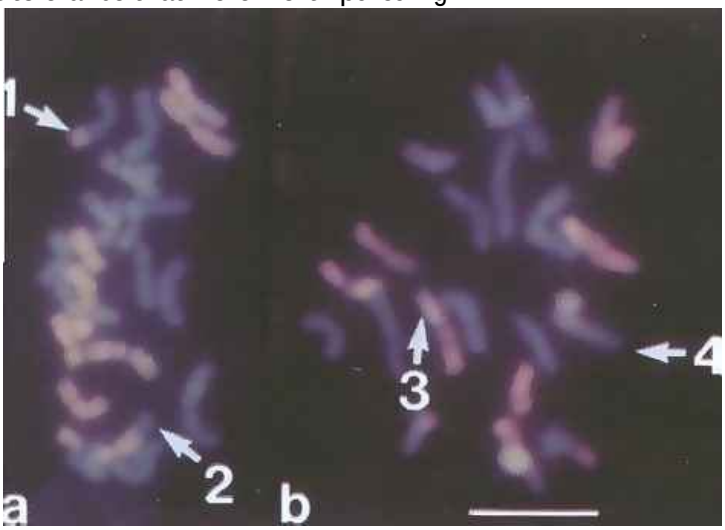

Fig 3. Genomic in situ hybridization (GISH) with L. multiflorum total genomic DNA as probe of mitotic chromosomes of two androgenic plants (2n = 21).The colours indicate: Pink - l. multiflorum chromosome; Mauve - Festuca pratensis chromosome; Blue - Festuca glaucescens chromosome. Interspecific recombinants are arrowed (e.g. 3 = Lm chromosome with Fp recombinant) Bar = 10 micrometers.

Demonstration of tissue culture

The starting point for all tissue cultures is plant tissue, called an explant. It can be initiated from any part of a plant - root, stem, petiole, leaf or flower - although the success of any one of these varies between species. It is essential that the surface of the explant is sterilised to remove all microbial contamination. Plant cell division is slow compared to the growth of bacteria and fungi, and even minor contaminants will easily over-grow the plant tissue culture. The explant is then incubated on a sterile nutrient medium to initiate the tissue culture. The composition of the growth medium is designed to both sustain the plant cells, encourage cell division, and control development of either an undifferentiated cell mass, or particular plant organs Manoj et al. (2010). .

The concentration of the growth regulators in the medium, namely auxin and cytokinin, seems to be the critical factor for determining whether a tissue culture is initiated, and how it subsequently develops.

The explants should initially form a callus, from which it is possible to generate multiple embryos and then shoots, forming the basis for plant regeneration and thus the technology of micropropagation. The first stage of tissue culture initiation is vital for information on what combination of media components will give a friable, fast-growing callus, or a green chlorophyllous callus, or embryo, root or shoot formation. There is at present no way to predict the exact growth medium, and growth protocol, to generate a particular type of callus. These characteristics have to be determined through a carefully designed and observed experiment for each new plant species, and frequently also for each new variety of the species which is taken into tissue culture. The basis of the experiment will be media and protocols that give the desired effect in other plant species, and experience.

Demonstration

The strategy for designing a medium to initiate tissue culture, showing how growth regulators and other factors modulate development, can be demonstrated using the African Violet, a popular house plant. Leaf sections are the source of explants. This demonstration is regularly carried out by a student class, and gives reliable results. Sterile supplies are provided from central facilities, and provision of sterile working areas (for example, in laminar flow hoods) is an advantage, although cultures can be initiated in an open laboratory with careful aseptic technique. The standard precautions used during any laboratory work involving chemicals or microbes should be adopted. If you are in any doubt about safety hazards associated with this demonstration, you should consult your local safety adviser.

Step 1 - Selection of the leaves

Leaves are cut from healthy plants, leaving a short length of petiole attached. They should be selected to each yield several explants of leaf squares with approximately 1 cm sides. The youngest and oldest leaves should be avoided.

Wash the dust off the leaves in a beaker of distilled water, holding the leaf stalk with forceps.

Step 2 - Surface sterilization and preparation of the explants

This part of the procedure should be carried out in a sterile working area, or with meticulous aseptic technique. The leaf, with the petiole still attached, should be immersed in 70% ethanol for 30 seconds, then transferred to a sterile petri dish. Sterile scissors and forceps are then used to cut squares from the leaf as explants, each with approximately 1 cm sides. The explants are transferred into a 10% hypochlorite bleach solution for 5 minutes, gently agitating once or twice during this time. They are then washed free of bleach by immersing in four successive beakers of sterile distilled water, leaving them for 2-3 minutes in each. Three explants are placed on each petri dish of growth medium (see table and below), with the upper epidermis pressed gently against the surface of the agar to make good contact. The petri dishes are sealed with plastic film to prevent moisture loss, and incubated at 25^{o}C in 16h light/8h dark.

Step 3 - Assessment of tissue culture development

The explants are incubated for 4 - 6 weeks, and inspected at weekly or fortnightly intervals. The growth of obvious bacterial or fungal colonies indicates contamination, and data from such cultures is obviously suspect. The development of dark brown tissue cultures can also be a consequence of contamination.

The media used in the demonstration are designed to show the effects of auxin, cytokinin, sucrose and mineral salts on development. The media were based on the well-known Murashige and Skoog inorganic medium, with additions as shown in this table Georgiev et al. (2009).

RESULTS

These pictures show typical results, after about 8 weeks on each medium. To summarise, multiple adventitious buds form on the **control** medium, leading to many small shoots on the upper surface where the leaf is not in contact with the medium.

Absence of **sucrose** inhibits this production. Shoot production is also limited on the low sucrose concentration, but comparable with the control at high sucrose.

At zero and low levels of **cytokinin**, callus forms where the leaf surface is in contact with the medium, while at high levels, shoot formation is stimulated.

At zero and low levels of **auxin** there is a stimulus to shoot formation, but at high concentrations, large numbers of roots are formed.

At low and zero levels of **MS** salts, there is no growth at all.

These very obvious variations demonstrate the importance of a carbon and inorganic salt source for plant growth, as well as the effect of the auxin:cytokinin ration on the control of plant development.

Fig 4. Initiating plantlets in Tissue Culture.

Table 1. Stock Preparation.

Component	Molecular weight	MS Basal media		B5 Basal media		SH Basal media	
		Mg l^{-1}	mM	Mg l^{-1}	mM	Mg l^{-1}	mM
MAJOR SALTS:							
NH_4NO_3	80.09	1650	20.6	-	-	-	-
KNO_3	101.1	1900	18.8	2500	25	2500	25
$CaCl_2.2H_2O$	147.02	440	3	150	1	200	1.4
$MgSO_4.7H_2O$	246.5	370	1.5	250	1	400	1.6
KH_2PO_4	136.09	170	1.25	-	-	-	-
$(NH_4)_2SO_4$	132.14	-	-	134	1	-	-
$NH_4H_2PO_4$	115.03	-	-	-	-	300	2.6
$NaH_2PO_4.H_2O$	119.98	-	-	150	1.1	-	-
MINOR SALTS:							
KI	166.01	0.83	0.005	0.75	0.0045	1	0.006
H_3BO_3	61.84	6.2	0.1	3	0.05	5	0.08
$MnSO_4.4H_2O$	223.09	22.3	0.1	-	-	-	-

The propagation of shoots or nodal segments is usually performed in four stages for mass production of plantlets through in vitro vegetative multiplication but organogenesis is a common method of micropropagation that involves tissue regeneration of adventitious organs or axillary buds directly or indirectly from the explants. Non-zygotic embryogenesis is a noteworthy developmental pathway that is highly comparable to that of zygotic embryos and it is an important pathway for producing somaclonal variants, developing artificial seeds, and synthesizing metabolites. Due to the single cell origin of non-zygotic embryos, they are preferred in several regeneration systems for clonal propagation, ploidy manipulation, gene transfer, and synthetic seed production. Nonetheless, tissue regeneration via organogenesis has also proved to be advantageous for studying regulatory mechanisms of plant development.

Table 2. Stock solution of growth regulation.

COMPOUND	mg/ 50 ml (1mM)
CYTOKININS;	
Benzyladenine	11.25
Isopentenyl Adenine	10.15
Kinetin	10.75
Zeatin	10.95
Dissolve cytokinin in 2.5ml of 0.5N HCl; heat gently and make to volume. Adjust pH to 5.0.	
AUXINS;	
Indole -3 acetic acid	8.25
Indole-3 butyric acid	10.16
1-Naphtaleneacetic acid	9.31
2,4- Dichlorophenoxyacetic acid	11.05
2,4,5- Trichlorophenoxyacetic acid	12.78
Picloram	12.06
Dissolve auxin in 2.5ml of 95% ethanol or 2.5ml of 1M KOH or NaOH ; Stir, heat gently; gradually add water to volume; adjust pH to about 5.0	
OTHERS;	
Thidiazuron	11
Silver nitrate	9
Dissolve in 5 ml of 95% ethanol; stir, heat gently and make to volume silver nitrate must be filter sterilized.	
Gibberillic acid	17.32
Abscisic acid	13.20

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