Future Options for the Genetic Improvement of Conifers

Part II: Longer-Term Technologies

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Summary

Developments in the areas of micropropagation, "rejuvenation", cryogenic storage, in vitro selection, somaclonal variation, molecular genetics and genetic engineering offer potential new tools for forest tree improvement. Because of the technologies involved, these techniques are not widely employed in tree improvement programmes at the present. This paper summarises the current state-of-the-art of these techniques and provides some suggestions as to how they might be employed in tree improvement programmes in the future.

Introduction

Part I of this paper (Thompson and Pfeifer, 1992) reviewed the current methods used in the genetic improvement of forest tree species, discussed several near-term technologies and suggested how they may be used in tree breeding in the near future (next 5 years). The purpose of this paper is to look further into the future, to review longer-term technologies and attempt to predict how and when they may affect tree breeding in the next 10 years and beyond.

Micropropagation

Micropropagation is a technique that straddles the border between Near-Term and Long-Term technologies. Some applications of micropropagation are already being successfully used in tree improvement programmes, however, their full application to tree improvement will require more time for improvement in the efficiency of the systems and proof of quality and uniformity of the plants produced.

Micropropagation employs the same basic principles as macropropagation (rooted cuttings) except for the fact that the size of the original plant part employed is much smaller (tissues, cells or ultimately a single cell). In micropropagation the small size of the original plant part (explant)

requires precise control of both its chemical and physical environment. Cells and tissues are fed with nutrients, sugars and growth regulators under controlled conditions of light and temperature. Plant growth is controlled by the applied plant growth regulators to stimulate shoot or root formation. By regulating the type, amount and sequence of the plant growth regulators, the type of plant growth can be controlled. These methods date back to the late 1930's, but only since the 1970's has there been interest in growing plant cells and tissue in culture for the commercial production of plants. Currently some 212 million plants are produced by micropropagation in western Europe (Pierik, 1990).

Under the general category of micropropagation there are two basic methods which can be used to multiply plants in culture or as it is also known, "in vitro". The first is by a process known as "organogenesis" in which plant organs (shoots and roots) are induced to form. This is basically a multi-step process consisting of (1) culture establishment, (2) bud induction, (3) shoot elongation, (4) root induction, (5) root elongation and (6) transfer to soil. Each step requires a different growth regulator treatment and all manipulations are done by hand in a sterile environment. This results in micropropagated plants which are more expensive to produce than by conventional seed or cutting propagation methods.

The main advantage of micropropagation is in the number of plants it can produce. Ideally with seeds or cuttings, each seed or cutting will produce only one plant. In micropropagation each culture produces several shoots, which can be divided and induced to multiply and produce more shoots. Typically woody plant multiplication rates in culture vary between 3 to 10 shoots per original shoot, depending on the species (oak=3, cherry=10). Another advantage is that the cycle can be repeated indefinitely. Thus, at least in theory, the number of plants produced from one original explant would be unlimited.

Organogenesis is usually most easily accomplished with juvenile tissues. Embryos are removed from ungerminated seed or very young (2 to 4 week-old) seedlings and used to initiate cultures. Unfortunately material of this age has not yet demonstrated it's genetic potential. Only by using seed resulting from controlled crosses of two parents that are known to produce high quality offspring can improved performance be assumed.

The major disadvantage of micropropagation is that it is a very labour intensive process. Labour typically accounts for 60 to 75% of the production cost of micropropagated plants. Of the total 212 million plants multiplied in tissue culture mentioned above, only about 1.3 million are forest trees, mainly broadleaf species (oak, cherry and birch) for horticultural purposes. Only very high value material or species that cannot be propagated by conventional means can afford to be propagated in this way. Commercial propagation by organogenesis is difficult to justify economically because of the low cost of conventional forest tree seedlings. There have been

attempts to automate the micropropagation process including the use of robotic manipulators to reduce labour costs, but this work is only in its early stages. It is both quite complex and very capital intensive.

In most cases the high production costs of plantlets produced by organogenesis means that they will not be used directly in the production of forestry planting stock on a large scale. One exception to this is the Monterey pine (*Pinus radiata*) organogenesis system employed by Tasman Forestry Ltd. in New Zealand (Gleed, 1991). Alternatively. micropropagated trees could be used to produce stock plants which are grown to provide cuttings for rooting in traditional ways (John and Mason, 1987). This would reduce the amount of time required to produce a large number of genetically improved plants for trials and also spread higher propagation costs over a larger number of plants.

Nevertheless, reasonable numbers of plantlets of a number of forest tree species have been produced since the mid 1970's. Currently about 1.25 million micropropagated birch plantlets are undergoing field trials in Finland (Jokinen *et al.*, 1990). Among the conifers there are probably more than 10,000 loblolly pine and 5,000 Douglas- fir plantlets in the U.S., several thousand maritime pine and coastal redwood plantlets in France and about 10,000 Monterey pine in New Zealand, all produced by organogenesis. In general, the field performance trials of this material have shown that after

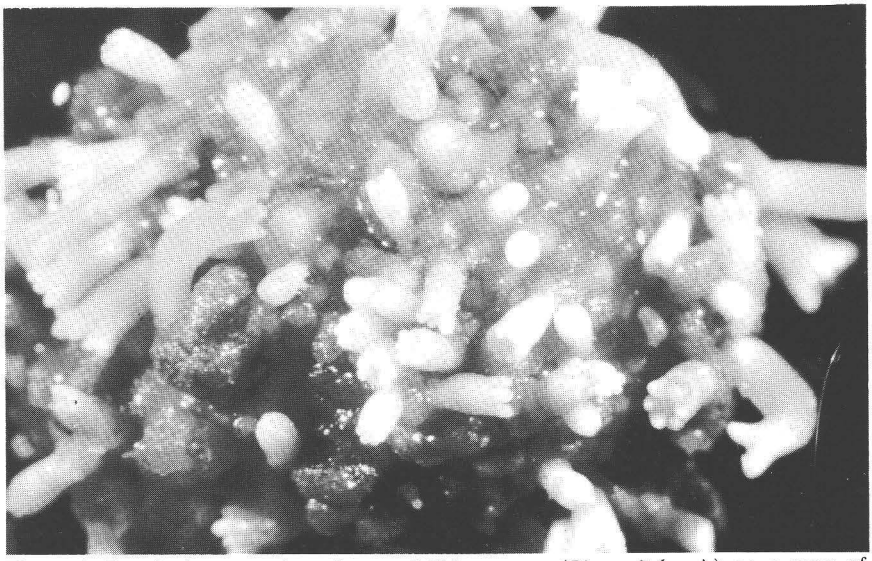

Figure 1. Developing somatic embryos of Sitka spruce (*Picea sitchensis*) on a mass of embryogenic callus initiated from a zygotic embryo.

a longer establishment time than seedlings, the micropropagated conifers grow at an annual growth rate comparable to the seedling controls (Ritchie and Long, 1986; Amerson *et al.*, 1987).

In addition to organogenesis, a second type of micropropagation is known as "somatic embryogenesis". In this process ordinary cells of the plant (somatic cells) are made to mimic the cells in a seed to produce structures that look and act like embryos. Although this process has been possible in some plant species since the late 1950's, it has only been possible in the conifers since the mid–1980's. In this process, zygotic embryos are removed from seeds and placed in culture. Under the influence of certain combinations of nutrients and growth regulators, special cells are formed which mimic zygotic embryo formation in seeds. Somatic embryos have shoots and roots which are formed in one step, rather than the several steps required in organogenesis. Because the process is simplified, production costs for somatic embryos should be less than for plants produced by organogenesis. Somatic embryos can also be produced in liquid media in bioreactors similar to those used to grow large amounts of bacteria. In Norway spruce it has been estimated that one liter of embryogenic cells would contain between 50,000 and 100,000 somatic embryos. Taken together the scale-up possibilities and the opportunity to reduce labour costs make this process very attractive. The process of somatic embryogenesis in conifers has been reviewed recently by Tautorus *et al.*, 1991 and its potential commercial application to forestry by Gupta *et al.*, 1993.

Currently there are small field trials of conifer somatic embryos of Norway, interior and Sitka spruce, Douglas-fir, loblolly pine and Monterey pine at various stages of testing in Europe, North America and New Zealand. None of these trials is more than a few years old and results to date show normal growth, but several years of field growth and detailed genetic analysis will be necessary to confirm that there are no problems with the propagation method. The largest field trials are with Norway spruce in the United States where some 3,000 somatic embryo plantlets are undergoing tests (Gupta *et al.*, 1993). At present this techniques is successful with embryos from seed and needle tissue form seedlings up to 12 months old (Ruaud *et al.*, 1992). Attempts to extend this ability to tissues from mature trees of proven superior traits are in progress.

"Rejuvenation"

Because both rooted cuttings and micropropagation (organogenesis and somatic embryogenesis) are most successful in very juvenile tissues (excised embryos or young seedlings), attempts have been made to try to "rejuvenate" tissues of mature, proven superior trees by returning them to their responsive, juvenile condition. In nature mature trees "rejuvenate" themselves when they flower and produce seed which is juvenile. It may

be that if mature tissues can be induced to form somatic embryos in culture they will have undergone "rejuvenation".

There are several claims of "rejuvenation" of mature tissues by growing them for extended periods of time in culture. These claims, however, are based on the morphological appearance of the plants. The fact that a plant "looks juvenile" is not enough to claim true rejuvenation. Aging or maturation is a change in a number of characteristics which include the ability to form roots, leaf or needle morphology, bark characteristics, growth rate, occurrence of plagiotropic growth, flowering ability and several other characteristics. These characteristics may change at different rates as the plant ages almost as if they were regulated by different internal clocks. For example, changes in leaf morphology usually occur long before the onset of flowering in many woody species. Claims of "rejuvenation" are usually based on a change in only one of these characteristics. In order for rejuvenation to be practical, all characteristics should be returned to the juvenile state. If complete rejuvenation is possible, then material from selected, tested superior individuals can be rapidly multiplied by vegetative propagation (rooted cuttings or micropropagation).

Cryogenic Storage

If true "rejuvenation" is not possible, either in the short or long term, then a system that would allow for storage of material in a state where it will not age or mature would provide an alternative solution to the problem. One way to accomplish this is by cryogenic storage of tissues at liquid nitrogen (−196° C) temperatures. In this way both organogenic tissues (Toivonen and Kartha, 1989) and embryogenic tissues (Kartha *et al.*, 1988) have been stored for extended periods of time. Using this method a tissue sample would be divided with one half used to regenerate plants by organogenesis or embryogenesis and the other half stored in liquid nitrogen. After the necessary period of field testing (6 to 15 years), the best individuals would be identified and tissues could be retrieved from cryogenic storage and multiplied to produce "field tested" superior material.

In Vitro Selection

In screening trials, large numbers of seedlings are exposed to a selective agent and the resulting "tolerant" or "resistant" individuals are isolated (De Souza *et al.*, 1990). An alternative method is to grow large numbers of cells in culture and expose them to a selective agent. Examples include selection for resistance to pathogens and stress. Families of larch have been screened in culture for resistance to the fungal pathogen *Mycophaerella* which correlated well with field performance results (Ostry *et al.*, 1991). Loblolly pine families have been screened in culture for the ability to survive drought which also correlated well with field performance of the

same families (Newton and van Buijtenen, 1986). In vitro screening could provide a first screening of genotypes which might help reduce the number of individuals in field trials. It is important to remember, however, that in vitro screening cannot replace field testing.

Somaclonal Variation

Plant cells when grown in an unorganized condition in culture for long periods of time may develop changes in gene expression for a variety of reasons. This type of variation is known as "Somaclonal" variation (*somatic* or body cell). These changes may result, in some cases, in useful new genotypes. An example of this is *Populus* resistance to a leaf spot disease caused by the fungi *Septoria* which was generated in culture (Ettinger *et al.*, 1985). Another example is the selection of cherry cells that are tolerant to salt and drought stress and the regeneration of tolerant plants (Ochatt and Power, 1989). Somaclonal variation may prove to be very useful in species where genetic variation is limited, such as many agricultural species. Its usefulness in genetic improvement of forest trees remains to be demonstrated, where it can be argued that there is still a large amount of under-utilized genetic variation in the "wild population". Thus the utility of somaclonal variation in tree improvement programmes will require additional time to demonstrate its potential.

Basic Genetic Knowledge

Our basic understanding and knowledge of the genetics of all forest tree species is very primitive compared to the situation in agricultural and some horticultural crops. We know how many chromosomes are present in the normal diploid level in all major species, but beyond that we know very little. The reason for this is that we are working with essentially a wild population much like all agricultural crops were several thousand years ago. Forest trees have not undergone the extensive inbreeding work to produce homozygous lines that has occurred in most agricultural crops.

One of the reasons why interest in biotechnology and molecular biology is so high in forestry is because, rather than being 50 to 100 years behind agriculture, forestry is perhaps only 10 or 20 years behind in biotechnology and the chance of keeping up is much better than with conventional breeding.

Molecular Genetics

Genetic selection probably first began when primitive man collected and planted seeds from plants that had characteristics he could use. The concept that information on chromosomes in the nucleus of the cell determines how a plant will look or act only helped to explain what man had already been doing for thousands of years. The discovery in the 1950's that DNA was the

key chemical mechanism for encoding this information has led to the age of biochemical or molecular genetics. In spite of these discoveries, advances in forest genetics and tree improvement have continued to progress at a slow pace. This is mainly because of the long time required to breed trees and the resulting lack of knowledge of the basic inheritance patterns of major traits in forest trees. In contrast, one of the plant species that is being used today in plant molecular biology experiments is *Arabidopsis thaliana* which can be grown from seed to flowering plant producing seed in 28 days.

Much work has been done in forestry using plant metabolites as indirect indicators of their genetic background. Terpene analysis allows the identification of population origins of certain species (Forrest, 1990).

The study of genetics at the molecular level offers an opportunity to study the mechanisms of inheritance at the finest level of detail. One aspect of this work attempts to develop libraries of genetic information. Because there is so much information in the DNA of living organisms it is necessary to cut it using enzymes into more manageable segments for study. To develop a library covering 99% of the human genome would require 800,000 DNA segments. In order to cover the genetic information of a black spruce (*Picea mariana*) at the same level, it would require 3.2 million DNA segments (Cheliak and Rogers, 1990). The conifer genome is one of the largest, probably as a result of its ancient ancestry.

The DNA in cells is "translated" into proteins which have either structural or regulatory functions. These regulatory proteins are known as enzymes. Comparing differences in the same enzymes between individual plants (known as isozyme or isoenzyme analysis) has been used as a tool in forest genetics for many years. Its major use is in identifying cultivars (Tobolski and Kemery, 1992) as well as mislabelled seed lots. It has also been used in checking the purity of seed orchard seed by detecting foreign genotypes that could only be accounted for by pollen from outside the orchard, as well as determining evolutionary relationships between species of forest trees (Szmidt and Wang, 1993).

Rather than attempting to look at the entire genome of the conifers it is also possible to look only at genes that are active during certain stages of development in the life of the tree. For example, it is possible to look at the genes or gene products (messenger RNA) which are active only when the tree is forming flowers or seed, forming roots, breaking buds, etc.

This information can also be used to compare differences in the DNA fragments and see how they differ between individuals. These fragments differ in size and can be separated by gel electrophoresis and will produce a pattern unique to that individual. Any change, such as a mutation, will result in a different pattern. This type of analysis is known as Restriction Fragment Length Polymorphisms or RFLP. Neale and Williams (1993) have recently published a review of RFLP technology and how it could be used in tree improvement.

RFLP techniques can be used to identify the location of specific genes within the genome. They cannot recognize the gene itself but can tell where it is located within the total genetic information of the individual. This information can produce a "genetic map" of a species by comparing differences in RFLP patterns between individuals with known different genetic traits. In human genetics similar maps provide the ability to estimate the relative risk of producing an offspring that would have a possibility of certain genetically controlled disorders.

In most plants, major traits are controlled not by one gene but by several different genes in different locations known as Quantitative Trait Loci (QTL). In tomato it has been found that 6 specific QTL control fruit size, 4 other QTL control the solid content of the fruit and 5 QTL regulate the acidity of the fruit. These are important characteristics in breeding tomato fruit with desired characteristics. Now that these QTL are known, breeding programmes can more effectively work to develop new tomato varieties with special characteristics. At present no QTL are known in any woody species, but within the next few years this could become an important tool in tree breeding.

RFLP technology can also provide a way to identify families, or clones by looking at their unique genetic "fingerprint". In this way it will be possible to check "superior" material is indeed what it is purported. Certain artificial sequences can be inserted into a genotype to act as "signature" or "logo" should there be a dispute over ownership or origin of genetic material. An example of this is the identification of *Cornus* genotypes using DNA patterns or "fingerprints" (Culpepper *et al.*, 1991) and to distinguish between species where morphological characteristics may not be reliable (Bobola *et al.*, 1992).

Genetic Engineering

The goal of all breeding is to identify beneficial genes and bring as many of them together in one individual as possible. Selective breeding is one way to accomplish this, but it is slow, especially with forest trees. Since the 1970's techniques have been developed that speed up this process. They involve the identification, localisation and isolation of genes for specific traits using some of the techniques previously described. Recent reviews of transformation and genetic engineering in woody species have been published by Manders *et al.* (1992) and Schuerman and Dandekar (1993).

Once a gene for a desired trait is known and has been isolated, it is necessary to insert it into the target plant. There are several methods to do this, one of which involves a naturally occurring soil bacterium called *Agrobacterium tumefaciens* which causes the crown gall disease on plants. This organism has developed a method to insert its genes into a plant host.

The desired gene is inserted into a special region of the bacterial genome which is inserted into the host plant genome when it is inoculated with *Agrobacterium*. Unfortunately some conifer species appear to be quite resistant to *Agrobacterium* infection, so other methods may be necessary.

Another method uses plants cells in culture which have had their cell walls removed by an enzyme. These plant cells, called protoplasts, are surrounded only by their cell membrane, and are either injected directly with the foreign DNA or, they are placed in an electric current which encourages the uptake of the foreign DNA (electroporation). The limiting factor with protoplasts is the ability to produce protoplasts which can be treated and then regenerate complete plants from the protoplasts. Currently this is not possible with all species of plants and the conifers are among the most difficult to regenerate from protoplasts.

Perhaps the most dramatic method for gene insertion is the "biolistic" or "gene gun". This method uses microscopic gold beads which are coated with the foreign DNA which are fired into the cells. The beads penetrate the cell and place the gene directly into the cell close to the nucleus. This method avoids problems of regeneration from protoplasts because almost any type of tissue can be treated including intact zygotic embryos. Thus, the method of inserting foreign DNA into cells is no longer the limiting factor in genetic engineering.

Figure 2. Transient GUS (ß-glucuronidase which produces a blue pigment in transformed cells) expression in white spruce (*Picea glauca*) zygotic embryos following gene insertion using particle accelation ("gene gun") methods.
(Photograph courtesy of Dr. David Ellis, Department of Horticulture, University of Wisconsin, Madison, Wisconsin, USA).

Genetic engineering, like so many things, is not as simple as it first appeared. At one time just getting the foreign DNA into the cell was believed to be the major stumbling block. Once this was accomplished getting the foreign DNA to insert itself into the host DNA became the problem. Once it was inserted, questions as to how to regulate the expression of the gene arose. Most processes in biological systems are "turned on" and "off" at the appropriate times during development and are not "on" at all times. For example, hybrid poplar trees that contained a foreign gene coding for proteinase inhibitors to confer resistance to *Septoria* leaf spot had significantly less total basal diameter growth as compared to the control plants after two years in the field (McNabb *et al.*, 1991). Just how to regulate expression of these foreign genes may prove to be a *very* formidable task.

Exactly which genes to insert into the engineered plant pose another set of problems. The obvious traits to incorporate into genetically engineered trees such as growth rate, wood production, disease resistance etc. are usually traits that are regulated by several genes. At present, methods for the insertion of foreign genes are limited to one gene at a time. Thus, in addition to the complexity of isolating the series of genes involved in a certain process, there is the technical problem of inserting them one at a time.

In the conifers, very few single gene traits are known. Among these are a gene for resistance to white pine blister rust, synthesis of specific monoterpenes and the narrow crown form of Norway spruce. Unfortunately, although there is evidence that each of these traits is controlled by a single gene, none of these genes has been identified or isolated. Most of the genes that have been inserted into woody species are marker genes used to demonstrate the successful insertion and expression of the gene such as antibiotic resistance genes ((Charest *et al.*, 1991), a gene that produces a blue pigment in cells (Duchesne *et al.*, 1991; Goldfarb *et al.*, 1991; and Stomp *et al.*, 1991) and the gene for firefly luciferase (Campbell *et al.*, 1992).

Genes for insertion into conifers are not be limited to genes isolated from conifers. A bacterial gene for resistance to the herbicide glyphosate resulted in tolerance to this herbicide when inserted into a hybrid poplar clone (Riemenschneider *et al.*, 1987). Protoplasts of conifer embryogenic cells have been inserted with foreign bacterial genes that serve as markers of transformation. To date, there has been only limited or "transient" expression of these genes in conifers (Tautorus *et al.*, 1991). Current genetic engineering research projects include insertion of genes for insect resistance (Strauss *et al.*, 1991), drought tolerance (Newton *et al.*, 1991) and wood formation (Whetten and Sederoff, 1991) as well as studies of genes resulting in maturation (aging) (Hutchinson and Greenwood, 1991) and photosynthesis (Gustafsson *et al.*, 1991).

Figure 3. Glyphosate resistance clearly demonstrated. Poplar plants with the resistant gene in the background. Normal stock in the foreground.

Conclusions

Due to limited space the techniques discussed in Part I and II of this paper were arbitrarily divided along the line of when a technique would likely be ready for use in tree improvement programmes. An arbitrary dividing line of within or beyond 5 years was selected. Some of the techniques that have fallen into this long-term group are already being used. Micropropagation is a good example of this. Micropropagated Monterey pine are already in the field in New Zealand and somatic embryos of interior spruce are in field trials in Canada. Most of the other techniques are only being used at the research level at this time, but predicting how long they will take to reach general use is difficult.

Cold storage or cryopreservation (storage at liquid nitrogen temperatures of −196° C) of organogenic or embryogenic cultures may permit storage of valuable genotypes in a stable, propagatable state while they are being field tested. In addition, if "rejuvenation" methods can be proven to be effective this will permit the propagation of mature tested superior individuals, initially by rooted cuttings, and perhaps later, by micropropagation.

In vitro selection techniques offer a more effective and faster way for the selection of genotypes with certain desired characteristics. The selection techniques need to be correlated with field trials to prove their effectiveness. The use of somaclonally derived variations will require more diligent evaluation. The fact that the variation originated during the culture period means that other undetected variation may also exist. This will require extensive field testing before it can be incorporated into propagation or breeding programmes.

Isozyme, and in the future, RFLP techniques will undoubtedly add to our basic understanding of conifer genetics. Isozyme pattern studies are presently used to determine the purity of seed being produced from seed orchards. They have also allowed studies of the inheritance patterns of Douglas-fir which led to the discovery that cytoplasmically inherited traits were passed through pollen, rather than the egg, as would be expected (Neale *et al.*, 1986). This rather unexpected observation is typical of the type of unexpected and potentially important information provided by molecular genetics. Unfortunately many of the advances made in agricultural crops such as the identification of Quantitive Trait Loci for important traits await information on inheritance patterns in conifers, which is presently lacking.

Plant genetic engineering will undoubtedly be among the last of the techniques discussed in this paper to become tools in tree improvement programmes. This is because of two main facts. The first is technical problems with the expression of foreign genes in conifers. Only transient expression has so far been observed. Whether these will in fact be temporary problems or long term problems remains to be seen. Conifers are very sensitive to changes in the amount of DNA and a doubling of the DNA levels (polyploidy), or reducing it by half (haploidy), results in severe reductions in

growth rate, unlike the response of angiosperms. Admittedly this is a rather large change of DNA levels in these cells. In Douglas-fir the addition of one additional chromosome results in a change in morphology, growth rate and reduction of flowering. The effect of inserting a single gene has yet to be demonstrated.

The second problem with genetic engineering will be the long time required for field testing of the engineered plant. Unlike agricultural crops where the life cycle of the plant requires only a few months, the time required to determine that there are no serious problems or side effects of the engineering process will take probably 15 to 20 years. Thus, a genetically engineered trait must be very beneficial to be useful after all the time required for testing. The ecological implications of biotechnology in forest ecosystems have recently been reviewed by Duchesne (1993).

The first paper in this series ended with a quotation from Faulkner (1981) saying that forest genetics could be likened to " . . . the use of the broadsword to crudely reduce the breeding base to a manageable size, then to use a scalpel to refine existing and develop new techniques . . ." for continued improvement. Certainly, when we think about the longer-term potential uses of biotechnology in forestry, we are approaching the level of the scalpel, if not the laser to refine our technique.

REFERENCES

Amerson, H. V., L. J. Frampton, R. L. Mott and P. C. Spaine. 1987. Tissue culture of conifers using loblolly pine as a model. IN: Hanover, J. W. and D. E. Keithley (eds.) Genetic Manipulation of Woody Plants, Plenum Press, New York (pp 117–137).

Bobola, M.S., R.T. Eckert and A.S. Klein. 1992. Restriction fragment variation in the nuclear ribosomal DNA repeat length within and between *Picea rubens* and *Picea mariana*. Can. J. For. Res. 22:255–63.

Campbell, M.A., C.S. Kinlaw and D.B. Neale. 1992. Expression of luciferase and ß-glucuronidase in *Pinus radiata* suspension cells using electroporation and particle bombardment. Can. J. For. Res. 22:2014–8.

Charest, P.J., Y. Devantier, C. Ward, C. Jones, U. Schaffer and K. Klimaszewska. 1991. Transient expression of foreign chimeric genes in the gymnosperm hybrid larch following electroporation. Can. J. Bot. 69:1731–6.

Cheliak, W.M. and D.L. Rogers. 1990. Integrating biotechnology into tree improvement programs. Can. J. For. Res. 20:452–63.

Culpepper, J.H., L.A. Sayavedra-Soto, B.J. Bassam and P.M. Gresshoff. 1991. Characterization of *Cornus* (Dogwood) genotypes using DNA fingerprinting. J. Amer. Soc. Hort. Sci. 116(6):1103–7.

De Souza, S.M., T.L. White, R.A. Schmidt, C.H. Young and R.L. Anderson. 1990. Evaluating fusiform rust resistance symptoms on greenhouse-grown slash pine seedlings to predict field resistance. Plant Dis. 74:969–74.

Duchesne, L.C. 1993. Impact of biotechnology on forest ecosystems. For. Chron. 69:307–13.

Duchesne, L.C. and P.J. Charest. 1991. Transient expression of the ß-glucuronidase gene in embryonic callus of *Picea mariana* following microprojection. Plant Cell Rep. 10:191–4.

Ettinger, T.L., P.E. Read, W.P. Hackett, M.E. Ostry and D.D. Skilling. 1985. Development of resistance in *Populus* to *Septoria musiva* utilizing somaclonal variation. IN: Caron, F., A.G. Corriveau and T.J.B. Boyle (eds.) Proc. of the 20th Meeting of the Canadian Tree Improvement Association, Quebec City, Quebec, Canada, August 19–22, 1985 (pp 83–90).

Faulkner, R. 1981. Tree improvement research and development- Some thoughts for the 1980's. IN: Pollard, D.F.W., D.G.W. Edwards and C.W. Yeatman (eds.) Proc. of the 18th Meeting of the Canadian Tree Improvement Association, Duncan, British Columbia, Canada, August 17–20, 1981 (pp 1–18).

Forrest, G.I. 1990. Distinguishing Sitka spruce seed origins. Research Information Note 176, Forestry Commission Research Division, 3 p.

Gleed, J.A. 1991. Towards clonal afforestation. IN: Miller, J.T. (ed.) Proceedings FRI/NZFP Forests Ltd. Clonal Forestry Workshop, FRI, Rotorua, New Zealand (p. 61).

Goldfarb, B., S.H. Strauss, G.T. Howe and J.B. Zaerr. 1991. Transient gene expression of microprojectile- introduced DNA in Douglas-fir cotyledons. Plant Cell Rep. 10:517–21.

Gupta, P.K., G. Pullman, R. Timmis, M. Kreitinger, W.C. Carlson, J. Grob and E. Welty. 1993. Forestry in the 21st century. BioTechnology 11:454–9.

Gustaffson, P., S. Jansson, J. Lidholm and A.-K. Lundberg. 1991. Structure and regulation of photosynthesis genes in *Pinus sylvestris* (Scots pine) and *Pinus contorta* (lodgepole pine). For. Ecol and Manag. 43(3–4):287–300.

Hutchinson,K.W. and M.S. Greenwood. 1991. Molecular approaches to gene expression during conifer development and maturation. For. Ecol and Manag. 43(3–4):273–86.

John A. and B. Mason. 1987. Vegetative propagation of Sitka spruce. Proc. Royal Soc. Edinburgh 93B:197–203.

Jokinen, K.J., J. Honkanen, P. Seppanen and T. Tormala. 1990. Biotechnology of the silver birch (*Betula pendula* Roth.). Agro-Industry Hi-Tech (pp 23–6).

Kartha, K.K., L.C. Fowke, N.I. Leung, K.L. Caswell and I. Hakman. 1988. Induction of somatic embryos and plantlets from cyropreserved cell cultures of white spruce (*Picea glauca*). J. Pl. Physiol. 132:529–39.

Manders, G., M.R. Davey and J.B. Power. 1992. New genes for old trees. J. Exp. Bot. 43(254):1181–90.

McNabb, H.S. Jr., N.B. Klopfenstein, R.D. Hanna, R.B. Hall, E.R. Hart, S.A. Heuchelin and R.W. Thornburg. 1991. A field trial of transgeneic hybrid poplar trees: Establishment and growth through the second season. IN: MacKenzie, D.R. and S.C. Henry (eds.), Biological Monitoring of Genetically Engineered Plants and Microbes: Proc. of the Int. Symp. on the Biosafety Results of Field Test of Genetically Modified Plants and Microorganisms, November 27- 30, 1990, Kiawah Island, South Carolina, U.S.D.A./C.S.R.S., Clemson, South Carolina (pp 155–59).

Neale, D.B. and C.G. Williams. 1991. Restriction fragment length polymorphism mapping in conifers and applications to forest genetics and tree improvement. Can. J. For. Res. 21:545–54.

Neale, D.B., N.C. Wheeler and R.W. Allard. 1986. Parental inheritance of chloroplast DNA in Douglas-fir. Can. J. For. Res. 16:1152–4.

Newton, R.J., H.S. Yibrah, N. Dong, D.H. Clapham and S. von Arnold. 1992. Expression of an abscisic acid responsive promoter in *Picea abies* (L.) Karst. following bombardment from an electric discharge particle accelerator. Plant Cell Rep. 11:188–91.

Newton, R.J., E.A. Funkhouser, F. Fong and C.G. Tauer. 1991. Molecular and physiological genetics of drought tolerance in forest species. For. Ecol. and Manag. 43(3- 4):225–50.

Newton, R.J. and van Buijtenen, J.P. 1986. Evaluation of stress resistance of loblolly pine with seedlings in controlled environment chambers and tissue culture. Tappi Res. and Devel. Conf, Appleton, Wisc. (pp 39–42).

Ochatt, S.J. and J.B. Power. 1989. Selection for salt and drought tolerance in protoplast-and explant-derived tissue cultures of Colt cherry (*Prunus avium* X *pseudocerasus*). Tree Physiol. 5:259–66.

Ostry, M.E., P.M. Pijut and D.D. Skilling. 1991. Screening Larch in vitro for resistance to *Mycosphaerella laricina*. Plant Dis. 75:1222–4.

Pierik, R.L.M. 1990. Commercial micropropagation in Western Europe and Isreal. IN: Deberg, P.C. and R.H. Zimmerman (eds.) Micropropagation, Technology and Application, Kluwer Academic Publishers, Dordrecht, the Netherlands (pp.155–65).

Ruaud, J-N., J. Bercetche and M. Paques. 1992. First evidence of somatic embryogenesis from needles of 1-year-old *Picea abies* plants. Plant Cell Rep. 11:563–6.

Riemenschnieder, D.E., B.E. Haissig and E.T. Bingham. 1987. Integrating biotechnology into woody plant breeding programs. IN: Hanover, J.W. and D.E. Keathley (eds.) Genetic Manipulation of Woody Plants, Plenum Press, N.Y. (pp. 433–49).

Ritchie G.A. and A.J. Long. 1986. Field performance of micropropagated Douglas fir. New Zealand J. of For. Sci. 16(3):343–56.

Schuerman, P.L. and A.M. Dandekar. 1993. Transformation of temperate woody crops: Progress and potentials. Sci. Hortic. 55:101–24.

Strauss, S.H., G.T. Howe and B. Goldfarb. 1991. Prospects for genetic engineering of insect resistance in forest trees. For. Ecol and Manag. 43(3–4):181–210.

Stomp, A.-M., A. Weissinger and R.R. Sederoff. 1991. Transient expression from microprojectile-mediated DNA transfer in *Pinus taeda*. Plant Cell Rep. 10:187–90.

Szmidt, A.E. and X.-R. Wang. 1993. Molecular systematics and genetic differentiation of *Pinus sylvestris* (L.) and *P. densiflora* (Sieb. et Zucc.). Theor. Appl. Genet. 86:159–63.

Tautorus, T.E., L.C. Fowke and D.I. Dunstan. 1991. Somatic embryogenesis in conifers. Can. J. Bot. 69:1873–99.

Thompson. D.G. and A.R. Pfeifer. 1992. Future options for the genetic improvement of conifers. Part I Near-term technologies. Irish Forestry 49(1–2):27-39.

Tobolski, J.J. and R.D. Kemery. 1992. Identification of red maple cultivars by isozyme analysis. HortSci. 27(2):169–71.

Toivonen, P.M.A. and K.K. Kartha. 1989. Cryopreservation of cotyledons of nongerminated white spruce (*Picea glauca* (Moench) Voss) embryos and subsequent plant regeneration. J. Pl. Physiol. 134:766–8.

Whetten, R. and R. Sederoff. 1991. Genetic engineering of wood. For. Ecol. and Manag. 43(3–4):301–16.