Project 3223
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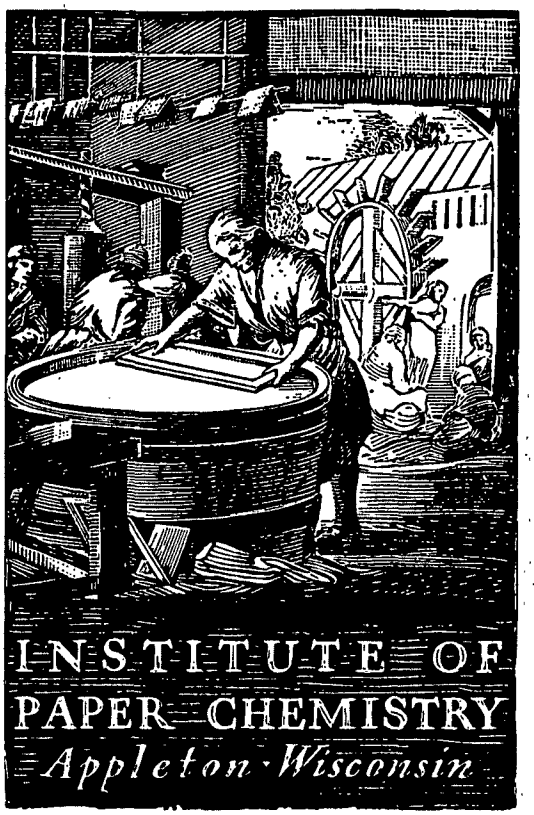

A BIOCHEMICAL TEAM APPROACH TO THE PRODUCTION OF TREE HYBRIDS FROM FUSED CONIFER PROTOPLASTS

Project 3223

2 Report One

A Progress Report

to

MEMBERS OF THE INSTITUTE OF PAPER CHEMISTRY

January 20, 1975

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Appleton, Wisconsin

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SUMMARY

The research described in the report that follows is centered in the Tissue Culture, Biochemistry and Electron Microscopy Laboratories. Although the work is closely related and closely coordinated, it is helpful to review activities and progress by members of the research team on a laboratory by laboratory basis as in the following summary of progress during the past six months.

Tissue Culture Laboratory investigations have concentrated on the production of dark- and light-grown callus for biochemical analysis, manipulation of callus environment for biochemical studies, and empirical studies on single-cell suspensions. Douglas-fir and loblolly pine have been the tree species involved. Appropriate light- and dark-grown callus are being supplied for biochemical analysis. Only a limited amount of cotyledon and cotyledon callus has been produced and plans are to step up production so that comparative chemical analyses can continue at a faster pace. Manipulation of callus environment is under way, including growth media, day length, light quality, and media pH changes. Perhaps the most interesting development during this first six months has been the production of leafy shoots on callus that had been subcultured for from four to six months. An equally important step forward has been the development of several treatments that initiate 50-80% embryonic tissue (E-cells). After initiation of E-cells, they are transferred to new adjusted media and, in at least one instance, 70-80% of the E-cells developed into medium-sized embryoids. Now that E-cells can be produced, the main problem is to consistently obtain large percentages of embryoids.

Biochemistry Laboratory research during the first six months since the program was initiated has centered around: (1) comparative compositional analysis of differentiated and undifferentiated tissue and (2) preliminary biochemical evaluation of callus-environment relationships. Analytical efforts to date have dealt primarily with four classes of compounds: amino acids, sugars, lipids and proteins. Arginine and lysine in callus grown on certain media are two of the most promising polar compounds under investigation. Work with nonpolar compounds in the organic phases of callus extracts turned up a number of differences between the callus extracts and extracts of young developing seedlings (seed embryos). The compounds involved behaved chromatographically as lipids and will receive further attention in upcoming investigations. The rapid conversion of sucrose to glucose and fructose by callus cells grown in suspension culture is also under investigation.

Another development of interest has been the ability of mesityl oxide (MO) to cause the formation of embryoids, when added to nondifferentiating callus in small amounts (drop tests). Drop test procedures, followed by cytological examination by Dr. Winton, are being used to evaluate the influence of a number of compounds on embryoid formation.

Electron Microscopy Laboratory investigations have been concerned with refining procedures for fixing, staining, and embedding callus cells and using the procedures developed to examine treatment changes. A much improved fixing schedule has been developed using a low temperature combined with the incorporation of 0.1M glucose to adjust the fixative osmolality. Embedding procedures utilizing paraffin and various epoxies received considerable attention with a "firm," low viscosity epoxy resin being adopted for future work. Several stains,

including the classical Pap stain for malignant animal cells are being tested for use with callus cells.

Noted in the process of examining treatment changes was the frequent occurrence of tannins in callus cells and the extreme variability between and within callus sources. Of particular interest was the observation that many cells in the callus tissue that produced leafy shoots were devoid of commonly seen lipid bodies (droplets). The latter were found instead as clustered or scattered droplets in the medium outside the cells.

INTRODUCTION

After a short period of preliminary investigations, Project 3223 was established on July 1, 1974, as a formally funded (internally funded) research project. Prior to establishment, the proposed program was reviewed with member company representatives at meetings in Atlanta, Georgia and Seattle, Washington. As a result of these meetings, the overall objective of the "Production of Valuable Conifer Hybrids Through Protoplast Fusion" was confirmed and a series of research priorities was assigned. Through input from IPC staff members and company representatives, it became clear that development of the "single cell — embryoid — plantlet" technique, which was part of the overall program, would be a major breakthrough and could be expected to greatly speed forest genetics progress.

As a result of the above-described research project review meetings, the established order of research priorities were as follows:

1. Differentiation of diploid callus into trees based upon biochemical comparison between differentiating and nondifferentiating tissue will receive major attention during the first year's program. Not only would the technique itself be valuable, but the biochemical data to be obtained are expected to be extremely valuable in making progress in the development of the "single cell — embryoid — plantlet technique."

2. Development of the "single cell — embryoid — plantlet technique" will also receive a major amount of attention during the first year. Intensive investigations, however, will be delayed until biochemical data from the callus differentiation studies become available for use in research with single cell suspensions.

3. Development of protoplast fusion techniques will follow as soon as appropriate progress is made in Steps 1 and 2 above. This phase of the work cannot be carried through to completion until it is possible to produce a tree from either callus or single cell suspensions. A large number of independent investigations are under way on protoplast fusion and these results are

expected to be available during the next two years. Preliminary work will be carried on in this field at a level that will make it possible for us to step in and capitalize on progress made by others.

4. Conifer hybridization — successful "nonsexual" hybridization hinges upon being able to fuse protoplasts and then grow the hybrid cells into trees. Attempts at hybridization will be made as soon as suitable progress is made on the basic problems cited in Items 1 through 3.

Because of the complex nature of the research program, a team approach was adopted. This research team consists of Dr. Lawson Winton and Ms. Shirley Verhagen from the Tissue Culture Laboratory, Dr. Morris Johnson and Mr. John Carlson from the Biochemistry Laboratory and Dr. Russell Parham and Ms. Hilkka Kaustinen from the Electron Microscopy Laboratory. The progress report that follows summarizes the activities and advances made in Project 3223 since its inception, with particular emphasis on progress during the past six months.

TISSUE CULTURE LABORATORY PROGRAM

INTRODUCTION

Our current objective is the aseptic propagation of Douglas-fir and loblolly pine from callus and cell cultures. Callus tissue is initiated from the cambial layer, just under the bark, on sterilized segments about 1-cm long from terminal branches (1). This derived callus tissue can be isolated from the parental branch segments and grown independently on a solidified nutrient medium containing major and minor salts, sugar, vitamins, and growth hormones. Callus has been initiated from several conifer tree species in our laboratory and maintained for 3-4 years by monthly subculturing to fresh medium. Our old callus is composed mainly of cells with only primary walls, and secondary thickenings are not normally observed. However, in callus initiated from Douglas-fir early in 1974, most of the firm green callus has cells with secondary-wall spiral thickenings, but we do not know yet if secondary wall thickenings are necessary for the initiation of shoots from callus tissue.

Plantlets and trees theoretically can be produced in two ways from callus and cell cultures. From aspen callus tissue, grown on solidified medium, clusters of cells form meristemoidal areas of differentiated cells that produce an adventitious shoot. Rarely, such a shoot might grow a root in place, but in hardwood species the shoots usually must be removed and rooted in a rooting medium (2). Only a half-dozen hardwood tree species have so far been reproduced from callus tissue (3), but no conifer forest tree has yet been grown by this method. A few shoots have been found on callus from Norway spruce, but these did not survive to form plants or trees.

The second method of tree production from culture is from small groups of cells in liquid suspensions. Usually a more simplified nutrient medium is necessary, and the solidifying agar is omitted. Agitation is necessary to provide aeration, usually in the form of shaking or rotating on a revolving drum. Other environmental factors also appear to affect cell differentiation, such as temperature and the type and duration of light. Usually one cell in a cluster will expand, divide, and form an embryo much like the one that forms in a seed. In herbaceous plants, several species have been reproduced by this method and grown into fully mature and reproducing plants (4). Spruce tree cells have been grown in suspension cultures and have formed embryoid-types of nonrandom cell-division masses, but did not develop into true embryos or plants (5). One of the same authors, however, was successful in growing elm cells in suspension, pouring them onto agar medium to form callus, then growing adventitious shoots that were rooted to form plants (6). These are only a few of the many indications that make us believe we are on the threshold of discovering the key to tree cell differentiation, and many workers feel it is only a matter of time and hard work before trees can be reproduced routinely from callus or cell cultures (7).

During the past six months in this laboratory, we have, in cooperation with the electron microscopist and biochemist, transformed undifferentiated callus tissue from Douglas-fir to callus cells having secondary spiral-wall thickenings. We have also initiated, in liquid cell suspensions, what we consider to be preembryonic cells (E-cells) from undifferentiated callus cells. By simplifying the medium, we have initiated up to 95% E-cells in 5-7 days, and have also been able to stimulate up to 50-70% of the E-cells to divide. Unfortunately, the dividing E-cells have "dedifferentiated" back to primary cells instead of forming embryos. In other experiments, we have recovered small and

medium-sized embryoids of up to 50-70 cells, but no true embryo has formed that has gone on to develop into a plant. In one dish of callus tissue we had an unusually high number of embryoids, whereas we usually only find occasional embryoids scattered among the other cells. Most embryoids are composed of E-cells, but we also find large cells with large vacuoles that occasionally divide and form an embryoid structure. However, we feel that the E-cell is the starting place for true embryos, because of their relatively small vacuole and large cytoplasm, indicating perhaps the capacity to produce large amounts of precursors necessary for embryo formation. So far we are mainly working with differentiation in cells from soft green callus of Douglas-fir, but we have also recovered some embryoids from soft yellow callus (grown in the dark) from lob-lolly pine and a few from other conifer species. Unless otherwise noted, all reported work will be with Douglas-fir callus initiated in December 1970, and maintained in the laboratory on our modification of Murashige and Skoog medium (1).

UNDIFFERENTIATED CALLUS TISSUE

Callus tissue is maintained in stock cultures for use (1) in experiments that attempt to initiate shoots from callus, also (2) as a source for cell suspensions in liquid medium, and (3) for biochemical analyses for comparisons with differentiated needle tissue. In the future, after we learn how to grow trees from callus tissue, callus cells will also be a source of cells for protoplast production by the enzymatic removal of cell walls. The eventual goal of the project is the nonsexual hybridization of trees by the fusion of protoplasts from different, but related species of conifers. At present, however, we are confining our attention to the production of plants and trees from callus or cell cultures.

Stock cultures now used in this project include the old callus of Douglas-fir and loblolly pine initiated in 1970 (1), new Douglas-fir callus initiated in 1974 according to the same method, and callus initiated from elongating cotyledons (primary leaves) of excised seed embryos in 1974. Some of the environmental factors are given in Table I, as well as some distinguishing characteristics of the important stock cultures.

SEEDLING TISSUE FOR BIOCHEMICAL ANALYSES

One of the approaches to this problem is for the biochemist to determine chemical differences between differentiated seedling tissue and undifferentiated callus tissue. Significant differences will then be applied by the tissue culture laboratory, in adapting the physical and chemical environment of callus, for cell differentiation and eventual embryo formation.

Embryos have been isolated from mature Douglas-fir seeds and grown aseptically on medium E-1, without auxin. The seedlings elongate normally and are harvested periodically and used for biochemical extracts. Several hundreds of seedlings have been grown to 5-10 cm in height and used for biochemical studies. Recently, a new source of seeds was received from a member company, collected from Douglas-fir, loblolly pine, and ponderosa pine. We plan to grow seedlings from these new sources also, and use them for analyses.

On medium with auxin, seedlings do not develop normally and the elongating cotyledons eventually form a callus mass. We have isolated callus from at least ten Douglas-fir seedlings and established clonal lines. In a method similar to this, Sommer, et al. (8) have grown buds on the elongating cotyledons of longleaf pine, loblolly pine, Virginia pine, pitch pine, eastern white pine, and Douglas-fir. The buds were isolated along with a section of cotyledon and grown

TABLE I

GROWTH CONDITIONS AND CYTOLOGICAL CHARACTERISTICS
OF IMPORTANT STOCK CULTURES

Tree Species:

Callus from branch segments in 1970

D = Soft green callus from Douglas-fir
K = D transferred to liquid then back to agar medium
Lob = Crumbly light-yellow callus from loblolly pine

Callus from branch segments in 1974

ND = New firm callus from Douglas-fir

Callus from seed cotyledons in 1974

CC = New soft-green callus from Douglas-fir

Light & Temperature:

L = Lab temperature of 23°C ± 1°C, 200-400 ft-c of cool white fluorescent light on for 16 hr light alternating with 8 hr dark

I = Incubator with 16 hr of 40-50 ft-c cw fluorescent light at 24°C, alternating with 8 hr dark at 15°C

Dark = Continuous darkness at 27°C ± 1°C

Nutrient Media:

3 = Medium NBA 3 (1) with 1 NOAA and 0.1 BAP[a]
10 = Medium NBA 10 (1) with 5 NOAA and 0.1 BAP
17 = Medium 3 with no vitamins or BAP, 0.5 2,4-D
E-1 = Medium 3 salts, 0.4 thiamine, 100 inositol, 40,000 sucrose

Callus Cultures:

Laboratory

D-L-3	Uniformly green, spherical, and undifferentiated cells
K-L-3	Soft green with occasional E-cells and small embryoids
K-L-10	Uniformly smaller cells than D-L-3 and undifferentiated
K-L-17	Light-green or clear cells, variable in size and shape
ND-L-10	Firm green with cells variable in size and shape

Light Incubator

D-I-3	Uniformly spherical and undifferentiated cells
D-I-10	Smaller and more uniform cells than D-I-3.
K-I-10	Uniform green cells with occasional E-cell or small embryoids
CC-I-10	Soft green callus with occasional tracheidlike cells

Dark Incubator

Lob-10	Medium-sized clear, spherical cells with starch
Lob-17	Variable in size and shape; will not survive with cytokinin
Lob-IPA	Small and uniform clear, spherical cells, easily dissociated
SL-10	Shortleaf: medium and large cells with tan deposits
WH-10	Western hemlock: small, uniform, spherical cells with deposits

[a]Amounts given in ppm (mg/l); NOAA = naphthoxyacetic acid; BAP = benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid; IPA = isopentenylaminopurine.

into complete plants, and the method is now being adapted for the commercial cloning of seeds.

We recently have found leafy shoots (Fig. 1) on four of ten lines of our Douglas-fir cotyledon callus subcultured for 4-6 months, and are currently attempting to root them to form complete plants. If successful, this would extend the method of Sommer one step further, and make possible the unlimited cloning of individual seeds. However, this is not the achievement of the project objective, which is to reproduce conifer trees from callus tissue initiated from branch cuttings. The two tissues apparently are not chemically identical, and we still have not produced shoots from undifferentiated branch callus.

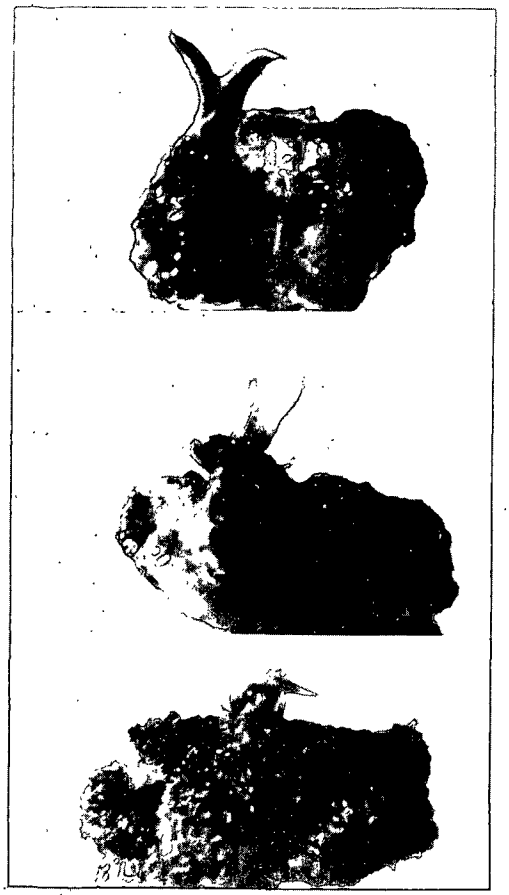

Figure 1. Leafy Shoots Initiated from Douglas-fir Seed Cotyledon Callus Subcultured Monthly for Six Months. Shoots are from Three Different Clonal Callus Lines. Four Times Actual Size

EMBRYOID INITIATION AND ENLARGEMENT

Embryoids are nonrandom masses of cells that have the appearance of embryolike structures, but which have not gone on to develop into true embryos and plants. These range from small 2-5 celled, linear or spherical groups of E-cells, up to large bodies composed of 40-50 cells. Occasional small to large embryoids have been observed in callus cultures on agar medium and in liquid cell suspensions, but our present objective is to produce high frequencies of large embryoids in repeatable experiments. So far this has been done only once, in one callus culture after many weeks, and has not yet been repeated.

We believe that true embryos will form from clusters of E-cells, so our work during the past six months has really been to initiate high frequencies of E-cells. We have not succeeded for callus cultures, but we have initiated up to 95% E-cells repeatedly in cell suspensions using several types of liquid media.

Much of our work has centered around simplifying the medium to the point of removing all vitamins and growth hormones, leaving only the salts and sucrose. However, we have found that the ratios are critical of the relative amounts of ammonium to nitrate nitrogen, as well as to the remaining salts. With our best simplified medium, we can start with undifferentiated D-I-10 stock callus and get 20-40% E-cells after 3 days and 80-95% E-cells after 5-7 days. The E-cells start dividing after 10-14 days, but so far they have "dedifferentiated" back to simple cells with large vacuoles and have not formed embryoids. Some preliminary experiments with additives indicate that we may be able to go through a sequence of (1) E-cell initiation, (2) division of E-cells, then (3) formation of embryoids from dividing E-cells. Some of the additives we have been trying include casein hydrolyzate, inositol, adenosine, thiamine, mesityl

oxide, adenine sulfate, and asparagine. Some additives definitely inhibit the induction of E-cells, while others seem to stimulate their formation.

Following the general approach of Halperin and Wetherell (9), we likewise have found that cytokinin inhibits both cell division and E-cell formation in liquid cultures. In addition, auxin seems to be the main inhibitor of differentiation in both agar and liquid cultures. We have initiated fairly high frequencies of E-cells by adding the antiauxin 2,4,6-T (2,4,6-trichlorophenoxyacetic acid) to liquid medium 17 made without auxin (10), but it does not do as well when added to our best simplified medium. We have also initiated E-cells by leaving, for 24 hours each, the cells first in medium with BAP as the only hormone followed by GA (gibberellic acid) alone, and then finally in medium with an auxin. For one of our stock cultures, just the reverse order of GA then BAP produced the best results. However, all of these were early results, and have not yet been tried with our simplified medium. Gibberellic acid will also be an additive to try after embryoids are formed, in order to get enlargement into embryos. BAP seems to inhibit embryoid development, but some combination of auxin with other additives may be successful.

Other factors we have tested include Gro-Lux (daylight) vs. cool-white fluorescent lights as well as the duration and intensity of the light-dark cycle. Gro-Lux seems to stimulate the production of chlorophyll, but our evidence to date indicates that continuous cool-white fluorescent light is best for E-cell initiation. All liquid cultures have been carried out on a revolving drum at 20 rpm, but we are expecting delivery of a slower drum of 1 rpm and a shaker to expand our testing program. We are also planning experiments of light stimuli to induce E-cells to develop into embryoids. Past trials of gentle centrifugation or settling as polarizing agents to induce embryoid formation may also

be tested again with our simplified medium. We now can induce high frequencies of E-cells by several different methods, so the next step will be the transformation of E-cells into embryoids and embryos.

A series of photographs is shown in Fig. 2-5 of occasional small, medium, and large embryoids of Douglas-fir observed in liquid cell suspensions. The linear embryoid of loblolly pine, shown in Fig. 6, is not the largest observed for this species, but clearly shows the thickened cytoplasm of the E-cells in contrast to the large vacuole and thin cytoplasm of undifferentiated cells.

The next series of photographs (Fig. 7-11) illustrates the continuous development, in simplified liquid medium of undifferentiated cells (Fig. 7), E-cells (Fig. 8), division of E-cells (Fig. 9), and "dedifferentiation" of E-cells into undifferentiated cells (Fig. 10). Figure 11 is an enlargement of one dividing E-cell, showing the pattern of development we anticipate for embryoid formation: that is, first the formation of a cluster of E-cells, followed by the enlargement of one cell into an embryoid. This is the apparent sequence leading to the formation of embryoids seen in Fig. 2-4.

ACCOMPLISHMENTS AND FUTURE PLANS

At the end of our first six months into the project, we have found how to repeatedly initiate high frequencies of E-cells in simplified liquid nutrient medium, on a revolving drum in continuous light. We believe this is a significant step in the progress of our work. This has been accomplished both as a result of empirical experiments, as well as the application of feedback from the biochemistry and electron microscopy laboratories. Several factors that have been studied in some depth have not been reported here, mainly because we need to repeat the experiments or do not yet have a clear indication of how the results relate to

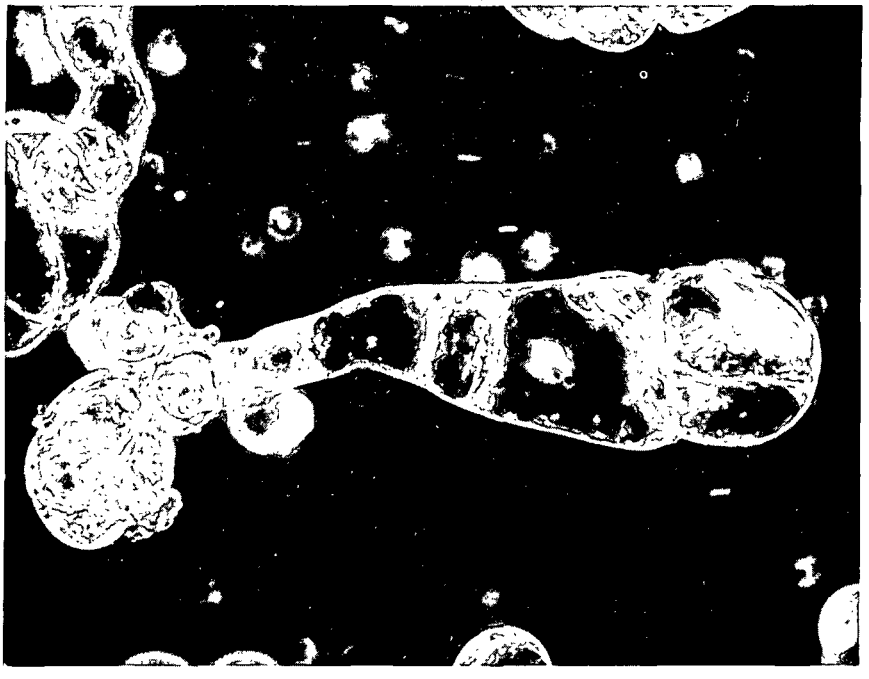

Figure 2. Medium-Sized Embryoid Emerging from a Cluster of Douglas-fir E-Cells. 110X

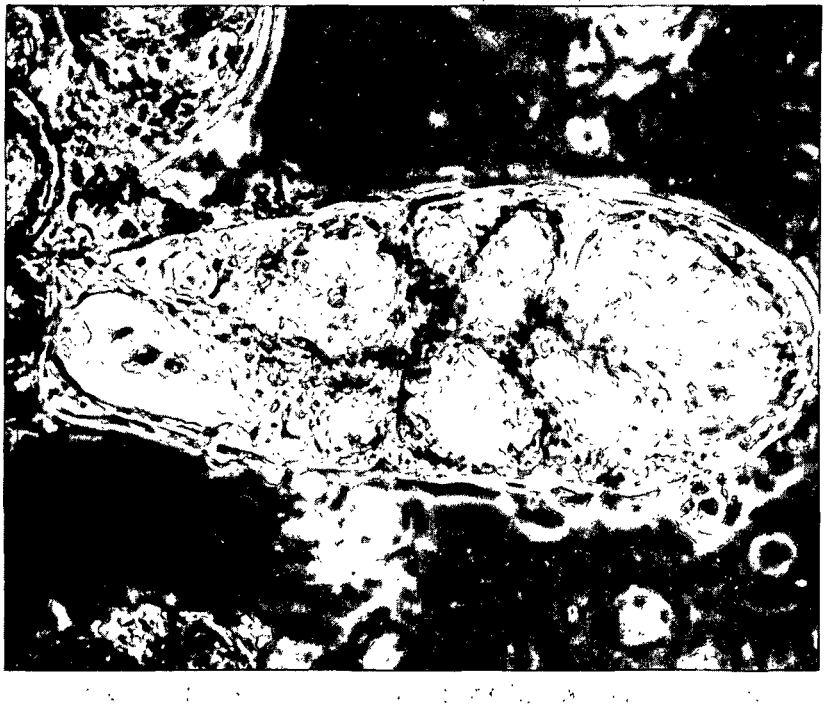

Figure 3. Large Embryoid Emerging from a Cluster of Douglas-fir E-Cells. 610X

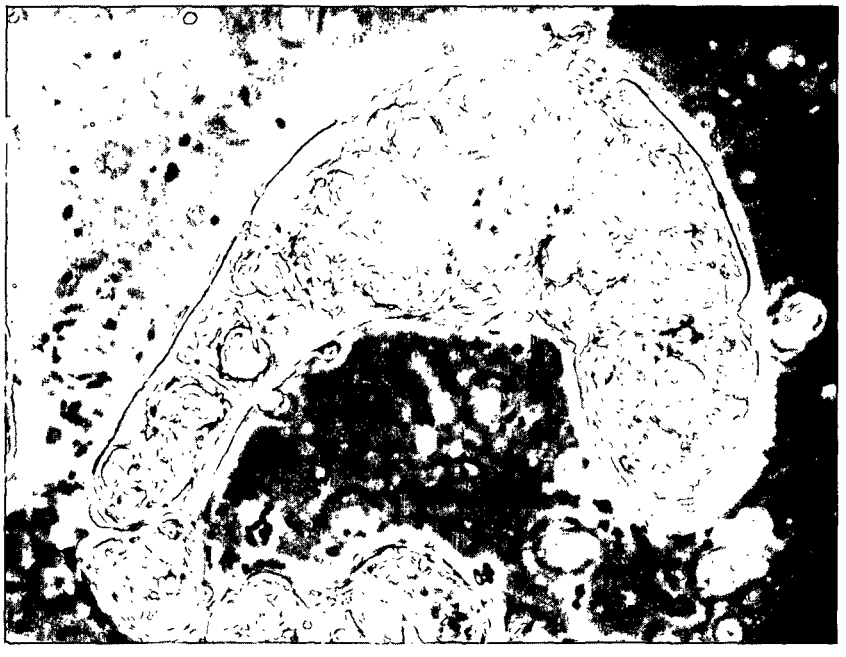

Figure 4. Very Large Embryoid Composed of Douglas-fir E-Cells. 490X

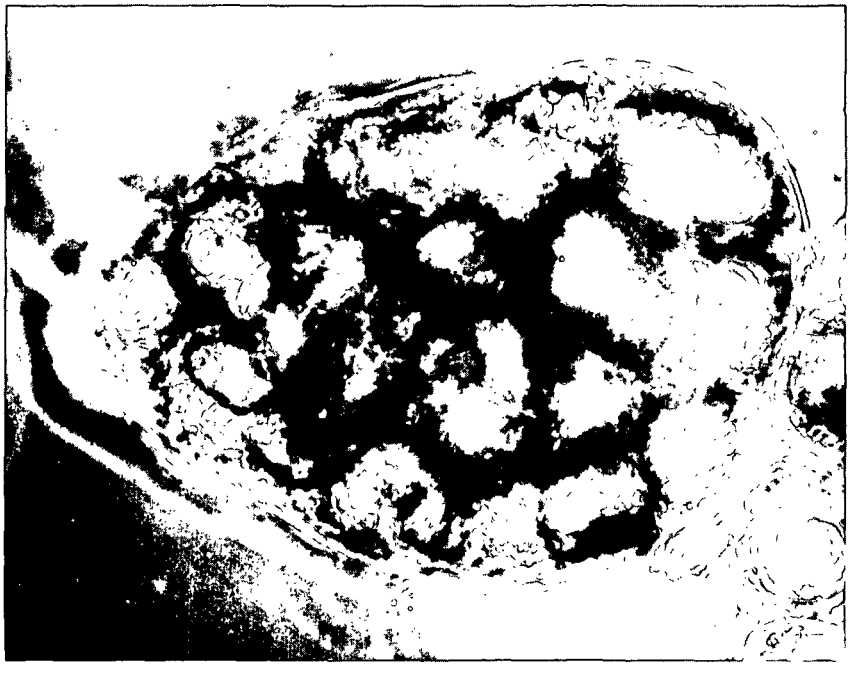

Figure 5. Large Embryoid of Douglas-fir Approaching the Heart-Shaped Stage Found in Normal Embryogenesis. 550X

our work. However, some of the results seem to point to areas of future investigation that may be useful in resolving the problem of E-cell development into embryoids and embryos. We hope that by the next report, many of the problems we are working on now can either be resolved or discarded as unimportant, as part of the key to cellular differentiation and development.

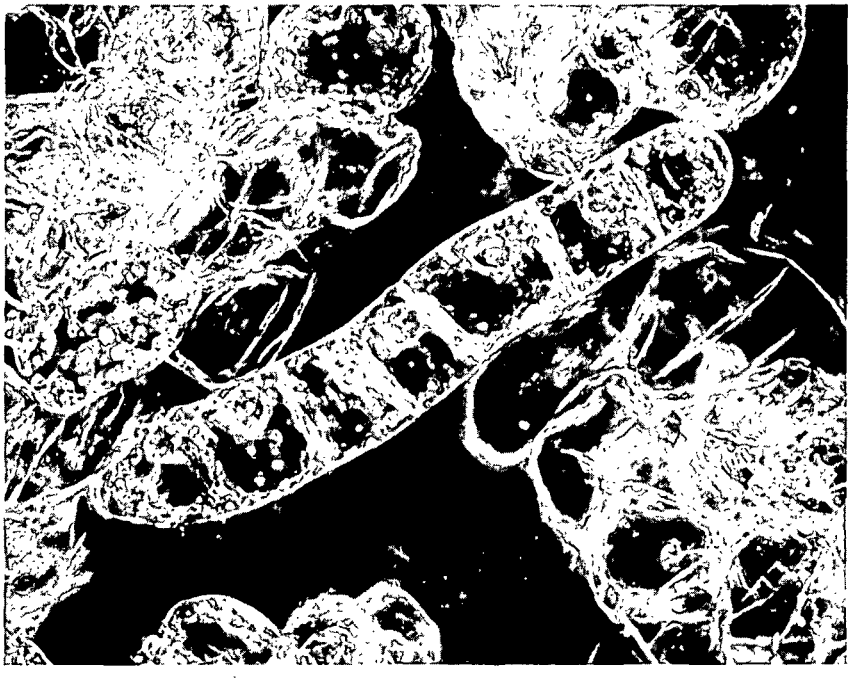

Figure 6. Linear Embryoid of Loblolly Pine Composed of E-Cells. 280X

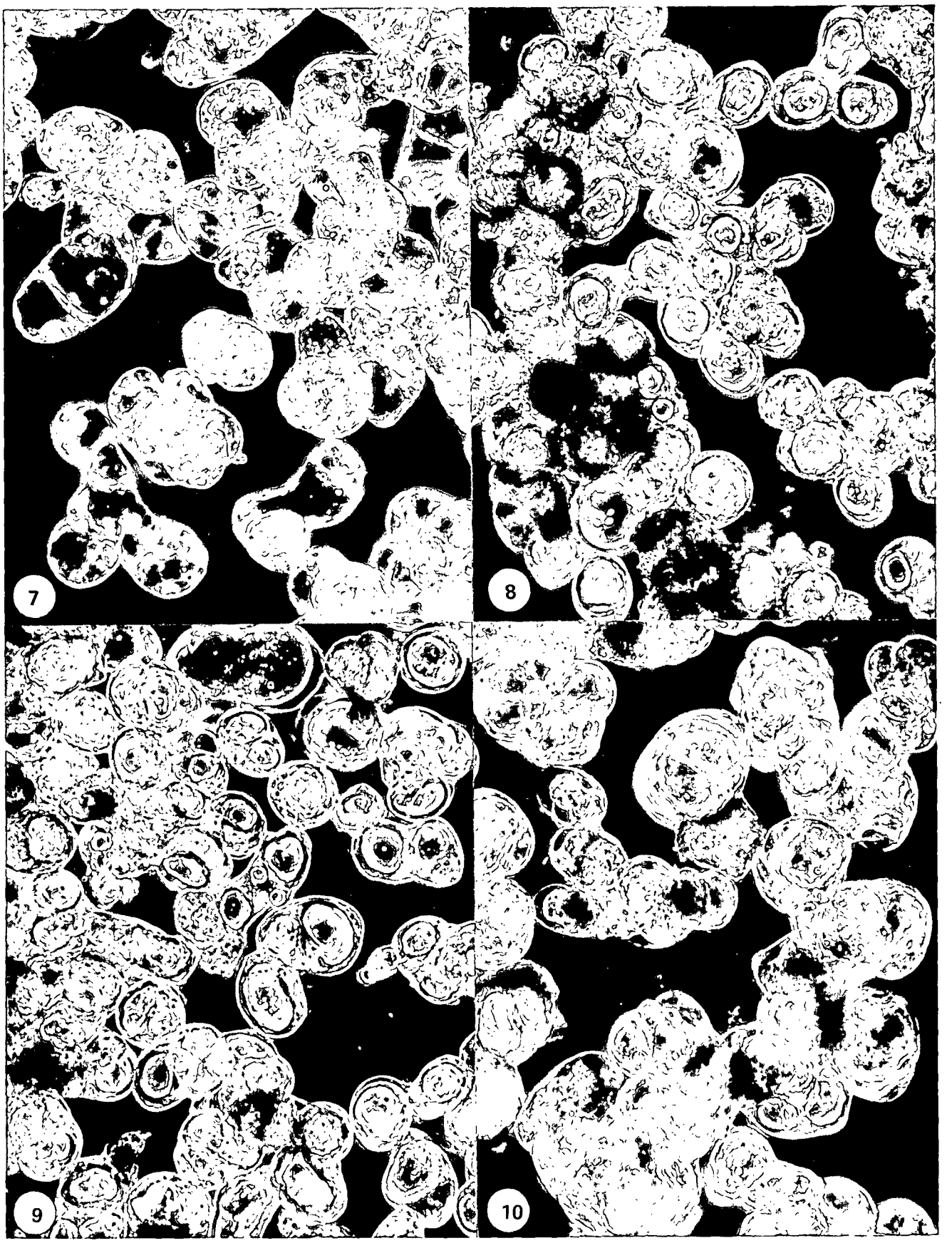
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Figure 7-10. (Opposite Page) Continuous Development in Simplified Liquid Medium of Undifferentiated Cells from Douglas-fir Callus D-I-10 (Fig. 7), A High Frequency of E-Cells (Fig. 8), Dividing E-Cells (Fig. 9), and Dedifferentiation of Dividing E-Cells into Undifferentiated Cells (Fig. 10). All Figures at 320X

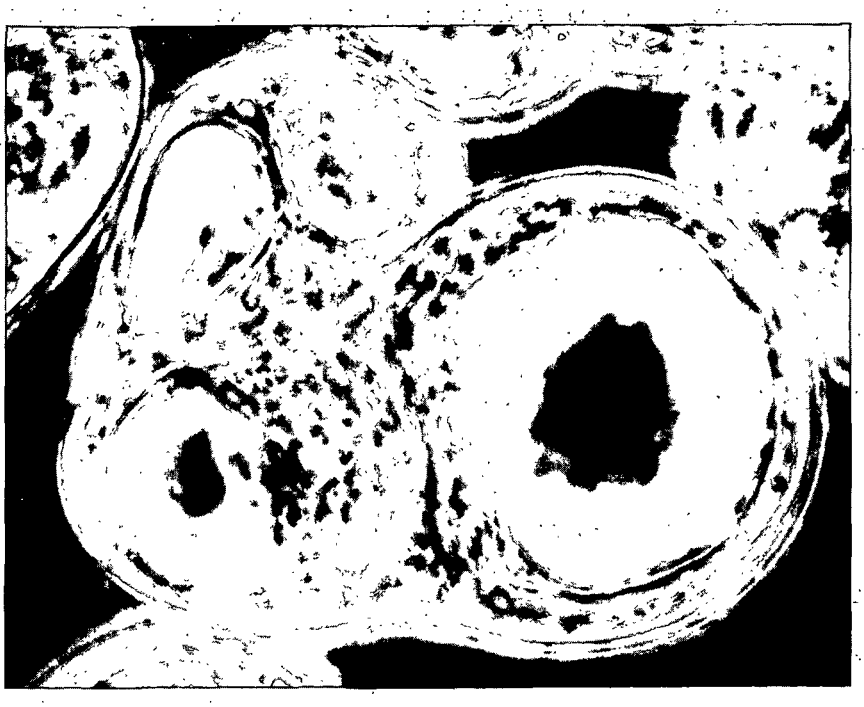


Figure 11. Small Mass of Cells from One Dividing E-Cell of Douglas-fir. 1350X

BIOCHEMISTRY LABORATORY PROGRAM

INTRODUCTION

The contributions of biochemistry in this team approach to protoplast fusion are analytical, experimental and interpretative with the goal of understanding some of the fundamentals of the differentiation process in tree cells. Unless we can come to grips with the latter unsolved biological phenomenon, fused protoplasts, though successful, might well remain an unexploitable curiosity. The process of differentiation is not only a puzzle for those of us now working with trees, but also for biologists concerned with the problem in any living system. As in many endeavors, researchers studying the bacteria probably can point to the most progress for their efforts; unfortunately, this is one problem where success with the procaryotic organisms may not be so easily extrapolated to the eucaryotes such as we have to deal with in trees. This can be appreciated by noting that structural differentiation or morphogenesis in procaryotes is meager relative to the complexities of eucaryotic structures. Nevertheless, control mechanisms like induction and repression are probably integral parts of more complex regulatory mechanisms operating in eucaryotes.

While differentiation is not a well-understood phenomenon, there are scattered sections of the puzzle in which some pieces seem to fit together. As a result, several investigators have tried their hand at proposing working hypotheses to guide attacks on parts or all of the problem. Practically all speculation focuses at some point on gene activation in terms of the current concepts of molecular biology (11). Therefore, much of the research in the field involves studies of the biological macromolecules, particularly proteins and nucleic acids, and their regulating mechanisms.

Mindful of the foregoing and in agreement that ultimate answers are likely to be obtained from studies on the control of biopolymer syntheses, the biochemical approach we have taken in this project begins a step further removed where the view is broader and where, hopefully, some assessment of possible relevance can be made before taking the plunge into specific areas. The number of kinds of just proteins in living plant tissue should be quite large and in some cases there will be only a few molecules of a particular kind per cell, making detection difficult. One way of sorting out, for example, those proteins which are relevant to the differentiation problem might be to determine by analysis which ones are unique to differentiated tissue and which are constitutive in both differentiated and undifferentiated tissue. Since the complement of low molecular weight organic constituents of any living system is largely determined by macromolecular constituents, we have taken the further tack that analyses of these smaller organic molecules, as present in differentiated versus undifferentiated tissue, may provide us with clues as to which genes were activated and how during the differentiation events. We refer to these analyses of both macro and micro species as "comparative biochemical analyses" in this project, taking some liberties with the usual meaning of that phrase.

There is the question of whether "trigger events" can be discerned by comparative analysis, i.e., might not the results of the events be found with little trace of what caused the events or indication of whether it was an early or late event. To some extent this should relate to whether differentiation requires only initiation or both initiation and maintenance. If only the former, the task would seem to be more difficult with our approach but not impossible nor necessarily more roundabout than the approaches of other workers. Furthermore, although the foregoing approach is the major thrust in this project, other

kinds of investigations are also under way based upon the observations of other scientists engaged in differentiation research.

One of the most promising research techniques brought into use in this initial report period (in cooperation with the Tissue Culture Laboratory) was temporal analysis of the media in which suspension cells were growing. The analysis of substances released by these cells may prove to be very useful, i.e., the released products of cells for which the immediate morphological past is on record can be analyzed. Also, the fact that cells in suspension culture are in intimate and uniform contact with the nutrient solution makes the conduct of feedback and reanalysis experiments feasible on a more reliable and shorter-term basis than similar efforts with callus on nutrient agar.

COMPOSITIONAL ANALYSES

The analytical effort to date has dealt primarily with four classes of compounds: amino acids, sugars, lipids and proteins. Other kinds of molecules have been encountered, but, for the most part, they will not be discussed in this report; however, they may appear in later reports pending further investigation. In the case of loblolly pine, only extracts of dark-grown callus have been examined which precludes any comparative analysis yet. For Douglas-fir, some data are available for extracts of differentiated as well as callus tissue and, in addition, for callus tissue grown under various conditions and for substances released by cells in suspension culture.

Low Molecular Weight Organic Compounds

Extractions of callus (undifferentiated tissue) and cultured seed embryos (differentiated tissue) for these compounds were conducted with a potential two-phase solvent system (12) which was subsequently induced to

separate into aqueous and nonaqueous phases; the aqueous phase was the source of polar compounds for analysis while the nonaqueous phase provided nonpolar compounds. Occasionally, a minute quantity of interphase material was seen; this has been discarded in work to date. Except for a small amount of spectroscopy and ion-exchange chromatography, analysis of these compounds has been by thin-layer chromatography (TLC) (13), mostly two-dimensional (2D-TLC) on silicic acid.

Water-Soluble Compounds

Free amino acids and sugars are the prominent compounds encountered in this category. With a few exceptions, other aqueous phase constituents that might be expected seem to be absent or in very low concentration such that concentration steps will be necessary prior to analysis. Among the amino acids, lysine and arginine have attracted the most attention because differentiated tissue generally yields massive quantities of these two amino acids while undifferentiated callus generally contains only modest amounts of free arginine and trace amounts of free lysine, as in the chromatograms of Douglas-fir extracts compared in Fig. 12 and 13 (only compounds under discussion are shown). It seems likely that similar observations may turn up in the case of loblolly pine, but, as of this writing, only loblolly dark-grown callus has been analyzed in this respect and found to contain only modest amounts of these two amino acids. Determination of the origin of the large amounts of arginine and lysine in differentiated tissue could be germane to the differentiation mechanism; metabolic experiments will be necessary, perhaps of a sophisticated nature. Factors which regulate arginine and lysine production would also be of extreme interest.

While a number of sugars appear to be present in aqueous phases of callus and cultured differentiated tissue extracts, most attention here has been devoted to sucrose, glucose and fructose, and particularly these three

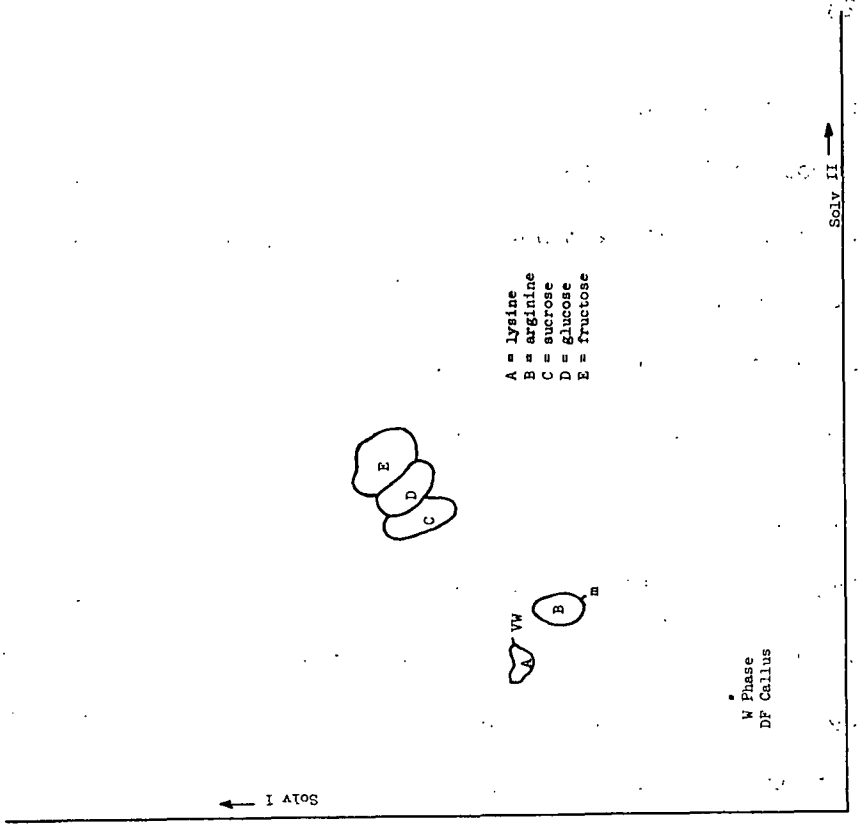


Figure 13. Some Amino Acids and Sugars in Extracts of Undifferentiated Tissue

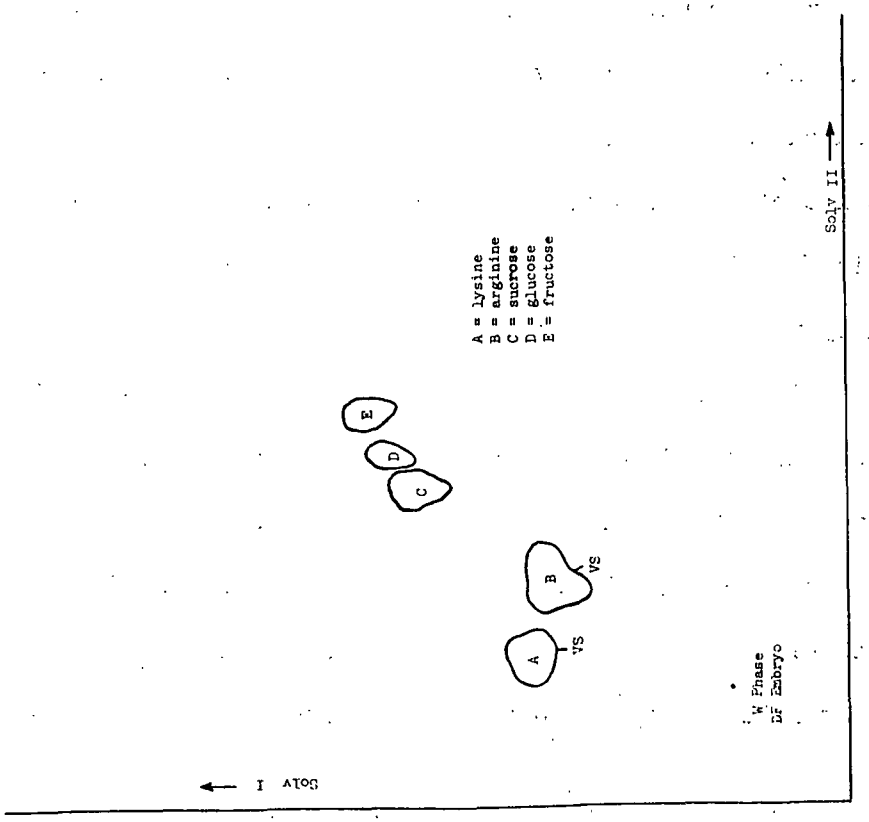


Figure 12. Some Amino Acids and Sugars in Extracts of Differentiated Tissue

sugars as they appear in cell suspension culture media. Extracts of callus and cultured differentiated tissue contain roughly equivalent amounts of sucrose but much more glucose and fructose appear in callus extracts than in those of differentiated tissue (Fig. 12 and 13). When callus cells are transferred to cell suspension culture, they quickly convert the sucrose carbon source of the suspension culture medium to glucose and fructose. The enzymes involved in this conversion and whether they are extracellular or intracellular enzymes are not yet determined with certainty, but available evidence suggests that the conversion is intracellular followed by leakage or secretion and that the breakdown of sucrose may be more involved than simple hydrolysis. Practically no sucrose (originally present at 3.5%) is found in the medium after five days, only glucose and fructose. Since there appears to be some correlation of these biochemical observations with morphological observations (e.g., E-cell formation), studies surrounding the utilization of these sugars and other carbon sources have become very active research areas.

Brief mention should also be made of a rather prominent water-soluble substance found to fluctuate in suspension media in a manner suggestive of a role in growth or differentiation. Its identity is still unknown, and it has come to be referred to as compound X.

Water-Insoluble Compounds

Nonpolar constituents found in the nonaqueous phase of tissue extracts present a rather complex picture, particularly for differentiated tissue. A comparison between extracts of differentiated (Fig. 14) and undifferentiated (Fig. 15) Douglas-fir tissue illustrates this complexity. These chromatograms were examined under visible and ultraviolet light before and after treatment with two chemical detection agents. Differences are apparent, but it will be some time

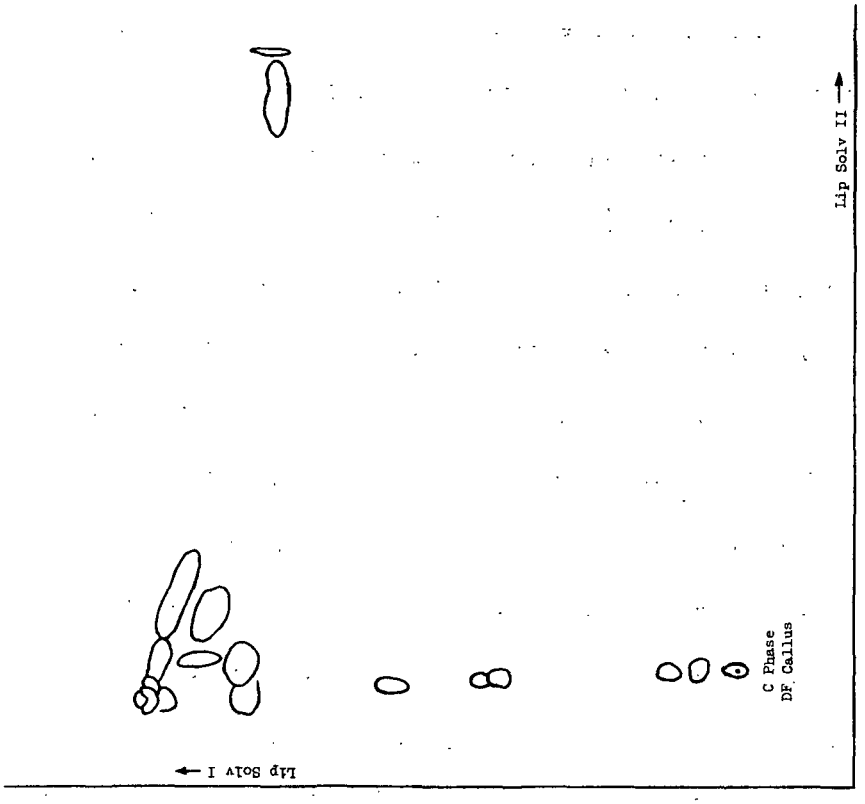


Figure 15. TLC of Some Nonpolar Compounds from Undifferentiated Tissue

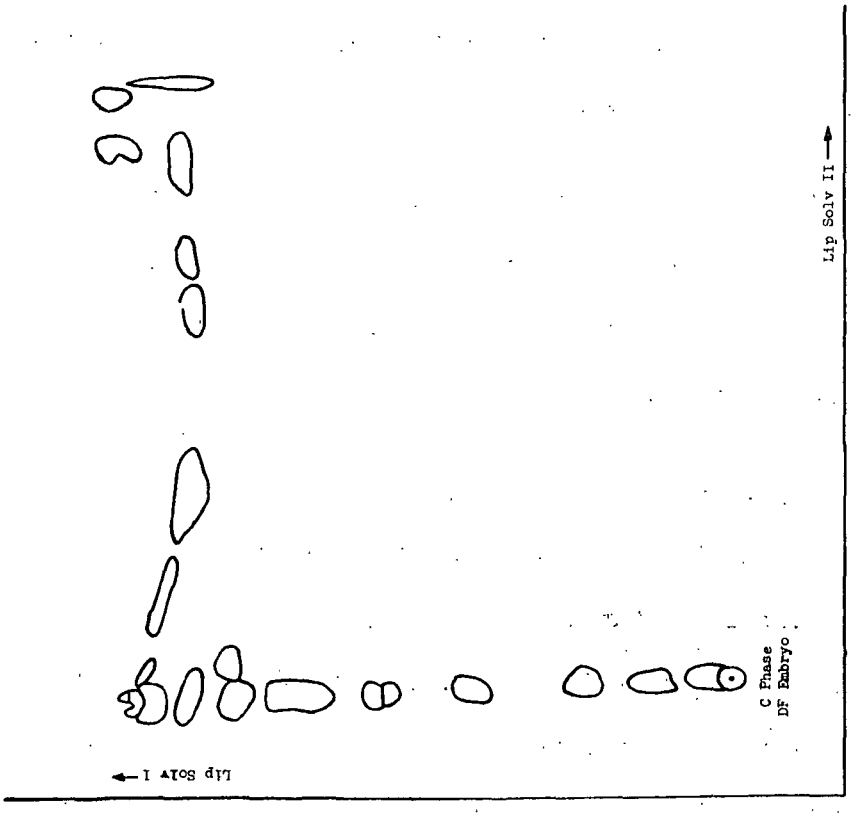


Figure 14. TLC of Some Nonpolar Compounds from Differentiated Tissue

before the significance of these data can be appreciated. Figure 16 presents the somewhat less formidable picture of dark-grown loblolly pine undifferentiated tissue grown on the same medium as used for the Douglas-fir in Fig. 15. Since there are some obviously and no doubt some not-so-obviously unresolved compounds on all of these chromatograms, spotted mixtures were probably even more complicated than represented in these figures. Some phospholipids of Douglas-fir differentiated and undifferentiated tissue are reasonably well resolved along the bottom of the TLC chromatograms in Fig. 17 and 18, respectively; some quantitative differences are apparent but qualitative differences seem few.

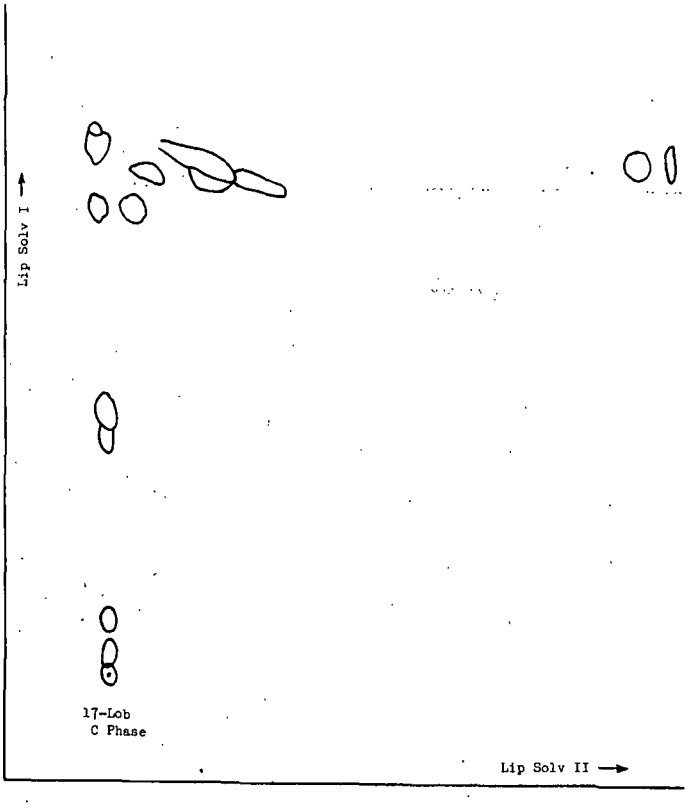


Figure 16. TLC of Some Nonpolar Compounds from Loblolly Pine Undifferentiated Tissue

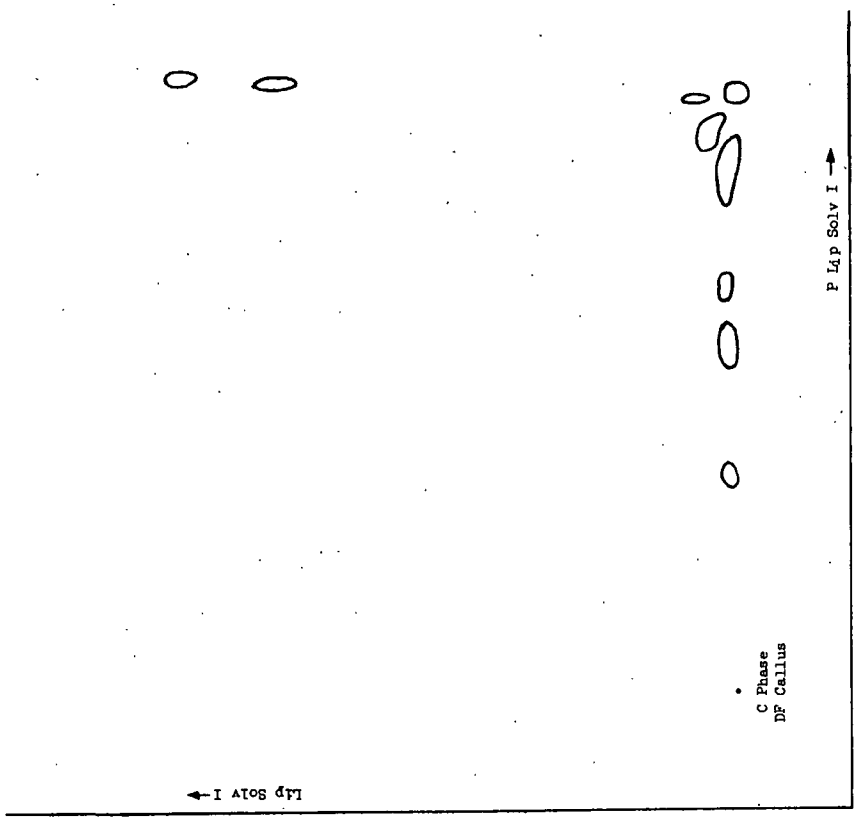


Figure 18. Some Phospholipids in Undifferentiated Tissue

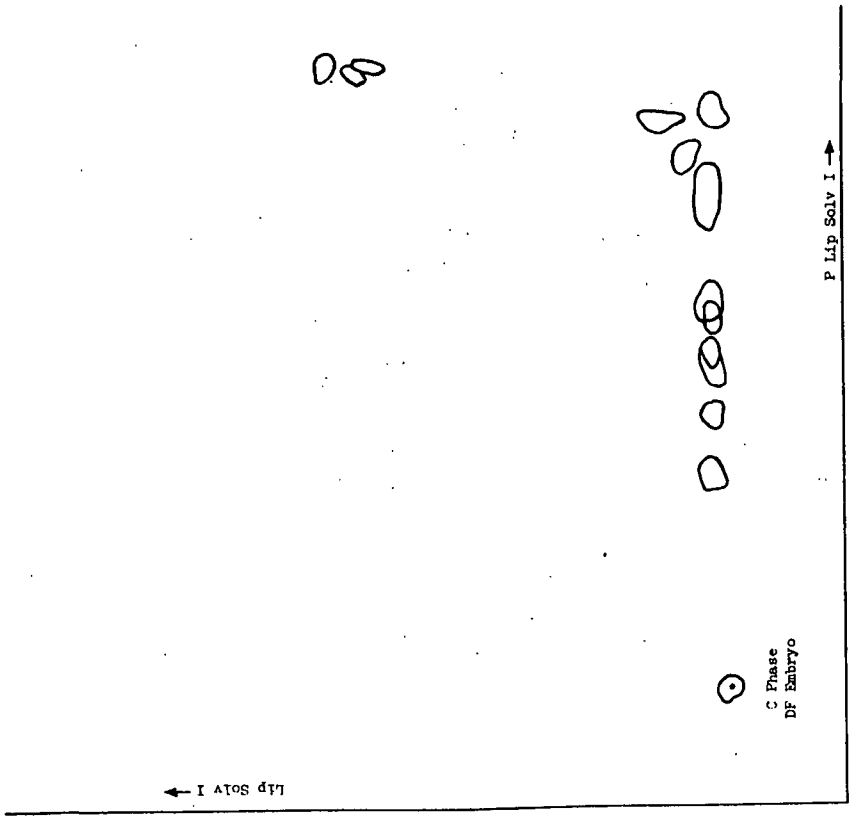


Figure 17. Some Phospholipids in Differentiated Tissue

High Molecular Weight Compounds

The only species to be discussed here are the proteins. No significant amount of effort has been expended on polysaccharides or nucleic acids yet. The extraction of water-soluble proteins must be carried out under conditions quite different from those employed for low molecular weight organic compounds because the proteins are subject to denaturation (in which case they are very apt to become insoluble in water and precipitate). In results conveyed here, the tissues were converted to acetone powders which were then extracted with phosphate buffer. Analysis of these extracts was primarily by the technique of disk electrophoresis (14) with minor contributions from chemical and spectroscopic analysis. In electrophoresis, mixtures of soluble proteins can be separated into multiple bands and banding patterns can then be compared. With gel scanning instrumentation these banding patterns can be scanned directly by ultraviolet absorbance or the bands can be stained and scanned by visible absorbance; in either case a densitometric trace results. An alternative to scanning is to stain the gels and provide schematic diagrams of the results or photographs. Although it is anticipated that many results will be presented as densitometric traces in these reports, all mentioned methods have certain advantages and, therefore, schematics and photographs may also be used.

Because proteins are subject to denaturation, the proteins extracted from a given tissue could reflect the effectiveness of the extraction procedure as much or more than the actual protein composition of the tissue. When proteins found by extraction of Douglas-fir callus directly with phosphate buffer are compared with proteins extracted from the same tissue which was first acetone-powdered and then extracted with phosphate buffer, substantial differences are apparent. Considerations such as these make it imperative that any comparisons

of protein banding patterns between differentiated and undifferentiated tissue be conducted with tissues extracted under identical conditions in so far as possible. In addition to extraction conditions, one must also investigate such factors as the influence of tissue growth conditions upon the banding patterns. For example, Fig. 19 shows that the complexity of the growth medium is reflected in the complexity of the banding pattern; extracts of callus grown on complex Medium 10 have bands that do not appear in extracts of callus grown on minimal Medium 17. Another set of conditions that must be maintained as constant as possible are those of the electrophoresis itself. With the number of possible variables that must be kept under control in order to make any valid comparisons related to differentiation state, more specific band identification will be useful in the future.

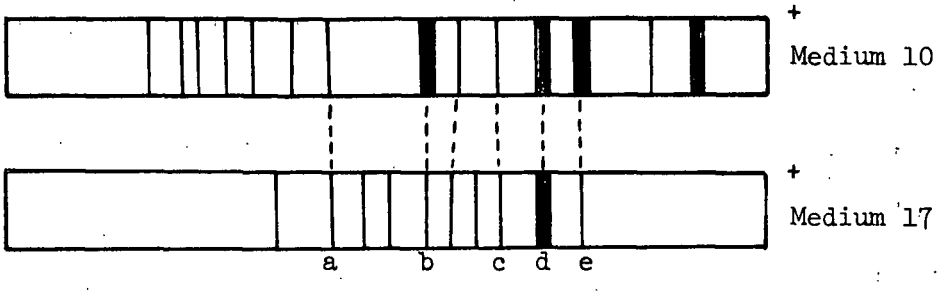
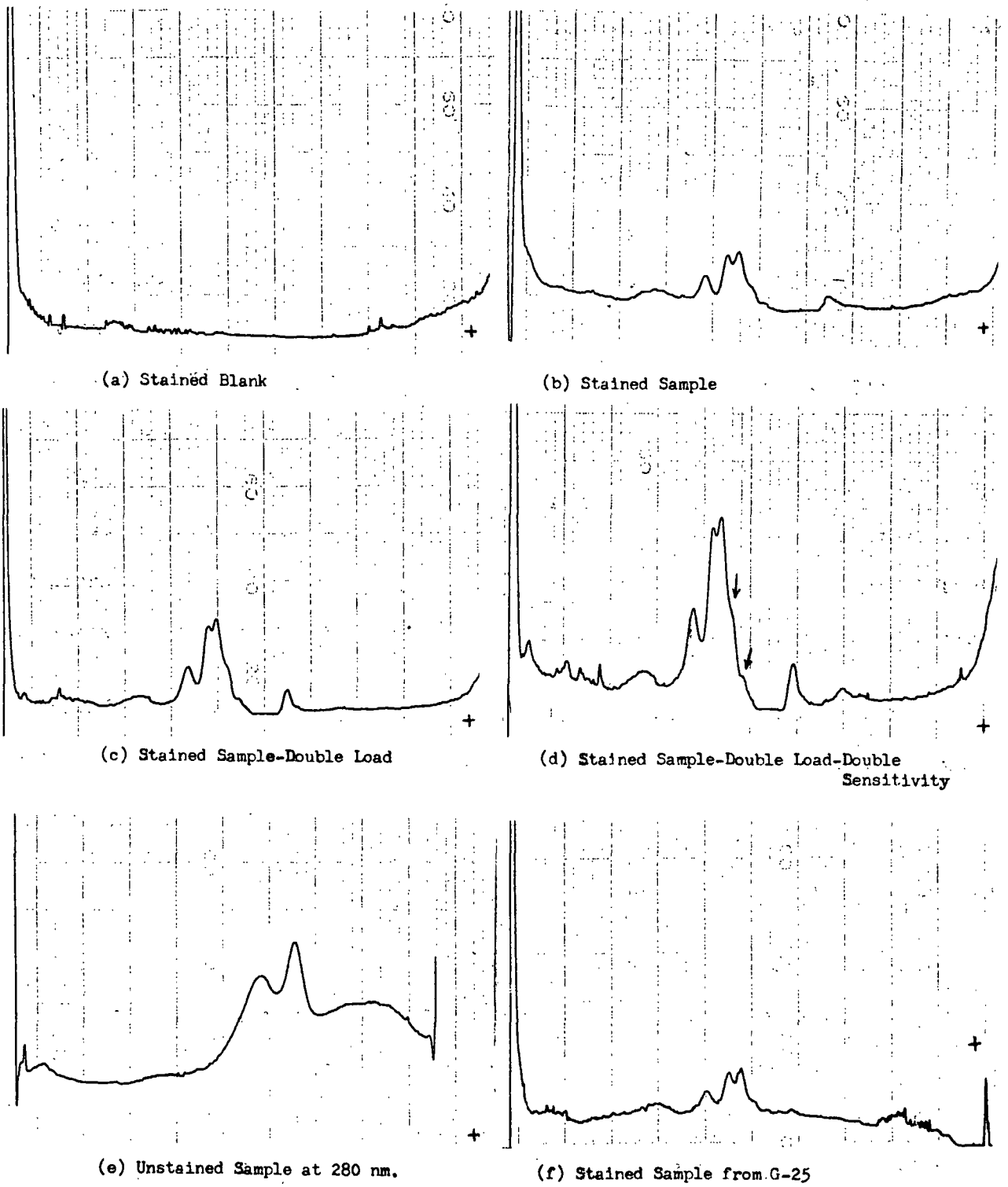


Figure 19. Protein Banding Patterns in Extracts of Douglas-fir Callus Grown on Two Different Media

Because of the need to research the foregoing considerations, it is not our intention to present any direct comparisons of differentiated and undifferentiated tissue in this report. Figure 20 (a), (b), (c) shows the banding pattern from stained gels on which two loading levels of extracts from differentiated tissue are compared with a stained blank. In Fig. 20 (d) is a rescan of gel (c) at greater sensitivity; one sees here that very weak bands are becoming



(a) Stained Blank

(b) Stained Sample

(c) Stained Sample-Double Load

(d) Stained Sample-Double Load-Double Sensitivity

(e) Unstained Sample at 280 nm.

(f) Stained Sample from G-25

Figure 20. Some Electrophoretic Banding Patterns of Extracts of Differentiated Tissue

detectable although such amplification will also increase the background noise level. Visual examination of these stained gels indicated that the two shoulders (arrows) on the large peak are significant. In Fig. 20 (e) is shown the pattern found by scanning a gel, loaded as in (b), for protein at 280 nm without staining. Note that this trace is not, for the most part, superimposable on (b); other data (not presented here) indicated that this is a matter of both specificity and sensitivity of the 280 nm scan. Often it is necessary to remove interfering low molecular weight materials from solutions of biopolymers by gel filtration. In Fig. 20 (f) is the banding pattern of protein recovered from a Sephadex G-25 column. The protein loaded on the column was that yielding the pattern in (b); it is seen to be essentially identical to (f) except that the latter is now free of low molecular weight contaminants and more dilute. One can also examine samples, electrophoresed as above, at another wavelength to spot nucleic acids.

Biopolymers appearing in media of suspension cultures are also being examined by the foregoing procedures. Some results should be available for the annual report.

FEEDBACK AND PERTURBATION RESEARCH

Real feedback experiments have not been very extensive because they depend upon results of compositional analyses which are, in many cases, still in progress. In view of the results of the amino acid analyses, some experiments were conducted in the Tissue Culture Laboratory employing lysine-enriched media. Much as expected, lysine has not caused differentiation, either alone or in combination with other variables; if anything, it has induced better and more uniform growth of undifferentiated callus. However, these feedback experiments led to the observation that the pH drops to rather acidic levels during callus

growth, and pH adjustments of media per se have had some effects with more embryoidal formations appearing at the more alkaline pH's. Continuation of these studies at the level of cell suspensions has shown that the pH drop is quite rapid. Attempts to prevent it by buffering have not been met with much success to date. Several possibilities for the cause of the pH drop are under investigation. Other investigators are encountering these pH effects associated with the action of plant hormones (15).

Several attempts have been made to screen chemical additives to callus tissue by direct dropwise applications (drop tests). Although Douglas-fir callus growing in the light is green, drop tests and other observations suggest that it is not photosynthetic to a degree sufficient to be completely autotrophic. A study of its sustaining metabolic processes is just beginning. Some "add-back" drop tests have produced interesting responses, such as the formation of tracheid-like cell structures. Complex mixtures of nonpolar compounds have been most effective in these endeavors. Further investigations of this type will be forthcoming, but there are some technical difficulties to be surmounted in this work. Mesityl oxide has induced the formation of embryoids in callus rather consistently; its use is based upon a very general hypothesis for the mechanism of differentiation. Some of the additives have recently been introduced to cells in suspension cultures by media incorporation. It is likely that this will be a favored approach in the future for reasons already noted.

FUTURE PLANS

Much of the research in this laboratory for the next six months will be a continuation of investigations already in progress, i.e., compositional analysis with feedback and perturbation experiments. There should be a gradual

shift in the direction of more feedback experiments as more comparative compositional data become available. Also, there will be some metabolic experiments, probably confined mostly to the in vivo level and based upon observations noted in this report. As projected in our report and meeting schedule, isotope tracers are apt to be brought into this research soon.

It is expected that cell suspensions may find increased use as the experimental "animal," hopefully facilitating data interpretation and increasing the rate of progress. Considerable attention will be given to temporal analysis of cell suspension growth media in combination with structural and ultrastructural observations of cytological changes. Compound X will receive further scrutiny.

ELECTRON MICROSCOPY PROGRAM

INTRODUCTION

The use of microscopical methods is an essential part of any investigation directed toward a comprehensive description of a biological system. Frequently, depending on the research objectives, light microscopy (LM) at the tissue and/or cell level is sufficient. However, if a knowledge of intracellular anatomy (i.e., ultrastructure) is to be gained, the electron microscope (EM) is required, yielding pictorial data down to essentially the molecular level. Since all imposed environmental stimuli, both chemical and physical, are directed at controlling the response of individual cells, it is desirable to record in some way the cellular changes induced by these stimuli, whether the latter are beneficial or not to the overall tissue composed of such cells. In the event that a tissue responds favorably to a given treatment, it will likely be possible to correlate such action with the ultrastructure of those cells involved. If there is any difficulty in repeating these same experiments, it is only via the electron microscope that one can judge (at least ultrastructurewise) as to how closely the conditions for repeated success are being approached. Of course, there is no guarantee that, if and when this cellular ultrastructure is matched to that of a once successful tissue, said tissue will respond identically. Still, there is much information at the cellular level that can be gained and put to use as a gage of the overall influence and/or effectiveness of the treatments selected by the cytochemist.

OBJECTIVES

The primary goal of the Electron Microscopy Laboratory during the past 6 months has been to develop schedules of microtechnique that will produce accurate

and reliable data on the ultrastructural state of various callus tissues and suspension-cell cultures of Douglas-fir. Such specimens are extremely sensitive to chemical treatments, including those imposed experimentally by the cytochemist or those by the microscopist for cell preservation. Consequently, chemical fixation of such tissue in a manner that will permit distinction between fact and artifact (from preparation) is not elementary. A further imposition here is the potential but natural variability in cell ultrastructure between and within tissue sources. Clarification of this variability is imperative for meaningful interpretation of typical cell ultrastructure and demands correlation of data from both light and electron microscopy.

REFINEMENT OF MICROTECHNIQUES

Since the initial consideration here was to observe via light and electron microscopy sections of the same cells/tissues, numerous fixation schedules, staining methods, embedding media, and other preparation variables were examined with respect to their compatibility for combined LM/EM studies. These procedures included both classical as well as more recent adaptations of plant cell microtechniques.

LIGHT MICROSCOPY

Recent literature (16-21) was available as a reference for some of the stains and other procedures already demonstrated useful for study of plant tissue culture and especially for coniferous species. One salient feature of such tissue is the frequent occurrence of tannins [various polyphenols (16,18)] and their possible correlation with cell viability (18,19) and culture conditions (16,17). Various stains were tried here, including the classical ferrous sulfate and nitroso reactions (22-24) as well as a novel application of the "Pap"

stain commonly used for illustrating hyperchromatic cell nuclei in human cells (25,26). Staining was carried out on whole cells, paraffin-embedded and sectioned cells, and epoxy-embedded and sectioned material. Although some schedules provided limited polychromasia within the cultured cells, these techniques were eventually labeled as either inconvenient, not consistently reproducible, or insufficiently graphic for our needs. More convenient for routine light microscopy of tissues destined for the EM were some stains utilized in conjunction with those already a part of the EM schedule. Figures 21-28 are TEM and light micrographs illustrating a variety of cell structures and inclusions observed in Douglas-fir callus.

It was found that utilization of p-phenylenediamine (PPDA) as a means of staining osmium-fixed cells (27) intensified already darkly stained tannin deposits. In phase contrast, these deposits were rendered orange in color and stood out clearly from the rest of the cell. In black and white, the tictorial effect is much less dramatic. The use of toluidine blue (28) enhances the normally thin cell wall of callus cells (see Fig. 21 and 23).

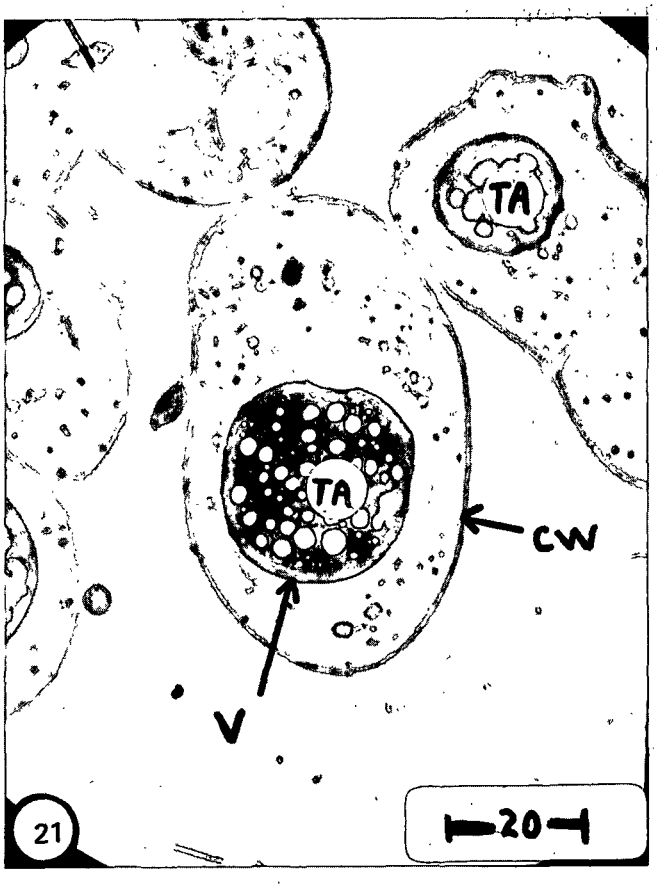
The only significant discovery in the EM lab via light microscopy has been that in Douglas-fir callus recently shown to produce shoots, the cells are essentially devoid of lipid bodies (droplets). The latter are clustered or scattered in the medium outside the cells, and a similar situation, though not as extensive, exists for suspension cultures derived from this same callus. The extruded lipids appear blue after staining with toluidine blue, and in phase contrast are easily distinguished outside the cell wall (see Fig. 24 and also 25-33). The significance of this finding will hopefully be deciphered after further study.

Figure 21. Healthy Green Callus of Douglas-fir. Note Tannin (TA) Deposits in all Vacuole (V); Cell (CW). Light Micrograph (LM). (Line Scale on all Micrographs is in Micrometers)

Figure 22. Callus Similar to that in Fig. 21; Nucleus (N). Transmission Electron Micrograph (TEM)

Figure 23. Dividing Callus Cells. Cell A: Nucleus is in the Form of Two Clusters of Chromosomes, Representing Early Telophase of Mitosis. Cells B and C: Here the Crosswall Between the Two Daughter Cells has Formed, and the Nuclei are Almost Reformed. The Chromatin Material in the Daughter Nuclei is Redispersed, and Cytokinesis (Cell Division) is Complete. LM

Figure 24. Callus from Cotyledon Tissue Which Gave Rise to Shoots. The Interesting Feature of These Cells is that Almost all Cell Lipid Bodies (L) Normally Located Throughout the Cytoplasm (Ground Plasm) are Situated Outside the Cells. LM


TA
TA
CW
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21
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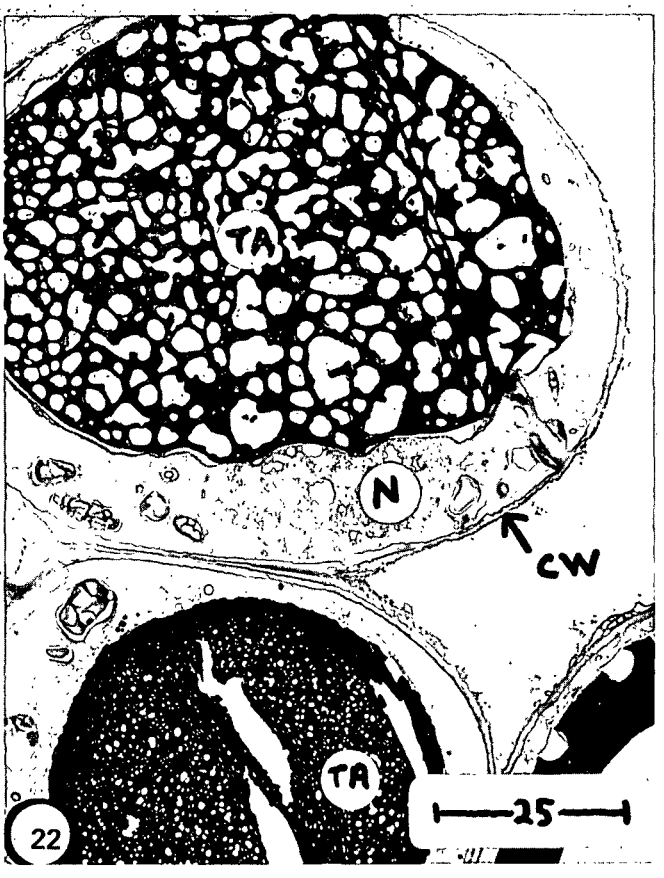
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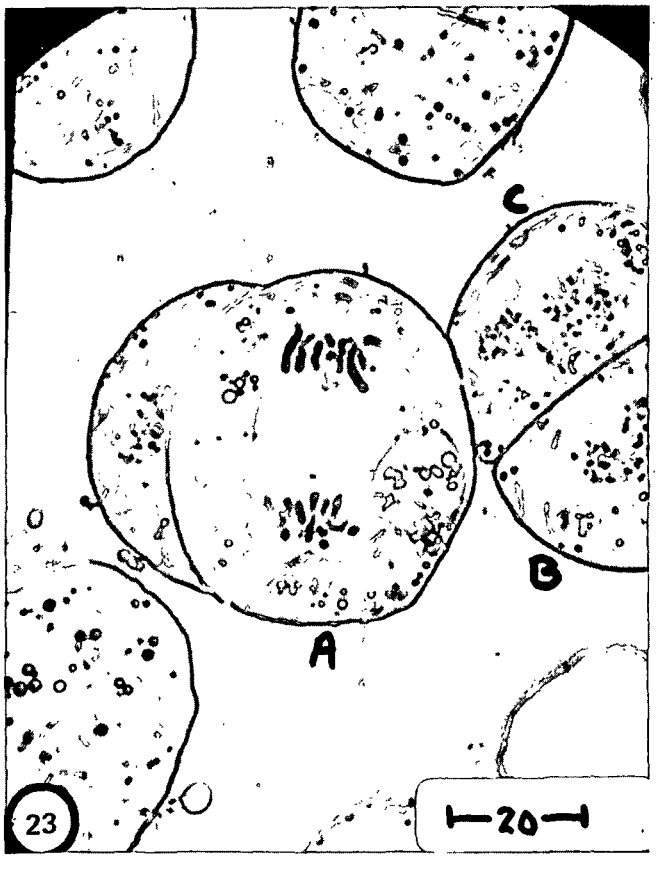
C
B
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L
L
L
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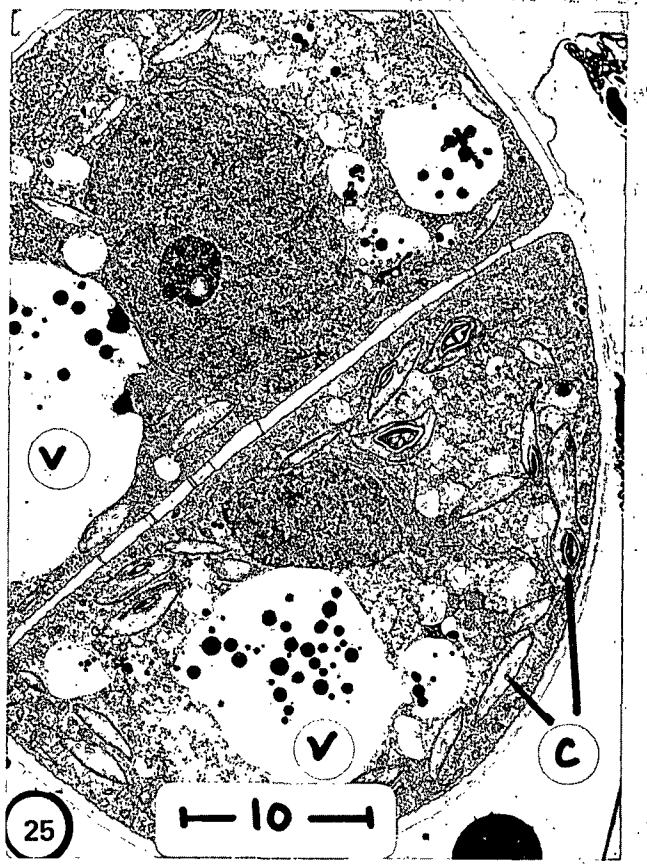
20

Figure 25. Cells Appear Normal with Variable Amounts of Tannin in the Vacuoles. Chloroplasts (C) Contain Some Starch (S). TEM. (Line Scale on all Micrographs is in Micrometers)

Figure 26. Numerous Mitochondria (M) that were Encountered in this Type of Tissue. Note Also the Starch-Storing Chloroplasts (C). Perhaps the Latter are Actually Amyloplasts at this Stage. TEM

Figure 27. Extruded Lipid Droplets (L), Plasmalemma (P), and Cell Wall (CW) at Periphery of Callus Cell. TEM

Figure 28. High Magnification View of What is Probably Lipid (L) Being Extruded from a Callus Cell


V
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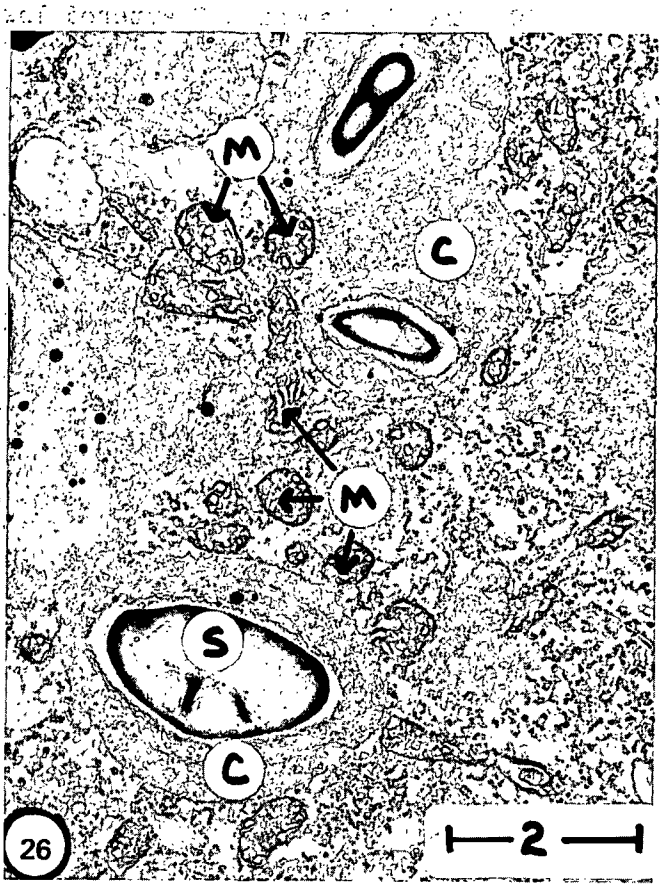
M
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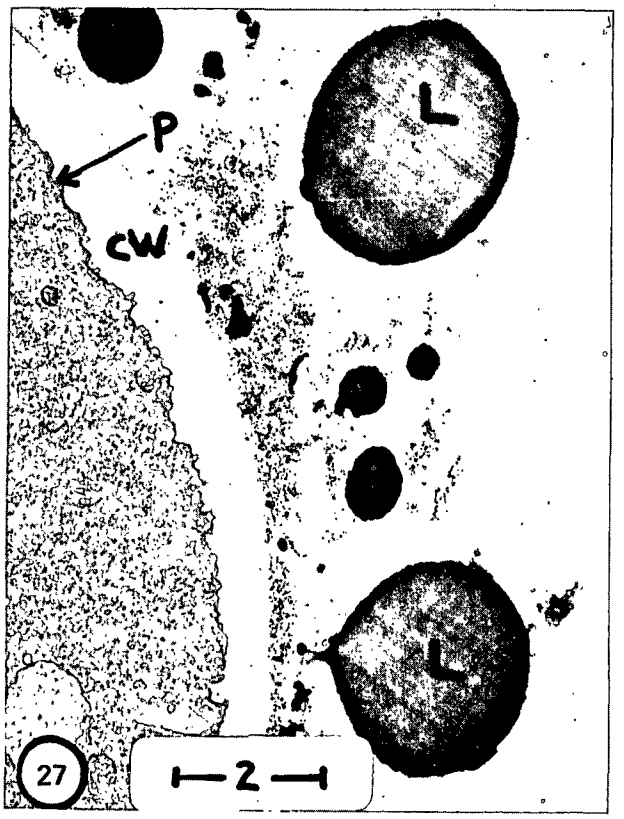
P
CW
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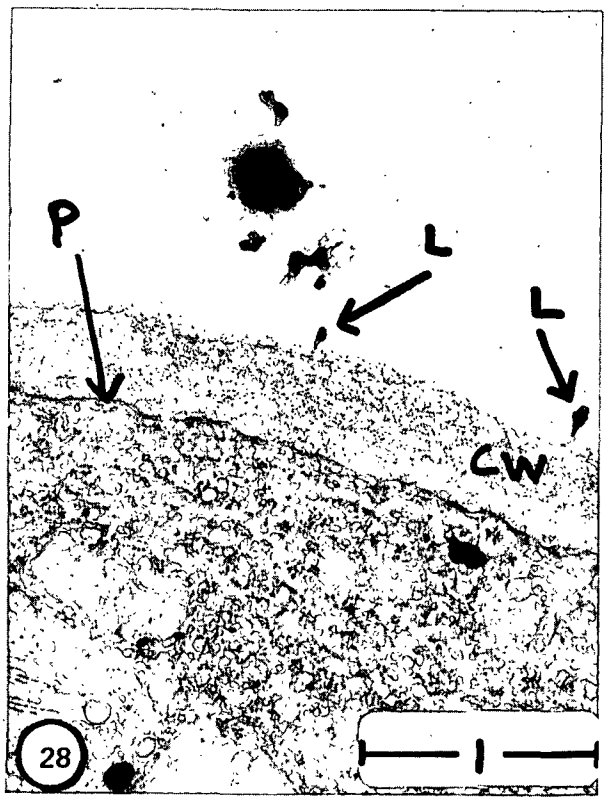
P
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1

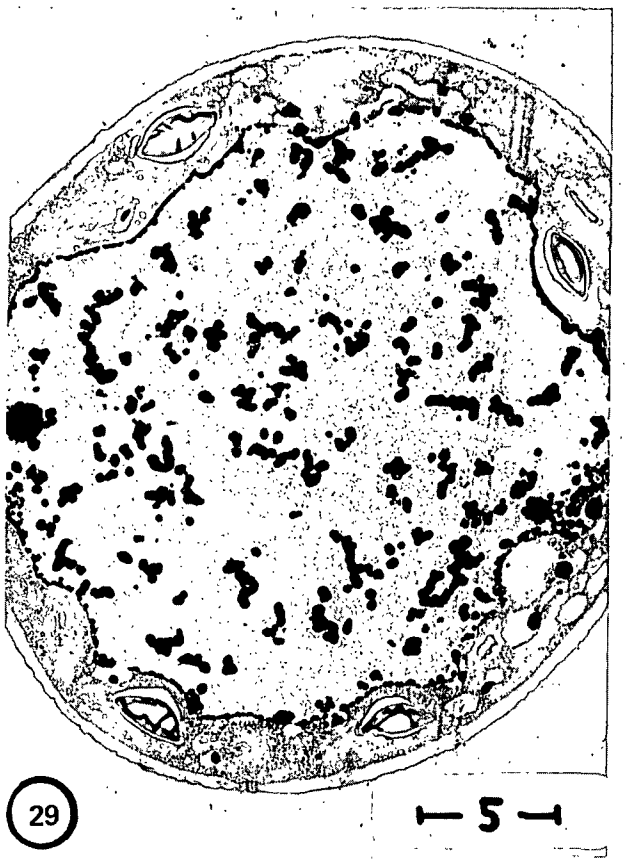
Figure 29. Tannin Deposits Scattered Inside and Lining a Large Vacuole. TEM. (Line Scale on all Micrographs is in Micrometers)

Figure 30-33. Suspension-Cultured Cells Derived from the Shoot-Producing Callus in Fig. 24-29.

Figure 30. Concomitant Accumulation of Starch and Tannin, a Phenomenon not Usually Attributed to Callus Cells. TEM

Figure 31. Higher Magnification of Fig. 30. Note the Aggregation of Microvesicles Between the Two Vacuolar Regions. It has been Suggested (16) that These Vesicles are Derived from the Endoplasmic Reticulum (ER) and Play Some Role in Secretion of Cell Tannin and/or Cell Degeneration. TEM

Figure 32. Junction of Three Cells in Suspension. Note the Rough Surface Where Cells do not Abut. Apparently, this Surface Roughness is a Feature of Cells Cultured Without Additional ATP in the Medium (See Also Later Fig. 45-48). TEM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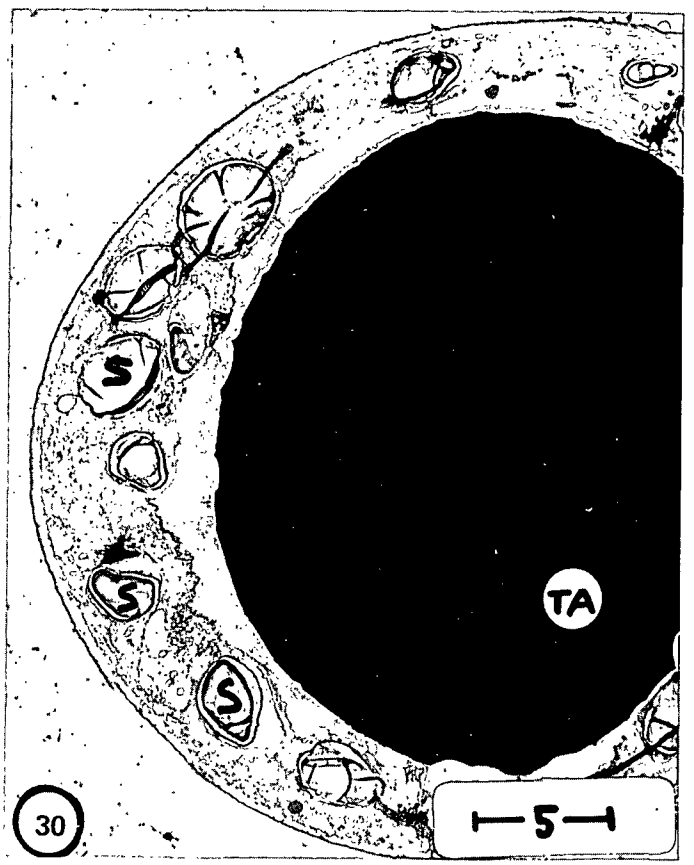
S
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30
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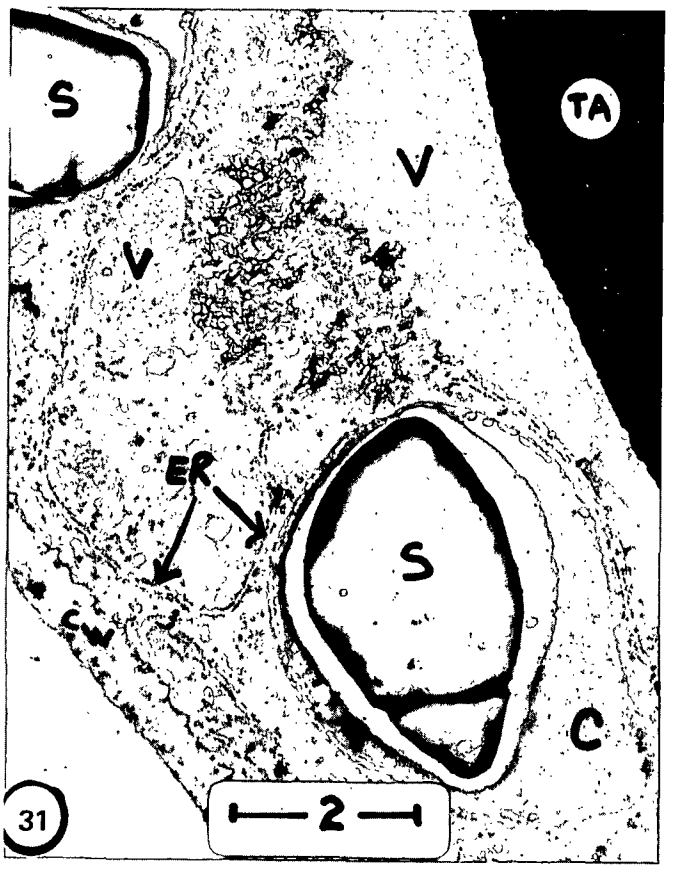
S
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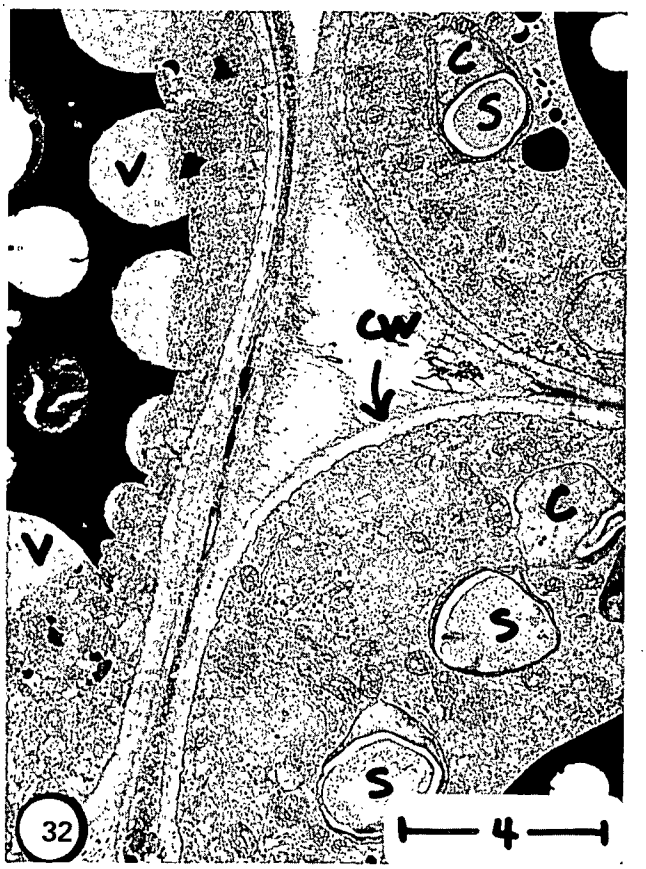
31
2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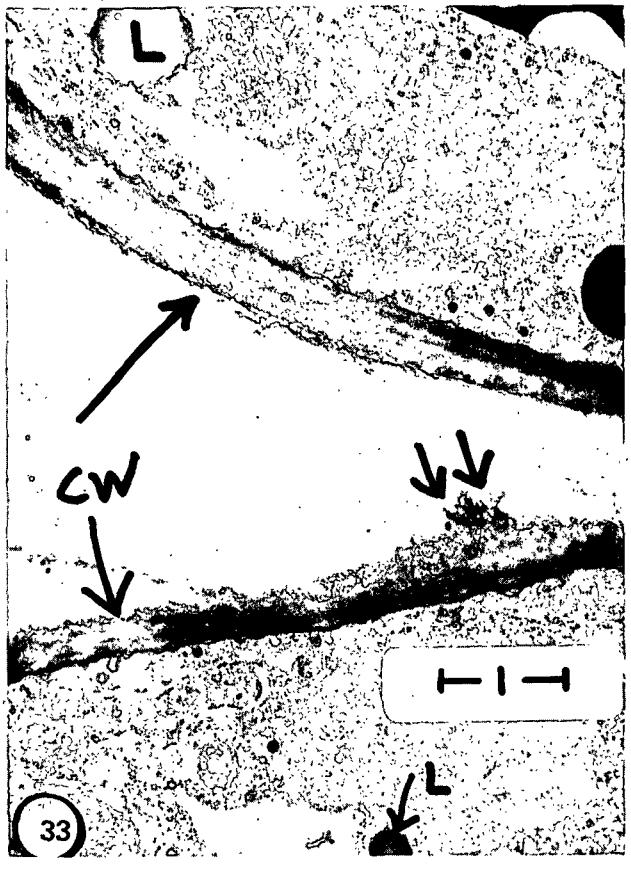
4

Figure 33. Same Cells as in Fig. 32. Note the Rough Cell Surface (Arrows). Some, but Minimal Lipid (L) Levels Retained by the Suspension Cells in Contrast to the Callus from Which These Cells were Derived. TEM. (Line Scale on all Micrographs is in Micrometers)

Figure 34. Different Callus Source Tested for Lipid Content. The Exact Same Preparation Procedures were Used here as for Those Cells (Fig. 25-33) with Extracted Lipid. Note that the Lipid (L) is Retained, Implying a Distinct Difference in the Ultrastructure of Callus Cells Giving Rise to Douglas-fir Shoots. The Nature and Cause of this Difference Remains to be Clarified. TEM

Figure 35. Dividing Nucleus in Douglas-fir Callus. Same Cells as Seen in Earlier Fig. 23, Cell A. Note Condensation of Chromosomes at Poles of Spindle Network. TEM

Figure 36. Chromosomes in Fig. 35 Mixed with Fragments of Nuclear Envelope and Endoplasmic Reticulum. The Latter Two Structures are Indistinguishable Here. TEM


L
CW
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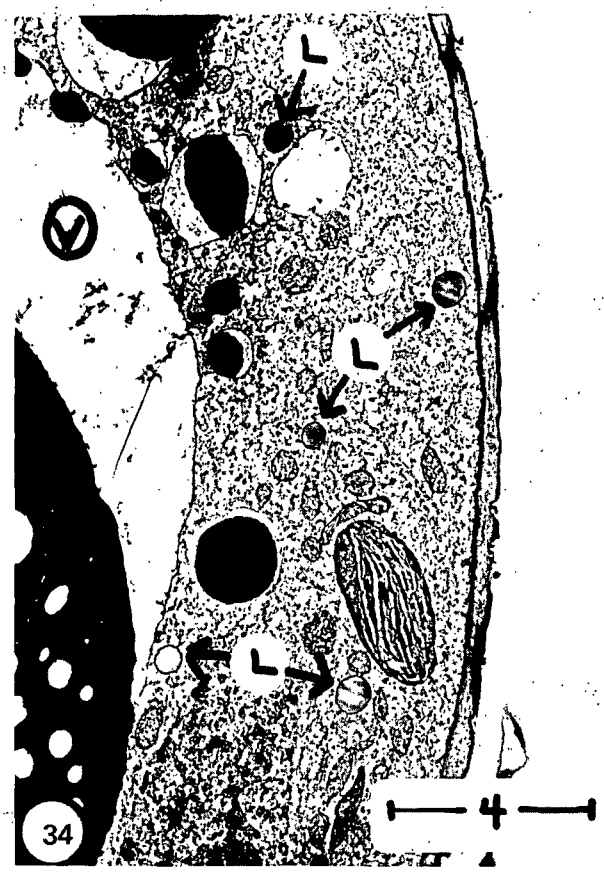
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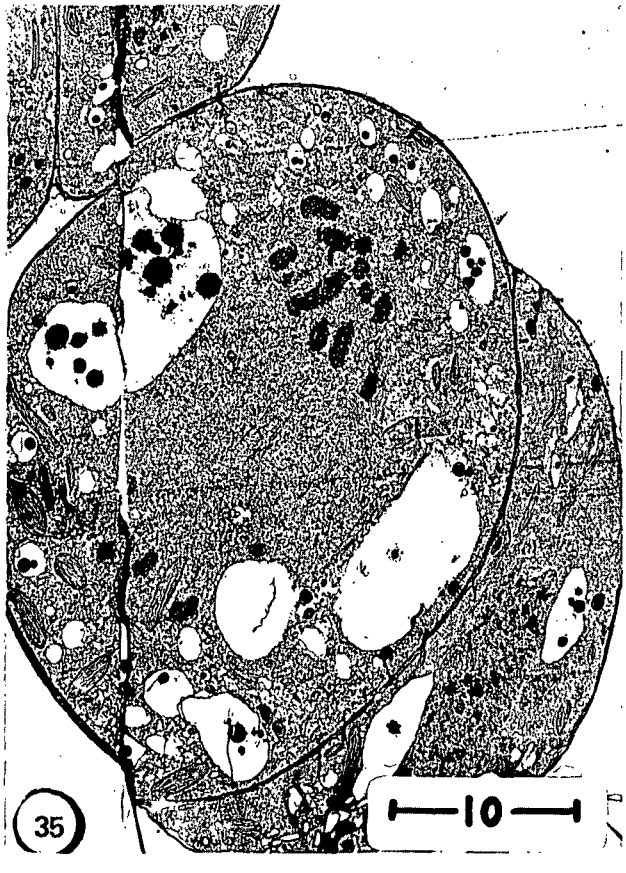
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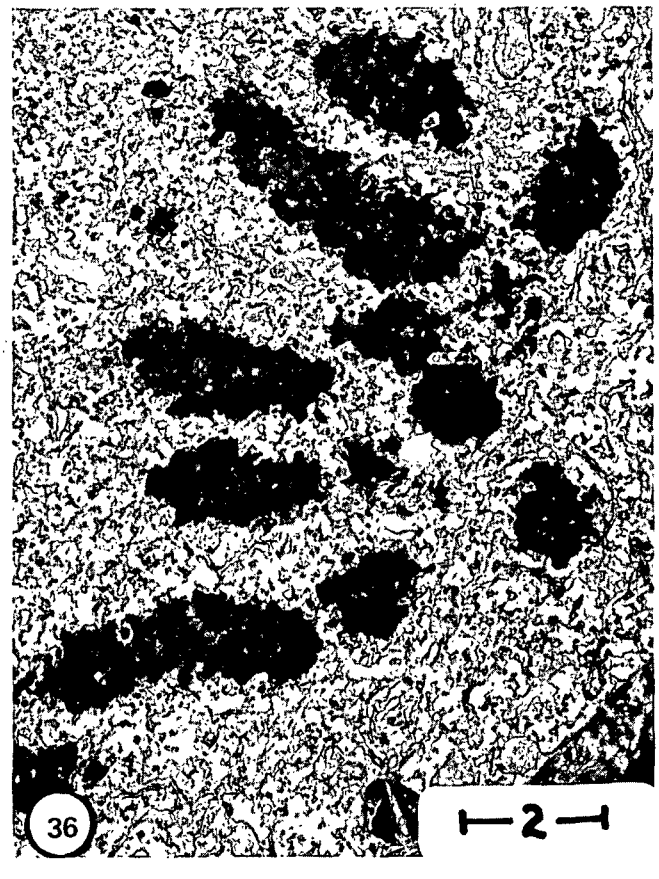
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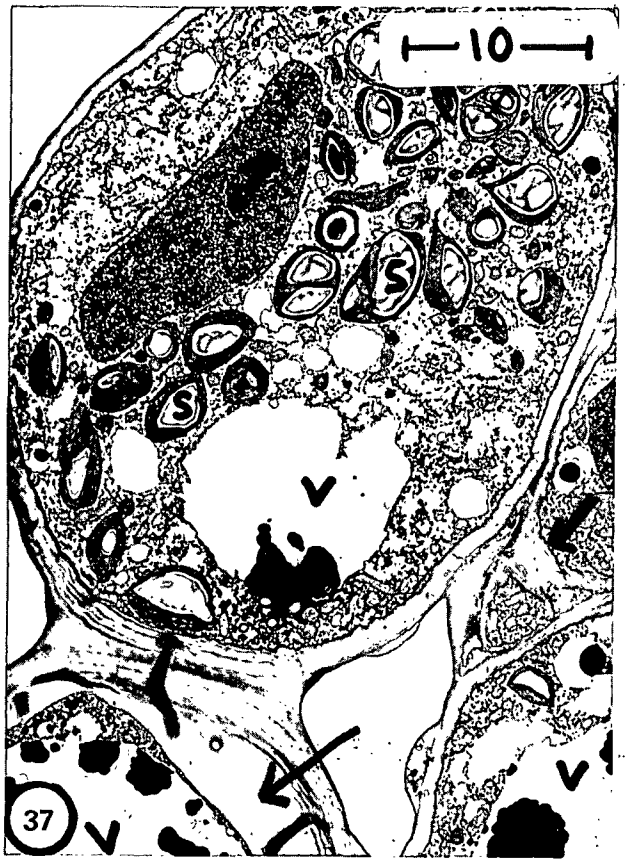
Figure 37. Thick-Walled, Firm but Green Callus. Note Abundant Starch, Relatively Little Tannin, and the Presence of Cell Wall Thickenings (Arrows). The Latter are Secondary in Nature and are Formed to the Inside of the Typically Thin and Primary Cell Wall of Nonvascularized Callus. These are Probably Cross Sections of Helical Wall Thickenings, an Inherent Anatomical Characteristic of Douglas-fir Fibers. Note Also the Abundant Intercellular Matrix at Various Locations Where Cells Touch. TEM. (Line Scale on all Micrographs is in Micrometers)

Figure 38. The Wall Thickenings (*) are Especially Prominent Around Pits Forming Between Contiguous Cells. TEM

Figure 39. In Cells Switched from a Dark Environment to a Day/Night Cycle, Callus Tannin Droplets that are Originally More or Less Spheroid Take on Additional and Smaller Deposits of Tannin at Their Periphery. The Overall Effect is Rounded but Bumpy Deposits of Tannin, or Else Smaller Secretions into the Vacuole as Seen in Fig. 40. Note Here Also the "Pustule" (Arrow) on the Outside of the Cell Wall. These were Common in the Very Firm Callus, Especially on Exposed Cell Surfaces and at Intercellular Spaces. TEM

Figure 40. Same Tissue as Fig. 39. Cytoplasm is Well Preserved but Cell Also has a Large Amount of Newly Secreted Tannin (TA). Just to the Left of the Lipid Droplet (L), Small Secretions of Tannin can be Seen Forming in Swollen Strands of the Endoplasmic Reticulum (ER). Ribosomes are Seen as Small Black Specks in the Ground Plasm and/or on the ER. TEM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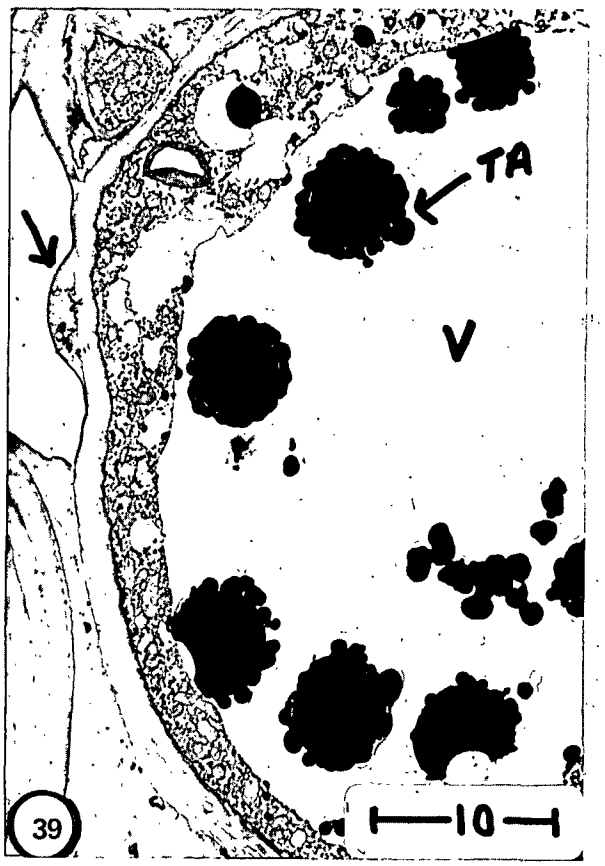
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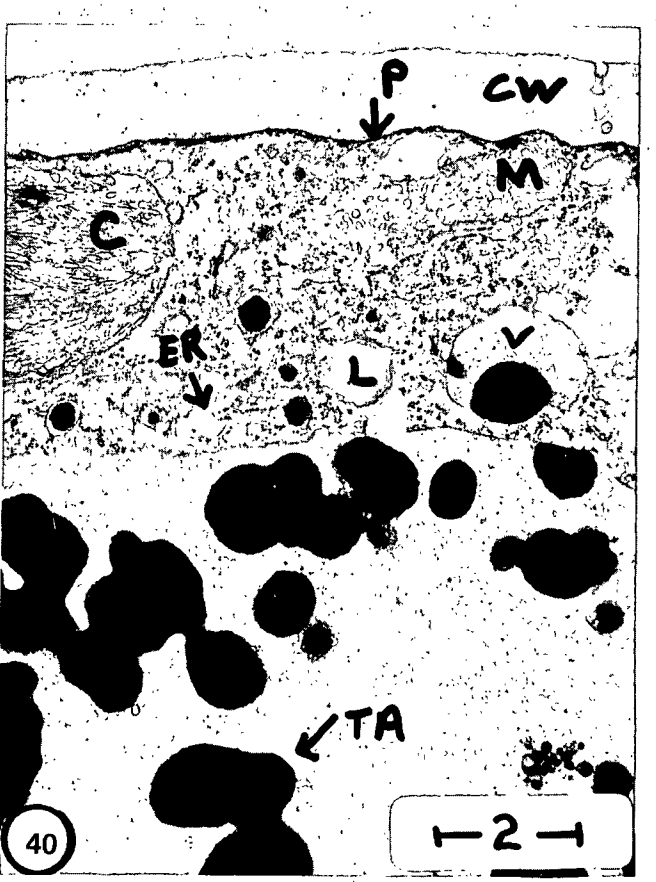
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The other staining methods applied thus far but without too much promise are listed in Ref. (19-35). Some other stains remain to be tested (36,37).

ELECTRON MICROSCOPY

Many of the remarks relevant to EM experiments apply also to the LM work, since the same tissue embedments were used for both phases.

After trying a variety of chemicals and schedules for tissue culture preservation, a combined fixative of 2% glutaraldehyde and 2% acrolein in 0.05M sodium cacodylate buffer (pH 7.0) for 10 hr at 4°C was deemed to yield the most reliable cell image. This treatment was followed by a postfixative soak in 1% osmium tetroxide (also in buffer) for 11 hr at 4°C. Dehydration via ethanol or acetone preceded embedment in a variety of epoxy formulations. Low-viscosity mixtures (38) of soft, firm, and hard plastic were tried before a suitable formulation was found that would permit both ultrathin (50 nm for EM) and semi-thick (0.5-1.5 μm for LM) sections of the same tissue region to be cut consecutively on the ultramicrotome while employing a diamond knife. Ultrathin sections were normally stained with uranyl acetate and then lead citrate prior to EM observation. The thicker sections were dried onto glass slides and processed for light microscopy of the same cells.

CALLUS CELL ULTRASTRUCTURE

Documentation of the internal cell anatomy of both callus and suspension cells has led to the conclusion that there exists a tremendous variability, even between adjacent cells. Figures 22 and 25-40 serve to illustrate typical cases of this sort. This factor will continue to receive special attention in the future.

It is hopeful that individual cells, or more likely, cell clusters (E-cells) can be processed individually with corresponding observations from both light and electron microscopy.

Some features of interest in Douglas-fir cells that have been revealed by EM study include:

(1) Secondary cell-wall thickenings (probably helical and characteristic of the species) in various cultures, indicating "vascularization" of some tissues (Fig. 37, 38).

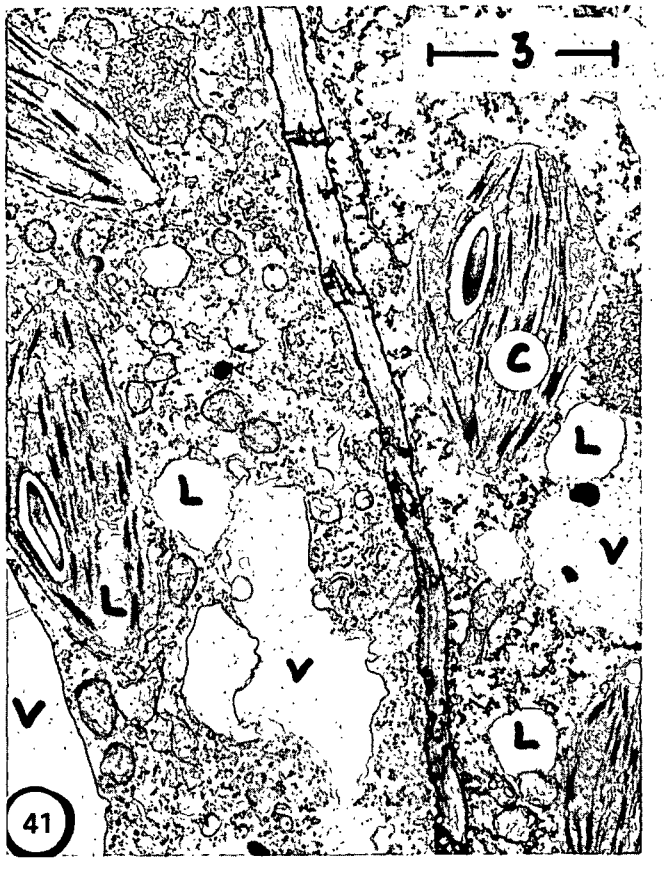
(2) "Pustules" on the external surface of some cells, probably those having an exposed face to the surrounding air (Fig. 37, 39).

(3) The presence of essentially all organelles normally necessary for functioning of healthy callus or suspension cells, as well as alterations in the morphology and number of those organelles with cell viability (especially chloroplasts and starch synthesis, mitochondrial sophistication and distribution, microbodies (24), ribosomes and indication of protein synthesis, lipid accumulation, and vacuolar tannin deposits and origin of the latter from the endoplasmic reticulum (see Fig. 39-44).

(4) External surfaces of suspension cells as seen after fixation, dehydration, and critical-point drying. Easily seen here are intercellular microfibrils and attachment within cell clusters as well as decorations on the cell surfaces. Apparently, the cell surfaces can be altered quite radically by the presence or absence of ATP (see Fig. 45-48).

All details recorded via the EM cannot be elaborated upon in this report nor are they of general interest. However, suffice it to say that the physiological state of both callus and suspension cells is definitely reflected and detectable at the ultrastructural level. Utilization of the EM as a "process control" tool has been minimal thus far, but with the determination in the tissue culture and biochemistry labs of those factors or stimuli that will induce significant (and favorable) macroscopic alterations in test specimens, it is anticipated that the EM lab will be able to successfully describe concomitant and corresponding

Figures 41-44. Variability Commonly Encountered in Callus Without (Fig. 41, 42) and with (Fig. 43, 44) High Proportions of "Embryoid" or E-Cells. It is not Rare to Find Very Healthy Cells Immediately Adjacent to Essentially Moribund Cells in Most Types of Functioning Callus. This Variation is Attributable to the Callus Itself and not to Preparation Procedures, Since Tissues Treated Identically Display Similar Preservation Except for This Easily Documented Variability. TEM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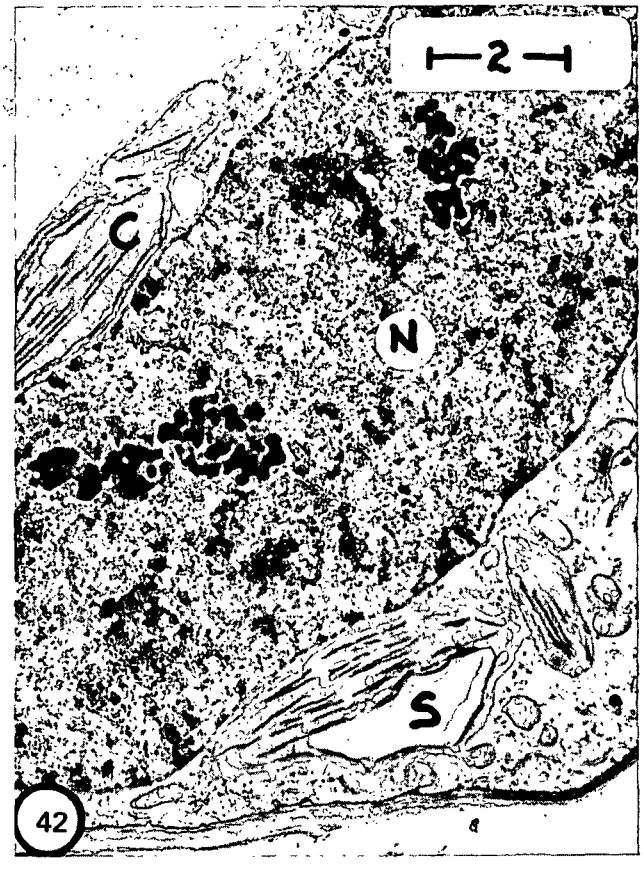
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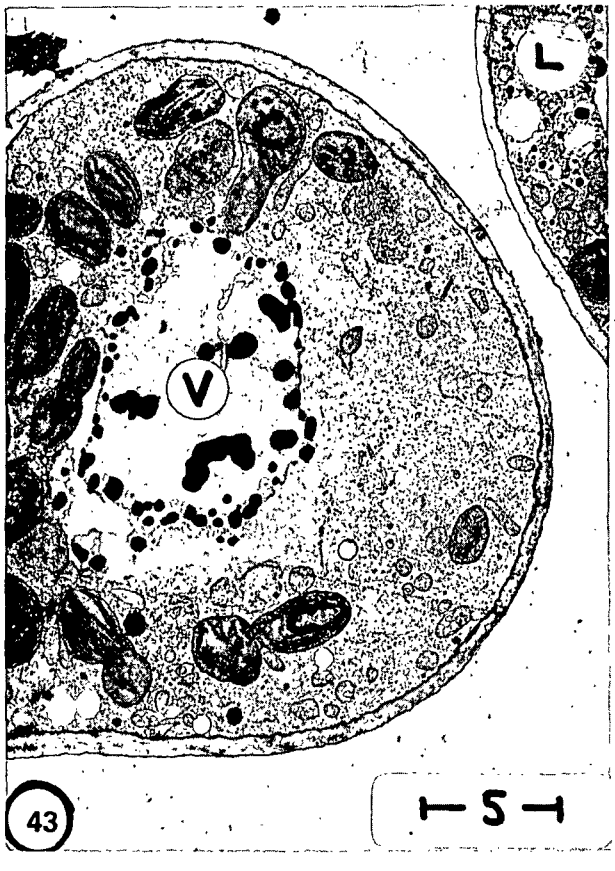
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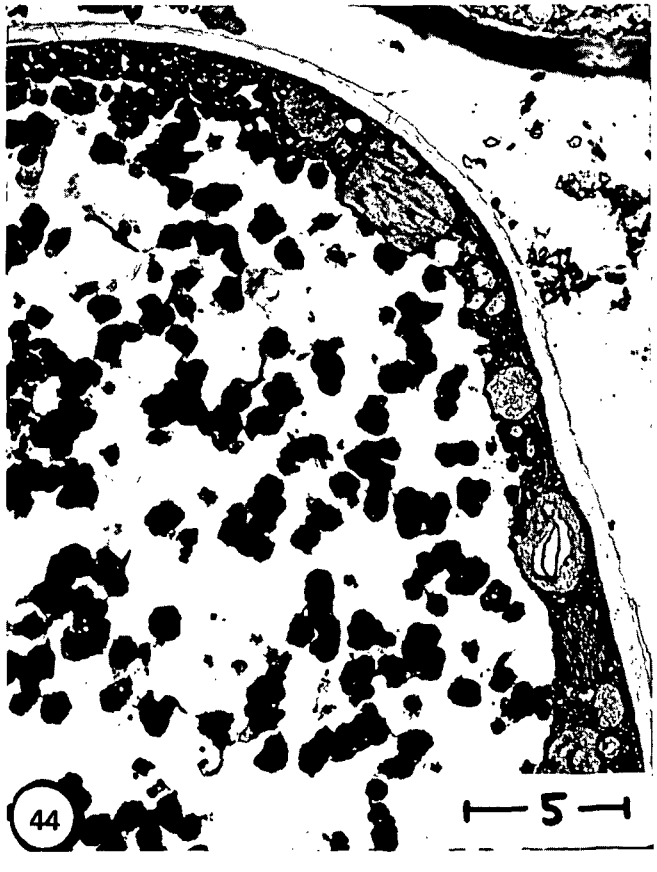
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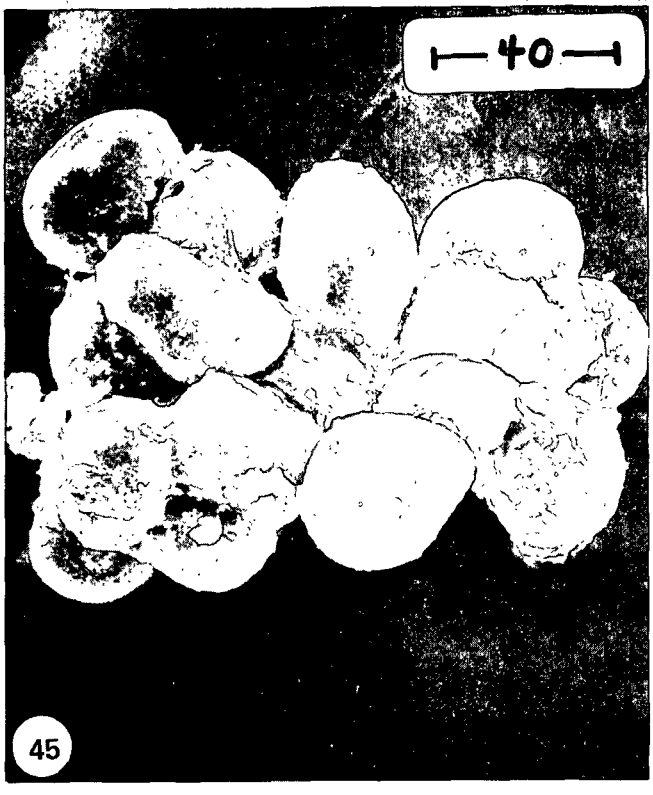
Figures 45-48. Suspension-Cultured Cells After Chemical Fixation and Critical-Point Drying. These Four Views are Scanning Electron Micrographs (SEM). (Line Scale on all Micrographs is in Micrometers)

Figure 45. A Cluster Supposedly of E-Cells Which are Physically Bonded by Cell Wall Material. SEM

Figure 46. An E-Cell Aggregate that has Apparently Developed Division Polarity and is Probably Forming an Embryoid. SEM

Figure 47. Same Cells as in Fig. 45. Note Here the Very Rough Cell Surface, Probably Due to the Omission of ATP in the Liquid Culture Medium. The Intercellular Connections (Arrows) are Easily Resolved Here. SEM

Figure 48. Higher Magnification of Intercellular, Microfibrillar Connections Depicted in Fig. 47. SEM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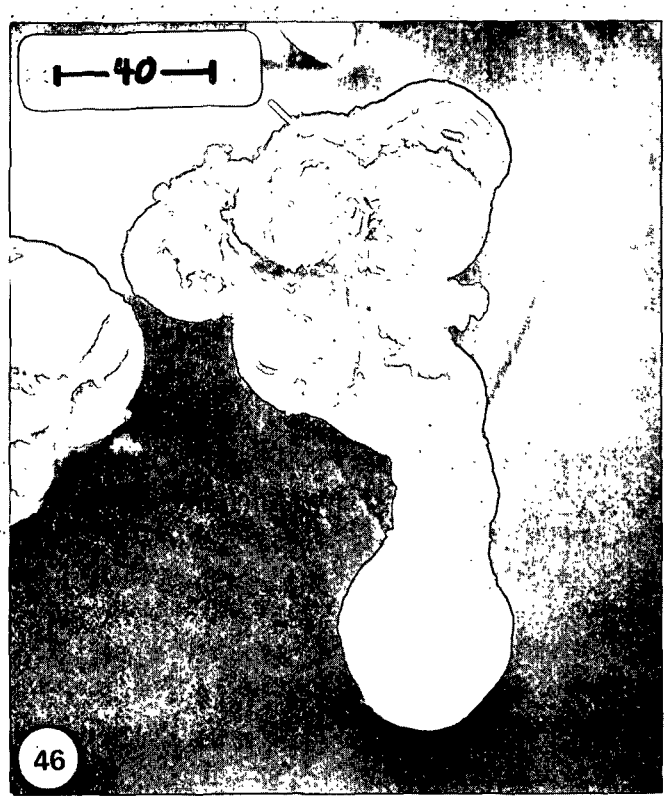
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changes within the participating cells. Further experiments may also allow us to suggest changes in treatment applications after monitoring either beneficial or nonbeneficial changes at the intracellular level.

FUTURE PLANS

Early research emphasis has concentrated on developing and refining techniques for fixing, embedding, and staining callus and single cells of Douglas-fir and loblolly pine. During the past three months, the first treatment difference was characterized using SEM, TEM, and light microscopy. During the next six months, studies conducted by the Electron Microscopy Lab will include:

(1) Additional TEM observations on control and treated callus from the tissue culture and biochemical laboratories.

(2) Additional SEM observations of callus cell surfaces and cell organization using newly acquired critical-point drying capabilities.

(3) Accumulation of enough TEM and SEM data to permit meaningful interpretation of biochemical and environmental treatments under way.

(4) Correlation of these data to light microscopy and the staining characteristics of the same callus tissue.

PLANS

A recent discussion by Winton, et al. (40) presents a useful overview of the potential that exists for increasing tree improvement gains by callus, cell, and protoplast culture. With the ultimate objective of parasexual (vegetative) hybridization in mind, the overall research plan calls for early emphasis to be placed on the intermediate objectives of: (1) differentiation of diploid callus into trees and (2) differentiation of diploid "single cells" in liquid suspensions into embryoids and trees. Biochemical comparisons of differentiated and undifferentiated callus from (1) above are expected to be useful in speeding progress in the development of the extremely valuable "single cell — embryoid — plantlet" technique. Light and electron microscopy are expected to assist in the evaluation of biochemical treatments.

Detailed plans for the next six-month period are presented as the last section of reports given by the three IPC laboratories involved in Project 3223.

ACKNOWLEDGMENTS

The authors of this report are indebted to individuals from the various paper companies who helped formulate the direction of this program. Special thanks also go to Shirley Verhagen of the Tissue Culture Laboratory, John Carlson of the Biochemistry Laboratory and Hilkka Kaustinen of the Electron Microscopy Laboratory for their assistance in carrying out many aspects of the program. Also acknowledged is the assistance of Marianne Harder in preparing this report.

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GLOSSARY

Amyloplast - A colorless plastid modified for starch storage.

Asexual reproduction - Reproduction without fertilization. New individuals may develop from vegetative parts such as tubers, bulbs or rooted stems, or from sexual parts such as unfertilized eggs or other cells in the ovule.

Auxins - A class of plant growth hormones of diverse makeup which cause cell enlargement, apical dominance and root initiation.

Callus culture - The dissociated component cells of a tissue growing on a nutrient medium.

Cell suspension - A liquid culture of small groups of cells agitated in some way to provide aeration and growing in a liquid nutrient medium.

Chloroplasts - A membrane-enclosed subcellular organelle containing chlorophyll. Chloroplasts are the sites where photosynthesis takes place. They contain DNA and polysomes and are capable of replication.

Clonal propagation - Propagation of a group of plants derived from a single individual (ortet) by asexual reproduction. All members (ramets) of a clone have the same genotype and consequently tend to be uniform.

Cytokinin - A class of plant growth hormones associated with cell division, assisting with the transmission of the genetic information from the genes to the proteins.

Differentiated callus - Result of the process whereby cells in a callus mass divide to give rise to dissimilar, more specialized tissues.

Diploid - Having two sets of chromosomes in the nucleus. One-half of the chromosomes are contributed by the female parent, one-half by the male parent. Many higher organisms are diploid except for their sex cells and associated tissue.

E-cells - Embryonic cells that have a relatively small central storage vacuole surrounded by a thick cytoplasm. Normal callus cells have a large vacuole and a small amount of cytoplasm.

EM - Electron microscope.

Embryo - A young plant developing from an egg cell, after fertilization, or without fertilization.

Embryoid - A cell group approximating an embryo, but with a more random cell arrangement.

Empirical method - Method based solely on experiment and observation.

Endoplasmic reticulum - A system of membranes (originating from the external membrane of the nuclear envelope) that permeates the cytoplasm and which may or may not be covered with ribosomes.

Enzyme - A protein molecule that catalyzes a specific chemical reaction.

Eucaryotes - The superkingdom containing all organisms that are, or are made up of, cells with true nuclei bounded by nuclear envelopes and which undergo meiosis.

Fertilization - Sexual fusion of male and female nuclei.

Gene - One of the units of inherited material carried on a chromosome; arranged in a linear fashion and indivisible.

Gene pool - Reservoir of genetic variability available for use in genetic improvement of tree species.

Genetic gains - Average improvement in progeny over the mean of the parents.

Genetic variability - The variation existing in a given population (species, for example) with respect to particular genes or arrangement of genes.

Groundplasm - Homogeneous plasma (matrix) remaining after cell organelles and particles have been excluded.

Haploid - Having the reduced chromosome number, i.e., having one set of chromosomes in the nucleus. This is normal in sex cells, which have only half the number of sets occurring in diploid vegetative cells.

Hormone - All classes of growth substances which are generally transported to the site of action and can stimulate growth or cell enlargement (auxins), cell division (cytokinins), stem elongation (gibberellins), or can retard growth as in the abscission of leaves (ethylene).

Hybrid vigor - The increase in vigor, size and fertility of a hybrid as compared with its parents, resulting from the union of genetically different gametes and assumed to be due to special recombinations of dominant and recessive genes (heterosis).

Hybridization - The production of offspring of genetically different parents.

Hydrophobic - Water repelling.

Interspecific hybrid - The progeny from matings between species.

Intraspecific hybrid - The progeny from matings within species.

Lipids - Any of a group of biochemicals which are variably soluble in organic solvents like alcohol and barely soluble in water.

Mitochondria - Small bodies in spaces of the ground cytoplasm. They are spherical, long rods, or threads, and are the sites of many important enzymatic processes. The inner layer of the wall is infolded into fingerlike processes.

Nonrelated species - Species that are members of different genera.

Organelles - Complex cytoplasmic structures of characteristic morphology and function, such as a mitochondrion or plastid.

Parasexual hybridization - Hybridization resulting from asexual fusion of cells; either diploid or haploid.

Plasmalemma - The semipermeable, unit membrane surrounding and containing the cell cytoplasm. In plant cells, it is pressed up against the inner surface of the cell wall.

Polyploidy - Having three or more times the haploid number of chromosomes.

Procaryotic - The superkingdom containing the viruses, bacteria, and blue-green algae, organisms lacking well-defined nuclei and meiosis.

Protoplast - Spherical cell protoplasm (cytoplasm + nucleus) bounded by a membrane but no cellulose wall.

Protoplast fusion - Union of two protoplasts into one cell.

Ribosome - A macromolecule containing protein and RNA. It is seen as a dense particle in electron micrographs. They are found in all types of cells in which protein is being synthesized.

SEM - Scanning electron microscope.

Somatic - Diploid body cells of an organism; those cells other than germ cells.

TEM - Transmission electron microscope.

Tissue culture - General term for callus and cell cultures of undifferentiated cells.

Ultrastructural - Sublight microscopic, intracellular structure.

Undifferentiated callus - Tissue — composed of undifferentiated cells. Cells — thin-walled, capable of division and generally spherical and uniform in size. Very little, if any, secondary wall thickening and no specialization such as characterizes fiber cells or conductive tissue. The chemical composition is generally that of xylem (wood) cambial cells found in trees.

Vacuole - A fluid-filled space in a cell. A single vacuole, taking up most of the volume of the cell is present in many plant cells, and contains a cell-sap which is isotonic with the protoplasm.

Vegetative cell - Nonreproductive cells such as haploid cells from female gametophytes of conifers or diploid somatic cells.

Vesicle - Small membrane-bound body in the cytoplasm.

Zygote - Fusion product of male and female sex cells or fusion product of protoplasts.

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