techniques and these are developed over years of trial and error along with help from various universities that are doing continuing research in this area.

Lab space and layout is another important aspect. One point that might be made is that there never seems to be enough space, even though initially the projected layout seems adequate.

CONIFER TISSUE CULTURE

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Abstract. Significant progress has been made during the last five years (1972-1977) in the commercial implementation of plant tissue culture technology; larger commercial nurseries have pioneered the application of this technology. More recently, several forest products industries have shown an interest in plant tissue culture. The current status of these forestry programs in conifer tissue culture, and some recent advances in basic technology, are reviewed.

TECHNOLOGY

Almost three decades have passed since the first experiments in plant tissue culture were reported (15), demonstrating the potential for vegetative propagation of selected plant tissues. This early work has been refined and extended to many plant species; among the most intensively studied has been the carrot and tobacco systems. At the time the original reports of these studies were being published, few in forestry could envision a significant impact in the field of domesticating forest trees. While this domestication is still under debate, many forest products companies have accepted tissue culture as a viable alternative to traditional reforestation practices (10).

The *in vitro* culture of conifer tissues has considerable significance for the forest products industry. Provided an effective tissue culture system is available, the technology can be implemented for mass propagation. *In vitro* vegetative propagation may be used to supplement an existing or planned program such as grafts or rooted cuttings. Similar to horticultural applications, tissue culture may be used as a tool in the forest products industry to eliminate pathogens from mother plants, or to augment an existing breeding program.

Tissue culture laboratories are now in existence at, or being planned by, Weyerhaeuser Company, International Paper Company, Crown Zellerbach, ITT-Rayonier and St. Regis. The first

two companies have the largest tissue culture programs at this time.

This paper will review some of the recent progress made in conifer tissue culture and discuss some results from our laboratory.

REVIEW OF LITERATURE

Conifer embryos were first cultured in sterile conditions by A. Schmidt in 1924 (14). For the next 40 years various researchers succeeded in culturing embryos, cambial tissue, megagametophytes, mature pollen, shoot tips, roots, and the callus derived from many of these explants. A detailed atlas of gymnosperms cultured *in vitro* from 1924-1974 provides considerable information on the subject (4).

Adventitious buds have differentiated in sterile culture from embryos of pine and Douglas-fir (5,11,13,16,17,18), cotyledons of Douglas-fir and western hemlock (7,8), buds of Douglas-fir (2,3), young shoots of balsam fir (1) and hypocotyls of white spruce (6). In many cases the induced buds developed into shoots which could be rooted.

Shoots have also differentiated from cotyledon-derived callus, subcultured needle callus and callus from seedling stem explants of Douglas-fir (18). All of the investigators reported low rooting percentages of tissue culture-derived shoots. In 1976 Boulay found that resting buds and shoot apices taken from trees two-years-old or less could regenerate viable shoots that would root (3).

The usefulness of plant tissue culture in tree improvement programs has been cited (9). The diverse applications of this technology to such programs include freeze preservation of gene pools, production of homozygous specimens, study of host-parasite relations, production of disease-free specimens and prediction of phenotypic expression. Others have cautioned against the over-emphasis on the use of this technology solely for the purpose of mass propagation. Two criteria are of foremost importance: 1) consistent differentiation of buds and roots, embryoids or plantlets must be achieved with a minimum of time to reduce the incidence of genetic change under artificial cultural conditions; 2) subsequent generations of plantlets or propagules must be easily attained for mass production of desirable genotypes. These criteria have not been met for optimizing commercial production (5).

MATERIALS AND METHODS

Douglas-fir seeds, obtained from Weyerhaeuser Company's seed production facility in Rochester, Washington, were germinated and 2- to-4-week-old seedlings were harvested. The seed-

ling tops were then surface sterilized in 10 percent Clorox for 6 minutes and washed three times in sterile deionized water. The cotyledons were cut into 2-to-4-mm pieces and placed on nutrient media described by Cheng (8). In some experiments cotyledon explants from individual seedlings were distributed uniformly on different culture media; each group of media containing explants from the same seedling will be referred to as a set.

Plantlets were obtained by placing the shoots in a nutrient medium containing 0.05 mg/L NAA solidified with agar. The root tips were then prepared histologically by the squash technique and acetocarmine staining for chromosomal analysis.

Growing shoot tips were removed from an 11-year-old grafted superior Douglas-fir tree during the period March through July; the original graft was taken from a 53-year-old tree. Denuded shoot tips, 3-5 cm long, were sterilized and placed on the same media in culturing the cotyledons. Leaves from these actively growing shoots were cultured separately.

RESULTS AND DISCUSSION

Somatic tissues exhibited organogenesis by forming bud-like outgrowths on the surface of the cotyledons after 2-4 weeks in culture. In most cases, these buds differentiated leaf primordia after 4-6 weeks on the initial medium (Figure 1) followed by shoot elongation (Figure 2). Alternatively, callus may develop from the same tissues which responded organogenetically, or callus growth may persist without any organized development. Shoots developed from cotyledon explants were excised, separated, and cultured on a medium lacking hormones to promote further growth. These were later transferred to solidified rooting medium, with only 2-3 mm of the basal end embedded in the medium. We have obtained, in six treatments including 3,300 shoots, a range of rooting response from 3 to 11 percent with an average of 5 percent (Figure 3).

Root tips from plantlets differentiated from somatic cells, were found to contain the diploid chromosome number ($2n = 26$), indicating chromosomal stability of these cells (Figure 4).

There is evidence that considerable variability exists in wild seedling material as demonstrated by the wide range of responses in bud development. When 10 sets of cotyledons from wild seedlings were tested on five different media the response of each set was found to be highly variable. The number of bud primordia produced ranged from 21 to 264 with an average of 97.6 per seedling. These data indicate a high level of genetic variance in terms of *in vitro* morphogenetic potential. In com-

paring overall response to the five media there was a slightly greater than two-fold difference between the medium effecting the least and most production of buds (Table 1).

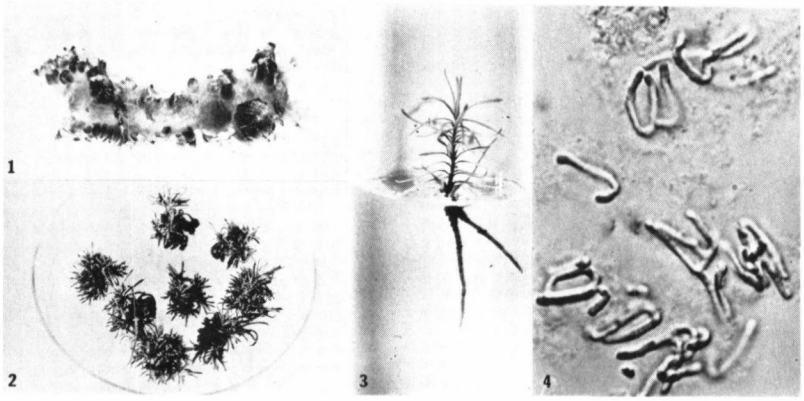

Figure 1. Cultured cotyledon explant of Douglas-fir showing adventitious bud formation (after 5 weeks of incubation). **Figure 2.** Large number of expanded shoots on the cotyledon explants. **Figure 3.** Douglas-fir plantlet produced *in vitro* after root induction and growth on agar rooting medium. **Figure 4.** Chromosome configuration (2n = 26) in root-tip cells of *in vitro* produced plantlets.

Table 1. Differentiation of bud primordia on Douglas-fir cotyledon explants *in vitro* as a function of wild seedling responses to 5 media.

	Seedling Number										
Medium	1	2	3	4	5	6	7	8	9	10	Total
3	71	54	3	18	0	32	32	34	18	0	208
4	38	0	14	0	76	7	43	31	25	0	234
5	45	11	0	0	4	11	17	15	7	2	112
6	61	15	9	1	6	0	25	38	17	10	182
7	49	28	15	4	24	55	30	16	10	9	240
Total	264	54	41	23	110	106	147	134	77	21	

We have also found that relative age of the tissue selected for culture is critical as was established with other tissue culture systems (12). Our data showed that there is a diminished responsiveness *in vitro* of cotyledons selected from 2- to 4-week-old and 7- to 8-week-old seedlings. Mature cotyledon tissue is far less responsive than immature tissue for both wild seedlings and genetically selected seedling stock (Table 2).

Results similar to that of Boulay (3) have been obtained with shoot tips of actively expanding spring growth of Douglas-fir. Removing the needles causes activation of axillary bud growth (Figure 5) and in certain media, the development of

adventitious buds (Figures 6 and 7). Leaves from mature trees have also formed buds and shoots (Figure 8), but aging of the host explant tissue resulted in degeneration of these shoots. Still, this represents a significant achievement toward propagation of somatic tissue from older, genetically improved forest trees.

Table 2. Percent bud induction on young (Y) and mature (M) Douglas-fir cotyledons from wild and selected seedlings after 6 to 8 weeks in culture.

	3 × 76		28 × 88		53 × 71		Wild	
Medium	Y	M	Y	M	Y	M	Y	M
2	51	9	89	13	59	15	43	10
3	61	4	85	4	54	1	6	1
4	50	9	97	7	45	2	—	—
5	54	2	49	5	77	2	86	—
6	63	4	83	6	68	7	26	1

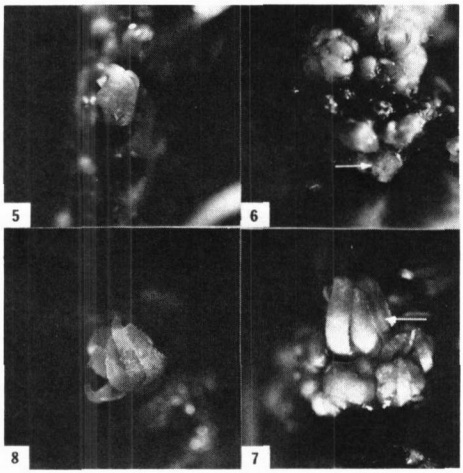

Figure 5. Activation of axillary bud growth on a denuded cultured stem. **Figure 6.** Adventitious bud formation and callus induction (arrow) of a 64-year-old Douglas-fir tree cultured on bud induction medium. **Figure 7.** Adventitious bud formation around the actively growing axillary bud (arrow) on a cultured stem taken from a flowering Douglas-fir tree. **Figure 8.** Adventitious bud formation associated with callus induction on a cultured needle from a mature tree.

CONCLUSION

When perfected, conifer tissue culture techniques will aid genetic tree improvement research and reforestation programs.

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DICK MAIRE, Moderator: We have time now for several questions for our speakers.

WES HUMPHREY: Dr. Uchimiya, part of the reason for asking you to come is because of the great amount of controversy that we see concerning DNA type of research in the newspapers. So much of it seems to be negative. Would you like to comment a bit on positive aspects of DNA type research?

HIRO UCHIMIYA: Much of what we know about genetic engineering at this moment is particularly for bacterial systems.

People are worrying about biological hazards such as transfer of cancer virus into bacterial systems, which is DNA taken outside of bacterial chromosome and can replicate in tremendous amounts, about 3,000 copies in only one night. So far this can only work only in the bacterial system. Nobody has shown that the same system works in animal or plant tissue. So people try to jump from lower organisms, such as bacteria, to higher plants or animals. Recently we are organizing some guidelines for the use of genetic engineering techniques, especially for plant systems. There is the idea of transfer of nitrogen fixation genes from soybean to rice or wheat; but we don't know if the techniques are hazardous or not. There is still much research to be done. I can only say that at this moment we can see some new ideas coming in several years.

BRUCE BRIGGS: Dr. Wochok, we know that juvenile shoots will root better than mature shoots. Do you have any ideas of techniques, whether physical or chemical, of getting plant material to revert back into juvenile wood. The second question is on your various tissue culture media. Was the variability in salinity or in hormones?

ZACHARY WOCHOK: Second one, first; the five media differed only in hormone composition. First question. We were having difficulty, as I understand, in vegetative propagation up to just recently with rooting adult Douglas fir cuttings and, as I understand it now, that has been overcome. The rooting percentages are very high. One method of vegetative propagation would be to establish hedging stock plants to maintain young material. This is being looked at in our work and, of course that information won't be coming out very soon because it takes a while to establish a hedging orchard. Now with woody material, I guess I didn't quite understand your question. Maybe you could be more specific.

BRUCE BRIGGS: We know that cuttings from wood in a juvenile stage root better than adult wood. Just because wood is young on a plant doesn't make it juvenile wood. That is the reason I was concerned. We know that you can have juvenile wood and adult wood all on the same plant. Is there some way it can all be converted back into juvenile wood? I noticed yesterday on the tour, seeing the eucalyptus which had mature wood on the tops, but near the root system there was juvenile wood, whose leaves were nice and blue.

ZACHARY WOCHOK: On the slide which I showed you, which is from 63-year-old material, the shoots which were regenerated in that instance must be considered juvenile material. That they are coming from mature tissue makes them mature, I don't hold to that. The fact of the matter is, the regenerative process in and of itself would indicate that you are not carrying

mature gene material into the new cells. There is no transmission of age into that material. Now one gets into this controversy of a meristem on a mature tree — is that considered juvenile? Some people will say, no, because it is a part of the mature tissue. Now strictly speaking the meristem is juvenile by definition. It can be influenced, however by the sub-tending cells. If you have older tissue that is now putting out a new flush of growth, that meristem can be influenced by the physiological conditions of the tissue of which it is a part. Hence, in a seedling, that meristem on the shoot tip or the leader is going to be different certainly than the meristem on the lateral branch.

VOICE: I have heard of embryo culture of certain palm species, I wonder if meristem culture or tissue culture has been applied to any of the palm species.

GARY GALLUP: We have tried applying tissue culture to several palm species. Keeline-Wilcox has done some work on this too. I don't know what their success has been. Palms are very slow in all stages of development; there has been work done but I don't know how far it has gone.

ESTHER LAWYER: Could you comment on any tissue culture media which you think would be especially good to promote formation of callus, which is necessary for formation of the graft union.

ZACHARY WOCHOK: I can't speak to the technique of grafting, but if you want to induce callus, the auxins, or auxins in combination with cytokinins, are the best way to do it. They could be applied in a paste, or what have you.

ESTHER LAWYER: We tried dipping our scions and our rootstock in various solutions to promote callus. One thing that we tried was vitamin C and we had positive results with this.

ZACHARY WOCHOK: What is the problem?

ESTHER LAWYER: The problem is to promote graft formation of callus for healing of the grafted materials.

ZACHARY WOCHOK: Then I would suggest you use auxins and cytokinins. Vitamin C then would possibly counter any problems you might have with browning or aging of the tissue, or building up of phenol compounds. That, together with the auxins, should do the trick. I think what you want to do is to screen compounds and probably the best ones to go with are indolebutyric acid or naphthaleneacetic acid; probably indolebutyric acid would be the first that I would use.

VOICE: I have two questions. The first to Mr. Gallup: What is the reason for reversion to original type in ferns; the second

one is for Dr. Wochok: What briefly is the method by which you are able to root the conifer cuttings.

GARY GALLUP: THe reason for the reversion back to the original type of fern is the same reason that it has "sported" to begin with. It is usually a chimera; a different tissue covers the meristematic tissue and you damage this through cutting and things of this sort. It reverts back to the original tissue or it will "sport" again into something entirely different, maybe better or worse. It is just unstable tissue. There are a lot of ferns that are stable — you can take the tissue and go on forever without any problems. But in the very unstable ferns, the very fancy Boston types, there is a continuing problem with "sports."

VOICE: Dr. Wochok, you mentioned that there had been a recent development; that it is now possible to root mature Douglas fir cuttings. What is the procedure?

ZACHARY WOCHOK: The technical report hasn't come out yet. So I can't go into great detail, but I can say it involves the traditional method of dipping in a rooting auxin compounds. I don't think that is anything different from what you are using now. That is not the critical issue, though. The critical issue has been to prime the material, if you wish, into its biorhythmic patterns because the tissue has to get into an environmental set of conditions. The environmental conditions could be photo-period, short-days or long-days, or whatever the plant needs. It will be different for every plant. So you have to find out what the best regimen is for the given plant that you are working with, such as the day-length versus night period, and, of course — temperature.

HUDSON HARTMANN: I want to refer back to Dr. Wochok's last response. Who is doing the work on biorhythms in rooting Douglas fir cuttings and where is it going on? Where and when will it be published?

ZACHARY WOCHOK: The work is being done in the Rochester, Washington, facility of Weyerhaeuser Co.