PROSPECTS FOR THE INTRODUCTION OF TRAITS IN FOREST TREES BY CELL AND TISSUE CULTURE

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ABSTRACT

Cell and tissue cultures offer the prospect of adding traits and producing genetic combinations which could not be obtained by sexual crossing. This would involve mutation, transcession, transduction, transformation, and somatic cell hybridisation. **In vitro** methods can also be used in forestry for preservation of gene resources, production of homozygous specimens, prediction of phenotypic expression, production of disease-free specimens, study of host-parasite relations and study of mycorrhizae. It is concluded that the techniques of cell and tissue culture have considerable scope for altering the genetic quality of trees.

INTRODUCTION

Since the resources of the world are rapidly being depleted, renewable resources, such as trees, will become increasingly important to meet the needs of the economy and environmental quality. One way to improve the quality of forests is to vegetatively propagate superior specimens and use the propagules for planting. The use of cell and tissue culture may allow the routine vegetative propagation of tree species—even those which cannot be propagated asexually at present by other means. The second way to improve forests is to develop better trees by adding new genes. Recent advances in molecular biology suggest ways to revolutionise the classical methods of adding traits by sexual crossing. This review considers only the second method of forest improvement.

A basic requirement in this approach is that tree material can be grown *in vitro* as undifferentiated cells and that trees can be regenerated from these undifferentiated cells. The fact that this has been accomplished with a tree species (Winton, 1970), as well as with many herbaceous species, suggests that the requirement will be met soon for many tree species.

IMPROVEMENT BY INTRODUCTION OF DESIRABLE TRAITS

When a new trait is introduced by non-sexual means, it will usually occur in a very small fraction of the treated population. For example, a recent experiment required an initial population of 2×10^7 plant cells (Carlson *et al.*, 1972). If the population being treated consists of seeds or whole plants, the logistics may make the project impractical. On the other hand, if the population is a cell suspension which can be

handled in the same way as bacteria and plated out on media on which only the altered cells grow, the project becomes relatively simple*.

The simplest way of introducing new characters is by mutagenic agents (Brock, 1971). Whether the mutations are induced in whole plants, seeds, pollen, or cell cultures, propagation of the mutant specimens by tissue culture would be feasible. For example, some *Acer negundo* seedlings obtained from X-ray irradiated seeds appear to have increased frost resistance (Privalov, 1965). These could be propagated *in vitro*. One problem in induced mutation studies is the fact that most mutations are recessive, at least with respect to observable characters, and hence are difficult to detect. The use of haploid material would allow the immediate expression of induced mutations. Haploid tissue cultures of trees have been reported (Tulecke, 1957; Borchert, 1968; Bonga and Fowler, 1970; Rohr, 1972), and haploid plants have been produced from haploid tissue cultures of herbaceous species, e.g., tobacco (Ohyama and Nitsch, 1972). Thus there is an encouraging basis for further studies in this line.

There is also a wide range of existing genes which cannot be introduced by sexual crossing because of interspecific fertility barriers. One possible means to overcome this would be to mix suspension cultures from two sources to form a chimera. The problem remains that, at least in animal cultures, one of the cell lines normally takes over. Preliminary experiments indicate that this occurs in mixed cultures of pine and spruce as well (Durzan and Lopushanski, unpublished). An alternative approach for blending characters, via somatic cell hybridisation, has recently gained considerable attention. Walls of plant cells can be removed to form protoplasts (Cocking, 1972). Although protoplasts will fuse spontaneously, the frequency is very low in the absence of an inducing agent. With animal cells, fusion can be induced by active and inactivated viruses, by chemicals such as lysolecithin and glycerol mono-oleate, and by microsurgery (Diacumakos and Tatum, 1972). For plant cells, sodium salts (particularly sodium nitrate) are the only effective agents discovered to date. When protoplasts of two different types are mixed in the presence of an inducing agent, fusions occur resulting in homokaryons (hybrid cells with nuclei and cytoplasm of the same types) and heterokaryons (hybrid cells containing nuclei and cytoplasms of different types). Although it has been possible to regenerate whole plants from protoplasts of several species (Takebe *et al.*, 1971; Grambow *et al.*, 1972), it is only recently that organs have been regenerated from hybrid protoplasts. In this experiment protoplasts were isolated from two species of tobacco, mixed, and induced to fuse. Next the protoplasts were cultured in a medium which allowed only the hybrid protoplasts to divide and form colonies. These colonies were then transferred to a medium which induced shoot and leaf formation. Although it was not possible to induce root formation, the shoots, when grafted onto the stem of one of the parent species, produced flowers and seed identical with those of the hybrid produced by sexual means. The scion represented a true hybrid and not merely a chimera. This was conclusively demonstrated by morphological, chemical, and cytological analysis (Carlson *et al.*, 1972).

* 10^7 pine cells, which would weigh less than 1 g, could be obtained from a small amount of tissue from a single tree, would be genetically uniform, and could be handled in a single small culture vessel. 10^7 jack pine (**Pinus banksiana** Lamb.) seeds would weigh about 36 kg, would be very difficult to collect and work with, and would be genetically heterogeneous.

It may be possible to introduce extrachromosomal characters to a species without altering its nuclear genome. The transfer of self-replicating subcellular particles such as mitochondria and chloroplasts from one species to another might improve the processes associated with them. For example, if the chloroplasts of a pioneer species such as jack pine were transferred to a species such as white spruce, which requires shade as a seedling, the latter might become more of a pioneer species. Conversely chloroplasts of white spruce transferred to jack pine might allow the pine to become more tolerant of shade.

Recent progress in the isolation of all types of sub-cellular particles in good condition has permitted the first steps to be taken in this direction. Protoplasts from mutant albino tobacco plants took up wild type chloroplasts from several different tobacco species. Whole green plants with functional chloroplasts were regenerated from these albino protoplasts (Carlson, 1973). An extreme example of the potential of this approach is a report of the entry of spinach and African violet chloroplasts into mouse liver cells *in vitro*. Division of the mouse cells was not affected by the presence of the chloroplasts, and the chloroplasts retained their structural integrity and photosynthetic capacity (Nass, 1969).*

Genes may also be introduced by transcession, transduction, and transformation (Hess, 1972). In transcession, plants incubated with bacterial suspensions acquire bacterial genes. That such genes are replicated and expressed is indicated by the production of bacterial enzymes by the treated plants. By means of transduction it has been possible to specifically introduce a single gene into cultured animal cells. This was done by producing a stock of the virus carrying the gene, and exposing the cells to the virus (Merril *et al., 1971).* Transduction may also be possible in higher plants. After *Hordeum vulgare* protoplasts were infected with bacteriophage T3, two enzymes, which are encoded for by the T3 genome, were produced by the protoplasts. This provides strong circumstantial evidence that bacteriophage genes can be transcribed and translated in plant cells (Carlson, 1973). Since cell walls tend to act as filters preventing the entry of some viruses into plant cells (Cocking, 1970), protoplasts will probably be the preferred recipients in transduction studies. In transformation, cells, seeds, or seedlings are exposed to deoxyribonucleic acid (DNA) isolated from another source. In this way genes for flower colour have been transferred from one strain of petunia to a colourless mutant. The character was inherited by subsequent generations (Hess, 1972, 1973). Although these experiments involved total extractable DNA, the isolation and synthesis of individual genes (Shapiro *et al.,* 1969; Khorana *et al.,* 1972) indicate the possibility of introducing genes more specifically. Protoplasts may be useful here as transformation recipients (Ohyama *et al.,* 1972).

A very dramatic goal of tree improvement by introduction of traits would be the introduction of nitrogen-fixing genes into the forest ecosystem. Since all known cases of nitrogen fixation involve prokaryotic organisms (Streicher *et al.,* 1972), the ability to form a symbiosis between *Rhizobium* and plant cells *in vitro* provides a good system with which to attack the problem (Holsten *et al.,* 1971). While it may be difficult to

* There are still problems to be solved since the chloroplasts in the mouse cells did not replicate, nor in fact did chloroplasts from genera other than **Nicotiana** replicate in tobacco protoplasts.

introduce nitrogen-fixing genes into trees (either the genes themselves or the ability to form nodules with *Rhizobium),* it may be possible to introduce the genes into mycorrhizal fungi. A different approach would be to increase the nitrogen-fixing bacteria in the rhizosphere. There is evidence that cereals and grasses establish a close relationship with free-living aerobic and anaerobic nitrogen-fixing micro-organisms in the soil and in the mucilaginous covering of the roots themselves (Phillips *et al.,* 1971). Perhaps such micro-organisms could be genetically altered in such a way that they would grow better in certain forest soils and form a close relationship with tree roots.

As mentioned previously, a major problem in the introduction of new characters is the occurrence of very few altered individuals in a large treated population. Carlson *et al.* (1972) were able to isolate 33 altered cells from a treated population of 2×10^7 because they had a medium on which only the altered cells grew. Attempts should be made to develop techniques for increasing the proportion of altered cells. For example, micromanipulation of individual protoplasts might allow directed fusion of protoplasts from different sources—and subsequent isolation of the hybrid. It may not be possible to increase the proportion of altered cells so techniques for isolating them should be developed. In many cases it will not be possible to select for the character directly. For example, if nitrogen-fixing genes were introduced into a cell population one could probably not isolate the nitrogen-fixation competent cells by plating the whole treated population on a nitrogen-free medium. The reason is that in most cases a morphological structure must be formed in order to maintain the anaerobic conditions required by the nitrogenase enzyme (Phillips *et al.,* 1971). Thus it would be necessary to find another character, associated with the presence of the nitrogen-fixing genes, which could be used for the selection. The cells might be more resistant to an inhibitor or not require a certain substance in the culture medium which the parent cells did. This approach has been used successfully to isolate mutants resistant to tobacco wildfire disease. Haploid tobacco protoplasts were exposed to a mutagen and then plated on a medium containing methionine sulfoximine. This latter compound causes lesions identical to those caused by the true bacterial toxin on tobacco leaves. Calluses which formed from resistant protoplasts on this medium were diploidised and regenerated into plants which proved to have increased resistance to the disease (Carlson, 1973). The selection of altered cells need not necessarily be based on nutrition. For example, algal photosynthetic mutants have been isolated by plating out the cells and identifying mutants visually by their increased fluorescence. Since most genes seem to be pleiotropic* (Wright, 1968) there will probably always be a basis for selection—the problem is to find it. This means that much more research in biochemistry and molecular biology will be required in order to determine the metabolic basis and inter-relationships of traits to be selected for.

OTHER APPLICATIONS OF TISSUE CULTURE IN FORESTRY

There are a number of additional values of tissue culture to forestry, which will be mentioned briefly.

Concern exists about the future existence of endangered species and more recently about conservation of gene resources. Genomes of trees could be preserved by freezing callus cultures, using the method of Latta (1971). In the distant future these could be revived and regenerated into trees suitable for further genetic crossing.

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* Having multiple phenotypic expressions.

Another aid to the tree breeder would be the production of homozygous specimens from haploid cultures. The value of this approach has been summarised by Sunderland (1970) and Melchers (1972).

A limitation of the diallel cross, used in tree breeding, is that each progeny can only be tested under one soil and environmental condition. If each progeny were replicated by tissue culture methods, much more information could be obtained from a single cross.

Another problem in tree breeding is that it may take many years for the full phenotypic expression of the progeny of a cross to become apparent. Tissue culture may be able to help as there is a report that the growth rate of callus could be used to predict the later growth rate of a tree (Mathes and Einspahr, 1965). Because of the pleiotropic nature of traits, one also should be able to assay for phenotypic characters which would not appear in callus (e.g., fibre length).

Tissue culture has been used to rid some agriculturally important plants of virus diseases (Hildebrandt, 1971). It is conceivable that this could be done in forestry as well (e.g., to produce elm free of phloem necrosis).

Tissue culture allows host-parasite relationships to be studied under simple and controlled conditions (e.g., Harvey *et al.,* 1971; Harvey and Grasham, 1971).

Isolated mycorrhizae have been cultured *in vitro* (Fortin, 1966). This should provide a good system with which to study this vital but poorly understood symbiosis.

CONCLUSION

Results with herbaceous plants and a few studies with forest trees indicate very great potential for obtaining new and improved forest tree species by introducing new traits by cell and tissue culture methods. This approach is, however, still novel to forestry.

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