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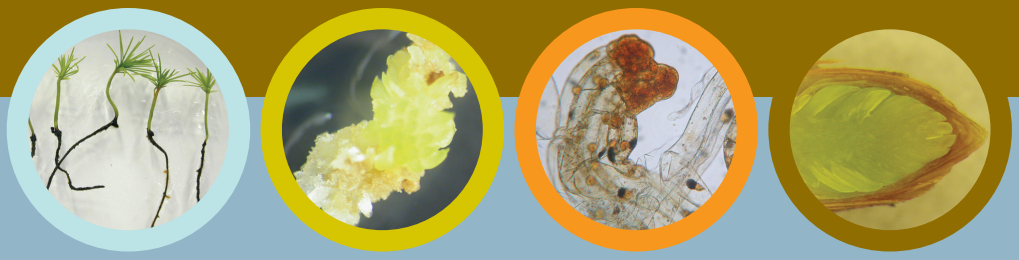
Tissue culture research at the CFS:

Its history, current status and potential benefits



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Tissue culture research at the CFS:
Its history, current status and potential benefits

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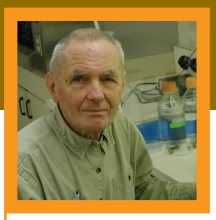
Yill Sung Park



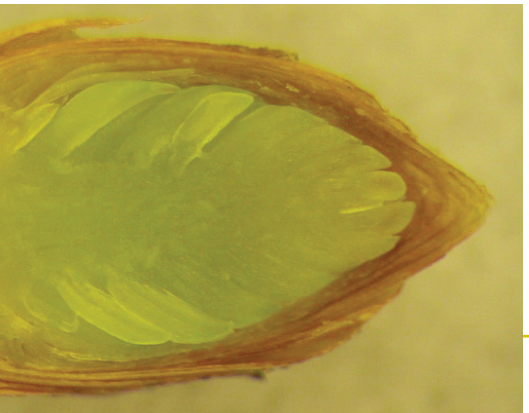
Jan M. Bonga

PREAMBLE

Pinus strobus somatic embryogenesis-derived trees, Valcartier, QC.

Modern forest management relies on extensive breeding and reforestation programs to support the sustainability of forest productivity and conservation of natural forests. Plantation forestry, with its increased forest productivity and improved wood quality, is likely to become an important source of wood products in the future. **Vegetative propagation** of superior coniferous forest trees through **biotechnology (tissue culture)** has the potential to deliver a stable supply of superior seedlings for forest plantations.

The objective of this report is to present an overview of the **tissue culture research** that has been carried out in Canadian Forest Service (CFS) centres over several decades and to outline the important impact this research has had worldwide. In addition, it is our intent to indicate in which direction this research, in our opinion, ought to go to further serve the industrial and scientific communities. This is not a complete review of all past research activities at the CFS. Only a general outline of major achievements will be presented and only a small part of the published literature will be discussed. Genetic engineering, which is generally carried out with tissues obtained in tissue culture, is largely outside the scope of this report and will not be discussed in detail.

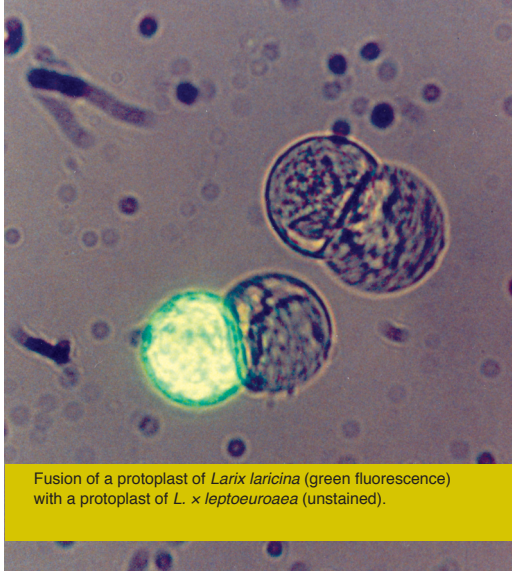


WHAT IS TISSUE CULTURE?

Tissue culture, or as it is also called *in vitro* culture or micropropagation, is a biotechnology by which small pieces of tissue are removed from the plant and cultured on a tissue culture nutrient medium. By manipulating the culture medium, in particular its levels of plant growth hormones and regulators, the plant cells transferred onto the nutrient medium will start to divide and produce cell masses that will eventually produce new plants, often in large numbers. However, producing new plants (clonal propagation) is not the only objective of tissue culture; there are many other applications as well. For example, this technology is used to introduce useful foreign genes into plants through genetic engineering or to screen and select cells or plantlets for resistance to biotic or abiotic stresses. These, and the many other useful applications of tissue culture, will be discussed in the following document, with an emphasis on the work that has been carried out in that field at the CFS.

History

Even though several attempts were made in the 19th and early 20th centuries to culture plant cells on a nutrient medium, it was not until the 1940s that proper cultures were established. The main purpose at that time was not clonal propagation but simply a demonstration that culture media could be concocted that would keep cells alive and dividing. In those days it was mostly just unorganized callus tissue that was being produced. It was deemed that a viable tissue culture had been established if the tissue continued to grow and could regularly be subdivided and transferred onto fresh nutrient medium over half a dozen times. The consensus was that after a number of subdivisions, any parental factors that would stimulate growth had been diluted to the point where they would no longer stimulate cell division, and all further growth was supported by the nutrients provided in the culture medium.



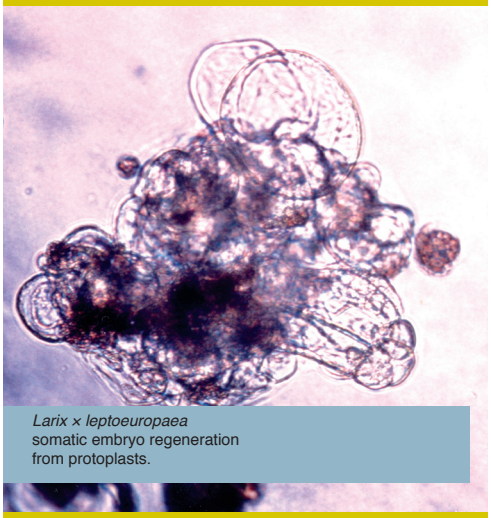
Fusion of a protoplast of *Larix laricina* (green fluorescence) with a protoplast of *L. × leptoeuroaea* (unstained).

In those days, it was discovered that some species were easy to culture *in vitro* while others remained recalcitrant. In that latter group were cereal crops, conifers and some hardwoods. For conifers, continuous cultures of a species were first established by White and Risser (1964) and Risser and White (1964) with white spruce (*Picea glauca*). Many of the plant growth regulators that are now used routinely were still unknown at the time. For example, for cytokinins, one depended on the natural and as yet unidentified ones supplied by coconut milk, while the growth hormones abscisic acid and gibberellic acid were still unknown. A major breakthrough occurred when Skoog and Miller (1957) found that auxin primarily stimulates rooting while cytokinin stimulates shoot formation. By having these compounds in proper proportion in the culture medium, both roots and shoots would grow, and new plants could thus be produced.

Another major event occurred when Murashige and Skoog (1962) published their MS culture medium. This medium proved to be suitable for the culture of a large number of species and is still being used for many of them. However, because there are no universal rules applicable to tissue culture, it should come as no surprise that for many species, including several conifers, different nutrient media had to be developed. Several of these new media originated in CFS centres.

Other significant events occurred subsequently. For conifers, in 1985, three research groups, one in Sweden, one in Czechoslovakia and one at the CFS's Atlantic Forestry Centre (AFC) in Fredericton, New Brunswick (Nagmani and Bonga 1985), obtained somatic embryogenesis (SE) in conifer tissue cultures. This important event resulted in greatly expanded research efforts worldwide aimed at developing industrial applications for the new SE technology.

Nowadays, several forest industries are using SE in their commercial operations. Among other applications, SE has led to genetic engineering of plants, including that of conifers and hardwood species. There is a worldwide frenzy of activity in this field at present, particularly with agricultural crops. Without proper SE technology, this development would not have been possible. The next major event to occur was the first report of regenerated plants that were large enough to survive transfer to soil from protoplasts (cells without cell walls).



Larix × leptoeuropaea
somatic embryo regeneration
from protoplasts.

This again was first achieved in a CFS laboratory (Klimaszewska 1989). The importance of regenerating plants from protoplasts is due to the fact that by fusing protoplasts from different species and regenerating plants from the fused protoplasts, one can often bypass hybridization limitations imposed by the sexual process.

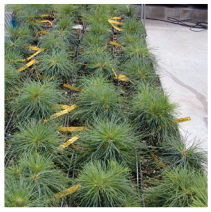
In the fruit industry, this technology has led to the creation of numerous new fruit varieties, especially within *Citrus* species. Unfortunately, research to explore the potential benefits of this technology has remained inactive at the CFS since 1998 (Pattanavibool et al. 1998).

These are the most significant achievements in the tissue culture field. However, many other avenues have been explored and have given potentially significant results as shown in the following sections.

CHRONOLOGY OF TISSUE CULTURE RESEARCH CARRIED OUT IN CFS LABORATORIES

In vitro culture of *Arceuthobium*

To our knowledge, no tissue culture research was conducted at the CFS prior to 1960, when Jan Bonga started working at the AFC. His task was to establish control methods for the eastern dwarf mistletoe (*Arceuthobium pusillum*), a parasite on spruce. This parasite was not of major economic concern in eastern Canada; however, dwarf mistletoes were and still are a major problem in Alberta and British Columbia. The rationale for starting this project at the AFC was that a research position was open in that laboratory and that control methods developed for the eastern dwarf mistletoe could presumably be applied in western Canada on the western species. One of the problems encountered when studying this parasite is that only a minute part (the flowers) appears on the surface of the host, with most of its mass growing inside the bark and wood of the host. Therefore, it was decided to try to obtain as much as possible of the life cycle of the parasite *in vitro*, thus making the parasite available for physiological studies separate from its host. This work resulted in a number of publications and finally culminated in obtaining the part of the parasite that grows inside the bark in tissue culture (Bonga 1974). Unfortunately, because of a shift in focus from national research priorities to regional ones, the project was prematurely terminated. Many years later it was taken up again at the CFS's Pacific Forestry Centre (PFC) in Victoria, British Columbia, by Simon F. Shamoun and co-workers. They tested the virulence of parasitic fungi on hemlock dwarf mistletoe (*Arceuthobium tsugense*) in tissue culture (Deeks et al. 2002) and found a couple that effectively destroyed the mistletoe.



In vitro culture of conifers at the Petawawa National Forestry Institute (early years)

In the mid-1960s, Don Durzan initiated the culture of conifer tissues at the Petawawa National Forestry Institute (PNFI) in Chalk River, Ontario. He obtained cultures of masses of single cells of white spruce and jack pine (*Pinus banksiana*). Some of these single cells divided and formed structures that looked like the earliest stages of somatic embryos (Durzan and Bennett 1968; Durzan and Steward 1968). However, these did not develop further into mature embryos and plantlets, a feat that would not be achieved until about 20 years later (see below).

Attempts were also made to obtain clonal propagation of selected superior mature conifer trees by means of *in vitro* culture of their shoot buds. This resulted in the formation of elongating shoots, but no roots were formed (Chalupa and Durzan 1973). Although attempting to obtain clonal propagation from such bud cultures was considered to be a reasonable and logical objective at the time, it was discovered in later years that it is very difficult to overcome problems associated with tree maturation.

This is due to physiological changes that occur from embryonic to adolescent to adult stages of growth in most tree species. As growth progresses, the developmental program becomes increasingly more fixed and, consequently, it becomes more difficult to induce cells to behave in an embryonic or juvenile fashion to form embryos or shoots and roots. Therefore, greater success was achieved in later years using much younger tissue sources (immature and mature zygotic embryos; see below).

Research at the PNFI was not restricted to conifers. Durzan and Lopushanski (1975) published a paper reporting on propagation from cell suspension cultures obtained from American elm (*Ulmus americana*) seedlings.

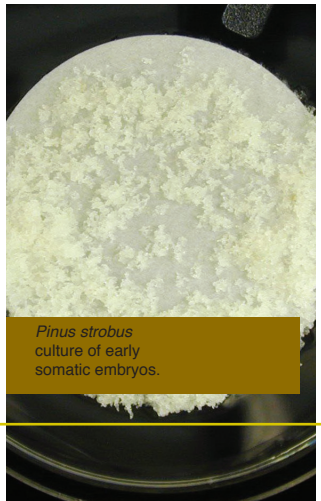
Tree biotechnology group at PNFI nursery, 1993.

1. Don Stewart – biologist
2. Chris Ward – technician
3. Tannis Beardmore – research scientist
4. Bao Xue – postdoctoral fellow
5. Chris Kaufeldt – technician
6. Denis Lachance – biologist
7. Jane Lego – technician
8. Marc Kalous – biologist
9. Cathy Overton – technician
10. Julie Derosier – technician
11. Chantal Côté – biologist
12. Bob Rutledge – research scientist
13. Madoka Mitsumuni – student
14. Yvonne Devantier – student
15. Pierre Charest – research scientist
16. Krystyna Klimaszewska – research scientist
17. Julie Mireault-Wiseman – student
18. Jessica Crew – student
19. Stephanie McInnis – Ph.D. student

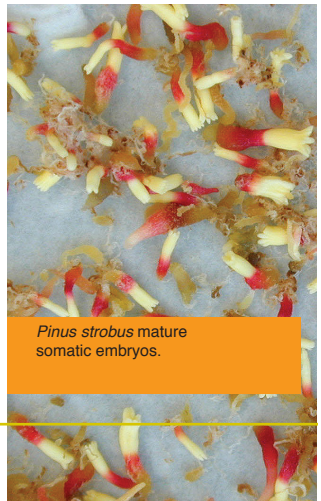
In vitro culture of conifers at the Atlantic Forestry Centre (early years)

Around 1970, the first attempts were made to obtain haploid cultures of conifers. At that point in time, considerable progress had been made in obtaining homozygous diploid lines from haploid cultures for several agricultural crops, in particular for cereals. Normally, near homozygous diploid lines are obtained through seven to ten cycles of in-breeding, a long-term and expensive process. By establishing haploid tissue cultures followed by artificial chromosome doubling, homozygous diploid cultures are obtained, from which homozygous diploid crop plants are regenerated. By following this procedure, the desired pure breeding lines are obtained rapidly in just one generation. For tree species, success with this approach would be even more important than with annual crops because of the long generation cycle of trees that makes obtaining homozygous plants by in-breeding totally impractical.

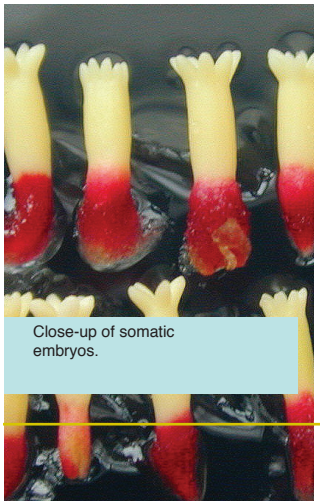
With agricultural crops, haploid cultures are obtained from pollen. With conifers, both pollen and the female gametophyte are available for use. The first attempt was made with red pine (*Pinus resinosa*) and resulted in haploid tissue growth from both the male and female gametophytes (Bonga and Fowler 1970). This work was later expanded to Austrian pine (*Pinus nigra* var. *austriaca*) and mugo pine (*Pinus mugo*), resulting in haploid callus formation from pollen as well as from the female gametophyte. Some of the pollen produced cellular structures that showed similarities with early zygotic embryos, but none of these developed into plants (Bonga 1974). Further work carried out with other species led to haploid callus formation in Norway spruce (*Picea abies*), white spruce, tamarack (*Larix laricina*) and European larch (*Larix decidua*) (Bonga 1981). In subsequent years, this work was supported by funding from the National Biotechnology Strategy (NBS), which led to the development of the technology that resulted in gametophytic as well as somatic embryogenesis (see below).



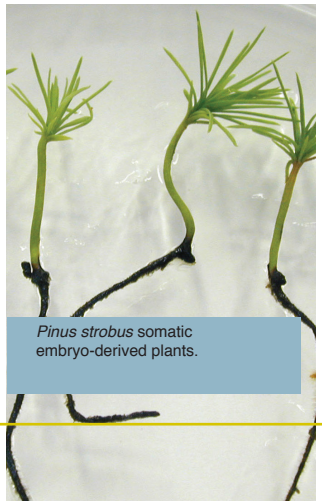
Pinus strobus culture of early somatic embryos.



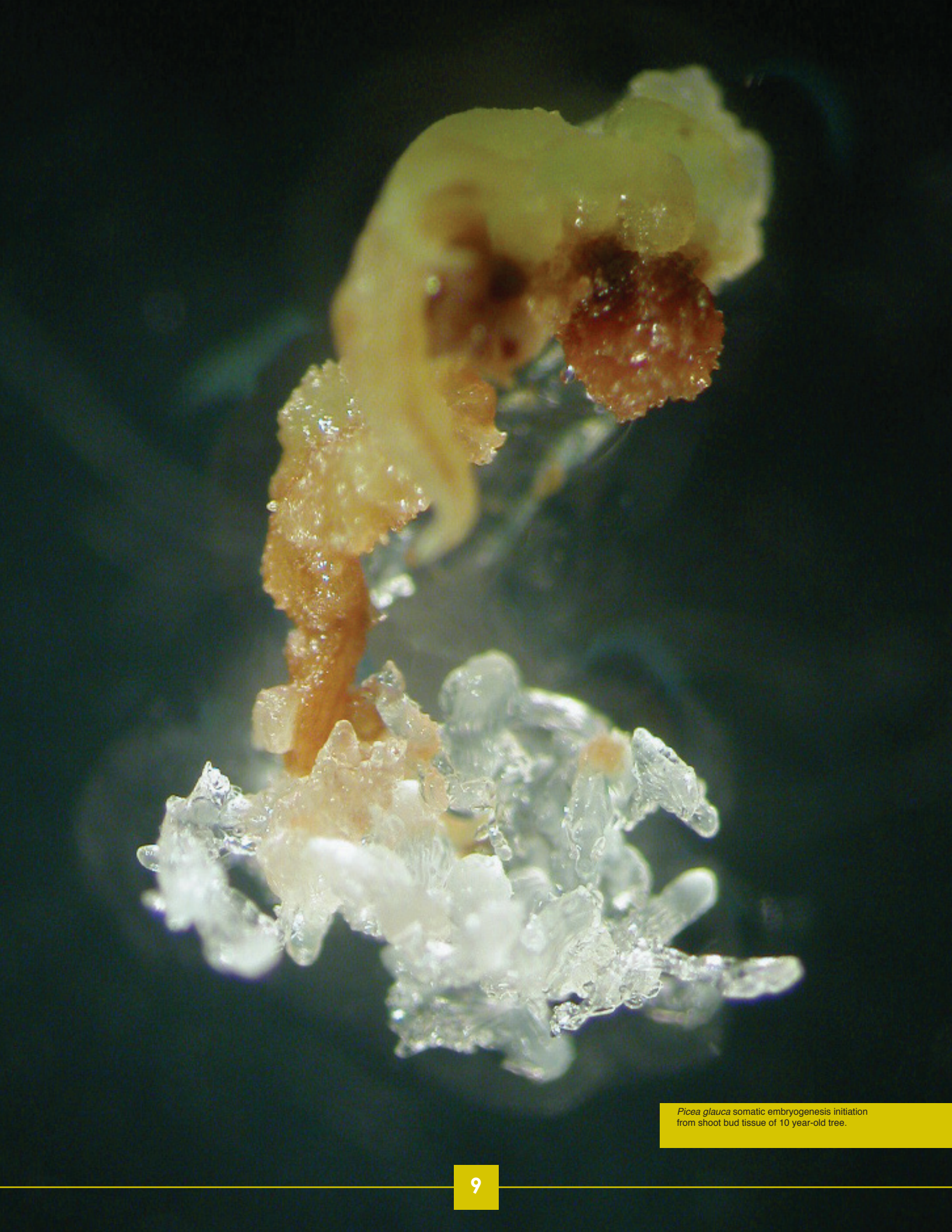
Pinus strobus mature somatic embryos.



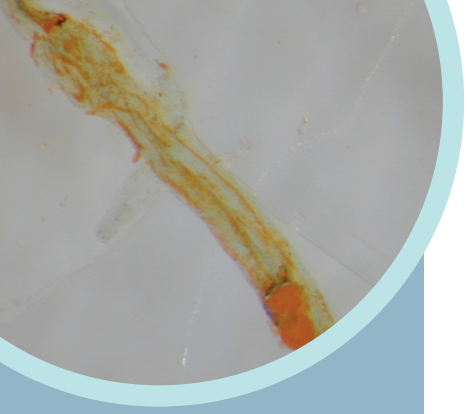
Close-up of somatic embryos.

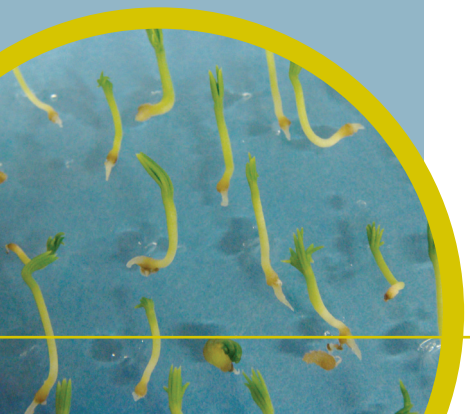


Pinus strobus somatic embryo-derived plants.



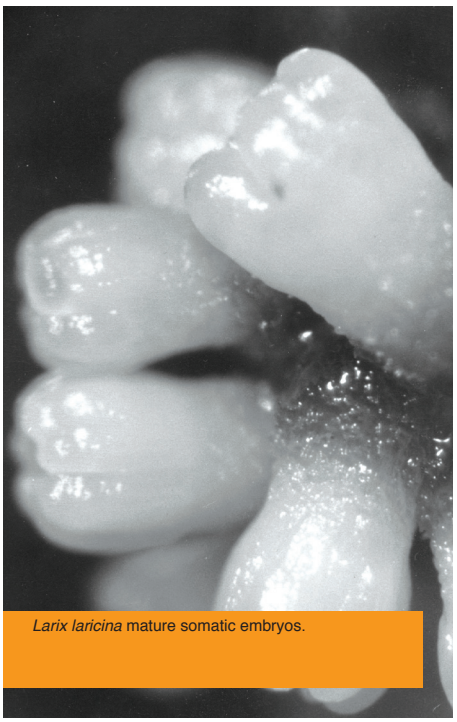
Picea glauca somatic embryogenesis initiation from shoot bud tissue of 10 year-old tree.





Attempts to obtain clonal propagation from tissues of superior mature conifer trees cultured *in vitro* resulted in the formation of clonal shoots but, unfortunately, with a few exceptions, these failed to form roots. A paper by Sommer et al. (1975) demonstrated that it was much easier to obtain clonal propagation from tissues of germinating embryos than from tissues of mature trees. Researchers worldwide took this as a signal to divert their attention from mature trees and focus on zygotic embryos or young seedlings. Both the AFC and PNFI, and later at the CFS's Laurentian Forestry Centre (LFC) in Quebec City, Quebec, work with mature trees continued on a part-time basis because the objective to achieve success in that field was still considered valuable.

Over the years, advances have been made (as reviewed in Bonga et al. 2010), but the ultimate goal of achieving mass clonal propagation by these means remains elusive.



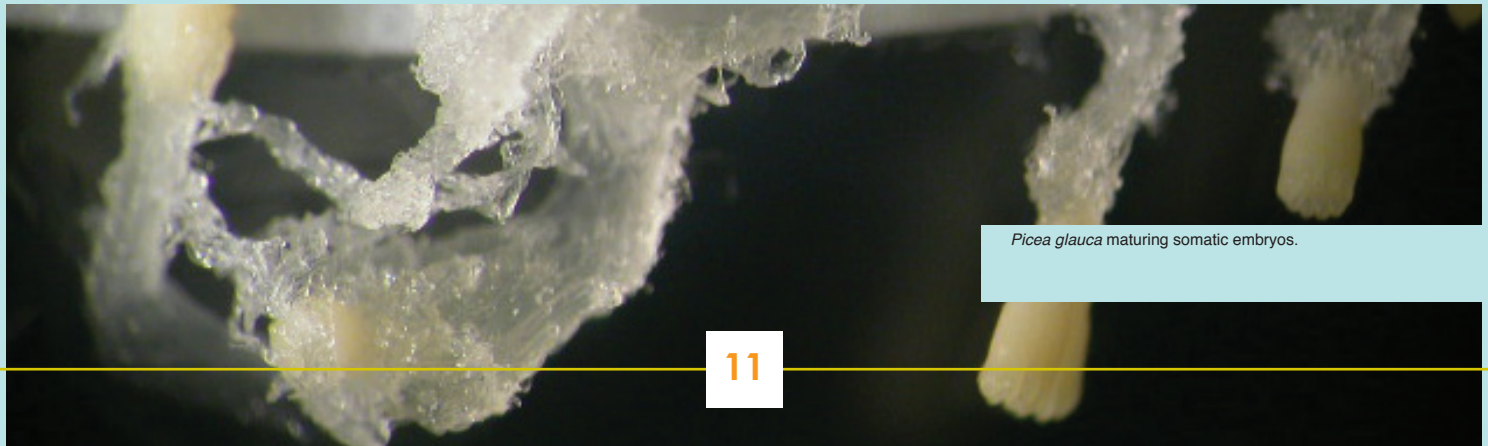
Larix laricina mature somatic embryos.

Discovery of somatic embryogenesis from seed embryos

In the early 1980s, the National Biotechnology Strategy (NBS) was initiated. The objective at the time was to fund long-term and high-risk research that showed potential for significant applications if successful. Five-year funding was given to the AFC, which was sufficient to re-equip the laboratory and hire a post-doctoral fellow for the 5-year period. The funding was provided to expand research on haploid tissues that had been going on at the AFC for some years. The ultimate goal was to produce clonal plants from haploid tissues, an objective that was considered difficult to achieve and that required a long-term commitment. As already indicated, the purpose of these studies was the production of homozygous diploid plants to be used for controlled hybridization. An additional objective was to fuse protoplasts from different conifer species and to regenerate plants from the product of this fusion. As already pointed out, this technology makes it possible to bypass sexual hybridization barriers and produce hybrids that cannot be obtained sexually. To our immense surprise, we succeeded in obtaining embryogenesis from haploid female gametophyte tissue within a couple of years. This result was published in 1985, and in the same year two other papers, one from a group of Swedish researchers and one by a Czechoslovakian investigator, reported on SE obtained from tissues of zygotic embryos. SE from female gametophytes and that from zygotic embryos both required the same culture protocol and followed the same pattern of development. Research carried out with haploid cultures resulted in several publications (the most recent being Pattanavibool et al. 1995). In another surprising development, one of our gametophytic cultures resulted in a haploid seedling that over time produced a homozygous diploid tree through spontaneous chromosome doubling (von Aderkas and Bonga 1993). Since conifers contain semi-lethal and lethal recessives, we were lucky to have found one genotype in our cultures that was low in these recessives, thus allowing the development of a functionally normal tree. Hence, we achieved the objectives that were stated when the NBS funding was first received.

After this initial success, it was decided to determine whether hybridization by protoplast fusion could be achieved. Unfortunately, after the first 5 years of funding, the objective of the NBS was changed from funding long-term research to supporting only research that would have immediate industrial applications.

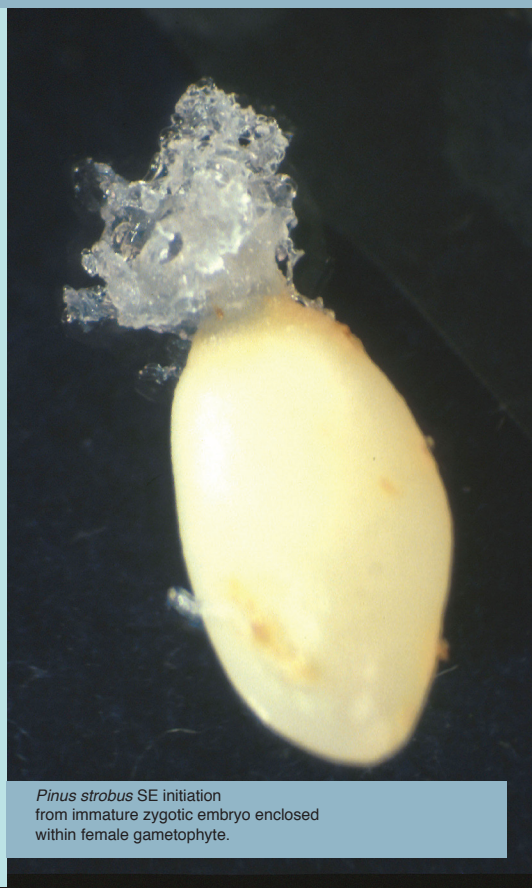
Therefore, the haploid research program had to be prematurely terminated. During the 5 years of NBS funding, we were able to hire two post-doctoral researchers; such assistance was subsequently no longer available.



Picea glauca maturing somatic embryos.

Improvement of somatic embryogenesis protocols

Soon after the first reports of SE in conifers, further research aimed at improving protocols and adapting the new technology to other species came into full swing at both the AFC and PNFI laboratories. In 1985 and 1986, two scientists, F. Tremblay and K. Klimaszewska, were hired by the PNFI to conduct research on SE of conifer species. The species that both laboratories initially worked with were European larch and hybrid larch (*Larix* x *eurolepis*), and these were soon followed by black spruce (*Picea mariana*), white spruce, and eventually white pine (*Pinus strobus*), jack pine and some other species (Klimaszewska 1989; Tremblay 1990; Klimaszewska and Smith 1997). After the initial success of SE, it became obvious that initiation often was the easy part. Embryo abnormalities were common and proper germination of the somatic embryos often failed to materialize. However, diligent work in both laboratories resulted in much improved culture protocols and good regeneration rates (Park et al. 2006). In the meantime, a number of interesting results were obtained that greatly improved the attractiveness of the techniques for the forest industry.



Pinus strobus SE initiation from immature zygotic embryo enclosed within female gametophyte.

Primarily at the PNFI and, to a lesser extent, at the AFC, experiments were carried out to determine if embryogenic cultures could be cryopreserved, i.e., stored in liquid nitrogen at -196 °C (Klimaszewska et al. 1992; Charest et al. 1996). Unexpectedly, it was found that embryogenic cultures were easier to store in liquid nitrogen than many other tissues. Cryopreservation makes the long-term storage of cultures possible, which in turn makes it possible to use part of each embryogenic clone for long-term field testing of somatic seedlings while the remaining part is kept in cryostorage until the end of the field testing period. At the end of the field test, the clones that behaved best are retrieved from cryostorage and used for mass production of planting stock. Whereas in traditional breeding experiments one can select for family average behaviour, breeding in combination with SE and cryopreservation allows selection within families. Since most conifers are genetically highly heterozygous, the combination of breeding, SE and cryopreservation will result in genetic improvement substantially above that obtained with breeding alone (Klimaszewska et al. 2007). Development of the proper protocols for cryopreservation in CFS and other laboratories has made SE technology highly attractive to the forest industry and, consequently, it has found worldwide applications (Cyr and Klimaszewska 2002; Klimaszewska et al. 2007).

Pinus banksiana mature somatic embryos.

In a very large-scale breeding–SE experiment conducted at the AFC, it was found that SE is a genetically controlled trait. It was discovered that the initiation of SE was under strong additive genetic control (Park et al. 1993), and that this genetic effect declined to a low level at subsequent proliferation, maturation and germination stages (Park et al. 1994). This means that by crossing a parent with a high capacity for SE with a parent with a low capacity for SE, one will obtain offspring with both the characteristics of the low SE capacity parent and the high SE capacity of the other parent. Thus, a large variety of genotypes with excellent qualities and high SE capacity were generated for testing (Park 2002). Another factor that needed to be determined was the genetic stability of clones in long-term cryopreservation. At both the AFC and PNFI laboratories, it was found that several years of cryopreservation did not affect the genetic make-up of the stored cultures nor their capacity to regenerate new embryos (Park et al. 1998; DeVerno et al. 1999). This indicated that the forest industry could safely use the technology for long-term field testing.

One aspect that turned out to be important for the proper maturation of conifer somatic embryos is water relations. This aspect was studied in detail by one of the AFC researchers together with researchers at the University of Victoria (Dumont-BéBoux et al. 1996). Further research on the tissue culture environment and the availability of water to somatic embryos in culture was carried out at the AFC and LFC (Klimaszewska et al. 2000; Cameron 2001, 2006). Treatments to improve desiccation tolerance during the SE process as well as its biochemical characterization were carried out at the PNFI and AFC (Beardmore and Charest 1995; Pond et al. 2002; Beardmore 2003).

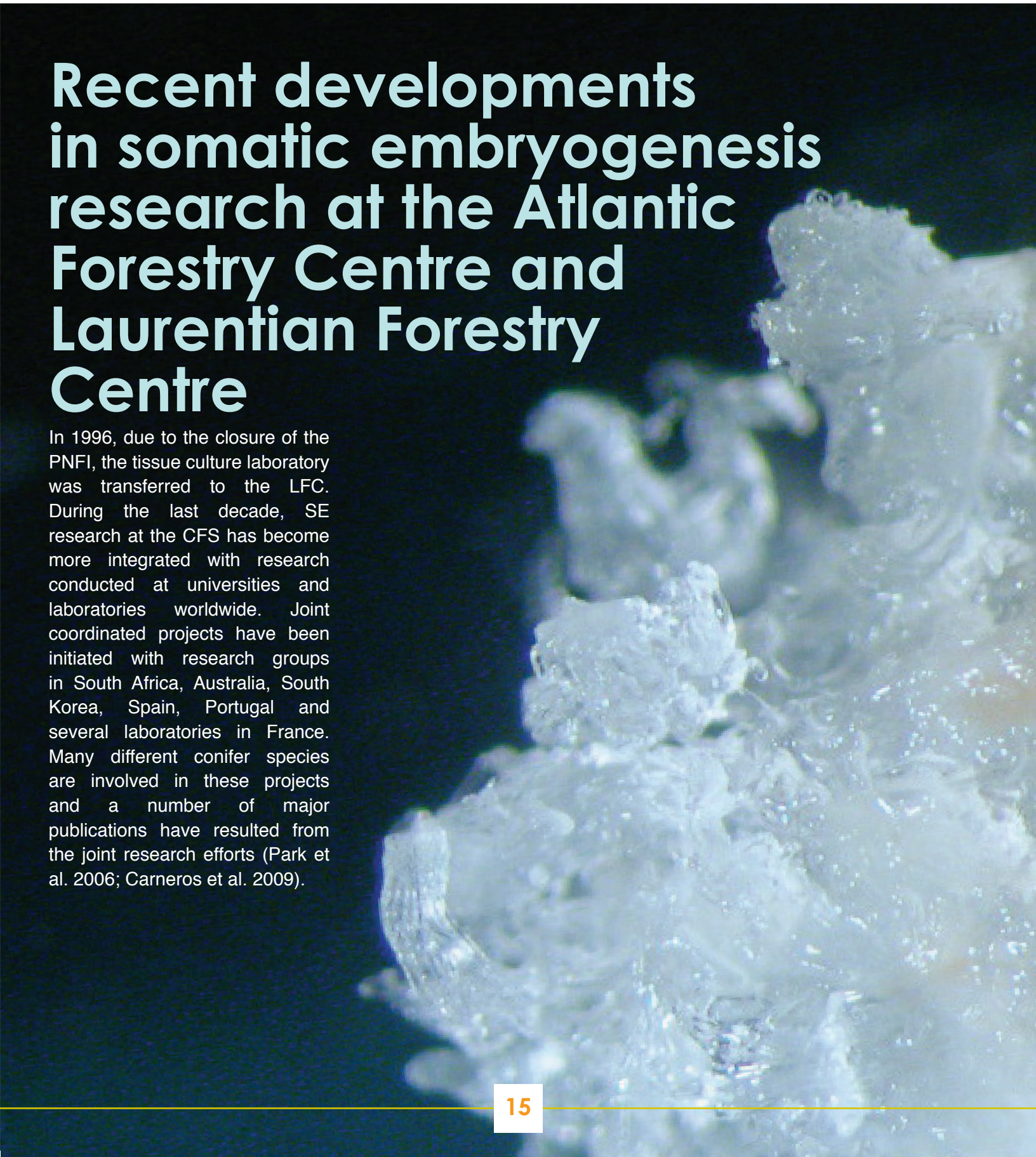
An important achievement was the development of a protocol that produced protoplasts from an embryogenic culture of hybrid larch, which subsequently regenerated somatic embryos and plantlets (Klimaszewska 1989). This potentially opened the way to achieving somatic hybridization through the fusion of protoplasts of different origins, thus creating new varieties that cannot be obtained by sexual means. Interspecies protoplast fusion and formation of mature somatic embryos from the fused protoplasts was achieved for larch species by a team of LFC and University of Victoria researchers (Pattanavibool et al. 1998). Of particular interest in that respect is the fact that SE was obtained from haploid megagametophytes at the AFC (see above). Fusion of haploid protoplasts would result in normal diploids instead of tetraploids (which is what occurs after fusion of diploid protoplasts).

A large-scale project with the objective of increasing taxol production in Canada yew (*Taxus canadensis*) by clonal propagation (S. Cameron) has been in progress at the AFC for over a decade, and a number of high-yielding cultivars have been found. Yew can easily be clonally propagated by rooting of cuttings. However, because of the advantages offered by SE and cryopreservation, and because SE provides the basis for maintaining clonal integrity and for improvement through genetic engineering, SE has been studied as part of this project. There has been some sporadic success (S. Pond) using zygotic explants, but the goal of using bud tissue as the initial explant has yet to be reached. SE is potentially important as a scale-up propagation method for producing somatic propagules of known pedigree derived from elite clonal plants selected for taxane production.

In a second tissue culture study, a method was found (by S. Cameron) whereby yew vegetative buds could be consistently induced to elongate into small shoots, a potentially important step in providing aseptic clonal material for genetic transformation and hairy root culture (see below).

Recent developments in somatic embryogenesis research at the Atlantic Forestry Centre and Laurentian Forestry Centre

In 1996, due to the closure of the PNFI, the tissue culture laboratory was transferred to the LFC. During the last decade, SE research at the CFS has become more integrated with research conducted at universities and laboratories worldwide. Joint coordinated projects have been initiated with research groups in South Africa, Australia, South Korea, Spain, Portugal and several laboratories in France. Many different conifer species are involved in these projects and a number of major publications have resulted from the joint research efforts (Park et al. 2006; Carneros et al. 2009).

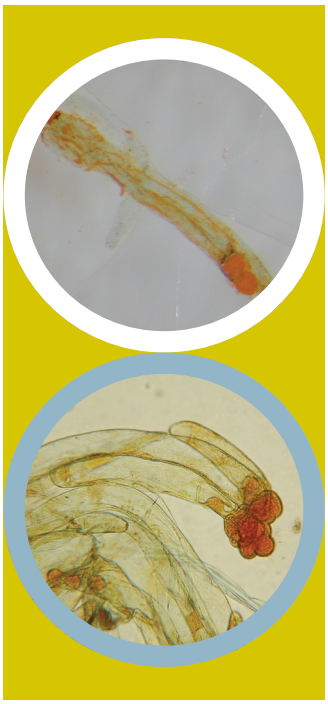


Somatic embryogenesis technology transfer

Joint projects have also been initiated with several forest industry and forest biotechnology organizations (J.D. Irving Ltd. in New Brunswick, ministère des Ressources naturelles et de la Faune du Québec, BC Research Inc./CellFor Inc. in British Columbia, Ontario Forest Research Institute). These projects involve various breeding and planting strategies with seedlings produced by SE. Furthermore, some of the CFS's SE projects have lately been transferred to the Canadian Wood Fibre Centre (CWFC) through the creation of the National Network of Somatic Embryogenesis Laboratories led by Y.S. Park. This has resulted in the establishment of a training program at the AFC and LFC that aims to familiarize technicians from provincial, industry and academic institutions with SE technology. At the LFC, a "Conifer Somatic Embryogenesis and Genetic Transformation Technology Platform" was created by K. Klimaszewska to accommodate national and international research collaborations in the areas of genetic transformation, gene expression, and basic research on SE. It also provides services (on a cost recovery basis) such as production of high-quality research materials for other Canadian scientists involved in conifer genomics research (such as Université Laval's Arborea project and the University of British Columbia's Treenomix project).

The role of somatic embryogenesis technology in multivarietal forestry

As already mentioned, there are many important applications for SE; however, the most important current application has to do with the implementation of multivarietal forestry (MVF), which is defined as the use of tested tree varieties in plantation forestry. Owing to the development and refinement of SE and cryopreservation techniques, the development of tree varieties in conifers has become a reality, something that was not possible previously with sexual reproduction. It became possible because embryogenic lines can be stored in liquid nitrogen indefinitely without changing the genetic make-up or losing viability while lengthy field tests are carried out. This provides an opportunity to produce genetically tested identical genotypes consistently over time, which is analogous to what can be done with agricultural and horticultural varieties. There are many advantages related to MVF; however, the most important ones are: (1) greatly increased genetic gain, (2) the flexibility to rapidly adapt to changing breeding goals and/or environment, and (3) the ability to manage genetic gain and diversity.



The flexibility in tree breeding offered by MVF is particularly important because modern tree breeders are faced with a new set of challenges for an uncertain future, including the demand for increased productivity, adaptation to climate change, changing product goals, and conservation and restoration of threatened species.

Pinus strobus early somatic embryo.

Picea glauca mature somatic embryos.

Now that its benefits and potential impact on plantation forestry have been recognized, the implementation of MVF is well underway in eastern Canada. For example, J.D. Irving Ltd., in collaboration with the CFS, plants about one million tested varietal trees every year in its prime site plantation forests. Furthermore, through the National Network of Somatic Embryogenesis Laboratories, the CWFC is producing embryogenic varietal lines for field testing at the AFC for various tree improvement programs, including the New Brunswick Tree Improvement Council, the Nova Scotia Tree Improvement Working Group, and Alberta Sustainable Resource Development. It is envisioned that when the field test results become available, these tree breeding programs will implement MVF. In addition, the CFS is collaborating with the Ontario Forest Research Institute to develop blister rust-resistant hybrid white pine and with the British Columbia Ministry of Forests, Mines and Lands to develop a SE system for Douglas-fir (*Pseudotsuga menziesii*), western red-cedar (*Thuja plicata*) and yellow-cedar (*Chamaecyparis nootkatensis*).

Somatic embryogenesis and genetic engineering

Discussion of the genetic engineering experiments carried out at the CFS is not within the scope of the present document. However, we wish to emphasize the important function that SE serves in genetic transformation procedures and, therefore, in functional genomics.

There are various methods of achieving genetic transformation in plants, but transformation of embryogenic cultures is by far the most common one. The first advantage of using SE for that purpose is that the process can be carried out in a strictly confined and controlled environment. Also, by including an antibiotic resistance gene, one can easily separate transformed from non-transformed cells. Finally, since SE in most species starts from single cells, one avoids ending up with chimera, i.e., individuals with both transformed and non-transformed cells.

Picea glauca SE-derived trees, Valcartier, QC.

Somatic embryogenesis from adult conifers

To clone adult (mature) conifer trees by means of tissue culture has been a cherished goal for as long as researchers have been experimenting with *in vitro* culture. The benefits derived from improving the genetic make-up of planting stock would be substantial if such clonal propagation could be achieved at a high success rate and without growth abnormalities induced by the culture techniques (seedlings free of growth abnormalities are called “true-to-type”). In most conifer species, genetic variation is enormous. Consequently, if seedlings derived from seed of good parents are used for planting, a mixture of seedlings is obtained with some having excellent growth potential and others a poor one, with the latter decreasing the average performance of the seedlings. If clonal propagules of adult trees with a good track record of growth, insect and disease resistance, good fibre quality, etc., could be produced true-to-type, a crop would be obtained that would be substantially better than that obtained from seed of superior adult trees.

Unfortunately, even though SE technology has worked well for many conifer species using zygotic embryos as starting material, the same cannot be said when SE is attempted with adult trees. Research to induce SE in tissues from adult trees has been carried out in several laboratories worldwide, but success so far has been very limited.

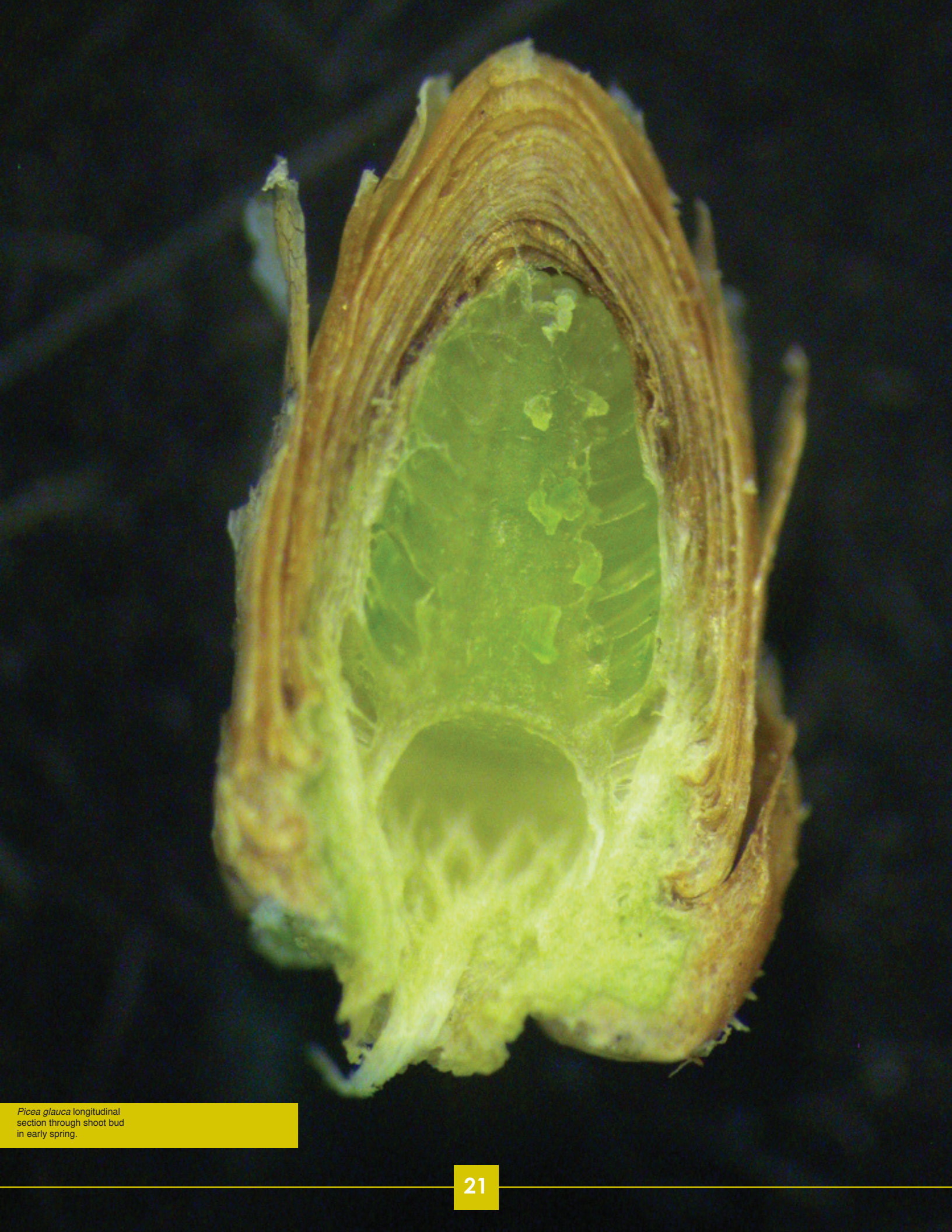
At the AFC, SE was obtained in tissues from adult European larch by a mechanism different from that normally shown in tissues from seed-derived embryos in culture. However, these embryos did not mature and germinate properly, mainly due to an underdeveloped root system (Bonga 2004). At the LFC, SE resulting in viable juvenile plantlets was obtained from shoot buds excised from 10-year-old white spruce trees that had been obtained from embryogenic cultures initiated from zygotic embryos (Klimaszewska et al. 2010). These somatic trees are presently the subject of genome-wide transcriptional analysis of an embryogenic versus non-embryogenic genotype.

Continued recalcitrance in conifer tissue cultures

Even though SE has been achieved in zygotic embryo cultures of many conifer species, in several others, success has been limited or absent. Two of our native pine species that have proven to be recalcitrant are jack pine and lodgepole pine (*Pinus contorta*). Jack pine has been studied at the AFC for about 15 years but has produced initiation rates of only 3-4%, which is too low for practical purposes. Recent work carried out at the LFC (in collaboration with the University of British Columbia) and at the AFC has also shown similar results in lodgepole pine. Recalcitrance has proven to be even more intractable when using adult trees as the source of tissues for culture. Considerable effort has gone into literature searches at both the AFC and LFC laboratories to find a theoretical basis for this recalcitrance. This has resulted in several extensive review papers dealing with the theoretical aspects of the problem, the last one having just been published (Bonga et al. 2010). Research aimed at reducing recalcitrance in several commercial species is underway at both centres.

Somatic embryogenesis and species/ germplasm preservation

Somatic embryogenesis, along with cryopreservation, provides another dimension for species conservation and restoration. For example, whitebark pine (*Pinus albicaulis*) is an ecologically important keystone species that is an integral part of the ecosystem in western North America; however, the species is seriously threatened due to its susceptibility to white pine blister rust and mountain pine beetle attacks. In collaboration with Alberta Sustainable Resource Development, SE of whitebark pine has been developed and is being adopted as a conservation strategy for the species. A similar SE system has been developed for limber pine (*Pinus flexilis*).



Picea glauca longitudinal section through shoot bud in early spring.

At the AFC, a number of hardwood species are being studied. Efforts are underway to obtain SE from buds of butternut (*Juglans cinerea*) and American beech (*Fagus grandifolia*) to attempt to achieve remedial genetic conservation since both species are plagued by pathogens. The use of buds is important to maintain the pedigree of the SE progeny, particularly if pathogen-resistant clones are found. Both species are difficult to work with but SE has been initiated from mature trees of both beech and butternut, and SE cultures have been cryopreserved. In butternut, SE has resulted in plant regeneration, and somatic embryos of beech have been matured and germinated (Pond 2007, 2008). Beech and butternut are threatened with extinction, and SE followed by cryopreservation would allow the development of an alternative means of conservation for these species. Pond has also achieved plant regeneration from individual lateral vegetative meristems of Cottet willow (*Salix cottetii*), a sterile triploid species, and three other willow species. Willow is a fast-growing species, a good source of biomass, and a potential source of bioproducts as well. Fast-growing, genetically modified willow needs to be sterile to prevent gene flow to surrounding native willow species. Tissue culture can be used to economically propagate genetically sterile hybrids, which are the starting material for genetic transformation, to incorporate useful traits and/or bioproduct genes.

High efficiency SE and plant regeneration have recently been achieved for the first time at the AFC in Spanish cedar (*Cedrela odorata*), a tropical hardwood species, using a heat shock treatment (Cameron 2010). This commercial mahogany species had until then been considered recalcitrant to regeneration by tissue culture techniques.

Many Canadian tree species that are considered threatened or endangered produce seed that cannot be stored in the long term (recalcitrant seed) (Beardmore and Vong 1998; McIlwrick et al. 2000). Tissue culture techniques have been developed by AFC researchers for conserving the germplasm of a selection of tree species that produce recalcitrant seed (Beardmore and Vong 1998; Marshall et al. 2000; Beardmore and Whittle 2005).

FURTHER CONTRIBUTIONS

International recognition

In addition to a large number of research papers, CFS employees have prepared numerous reviews published in peer-reviewed journals and encyclopedias, and as book chapters. This has made information widely available. Several multiauthor textbooks have been edited by CFS researchers. One multiauthor three-volume series edited by CFS scientists was included in two international surveys of forestry-related books and monographs (McDonald 1996). In one survey, these three volumes were ranked number 6, and in the other survey they were ranked number 8 out of 1,053 titles entered. A textbook dealing with *in vitro* culture of trees was written by a CFS researcher and his post-doctoral fellow (Bonga and von Aderkas 1992). Furthermore, a joint US Patent (6,200,809 B1, "Maturation of somatic embryos", March 13, 2001) was awarded to a CFS employee and co-workers at BC Research Inc. (Klimaszewska et al. 2001). Staff members have also frequently acted as guest speakers and session chairs at international meetings. To further promote international collaboration on the research and use of SE in connection with tree breeding around the world, the International Union of Forest Research Organizations (IUFRO)'s Working Party 2.09.02 on Somatic Embryogenesis of Tree was created and is coordinated by Y.S. Park.

Awards and other forms of recognition

Jan Bonga

CFS-AFC 1960 - 1995 retired
CFS-AFC 1996 - 2004 part time
CFS-AFC 2005 - 2011 volunteer

- Forest Biology Project Advisory Committee: Institute of Paper Chemistry, Appleton, Wisconsin; 1986-1988
- Canadian Forest Service Award; 1993
- Canadian Forestry Scientific Award: Canadian Institute of Forestry; 1995
- NSERC: Plant Biology Grant Selection Committee, Forestry Sub-Committee; 1989-1992

Krystyna Klimaszewska

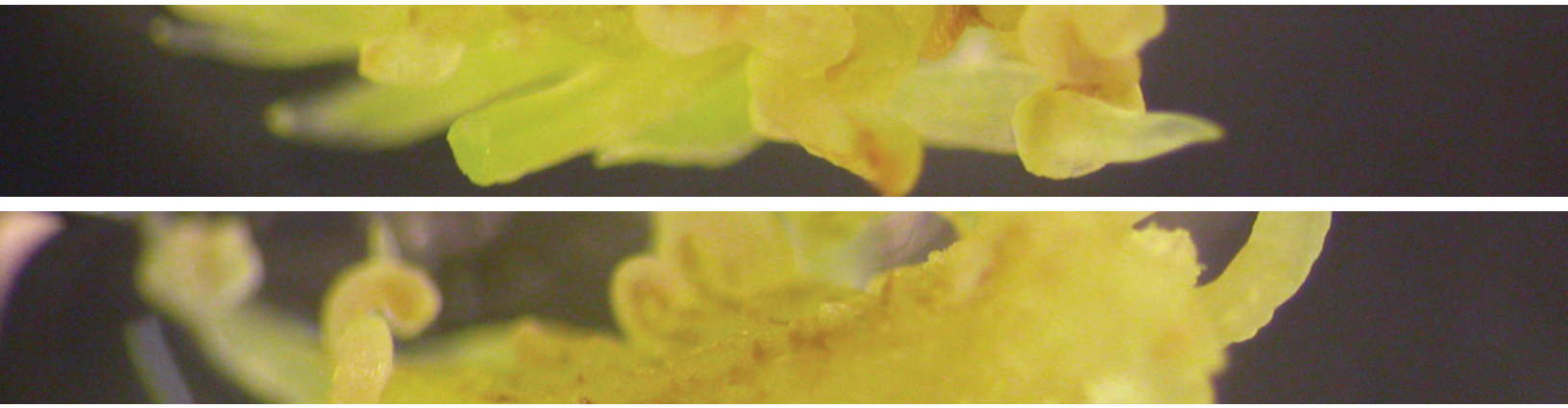
PNFI 1986 - 1996
BC Research Ltd. 1997-1998
CFS-LFC 1999 - present

- Forestry Canada: "Honorary Award, Excellence in Science" for International Achievement in Forest Biotechnology; 1990
- Natural Resources Canada: "Merit Award" for Creativity and Innovation in Scientific Research; 1998
- Public Service of Canada: "Award of Excellence"; 1998
- Governor General of Canada: "Meritorious Service Medal" (civil division); 1999
- Foreign Research Scientist hosted by le STUDIUM® (research agency hosting foreign research associates), Orléans, France; 2004-2005
- Adjunct Professor, Laurentian University, Sudbury, Ontario; 2010-…

Yill-Sung Park

CFS-AFC 1978 - present

- Technical Advisor, New Brunswick Tree Improvement Council and Nova Scotia Tree Improvement Working Group; 1979-…
- Adjunct Professor, University of New Brunswick; 1994-…
- Coordinator, IUFRO Working Party 2.09.02: Somatic Embryogenesis of Trees; 2008 -…
- Deputy Coordinator, IUFRO Unit 2.09.00: Tree Seed, Physiology and Biotechnology; 2008 -…



PROPOSED FUTURE RESEARCH OBJECTIVES

- **Adapt SE technology to more species**
  SE works well for some spruce, pine and larch species, but not for other conifers. SE is generally difficult, or has not yet been achieved, with most other conifers. Because of the enormous potential of tree breeding combined with SE and cryopreservation to improve planting stock, the expansion of the range of species for which SE can be used effectively is a worthy objective. Past experience has shown that this is not going to be easy and a long-term commitment to funding this research is needed.

- **Assist the industry and others interested in adapting SE to their enterprise**
  If CFS research efforts could improve the effectiveness of SE and its application to a wider range of conifer species, our attempts to help the industry and others would be greatly expanded. To date, transfer of SE technology to others has been effective and is much appreciated. Continued efforts are worthwhile.

- **Find useful molecular markers in SE cultures that will improve clonal selection**
  If reliable markers could be found for growth rates, fibre quality, resistance to pathogens and adverse environmental conditions, etc., the testing phase of SE could be greatly reduced.

- **SE and genetic engineering**
  Since SE serves as the basis for much of the genetic engineering research that is going on, further investigation as to how to combine these two branches of research is needed.

- **SE and embryo rescue**
  As outlined above, new hybrids have been obtained in citrus and other fruit trees by excising the immature embryo before it aborts and culturing it to maturity on a tissue culture medium. This could be attempted with crosses between related conifer species that are normally genetically incompatible. This would be particularly effective if the rescued embryo could be clonally propagated by SE.

- **Regeneration from protoplasts and protoplast fusion**
  Protoplasts are useful for genetic engineering since DNA can be introduced by electroporation. This circumvents the need to use disarmed *Agrobacterium tumefaciens*, which is the usual means of obtaining genetic transformation. Bypassing sexual barriers through protoplast fusion is an attractive option in creating new hybrids.

This is of particular interest since research at the CFS has already demonstrated that SE can be obtained in haploid megagametophyte tissues. Fusion of haploid protoplasts followed by regeneration by means of SE would result in normal diploid offspring. Attempts to fuse diploid protoplasts would probably be less rewarding since conifers generally do not tolerate tetraploidization. This type of research would require a long-term commitment.

- **Hairy root culture in yew**

With the discovery of elite clones and the current industrial interest in bioreactor-based taxane production, hairy root culture is a potentially attractive method to produce paclitaxel. High and low producing clones are being transformed using conventional wild-type *Agrobacterium rhizogenes* and standard tissue culture-based co-cultivation methods.
The immediate goal is to determine whether hairy root cultures continue to exhibit the different capabilities of their parent clones to produce taxanes, followed by focused genomic analysis for taxane production markers using RTQ-PCR.

- **Attempts to obtain SE in tissues from adult trees**

The responsive white spruce genotype presently used for induction of SE from vegetative buds that have already reached the reproductive state will provide a unique opportunity to prove or disprove the existing hypothesis that the lack of SE potential in an adult conifer is caused by biochemical and molecular modifications associated with aging and phase change. For the first time in conifer tissue culture history, thanks to recent discoveries made at the LFC, researchers have an opportunity to separate the assumed influence of phase change-associated molecular events from genotype recalcitrance, as the latter is known to play a decisive role in the tissue culture response.

- **Hardwood trees**

Research should continue on the SE of beech and butternut and be expanded to other endangered hardwood species. SE tissue can be cryopreserved to conserve germplasm from the remaining healthy trees and provide a means of mass propagating disease-resistant trees.

If no naturally resistant trees are found, the potential exists to use genetic engineering to insert genes in SE tissues to confer disease resistance. At the AFC, researchers have already succeeded in inducing aseptic willow plantlet formation directly in tissue culture (without an intervening callus stage) by rapid rooting of lateral buds in as little as 3 weeks. Efficiency varies depending on the species but can be up to 85% for the four species studied thus far. Since there can be more than 50 lateral buds per meter on a single whip, this may be an efficient method to upscale the initial stages of hybrid propagation (followed by conventional rooting of cuttings) compared with conventional methods. This may be the only way to do so for reproductively sterile triploid hybrids, which do not produce seed. The sterile plantlets are also intended to be used in conventional transfection to produce willow clones for bioproduct production. Research should continue on tissue culture of willow and other reproductively sterile species that can be genetically enhanced to increase biomass production or to produce bioproducts. Tissue culture will be the most productive way of propagating these species.

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All photos in this publication were taken by Krystyna Klimaszewska, except the one on page 7, which was provided by the PNFI.