TISSUE CULTURE PROPAGATION OF CONIFERS: CURRENT AND FUTURE

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Abstract -- Tissue culture methods of propagation are already a commercial success for a variety of horticultural crops. These methods are also central to the recent speculative interest in "genetic engineering" applied to plants. On this basis we may be pardoned for being very optimistic about what tissue culture and associated methodologies will do for the forest industry. The question is, when? Technology follows steps in its advancement to commercial use. First, the process is demonstrated in the laboratory, then generalized and broadened to more than one species, then made dependable and more efficient as the product quality is evaluated. Cost effectiveness can then be determined to dictate commercial use. Propagation of small conifer clones (5-20 members) from seedling cotyledons is well advanced along this path to the point where propagule quality is being evaluated. Recent success with recycling propagules, to make more propagules from each, promises to significantly increase the clone members, but the clones must originate from young, unproven seedlings rather than mature elite trees. The methods for elite trees include use of buds (terminal, axillary, fascicle) and plantlet production from stem callus cultures (organogenesis) as well as artificial embryo production from such callus cultures (somatic embryogenesis). Current capability is described for each approach, which is now at or near the initial laboratory demonstration step. We expect substantial application of knowledge from the seedling system to speed development of the other systems. The possible applications of the emerging methods and knowledge to problems other than mass propagation, i.e., selection of disease resistance, protoplast fusion and genetic engineering, are discussed relative to present capabilities.

Additional Keywords: Mass propagation, early selection, genetic engineering

Substantial advances have been made in yield and quality of the forest crop by breeding programs and refined management strategies. Mass vegetative propagation by tissue culture will give us a new dimension, and it is on the door step. The urgent and practical questions concerning the real value of vegetative propagation and its proper deployment for the forest crop are being addressed. We can look to the horticultural industry for a model of tissue culture use in vegetative propagation, but we should also be aware of the emerging methodology of recombinant DNA and gene manipulation in cultured animal and plant cells.

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The technology of test tube genetics offers a more efficient approach to breeding programs, and more efficient methods to map the location and interaction of genes on the chromosomes. This is in addition to the eye-catching accomplishments of engineering bacteria to produce insulin or interferon which have recently made headlines. The potential value of this technology for agricultural crops such as tomato or wheat has sparked an influx of speculative money and new companies almost without precedent. This manifest appraisal of worth for agricultural crops that already have vegetative propagation and rapid breeding programs should not go unnoticed by those of us who seek to improve the conifer crop with its delayed flowering and slow growth to maturity for progeny selection. This paper will attempt to sketch the flow of the emerging technology, then assess the relative position of conifer research in that flow of possibilities.

EMERGING GENERAL TECHNOLOGY

The horticultural industry has recognized that more uniform plants emerge from tissue culture propagation if the cells are not kept too long in culture, and if new shoots are produced directly from existing shoots or leaves rather than from an intervening disorganized callus step (Murashige, 1974). The process is more efficient and less labor-intensive if callus or cell suspensions can be multiplied,then induced to form shoots or embryos. However, variability in the plants so produced can be a problem for many species. Since horticultural crops have high value, the less efficient and more reliable route which avoids callus has received the attention.

Vegetative propagation by tissue culture is preferred commercially for many ferns, foliage plants, woody ornamentals and flower crops. The tissue culture micropropagation methods differ from crop to crop, but they are based on identified general principles (Murashige, 1974). The cultured stocks can be kept for long periods in refrigerators. At the proper season, propagules can be generated in large numbers from these stocks and placed directly in the greenhouse for crop production. The energy and labor-consuming greenhouse space that otherwise would have been used through the year to maintain traditional propagation stock plants is thus released for continuous crop production. With adequate precautions, the tissue culture propagules enter the greenhouse free of viruses and other diseases. Large stocks can be built up quickly from a few select plants, and the uniformity among plants within a tissue culture clone insures that all will be ready for harvest at one time. Greenhouses may thus be emptied on schedule for the next crop. Generalized estimates of required laboratory facilities and costs for commercial clone production have been made (Anderson et al., 1977, Hartman, 1979; Barnes, 1979). Minimal equipment and initial supply costs for a modest production laboratory capable of producing 10,000 plants per week appear to be in the range of $25,000 and the plants produced may cost S0.15 to $0.25 each. Of course these figures depend on expected volume and do not factor in the cost of the research and development of the methodology to be used. Forest clone production, with its stable and continuous market for vast numbers of propagules that must compete with the low cost of seedlings, places strong emphasis on efficiency and economy. This use then departs from the commercial forces that have shaped horticultural clone production. We likely must face the modest but nagging variability problems of callus culture that horticulture could ignore by choosing a different route.

We are not alone, however. Recombinant DNA technology will require callus or cell suspension cultures as the vehicle for new gene insertion into plants. After insertion the callus must regenerate plants which faithfully display their genetic constitution. Others, working along these lines on many different crops, will also focus on understanding and eliminating the small but irritating degree of variability in plants coming from callus cultures. This much effort, on many species, focussed on one problem must surely succeed. The existing variability has even been turned to constructive ends as a source of variant-improved varieties in vegetatively propagated crops such as potato (Shepard et al., 1980). These variants likely represent changes in regulation of existing genes and a new door to useful genetic alteration may have been opened. Time will tell.

The recombinant DNA technology and its logical extensions will serve the forest industry in even more direct ways. Large amounts of particular plant genes can now be manufactured in bacteria. Bacteria contain extra pieces of DNA (plasmids), perhaps viral in origin. Gene sequences from plants can be attached to these pieces and, after they are multiplied within bacteria, one can harvest the bacteria and retrieve many identical copies of the desired plant gene sequence. Now with enough copies, the researcher can set about the task of exposing plant cells to these copies in such a way that the gene sequence becomes incorporated into the permanent genetic composition of the plant cells. If plants are then made from the cells, the cloned plants have the desired gene and thus the new, desirable trait. If the gene imparts disease resistance, the plant now has become a disease resistant variety. Many obstacles exist on the way to commercial application for trees, but the thrust is clear, and the obstacles are being overcome in other crops.

Armed with many copies of a particular gene sequence it is also possible to locate the natural position of that gene on the plant chromosomes. Genetic maps of the chromosomes can be constructed from such test tube manipulation without waiting 6 years for cone and seed production on the tree. Such maps of gene location and linkage with other genes are of great help to the breeder. In addition, when the cell wall is removed from cultured plant cells, the resulting naked protoplasts can be made to fuse. Protoplasts from two plants may thus fuse to produce a hybrid containing the DNA from both parent cells. In principle, we have then accomplished what normally occurs when pollen fertilizes the egg in the pine 'cone -- but again without the need for pollen or egg. The hybrid plants generated from the cells by way of callus may themselves be useful improvements. Alternatively, as cells divide in callus, most of the chromosomes of one or the other parent may be lost, leaving the chromosomes of one parent with but one chromosome exchanged for its counterpart from the other parent. If desired genes reside in that chromosome, we have made an improvement. Also, since we have only one foreign chromosome, any new traits gained can be attributed to genes on that particular chromosome. Genetic maps can be made by this device as well. Such mapping was conducted effectively for all human chromosomes using hybrid cells between man and mouse. Recent non-technical reviews of this work with human cell cultures (Ruddle and Kucherlapati, 1974) and with gene location on chromosomes (Chambon, 1981) give an appreciation of the possibilities which might be applied to conifers.

Further elaboration of this emerging technology is probably premature, and it is certainly beyond the scope of this paper. It is enough that we recognize that different and powerful capabilities are being developed which will aid tree breeders. Callus and suspension cultures are central to the new technology. Mass vegetative propagation from callus is close at hand. The next and necessary step is to couple this with the advancing technology of genetic manipulation. In this way, rapid breeding programs can generate the new trees that will be worth cloning. I think it appropriate to mention here that it is the business of the forest industry to keep alert to the rules regarding gene-splicing and recombinant DNA now being considered by federal governments. These rules govern whether genetically altered plants and other organisms can be deployed in the field. The forest industry and tree breeders have a stake in the deliberations which will yield the rules.

CONIFER TECHNOLOGY

The technology now exists for the clonal propagation of most conifers from embryonic or seedling materials. Some ten pine species, two spruces, Sequoia, Douglas-fir, Western red cedar, Western hemlock, Cupressus species and others have all shown this potential (Mott, 1981). A specific and detailed step-wise procedure has been developed for loblolly pine (Mott and Amerson, 1981). Seedling cotyledons are used and the method has been tested across many seed families. It extends to other pines including white pine (Mott and Amerson, in press). Clones of 10 or so rooted plants can be expected on the average, but exceptional seedlings can yield much greater numbers approaching 100. Exceptional clones can yield high numbers in radiata pine as well (Aitken et al., 1981), and schemes to recycle and prolong shoot generating cultures show promise to increase numbers with even the less exceptional clones of these species. These sets of propagation methods, applicable to conifers in general, all add up to about what is generally used in the horticultural industry. Clones are produced directly from excised plant parts without intervening callus. The difference is that with conifers, only young seedling materials may be used, not mature trees, and the shoot generation can not yet be prolonged indefinitely. Therefore, the clone numbers are small. Some schemes have been developed (Boulay and Franclet, 1977) which avoid even adventitious buds and relay on axillary bud break to produce new shoots which again produce axillary buds, etc. These schemes of course can be carried on indefinitely to produce large clone numbers which retain juvenility, but the method is labor intensive, and the clones are much too expensive for reforestation.

Small clones of some species have been planted in soil for observation, eg., loblolly pine, radiata pine, Sequoia, Douglas-fir, and Western hemlock. But with the exception of loblolly pine (Kelly, 1978; Leach, 1978, 1979) little public information on performance of the clones in soil is available. It is, therefore, not possible to make confident statements about clonal fidelity in the field for tissue-culture-produced conifer plants. Our research programs at North Carolina State University address this need to carry on through from the lab bench discovery of methods, to production-oriented improvement and ultimately to greenhouse and field testing of the product trees. This program is well underway with clones in the greenhouse being made ready for field plantings. Cooperative support for this work comes from the University and from an increasing number of companies, currently twelve, within the forest industry.

The current technology is thus sufficient for small clone production, and it is being used to research the fidelity and worth of such propagules in the field. Further improvement is likely, such that one can envision shoot production and growth from excised cotyledons using exchangeable liquid medium to reduce handling costs and rooting of the shoots directly in soil as plants grow to planting size under greenhouse conditions. The costs per plant will be reduced accordingly, but the drawback will still be that seedling material must be used when clones from elite mature trees would be far more desirable. Even so, the methodology developed for seedling material will apply to clone production from mature-tree parts when that comes along.

Laboratory demonstrations of clone production from the terminal buds (Arnold and Eriksson, 1979) or from fascicles (Mehra-Palta et al., 1978) of older mature trees have been reported. Adequate surface sterilization of buds and fascicles from the field presents a major problem as does selection at the proper season. In the absence of prolonged shoot generation in culture, the above problems limit clone sizes available by this route, but the clones are from mature trees. Major improvements will be necessary before this avenue is reliably available. Considering the obstacles it is more likely that economical clone production from mature trees will eventually come via intermediate callus obtained from stems or buds. Some shoot production (organogenesis) has been reported from a callus-like culture of Pinus wallichiana initiated from embryos (Konar and Singh, 1980). An imprecise method for early stages of embryogenesis from suspension cultures initiated from Douglas-fir cotyledons was reported as a U. S. Patent No. 4, 217, 730, August 19, 1980. Although both started with embryo or seedling materials they deal with occurrences that have relevance for clone production from mature trees. It will not be long before the discoveries are made which make economical clone production from mature trees a reality. When this occurs, much of the methodology will already have been mastered for handling the shoots or embryos to ensure growth to plantlets ready for the field. We expect the clonalfidelity studies in the field to show faithful clonal traits and consequently we anticipate that mass propagation will faithfully capture for our use the non-additive genetic variance in present tree populations.

Once plantlet regeneration from callus is reliably achieved, whether by organogenesis or embryogenesis, the way is open for genetic manipulations to aid breeding programs. Genetically altered callus can then be made to regenerate genetically altered plants. New and better trees will be generated by current, traditional breeding programs and subsequent mass vegetative propagation will yield second generation capture of non-additive tree improvement. Selection in culture for desirable tree traits will be an important part of mass propagation of these trees, and an even more critical part of in vitro genetic DNA manipulation. If a gene sequence or a chromosome is inserted into a cell, one must be able to recognize its presence in culture by observing the associated trait now acquired by the cell. In this way, the correctly altered cells may be selected for use in subsequent plantlet regeneration. Work must be started now to identify traits which can be recognized at the cellular or callus level in culture. A catalog of these genetic marker traits must be assembled to support the genetic manipulation work to come, if conifers are to take a rightful place in the wave of advancing technology. Foresters and tree breeders should become educated to the nature of markers useful for this purpose, for it is the people who work with the trees

that represent the library from which the catalog must be constructed. Things like albino seedlings, or trees with characteristic terpenes and lignins come to mind.

We have developed procedures for axenic culture of the fusiform rust fungus (Amerson and Mott, 1978) and the blister rust fungus, which are being used to study conifer host resistance to these diseases in culture. This may provide marker traits which can be seen at the cellular level, but it also brings the power of cloned host plus cloned pathogen to bear on the study of the control of disease resistance. It is evident that the emerging tissue culture technology can have practical impact beyond mass propagation. There is potential for impact even with symbiotic organisms. Nitrogen-fixing bacteria were inserted <u>into</u> a conifer mycorrhizal fungus, and the fungus gained the capacity to fix nitrogen (Giles and Whitehead, 1977). Successful mycorrhizal association with pine in the soil and nitrogen fixation beneficial to the pine have yet to be reported, but the example is served.

CONCLUSIONS

Reliable tissue culture propagation for conifers now exists to produce clones of ten to one hundred members from juvenile materials. These clones are being used to develop better methods and to evaluate fidelity in the field. Field performance is promising at this early stage. The economics of reforestation foster a thrust toward plantlet regeneration from an intermediate callus culture. This thrust also fits in with a developing genetic engineering technology which can greatly aid tree breeding programs. Mass clonal propagation from elite mature trees is close at hand and will be aided by preliminary work with clones of juvenile material. As the achievement of mass propagation draws near, the potential of test-tube genetics and test-tube selection for desirable traits stands out and calls for some preliminary action. Conifer tissue culture thus seems poised and ready to move with the leading edge of an emerging technology seemingly without bounds.

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