THE POTENTIAL FOR CLONING WHITE SPRUCE VIA TISSUE CULTURE

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ABSTRACT.-- When hypocotyl segments of white spruce were placed with their apical ends in an agar medium containing 10^{-5} M 1-naphthaleneacetic acid (NAA) but no 6-benzylaminopurine (BAP), 50 percent formed roots. Almost all segments placed with their basal end in a medium containing 10^{-5} M BAP with 10^{-7} M NAA formed scalelike organs. When explants with the scalelike organs were transferred to media containing neither BAP nor NAA, the organs grew into needles, buds developed, and elongated, branched shoots were obtained from these buds. A number of shoots have been obtained from a single hypocotyl segment. One such shoot has rooted. These results strengthen the hypothesis that a small explant could be used to mass propagate a superior tree.

There will probably be a massive shortage of wood in the world market within 20-25 years (Keays 1974)2,3/ this reason there is an urgent need to increase the productivity of our forests.

One means of doing this is by reforesting with superior genotypes that grow faster, larger, straighter, are responsive to silvicultural practices, and are resistant to diseases and pests. The question is, "How to get the superior genotypic material for planting?"

One way is through breeding, as has been done successfully for many agricultural crops. Unfortunately, the long life cycle of trees makes obtaining superior varieties a lengthy process. In Ontario, 30 million white spruce seedlings are outplanted each year but only 10 percent are from seed collected in seed orchards and production areas. Because the

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[3] Jones, P. 1975. Address to the Canadian Pulp and Paper Association, Montreal. March 25.

trees in the present orchards are not the progeny of controlled breeding programs and have not been tested, the genetic gain in using seed from them is probably small. Thus it will be some time before genetically improved and proven trees are available in sufficient numbers and are mature enough to produce useful quantities of improved seed.

Another way to reforest with superior genotypes is to take advantage of the variation in the natural population and vegetatively propagate superior specimens. This could allow immediate productivity gains. The problem here is that by the time a tree is old enough to demonstrate that it is superior, it is difficult to propagate it vegetatively by rooting cuttings. As the age of the ortet of spruce increases beyond 10 years, rooting ability decreases rapidly. "Percent rooting, speed of rooting, root length and number, survival, and growth in and after the year of rooting, all decrease with increasing age of the parent tree" (Girouard 1974). Another problem is that rooted lateral shoots must undergo a transition from plagiotropic to orthotropic growth and this occurs less readily with increasing ortet age (Girouard 1974). A final problem is that cuttings may grow slower than seedlings. For the first 5 years at least, the stem volume growth of Pinus radiata cuttings was considerably less than that of seedlings (Sweet and Wells 1974, Shelbourne and Thulin 1974, Libby 1974). If these differences continue beyond 5 years, they are probably associated with meristem aging. This could cancel out gains expected from the use of cuttings rather than seedlings from superior genotypes.

In an attempt to bypass some of the problems mentioned above, we have been investigating the use of tissue culture for the vegetative propagation of forest tree species.

Having obtained suitable media for the continuous culture of callus and suspensions of white spruce, jack pine, and American elm, we have recently begun attempts to induce differentiation in our cultures. The present work utilizes aseptic hypocotyl segments from 6 to 12-day-old white spruce seedlings (fig. 1). This is a model system because practical application would require the use of tissue from trees old enough to have demonstrated superior qualities. But the model system is useful at this stage for several reasons: (1) it is easier to obtain large numbers of sterile explants by surface-sterilizing seeds and then germinating them aseptically than it is to sterilize tissue from trees in the field; (2) there are no seasonal variations in the starting material because seeds can be germinated in a growth chamber any time; and (3) tissue from juvenile plants is often easier to induce to differentiate, so it makes more sense to start here and proceed to the more difficult later.

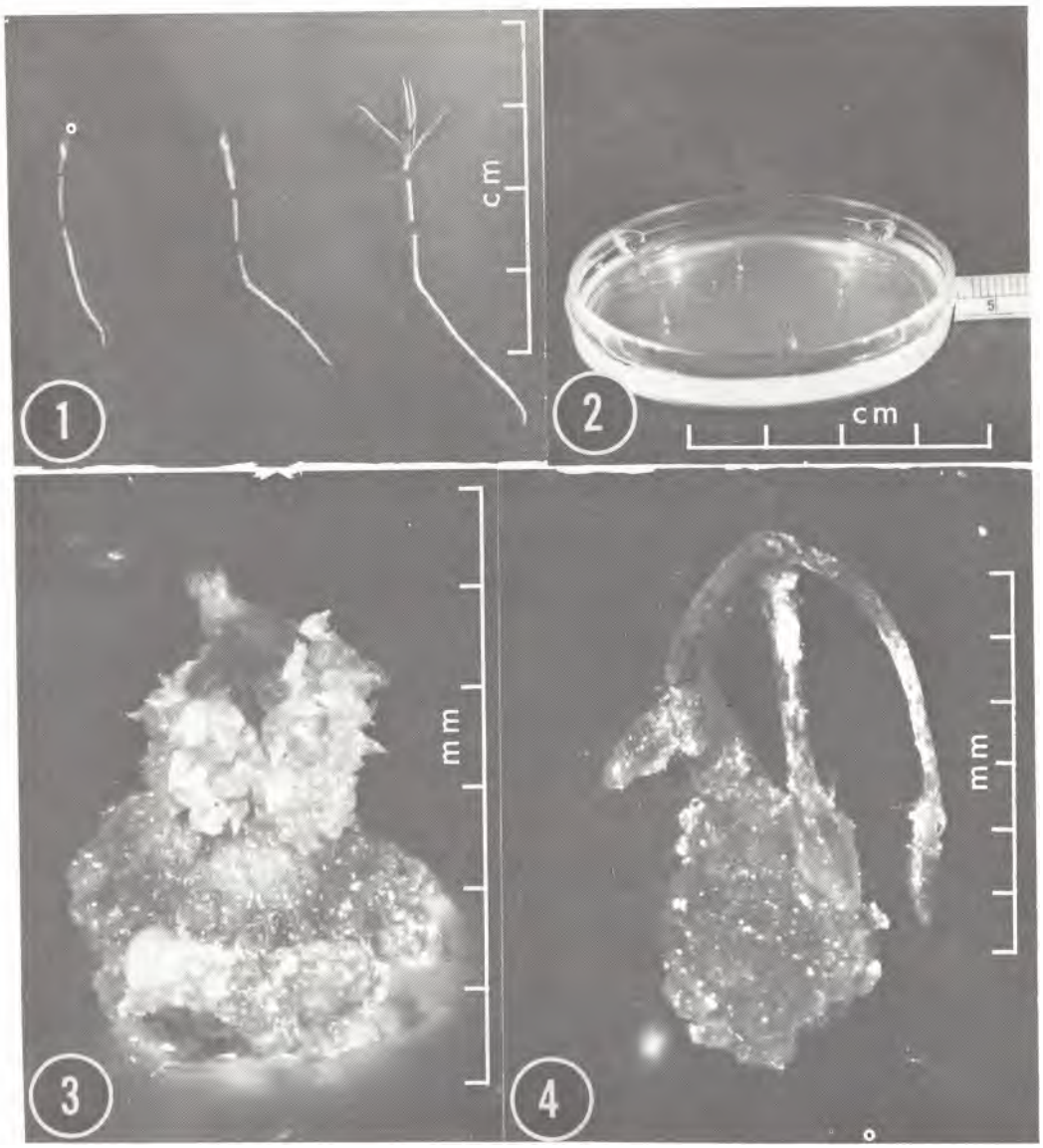

Figure 1.--Six to 12-day-old white spruce seedlings. A 5 to 7 mm segment of hypocotyl has been excised from each seedling taking care to exclude the apical meristem.

Figure 2.-- Hypocotyl segments with one end embedded in agar medium in culture dish.

Figure 3.--Hypocotyl segment after incubation on a medium containing a high level of BAP and low NAA. There is a basal callused region in contact with the agar medium, a swollen intermediate region covered with scalelike organs, and an unswollen tip. There has been little longitudinal growth.

Figure 4.--Hypocotyl segment after incubation on a medium containing a low level of BAP and a high level of NAA. There is a basal callused region and above this a portion of unchanged hypocotyl bearing three roots. There has been considerable elongation of the explant (cf figure 3).

METHODS AND RESULTS

Detailed methods and media have been reported elsewhere (Campbell and Durzan 1975). Hypocotyl segments used were 5 to 7 mm long and were cut carefully to exclude the apical meristem (fig. 1). The segments were cultured in an agar medium in 50-mm petri dishes in a vertical position with the end formerly attached to the radicle embedded in the medium (fig. 2). The segments differentiated by expanding laterally but very little vertically (fig. 3). There is some callusing at the base, i.e., the portion in contact with the medium. Above this there is a portion that is swollen and has scalelike outgrowths and at the top there is an unswollen portion. The fact that the scalelike outgrowths are below the tip, together with the cutting procedure, demonstrates clearly that the outgrowths did not arise from a pre-existing apical meristem.

The key factor controlling this differentiation is the cytokinin-auxin balance (table 1). In this experiment, segments were cultured on media containing different amounts of the plant growth regulators: 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). The frequency of induction increased with increasing BAP and decreasing NAA concentration.

Table 1.--Explants that had produced at least one scalelike organ after 20 weeks in culture under varying conditions, [1] as a function of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) concentration in the medium[2]

(In percent)

BAP (M)	NAA (M)		
	10^{-5}	10^{-7}	0
10^{-5}	14	64	69
10^{-7}	0	0	0
0	0	0	0

1 13.5 hours light at 20°C and 10.5 hours of dark at 7°C.

2 The number of segments per treatment ranged from 11 to 17 (differences due to contamination losses).

The active range and different environmental conditions were examined in more detail in a further experiment (table 2). Induction occurred better and faster under constant than under varying conditions but by

98 days the number of explants that had produced organs had reached a maximum in both environmental conditions. The explants cultured under varying conditions were inhibited by the highest level of BAP whereas those under constant conditions could not be induced to develop further with subsequent treatments.

Table 2.--Explants that had produced at least one scalelike organ after 14 weeks in culture, as a function of NAA and BAP concentration in the medium [1]

(In percent)

	NAA (M)			
BAP (M)	10^{-5}	10^{-6}	10^{-7}	0
10^{-4}	25(13)[2]	78(38)	82(28)	89(19)
10^{-5}		75(50)	84(56)	86(58)
10^{-6}		45(22)	59(50)	53(38)

[1] The number of segments per treatment ranged from 20 to 36

[2] The figures in parenthesis were obtained under varying conditions (13.5 hours light at 20C and 10.5 hours dark at 7C). The figures not in parenthesis were obtained under constant light and temperature at 20C.

Those induced by lower levels of BAP did develop. Thus, the optimum induction occurs under constant environmental conditions with 10^{-5} M BAP and 10^{-7} M or 0M NAA.

In the previous experiments, the explants were placed right side up. When they were inverted (i.e., the end formerly attached to the apical meristem was placed in the medium) organs were again induced at about the same frequency with the same BAP-NAA optima. The difference was that roots could also be induced (fig. 4, table 3). The requirement for root induction was high NAA and low BAP. This is opposite to that for induction of the scalelike organs and thus follows the pattern of differentiation control reported by Skoog and Miller (1957).

Having induced roots and scalelike organs, attempts were made to control further development. The roots did not develop much beyond that shown in figure 4 and eventually died, although a number of different media were tried. The scales did develop if the explants were left on the same media for a period or were transferred to a medium containing neither BAP nor NAA (this was probably the same treatment because the

growth regulators likely got depleted in the first instance). The scales became elongated and more needlelike (fig. 5). When explants at this stage were broken up and cultured for a further period on medium lacking NAA and BAP, numerous elongated shoots bearing normal-looking needles developed (fig. 6).

Table 3.--Explants that had produced at least one root after 12 weeks in culture, as a function of NAA and BAP concentration in the medium

(In percent)

	NAA (M)		
BAP (M)	10^{-5}	10^{-7}	0
10^{-5}	15(19)[2]	0(0)	0(0)
10^{-7}	28(28)	0(0)	0(0)
0	47(25)	0(0)	0(0)

[1] The number of segments per treatment ranged from 25 to 36.

[2] The figures in parenthesis were obtained under varying conditions (13.5 hours light at 20C and 10.5 hours dark at 7C). The figures not in parenthesis were obtained under constant light and temperature at 20C.

All of the previous steps have been repeated and occur with a high frequency. The final step, the rooting of induced shoots to regenerate a whole plant, has only occurred recently so its frequency, control, and repeatability have not been fully determined. The shoot in figure 7 produced roots following shoot elongation on the medium lacking BAP and NAA. It has been transferred to a sand-sphagnum mixture in a pot and is thriving.

DISCUSSION

We have demonstrated that it is possible to regenerate whole white spruce plants from a small piece of tissue. Similar results have also been recently reported for longleaf pine (Sommer et al. 1975), Cryptomeria japonica (Isikawa 1974), and Douglas fir (Rediske 1975). Once the process has been optimized, the next step would be to attempt to repeat it with tissue from a mature tree. Because the shoots in the present work apparently arose from epidermal tissue (Campbell and Durzan 1975), a

Figure 5.--Hypocotyl segments after a prolonged incubation on a medium containing high BAP and low NAA. Depletion of the growth regulators in the medium has allowed the scalelike outgrowths to elongate.

Figure 6.--A number of shoots obtained by dividing up an explant, such as those in figure 5, and incubating the pieces on a medium lacking BAP and NAA.

Figure 7.--An explant bearing two induced shoots that have rooted (arrows).

logical tissue to try would be needles. Further justification for this choice comes from the fact that leaf epidermal tissue from mature herbaceous plants has been readily induced to form shoots (Tran Than Van and Drira 1971, Chlyah and Tran Than Van 1971). If each needle could produce 10 shoots and a single bud has 100 to 200 needles, the number of potential propagules from a single tree would be enormous. It should also be noted that because the plantlets induced from tissue cultures do not arise from pre-existing apical meristems, their apical meristems may be rejuvenated. Slow growth, plagiotropic growth, and poor rooting may not be a problem with such vegetative propagules.

A slightly different way of using tissue culture in propagation would be through suspension cultures. Tissue from many plant species can be grown in an agitated liquid medium as single cells and small clumps--much like bacteria. We have done this in our laboratory with white spruce, jack pine, and American elm. The doubling time of such cultures is less than a week, so a tremendous number of cells and clumps can be generated in a short time. For example, starting with 1 gram of tissue, 1 kilogram could be generated in 10 weeks and 1,000 kilograms in 20 weeks. With a number of plant species it has been possible to induce the free cells and clumps to form embryos that develop into normal plants capable of producing viable seed (Steward et al. 1964). The best studied example is carrot. If white spruce embryos could be induced in suspensions at the same rate as carrot embryos (Halperin 1967), then in only 100 liters of medium, 73 million plantlets could be produced and raised in containers for field planting. This is more than enough to plant 100,000 acres (the predicted rate of white spruce planting in Canada to 1985 at 8 by 8 spacing (Carlisle and Teich 1971).

Is this possible? We can grow spruce tissue as suspensions. A number of herbaceous species have been induced to form embryos in suspensions (Halperin 1967, Thomas and Street 1970). White spruce tissue cultures have been induced to regenerate whole plants. Cambia and vascular tissue have been induced in white spruce suspensions (Durzan et al. 1973). Thus, the probability is high that it is only a matter of time and effort to achieve effective vegetative propagation of spruce by tissue culture.

The predicted world wood shortage by the year 2000 makes it seem mandatory that all methods of increasing forest productivity be fully explored immediately. Through tree improvement and genetics, Canada will be able to increase the productivity of its forests and thus fulfill its responsibility to help minimize the predicted world wood shortage.

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