PART 3

VEGETATIVE PROPAGATION BY TISSUE AND ORGAN CULTURE

VEGETATIVE PROPAGATION: TISSUE AND ORGAN CULTURE AS AN ALTERNATIVE TO ROOTING CUTTINGS

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ABSTRACT

Rooting cuttings is often difficult, and alternative methods of vegetative propagation should be considered. A number of herbaceous plants have been propagated effectively using various techniques of tissue and organ culture. For tree species the potential of tissue and organ culture is evident, but success so far has been limited. Cell-suspension culture may potentially be the best method for large-scale propagation of tree species. There are a number of problems associated with this technique, however, that may take several years to solve. **In vitro** culture of buds, although missing some of the advantages of cell-suspension cultures, may become a practical method of vegetative propagation for most tree species, much sooner and with less research effort than cell-suspension culture. It therefore deserves immediate consideration.

Tissue and organ cultures are good techniques for studying the physiology of root initiation and growth. Much of the knowledge gathered in this manner will help to improve methods of tree propagation, not only by tissue and organ culture but also by conventional rooting of cuttings.

INTRODUCTION

The aim of most tree breeding programmes is to mass-produce genetically superior populations or clones. Trees are mostly propagated sexually because vegetative propagation of trees old enough to have demonstrated their superior characteristics is often difficult, particularly if they are conifers (Cameron, 1968; Thulin and Faulds, 1968). Vegetative propagation, however, is preferred because superior characteristics are maintained better than by sexual propagation. It is opportune therefore to look for new methods of vegetative propagation to be used where conventional procedures are unsatisfactory.

Before discussing other techniques, some attention will be given to rooting cuttings,

at present the most common method of vegetative propagation, because much of the knowledge in this field is applicable to the newer methods. Furthermore, a discussion of some of the limitations inherent in the rooting of cuttings technique will suggest why, and in what instances, other methods could be more effective.

ROOTING OF CUTTINGS

The most dramatic advance in the field of vegetative propagation was made when auxins were first used to stimulate root formation in cuttings (*see* Hartmann and Kester, 1968), auxins being one of the main natural factors involved in root formation (Smith and Wareing, 1972a). However, auxins are often not the only missing factors. Others that have attracted attention are carbohydrates (Nanda and Jain, 1972), phenolic rooting cofactors (Hess, 1964; Girouard, 1969), terpenic lactones (Shibaoka *et al.,* 1967), lipid-like compounds (Heuser and Hess, 1972) and abscisic acid (Basu *et al.,* 1970; Chin and Beevers, 1969). These compounds, however, may not be universally applicable to cuttings, particularly for species with cuttings that are slow-rooting, because these organic compounds are decomposed by micro-organisms in the rooting medium. Although auxins are also subject to microbial decomposition, they have been successful in root induction because they act relatively quickly in many morphogenetic systems, including root induction (Gautheret, 1969). Bonner (1965) and Romberger (1966) consider that they are gene activators, i.e., that they act as triggers, and that their continuous presence is not required, although this point of view is somewhat controversial (Steward and Krikorian, 1971). However, many other organic rooting factors may have to be applied continuously, and at much higher concentrations and over longer periods of time than auxins to elicit a rooting response in slow or hard-to-root cuttings. As a result, it may be necessary to use aseptic techniques, i.e., the specimens will have to be surface-sterilised and placed on a sterilised rooting medium in closed containers. These requirements limit the usefulness of stem cuttings as propagation material and lead to the consideration of tissue and organ culture techniques as means of large-scale vegetative propagation.

TISSUE AND ORGAN CULTURE

Over the last 20 years or so, considerable progress has been made in applying tissue and organ culture techniques in breeding programmes of horticultural and agricultural crops (Nickell and Torrey, 1969; Bajaj and Bopp, 1971; Hildebrandt, 1971). Of course the potential of tissue culture is not limited to vegetative propagation: embryo culture has been used to produce otherwise incompatible hybrids (Bajaj and Bopp, 1971), haploid cultures have resulted in the production of homozygous diploids (Vasil, 1972), hybridisation through protoplast fusion is being considered (Cocking, 1972), and meristem culture has yielded virus-free clones (Smith and Murashige, 1970; Bajaj and Bopp, 1971; Hildebrandt, 1971).

Successful propagation of tree species using tissue culture has, so far, been limited (Winton, 1972a, b), but, if the success achieved with agricultural and horticultural crops is any indication, the potential is immense. Tree species that have been propagated from a callus tissue to a stage larger than mere seedlings are the triploid quaking aspen and tetraploid European aspen (Winton, 1971), *Populus* × *canadensis* (Berbee *et al.,* 1972), and several citrus species (Rangan *et al.,* 1969; Mitra and Chaturvedi, 1972). Rangan *et al.* (1969) make the interesting comment that, in contrast to clones established

from cuttings, those derived from a callus of nucellus origin are free of most viruses and are more juvenile. A few other tree species have yielded either a few small plantlets or either shoots or roots from callus in culture (Jacquiot, 1966; Winton, 1972a, b). In embryo cultures of *Biota orientalis* (Konar and Oberoi, 1965) and *Ilex aquifolium* (Hu and Sussex, 1971), the cotyledons produced embryoids (embryo-like structures arising in cell and tissue cultures). Embryoids also formed in callus cultures of *Santalum album* (Rao, 1965). Small organised structures, bearing some resemblance to pro-embryos, were found in callus tissues of *Pinus* (Konar, 1963; Durzan and Steward, 1970; Bonga, in prep.). Generally, however, little or no organisation occurred in callus cultures of tree species (Haissig, 1965; Konar and Guha, 1968; Winton, 1972a, b). However, further refinement of techniques undoubtedly will eventually lead to large-scale propagation using these calluses. Since it is probable that methods similar to those used successfully for some herbaceous plants will eventually be used for the large-scale vegetative propagation of commercially important forest tree species, a short description of some of the techniques used for these herbaceous plants will be presented here.

By far the most effective method of large-scale vegetative propagation has been cell-suspension culture. A small piece of callus is transferred to a liquid medium and, by gentle shaking, single cells and small clumps of cells are released into the medium. After transfer of the cell suspension to agar plates, many of the single cells and cell clumps form embryoids, which later often grow to maturity and germination. Vasil and Vasil (1972) found up to 100,000 embryoids on some agar plates. There are, however, a number of problems inherent in this technique. It has been tried with many species but only a limited number of herbaceous plants have been responsive so far. Often, even species in the same family as one propagated successfully will not form embryoids in culture (Steeves and Sussex, 1972). Obviously, a delicate balance of nutrients and other factors, differing from one species to the next, is required for embryogenesis, and for many species it may be a formidable task to determine these requirements. This is further complicated because a succession of differently composed nutrient media is often needed, each medium being designed to initiate and sustain a particular stage in embryogenesis (Steward and Mapes, 1971). A further problem is that there is often a high incidence of endopolyploidy, aneuploidy, and other genetic aberrations in cell and callus cultures (Murashige and Nakano, 1967; Sacristan, 1971), even in those derived from a single cell (Sievert and Hildebrandt, 1965). This results in genetic variation in the embryoids and plants derived from these cultures (Lutz, 1971; Murashige *et al.,* 1972). However, the chromosomal characteristics of a callus can often, to a considerable extent, be controlled by manipulation of the culture medium (Torrey, 1959, 1961).

In spite of these problems it may eventually be possible to propagate most species, including forest tree species, using cell-suspension culture techniques. For most species, however, this may take many years to accomplish and other techniques should therefore be considered. The best alternative method, which misses some of the potential advantages of cell-suspension cultures but is likely to lead to vegetative propagation sooner and more easily, is the bud-culture technique.

Comparing bud cultures with callus and cell-suspension cultures, the following advantages become apparent: (1) Buds are well protected by bud scales and are therefore

easy to surface-sterilise. After surface-sterilisation the bud scales can easily be removed aseptically if desired. (2) By starting with a bud, a total shoot is *a priori* present and does not have to be initiated in culture. (3) In trees, the buds are one of the main production sites of various root promoters (Bouillenne, 1964; Hartmann and Kester, 1968; Smith and Wareing, 1972a, b). One would therefore expect buds to be relatively easy to root. (4) The cells of the shoot apex are more uniformly diploid than those of many calluses (Murashige and Nakano, 1965; Murashige *et al.,* 1972). For that reason, Murashige *et al.* (1972) dissected meristematic domes with a few leaf primordia from buds, and implanted these on the culture medium thus avoiding genetic variation within the clones obtained. Dissection and culture of these minute meristematic areas, however, is a delicate procedure. Bud inoculations are a lot easier and shoots arising from rooted buds will probably show the same genetic uniformity as those arising from shoot meristems in culture.

Buds have not been cultured sufficiently often to really test to what extent the four points presented above have general validity. However, for species which are difficult or impossible to propagate by the well-established technique of rooting stem cuttings, priority probably should be given to experiments designed to explore the potential practicability of bud cultures for propagation purposes.

In the following paragraphs, some of the culture work performed with buds and related structures will be discussed briefly. Rooting of buds and apices and their growth into normal plants has been achieved for some herbaceous species, but not for others (Butenko, 1968; Street, 1969). Often, however, this type of work was not done with the primary intention of vegetative propagation, since this was often possible simply by rooting of cuttings. Instead, its purpose was to obtain clones free of virus and other diseases (Smith and Murashige, 1970; Hildebrandt, 1971). For woody plants, Hackett (1969) reported rooting of shoot tips of both juvenile and adult *Hedera helix.* Rooting of shoot tips of the adult form is particularly interesting, because the adult form is difficult to propagate if the conventional approach of rooting stem cuttings is used (Hess, 1964). Rooting buds of woody plants has also been reported for roses (Kofler, 1945), gooseberry (Jones and Vine, 1968), apple (Walkey, 1972), and black cottonwood (Bawa and Stettler, 1972). Shoot apical meristems and buds of several conifers have been cultured, however, without producing roots. Romberger *et al.* (1970) and Romberger and Tabor (1971) cultured shoot apical meristems and embryonic shoots (buds minus nodal diaphragm) of *Picea* on a relatively simple nutrient medium. This was not to explore the possibilities of vegetative propagation, but primarily to study the mechanisms that control shoot morphogenesis, including flowering. Al-Talib and Torrey (1959, 1961), with dormant terminal buds of *Pseudotsuga,* and Chalupa and Durzan (in press) with those of *Picea* and *Abies,* obtained shoot and needle elongation and callus formation at the shoot base. I have obtained similar results with *Picea* and *Abies* buds (unpubl. data), except that growth only occurred in buds on the verge of budbreak (this was induced by placing twigs with buds in water at about 21°C and 16 h to 24 h fluorescent light, ca. 5380 lux, for several weeks). Finally, the needle fascicles found in some conifers should be mentioned, because they could be a good source of buds. Needle fascicles root occasionally using the conventional techniques (Girouard, 1971), and their buds may be relatively easy to root in culture *in vitro.*

PHYSIOLOGY OF ROOT INDUCTION

To assess the prospects of inducing roots in callus and bud cultures, a few remarks should be made about some of the physical and chemical factors involved in root induction and growth. The literature on this subject is extensive (for reviews *see:* Torrey, 1965; Cameron, 1968; Hartmann and Kester, 1968; Street, 1969), and therefore I will limit myself to only a few aspects, mainly indicating the complexity of the rooting process. At present, the rooting theory postulated by Bouillenne (1964) is the one generally accepted. Apparently specific *o*-dihydroxyphenols (rooting cofactors) produced in the leaves and buds are translocated to the rooting region where, with auxin and polyphenoloxidases, they give rise to a root-stimulating complex (Bouillenne, 1964; Hartmann and Kester, 1968; Girouard, 1969). This root-stimulating complex, with many other endogenous and environmental determining factors, leads to the initiation of root primordia and root growth. However, to avoid excessive induction and proliferation of new roots, inhibitors of root initiation are formed in the tips of growing roots themselves (Libbert, 1957; Torrey, 1959), and in the foliar organs (Wells and Riopel, 1972).

In callus and suspension cultures, a proper balance is essential between auxin and cytokinin, and between gibberellin and cytokinin, for a proper balance between shoot and root induction and growth (Skoog and Miller, 1957; Skoog, 1971). Also certain combinations of light, temperature, minerals, gibberellic acid, auxins, and carbohydrates (Gautheret, 1966, 1969) were found to be operating at specific locations in the tissue and at specific stages of root initiation and development. Glucose appeared to be a prime factor during the initial stages, possibly leading to the appearance of an unstable rooting factor. Auxin, on the one hand, is required to help express the action of this rooting factor, but, on the other hand, also destroys it. This destruction is hindered by light (low intensity) and gibberellic acid. Minerals in the rooting medium have a very strong effect (Gautheret, 1969; Tripathi, 1971). Relatively high concentrations of calcium and nitrogen are essential; potassium, on the other hand, inhibits root formation. The stimulating effect of calcium is particularly interesting because it has been found to slow down the process of senescence in callus cultures of sunflower crown gall (Stonier, 1971). Calcium prevents leakage of auxin protectors from the tissue into the nutrient medium. At least some of the auxin protectors are *o*-dihydroxyphenol polymers, and their main function is to keep tissues in a reduced state, i.e., they act as antioxidants and thus maintain a low redox potential, a condition associated with juvenility (Stonier *et al.,* 1970). As was pointed out earlier, *o*-dihydroxyphenols are active rooting promoters, present naturally in juvenile cuttings. We may now speculate that they act by maintaining the cuttings in a juvenile condition, i.e., a condition capable of rooting.

CONCLUSIONS

Tissue and organ cultures are techniques that have already found practical application in the propagation of many herbaceous plants. Large-scale propagation of forest tree species by tissue and organ cultures is not to be expected within the next few years, but will eventually become a reality. Tissue and organ cultures have proven to be excellent methods of studying the physiology of root induction and growth. The results from further studies not only will help to improve methods of forest tree propagation by tissue and organ cultures, but also will be applicable to conventional techniques of

rooting cuttings. Undoubtedly these techniques will be practised for years to come, each benefiting from experience and knowledge gained in the other fields.

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