7/1/93
LCMR Final Status Report-Summary-Research

I Aspen Hybrids and New Tissue Culture Techniques.
Forestry 19.

Program Manager: Carl A, Mohn
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Department of Forest Resources
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A. M.L. 91, Ch 254 , ART. 1, Sec.14, Subd. 7(f)
Appropriation: $70,000
Balance: $ 925 (estimate for 7/1/93)

Aspen Hybrids and New Tissue Culture Techniques: This appropriation is to the University of Minnesota, Department of Forest Resources to research tissue cultured aspen and hybrid aspen clones.

B. Compatible Data: NA

C. Match Requirement: None

II.NARRATIVE. This project's goal is to continue to develop the production efficiency of two new tissue culture (cloning) techniques and to examine clonal fidelity (trueness-to-type) of materials produced under these systems. The investigation will compare a method involving a tissue from which plants are derived naturally (plants derived from root cultures) versus a technique involving tissue from which plants are not derived naturally (plants produced from very thin leaf midvein sections). Both techniques have been developed by scientists at the University of Minnesota and are at the stage indicating promise as commercial propagation methods. This grant will provide funding for a joint research effort between the University of Minnesota and Minn Vitro, Inc., an independent commercial tissue culture lab. The University of Minnesota will continue research on isolated root cultures. Minn Vitro, Inc. will assess the commercial production efficiency of the systems and develop labor efficient modifications. Clonal fidelity evaluation parameters will be jointly developed and used by both organizations.

The relatively high cost of conventional shoot multiplication tissue culture techniques is acceptable for some high value per unit crops. Large-scale clonal forestry planting of selected aspen and hybrid aspen has been limited by the relative high cost per tissue cultured plant which is in the $450/1,000 range. This is too costly to fulfill projected Minnesota forestry demand for aspen of 5 million trees per year even with the anticipated reduction in cost based on economics of scale.

Clones are desirable because they retain desirable characteristics such as disease resistance, fast growth, etc. of the parent plant. Cost reduction research, to date, has concentrated on automation and robotics. This grant proposes to investigate the biological components of adventitious propagule production, an area not yet scrutinized in any detail.

III.OBJECTIVES.

A. Increasing the biological efficiency of the root culture system.

1.Narrative: Approaches to increase efficiency will build on previous work on the in vitro culture of aspen root masses and shoot induction on small segments of these masses.

2.Procedures: Isolated root cultures are established from in vitro derived shoots which have been induced to form roots. These roots are excised and transferred to a liquid growing medium (Woody Plant Medium-WPM) supplemented with 3% sucrose and 1 uM auxin (naphthaleneacetic acid-NAA). The culture vessels are roller bottles that rotate at 1/4 rpm. Four clones have been used in preliminary trials. An "easy" and a "recalcitrant" clone have been identified and will be used for subsequent trials.

Three areas will be investigated:

a.Nitrogen. Results to date indicate that isolated root growth is enhanced by manipulating the form and/or quantity of nitrogen, i.e., ammonium:nitrate ratio and quantity.

Optimal nitrogen configuration will be tested by varying the nitrate:ammonium ratio on a 1:1, 1:2, 2:1 ratios at 1X and 2X the total N. Each vessel will contain 4, 2 cm root segments with 5-50 ml vessels per treatment. Growth of roots will be measured by weight gain, length and branching pattern.

b. Shoot production from root segments. Currently, root segments can be induced to form shoots. Conditions for optimal and reliable production have yet to be determined.

Enhancement of adventitious bud initiation on the root segments, i.e., in vitro suckering, will be tested by varying the length of the root segment and type of cytokinin. Root segments of 0.5, 1.0, 2.0 and 4.0 cm will be placed on solid basal medium with 0, 0.5, 1.0 and 1.5 mg/l BA (benzyladenine) or 0, 0.01, 0.1 and 1.0 mg/l Thidiazuron for 1, 2, 3 or 4 weeks. Twenty root segments per treatment will be tested. Data collected are the number of adventitious shoots per root segment and size (length) of shoot. These microshoots obtained will be used in "c" below.

c. Subsequent rooting and plantlet development of the shoots derived from in vitro suckering. These processes have not yet

been attempted. The rooting and development of the above microshoots will be assessed using an aseptic (Sorbarod) plug system and ex vitro, nonaseptic (Technic ulture) plug system. The Technic ulture system has been used commercially with hybrid aspen microshoots with moderate success. The primary problem is death of microshoots from pathogens during the rooting phase. The Sorbarod system has not yet been tested for aspen. Because it is aseptic, the problem of pathogens during rooting should be eliminated. It also allows the transition between aseptic to nonaseptic conditions, i.e., acclimation, to occur after the critical rooting stage. Survival and growth characteristics will be measured during this stage.

Experimental design. Treatments will be replicated. Differences in means will be determined using the HSD (Honest Significant Difference) test.

3. Budget:

a. Amount Budgeted: $26,000
b. Balance: $ 925

4. Timeline for Tasks

	July 91	Jan 92	June 92	Jan 93	June 93
a. Nitrogen study	**********				
b. Shoot production		**********	**********		
c. Rooting and acclimation			**********	**********	
d. Reporting	**	**	**	**	

5. Status:

a. Root growth. Results of experiments testing the effects on root growth of changing the form and quantity of nitrogen in media were not consistent with earlier observations. The ratio and concentration found in MS media proved superior and standard MS media is recommended.

Media, pH, light, auxin level and type of explant (root alone, root + stem, etc.) all were found to impact root growth. Media pH. of 5 - 6 gave the most rapid growth in roller culture. Light inhibited the growth of excised root segments but, over time, increased the growth of roots with stem segments attached. In experiments in which different basal media were compared, MS consistently gave the best development. Studies designed to find the optimum level of NAA (auxin) for excised root grouth identified values between 10^{-6}M and 10^{-7}M as the most beneficial.

Growth of roots on solid media or on filter paper supports in liquid media exceeded that observed in roller bottles. Using the best combination of auxins, light and roots attached to stems with leaves, root growth of about 15mm/day over 28 days was obtained. Assuming a production of 1 plant per cm of root this

would gives a multiplication factor of about 40 per cycle which is marginal for commercial propagation. Efforts to improve growth rate will continue.

In addition to the marginal rate of root growth achieved, other limitation to applying this and other system are: (1) a buildup of bacterial contamination with time in materials in culture and (2) the variation in the response of clones to culture. At present the only solution to contamination problem is avoidance via frequent initiation of new cultures, This is an additonal cost. The poor response of some clones in culture means that either specific protocols (most likely involving modification of growth regulator levels) be developed for recalcitrant clones or that only clones with favorable propagation characteristics be used. This will increase costs and/or reduced the availability of clones with desirable characteristics.

b. Shoot production from root segments. We identified thidiazron as the cytokinin of choice and MS as the most promising basal media. Our studies indicated that a thidiazron level between .01 and .1 mg/l was the most satisfactory. While auxin is always necessary in initiation media for leaf micro cross-sections, benefits for either the stimulation of buds or subsequent shoot development was noted when using root segments was clone dependent.

Small segments (1-2mm) were found to produce many more bud clusters per unit of root than the the .5/cm produce on the 1 cm segments initially. Shoot yields per unit of root increased by a factor of approximately 10 when smaller segments were used.

The development of shoots from bud clusters is critical in the production of plants. The production of useable shoots per bud cluster has ranged from .3 to over 2. Variation can be attributed in part to clone.

Rooting of shoots derived from roots has been successful and under optimal conditions over 90 percent of shoots produce vigorous plants. Ex vitro systems were satisfactory.

Bud and shoot production in experiments varied widely. Larger scale trials using the treatment which has been most effective to date are in progress. These tests were delayed by culture contamination and will not be completed until late 1993. They should provide a clearer indication of root production, plant yield per unit of root and other data needed for cost estimation.

6. Benefits: Optimization of the biological elements of this system will increase its overall efficiency. This will impact costs directly by reducing the required investment in equipment and labor per plant produced.

B. Optimize and evaluate the regeneration of adventitious plantlets using the leaf micro-section culture system.

1. Narrative: This objective will evaluate the commercial feasibility of high volume clonal production of 2 aspen, 2 big tooth aspen and 4 hybrid aspen genotypes using the leaf micro-section system.

2. Procedures: A new technique of micro-cross section (MCS) has been developed by scientists at the University of Minnesota to increase the multiplication efficiency of Populus clone NE299 and two other hybrid poplar clones. MCS has increased the potential of explants to form adventitious shoots by 25 times when compared to the standard explants used for adventitious shoot cultures. Additionally, the startup time is reduced from 6-12 months to 1-2 months. The increased number of shoots per explant, the increased number of explants per donor plant plus the shorter startup time increases the overall multiplication efficiency significantly. This efficiency is especially critical when donor plant material and time is limiting, a common occurrence when new hybrids are introduced.

The technique of MCS consists of cutting the explant into very thin cross sections using a Lancer Vibratome. This microtome vibrates as it slices tissue which results in minimal tissue damage. Tissue cross section of specific thickness can be readily prepared in this manner. Researchers at the University of Minnesota tested several thicknesses of cross sections 100, 200, 300, 400 and 500 Êm as well as a 1 cm control. The most efficient thickness (400 Êm) averaged more than 4 uniform shoots per MCS. This is equal to the production efficiency of the 1 cm control. However, using MCS, 25-400 Êm sections can be realized from 1 cm of donor material and results in a 25 times increase in productivity. Only NE299 and two other clones have been tested by the University of Minnesota.

Minn vitro, Inc. has been funded by the USDA-SBIR (Small Business Innovative Research) program, Phase I to test four hybrid aspen and two aspen clones using MCS. Preliminary results indicate a strong genotype response demonstrating that the protocol that was established for NE299 does not necessarily work well with all hybrid and non-hybrid aspen. Modifications to the established protocol, i.e., basal medium differences and plant growth regulator variations, are underway. The SBIR project should establish the MCS protocol for the four hybrid aspen and two aspen selections. LCMR funds would be used to establish the protocol for Big Tooth Aspen and to continue work after termination of the SBIR grant that would allow the four hybrid aspen and two aspen to be tested on a larger scale.

Based on the outcome of results from the SBIR project, a single medium composition for each clone will be selected. Two hundred jars of 10 MCS each will be initiated per clone. This should produce a projected 8,000-10,000 plants per clone. The plantlets will then be field tested and used for Objective C. Time and

cost data will be collected during all stages of production, i.e., a) initiation, b) adventitious bud induction and development, c) rooting, d) acclimation and e) establishment.

3. Budget:

Amount budgeted:	$25,500
Balance:	$ 0

4. Timeline for Tasks

	July 91	Jan 92	June 92	Jan 93	June 93
a. MCS establishment	**********	**********			
b. Shoot production		**********	**********		
c. Rooting and acclimation			**********	**********	
d. Reporting	**	**	**	**	**

5. Status: Work on the pilot propagation study of selected clones was completed. Data were collected for 5 clones: three hybrids (MINN VITRO's #1,#8 and #22) and two trembling aspen clones (U of M. #3 and #17A). The fourth hybrid clone was not responsive to the MCS system. Two bigtooth aspen (Populus grandidentata) clones were to be included in this study but uncontaminated cultures could not be derived from field materials.

Studies designed to refine the initiation medium for selected clones indicated that media with .05 mg/l thiodiazron and .05 mg/l NAA was most effective in inducing shoots in the clones tested.

Clonal differences in shoot development which must be accommodated by a propagation system were noted. For some recalcitrant clones a longer period on developmental medium resulted in good microshoot development. However, the poor response of some clones is a limitation of the proposed propagation system.

Cold storage is critical in controlling timing in a production system. Studies indicated that microcross sections with bud cluster can be successfully stored. However, contamination and mortality will decrease the number of microshoot produced. Cold storage does not reduce the vigor of the resulting microshoots.

Rooting and acclimation trials utilizing shoots from the MCS studies indicate that rooting varied by clone with a range from 64 to 92 percent. Survival of the rooted shoots through the acclimation process was 95 percent. On average about 85 percent of the microshoots produce useable plantlets. These observations and those from rooting experiments with shoots from in vitro root explants indicate that the production of plants capable of surviving "ex vitro" from microshoots is not a serious obstacle to the development of an efficient and economical propagation system.

6.Benefits: Projected increases in the efficiency of the leaf MCS system indicates a reduction in the need for donor tissue by 100 times, a reduction in culture time, vessels and overhead charges by a factor of 8. MCS also has potential for use with other plants that are currently produced by conventional shoot tissue culture, such as blueberries, basswood, birch, etc.

C.Comparison of production efficiency and clonal fidelity (trueness-to-type) to plants produced from objectives A and B.

1.Narrative: Production efficiency will be evaluated using the model developed by Minn vitro, Inc. This model takes into account all factors of commercial importance for research and production and is based on six years of commercial production of 100+ different kinds of plants, including two years of commercial tissue culture of hybrid aspen plants that were produced using conventional shoot culture techniques. Clonal fidelity evaluation criteria will be developed and based on established parameters such as growth rates, branching habits, leaf shape, etc. for the clones being used.

2.Procedures: Production efficiency for plants using methods developed in Objectives A and B will be assessed using the Minn vitro, Inc. cost analysis model.

Trueness-to-type (genetic fidelity) will be assessed with established parameters. Height and stem caliper at the soil line of 100 randomly selected trees per clone will be measured at planting time and at 6 week intervals during the growing season. Mid-season leaf shape will be assessed by measuring width and length of the lamina of ten leaves per tree of ten randomly selected trees of the 100 from above. The first fully expanded leaves will be measured at 4 feet above ground level. Leaf margins variations such as the number of serrations per cm will be recorded. The entire planting will be surveyed for unusual trees. These trees will undergo ploidy level analysis. A change in ploidy level is currently the most common test for <u>Populus</u> variants.

All differences in data means will be assessed using HSD.

3.Budget:
Amount Budgeted: $18,500
Balance: $ 0

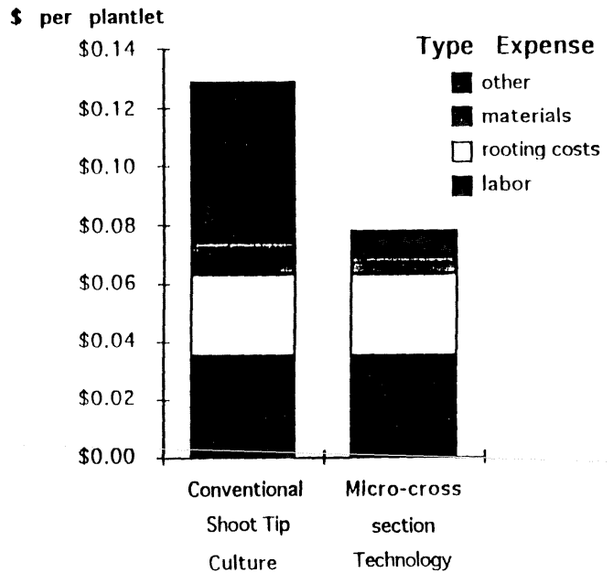
4.Timeline for Tasks

	July 91	Jan 92	June 92	Jan 93	June 93
a.Develop clonal fidelity criteria	********				
b.Collect data		**********	**********		
c.Reporting		**	**	**	**

5.Status:

a.Production efficiency: Production efficiency of the MCS system was evaluated by comparing the costs of producing of 20,000 rooted microshoots for each of the six productive clones in the pilot study (see B above) to those of traditional shoot tip culture. Cost as estimated by Minn vitro are summarized below:

Comparative Costs Convention vs. MCS System



Labor and rooting costs under the two systems are equal. The slicing machine used was inefficient and as much labor was required to put MCS's into culture as was required to do the transfers in a traditional shoot tip system. The MSC system can produce plantlets at a substantially reduced cost (39% lower based on Minn Vitro estimates) because the reduced production time (2 vs 11 months) lowers adminstrative, overhead and materials expenses. The reduction in time is a function of the availability of large numbers of explants.
The cost of producing a plantlet from <u>in vitro</u> root segment was not estimated. A very small amount of root tissue (5mm) per explant is required to initiate cultures and the potential for rapid expansion using this system is great. However, an efficient method for growing roots <u>in vitro</u> did not evolved from these studies. Lacking an efficient root production system the

time, materials and labor requirements for producing rooted plantlets using in vitro grown root segments are approximately those of conventions shoot tip culture and the costs are equal.

Labor costs in all systems are a major cost component. Automation of the MCS or in vitro root system is feasible and this would significantly reduce costs. Investigations of automation were beyond the scope of this project.

It is important to note that the above costs are for the production of small acclimated rooted plantlets. These plants are not large enough to go directly into field plantings and additional expenditures would be required to develop plantable stock.

b. Clonal Fidelity: Two hundred eighty-six plants representing two trembling aspen clones (3 and 17A) produced by procedures A and B were grown in the greenhouse to a height of approximately 1 meter. Differences between clones were obvious but within clones all stems were uniform. No variation was observed that suggested a lack of genetic fidelity. These plants and a small number of ramets from each of the three hybrid clones used in the pilot propagation study (see B above) were moved to the University's nursery at the North Central Experiment Station in Grand Rapids in June of 1993. They will be observed over the next two growing seasons. Approximately 150 additional tissue culture plants from clone 17A (a select trembling aspen) will be included in tests of the Aspen-Larch Cooperative and they will be monitored.

Stem and leaf characteristics of the 286 large plants referred to above were measured in the greenhouse. Variation within the 4 clone-propagation system combinations was examined graphically using eight measured and derived variables. No valid statistical tests for differences within clones could be made. This evaluation was used to identify fourteen individual stems, seven from each clone, which represented the range of observed variation within clones.

Explants from each of these 14 stems were taken to develop "sub-lines" of 15-20 stems. Statistical comparisons among the "sub-lines" of a clone to evaluate clonal fidelity are planned. Sub-lines plants of the seven ramets of one clone (17A) are now growing in the greenhouse but will not be large enough to measure and transfer to the nursery until mid-July. Unexpected difficulties were encountered in the production of sub-lines from clone 3. This clone was included in our studies because of its favorable response in shoot-tip culture systems and there is no obvious explaination for the propagation difficulties no being experienced. It may be related to the physiological state of the plants growing in the greenhouse from which explants were derived, a change in culture protocol or a fundamental change associated with long term in vitro culture. New cultures of the clone 3 sublines have been established in order to confirm or

refute the apparent change in propagation characteristics. Assuming sublines can be developed, measurements and comparisons to evaluate fidelity will be completed for this clone.

Our observations of the growth and development of plants from tissue culture suggest a high level of the clonal fidelity. At the morphological level there is no evidence of genetic changes associated with tissue culture which would limit the usefulness of the propagation technique. The continued observation of materials from this project and planned evaluations of clonal sub-lines will provide further insight into clonal fidelity as reflected in morphology, growth and development.

In addition to the observations of physical characteristics, isoenzymes from leaf samples of from 72 plantlets each of clone 3 and 17A were examined. Strach gel electrophoresis was used to separate enzymes and allozymes were examined using 11 stains. This allowed evaluation of about 18 loci (genes). The techniques provides a quick and inexpensive assessment of genetic differences. It is not a powerful tool for this type of investigation because of the relatively small number of loci that can be examined. The probability of detecting a genetic change in a clone which was induced by tissue culture is extremely small. No allozyme variation among ramets of clone 17a was observed.

Unexpectedly, eight of the 72 samples from ramets of clone 3 had apparently different allozymes at loci of two enzymes (Lucine aminopeptidase and Catalase). Deviant plants came from both the MCS and in vitro root explants. Their banding patterns were similar. These results suggest a genetic change. However, this can not be concluded with any certainity. Possible sources of error are variation in isozyme expression related to sample variablilty, inaccurate scoring of gels because of the poor resolution associated with the use of leaf tissue and improper labeling sometime during the five years this clone has been used in our laboratory and greenhouse.

Both the recent propagation difficulties and the result of isozyme evaluations of clone 3 raise questions. Ongoing propagation work to develop sublines of clone 3 will help define problems related to changes in propagation characteristics. The allozyme analysss of will be repeated using tissue from dormant buds collected from the nursery in the winter of 1993-94. Resolution and sample uniformity will be improved. If results are repeatable, the possiblity of mislabeled materials must be discounted and the practical implications of this variation explored.

Because morphologically unique trees were not identified analyses of ploidy level (chromosome counts) were not undertaken.

6. Benefits: Use of clonal aspen forestry has been limited by the lack of low cost propagules of selected superior plants. If these new techniques prove to be commercially feasible and the propagules genetically true, a large number of selected superior aspen and hybrid aspen plantlets could be produced at an economically feasible price.

IV. Evaluation

Results from Objective A should provide adequate information to begin commercial testing for isolated root cultures as a new large-scale technique for commercial propagation.

Results from Objectives B and C should provide an adequate number of trees to initiate an operational sized field test of the new hybrid aspen and selected aspen clones. The final evaluation will be the acceptance by the forestry industry of one of the tissue culture procedures as a method of clonal propagation.

V. Context

A. Current and previous work in the tissue culture of aspen has focused on conventional shoot multiplication micro-propagation. This method of clonal propagation has a high cost/plantlet. MCS is currently being investigated for its commercial feasibility but is not yet in commercial production. Root culture is not yet ready for a commercial feasibility study.

B. This project would advance MCS to commercial production levels and root culture to commercial feasibility testing.

C. University of Minnesota: Program history: This project will continue research initiated jointly by the Departments of Horticultural Science and Forest Resources with the support of an ORTTA administered Blandin Foundation Early Stage Technology Development Grant ($17,650; 7/1/89-6/30/90). The basic techniques for the in vitro culture of root masses were developed earlier as a part of research projects on hybrid poplar clones. MCS was developed as a part of a cooperative agreement with USDA Forest Service North Central Forest Experiment Station.

Development of effective propagation techniques will complement work of the Hybrid Aspen Cooperative which is supported by the Minnesota DNR and Department of Forest Resources. It will also increase the effectiveness of aspen genetics research being carried out in the Departments of Forest Resources and Plant Pathology, University of Minnesota, and the North Central Forest Experiment Station.

Budget History (since 1989).

Source	July 89	July 90	July 91
Agricultural Experiment Station Funds-Dept. Forest Resources $8,500 (estimated)		**********	
Blandin Foundation Early Stage Technology Grant $17,650 (ORTTA Administered)		**********	

Minn vitro, Inc. Program History: During 1989 we conventionally tissue culture propagated 37,000 hybrid aspen for the Minnesota DNR. We are contracted to produce 30,000 more during 1990. Current numbers are limited by the high cost per plantlet.

Budget History: A limited version of Objective B is currently being funded by the USDA-SBIR (Small Business Innovative Research) Program. The SBIR grant will enable us to begin to assess the leaf micro-section technique (May 1990 through Nov. 1990). LCMR funding would be used to more fully assess a larger number of aspen genotypes for a longer period of time.

D. NA

E. Biennial Budget System Program Title and Budget: Not available at this time.

VI. Qualifications

A. Program Manager: Carl A. Mohn
Professor
Department of Forest Resources
University of Minnesota
Ph.D. Forest Genetics
University of Minnesota, 1969
M.S. Forest Genetics
University of Minnesota, 1964

Dr. Mohn's research involves investigation of genetic variation in major timber species (primarily Populus species, black spruce, white spruce, jack pine, and red pine). His current emphasis is selection, seed orchard management, the development of advanced generation population, estimation of genetic parameters and isozyme analysis. He teaches an undergraduate course in dendrology and a graduate course in forest genetics. He also reviews manuscripts for the Canadian Journal of Forest Research, Forest Science, Northern Journal of Applied Forestry, and several other agencies. In addition, Dr. Mohn serves on advisory committees and/or the Board of Directors for the Minnesota Tree Improvement Cooperative, American

Chestnut Foundation and the Wilderness Research Foundation. Dr. Mohn's primary role will be as program manager and to assist in developing the clonal fidelity parameters.

B.Major Cooperators:

1. Dr. Wesley P. Hackett
Professor
Department of Horticultural Science
University of Minnesota
Ph.D. Plant Physiology
University of California, 1962
M.S. Horticulture
University of California, 1959

Dr. Hackett's research has focused on the physiology of flowering, adventitious bud and root initiation, juvenility and reproductive maturation, tissue culture and vegetative propagation by cuttings. He, along with colleagues, was responsible for developing the MCS approach to adventitious propagule production and the isolated root culture concept for adventitious propagules production. He was the first person to demonstrate the biological feasibility of the shoot multiplication tissue culture concept.

Dr. Hackett's primary role will be to supervise the testing of biological efficiency parameters for both the MCS and isolated root culture propagation systems.

Relevant publications:

Lee-Stadelmann, O. Y., S. W. Lee, W. P. Hackett, and P. E. Read. 1989. The formation of adventitious buds in vitro on micro-cross sections of hybrid Populus leaf midveins. Plant Science 61:263-272.

Lee-Stadelmann, O. Y., S. W. Lee, H. Chung, Q. Gus, M. Kim, C. Pak, and W. P. Hackett. 1989. Optimizing potential for adventitious shoot organogenesis in hybrid Populus explants in vitro with wound treatment and micro-cross sections. In Proc. NATO ASI Series Meeting, Woody Plant Biotechnology, (ARW 89/092), ed. M. R. Ahuja.

2. Dr. Kathryn Louis
Research Director/CFO
Minn vitro, Inc.
Ph.D. Horticultural Science
University of Minnesota, 1990
M.S. Horticultural Science
University of Minnesota, 1984

Dr. Louis is co-owner, co-founder of Minn vitro, Inc. an independent commercial tissue culture laboratory. Dr. Louis' research concentrates on in vitro physiology and development of adventitious bud formation in woody plants such as Populus, Picea

and Pinus species. She developed the commercial protocol for hybrid aspen axillary bud cultures that is used to propagate the hybrid aspen for the Minnesota Department of Natural Resources and developed a cost analysis model for commercial micropropagation. She is currently administering the USDA-SBIR grant at Minn vitro, Inc. Dr. Louis' primary role will be to conduct all phases of Objective B research at Minn vitro, Inc., provide plants for Objective C, perform all cost analyses and assist with the preliminary commercial feasibility of Objective A.

Supplemental Information: Minn vitro, Inc. was founded in 1983 as an independent commercial tissue culture laboratory dedicated to propagation production research and large-scale micropropagation. It has demonstrated the capacity to produce one million tissue culture units per year. It is licensed by the State of Minnesota, Department of Agriculture and has customers throughout the Upper Midwest, California, New York and Canada as well as Chile, Japan, Sweden and Russia.

VII.Reporting Requirements

Semi-annual status reports will be submitted no later than January 1, 1992; July 1, 1992; January 1, 1993; and a final status report by June 30, 1993.

1991 Research Project Abstract
For the period ending June 30 1993
This project was supported by MN Future Resources Fund (MS 116.13)

TITLE:	Aspen Hybrids and New Tissue Culture Techniques.
PROGRAM MANAGER:	Carl A, Mohn
LEGAL CITATION:	M.L. 91, Ch 254 , ART. 1, Sec.14, Subd. 7(f)
APPROP. AMOUNT:	$70,000.

STATEMENT OF OBJECTIVES

To continue to develop the biological efficiency of two new tissue culture (cloning) techniques, examine clonal fidelity (trueness-to-type) of materials produced under these systems, and assess the commercial production efficiency of the systems.

RESULTS

1. Root culture system: Medium and conditions were defined which gave in vitro root growth at a rate marginal for commercial production. The combination of growth regulator levels, basal medium, root segment size and time of treatments which gave a satisfactory level of microshoot production from roots was defined. Plantlets were successfully produced from microshoots.
2. Micro-cross section (MCS) system. A pilot propagation study with five clones was completed. The best medium and timing of treatments to stimulate microshoot on MCS of these clones was defined. Tests of cold storage techniques necessary to control timing in the production system were positive. Conversion of microshoots to plantlets with roots had a success rate of approximately 85 percent.
3. Genetic fidelity. Our observations of the growth and development of plants from tissue culture indicated no difficulty with clonal fidelity. Monitoring of these plants, now growing in an experimental nursery, will continue. Analysis at the molecular level (allozyme evaluation) suggested genetic changes within one clone, this work will be repeated when uniform samples of more suitable tissue are available.
4. Commercial production efficiency. Under a likely scenario, propagation of 100,000 microshoots (85,000 plantlets) using a standard tissue culture system (shoot tip) would require 11 months and cost approximately $0.129 per plantlet. If a root culture system were used, because of the modest rates of in vitro root growth achieved, time and costs would be roughly the same. The MCS system costs would be $0.078 per plantlet. This 39% cost reductions is attributable to reduced materials, overhead and administrative cost. As much labor was required to put MCS's into culture as was required to do the transfers in a traditional shoot tip system. Note that the costs cited are for the production of small acclimated rooted plantlets. Additional expenditures would be required to develop plantable stock.

PROJECT RESULTS USE AND DISSEMINATION

Two presentation of project results were made at the 12th North American Forest Biology Workshop in August of 1992. Abstracts were published in the proceedings. Work on the in vitro root propagation system was summarized at the meetings of the International Plant Propagators Society in December of 1992 and will be published in those proceedings. Contacts have been maintained with the Aspen/Larch Cooperative and potential aspen growers in Minnesota. The techniques are now being used in aspen research at the U of M. Commercal use of the results depends on acceptance by the forestry industry of one of the tissue culture procedures as a method of clonal propagation.

7/1/93
LCMR Final Status Report-Detailed for Peer Review-Research

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The relatively high cost of conventional shoot multiplication tissue culture techniques is acceptable for some high value per unit crops. Large-scale clonal forestry planting of selected aspen and hybrid aspen has been limited by the relative high cost per tissue cultured plant which is in the $450/1,000 range. This is too costly to fulfill projected Minnesota forestry demand for aspen of 5 million trees per year even with the anticipated reduction in cost based on economics of scale.

Clones are desirable because they retain desirable characteristics such as disease resistance, fast growth, etc. of the parent plant. Cost reduction research, to date, has concentrated on automation and robotics. This grant proposes to investigate the biological components of adventitious propagule production, an area not yet scrutinized in any detail.

III.OBJECTIVES.

A. Increasing the biological efficiency of the root culture system.

1.Narrative: Approaches to increase efficiency will build on previous work on the in vitro culture of aspen root masses and shoot induction on small segments of these masses.

2.Procedures: Isolated root cultures are established from in vitro derived shoots which have been induced to form roots. These roots are excised and transferred to a liquid growing medium (Woody Plant Medium-WPM) supplemented with 3% sucrose and 1 uM auxin (naphthaleneacetic acid-NAA). The culture vessels are roller bottles that rotate at 1/4 rpm. Four clones have been used in preliminary trials. An "easy" and a "recalcitrant" clone have been identified and will be used for subsequent trials.

Three areas will be investigated:

a.Nitrogen. Results to date indicate that isolated root growth is enhanced by manipulating the form and/or quantity of nitrogen, i.e., ammonium:nitrate ratio and quantity.

Optimal nitrogen configuration will be tested by varying the nitrate:ammonium ratio on a 1:1, 1:2, 2:1 ratios at 1X and 2X the total N. Each vessel will contain 4, 2 cm root segments with 5-50 ml vessels per treatment. Growth of roots will be measured by weight gain, length and branching pattern.

b. Shoot production from root segments. Currently, root segments can be induced to form shoots. Conditions for optimal and reliable production have yet to be determined.

Enhancement of adventitious bud initiation on the root segments, i.e., in vitro suckering, will be tested by varying the length of the root segment and type of cytokinin. Root segments of 0.5, 1.0, 2.0 and 4.0 cm will be placed on solid basal medium with 0, 0.5, 1.0 and 1.5 mg/l BA (benzyladenine) or 0, 0.01, 0.1 and 1.0 mg/l Thidiazuron for 1, 2, 3 or 4 weeks. Twenty root segments per treatment will be tested. Data collected are the number of adventitious shoots per root segment and size (length) of shoot. These microshoots obtained will be used in "c" below.

c. Subsequent rooting and plantlet development of the shoots derived from in vitro suckering. These processes have not yet been attempted. The rooting and development of the above microshoots will be assessed using an aseptic (Sorbarod) plug system and ex vitro, nonaseptic (Techniculture) plug system. The Techniculture system has been used commercially with hybrid aspen microshoots with moderate success. The primary problem is death of microshoots from pathogens during the rooting phase. The Sorbarod system has not yet been tested for aspen. Because it is aseptic, the problem of pathogens during rooting should be eliminated. It also allows the transition between aseptic to nonaseptic conditions, i.e., acclimation, to occur after the critical rooting stage. Survival and growth characteristics will be measured during this stage.

Experimental design. Treatments will be replicated. Differences in means will be determined using the HSD (Honest Significant Difference) test.

3. Budget:

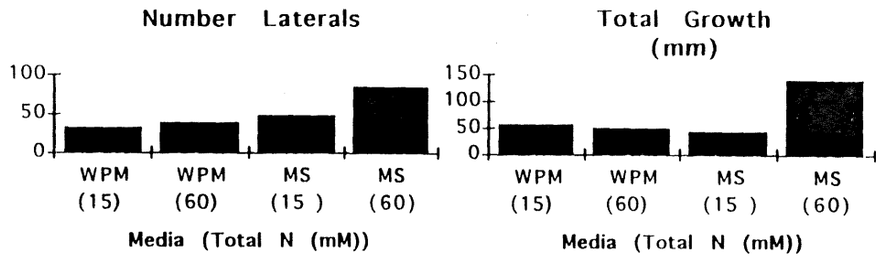
a.Amount Budgeted: $26,000
b.Balance: $ 625. (estimated 6/25/93)

4.Timeline for Tasks

	July 91	Jan 92	June 92	Jan 93	June 93
a.Nitrogen study	**********				
b.Shoot production		**********	**********		
c.Rooting and acclimation			**********	**********	
d.Reporting	**	**	**	**	**

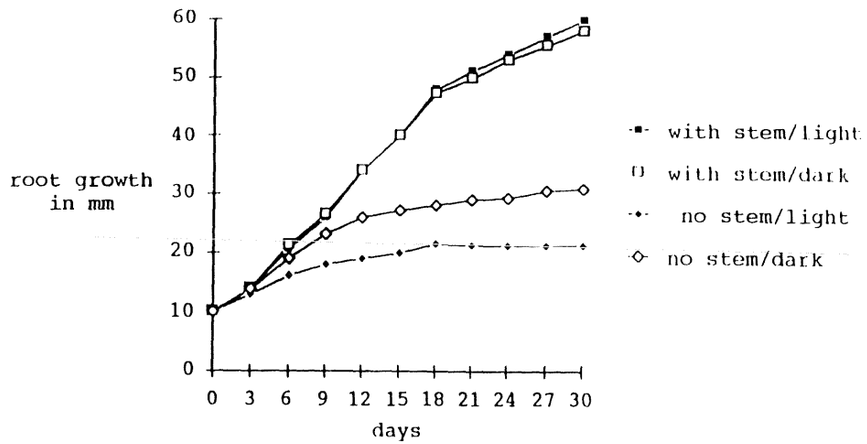
5.Status:

a.Root growth. Results of experiments testing the effects on root growth of changing the form and quantity of nitrogen in media were not consistent with observations made previous to the grant. The ratio and concentration found in MS (Murashige and Skog) media proved superior ($1NH_4:2NO_3$; 60μM total N) and standard MS media is recommended. Typical results of the results of media-nitrogen experiments is the growth of root lateral roots on 1cm explants with their apical tips removed (10/treatment) shown below:



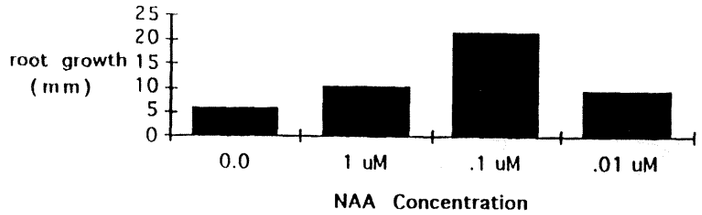
Media, pH, light, auxin level and type of explant (root alone, root + stem, etc.) all influenced root growth. As illustrated by the above figure, MS media consistently gave the best development in experiments in which basal media were compared.

The figure below illustrates the inhibition by light of the growth of excised root segments and the benefit of leaving small amounts of stem attached to roots.



Media pH of 5 - 6 (initial value) gave the most rapid root growth. A standard media pH of 5.7 before autoclaving was adopted following numberous experiments.

Several studies designed to define the optimum level of NAA (auxin) for excised root growth showed that values between 10^{-6} and 10^{-7}M were the most promising. Typical date were:



Growth of roots on solid media or on filter paper supports in liquid media exceeded that observed in roller bottles. Using the best combination of basal media, auxins concentration, light and leaving a sement of stem attachted to roots produced gave growth of about 15mm/day over 28 days (clone 3). Assuming a production of 1 plant per cm of root this would gives a multiplication factor of about 40 per cycle which is marginal for commercial propagation. Efforts to improve growth rate will continue as part of a MS thesis project.

In addition to the marginal rate of root growth, other limitation to applying this and other system are: (1) a buildup of bacterial contamination over time in materials in culture and (2) the variation in the response of clones to culture. At present the only solution to contamination problem is avoidance by frequently initiating fresh root cultures, This is an additonal cost. The poor response of some clones in culture means that either specific protocols (most likely involving modification of growth regulator levels) be developed for recalcitrant clones or that only clones with favorable propagation characteristics be used. This will increase costs and/or reduced the availability of clones with desirable characteristics.

b. Shoot production from root segments. The number of bud clusters forming on root segments and number of shoots developing from clusters in response to various induction treatments was examined. We identified thidiazron (TZD) as the cytokinin of choice and MS as the most promising basal media. Results were extremely variable but the weight of evidence from several experiments is that thidiazron levels between .01 and .1 mg/l are the most satisfactory for bud induction.. While auxin is necessary in initiation media for leaf micro cross-sections, with roots the response seems to be clone dependent. Typical experimental results are:

Induction Treatment		Mean nr. adventitious bud clusters		Mean num of microshoots	
level TDZ (mg/l)	.01 mg/l NAA	Clone 3	Clone 17	Clone 3	Clone 17
0.01	no	.18	.10	.20	.04
0.01	yes	.11	.13	.06	.04
0.10	no	.19	.38	.19	.12
0.10	yes	.19	3.31	.06	.16
1.00	no	.30	.39	.06	.82
1.00	yes	.26	.31	.12	.42

A trend toward more and larger shoots with increased time on the initiation and development media was noted The number and size of shoots produced by the best treatment (4 weeks initiation, 8 weeks development) is compatible with large scale propagation efforts. The incidence of systemic bacterial contamination increases with the length of time explants remain in culture and this contributes to inconsistencies in the data. The following are examples of the numbers and lengths of shoots produced 4 and 8 weeks after four periods on initiation:

Duration initiation treatment	Number bud clusters	Average number of microshoots per bud cluster		Mean length of microshooots (mm)	
		at 4 weeks	at 8 weeks	at 4 weeks	at 8 weeks
1 week	143	.014	.322	6.0	10.5
2 weeks	128	.164	1.120	10.2	13.4
3 weeks	195	.036	.421	6.4	12.2
4 weeks	72	.650	4.350	10.4	19.2

Small segments (2-3mm) were found to produce more bud clusters per unit of root than 1 cm segments. The following data show how shoot yields per unit of root increase as explants size decreased.

	Average Segment Length		
	2.5 mm	7.5 mm	15 mm
# bud clusters/unit	2.4	2.7	2.3
% segments with buds	65	86	73
# buds/mm root	.96	.36	.21
# units observed	215	100	66

Rooting of shoots derived from roots has been successful and in the best trial over 90 percent of the shoots produce vigorous plants. Ex vitro systems were satisfactory.

Response in these experiments has varied widely and the reliable data needed to project costs are not available. Larger trials using the treatment which has been most effective to date are in progress. These tests were delayed

by culture contamination and will not be completed until late 1993. They should provide a clearer indication of root production, plant yield per unit of root and other data needed for cost estimation.

6. Benefits: Optimization of the biological elements of this system will increase its overall efficiency. This will impact costs directly by reducing the required investment in equipment and labor per plant produced.

B. Optimize and evaluate the regeneration of adventitious plantlets using the leaf micro-section culture system.

1. Narrative: This objective will evaluate the commercial feasibility of high volume clonal production of 2 aspen, 2 big tooth aspen and 4 hybrid aspen genotypes using the leaf micro-section system.

2. Procedures: A new technique of micro-cross section (MCS) has been developed by scientists at the University of Minnesota to increase the multiplication efficiency of Populus clone NE299 and two other hybrid poplar clones. MCS has increased the potential of explants to form adventitious shoots by 25 times when compared to the standard explants used for adventitious shoot cultures. Additionally, the startup time is reduced from 6-12 months to 1-2 months. The increased number of shoots per explant, the increased number of explants per donor plant plus the shorter startup time increases the overall multiplication efficiency significantly. This efficiency is especially critical when donor plant material and time is limiting, a common occurrence when new hybrids are introduced.

The technique of MCS consists of cutting the explant into very thin cross sections using a Lancer Vibratome. This microtome vibrates as it slices tissue which results in minimal tissue damage. Tissue cross section of specific thickness can be readily prepared in this manner. Researchers at the University of Minnesota tested several thicknesses of cross sections 100, 200, 300, 400 and 500 Êm as well as a 1 cm control. The most efficient thickness (400 Êm) averaged more than 4 uniform shoots per MCS. This is equal to the production efficiency of the 1 cm control. However, using MCS, 25-400 Êm sections can be realized from 1 cm of donor material and results in a 25 times increase in productivity. Only NE299 and two other clones have been tested by the University of Minnesota.

Minn vitro, Inc. has been funded by the USDA-SBIR (Small Business Innovative Research) program, Phase I to test four hybrid aspen and two aspen clones using MCS. Preliminary results indicate a strong genotype response demonstrating that the protocol that was established for NE299 does not necessarily work well with all hybrid and non-hybrid aspen.

Modifications to the established protocol, i.e., basal medium differences and plant growth regulator variations, are underway. The SBIR project should establish the MCS protocol for the four hybrid aspen and two aspen selections. LCMR funds would be used to establish the protocol for Big Tooth Aspen and to continue work after termination of the SBIR grant that would allow the four hybrid aspen and two aspen to be tested on a larger scale.

Based on the outcome of results from the SBIR project, a single medium composition for each clone will be selected. Two hundred jars of 10 MCS each will be initiated per clone. This should produce a projected 8,000-10,000 plants per clone. The plantlets will then be field tested and used for Objective C. Time and cost data will be collected during all stages of production, i.e., a) initiation, b) adventitious bud induction and development, c) rooting, d) acclimation and e) establishment.

3. Budget:

Amount budgeted:	$25,500
Balance:	$ 0

4. Timeline for Tasks

	July 91	Jan 92	June 92	Jan 93	June 93
a. MCS establishment	**********	**********			
b. Shoot production		**********	**********		
c. Rooting and acclimation			**********	**********	
d. Reporting	**	**	**	**	**

5. Status: Work on the pilot propagation study of selected clones was completed. Data were collected for 5 clones: three hybrids (MINN VITRO's #1,#8 and #22) and two trembling aspen clones (U of M. #3 and #17A). The fourth hybrid clone was not responsive to the MCS system. Two bigtooth aspen (Populus grandidentata) clones were to be included in this study but uncontaminated cultures could not be derived from field materials.

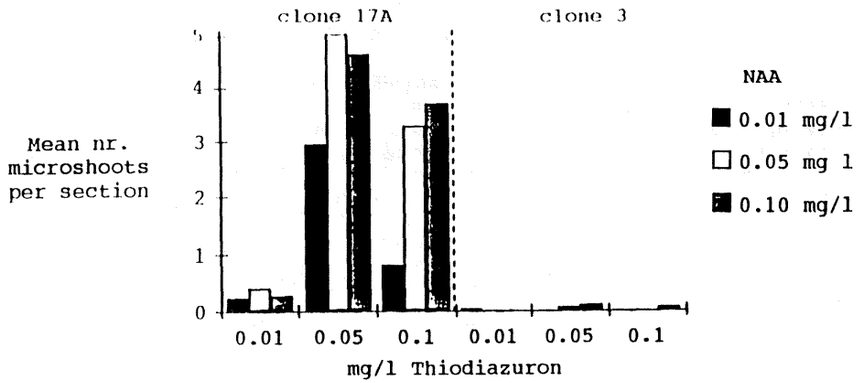
Because materials for initiating cultures are almost unlimited under the MCS system, a two month cycle can be used. MCS are first placed on initiation media. After one month, sections with adventious buds are transfered to elongation media for one month and then the shoots are rooted. Under a traditional shoot tip system materials needed to initiated cultures are limited and additonal time for shoot proliferation-is required. The data following reflect our experience with the two systems:

Clone	MCS System Average nr. microshoots per MCS	Shoot Tip System Microshoots per jar
MV #1	2.94	7.12
MV #8	1.66	3.50
MV#22	8.09	2.63
UM #3	.11	6.42
UM #17A	5.01	4.73

The MCS system data are for a 2 month cycle with 10 microcross sections in each initiation jar. To produce 100,000 microshoots (20,000/clone) aproximately 11,700 culture jars are required (4,350 for initiation and an additional 7350 for enlongation). In the traditional system a one month initiation period and ten months for proliferation-elongation were needed to reach this goal and approximately 23,500 culture jars were required. On a microshoot per jar basis the MCS system is approximately twice as efficient (8.5 vs 4.3).

The initiation media used in the microshoot production study contained .05 mg/l thiodiazron and .05 mg/l NAA. On average, this media was most effective in inducing shoots from the clones tested. Typical results of test to define media were:

Microshoot production from microcross sections of clones 3 and 17A.



Clonal differences in shoot development which must be accommodated by a propagation system were apparent in all studies. For some recalcitrant clones a longer period on developmental medium resulted in good microshoot development. However, the poor response of some clones indicates

limitations of the proposed propagation system.

Cold storage is critical in controlling timing in a production system. The results of a storage test of approximately 1800 uncontaminated MCS from each of three hybrid clones are presented below. They indicate that microcross sections with bud cluster can be successfully stored. However, contamination and mortality decrease the number of microshoot produced. Cold storage does not reduce the vigor of the resulting microshoots.

	% MCS contaminated	% MCS dead*	Mean Nr Microshoots per MCS	Mean Length Microshoots (mm)
Clone MV #1				
Cold Storage	44	47	.43	13.3
No storage	0	0	2.69	15.0
Clone NV #8				
Cold Storage	0	82	.80	11.9
No storage	0	00	>2.00	8.6
Clone MV #22				
Cold Storage	19	31	.39	18.5
No storage	0	0	>3.00	20.5

*includes contaminated MCS

Rooting and acclimation trials utilizing shoots from the MCS studies indicate that rooting varied by clone with a range from 64 to 92 percent. Survival of the rooted shoots through the acclimation process was 95 percent. In the pilot propagation approximately 85 percent of all shoots became plantlets. These observations and those from rooting experiments with shoots from in vitro root explants indicate that the production of plants capable of surviving "ex vitro" from microshoots is not a serious obstacle to the development of an efficient and economical propagation system.

6. Benefits: Projected increases in the efficiency of the leaf MCS system indicates a reduction in the need for donor tissue by 100 times, a reduction in culture time, vessels and overhead charges by a factor of 8. MCS also has potential for use with other plants that are currently produced by conventional shoot tissue culture, such as blueberries, basswood, birch, etc.

C. Comparison of production efficiency and clonal fidelity (trueness-to-type) to plants produced from objectives A and B.

1. Narrative: Production efficiency will be evaluated using the model developed by Minn vitro, Inc. This model takes into account all factors of commercial importance for research and production and is based on six years of commercial production

of 100+ different kinds of plants, including two years of commercial tissue culture of hybrid aspen plants that were produced using conventional shoot culture techniques. Clonal fidelity evaluation criteria will be developed and based on established parameters such as growth rates, branching habits, leaf shape, etc. for the clones being used.

2. Procedures: Production efficiency for plants using methods developed in Objectives A and B will be assessed using the Minn vitro, Inc. cost analysis model.

Trueness-to-type (genetic fidelity) will be assessed with established parameters. Height and stem caliper at the soil line of 100 randomly selected trees per clone will be measured at planting time and at 6 week intervals during the growing season. Mid-season leaf shape will be assessed by measuring width and length of the lamina of ten leaves per tree of ten randomly selected trees of the 100 from above. The first fully expanded leaves will be measured at 4 feet above ground level. Leaf margins variations such as the number of serrations per cm will be recorded. The entire planting will be surveyed for unusual trees. These trees will undergo ploidy level analysis. A change in ploidy level is currently the most common test for Populus variants.

All differences in data means will be assessed using HSD.

3. Budget:
Amount Budgeted: $18,500
Balance: $ 0

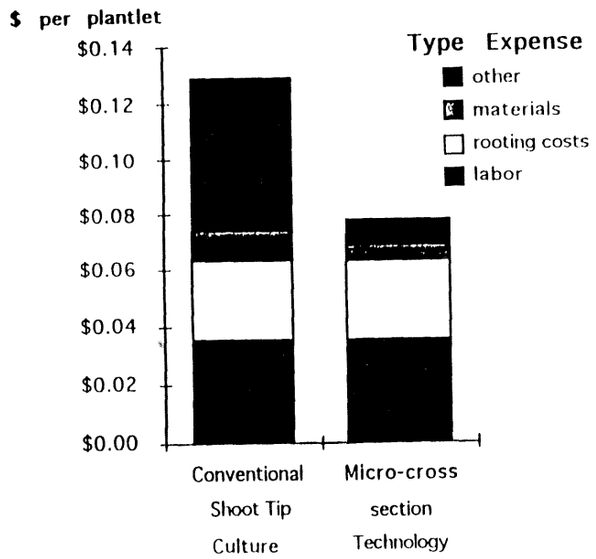
4. Timeline for Tasks

	July 91	Jan 92	June 92	Jan 93	June 93
a. Develop clonal fidelity criteria	********				
b. Collect data		**********	**********		
c. Reporting		**	**	**	**

5. Status:

a. Production efficiency: Production efficiency of the MCS system was evaluated by comparing the costs of producing of 20,000 rooted microshoots for each of the six productive clones in the pilot study (see B above) to those of traditional shoot tip culture. Cost were estimated using procedures employed by Minn Vitro. Results are summarized below:

Comparative Costs Convention vs. MCS System



Labor and rooting costs under the two systems are equal. The slicing machine used was inefficient and as much time was required to put MCS's into culture as was required to do the transfers in a traditional shoot tip system. The MSC system can produce plantlets at a substantially reduced cost (39% lower based on Minn Vitro estimates) because the reduced production time (2 vs 11 months) lowers adminstrative, overhead and materials expenses. The reduction in time is a function of the availability of large numbers of explants.

The cost of producing a plantlet from in vitro root segment was not directly estimated. A very small amount of root tissue (5mm) per explant is required to initiate cultures and the potential for rapid expansion using this system is great. However, an efficient method for growing roots in vitro did not evolved from these studies. Lacking an efficient root production system the time, materials and labor requirements for producing rooted plantlets using in vitro grown root segments are approximately those of conventions shoot tip culture and the costs are equal.

Labor costs in all systems are a major cost component. Automation of the MCS or the in vitro root system is feasible and this would significantly reduce costs. Investigations of

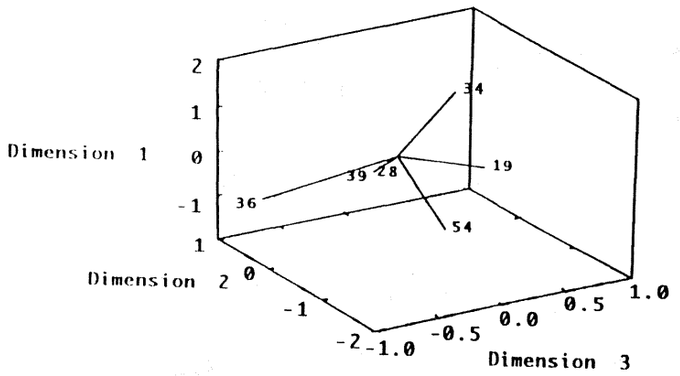
automation were beyond the scope of this project.

It is important to note that the above costs are for the production of small acclimated rooted plantlets. These plants are not large enough to go directly into field plantings and additional expenditures would be required to develop plantable stock.

b. Clonal Fidelity: Two hundred eighty-six plants representing two trembling aspen clones (3 and 17A) produced by procedures A and B were grown in the greenhouse to a height of approximately 1 meter. Differences between clones were obvious but within clones all stems were uniform. No variation was observed that suggested a lack of genetic fidelity. These plants and a small number of ramets from each of the three hybrid clones used in the pilot propagation study (see B above) were moved to the University's nursery at the North Central Experiment Station in Grand Rapids in June of 1993. They will be observed over the next two growing seasons. Approximately 150 additional tissue culture plants from clone 17A (a select trembling aspen) will be included in tests of the Aspen Larch Cooperative and monitored.

Stem and leaf characteristics of the 286 large plants referred to above were measured in the greenhouse. Variation within the four clone-propagation system combinations was examined graphically using eight measured and derived variables with multidimensional scaling (illustrate by figure below).

Multi Dimensional Scaling, 3 Dimension (Standardized)
Vector Origins 0,0,0
Selected Ramets of Clone 17, MCS system



No valid statistical tests for differences within clones could be made. This type of graphic description of variation was used to identify fourteen individual stems, seven from each

clone, which represented the range of variation within clones.

Explants from each of these 14 stems were taken to develop "sub-lines" of 15-20 stems. Statistical comparisons among the "sub-lines" of a clone to evaluate clonal fidelity have not yet be made. Sub-lines plants of the seven ramets of one clone (17A) are now growing in the greenhouse but will not be large enough to measure and transfer to the nursery until mid-July. Difficulties were encountered in the production of sub-lines from clone 3. This clone was included in our studies because of its favorable response in shoot tip culture and there is no obvious explaination for these propagation difficulties. It may be related to the physiological state of the plants growing in the greenhouse from which explants were derived, changes in culture protocol or a fundamental change associated with repeated tissue culture. New shoot tip cultures of the clone 3 sublines have been established in order to confirm or refute the apparent change in propagation characteristics. Assuming sublines can be developed, measurements and comparisons to evaluate fidelity will be completed for this clone.

Our observations of the growth and development of plants from tissue culture suggest a high level of the clonal fidelity. At the morphological level there is no evidence of genetic changes associated with tissue culture which would limit the usefulness of the propagation technique. The continued observation of materials from this project and planned evaluations of clonal sub-lines will provide further insights into clonal fidelity.

In addition to the observations of physical characteristics, isoenzymes from leaf samples of from 72 plantlets each of clone 3 and 17A were examined. Strach gel electrophoresis was used to separate enzymes and allozymes were examined using 11 stains. This allowed evaluation of about 18 loci (genes). The techniques provides a quick and inexpensive assessment of genetic differences. It is not a powerful tool for this type of investigation because of the relatively small number of loci that can be examined. The probability of detecting a genetic change in a clone which was induced by tissue culture is extremely small. No allozyme variation among ramets of clone 17a was observed.

Unexpectedly, eight of the 72 samples from ramets of clone 3 had apparently different allozymes at loci of two enzymes (Lucine aminopeptidase and Catalase). Deviant plants came from both the MCS and <u>in vitro</u> root explants. Their banding patterns were similar. These results suggest a genetic change. However, this can not be concluded with certainity. Possible sources of error are variation in isozyme expression related to sample variablilty, inaccurate scoring of gels because of the poor resolution associated with the use of leaf

tissue or improper labeling during the five years this clone has been used in our laboratory and greenhouse.

Both the recent propagation difficulties and the result of isozyme evaluations of clone 3 raise questions which should be addressed. Ongoing propagation work to develop sublines of clone 3 will help define problems related to changes in propagation characteristics. The allozyme analysss of will be repeated using tissue from dormant buds collected from the nursery in the winter of 1993-94. Resolution and sample uniformity will be improved. If results are repeatable, the possiblity of mislabeled materials must be discounted and the practical implications of this variation explored.

Because morphologically unique trees were not identified analyses of ploidy level (chromosome counts) were not undertaken.

6. Benefits: Use of clonal aspen forestry has been limited by the lack of low cost propagules of selected superior plants. If these new techniques prove to be commercially feasible and the propagules genetically true, a large number of selected superior aspen and hybrid aspen plantlets could be produced at an economically feasible price.

IV. Evaluation

Results from Objective A should provide adequate information to begin commercial testing for isolated root cultures as a new large-scale technique for commercial propagation.

Results from Objectives B and C should provide an adequate number of trees to initiate an operational sized field test of the new hybrid aspen and selected aspen clones. The final evaluation will be the acceptance by the forestry industry of one of the tissue culture procedures as a method of clonal propagation.

V. Context

A. Current and previous work in the tissue culture of aspen has focused on conventional shoot multiplication micro-propagation. This method of clonal propagation has a high cost/plantlet. MCS is currently being investigated for its commercial feasibility but is not yet in commercial production. Root culture is not yet ready for a commercial feasibility study.

B. This project would advance MCS to commercial production levels and root culture to commercial feasibility testing.

C. University of Minnesota: Program history: This project will continue research initiated jointly by the Departments of Horticultural Science and Forest Resources with the support of an ORTTA administered Blandin Foundation Early Stage Technology Development Grant ($17,650; 7/1/89-6/30/90). The basic techniques

for the in vitro culture of root masses were developed earlier as a part of research projects on hybrid poplar clones. MCS was developed as a part of a cooperative agreement with USDA Forest Service North Central Forest Experiment Station.

Development of effective propagation techniques will complement work of the Hybrid Aspen Cooperative which is supported by the Minnesota DNR and Department of Forest Resources. It will also increase the effectiveness of aspen genetics research being carried out in the Departments of Forest Resources and Plant Pathology, University of Minnesota, and the North Central Forest Experiment Station.

Budget History (since 1989).

Source	July 89	July 90	July 91
Agricultural Experiment Station Funds-Dept. Forest Resources $8,500 (estimated)		**********	
Blandin Foundation Early Stage Technology Grant $17,650 (ORTTA Administered)		**********	

Minn vitro, Inc. Program History: During 1989 we conventionally tissue culture propagated 37,000 hybrid aspen for the Minnesota DNR. We are contracted to produce 30,000 more during 1990. Current numbers are limited by the high cost per plantlet.

Budget History: A limited version of Objective B is currently being funded by the USDA-SBIR (Small Business Innovative Research) Program. The SBIR grant will enable us to begin to assess the leaf micro-section technique (May 1990 through Nov. 1990). LCMR funding would be used to more fully assess a larger number of aspen genotypes for a longer period of time.

D. NA

E. Biennial Budget System Program Title and Budget: Not available at this time.

VI. Qualifications

A. Program Manager: Carl A. Mohn
Professor
Department of Forest Resources
University of Minnesota
Ph.D. Forest Genetics
University of Minnesota, 1969
M.S. Forest Genetics
University of Minnesota, 1964

Dr. Mohn's research involves investigation of genetic variation in major timber species (primarily Populus species, black spruce, white spruce, jack pine, and red pine). His current emphasis is selection, seed orchard management, the development of advanced generation population, estimation of genetic parameters and isozyme analysis. He teaches an undergraduate course in dendrology and a graduate course in forest genetics. He also reviews manuscripts for the Canadian Journal of Forest Research, Forest Science, Northern Journal of Applied Forestry, and several other agencies. In addition, Dr. Mohn serves on advisory committees and/or the Board of Directors for the Minnesota Tree Improvement Cooperative, American Chestnut Foundation and the Wilderness Research Foundation. Dr. Mohn's primary role will be as program manager and to assist in developing the clonal fidelity parameters.

B.Major Cooperators:

1. Dr. Wesley P. Hackett
   Professor
   Department of Horticultural Science
   University of Minnesota
   Ph.D. Plant Physiology
   University of California, 1962
   M.S. Horticulture
   University of California, 1959

Dr. Hackett's research has focused on the physiology of flowering, adventitious bud and root initiation, juvenility and reproductive maturation, tissue culture and vegetative propagation by cuttings. He, along with colleagues, was responsible for developing the MCS approach to adventitious propagule production and the isolated root culture concept for adventitious propagules production. He was the first person to demonstrate the biological feasibility of the shoot multiplication tissue culture concept.

Dr. Hackett's primary role will be to supervise the testing of biological efficiency parameters for both the MCS and isolated root culture propagation systems.

Relevant publications:

Lee-Stadelmann, O. Y., S. W. Lee, W. P. Hackett, and P. E. Read. 1989. The formation of adventitious buds in vitro on micro-cross sections of hybrid Populus leaf midveins. Plant Science 61:263-272.

Lee-Stadelmann, O. Y., S. W. Lee, H. Chung, Q. Gus, M. Kim, C. Pak, and W. P. Hackett. 1989. Optimizing potential for adventitious shoot organogenesis in hybrid Populus explants in vitro with wound treatment and micro-cross sections. In Proc. NATO ASI Series Meeting, Woody Plant Biotechnology,

(ARW 89/092), ed. M. R. Ahuja.

2. Dr. Kathryn Louis
   Research Director/CFO
   Minn vitro, Inc.
   Ph.D. Horticultural Science
   University of Minnesota, 1990
   M.S. Horticultural Science
   University of Minnesota, 1984

   Dr. Louis is co-owner, co-founder of Minn vitro, Inc. an independent commercial tissue culture laboratory. Dr. Louis' research concentrates on in vitro physiology and development of adventitious bud formation in woody plants such as Populus, Picea and Pinus species. She developed the commercial protocol for hybrid aspen axillary bud cultures that is used to propagate the hybrid aspen for the Minnesota Department of Natural Resources and developed a cost analysis model for commercial micropropagation. She is currently administering the USDA-SBIR grant at Minn vitro, Inc. Dr. Louis' primary role will be to conduct all phases of Objective B research at Minn vitro, Inc., provide plants for Objective C, perform all cost analyses and assist with the preliminary commercial feasibility of Objective A.

   Supplemental Information: Minn vitro, Inc. was founded in 1983 as an independent commercial tissue culture laboratory dedicated to propagation production research and large-scale micropropagation. It has demonstrated the capacity to produce one million tissue culture units per year. It is licensed by the State of Minnesota, Department of Agriculture and has customers throughout the Upper Midwest, California, New York and Canada as well as Chile, Japan, Sweden and Russia.

VII.Reporting Requirements

Semi-annual status reports will be submitted no later than January 1, 1992; July 1, 1992; January 1, 1993; and a final status report by June 30, 1993.