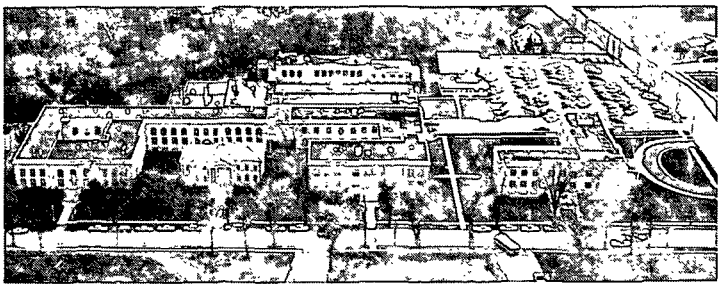

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FOREST BIOTECHNOLOGY: GROWING CONIFERS FROM CELLS

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FOREST BIOTECHNOLOGY: GROWING CONIFERS FROM CELLS

Michael R. Becwar and Steven R. Wann

Conifers, the main source of fiber for paper production, are propagated sexually through seeds. Considerable genetic gains have been realized through conventional tree breeding programs. The increment of genetic gain per each generation is small, because sexual reproduction preserves only some of the desired genetic traits of a select tree. In contrast, vegetative propagation of conifers holds the potential for obtaining much larger and faster increments of genetic gain. This is because all of the genetic characteristics of a select tree are "cloned" and expressed in the next generation. Conventional methods of clonal propagation, such as grafting or rooting of cuttings, are useful techniques for tree breeders but are not practical methods of mass propagation of conifers. In this paper we discuss forest biotechnology techniques being developed for mass propagation of conifers. Particular emphasis is given to the process of somatic embryogenesis. We provide an overview of our research effort at The Institute of Paper Chemistry to establish a somatic embryogenic system for efficient mass propagation of conifers important to the pulp and paper industry.

The propagation techniques we discuss involve methods of tree tissue and cell culture. These in vitro (test-tube) techniques differ from conventional vegetative propagation in that small tissues or even individual conifer cells are used as the starting material. There are several advantages to using in vitro techniques, including that much less space is required to grow cells in a laboratory flask than is required to maintain seed production orchards. Also the laboratory cell culture systems lend themselves well to automation, enabling the efficient production of millions of propagules. Tissue culture methods also offer possibilities for the production of interspecific hybrids by cell fusion

techniques. Lastly and perhaps most importantly, working with conifer cells opens the way for applications of the biotechnology techniques of genetic engineering. With these techniques it may be possible to engineer desirable traits (e.g., insect and disease resistance) into conifers grown from cells that could not easily be obtained in a conventional tree breeding program.

SOMATIC EMBRYOGENESIS

Somatic embryogenesis, the production of embryos in test-tube conditions, was first discovered in carrot in the 1950's.[1] Although it has been found to occur in most agronomically important crop plants, it was not until 1985 that reliable reports of somatic embryogenesis in conifers were published.[2,3] At present, the only route available for the initiation of the embryogenic process under test-tube conditions in conifers involves the utilization of juvenile (embryonic) tissue as starting material. Apparently, cells derived from embryos have the capacity imprinted in their DNA for initiating the somatic embryogenesis process. When grown in the appropriate nutrient medium and growth hormones, these "starting cells" divide and give rise to a tumor-like growth of cells referred to as callus. The cells of the callus express the genetic information necessary to undergo the embryo initiation process and form somatic embryos. The test-tube embryos are called somatic because they are derived from vegetative (somatic) cells. In contrast, seed embryos are derived from the fusion of the male and female sexual cells.

The reason for the extensive research interest in developing somatic embryogenesis of conifers is that the process lends itself well to automation and it by-passes many of the labor intensive steps required in other micropropagation systems. Because the somatic embryos contain both root and shoot growth centers, they are capable of growing directly into plants when taken out of

test-tube conditions. Thus many of the sequential steps required for micropropagation of conifers are avoided. Somatic embryos also lend themselves well to encapsulation as artificial seeds, which could be sown as conventional seeds in nursery beds.

NORWAY SPRUCE MODEL SYSTEM

We have established callus cultures of Norway spruce that are highly embryogenic.[4] A gram of the callus from our best lines contains over 1000 somatic embryos. The potential utility of this method for mass propagation of conifers becomes evident when one considers that a single culture plate as shown in Figure 1, which contains about 3 grams of embryogenic callus, will yield over 3000 somatic embryos. We have developed an efficient method for determining the density of somatic embryos among different callus lines.[5] This has enabled us to identify preferred callus lines with a high level of embryogenic capacity. It would be feasible to grow these lines en masse and have available millions of somatic embryos for propagation purposes.

Somatic embryos removed from the embryogenic callus are strikingly similar to those removed from seeds of immature cones of Norway spruce. Figure 2 shows the similarity between an early stage somatic embryo (A) and the natural counterpart (B) taken from a seed. Both embryos have characteristic dense embryonal heads (left) and elongated suspensor regions (right). Figure 3 shows a more fully developed somatic Norway spruce embryo (A) and the seed-embryo (B) at a similar stage of development. Note how both somatic and seed-embryos have several developing cotyledonary (seed) leaves. In the somatic embryo (A) the region of the shoot apex is visible in the center of the ring of seed leaves. When the primary root of the somatic embryo has developed, these test-tube

derived embryos can be transferred to a soil mix for further development. Our results to date show that somatic embryos "germinate" and grow to the seedling stage.[4] We currently have several Norway spruce somatic embryo derived plants growing in soil and plan to transplant them to the field in the spring of 1987 for evaluation of growth and uniformity. One of these somatic embryo plants is shown in Figure 4.

BIOCHEMISTRY OF SOMATIC EMBRYOGENESIS

Although Norway spruce is an important source of softwood fiber on a worldwide basis, it is of little importance in the United States. Therefore, in order for forest biotechnology to have an impact on the U.S. pulp and paper industry the process of somatic embryogenesis will have to be extended to pines - particularly to the so-called hard pines grown in the southeastern United States. A recent report of somatic embryogenesis in a soft pine, sugar pine, suggests that somatic embryogenesis will be a property of the genus Pinus.[6]

To extend somatic embryogenesis to other coniferous species, notably loblolly pine and Douglas-fir, The Institute of Paper Chemistry has taken a biochemical approach to somatic embryogenesis. A study of the biochemistry of somatic embryogenesis might be expected to supplement a conventional tissue culture approach in several ways. For example, the capacity for embryogenesis may only be present in a portion of the cell population comprising the callus. Knowledge of the metabolic state corresponding to an embryogenic condition may serve as a marker to identify the requisite cells in the culture. In addition to providing an assay for embryogenic potential, biochemical analysis may define metabolic pathways critical to the growth and development of somatic embryos into plants. It should be appreciated that a distinct advantage of the test-

tube environment is its ability to achieve rapid multiplication or proliferation of tissues. However, at some point a drastic change in the form of growth must occur such that the proembryos proliferated embark on a pattern of organized growth that leads to the production of seedlings. The switch, in the form of growth from a relatively unorganized fashion to a highly regimented, organized fashion, is not always a smooth transition. Often, proembryos emerge from the proliferation cycle still possessing characteristics of the unorganized pattern of growth which can effectively thwart normal plant development. Biochemical analysis of this crucial transition can assist in ensuring that the majority of proembryos emerging from the test tube will grow into viable plants.

In studying the biochemistry of somatic embryogenesis in Norway spruce, we have taken advantage of the observation that both embryogenic and nonembryogenic callus can be initiated from certain donor tissues.[7] The two callus types can be readily separated from each other on the basis of color and texture - embryogenic callus being white and mucilaginous, while nonembryogenic callus is green and compact. Once segregated, the two callus types were maintained independently and subjected to biochemical analysis. The macroscopic differences between the two callus types persist at the cellular level in the striking differences in metabolism between the two types. Embryogenic callus, when compared to nonembryogenic callus appears to (1) synthesize protein at 20-fold faster rate, (2) evolve 10 times less ethylene, and (3) contain only a half to a tenth the amount of chemical reducing agents.

In order for a biochemical feature to find utility as a marker of embryogenic potential, it should meet several requirements. Aside from being unambiguously characteristic of an embryogenic state, the assays should be easy and rapid to measure and require small amounts of tissue. The latter features

are a necessity because often the deterministic events that impart the capacity for somatic embryogenesis occur over a brief time frame. Usually this time frame is soon after initiation, when the cultures are still quite small. All of the biochemical features analyzed above meet the above mentioned criteria, with the added bonus that measurement of the ethylene evolution rate is nondestructive - enabling the reuse of the culture in other experiments.

While the biochemical markers we have developed assist in the identification of embryogenic tissues, they only serve to define metabolic pathways associated with somatic embryogenesis. To test whether a metabolic pathway is not only associated with, but necessary for embryogenesis, further examination is required. A common method employed to examine the relative importance of a metabolic pathway is to block the pathway and examine the effect on somatic embryogenesis. The blockage is usually accomplished by selectively inhibiting a key enzyme in the pathway. By choosing the appropriate culture media in which to substitute the inhibitor, we can examine the role that certain metabolic pathways have in either the events responsible for embryo production or the events critical to the growth of embryos into plants.

To date, we have found that inhibition of the enzymes responsible for the production of nonspecific and specific reducing agents have an influence on the development of somatic embryos into plants, but have little effect on the events that lead to the production of the embryos. The result of the enzymic inhibition is a smoother and more uniform transition between the two forms of growth.

PROSPECTS AND APPLICATIONS

The ultimate goal of our research program is to apply the somatic embryogenesis technique we have discussed to mature elite conifer trees important to the pulp and paper industry. This would enable "true-to-type" cloning and mass propagation of proven trees. To achieve this goal, two specific areas of research need to be addressed: 1) obtaining somatic embryogenesis from mature rather than juvenile starting tissue and 2) increasing the list of species where somatic embryogenesis can be obtained to include conifers of most economic importance - loblolly pine and Douglas-fir.

With regard to the first area of research, progress is being made at broadening the "window" or time-span in which somatic embryos can be induced from starting tissue of conifers. We have been able to obtain embryogenic callus very efficiently from mature Norway spruce seeds. A recent report extended the window even further by using tissue for germinating seeds.[8] Although these examples are still with juvenile starting tissue, they clearly demonstrate that somatic embryo induction in conifers is not restricted to a narrow time window during immature embryo development. Therefore, they offer encouragement toward extending the window even further to mature tissue. With regard to obtaining somatic embryogenesis in species other than Norway spruce, we are devoting a major effort to obtaining somatic embryogenesis in loblolly pine and Douglas-fir. One approach, as we have discussed, is to use biochemical markers to identify cells with embryogenic potential. It is also encouraging that all recent reports of somatic embryogenesis in conifers, regardless of species, describe a type of embryogenic callus similar to the embryogenic Norway spruce callus we described earlier: a white callus with somatic embryos dispersed within a mucilaginous matrix. Having these distinct

visual and tactile markers should be useful in attempting to identify embryogenic callus in species that have thus far been recalcitrant.

The above discussion provides just one example of how biochemistry can assist in making plant tissue culture more of an exact and less of a descriptive science. We are also initiating research in the area of molecular biology - both as a tool to characterize the embryogenic state and for purposes of genetic engineering. Interdisciplinary approaches like those being undertaken at The Institute of Paper Chemistry will be required to fully exploit applications of biotechnology in forestry.

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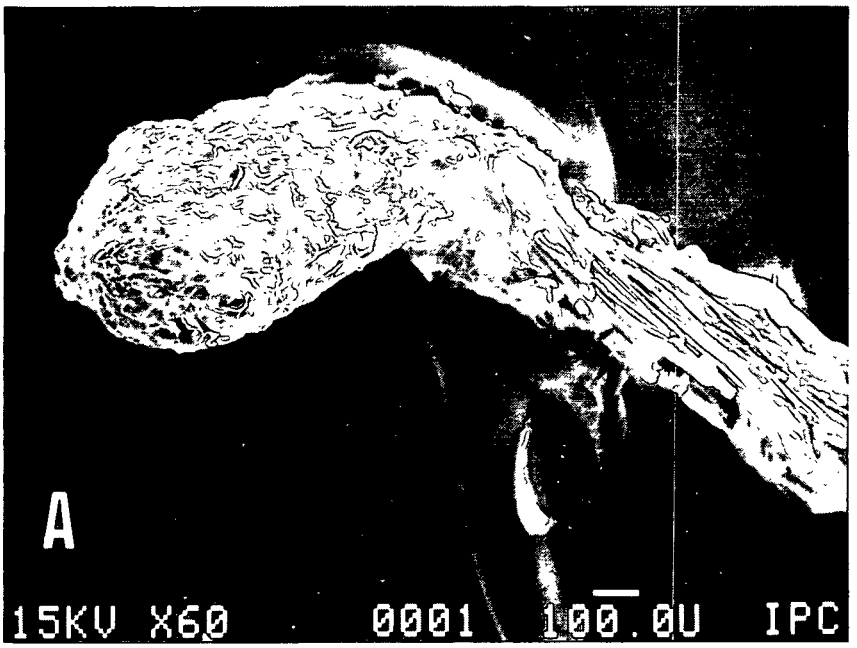
Figure 1. Conifer cells are grown and maintained as embryogenic callus.



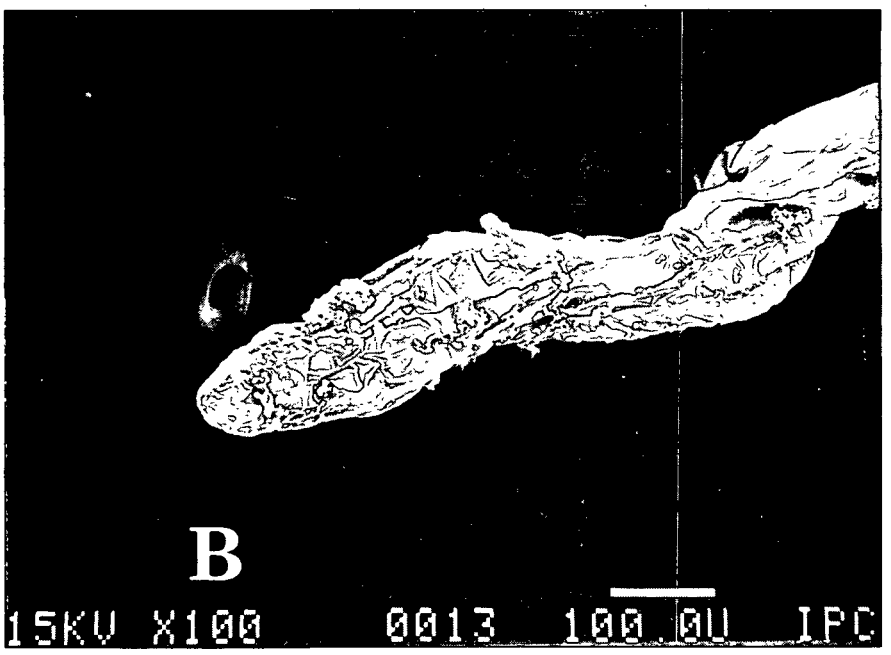


Figure 2. Electron micrographs of Norway spruce somatic embryo (A) removed from embryogenic callus and natural seed-embryo (B) removed from immature Norway spruce seed.

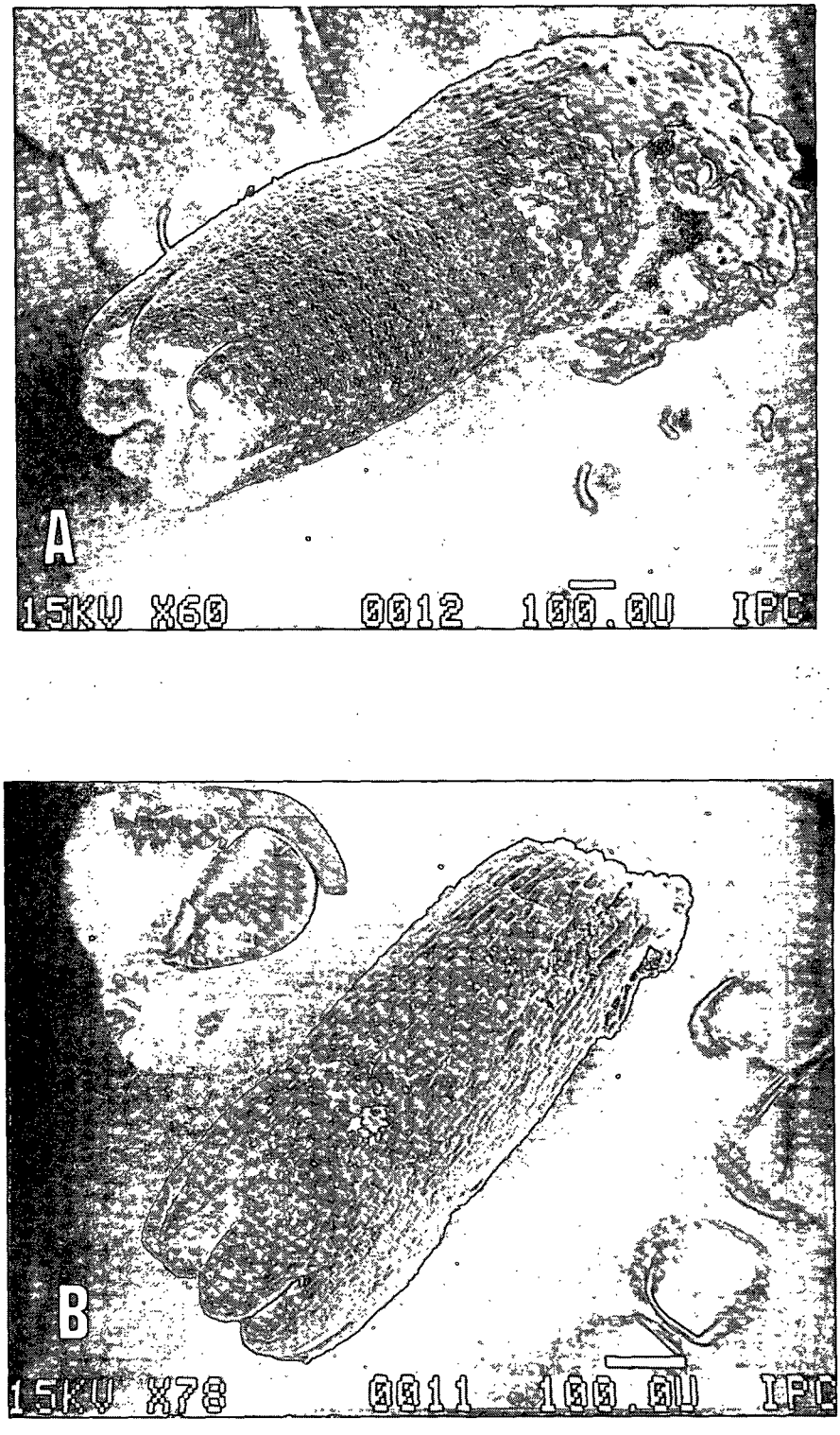


Figure 3. Electron micrographs of fully developed Norway spruce somatic embryo (A) and natural seed-embryo (B).

Figure 4. Norway spruce somatic embryo plant growing in soil mix. The plant is 3 months old and approximately 3 cm in height.