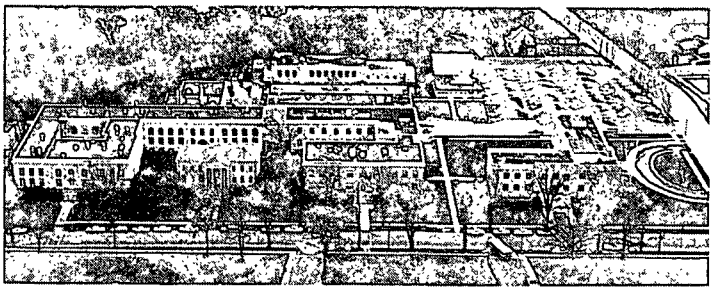

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

Status Report

to the

PROJECT ADVISORY COMMITTEE
ON
FOREST GENETICS

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

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

October 4-5, 1984
The Institute of Paper Chemistry
Appleton, Wisconsin 54912

THE INSTITUTE OF PAPER CHEMISTRY
Post Office Box 1039
Appleton, Wisconsin 54912
Phone: 414/734-9251
Telex: 469289

September 12, 1984

TO: Members of the Forest Genetics PAC

Enclosed is advanced reading material for the October 4-5 meeting of the Forest Genetics Project Advisory Committee (PAC). Included is the status report for Project 3223, a tentative agenda, a current membership list, and a description of research strategies for embryogenesis.

The meeting will be held in the Calder Student Center (just South of the Continuing Education Center) and meals will be provided as stated on the Agenda. Please be reminded that because of a conflict with a continuing education course, we will not be able to provide rooms for the committee on October 3 or 4. Please make your own reservations. The Institute has blocks of rooms reserved in the Institute's name in both the Midway Motor Lodge (414-731-4141) and the Amora Villa Motor Lodge (414-735-2733). Please indicate when you make your reservations that you would like to use the rooms reserved in the Institute's name. Recent progress and research plans for the coming year will be the topics featured the first day. The morning of the second day will be reserved primarily for committee deliberations. Please call Becky Dietzen (414-738-3448) if you have problems with your reservations.

We look forward to meeting with you on October 4-5.

Sincerely,

Dean

Dean W. Einspahr
Director
Forest Biology Section

DWE/bd
Enclosure

TABLE OF CONTENTS

AGENDA

PROGRAM ADVISORY COMMITTEE
FOREST GENETICS

October 4-5, 1984

The Institute of Paper Chemistry
Calder Student Center
Appleton, Wisconsin

Thursday, October 4, 1984
Calder Center, Seminar Room

8:00 a.m.	Introduction and Overview	Einspahr
	I. Review of Previous PAC Recommendations	Einspahr
	II. Recent Publications	Einspahr
	III. Committee Business	Einspahr and Lazar
9:45	Coffee Break	
10:00	Introduction and Overview of Open Meeting	Einspahr
10:10	Project 3223 - Mass Production of Conifer Hybrids	
	I. Project Objectives and Subobjectives	Einspahr
	II. Model Systems Research	
10:20	Biochemical Perspectives of Recent Results, Directions and Needs	Johnson
11:00	ATP and Energy Charge	Johnson
11:30	Ascorbic Acid	Johnson
12:00	Lunch (CEC Dining Room)	

1:00	Polyamines and Embryogenesis	Feirer
	III. Objective I Research - "Generating and Maintaining Quality Cell Suspensions	
1:30	Progress in Obtaining New Cell Lines, and Emphasis on Auxins	Litvay
2:00	Phenolics as a Measure of Cell Line Quality	Noland
2:15	Investigations of the Fate of Labeled Methionine in Pine and Wild Carrot Cultures	Phythyon
	IV. Objective II Research - "Somatic Embryogenesis"	
2:30	Current Status and Plans for Addback Investigations with Extracts of Immature Loblolly Pine Seeds	Johnson
	V. Prospectus for Expanding Research	
3:00	Prospectus	Posner
	Strategies and Models for Embryogenesis	Litvay
5:00	Cocktails and Dinner (CEC Dining Room)	
6:30	VI. Additional Discussion of Prospectus	Litvay
7:30	VII. Summary of Recent Progress	Einspahr
8:00	VIII. Research Plans	

Friday, October 5, 1984
Calder Center Seminar Room

8:00	I.	PAC Deliberations	Lazar
11:30	II.	Adjourn	
11:30	III.	Lunch (CEC Dining Room)	

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THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

Status Report

to the

FOREST GENETICS

PROJECT ADVISORY COMMITTEE

Project 3223

THE MASS PRODUCTION OF CONIFER HYBRIDS

October 4-5, 1984

PROJECT TITLE: The Mass Production of Conifer Hybrids

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

PRIMARY AREA OF INDUSTRY NEED: Raw Materials

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

Date:	6-1-83
Budget:	$420,000
Period Ends:	6-30-84
Project No.:	3223
Approved by VP-R:	

PROGRAM GOAL: Mass production of conifer hybrids

PROJECT OBJECTIVE/GOAL:

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

PROJECT RATIONALE:

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

RESULTS TO DATE:

Appropriate new media have been developed for initiating callus production and for growing cells in suspension. Procedures for establishing appropriate cell lines have been developed. Biochemical and morphological characterization of embryogenesis is under way. Model systems on wild carrot somatic embryogenesis and natural Douglas-fir and loblolly pine embryo development have assisted in establishing media change requirements and in developing needed biochemical markers. Excised conifer embryo investigations have been used to determine the nutrient requirements of developing embryos. Inhibitor studies have demonstrated the importance of several polyamines in embryogenesis and determined that polyamine synthesis was controlled mainly through the arginine-agmatine pathway. Alternative procedures for modifying free amino acid and polyamine levels have been determined.

PLANNED ACTIVITY FOR THE PERIOD:

Research is planned in the areas of model systems investigations, generating and maintaining competent cell lines and embryogenesis. More specifically under the model system we plan to: (1) determine the influence of gibberellins on wild carrot somatic embryogenesis, (2) determine the changes in growth regulator levels during natural conifer and wild carrot somatic embryogenesis, and (3) determine the critical factors important in the conversion of arginine to polyamines. Research on generating and maintaining competent cell lines is expected to include: (1) generation of new cell lines from immature embryos, protoplasts and microsporophyll tissue, (2) determining the influence of

nitrogen sources, polyamines, gibberellins and cytokinins on conifer cell line quality, (3) determining the influence of natural conifer extracts on conifer cell line quality. Conifer somatic embryogenesis studies are expected to include: (1) establishing monitored launch experiments* that have as objectives the correction of apparent deficiencies and the production of inhibitors, (2) conducting occasional unmonitored launch experiments incorporating a combination of promising factors, (3) running monitored launch experiments using promising new cell lines, and (4) studies with the objective of determining the influence of natural extracts on conifer embryogenesis.

POTENTIAL FUTURE ACTIVITIES:

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

SHORT TERM GOALS:

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

1. Complete the biochemical characterization (free amino acids, polyamines, and proanthocyanidin) of:

   (a) Wild carrot somatic embryogenesis model system, and

   (b) The conifer natural embryogenesis model system.

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

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

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

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

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

The above short term goals 1 through 4 are expected to be completed during the coming year with the current level of funding. A major increase in funding will mean an increase in the rate of progress in achieving these goals. In addition, increased funding would allow us to begin much needed growth regulator research. Growth regulator levels appear to be critical during the early stages of embryogenesis. Short term goals 5 and 6 provide additional details on the first phases of a planned growth regulator research program.

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

PROJECT TITLE: The Mass Production of Conifer Hybrids

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

PRIMARY AREA OF INDUSTRY NEED: Raw Materials

PROGRAM AREA: Increased wood production by embryogenesis and bioengineering

Date:	6-1-84
Budget:	$570,000
Period Ends:	6-30-85
Project No.:	3223
Approved by VP-R:	

PROGRAM GOAL: To increase significantly the productivity of our forests by the propagation of superior trees.

PROJECT OBJECTIVE/GOAL:

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

PROJECT RATIONALE:

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

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CONIFER TISSUE CULTURE PROGRAM

Recent Progress (March 1, 1984 - August 31, 1984)

During the five months since our last meeting several very interesting developments have occurred. These developments along with a certain amount of routine progress are summarized in the comments that follow.

1. Studies with wild carrot cell suspension confirmed earlier results which demonstrated that the use of extracts from immature loblolly pine seeds improved embryogenesis.

2. Preliminary studies with loblolly pine indicate polyamine markers were improved when extracts from immature seeds were added to the medium.

3. A new series of loblolly pine cone collections were made, and extracts were prepared from developing seeds. This new "lot" of extract has been tested and found as active as last years extract, allowing continued research using this approach.

4. Polyamine inhibitor studies again demonstrated the importance of polyamines in morphogenesis. The most recent results indicate that spermidine may be the polyamine most involved in plant development.

5. Research into energy charge levels of the wild carrot system and loblolly pine cell lines revealed that pine cells in culture should have no problem with the availability of ATP to drive biosynthesis.

6. Ascorbic acid investigations to date indicate that wild carrot model cells in culture may have more control of their internal redox status than do pine cells, particularly old cell lines.

7. Investigations on screening synthetic auxins in our search for an alternative for 2,4-D have shown that only one or two compounds have promise. Followup research will involve checking our markers on cell lines with the most promising growth regulators.

8. New loblolly pine cell lines have been initiated from embryos collected at various stages of maturity. After a modest cell line buildup period, many of these lines will be used in a series of "launch" experiments.

9. Additional red pine cone collections were made during the 1984 growing season in our continuing evaluation of biocemical markers associated with natural pine embryogenesis. These collections are providing information on polyamines, proteins, ascorbic acid, energy and glutathione. This new data tends to confirm data collected in previous years.

10. An updated research plan for expanding the program and accelerating progress has been developed and the first steps aimed at implementing the plan have been taken.

STATUS OF RECENT PUBLICATIONS

1. Monroe, Steven H., and Morris A. Johnson.
A membrane-bound-o-methyltransferase from Douglas-fir needle callus and its role in the nature of endogenous phenolics. Phytochemistry 23(8):1541-43. 1984.

2. Feirer, R., G. Mignon, and J. Litvay.
Arginine decarboxylase and polyamines required for embryogenesis in wild carrot. Science 233:1433-35, 1984.

3. Feirer, R., G. Mignon and S. Wann.
Effect of spermidine synthesis inhibitors on in vitro plant development. Plant Physiol. 75:103 (1984) Supplement (Poster).

4. Feirer, R., S. Wann and D. Einspahr.
Effect of spermidine synthesis inhibitors on plant development. Submitted to Plant Growth Regulation.

5. Einspahr, D. W.
Tissue culture in forestry, current status. Proceedings, Second Regional Technical Conference, Soc. of Am. Foresters, Charlotte, NC. Jan. 26-27, 1984. p. 8-16.

6. Einspahr, D. W., J. D. Litvay, M. A. Johnson, and R. P. Feirer
Challenges of somatic embryogenesis in conifer tissue culture. Proceedings, International Symposium of Recent Advances in Forest Biology. Traverse City, Michigan, June 10-13, 1984. (In press).

7. Litvay, John D.
The Institute of Paper Chemistry approach to propagation of forest trees using somatic embryogenesis. Proceedings, TAPPI R&D Division Conference, October 1-3, 1984. (In press).

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

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

10. Litvay, John D., Morris A. Johnson, Devi C. Verma, and Dean W. Einspahr
A new tissue culture medium based on the analysis of conifer ovules. (Being revised for Plant Cell Reports).

11. Wann, Steven R. and D. W. Einspahr
Reliable plant formation from seedling explants of Populus tremuloides. (Submitted to Canadian Journal of Forest Research).

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

Status Report

to the

FOREST GENETICS

PROJECT ADVISORY COMMITTEE

RESEARCH STRATEGIES

The following is part of our internal discussion of research strategies, including the use of model systems. We will send you more material later.

October 4-5, 1984

STRATEGIES

For the past several years we have defined and worked on two

Objectives:

1) Production of competent cell lines
2) Inducing embryogenesis

The basic philosophical problem with these definitions is the evaluation of competency. We cannot be certain that any cell line is competent until it does, in fact, produce embryos. The definitions have been, and are, helpful in delimiting the scope of the work, i.e. to state that, at this time, we are not concerned about plantlet transfer, fidelity testing, genetically engineered improvements, etc. But, accepting these limitations, and the fact that we can now produce cell lines, which can be subcultured many times and continue to appear healthy, the distinction between the two objectives is not useful. *The real objective is to induce embryogenesis.*

There is still a need to define distinct and useful intermediary goals and objectives. A crude but seemingly workable split of objectives is based on the two hypotheses of totipotency:

1) **Very few**, specialized cells, possibly only from certain types of explants, have the potential to form an exact copy of the parent
2) **Most** cells have the potential to form an exact copy of the parent.

These two hypotheses lead to two different strategies for achieving the real objective, i.e. somatic embryogenesis in conifers:

Strategy number 1, which is consistent with hypothesis number 1, emphasizes *Enhancement of cell lines* by growth regulators etc., *Stress and Selection* in the initiation step, so that only the competent cells can divide and grow.

Strategy number 2, consistent with hypothesis number 2, is to *Search for the Switches*, i.e. those procedures or compounds or combinations, which will enable the cells to express their potency to form embryos in the induction step.

STRATEGY 1 - ENHANCEMENT, STRESS AND SELECTION IN THE INITIATION STEP

In tissue culture systems the four main variables are:

1) cell source
2) light
3) medium
4) growth regulator.

Using the strategy of enhancement of cell lines or selection pressure in the initiation step, two of these four variables, *cell source and light, can be almost fixed.* We may elect to work only with cells from the growing immature embryo in the seed and with mature embryos. Haploid and diploid lines can be obtained as well as cells which are young in all respects. It would be sufficient, in this context, to make experiments under only two different lighting conditions, e.g. complete darkness and 16 hour/day light conditions.

The *method for evaluating success is also nearly fixed.* The cells would be induced using LM medium. The growth regulator, '2,4-D', will be added in 2 or 3 different concentrations and the inoculation density will be set at 2 or 3 different levels. The number of levels of growth regulator concentration and inoculation density used will depend on anticipated chance of success as well as on team capacity.

The two variables that need experimentation are the types of growth regulator and culture medium used in the initiation process. If competent cells are competent because they are meristematic, i.e. can produce their own growth regulators, induce influx of suitable nutrients, divide actively, etc., then experiments can be designed which take advantage of their vitality to put selection pressure on the other cells, just as is done when selecting for disease or herbicide resistance. In this case, of course, selection will be made on the basis of embryogenic characteristics. This should also enable us to learn to recognize embryonic cells, i.e. to establish good markers for such cells. Hence, the main question is:

What are the characteristics of cells that can form embryos ?

Specific examples of experiments relating to this question are:

1) In the coffee system, white colored embryos emerge from brown phenolic masses of cells; is this a form of selection pressure? If so, can high levels of phenolics be used in our system to select for embryonic cells that can tolerate such an environment?

2) Can calcium be used to select for cell lines efficient in their utilization of calcium? Such cell lines should be embryonic.

One base case under strategy no. 1 will be use of variants of the wild carrot medium. Criteria to be used include functionality of the conifer cells and measurable and significant deviations in this respect. Many of the answers concerning mechanisms as well as markers will be obtained by making changes to the medium and characterizing the systems and the results using techniques from biochemistry and molecular biology. Specific questions, which should be answered with the help of biochemistry include:

STRATEGY 2 - THE SEARCH FOR SWITCHES to INDUCE EMBRYOGENESIS

Embryogenesis of gymnosperm cells has not yet been obtained. We have worked with cell lines, many of which originated from explants with proven competency in organogenic systems. The cell lines appear to be healthy, divide vigorously, and can be subcultured many times. We have been using cells from very immature as well as other sources. We are using a culture system, which has been proven successful with wild carrot.

At the root of the embryogenesis problem is probably the requirement that, once the path of embryogenesis is induced, a specific, sequential pattern of cellular events must take place. Specific genes must be turned "off" or "on" (colloquially: "to throw the switches") at specific times during this sequence. Because our gymnosperm cells do not form embryos the correct genes have not been turned "on", or genes that should be "off" are actually "on".

To go into a cell and resequence the coding of the genes is a much tougher problem than merely releasing a cell to "do its thing", which is what is attempted under strategy no.1. A real problem here is the scarcity of data concerning the effects of growth regulators, light, medium, etc., on gene activation and repression. However, there are some techniques available, which might facilitate embryogenesis. Work done with animal systems might point the way; technologies such as cell fusion, transdifferentiation and nuclear transplantation, are successfully used in animal systems. (See for instance Science: June 1, 1984, page 946.) The corresponding techniques in plants include protoplastology, use of extracts from seeds and immature embryos, and microinjection.

Some of the pertinent questions, therefore, deal with causal relationships, notably concerning crucial stimuli. It is also necessary to determine the qualitative nature of at least some of the most crucial events that must take place for embryogenesis to occur, and some factors controlling these events. But we must also deal with techniques. Is the protoplast or the micro-injection technique to be preferred? Which one holds the most promise for genetic manipulation? Lacking expertise in the molecular biology field the Institute has not yet been able to take advantage of these techniques.

Application of New Techniques

The following paragraphs exemplify techniques, at least some of which will have to be developed if full advantage of gymnosperm cell biotechnology is to be realized. More important, however, is the fact that the specific techniques are real, proven alternatives to conventional tissue culture techniques for attaining the goal. Some techniques appear to lend themselves readily to mass propagation schemes while others should be more useful as models or for achieving competency in recalcitrant cells.

Protoplastology

There are two ways to get foreign material into a cell, namely protoplastology and microinjection. Previous work with protoplasts from other plant species shows that recalcitrant cell lines may be made competent if first

reduced to protoplasts. Also, fusions between recalcitrant and competent cells may become competent. Protoplastology has the potential for treating millions of cells simultaneously.

Microinjection

Microinjection has all the potential of protoplastology but not some of the problems. A fine bore needle is used to inject into suspension cells exactly what is required at any specific site. Because whole suspension cells are used, the problem of regrowing the cells back to callus does not exist. Even a small number of successfully micro-injected cells growing into embryos or plants could serve as a very useful model system. Microinjection is very laborious. Each cell has to be treated individually. If the technique is to be used for the purpose of mass propagation then the cells must be put through a proliferative process after injection.

In-vitro Fertilization

Controlled fertilization of plants, similar to what is currently being achieved in animal systems, would allow a number of questions to be answered and would, potentially, allow at least partial attainment of the project goal.

Using this technology single cells could be grown into embryos, but it would probably not be possible to do this en masse. This technique should provide us with a very good model system, and should provide some good markers. In conjunction with microinjection, it should be possible to grow a single engineered cell into an embryo. Some of the critical issues, which this work would relate to are:

1) The conditions required to grow a single cell into an embryo.

2) the critical roles that cytoplasm, nuclear and other substances play in embryo development.

Establishment of Haploid Cultures

If haploid lines could be established, then material would be made available, which, through microinjection, cell fusion or polyploidy, might develop into embryos. One added benefit from these pseudo-fertilization techniques would be that work could be done with tissue from mature, proven sources.

The Need for Additional Information

In addition to new techniques there is also a need to collect pertinent data to form the basis for decisions as the project progresses. There is a need for very basic information in two main areas, namely:

1) the role of growth regulators in gymnosperm cultures
2) the effects of various stimuli on gene expression, specifically on embryonic proteins and RNA.

Plant growth regulators purportedly control not only the amount but also the type of growth (embryonic versus callus), and we need such information in order to control properly the development of individual cells. Without it, application and use of growth regulators will remain rather empirical, implying a relatively small chance of achieving success in a timely fashion. It has become increasingly clear, however, that information in this area, obtained from other plant species such as corn, wild carrot, tobacco, etc., is not always relevant to gymnosperm tissue culture. It is required, therefore, that we obtain basic information on such factors as transport processes, breakdown of metabolic pathways, and effects of key enzyme, nuclear, and alternative regulatory systems.

The ultimate answers to our questions are to be found at the DNA level. The levels of activity and dissemination of information from work on animal systems are high enough to indicate that the time is right to enter these fields, build up our competence, and start to collect information; specifically in the following two areas:

1) Use of the DNA regulation hypothesis of methylation, acetylation and phosphorylation on gene expression and cell differentiation.
2) Searching for key embryonic RNA or protein markers.

The development of specific DNA patterns or the occurence of specific embryonic molecules would not only be the ultimate "marker", but with current technology could allow for some backtracking to determine key stimuli controlling cell suspension development.

Methods of Attack under Strategy 2

Only cell lines showing a lot of promise would be used. Our biochemical and other markers would be used as indicators in this respect. The measure of success under strategy 2 is whether embryos or other well organized structures develop, i.e. a rather straightforward Yes/No response. Hence, the number of experiments can be quite large. Five main lines of attack are envisioned:

1. Induction of embryogenesis by *Stress Mechanisms*:.

   - Light
   - Inoculation density
   - Growth regulator type and concentration
   - Type of medium
   - pH
   - Osmolarity
   - Perturbation chemicals (extracts, inhibitors, etc.)
   - Temperature
   - Physical stress

2. Induction of embryogenesis by changing *environmental conditions* in accordance with biochemical models.

   - type, concentration, time of application of cytokinin and auxin
   - additions of polyamines, extracts, gases

3. Induction of embryogenesis through *protoplastology*:.

- Isolation of protoplasts from cell lines (and explants later)
  - medium type and concentration
  - enzymes
- Regeneration of callus
  - medium type and concentration
  - growth regulator type and concentration
  - osmolarity
- Regeneration of plants
  - inoculum density
  - medium type and concentration
  - growth regulator type and concentration
- Fusion of cell lines
  - methodology
  - cell lines (some may be from explants)
  - regeneration of callus and plants
    - osmolarity
    - medium type and concentration
    - growth regulator type and concentration

4. Alteration of cell types and induction of embryogenesis by *micro-injection.*

Development of techniques
- de-nucleation and re-injection
- regeneration of callus from low inoculation density suspensions
  - cell line, light
  - medium type and concentration
  - growth regulator type and concentration
  - inoculation density

Other sources of cells
- in-vitro ovule culture
- haploid tissue, ovules, pollen

Re-nucleate de-nucleated material and grow into callus
- growth regulator type and concentration
- medium type and concentration
- check for alterations

Induction of re-nucleated callus using wild carrot and other protocols

5. Induction of Embryogoenesis by *in-vitro fertilization* and *embryo rescue* techniques.

The starting materials are unfertilized ovules and pollen

Ovule isolation
- media, storage conditions, time

Pollen germination
- media, growth regulator, time

Fertilization
- media, markers, systems for

Development of embryos fertilized in-vitro
- media, growth regulator type and concentration, temperature, osmolarity
- embryo rescue techniques
- tests with natural ovules and immature embryos

Repeat successful process using micro-injected pollen and ovules as starting materials to produce genetically altered embryos.

STRATEGY 1 VS. STRATEGY 2

The two strategies parallel two main questions in tissue culture, namely a) what cells form embryos? and b) what stimuli cause cells to form embryos? A major task for biochemistry and molecular biology is to answer at least one of these questions, thus allowing us to concentrate on the most relevant area. Until we have at least one answer we must do research in both areas - diluting our effort - or, using some convincing argument, decide to concentrate on only one of the two areas.

For the time being, we have elected to do research in both areas:

1) Initiating new cell lines to obtain cells, whose competency will be exhibited through the use of stimuli similar to those used in the wild carrot system.

2) New techniques for stimulation of cell populations to produce embryos.

We believe that we have to work along both these lines, but with heavy emphasis on molecular biology and biochemistry, until we can see clearly how to pick our way through this maze which is so rich on options. What is quite clear at this point is that increased resources are necessary in order to make rapid and timely progress.

THE MODEL SYSTEMS APPROACH

Biology as a subject of learning has been and, to a large extent still remains, descriptive. Very rapid progress is now being made towards mechanistic understanding of many biological processes. Still, the number of variables is virtually infinite, and progress towards mechanistic understanding does not come easily. Therefore, much research is still of a very empirical nature (colloquially: "Spray & Pray"). An "Analysis & Synthesis" approach would be highly desirable but is rarely possible because of the large number of variables involved and the lack of knowledge of the functions of most compounds.

The next best possibility appears to be "Analysis and Synthesis by Analogy". This involves comparisons, in many respects, between functioning and non-functioning systems in order to discover valid "markers", which are characteristic of the functioning but not of the non-functioning systems. Many scientific reports point to similarities and dissimilarities between different

species of plants, between woody and non-woody plants, between animals and plants, etc. Our knowledge of the conditions and processes leading to growth, proliferation, organized structures, and developed plants is very limited - but not nil. The interesting point is that there are numerous similarities between highly dissimilar organisms. This fact has led The Institute to adopt the "Model Systems" approach, which, in fact, is a version of "Analysis and Synthesis by Analogy". Very substantial progress in the use of this technique has been made over the past several years, but proof of its validity for conifers will have to await repeatable embryogenesis. The main markers being used now are:

1. Occurrence of embryogenesis
2. Development of cell mass as a function of time.
3. Histology (cell size and structure, cell organization)
4. Biochemical markers:
   - Polyamines
   - Phenolics
   - Free amino acids
   - Lipids
   - ATP (Adenosine triphosphate, energy carrier in cells)

It is hoped that, as the program progresses, the number of valid markers will continue to grow, and the complexity and time consumed in using them will diminish. It will then become increasingly feasible to speed up the rate of progress by early termination of unsuccessful experiments and improved focusing on successful approaches.

Several of these markers have been well described in the literature for many species, animal as well plant species, and hence, seem to be quite generally valid. We are also using, current, living models, notably wild carrot and natural embryos. Other plants, e.g. coffee and aspen, are being studied for evaluation of their potential.

Continued improvements to our Model Systems approach, both with respect to markers, models, and techniques are parts of the proposed strategy for attaining somatic embryogenesis in conifers.

Diagram 1

OVERVIEW OF STRATEGIES

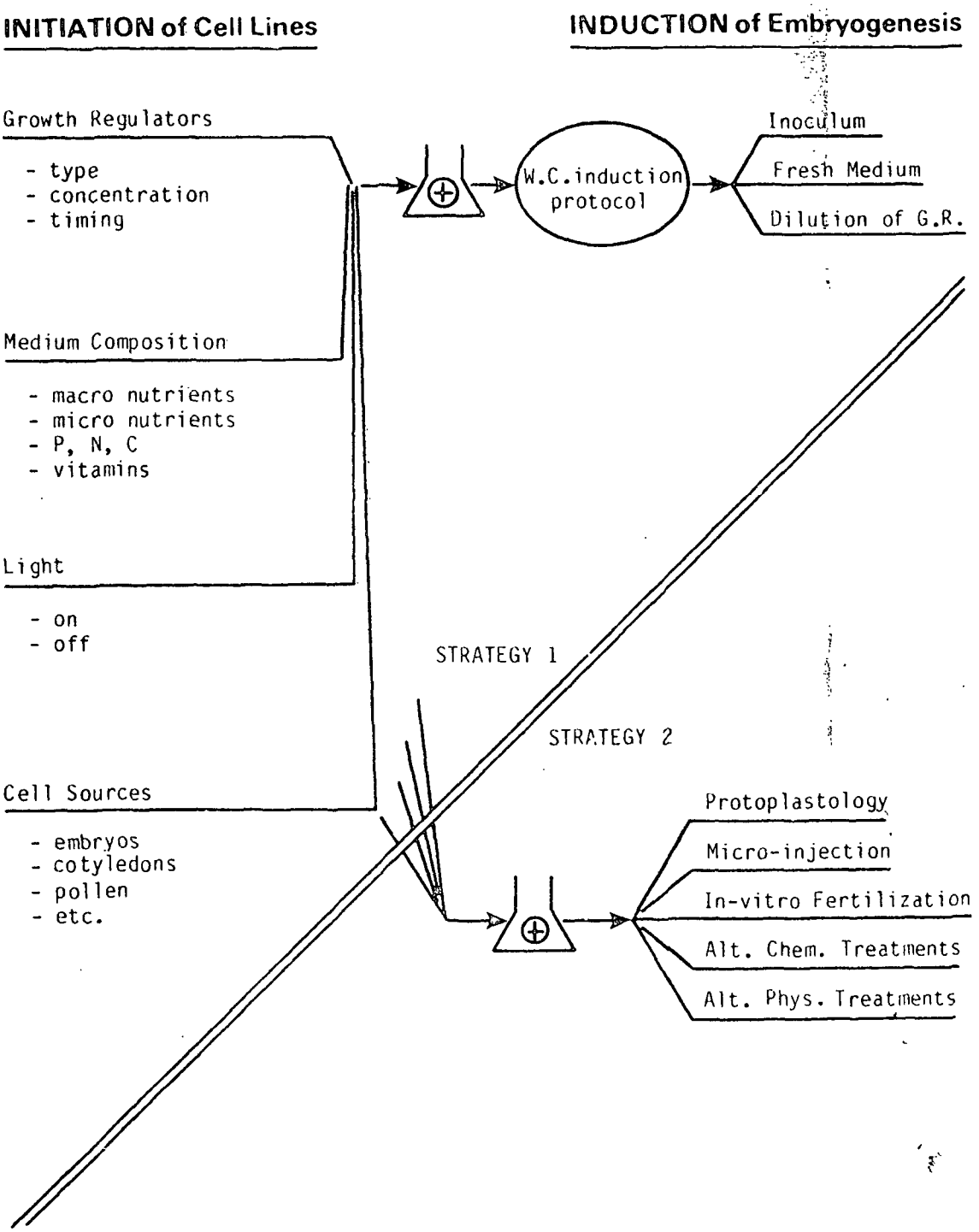

Diagram 2

CURRENT and PAST IPC Efforts