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PLANT PROPAGATION THROUGH TISSUE CULTURE

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INTRODUCTION

In plant propagation various methods are used, depending on the objective.

To reproduce a botanical species of plant, seedlings are generally used even if there is a certain amount of variation amongst the daughter plants. This variation, which results from the combination of different genes from the two parent plants may be quite acceptable in the propagation of certain ornamental plants where one simply wishes to reproduce the general species characteristics.

However in many cases one is interested in reproducing certain specific characteristics of a plant that are of great importance from a horticultural point of view, e.g. the colour of a rose flower, or the shape, size and texture of a specific fruit.

Where one has a group of plants which all have identical desirable characteristics that can be reproduced when propagated, the group is referred to as a cultivated variety or cultivar (Anon, 1969).

Some cultivars e.g. of annual flowers and vegetables come true-to-type when grown from seed because their chromosome pairs have fairly uniform genes (homozygous). By culling off-type plants before flowering, the desirable characteristics of the 'line' are retained when it is propagated by seed.

Many other plants, especially perennials, have very variable gene-pairs (i.e. they are heterozygous) and seedlings grown from them do not reproduce the horticulturally desirable characteristics. In such cases it is essential to propagate the cultivar by some asexual or vegetative method to retain the desirable, characteristics, in the daughter plants. This is possible with plants where, in the normal process of cell division, the exact genetic constitution (genotype) is duplicated in every daughter cell. With plants it is also possible to induce the differentiation of different organs from other parts of the plant, e.g. roots from stems, and buds on roots.

Thus there are numerous methods of vegetative propagation varying from naturally detachable structures (bulbs & corns), through many kinds of cuttings to methods of budding and grafting — all of which lead to the production of genetically uniform plants i.e. a clone.

However a danger inherent in all methods of vegetative propagation is that if the plants become infected with virus diseases the virus that is contained within the cells, can be propagated along with every daughter plant. Besides the undesirable visual symptoms

that may arise from virus infection, the growth and production of infected plants can be severely depressed. Hence the importance of the 'Superplant' scheme in the deciduous fruit industry and the Citrus Improvement Programme, where material free of specific viruses is utilized as far as is possible.

Another problem in vegetative propagation is that with some plants only a few daughter plants can be produced in a year or more. Hence it is very difficult to produce large numbers of a new clone in a short period of time to supply an anticipated demand.

PLANT TISSUE CULTURE

From a horticultural point of view the most exciting developments involve the rapid clonal propagation of plants that are possibly free of infection by a harmful virus. Both these aspects are of vital interest to nurserymen. The potential was soon exploited commercially overseas and today there are many nurseries in the USA & Europe that have their own tissue culture laboratories for the clonal propagation of specific plants on a vast, previously undreamt of, scale. Researchers have calculated that it is possible to. produce thousands of plantlets, within a single year from one original piece (explant) of a plant, by repeated sub culturing.

While it is possible to obtain plants free of a specific disease through tissue culture, it is a fallacy to believe that all tissue-cultured plants are disease-free. However one of the important aspects is that it is sometimes possible through tissue culture, to eliminate a virus from plants that were originally infected. However it is only after the daughter plants have been tested (indexed) and shown to be free of a specific virus that they can be spoken of as such.

TYPES OF TISSUE CULTURE

Callus Cultures

When plant tissue is injured the tissue that grows over the wound is referred to as callus. In 1939 three investigators, Nobecourt & Gautheret in France, and White in the U.S.A., reported independently the indefinite culture of plant callus tissue in a synthetic medium (Wimber, 1963). The calluses increased in size by continuous cell division and were sectioned and sub cultured periodically to produce additional calluses. In a classic experiment, Skoog & Miller (1957) demonstrated that the regulation of plant organ formation (órgano génesis) in tobacco callus was regulated by interactions between two plant hormones viz. auxin and cytokinin.

Callus cultures have been maintained for many years and, with the correct concentrations and ratio of growth substances, it has been possible to induce the differentiation of shoots and roots from them, thereby producing new plants.

Unfortunately callus cultures are unstable and, during the cell divisions there is a tendency for chromosome numbers to be increased in daughter cells resulting in polyploidy, with atypical daughter plants.

Seed germination

Orchid seeds, which normally fail to germinate because they contain little reserve food, were cultured on a nutrient medium, under sterile (aseptic) conditions by Knudson (1922). This technique had an important influence on orchid production but, because of heterozygosity, the seedlings were variable and growers were unable to guarantee the colour or shape of flowers produced by daughter plants (Sagawa, 1976).

Embryo culture

Immature or rudimentary embryos of other seeds have also been germinated under similar sterile conditions to orchid seeds, after excising the embryo from the seed. This technique has enabled seeds which normally require a period of months or even years to germinate (e.g. African oil palm *Elaeis guineensis),* to develop rapidly (Hartmann & Kester, 1975).

Pollen grain culture

Anthers taken from young flower buds of some plants, e.g. tobacco, and placed in an aseptic culture solution have developed into haploid plants (i.e. with only one set of chromosomes). Treatment of such plants with colchicine solution (Nitsch & Nitsch, 1969) has resulted in the production of homozygous diploid plants (i.e. with pairs of identical chromosomes).

Shoot-apex culture

In 1946 Ball was able to produce complete plants of *Lupinus* and *Tropaeolum* (Nasturtiums) through shoot apex culture and this method has been widely used since then (Murashige, 1974; Sagawa, 1976).

The technique consists in carefully dissecting out the minute growing point (apical meristem) together with the first one or two leaf primordia, at the tip of a shoot. The larger, developing leaf primordia which have enclosed and protected the growing point have often prevented any external infection from reaching the growing point. Thus the shoot apex can be carefully transferred to a sterilized nutrient medium where it may continue growth without any fungal or bacterial infection. The apical meristem plus two leaf primordia may be only about 0,1 mm in length but many workers have used a longer shoot tip about 5 to 10 mm long.

A major advance was when Morel and Martin (1952) showed that it was possible to obtain virus-free dahlias through shoot apex culture. Because the conducting tissue in a plant stem has not yet differentiated in the growing point at the tip of a shoot, there tends to be less virus at the tip than lower down the stem. Thus by taking the tiny terminal piece it may be possible to avoid virus infection that is present lower down in the plant. The smaller the explant, the less the chance of virus infection.

In fact Walkey (1968) showed that even though a virus was present in a shoot apex, it is sometimes eliminated by culturing the shoot apices.

In attempting to produce plants free of a specific pathogen e.g. virus, actively growing shoot apices should be used, and their growth may be enhanced by application of GA_3, etiolation, fertilization and irrigation etc. Heat treatment of the parent plant (i.e. growing it at a temperature of 35—40°C from a few hours to a few years) gives a better chance of having a larger separation between infected and uninfected plant tissues. However such work is usually performed at a research institute. Plants freed of these viruses have produced several hundred per cent more crop than virus-infected plants of the same clone.

It has been estimated that millions of strawberry plantlets can be produced within a year from a few mother plants by tissue culture techniques. However over 50 viral and 8 mycoplasmal diseases have been recorded on strawberry and few studies have resulted in the production of strawberry plants free of some of the viruses. Virus free plantlets derived from meristems have been cold stored successfully and more recently cryopreservation of strawberry plantlets in liquid nilrogen (-196°C) has been shown to be possible. Such a step should further improve the chances of elimination of viruses (Kartha, Leung & Pahl, 1980).

Shoot-apex grafting

A further method of producing virus-free citrus clones is to micro graft virus-free shoot apices onto pathogen-free seedling rootstock plants growing in sterile culture (Murashige, Bitters, Rang an, Nauer, Roistacher & Holliday, 1972). In this way the problem of juvenility associated with the use of nucellar seedlings is eliminated. This technique is also useful with woody plants that are not root able.

Rapid propagation

In 1960 Morel demonstrated that shoot apex culture was a potential method for the rapid propagation of orchids. From a single shoot tip it was possible to induce the development of a protocorm. If this protocorm was cut into sections before it produced a root and shoot, or if it was kept agitated in a liquid culture solution, many more protocorms were produced. When plantlets were desired, the subculturing or shaking were stopped and complete plants developed.

This shoot apex technique, referred to as 'mericloning', has been used successfully with various ornamental plant species e.g. carnation, chrysanthemum, dahlia, potato and asparagus.

With woody plant species early attempts were not successful. However in recent years success has been achieved with several species. As an example Jones, Pontikis & Hopgood (1979) estimated that it is possible to reproduce 1 - 3 000 apple rootstock plantlets in eight months from a single 5 - 8 mm shoot-apex explant. In their technique they claimed that the use of phlorglucinol induced the development of the lateral buds in the axils of the developing leaf primordia, and the cultures were consistently multiplied between four and five fold every three weeks.

A large number of reports are currently published each year on a wide range of plants

that have the potential of being propagated through plant tissue culture.

Current methods of plant propagation *in vitro* involve two basic approaches viz:-

a. the induction of adventitious shoots from various types of explants, including stem tips, followed by rooting once the new shoots have been produced,

b. the induction of axillary branching from buds in the leaf primordia axils of stem tip explants by overcoming apical dominance. Once new shoots have developed, roots can be induced to form.

In addition a third approach is somatic cell embryogenesis i.e. embryos arising from cells other than the zygote (fertilized egg). This has been done for example in mono-embryonic citrus (Rangan, Murashige & Bitters, 1968) and in grapes where adventitious embryos were induced to form in tissue cultured from the nucellus. A review on the physiology of asexual embryogenesis has recently been published by Sondahl, Caldas, Maraffa and Sharp (1980).

Other uses of tissue culture

Scientists have also used tissue culture to produce hybrids by pollinating ovules artificially *in vitro.* Geneticists are interested in the fusion of protoplasts from genetically different species in the development of new cultivars. Protoplast work pertaining to the study of extra chromosomal inheritance, manipulation of symbiotic nitrogen fixation, etc. is also promising.

There have been major developments in the achievement of secondary products in plant cell cultures and there is reason to be hopeful that commercial utilization of these possibilities is near at hand. In addition, by feeding precursors, the synthesis of desired compounds may be possible (Dougall, 1979).

Existing germplasm banks consist of seed germplasm collections, but these are usually variable and the plants are not necessarily true to the mother plant. Recent work on freeze-storage of plant tissue cultures at — 196°C has shown that entire plants can be regenerated from cell suspensions, meristem tips and pollen embryos. The establishment of germplasm banks by freeze-storage of plant tissue cultures would ensure genetic uniformity of material and should be seriously considered for the vegetatively propagated plants (Bajaj, 1979).

Attempts are being made to produce certain Pharmaceuticals *in vitro.* The possibility of cell farming to produce more food e.g. apple pulp in a smaller space *in vitro* is also being investigated. However there are problems in that the raw materials required (e.g. sugar) may be too expensive, while the full flavour of the fruit is not developed *in vitro.*

While these techniques may be of value in the future the most important aspects of plant tissue culture for nurserymen involve the rapid propagation of plants free of specific viruses or pathogens.

COMMERCIAL DEVELOPMENT

Research on plant propagation through tissue culture of many floricultural and horticultural crops has been conducted at various universities and research institutes (Murashige, 1974; Sagawa, 1976). The outcome of this research has been a boom in the development of privately owned plant tissue culture laboratories during the 1970's. Murashige (1979a) stated that there are at least 100 facilities engaged in commercially propagating a variety of plants through tissue culture. Most of these have been established in the last six years. With additional laboratories being completed each year in various countries, tissue culture propagation methods have become standard nursery operational procedures (Jones, 1979).

Lists of plants that are propagatable through tissue culture are given by Murashige (1979a).

However as plant tissue culturists are making serious attempts at commercialization, we are faced with the need to separate scientific hopes from commercial realities (Smith & Oglevee-O'Donovan, 1979). Unfortunately many people have false impressions of the ease of commercialization of plant tissue culture systems. While scientists usually qualify their results with the proper disclaimers, many people involved in commercial floriculture have the naive impression that with any system of plant tissue culture, thousands of uniformly high-quality, commercially saleable, pathogen-free plants can be obtained in a relatively short period of time, regardless of the genetic stability of the crop or its disease problems. Furthermore, it is often assumed that any commercialization of a tissue culture system, no matter how specialized in its intent, will achieve all of these goals, which unfortunately is not correct (Smith & Oglevee-O'Donovan, 1979).

Uniformity

Smith & Oglevee-O'Donovan (1979) cite the example of geraniums, in discussing the above concepts. Regarding uniformity, they point out that in any method of commercial vegetative propagation, the daughter plants vary in their growth and morphological characteristics. This variability should not be confused with mutability, which creates the easily recognized aberrant plant. Major mutations normally arise at relatively low rates under commercial vegetative propagating conditions including shoot-apex cultures, which are in reality only minute commercial cuttings. Selection, which is practised to eliminate normal variability in vegetatively propagated crops to obtain a more uniform and desirable product, is also necessary in tissue culture propagation. They illustrated marked differences in size and vigour of two geranium plants grown from shoot apices taken from the same plant and both found to be free of recognized viruses. They have found this degree of variation to be quite common in shoot apex culture derived clones from over 25 geranium cultivars compared in commercial flowering trials. Whether this variation, which is consistent in cuttings taken from these plants, is due only to selection from within the normal range seen in vegetatively propagated geraniums, or is due to the fact that tissue-cultured plant cells are characterized by instability of chromosome number and structure, is still unknown.

Fortunately this degree of variability is not encountered in all plants propagated by

tissue culture techniques, but it is important that possible variation be borne in mind.

Pathogen-free

The term pathogen-free which is sometimes used to describe plants grown in tissue culture is both illogical and of questionable legality. Pathogen-free implies that the plant material is free of all recognized and non-recognized pathogens, when, in fact, the only statement that can actually be made is that the material probably is free from only those pathogens for which it has actually been indexed or tested. Also any test is only as good as the sensitivity and reliability of the available indexing or testing systems. One must bear in mind that non-recognized pathogens may, in fact, still be present.

Murashige (1979a) listed Specific Pathogen Free (SPF) plants that have been attained *in vitro* from infected plants (Table 1). This list contains only virus diseases. Examples of plants freed from fungi, bacteria, nematodes and other pathogens by tissue culture methods are not included.

Time

The concept that tissue culture systems will produce a saleable product within a short period of time may be quite misleading, particularly when dealing with shoot apex cultures from virus-infected plants. Any commercial firm interested in producing quality plants through shoot-apex culture from virus-infected stock must be prepared to invest in approximately two to five years work before selling their first plants (Smith & Oglevee-O'Donovan, 1979).

Virus infection

As with other vegetatively propagated crops that have not been exposed to a virus indexing system, over 95 percent of commercial geranium cultures were found to be virus-infected. While some viruses that induced dramatic foliar symptoms were undoubtedly rogued out of commercial propagation, other viruses, that showed no foliar symptoms, severely affected flowering. Thus the removal of these viruses in geraniums would be commercially worth while.

Smith & Oglevee-O'Donovan (1979) state that a major decision must be made about the objectives of shoot-apex culture i.e. is it to be used as a means of eliminating certain viruses only, or is it also to be used as a method of rapid propagation as well. In view of the marked variation in shoot-apex cultured geraniums they decided that, once indexed plants had been produced, they would increase these superior plants by normal cutting production.

Virus elimination is a complex and variable process involving heat treatment of plants that have first been freed of vascular wilt pathogens, followed by shoot-apex culture and then indexing.

When young plantlets are being indexed for known viruses they must be kept in individual isolation to prevent re-infection. Those plants that index negatively must then

undergo extensive selection for plant quality, with only the highest quality plants being retained for immediate use. Thorough selection for horticultural and commercial qualities is essential.

The selected 'Elite Mother Plants' will form the source of a 'Nucleus Block' which must be maintained in an isolated greenhouse with periodic virus indexing.

Cuttings taken from the 'Nucleus Block' form an 'Increase Block' from which cuttings are taken for 'Stock Plants' that will eventually provide cuttings that are propagated and sold to the commercial grower. New 'Elite Mother Plants' must be produced each year.

Many research workers have produced shoot-apex cultured and virus indexed negative plants. However unless these plants can be multiplied commercially and be shown to be of superior quality, while preventing their reinfection by viruses, their production will have been largely an academic exercise (Smith & Oglevee-O'Donovan, 1979).

Other organisms

During the cultural processes one can easily detect most bacterial and fungal organisms, and those infected cultures are discarded without proving the pathogenicity of the contamination. Among the more persistent contaminating organisms have been *Erwinia, Pseudomonas,* and *Bacillus* spp. Unfortunately it has not always been possible to detect fungal and bacterial contamination at an early stage and apparently clean cultures may develop symptoms in subsequent subcultures e.g. a milky white haze in the agar surrounding the explant; a cloudy liquid medium, or a rapid decline in vigour of the plant in culture (Jones, 1979).

Once a culture of an ornamental tropical foliage plant has been established in culture and found to be free of known pathogens it may be advantageous to maintain mother block stock *in vitro,* although the problem of variability must be carefully watched. Many of the plants listed in the tables have had significant increases in commercial value because of a more commercially desirable form, (increased vigour, more prolific branching, more shoots terminating in flowers, earlier flowering, denser foliage), following tissue culture propagation. These improved qualities may be due to the elimination of bacterial and fungal pathogens (Jones, 1979) as well as viruses.

In many of the commercial nurseries where tissue culture is being used for mass multiplication, no attempts are being made to obtain clones free from specific pathogens that could be having a detrimental effect on plant growth, production and quality. Commercial companies must recognize the need for, and have the desire to produce better quality plant material than is presently available. Lack of knowledge, training, and research all contribute to the situation where poorer quality plants are produced because the potential of producing true-to-type plants free of specific detrimental pathogens is not developed to its full capacity.

Certain sections of the industry have developed tissue culture to its full potential e.g. carnations, chrysanthemums, strawberries, with consequent economically beneficial effects on production and quality. Local flower growers import indexed 'Elite Mother Plants', grown from shoot apices, from overseas and then multiply them by normal cutting techniques locally.

There is great scope for plant propagation via tissue culture in South Africa but further developments will be dependent on adequate research and knowledge.

TABLE 1: Specific pathogen-free (SPF) plants that have been attained *in vitro* from infected plants (Murashige 1979a)

Crop	Pathogen Excluded
Allium sativum L.	Garlic Mosaic virus
Armoracia lapathifolia Gilib.	Turnip Mosaic virus
Asparagus officinalis L. (51)	Viruses A, B and C
Brassica oleracea L. (52)	Cauliflower and Turnip Mosaic viruses
Caldium hortulanum Birdsey	Dasheen Mosaic virus
Cattleya hybrids (53)	Cymbidium Mosaic virus
Chysanthemum morifolium Ramat.	Chrysanthemum B, Vein Mottle, Green Flower viruses; Chrysanthemum Stunt viroid
Citrus sp. (54)	Exocortis viroid; Stubborn Spiroplasma; Cachexia, Concave Gun, Dweet Mottle, Infectious Variegation, Psorosis A, Psorosis B, Seedling Yellow, Tristeza, Vein Enation, and Yellow Vein viruses
Colocasia esculenta (L.) Schott.	Dasheen Mosaic virus
Cymbidium sp.	Cymbidium Mosaic virus
Dahlia sp.	Dahlia Mosaic virus
Dianthus barbatus L. and *D. caryophyllus* L.	Carnation Latent, Mottle, Ringspot, Vein Mottle, Etched Ring and Streak viruses
Fragaria grandiflora Ehrhart	Strawberry Mottle virus
Fragraria vesca L.	Strawberry Latent A, Latent C, Crinkle, Mottle, Pallidosis, Vein Banding, Vein Chlorosis, and Yellow Edge viruses
Humulus lupulus L.	Hop Mosaic, Hop Latent & Prunus Necrotic Ringspot viruses
Hyacinthus sp.	Hyacinth Mosaic virus
Ipomoea batatas (L.) Lam.	Internal cork, Rugouse and Feathery Mottle viruses
Iris Xiphium L.	Iris Mosaic virus
Lilium sp.	Lily Symptomless, Lily Mosaic, Cucumber Mosaic viruses
Lolium multiflorum Lam. (55)	Ryegrass Mosaic virus
Manihot esculenta Covaritz. (56)	Cassava Mosaic virus
Narcissus tazetta L.	Arabis Mosaic and Narcissus Degeneration viruses
Nicotiana rustica L. (57)	TMV, Cucumber Mosaic and Alfalfa Mosaic viruses
Nicotiana tabacum L. (57)	TMV
Petunia sp.	TMV
Rheum rhaponticum L.	Arabis, Cucumber & Turnip Mosaic; Cherry Leaf Roll; & Strawberry Latent viruses
Ribes sp.	Vein Banding virus
Saccharum officinarum L. (58)	Sugarcane Mosaic virus
Solanum tuberosum L.	Potato virus A, G, M, S, X, Y, and Leaf Roll; Spindle Tuber viroid
Trifolium repens L. (59)	Peanut Stunt, Alfalfa Mosaic, White Clover Mosaic and Clover Yellow Vein viruses
Xanthosoma sagittifolium (L.) Schott.	Dasheen Mosaic virus

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