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## ***FOREST BIOLOGY***

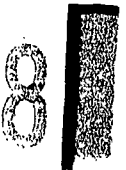
### ***ANNUAL RESEARCH REVIEW***

#### ***HANDOUT BOOK***

***April 1, 1991***



*Atlanta, Georgia*



**FOREST BIOLOGY  
HANDOUT BOOK**

**APRIL 1, 1991**

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**FOREST BIOLOGY**  
**PROJECT ADVISORY COMMITTEE MEETING**  
**CRESCENT ROOM, WYNDHAM HOTEL**  
**TENTATIVE AGENDA - APRIL 2, 1991**

<b>08:00 - 08:15 am</b>	<b>Welcome &amp; Introductions Anti-Trust Statement Approval of Minutes Groundrules</b>	<b>Dinus/Stanton</b>
<b>08:15 - 08:45</b>	<b>Overview of Projects RAC Recommendations Goals &amp; Budgets</b>	<b>Malcolm/Dinus</b>
<b>08:45 - 10:45 *</b>	<b>Softwood Discussion</b>	<b>Webb/Nagmani</b>
	<b>Loblolly Pine Initiation</b>	
	<b>Loblolly Seed Composition</b>	
	<b>Embryo Maturation Loblolly Pine Douglas-fir</b>	
<b>10:45 - 12:15 pm</b>	<b>Hardwood Discussion</b>	<b>Staff</b>
	<b>Student Project, Auxin Transformation</b>	
	<b>Progress to Date Projected Activity</b>	
	<b>Leaf Section System, Plans</b>	
	<b>Culture Establishment &amp; Inventory</b>	

**Herbicide Tolerance**

**Selection in Culture  
Transformation  
Monsanto Interactions**

**12:15 - 01:00**

**Committee Deliberations**

**All**

**Project Goals  
FY 90-91  
FY 91-92  
FY 92-93**

**Minutes, Alternates**

**Other Business**

**Next Meeting**

**01:00\*\***

**Lunch & Adjournment**

**\* Coffee Break will be taken at a convenient stopping point during or after discussion of these topics.**

**\*\* Can continue if desired or necessary.**

FOREST BIOLOGY  
PROJECT ADVISORY COMMITTEE

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**PROJECT 3223-00 SUMMARY FORMS  
MASS CLONAL PROPAGATION OF IMPROVED CONIFERS**

**DUES-FUNDED PROJECT SUMMARY FORM**  
**FY 90-91**

**Project Title:** MASS CLONAL PROPAGATION OF IMPROVED CONIFERS  
**Division:** Chemical and Biological Sciences  
**Project Code:** SFTWD  
**Project Number:** 3223-00  
**Project Staff:** Ron Dinus, Jim Mathis, Nagmani Rangaswamy, Dave Webb  
**FY 90-91:** \$500,000

**PROGRAM OBJECTIVE:** Develop reliable cell and tissue culture systems for mass clonal propagation of genetically improved softwoods.

**PROGRAM AREAS:** Reduced Operating Costs, Capital Effectiveness, Fiber Availability, Quality, and Cost, End-Use Performance.

**FY 90-91 Goals:**

- 1) Raise frequencies for initiation of embryogenic cultures in loblolly pine and Douglas-fir; obtain additional cultures for accelerated work on embryo maturation.
- 2) Increase frequencies of embryo maturation and seedling conversion in Norway spruce model system; extend best treatments to Douglas-fir and loblolly pine.
- 3) Establish guideposts for manipulating somatic materials via documenting course of zygotic embryo development, maturation, and germination as well as early growth and development of zygotic seedlings.
- 4) Explore initiation of embryogenic cultures from explants of more mature plant materials.

**ACCOMPLISHMENTS TO DATE:**

Past research on cell and tissue culture systems has brought somatic embryogenesis, one method of mass cloning, closer to commercialization. Embryogenesis in Norway Spruce, a model system, is not reproducible and straightforward. Embryogenic cultures can be obtained from immature and mature seeds and from tissues of newly germinated seedlings. Last efforts to produce mature embryos showed that using glutamine in lieu of ammonium nitrate as well as

Successful maturation of loblolly embryos via substitution of maltose or glucose for sucrose infers that a similar tack would work for Douglas-fir. Informal experiments generally confirmed this hypothesis, but maltose proved more effective than both sucrose and glucose. Maltose effects varied with concentration. More embryos were produced at higher levels, but they did not develop as well as at low concentrations. Several additional modifications have been or are being evaluated: changing from solid to liquid media, reducing nutrient salts to half-strength, and eliminating casein hydrolysate. The best treatment encountered to date yielded 82 cotyledonary embryos per 50 ml of liquid culture.

Planning, conduct, and interpretation of research on somatic embryogenesis is being aided by deployment of a consistent system for classifying embryo developmental status. The system was derived from observations of developing Douglas-fir embryos over the course of a summer, literature on pine embryology, and consultation with coworkers, including member company scientists. This new tool will facilitate our selecting explant types, quantifying experimental results, and communicating research findings.

**RELATED STUDENT RESEARCH:** In progress

David Barzyk - M.Sc., Development of a fiber optic system to determine the in vivo pH of developing Pinus taeda seeds. Advisor, Dinus.

Rene Kapik - Ph.D., Completing A390 Problems; Likely dissertation topic, Growth regulators in developing zygotic embryos. Advisor, Dinus.

higher concentrations of abscisic acid foster maturation. Numbers of mature embryos, averaged over all treatments, were 130 per g of culture tissue; the best treatment yielded 750 per g. Summary of Results Since Last Report:

Work on Norway spruce, our model system, has been further reduced. Samples for microscopy are being held in storage pending receipt of results from growth regulator assays. This strategy made available more resources for our target species. Cessation of spruce work, with possible exception of that on conversion to seedlings and genetic fidelity, seems best in view of advances made by other laboratories.

A major accomplishment in recent months has been increased success with and understanding of initiation in loblolly pine. Restriction of explant type and mother trees to those that worked best in past years allowed testing an enlarged variety of media and growth regulators. Explants, whole ovules containing precotyledonary embryos, were secured from open-pollinated cones of two mother trees and control-pollinated cones from the cross between them. Explants from both mother trees responded well, but those from the cross were even more responsive. DCR and MSG media performed well for initiation, but only DCR did so for maintenance. Growth regulators were not essential for initiation, but 2,4-D improved initiation and maintenance. Raising auxin levels improved initiation, but low levels were best for maintenance. Addition of cytokinin with low auxin levels somewhat improved initiation, but was detrimental to maintenance. Overall initiation frequency was much greater than any earlier report, eight percent as opposed to less than one percent. Forty embryogenic cultures were obtained. Six have grown rapidly, have been added to our culture bank, and can soon be used in work on maturation. Another 16 are growing quite well. Ten others are growing reasonably well, but utility will not be certain for some time. The remaining eight have shown little or no growth, and have since been discarded. Work to extend these findings to a wider array of genetically improved pines is underway with cones imported from Brazil.

In related research on media suitability, additional data was gathered on fresh and dry weights, moisture content, and cation and anion composition of loblolly ovules from developing and fully ripened cones. Results thus far indicate presence of 13 metallic elements (cations) in all ovules. Potassium, phosphorus, and magnesium were abundant, with potassium concentrations being particularly high. Manganese, calcium, zinc, and iron were present at intermediate levels, boron, copper, sodium, and nickel in low amounts, and molybdenum and cobalt at very low concentrations. Of seven detectable anions, phosphate, chloride, sulphate, and oxalate predominated. Two others could not be identified. Nitrate was detected only in ovules from mature cones.

We continue to seek confirmation of benefits associated with substituting maltose/glucose for sucrose in the maturation protocol for loblolly pine. Difficulties with contamination and slower than desired culture growth have persisted, but some progress has been made. New cultures initiated this past summer may be used to expedite this important effort. Experiments being planned or in progress will test more carbohydrate concentrations, monitor osmotic effects, document the role of ABA, and confirm the need for IBA.

**DUES-FUNDED PROJECT SUMMARY FORM**  
**FY 91-92**

**Project Title:** MASS CLONAL PROPAGATION OF IMPROVED CONIFERS  
**Division:** Chemical and Biological Sciences  
**Project Code:** SFTWD  
**Project Number:** 3223-00  
**Project Staff:** Ron Dinus, Jim Mathis, Nagmani Rangaswamy, Dave Webb  
**FY 91-92:** \$360,000

**PROGRAM OBJECTIVE:** Develop reliable cell and tissue culture systems for mass clonal propagation of genetically improved softwoods.

**PROGRAM AREAS:** Reduced Operating Costs, Capital Effectiveness, Fiber Availability, Quality, and Cost, End-Use Performance.

**FY 91-92 Goals:**

- 1) Obtain additional loblolly pine and Douglas-fir cultures for accelerated research on embryo maturation.
- 2) Increase embryo maturation frequencies in loblolly pine and Douglas-fir; secure first seedlings in loblolly pine.
- 3) Improve frequencies for initiation of embryogenic cultures in loblolly pine to 10 percent.
- 4) Explore initiation from explants of more mature trees.

**ACCOMPLISHMENTS TO DATE:**

Past research on cell and tissue culture systems has brought somatic embryogenesis, one method of mass cloning, closer to commercialization. Embryogenesis in Norway Spruce, a model system, is not reproducible and straightforward. Embryogenic cultures can be obtained from immature and mature seeds and from tissues of newly germinated seedlings. Last efforts to produce mature embryos showed that using glutamine in lieu of ammonium nitrate as well as higher concentrations of abscisic acid foster maturation. Numbers of mature embryos, averaged over all treatments, were 130 per g of culture tissue; the best treatment yielded 750 per g. Summary of Results Since Last Report:

Work on Norway spruce, our model system, has been further reduced. Samples for microscopy are being held in storage pending receipt of results from growth regulator assays. This strategy made available more resources for our target species. Cessation of spruce work, with possible exception of that on conversion to seedlings and genetic fidelity, seems best in view of advances made by other laboratories.

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In related research on media suitability, additional data was gathered on fresh and dry weights, moisture content, and cation and anion composition of loblolly ovules from developing and fully ripened cones. Results thus far indicate presence of 13 metallic elements (cations) in all ovules. Potassium, phosphorus, and magnesium were abundant, with potassium concentrations being particularly high. Manganese, calcium, zinc, and iron were present at intermediate levels, boron, copper, sodium, and nickel in low amounts, and molybdenum and cobalt at very low concentrations. Of seven detectable anions, phosphate, chloride, sulphate, and oxalate predominated. Two others could not be identified. Nitrate was detected only in ovules from mature cones.

We continue to seek confirmation of benefits associated with substituting maltose/glucose for sucrose in the maturation protocol for loblolly pine. Difficulties with contamination and slower than desired culture growth have persisted, but some progress has been made. New cultures initiated this past summer may be used to expedite this important effort. Experiments being planned or in progress will test more carbohydrate concentrations, monitor osmotic effects, document the role of ABA, and confirm the need for IBA.

Successful maturation of loblolly embryos via substitution of maltose or glucose for sucrose infers that a similar tack would work for Douglas-fir. Informal experiments generally confirmed this hypothesis, but maltose proved more effective than both sucrose and glucose. Maltose effects varied with concentration. More embryos were produced at higher levels, but they did

not develop as well as at low concentrations. Several additional modifications have been or are being evaluated: changing from solid to liquid media, reducing nutrient salts to half-strength, and eliminating casein hydrolysate. The best treatment encountered to date yielded 82 cotyledonary embryos per 50 ml of liquid culture.

Planning, conduct, and interpretation of research on somatic embryogenesis is being aided by deployment of a consistent system for classifying embryo developmental status. The system was derived from observations of developing Douglas-fir embryos over the course of a summer, literature on pine embryology, and consultation with coworkers, including member company scientists. This new tool will facilitate our selecting explant types, quantifying experimental results, and communicating research findings.

**RELATED STUDENT RESEARCH: In progress**

David Barzyk - M.Sc., Development of a fiber optic system to determine the in vivo pH of developing Pinus taeda seeds. Advisor, Dinus.

Rene Kapik - Ph.D., Completing A390 Problems; Likely dissertation topic, Growth regulators in developing zygotic embryos. Advisor, Dinus.

**PROJECT 3223-02 SUMMARY FORMS  
BIOCHEMISTRY OF CLONAL PROPAGATION**

# ***DUES-FUNDED PROJECT SUMMARY FORM***

***FY 90-91***

**Project Title:** BIOCHEMISTRY OF CLONAL PROPAGATION  
**Division:** Chemical and Biological Sciences  
**Project Code:** BIOCM  
**Project No.:** 3223-02  
**Project Staff:** Ron Dinus, Jim Mathis  
**FY 90-91 Budget:** \$150,000

## **PROJECT OBJECTIVE:**

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

Program Areas: Reduced Operating Costs, Capital Effectiveness, Fiber Availability, Quality, and Cost, Environmental Impact, End Use Performance.

## **FY 90-91 GOALS:**

- 1) Complete restaffing and equipping of laboratory.
- 2) Renew work on biochemical similarities/differences of developing somatic and zygotic embryos, with emphasis on storage proteins and lipids.
- 3) Develop techniques for obtaining herbicide tolerance in selected hardwood species via genetic transformation and/or somaclonal variation/selection.
- 4) Adapt molecular techniques for verifying genetic fidelity and gene expression.

## **ACCOMPLISHMENTS TO DATE:**

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

Earlier work on the biochemistry of embryogenesis yielded useful data on differences between embryogenic and nonembryogenic cultures, and some knowledge of factors affecting the process. Such differences and associated markers can be 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 adapted for use with forest trees. These are now available for application toward increasing initiation and maturation frequencies, facilitating conversion to seedlings, and evaluating seedling performance and fidelity.

Actions needed to secure the laboratories for recombinant DNA research were completed, and we have been certified as in compliance with all applicable USDA and NIH regulations. Permits for acquisition, storage, and handling of Agrobacterium tumefaciens and related materials have been secured. Acquisition of new equipment and supplies has been completed as well.

### **SUMMARY OF RESULTS SINCE LAST REPORT:**

Experiments on biochemical and molecular mechanisms underlying beneficial effects of maltose on somatic embryo maturation have been planned and readied for implementation. These will be executed in concert with and in direct support of major thrusts undertaken on this important front in Project 3223-00. Analyses of proteins and lipids in maturing somatic embryos as well as genetic fidelity of somatic embryos and seedlings is also being planned in support of that project. Further information is given on the Project 3223-00 Summary Form.

Projected research on herbicide tolerance in hardwoods is well underway. Efforts are focused on gene transfer with somaclonal variation/selection as insurance. The working dialogue with Monsanto Corporation has been expanded. Detailed discussions are underway in connection with our securing their new gene for glyphosate tolerance. In addition, a Monsanto Corporation genetic construct for enhanced auxin synthesis is being used in a student research project on hardwood transformation. Much progress has been made. When student work concludes, permanent staff will continue this promising effort. Successful transformation should result in Monsanto Corporation providing another construct that will ensure expression of enhanced auxin synthesis only in tissues of importance to the industry.

### **Related Student Projects:**

#### In Progress

- James Bond - Ph.D., A Ramin microspectroscopic investigation of the patterns of molecular order in secondary walls of southern pine tracheids. Complete participation, Dinus.
- Colleen Walker - Ph.D., Development of a biomimetic approach for pulp bleaching. Advisor, Dinus.

# ***DUES-FUNDED PROJECT SUMMARY FORM***

***FY 91-92***

**Project Title:** BIOCHEMISTRY OF CLONAL PROPAGATION  
**Division:** Chemical and Biological Sciences  
**Project Code:** BIOCM  
**Project No.:** 3223-02  
**Project Staff:** Ron Dinus, Jim Mathis  
**FY 91-92 Budget:** \$115,000

## **PROJECT OBJECTIVE:**

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

Program Areas: Reduced Operating Costs, Capital Effectiveness, Fiber Availability, Quality and Cost, Environmental Impact, End Use Performance.

## **FY 91-92 GOALS:**

- 1) Develop techniques for:
  - a) Monitoring and manipulating gene expression during somatic embryo maturation.
  - b) Testing genetic fidelity of cloned trees.
  - c) Effecting and verifying genetic transformation.
  - d) Generating, selecting, and testing useful somaclonal variants.

## **ACCOMPLISHMENTS TO DATE:**

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

Earlier work on the biochemistry of embryogenesis yielded useful data on differences between embryogenic and nonembryogenic cultures, and some knowledge of factors affecting the process. Such differences and associated markers can be 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 adapted for use with forest trees. These are now available for application toward increasing initiation and maturation frequencies, facilitating conversion to seedlings, and evaluating seedling performance and fidelity.

Actions needed to secure the laboratories for recombinant DNA research were completed, and we have been certified as in compliance with all applicable USDA and NIH regulations. Permits for acquisition, storage, and handling of Agrobacterium tumefaciens and related materials have been secured. Acquisition of new equipment and supplies has been completed as well.

### **SUMMARY OF RESULTS SINCE LAST REPORT:**

Experiments on biochemical and molecular mechanisms underlying beneficial effects of maltose on somatic embryo maturation have been planned and readied for implementation. These will be executed in concert with and in direct support of major thrusts undertaken on this important front in Project 3223-00. Analyses of proteins and lipids in maturing somatic embryos as well as genetic fidelity of somatic embryos and seedlings is also being planned in support of that project. Further information is given on the Project 3223-00 Summary Form.

Projected research on herbicide tolerance in hardwoods is well underway. Efforts are focused on gene transfer with somaclonal variation/selection as insurance. The working dialogue with Monsanto Corporation has been expanded. Detailed discussions are underway in connection with our securing their new gene for glyphosate tolerance. In addition, a Monsanto Corporation genetic construct for enhanced auxin synthesis is being used in a student research project on hardwood transformation. Much progress has been made. When student work concludes, permanent staff will continue this promising effort. Successful transformation should result in Monsanto Corporation providing another construct that will ensure expression of enhanced auxin synthesis only in tissues of importance to the industry.

### **Related Student Projects:**

#### In Progress

- James Bond - Ph.D., A Ramin microspectroscopic investigation of the patterns of molecular order in secondary walls of southern pine tracheids. Complete participation, Dinus.
- Colleen Walker - Ph.D., Development of a biomimetic approach for pulp bleaching. Advisor, Dinus.

**PROJECT 3223-03 SUMMARY FORMS  
MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED  
AND ENGINEERED HARDWOODS**

# **DUES-FUNDED PROJECT SUMMARY FORM**

**FY 90-91**

**Project Title:** MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS  
**Division:** Chemical and Biological Sciences  
**Project Code:** BIOCM  
**Project No.:** 3223-03  
**Project Staff:** Ron Dinus, Jim Mathis  
**FY 90-91 Budget:** \$125,000

## **PROJECT OBJECTIVE:**

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

Program Areas: Reduced Operating Costs, Capital Effectiveness, Quality and Cost, End Use Performance.

## **FY 90-91 GOALS:**

- 1) Complete construction and equipment of greenhouse.
- 2) Secure additional plant materials and establish "clean" greenhouse populations.
- 3) Expand existing cultures, and initiate or obtain and stabilize additional ones.
- 4) Refine technologies for mass propagation; ensure suitability for genetic transformation and/or somaclonal variation/selection.
- 5) Accelerate research on gene transfer and expression.

## **ACCOMPLISHMENTS TO DATE:**

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 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.

Since its recent founding, most effort in this project has been devoted to acquiring research materials and evaluating cloning methods. Significant numbers of cottonwood clones now reside in our greenhouse and culture bank. A "Leaf Section System" for cloning was developed that yields significant numbers of normal, vigorous trees within 12 to 14 weeks of placing explants in culture. Cottonwood suspension cultures were also developed and characterized. Such cultures represent an alternative route to herbicide tolerant trees, and are also essential for studying fiber formation (xylogenesis) in culture. Growth analyses showed that cell population sizes double every three days. Transfer of cells from rapidly growing cultures to shoot induction and rooting medium, similar to that used in leaf section research, yielded vigorously growing trees.

#### **SUMMARY OF RESULTS SINCE LAST REPORT:**

In accordance with earlier plans, this project seeks to develop technologies for transferring genes for herbicide tolerance into commercially important species, and for effective mass propagation, testing, and release of genetically modified plant materials. Herbicide tolerance is also sought, as a matter of insurance, via somaclonal variation and selection.

Greenhouse populations have been further increased to ensure availability of genetically improved clones, particularly of southern origin. Eight new clones, four from James River Corp. and four from Westvaco Corp., have been received and planted.

Additional shoot cultures were also established and added to our bank. A large scale experiment testing our earlier protocol against one derived from cooperators at Oregon State University and the University of Nebraska, Lincoln, has been installed. Results thus far are encouraging, with shoots forming on explants of most clones in response to one or more treatments. Primary objective is provision of new materials for generalization of the Leaf Section System. Representative samples of aspen and sweetgum cultures are also being maintained.

Work on extending the Leaf Section System to additional clones has accelerated, and several trials are in progress or soon to be executed. Refinements in the course of student research have shown that 30 percent of leaf section explants form shoots and that each responsive explant produces an average of 8 shoots. Tests of the suitability of this system for transformation with a gene for auxin synthesis are underway. Sensitivities of both cottonwood and Agrobacterium tumefaciens to various antibiotics have been completed or started.

Our working relationship with Monsanto Corp. has been expanded. Detailed discussions concerning technical and legal agreements necessary for our acquiring their new gene for glyphosate tolerance are in progress.

Plans for obtaining herbicide tolerance via somaclonal selection/selection were implemented on schedule. Addition of glyphosate herbicide to rapidly growing suspension cultures quickly killed most cells. Small numbers of cells, fewer than 10 percent, nevertheless survive challenge. These are being expanded as new cultures for further challenge, after which they will be plated onto solid media in efforts to secure glyphosate tolerant calli, shoots, and, eventually, trees.

## **RELATED STUDENT PROJECTS:**

### **In Progress**

- Lois Forde - M.Sc., Phenylalanine ammonia lyase and lignin biosynthesis. Advisors, Conners and Dinus.
- Jim Kramer - M.Sc., Pulping and papermaking properties of Florida-grown and papermaking properties of Florida-grown Eucalyptus amplifolia. Advisors, Dinus and McDonough.
- Tom Ptacek - M.Sc., Variability of wood, fiber, and pulping properties as affected by cloning. Advisor, Dinus.
- Peasely Shorter - M.Sc., Promotion of additional auxin synthesis in Populus deltoides via transformation with Agrobacterium tumefaciens. Advisor, Webb.

## ***DUES-FUNDED PROJECT SUMMARY FORM***

***FY 91-92***

**Project Title:** MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS  
**Division:** Chemical and Biological Sciences  
**Project Code:** BIOCM  
**Project No.:** 3223-03  
**Project Staff:** Ron Dinus, Jim Mathis  
**FY 91-92 Budget:** \$125,000

### **PROJECT OBJECTIVE:**

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

Program Areas: Reduced Operating Costs, Capital Effectiveness, Fiber Availability, Quality and Cost, End Use Performance.

### **FY 90-91 GOALS:**

- 1) Extend methods for mass clonal propagation to a wider array of trees; emphasize genetically improved materials.
- 2) Adapt said methods for production of herbicide tolerant trees via genetic transformation and/or somaclonal variation/selection.
- 3) Complete first work on production of herbicide tolerant trees.

### **ACCOMPLISHMENTS TO DATE:**

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 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.

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In accordance with earlier plans, this project seeks to develop technologies for transferring genes for herbicide tolerance into commercially important species, and for effective mass propagation, testing, and release of genetically modified plant materials. Herbicide tolerance is also sought, as a matter of insurance, via somaclonal variation and selection.

Greenhouse populations have been further increased to ensure availability of genetically improved clones, particularly of southern origin. Eight new clones, four from James River Corp. and four from Westvaco Corp., have been received and planted.

Additional shoot cultures were also established and added to our bank. A large scale experiment testing our earlier protocol against one derived from cooperators at Oregon State University and the University of Nebraska, Lincoln, has been installed. Results thus far are encouraging, with shoots forming on explants of most clones in response to one or more treatments. Primary objective is provision of new materials for generalization of the Leaf Section System. Representative samples of aspen and sweetgum cultures are also being maintained.

Work on extending the Leaf Section System to additional clones has accelerated, and several trials are in progress or soon to be executed. Refinements in the course of student research have shown that 30 percent of leaf section explants form shoots and that each responsive explant produces an average of 8 shoots. Tests of the suitability of this system for transformation with a gene for auxin synthesis are underway. Sensitivities of both cottonwood and Agrobacterium tumefaciens to various antibiotics have been completed or started.

Our working relationship with Monsanto Corp. has been expanded. Detailed discussions concerning technical and legal agreements necessary for our acquiring their new gene for glyphosate tolerance are in progress.

Plans for obtaining herbicide tolerance via somaclonal selection/selection were implemented on schedule. Addition of glyphosate herbicide to rapidly growing suspension cultures quickly killed most cells. Small numbers of cells, fewer than 10 percent, nevertheless survive challenge.

These are being expanded as new cultures for further challenge, after which they will be plated onto solid media in efforts to secure glyphosate tolerant calli, shoots, and, eventually, trees.

#### **RELATED STUDENT PROJECTS:**

##### In Progress

- Lois Forde - M.Sc., Phenylalanine ammonia lyase and lignin biosynthesis. Advisors, Conners and Dinus.
- Jim Kramer - M.Sc., Pulping and papermaking properties of Florida-grown and papermaking properties of Florida-grown Eucalyptus amplifolia. Advisors, Dinus and McDonough.
- Tom Ptacek - M.Sc., Variability of wood, fiber, and pulping properties as affected by cloning. Advisor, Dinus.
- Peasely Shorter - M.Sc., Promotion of additional auxin synthesis in Populus deltoides via transformation with Agrobacterium tumefaciens. Advisor, Webb.

INITIATION OF EMBRYOGENIC CALLUS FROM  
MEGAGAMETOPHYTES OF LOBLOLLY PINE

D. T. WEBB - PRINCIPAL INVESTIGATOR

D. EVANS, Y. POWELL, C. STEPHENS - TECHNICAL ASSISTANTS

# ***INITIATION***

**I. WESTVACO CLONES [7-34 & 9-11] --> EMBRYOGENIC CALLUS**

**A. EQUAL FREQUENCIES**

**B. 7-34 x 9-11 --> HIGHER FREQUENCY VS PARENTS**

# *INITIATION*

## II. MEGAGAMETOPHYTES --> GOOD EXPLANTS

A. HIGHER FREQUENCIES vs EARLIER WORK

B. SUBOPTIMAL vs % FERTILITY & E. Wt. PINE

# ***INITIATION***

**III. ALL MEDIA --> EXTRUDED CALLUS**

**A. MSG > or = DCR >> BM**

**B. ONLY DCR --> CALLUS LINES**

# ***INITIATION***

## **IV. PHYTOHORMONES NOT REQUIRED --> EXTRUDED CALLUS**

**A. 2,4-D ENHANCES [1 < 5  $\mu$ M]**

**B. BA ENHANCES WITH 2,4-D [2:1; 2,4-D:BA]**

# ***INITIATION***

## **V. MAINTENANCE REQUIRES 2,4-D**

**A. SEVERAL LEVELS --> SUCCESS**

**B. LOW CONCENTRATION > HIGH**

**C. ONE CONCENTRATION [1.1  $\mu\text{M}$ ] --> ALL LINES**

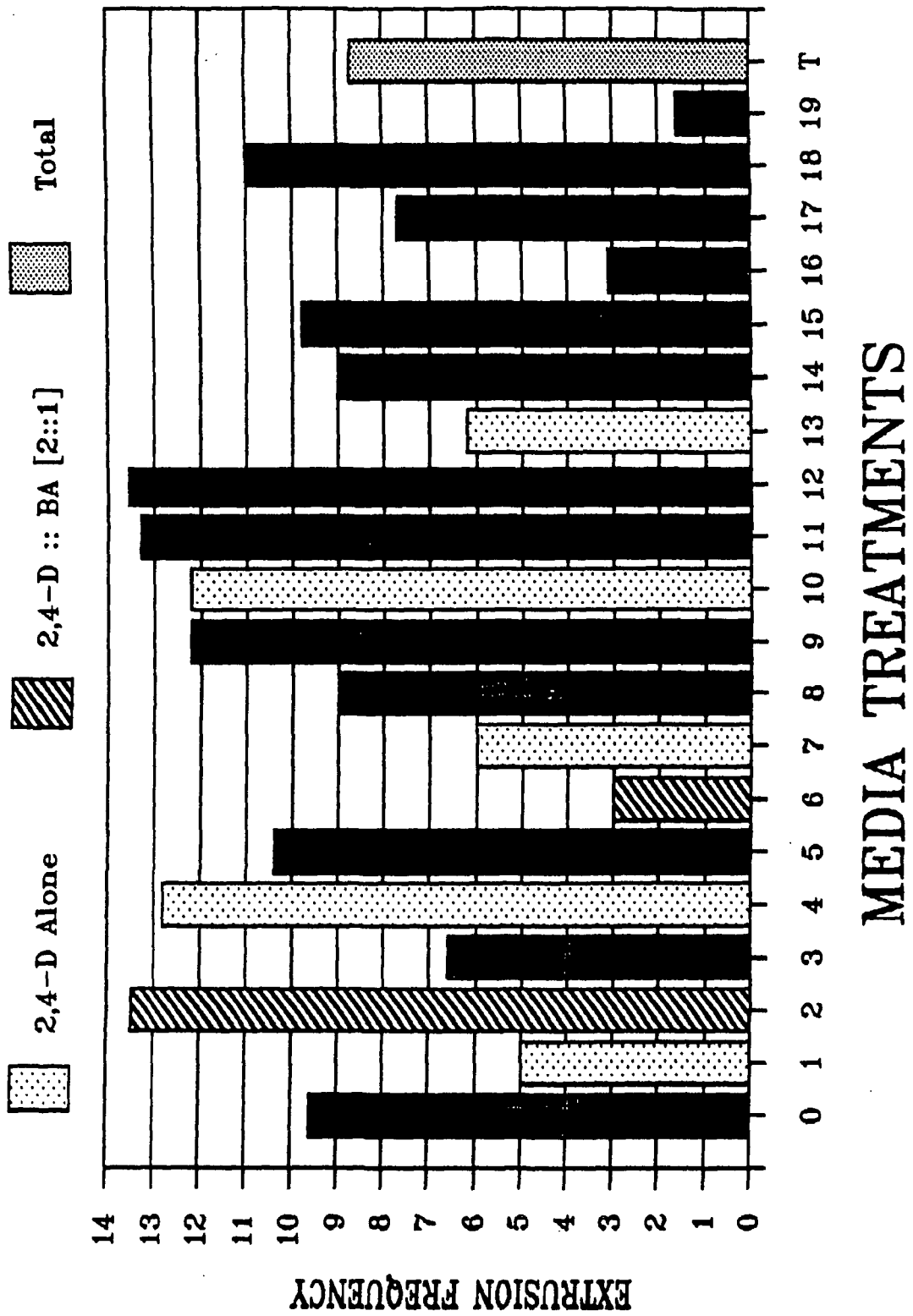


FIG. 4 FORMATION OF EXTRUDED CALLUS ON MSG MEDIUM AFTER 8 WEEKS

## INITIATION - PRESENT EXPERIMENTS

A. UNRELATED SOURCE TREES [BRAZIL]

B. 2,4-D [1.1, 2, 3, 5  $\mu$ M]

C. 2,4-D : BA [2 : 1]

D. DCR MEDIUM [FULL vs HALF-STRENGTH]

E. MEDIA SEQUENCE

GOOD INITIATION --> GOOD MAINTENANCE

## **INITIATION - FUTURE EXPERIMENTS**

**A. ALTERNATE SYNTHETIC AUXINS**

**B. ALTERNATE CARBOHYDRATES**

**C. ALTERNATE SUBSTRATES**

**D. OSMOLARITY**

**E. OXYGEN LEVELS**

**F. MEDIUM COMPONENTS**

**1] MACRONUTRIENTS**

**2] NITROGEN SOURCES**

**G. GENETICS OF SOURCE TREE**

**H. ALTERNATE EXPLANTS**

ELEMENTAL COMPOSITION OF DEVELOPING OVULES OF LOBLOLLY  
PINE ..... A MECHANISTIC APPROACH

NAGMANI, R.

RON HOOPER  
JACINTA CASTELLINO

RON DINUS

OBJECTIVES

REVIEW PAST I.P.S.T RESEARCH

COMPLETE DATA ON OVULE COMPOSITION

COMPARE WITH EXISTING SYNTHETIC CULTURE  
MEDIA

RECOMMEND PROTOCOL CHANGES IF POSSIBLE

METHODS

ICP - EMISSION SPECTROMETRY

ION CHROMATOGRAPHY

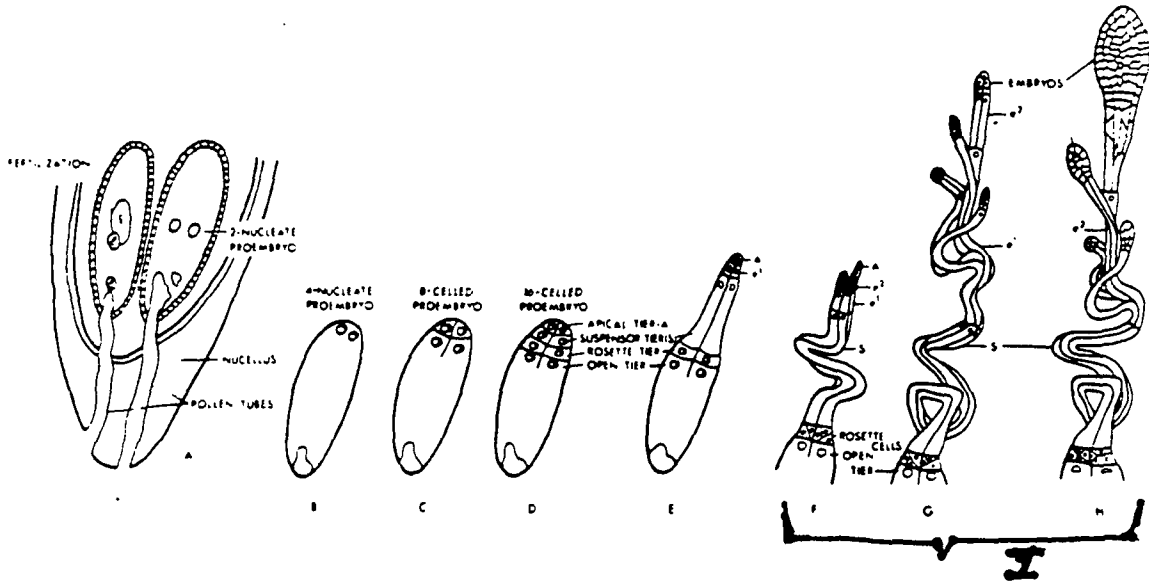


Figure 7.2 Fertilization and cleavage polyembryony in Pinus (from Owens and Molder 1984b).

*Development of Seeds and Embryos in Pinus ponderosa*

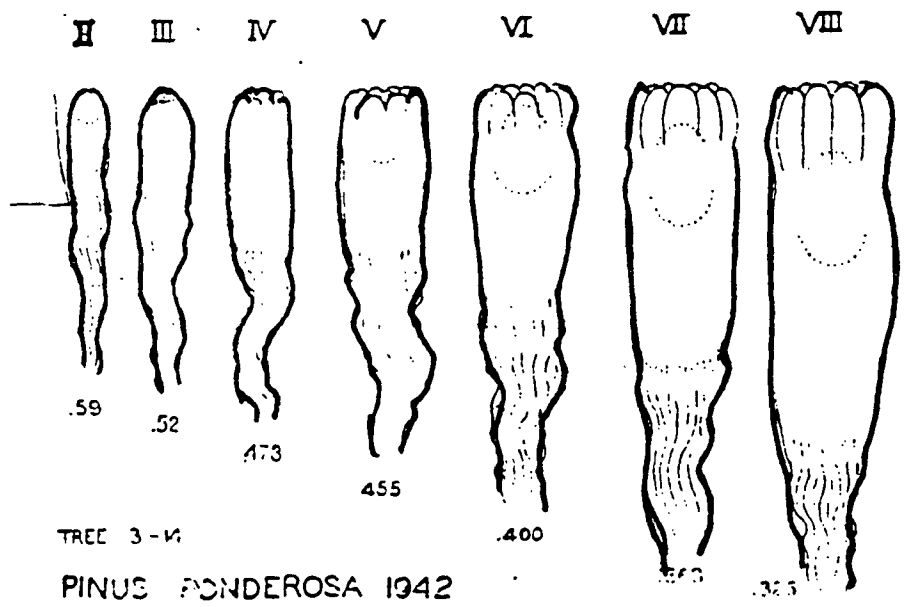


FIG. 1.— Embryos and parts of suspensors in various stages (X 12).

Figure 1. Developmental stages of conifer embryo (Owens & Molder, 1984 ; Buchholz & Stiemert, 1945)

Table 1: Weight and Moisture content of developing loblolly pine seed

Parameter	Date of seed cone collection			
	7/2/90	7/16/90	Dry	9/15/90* imbibed
Fresh Wt. g/seeds	1.779 g (575)	3.283 g (545)	4.449 g (230)	4.623 g (223)
Dry Wt. g/seeds	0.4408	1.4480	4.166	3.184
Wt. Water g	1.3397	1.8352	0.283	1.439
Moisture % content	75.3	55.9	6.36	31.12

\* Seeds at the time of harvest (mature seeds)

Table 2: Weight and sample concentration of loblolly pine seeds for Anion Analysis

Parameter	Date of seed cone collection			
	7/2/90	7/9/90	7/16/90	9/15/90*
Fresh Wt. g/seeds	0.226 g (36)	0.187 g (36)	0.277 g (36)	0.549 g (36)
Initial sample concentration	226mg/25ml	187mg/25ml	277mg/25ml	549/25ml

Final concentration of the sample injected to IC in the ratio of 1:1 to 1:10 of Sample: de-ionized water

\* Seeds at the time of harvest (mature)

RESULTS

TABLE 3: ELEMENTAL ANALYSIS OF LOBLOLLY PINE OVULES  
(CATION ANALYSIS)

MACROELEMENTS	μg/gram of oven dried samples; Dry wt.		
	7/2/90 *	7/16/90 *	9/15/90 *** DRY           IMBIBED
CALCIUM	260	320-350 **	
MAGNESIUM	11,900	9,300-9,600 **	
PHOSPHORUS	17,300	18,200-21,000 **	
POTASSIUM	36,900	15,200-15,400 **	Analysis in Progress
SODIUM	13.2	6.21-7.77 **	

\* . COLLECTION DATES OF SEED CONES FROM LYONS, GA

\*\* DATA FROM DUPLICATE SAMPLES

\*\*\* SEEDS AT THE TIME OF HARVEST

RESULTS

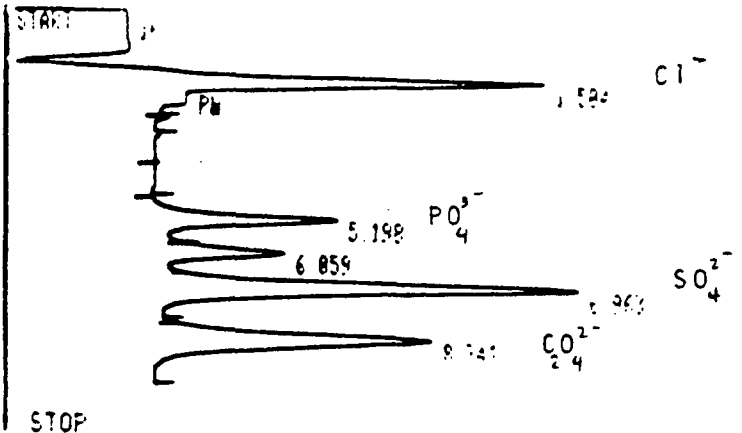
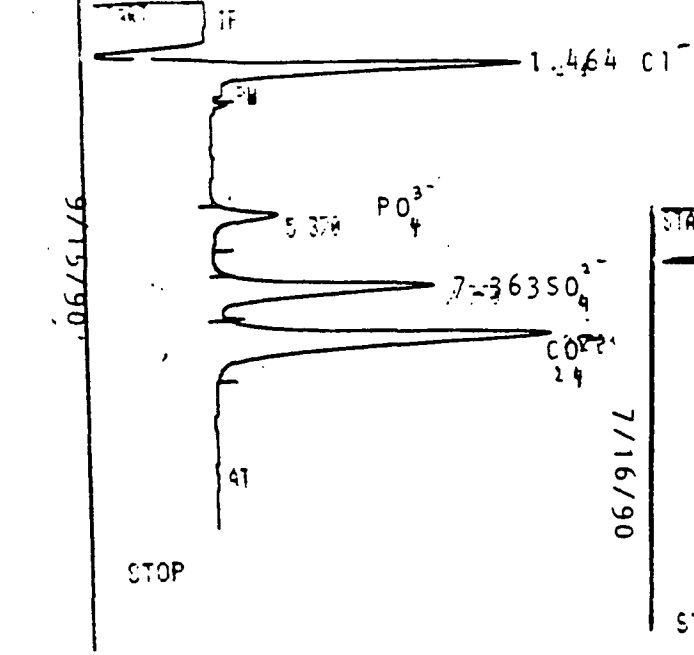
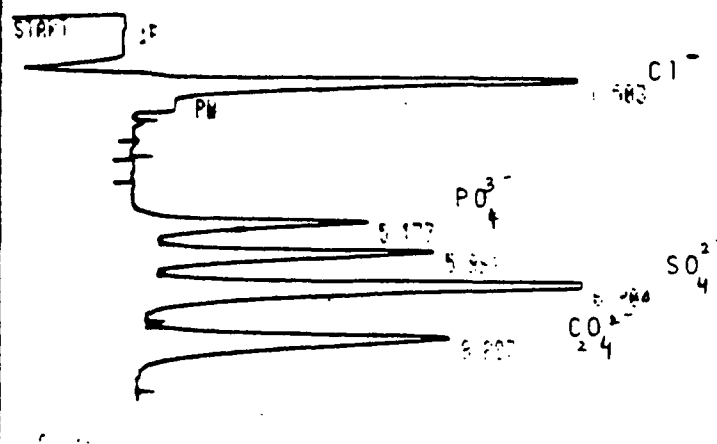
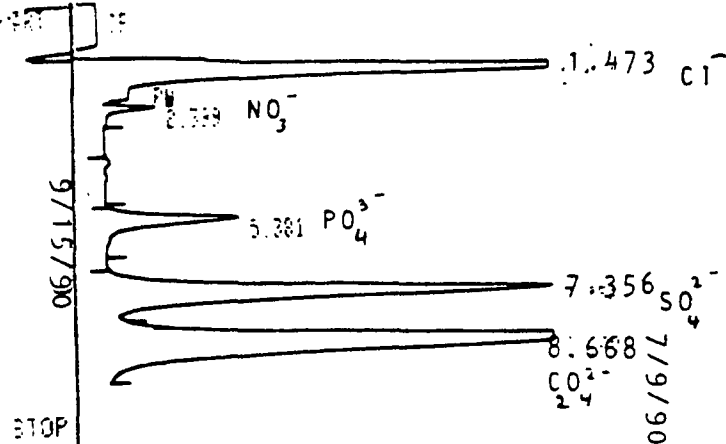
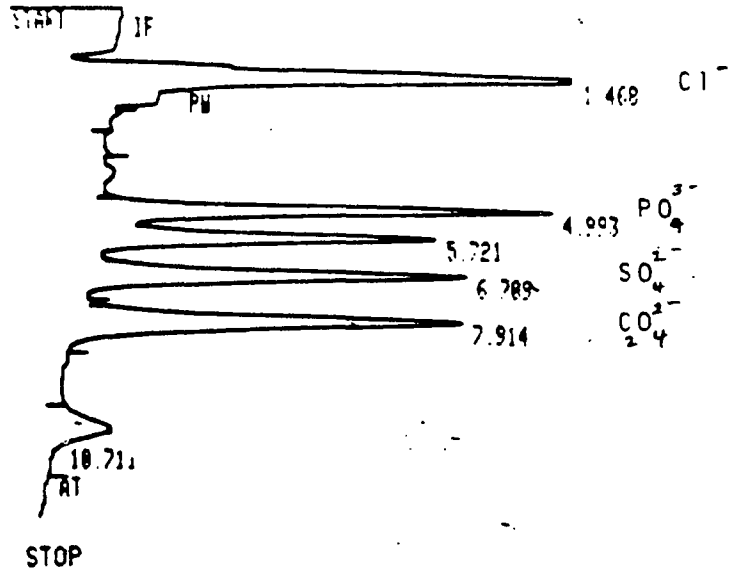
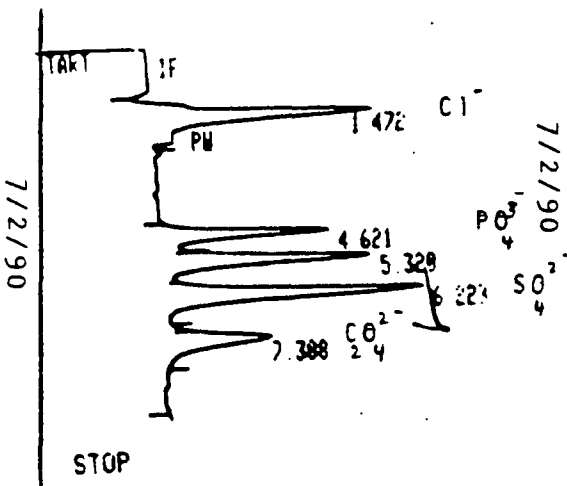
TABLE 3: ELEMENTAL ANALYSIS OF LOBLOLLY PINE OVULES  
(CATION ANALYSIS)

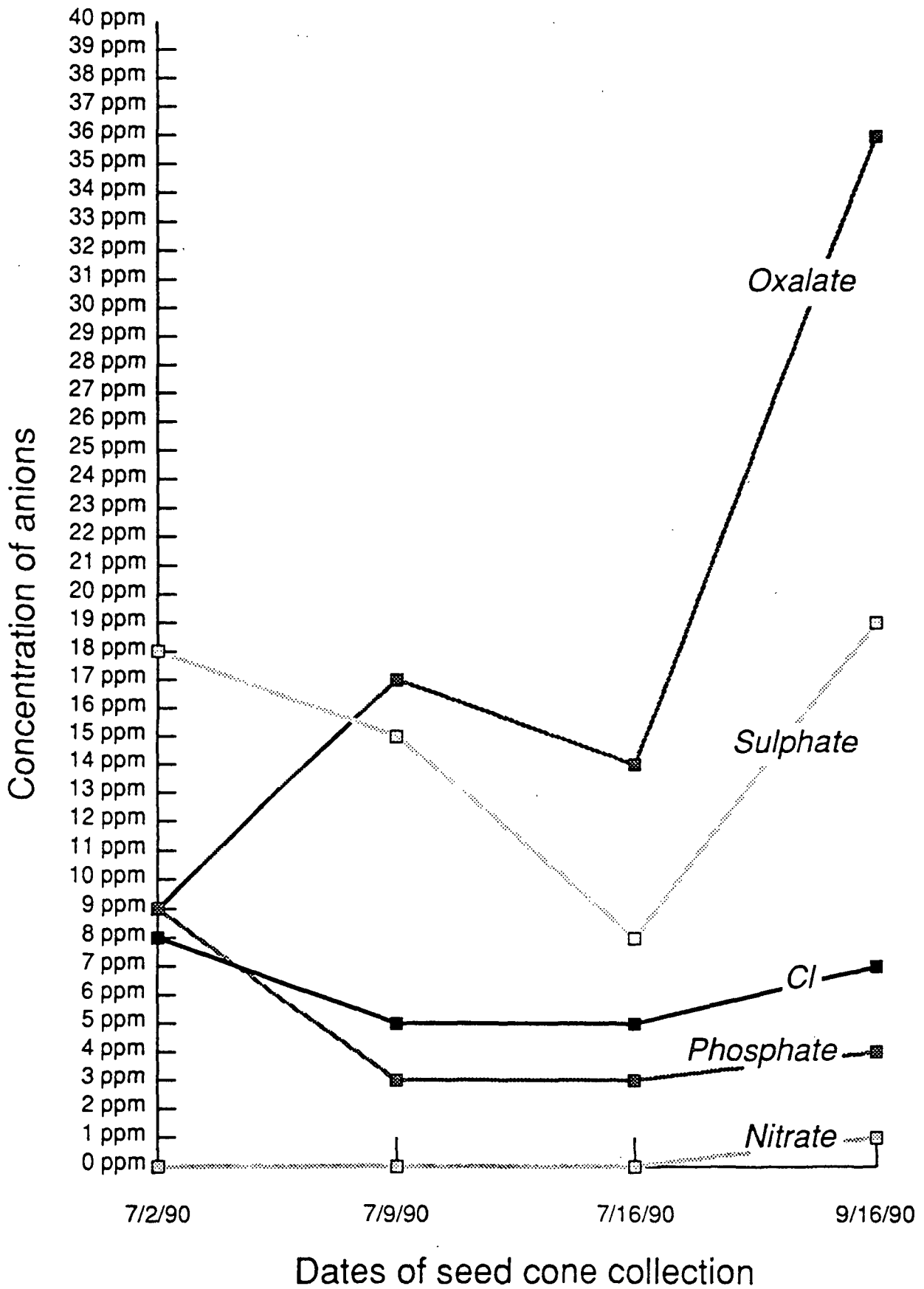
MICRO-ELEMENTS	ug/gram of oven dried samples; Dry wt.		
	7/2/90 *	7/16/90 *	9/15/90 ** DRY            IMBIBED
BORON	70	43-48 **	
MANGANESE	280	420-430 **	
ZINC	237	210-240 **	
MOLYBDENUM	<0.125	<0.125	Analysis in Progress
COPPER	19	27-26 **	
COBALT	<0.075	<0.075	
NICKEL	8.4	6.88-7.28 **	
IRON	110	92-99 **	

\* COLLECTION DATES OF SEED CONES FROM LYONS, GA

\*\* DATA FROM DUPLICATE SAMPLES

\*\*\* SEEDS AT THE TIME OF HARVEST





Anion Composition in Developing Ovules of Loblolly Pine

TABLE 4: ANION COMPOSITION OF DEVELOPING OVULES OF LOBLOLLY PINE

ANIONS	DATE OF SEED CONE COLLECTION				
	7/2/90 g/l	7/9/90 g/l	7/16/90 g/l	9/15/90* g/l	9/15/90** g/l
CHLORIDE (Cl <sup>-</sup> )	0.0080	0.0057	0.005	0.0077	0.007
PHOSPHATE (PO <sub>4</sub> <sup>3-</sup> )	0.0092	0.0033	0.0036	0.0048	0.004
SULPHATE (SO <sub>4</sub> <sup>2-</sup> )	0.0186	0.0158	0.0080	0.0241	0.014
OXALATE (C <sub>2</sub> O <sub>4</sub> <sup>2-</sup> )	0.0098	0.0174	0.0140	0.0361	0.037
NITRATE (NO <sub>3</sub> <sup>-</sup> )	-----	-----	-----	-----	0.001

\* SEED SAMPLE AT THE TIME OF HARVEST

\*\* DUPLICATE SAMPLES

**SOMATIC EMBRYO MATURATION**

ROLE OF CARBOHYDRATES AND GROWTH  
REGULATORS IN THE MATURATION AND  
DEVELOPMENT OF LOBLOLLY PINE  
SOMATIC EMBRYOS

D. T. WEBB, J. N. MATHIS - PRINCIPAL INVESTIGATORS

Y. POWELL, D. EVANS - TECHNICAL ASSISTANTS

## DEVELOPMENT & MATURATION

### A. SUCROSE DOES NOT SUPPORT MATURATION & DEVELOPMENT

### B. MALTOSE & GLUCOSE

- 1] STAGE 3 SOMATIC EMBRYOS
- 2] STAGE 7-8 (COTYLEDONARY) EMBRYOS
- 3] MALTOSE > GLUCOSE
- 4] EFFECTIVE CONCENTRATIONS (0.33 - 0.5 M)

### C. ABSCISIC ACID

- 1] REQUIRED
- 2] EFFECTIVE CONCENTRATIONS (20-30  $\mu$ M)

# **DEVELOPMENT & MATURATION**

## **CURRENT EXPERIMENT**

**A. EFFECTS OF SUCROSE, GLUCOSE & MALTOSE**

**B. IPC LINES**

**C. IPST LINES**

**D. FILTER-STERILIZED vs AUTOCLAVED SUGARS**

## ***DEVELOPMENT & MATURATION***

### **FUTURE EXPERIMENTS**

- A. ABA <--> CARBOHYDRATE INTERACTION**
- B. ABA <--> OSMOTIC <--> CARBOHYDRATE INTERACTIONS**
- C. LIGHT vs DARK**
- E. IBA**

DEVELOPMENT AND MATURATION OF SOMATIC EMBRYOS  
DOUGLAS-FIR

NAGMANI, R.

OBJECTIVES

ESTABLISH SUSPENSION CULTURES

PROMOTE DEVELOPMENT & MATURATION OF  
SOMATIC EMBRYOS IN SUSPENSION CULTURES  
IN LARGE NUMBERS

TO DEVELOPE A PROTOCOL OPTIMAL FOR  
ATLEAST 3 EC LINES REPRESENTING 3  
GENOTYPES

EXPERIMENTS

1. TO TEST THE EFFECT OF CASEIN HYDROLYSATE  
AS NITROGEN SOURCE ON DEVELOPMENT & MATURATION
2. TO TEST THE EFFECT OF MALTOSE vs SUCROSE
3. TO TEST THE EFFECT OF AUTOCLAVED CHO vs FILTER  
STERILIZED
4. TO TEST THE EFFECT OF DIFFERENT LEVELS OF ABA

Table 1. Basal media, supplements, and growth regulator combinations used for initiation and maintenance, development & maturation of somatic embryos. Composition of standard MS medium is shown for comparison.

Components, mg <sup>l</sup> <sup>-1</sup>	MS	MSCG 5/0	MSCG5/2.5	mMSCG2/0	mMSG *
NH <sub>4</sub> NO <sub>3</sub>	1650	--	--	--	--
KNO <sub>3</sub>	1900	100	100	950	950
MgSO <sub>4</sub> •7H <sub>2</sub> O	370	370	370	185	185
KH <sub>2</sub> PO <sub>4</sub>	170	170	170	85	85
CaCl <sub>2</sub> •2H <sub>2</sub> O	440	440	440	220	220
Ca(NO <sub>3</sub> ) <sub>2</sub> •4H <sub>2</sub> O	--	--	--	--	--
KCl	--	745	745	--	--
KI	0.83	0.83	0.83	0.41	0.41
H <sub>3</sub> BO <sub>3</sub>	6.2	6.2	6.2	3.1	3.1
MnSO <sub>4</sub> •H <sub>2</sub> O	22.3	22.3	22.3	11.15	11.15
ZnSO <sub>4</sub> •7H <sub>2</sub> O	8.6	8.6	8.6	4.3	4.3
Na <sub>2</sub> MoO <sub>4</sub> •2H <sub>2</sub> O	0.25	0.25	0.25	0.125	0.125
CuSO <sub>4</sub> •5H <sub>2</sub> O	0.025	0.025	0.025	0.0125	0.0125
CoCl <sub>2</sub> •6H <sub>2</sub> O	0.025	0.025	0.025	0.0125	0.0125
NiCl <sub>2</sub> •6H <sub>2</sub> O	--	--	--	--	--
FeSO <sub>4</sub> •7H <sub>2</sub> O	27.8	27.8	27.8	27.8	27.8
Na <sub>2</sub> EDTA	37.3	37.3	37.3	37.3	37.3
Inositol	100	100	100	100	100
Glycine	--	--	--	--	--
Nicotinic acid	0.5	0.5	0.5	0.5	0.5
Pyridoxine	0.1	0.1	0.1	0.1	0.1
Thiamine HCl	0.1	0.1	0.1	0.1	0.1
Sucrose	30,000	30,000	30,000	30,000	30,000
Glutamine (G)	--	500	500	500	500
Casein	--	--	--	--	--
Hydrolyzate(C)	--	1000	1000	1000	--
Agar	0.8%	0.8%	0.8%	0.8%	--
<u>Growth Regulators</u>					
2,4-D	--	5	5	2	--
BA	--	--	2.5	--	--
Kinetin	--	--	--	--	--
ABA	--	--	--	--	2.6

\* Development and Maturation media with sucrose or maltose or glucose at various levels.

TABLE 2: Effect of maltose and ABA on somatic embryo development in Douglas-fir suspension cultures

Treatments	Number of Somatic embryos per 50 ml	Developmental stage of somatic embryos
mMSG + Maltose 0.08 M	82	Green, cotyledonary, mature embryos (Stage 5-7)
mMSG + Maltose 0.17 M	55	" "
mMSG + Maltose 0.31 M	60	" "
mMSG + Maltose 0.49 M	49	" "
mMSG + Maltose 0.08 M + ABA 10 $\mu$ M	151	Stage 3 embryos

## SUMMARY

PRELIMINARY EXPERIMENTS HAVE INDICATED:

CAESEIN HYDROLYSATE IS NOT NECESSARY FOR  
DEVELOPMENT & MATURATION ?

MALTOSE ALONE PROMOTES DEVELOPMENT & MATURATION

MALTOSE WITH ABA AT 10 $\mu$ M ENHANCES SYNCHRONOUS  
DEVELOPMENT OF PRE-COTYLEDONARY EMBRYOS IN  
LARGE NUMBERS

## FUTURE PLANS

1. EFFECT OF CARBOHYDRATES; GLUCOSE, MALTOSE  
& SUCROSE AT 1/2 ; 3, 6, 9 & 12%
2. OPTIMAL SOURCE OF CHO & OPTIMAL LEVEL +  
ABA ( 0,2,5,10,20,40 & 80  $\mu$ M )
3. STATISTICAL EVALUATION OF DATA

**HARDWOODS, STUDENT PROJECT  
AUXIN TRANSFORMATION**

PROMOTION OF ADDITIONAL AUXIN BIOSYNTHESIS  
IN EASTERN COTTONWOOD THROUGH GENETIC  
ENGINEERING

J. N. MATHIS, D. T. WEBB - PRINCIPAL INVESTIGATORS

C. STEPHENS - TECHNICAL ASSISTANT

P. SHORTER - M. Sc. STUDENT

# RESULTS

A. IMPROVED LEAF SECTION REGENERATION SYSTEM

B. ANTIBIOTIC SCREENING

C. GENES & AGROBACTERIUM STRAINS

D. COCULTIVATION

---

Summary of *P. deltoides* Tissue Culture Regeneration Systems.

---

Leaf Disk:

<u>Steps</u>	<u>Function</u>	<u>Explant</u>	<u>Growth Medium</u>	<u>Environment</u>
1	Shoot Initiation	Leaf Disks	DKW + B1N1	3 wks. Dark
2	Shoot Elongation	Leaf Disks	DKW + B1N1	3-6 wks. Light
3	Root Initiation	Shoots	WPM + IBA	4-6 wks. Light

Internode:

<u>Steps</u>	<u>Function</u>	<u>Explant</u>	<u>Growth Medium</u>	<u>Environment</u>
1	Shoot Initiation	Internodes	DKW + Z	3-4 wks. Light
2	Root Initiation	Shoots	WPM + IBA	4-6 wks. Light

Shoot Tip:

<u>Steps</u>	<u>Function</u>	<u>Explant</u>	<u>Growth Medium</u>	<u>Environment</u>
1	Shoot Elongation	Shoot Apex	DKW + Z	3 wks. Light
2	Root Initiation	Shoots	WPM + IBA	4-6 wks. Light

Tissue Culture Media:

DKW = Cottonwood Maintenance Medium (McGranahan, Driver, and Tulecke 1987)

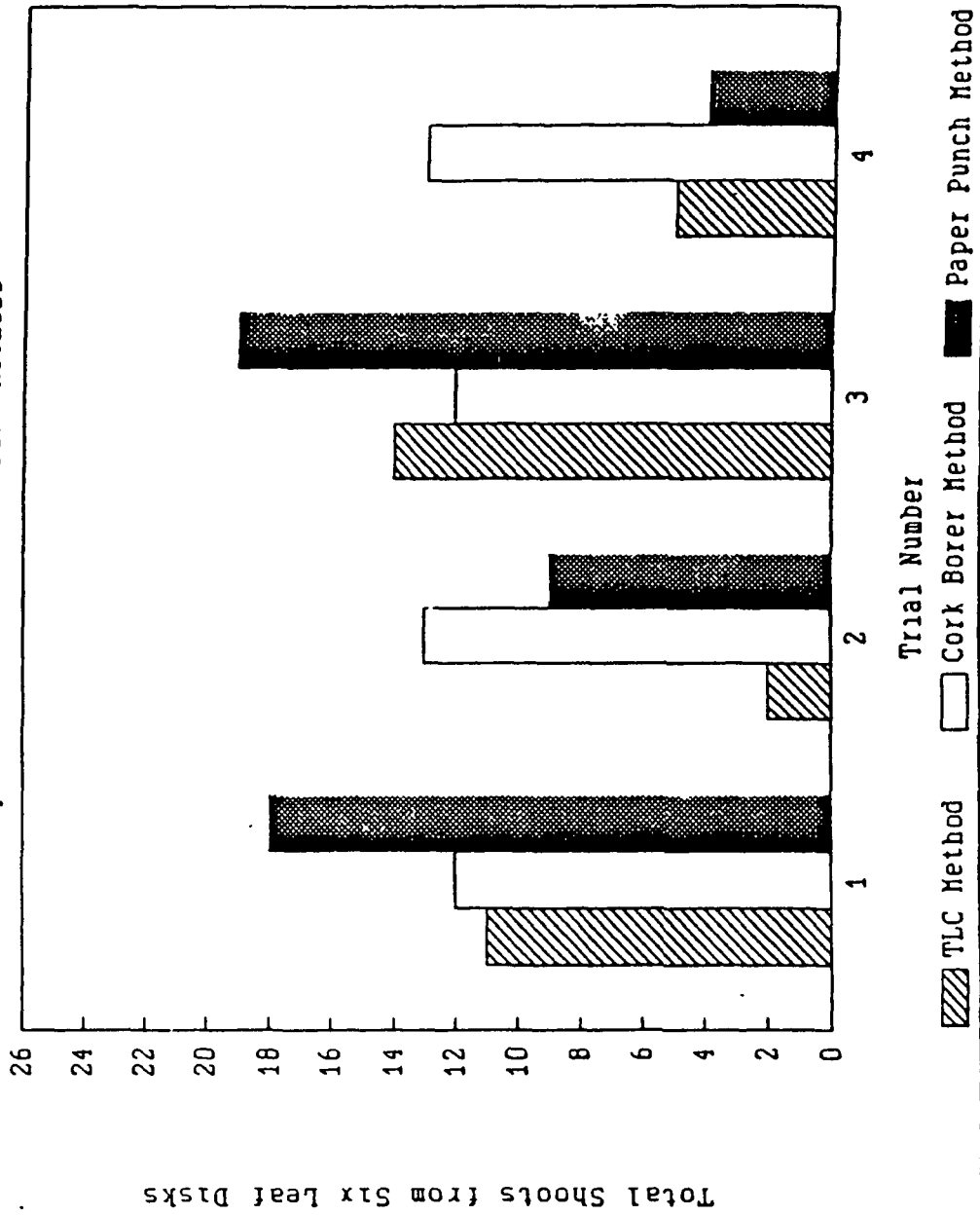
WPM = Woody Plant Medium (Lloyd and McCown 1980)

Growth Regulators or Hormones (Uddin pers. com. 1990):

B1	=	1 uM 6-benzylaminopurine (BA)
N1	=	1 uM naphthaleneacetic acid (NAA)
IBA	=	0.1 mg l <sup>-1</sup> indole-3-butyric acid (IBA)
Z	=	0.25 mg l <sup>-1</sup> zeatin (Z)

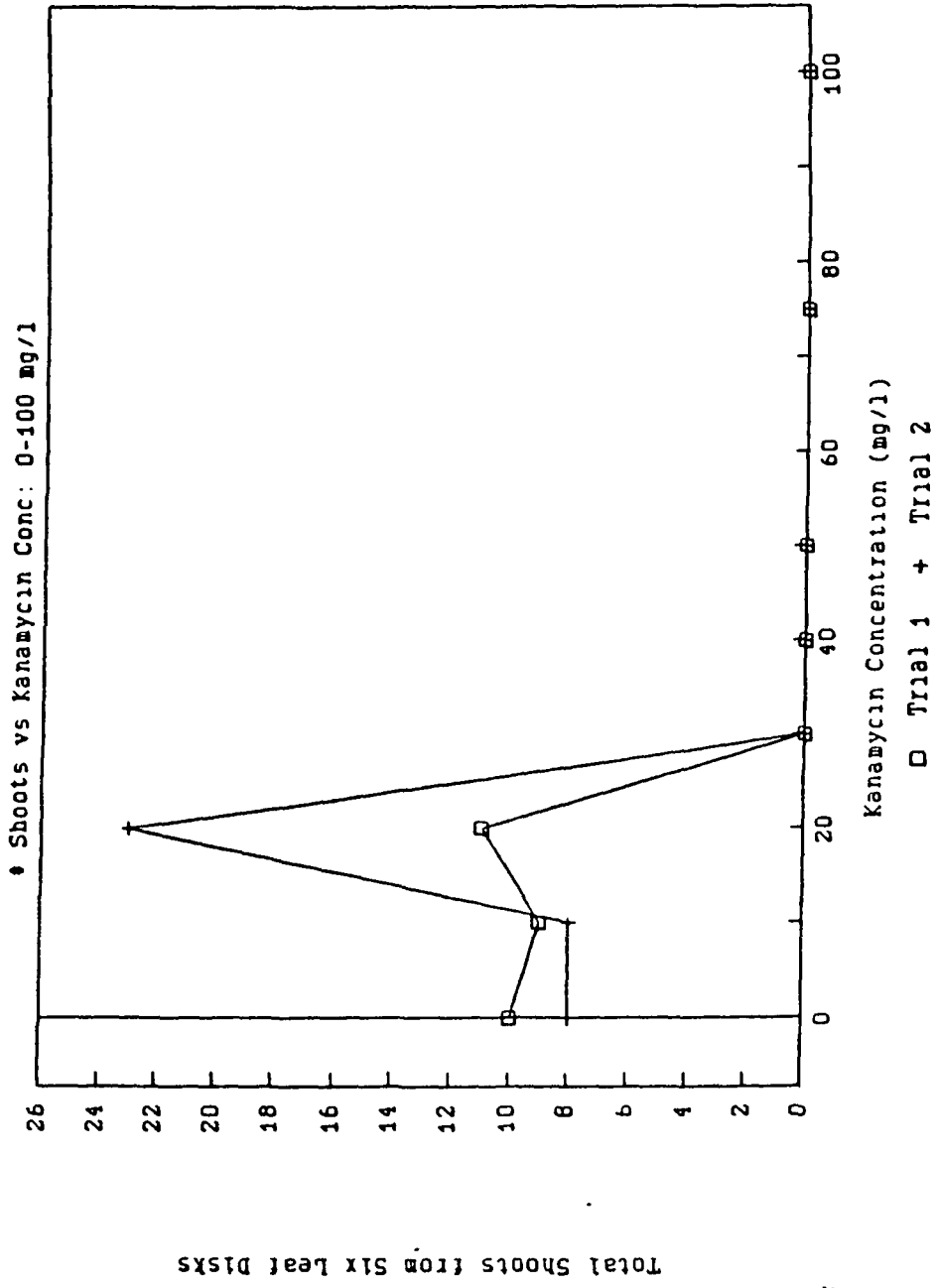
# LEAF DISK PREPARATION EXPERIMENT

Comparison of Three Leaf Disk Methods

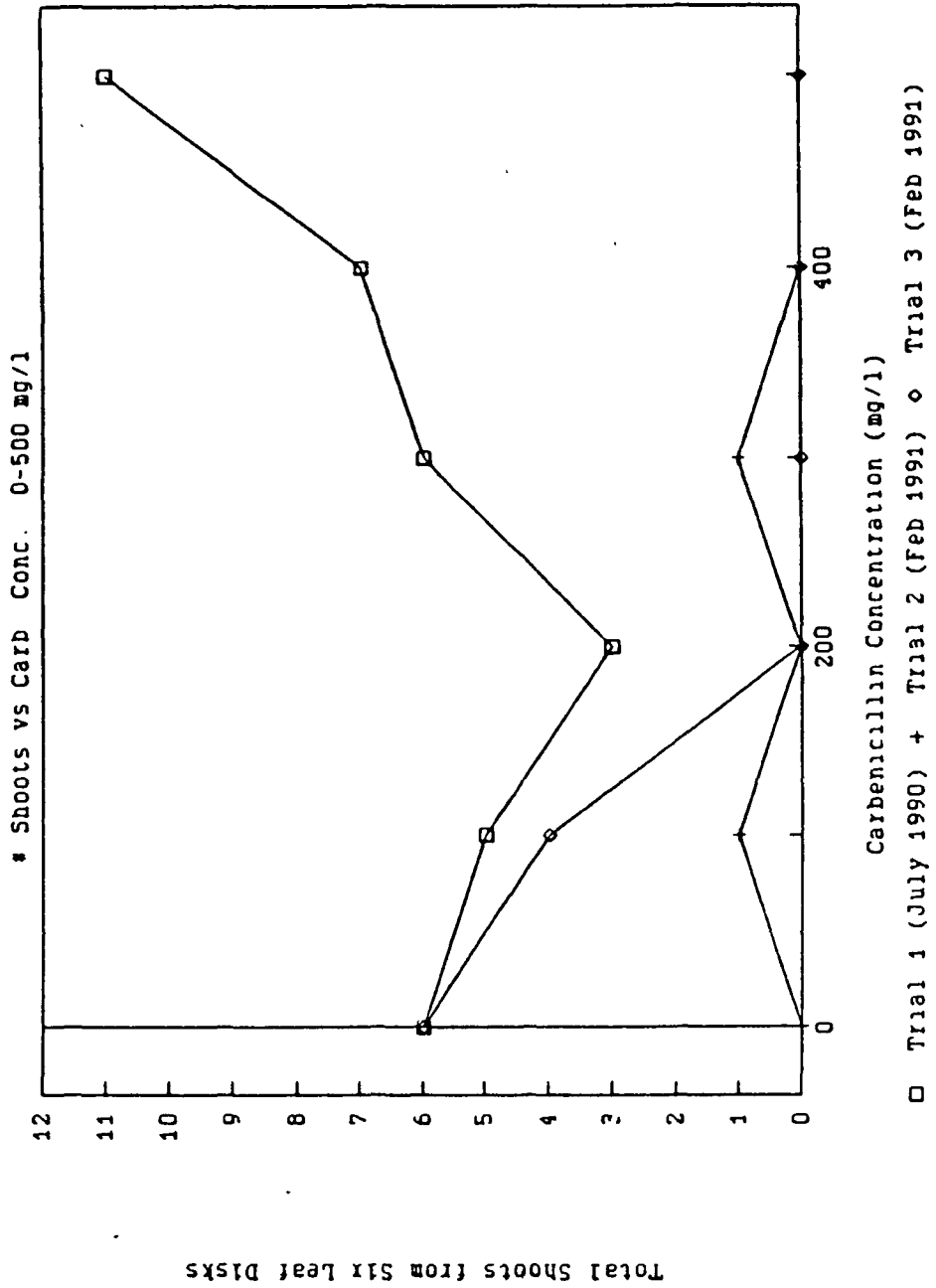


Comparison of shoot production from TLC, cork borer, and paper punch

# LETHAL DOSE ASSAY OF KANAMYCIN

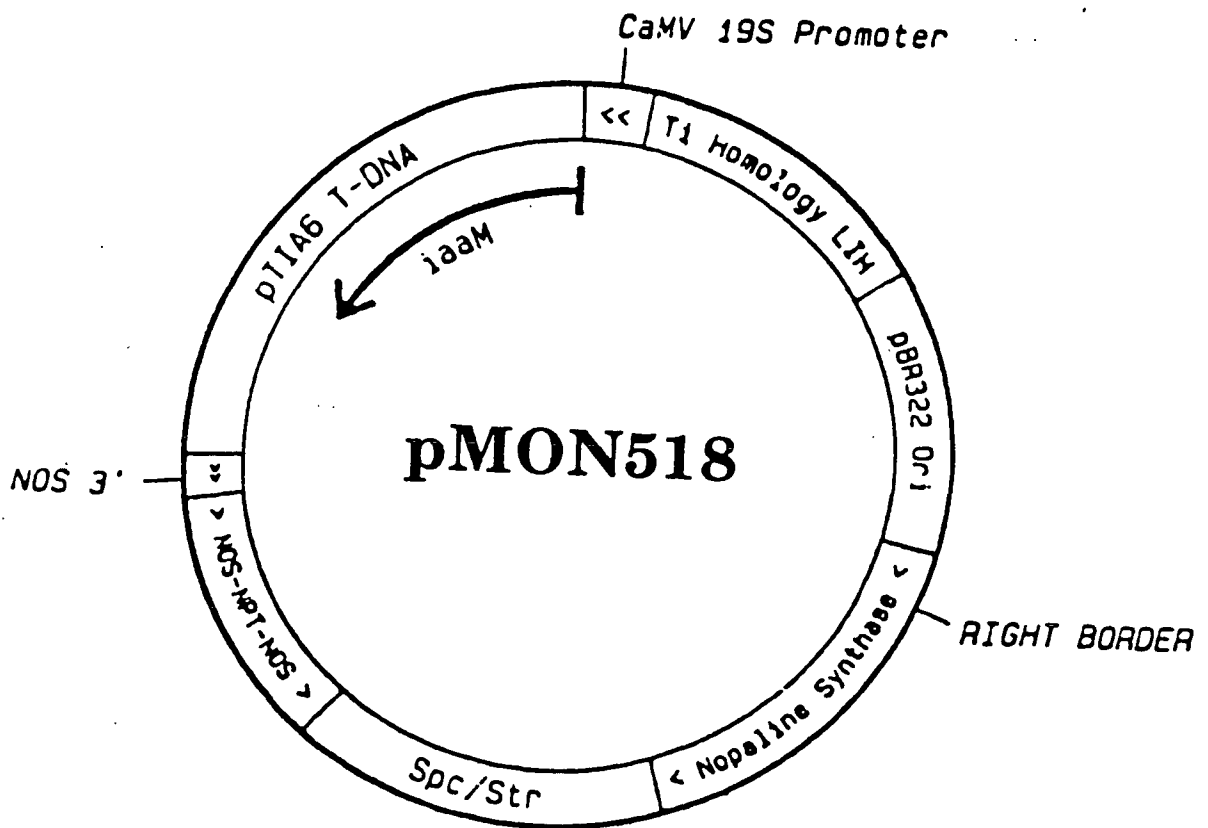


# LETHAL DOSE ASSAY OF CARBENICILLIN



Plot of carbenicillin lethal dose assay.

***Agrobacterium tumefaciens* strain  
pMON518 provided by Monsanto  
has *iaa* M gene -- enhanced auxin  
synthesis**



## **Methods for Auxin Transformation**

***A. tumefaciens* pMON518 and leaf discs will be co-cultivated for 96 hours**

**The leaf disc will then be placed in the dark for 3 weeks on media described previously.**

**Selection of transformants will then be carried out on kanamycin and carbenicillin.**

**Reported transformation frequencies in the literature for *Populus* have varied between 1 and 20%.**

# **Methods for Final Verification of Transformation with pMON518**

**1.) Southern blot**

**2. ) Chemical analysis by HPLC  
for indole-3-acetamide and  
indole-3-acetic acid**

**Future work will involve  
cambium specific promoters.**

**Two sources have been located:**

**1) Monsanto**

**2) Dr. J. Choi at Georgia Tech**

**This will help eliminate previous  
difficulties in plant development  
encountered due to systemic  
auxin synthesis.**

**LEAF SECTION SYSTEM**

**C175 PROTOCOL IMPROVED IN STUDENT PROJECT**

**NEEDS/OPPORTUNITIES:**

**EXTEND TO OTHER CLONES, GENETICALLY  
IMPROVED**

**SHORTEN TIME REQUIRED**

**IMPROVE ELONGATION**

**INCREASE YIELDS**

**PLANS:**

**TEST EXISTING PROTOCOL**

**ADD BEST TREATMENTS FROM**

**CULTURE ESTABLISHMENT EXPERIMENT**

**(COLEMAN & ERNST 1989, 90)**

**(PRAKASH & THIELGES 1989)**

**ALSO, BORROW FROM WORK ON POPULUS  
HYBRIDS**

**(FILLATTI ET AL. 1987)**

**(LEE-STADELMANN ET AL. 1989)**

**(MCCOWN & MCCOWN 1987)**

**EXPLANTS FROM IN VIVO & INVITRO**

**LAUNCH IN MID-APRIL**

SHOOT CULTURES OF *P. deltoides*

Ron Dinus  
Shannon Johnson  
Jim Mathis  
Sonja Ozturk  
Cammie Stephens

## OBJECTIVES

### PRIMARY:

Increase number and availability of cottonwood clones for research.

Improve past IPST protocols.

### SECONDARY:

Document roles and importance of growth regulators.

Examine effects of explant age (position on branch).

## EXPERIMENTAL DESIGN

Clones = 6

Treatments = 6

Replications = 6

## CLONES

CLONES	SEX	ORIGIN/ PROVIDER*	IMPROVED	DESCRIPTION
C175	M	Wabash Co., MN Univ. of Nebraska	No	Experimental model
K417	?	Fulton Co., KY Univ. of Kentucky	No	Experimental model
ST66	M	Issaquena Co., MS James River Corp.	Proven	Best in volume, sp gr, & cellulose content**
ST70	?	Issaquena Co., MS James River Corp.	Proven	20% > average on volumetric basis**
ST72	F	Issaquena Co., MS James River Corp.	Not sure	Selected for rapid growth
ST75	M	Issaquena Co., MS James River Corp.	Proven	20% > average on volumetric basis**

\* All in public domain

\*\* Olson *et al.*, 1985

## SIX TREATMENTS

IPST STANDARD:

DKW + 0.1  $\mu$ M Thidiazuron

DKWT

BASAL CONTROL MEDIUM:

WNA (no growth regulators)

WNA

CALLUS INDUCTION MEDIUM (CIM):

WNA + 0.5 mg/L 2,4-D

CIM 1 day

CIM 4 days

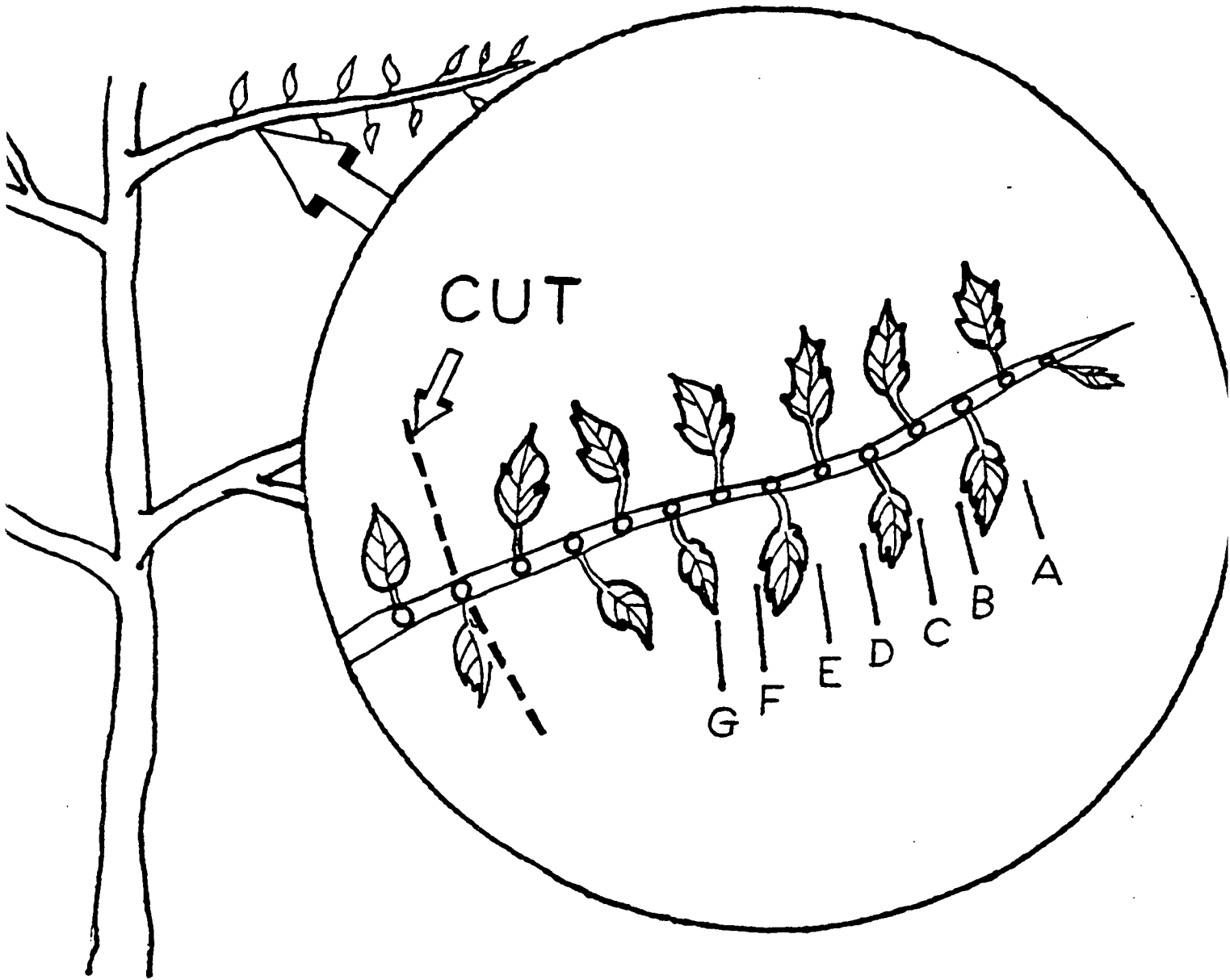
CIM 8 days

SHOOT INDUCTION MEDIUM (SIM):

WNA + 0.5 mg/L Zeatin

CIM 0 days = SIM

# EXPLANT COLLECTION

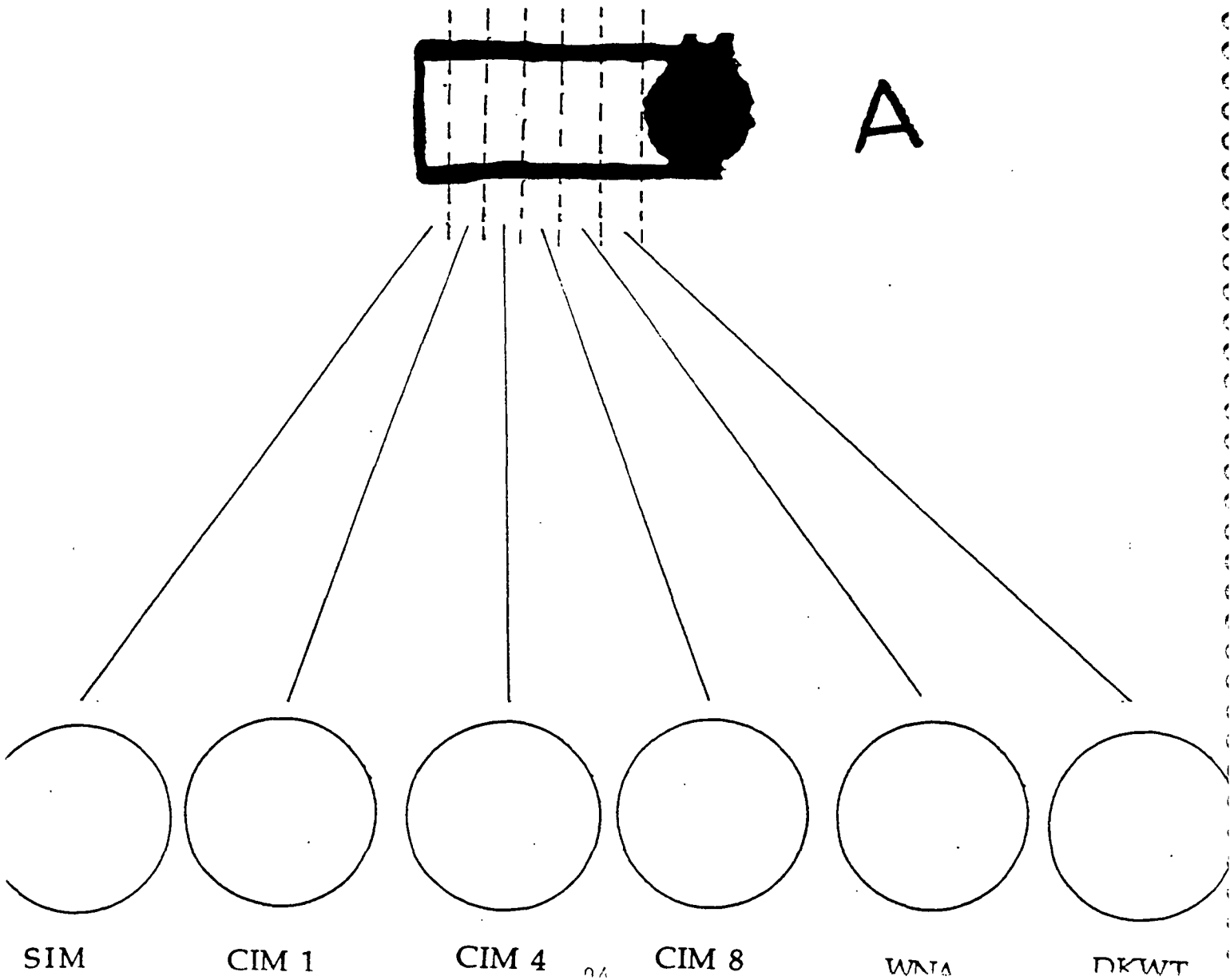
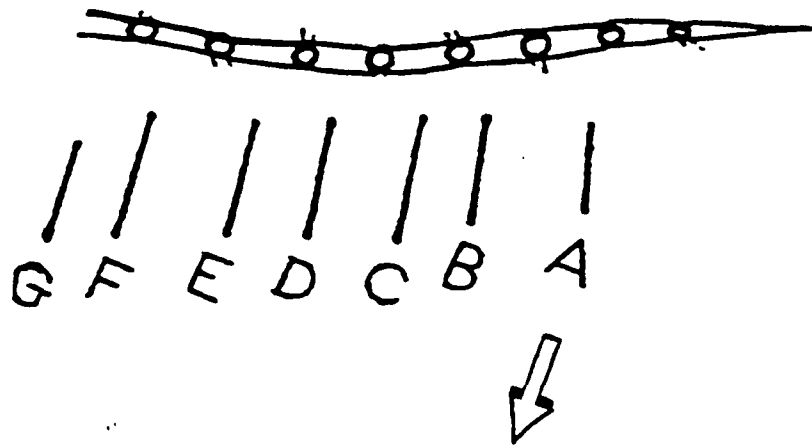


COLONE X

## STERILIZATION

- |                                      |                  |
|--------------------------------------|------------------|
| 1) 1% TWEEN                          | 15 minutes       |
| Sterile Water Rinse (3 X)            | 5 minutes/rinse  |
| <br>                                 |                  |
| 2) 20% BLEACH                        | 20 minutes       |
| Sterile Water Rinse (5 X)            | 5 minutes/ rinse |
| <br>                                 |                  |
| 3) WNA Liquid Medium                 | 24 hours         |
| + 500 $\mu\text{g/ml}$ carbenicillin |                  |
| + 50 $\mu\text{g/ml}$ tetracycline   |                  |
| + 15 $\mu\text{g/ml}$ rifampicin     |                  |
| Sterile Water Rinse (3 X)            | 5 minutes/rinse  |

# EXPLANT ALLOCATION



## PROTOCOL

DAY 0 Explants to all treatments w/carbenicillin  
in dark

DAY 1 Treatment CIMC1 → SIMC

DAY 4 Treatment CIMC4 → SIMC

DAY 8 Treatment CIMC8 → SIMC

DAY 10 All treatments to light

DAY 28 All treatments to fresh media minus  
carbenicillin.

DAY 60 End observations?

All replications were observed and selected explants  
photographed every seven days.

## VARIABLES

Contamination

Mortality

Pith--color, swelling

Lenticels--color, swelling, extrusions

Growth--color, origin, amount, form, condition

## RESULTS I

### Contamination After 28 Days in Culture

Bacterial contamination over entire experiment:  
10.3%

Fungal contamination over entire experiment:  
0.5%

Best Reps: Reps 1, 3 and 4, with 0.0% contamination

Worst Rep: Rep 6, with 24.0% contamination

Best clone: St 66, with 0.0% contamination

Worst clone: ST70, with 41.9% contamination\*

\* 1.6% of this contamination was fungal

## RESULTS II

**% of explants with primordia after 28 days in culture**

	CIMC 0	CIMC 1	CIMC 4	CIMC 8	WNA	DKW
K417	63%	66%	47%	15%	28%	23%
C175	41%	48%	42%	5%	33%	50%
ST66	8%	4%	0%	0%	0%	0%
ST70	4%	10%	0%	6%	0%	0%
ST72	6%	0%	0%	0%	9%	14%
ST75	0%	0%	0%	0%	42%	36%

## **NEXT STEPS:**

Complete observations.

Analyze results.

Compare results of Coleman/Ernst treatments to IPST treatment.

Foster shoot elongation.

Harvest shoots: Root and/or induce stabilized shoot cultures.

**Development of Glyphosate  
Resistant Somaclonal Variants  
of *Populus deltoides* C175**

**Somaclonal variant -**

**a genetic variant arising from cultured  
somatic cells**

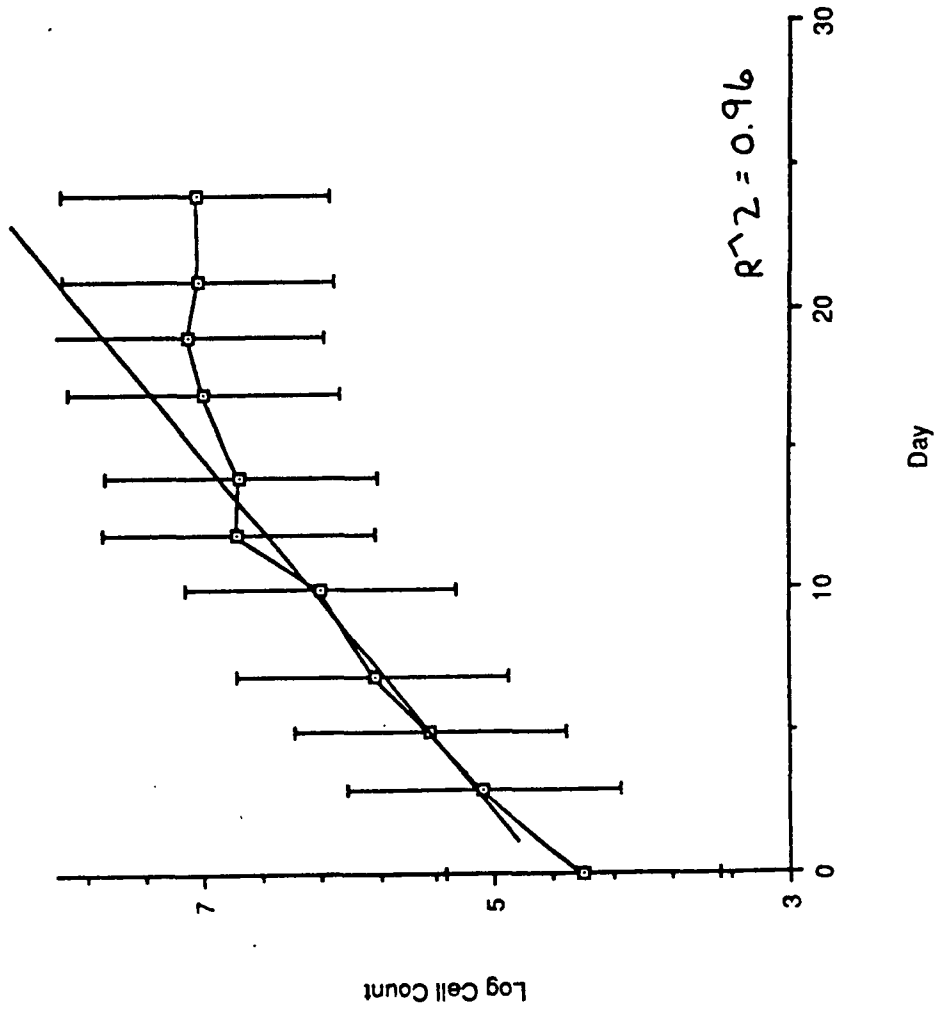
**In order to develop somaclonal  
variants we need some idea as to the  
growth kinetics of our cell cultures**

## **Experimental Approach**

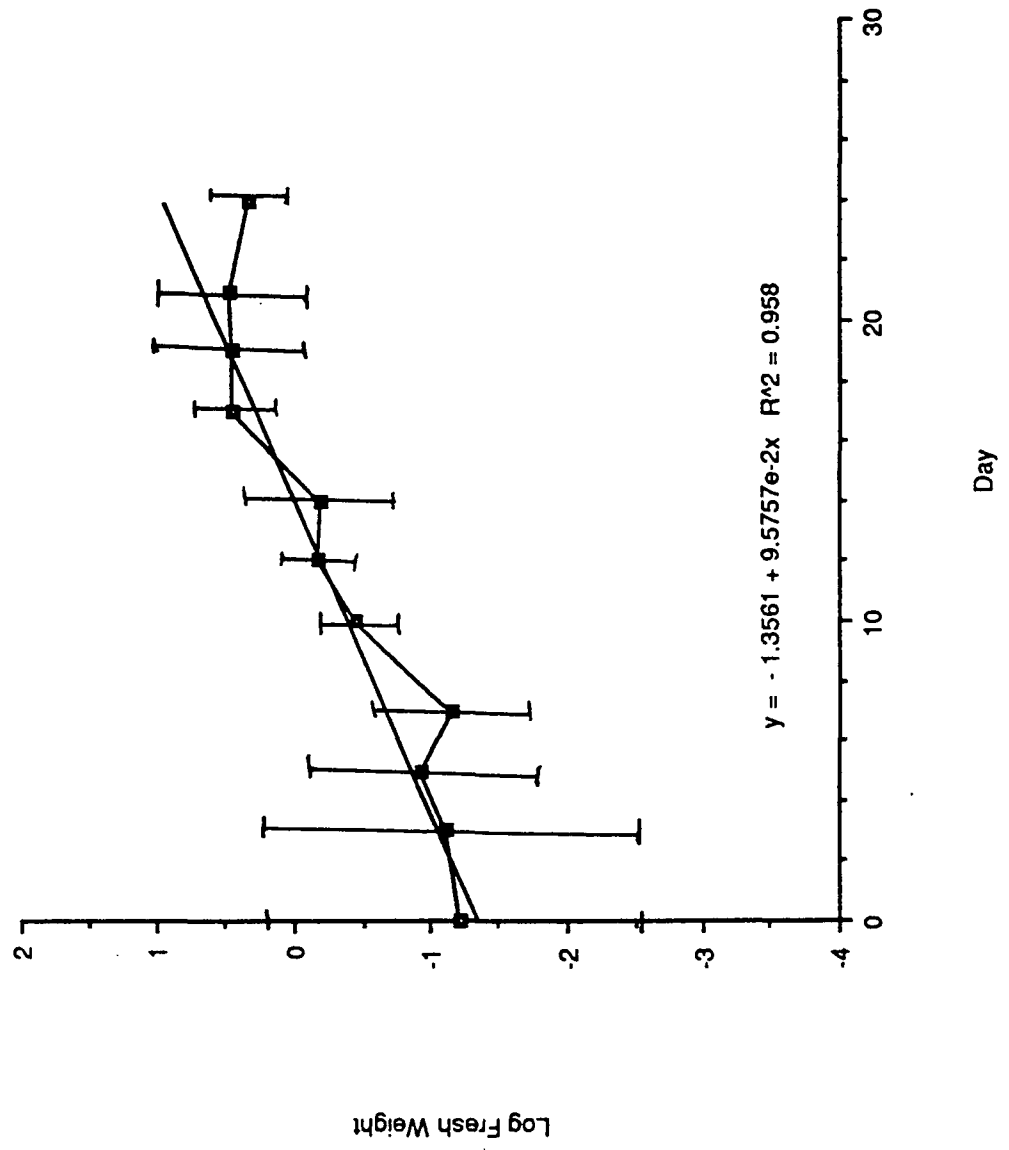
**Measure cell growth in five different ways with five replicate cultures and compare each method**

- 1. Cell Number**
- 2. Fresh Weight**
- 3. Dry Weight**
- 4. Total Protein by Lowry**
- 5. Settled Cell Volume**

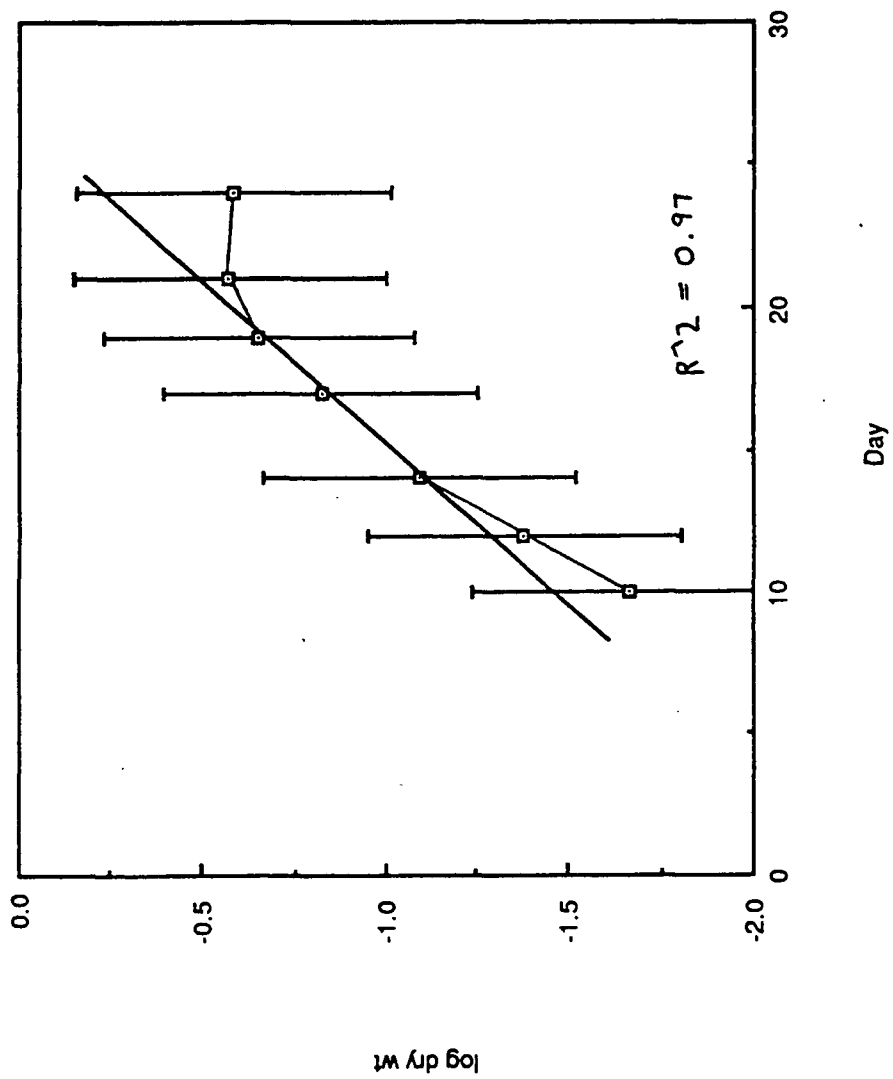
Log Cell Count vs. Time



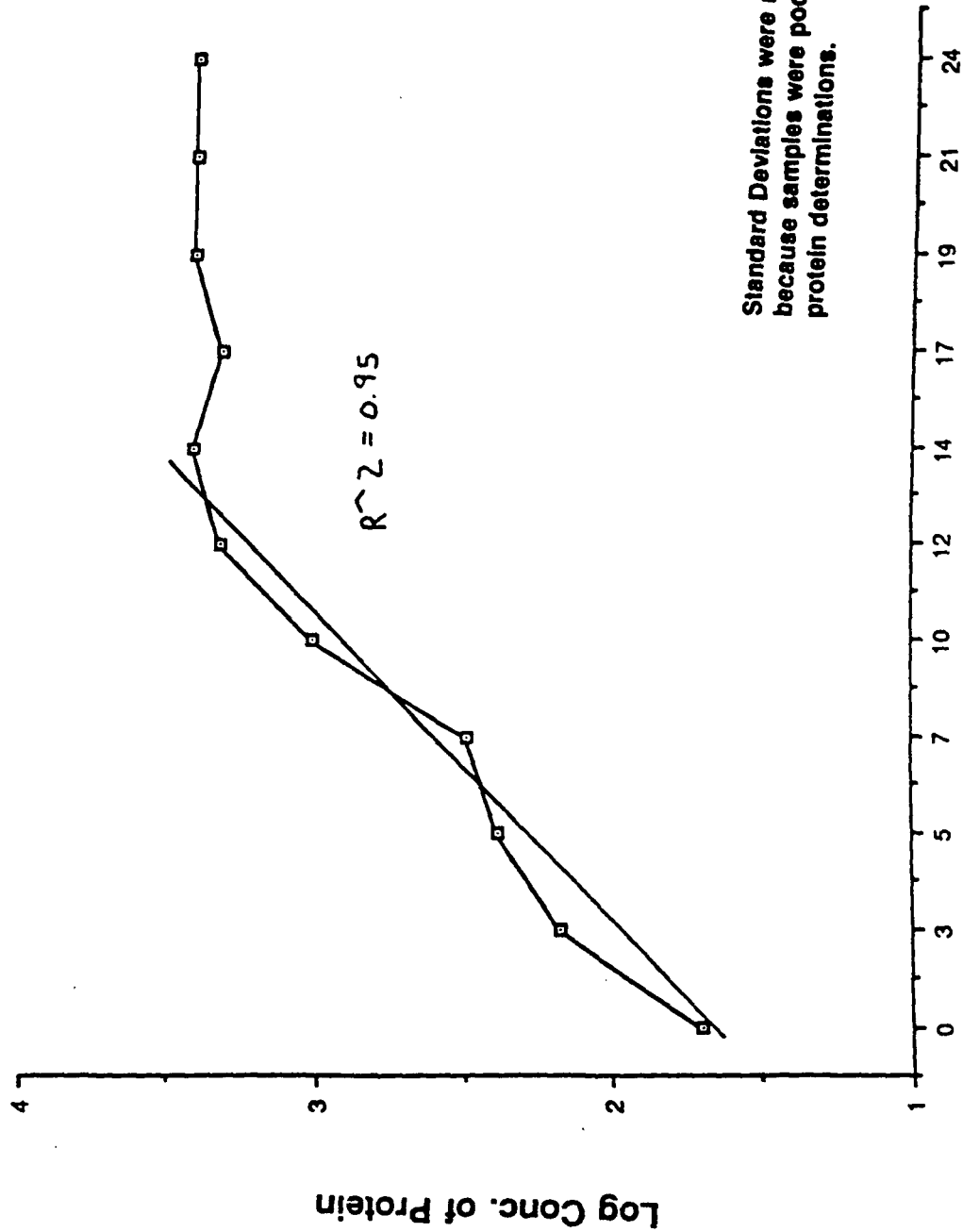
Log Fresh Weight vs. Time



Log Dry Weight vs. Time

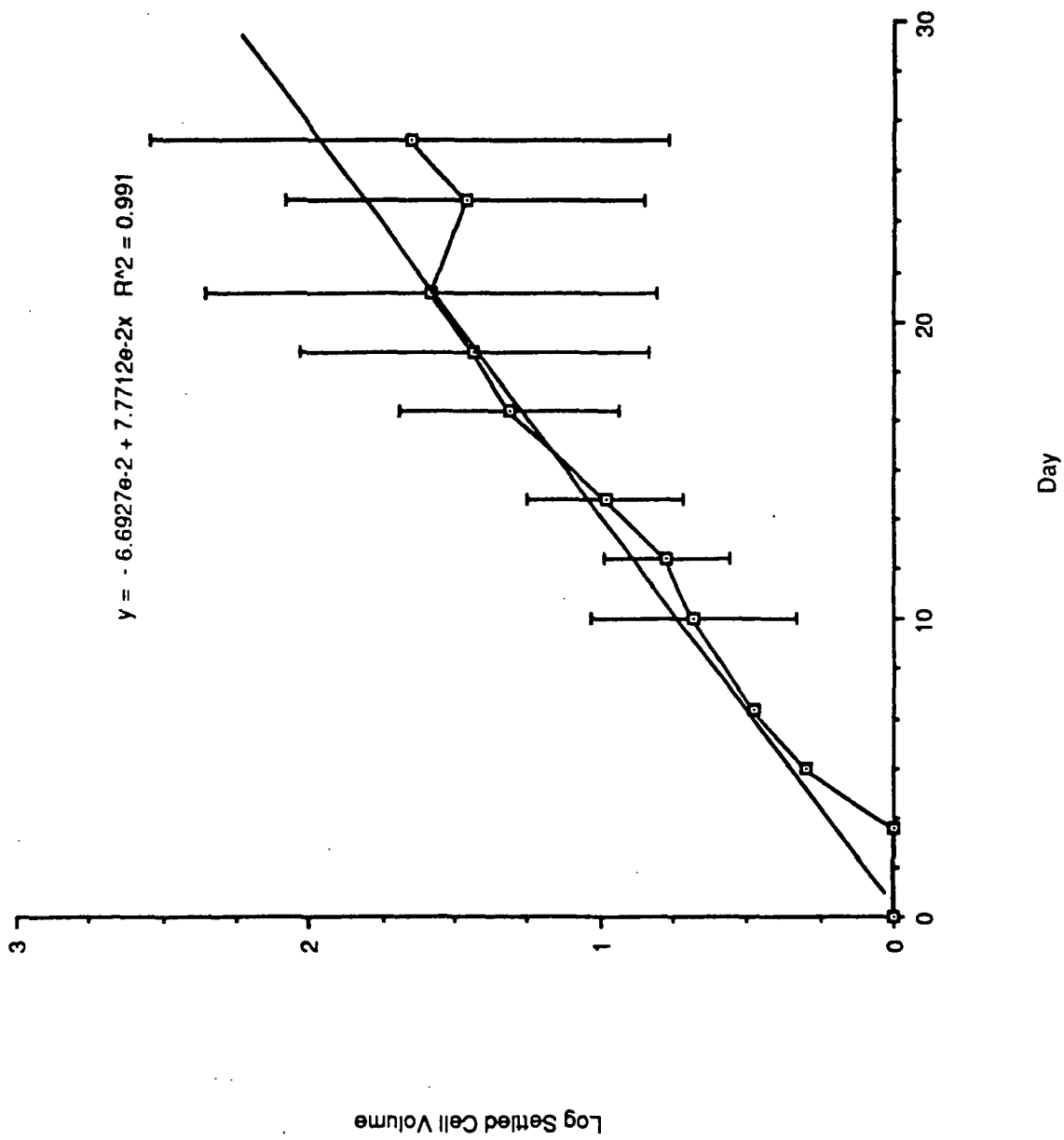


# Log Concentration of Protein vs. Time



Standard Deviations were not calculated because samples were pooled for Lowry protein determinations.

# Log Settled Cell Volume vs. Time



## **Conclusions**

<b>generation time under specific conditions</b>	<b>3 days</b>
<b>exponential growth phase</b>	<b>7-19 days</b>

## Effect of Glyphosate Treatment on Fresh and Dry Weight of Suspension Cultured *Populus deltoides* clone C175 Cells.

$\mu\text{M}$ Glyphosate	Fresh Weight g/150ml Culture	Dry Weight g/150ml Culture
0	40.6	1.4
8	38.9	1.4
16	26.6	1.5
32	23.7	1.6
64	19.4	1.5
128	17.8	1.5
256	9.0	0.9
512	4.0	0.5
initial inoculum	3.0	0.3

## **Conclusions**

- 1) Glyphosate treated cells are elongated and may vary in osmotic tolerance**
- 2) 256 and 512  $\mu\text{M}$  doses of glyphosate result in the death of greater than 90% of the treated cells**

## **Future experiments**

- 1) Rechallenge survivors of 256  $\mu\text{M}$  and 512  $\mu\text{M}$  glyphosate with higher levels of glyphosate**
- 2) Determine  $\text{LD}_{100}$  by raising concentration of glyphosate to 1024, 2048, 4096, and 8192  $\mu\text{M}$**
- 3) Develop glyphosate resistant trees from somaclonal variants**

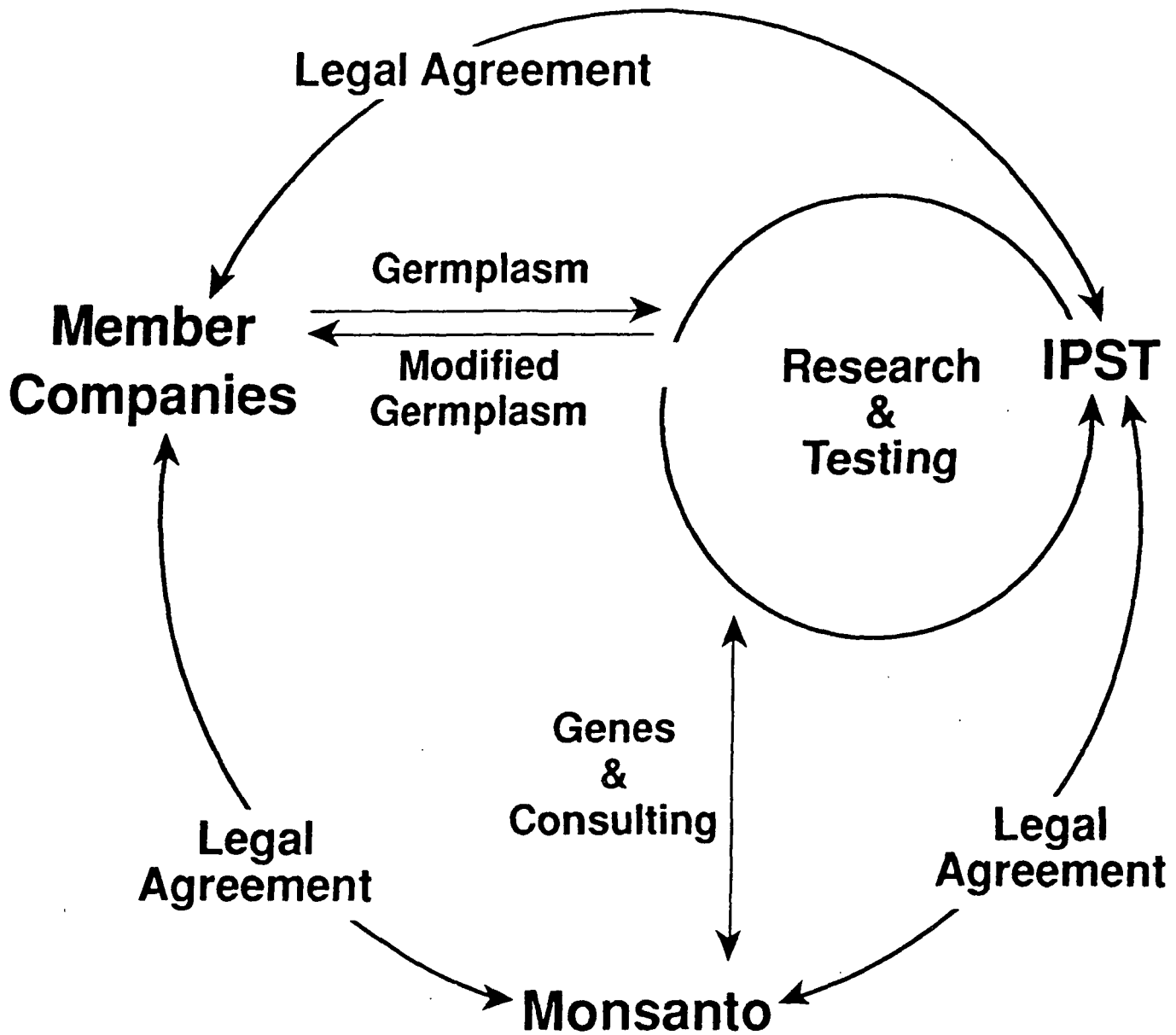
## **Herbicide Tolerance**

**Glyphosate inhibits EPSP-synthase (a key enzyme in the synthesis of aromatic amino acids).**

**Monsanto has developed an *Agrobacterium tumefaciens* vector which will place an altered (by one amino acid) EPSP-synthase in plants.**

**The altered EPSP-synthase will not bind glyphosate there by imparting resistance to glyphosate to its host plant.**

# Herbicide Tolerance Gene: Status & Process



## **MONSANTO INTERACTIONS**

### **\* PAST & CURRENT - STUDENT PROJECT**

**CONSTRUCT FOR ENHANCED AUXIN SYNTHESIS**

**STAFF WILL CONTINUE**

**PROSPECTS FOR XYLEM-SPECIFIC PROMOTER**

## **MONSANTO INTERACTIONS**

### **\* CURRENT & FUTURE - GLYPHOSATE TOLERANCE**

**SEEKING PARTNERS IN "SMALLER" MARKETS**

**WILLING TO PROVIDE "NEW" CONSTRUCT**

**BUT, NEED FURTHER ASSURANCES & INFORMATION**

### **\* ISSUES/NEEDS**

**MEMBERS WAIVE ALL RIGHTS TO MONSANTO MATERIALS,  
TECHNOLOGY, ETC. IN IPST DUES-FUNDED PROJECT**

**MEMBERS DEVELOP INDIVIDUAL COMMERCIAL LICENSING  
AGREEMENTS WITH MONSANTO/IPST WHEN READY**

**COMMENTARY ON CONTINUED USE OF HERBICIDES,  
WILL PUBLIC OUTCRY VOID PROJECTED MARKET?**

**NATIONAL MARKET DATA, NEED FURTHER INFORMATION  
ACREAGE PLANTED ANNUALLY TO CW + TRENDS  
SAME FOR ALL HARDWOODS**

### **TESTING/RELEASE**

**LAND FOR & ASSISTANCE WITH ACTUAL TRIALS**

**DATA ON AGE TO & ABUNDANCE OF FLOWERING IN  
PLANTATIONS THROUGH ROTATION AGE**

**WORK WITH MALE CLONES, POSSIBILITY OF  
CONSTRUCT FOR MALE STERILITY**

**WILL MEMBERS HELP ADDRESS THESE ISSUES/NEEDS?**

**RELATED STUDENT RESEARCH**

## STUDENT RESEARCH - COMPREHENSIVE LIST

### Completed in 1990

Michael Wood - M.Sc., Effect of cold shocking on cell cultures of Larix decidua. Advisor, Dinus.

### In Progress

Teri Ard - Special Student, Dinus. A379, Special Topics Course, Literature review and report on Biomonitoring of pulp and paper mill effluents for chronic toxicity.

David Barzyk - M.Sc., Development of a fiber optics system to determine the in vivo pH of developing Pinus taeda seeds. Advisor, Dinus.

James Bond - Ph.D., A raman microspectroscopic investigation of the patterns of molecular order in secondary walls of southern pine tracheids. Committee participation, Dinus.

James Bradburne - Ph.D. (GT), Molecular characterization of ineffective Bradyrhizobium japonicum USDA 110 variants and differences in signal transduction pathways between effective and ineffective Bradyrhizobium japonicum USDA 110 variants. Advisor, Mathis.

Rebecca Champion - Ph.D. (GT), Strain X cultivar interactions; Effects of nitrogen-fixing and non-nitrogen-fixing Bradyrhizobium japonicum USDA 110 on nodulation and nitrogen fixation. Advisor, Mathis.

Lois Forde - M.Sc., Phenylalanine ammonia lyase and lignin biosynthesis. Advisors, Connors and Dinus.

Robert Golden - M. Sc. (GT), Development of a rapid clinical diagnostic procedure for the most common cystic fibrosis gene. Advisor, Mathis.

Rene Kapik - Ph.D., Completing A390 problems; Likely dissertation topic, Growth regulator in developing zygotic embryos. Advisor, Dinus.

Jim Kramer - M.Sc., Pulping and papermaking properties of Florida-grown Eucalyptus amplifolia. Advisors, Dinus and McDonough.

Tom Ptacek - M.Sc., Variability of wood, fiber, and pulping properties as affected by cloning. Advisor, Dinus.

Peasely Shorter -

M.Sc., Promotion of additional auxin synthesis in Populus deltoides via transformation with Agrobacterium tumefaciens.  
Advisor, Webb.

Colleen Walker -

Ph.D., Development of a biomimetic approach for pulp bleaching.  
Advisor, Dinus.

Michael Wood -

Ph.D., Completing A390 problems; Likely dissertation topic, Genetic changes associated with forest management practices.  
Advisor, Dinus.

**COOPERATIVE INTERACTIONS**

### COOPERATIVE INTERACTIONS

North Carolina State University - Continuing exchange of information on design and analysis of experiments on somatic embryogenesis: initiation, maintenance, and maturation.

University of Florida, Leesburg - Joint research with Dr. D. Gray on desiccation as a method of preparing Norway spruce somatic embryos for storage and germination.

Joint research on related topics is in progress or being negotiated with Drs. J. Cutting, University of Stellenbosch and A. Bayley, SAPPI, RSA; Drs. K. Eriksson and J. Dean, University of Georgia; and Dr. D. Neale, USFS, Berkeley, CA.

Additionally, plant materials and protocols have been secured from and/or shared with Dr. J. Choi, Ga. Tech; Dr. G. Coleman, Oregon State University; Dr. S. Ernst, University of Nebraska, Lincoln; Dr. H. Klee, Monsanto Corp., St. Louis, MO; Dr. C. Michler, USFS, Rhinelander, WI; Dr. C. Prakash, Tuskegee University; and Drs. R. Sederof and R. Whetten, North Carolina State University.

## **PROJECT GOALS**

## **PROJECT (KEY) GOALS**

**PROJECT 3223-00: Mass Clonal Propagation of Improved Softwoods**

**FY 90-91 Goals:**

- 1) Raise frequencies for initiation of embryogenic cultures in loblolly pine and Douglas-fir; obtain additional cultures for accelerated work on embryo maturation.**
- 2) Increase frequencies of embryo maturation and seedling conversion in Norway spruce model system; extend best treatments to Douglas-fir and loblolly pine.**
- 3) Establish guideposts for manipulating somatic materials via documenting course of zygotic embryo development, maturation, and germination as well as early growth and development of zygotic seedlings.**
- 4) Explore initiation of embryogenic cultures from explants of more mature plant materials.**

**FY 91-92 Goals:**

- 1) Establish additional loblolly pine and Douglas-fir cultures for accelerated research on embryo maturation.**
- 2) Increase embryo maturation frequencies in loblolly pine and Douglas-fir; secure first seedlings in loblolly pine.**
- 3) Improve frequencies for initiation of embryogenic cultures in loblolly pine to 10 percent.**
- 4) Explore initiation from explants of more mature explants.**

## **PROJECT 3223-02: Biochemistry of Clonal Propagation**

### **FY 90-91 Goals:**

- 1) Complete restaffing and equipping of laboratory.**
- 2) Renew work on biochemical similarities/differences of developing somatic and zygotic embryos, with emphasis on storage proteins and lipids.**
- 3) Develop techniques for obtaining herbicide tolerance in selected hardwood species via genetic transformation and/or somaclonal variation/selection.**
- 4) Adapt molecular techniques for verifying genetic fidelity and gene expression.**

### **FY 91-92 Goals:**

- 1) Develop techniques for:**
  - a) Monitoring and manipulating gene expression during somatic embryo maturation.**
  - b) Testing genetic fidelity of cloned trees.**
  - c) Effecting and verifying genetic transformation.**
  - d) Generating, selecting, and testing useful somaclonal variants.**

**PROJECT 3223-03:            Mass Clonal Propagation of Genetically Improved and Engineered Hardwoods**

**FY 90-91 Goals:**

- 1) Complete construction and equipping of greenhouse.
- 2) Secure additional plant materials and establish "clean" greenhouse populations.
- 3) Expand existing cultures, and initiate or obtain and stabilize additional ones.
- 4) Refine technologies for mass propagation; ensure suitability for genetic transformation and/or somaclonal variation/selection.
- 5) Accelerate research on gene transfer and expression.

**FY 91-92 Goals:**

- 1) Extend methods for mass clonal propagation to a wider array of trees; emphasize genetically improved materials.
- 2) Adapt said methods for production of herbicide tolerant trees via genetic transformation and/or somaclonal variation/selection.
- 3) Complete first work on production of herbicide tolerant trees.

## **COMMITTEE OPERATIONS**

## **COMMITTEE OPERATIONS**

- \* TERMS OF OFFICE**
- \* OFFICERS**
- \* ALTERNATES/SUBSTITUTES**
- \* EXTERNAL SPECIALISTS**
- \* MINUTES**
- \* FUTURE MEETINGS**

**TERMS OF OFFICE**

<b>MEMBER CATEGORY</b>	<b>MEMBER (COMPANY)</b>	<b>TERM (YRS)</b>	<b>APPOINTED ----- YR -----</b>	<b>EXPIRES</b>
R & D, TECH.	DAVE CANAVERA (WESTVACO)	3, 3	1990	1993, 96
	BOB LAZAR (UNION CAMP)	1, 3	1990	1991, 94
	GERRY PULLMAN	2, 3	1990	1992, 95
APPLICATIONS	STEVE COLEMAN (BOISE CASCADE)	3, 3	1990	1993, 96
	BRIAN STANTON (JAMES RIVER)	3, 3	1990	1993, 96
	JIM RYDELIUS	1, 3	1990	1991, 94
POLICY	WALT JARCK (GEORGIA PAC)	2, 3	1990	1992, 95
	GREG LEACH (CHAMPION)	2, 3	1990	1992, 95
	SHARON MILLER	1, 3	1990	1991, 94

## **OFFICERS**

**CHAIR,**

**BRIAN STANTON - 1990 - 92**

**VICE CHAIR,**

**DAVE CANAVERA - 1990 - 92**

## **ALTERNATES/SUBSTITUTES**

**REMINDER: APPOINT, IN WRITING ALTERNATE OR SUBSTITUTE**

**ENSURE CONSTANT REPRESENTATION!**

**WE VALUE INPUT**

**MUST HAVE QUORUM (51%)**

**REMEMBER: ALTERNATE = VOTING RIGHTS**

**SUBSTITUTE = INFO ONLY**

**ALTERNATES NAMED BY: CHESAPEAKE**

**JAMES RIVER**

**WESTVACO**

**NEED TO KNOW INTENTIONS OF OTHER MEMBERS**

**EXTERNAL SPECIALISTS**

**CONCUR WITH COMMITTEE RECOMMENDATIONS**

**DESIRE AT LEAST TWO SPECIALISTS**

**1) SOMATIC EMBRYOGENESIS**

**STRESS EXPERTISE NOT CROP**

**2) MOLECULAR GENETICIST**

**STRESS FUNDAMENTALS NOT  
TRANSFORMATION**

**3) POSSIBLE THIRD**

**CONIFER PHYSIOLOGIST - MATURATION**

**ROTATE TO MEET CHANGING NEEDS**

**WILL SEEK CONFIDENTIALITY AGREEMENTS AS NEEDED**

**PROTECT INTELLECTUAL PROPERTY RIGHTS**

**DIMINISH RISK OF HEIGHTENED COMPETITION**

**EXTERNAL SPECIALISTS (continued)**

**RAC DEBATE ON GOALS DELAYED ACTION**

**WILL COMPLETE BEFORE NEXT MEETING**

**STAFF DISCUSSION**

**MEMBER CONSULTATION**

**YESKE APPROVAL**

## **MINUTES/REPORTS**

**MINUTES = REPORT OF MEETING**

- 1) MEMBER INPUT TO CHAIR (2 WEEKS)**
- 2) PAC CHAIR & SPECIAL ASSISTANTS**
- 3) IPST LIAISON**
- 4) VP-RAA**
- 5) LEGAL COUNSEL**
- 6) IPST LIAISON**
- 7) CIRCULATION TO MEMBERS**
- 8) REVIEW & APPROVAL AT NEXT MEETING**

**FORMAL REPLY NOT NEEDED; BUT SPECIAL ISSUES ADDRESSED**

**PROJECT SUMMARY FORMS DISTRIBUTED IN ADVANCE**

**HANDOUTS AVAILABLE AT MEETINGS**

**NEXT MEETING**

**IPST RECOMMENDS OCTOBER, BUT?**

**WHEN & WHERE, OUR DECISION**

**OTHERS - NEEDED, WHEN & WHERE**