"This information represents a review of ongoing research for use by the Project Advisory Committees. The information is not intended to be a definitive progress report on any of the projects and should not be cited or referenced in any paper or correspondence external to your company."

Your advice and suggestions on any of the projects will be most welcome.

March 26-27, 1984
The Institute of Paper Chemistry
Appleton, Wi  54912
TO: Members of the Forest Genetics PAC

Enclosed is advance reading material for the March 26-27 meeting of the Forest Genetics Project Advisory Committee (PAC). Included are status reports for active projects, a tentative agenda, and a current membership list.

Rooms have been reserved in the Continuing Education Center, and meals will be provided as stated on the agenda. Meeting attendance will be limited to Committee Members. Recent progress and research plans for the coming year will be the topics featured the first day. The morning of the second day will be reserved primarily for committee deliberations. Please call Becky Dietzen (414) 738-3448 to make your reservations.

We look forward to meeting with you on March 26-27.

Sincerely,

Dean W. Einspahr
Director
Forest Biology Division

DWE/bd
Enclosure
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AGENDA

PROJECT ADVISORY COMMITTEE
FOREST GENETICS

March 26-27, 1984
The Institute of Paper Chemistry
Continuing Education Center
Appleton, Wisconsin

Monday, March 26, 1984
Continuing Education Center, Seminar Room

12:00 p.m. Lunch PAC Members (CEC Dining Room)

1:00 Introduction and Meeting Overview  

1:10 Conventional Forest Management  
I. Aspen Genetics  
II. Genetic Improvement of Larch

1:30 Project 3223 - Mass Production of Conifer Hybrids  
I. Objectives and Subobjectives  
II. Project Funding and Staffing

2:15 III. Model System Research  

3:30 Break

3:45 IV. Objective I Research -- Generating and Maintaining Quality Cell Suspensions

5:00 Social Hour

5:30 Dinner (CEC Dining Room)
6:30  V. Objective II Research -- Somatic Embryogenesis  Johnson

7:15  VI. Student Research  Johnson

7:30  VII. Summary of Progress  Einspahr

Tuesday, March 27, 1984
CEC Seminar Room

7:00 a.m.  Breakfast - CEC Dining Room

8:00  Forest Biology Section Research Plans

Conventional Forest Genetics Research  Einspahr

Conifer Tissue Culture Research  Litvay

Model Systems
Objective I
Objective II
Objective III

9:00  PAC Deliberations  Eudy

11:30  Adjourn

11:30  Lunch - CEC Dining Room
Project Advisory Committee

FOREST GENETICS

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THE INSTITUTE OF PAPER CHEMISTRY
Appleton, Wisconsin

Status Report
to the
FOREST GENETICS
PROJECT ADVISORY COMMITTEE

Project 3501
DEVELOPMENT OF ANALYTICAL TECHNIQUES FOR CHARACTERIZING
WOOD & FIBER AND RELATED EXPLORATORY RESEARCH

March 1, 1984
PROJECT TITLE: Development of Analytical Techniques for Characterizing Wood & Fiber and Related Exploratory Research

PROJECT STAFF: Section 30

PRIMARY AREA OF INDUSTRY NEED: Raw Materials

PROGRAM AREA: Special Competence

PROGRAM GOAL: Increase the supply and improve the utilization of the fibrous raw materials.

PROJECT OBJECTIVE:

Evaluate and/or develop the analytical techniques required to meet Institute and member company demands for characterizing wood and fiber. Investigate novel ideas and develop supporting information to justify funded projects having the objective of expanding the industries source of useable fibers.

PROJECT RATIONALE:

Improved utilization of the available supply and investigations into new sources of fiber requires having adequate analytical techniques for characterizing the original untreated source of fiber and the influence that chemical and mechanical action has on the usefulness of these new sources of fiber. These same techniques are expected to be useful in evaluating present and new pulping, bleaching, and refining procedures. Additionally, researchers are encouraged to devote part of their time to developing novel ideas that will result in new analytical techniques and/or new research projects. These exploratory studies are expected to be in the wood and fiber science, tree physiology, biochemistry and forest genetics area.

RESULTS TO DATE:

Progress in the wood and fiber science area relates primarily to improving our capabilities in characterizing cellulose fibers at the molecular level. The electron microscopy laboratory has recently been renovated. The laboratory has two state-of-the-art electron microscopes: a scanning electron microscope (SEM) and a scanning transmission electron microscope (STEM). Besides offering high resolutions imaging capabilities, both scopes can provide qualitative and quantitative elemental analyses. The SEM can detect and measure all elements having an atomic number equal to or greater than that of boron (5) by using an energy dispersive spectrometer (EDS) or a wavelength dispersive spectrometer (WDS). The STEM can determine all elements having an atomic number greater than that of sodium (11) by using an EDS. Work during the past year brought all the equipment on line and established all basic procedures.

Isozyme procedures employing the peroxidase enzyme system demonstrated the potential of determining the "relatedness" of parent trees and suggested the need for additional research to make the approach as definitive as required. Of 25 enzyme systems evaluated, two now appear very promising; peroxidase and acid phosphatase. Additionally, banding patterns of total proteins show promise for use in defining relatedness. A biological similarity index system has been modified for statistically comparing enzyme banding patterns and providing an estimate of relatedness of parent trees.
Mycorrhizae investigations have resulted in the development of a suitable inoculation procedure and the establishment of two organisms (*Pisolithus tinctorius* and *Suillus grevillei*) on larch that appear worthy of field testing.

PLANNED ACTIVITIES FOR THE PERIOD:

A. For the wood and fiber science area, preliminary molecular level characterization of fiber surfaces that have been treated in several ways (pulping and refining) will be evaluated.

B. Isozyme procedures for determining relatedness of parent trees will be continued by trying additional enzyme systems on trees of known origin and relatedness. Also, the possibility of using dormant season buds as a source of enzyme activity, instead of late summer needles, will be investigated.

C. Mycorrhizae research will be continued by field testing Japanese larch that have been inoculated using the two previously listed organisms.

POTENTIAL FUTURE ACTIVITIES:

A. Evaluate the potential of:

1. High resolution imaging of fiber surfaces which have undergone mechanical or chemical degradation.

2. Quantification and Z-direction distribution of inorganic fillers and coating materials in paper, and

3. Determination of lignin distributions in fibers using the bromination reaction.

B. Examine the usefulness of the isozyme approach to "relatedness" using full-sib progeny and additional half-sib populations.

C. Establish a field test with European and/or hybrid larch that would evaluate the growth increases associated with the presence of mycorrhizae.
THE INSTITUTE OF PAPER CHEMISTRY
Appleton, Wisconsin

Status Report
to the
FOREST GENETICS
PROJECT ADVISORY COMMITTEE

Project 3223
THE MASS PRODUCTION OF CONIFER HYBRIDS

March 1, 1984
PROJECT TITLE: The Mass Production of Conifer Hybrids

PROJECT STAFF: D. Einspahr, J. Litvay, M. Johnson

PRIMARY AREA OF INDUSTRY NEED: Raw Materials

PROGRAM AREA: Increase wood production ... by embryogenesis and bioengineering

PROGRAM GOAL: Mass production of conifer hybrids

PROJECT OBJECTIVE/GOAL:

The overall objective is the mass production of conifer hybrids. The near-term objective is the development of a procedure for producing plantlets from single cells or small groups of cells.

PROJECT RATIONALE:

Major increases can be obtained in tree growth and forest production through the clonal propagation of "elite" trees and through the creation of new genetic combinations. Planned genetic combinations are ones that are difficult to produce using conventional techniques but are anticipated to result in individuals of exceptional disease resistance and special site and/or climatic adaptability. Production of plantlets from cell suspensions will open the way to the badly needed genetic gains described above through genetic engineering. The cell suspension approach forms the basis for a second-generation technology that is considered to some day replace the existing practice.

RESULTS TO DATE:

Appropriate new media have been developed for initiating callus production and for growing cells in suspension. Procedures for establishing appropriate cell lines have been developed. Biochemical and morphological characterization of embryogenesis is under way. Use of wild carrot somatic embryogenesis and natural Douglas-fir and loblolly pine embryo development as model systems, have assisted in establishing media change requirements and in developing needed biochemical markers. Excised conifer embryo investigations have been used to determine the nutrient requirements of developing embryos. Inhibitor studies have demonstrated the importance of several polyamines in embryogenesis and determined that polyamine synthesis in wild carrot was controlled mainly through the arginine-agmatine pathway. Alternative procedures for modifying free amino acid and polyamine levels have been determined. Studies of natural pine embryogenesis indicate polyamines play an essential role in the development of pine embryos in maturing cones. Tests of the new LM medium for growing conifer cells in suspension demonstrated the medium can also be used to produce somatic embryogenesis in wild carrot. New immature embryo cell lines have been produced and they are being monitored to determine if they have an improved potential for embryogenesis. The immature seed extracts that improved wild carrot embryogenesis are being evaluated on loblolly pine cell lines. A comprehensive,long-range research plan has been developed that includes developing plantlet transfer techniques, methods of checking plant material fidelity and the eventual application of genetic engineering techniques to conifer cell lines.
PLANNED ACTIVITY FOR THE PERIOD:

Research is planned in the areas of model systems investigations, generating and maintaining competent cell lines and embryogenesis. More specifically under the model system we plan to: (1) determine the influence of gibberellins on wild carrot somatic embryogenesis, (2) determine the changes in growth regulator levels during natural conifer and wild carrot somatic embryogenesis, and (3) determine the critical factors important in the conversion of arginine to polyamines. Research on generating and maintaining competent cell lines is expected to include: (1) generation of new cell lines from immature embryos, protoplasts and microsporophyll tissue, (2) determining the influence of nitrogen sources, polyamines, gibberellins and cytokinins on conifer cell line quality, (3) determining the influence of natural conifer extracts on conifer cell line quality. Conifer somatic embryogenesis studies are expected to include: (1) establishing monitored launch experiments that have as objectives the correction of apparent deficiencies and the production of inhibitors, (2) conducting occasional unmonitored launch experiments incorporating a combination of promising factors, (3) running monitored launch experiments using promising new cell lines, and (4) studies with the objective of determining the influence of natural extracts on conifer embryogenesis.

POTENTIAL FUTURE ACTIVITIES:

Future emphasis, when somatic embryogenesis is a reality, will be on plantlet transfer to soil and research related to genetic engineering (i.e., production of haploid cell suspension, protoplast fusion, etc.).

SHORT TERM GOALS:

Short term goals in the conifer tissue culture program will be to:

1. Complete the biochemical characterization (free amino acids, polyamines, and proanthocyanidin) of:
   (a) Wild carrot somatic embryogenesis model system, and
   (b) The conifer natural embryogenesis model system.

2. Determine factors critical to the conversion of arginine to polyamines.

3. Work out methods of controlling polyamine and proanthocyanidin levels in rapidly growing cell lines.

4. Generate and evaluate several new, very juvenile cell line sources including immature embryos, male flower parts, and protoplasts for their embryogenetic capability.

5. Evaluate and select appropriate methods for determining growth regulator levels (IAA, IBA, GA, NOA, BAP, etc.) in tissue culture samples.

6. Establish patterns of change in growth regulator levels present during natural conifer and wild carrot somatic embryogenesis.

The above short term goals 1 through 4 are expected to be completed during the coming year with the current level of funding. Increased funding would allow us to begin much needed growth regulator research. Growth regulator levels appear to be critical during the early stages of embryogenesis. Short term goals 5 and 6 provide additional details on the first phases of a planned growth regulator research program.

*Launch experiments are studies which attempt to cause cells in suspension to go through the necessary steps to produce embryos.
MODEL SYSTEMS RESEARCH

During the past year, model systems research was an important part of the overall tissue culture research effort. Studies on the polyamine levels in wild carrot and in developing pine cone embryos represent just a part of our research effort in this area. As a follow-up to earlier reported metabolic pathway/inhibitor research, a study was established in which the role of arginine decarboxylase (ADC) and polyamines in wild carrot embryogenesis was investigated. The results of these experiments demonstrated that the inhibitor α-difluoromethylarginine (DMFA) caused a 50% reduction in wild carrot embryo formation. Additions of putrescine restored embryogenesis. The inhibition of development by DFMA, which acts by decreasing ADC activity and lowering putrescine and spermidine levels in the cells, demonstrates that putrescine and spermidine are among factors essential for embryogenesis in wild carrot.

Additional inhibitor studies which employed dicyclohexylammonium sulfate (DCHA) and methylglyoxal bis-guanyl hydrazone (MGBG) seem to indicate that spermidine, rather than putrescine may be the polyamine most required in wild carrot embryo development.

Studies conducted on polyamine levels in pine ovules collected from developing cones demonstrated that polyamines rose at times of suspected fertilization and embryo development. Spermidine levels rose more than either putrescine or spermine, providing an additional example of the similarity between wild carrot and pine embryogenesis.
As part of our research on testing the use of "undefined media" to stimulate embryogenesis, extracts of developing pine seeds were prepared and first tested to determine their influence on wild carrot embryogenesis. Certain fractions are now undergoing similar tests with conifer cell lines. Preliminary results indicate that a high molecular weight fraction has been isolated that promotes wild carrot embryogenesis. The low molecular weight fraction used appears to have a toxic influence on embryogenesis and its future use will require removal of that part of the low molecular weight fraction causing the toxicity.

Two additional model system studies were established to check on the ability of the new LM to initiate competent cell lines and to determine if there was anything inherent in the make up of LM that prevented embryogenesis in competent cell lines. The wild carrot model system was used and the results demonstrated that LM could be used to initiate and maintain competent cell lines and that upon the removal of 2,4-D, satisfactory embryogenesis occurred.

Concern over possible changes that may occur during transport and storage of pine cones resulted in the establishment of two studies, one to examine the effect of cone storage on biochemical parameters and a second on the effect of storage on the organogenesis of explants. These two studies demonstrated that free amino acid levels generally increased during storage. Putrescine levels, which are considered sensitive to pH and osmotic stress in plant tissue, also increased quite dramatically with storage. Four day storage, however, had no influence on immature embryo explants as a source of tissue for organogenic studies.
Growth regulators, particularly IAA, are important in the growth and differentiation of plant structures and we need to be able to accurately measure growth regulator levels in order to have an appropriate understanding of their importance in embryogenesis. Despite the limitations of current measurement techniques, there appears to be an early increase in IAA content (day 12-14) in both proliferating and organizing wild carrot cells and a second IAA increase during a more advanced stage of wild carrot embryo development (at approximately 3 weeks). Only preliminary data are available on IAA levels in loblolly pine cell suspensions.

OBJECTIVE I RESEARCH

Objective I research which centers on initiating and maintaining good quality cell suspensions, also received a major amount of emphasis during this past year. One media study investigated phosphate uptake of wild carrot under plus growth regulator (proliferative) and minus growth regulator (embryogenesis) growth conditions. Our standard 10-D cotledon loblolly pine line was also evaluated under similar growth regulator conditions. Wild carrot cultures were found to either (1) remove phosphate prior to growth or (2) remove phosphate very rapidly and as a result depleted the phosphate in the wild carrot medium by day 9. Higher levels of phosphate in LM delayed depletion until day 14. Loblolly pine cultures, with an inoculum density of 10 µL/mL and "plus" growth regulator, did not deplete the phosphate until day 14. Thus, subculturing 10-D Cot at 10-day intervals is expected to result in adequate cell line phosphate levels (and appropriate energy levels) when transferred into "minus" growth regulator (embryogenesis) conditions. One possible reason for the slow growth we
OBJECTIVE II RESEARCH

Objective II research includes studies designed to induce embryogenesis from cells growing as cell suspensions. Studies undertaken this past year in this phase of our program included a series of unmonitored launch experiments aimed at embryo production, a study of tannin buildup in loblolly pine cell suspensions, and an interesting study in which wild carrot and loblolly pine cells were grown as a mixed cell suspension. This latter study was an attempt to induce loblolly pine embryogenesis by a "co-culture" technique. Results of the co-culture work demonstrated that (1) wild carrot and loblolly pine cells can be grown together, and (2) co-culturing did not inhibit embryogenesis in wild carrot nor did it stimulate embryogenesis in the loblolly pine cells.

The research on tannin in loblolly pine cell suspensions consisted of a series of four experiments designed to (1) determine the relationship between tannin production and the activity of the enzyme, phenylalanine ammonia lyase (PAL) and (2) determine if inhibition of PAL activity was a feasible approach for controlling tannin production. The results of these four experiments demonstrated that (1) there was no strong correlation between PAL activity and tannin production, (2) tannin itself was not solely responsible for the failure of pine cells to grow in a 2,4-D free media and (3) that although the use of PAL inhibitors prevented the appearance of tannins, this did not result in growth response. Of particular interest in this series of studies was the last study in which, by proper selection of the age of the inoculum, some growth occurred and tannin buildup was delayed until about day eight. This suggests that by the proper use of inhibitors along with careful selection of the inoculum age, growth in 2,4-D-free media without tannin buildup may be feasible.
Plans for the coming year include additional model systems research on:
(1) polyamine levels and associated enzyme activity during the early stages of
natural pine embryogenesis, (2) growth regulator changes during natural conifer
and wild carrot somatic embryogenesis, (3) the factors important in the conver-
sion of arginine to polyamines, and (4) growth rate and cell division during
early stages of embryogenesis. Research on generating and maintaining competent
cell lines is expected to include: (1) generation of new cell lines from im-
mature embryos, protoplasts and microsporophyll tissue, (2) determining the
influence of nitrogen sources, polyamines, gibberellins and cytokinins on
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porating a combination of promising factors, (3) running monitored launch
experiments using promising new cell lines, and (4) studies with the objective
of determining the influence of natural extracts on conifer embryogenesis.

For additional details on recent progress see Project 3223, Progress
RECENT PUBLICATIONS

   Arabinogalactan - proteins from Douglas-fir and loblolly pine.
   Phytochemistry 22:1500-3(1983). (Reprint available on request.)

2. Eddo Rugini and D. C. Verma.
   Micropropagation of difficult-to-propagate almond (Prunus amygdalus,
   available on request.)

   A membrane-bound-o-methyltransferase from Douglas-fir needle callus and
   its role in the nature of endogenous phenolics. (Accepted by Phyto-
   chemistry.)

   Conifer tissue culture and how it may impact the pulp and paper industry.

5. R. D. Teasdale and E. Rugini.
   Preparation of viable protoplasts from suspension-cultured loblolly pine
   (Pinus taeda, L.) cells and subsequent regeneration to callus. (IPC
   Technical Paper Series No. 134.) Plant Cell, Tissue, and Organ Culture

6. Russell Feirer, John D. Litvay, and Gail Mignon.
   The role of arginine decarboxylase and polyamines in somatic embryogenesis

   Tissue culture in forestry, current status. Proceedings Appalachian

   Study of regenerating cell wall material on Douglas-fir protoplasts. I.
   Proline and glucose metabolism. II. Cell wall glycoprotein charac-
   terization. (Submitted to Zeitschrift fur Pflanzenphysiologie.)

9. R. Feirer, G. Mignon, and J. Litvay. Arginine decarboxylase and polyamines

    Influence of a gymnosperm culture media and its components on growth and
    somatic embryogenesis of wild carrot, (Daucus carota, L.). (Submitted to
    Plant Cell Reports.)

11. John D. Litvay and Hilkka M. Kaustinen.
    Gymnosperm embryo development - tissue culture implications for 'r
    gymnosperms. (Being revised for Plant Cell Reports.)
