

TISSUE CULTURE AS A METHOD FOR VEGETATIVE PROPAGATION OF FOREST TREES

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ABSTRACT

A review of the world's literature indicates that almost all parts of a plant can be induced to form callus if given the proper stimulus. For some plants the callus can be redifferentiated to form shoots and roots. For others, it is possible to initiate embryoids. There appears to be no reason why forest trees can not be propagated by means of tissue culture.

INTRODUCTION

Research in propagation and breeding has made tree breeders more aware of their inability to propagate many common tree species vegetatively. At the same time, failure to produce a number of desirable interspecific and intergeneric crosses due to inadequate development of ovules or ovaries has raised interest in the technique of tissue culture. Potentially this technique can enable the plant breeder to produce and multiply valuable clonal stocks and hybrids for testing and for production planting.

Methods of clonal propagation in forestry, like those of agriculture and horticulture, are very traditional. Practices such as grafting, cutting, air-layering and bench propagation have not changed in centuries. However, these techniques cannot be successfully applied to all plants, and additionally several of the practices need clearer scientific understanding if they are to be successful. For example, the physiological causes responsible for differences in rooting responses of shoots taken from various regions of trees are not known. There are other factors like the time of sampling of the explants, age of the donor plant and seasonal variation in the rooting ability of woody cuttings which also make these techniques quite problematic. Further, there are many tree species and varieties whose cuttings do not root or root with extreme difficulty.

While the age-old conventional methods of plant propagation will continue to hold good for years to come, new techniques like those of tissue culture have also to be adopted if progress is to be expected.

Methods of establishing and maintaining tissue cultures have been well described by several authors, including Gautheret (1959) and White (1963). Fundamentally, the principles of sterile culture technique are followed. There is no one method of growing explants *in vitro*. The basic approach is simply to place a living sterile tissue on a medium that will induce cell division.

The main effort in vegetative propagation of forest trees should aim at growing

isolated plant parts to obtain initially a subculturable callus*. Often this callus can be made to shoot or root under appropriate chemical stimulation. It has also proved possible to induce callus to form embryoids.† In certain cases all three reactions can be induced on the same callus. The plants that are obtained by these methods are genetically similar to the parental ones and hence this technique can be an ideal method for vegetative propagation.

This communication sets out to highlight the achievements of tissue culture and to show the various ways plant organs or parts can respond to this technique and yield new plants vegetatively. The forestry scientist can utilize the method most suitable to his particular need.

HISTORICAL SURVEY

The culture of isolated plant cells was first attempted by Haberlandt (1902), when he tried to grow mature palisade cells of *Lamium purpureum* and hair cells of *Tradescantia* and *Pulmonaria* in Knop's solution with 1 to 5% sucrose. Since then considerable progress in raising tissues *in vitro* has been made, though much more remains to be done.

The first serious attempt to raise tissue in culture came from Kotte (1922), a pupil of Haberlandt, who succeeded in culturing isolated roots in an artificial medium. Simultaneously Robbins (1922) grew seeds in aseptic conditions, and transferred the excised root tips to a nutrient medium, where the roots grew abundantly. It was noticed however that with each successive transfer there was a progressively diminishing rate of growth. White (1934) obtained continuous growth of roots *in vitro* by supplying to the growing roots, besides sucrose and mineral salts, three other substances, thiamine, pyridoxine and niacine.

Since then almost all parts of the plant, i.e., root, stem, leaf, cambium, sepals, petals, stamens, carpels and even pollen and ovules have been used to produce callus in culture.

CALLUS INDUCTION AND RE-DIFFERENTIATION

(a) *Root Tissue*

Callus can be induced by chemical stimuli (including synthetic and natural auxins and cytokinins) and by this means it has been induced in tissues which do not normally form a callus on injury. On subculturing, these calluses frequently grow and enlarge. It was Gautheret (1939) who for the first time raised such a culture from *Daucus carota* roots.

Bonner (1942) reported the growing of isolated roots of *Acacia melanoxylon*. This was soon followed by Slankis (1948a, b: 1949) who was able to grow excised roots of *Pinus sylvestris*. He demonstrated that by using naphthalene-acetic acid (NAA) it was possible to obtain dichotomously-branched dwarf roots which resembled *in vivo* dwarf roots (Slankis, 1950). The effect of various sugars as carbon sources for growth of excised roots of *Robinia* was studied by Toda and Ishikawa (1951). Barnes and Naylor

* Injuring a plant organ such as a stem or root results in the parenchymatous tissue around the wound becoming meristematic and giving rise to an undifferentiated cell mass called a callus. Calluses generally show irregularity of growth and cell division.

† Embryoids are structures resembling embryos which may germinate to form a shoot and root. They may arise from any somatic cell except the zygote.

(1958) raised callus from the isolated roots of *Pinus serotina* and used the callus for metabolic studies (see also Barnes and Naylor, 1959). Further, they isolated cells from the callus to study the effect of various nitrogenous salts on their growth. Roots of *Acer rubrum* and *Eucalyptus camaldulensis* have been raised *in vitro* by Batchelard and Stowe (1963).

White (1943) reported that shoot formation could occur from isolated dandelion roots. Excised cultured roots of *Lycopersicon peruvianum* (Norton & Boll, 1954), *Isatis tinctoria* (Danckwardt-Lilliestrom, 1957) and *Convolvulus* (Torrey, 1954) also gave rise to shoots. In the case of *Isatis*, the roots failed to produce shoot buds after the third transfer. But this could be restored even after the sixteenth transfer if kinetin was incorporated in the medium. Seeliger (1956, 1960) was able to grow isolated roots of *Robinia pseudoacacia* and get bud formation on them. In *Atropa belladonna* the roots initially have a high growth rate which gradually declines; this is followed by a reduction in the capacity for the formation of shoot buds if the roots are not subcultured (Thomas and Street, 1972). It has been found that roots even after three years of subculturing may, under appropriate conditions, produce shoot buds. They further reported that cell suspension cultures of *Atropa belladonna* raised from excised segments of roots are capable of prolific root formation, and these suspensions during subculturing undergo a changing morphogenetic pattern. Konar *et al.* (1972) report a range of morphogenetic pattern in root callus suspension of *Atropa* which has not yet been reported for any other root. The root callus was grown on a liquid medium containing 2% sucrose, the inorganic salts of White (1934) along with Wood and Braun (1961), FeEDTA, White's vitamins, 100 mg/l mesoinositol, 2 mg/l NAA and 0.1 mg/l kinetin.

The cell suspension showed both single cells and colonies of cells. The colonies consisted of a central core of compactly arranged cells surrounded by loosely arranged cells. Under conditions conducive to root initiation, a continuous peripheral meristem develops. Initially this is single-layered, but becomes multilayered by periclinal divisions. This meristem becomes ridged and finally gives rise to numerous endogenous roots. These roots finally separate from the parent aggregate and float free in the medium. Alternately, the tissue in suspension forms root nodules which develop into roots. As they grow they also get detached from the parent callus.

Roots formed by either means may develop in various ways. In some cases the basal end shows an active callus which continues to proliferate and release cell aggregates. The roots continue to grow and occasionally may develop laterals. In other cases, the small basal callus does not proliferate actively but gives rise to shoot buds. These develop foliar structures which gradually turn green. These buds normally show two leaf-like structures, but sometimes there may be more, or in other instances just a single leafy structure. In most cases there is a balanced development of the root and shoot giving rise to plantlets which tend to show abnormal growth if left in suspension culture.

The same type of floating cell aggregates from which roots are initiated may also give rise to embryoids—in fact aggregates forming embryoids are often present in the same culture flask as aggregates developing either roots or plantlets. The embryoids develop from any cell of the aggregate. These have well developed suspensors.

Adventive embryos originate from the suspensor of the embryoids. The root callus

of *A. belladonna* is unusual as it shows both organogenesis and embryogenesis in the same culture medium.

(b) *Shoot Tissue*

Gautheret (1934, 1935) reported that cambial explants of *Salix caprea*, *Populus nigra* and a few other trees formed callus when transferred to a semisolid medium containing Knop's solution, dextrose and cystein hydrochloride. Gautheret (1959) lists eight angiosperms and ten gymnosperms which have been raised in culture. Jacquiot (1959) isolated cambial tissues from trunks of 25 forest tree species and obtained viable callus cultures from *Populus tremula*, *Betula verrucosa*, *Castanea vesca*, *Ulmus campestris*, *Prunus avium*, *Tilia parvifolia* and *Fraxinus excelsior* on half-strength Knop's solution supplemented with various vitamins and sugar. Wolter and Skoog (1966) studied the nutritional requirements of *Fraxinus pennsylvanica* callus. They could maintain a continuous growth of callus on a modified Reinert and White (1956) inorganic nutrient solution with 5 mg/l of iron as NaFeEDTA supplemented with myo-inositol, pyridoxine hydrochloride and kinetin along with 2,4-D. However, none of these cultures showed any organogenesis.

Ball (1950) was able to obtain a continuous culture from young adventitious shoots growing on the burls of *Sequoia sempervirens*. In the callus, marginal meristems and cambium-like meristems could be distinguished in the callus formed around groups of tracheids and mature parenchyma cells.

Reinert and White (1956) cultured the normal and tumorous tissues of *Picea glauca* on a complex medium with a view to understanding the degree of malignancy of the cells and the biochemical characteristic of the tissue (for detail see Konar and Guha, 1968). Later, White and Risser (1964) and Risser and White (1964) studied the conditions necessary for the best growth of these cells. They were able to culture the cells on a simpler medium than used earlier (Reinert and White, 1956). Harvey (1967) defined a medium for successful growth of callus from the stem of *Pinus monticola* and Brown and Lawrence (1968) were able to get callus from several pines growing in the U.S.A.

In an effort lasting more than seven years, Konar (1972) made a study of the ability to grow in culture of some of the pines growing in the Himalayan region. The species tried were *P. gerardiana*, *P. roxburghii* and *P. wallichiana*. It was found that *P. gerardiana* was the most responsive. After investigating 18 well known media with numerous supplements it was found that the best two media were White's basal (WB) and Murashige and Skoog's (MS). However, when these two media were compared with supplements, the growth of the callus on WB with coconut milk (CM), casein hydrolysate (CH) and 2,4-D was statistically significantly better than MS's medium with the same supplements.

Subculturing the callus was necessary after every four to five weeks. In study of the growth parameters of this callus it was found that glucose, mannose and sucrose can equally effectively serve as the carbon source. When the amino acids and amides were individually tried, glutamine at 10^{-3} M was found to be most effective. When this chemical was tried with other additives at this concentration, best growth of the callus was obtained with 2,4-D. Interaction of indole-acetic acid (IAA), 2,4-D and kinetin was also studied. Earlier, Konar (1963) reported the growth pattern of suspension cultures of *P. gerardiana*. The effect of cell density at two densities, viz., 50×10^{-3} and

20×10^{-3} cells/ml, on the growth of cells in suspension culture was investigated. It was also shown that the cell suspensions could be plated out and new clones obtained. Under suitable cultural conditions these could be made to root or produce a shoot-like structure. The latter has the anatomy of a shoot apex but does not grow beyond a few mm in length (Konar, 1972).

Among the angiosperms, Winton (1970) reported the production of trees from firm white callus of a triploid *Populus tremuloides*. The callus was initiated on a modified Wolter and Skoog's (1966) medium and subcultured monthly for two years. When transferred to a medium with 0.15 mg/l of 6-benzylamino-purine (BAP) numerous stunted shoots appeared on the calluses. On lowering BAP concentration, a few vigorously growing shoots appeared, and 7 rooted (2 in dark with BAP and 5 in 2150 lux light with 0.04 mg/l of 2,4-D). When transferred to soil four of these grew to trees. The author states that while this is not a good return from the thousands of inocula, it is perhaps the first case of trees produced from undifferentiated callus. Leafy shoots and roots have been obtained on pieces of subcultured tissue from European aspen and *Betula alba* (Jacquiot, 1964; 1966) and triploid *Populus tremuloides* (Mathes, 1964; Winton, 1966). Both Wolter (1968) and Mathes (1964) could get shoots after they were excised from the callus but no shoot developed beyond a few cm. Four complete plants that grew in culture died on transference to soil. Aneja and Atal (1969) report that callus from lignotubers of *Eucalyptus citriodora* produced plantlets.

(c) Shoot Apex Culture

Indefinite production of organs in higher plants during the vegetative phase is attained by continued meristematic activity of the apical meristem. This activity determines the growth patterns of trees. The cells in these tissues are perpetually young, pliable and responsive to cultural conditions. Hence, if they could be isolated and raised *in vitro*, the morphogenetic influence would be clearly evident in new organs and tissues formed. Romberger *et al.* (1970) have been able to isolate meristems by microdissection and have raised numerous cultures of *Picea abies* on a relatively simple medium. Their aim has been to learn how the growth and development of shoot apices can be controlled by manipulation of the biochemical and physical environment.

Al-Talib and Torrey (1959) tried to culture the dormant terminal buds of *Pseudotsuga taxifolia*. They found that urea (10^{-1} M) could help break dormancy to some extent. These two studies are the only reported work on shoot apex cultures of forest trees.

Shoot meristem cultures of several herbaceous plants like *Asparagus* (Loo, 1945), *Cuscuta* (Loo, 1946; Baldev, 1962), *Perilla* (Chailakhian, 1961), *Tropaeolum* (Ball, 1946) have been grown. Remarkable success has been achieved with orchids like *Cymbidium*, *Cattleya*, *Miltonia* and *Vanda* to mention a few (Morel, 1960, 1964, 1966; Marston, 1967; Lindemann *et al.*, 1970). In sympodial forms of orchids the main meristem degenerates very early; hence studies are restricted to the lateral buds. The apices when excised grow *in vitro*. In *Cymbidium*, the development starts with a swelling between the two largest leaf primordia. Diffuse growth then initiates resulting in random cell division followed by cell enlargement. The apex is consumed in this process. A little later, small areas of intense cell division can be recognized on the surface of the explant. Small spherical bulges are produced with rhizoids at the base and a few scales on the top. These are termed 'protocorms'. When they reach a diameter of 3-4 mm they can be excised from the parent tissue and cut into small slices and placed on a Knudson's

medium. On this medium the segments proliferate and give rise to 3-5 new protocorms. In *Cattleya* and related genera like *Laelia* and *Brassavola* the morphogenesis of the bud is rather different. Here the nutrient media should be supplemented with auxin and coconut milk. Initially, around the cut surface, a wound reaction is noticed. This is followed by the appearance of limited areas of small meristematic cells which grow very slowly around the vicinity of the necrotic tissue or adjacent to the conducting system. Simultaneously, the leaf base primordium undergoes intensive swelling and becomes corrugated. A little later, small bulges appear on the swollen surfaces and these finally become individual protocorms with rhizoids.

Several months later bud initiation occurs. The buds appear mostly between the protocorms or at their bases, but sometimes at their tips as in *Cymbidium*. Protocorms appear to have the property to regenerate plants almost indefinitely.

(d) *Floral and Floral Apex Culture*

Floral apices have also been used in study of morphogenesis. The early work with floral buds was carried out by La Rue (1942). Blake (1966) achieved complete flower development in two species of *Viscaria*—*V. candida* and *V. cardinalis*. Recently, Porath and Galun (1967) succeeded in culturing bisexual flower buds of *Cucumis melo* leading to the formation of fully developed pollen grains and ovaries. Tepfer *et al.* (1963, 1966) in their critical studies of *Aquilegia formosa* indicated that a culture medium containing mineral salts, vitamins, CM, IAA, gibberellic acid (GA_3) and kinetin supports the growth of buds, sepals and carpels. Konar and Konar (1966) excised the flower buds of *Pblox drummondii* when they had primordia of sepals and petals only. On WB + 10% CM + 2% sucrose, the flower buds produced a fast-growing callus which subsequently differentiated into shoots and roots and established new plants.

In a series of papers, Konar and Nataraja (1964, 1965a, b; 1969) report on the culture of floral apices of *Ranunculus sceleratus* isolated at three stages of development. The explants grow well on WB + 2% sucrose or on WB + 10% CM + 2% sucrose and give rise to profuse callus. This callus then develops shoot and root buds which finally give rise to plantlets. Embryoids also differentiated on this callus. These may germinate *in situ* or on a fresh medium. If a portion of this callus was placed in a liquid medium and shaken, it dissociated into single cells and cell aggregates. The latter formed the embryoids which finally gave rise to plantlets. The cell suspensions could be plated like a bacterial suspension and fresh clones could be raised. These then could be differentiated at will. In the case of *Ranunculus sceleratus* individual sepals, petals, stamens and carpels, and also root, stem, leaf and petiolar fragments respond to culturing and give rise to new plants.

The flower culture technique, as also the ovary culture technique, was initiated by La Rue (1942) and followed up by Jensen and Bonner (1949) and Nitsch (1949, 1951). Several workers at the University of Delhi have had success with herbaceous plants like *Tropaeolum majus*, *Linaria maroccana*, *Zephyranthes*, *Iberis amara*, *Hyocyanus niger* and *Allium cepa*.

In some cases where ovary culture was not successful the ovules have been dissected out and raised in culture. Withner (1942, 1943) using ovules of some orchids was able to shorten the interval between pollination and maturation of seeds and hasten the production of seedlings. The ovule of *Gossypium hirsutum* with only a 12-celled proembryo was brought to maturity by Joshi (1962).

(e) *Nucellus, Embryo and Endosperm Culture*

In several flowering plants, particularly varieties of *Citrus*, *Mangifera* and *Syzygium*, nucellar embryos are produced within the seed. The apomictic embryos arise by the development of a diploid cell of the nucellus bordering the embryo sac. These embryos are known to provide genetically uniform seedlings which reproduce the maternal genotype without the variation caused by segregation in sporogenesis or by recombination in fertilization.

Rangaswamy (1968) isolated nucellus tissue of *Citrus microcarpa* that had begun to develop proembryos and placed it on White's (1943) medium modified by the addition of trace elements (Nitsch, 1951) and cobalt chloride (0.025 ppm). The nucellus failed to grow but after the medium was supplemented with 400 ppm of CH (acid hydrolysed, neutralized and vitamin-free) there was profuse proliferation of nucellar cells. In about 6-8 weeks they produced abundant masses of tissues called pseudobulbils—a term coined by La Rue (1954). These were parenchymatous, irregular and pearly white. On treatment with 40 ppm of adenine and 20% coconut milk, the pseudobulbils differentiated into embryos with cotyledons, radicle and stem tips. Hence, by culturing nucelli it is possible to obtain an unlimited number of plants breeding true to their maternal parents.

The embryos can be isolated and grown on a nutrient medium. Konar and Oberoi (1965) grew the excised embryos of *Biota orientalis* on Butenko's medium. Within a week, the cotyledons unfolded and formed a short root. This was followed by slight callusing of the hypocotyl and an appreciable swelling of the cotyledons. Such cultures failed to grow further, but in about 8 weeks 5-7 small swellings appeared on the inner face of the cotyledons which finally differentiated into embryoids. These later germinated to form shoots and normal leaves.

Embryonal proliferation and its further reconstitution into accessory embryoids has also been observed in cultures of *Foeniculum vulgare* (Sehgal, 1964), *Anethum graveolens* (Johri and Sehgal, 1963), *Cuscuta reflexa* (Maheshwari and Baldev, 1962), *Dendrothoe falcata* (Johri and Bajaj, 1963), *Daucus carota* (Steward *et al.*, 1964) and *Cichorium endivia* (Vasil, *et al.*, 1964). In *Santalum album*, Rao (1965) found that in 10-15% of the seeds cultured on WB + 5 ppm of kinetin, 400 ppm of CH + 2 ppm 2,4-D, the embryo proliferated into an unlimited mass of tissue and the endosperm was completely obliterated. In about 12 weeks several accessory embryoids differentiated from this callus.

The nutritive tissue for the fertilized egg is the endosperm which is triploid in angiosperms and haploid in gymnosperms. It was La Rue (1949) who showed that this tissue also possesses potential for unlimited growth. The first convincing report on organ formation from endosperm was that of Bhojwani (1969) on *Exocarpus cupressiformis*. The seeds without seed coats were cultured on WB containing CH, IAA and kinetin. These developed shoot buds all round the endosperm. These buds had distinct shoot apices, well differentiated vascular structure and were triploid. Mature endosperm of *Scorulla pulverulenta* was also reported to develop shoot buds when exogenous cytokinin was supplied. In the case of *Dendrothoe falcata* however both cytokinin and an auxin were needed.

(f) *Haploid Gamete Culture*

In recent studies it has been shown that not only diploid and triploid tissues have

the property to proliferate and give rise to plants but also the haploid gametic tissue like pollen has the capacity to proliferate, and in some cases to give rise to haploid plants. Since these plants will be genetically homozygous they have attracted the attention of the geneticists. Haploid tissues from the pollen of gymnosperms were reported by Tulecke (1953, 1957), Konar (1963) and Tulecke and Sehgal (1963). However, none of the gymnosperm tissue is known to differentiate. Guha and Maheshwari (1964) accidentally discovered haploid embryos from the pollen of *Datura*. Since then haploid embryos have been reported from several plants which include *Nicotiana* (Bourgin and Nitsch, 1967; Nakata and Tanaka, 1968; Nitsch and Nitsch, 1967; Nitsch and Hamon, 1968; Nitsch, 1969; Sunderland and Wicks, 1969, 1971), and *Oryza* (Nizeki and Oono, 1968; Guha *et al.*, 1970; Guha-Mukherjee, 1973). The other plants in which androgenic haploids have been achieved are *Brassica-Lolium-Festuca* hybrid, *Lycopersicon esculentum*, *Atropa belladonna*, *Petunia*, *Asparagus*, *Aegilops* and *Arabidopsis*. In *Populus sieboldi* and *P. grandidentata*, Sato (1972) reports the regeneration of shoots in the dark in the presence of NAA and benzylaminopurine. Rooting of these has been obtained after transference to the light, but the ploidy of the plantlets is yet to be determined. He further reports raising anther cultures of *Alnus tinctoria* var. *obtusiloba*, *Prunus apetala*, *P. edoensis* and *P. lannesiana* but no shoot formation has yet been reported.

CONCLUSIONS

It is clear from the foregoing discussion that:

- (a) Almost all parts of the plant, given the proper stimulus, will form callus.
- (b) It is possible to redifferentiate this callus to form shoot and root. The plantlets thus obtained are similar to the parent stock and hence this is a useful method for vegetative propagation.
- (c) It is also possible to initiate embryoids on this callus. These embryoids can be made to grow into plants. Unlike seed embryos which are heterozygous, these are homozygous and therefore would be true to parent. Since hundreds of these embryoids can be made to appear on a callus this would be another method for vegetative propagation.
- (d) It is possible to dissociate the callus into single cell and cell aggregates, and obtain new plants either through the regeneration of roots/shoots or embryoids in suspension.
- (e) Several studies have shown that haploid and triploid plants can also be produced by tissue culture.

The technique of tissue culture thus offers immense possibilities for vegetative propagation of forest trees by methods enumerated above. Until now success has mostly been restricted to herbaceous plants, but there is no reason to expect that forest trees should not yield the same results. It may be possible to maintain 'Tissue Banks' of good clones of trees from which at any time when needed new plants could be raised on synthetic media. This has already been possible in the case of orchids where clonal propagation by tissue culture permits these plants to be propagated at a very rapid rate. Earlier, the entire process of orchid propagation was highly laborious, costly and permitted the grower at best to double the number of plants per year. Today, thousands of these plants could be raised within months. Several horticultural plants like *Lilium*, *Hyacinthus*, and *Chrysanthemum* have been also raised this way. It is to be hoped

that the day is not far off when the same can be done with some of our important valuable timber species.

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