THE INSTITUTE OF PAPER SCIENCE AND TECHNOLOGY

Atlanta, Georgia

Status Report
to the
FOREST GENETICS
PROJECT ADVISORY COMMITTEE

March 28, 1990

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FOREST GENETICS

PROJECT ADVISORY COMMITTEE

March 28, 1990

March 14, 1990

TO: The Forest Genetics Processes Project Advisory Committee

As indicated in my recent letter of inivitation, we are forwarding an agenda and a status report in preparation of our spring Project Advisory Committee meeting.

We look forward to sharing results with you, and showing you our new facilities on March 28 and 29. Please remember to register if you have not already done so. Best wishes for a safe trip.

Sincerely,

Ron

Dr. Ronald J. Dinus
Director
Forest Biology Group

RJD/cw

Enc.

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AGENDA

FOREST GENETICS PROJECT ADVISORY COMMITTEE

Institute of Paper Science and Technology
Radisson Conference Center
Atlanta, Georgia

Wednesday, March 28, 1990

8:00 a.m.	Registration, Coffee and Doughnuts	
8:30 a.m.	Welcome and Introductions Overview and PAC Recommendations	Dinus
9:00 a.m.	Somatic Embryo Maturation Update on Loblolly Pine	Uddin
9:25 a.m.	Embryo Classification and Germination	Webb
9:45 a.m.	Coffee Break	
10:00 a.m.	Zygotic Embryo Composition	Nagmani
10:40 a.m.	Student Presentation	Wood
11:00 a.m.	Hardwood Regeneration Leaf Section System Cell Suspensions	Uddin
11:30 a.m.	Summary and Near-Term Plans	Dinus/Malcolm
Noon	Lunch, IPST Conference Room	
1:30 p.m.	Laboratory and Greenhouse Tour	Committee
	Laboratories Open and Personnel Available	
5:00	Reception, IPST Conference Room	
6:00	Group Dinner, Dutch Treat	

Agenda, Cont'd

Thursday, March 29, 1990

8:00 a.m.	Agenda for Morning	Leach/Dinus
8:15 a.m.	PAC Operations	Yeske/Malcolm
9:15 a.m.	Discussion/Deliberations	Committee
10:00 a.m.	Coffee Break	
10:20 a.m.	Discussion/Deliberations	Committee
11:00 a.m.	Closing Remarks	Leach, Dinus, and Malcolm
11:30 a.m.	Adjournment	

FOREST GENETICS

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Projects 3223-00,02,03 Status Report

COOPERATIVE INTERACTIONS

University of Florida, Leesburg - Investigation by Dr. D. Gray of desiccation as a method of preparing Norway spruce somatic embryos for storage and germination.

SAPPI Forests Ltd. and Stellenbosch University - Joint assay with Drs. Barbour and Cutting, of respectively, of growth regulators in embryogenic and nonembryogenic cultures and in developing zygotic and somatic embryos.

University of Nebraska - Cottonwood cultures and protocols supplied by Dr. S. Ernst.

Tuskeegee University - Cottonwood cultures, cuttings and protocols supplied by Dr. C. Prakash.

Joint research arrangements are also being sought or negotiated with Dr. J. Caruso, University of Cincinnati; Drs. J. Choi and J. Mathis, Georgia Tech; Dr. K.E. Eriksson, University of GA; Dr. D. Neale, US For. Serv., Berkeley, CA; and Dr. S. Strauss, Oregon State University.

RELATED STUDENT RESEARCH:

Completed in 1989

Lisa T. Dudek - M.S., "Encapsulation of zygotic and somatic embryos of confier species." Advisor, N. Rangaswamy.

Patricia Exarhos - M.S., "Electron microscopy study of ultra-structure on Picea abies plants obtained via somatic embryogenesis." Advisor, T. E. Conners.

Frederick Lang - M.S., "Application of recombinant DNA technology in construction of a gene library." Advisor, R. J. Dinus.

Lorrain Logsden - M.S., "Patterns of gene expression in maturing and germinating tree seeds." Advisor, R.J. Dinus.

Mary Kay Lynde-Maas - M.S., "Fructose utulization by embryogenic and nonembryogenic suspension cultures of Norway spruce." Advisor, M.A. Johnson.

Colleen Walker - M.S., "Optimization and quanitification of embryogenic cultures of several conifer species in bioreactors." Advisors, M.R. Becwar and R.J. Dinus.

In Progress

Lois Forde - M.S., "Phenylalanine ammonia lyase and lignin biosynthesis." Advisors, T.E. Conners and R.J. Dinus.

Peasely Shorter - M.S., "Promotion of additional auxin synthesis in Populus deltoides via transformation with Agrobacterium tumefaciens." Advisor, D.T. Webb.

Colleen Walker - Ph.D., Tenative Dessertation Topic: "Comparison of biological and other methods for delignification of kraft pulps." Advisor, R.J. Dinus.

Michael Wood - M.S., "Effect of cold shocking on cell cultures of Larix decidua." Advisor, R.J. Dinus.

Others of Potential Interest

Ingegerd Uhlin - Ph.D., "Influence of hemicelluloses on structure of bacterial cellulose." Committee participation, R.J. Dinus.

James Bond - "A Raman microspectroscopic investigation of the patterns of molecular order in secondary walls of southern pine tracheids." Committee participation, R.J. Dinus.

PROJECT SUMMARY FORM

DATE: March 28, 1990

PROJECT NO. 3223-00: MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

PROJECT LEADERS: R.J. Dinus, N. Rangaswamy, M. R. Uddin, D. T. Webb

IPST GOAL: To develop and assure low-cost supplies of quality fiber

OBJECTIVE:

To develop reliable cell and tissue culture systems for mass clonal propagation of improved conifers.

CURRENT FISCAL YEAR BUDGET: $500,000

PROJECT RATIONALE:

Major increases can be obtained in fiber production, quality, and uniformity via mass cloning of improved trees. Reliable cell and tissue culture systems will also open the way for genetic engineering and production/delivery of new genetic combinations having exceptional growth, increased pest resistance, special fiber properties, and enhanced site and/or climatic adaptability. Screening for and selecting useful variants in culture could also lower costs and accelerate the pace of conventional tree breeding.

Improved growth will reduce raw material costs and increase returns on capital invested in land and equipment. Greater uniformity of clonal plantations can lower both woodlands and mill operating costs as well as enhance end-use performance and foster development of value-added or new products.

PRIOR RESULTS:

Past research on cell and tissue culture systems has brought somatic emryogenesis, one method of mass cloning, closer to commercialization. Embryogenesis in Norway spruce, our model system, is now reproducible and straightforward. Embryogenic

cultures can be obtained from immature and mature spruce seed and from tissues of newly germinated seedlings.

In accordance with earlier plans, Norway spruce work remains focused on somatic embryo development and maturation. The archive detailing culture orgin, past treatment, and current composition is nearing completion. Action to date has streamlined our culture bank, reduced workloads associated with subculturing, and established bases for both future research on embryo maturation and seedling production. Research continues on gauging embryo maturity and documenting germination events. Collective results should yield improved procedures for maturation, germination, and acclimatization. Somatic seedlings derived from past research are alive and well.

Available loblolly pine embryogenic cultures have been and are being used to improve maturation frequencies. Efforts center on extending recent success with carbohydrates and abscisic acid. A factorial experiment has been installed to test several levels of three carbohydrates and varying concentrations of abscisic acid. Cultures are also being expanded for a second factorial involving best carbohydrate and abscisic acid treatments and differing levels and durations of exposure to Indolebutyric acid. Several exploratory trials are also planned.

In Douglas-fir, efforts to initiate new embryogenic cultures from summer cone collections gave mixed results. Relocation to temporary quarters and later to present facilities inhibited our processing cones as quickly and effectively as desired. In addition, a number of cultures appeared promising at the outset, but later deteriorated or became nonembryogenic. Even so, four new embryogenic lines were obtained. Growth rates, however, have been slow and numbers are insufficient for maturation research at this time. Hence, we are attempting to initiate yet new cultures from mature cones collected last summer in the US and this winter in New Zealand. Most cultures from past years remain viable, but generally slow growth rates are hampering all but exploratory work in maturation.

Difficulties associated with initiation and proliferation of Douglas-fir and loblolly pine prompted a survey of past Institute work on physical and chemical properties of zygotic embryos, gametophytes, and seed. Preliminary findings suggest that conventional culture media differ from environments afforded by developing seed. Importance of deviations will be evaluated as quickly as surveys of institute databases and available literature are completed. The outcome may justify adjustment of current media composition. In any event, new research concerning effects of anionic composition seems warranted, and a grant proposal has been submitted to the National Science Foundation.

PLANNED ACTIVITY FOR THE PERIOD:

Complete staffing plan. Complete renovation of laboratory, office, and greenhouse facilities. Raise initiation frequencies in and obtain additional embryogenic cultures of Douglas-fir and loblolly pine. Improve maturation frequencies and raise efficiency of conversion of seedlings; extend best treatments to target species. Document course of zygotic embryo development, maturation, and germination as well as early growth and development of zygotic seedlings so as to establish guideposts for manipulating somatic materials.

POTENTIAL FUTURE ACTIVITIES:

Renew research on suspension cultures and initiation from explants of trees mature enough to have been proven genetically superior. Adapt cell and tissue culture techniques to study xylogenesis in vitro; explore factors affecting fiber formation and lignification.

Project 3223-00 Status Report

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

PROJECT 3223-00

GENERAL SUMMARY OF RECENT ACTIVITIES

March 28, 1990

This project is designed to develop reliable cell and tissue culture systems for rapid, low-cost mass clonal propagation of genetically improved softwoods. All IPST member companies rely on US forests for raw material, and most operate intensive forest management programs on their own woodlands. The management programs typically include aggressive tree breeding efforts. Economic returns from tree breeding are sizeable, accruing from increased growth and shorter rotations as well as improved quality. Much progress has been made toward ensuring lower cost and stable supplies of raw material.

Such benefits could be increased even further by a cost-effective method of mass clonal propagation. Cloning would maximize genetic gain from existing breeding programs, permit tailoring of planting materials to specific sites and markets, and greatly decrease variability in raw material size and quality.

Somatic embryogenesis, one approach to cloning, has been the focus of this project since its start a little over 10 years ago. The process involves production of "artificial seeds" in sterile culture from a small amount of cells or tissue collected from a donor tree of value. The method is preferred over others, e.g., rooting cuttings, because of its multiplying power. Successfully deployed, the process can provide millions, not tens or thousands, of genetically identical seedlings for planting. Mass cloning only the very best genetically improved materials will thus enable member to maximize returns from their considerable investments in land, forest management, and tree breeding. In addition, such seedlings are expected to suffer minimally, if at all, from the lingering age and position effects commonly encountered in plants cloned by other means.

Reliable cell and tissue culture systems will also open the way for eventual development and application of several other technologies: "genetic engineering" and delivery of new genetic combinations having exceptional properties not otherwise obtainable; screening for and selecting useful variants in culture to lower costs of and accelerate the pace of conventional tree breeding; and investigation under controlled conditions of the factors regulating fiber length, diameter, flexibility, and other properties affecting pulping and papermaking properties.

RESULTS TO DATE:

Initially, project activities focused on studies of natural conifer seed development and manipulation of cells and tissues in culture. An in-depth database and considerable expertise were developed. Other, non-conifer systems; e.g., carrot and coffee, were also investigated with the aim of extending somatic embryogenesis from them to conifers. The phenomenon, however, was not observed in conifers until 1985. At that time, Swedish workers obtained low levels of somatic embryogenesis in Norway spruce, and IPST scientists quickly adopted the species as a model system. The coupling of the in-depth IPST experience with a reasonably workable model engendered rapid process. IPST scientists have since optimized many process steps in the model system, and have extended the process across three related species to the target species, Douglas-fir and loblolly pine.

The process is best viewed as consisting of four major steps: Initiation, Maintenance, Maturation, and Conversion to Seedlings. In the first or Initiation Step, a few cells or small amount of tissue are collected and established in sterile culture. At present, embryos from developing or ripened natural seed are the usual starting material, but the process is gradually being extended to older materials; e.g., the first leaves or needles of newly germinated seedlings. Extension to older materials is an important part of ongoing and future research as commercialization depends on cloning trees old enough to have been certified as genetically superior. Once established in culture, cells or tissues are given a mix of growth regulators and nutrients to foster rapid cell division and growth. Within a few weeks, certain cells within the enlarging mass cease normal growth and differentiate into embryos. The initiation of this embryogenic state is straightforward and reproducible for the model system, Norway spruce. Frequencies of initiation now average 80 percent for cultures started from developing embryos, 25 percent for embryos from ripened seed, and 1 percent for the first needles from germinating seedlings. The target species are more difficult to manipulate, and success rates currently average less than one percent. A major portion of current project resources is thus devoted to optimizing conditions for Douglas-fir and loblolly pine.

In the Maintenance Step, embryogenic cultures are transferred to culture media designed to maintain the embryogenic state, foster culture growth, and promote proliferation of embryo numbers. The medium typically is similar to that used for initiation, but often contains a different mix or concentration of growth regulators. Maintenance of Norway spruce cultures has proven rather easy, and major effort has not been devoted to optimizing it. Culture numbers sufficient for research on subsequent steps generally have been obtained without difficulty. Thus, over 50 percent of embryogenic Norway spruce cultures can be maintained. In addition, growth rates are such that culture numbers double every two weeks, and developing embryos number 750 or more per gram of culture.

Somewhat similar success has been achieved with loblolly pine. Maintenance frequencies average 80 percent, and growth rates, though slower, are adequate. Loblolly pine cultures, however, contain far fewer embryos per gram, and much effort is being given to understanding and remedying the cause of this important difference.

Maintenance of Douglas-fir is more difficult, even though roughly 50 percent of the embryogenic cultures can be maintained for reasonable lengths of time. Growth rates are slow; doubling times average three or more weeks. In addition, perhaps 25 percent of the cultures deteriorate or revert to a nonembryogenic condition within a year or so. This is in direct contrast to Norway spruce and loblolly where cultures have remained embryogenic for five and three years, respectively. This problem has retarded research on subsequent steps, and is receiving and will continue to receive major attention.

Manipulations in the Maturation Step are structured to slow overall growth of the cultures, stop proliferation of new embryos, and stimulate growth, development, and maturation of individual embryos. The objective is to grow the many small embryos produced in cultures during the Initiation and Maintenance steps to a size and organizational state equal to that of natural embryos in ripened seed. Toward these ends, growth regulators used to foster growth and proliferation must be removed, and a new mix substituted in their place. Recent findings also indicate that the nature and level of both nitrogen and carbohydrate sources play significant roles in maturation.

Embryo maturation has proven a difficult step, regardless of species. Fully mature Norway spruce embryos were produced at IPST several years ago, but numbers were low and the results sporadic until recently. Increased reliability was secured only after a lengthy series of experiments testing various growth regulators and nitrogen sources. The most recent experiment, completed in 1989, raised mature embryo yields five-fold. On the average, each gram of culture now yields 130 embryos, mature and ready for germination. Best treatments yield roughly 350. Despite this improvement, however, conditions are far from optimal in that each gram contains 750 potential embryos. Further improvement to this key step is critical to realizing the tremendous mulitplying power of somatic embryogenesis.

Considerable effort has also been devoted to extending such results to the target species. Small but significant numbers of mature embryos have been obtained in both loblolly pine and Douglas-fir. In loblolly pine, the very best treatments produce two or three mature embryos per gram. Results are reproducible, and microscopic assays indicate that the embryos are structurally identical to their natural counterparts. Reasons for the low yields are not yet clear, but several factors have been implicated. Firstly, loblolly pine embryogenic cultures contain fewer embryos in the Initiation and Maintenance Steps. Secondly, the few that are present apparently require stimuli different from those effective in Norway spruce. As an example, preliminary trials suggested that different carbohydrate sources and concentrations were needed. Thus, comprehensive tests of

the interaction between growth regulators, nitrogen regimes, and carbohydrates are underway. Prospects for overcoming the barrier posed by the Maturation Step clearly have improved in recent times. It nevertheless remains a serious obstacle, and major effort will be required to resolve it.

The last step, Conversion to Seedlings, entails harvesting mature embryos, germinating them, and moving the newly germinated embryos through a series of controlled environments that condition them for transfer to a greenhouse or nursery and eventually to field trials. Having developed under protected conditions, somatic embryos and seedlings are likely to be quite sensitive to natural levels of and changes in temperature, humidity, and moisture. The transition to natural conditions must therefore be gradual, with embryos germinated in sterile culture and planted in closed containers of soil. Acclimatization can then be accomplished by slowly reducing the frequency of watering and gradually opening the containers to ambient temperatures and humidities. Fertilizer and fungicide applications may also be necessary, but little is known about specific requirements.

Work on this step has to date involved only Norway spruce. Varying numbers of seedlings have been produced over the last four years, but they have been more the by-product of research on other steps than a goal in and of themselves. Thus, experiments on germination and acclimatization have been conducted on an ad hoc basis. Germination trials initially gave poor and highly variable results. Important factors were identified, and more recent trials have given more uniform results. Germination currently averages 19 percent, considerably higher than in most earlier trials but still far too low.

The first few seedlings obtained at IPST are now entering their fifth growing season. Throughout their lives, they have started and stopped growth in synchrony with their natural standards, and have remained similar in most other external appearances as well. The only obvious difference has been a tendency for the somatic seedlings to have fewer and finer branches. Summed over all years, roughly a hundred seedlings of various ages and in different stages of development have been produced. Regrettably, these represent only 10 to 25 percent of the embryos that germinated and were planted in soil. Difficulties with acclimatization were planted in soil. Difficulties with acclimatization were numerous and critical factors are not understood. Clearly, more reliable procedures for both germination and acclimatization are needed. Recent improvements to the Maturation Step should enable us to produce sufficient mature embryos for definitive trials. These will be designed to improve the overall conversion process, and establish replicated greenhouse and field trials.

NEAR-TERM PLANS:

Mass clonal propagation of genetically improved conifers has been moved closer to commercialization in recent years. As noted above, however,

significant obstacles remain. Over the near-term, project efforts and resources will be focused as follows:

Model System -
- Document events in natural systems so as to develop guidelines for manipulating somatic materials
- Improve maturation frequencies
- Raise efficiency of conversion to seedlings
- Adapt best treatments and methods for application to target species
- Develop methods for initiation from older materials

Target Species -
- Raise initiation frequencies
- Obtain sufficient embryogenic cultures for work on later steps
- Improve maturation frequencies
- Initiate research on conversion to seedlings

POSSIBLE FUTURE DIRECTIONS:

* Establish replicated greenhouse and field trials of seedlings from the model system; use the results to accelerate production and testing of seedlings from the target species.

* Utilize techniques developed in Project 3223-02 to evaluate genetic fidelity of somatic embryos and seedlings.

* Evaluate liquid suspension cultures as a means for increasing embryo production

* Investigate feasibility of and techniques for constructing "synthetic seeds", ie., encasing somatic embryos in a nutrient medium and polymer coating that will permit easy handling, storage, and nursery sowing.

* Adapt cell and tissue culture methods for study of factors affecting lignification, fiber dimensions, and other aspects of fiber formation.

* Devise methods for screening for and selection of novel variants in culture.

* Initiate research on "genetic engineering"; genes of possible interest include those regulating lignin biosynthesis, production of growth regulators and other factors affecting fiber formation, and resistance to diseases and insects.

PROJECT SUMMARY FORM

DATE: March 28, 1990

PROJECT NO. 3223-02: BIOCHEMISTRY OF CLONAL PROPAGATION

PROJECT LEADERS: Vacant (2)

IPST GOAL: To develop and assure low-cost supplies of quality fiber

OBJECTIVE:

Develop an improved understanding of biochemical mechanisms controlling embryogenesis and other cloning methods, and devise procedures for raising the effectiveness and efficiency of mass cloning methods.

CURRENT FISCAL YEAR BUDGET: $150,000

PROJECT RATIONALE:

Improved understanding of biochemical mechanisms controlling embrygenesis and other cloning methods will shorten the time to commercial application of clonal forestry, raise their efficiencies, and facilitate extension to trees mature enough to have been proven genetically superior.

PRIOR RESULTS:

Past Institute efforts have been made somatic embrygenesis in Norway spruce, our model system, straightforward and reproducible. Embryo numbers can be quantified, and seedlings have been recovered. Somatic embryogenesis has also been obtained in our target species, loblolly pine and Douglas-fir, but initiation frequencies remain low and seedlings have not been recovered.

Earlier work on the biochemistry of embryogenesis yielded useful data on differences between embrygenic and nonembrygenic cultures, and some knowledge of factors affecting the process. Such differences and associated markers are used to screen cultures for embryogenic potential, and monitor effects of modified or new

protocols. In addition, techniques for isolating, purifying, and characterizing proteins, lipids, enzymes, RNA, and DNA have been developed or refined. These are now available for use in increasing initiation and maturation frequencies, facilitating conversion to seedlings, and evaluating seedling performance and fidelity.

Investigation of biochemical and molecular properties of zygotic and somatic embryos slowed upon relocation as all involved personnel chose not to move. Efforts since then have focused on completing publications and internal documents. Much effort has also been given to readying facilities, unpacking equipment, and recruiting new employees. Once new personnel are hired, work on using biochemical and molecular methods to promote embryo maturation will be renewed and accelerated. Efforts to quantify and stimulate biosynthesis of proteins and lipids will receive particular attention. In anticipation of renewed research, numerous cultures from selected experiments have been and are being frozen and stored.

Work continues on quantifying levels of growth regulators, especially abscisic acid, in developing embryos. Cooperative arrangements for assay, using monoclonal antibodies, have been finalized with industril and university scientists, in the Republic of South Africa. To ensure successful completion, similar arrangements are being negotiated with several US scientists. Growth regulators are major factors in maturation, and successfully documenting their ebb and flow should enable us to improve maturation frequencies.

Recrutiting/hiring is underway and a number of likely candidates have been identified.

PLANNED ACTIVITES FOR THE PERIOD:

Complete recruiting/hiring, and ready facilities for use. Renew work on similarities/differences of zyotic an somatic embryos, with emphasis on proteins and lipids as well as on using substrates and inhibitors to stimulate maturation. Execute cooperative efforts to quantify abscisic acid levels in developing embryos. Direct and facilitate student project on documenting onset and course of lignification.

POTENTIAL FUTURE ACTIVITIES:

Refine and apply methods for certifying genetic fidelity of seedlings derived from cloning of softwoods and hardwoods. Document differences between mature and immature tissues, compare characteristics of mature tissues to those of explants known to have embryogenic potential, and devise means for rendering mature tissues more easily manipulated in culture. Refine and develop promising new techniques. Explore procedures for early selection and testing.

Project 3223-02 Status Report

BIOCHEMISTRY OF CLONAL PROPAGATION

GENERAL SUMMARY OF RECENT ACTIVITIES

March 28, 1990

This recently established project was originally part of Project 3223-02, Mass Clonal Propagation of Improved Conifers, and work specifically supported that project. Separation was effected to ensure more precise direction, encourage exploratory research in other areas, and provide support to newly chartered Project 3223-03, Mass Clonal Propagation of Genetically Improved/Engineered Hardwoods. Improved understanding of and ability to manipulate biochemical and molecular mechanisms affecting clonal propagation remain the primary goal. Results are expected to make research on cloning less empirical and more efficient, hasten commercialization, and facilitate extension to trees mature enough to have been proven genetically superior.

RESULTS TO DATE:

Earlier work on the biochemistry of somatic embryogenesis yielded useful data on differences between embryogenic and nonembryogenic cultures and on factors affecting the process. Such differences and associated markers are being used to screen cultures for embryogenic potential and monitor effects of changes in culture media. Techniques for isolating, purifying, and characterizing proteins, fats, and enzymes, RNA, and DNA were also adapted or developed. Several are now available for use in increasing production of embryogenic cultures and fostering maturation of somatic embryos. Further work on others is essential before they can be applied routinely, especially in verifying genetic fidelity of somatic embryos and seedlings and in monitoring gene expression in genetically engineered hardwoods.

Efforts in recent years centered on stimulating biosynthesis of storage products, proteins and fats, in developing embryos. Types and quantities of such compounds were first followed across developmental stages in natural embryos. Analyses were then extended to somatic embryos. Comparisons showed that somatic embryos accumulated the same compounds but in smaller amounts and somewhat later. Attempts were then made to stimulate biosynthesis and thereby promote embryo maturation and readiness to germinate. Some improvement was obtained, but the research is on hold since involved personnel chose not to move with IPST. Such products provide the wherewithal for embryo germination and early seedling growth, and ability to control their biosynthesis is critical to success with somatic embryogenesis. The research will therefore be activated and

given high priority as new personnel are put in place. In anticipation of renewed effort, cultures and embryos of interest are being harvested, frozen, and stored for later analysis.

Work is proceeding apace on quantifying levels of growth regulators in devloping somatic embryos. Cooperative arranements for assay habe been finalized with an internationally recognized team of scientists, and similar arrangements are being negotiated with others as insurance. Growth regulators are major factors in embryo maturation, and documenting their ebb and flow should enable us to improve several steps in the process of somatic embrygenesis.

NEAR-TERM PLANS:

*Renew development of molecular and biochemical techniques
*Reactivate analyses of protein and lipid biosyntheses
*Initiate research on use of inhibitors and substrates to stimulate embryo maturation
*Execute cooperative assay of growth regulator levels
*Initiate research on hardwood genetic engineering

POSSIBLE FUTURE DIRECTIONS:

*Refine molecular methods for verifying genetic fidelity and gene expression
*Devise means for rendering tissues from older trees more easily mainpulated in culture
*Develop methods for early selection and testing
*Acquire or develop new techniques
*Explore opportunities for influencing fiber formation in culture

PROJECT SUMMARY FORM

DATE: March 28, 1990

PROJECT NO. 3223-03: MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED/ENGINEERED HARDWOODS

PROJECT LEADERS: R.J. Dinus, M.R. Uddin

IPST GOAL: To develop and assure low-cost supplies of quality fiber

OBJECTIVE:

To develop reliable cell and tissue culture systems for mass clonal propagation of genetically improved and/or engineered hardwoods.

PROJECT RATIONALE:

Major increases can be obtained in fiber production, quality, and uniformity via mass cloning. Reliable cloning systems will also open the way for genetic engineering and production/delivery of new genetic combinations having exceptional growth, greater pest resistance, special fiber properties, and enhanced site and/or climatic adapatability. Screening/selection for useful variants in tissue culture holds promise for raising the pace and efficiency of conventional tree breeding.

Accelerated growth will ensure reliable raw material supplies, reduce their costs, and raise returns on capital invested in land and equipment. Greater uniformity can lower both woodlands and mill operating costs as well as enhance properties related to end-use performance. Better or new fiber properties can improve end-use performance and foster development of value-added or new products.

PRIOR RESULTS:

Considerable hardwood research has been done at the Institute in past years. This work resulted in production of plants from tissue culture, and successful application of polyploidy to forest tree breeding. Other exploratory work at the Institute suggested that tissue culture methods can be used to test for disease resistance. Results from these efforts and those of other organizations indicate that hardwood tissues, cells, and protoplasts can be

manipulated in culture with relative ease. In addition, the first demonstration of gene transfer and expression in forest trees was accomplished with a hardwood. Still other work infers that novel variants can be produced in culture, isolated, and used to introduce new traits into breeding and/or planting stock.

In accordance with earlier plans, this project seeks to develop technologies for transferring genes for herbicide tolerance into commercially important species, and for efficient mass propagation, testing and release of genetically modified plant materials. Herbicide tolerance may also be sought, more on an exploratory or insurance basis, via somaclonal variation and selection. Since project plans were finalized, most efforts have been devoted to selecting research approaches, collecting suitable plant materials, and establishing stable cultures.

Both diploid and haploid explants of cottonwood and aspen were obtained from IPST sources and cooperating organizations, and used to establish cultures for subsequent expansion and manipulation. Stable cultures and protocols for adaptation to our needs were also obtained from collaborators at the University of Nebraska and Tuskeegee University. Cuttings of additional elite trees will be provided in the near future by a member company, Tuskeegee University, and the University of Minnesota.

In cottonwood, several donor trees are represented by stable diploid and/or haploid cultures. A system suitable for tranformation and easy regeneration has been developed with cultures from one of the aforementioned donor trees. Leaf sections were isolated from stabilized shoot cultures, surface sterilized, and plated on modified Woody Plant Medium containing Naphthaleneacetic acid and 6-Benzylaminopurine. Adventitious buds formed within several weeks, and shoot elongation occurred after transfer to the same basal medium with half-strength growth regulators. Excised shoots root readily on the basal medium supplemented with Indolebutyric acid. Significant numbers of rooted plants have been produced and are ready for acclimatization and transfer to the greenhouse. The system is also being used for a student project on genetic transformation.

Suspension cultures have also been established from leaf callus of two donor trees. Successful regeneration from suspensions will provide another avenue for attack on transformation and/or somaclonal variation/selection. In an effort to regenerate plants, samples of the suspensions were plated on solid medium and manipulated to form callus. Cultures from one of the donor trees yielded micro-calli, and these are being used to obtain regeneration with this system.

Aspen shoot cultures were stablized with relative ease, and diploid cultures of two elite aspens and one triploid hybrid are available. Also, cultures from past exploratory work on tetraploid aspen and native sweetgum are being maintained.

PLANNED ACTIVITIES FOR THE PERIOD:

Expand existing cultures, and initiate or secure and stablize additional cultures. Complete greenhouse construction, secure additional plant materials, and establish "clean" greenhouse populations. Refine technologies for mas propagation, and ensure that resultant systems are suitable for genetic transformation. Direct and facilitate student project on genetic transformation. Complete recruiting key personnel, and accelerate research on gene transfer and expression.

POTENTIAL FUTURE ACTIVITIES:

Explore novel methods for accelerating conventional tree improvement by early testing and selection in culture, generating haploid cultures and dihaploids, effecting protoplast fusion, and creating polyploid or hybrid individuals.

Project 3223-03 Status Report

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED/ENGINEERED HARDWOODS

GENERAL SUMMARY OF RECENT ACTIVITIES

March 28, 1990

Demand for high quality hardwoods has increased dramatically in recent years. Conversion of mills from manufacturer of commodity grade products to those having higher profit margins has been the principle driving force, especially in the Southeastern Coastal Plain. In the Pacific Northwest, escalating export demand has also played a significant role. In yet other regions, supplies are adequate but are located in wet or mountainous areas, economically harvestable volumes are scattered, or stands are situated at sizeable haul distances from mills. In addition, the products in question typically require fiber of consistently high quality, but wood deliveries are highly variable in terms of species, age, and quality. Thus, significant numbers of IPST member companies require more and better hardwood in a time of tightening supplies and rising costs. Many have become convinced that breeding, planting, and management of hardwoods is warranted.

Planting and management, however, can be costly. Sites suitable for prime species; e.g., cottonwood and aspen, are fertile and support a variety of competing vegetation. Commercially available herbicides cannot be used to control competition because managed species are as susceptible as competitors. As a result, competition control is either not done and volume productivity is reduced or control is effected by mechanical means and establishment costs rise to near prohibitive levels. Sensing an opportunity to help member companies, IPST recently chartered the present project which seeks to develop technologies for transferring genes for herbicide tolerance into commercially important hardwoods, and for efficient mass propagation, testing, and release of genetically transformed trees.

RESULTS TO DATE:

Considerable hardwood research has been done at IPST in past years. This work resulted in production of improved trees from tissue culture, and successfully demonstrated that tissue culture could benefit tree breeding. Other exploratory work at IPST suggested that tissue culture methods can be used to test for disease resistance. In addition, the first demonstration of genetic engineering in forest trees was accomplished with a hardwood. Still other work infers that novel variants can be produced in culture, and used to introduce new traits into breeding and/or planting stock. These collective results indicate that hardwood cells and tissues can be manipulated in culture and genetically engineered with relative ease.

Initially, most project efforts were devoted to selecting research approaches, collecting suitable plant material, and establishing stable cultures. Tissue samples of cottonwood and aspen were obtained from cooperating member companies and universities, and used to establish cultures for subsequent expansion and manipulation. Stable cultures and procedures were also obtained from collaborators at several universities.

More recently, work has focused on production of intact plants from culture, and a system suitable for genetic engineering and easy regeneration has been developed. Briefly, leaf sections are isolated from stabilized shoot cultures and placed on conventional culture medium supplemented with a unique mix of growth regulators. Numerous buds form within several weeks and these elongate into shoots after transfer to culture medium containing a different mix of growth regulators. Excised shoots can be rooted with ease, and significant numbers of intact plants have been produced for transfer to the greenhouse. The system is also being used for a student project on genetic engineering.

Liquid cells suspensions of cottonwood have also been established with relative ease. Addition of normally lethal doses of herbicides to such suspensions may result in isolation of one or a few cells with above average tolerance. Availability of rapidly growing cell suspension allows this approach to be tested, on an exploratory or insurance basis, as another route to herbicide tolerance.

Aspen cultures have also been stabilized with relative ease, and cultures of two elite and one hybrid aspen are available. Also, cultures from past exploratory work with a polyploid aspen tree and several sweetgum selections are being maintained for future use.

NEAR-TERM PLANS:

* Increase number and availability of existing cultures
* Initiate or secure additional cultures
* Secure additional plant materials for creation of a reserve greenhouse population
* Refine technologies for mass propagation
* Accelerate research on genetic engineering
* Regenerate genetically engineered trees

POSSIBLE FUTURE DIRECTIONS:

* Verify herbicide tolerance of genetically engineered trees
* Explore creation of novel variants and hybrids in culture
* Examine feasibility of studying factors affecting lignification, fiber dimensions, and other aspects of fiber formation in culture

* Explore opportunities for genetically engineering other traits; e.g.; lignification, growth regulator production, other fiber characteristics, and pest resistance

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