

THE MASS PRODUCTION OF CONIFER
TREE HYBRIDS

Project 3223

Report Seven

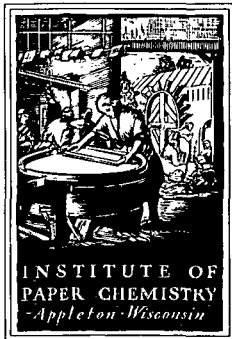
EMBRYOGENESIS IN CELL SUSPENSIONS OF
DOUGLAS-FIR AND LOBLOLLY PINE

A Progress Report

to

MEMBERS OF THE INSTITUTE OF PAPER CHEMISTRY

July 3, 1979



THE INSTITUTE OF PAPER CHEMISTRY
Post Office Box 1039
Appleton, Wisconsin 54912
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July 17, 1979

TO: MEMBERS OF THE INSTITUTE OF PAPER CHEMISTRY

PROJECT 3223 - PROGRESS REPORT SEVEN

THE MASS PRODUCTION OF CONIFER TREE HYBRIDS

EMBRYOGENESIS IN CELL SUSPENSIONS OF
DOUGLAS-FIR AND LOBLOLLY PINE

Please find enclosed Report Seven of The Mass Production of Conifer Tree Hybrids. This year's progress report deals with the induction and formation of embryos in cell suspension cultures of Douglas-fir and loblolly pine.

As far as we are aware, the induction and formation of embryo-like structures from cells taken from the body of coniferous trees has never before been observed or accomplished. Our report represents a breakthrough in this area. The extent of development obtained in culture flasks is illustrated on page 9. The details of our experimental work will be forthcoming in subsequent reports.

This year's list of publications is found on page 24 and they are available at no charge to members of The Institute of Paper Chemistry.

Yours Sincerely,

D. J. Durzan
Group Leader
Biochemical Systems
Forest Biology Section

DJD/fa
Enclosure

REPLY SHEET FROM MEMBER COMPANIES

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The Institute of Paper Chemistry
P. O. Box 1039
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THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

EMBRYOGENESIS IN CELL SUSPENSIONS OF
DOUGLAS-FIR AND LOBLOLLY PINE

SUMMARY

Our seventh report to the industry for 1978/1979 describes a significant breakthrough that deals with the induction and development of embryo-like structures from cell suspension cultures. The research clearly indicates that our major aim of mass producing superior trees from cells of Douglas-fir and loblolly pine is not only realistic but also a most desirable goal for the industry.

A key development in the project is the shift from largely empirical studies to a new effort where the parameters of our cloning technology can be identified, quantified, field tested and reported to the membership. The shift in emphasis, we feel, is a positive sign of progress. Nevertheless, the need for new technology to monitor and quantify the critical variables together with the current complexity associated with the biochemistry and physiology of developing tissues means that we may still be several years away from having available to our supporters a successful technology for mass propagation of conifers.

Future progress will depend on the support we can obtain to design and test systems that will monitor the development of our prospective trees and adjust the growing conditions so that abortive situations are minimized. Our preliminary results indicate that the young, embryo-like structures are very sensitive to nutritional changes and to levels of plant growth regulators used to encourage continued development. This sensitivity makes it difficult to achieve control over development in the short run and will require a truly transdisciplinary effort. In keeping with this projection our research team has established preliminary working relationships with similar research teams in the U.S.A. and abroad to foster continued progress by minimizing overlap of research effort.

RESEARCH OPERATIONS

INTRODUCTION

The successful scale-up of our cell suspensions of Douglas-fir and loblolly pine described in Report Six had set the stage for this year's effort. In 1978 and 1979 we aimed at the induction of embryos from cells taken from the body of the mother tree. This goal is considered unique and as we will see, its attainment represents a new landmark aimed at the mass production of conifer hybrids.

From the start, the experimental situation had to be right, the material available, the concepts in place, the support in hand and the overall effort integrated. The experimental system used for the scale-up and production of cells is shown in Fig. 1 and will orient the reader to the stages at which experimental data have been generated. Most of this year's report will deal with the control over the development of cells in the cloning cycle that our group has developed (Fig. 2).

Three new ingredients in our approach are: first, cells should be derived from more mature donors. Currently, nearly all of the work reported in the literature used juvenile tissues obtained largely from germinating seeds. Second, the induction of embryogenesis was modeled after classical systems such as the wild carrot. Third, a new medium was developed to imitate the natural environment of the real conifer embryo. Currently, most investigators are using nutrient balances that have been designed for agricultural or horticultural plants or in some cases have modified these formulations in an attempt to grow conifer cells.

This year's report describes exploratory studies of a preliminary nature that will tell us if somatic cell embryogenesis is feasible for Douglas-fir and loblolly pine. While we can anticipate that the answer is a resounding yes we

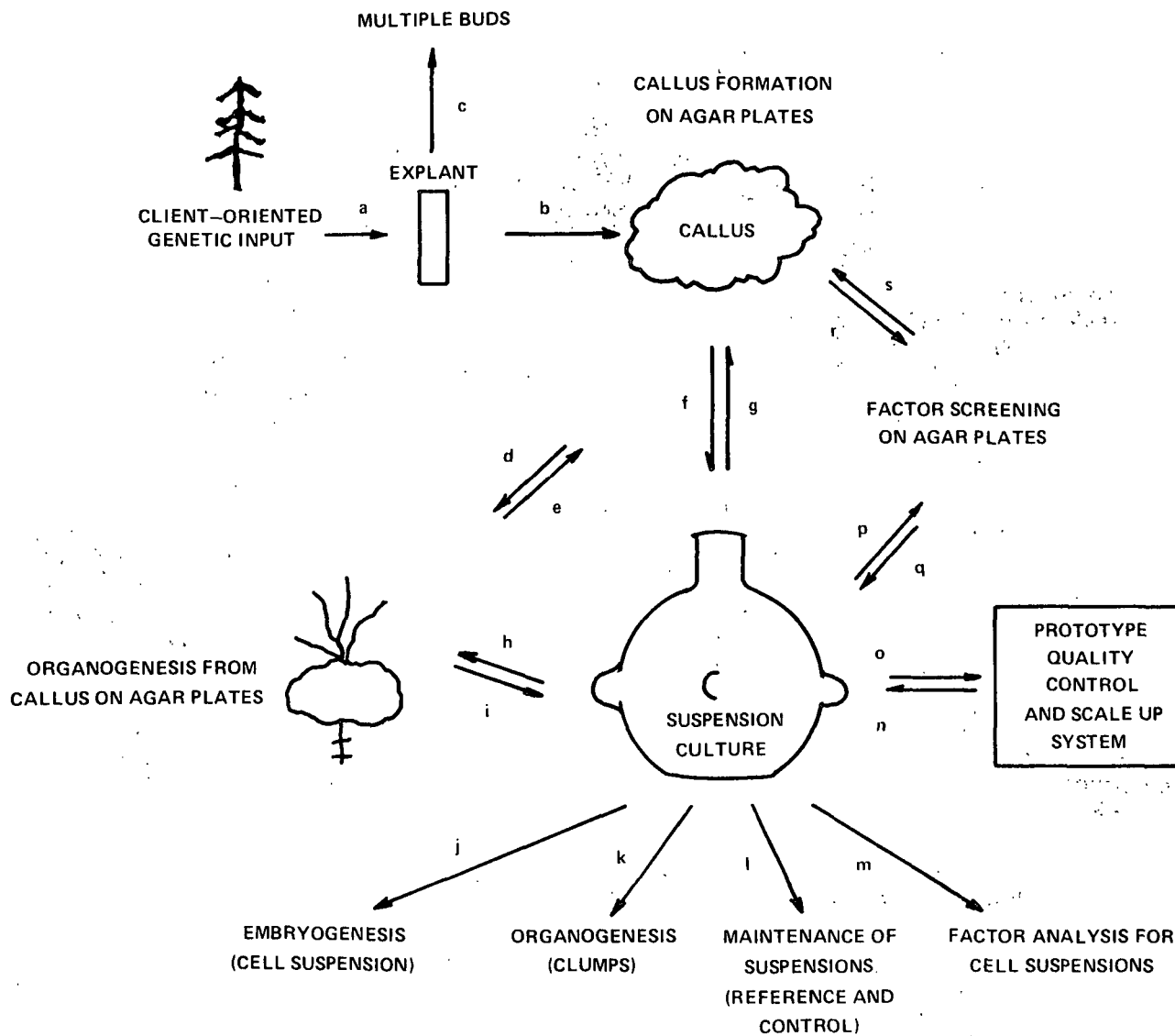


Figure 1. Orientation Model to Identify Stage at Which Experimental Data Are Generated. From Suspension Cultures, Cells Are Fed into the Cloning Cycle (Figure 2)

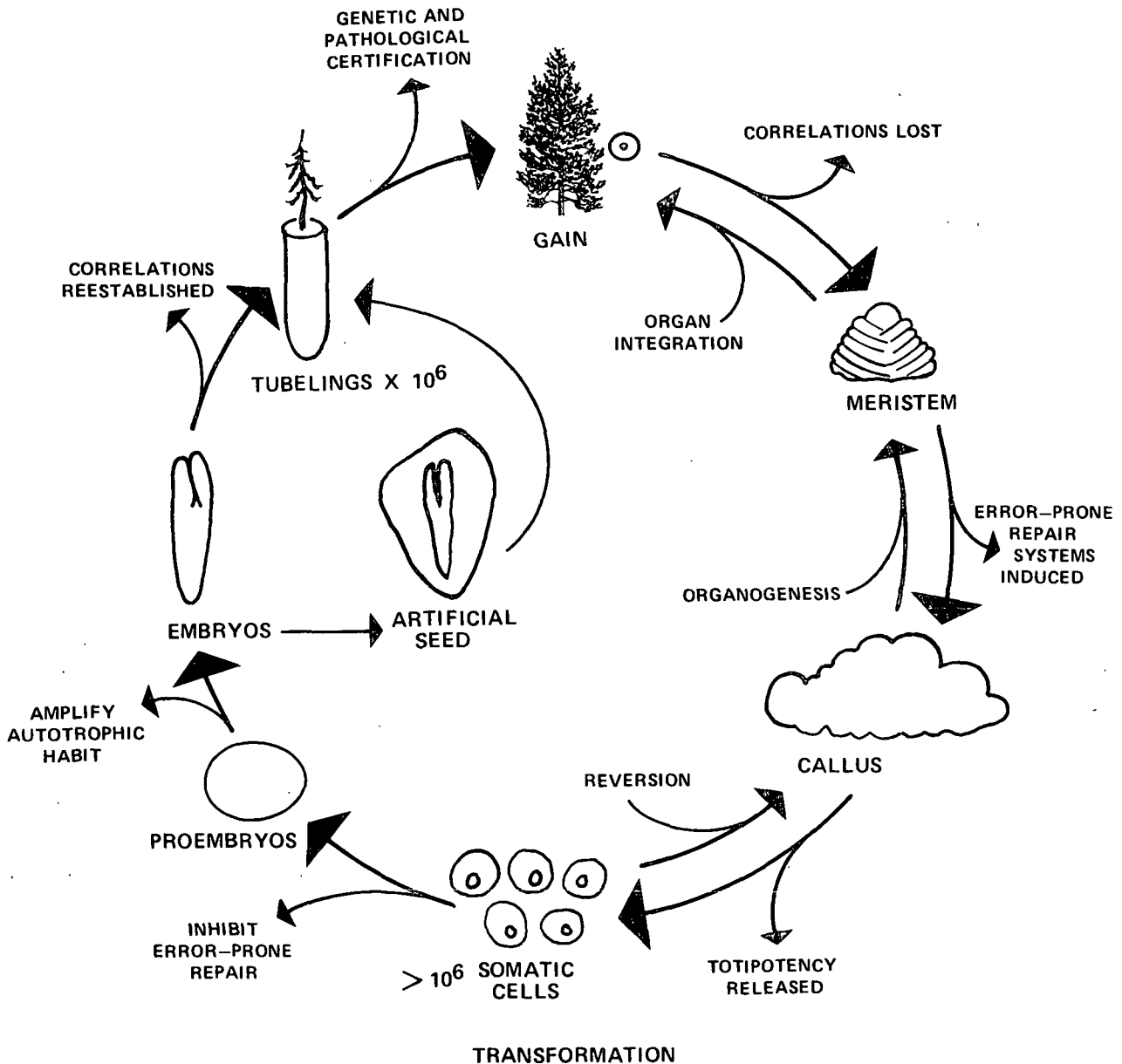


Figure 2. A Cloning Cycle Based on Cellular Growth and Development to Regenerate Massive Quantities of Trees from a Small Piece of Donor Tissue from Elite Mother Tree. The Tissue is Reduced to Cells Which Are then Grown Like Microorganisms. Each Multiplied Cell is then Induced to Form an Embryo Which can then be Fed Back into the Propagation System Either as an Artificial Seed (Under Development) or Directly Through Container Planting. This Approach is One of Several Alternative Processes in the Propagation System

must emphasize that more critical and comprehensive studies will have to be designed so as to repeat and identify the critical parameters in our system, otherwise our credibility will be lost. In other words, we will eventually need to design and monitor the developmental idiosyncracies of our new somatic structures.

Specific details of our research that were available during 1978 and 1979 are listed under PROJECT OUTPUT. The published reports by team members represent original research that has been accepted internationally after critical scrutiny by impartial scientific peers serving on the editorial boards of the journals cited. This information is readily available to the supporters of the Institute.

RESEARCH PLAN

General: The Cloning Cycle

The project's objectives are first, to provide our clients in industry with the information, technology, and manpower specifically related to the mass propagation of coniferous trees to maximize genetic gains. The second objective is to improve the genetic base by the creation of new hybrids suited to industry's needs through bioengineering using protoplasts and the newer developments in molecular biology and genetic engineering. The specific subobjectives of our research plan were identified in the Sixth Annual Report.

Our first goal is to obtain plants from cells. The achievement of this goal will demonstrate that the cloning cycle, outlined in Fig. 2, is indeed feasible. In our work, cloning means the mass production of individuals from cells or tissues of trees with desirable properties. The process we have outlined is set in an ideal experimental system and offers unique advantages (Table I).

TABLE I

CHARACTERISTICS OF AN IDEAL SYSTEM USED IN MOLECULAR GENETIC
STUDIES AND ADVANTAGES OF CLONING FROM CELL SUSPENSIONS

A. Characteristics of an Ideal System

1. Uniform population of cells
2. Imposed controls that isolate a factor and maintain the critical variables
3. Sufficient levels of growth regulators for purification and characterization
4. Wide fluctuations in the components that are affected by nutritional and environmental variables
5. Base-line monitoring and evaluative capacity
6. Parental trees or mutants are available to study the molecular specificity of the trait
7. Well-defined nutrients or simple methods for perturbing the critical factor
8. Easy manipulation with isotopic tracers
9. Demonstrable correlations between results in the system and events in nature or under field conditions

B. Advantages of Cloning from Cell Suspensions

1. Few cells are needed as starting material
2. Mother tree can be used for other purposes
3. Cells breed like bacteria and are totipotent (i.e., regenerate whole plants)
4. Space and study systems are economical and controlled
5. Refined technology from microbiology and molecular genetics can be employed
6. Mutations can be introduced and identified
7. Large populations of cells can be screened and selected for elite traits
8. Efficiency can be increased by a considerable reduction in time (cell to nursery 18 months) and cost of conventional breeding
9. With haploids, mutant traits are not masked as in diploids. Diploidization of haploid cells allows for genetic analysis and improvement (hybrid vigor)
10. Cell walls can be removed to produce protoplasts for genetic engineering

The progress described in the Research Highlights are keyed to Fig. 2 and aimed at completing the cloning cycle. If this cycle were used as an indicator of progress then this year's work has taken us to approximately three-quarters of the way to reaching our goal. The induction of somatic structures from cell suspensions is, in itself, a highly significant breakthrough (Fig. 3).

PERSONNEL 1977-1978

Principal Investigators

Dr. D. J. Durzan: Project Leader (Ph.D., Cornell; B.S., McMaster)

Don comes from Environment Canada where he served as Senior Advisor, Research Director, and Research Scientist over a period of 16 years. He is an Associate Editor of the Canadian Journal of Forest Research and has published over 100 scientific articles in environmental sciences, analytical biochemistry, tree biology, insect physiology and biochemistry, nutrition, seed physiology, and tissue culture.

Dr. D. W. Einspahr*: Forest Geneticist (Ph.D., M.S., B.S., Iowa State)

Dean is Division Director of the Forest Biology Section and provides the genetic input and field trials for our project. Dean has published over 50 papers in forestry journals and books and has considerable experience with the pulp and paper industry.

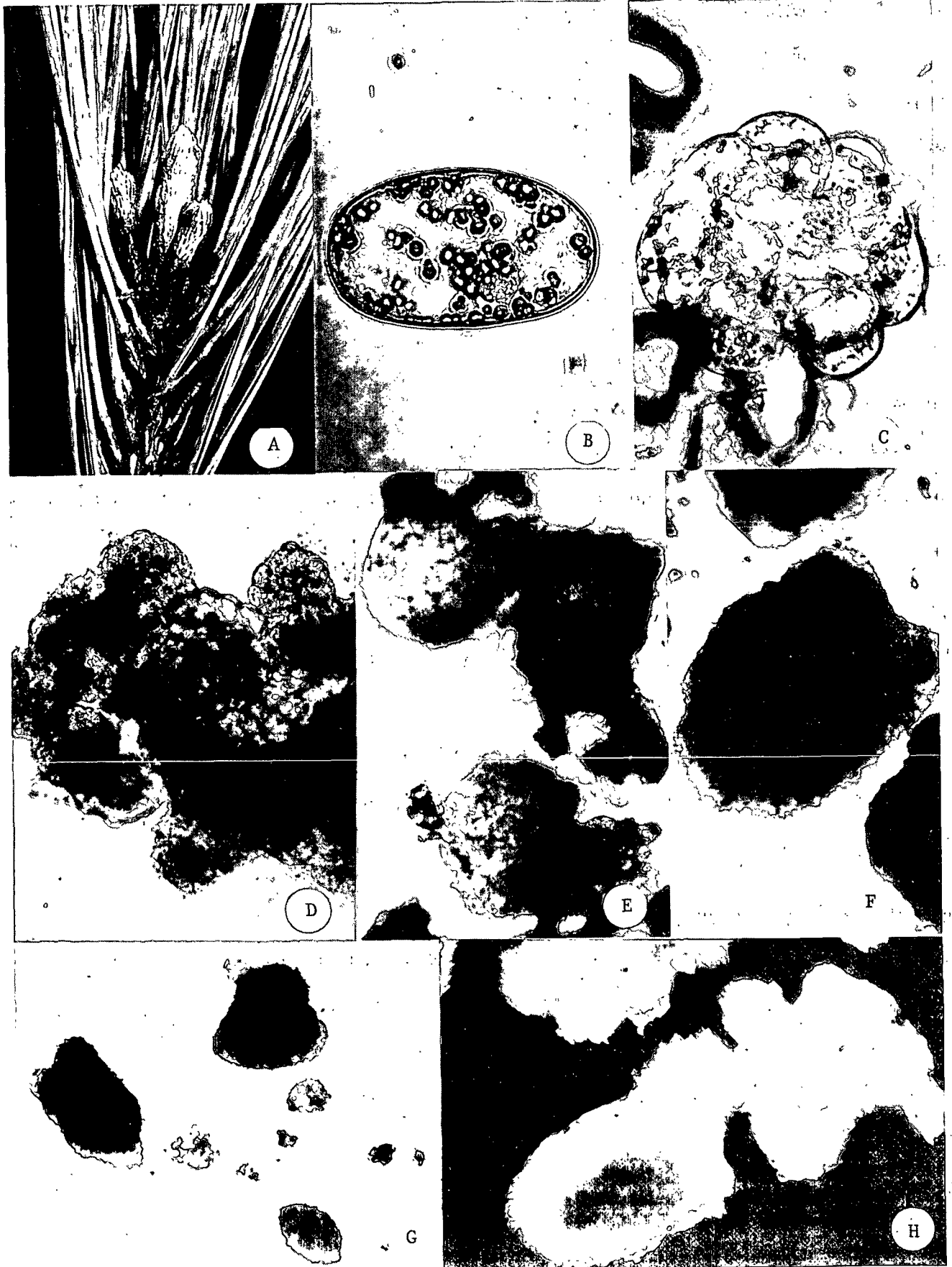
Dr. M. A. Johnson: Biochemist (Ph.D., Oregon State; M.S., B.S., North Dakota State)

Morris is concerned with developing the biochemical basis for our observations. His studies deal with molecular mechanisms that predict and control morphogenesis in our tissue culture systems. He has 12 publications.

*Part-time or service commitment.

Figure 3. Sequence of Events in the Somatic Embryogenesis of Coniferous Cells

- A. Shoot from elite loblolly pine. The shoot tip and the terminal lateral buds are used as donor tissue in the cloning cycle.
- B. The donor tissue or explant is placed on agar plates containing a chemically defined nutrient medium with plant growth regulators to produce a callus. The callus is fragmented in rotating flasks to produce cell suspension cultures. Illustrated is a Douglas-fir cell from suspension culture. The cell is approximately 60 micrometers in length.
- C. After removal of the callus growth stimulus and dedifferentiation of cells in darkness, the cell suspension is transformed after one month into slowly growing embryo-like clusters.
- D. The polyembryonic clusters are attached to one another by cells that resemble a suspensor.
- E. After two months the embryo-like clusters continue to grow in a coherent pattern. The cellular clusters can green up in darkness. This is a special trait of conifer embryos and encourages us to believe that somatic cell embryogenesis has been initiated.
- F. After another month of growth, the axis of the somatic structures elongates and a polarity emerges with one end forming rudiments where the seed leaves would appear as in normal zygotic embryogenesis.
- G. Several intermediate stages of growth, associated with the generation of somatic embryos from Douglas-fir are illustrated in a sample taken from a suspension culture.
- H. A larger elongated embryo (ca. 1 mm in length) with multiple primordia at the top where the seed leaves should appear is the next stage of development. Note the elongated dark central core of cells that has been established where the future wood-producing cells will arise.



Dr. J. Litvay*: Fiber Scientist (Ph.D., M.S., Oregon State).

John, who joined us from the University of Maine at Orono, is the newest member of the team. He interprets the cellular development and cell wall structures of our cell and tissue cultures with the aid of the transmission and scanning electron microscopes. John has three publications on cell ultrastructure to his credit.

Dr. D. C. Verma: Tissue Culture Scientist (Ph.D., State University of New York at Buffalo; M.S., University of California, Davis)

Devi is a recent addition to the team and comes to us from the W. Alton Jones Cell Science Centre at Lake Placid, NY. Devi has eight publications dealing largely with model tissue culture systems. Devi and his staff maintain tissue and cell cultures for studies dealing with embryogenesis and organogenesis in Douglas-fir and loblolly pine.

Visiting Scientist

Dr. J. Pardos: Departamento de Fisiologia Vegetal (Instituto Nacional de Investigaciones Agrarias, Madrid, Spain)

Jose is Professor of Forestry at the School of Forestry at the University of Madrid. He will spend a four-month term studying growth regulators in our tissue cultures.

Research Fellows and Associates

J. Carlson*: Biochemistry, Research Fellow (B.S., Minnesota)

G. Dawson*: Chemistry, Research Assistant (B.S., Northland College, Wisconsin)

R. Feirer: Research Fellow (M.S., Iowa State)

S. Hwo: Research Fellow (M.S., University of Connecticut)

S. Verhagen: Tissue Culture Research Assistant

E. Foxgrover*: Tissue Culture Assistant

*Part-time or service commitment.

Student Research

J. Bobalek: Ph.D. Program, Advisor, M. Johnson

B. Cann: A-291 Independent Study, Advisor, D. J. Durzan

S. Monroe: Ph.D. Program, Advisor, M. Johnson

K. W. Robinson: Ph.D. Program, Advisor, M. Johnson

M. Smits: Ph.D. Program, Advisor, M. Johnson

RESEARCH ADVISORY PROGRAM COMMITTEE (FOREST GENETICS)

Dr. R. H. Smeltzer
(Chairman, terminates 1980)

International Paper Co., Natchez Forest
Research Centre, Route 3, Box 312-B,
Natchez, Mississippi 39120

Dr. R. W. Ritzert

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Senior Biochemist

Dr. Roger L. Blair

Potlatch Corporation

Dr. William Gladstone

Weyerhaeuser, Tacoma, Washington

Dr. Greg Landry

St. Regis Paper Company

Dr. I. Bruce Sanborn

Consolidated Papers, Inc.

Two meetings with the Institute team were held in Appleton on October 31,
1978 and on March 22 and 23, 1979.

RESEARCH PROGRESS

INTRODUCTION

A very important factor in this year's report is the strong support received from the Institute in terms of continued encouragement and in funds to establish a clean, reliable and productive tissue culture laboratory. In this annual report, only the highlights of the research program are outlined by discipline and presented in a transdisciplinary context aimed at obtaining plants from cells.

We feel the progress has been remarkable considering the turnover of personnel and temporary reduction in postdoctoral fellows. Steps are currently underway to recruit people who can contribute significantly to this challenging project.

GENETICS

Mission

Our mission involves the selection of a genetic base best suited to client needs (Subobjective One, see page 10 of Report Six). This has involved obtaining genetically diverse seed sources and specific clones of documented origin that provide several types of starting material (seeds, cones, shoots, etc.) for use in initiating cell suspensions. See Fig. 1 and 2.

Factors

The ultimate goal is to provide industry with a reliable procedure that allows the mass propagation of elite trees; conventionally produced hybrids and hybrids produced by protoplast fusion, thus greatly increasing the chances for a

variety of types of genetic gains (growth rate, insect and disease resistance, climatic adaptability, wood quality).

Research Highlights

Genetic input has been modest during this past year. This resulted because the emphasis of the program has been on the development of reliable mass production techniques rather than on the large-scale production of "elite" trees. The desire to capture known genetic gains available in existing, outstanding parent trees or genetically desirable hybrids has "realistically" not been attempted. Instead, three additional seed sources and thirteen clones have been obtained. These materials have been used in biochemical studies and in the development of cell suspension - embryoid - plantlet procedures (CSEP).

Progress Report Six summarized the seed lots that were obtained prior to July 1, 1978. The following additional sources of plant material (seeds and clonal material) have been secured and introduced into the CSEP research.

Seeds

1. Seed Lot IPC-3223-7; Reid, Collins and Associates Ltd., Vancouver, Wash. Douglas-fir seed from Courtenay, B.C., for use in CSEP studies.
2. Seed Lot IPC 3223-8; Brown Seed Company, Vancouver, Wash. Digger pine (Pinus sabiniana) from near Red Bluff, California, for use in seed morphology and embryo development studies.
3. Seed Lot IPC-3223-10; Weyerhaeuser; 1500 ft elevation, east of Roseburg, Oregon, Umpqua River. This is Weyerhaeuser Seed Lot 491-15-1 (received June 1, 1978) and believed to be the same as IPC 3223-9, received several years earlier. Collection year may not be the same, however.

Clonal Material

1. IPC-3223-101 through 109; Weyerhaeuser; nine Douglas-fir clones received (6/27/78) as unrooted shoots from Dr. Zachary Wochok (Centralia, Wash.). Clones originated from seed cotyledons from nine superior sources. Eight clones survived (No. 102 did not) and were used in biochemical studies and CSEP research.

2. IPC-3223-110; Dr. J. P. van Buijtenen, Texas Forest Service. This loblolly pine clone was developed from a second-generation, 8-year-old selection that was being used in rooting experiments. The clone has a Texas Forest Service No. 691, R-18, T21-23. Shown in Fig. 4 is the clone being hedged to produce shoots for rooting experiments.



Figure 4. A Texas Forest Service Loblolly Pine Clone Used as a Donor for the Production of Cell Suspensions and Study of Somatic Cell Embryogenesis. The Clone Illustrated is Being Hedged to Produce Shoots for Rooting Experiments

3. IPC-3223-111; Dr. J. P. van Buijtenen, Texas Forest Service. A loblolly pine clone developed from a second-generation, 8-year-old selection that was being used in rooting experiments. The clone has a Texas Forest Service No. 700, R-11, T21-22.

4. IPC-3223-112; International Paper Company; loblolly pine clone No. D-HO-PT-12. Developing cones, male flowers, and shoots were received in the spring of 1979 from grafts located in the International Paper Company seed orchard near Natchez, Mississippi. Original tree located in Franklin County, Miss. was 48 years of age. Material used to produce diploid and haploid tissue for use in CSEP program.

5. IPC-3223-113; International Paper Company; loblolly pine tree selected from three-year-old regeneration growing at edge of plantation near Fayette, Jefferson County, Miss. Shoots were received in the spring of 1979 and used in CSEP developmental work.

Plans

For the next year, genetic efforts will involve: (1) continued emphasis on techniques rather than on the production of elite material, (2) use of well-defined clonal material of diverse origin, (3) tests for best tissue sources (dormant/mature shoots, needles, etc.) from older trees, and (4) development of techniques for an ultimate transfer of plantlets from the suspension system.

TISSUE CULTURE

Mission

Among the 12 subobjectives outlined in the transdisciplinary research plan (see page 10 of Report Six), the Tissue Culture Laboratory focused on the following:

- A. Establishment of tissue and cell culture systems.
- B. Control of the mass production of totipotent cells.
- C. Control over morphogenesis in Douglas-fir and loblolly pine.

Factors

With the above objectives in view, preliminary experiments were conducted in an attempt to demonstrate somatic embryogenesis in loblolly pine and Douglas-fir suspension cultures with some of the explant sources listed in Table II.

TABLE II.

EXPLANT SOURCES FOR CONIFER CULTURES

A. Somatic (Nonreproductive) Diploid Cells

Seed: whole embryo

Seedling: cotyledon, hypocotyl, epicotyl

Tree: veg. bud -- needle primordia & dome branch --
cambium, cortex, pith root -- apical meristem

B. Gametophytic (Reproductive) Haploid Cells

Male: microspore -- pollen grain -- gamete

Female: megaspore -- megagametophyte

It was clear that high salt content was a complication in the medium developed by Murashige and Skoog (MS medium) inasmuch as it did not support sub-culturability or morphogenesis of loblolly pine callus and suspension cultures. Hence, the MS medium was studied at half its strength.

A new nutrient medium was reconstituted (designated as the R-medium). It was based on the chemical composition of the female gametophyte of Douglas-fir and loblolly pine. Both R and MS media were evaluated against the classic wild carrot medium. The chemical composition of this wild carrot medium is given in Table III.

TABLE III

WILD CARROT MEDIUM NOW BEING TESTED AND MODIFIED FOR THE STUDY OF
MORPHOGENESIS IN CELL SUSPENSION CULTURES OF
DOUGLAS-FIR AND LOBLOLLY PINE^a

Original Formulation	mg/L	mM
KNO ₃	4,000	39.56
NH ₄ Cl	540	10.10
MgSO ₄ · 7H ₂ O	185	0.75
CaCl ₂	166	1.50
KH ₂ PO ₄	68	0.50
Na ₂ EDTA	18.6	0.05
FeSO ₄ · 7H ₂ O	13.6	0.05
MnSO ₄ · H ₂ O	7.0	0.04
ZnSO ₄ · 7H ₂ O	4.0	0.01
H ₃ BO ₃	2.4	0.04
(NH ₄) ₆ Mo ₇ O ₂₄ · 4H ₂ O	0.01	8x10 ⁻⁶
KI	0.38	2x10 ⁻³
CuSO ₄ · 5H ₂ O	0.015	6x10 ⁻⁵
Thiamine · HCl	3.0	9x10 ⁻³
Sucrose	20,000	58.43
2,4-D	0.5	2x10 ⁻³

^aWetherell, D. F., Plant Physiology 44:1734-7(1969).

Research Highlights

It was recognized that to express totipotency in cultured cells, one needs to reduce the potentially totipotent cells to a completely dedifferentiated state. To achieve this, it was imperative that the cultures were either grown in darkness or exposed to darkness for a suitable length of time. For this purpose a second rotary apparatus with a capacity to hold 60 nipple flasks and 130 t-tubes was built in a dark compartment located in our laboratory with controlled environment.

We have been successful in maintaining by repeated subculturing the loblolly pine suspension cultures in a dedifferentiated state. The use of our improvised wild carrot medium and conditions of darkness or diffused light at very low intensities are the key factors responsible for this achievement. When these suspensions were given treatments identical to those which would permit expression of somatic embryogenesis in wild carrot cell suspensions, our system manifested neither growth nor embryogenesis. The culture conditions were, therefore, significantly modified.

Structures reminiscent of early (globular) stage of zygotic embryology have now been induced in both loblolly and Douglas-fir suspension cultures using the R- and modification of wild carrot-media (Fig. 3). It must be emphasized that these studies are still preliminary in nature. Much work remains to be done to reproducibly identify the various inductive treatments before we can make more definitive statements on the nature of events taking place in our cultures vis-à-vis the wild carrot cultures. Removal of exogenous auxin such as 2,4-D from the loblolly pine or Douglas-fir suspension cultures does not create a permissive condition for induction and development of somatic embryoids. Indications are strong that the conifer tissue cultures are extremely sensitive and complex systems and there is a need of sequential treatments with some degree of control over reactions in the culture vessels. Attempts will be made to tackle some of these

problems during the next year. Nevertheless, the progress of the current year's rather exploratory investigations into somatic embryogenesis of Douglas-fir and loblolly pine is very remarkable.

Plans

During the next year, concerted efforts will be maintained on the variables evoking somatic cell embryogenesis. The R- and wild carrot-media will be refined in a systematic way to encourage further development of the embryoids. The roles and interaction of exogenously added growth regulators on somatic embryogenesis and organogenesis of coniferous tissue culture will be studied and the abortive patterns identified. This means that a large-scale factorial screening system will have to be employed and integrated with other subobjectives of the project. A temporary reduction in manpower has meant that the current emphasis on two species will be allocated more to Douglas-fir to help maintain progress. When the postdoctoral fellows become available, full effort can be reallocated to both species (Douglas-fir and loblolly pine).

MORPHOLOGY AND FIBER SCIENCE

Mission

The morphology and fiber science group has as their primary mission the development of a theoretical and structural basis for morphogenesis in cell suspension cultures.

Factors

The basic philosophy and major contribution of the wood and fiber group is to provide specimen preparation, analysis and interpretation for light, and scanning and transmission electron microscopy. Under the assumption that gymnosperm somatic cell embryogenesis will develop along the same pathways as shown by

natural development in the seed, our primary goal will be to determine and use the information of normal embryo development, to help monitor the progress and results of the in vitro experiments.

If deviations in development occur, a secondary goal will be the exploration and interpretation of such events.

Research Highlights

A revitalization of the research effort in this area is currently in the planning stages. A major portion of our work will be the application of new and specifically modified techniques to characterize and study the developmental patterns of somatic cell embryogenesis of loblolly pine and Douglas-fir. The application of the above techniques for routine monitoring of treatment effects on embryogenesis has been initiated and will be described in a future annual report.

Plans

Future plans include work in the following areas:

1. The morphological characterization of somatic cell embryogenesis.
2. The refinement of our microscopy techniques for the handling and analysis of protoplasts and for the description of changes in the cell walls associated with morphogenesis.
3. Refinement of our present microscopy techniques, so that more accurate (quantitative) and potentially more helpful information aimed at the control of events in the culture flask may be obtained.
4. The continued monitoring of the development of our newly developed somatic embryos and their abortive expressions.

BIOCHEMISTRY

Mission

The biochemistry laboratory has primary missions which fall within two subobjectives of the research plan:

- (5) Development of change agents and indicators for morphogenesis, and
- (6) Control over morphogenesis.

Input is provided for the theoretical basis for morphogenesis, Subobjective (4), (see page 10 of Report Six).

Factors

Factors under investigation during this period relative to Subobjectives (5) and (6) were primarily isozymes and phenolics. Most of the research was conducted with callus at Step d of Fig. 1 but with intended applicability at Step m. Input from these investigations also contributed to the working hypotheses leading to the fulfillment of Subobjective (4).

Research Highlights

Our results are interpreted in terms of the peroxide and IAA oxidase hypotheses (cf. Progress Report Five, p: 15-16). Continued isozyme screening of callus clones, including elite clones obtained from Weyerhaeuser Co., showed that, contrary to previous indications, IAA oxidase patterns characterized by a substantial activity in high mobility anionic isozymes were not reliable indicators of organogenic competence among our cultures. Only one of eight elite clones produced shoots under our ordinary callus culture conditions, yet at least four of the other clones had much "stronger" isozyme patterns but failed to produce any shoots. The picture was further muddled by recent observations that total IAA oxidase activity (wet assay of unfractionated extracts) of the competent clone

can be substantial even though the isozyme pattern is weak. It may be significant that extracts of the competent clone are much slower to turn brown (phenolic oxidation?) than those of the other clones.

In related research it was found that the high mobility isozymes are precipitated by polycations (expected) and stimulated by treatment of extracts with ribonuclease prior to electrophoresis (unexpected). The latter reproducible outcome suggests that nucleic acids and phenolics in crude extracts may modify isozyme patterns. Therefore, the issue of whether the isozymes are distinct proteins or merely various complexes of one or two proteins with other substances needs resolution if these patterns are ever to become reliable markers.

A survey of the literature prompted a cold treatment of Douglas-fir callus as an in vivo test of our working hypotheses. Omran reported that lowering the temperature of cucumber cotyledons decreased the catalase activity, increased peroxide concentrations, and increased IAA oxidase activity. No change in peroxidase activity was observed. In an abbreviated version of that experiment with needle callus, peroxidase and IAA oxidase activity increased with cold treatment. Although catalase activity was not determined in this experiment, several other interesting observations were made. For example, both quantitative and qualitative changes were noted in both proteins (Fig. 5) and free amino acids in the callus. Cold-treated callus yielded a qualitative test for lignin while control callus kept at 24°C did not. Changes were noted in the spectra of acetone extracts, and a putative degradation product of cytochrome P-450 (P-420) appeared in the cold treated callus (Fig. 5). Responses to the cold treatment reflect differences in aromatic metabolism, perhaps precluding vacuolar accumulation of toxic products. Another experiment extending beyond a single subculture and including quantitative analysis of total catalase and total IAA oxidase activities is currently underway.

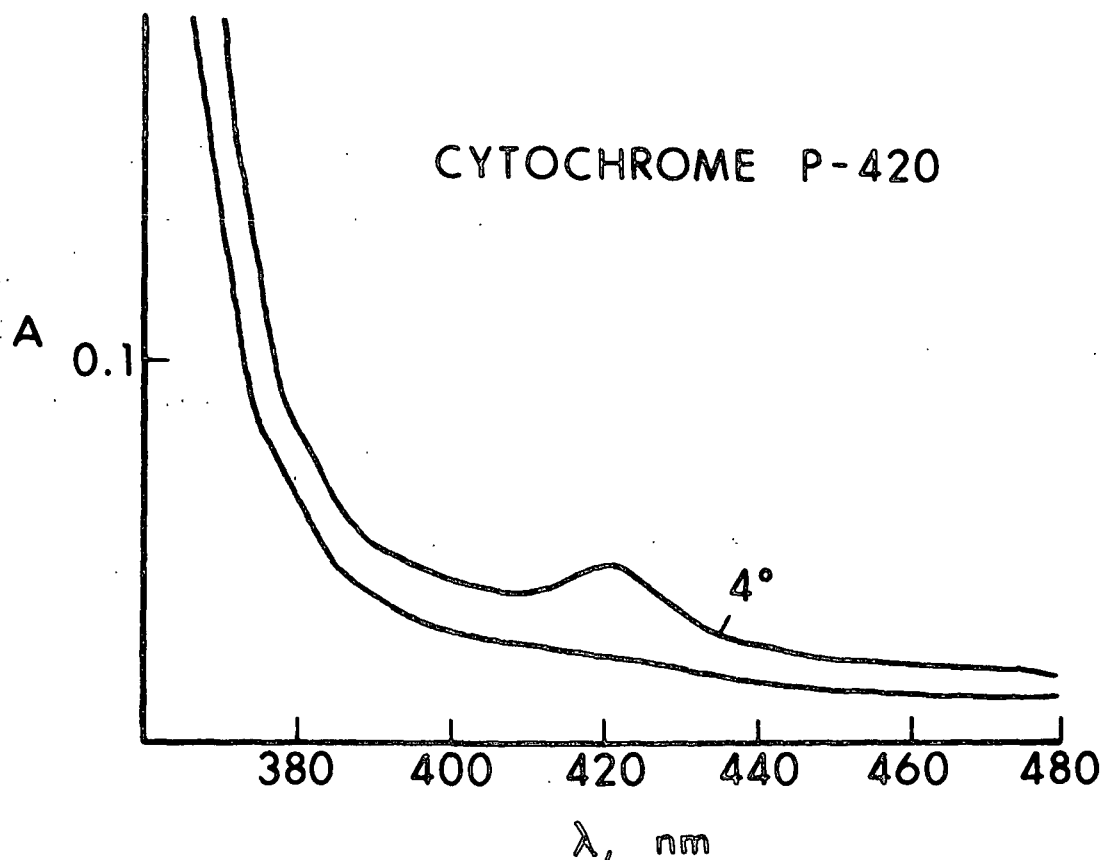
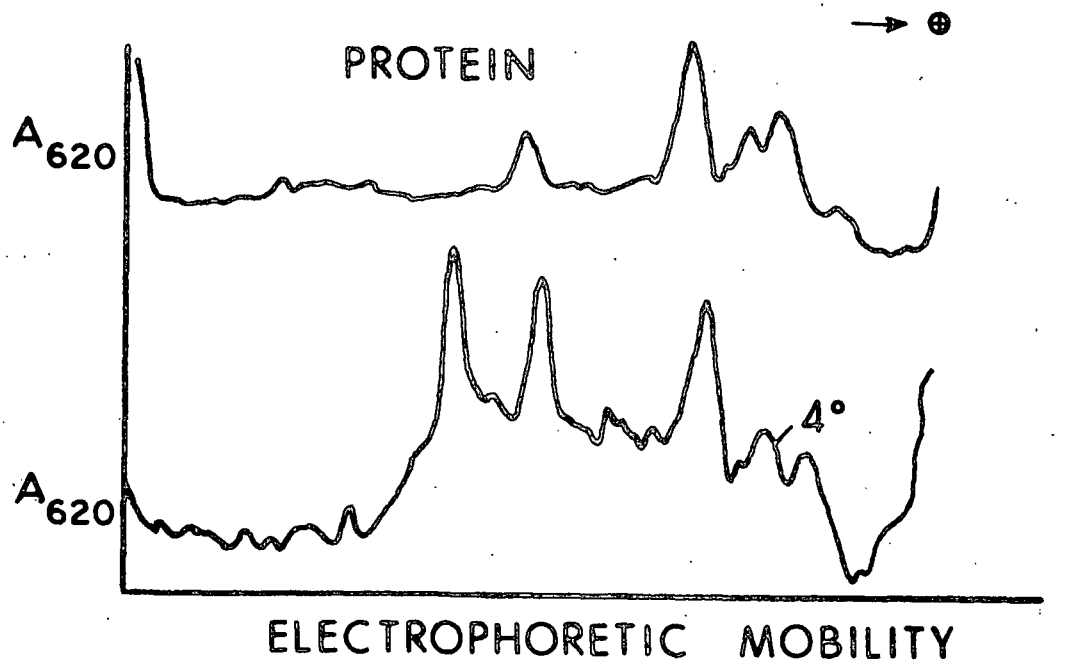


Figure 5. Evidence of Molecular Changes Induced by Cold Treatment of Douglas-fir Needle Callus

The cloudy factor (e.g., Project Report Six, p. 26) reacts strongly with phloroglucinol, thereby adding lignin-related structures to the known components of this complex material. Taxifolin, which may contribute substantially to the UV spectrum of some Douglas-fir callus extracts, was not detected in loblolly pine callus. To the extent that products of secondary metabolism may influence developmental processes, the two species may differ considerably.

Results obtained this past year helped to sharpen our existing hypotheses and suggested possible new morphogenic indicators such as cytochrome P-450 which, incidentally, may be related to peroxidase. While significant testing of these hypotheses is yet to be conducted in suspensions, it is anticipated that the insight to be gained may be crucial for full realization of "plants from cells."

Plans

In line with the current overall emphasis on embryogenesis and control over reactions in suspension cultures, increased biochemical monitoring of developmental progress can be expected. In some cases the analytical capability is available, including the ability to deal with small samples. On the other hand, there are cases where increased efficiency and sensitivity are needed leading eventually to on-line automation. Some aspects of the working hypotheses may be amenable to testing relative to embryogenesis in the near future. It appears, for example, that a cold treatment may be beneficial at some stage of development.

PROJECT OUTPUT: SUMMARY FOR 1978-1979

INFORMATION

Annual Report, Project 3223, July 17, 1978

RAC Reports, October 31, 1978, March 22 and 23, 1979

Publications

1. Johnson, M. A. and J. A. Carlson. 1979. Indoleacetic acid oxidase and related enzymes in cultured and seedling Douglas-fir. *Biochemie und Physiologie der Pflanzen* 174:115-27.
2. Winton, L. L. 1978. Morphogenesis in clonal propagation of woody plants. In Thorpe's *Frontiers of plant tissue culture*. Int. Assoc. Plant Tissue Culture, Univ. Calgary. p. 419-26.
3. Durzan, D. J. 1979. Progress and promise in forest genetics. Proc. of the Forty-third Executives' Conference, Appleton, Wisconsin, The Institute of Paper Chemistry (in press).

Papers at Scientific Meetings (Published Abstracts)

- Durzan, D. J. and J. Bonga. 1978. Propagation in woody plants. A summary of roundtable discussion. In Thorpe's *Frontiers of plant tissue culture*. Int. Assoc. Plant Tissue Culture, Univ. Calgary. p. 481-2.
- Johnson, M. A. and J. A. Carlson. 1978. IAA oxidase in Douglas-fir development. Proc. 4th Int. Congress Plant Tissue Culture, Calgary, Alberta, Abstr. 1732, p. 515.
- Winton, L. A. and S. A. Verhagen. 1978. Morphogenesis in Douglas-fir cultures. Proc. 4th Int. Congress Plant Tissue Culture, Calgary, Alberta, Abstr. 1731, p. 515.
- Durzan, D. J., D. W. Einspahr, M. A. Johnson, R. A. Parham, and L. L. Winton. 1978. Differentiation in cell suspension cultures of Douglas-fir and loblolly pine. Proc. 4th Int. Congress Plant Tissue Culture, Calgary, Alberta, Abstr. 1730.

Courses Related to the Project

A- 70 Biology (Winton)

A-171 Biochemistry (Johnson)

A-175 Wood and Fiber Biology (Litvay)

- A-371 Plant Biochemistry (Johnson)
- A-375 Special Topics in Plant Physiology (Durzan)
- A-377 Forest Genetics (Winton)
- A-374 Principles of Forest Management (Einspahr)
- A-378 Plant Tissue Culture (Winton)

Miscellaneous

The research effort was described and illustrated by Chemical and Engineering News, June 4, 1979 on page 26 under the title "Geneticists aim to develop taller, hardier trees."

Drs. D. J. Durzan and D. Einspahr visited International Paper (Tuxedo Park, NY) on November 30, 1978, to discuss client-oriented requirements for the tissue culture-genetics project. Dr. Durzan visited Oregon State University to discuss research progress on Douglas-fir with Drs. Gordon, Mapes, Morris, and Zaerr.

Executives of several member companies toured our laboratories during the Institute's Annual Executives' Conference, May 8-10 in Appleton. The research team contributed several scenes to a proposed television program, "Trees are more than wood," to be produced by the Public Broadcasting System for fall viewing in 1979.

On August 16, 1978, the Milwaukee Sentinel carried a front page article on cloning and illustrated the article with a picture and a review of the tissue culture project. On May 10, 1979 The Appleton Post-Crescent described the tissue culture progress reported at the Institute's 50th Anniversary Symposium: Pulp and Paper Technology: The Cutting Edge. Team members also participated in other local radio and television programs.

TECHNOLOGY

Representatives of member companies are welcome to see the rotating cell culture apparatus constructed at The Institute of Paper Chemistry. Plans for construction of the apparatus were sent to Dr. W. Eudy, International Paper, Tuxedo Park, NY.

MANPOWER

Dr. Lawson Winton, a long-standing, and well-known member of the research project at The Institute of Paper Chemistry has completed his research and will move to a new position this summer.

Dr. Pete Parham, formerly head of the Fiber Science Section is now with ITT Rayonier at Sheldon, Washington.

THE INSTITUTE OF PAPER CHEMISTRY



D. J. Durzan
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Biochemical Systems
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