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ANNUAL RESEARCH REVIEW

FOREST BIOLOGY

March 27, 1992

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Atlanta, Georgia

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INSTITUTE OF PAPER SCIENCE AND TECHNOLOGY

Atlanta, Georgia

ANNUAL TECHNICAL REVIEW REPORT

FOREST BIOLOGY GROUP

March 27, 1992

TABLE OF CONTENTS

PROJECT 3223-00

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

RESEARCH REVIEW

MARCH 27, 1992

Ron Dinus
Dave Webb

TECHNICAL PROGRAM REVIEW SUMMARY

PROJECT TITLE: MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

PROJECT STAFF: Ron Dinus, Dave Webb, Vacant Position

BUDGET (FY 91-92): $360,000

REPORTING PERIOD: January 1, 1991 - December 31, 1991

DIVISION: Chemical and Biological Sciences

PROJECT CODE: SFTWD

PROJECT NO.: 3223-00

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

GOAL: Build reliable and reproducible techniques for eventual commercialization of somatic embryogenesis as a technology for mass cloning of softwoods.

SUMMARY: Research with Norway spruce, our former model system, has been stopped to permit increased attention to and progress with our main target species, loblolly pine. Small numbers of responsive spruce cultures are being maintained in our culture bank for possible work on conversion to seedlings, genetic fidelity, or similar issues in future.

More than 30 embryogenic loblolly pine cultures were produced in summer 1990. Some differences in growth have occurred depending on media composition and culture vessel. Tests of these factors, as they affect maintenance and expansion, are yielding valuable results. Detailed microscopic assays are being performed, with the best and most stable cultures being added to our culture bank and/or screened for use in maturation trials. The Brazilian culture obtained in winter 1991 has also been banked.

Initiation research in summer 1991 focused on extending accomplishments of 1990. Several more successful treatments were repeated, along with a number of important modifications. In line with established goals, the work also includes an enlarged number of mother trees. The two used in 1990 were included, along with eight others, several of which have never been tested before. Gerogia Pacific, Union Camp, and Westvaco kindly provided developing cones. Approximately 80 embryogenic cultures have been obtained, and 9 of the 10 mother trees produced at least on embryogenic culture. Initiation frequencies in summer 1991 generally exceed those from earlier years regardless of mother tree. As for 1990 cultures, the best and most stable are being added to our culture bank and screened for maturation research.

Work on mineral composition of developing seed has been stopped for the present. The last cone collections and chemical analyses were completed. All available data have been secured, and are being held pending a decision on direction and emphasis given.

In a related vein, a student is examining the role of growth regulators in developing zygotic embryos of loblolly pine. Changing levels of ABA and IAA will be tracked via antibodies and mass spectroscopy, and related to important developmental events. Developing cones are provided by Union Camp, Westvaco, and Weyerhaeuser. In adition, the student is being aided by collaborating scientists at Westvaco and the University of Georgia. To date, techniques for extraction and purification have been developed; high recovery rates and minimal degradation have been confirmed. Methods for methylation and mass spectroscopy have also been tested. Quantifiable amounts are: ABA = +/- 250 picograms and IAA = +/- 125 picograms. Efforts to optimize the antibody technique, a faster and less expensive assay method, are underway.

Experiments on embryo maturation have advanced faster than anticipated. For some time, contamination and slow growth have limited experimentation. While we have not eliminated such problems, essential experiments, though somewhat smaller and simpler than desured, are in progress. All cultures are producing some precotyledonary stage embryos; two are yielding cotyledonary stage embryos. Maltose and culture in darkness remain the most important factors. Inclusion of our best cultures from 1990 and 1991 initiation trials is expected to accelerate progress.

Anticipating somatic seedlings from the aforementioned work, first experiments on conversion to seedlings have been started. Procedures and containers are being tested for germinating, acclimatizing, and moving somatic materials from in vitro to greenhouse conditions. Preliminary results with intact seed and excised zygotic embryos indicate high frequency germination and normal development on DCR medium (full or half strength with 3% sucrose). Transfer of germinants to "rockwool" plugs in Magenta boxes, followed by a brief acclimatization period in the culture room, appears to offer a workable means for transition from culture to greenhouse. Available resources were leveraged by efforts of a highschool science intern, who has established several seedling populations in the greenhouse to provide baseline data on early developmental and growth patterns. As a further preparatory measure, supplementary lighting, misting, and fogging systems have been installed in the greenhouse.

Work on Douglas-fir embryo maturation was stopped, at least for the present. Ongoing experiments, nevertheless, were brought to conclusion, and last results were published in the proceedings of a 1991 regional conference. Cultures are being maintained, pending a decision of future direction and intensity of research with this species.

Genetic fidelity is being addressed via a student project. Techniques for extracting and purifying DNA from several types of pine tissue have been refined. Methods for quantifying yields and purity have also been adapted for routine use. First applications of the polymerase chain reaction system have been successful.

LOBLOLLY PINE

INITIATION

EFFECTS OF GENETIC DIVERSITY, MEDIUM STRENGTH AND CARBOHYDRATES ON INITIATION OF EMBRYOGENIC CALLUS FROM CULTURED MEGAGAMETOPHYTES OF LOBLOLLY PINE

Principal Investigator

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EFFECTS OF GENETIC DIVERSITY, MEDIUM STRENGTH AND CARBOHYDRATES ON INITIATION OF EMBRYOGENIC CALLUS FROM CULTURED MEGAGAMETOPHYTES OF LOBLOLLY PINE

ABSTRACT

Seventy eight embryogenic callus lines were produced from nine of ten half-sib mother trees. Cultured megagametophytes containing early-stage pre-cotyledonary embryos gave the best results. The best overall response by a seed family was 3%. The highest frequency for embryogenic callus production for an individual experiment was 5%. The highest frequency for individual treatments within several exeriments was 12.5%. Initiation involves two phases. Materials were extruded onto the medium in phase 1. Some of these continued to proliferate as embryogenic callus lines in phase two. Extrusions were grouped into five classes. Embryogenic callus developed from each extrusion class, but most of the callus lines developed from extrusion class A which was morphologicallly similar to proliferating embryogenic callus. The production of class A extrusions was strongly correlated with the developmental stages present in the different seed famlies, and seeds with stage 2-3 pre-cotyledonary embryos gave the best response. A similar trend was evident for the production of embryogenic callus lines, but the correlation was not as strong. Equivalent results were obtained with full-strength and half-strength basal media. Embryogenic callus formed in the absence of plant hormones, but was promoted by low

auxin levels. The addition of a cytokinin did not stimulate embryogenic callus production. Maltose was better than sucrose for the production of embryogenic lines, and cold-sterilized sugars gave the best results. Callus lines grew well on media containing a low auxin level. Growth was better on media containing maltose compared to medium contianing sucrose.

INTRODUCTION

A thorough literature review of embryogenic callus initiation for conifers, particularly Pinus species, was presented in the 1991 Annual Review, and only the most pertinent articles will be discussed in this report. The process of embryogenic callus initiation consists of two phases, extrusion and proliferation. The results from 1990 initiation studies [1991 Annual Review] indicated that DCR medium was superior for initiation. While callus extrusion occurred with other media, calli failed to proliferate unless they were transferred to DCR medium. Extrusion occurred in the absence of plant hormones but was apparently enhanced at high 2,4-D levels. The addition of a cytokinin [BA] stimulated extrusion at low 2,4-D levels when the ratio of 2,4-D to BA was 2:1. However, only 8% of all cultured megagametophytes produced extruded callus even though 90% of them contained zygotic embryos. The best extrusion frequencies for individual treatments were approximately 15%. Most of the extruded calli became brown and moribund. While a high proportion of calli proliferated on DCR medium containing 2,4-D [1.1 mg/l],

approximately half grew slowly. These experiments used seeds from two Westvaco half-sib clones, and a cross of the two. The seeds were very uniform and contained stage 2-4 pre-cotyledonary embryos [1991 Annual Report] which were thought to be ideal for initiation [2, 10]. These clones had been used in the past to study embryogenic callus initiation at the IPST. The 1990 results were better than those previously obtained with the same clones, and the cross gave the best response [Table. 1]. However, because of the limited gene pool represented in these experiments it was not possible to know how well other seed families would respond to these treatments.

The major goal of the present work was to determine how a wide array of genetically distinct seed families would respond to the same initiation treatments. This involved clones previously tested at the IPST, and hitherto untested clones. The effects of 2,4-D levels, 2,4-D + BA combinations, full- vs half-strength medium, plus different carbohydrate treatments were also studied.

MATERIALS AND METHODS

Loblolly pine seeds were obtained from Westvaco, Union Camp and Georgia Pacific in early July, 1991. The Westvaco clones were 11-9, 7-34 and "seed source A", hereafter designated as W1, W2 and W3. The Union Camp clones were 10-105, 10-1003 and 10-1018,

hereafter designated as U1, U2 and U3. The Georgia Pacific clones were 8-74, 10-14, 27-10 and 27-9, hereafter designated as G1, G2, G3 and G4. Embryo development was monitored for several clones, and the cone collections were scheduled so that uniform developmental stages would be present in all collections. The aim was to have stage 2-4 zygotic embryos in the seeds Fig. 1. However, the developmental stages present in the clones differed considerably in terms of their average stage and their range of stages [Figs. 1-3]. Clones W1, W2, W3, U1 and G2 contained the desired embryo stage distributions. The other collections contained some pre-cotyledonary embryos, but also had cotyledonary stages which are thought to be less responsive [2, 10, 1991 Annual Report].

Cones were stored at 4 ^{o}C in plastic boxes prior to inoculation. Seeds from the middle two-thirds of each cone were removed and disinfected in with 20% commercial bleach containing 0.1% [v/v] detergent [Tween 20]. A minimum of three cones from each clone was used for each experiment, and 24 seeds were inoculated for each treatment. Statistical tests were performed by following standard protocols [5]. Megagametophytes were aseptically excised from washed seeds [3 times with sterile water] and placed horizontally on test media in 12-well culture plates. Each well contained 3 ml of medium. DCR basal medium was used in all cases. Half-strength medium contained half-strength macronutrients, micronutrients and nitrogen sources [glutamine and casein hydrolysate], and full-strength inositol and vitamins. The plant

hormones, 2,4-dichlorophenoxyacetic acid [2,4-D] and benzylaminopurine [BA] were added prior to medium sterilization at 121 ^{o}C for 20 min in an autoclave. Sucrose [3% w/v] was routinely used as a carbon source. In some cases, maltose was used instead of sucrose. Carbohydrates were generally added prior to autoclaving. However, in one experiment cold-sterilized sugars were compared with autoclaved sugars. Glutamine was cold-sterilized and added after autoclaved media had cooled to approximately 60 ^{o}C.

Cultures were maintained in darkness at 25 $^{+}/_{-}$ 2^{o}C. Subcultures were made every two weeks for the first eight weeks when initial results were tabulated. Promising extrusions were removed at this time and transferred to D1 medium [DCR with 1.1 mg/l 2,4-D] for further proliferation. These were subcultured every two weeks. The original explants for all treatments were retained in case additional extrusions occurred. These residual explants were left on initiation media and were not subcultured. Subsequent extrusions were recorded and treated as above. Original explants were gradually discarded.

Six experiments were performed. Seeds from all ten clones were used in Experiments 1-5. However, only five clones were used in Experiment 6.

Experiment 1 - The effects of 2,4-D at 0, 1.1, 2, 3, 5 and 11 mg/l were tested with full-strength basal medium in Experiment 1.

Experiment 2 - The effects of BA with the above 2,4-D levels at a 2,4-D : BA ratio of 2:1 was tested in Experiment 2. The 2,4-D : BA combinations were 1.1 : 0.55; 2 : 1; 3 : 1.5; 5 : 2.5; 11 : 5.5 mg/l].

Experiment 3 - Experiment 3 was the same as Experiment 1, except that half-strength medium was used.

Experiment 4 - Experiment 4 was the same as Experiment 2, except that half-strength medium was used.

Experiment 5 - The effects of maltose were compared with sucrose for 0, 1.1 and 5 mg/l 2,4-D.

Experiment 6 - The effects of cold-sterilized maltose were compared with sucrose for 0, 1.1 and 5 mg/l 2,4-D.

RESULTS

Extrusions were evident after two to four weeks of culture, and reached maximal levels by week eight. Various types of extrusions were observed. These were categorized as follows. Class A extrusions resembled embryogenic callus [Fig. 4]. These were glassy and occasionally contained stage 2-3 embryos. Class B extrusions were composed of one-several stage 4-5 [pre-

cotyledonary] embryos [Fig. 5]. Class C extrusions were highly elongated, often rigid, solitary structures [Figs. 6, 7]. In some cases a pre-cotyledonary embryo head was present at the distal tip of these extrusions, but this was not always the case. Occasionally, embryogenic callus similar to that seen with class A extrusions developed at the tip of class C extrusions [Fig. 7]. Class D extrusions were quasi-elongate structures composed by soft, loose callus-like material [Figs. 8-11]. In most cases solitary extrusions were observed, but occasionally two separate extrusions were encountered [Fig. 9]. In some cases, class D extrusions were not elongated and formed a soft, round lump. Glassy cells and calli were occasionally observed at the distal ends of these extrusions [Figs. 10, 11]. Class E extrusions contained entire cotyledonary embryos or their hypocotyls [Figs. 12-15]. Glassy embryogenic-like callus sometimes occurred at the distal end of these extrusions [Fig. 12]. In addition, mixtures of early-stage embryos and suspensors were pushed out of the micropyle by extruding cotyledonary embryos [Fig. 13]. In some cases, several embryos of different stages were extruded from one megagametophyte. These were separated so that the origin of an embryogenic callus from each explant could be discerned. A loose callus which resembled class D extrusions frequently developed from the root and hypocotyl of extruded cotyledonary embryos [Figs. 14, 15].

The overwhelming majority of embryogenic callus lines [56%] developed from class A extrusions [Fig. 16]. Class B accounted

for the next greatest number of callus lines [11.5%]. Less and nearly equivalent results were obtained from classes C, D and E [5.1, 7.7 and 5.1%, respectively. Embryogenic calli which developed after the extrusion of cotyledonary embryos originated from the mass of smaller embryos and suspensor-like material extruded in advance of the larger embryos. It was difficult to determine the origin of embryogenic callus in 14 % of the cases [Fig. 16]. This occurred with cultures that were retained beyond the initial eight weeks, and which were observed irregularly. In these instances numerous growths could be present on the medium, and while it was clear that the embryogenic callus had not arisen from the megagametophyte, it was sometimes impossible to its precise origin.

Class A extrusions were relatively infrequent and occurred with an overall frequency of 2.9% [Fig. 17]. Class B extrusions had a frequency of 7.2% while Class C, D and E occurred at 11.4, 3.4 and 2.2%, respectively [Fig. 17]. The average frequency for all extrusions was 30.5%, and varied from 18% [G1] to 41.6% [U2; Table 2]. There was a strong correlation between the developmental stages present in the different clones and their class A extrusion frequencies [Fig. 18]. The best production of class A extrusions occurred with clones that had the most immature developmental stages present in their seeds. The worst results occurred in cases where the embryos were more advanced. The Spearman's r value for a correlation between developmental stage and class A extrusions was -0.75. The results with clone W1

were biased because the W1 explants for Experiment 1 were inoculated one month later than those of the other clones. Consequently, an even stronger correlation would have probably been obtained if this data was removed.

Embryogenic lines were obtained from nine of the ten clones [Figs. 19-21]. Most lines were produced from Experiments 1-4 Figs. 19, 21]. Few embryogenic calli were derived from experiments 5 and 6. This was reflected by a decrease in the initiation frequency for most of the clones in Experiments 5 and 6. However, W2 and U1 had initiation frequencies of 4.9 and 3.6% in Experiment 6. This caused an increase in embryogenic callus formation compared to Experiment 5 [Fig. 19]. Clone U1 was the only one to show an overall increase in embryogenic callus production from Experiments 1-4, to 5 and 6. Most of the embryogenic lines [68%] came from clones W1, W2 and U1 [Fig. 20]. However, many lines were obtained from G4, G1 and U2, and several lines arose from U3 and W3. Only one embryogenic line was produced from G2.

The highest frequencies of embryogenic callus formation came from clones in which the average embryo developmental stage was 3 or less [Figs. 3, 21]. There was a negative correlation between the developmental stages present in the seeds of different clones and their production of embryogenic callus. When all clones were considered for experiments 1-5, in which they received identical treatments, a Spearman's r value of -0.42 was obtained. However,

clones G2 and W3 which contained very immature embryos produced few embryogenic calli compared to W1 and W2 which had embryos of approximately the same stages [Figs. 3, 21]. A negative correlation of -0.88 was obtained when G2 and W3 were deleted from the analysis.

Embryogenic callus was produced on hormone-free medium in Experiments 1 and 2 [Fig. 22]. The addition of 2,4-D to the medium stimulated embryogenic callus formation at 1.1, 2, 3 and 5 mg/l. A maximal stimulation of 250% occurred at 2 - 3 mg/l. Embryogenic callus production was inhibited at 11 mg/l. The addition of BA did not stimulate embryogenic callus formation and appeared to be inhibitory [Figs. 22, 23].

No embryogenic callus developed on hormone-free medium in Experiments 3 and 4 in which half-strength medium was used [Fig. 23]. Embryogenic callus production was stimulated by 2,4-D at all concentrations, and maximal results were obtained at 2-3 mg/l. The magnitude of these results was similar to that obtained in Experiments 1 and 2, when the results obtained on hormone-free medium were subtracted from those obtained with 2,4-D [Fig. 24]. The addition of BA to half-strength medium stimulated embryogenic callus formation with 1.1 mg/l 2,4-D [Fig. 23]. A small stimulation also occurred with 2 mg/l 2,4-D, but no stimulation was evident at higher 2,4-D levels.

Similar overall results were obtained with full- and half-strength media when 2,4-D was used alone [Fig. 24]. Elevated frequencies were observed with full-strength medium at all 2,4-D levels except 11 mg/l. In addition, there was a significant amount of embryogenic callus formation on hormone-free full-strength medium, but none with half-strength medium [Figs. 22-23]. Irregular results were obtained when BA was used with either full- or half-strength media [Fig. 25]. There was little difference between the use of full- versus half-strength media, and there was no clear benefit in using half-strength medium.

Embryogenic callus developed on hormone-free medium when maltose was used but none formed with sucrose in Experiment 5 [Fig. 26]. Equivalent results were obtained with sucrose and maltose at 1.1 mg/l 2,4-D. Embryogenic callus formation occurred at 5 mg/l 2,4-D with sucrose, but not with maltose. Better results occurred when filter-sterilized sugars were tested with five clones in experiment 6 [Fig. 27]. Embryogenic callus production occurred on hormone-free media, and with both 2,4-D concentrations. Maltose produced superior results in all cases.

Results obtained with five clones in experiment 6 were compared to the same clones in experiment 5 [Fig. 28]. Superior results were obtained with filter-sterilized carbohydrates in all cases. Furthermore, filter-sterilized maltose consistently gave the best results.

DISCUSSION

A greater variety of extrusions were encountered in these experiments than in last year's work. However, the bulk of embryogenic calli arose from class A and B extrusions which were the only types observed in 1990. The increased complexity of extrusions was probably due to the presence of more advanced developmental stages in the seeds of different clones.

A dominant embryo with a large suspensor develops early within the megagametophyte of loblolly pine. The suspensor pushes the dominant embryo's head to the chalasal end of the megagametophyte opposite the micropyle. A tangle of secondary embryos and their suspensors usually occupy the micropylar end of the corrosion cavity [3]. As the embryo matures, it develops basipetally towards the micropyle collapsing the structures in its path. The first step in germination is the elongation of the root and hypocotyl which emerge through the micropyle. The seed coat normally restrains the release of materials through the micropyle and prevents precocious germination. Because the micropyle is open, removal of the seed coat and the nucellus relieves most of the physical constraint, and probably allows the dominant embryo to push out the contents of the corrosion cavity, and to emerge prematurely. Both of these processes probably account for most class D and E extrusions, and may be involved in other types of extrusions. The loose callus-like class D extrusions probably reflected proliferation by the root and hypocotyl of cotyledonary

embryos. This type of callus was seen to develop when immature cotyledonary embryos were completely extruded onto the culture medium. However, the linearity of some class D extrusions suggested that they may also have been derived from elongating pre-cotyledonary embryos which protruded from the micropyle. Many class C extrusions had embryo heads at their apex. This indicated that they represented pre-cotyledonary embryos with unusually elongated suspensors. However, the rigidity of some class C extrusions suggested that the suspensor-line structure might have been a highly elongated root cap. Conifers typically has a massive root cap, and conditions in the cultures may have promoted its elongation. Such a structure could be rigid. Class B extrusions probably reflected the extramural development of a few secondary embryos located at the micropyle.

Type A extrusions resembled a proliferation of the stage 2-3 zygotic embryos and their suspensors. Discrete stage 2 embryos could often be seen within the extruded mass which grew into a less differentiated embryogenic callus. Larger more discrete stage 4-5 embryos composed class B extrusions. The embryo heads were very distinct in these, and they usually became brown. Embryogenic callus typically arose from the suspensor[s] of these embryos. Many class C extrusions resembled stage 3-5 embryos which had undergone an abnormally large amount of suspensor elongation. Embryogenic callus tended to form at the distal tip of these extrusions, and presumably came from the embryo head or cells proximal to it. However, some of these extrusions pushed

less developed embryo-suspensor complexes ahead of themselves, and these could have independently given rise to embryogenic callus. Similar events occurred with type D and E extrusions. Consequently, the origin of embryogenic callus with Class C-E extrusions was uncertain. The genetic and developmental complexity of the embryo-suspensor matrix which occupies the micropyle creates the possibility that extruded callus is genetically heterogeneous. This could result in the production of chimeric calli composed of several genotypes. It has been shown that this can occur with loblolly pine embryogenic callus. However, the large majority of these calli did not show evidence of being chimeras [3].

Prior work has shown that the culture of megagametophytes containing stage 3 zygotic embryos was superior compared to direct embryo culture for the initiation of embryogenic callus with Pinus species, including loblolly pine [2, 8, 11]. The present results generally support the hypothesis that stage 2-3 embryos are more embryogenic than later stages. This was especially evident for class A extruded callus which gave rise to most embryogenic lines. However, contrasting results were obtained for embryogenic line production with clones W1 and W2 compared to W3 and G1. The average developmental stages present in these four clones were between stage 2 and 3. The first two clones had the highest frequencies of embryogenic callus production while the latter two were among the worst. It is possible that genetic factors may have accounted for the

differential response of these clones. Seeds from W1 and W2 have produced good results over a long span of experiments, and may be genetically disposed to respond *in vitro*. Furthermore, the reaction of clones U2 and U3 were similar to those obtained with these in the past. It is well established that the ability of some crops to form embryogenic callus is inherited as a Mendelian trait controlled by approximately two genes [4, 6, 7]. It is noteworthy that a cross between W1 and W2 produced the best results in 1990 trials. Based on the above, it is reasonable to hypothesize that some clones may be genetically disposed to somatic embryogenesis while others may not be as competent. If this is the case, embryogenic potential could be transferred by pollination with responsive genotypes. This approach has worked with several crop species [4, 6-7].

These results confirm that DCR is a suitable medium for embryogenic callus initiation and proliferation [2, 3]. There was no apparent benefit from the use of half-strength medium, and better results were obtained with full-strength medium. The experiments on sucrose and maltose indicated that autoclaving carbohydrates with other media components was less effective than using cold-sterilized sugars. Furthermore, filter-sterilized maltose was the best carbohydrate treatment. Maltose was also superior to sucrose for the proliferation of new embryogenic callus lines. The deleterious effects of using autoclaved sucrose could be related to its hydrolysis into glucose and fructose, and the consequent production of a toxic product from fructose

[1, 8, 9]. Maltose is composed two glucose molecules, and glucose is not known to produce toxic by-products during autoclaving.

Extrusion of embryogenic callus occurred in the absence of plant hormones. However, 2,4-D stimulated embryogenic callus production. The optimal 2,4-D level was 2 - 3 mg/l. The addition of BA failed to stimulate embryogenic callus production with full-strength medium. Some stimulation was observed with half-strength medium containing 1.1 mg/l 2,4-D. BA generally had no effect or was inhibitory with 5 and 11 mg/l 2,4-D. These results were similar to those obtained in 1990.

Despite the unequal results obtained with different seed families, embryogenic calli were produced from nine of the ten clones, and class A calli were produced by all clones. Considering all the clones and treatments, the average frequency of embryogenic callus formation was 1%. The lowest overall clonal frequency was 0.15% [G2], while the highest was 2.95% [W2]. Frequencies of 12.5% were attained with individual treatments. Based on the results of this work, it should be possible to increase the frequency of embryogenic callus production by choosing the optimal embryo stage, choosing responsive clones, and making adjustments to the carbohydrate component of the medium. Consequently, it should be possible to obtain embryogenic callus from an extremely wide array of mother trees.

CONCLUSIONS

This research has shown that embryogenic callus can be obtained from the progeny of genetically diverse mother trees. Clones used in prior work responded as well as, or better than they did in the past. The use of intact megagametophytes containing stage 3 embryos produced the highest frequencies of extruded embryogenic callus. Initiation success can be determined early because most of the stable callus lines arose from class A extrusions which closely resembled proliferating embryogenic calli. Other types of extrusions also yielded embryogenic callus at much lower frequencies, but the origin of these calli was less certain, and this process encompassed a long time-frame. Embryogenic callus extrusion occurred in the absence of plant hormones. However, the addition of 2,4-D stimulated initiation at 1-3 mg/l. The use of BA did not stimulate initiation. Embryogenic calli were grown on medium which contained only 2,4-D at 1.1 mg/l. Full-strength DCR medium supported the initiation and proliferation of embryogenic callus. Half-strength medium produced equivalent or inferior results. The use of filter-sterilized carbohydrates, especially maltose, improved initiation frequencies. Maltose was also superior for growing embryogenic callus.

Because of the many uncontrolled physiological, developmental and genetic variables associated with using seeds for initiation work, and because seeds containing stage 3 embryos are only available once each year, it would be best to use somatic embryos

for future initiation experiments. This should be possible soon due to the progress being made with the maturation and development of loblolly pine somatic embryos. The use of somatic embryos would allow the harvesting of early- or late-stage embryos at any time of the year. Furthermore, the genetic diversity of somatic embryos would be greatly diminished compared to zygotic embryos. Even if callus lines were chimeric, genetic diversity would probably be significantly reduced if somatic embryos are used as explants. In addition, the use of isolated embryos would shed more light on the optimal developmental stage for initiation, as well as the site of embryogenic callus origination. Results obtained with somatic embryos could then be tested on zygotic embryos in focused trials.

Future work with seeds should focus on the genetic aspects of embryogenic callus formation. Several responsive clones have been identified as a result of work at the IPC and IPST. Preliminary results suggested that embryogenic potential may have been inherited in a full-sib cross of two responsive mother trees. If embryogenic potential is heritable, like it is with flowering plants, this trait could be bred into trees as part of a tree improvement program. It would also be important to evaluate the effects of maltose on initiation. The method of carbohydrate sterilization also needs attention.

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10] Laine, E. and A. David. 1990. Somatic embryogenesis in immature embryos and protoplasts of Pinus caribaea. Plant Sci. 69: 215-224.

Table 1. Results of Initiation Experiments with Seeds from Westvaco Clones Used in Prior and Current Experiments

	Frequency of Embryogenic Callus Production			
Clone	1986	1987	1990	1991
11-9	0.67	0.7	1.8	2.5
7-34	1.8	0.7	1.7	3
11-9 x 7-34			3	

Table 2. Frequencies of Different of Extrusions Formed by All Clones

Clone		Extrusion Class A	B	C	D	E	Total
W1	E 1-4[1]	2.6	6.1	8.7	3	7.5	27.4
	E 1-5[2]	2.4	5.5	7.4	3.1	9	27.4
	E 1-6[3]	2.4	5.5	7.4	3.1	9	27.4
W2	E 1-4	7.3	12.7	4.7	1.6	0.2	26.5
	E 1-5	6	12.6	4.4	2.3	1.9	27.2
	E 1-6	5.9	12.2	6.2	2.4	2.7	29.4
W3	E 1-4	3.6	6	14.7	2.7	0	27
	E 1-5	2.7	5.7	13.7	3.4	1.7	27.1
	E 1-6	2.7	5.7	13.7	3.4	1.7	27.1
U1	E 1-4	5.6	13.8	13.9	3.3	0.9	37.5
	E 1-5	4.6	13.2	13.9	3.2	2.7	37.6
	E 1-6	4.7	12.9	13.7	2.7	3.7	37.7
U2	E 1-4	2.9	6.2	25	5.3	2	41.4
	E 1-5	2.3	5.2	24.7	5.5	4.3	42.1
	E 1-6	1.9	5.3	24	5.4	5.9	42.6
U3	E 1-4	2.9	9.2	13.8	2.9	1.8	30.9
	E 1-5	2.5	7.6	14	4.4	4.7	33.4
	E 1-6	2.5	7.6	14	4.4	4.7	33.4

G1	E 1-4	2.6	4.7	3.3	1.6	1.6	13.9
	E 1-5	2.3	1.3	3.8	1.4	6.4	18
	E 1-6	2.3	1.3	3.8	1.4	6.4	18
G2	E 1-4	5.2	18.7	15.9	2.4	1.6	44
	E 1-5	4.2	15.4	17.3	4.1	3.7	44.7
	E 1-6	3.6	13.5	18.2	4.6	4.9	44.8
G3	E 1-4	1.4	4.8	9.3	4	4.8	24.3
	E 1-5	1.1	4	8.6	4.1	8.9	26.7
	E 1-6	1.1	4	8.6	4.1	8.9	26.7
G4	E 1-4	1.2	7.7	10.2	5.2	5.7	30
	E 1-5	1.2	6.8	10.4	6.4	10.3	35.1
	E 1-6	1	6.2	10.6	6.4	10.8	35.1

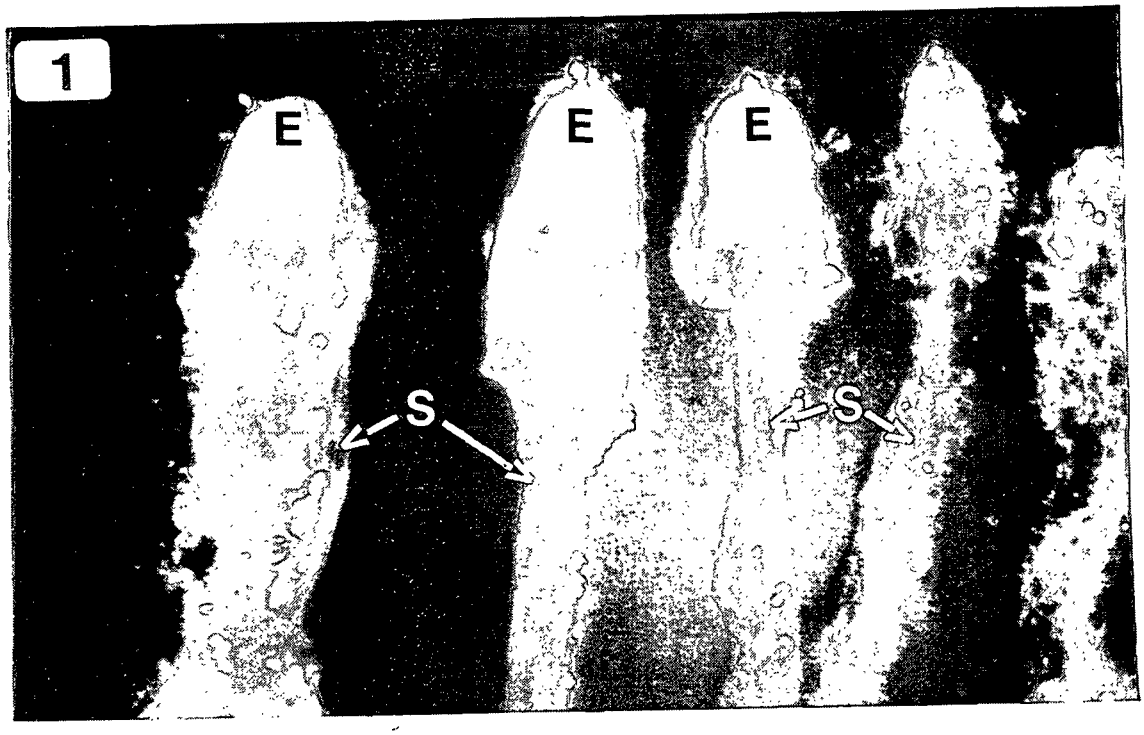
[1] Experiments 1-4

[2] Experiments 1-5

[3] Experiments 1-6

Figure 1. Stage 2-3 zygotic embryos from clone G2. Note the translucent embryo heads [E] and suspensors [S]. Magnification = 82X

Figure 2. Stage 3-7 zygotic embryos from clone U3. Numbers indicate the developmental stages. Note the cotyledons [C] on late-stage embryos. Magnification = 29X


1
E
E
E
S
S

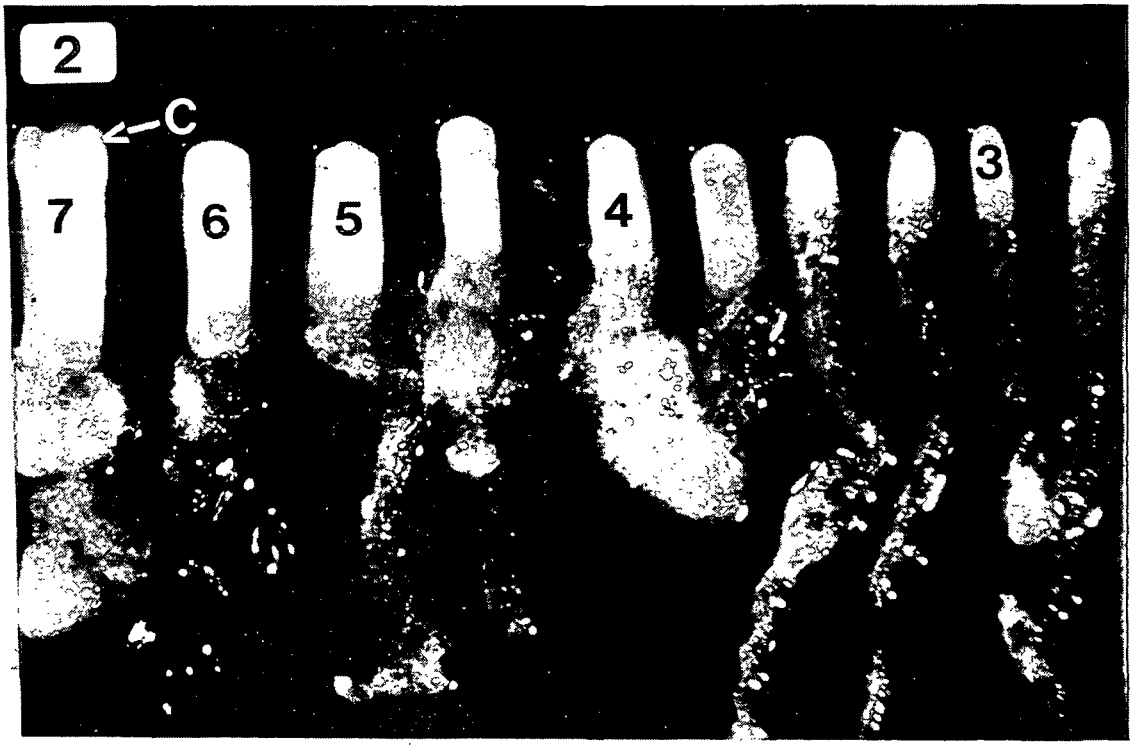

2
C
7
6
5
4
3

Figure 3. Developmental stages present in the seeds from different clones. Vertical lines about the means indicate the standard error of the mean.

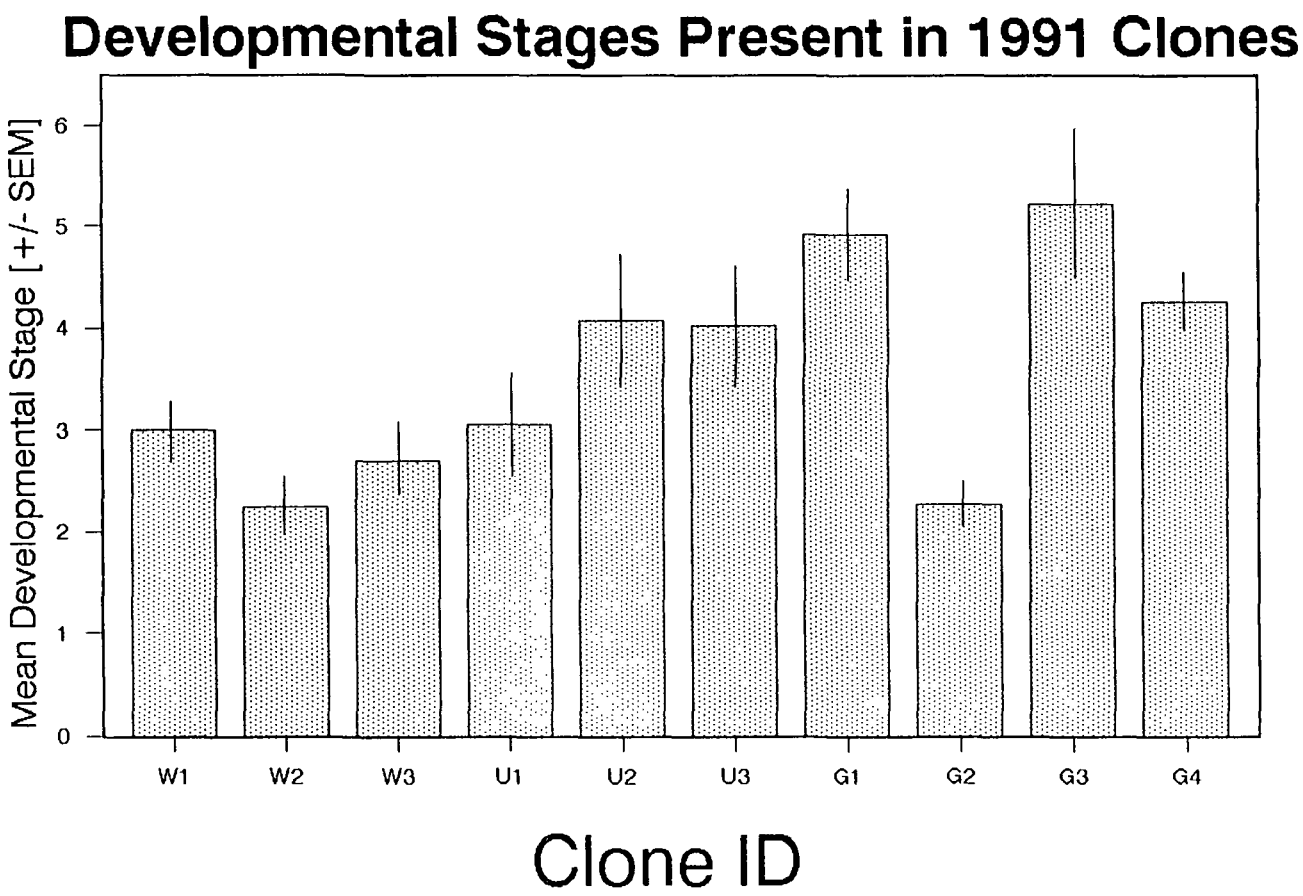
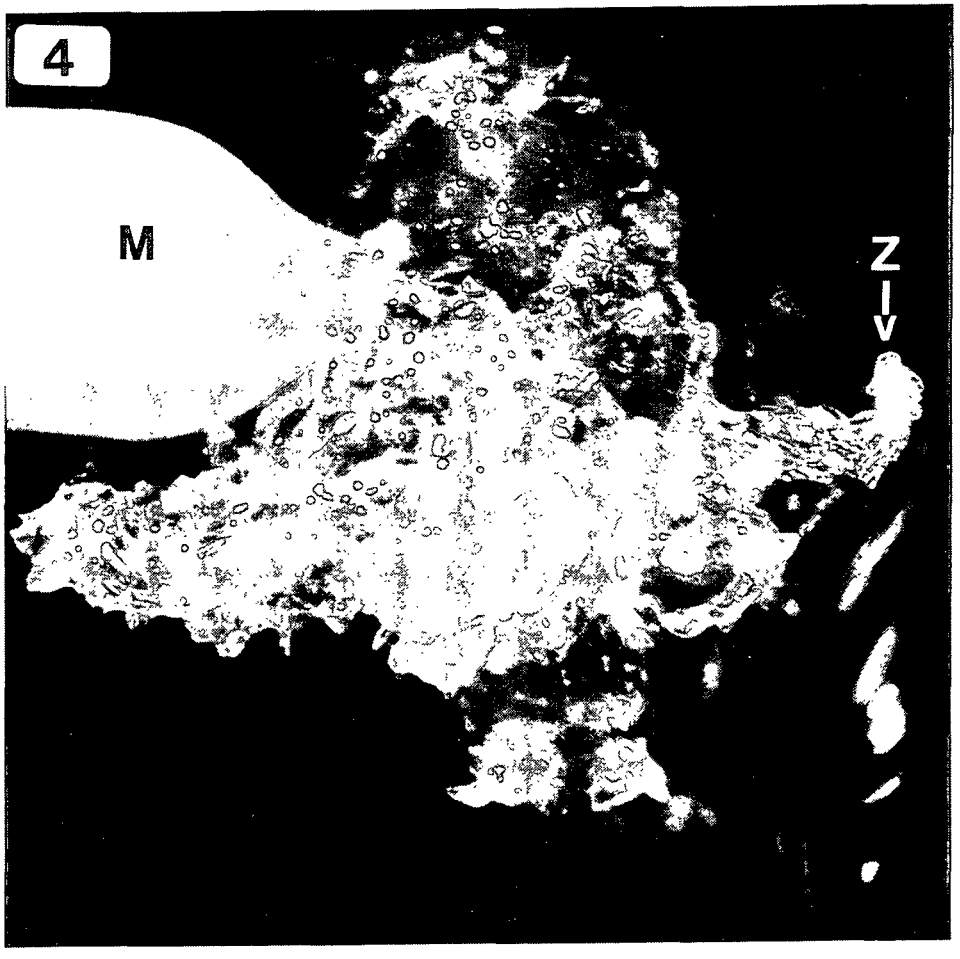

Developmental Stages Present in 1991 Clones
Mean Developmental Stage [+/- SEM]
0
1
2
3
4
5
6
W1
W2
W3
U1
U2
U3
G1
G2
G3
G4
Clone ID

Figure 4. Class A extruded embryogenic callus. Note the stage 2 zygotic embryo [Z] at the edge of the callus. M = megagametophyte, Magnification = 29X

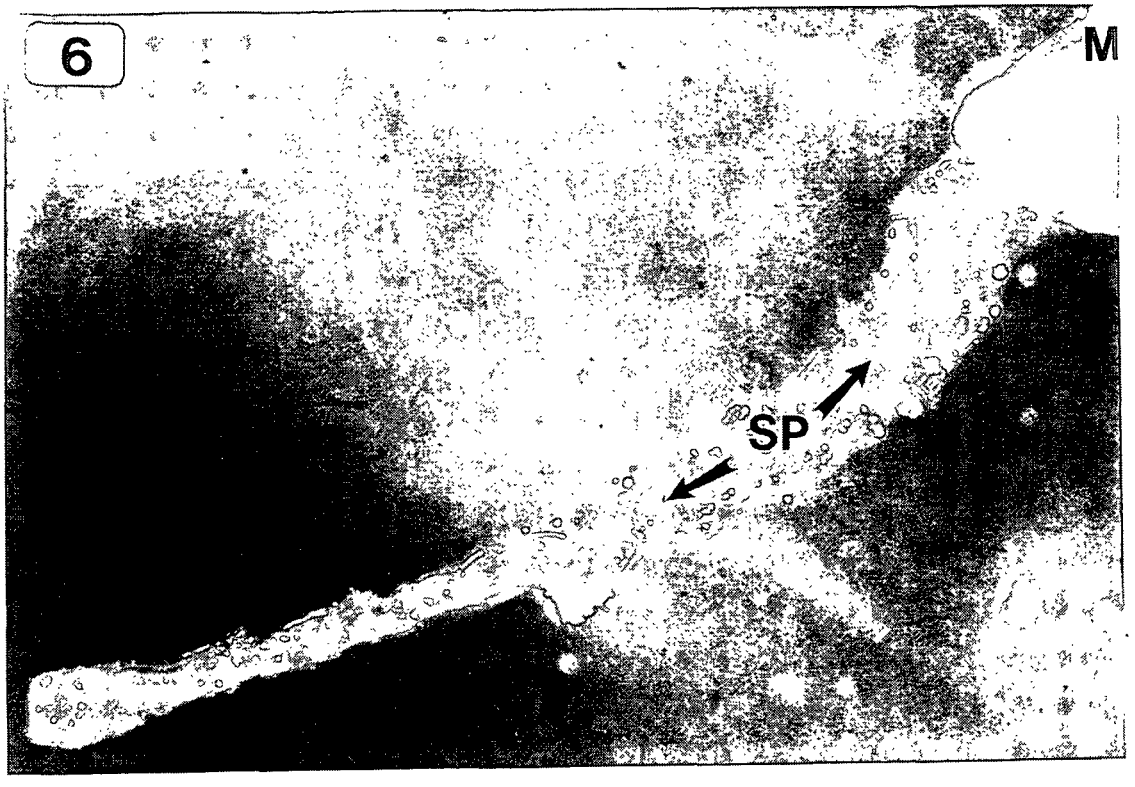
Figure 5. Extruded stage 4 zygotic embryo [Z] illustrating a class B extrusion. Note the embryo head [E] and suspensor [S]. M = megagametophyte, Magnification = 31X

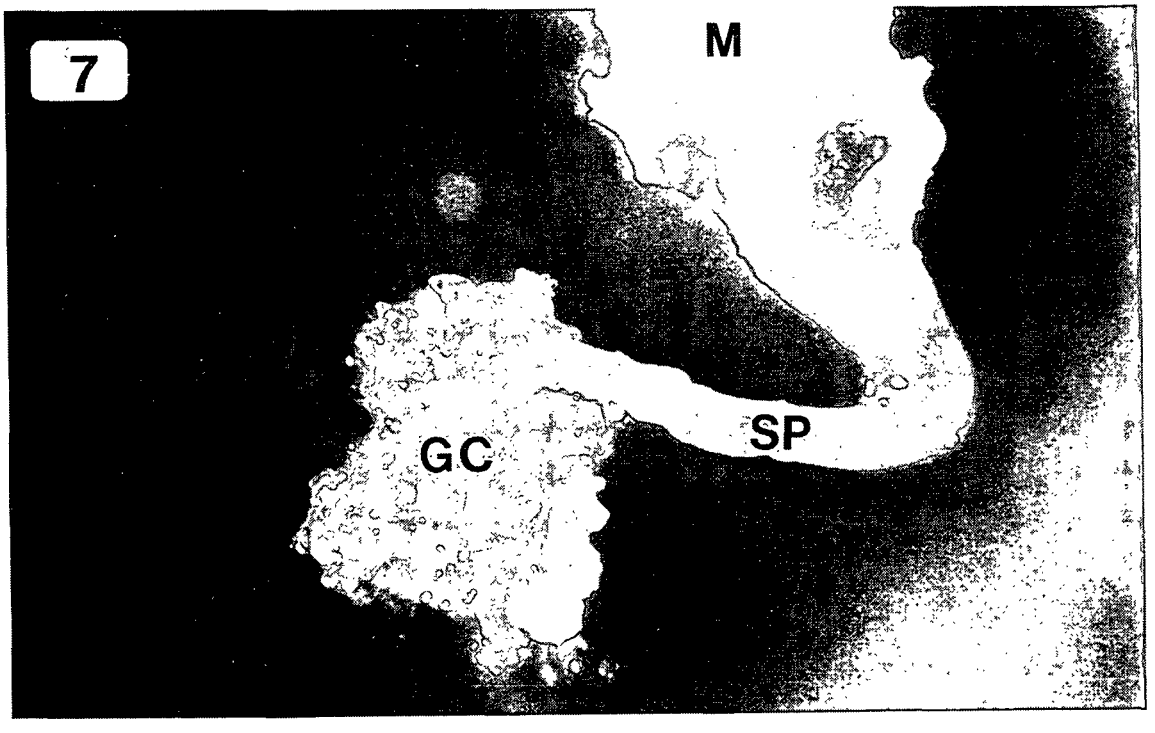

4
M
Z
I
V


5
E
S
M

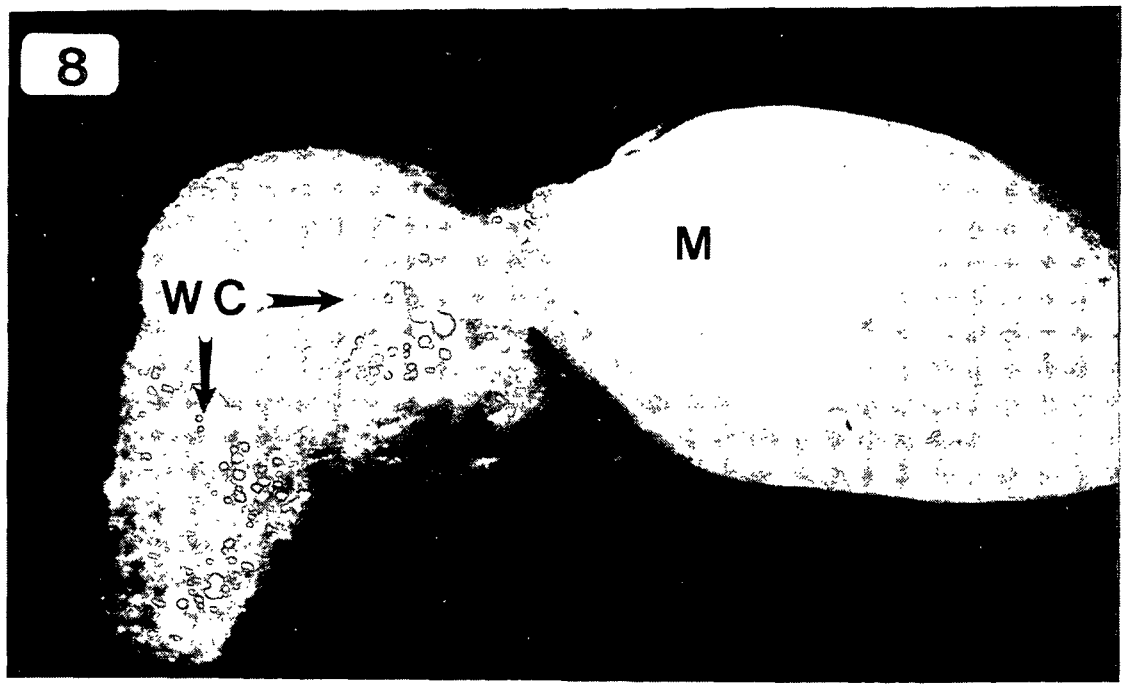
Figure 6. Class C extrusion showing highly elongate suspensor-like protrusion [SP]. M = megagametophyte, Magnification = 14X.

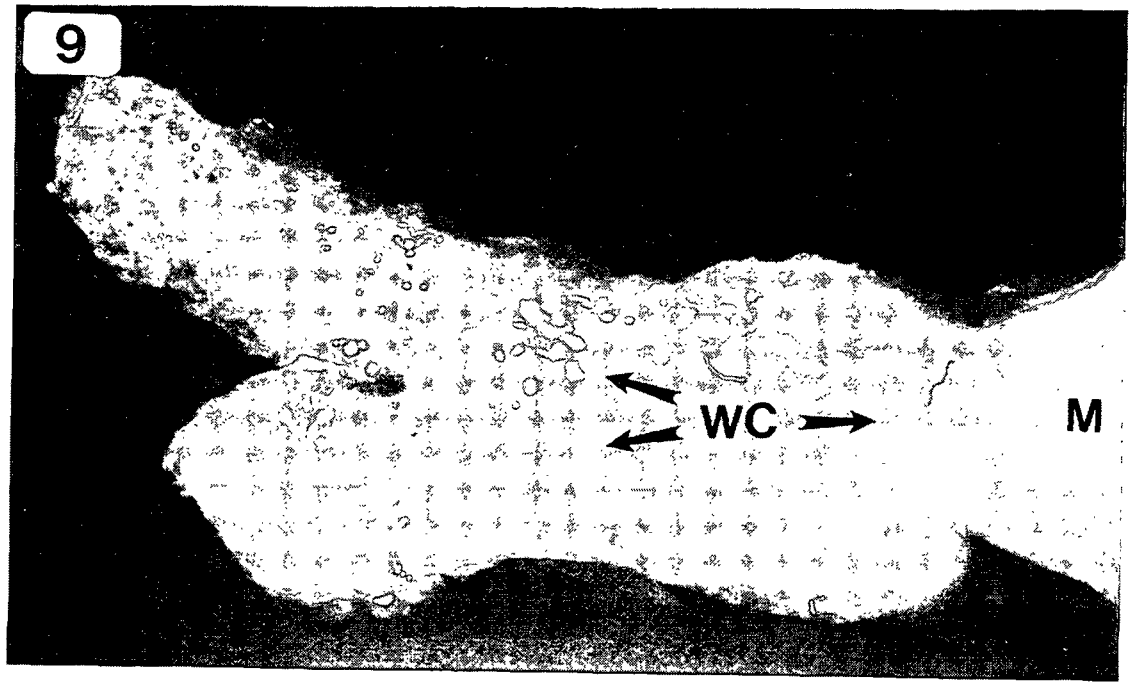
Figure 7. Class C extrusion with glassy embryogenic callus [EC] forming at the distal tip. M = megagametophyte, Magnification = 20X.


6
M
SP

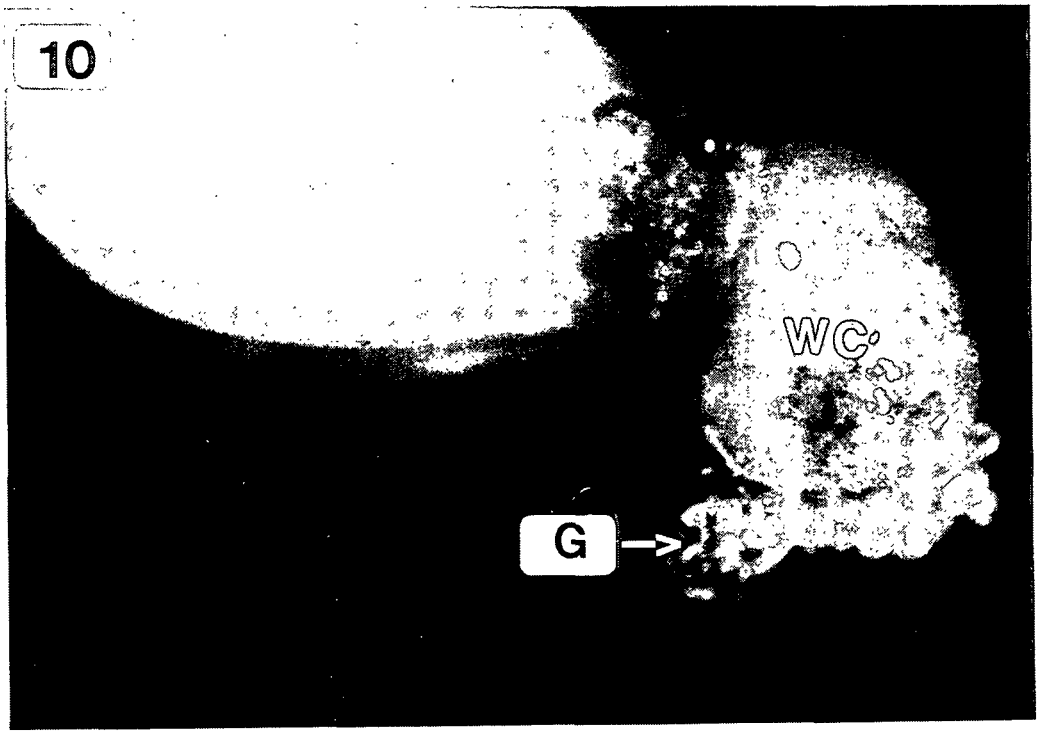

7
M
GC
SP

Figures 8 and 9. Class D extrusions. Note the loose, watery appearance of the extruded calli [WC] and the absence of embryo heads. M = megagametophyte, Magnifications = 26X [Fig. 8] and 41X [Fig. 9].


8
M
WC


9
WC
M

Figures 10 and 11. Same as Figs. 8 and 9, except for the glassy cells [G] at the distal tips of the extrusions. Magnifications = 31X.


10
WC
G

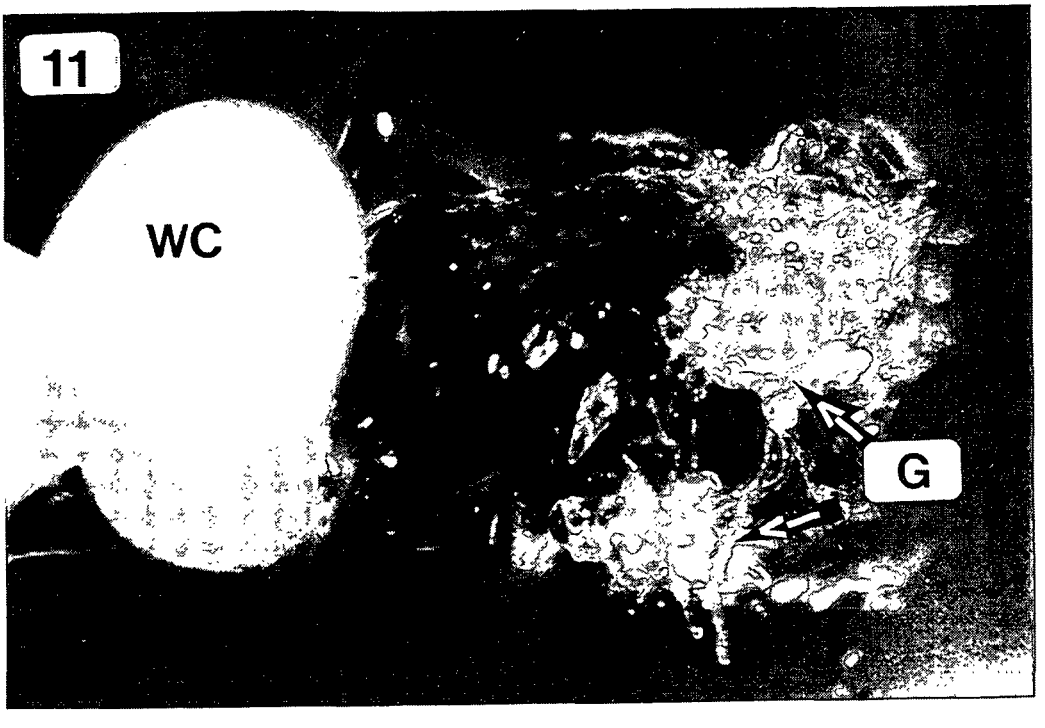
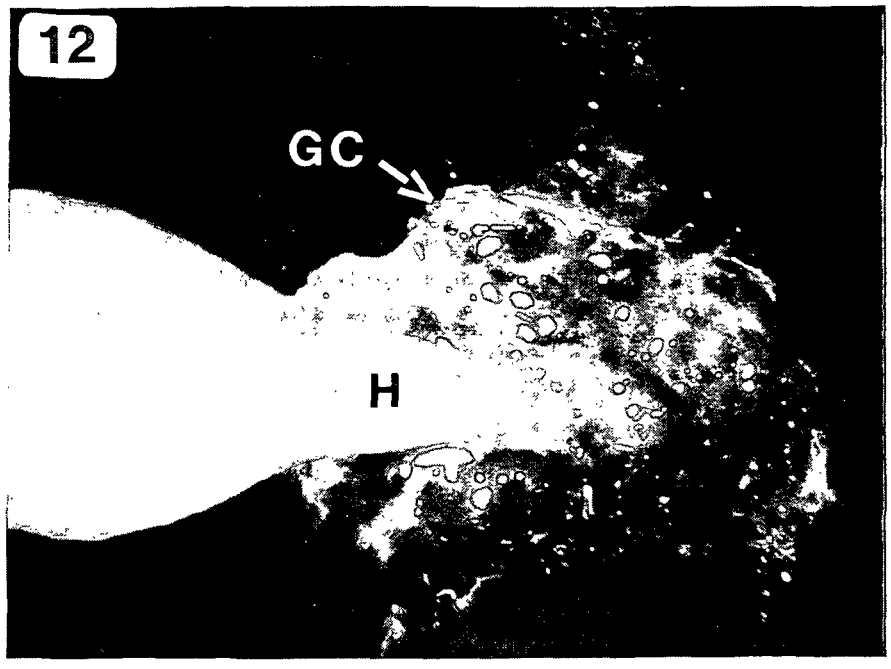
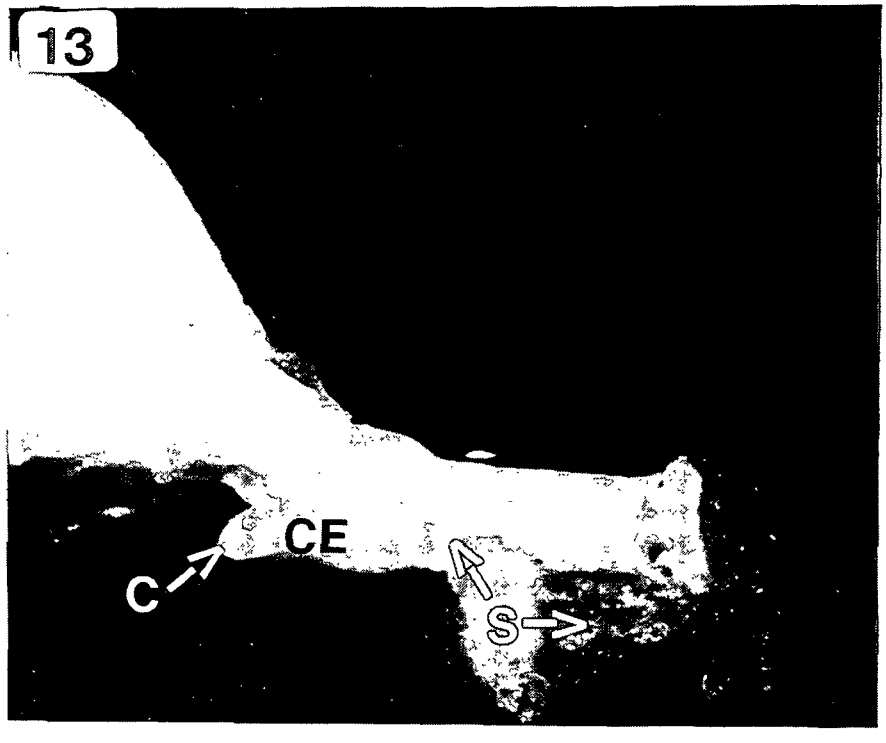

11
WC
G

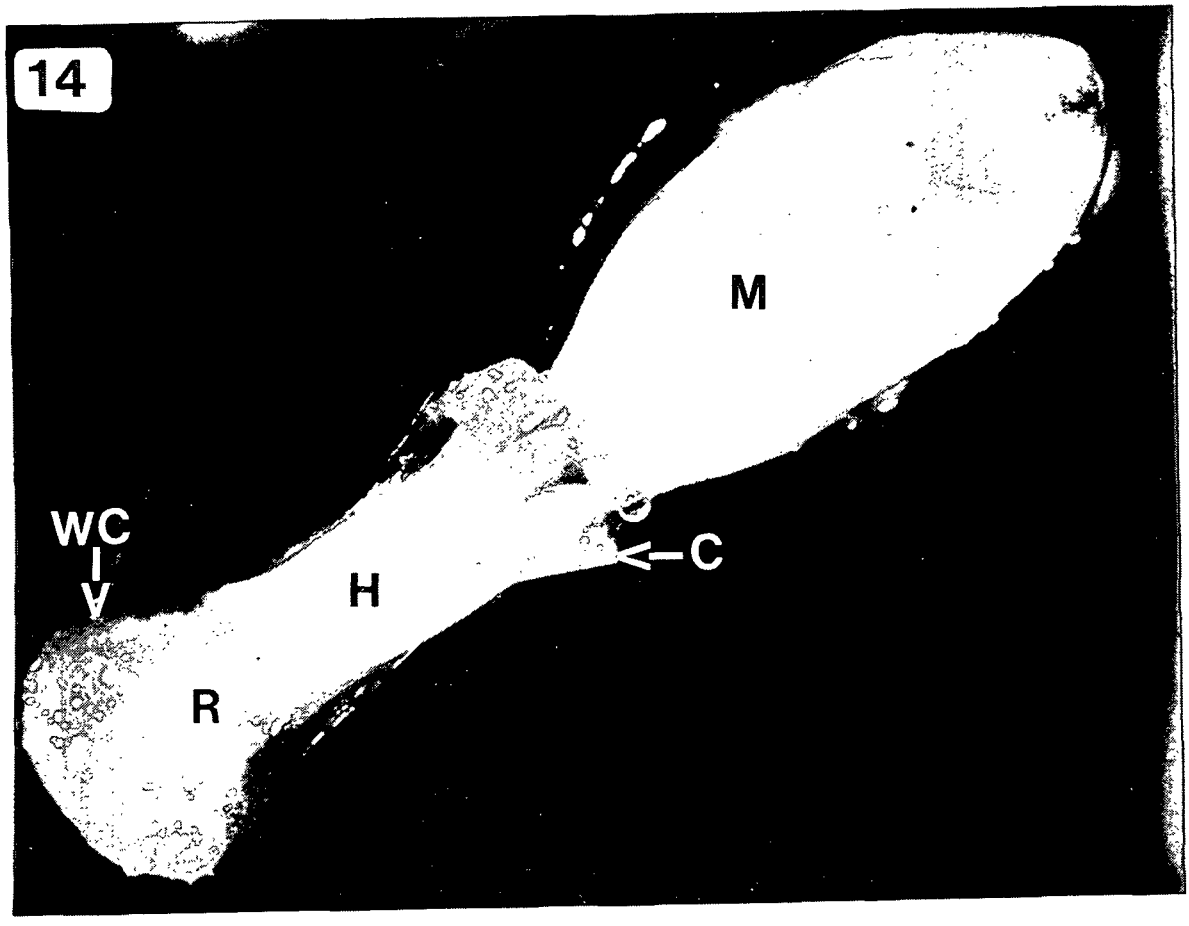
Figure 12. Class D extrusion with hypocotyl [H] of germinating embryo protruding from the megagametophyte. Note the glassy callus [GC] around the distal portion of the hypocotyl. Magnification = 26X.

Figure 13. Fully extruded early cotyledonary embryo [CE] extruded from the megagametophyte [Class D extrusion]. Note the glassy suspensor [S] extruded as part of the embryo. Magnification = 31X.


12
GC
H


13
CE
C
S

Figures 14 and 15. Extruded cotyledonary embryos [Class D extrusions]. Note the loose watery callus [WC] developing around the root [R, Fig. 14] and hypocotyl [H, Fig. 15]. Magnifications = 26X.


14
M
WC
C
H
R

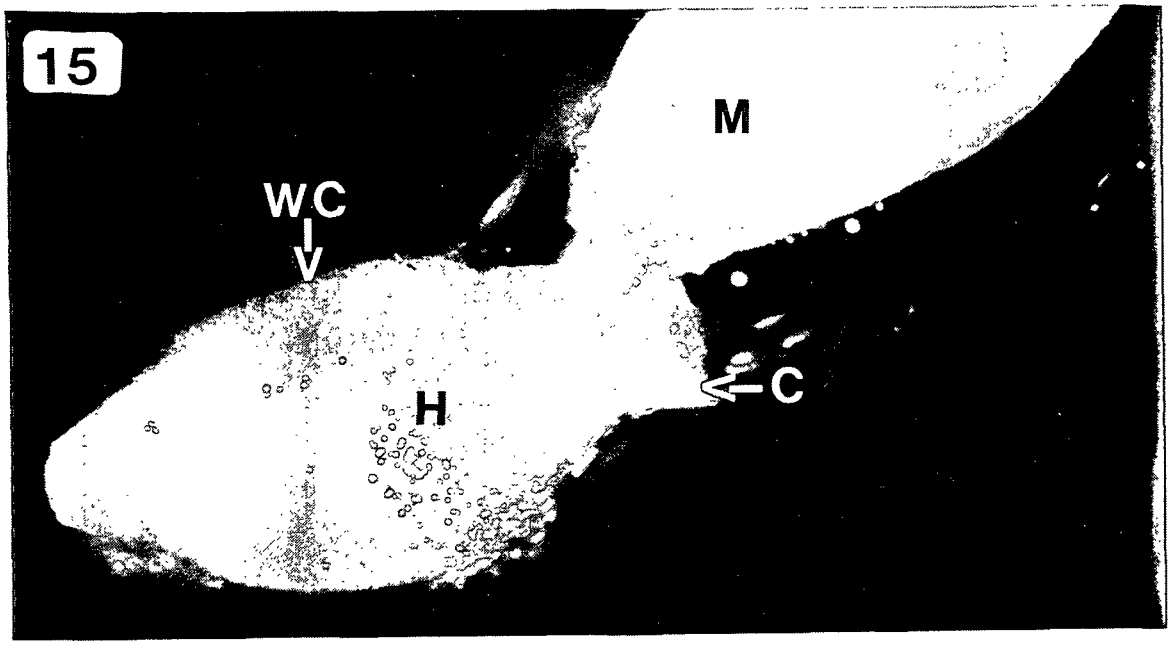

15
M
WC
C
H

Figure 16. Contribution of different extrusion classes to the total number of embryogenic callus lines obtained in experiments 1-6.

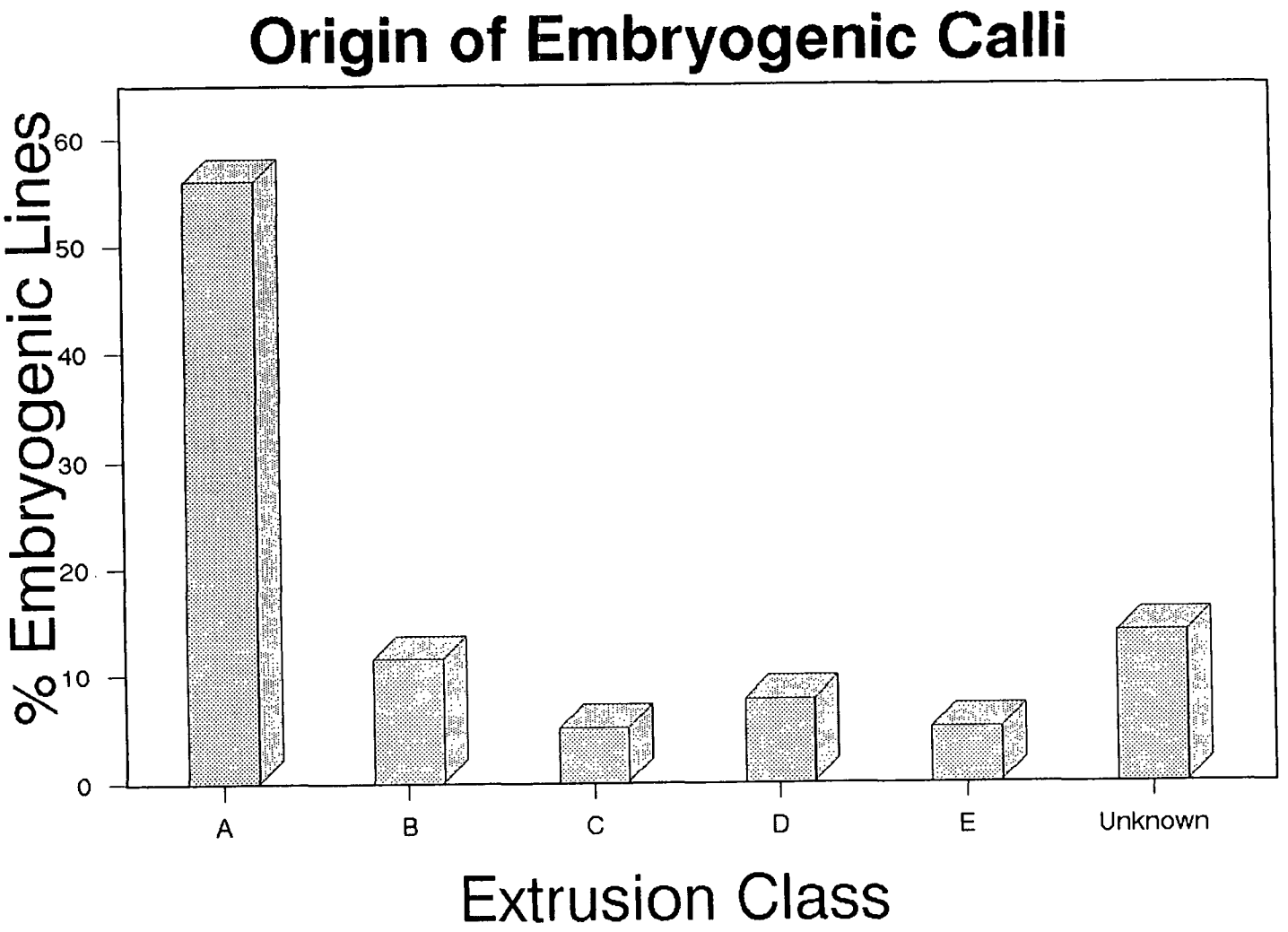

Origin of Embryogenic Calli
% Embryogenic Lines
0
10
20
30
40
50
60
A
B
C
D
E
Unknown
Extrusion Class

Figure 17. Frequencies of different extrusion classes formed in experiments 1-4, 1-5 and 1-6.

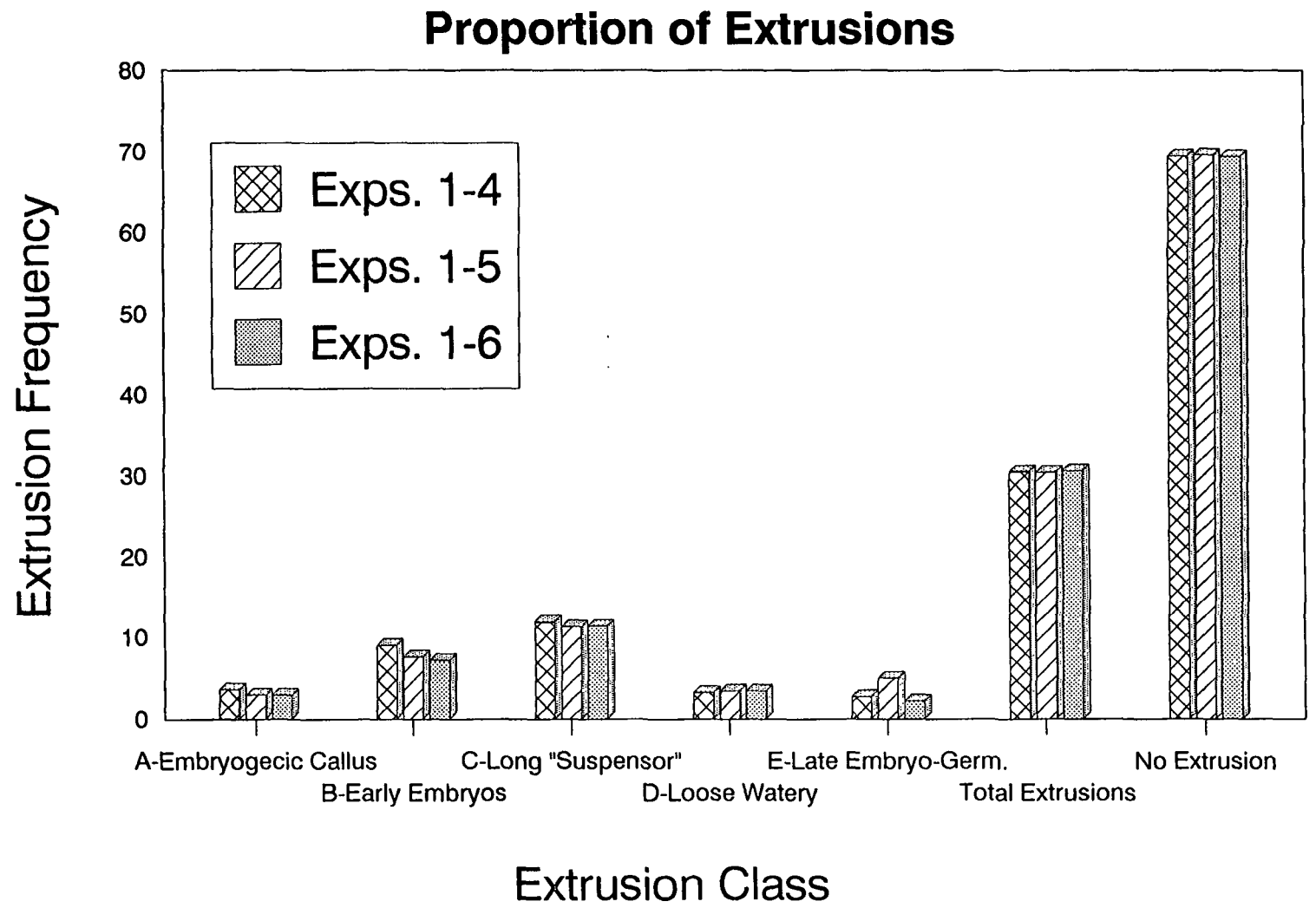

Proportion of Extrusions
Extrusion Frequency
80
70
60
50
40
30
20
10
0
Exps. 1-4
Exps. 1-5
Exps. 1-6
A-Embryogecic Callus
B-Early Embryos
C-Long "Suspensor"
D-Loose Watery
E-Late Embryo-Germ.
Total Extrusions
No Extrusion
Extrusion Class

Figure 18. Frequency of class A extrusions formed in all experiments, plus the average developmental stage present in seeds of different clones. Clones are arranged along the x-axis in descending order of class A extrusion frequency.

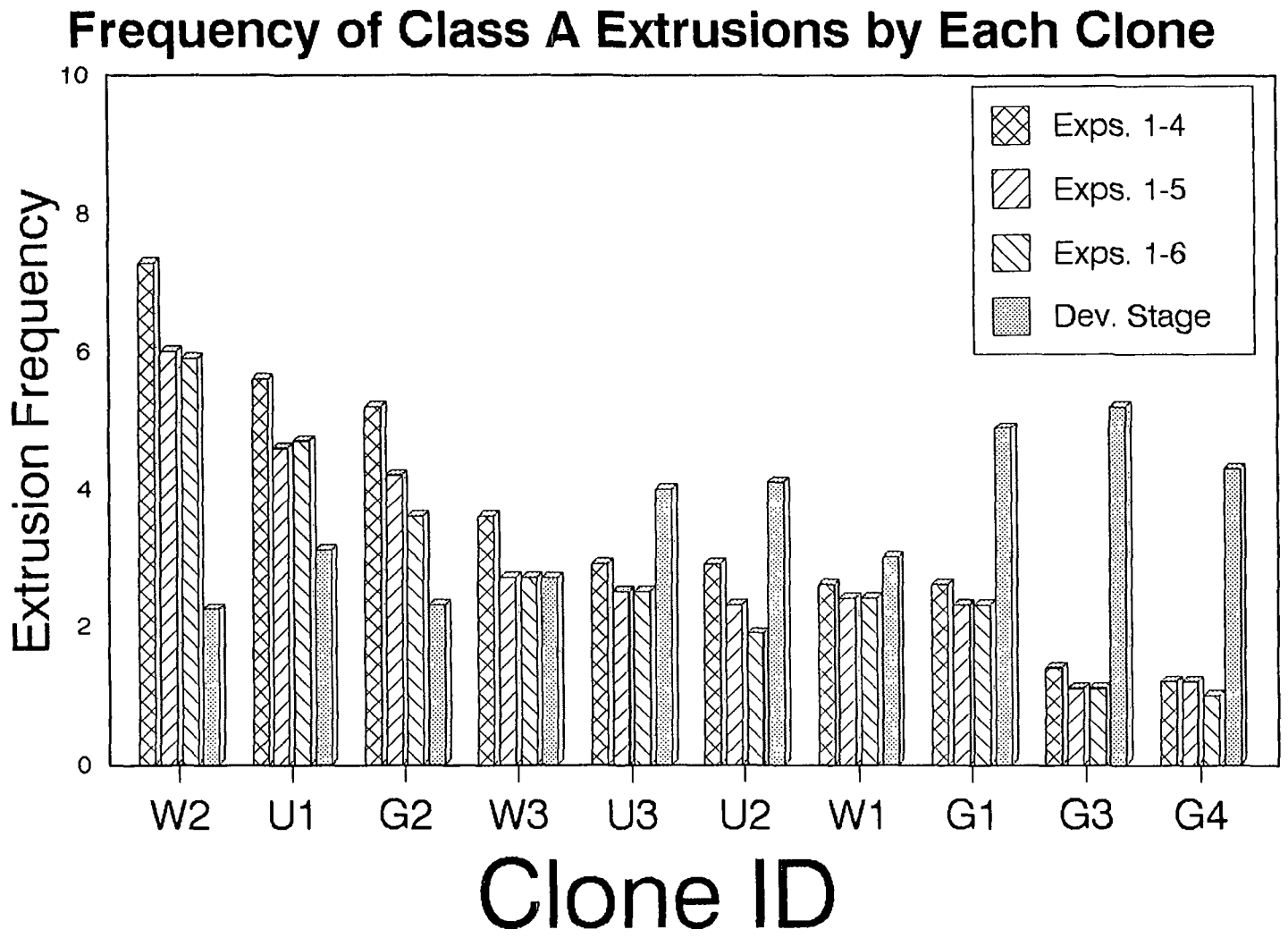

Frequency of Class A Extrusions by Each Clone
Extrusion Frequency
10
8
6
4
2
0
Exps. 1-4
Exps. 1-5
Exps. 1-6
Dev. Stage
W2
U1
G2
W3
U3
U2
W1
G1
G3
G4
Clone ID

Figure 19. Production of embryogenic callus lines by seeds from different clones in all experiments. The * indicates a clone which was tested in experiment 6. Clones were arranged along the x-axis in descending order according to their production of embryogenic callus lines. No callus lines developed with clone G3.

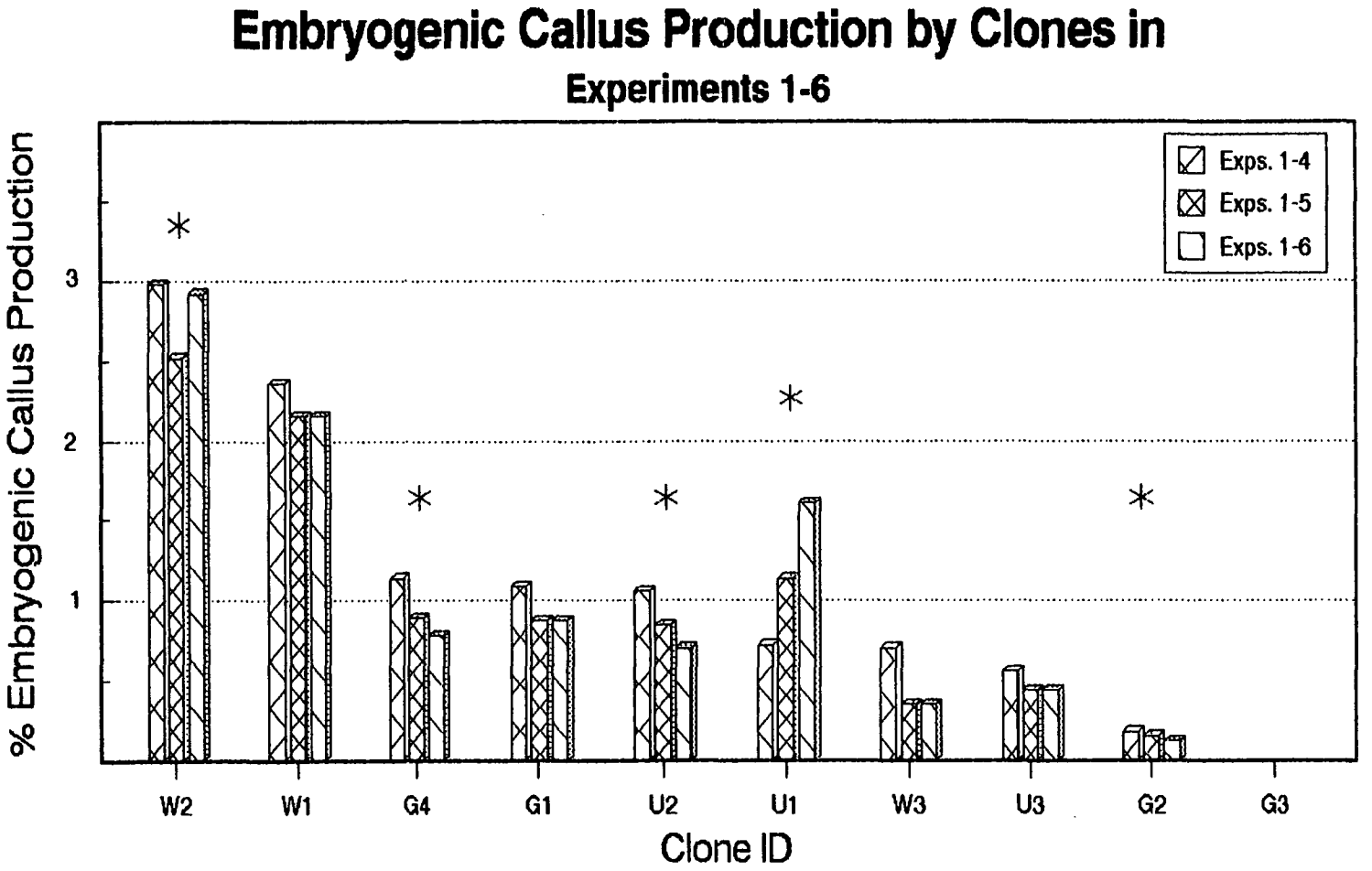

Embryogenic Callus Production by Clones in
Experiments 1-6
% Embryogenic Callus Production
3
2
1
Exps. 1-4
Exps. 1-5
Exps. 1-6
W2
W1
G4
G1
U2
U1
W3
U3
G2
G3
Clone ID

Figure 20. Proportion of embryogenic callus lines obtained from each clone in all experiments.

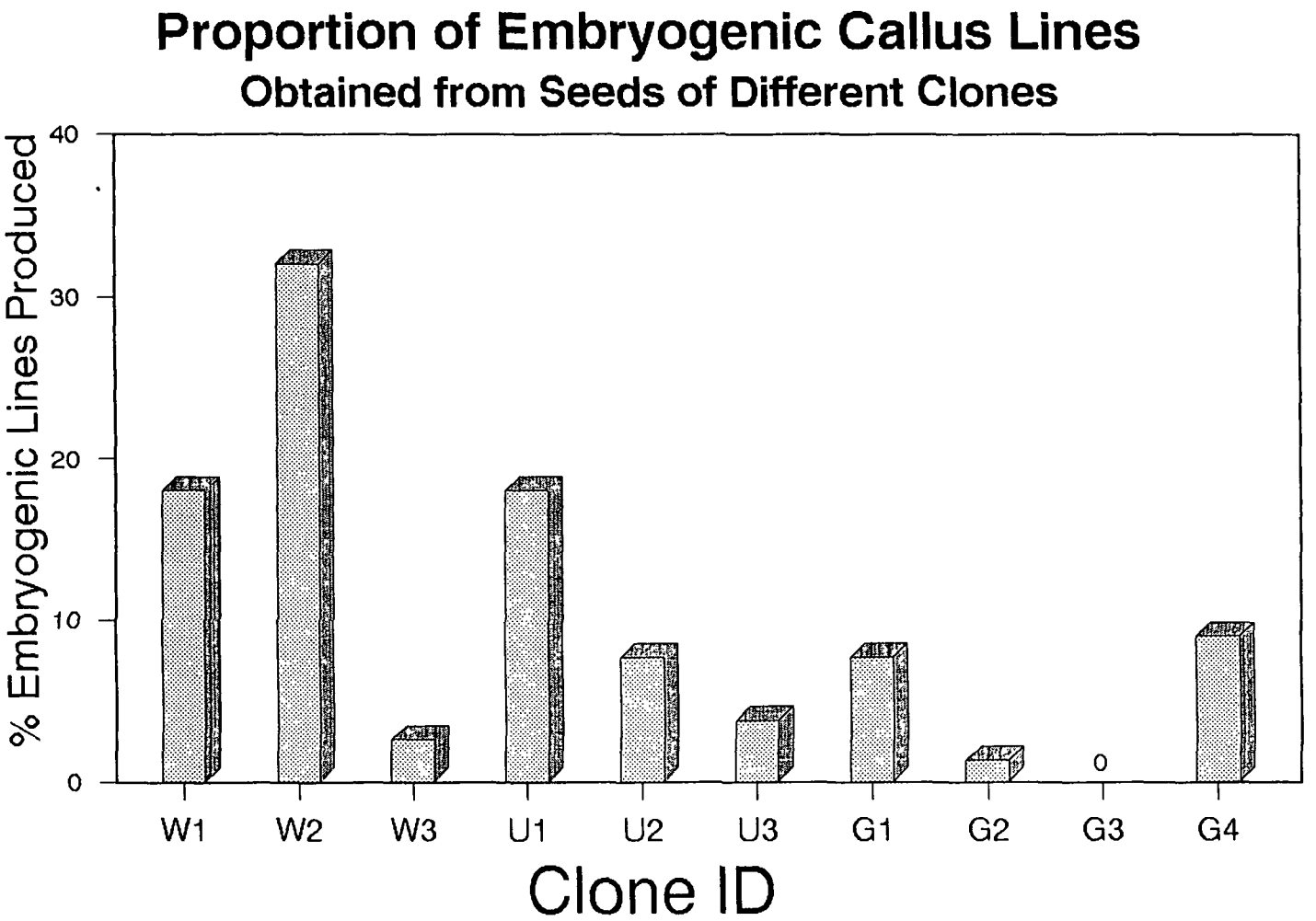

Proportion of Embryogenic Callus Lines
Obtained from Seeds of Different Clones
% Embryogenic Lines Produced
40
30
20
10
0
W1
W2
W3
U1
U2
U3
G1
G2
G3
G4
0
Clone ID

Figure 21. Effects of embryo developmental stage on the production of embryogenic callus lines. The clones are arranged along the x-axis in ascending order with relation to the average developmental stage present is the seeds of each. The scale on the y-axis is identical for average developmental stage and the frequency of embryogenic callus production.

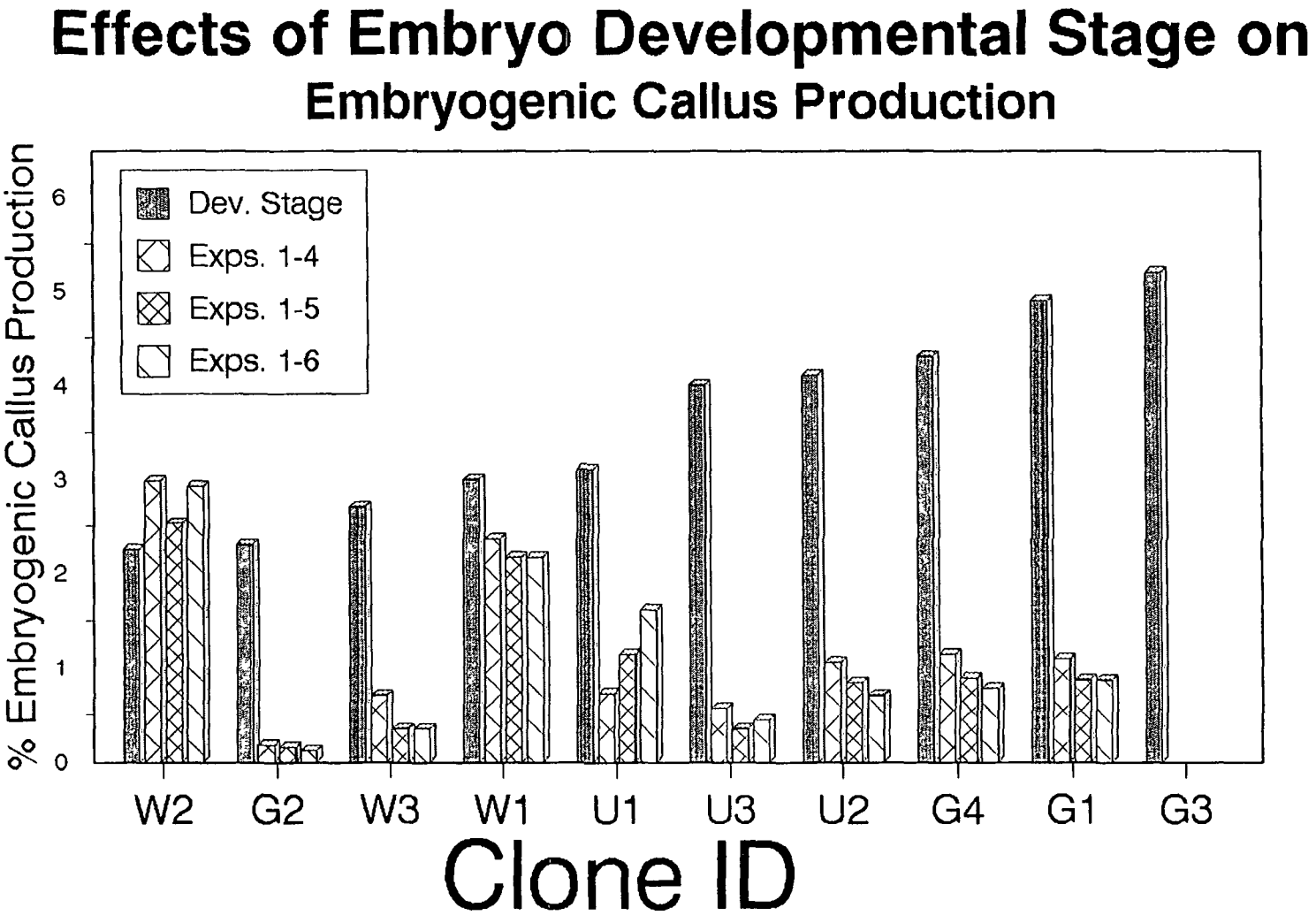

Effects of Embryo Developmental Stage on
Embryogenic Callus Production
% Embryogenic Callus Production
0
1
2
3
4
5
6
Dev. Stage
Exps. 1-4
Exps. 1-5
Exps. 1-6
W2
G2
W3
W1
U1
U3
U2
G4
G1
G3
Clone ID

Figure 22. Effects of 2,4-D with and without BA on embryogenic callus line formation in experiments 1 and 2 with full-strength basal medium.

Effects of 2,4-D with & without BA on

Full-Strength Medium Exps. 1 & 2

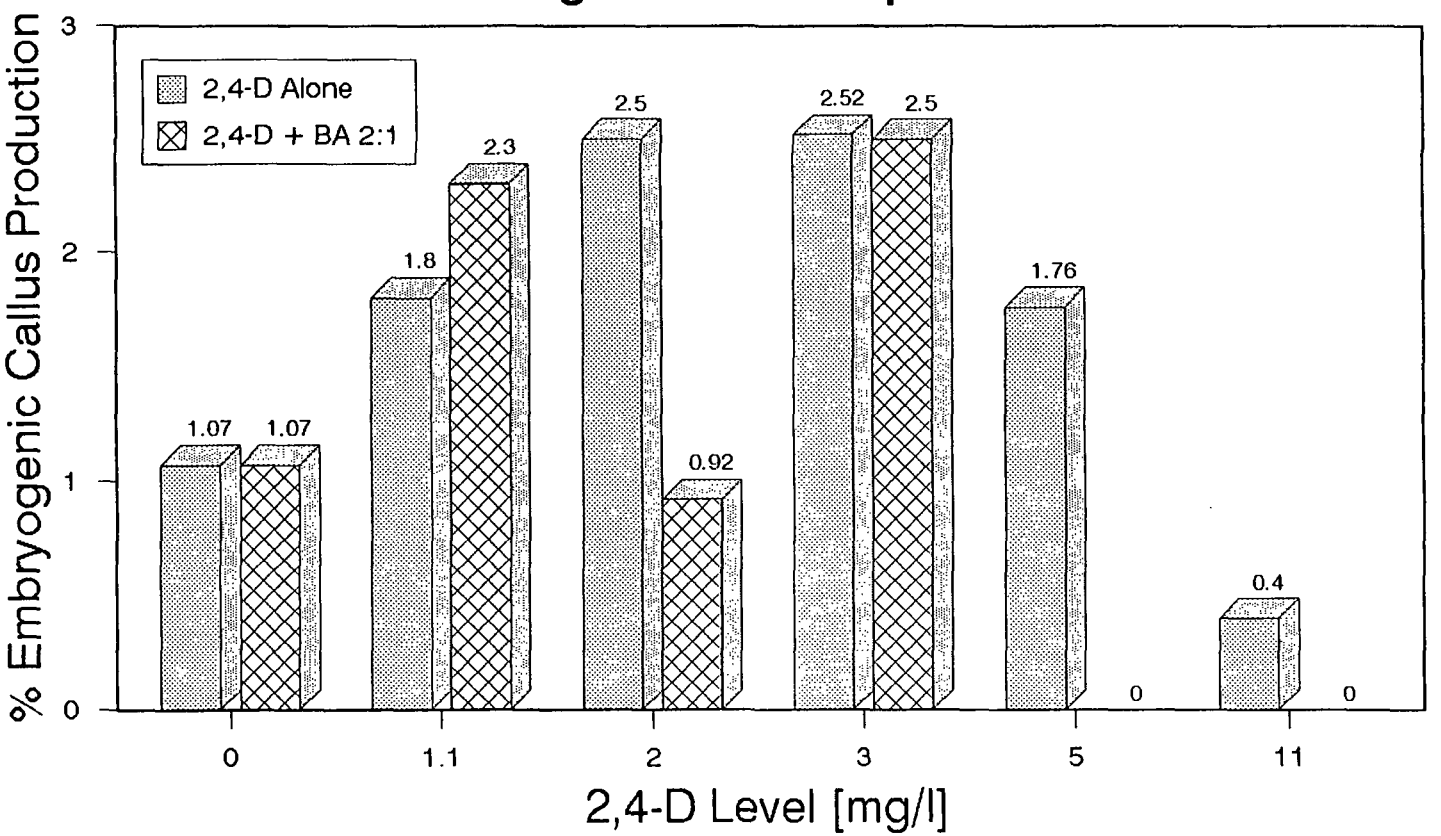


Figure 23. Effects of 2,4-D with and without BA on embryogenic callus line production in experiments 2 and 3 with half-strength basal medium.

Effects of 2,4-D with & without BA on Half-Strength Medium

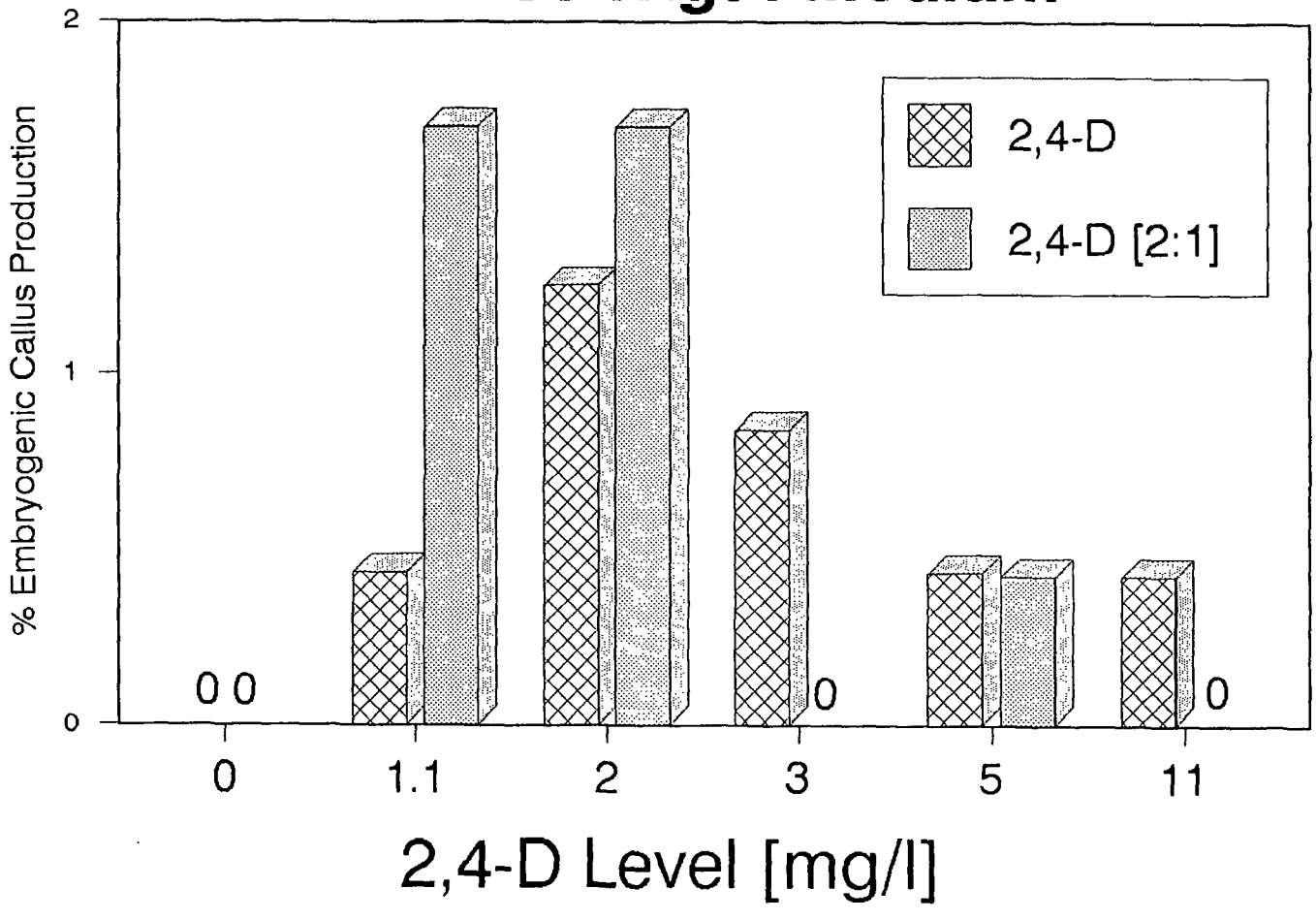


Figure 24. Comparison of embryogenic callus line production with 2,4-D alone on full- and half-strength basal medium. Callus production on hormone-free media has been subtracted from the total formed on media with plant hormones.

Effects of Full- & Half-Strength Media on

Embryogenic Callus Production with 2,4-D Alone

Minus Control [2,4-D = 0 mg/l]

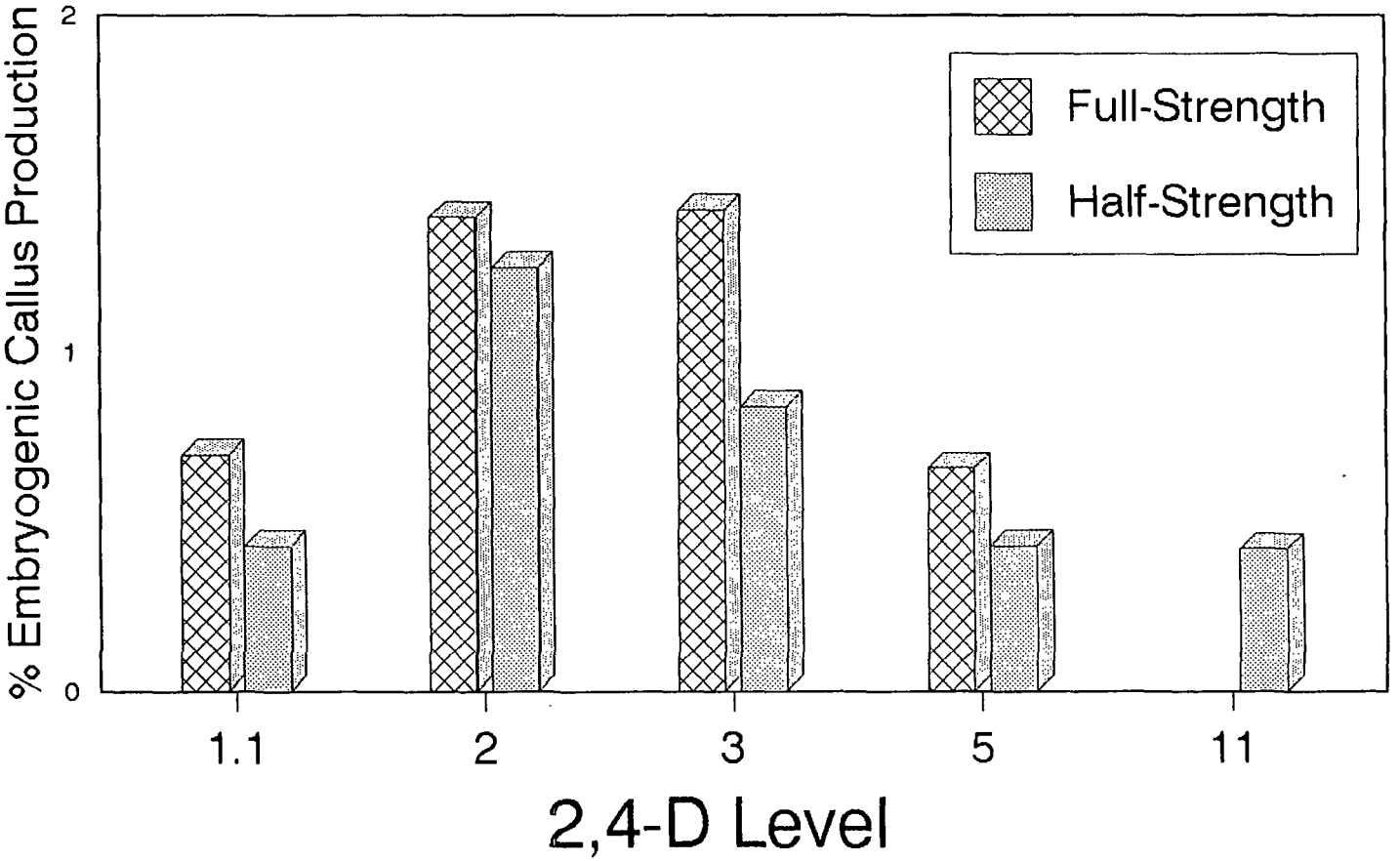


Figure 25. Embryogenic callus line production on full- and half-strength medium containing 2,4-D and BA in a 2:1 ratio. Callus formation on hormone-free media has been subtracted from the total produced on media with plant hormones.

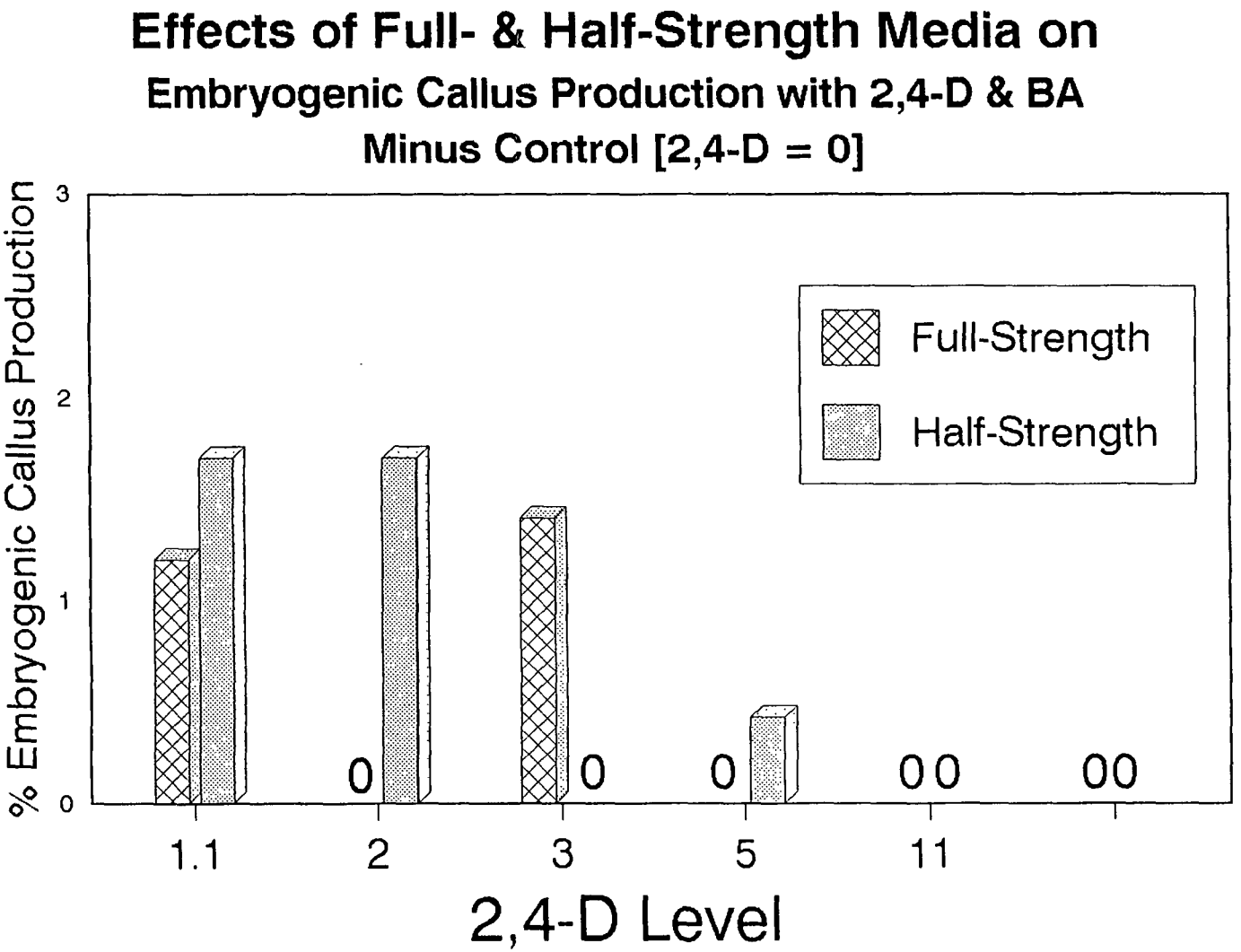

Effects of Full- & Half-Strength Media on
Embryogenic Callus Production with 2,4-D & BA
Minus Control [2,4-D = 0]
% Embryogenic Callus Production
3
2
1
0
Full-Strength
Half-Strength
0
0
0
0 0
0 0
1.1
2
3
5
11
2,4-D Level

Figure 26. Production of embryogenic callus lines on media with 2,4-D alone containing either autoclaved sucrose or maltose at 3%.

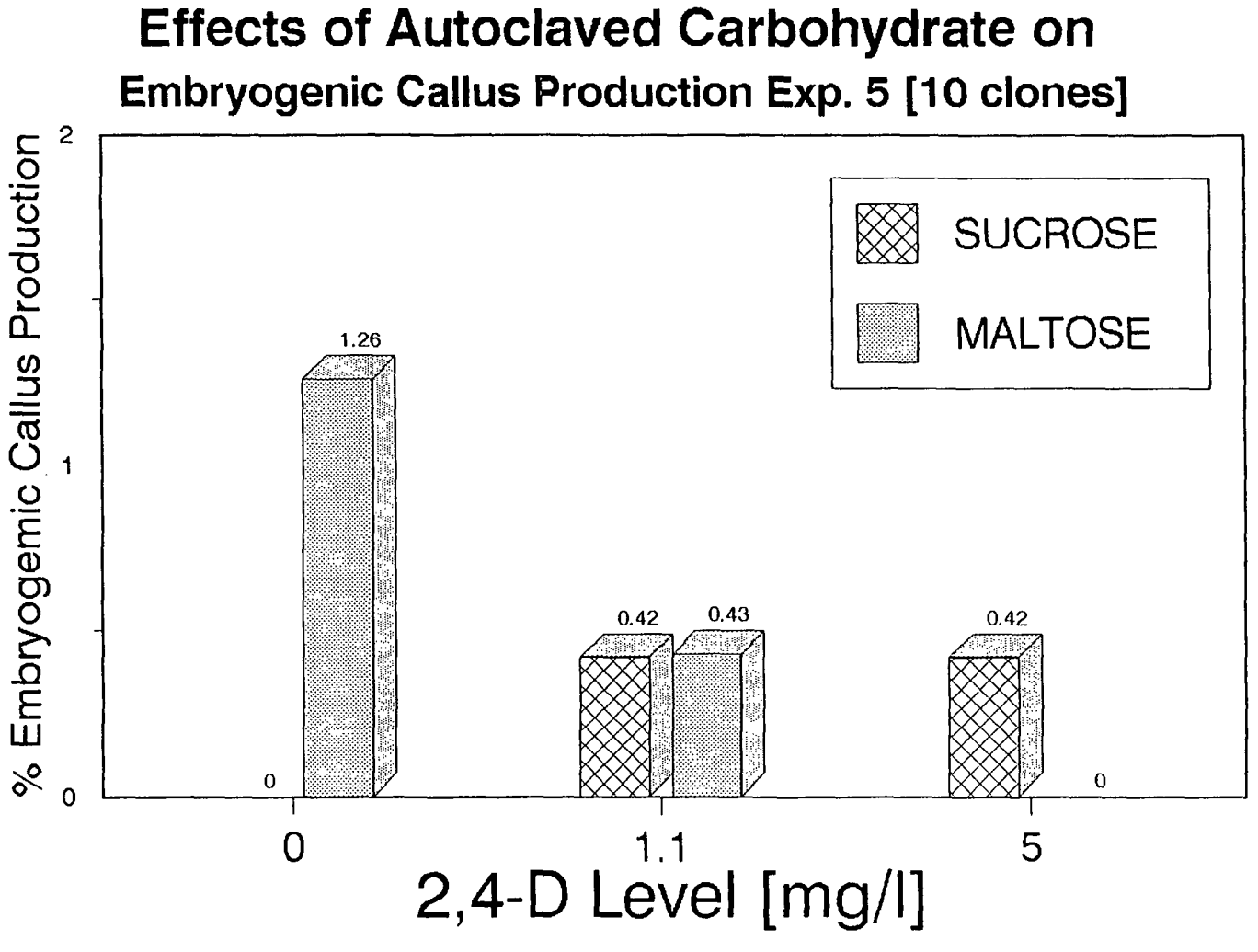

Effects of Autoclaved Carbohydrate on
Embryogenic Callus Production Exp. 5 [10 clones]
% Embryogemic Callus Production
2
1
0
SUCROSE
MALTOSE
1.26
0
0.42
0.43
0.42
0
0
1.1
5
2,4-D Level [mg/l]

Figure 27. Production of embryogenic callus lines on media containing 2,4-D alone with either cold-sterilized sucrose or maltose at 3%.

Effects of Filter-Sterilized Carbohydrates on

Embryogenic Callus Production Exp. 6 [5 clones]

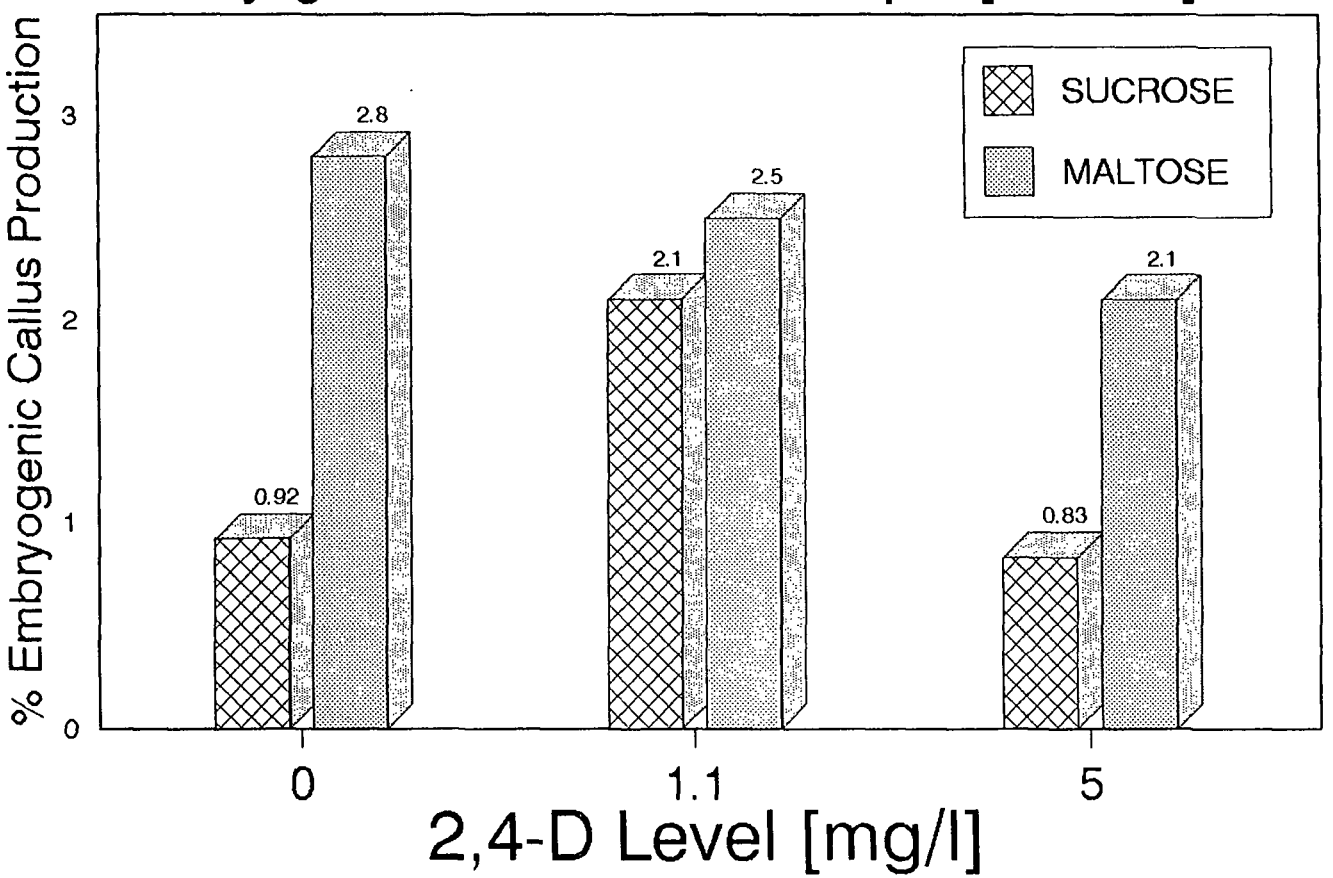
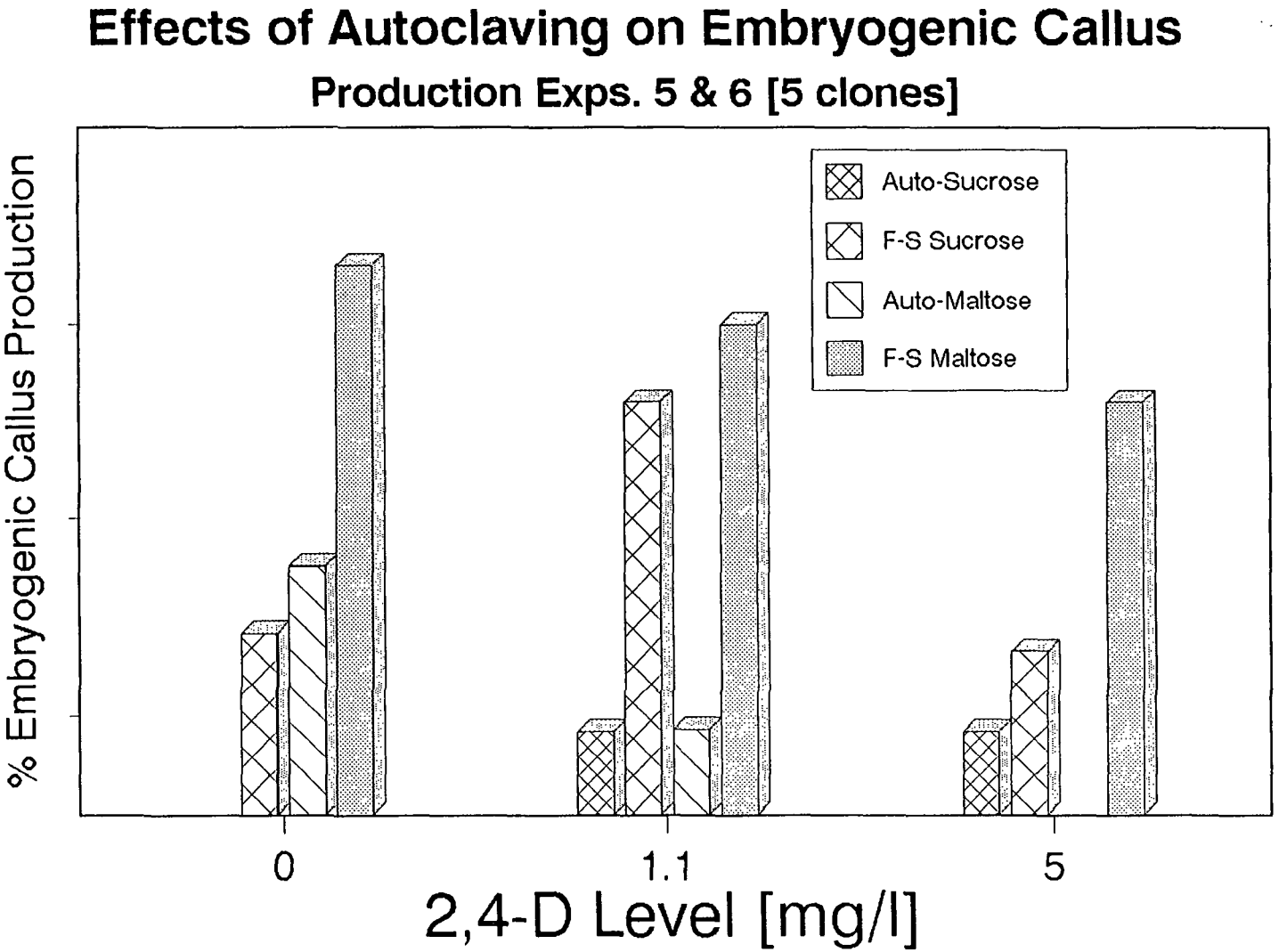


Figure 28. Comparison of embryogenic callus line production by the 5 clones tested in both experiments 5 and six. Auto-Sucrose = Autoclaved Sucrose, F-S Sucrose = Filter [Cold]-Sterilized Sucrose, Auto-Maltose = Autoclaved Maltose, F-S Maltose = Filter-Sterilized Maltose.


Effects of Autoclaving on Embryogenic Callus
Production Exps. 5 & 6 [5 clones]
% Embryogenic Callus Production
Auto-Sucrose
F-S Sucrose
Auto-Maltose
F-S Maltose
0
1.1
5
2,4-D Level [mg/l]

LOBLOLLY PINE

MATURATION

EFFECTS OF MALTOSE, SUCROSE, LIGHT, DARK, CHARCOAL AND CULTURE VESSEL ON THE FORMATION OF LOBLOLLY PINE SOMATIC EMBRYOS

Principal Investigator

David T. Webb

Technical Support

Y. Powell

ABSTRACT

Callus from eight lines developed at the Institute of Paper Chemistry were tested for their ability to form somatic embryos. These lines represented five genotypes, plus three lines of one genotype. Calli were transferred from proliferation medium to medium containing either 0.5 or 1% charcoal. These were subsequently transferred to petri plates or well-plates, with media containing either sucrose or maltose in light or darkness. Three of the genotypes formed somatic embryos. Two of these produced significant numbers of pre-cotyledonary and cotyledonary embryos. Pre-cotyledonary embryos developed with sucrose or maltose, but maltose was superior for the production of cotyledonary embryos. Both charcoal treatments produced equivalent results. Light inhibited somatic embryo development. Petri plates were superior for somatic embryo production.

INTRODUCTION

Prior results have shown that embryogenic callus of loblolly pine forms pre-cotyledonary and cotyledonary embryos on media containing abscisic acid [ABA] and maltose [Annual Review, 1991]. While pre-cotyledonary embryos developed in the presence of sucrose, cotyledonary embryos were not produced. The results of several independent trials had shown that cotyledonary embryos developed with 6% maltose and 20 uM ABA. These experiments were done with a small number of callus lines. In addition, all of these lines were old in terms of their origination, and needed to be reevaluated. Loblolly pine embryogenic callus was routinely grown in the dark, however, work at the IPC and IPST used light

during maturation and development. However, light was shown to be inhibitory to this process elsewhere [1]. The maturation and development process uses an initial transfer to hormone-free medium containing activated charcoal before calli are placed on medium containing ABA and carbohydrate. It was unclear whether 0.5% or 1% charcoal was the best concentration. Embryogenic calli were routinely multiplied in standard petri plates. However, calli were also grown in six-well plates to protect them from contamination before and during maturation and development. It was unclear whether there would be a different response in each culture vessel. The major goal of this work was to screen all loblolly pine lines obtained at the IPC for their ability to form somatic embryos in the presence of maltose or sucrose with 20 uM ABA. The effects of 0.5% versus 1% charcoal, light versus dark and petri plates versus six-well plates were examined.

MATERIALS AND METHODS

Embryogenic callus lines were grown in six-well plates or standard petri plates containing DCR medium, 3% sucrose, 0.6% Bactoagar [Difco], 3 mg/l 2,4-dichlorophenoxyacetic acid [2,4-D] and 0.5 mg/l benzyladenine [BA] in darkness at 25 ^{o}C =/- 2 ^{o}C. In one case [line 7CM, Table 1] callus was grown on MSG medium containing the same sucrose and agar levels as above, but with 2 mg/l 2,4-D and 1 mg/l BA. These were transferred to fresh medium every two weeks. The lines used in this study are given in Table 1. Hereafter only the IPST code will be used to refer to these lines. Line 7 was tested for maturation and development at the

IPC. During the move to Atlanta, backup lines were sent to Westvaco and Union Camp in case our lines became contaminated or died from stress. Line 7B was sent to Westvaco. It was returned to the IPST because of contamination problems. However, the original line 7 recovered and was grown on both DCR [Line 7CD] or MSG [Line 7CM] media. These lines were kept separate in case any culture induced variation had occurred. Consequently, all three of the lines were tested individually.

Calli were transferred to standard hormone-free BLG medium containing 2% sucrose and 0.6% Bactoagar in standard petri plates. This step lasted one week and was always done in the light. Light was supplied by Phillips Agro Lite fluorescent tubes [80-100 ft-c; 16 hr. photoperiod]. Calli were then placed on MSG medium containing 20 uM ABA and either 6% maltose or 6% sucrose. Half were grown in standard petri plates or in six-well plates. Each of these populations was further subdivided, half remained in the light and half were placed in darkness.

Proliferating calli had no macroscopically visible somatic embryos [Fig. 1]. Calli were scored for the presence of macroscopically visible pre-cotyledonary and cotyledonary somatic embryos after six and twelve weeks.

RESULTS

Calli were either creamy white or off-white on proliferation medium. There were no somatic embryos visible on the surface of

these calli [Fig. 1]. Most calli grew somewhat after transfer from charcoal medium to maturation and development media. However, lines 7B, 7CD and 7CM grew considerably on maturation and development media and had to be subdivided because of their increased size.

Singulated macroscopic structures were evident on the surface of the callus after six weeks on maturation and development media [Figs. 2-6]. Many of these were somatic embryos, but some were suspensor-like projections without obvious embryo heads [Fig. 7]. There were fewer somatic embryos visible after twelve weeks, but cotyledonary stages were evident [Figs. 3-4, 10-15]. However, pre-cotyledonary embryos were still present in low abundance [Figs. 2-4].

Embryo development followed the conifer pattern which occurs in pine seeds. The earliest pre-cotyledonary embryos were translucent with glassy, elongate suspensors and small, slightly opaque embryo heads [Fig. 5]. Enlarged embryo heads were observed at the distal end of massive, glassy, loosely-organized suspensors [Fig. 6]. Sometimes large suspensors were present without well organized embryo heads [Fig. 7]. It was difficult to tell whether the heads became organized first [Fig. 5], or whether the heads originated after the massive suspensors had developed. Some embryos proceeded to develop further but many regressed back to callus at this stage.

The next step in maturation was the organization of the suspensor at the root pole of the embryo [Fig. 8]. The suspensor lost its

loose organization and could be seen to consist of organized, highly elongated cells. The shoot apex was the next structure to appear [Fig. 9], followed by the emergence of the cotyledons [Fig. 10]. The cotyledons continued to elongate [Figs. 11-12], and eventually the hypocotyl elongated [Fig. 13].

Lines 5, 7B and 10 formed somatic embryos but the other lines did not. Line 10 produced a few embryos sporadically while the other two lines produced numerous embryos. Consequently, only data from lines 5 and 7B are presented. Lines 7CM and 7CD grew extremely well on maturation and development media but formed no visible somatic embryos.

Line 7B produced somatic embryos with much higher frequencies than line 5 [Figs. 14-19]. Overall embryo production was higher with maltose compared to sucrose. While pre-cotyledonary embryos were formed on sucrose, in general, cotyledonary embryos only developed with maltose. However, a few calli had cotyledonary embryos in dark-grown petri plates with line 5, 0.5% charcoal after six weeks [Fig. 14]. The frequency of somatic embryo production tended to increase from week six to week 12. This was especially the case for cotyledonary embryos. However, there were some cases in which the frequency declined. The latter was due to recallusing and the apparent demise of many large pre-cotyledonary embryos. This was especially the case with line 5, and brown pre-cotyledonary embryos could be seen on the callus surface after 12 weeks.

Light inhibited somatic embryo production [Figs. 14-19]. This was especially the case for cotyledonary embryos. Light-exposure led to anthocyanin production and the calli were more granular appearance compared to those in the dark. Pre-cotyledonary embryos were abundantly produced in well-plates and petri plates. However, the production of cotyledonary embryos was enhanced in the petri plates. The relative effects of the two charcoal levels were ambiguous, and it was not clear that either was better than the other.

Because embryos tended to become brown when left on the callus, pre-cotyledonary and early-stage cotyledonary embryos were removed and cultured individually on DCR medium containing 20 uM ABA and 6% maltose. Pre-cotyledonary embryos developed cotyledons, and cotyledonary embryos elongated and produced larger cotyledons. However, there was a tendency for these isolated embryos to become vitrified and produce callus, and none developed to a stage suitable for germination.

DISCUSSION

These experiments confirmed earlier results which showed that pre-cotyledonary embryos developed with sucrose and maltose, but cotyledonary embryos developed preferentially with maltose. It is known that sucrose is hydrolyzed into glucose and fructose during autoclaving [see 1991 Annual Review]. Furthermore, fructose can be converted into a toxic metabolite by autoclaving [2]. Maltose is a dimer of glucose and its hydrolysis yields only glucose. Consequently, it is possible that the inferior results obtained

with sucrose were due to autoclave-catalyzed toxin production. In addition, maltose has a tendency to lower the medium pH, relative to sucrose, following autoclaving [3]. The pH effects vary markedly with different media and can be significant. It is noteworthy that embryogenic callus proliferated more rapidly on maltose versus sucrose [see this Annual Review], and that filter-sterilized carbohydrates were more favorable for embryogenic callus production than autoclaved sugars.

These results clearly showed that petri plates work as well as, or better than well-plates, and that darkness was preferable to light-exposure. The original charcoal treatment with light was used in all the cases. The effects of this step were ambiguous as both 0.5% and 1% gave equivalent results. The role of charcoal and its interaction with light are unknown with regard to this process. It is possible that the charcoal step is unnecessary or even inhibitory. One line from the 1990 maturation and development screening tests formed embryos more readily without a charcoal step than with one. This step will be included until an experiment can be done to verify its importance and potential interaction with light.

While these conditions may not be optimal, they have supported the production of pre-cotyledonary and cotyledonary embryos from several lines. Consequently, we are using DCR medium containing 20 uM ABA and 6% maltose to screen for somatic embryo production with lines obtained in 1990 and 1991. Several positive lines have been identified from the 1990 cultures, and one of these is very

robust. The testing of lines initiated last summer [1991] has just begun, and several lines gave positive results in a small trial.

Future work will focus on obtaining optimal maltose and ABA concentrations. Experiments are in progress with lines 5 and 7 to determine the optimal ABA level [10-50 uM], and maltose concentration [3-12%]. The interactions between ABA and maltose at 20-40 uM ABA and 6-12% maltose are being tested in a factorial design. The results of this experiment will be used to refine experiments for determining optimal conditions for new lines. The best lines, like line 7, will be used to develop suspension cultures which will also be used to study maturation and development.

It is well known that cultures develop genetic variation in vitro. It is most probable that the stress caused by the transfer of callus lines to Atlanta, combined with poor culture facilities here, caused variations within line 7 which led to the loss of regenerative potential in the line we transferred here from Appleton. Lines 7B, 7CD and 7CM grew the same and had the same coloration but only 7B which was stored at Westvaco produced embryos.

The maltose effect probably involves osmotic as well as nutritional effects. Work at Weyerhaeuser [1] has definitely shown that the osmolarity of the medium influences somatic embryo development. The osmolarity of media used in our present experiments is being analyzed, and the osmolarity of future media

will be measured. The effect of autoclaved versus filter-sterilized carbohydrates on medium pH will also be examined.

REFERENCES

1] Gupta, P. K., and G. R. Pullman. 1990. Method for reproducing coniferous plants by somatic embryogenesis. U.S. Patent No. 4,957,866, September 18, 1990,

2] Hsiao, K-C., and C. H. Bornman. 1991. Further studies on autoclave-induced toxicity in tissue culture media: gauging sugar breakdown by spectrophotometry. Physiol. Plant. 82: 261-265.

3] Owen, H. R., D. Wengerd and A. R. Miller. Culture pH is influenced by basal medium, carbohydrate source, gelling agent, activated charcoal, and medium storage method. 1991. Plant Cell Reps. 10: 583-586.

Table 1. Lines Used to Test for Somatic Embryo Production

IPST Code	IPC Code
2	[672 LP 7D]3
3	[672 LP 7D]4
5	[672 LP 7H]1
6	[666 LP 4H]1
10	[575 LP 7F]1
7B[1]	[574 LP 2G]1
7CD[2]	[574 LP 2G]1
7CM	[574 LP 2G1]1

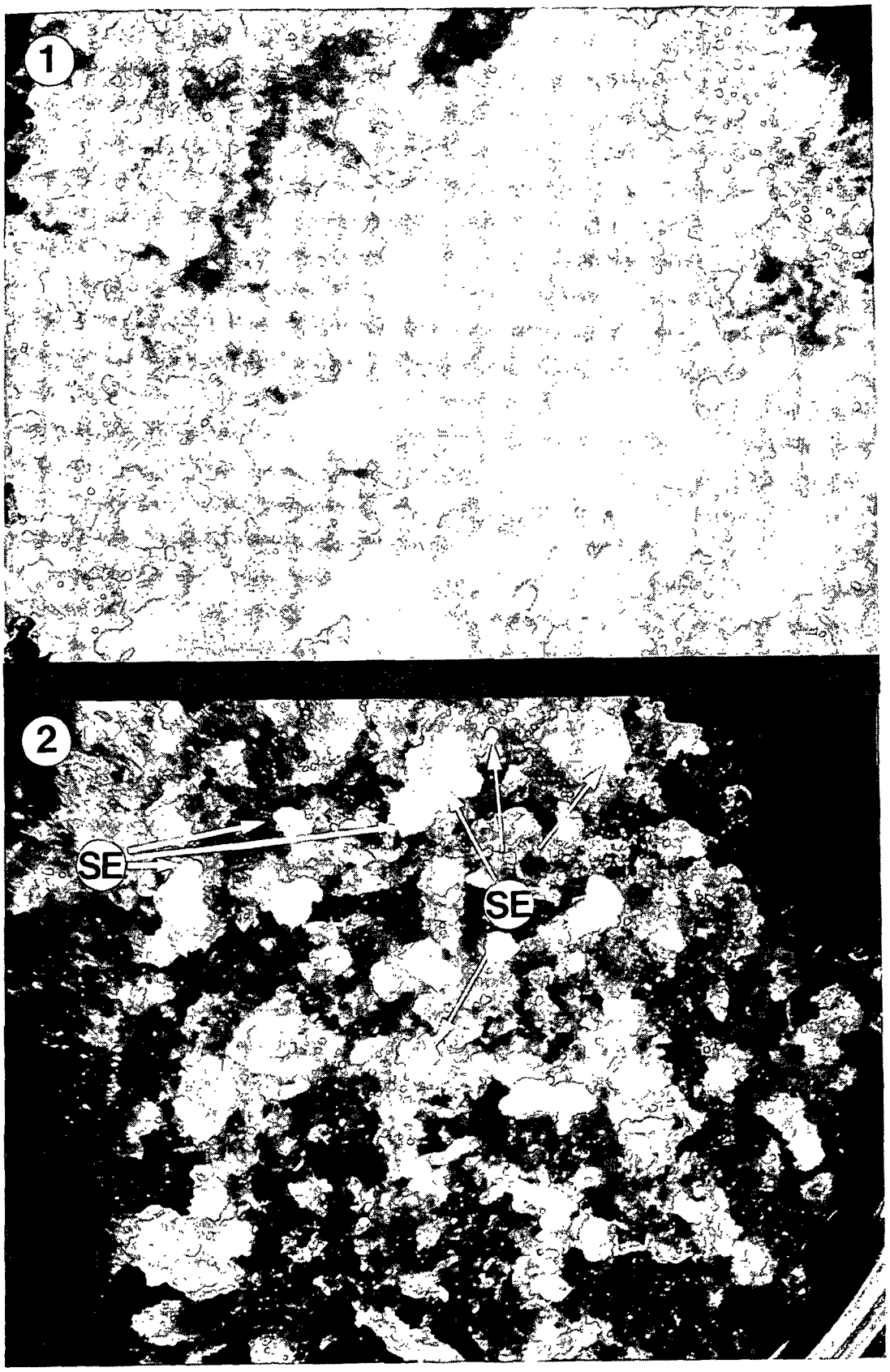
[1]Original IPC line stored at Westvaco during Atlanta move

[2]Original IPC line grown on DCR medium, transferred directly to Atlanta

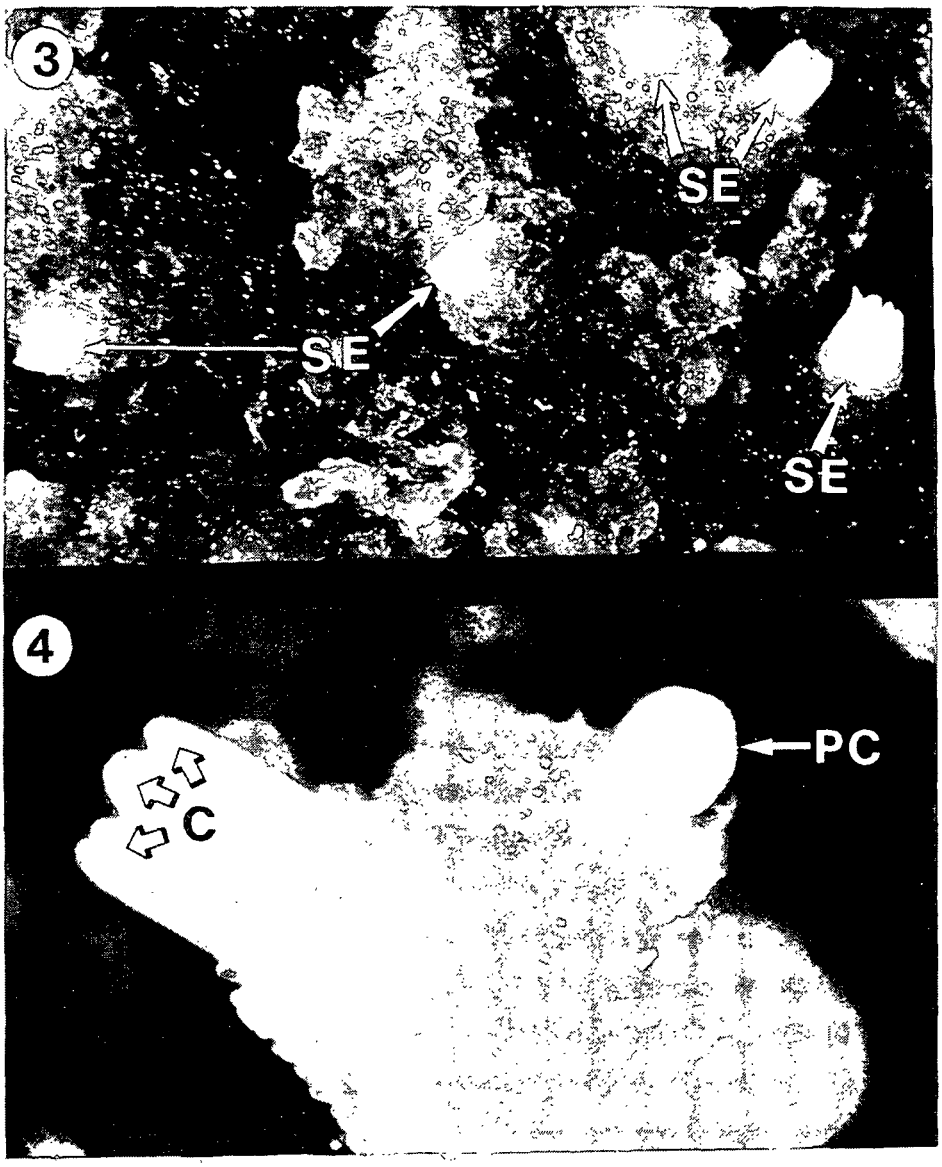
[3]Original IPC line grown on MSG medium, transferred directly to Atlanta

Figure 1. Friable callus from line 7 after charcoal step. Magnification = 6X.

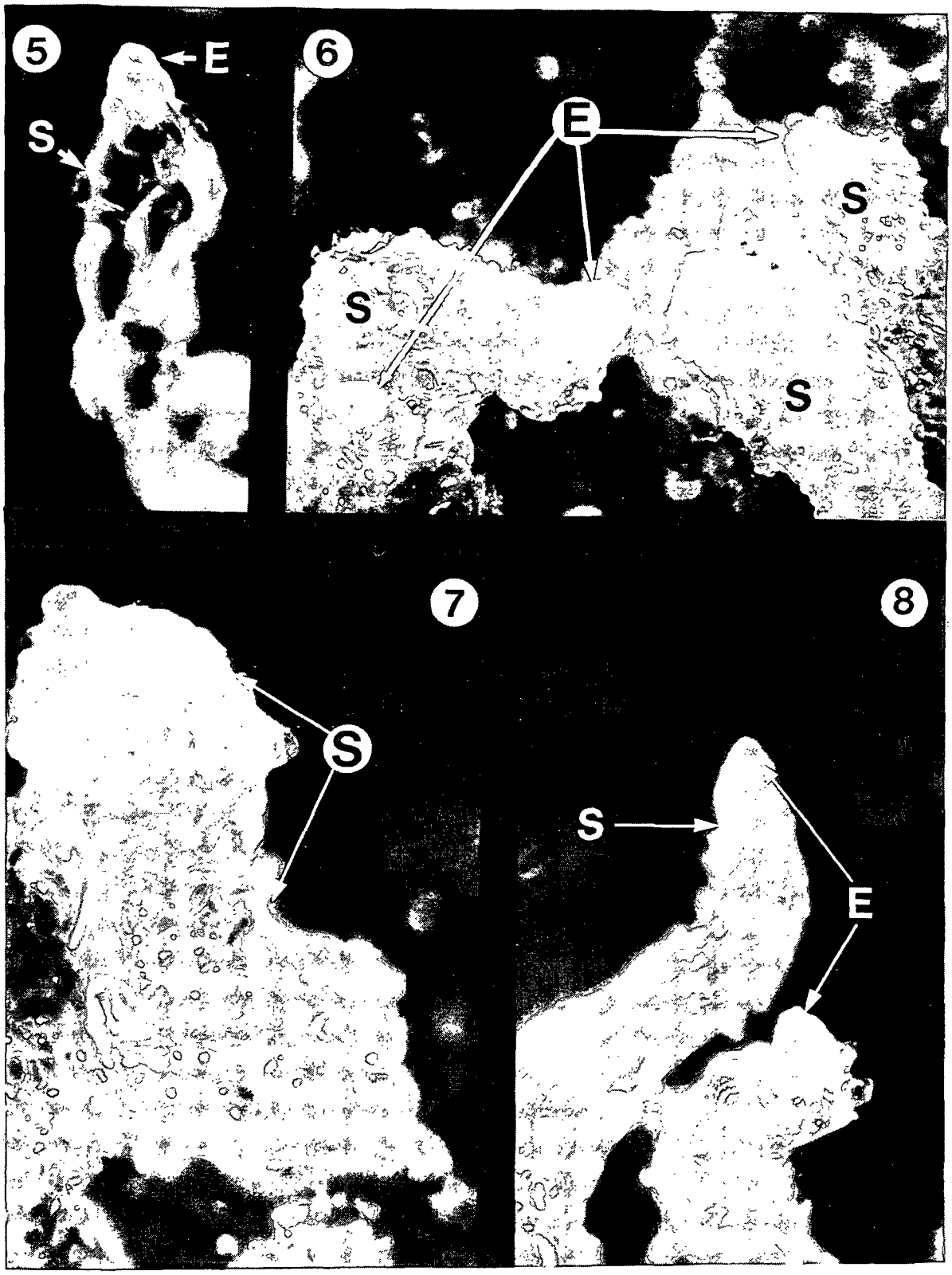
Figure 2. Same as Figure 1 but after 12 weeks on DCR with 20 uM ABA and 6% maltose. Note the many somatic embryos [SE]. Magnification = 6X.


1
2
SE
SE

Figures 3 and 4. Same as Figure 2, except at higher magnification. Note the pre-cotyledonary [PC] somatic embryos and embryos with cotyledons [C]. Magnifications = 11.5X [Fig. 3] and 44X [Fig. 4].


3
SE
SE
SE
4
PC
C

Figures 5-8. Structures protruding from the surface of calli after 12 weeks on maturation and development media. Fig. 5. Early pre-cotyledonary embryo. Note the glassy suspensor[S] and opaque embryo head [E]. Magnification = 70X. Fig. 6. Same as Fig. 5 except for greatly enlarged suspensors and larger embryo heads. Magnification = 26X. Fig. 7. Enlarged suspensor without prominent embryo head. Magnification = 42X. Fig. 8. Same as Figs. 5-6 but showing a more highly organized suspensor [S]. Magnification = 70X.


5
E
S
6
E
S
S
S
7
S
8
S
E

Figures 9-13. Stages of somatic embryo development from calli on maturation and development media. Fig. 9. Pre-cotyledonary embryo showing the shoot apex primordium [A] and hypocotyl [H]. Magnification = 70 X. Fig. 10. Embryo showing the first sign of cotyledon [C] development along the flank of the shoot apex. Magnification = 70X. Fig. 11. Same as Fig. 10, except with larger cotyledons. Magnification = 57X. Fig. 12. Same as 11 buy with larger cotyledons and elongating hypocotyl. Magnification = 46X Fig. 13. Same as Fig. 12 but with elongated hypocotyl. Magnification = 38X.

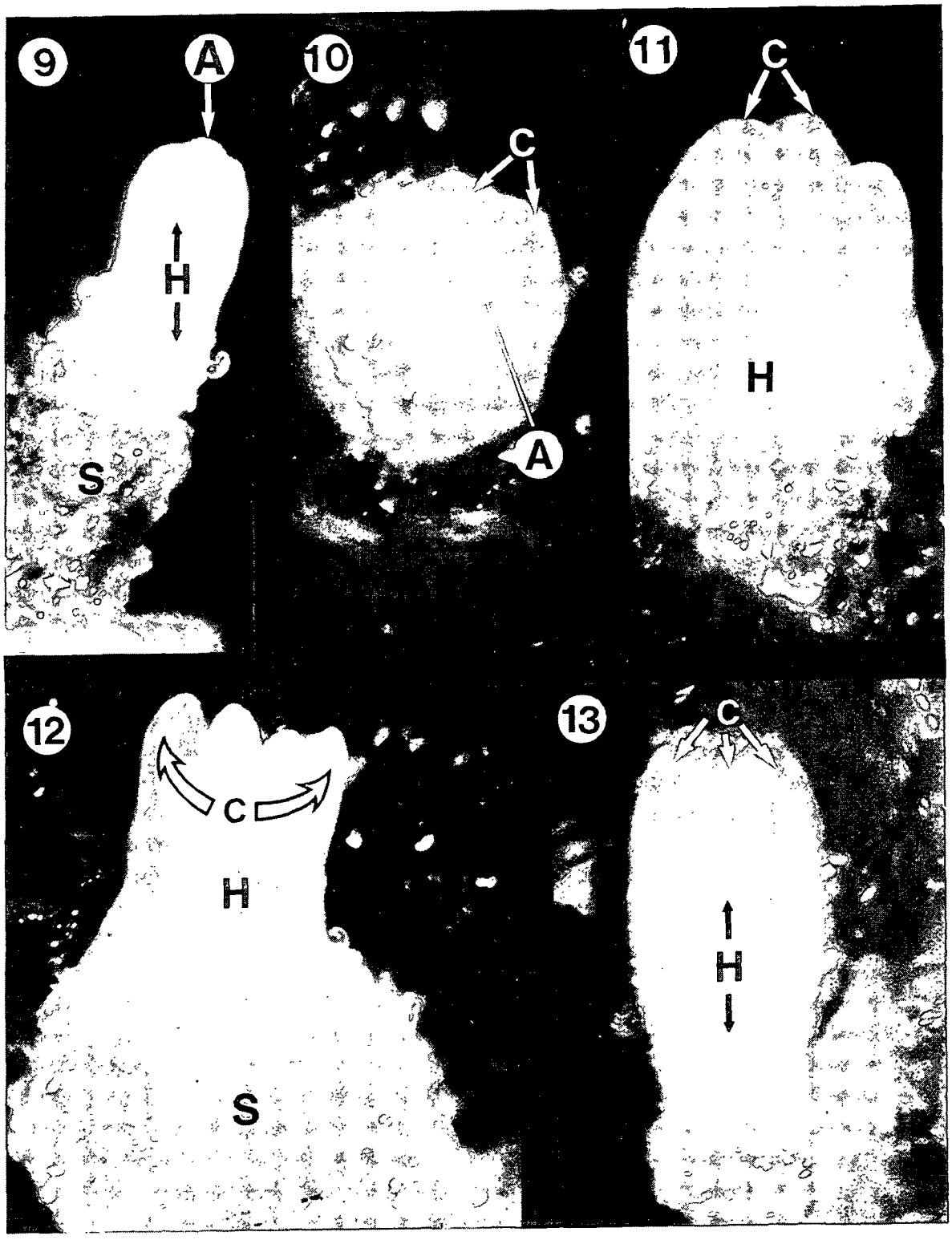

9
A
H
S
10
C
A
11
C
H
12
C
H
S
13
C
H

Figure 14. Effects of maltose and sucrose on the production of somatic embryos by line 5 after 0.5% charcoal and 6 weeks on maturation and development media which all contained 20 uM ABA.

[1]MS6-Well Plate Lt. = 6% sucrose, well-plates in the light

MS6-Petri Plate Lt. = 6% sucrose, petri plates in the light

MS6-Well Plate Dk. = 6% sucrose, well plates in the dark

MS6-Petri Plate Dk. = 6% sucrose, petri plate, in the dark

[1]MM6 are the same as MS6 but contain 6% maltose.

Production of Pre-Cotyledonary & Cotyledonary

Somatic Embryos with Maltose or Sucrose [6%]

After 6 Weeks Line 5 [0.5% Charcoal]

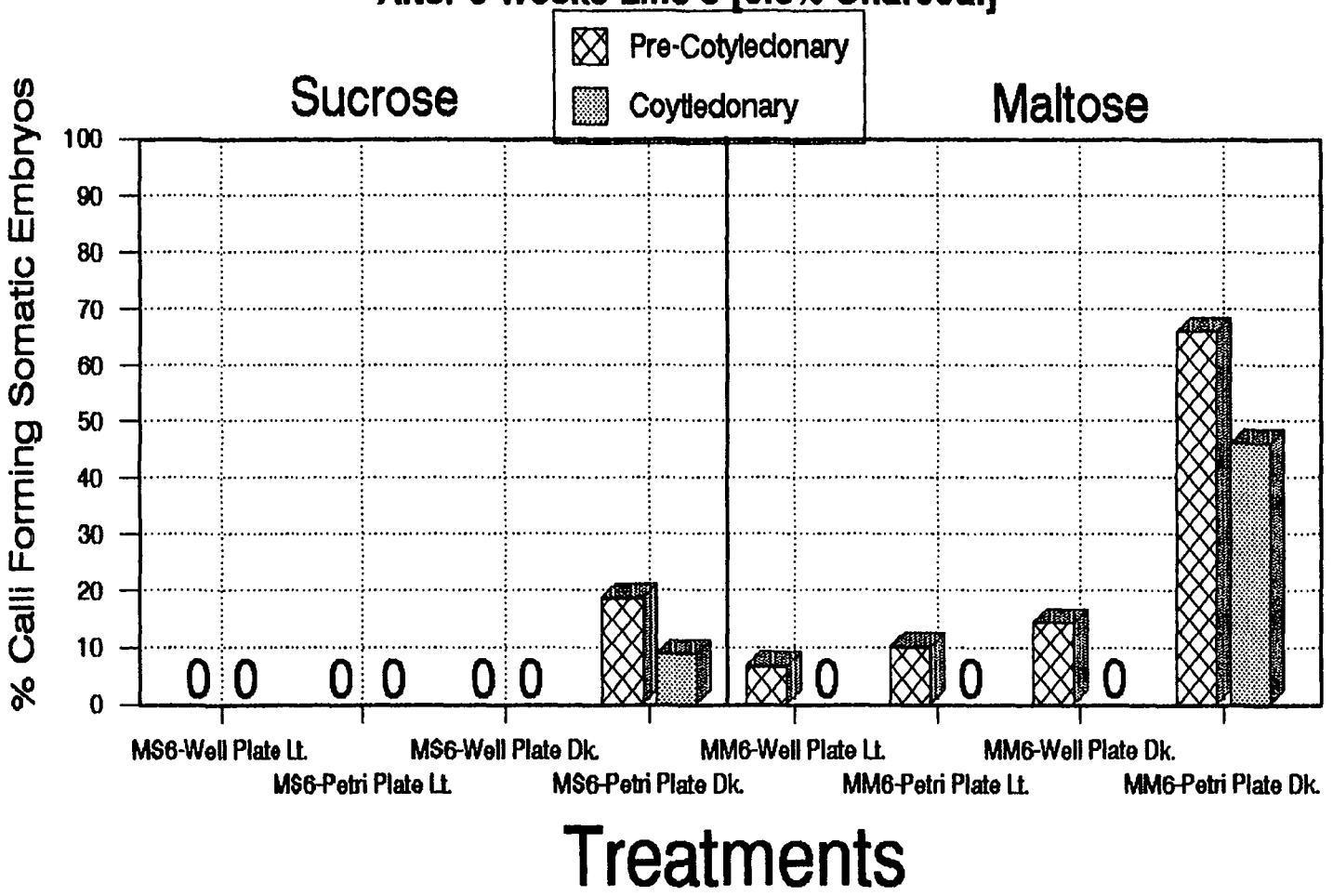


Figure 15. Same as Fig. 14, except with 1% charcoal pretreatment.

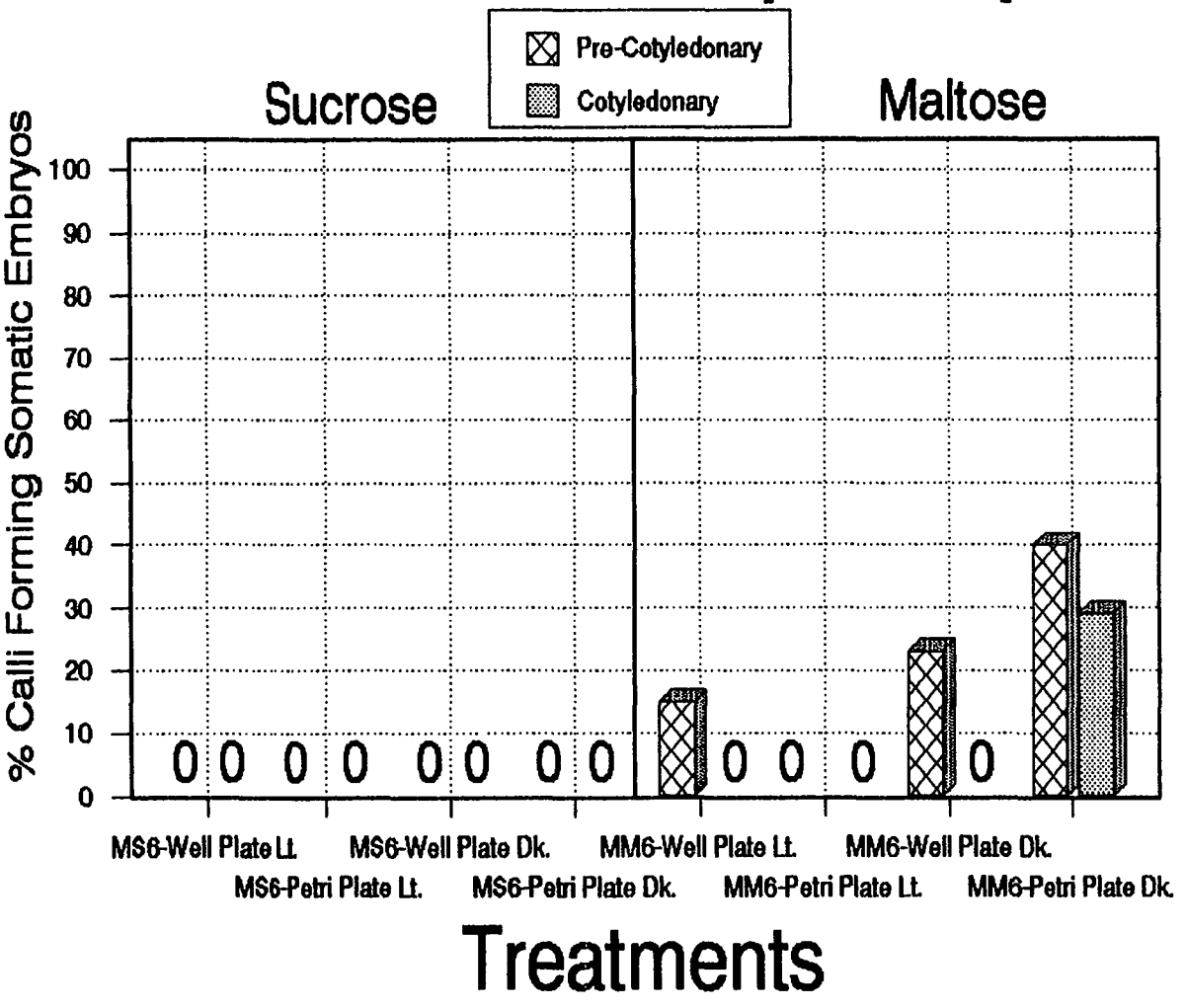
[1]MS6-Well Plate Lt. = 6% sucrose, well-plates in the light

MS6-Petri Plate Lt. = 6% sucrose, petri plates in the light

MS6-Well Plate Dk. = 6% sucrose, well plates in the dark

MS6-Petri Plate Dk. = 6% sucrose, petri plate, in the dark

[1]MM6 are the same as MS6 but contain 6% maltose.


Production of Pre-Cotyledonary & Cotyledonary
Somatic Embryos with Maltose or Sucrose [6%]
After 6 Weeks Line 5 [1% Charcoal]
Pre-Cotyledonary
Cotyledonary
Sucrose
Maltose
% Calli Forming Somatic Embryos
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40
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0
0 0 0 0 0 0 0 0 0 0 0 0
MS6-Well Plate Lt.
MS6-Petri Plate Lt.
MS6-Well Plate Dk.
MS6-Petri Plate Dk.
MM6-Well Plate Lt.
MM6-Petri Plate Lt.
MM6-Well Plate Dk.
MM6-Petri Plate Dk.
Treatments

Figure 16. Same as Fig. 14, except after 12 weeks on maturation and development media.

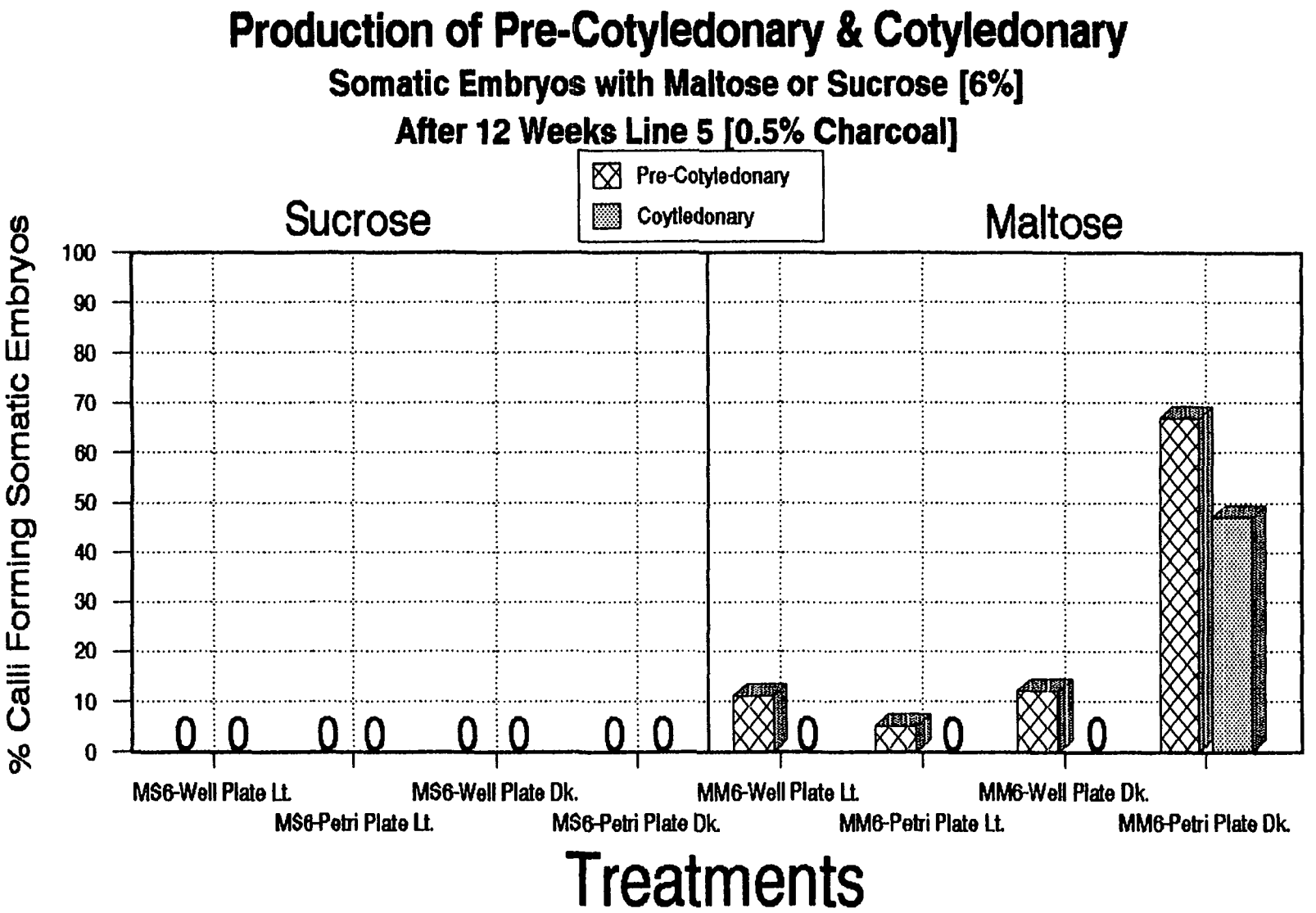
[1]MS6-Well Plate Lt. = 6% sucrose, well-plates in the light

MS6-Petri Plate Lt. = 6% sucrose, petri plates in the light

MS6-Well Plate Dk. = 6% sucrose, well plates in the dark

MS6-Petri Plate Dk. = 6% sucrose, petri plate, in the dark

[1]MM6 are the same as MS6 but contain 6% maltose.


Production of Pre-Cotyledonary & Cotyledonary
Somatic Embryos with Maltose or Sucrose [6%]
After 12 Weeks Line 5 [0.5% Charcoal]
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Coytledonary
Sucrose
Maltose
% Calli Forming Somatic Embryos
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MS6-Petri Plate Lt.
MS6-Well Plate Dk.
MS6-Petri Plate Dk.
MM6-Well Plate Lt.
MM6-Petri Plate Lt.
MM6-Well Plate Dk.
MM6-Petri Plate Dk.
Treatments

Figure 17. Same as Figure 15, except after 12 weeks.

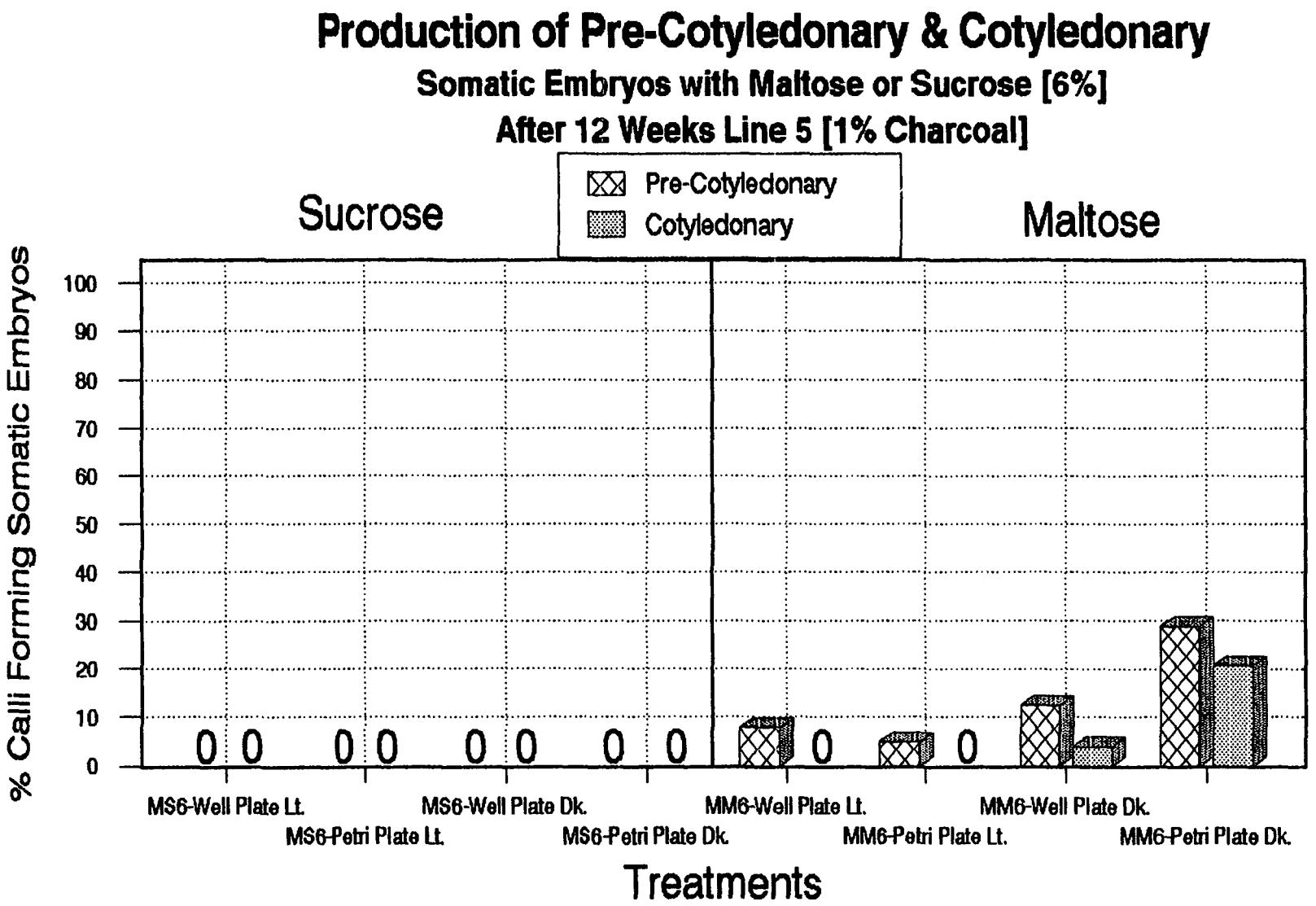
[1]MS6-Well Plate Lt. = 6% sucrose, well-plates in the light

MS6-Petri Plate Lt. = 6% sucrose, petri plates in the light

MS6-Well Plate Dk. = 6% sucrose, well plates in the dark

MS6-Petri Plate Dk. = 6% sucrose, petri plate, in the dark

[1]MM6 are the same as MS6 but contain 6% maltose.


Production of Pre-Cotyledonary & Cotyledonary
Somatic Embryos with Maltose or Sucrose [6%]
After 12 Weeks Line 5 [1% Charcoal]
Pre-Cotyledonary
Cotyledonary
Sucrose
Maltose
% Calli Forming Somatic Embryos
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MS6-Well Plate Lt.
MS6-Petri Plate Lt.
MS6-Well Plate Dk.
MS6-Petri Plate Dk.
MM6-Well Plate Lt.
MM6-Petri Plate Lt.
MM6-Well Plate Dk.
MM6-Petri Plate Dk.
Treatments

Figure 18. Production of somatic embryos with line 7B after 12 weeks on maturation and development media following 0.5% charcoal pretreatment.

[1]MS6-Well Plate Lt. = 6% sucrose, well-plates in the light

MS6-Petri Plate Lt. = 6% sucrose, petri plates in the light

MS6-Well Plate Dk. = 6% sucrose, well plates in the dark

MS6-Petri Plate Dk. = 6% sucrose, petri plate, in the dark

[1]MM6 are the same as MS6 but contain 6% maltose.

Production of Pre-Cotyledonary & Cotyledonary

Somatic Embryos with Maltose or Sucrose [6%]

After 12 Weeks Line 7B [0.5% Charcoal]

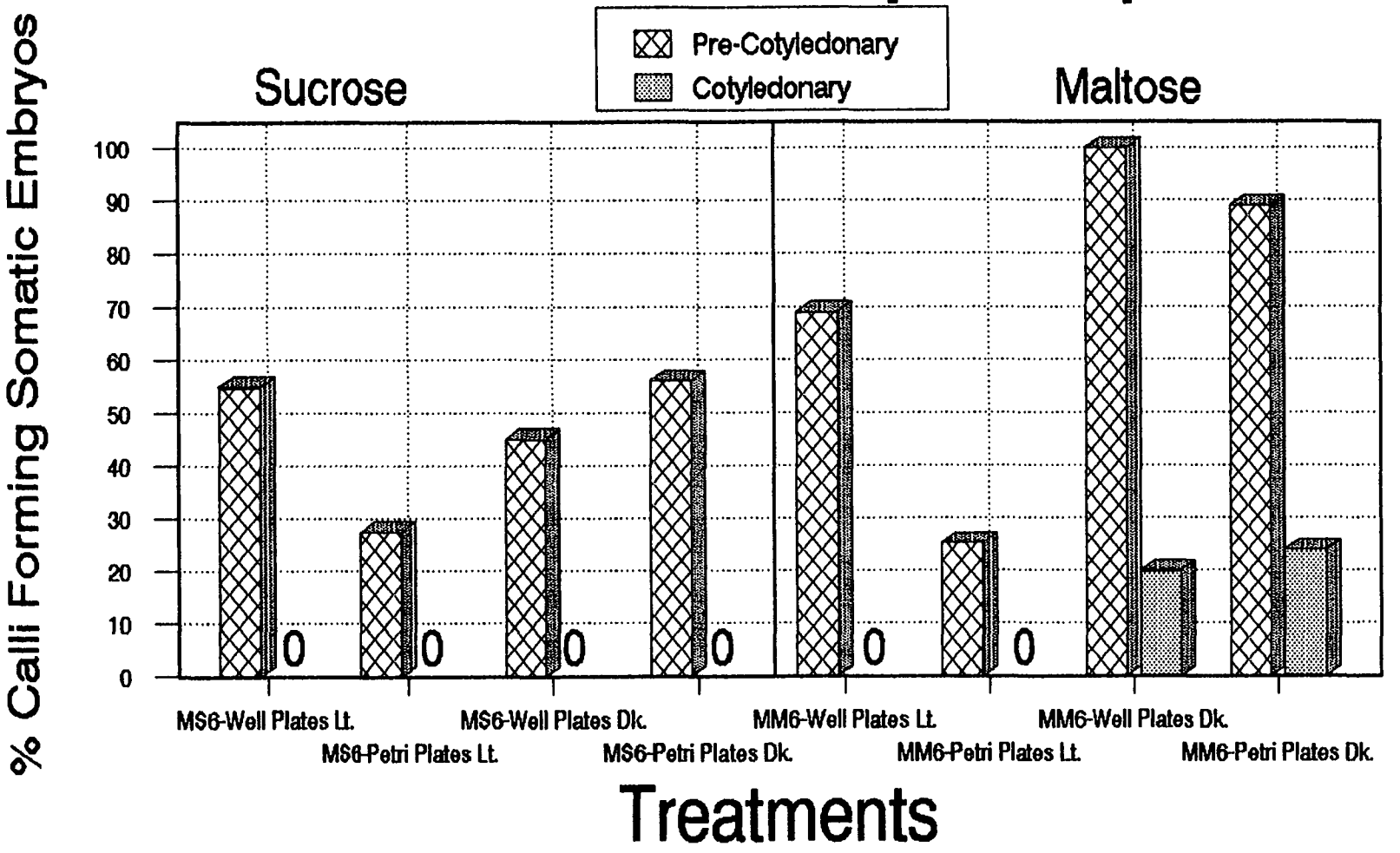


Figure 19. Same as Fig. 18, except with 1% charcoal pretreatment.

[1]MS6-Well Plate Lt. = 6% sucrose, well-plates in the light

MS6-Petri Plate Lt. = 6% sucrose, petri plates in the light

MS6-Well Plate Dk. = 6% sucrose, well plates in the dark

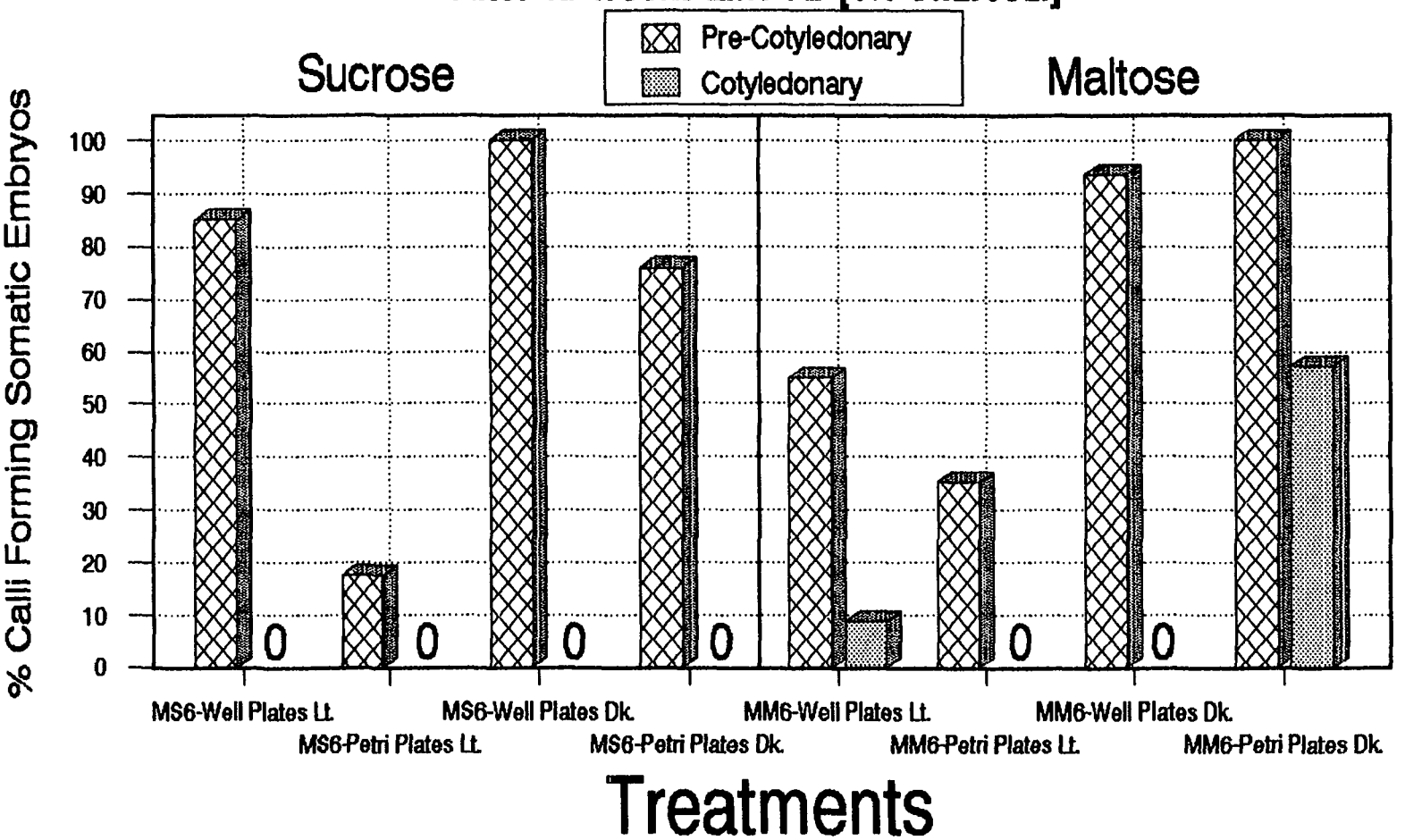
MS6-Petri Plate Dk. = 6% sucrose, petri plate, in the dark

[1]MM6 are the same as MS6 but contain 6% maltose.

Procuction of Pre-Cotyledonary & Cotyledonary

Somatic Embryos with Maltose or Sucrose [6%]

After 12 Weeks Line 7B [1% Charcoal]



LOBLOLLY PINE

CONVERSION

CONVERSION OF LOBLOLLY PINE SEEDLINGS AND EMBRYOS

Principal Investigators

D. Webb & R. Dinus

Technical Assistance

Barbara Johns

Kevin Heit[1]

[1] Intern, Fulton County Board of Education, Gifted Department Internship Program

ABSTRACT

Seeds of loblolly pine were germinated in various substrates under *in vitro* and *ex vitro* conditions. Nearly equivalent results were obtained in terms of germination rate, hypocotyl and cotyledon length for all treatments. The best results occurred when seeds were germinated in the greenhouse with no initial dark period. Seedlings which originated from the lighted culture room tended to be taller but were less sturdy than those grown directly in the greenhouse. Sorborods and Rockwool plugs were suitable germination substrates, however, tap roots tended to grow through Sorborods without branching, and roots took a long time to emerge from Rockwool. Half-strength Schenk and Hildebrandt medium supported the germination and growth of seedlings. Zygotic embryos germinated rapidly on agar-solidified DCR medium. Embryos placed on the surface grew better than those with their cotyledons inserted into the medium. Embryos germinated in the light had curled hypocotyls which made them unsuitable for further study. This was not a problem with embryos germinated in the dark for five to seven days. Hypocotyl elongation preceded root growth, but roots grew rapidly and reached one centimeter in length by day ten. These were transferred to Rockwool plugs for further development.

INTRODUCTION

The term conversion has recently been used to signify the successful germination and establishment of autotrophic plants from somatic embryos. The term embling has recently been used to describe plants produced from zygotic or somatic embryos [23]. The goals of this work are to define conditions for the germination and acclimatization of zygotic embryos of loblolly pine *in vitro* and *ex vitro*. This knowledge will be applied to the conversion of loblolly pine somatic embryos into somatic emblings [23]. In addition, seeds will be used to test germination media, substrates, containers and acclimatization conditions. Seedling and embling organs will be measured to develop the phenology of germination and provide comparative growth measurements for somatic emblings.

Little information is available regarding the germination of gymnosperm embryos *in vitro* [8]. The few articles in the literature deal with late-stage cotyledonary embryos and do not contain sufficient data to deduce conditions suitable for the culture of early-stage embryos. There is virtually no data on the successful culture of pre-cotyledonary conifer embryos [8]. Prior results with mature cotyledonary embryos of *Pinus* have used either White's, Knop's or Murashige-Skoog medium [1, 3, 4, 16]. Half-strength Schenk and Hildebrandt [17] medium was used to culture mature *Pinus strobus* embryos [22]. In this case, the root and hypocotyl grew normally, but their overall elongation was inferior compared to

intact seeds. Furthermore, the epicotyl did not emerge during the 28 days these embryos were in culture.

White's medium with various modifications was used unsuccessfully to culture mature Picea abies embryos [Webb, unpublished]. The best medium was half-strength HM which was the basal medium used during the maturation and development of spruce somatic embryos [23]. Hogland's medium has been used as the post-germination medium with white spruce somatic embryos [23]. Litvay medium [LM] was used to germinate black spruce somatic embryos [21].

MSG medium is used for the maturation & development of loblolly pine somatic embryos in the present IPST protocol, and was used successfully for the germination of larch somatic embryos [12]. Preliminary results, presented later, have shown that loblolly pine zygotic embryos germinated rapidly on both full-strength and half-strength DCR medium.

Consequently, a large number of media appear to be suitable for loblolly pine embryo culture. In order to produce autotrophic plants, and avoid destructive microbial infestations, it would be desirable to use a single medium in which the organic components could be progressively diluted or deleted without adversely affecting growth. The basic concept is to gradually change the medium composition so that physiological shocks will be minimized. At the end of this process the emblings should be grown in a substrate and nutrient medium which is similar to that used to grow pine seedlings in a nursery.

The criteria used in selecting test media, substrates and environmental conditions for future testing are discussed below. The results of initial trials concerning the germination of seeds and isolated zygotic embryos is presented.

CRITERIA USED TO SELECT TEST MEDIA, SUBSTRATES AND ENVIRONMENTAL CONDITIONS

Hogland's solution can be used reference medium because it has worked well in the final stage of conversion with spruce somatic embryos [23]. Furthermore, it is often used as a macronutrient solution in hydroponics, and has formed the basis for a mineral nutrition study with P. taeda seedlings [15]. Urea was the best nitrogen source and a total nitrogen level of 75 ppm gave good results, while 10 ppm produced deficiency symptoms. Nitrate at 75 ppm led to extensive root system development. Use of ammonium nitrate led to mortality at 50 and 125 ppm. However, 75 ppm ammonium chloride gave good results, as did 156 ppm ammonium sulfate. Ammonium mortality was thought to be exacerbated by low light and was a problem when the levels of ammonium and nitrate were equal. Ammonium chloride [75 ppm] gave the best shoot color, while nitrate alone produced pale green shoots. Urea gave the best lateral root production, followed by nitrate and ammonium. Nitrate alone gave the best root:shoot ratio at 75 ppm.

Ingestad [11] tested the nitrogen, potassium, phosphorous, calcium, magnesium requirements of Pinus silvestris, Pinus nigra [var. maritima] and Picea abies. The optimal ratio of

nitrate:ammonium was approximately 60:40. The recommended total amount of nitrogen in the medium was 1.5 - 3.5 mM. However, seedling growth did not show a positive effect until 400 ppm [28 mM] was used. Toxicity occurred with 800 ppm [56 mM] total nitrogen. Consequently, levels between 3.5 and 28 mM should be effective, while higher levels could be supraoptimal.

An optimal macroelement formulation has been proposed for Pinus strobus seedlings [5]. Compared to studies with other species, the recommended nutrient levels for P. strobus were very high, and the ratios of macroelements to nitrogen were all high.

Based on all of the above, an effective medium for growing loblolly pine seedlings should contain both ammonium and nitrate with relatively more nitrate compared to ammonium. An organic source of nitrogen could be a good substitute because urea gave better overall results than ammonium or nitrate. The total nitrogen level should be between 3.6 to 29 mM. The ratio of major elements should approximate those of Ingestad.

Overall, half-strength tissue culture media have produced better germination results with conifer embryos. Based on various studies concerning in vitro nutrient requirements of conifers [2, 7], the macronutrients are probably responsible for most media-related effects. Consequently, the primary focus should be on the macroelement composition of each candidate medium. Because the nitrogen supply is usually the most abundant and critical factor in culture media, this aspect of medium composition must receive special attention. The nitrogen supply is also important because

organic chemicals are often used to supplement or replace inorganic nitrogen sources. One complete microelement mixture can be selected for all test media [18-20].

None of the above media meet all of the suggested criteria. MSG is unusual because it contains no ammonium and little nitrate. This makes comparisons to other media difficult. The total nitrogen level is relatively low and is within the suggested range for full- and half-strength media. The nitrate to ammonium ratio is zero because no ammonium is present. The nitrate to glutamine ratio is exceedingly low. This could result in poor root growth. However, if glutamine acts like urea, root growth could be good. The ratio of macronutrients to total nitrogen compares well with Ingestad's recommendations. However, the calcium level is high. MSG should be tested for germination because it is currently used for the maturation of somatic embryos [see this Annual Review]. However, either a switch to another medium must be made after germination or a rational method for replacing glutamine with ammonium and nitrate must be devised.

DCR medium has several inorganic and organic nitrogen sources. Inorganics account for 67% of the total nitrogen. Consequently, the problems associated with eliminating organic nitrogen are significantly reduced compared to MSG. The total nitrogen level is slightly higher than MSG but is still moderate in full- and half-strength media. The nitrate:ammonium ratio is good [2.6:1]. However, when all nitrogen sources are considered the ratio is 1:1. This may not matter because organic nitrogen may only be supplied

during germination. DCR has a high potassium level and a low phosphorous level compared to Ingestad's recommendations. DCR has approximately the same salt levels as MSG and is on the low end of the spectrum close to Hogland's solution. However, the presence of casein hydrolysate probably increases the osmolarity of the medium slightly. Preliminary experiments have shown that mature loblolly pine embryos germinate readily on full- and half-strength DCR. In addition, ovules cultured on DCR produced seedlings which germinated normally. DCR is also used to grow loblolly pine embryogenic callus and has been used for somatic embryo maturation with P. strobus [6]. Organic nitrogen can be omitted after germination and should be exhausted by the time seedlings are removed from axenic conditions. DCR will be studied for germination and post-germination steps.

Hakman's medium [HM] has worked extremely well with spruce somatic and zygotic embryos [23]. In addition, it is a modified Murashige-Skoog medium and should closely resemble the medium used to germinate loblolly pine embryos in vitro [4]. The total amount of nitrogen in HM is twice as high as other media, except for Litvay's medium. This is largely due to the amount of nitrate in the medium. Half-strength HM has a total nitrogen level which is just within the upper limit proposed by Ingestad. The total nitrogen level in full-strength HM is probably in the inhibitory range. The nitrate:NH_3 ratio is good and there are no organic nitrogen sources. HM macronutrients have the best overall agreement with Ingestad's nutrient ratios. HM should be tested for germination because it has been very successful with spruce somatic and zygotic

embryos. In addition, it is a modified Murashige-Skoog medium and contrasts with MSG and DCR. Furthermore, it has a good nitrate:ammonium ratio, and has no organic nitrogen. Because of the relatively high salt concentration of HM, 1/3-1/4 strength media could be tested if poor results occur with half-strength medium. HM could also be used in post-germination steps.

Litvay medium [LM] is extreme in many respects. It has an exceedingly high total nitrogen level. Litvay et al. [15] adopted the Murashige-Skoog levels of ammonium nitrate and KNO_3 without testing or modification, and this accounts for the high nitrate and ammonium levels. Furthermore, the total nitrogen level for half-strength medium is very high. The nitrate:ammonium ratio is good. However, the macroelement ratios for phosphorous, calcium and magnesium are very different than those suggested by Ingestad. The calcium level in LM is extremely low and had to be greatly increased along with other components to successfully grow P. radiata suspension cultures [20]. LM also has the highest total salt content of all candidate media. Because LM and HM are modified MS media, and because we are planning to test HM, adding LM to the experiment would be somewhat redundant. In addition, because some of its components are used at unusually high or low levels in LM, complex modifications might need to be made in order for it to work well. If other media prove unsuccessful, LM will be tested.

Schenk and Hildebrandt [SH] medium has several inorganic nitrogen sources and is similar to DCR and MSG for total nitrogen. Most of

the nitrogen is added as KNO_3 which results in a high potassium level. The nitrate:ammonium ratio is high, but the high nitrate concentration could be favorable for root development. This could be important for successful acclimatization of emblings. SH conforms to Ingestad's macroelement ratios, except for the high potassium concentration. The total salt level is relatively high. SH should be tested for germination and post-germination steps. It has most of the attributes of "the best theoretical" medium for growing pine seedlings, except for the high potassium levels. In addition, it is a totally inorganic medium and will not require drastic modifications to produce an autotrophic medium. Furthermore, prior results have shown that SH can be successfully used to culture mature pine embryos [22], and worked well with seedlings in this project.

Modified Hogland's solution [MHG] has been used during the later stage of conversion with spruce somatic embryos and could be used in this study. MHG has relatively low levels of all inorganics and does not contain organics. The total nitrogen level of MHG compares well with half-strength MSG, DCR and SH media. Its nitrate:ammonium ratio is very high which could be good for root development. The phosphorous, calcium and magnesium levels in MHG do not match Ingestad's ratios. MHG has a moderate salt level which is similar to half-strength SH. MHG will not be used for germination but could be tested later if other media are unsuccessful in the final conversion steps.

Sucrose will be used for germination, but removed for subsequent steps. Somatic embryos will most likely be deficient in storage products, and will probably require a carbon source for germination and early growth. Mature zygotic embryos are packed with lipids and may not require sucrose. In addition the megagametophyte provides a strong growth impetus. Consequentially, the effects of sucrose on the conversion of mature zygotic embryos need to be evaluated so that we can identify favorable conditions. Sucrose is the carbohydrate generally used for pine embryo culture. Most authors have used 2% [3, 16], however, 4% and 6% have produced superior results [1]. Furthermore, 3-4% sucrose was optimal with isolated loblolly pine embryos [4]. Sucrose is normally autoclaved with the other medium components. However, Trembley [unpublished] reported a two-fold increase in somatic embryo production when filter-sterilized sucrose was used. Loblolly pine embryogenic calli grow better with maltose than sucrose [see this Annual Review]. Furthermore, maltose is better for loblolly pine somatic embryo maturation compared to sucrose. While mature embryos grow well with autoclaved sucrose, immature zygotic and somatic embryos may be more sensitive. Consequently, sugar type, concentration and mode of sterilization need to be considered. If sucrose is the only carbohydrate tested it should be filter-sterilized to avoid the production of any toxins [9, 10].

Glutamine and casein hydrolysate will be used for germination but should be deleted or greatly reduced in subsequent steps. Inositol and vitamins will be treated the same way. Half-strength medium will be used for germination, but 1/4-strength or more dilute

concentrations will be used thereafter. The level of nutrients should be gauged against the growth of the seedlings. Once rapid growth occurs, stronger doses could be used. Established seedlings will receive the normal fertilizer schedule for container-grown pine seedlings.

Embryo orientation in terms of cotyledon and root contact with the medium can be important [1, 4]. Sacher [16] obtained satisfactory growth by laying embryos on the surface of agar slants. However, submerged embryos developed abnormally. The best growth occurred when the cotyledons were inserted into the culture medium of inverted tubes. Horizontally oriented tubes produced inferior results in the light. However, opposite results were obtained in the dark [1]. In one study [4] cotyledons were inserted vertically into agar-solidified medium in petri plates which were inverted so that the roots pointed downwards. This resulted in good root growth, however, the roots were extremely. Spruce somatic embryos germinated unexpectedly well with their roots embedded in the medium [23]. Embryo orientation and contact with the medium must be considered in developing a strategy for embryo conversion.

Seeds normally germinate in darkness. Better overall growth of excised pine embryos occurred in darkness [1]. Light stimulated germination of pine seeds [4]. Sacher [16] germinated pine embryos in continuous light. Red light promoted root elongation by unstratified or partially stratified loblolly pine embryos in vitro [4]. However, fully stratified embryos were unaffected by light.

Spruce somatic embryos are typically germinated in the light [23]. Consequently, the effects of light must be considered.

Agar has been routinely used as a substrate for conifer embryo culture. However, agar is an impure and undefined mixture of organic and inorganic chemicals. Gelrite is an effective agar substitute and is a defined mixture of carbohydrates. It may be preferable to agar and should be considered if germination proves difficult on agar. Both of these are translucent. Root growth is usually inhibited by light and consequently, these may not be desirable substrates after the initial phase of germination. In addition, delicate roots can be damaged when plants are removed from them.

Sorborods and Rockwool plugs are inert, opaque, fibrous materials developed for horticulture. Rockwool has been used for the containerized growth of Norway spruce seedlings [13]. They have both been used for the rooting of various types of cuttings *in vitro* and *ex vitro*, and can be directly transplanted into leach tubes. They are also suited for automated planting.

Acclimatization procedures developed for spruce somatic embryos [23] will serve as a guideline for establishing plants in the greenhouse.

MATERIALS AND METHODS

Seeds from a Union Camp bulk collection were scarified and exposed to 1% hydrogen peroxide for five days. The peroxide solution was replaced each day. Seeds were then used for seedling trials, or

embryos were removed from them for embling trials. Seeds were either transferred directly to the greenhouse for germination or were placed in the dark at 25+/- 2 ^{o}C. Natural light in the greenhouse was supplemented by high-pressure sodium, incandescent and fluorescent light to provide a 16 hr photoperiod. Light readings fluctuated between 700 - 3,400 ft-c depending on the time of day and cloud cover. Phillips Agro-Lite fluorescent tubes provided 80 ft-c of light [16 hr. photoperiod] in the culture room.

SEEDLINGS

Trial 1

Seeds were aseptically germinated with sugar-free, quarter-strength SH medium in large Magenta boxes containing Jiffy 7 peat plugs [Treatment JPCR], 20 x 40 mm Sorborods [Treatment SRCR], 20 x 40 mm Rockwool plugs [Treatment RWCR], or Farfard #2 potting mix in leach tubes [Treatments MBCR & MBGH]. Two Magenta boxes were joined with an adapter to accommodate the leach tubes. All of the above, except MBGH were placed in the dark for 7 days, then moved to the light in a culture room. After 7 days in the light, the lids were cracked. After an additional 7 days the lids were removed. One week later the Magenta boxes were transferred to the greenhouse under the light conditions specified above. The Magenta boxes containing treatment MBGH were placed in the greenhouse at time zero and never received a dark period.

Trials 2 and 3

Seeds were germinated in leach tubes containing potting mix. There were four treatments in each trial. In treatment 1 [MBCR], seeds were germinated inside double Magenta boxes under axenic conditions in the culture room. Treatment 2 [MBGH] was identical except that the boxes were placed directly in the greenhouse without a dark period. In treatments 3 and 4 [GHRK and GHRKT] leach tubes were placed in racks under greenhouse conditions at time zero. A plastic tent was used to create a high humidity environment for treatment GHRKT. Seeds for treatment MBCR were treated as outlined above for Trial 1.

Seedlings in the greenhouse were watered as required, and were initially fertilized with 1/4-strength SH medium which contained only inorganic nutrients. Full-strength SH was used to fertilize established seedlings. The tubes were regularly flushed with water to prevent salt accumulation.

Germination was recorded weekly. Final hypocotyl and cotyledon lengths were measured after these had ceased elongating [5-6 wks. after sowing].

EMBLINGS

Excised embryos were germinated on full-strength or half-strength DCR medium containing autoclaved 3% sucrose and 0.6% washed agar in a culture room with light or darkness as specified above. In one trial, germinated emblings were transplanted to 20 x 40 mm Rockwool plugs moistened with 1/2-strength DCR medium and grown under fluorescent light in the culture room. After four weeks, sugar-free

half-strength DCR was used to replace the germination medium, and half of the Magenta boxes were transferred to the greenhouse. After another four weeks, the cultures received a second application of sugar-free medium and all boxes were moved to the greenhouse. The Magenta lids were cracked after two additional weeks and replaced with polypropylene Sun caps two weeks later.

RESULTS

SEEDLINGS

Trial 1

Germination ranged from 85 - 100% [Fig. 1]. Maximal germination occurred after 27 days with treatment MBGH but was delayed until day 41 with the other treatments. Hypocotyl elongation ranged from 44 - 57 mm [Fig. 2]. The greatest elongation occurred in treatments JPCR and MBCR. There was a narrow range of final cotyledon lengths [28 - 32 mm], and consequently there was little difference among the treatments.

Trials 2 and 3

The final germination frequency ranged from 75 to 80% in Trial 2 [Fig. 3], and from 70 to 95% in Trial 3 [Fig. 4]. Maximal germination occurred after 22 days with treatments GHRK and GHRKT in Trial 2. The other two treatments attained maximal germination after 28-30 days. A similar germination pattern was observed with Trial 3 except that overall germination was retarded by 7 days, regardless of treatment. Germination was particularly delayed with

treatment MBGH. This treatment became dry because it did not contain an adequate moisture supply at the outset. Once additional medium was added, germination commenced rapidly.

Final hypocotyl length was similar in Trials 2 and 3, and the range was similar to that obtained in Trial 1. Treatment MBCR produced the longest hypocotyls in all three Trials [Figs. 2, 5, 6]. There was greater variation in cotyledon length in Trial 2 compared to the other two Trials but no obvious pattern was apparent when all three trials were considered.

EMBLINGS

Excised embryos [Fig. 7] were placed on the surface of agar-solidified medium and oriented vertically so that the root pointed downwards. These germinated after five to seven days on full-strength and half-strength DCR medium in light or darkness [Fig. 8]. Hypocotyl elongation was prominent and the root was just visible on the medium after seven days. While there was little difference in the size of embryos germinated in the light or in the dark, embryos grown in darkness were relatively straight compared to those in the light. Light-exposure caused excessive hypocotyl curling on most embryos, and made them unsuitable for further use. Root elongation occurred rapidly from day seven to day ten, and reached 1 cm or more in length [Fig. 9]. At this time emblings could be transferred to Rockwool plugs, Sorborods or potting soil.

The effects of inserting the cotyledons into the medium were evaluated in two trials with half-strength DCR medium in light and

darkness. Embryos were treated exactly as outlined above, except that the cotyledons were inserted just below the surface of the medium while the rest of the embryo remained in contact with the surface. Germination was delayed and abnormal emblings developed when the cotyledons were inserted into the medium. This was observed with both vertical and horizontal orientations in light and darkness. Hypocotyl curling was once again more of a problem in the light.

Emblings transferred to Rockwool plugs rapidly completed hypocotyl and cotyledon elongation [Fig. 10] Although accurate measurements have not been taken, these organs are approximately half the size of seedling hypocotyls and cotyledons. Epicotyl growth occurred after the germination medium was replaced with sugar-free medium, and the Magenta boxes were transferred to the greenhouse.

DISCUSSION

These experiments have shown that loblolly pine seedlings germinate rapidly and become established under a variety of environmental conditions. Half-strength SH medium was suitable for these processes. In addition, all of the substrates were adequate for seedling growth and establishment. However, the primary root tended to grow straight through the Sorborods rather than branching to form lateral roots. Roots took a long time to emerge from the Rockwool plugs. Both of these observations could pose problems because the development of an expansive, branched root system is important for early seedling establishment. In the future, smaller Sorborods and Rockwool plugs could be employed for the initial phase of germination, and used to transfer germlings to potting mix in leach tubes for root establishment. While SH medium works well for seedlings, results with it will be compared to those with other media once we have established which media are most effective for embryo germination.

There was little difference in final organ size when seedlings exposed to different environmental regimes were considered. This was not very surprising because hypocotyls and cotyledons have determinate growth and stop elongating at specific points in development. The increased elongation seen with treatment MBCR was probably due to partial etiolation caused by the low light levels encountered in the culture room during the early growth phase. In general, seedlings raised in the culture room had more elongated hypocotyls than those grown directly in the greenhouse. However,

these long hypocotyls were not as sturdy as those on plants reared in the greenhouse. Epicotyl development also appeared to lag with seedlings grown in the culture room. Consequently, the best course of action would be to move germinants as quickly as possible to the greenhouse.

The data gathered on hypocotyl and cotyledon growth provides a good baseline for comparison with germinated embryos. Further data on epicotyl growth is being taken to complete the picture of early seedling growth. In addition, the acclimatization processes which are under investigation will help to develop protocols for emblings.

Zygotic embryos germinated rapidly on full-strength and half-strength DCR medium containing sucrose. Normal seedling-like emblings developed upon transfer to rockwool plugs moistened with sugar-free DCR medium. Consequently, DCR medium is suitable for continued work on embryo conversion. Other basal media will also be evaluated now that the timing and pattern of embryo germination is known for DCR. The shorter hypocotyls and cotyledons observed with emblings were probably due to the absence of the megagametophyte. However, this may not be a significant problem as long as the epicotyl becomes active and starts to produce the shoot system. It is possible that other carbohydrates like maltose, or higher sugar concentrations might provide for better germination and early growth. In addition, it is not clear how long the embryos need to be exposed to carbohydrates to achieve maximal growth. Embryos may require sugars beyond the initial ten days of culture. However, the

delay in epicotyl activation observed with emblings could have been due the presence of sucrose during the first five weeks of culture. This aspect of embryo culture will require considerable study to achieve maximal results. The delay in epicotyl growth could also have been related to the low light levels in the culture room.

While the initial rates of germination and organ elongation were similar in light and darkness, straight growth only occurred when the first five to seven days of incubation occurred in darkness. It is clear that zygotic embryos should be germinated in the dark, however, somatic embryos could react differently. Given the importance of light in seedling growth and development, the role of light during germination of somatic embryos should be evaluated early in the program.

Embryos germinated well when they were placed on the surface of agar-solidified medium and oriented vertically. Immersing the cotyledons in the medium produced irregular and distorted germination. While these results were unexpected, it was very evident that placing the cotyledons in the medium had clear adverse effects. Consequently, embryos will routinely be germinated on the surface of agar-solidified medium. The problem of embryo contact with the medium will need to be addressed during the testing of Sorborods and Rockwool plugs. Embryos will be placed at different depths inside these substrates and the consequent growth responses will be determined.

There are advantages in germinating embryos in small Sorborods or Rockwool plugs. These can be handled much more easily, and

germinants can be transferred without risking damage to the delicate tap root. An evaluation of the effects of different substrates on embryo germination has a high priority in our future work. We plan to use small plugs for the first 10 days of germination, then transfer them to leach tubes containing soil. This should minimize the root branching problems that were observed in the initial trials with Rockwool. The tap root should quickly emerge from the plugs and enter the soil. This should be conducive to root branching.

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Figure 1. Frequency of Seed Germination in Trial 1.

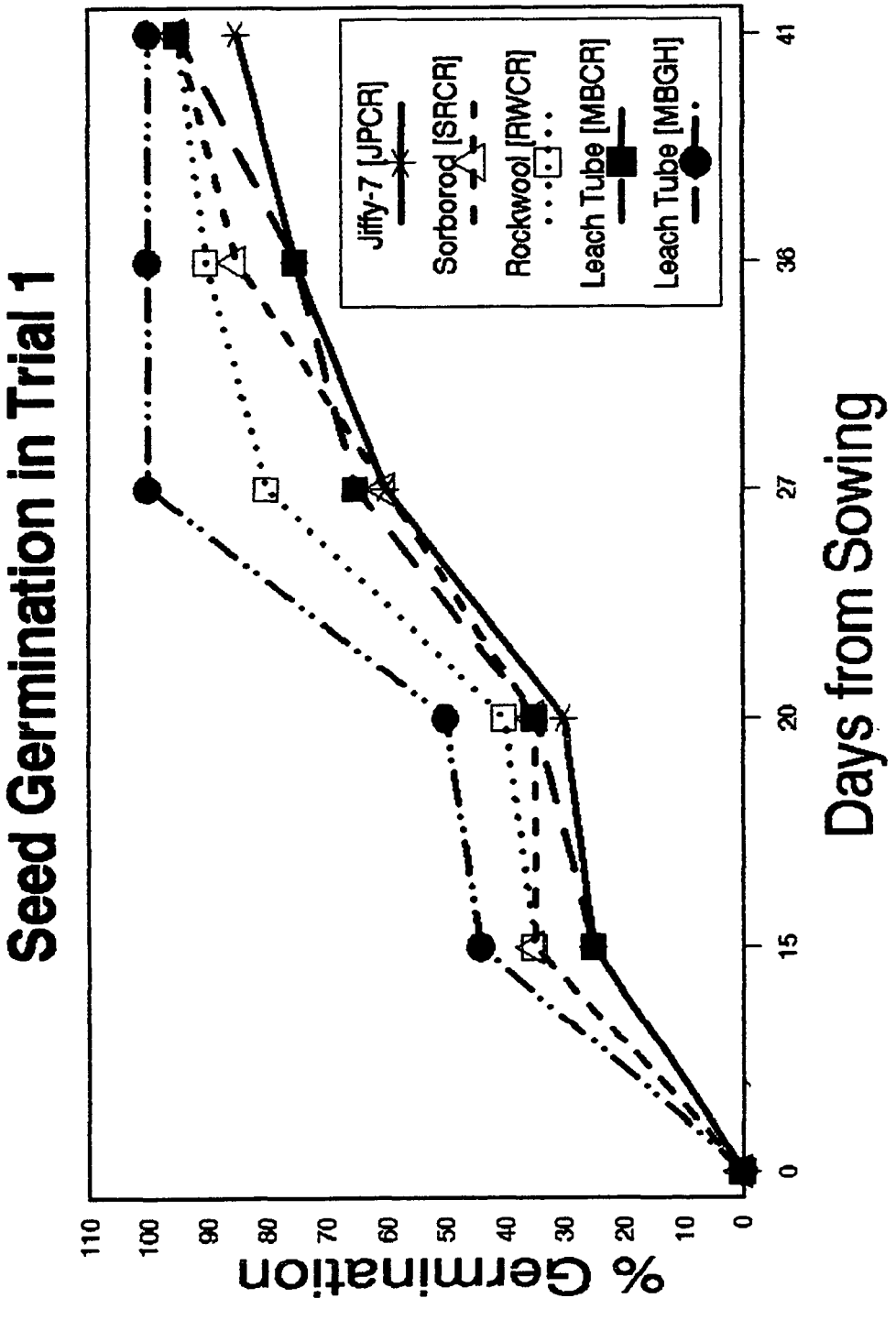

Seed Germination in Trial 1
% Germination
Days from Sowing
Jiffy-7 [JPCR]
Sorboroq [SRCR]
Rockwool [RWCR]
Leach Tube [MBCR]
Leach Tube [MBGH]
0
10
20
30
40
50
60
70
80
90
100
110
0
15
20
27
36
41

Figure 2. Final Hypocotyl and Cotyledon Length in Trial 1.

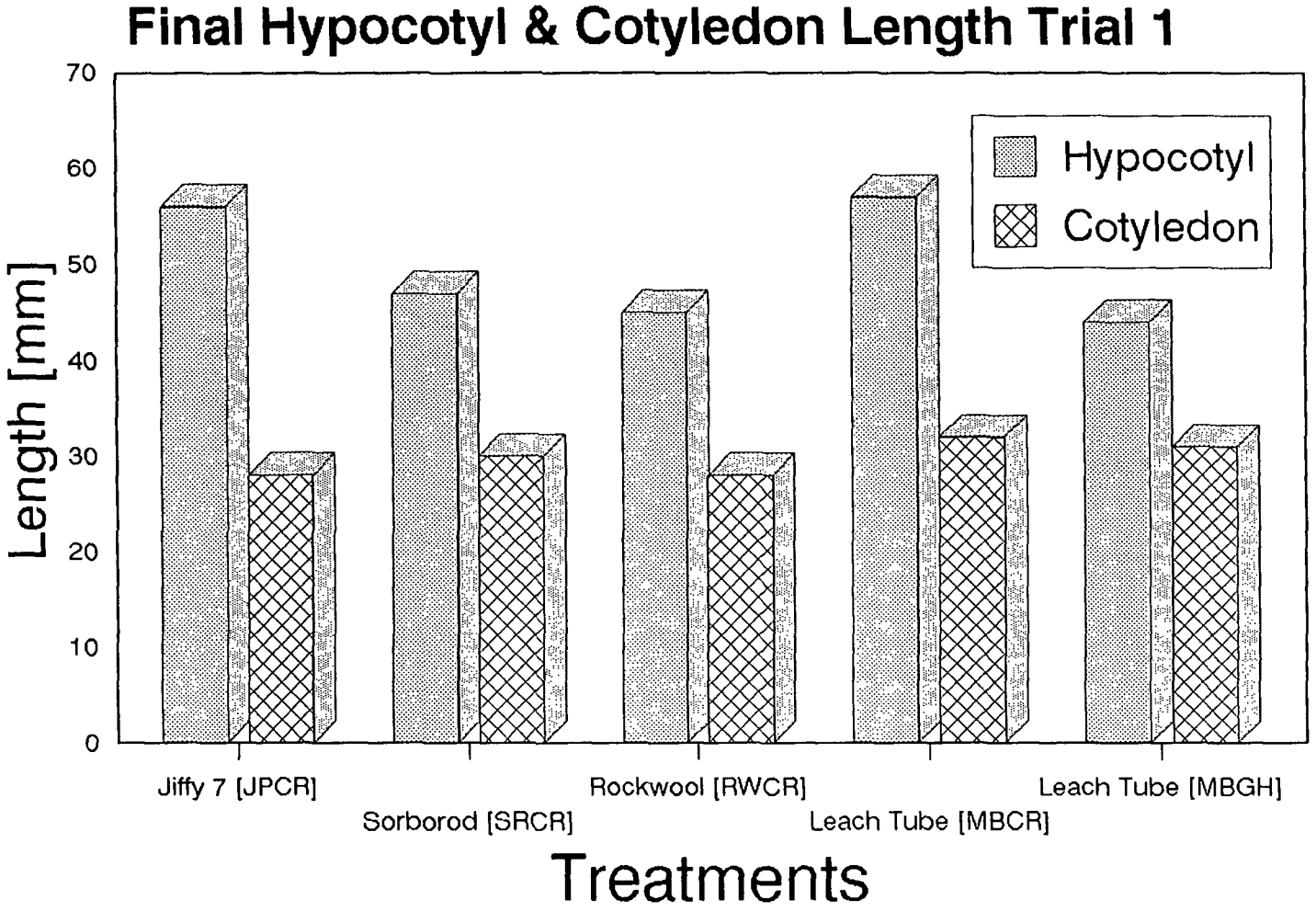

Final Hypocotyl & Cotyledon Length Trial 1
70
60
50
40
30
20
10
0
Length [mm]
Hypocotyl
Cotyledon
Jiffy 7 [JPCR]
Sorborod [SRCR]
Rockwool [RWCR]
Leach Tube [MBCR]
Leach Tube [MBGH]
Treatments

Figure 3. Frequency of Seed Germination in Trial 2.

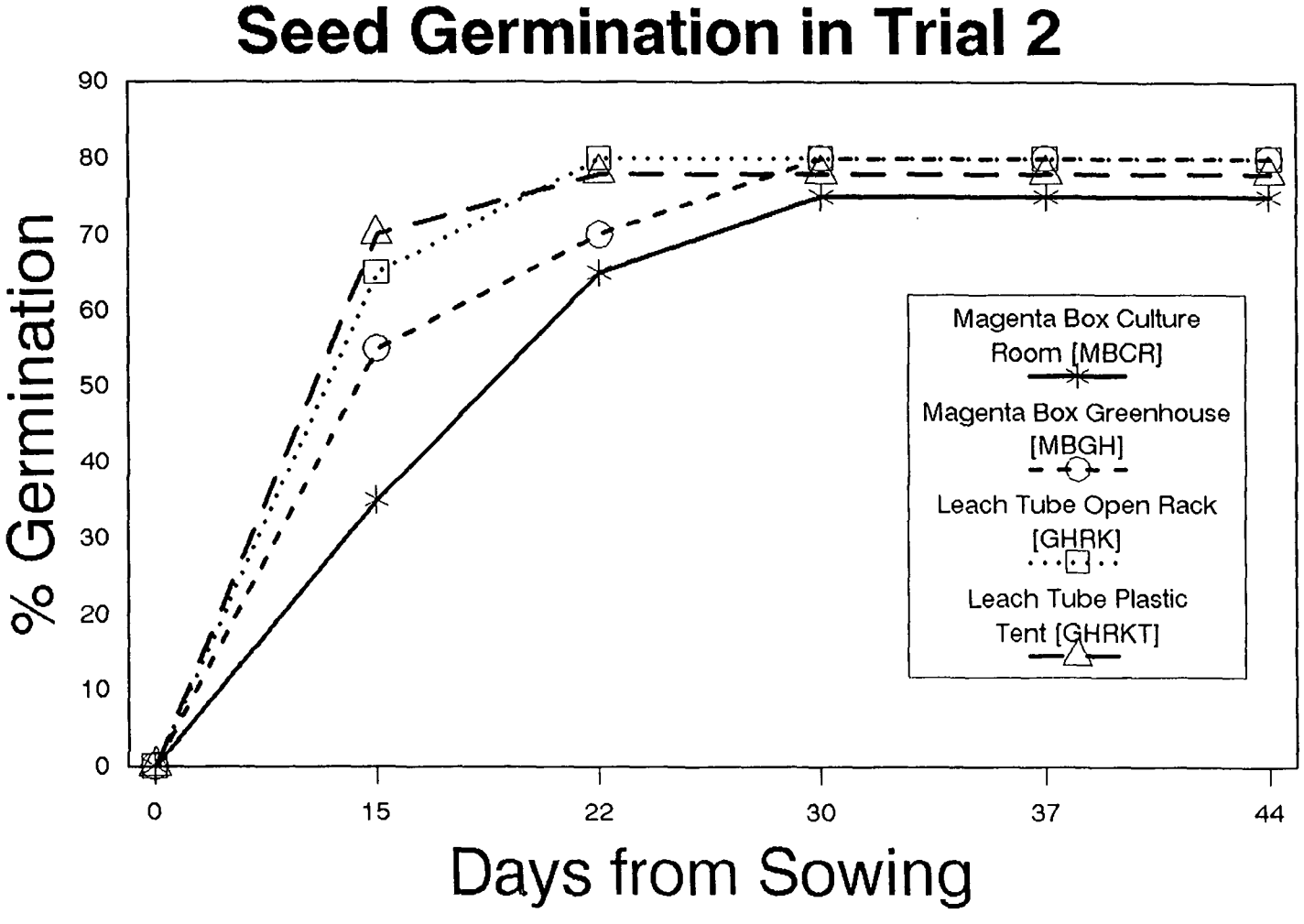

Seed Germination in Trial 2
% Germination
90
80
70
60
50
40
30
20
10
0
0
15
22
30
37
44
Days from Sowing
Magenta Box Culture Room [MBCR]
Magenta Box Greenhouse [MBGH]
Leach Tube Open Rack [GHRK]
Leach Tube Plastic Tent [GHRKT]

Figure 4. Frequency of Seed Germination in Trial 3.

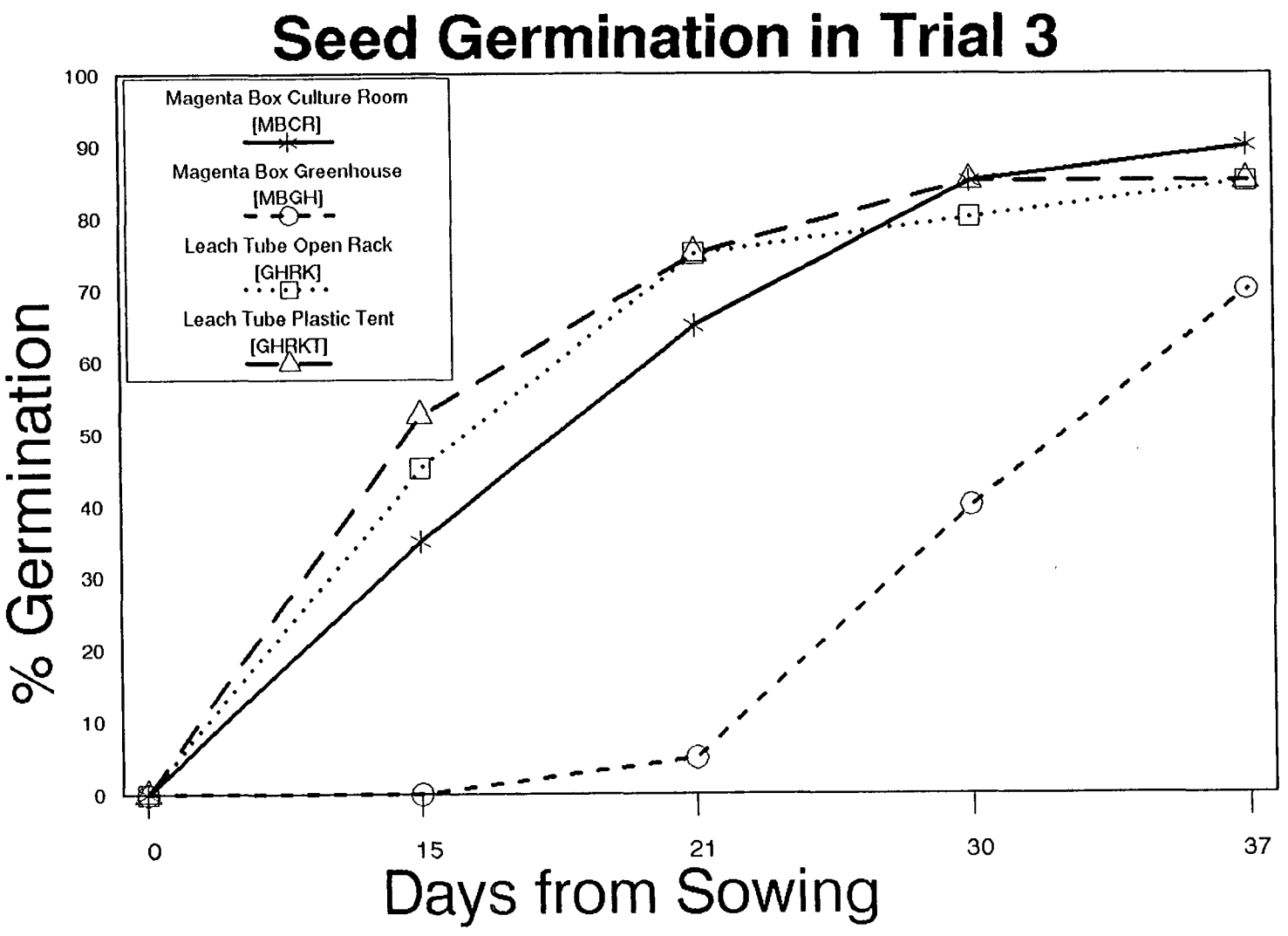

Seed Germination in Trial 3
Magenta Box Culture Room
[MBCR]
Magenta Box Greenhouse
[MBGH]
Leach Tube Open Rack
[GHRK]
Leach Tube Plastic Tent
[GHRKT]
% Germination
100
90
80
70
60
50
40
30
20
10
0
0
15
21
30
37
Days from Sowing

Figure 5. Final Hypocotyl and Cotyledon Length in Trial 2.

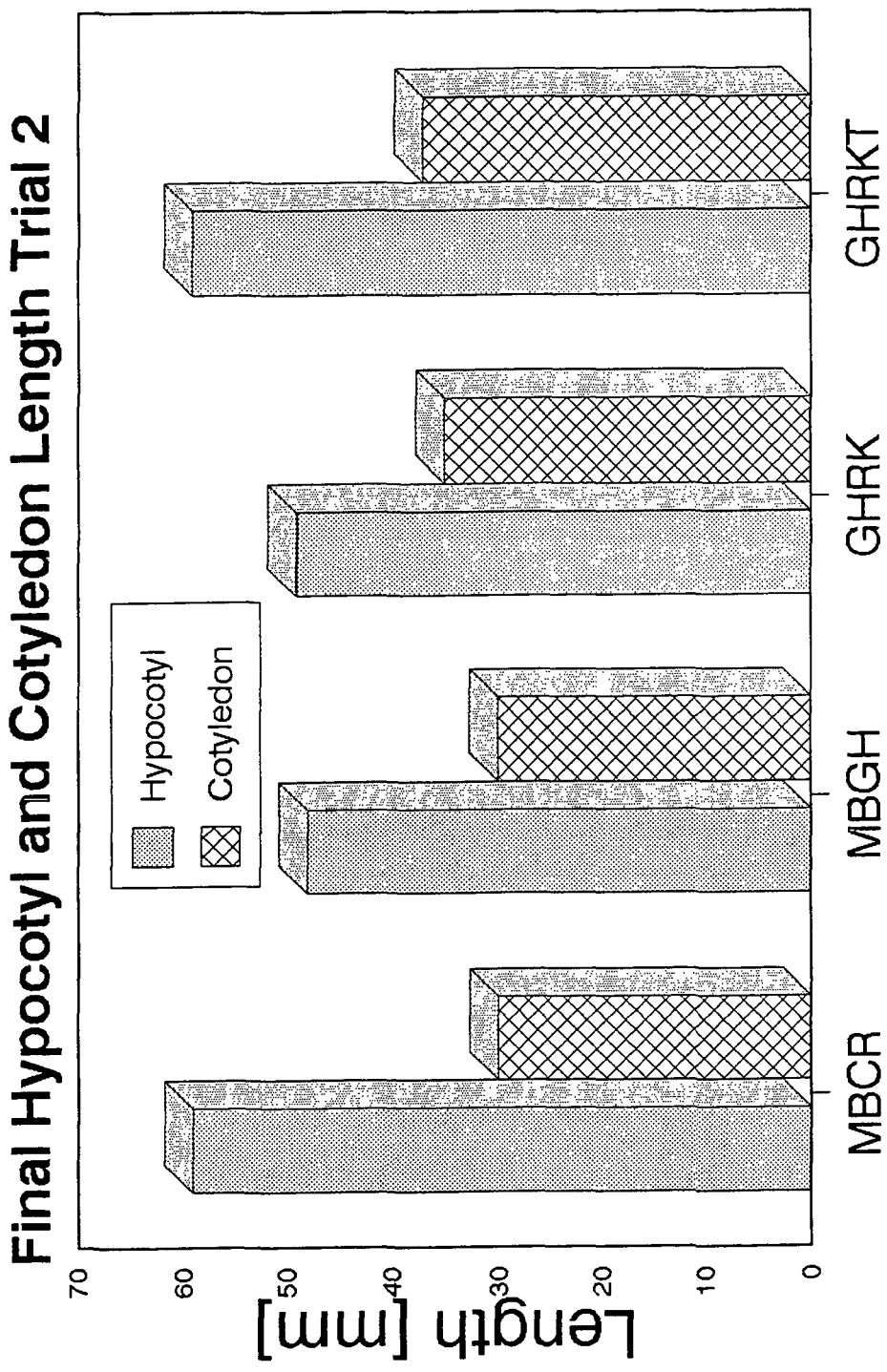

Final Hypocotyl and Cotyledon Length Trial 2
Length [mm]
0
10
20
30
40
50
60
70
Hypocotyl
Cotyledon
MBCR
MBGH
GHRK
GHRKT
Treatments

Figure 6. Final Hypocotyl and Cotyledon Length in Trial 3.

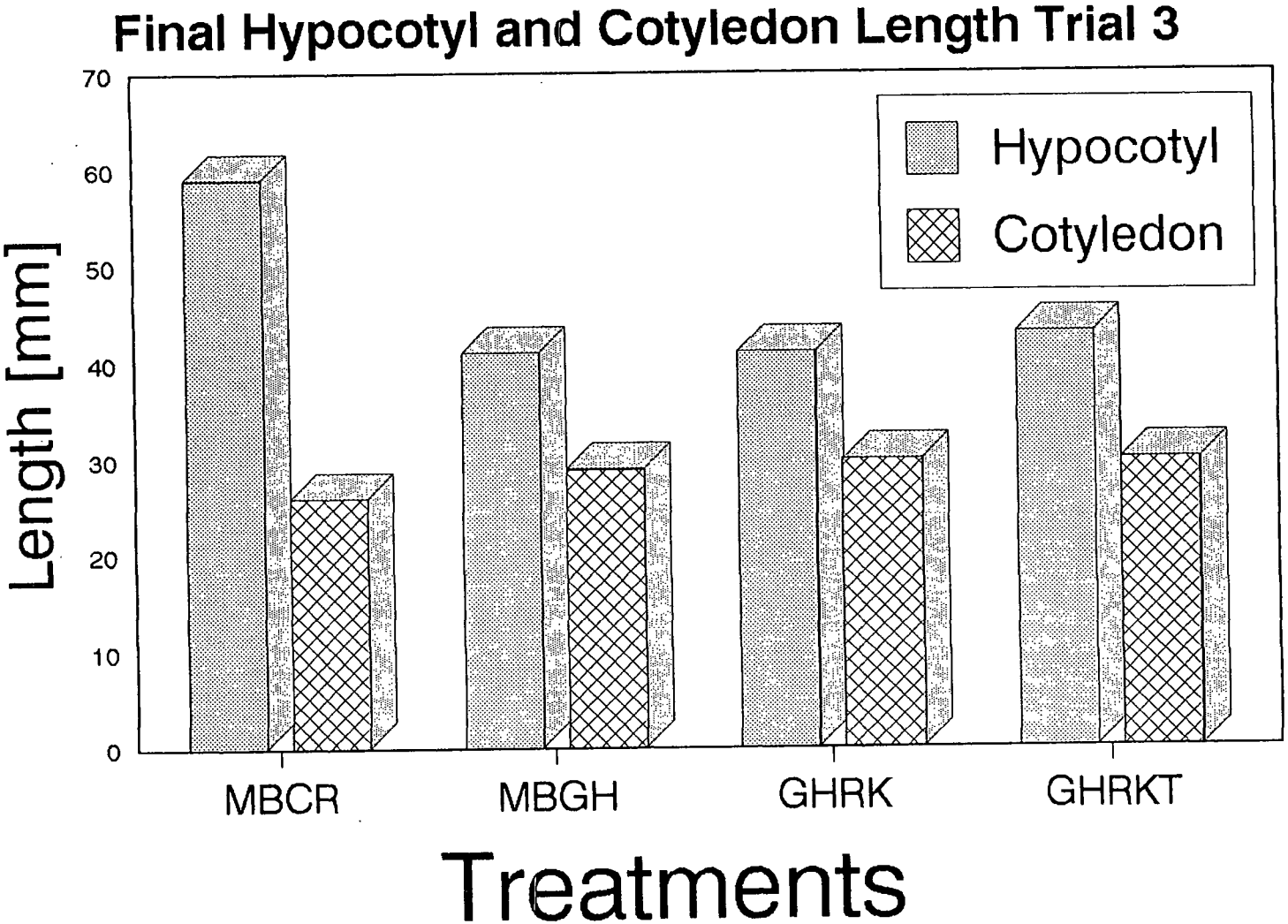
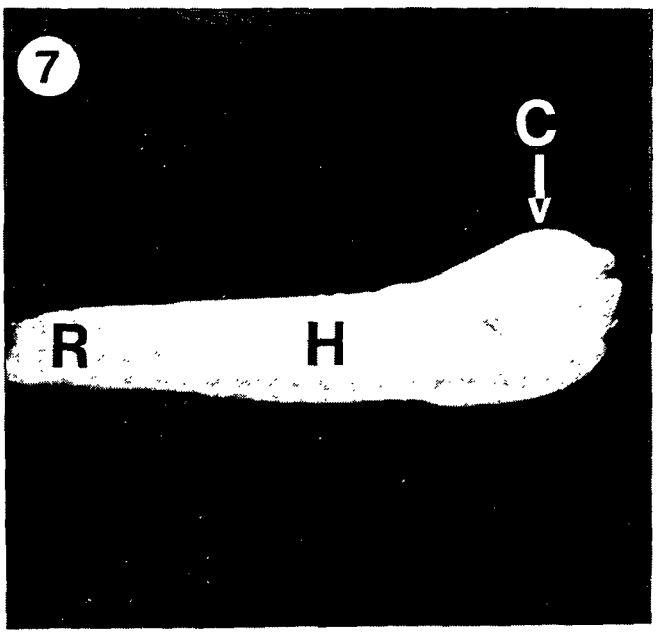

Final Hypocotyl and Cotyledon Length Trial 3
Length [mm]
70
60
50
40
30
20
10
0
Hypocotyl
Cotyledon
MBCR
MBGH
GHRK
GHRKT
Treatments

Figure 7. Mature loblolly pine embryo excised from seed prior to culture. Note the cotyledons [C], hypocotyl [H] and root pole [R]. Magnification = 7X.

Figure 8. Germinated embling after five days on half-strength DCR medium in the light. Note the elongated cotyledons [C] and hypocotyl [H]. Also note the emergence of the root tip [R]. Magnification = 3.5X.


7
C
R
H

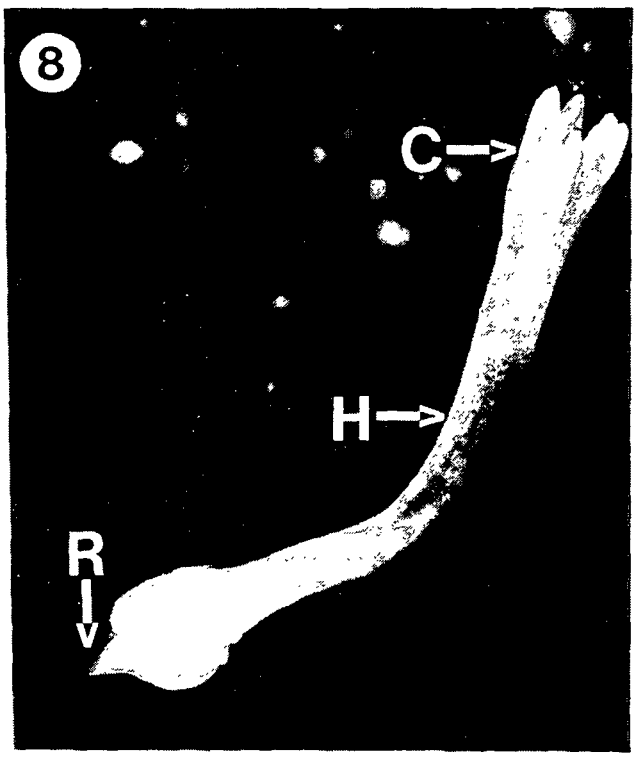
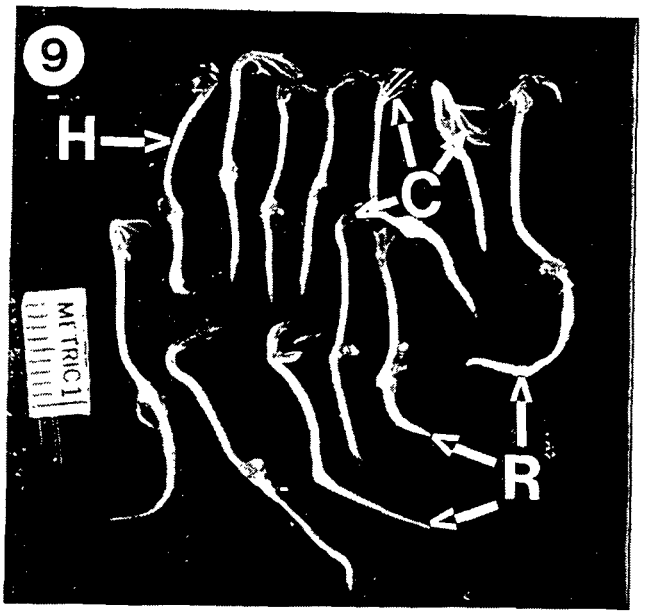

8
C
H
R

Figure 9. Germinated emblings after seven days in the dark and five days in the light. Note the highly elongated roots [R] and hypocotyls [H]. Also note the straight growth of the hypocotyls and the expanding cotyledons [C]. Scale bar = 1 cm.

Figure 10. Germinated emblings similar to those in Fig. 9 after two weeks in Rockwool plugs [Rw] moistened with half-strength DCR containing 3% sucrose. Note the elongated hypocotyls [H] and expanded cotyledons [C]. Magnification = 1X.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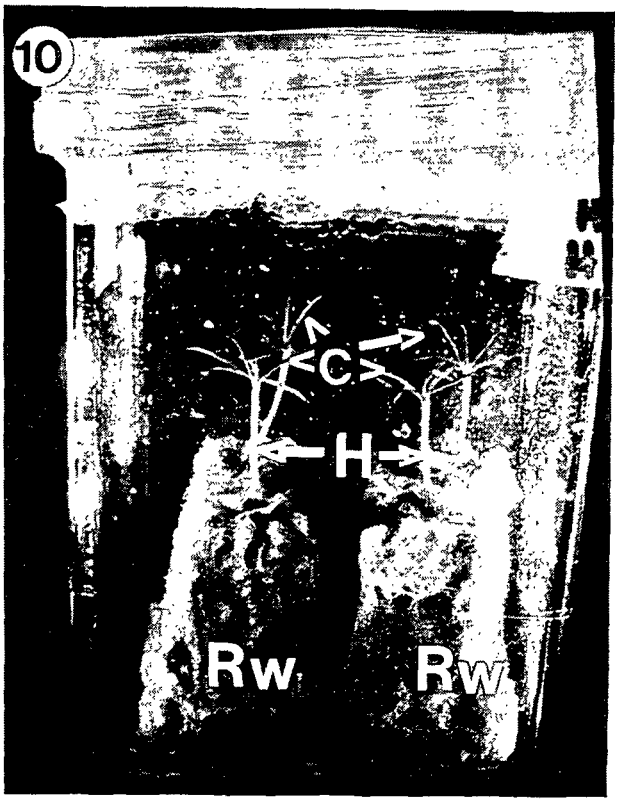
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PROJECT 3223-02

BIOCHEMISTRY OF CLONAL PROPAGATION

RESEARCH REVIEW

MARCH 27, 1992

Ron Dinus

TECHNICAL PROGRAM REVIEW SUMMARY

PROJECT TITLE: BIOCHEMISTRY OF CLONAL PROPAGATION

PROJECT STAFF: Ron Dinus, Vacant Position

FY 91-92 BUDGET: $115,000

REPORTING PERIOD: January 1, 1991 - December 31, 1991

DIVISION: Chemical and Biological Sciences

PROJECT CODE: BIOCM

PROJECT NO.: 3223-02

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

GOAL: Develop techniques to improve efficiency of cloning research and development.

SUMMARY: Experimental designs, cultures, and methods have been assembled for research on biochemical and molecular mechanisms underlying beneficial effects of maltose on somatic embryo maturation. Materials for work on lipid and protein assays of maturing somatic cmbryos are still being collected. Some delay on both fronts was incurred as a result of problems with culture numbers and responsiveness. Further information is given on the Project 3223-00 Summary Form.

The issue of genetic fidelity is being addressed via a student project. Polymerase Chain Reaction technology is being evaluated as a means for determining fidelity as well as hybridity and paternity in southern pine breeding programs. Techniques for extracting and purifying DNA from several tissue types have been refined. Methods for quantifying yields and pruity have also been adapted for routine use.

The drive to refine/develop techniques for genetic transformation of hardwoods moved forward. Efforts launched by a former student were accelerated by permanent staff members, and our first putatively transformed cultures have been obtained. Detailed findings are presented on the Project 3223-03 Summary Form.

Some progress, albeit limited, was made on techniques for generating and recovering somaclonal variants tolerant of glyphosate. Potentially useful variants can be obtained, but regeneration of

intact plants remains a serious problem. A more complete discussion of results, status, and prognosis is provided on the Project 3223-03 Summary Form.

NOTE: This project will end June 30, 1992. Needed research bearing on softwoods and hardwoods will be conducted within Projects 3223-00 and -03, respectively.

GENETIC TRANSFORMATION OF EASTERN COTTONWOOD: ENHANCED AUXIN SYNTHESIS

Ronald J. Dinus and Camille J. Stephens

INTRODUCTION

Current hardwood research at the Institute of Paper Science and Technology (IPST) centers on techniques for producing and actual production of useful variants of eastern cottonwood (Populus deltoides). Genetic transformation via Agrobacterium tumefaciens (At) is one approach being evaluated.

Traits of interest include, but are not limited to, herbicide tolerance and enhanced auxin synthesis. Herbicide tolerant planting stock should lower plantation establishment costs and increase volume productivity. Enhanced auxin synthesis has potential for altering fiber numbers and/or dimensions (Klee et al., 1987). Increasing fiber numbers could raise productivity; changing fiber dimensions, e.g., growing longer fibers with thinner walls, could lead to improved or new paper qualities. Also, ability to control auxin status at will in cell and tissue cultures and/or trees will permit research on mechanisms underlying fiber formation.

Klee et al. (1987) transformed Petunia hybrida VR with a gene for enhanced auxin synthesis (iaaM), and observed numerous changes in morphological and anatomical features. Leaves were much smaller, thinner, and curlier than those of normal, wild-type petunia plants. Adventitious roots formed on many plants, even on leaves and stems. In addition, transformants produced twice the normal number of secondary xylem and phloem cells, had elongated and woody stems, and exhibited extreme apical dominance. Transformants were fertile, and selfed offspring had the same phenotypes as parents. The various effects were attributed to overproduction of auxin, as transformed plants were found to contain 10-fold more auxin than wild-type plants.

First IPST attempts at genetic transformation involved transfer of the iaaM gene for enhanced auxin synthesis into a cottonwood clone (C175), noted for ease of manipulation in culture. The Leaf Section System for cloning cottonwood, developed earlier at IPST (Uddin et al., 1990 (See also IPST Annual Technical Review, Forest Genetics, 1991, pp. 130-144) was used to effect transformation with At and regenerate plants. The genetic construct and At were secured for research purposes from Monsanto Corporation.

MATERIALS AND METHODS

Development of useful variants requires ready access to sterile explants from trees of interest; reliable methods for establishing, maintaining, and multiplying cultures; efficient means for effecting genetic transformation and selecting somaclonal variants; reproducible protocols for regenerating plants from culture; and techniques for confirming genetic change, stability, and utility.

Earlier IPST research has made available most materials and methods required for work on transformation. Useful clones have been secured, and are maintained in the IPST greenhouse. The Leaf Section System provides means for effecting transformation, and systems similar to it have been used for transformation via At in various plant species, including Populus hybrids (Fillatti et al., 1987; Sellmer and McCown 1989). Such systems obviate need for protoplast or more complicated approaches (McCormick et al., 1986). Transformation of leaf disks, followed by development and rooting of adventitious shoots, is considered straightforward and efficient (Horsch et al., 1985).

C175, a clone easily manipulated via the Leaf Section System, was obtained from Dr. S.G. Ernst, University of Nebraska - Lincoln, and maintained as a continuous shoot culture. Reserve materials were rooted and held in the IPST greenhouse.

The Leaf Section System was further refined by an IPST M.Sc. student (Shorter, 1991) to produce large numbers of plants and, in turn, the leaf sections needed for transformation.
Second, third, and fourth leaves of C175 were collected and surface sterilized. Leaf sections were isolated with a standard paper hole punch, taking care that each section contained a portion of the midvein. This procedure proved more efficient than cutting individual sections with a scalpel, and further ensured that sections were wounded on all sides.

Sections were placed abaxially on 25 ml of DKW-C Medium (McGranahan et al., 1987) containing 1 μM 6-benzylaminopurine and 1 μM naphthalenacetic acid (B1N1 Medium) in 100 X 15 mm petri plates. Six sections were placed in each plate. Plates were incubated in darkness 22° C for 21 days, and then transferred to fresh medium and cultured in the light for 21 - 42 days, or until all observations were taken or callus and/or shoots were harvested. Unless noted otherwise, this procedure was followed in all steps.

The genetic construct and At strain had been used previously to transform petunia plants (Klee et al., 1987). The construct (pMON518), contains the iaaM gene for enhanced auxin synthesis fused to a strong constitutive promoter (CaMV 19S), and also contains genes for kanamycin resistance (NPT) and nopaline synthesis (Figure 1). Inclusion of the last two genes provides means for selecting putative transformants and verifying transformation.

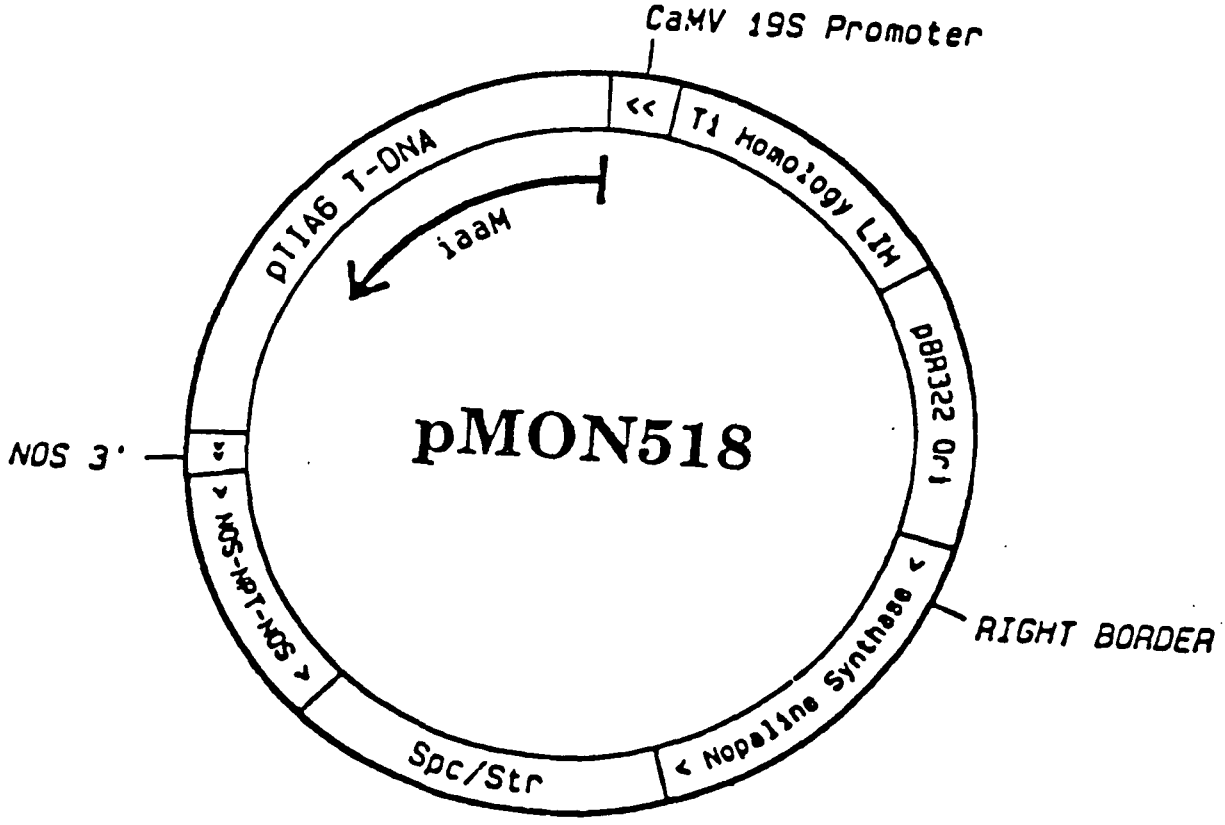

Figure 1. Genetic Construct Used to Introduce Tryptophan Monooxygenase (iaaM) into Cottonwood. pMON518 Contains the iaaM Gene Fused to the CaMV 19S Promoter, and Genes for Nopaline Synthase and Kanamycin Resistance (NPT).

Before transformation could be attempted, lethal dose assays for the antibiotics, carbenicillin and kanamycin, were necessary. Carbenicillin is used to clear cultures of the transforming agent, At, and knowing sensitivity of leaf sections to it was necessary to avoid harming putatively transformed leaf sections or inhibiting shoot formation. Kanamycin normally is toxic to plants, and presence of resistance to it provides a means for eliminating non-transformed materials and selecting putative transformants for further work.

Experiments were therefore performed to determine antibiotic levels that would suppress shoot formation and/or kill leaf sections. Sections were cultured as described above, but on B1N1 Medium supplemented with various concentrations of antibiotics. Carbenicillin was evaluated at concentrations of 0, 100, 200, 300,

400, and 500 mg/l. Kanamycin was tested at levels of 0, 10, 20, 30, 40, 50, 75, and 100 mg/l. Three trials were executed with each antibiotic. Survival, callus formation, and shoot production were noted periodically.

For transformation trials, sections were prepared as above and co-cultivated with At, containing the genetic construct. Prior to co-cultivation, At was grown overnight in liquid Luria-Bertani medium (LB Medium) (Sambrook et al. 1989) supplemented with 50 mg/l kanamycin to ensure maintenance of the construct. Sections were prepared in lots of 30, placed in an empty petri plate, and immediately exposed to 25-30 ml of At suspended in LB Medium. Plates were shaken at 50 rpm for 5 min to ensure that wounded edges were contacted by the bacterium. After shaking, sections were removed from the bacterial suspension and blotted on sterile filter paper until visibly dry. They were then transferred to 100 X 20 mm Petri plates containing 25 ml of B1N1 Medium and incubated in darkness for 3 days.

Following the 3-day co-cultivation period, sections were transferred to plates containing fresh B1N1 Medium supplemented with 500 mg/l carbenicillin and 150 mg/l kanamycin. Carbenicillin was added to kill residual bacteria, and kanamycin to select putative transformants. Sections remained on this selection medium in darkness for an additional 18 days (total of 21 days in darkness as per the Leaf Section System protocol). They were then transferred to fresh selection medium, and cultured in the light for 21 - 42 days, or until usable callus or harvestable shoots were observed. A total of 485 sections were used in two separate transformation trials.

Callus and/or shoots, derived from these cultures, were subcultured repeatedly on selection medium for use in verifying transformation via assays for nopaline production, Southern blotting, and/or analyses of indole-3-acetamide or indoleacetic acid (products of the enhanced auxin synthesis gene).

RESULTS AND DISCUSSION

Three trials were performed to determine sensitivity of C175 leaf sections to carbenicillin and kanamycin. Carbenicillin levels were needed that would eliminate At without inhibiting shoot production. The antibiotic was not expected to inhibit shoot production or kill leaf sections, but verification was essential. Results showed that shoot production was not affected by levels as high as 500 mg/l, and this concentration was considered more than adequate to kill residual At.

Using kanamycin resistance for selection requires adding it in quantities sufficient to eliminate nontransformed materials while permitting putative transformants to survive and form callus or shoots. Assays showed that C175 leaf sections were quite sensitive to kanamycin. Sections survived and shoots were produced at 10 and 20 mg/l, but levels of 30 mg/l or more proved lethal. To ensure

efficient selection, 150 mg/l were added to the selection media used in transformation trials.

In the transformation trials, one percent of the leaf sections survived, producing either callus or shoot-like structures (Table 1). Though lower than desired, this level of putative transformation is in line with the literature (e.g., Fillatti et al., 1987).

Table 1. Transformation of cottonwood clone C175 with the iaaM gene for enhanced auxin synthesis. Putative transformants from first trials, March - June, 1991.

Explant Type	Number	Percent
Transformed	5*	1.0
Shoot-Like	3	0.5
Callus	3	0.5
Attempted	485	100.0

* One putatively transformed explant produced both shoot-like structures and callus.

Plants have not yet been recovered, but cultures continue to grow on B1N1 Medium, supplemented with 500 mg/l carbenicillin and 150 mg/l kanamycin. In addition, the cultures have phenotypes similar to nontransformed ones given high exogenous levels of auxin; e.g., formation of numerous adventitious roots. The strong constitutive promoter presumably is causing continuous expression of the iaaM gene, and the cultures are responding to auxin over-production. These lines of evidence suggest successful transformation for both kanamycin resistance and auxin synthesis, but further work is needed to verify presence of the iaaM gene. Efforts to stimulate shoot production and recover plants by altering exogenous growth regulator regimes are underway. Even if plants are not obtained, transformation will be confirmed by Southern blotting and comparing indole-3-acetamide and indoleacetic acid levels in transformed and nontransformed cultures.

Cloning C175 via the Leaf Section System normally yields large numbers of vigorous shoots. In preparing for transformation trials, Shorter (1991) obtained shoot formation on 30 percent of explants, and harvested an average of 8 shoots per productive explant. That shoots and/or plants have not been recovered after transformation suggests that cottonwood is quite sensitive to auxin over-production. Transformed materials have thus far not responded to procedures typically causing shoot formation. In contrast, petunia plants, though abnormal, were recovered after transformation with the same gene (Klee et al., 1987). Transformed plants proved fertile, and selfed offspring had the same phenotypes as the parents. The 10-fold increase in endogenous auxin levels

and associated morphological changes did not prevent growth or alter reproductive capacity. The differential response of cottonwood demonstrates the danger of using constitutive promoters, and emphasizes need to employ a controllable promoter in future work.

SUMMARY, RECOMMENDATIONS, AND FUTURE WORK

This research indicates that the Leaf Section System is suitable for work on genetic transformation. Putative transformants have been obtained. Efforts are underway to stimulate shoot production by altering exogenous growth regulator regimes. Even if plants are not recovered, advantage will be taken of opportunities for confirming transformation. That is, cultures now in hand will be expanded and used for assays of nopaline content, Southern blotting, and analyses of indole-3-acetamide and indoleacetic acid levels.

To avoid problems caused by the constitutive promoter; ie., apparent constant expression of the iaaM gene, future work should be conducted with a genetic construct containing the iaaM gene coupled with a controllable promoter. An appropriate construct, containing a heat shock promoter, has been acquired from Monsanto Corporation. This should hasten confirmation of transformation and recovery of transformants. In addition, we are seeking cooperation with other laboratories that possess promoters specific to xylem. Recovery of plants and ability to control endogenous auxin levels in specific tissue or cell types should lead to a greater understanding of the role of auxin in tree growth and morphogenesis.

Efforts to extend the technology to other, more valuable clones will also be undertaken. This last issue is of extreme importance. Model clones; e.g., C175, are attractive because of the ease with which they can be cultured, but results will not be useful until the technology is workable with elite clones used or slated for use in breeding and planting programs.

ACKNOWLEDGEMENTS

Support of the Institute of Paper Science and Technology and its member companies is gratefully acknowledged. The authors also wish to thank James N. Mathis, former IPST Assistant Professor; Matthew P. Shorter, recent IPST M.Sc. graduate; Andria Costello and Laura Willis, Ga Tech undergraduate students; Rafique M. Uddin, former IPST Post Doctoral Fellow; David T. Webb IPST Associate Professor, and others in the IPST Forest Biology Group for their fine work and kind assistance.

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PROJECT 3223-03

MASS CLONAL PROPAGATION OF IMPROVED AND ENGINEERED HARDWOODS

RESEARCH REVIEW

MARCH 27, 1992

Ron Dinus

TECHNICAL PROGRAM REVIEW SUMMARY

PROJECT TITLE: MASS CLONAL PROPAGATION OF IMPROVED AND ENGINEERED HARDWOODS

PROJECT STAFF: Ron Dinus, Vacant Position

FY 91-92 BUDGET: $125,000

REPORTING PERIOD: January 1, 1991 - December 31, 1991

DIVISION: Chemical and Biological Sciences

PROJECT CODE: HRDWD

PROJECT NUMBER: 3223-03

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

GOAL: Build systems for producing hebicide tolerant or otherwise genetically altered strains of commercial hardwoods.

SUMMARY: In accordance with earlier plans, this project seeks to develop technologies for transferring genes for herbicide tolerance and other valuable traits into commercially important species, and for effective mass propagation, testing, and release of genetically modified trees. Herbicide tolerance is also being sought, as insurance, via somaclonal variation and selection.

Cottonwood clones maintained in our greenhouse continue to grow well. All materials, especially those used in current research, are hedged at least monthly to reduce size, ensure active growth, and foster proliferation of shoots/leaves used in research. To save time and expense, clone and ramet numbers have been reduced to minimum levels needed for current and projected work. Occasional additions of new clones will be made in a continuing effort to secure genetically improved materials that are easy to manipulate in culture.

Establishment and banking of shoot cultures has gone well. The large experiment undertaken last spring was repeated to document, with greater clarity, treatment internode position, and clonal effects. Results indicate that abundant shoot primordia form regardless of clone. Explants from near growing tips appear less useful than lower ones. Elongation to usable shoot size, however, varied greatly among clones, with only the northern (C175) and southern (K417) model clones yielding large numbers. Treatment effects were significant, with utility varying greatly among clones. Usable shoots nevertheless were obtained, rooted, and banked from all

clones. The more effective treatments were also used to secure cultures from internodes of the four Westvaco clones acquired last spring. Microscopic assays are being done to document origin of primordia and shoots; findings will be used to support publication of results from the several trials. Cultures established to date are being used to extend our Leaf Section System to genetically improved clones. The work also made possible addition of our southern model clone (K417) to work on genetic transformation and regeneration via the Leaf Section System. Representative cultures of aspen and sweetgum are being maintained as well.

Work on extending the Leaf Section System to more clones was slowed to permit completion of the trials noted above and to ensure adequate resources for seasonal work on loblolly pine initiation and maturation. Earlier informal trials, however, were completed. Results suggest that the six to nine weeks required for shoot formation and elongation can be reduced somewhat and that rooting can be effected at auxin levels below those used earlier and less likely to provoke unwanted side effects. Efforts to root, reroot, and reroot sprouts from shoots first rooted last March were successful. The approach shows promise for maintaining clones inSwulture, and may have potential as a means of multiplication. Findings from preliminary trials with additional clones were successful in that large numbers of primordia formed on leaves from all clones. Elongation, however, occurred only for C175 and K417.

First attempts to transform C175 leaf disks with the Monsanto gene for enhanced auxin synthesis appear successful. Several calli and shoots resistant to the selective antibiotic were obtained. In addition, the putative transformants all have the appearance of cultures given high exogenous doses of auxin, another, albeit indirect, line of evidence for transformation. Regeneration has not yet been possible, presumably due to excess auxin production. Efforts to offset this presumed difficulty by changing growth regulator regimes continue. Experiments to verify transformation via auxin assay and/or Southern blotting are being planned. As a further measure, we expanded our working relationship with Monsanto, and secured a second genetic construct bearing the enhanced auxin synthesis gene and a controllable promoter. We are seeking means to move the construct from its E. coli host to Agrobacterium tumefaciens and attempt transformation. This construct should allow us to control gene expression, and secure regeneration with less difficulty than encountered with the first construct. In addition, shoot production and expansion protocols were modified to yield more and larger leaves from our two model clones, C175 and K417. This should permit larger transformation trials in the future. Discussions concerning acquisition of the Monsanto gene for glyphosate tolerance, and possibly one for male sterility, are continuing.

Earlier cell suspension work yielded somaclonal variants tolerant of high glyphosate concentrations. These were expanded for additional challenges with the herbicide, and subsequent plating on solid media for callus induction and shoot formation. Calli formed with relative ease, but efforts ,based on work completed last year, to stimSwate shoot formation failed. We have reevaluated the protocol, and are executing experiments to test various modifications.

SHOOT INDUCTION FROM INTERNODES OF ELITE POPULUS DELTOIDES CLONES

Ronald J. Dinus, Shannon M. Johnson
Sonja J. Ozturk, and Camille J. Stephens

INTRODUCTION

The Institute of Paper Science and Technology (IPST) has conducted research in cell and tissue culture for over two decades. First efforts dealt with propagation of aspen (Populus tremuloides), and plants were produced from culture in the 1960s (Winton 1968). Since the 1970s, however, major emphasis has been on somatic embryogenesis of commercially important softwoods. Research on hardwoods was renewed recently. Focus of the new hardwood program centers on producing useful variants of eastern cottonwood (Populus deltoides) via genetic transformation and somaclonal variation/selection. Traits of interest include herbicide tolerance and enhanced auxin synthesis.

Herbicide tolerance promises substantially lower plantation establishment costs and increased growth. Enhanced auxin synthesis is expected to influence fiber numbers and/or dimensions (Klee et al. 1987). Greater fiber numbers could raise productivity, while altered dimensions, e.g., longer fibers with thinner walls, could raise product quality and variety. Also, ability to alter at will, auxin status in cultures and/or plants will permit investigation of mechanisms underlying fiber formation.

Development of useful variants requires ready access to clean explants from plants of interest; reliable methods for establishing, maintaining, and multiplying cultures; efficient means for effecting genetic transformation and selecting somaclonal variants; reproducible protocols for regenerating plants from culture; and techniques for confirming genetic change, stability, and utility.

The present research sought to develop and/or adapt methods for establishing cultures of elite clones. Model clones and systems are useful, but protocols eventually must work with elite clones of value to the pulp and paper industry and adapted to prospective planting sites. Cultures of such materials are needed to supply the large numbers of explants required for producing and regenerating useful variants.

Internodes were the explant of choice, and the approach was patterned after that used by Coleman and Ernst (1989, 1990b). Several treatments used by these authors, and a novel growth regulator/media combination were tested to discern utility of the approach, determine responsiveness of four elite clones, and secure cultures of elite genotypes for further research.

METHODS AND MATERIALS

Plant Material

Two model clones, K417 and C175, known for ease of manipulation in culture were used along with four elite clones. K417 (Dr. C. S. Prakash), was chosen for ease of manipulation (Prakash and Thielges 1989, Coleman and Ernst 1990a), southerly origin (Fulton Co., KY), and suitability for eventual testing on sites of immediate interest - bottomlands along the southern Mississippi and lower Columbia Rivers. The second model, C175, was supplied as a continuous shoot culture by Dr. S. G. Ernst (University of Nebraska-Lincoln). Although of northern origin (Wabasha Co., MN), this clone is noted for ease of manipulation (Coleman and Ernst 1989, 1990a,b). Elite clones St 66, 70, 72, and 75 from Issaquena Co., MS (Mohn et al. 1970) were supplied by Dr. B. J. Stanton (James River Corporation, Camas, WA). ST 66, 70, and 75 (Table 1) were chosen for site adaptability, superior volume productivity, average or better specific gravity, and variable alpha-cellulose content (Olson et al. 1985). ST72 was included for its site adaptability and superior productivity.

Table 1. Attributes of elite cottonwood clones St 66, 70, and 75 after 3 growing seasons in a Carlisle Co., KY, trial (1).

Clone No.	Volume (cu m)	Specific Gravity	a-Cellulose (%)
St 75	0.0694	0.32	53.7
St 66	0.0674	0.34	51.3
St 70	0.0665	0.32	49.5
Test Mean (2)	0.0490	0.33	51.1

(1) Adapted from Olson et al. 1985.
(2) A total of 75 elite clones were tested.

Whether received as dormant non-rooted cuttings or cultures (C175), ramets of each clone were rooted and grown in 2-gal pots containing a commercial soil-less potting mix of peatmoss, perlite, and vermiculite (1:1:1 vol). Water was provided as needed, and fertilizer (Peters 20-20-20 with micronutrients) was applied weekly. Two commercial fungicides (Captan or Benelate) and two insecticides (Avid or Mavrik) were applied alternately across weeks to prevent and/or remedy pest problems. Ramets were hedged every four to six weeks to maintain modest size, maximize explant numbers, and facilitate pesticide application. These measures plus day/night temperature (20-40 C) and photoperiodic (16/8 hr) regimes suited to year-round growth were used to ensure availability of explants. Natural photoperiods were supplemented with 34W cool white florescent tubes and 150W/55V high pressure sodium lamps.

Explants

Stems were cut immediately below the eleventh node. Leaves were removed and stems rinsed with tap water to remove debris and possible pesticide/fungicide residues. The two uppermost (Coleman and Ernst 1989) and lowermost internodes were discarded. Stem segments three through nine (seven total) were used as experimental material, and numbered 3 through 9, respectively.

After cutting into individual stem segments, all materials were placed in a sterile 1% (v/v) solution of Tween 20 (Sigma Chemical Co., St. Louis, MO) in deionized water and shaken (100 rpm) for 15 minutes to remove debris. Buds at the top of each segment served to ensure correct polarity of individual explants. Segments were then rinsed three times in sterile deionized water (5 min per rinse), and placed in a 20% (v/v) solution of commercial bleach (final concentration of 1.05% (w/v) NaOCl), shaken again for 20 min, and rinsed 5 times in sterile deionized water. They were next placed in liquid WNA medium (Coleman and Ernst 1989) supplemented with three antibiotics (500 mg/l carbenicillin, 50 mg/l tetracycline, and 15 mg/l rifampicin), and shaken in the dark for 24 hours to minimize systemic bacterial contamination (Coleman, personal communication).

Finally, segments were rinsed three times in sterile deionized water, and aseptically dissected into 5 mm internodal explants. Basal ends of the explants were inserted vertically in culture media. Nodal sections were used in separate trials or discarded.

Media

Internodal explants were cultured on either of two basal media: WNA medium, which is Woody Plant Medium (Lloyd and McCown 1980) as modified by Coleman and Ernst (1989), or DKW Medium (McGranahan et al. 1987). Alterations to Woody Plant Medium consist of an increase in $Ca(NO_3)_2 \cdot 4H_2O$ to 2000 mg/l, NH_4NO_3 to 1650 mg/l and addition of 500 mg/l casein hydrolysate. WNA was supplemented with 0.5 mg/l 2,4-D to induce callus formation (CIM), and with 0.5 mg/l zeatin to induce shoots (SIM) (Coleman and Ernst 1990b). DKW Medium was supplemented with 0.1 mg/l thidiazuron (NOR-AM Chemical Company, Wilmington, DE) to stimulate shoot production. This last combination (DKWT) was used to induce shoot formation on cottonwood leaf sections (Uddin et al. 1989). In addition, thidiazuron stimulated shoot formation on callus from cottonwood leaves (Prakash and Thielges 1989) and on callus derived from leaf protoplasts of a Populus hybrid (Russell and McCown 1986).

Individual media were brought to pH 5.8, supplemented with a solidifying agent, 0.25% Gelrite (Merck & Co. Inc. [Rawhay, NJ], Kelco Division), and autoclaved at 121°C and 1.4 kg/cm^2 for 20 min. For the first 28 d of culture, carbenicillin (500 mg/l) was

included in all media, regardless of medium or growth regulator content, to control possible systemic bacterial contamination. Zeatin and carbenicillin were filter sterilized, and added after autoclaving. Media were dispensed into 100 X 15 mm polystyrene Petri plates, with each dish receiving 20 ml. Larger plates (100 X 20 mm containing the same quantity of media were used for subculture.

Design and Data Collection

The entire experiment was replicated six times. For each replication, one branch from a given ramet of each clone was used to secure the required seven internodes. Individual internodes were cut into six 5 mm explants, with one explant allocated at random to each of the six media and growth regulator combinations. Each combination of media and growth regulator, clone, and replication (one petri plate) with seven explants thus constituted the experimental unit, and subsequent observations were based thereon. Internode position (age) of each explant was coded to permit monitoring internode position effects in addition to those of clone and growth regulator. All work on a replication was completed with one branch from a particular ramet of a each clone, performed on a given day, and executed by a particular array of operators. This arrangement proved efficient from a logistic standpoint and provided the potential for reducing experimental error by associating any variability caused by stock plant condition or explant processing with replications (blocks).

The six treatments consisted of WNA basal medium (control), DKWT, and 0, 1, 4, or 8 days on CIM followed by transfer to SIM. All materials were cultured in darkness at 22 °C for the first 10 days, and then transferred to a 16 hour/day photoperiod at 22 °C with illumination from 40W preheat- rapid start agro-lite lamps (15 μmoles M^{-2} s^{-1} of photosynthetically active light). Explants were moved to fresh media every 28 days. Observations of contamination, callus production, and shoot formation and/or elongation were recorded every 7 days over the 91-day trial. Selected explants were photographed weekly with a microflex UFX-II mounted on a Nikon SMZ stereoscopic microscope through day 56 to document origin, formation, and growth of callus and shoots. Harvestable shoots (5 mm or longer) were counted, and collected for other research on days 63 and 91. At the latter time, selected explants were also fixed, embedded, and sectioned for photomicrography, an additional effort to document origin and nature of callus and differentiating shoots.

Data Analysis

For statistical analyses, observations were summarized as percentages of explants forming shoots (FS) on day 63, and as percentages of explants having elongated shoots (ES) through day 91 for each combination of replication, clone, and treatment.

Day 91 data were used in order to allow greater opportunity for elongation on less responsive clones, and to maximize numbers of shoots harvested and made available for other research. Data were subjected to analyses of variance (Steel and Torrie 1960) for the Randomized Block Design with 6 replications (blocks), 6 clones, and 6 treatments, using the general linear models procedure of SAS Institute (1985). Similar analyses were performed for the Completely Randomized Design to determine if blocking, as described above, improved precision.

To quantify significance of internode position, ES and FS were estimated by summarizing explant condition across replications for all combinations of clones, treatments, and internode positions. Findings were evaluated via analysis of variance for the Completely Randomized Design having 6 clones, 6 treatments, and 7 internode positions nested within clones.

When main effects were significant, regardless of variable or analytical format, means were compared using Duncan's multiple range test. Standard errors of means for all clone/treatment combinations were also computed. Tests of significance were made at the 0.05 level of probability.

RESULTS AND DISCUSSION

On average, callus formation began after roughly 28 days of culture and continued with declining frequency through day 63. Explants of the more responsive model clones, however, formed callus as early as day 14. The least responsive clone, St 72, was the last to form callus, regardless of treatment. Compact green callus eventually covered the entire upper surface of many explants, especially those cultured on CIM 4 and 8. Explants from internodes 3 and 4 formed callus less frequently than others.

Callus developed after swelling and division of cortical cells located external to the cambium (Figure 1A). The callus contained thin-walled, isodiametric parenchyma cells, compactly arranged to form an homogeneous mass (Figure 1B). Periclinal divisions predominated at the growing edge, giving rise to radial organization. Eventually, vascular traces from developing adventitious shoots traversed the callus.

A. Transverse section of typical explant showing early callus development; thin ring of callus (C) forming from cortical cells just outside the cambium, 12.5X.

B. Transverse section showing callus (C), vascular system of explant (V), and vascular traces of adventitious shoots (T), 175X.

C. Numerous adventitious shoots from callus totally covering upper surface of explant, 18X.

D. Explant covered with callus (C) and bearing typical elongated shoot, 8.25X.

E. Longitudinal section through an elongating (E) adventitious shoot and forming shoot (F) and leaf (L), vascular tissue in shoot (V), and its connection with vascular trace (T) in callus, 175X.

Adventitious shoots formed subdermally from outermost layers of the callus (Figure 1B-C), without further dedifferentiation of the callus. Shoots formed from callus of model clones within 28 days (Figure 1B-C). Most responsive explants formed shoots by day 35 or 42, with rates of formation diminishing thereafter. Few explants, mainly from St clones, formed shoots after that day.

Shoots, especially for model clones, began elongating within 7 days of forming (Figure 1D). These structures were fully differentiated, complete with subtending leaves and normal apical meristems (Figure 1E). They were not associated with vascular system of explants, but vascular connections developed between the two systems as shoots elongated.

On average, harvestable shoots were first noted in quantity on day 56; most elongated within the first 63 days. Shoots from elite clones often failed to elongate, and yields of harvestable shoots were low compared to those from model clones (Table 2). Elongation on elite clones nevertheless continued through day 91. Harvesting shoots did not greatly influence on elongation of residual shoots. Proportions of shoots elongating between days 63 and 91 for individual clones and treatments were such that analyses of data from days 63 and 91 yielded the same outcomes with regard to significance of main effects and differences among means.

(1) Clone means represent mean percentages of explants forming (FS) or elongating (ES) shoots across all treatments for individual clones after 63 and 91 days in culture, respectively. Means followed by different letters differ significantly from others for same variable according to Duncan's multiple range test at $p = 0.05$.

(2) Means (+/- standard errors) for individual clone X treatment combinations.

(3) Treatment means represent mean percentages of explants forming (FS) and elongating (ES) shoots across all clones after 63 and 91 days in culture, respectively. Means followed by different letters differ significantly from others for same variable according to Duncan's multiple range test at $p = 0.05$.

Despite intensive sterilization procedures, some explants were lost because of contamination from residual bacteria and fungi. Contamination appeared within four days of culture,

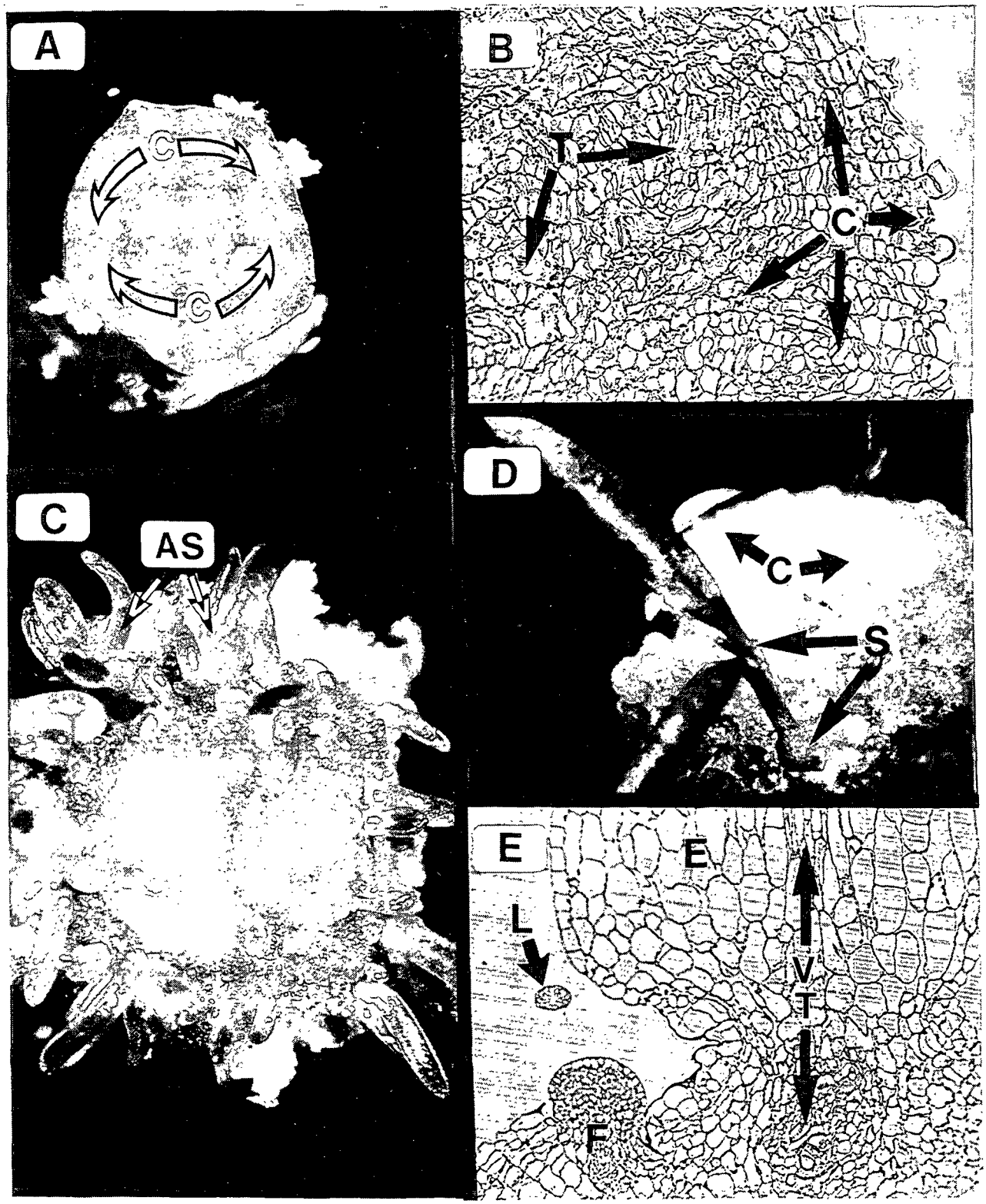

Figure 1. A-E. Callus Development, Shoot Formation, and Shoot Elongation on Cottonwood Internode Explants.

peaked rapidly, and was minimal after day 28. Overall, 10.8 percent of the explants were lost to contamination; 10.3 percent to bacteria and 0.5 percent to fungi. No complete clone X treatment combinations were lost.

Analyses of variance indicated that replication (block) effects were not significant. Error mean square terms from analyses for the Randomized Block Design were only marginally lower than those from analyses for the Completely Randomized Design. Coefficients of variation were reduced slightly. Outcomes from tests of significance for clone, treatment, and interaction effects as well as Duncan's multiple range tests were the same for both types of analysis. Although blocking did not improve statistical precision, the arrangement is so logistically convenient and efficient that we will continue the practice.

Overall clone and media/growth regulator effects were significant. K417 proved significantly more responsive than other clones, and gave the highest percentages of explants with harvestable shoots (Table 2). Modest percentages of K417 explants produced shoots even on control medium. Such responsiveness was expected in view of earlier research. Treatment with Z stimulated shoot production from internode explants (Coleman and Ernst 1990a). Abundant shoots also formed on leaf callus in response to incubation with thidiazuron (Prakash and Thielges 1989).

C175 was significantly less responsive than K417, but more responsive than other clones (Table 2). This clone was tested in similar experiments by Coleman and Ernst (1990 a,b), and our findings generally agree with their results. Shoots formed and elongated on fair to large percentages of explants following incubation on CIM 1, 4, and 8, but lesser numbers formed in response to CIM 0 and control medium. Percentages of C175 explants forming shoots in response to DKWT were similar to those of other treatments. DKWT, however, stimulated elongation more than control and CIM 0 media.

Though not differing one from another, elite clones proved significantly less responsive than K417 and C175 (Table 2). Depending on treatment, 3 elite clones, St 66, 70, and 75, formed shoots with some frequency, but all 4 performed poorly as regards elongation. Percentages of explants producing harvestable shoots ranged from only 0.6 to 8.8 percent, with St 66 tending to be more productive across all treatments. Interestingly, the three elite clones that yielded harvestable shoots are males. The least responsive, St 72, is female.

Differential responses between sexes have been noted in culture of other Populus species. Cultured leaf discs of female Himalayan poplar (P. ciliata) formed multiple shoots (Mehra and Cheema 1980). Discs of male trees, cultured under the same conditions, failed to produce shoots. The same results were obtained with leaf discs of P. alba (Mehra and Cheema 1985).

Nodal and internode explants from male and female trees of these species also differed in shoot productivity. In P. ciliata, internodes from male trees produced larger numbers of shoots than those from females. When cultured on basal medium lacking growth regulators, however, internode explants from female trees of P. alba produced shoots but male explants did not respond. Differential response in absence of exogenous growth regulators suggest that stem tissues of female and male Populus trees differ in levels of natural growth regulators. Definitive data on responses of the sexes in cottonwood might explain why many genotypes are difficult to manipulate in culture, and could thereby facilitate future work.

Treatment differences were more pronounced for shoot elongation than formation. As regards formation, CIM 1 and DKWT tended to give better results than other treatments, but differed significantly only from CIM 8 and WNA. Longer CIM treatments and DKWT caused higher frequencies of elongation, significantly more so than CIM 0 and WNA. CIM 1 and 4 produced harvestable shoots on explants from 4 and 5 of 6 clones, respectively. DKWT induced shoot formation on these same clones, but failed to stimulate elongation on a par with the better CIM treatments. This was especially true for elite clones, where shoots formed in response to DKWT for three clones at rates equal to (St 70 and 75) and better than (St 72) other treatments. Explants from St 75, however, were the only ones yielding harvestable shoots.

Another aspect of the response to DKWT is also noteworthy. Responding explants, regardless of clone, typically formed numerous shoots in clusters near the base of explants, ie., near surface of the media. Elongating shoots also occurred in this area rather than on the upper surface as for other treatments. Reasons for this behavior are uncertain, but may have to do with the ease with which thidiazuron is transported in stems relative to that for 2,4-D and Z. Regardless of the cause, further trials with different levels of thidiazuron seem warranted in view of its considerable effect on shoot formation.

The clone X treatment interaction was also significant; treatments best for one clone were not necessarily good for others (Table 2). Such findings underscore importance of genotype, and the need, at least for the present, to tailor protocols to individual clones. Differential responses occurred for even the model clones, especially in terms of elongation. K417 tended to yield more harvestable shoots after shorter CIM exposures, whereas C175 tended to perform better after longer exposures.

Differential responses were also found for elite clones. CIM 4 and 8 gave somewhat higher frequencies of elongation with St 66, while CIM 1 tended to work better with St 70 and 75. DKWT failed to stimulate formation or elongation on St 66 explants, promoted formation but not elongation on St 70 and 72, and stimulated St 75 to produce some harvestable shoots.

Table 2. Mean Percentages of Cottonwood Internode Explants Forming (ES) and Elongating (ES) Shoots After Incubation with 2,4-D (0.5 mg/l) for Various Lengths of Time before Exposure to Z (0.5 mg/l) (CIM 0, 1, 4, and 8) or Control Medium and DKWT. Data were Collected 63 (FS) and 91 (ES) Days After Start of Culture.

Clone No.	Variable FS(±SE) ES(±SE)	Clone Means	Control (Basal Medium)	CIM Treatments: Days of incubation on 2,4-D before exposure to Z 0	1	4	8	DKWT
				%				
K417	FS	77.3a[1]	31.0 (15.4)[2]	86.9 (5.1)	92.5 (3.4)	86.9 (8.7)	66.7 (13.6)	95.0 (5.0)
	ES	72.7a	23.0 (10.2)	79.8 (6.3)	86.5 (5.1)	83.6 (4.4)	69.4 (10.9)	88.3 (7.2)
C175	FS	82.4a	75.0 (25.0)	80.6 (7.9)	94.4 (3.5)	79.8 (10.8)	86.7 (8.2)	66.7 (33.3)
	ES	41.3b	0.0 (0.0)	13.5 (6.7)	50.6 (11.8)	56.7 (16.4)	47.5 (20.6)	66.7 (33.3)
ST66	FS	21.2b	0.0 (0.0)	30.6 (7.6)	38.4 (21.5)	21.9 (6.5)	20.7 (12.0)	0.0 (0.0)
	ES	8.1c	0.0 (0.0)	7.5 (5.0)	0.0 (0.0)	18.6 (8.0)	13.6 (9.4)	0.0 (0.0)
ST70	FS	21.0b	16.7 (16.7)	13.8 (7.1)	20.2 (8.7)	30.6 (17.4)	12.5 (12.5)	33.3 (33.3)
	ES	5.5c	0.0 (0.0)	0.0 (0.0)	23.2 (9.6)	5.6 (5.6)	0.0 (0.0)	0.0 (0.0)
ST72	FS	23.0b	5.0 (28.9)	30.1 (10.5)	25.6 (9.5)	4.8 (3.0)	2.8 (2.8)	45.8 (26.7)
	ES	4.9c	0.0 (0.0)	4.8 (4.8)	12.7 (8.1)	2.4 (2.4)	0.0 (0.0)	8.3 (8.4)
ST75	FS	15.16	16.7 (16.7)[3]	12.5 (6.0)	8.3 (8.3)	0.0 (0.0)	2.8 (2.8)	48.3 (18.3)
	ES	0.5c	0.0 (0.0)	2.8 (2.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Treatment Means	FS	40.2	26.3c	42.4ab	47.9a	37.8abc	31.4bc	54.3a
	ES	22.9	5.0c	18.11	30.8a	27.8a	22.8ab	29.4a

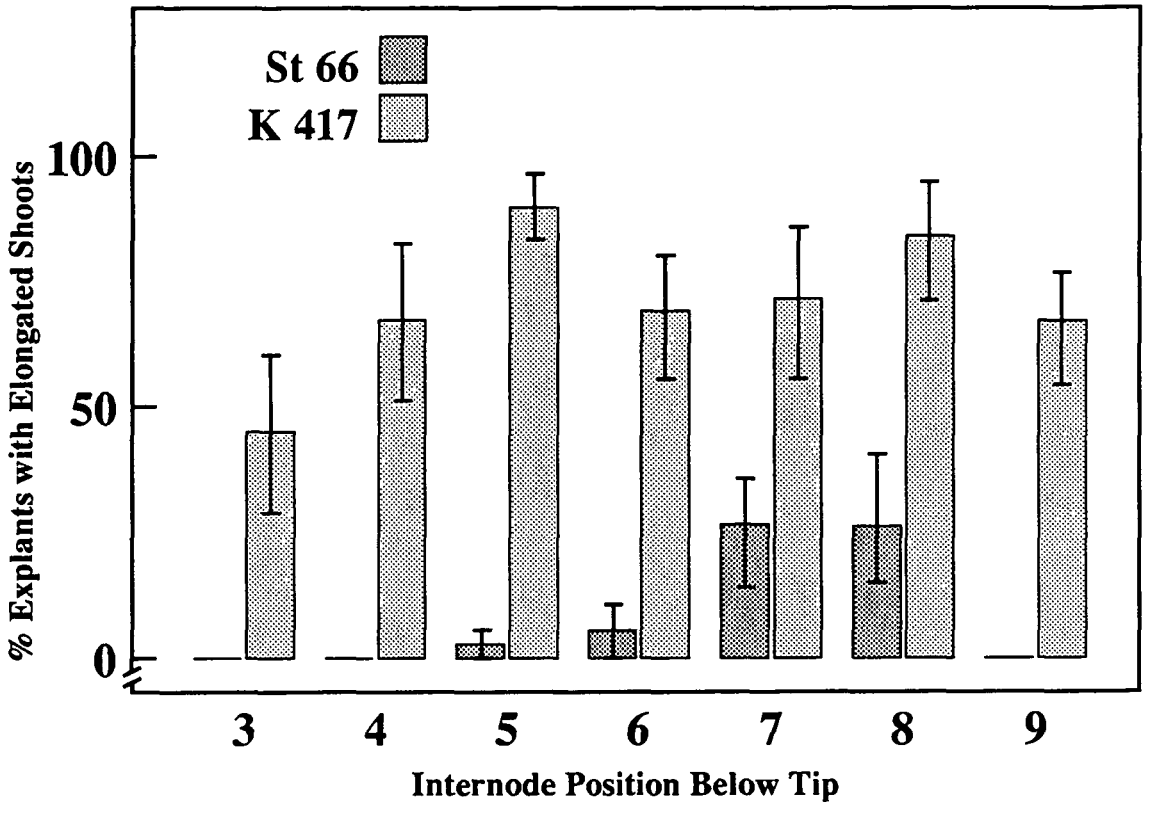

Figure 2. Effect of Internode Position for Most Responsive Model (K417) and Elite (St 66) Clone on Percent of Explants with Elongated Shoots Averaged Over All Media/Growth Regulator Treatments.

The ratio of explants forming shoots to those elongating shoots, a potential index of efficiency, differed markedly among clones and treatments. Depending on clone, some treatments stimulated shoot formation, but failed to cause useful numbers to elongate. K417 had a similar ratio for all treatments, except for the control (Table 2). For other treatments, most explants that formed shoots also produced harvestable shoots. The ratio for C175 was highest for CIM 4 and DKWT. Elite clones exhibited yet different patterns, both from model clones and from one another. In response to short CIM exposures, St 66 explants formed shoots readily, but few produced harvestable shoots. In response to longer exposures, however, fewer explants formed shoots but more of them eventually yielded harvestable shoots. Somewhat the opposite occurred for St 75; the highest ratio occurred for CIM 1 and 4. Viewed in this manner, results highlight the better clone X treatment combinations, and also suggest that the process may consist of two steps, induction and elongation. Thus, optimization of each such step may be required to raise protocol efficiency and reliability.

Effects of internode position were also significant (Table 3), perhaps providing a means for raising efficiency with even the more recalcitrant clones. When averaged over all clones and treatments, explants from internodes 3 and 4 generally produced fewer harvestable shoots than others. Explants from lower internodes that formed shoots also tended to produce harvestable shoots more often than those from upper internodes. Similar results with a Populus hybrid were reported by Douglas (1984), who attributed the effect to differential distribution of natural growth regulators or, possibly, gradients in tissue differentiation.

FOOTNOTES FOR TABLE 3:

(1) Clone means represent mean percentages of explants forming (FS) or elongating (ES) shoots across all treatments and internodes for individual clones after 63 and 91 days in culture, respectively. Means followed by different letters differ significantly from others for same variable according to Duncan's multiple range test at $p = 0.05$.

(2) Individual clone X internode means (+/- standard errors) averaged over all treatments.

(3) Internode means represent mean percentages of explants forming (FS) and elongating (ES) shoots averaged across all clones and media/growth regulator combinations after 63 and 91 days in culture, respectively. Means followed by different letters differ significantly from others for same variable according to Duncan's multiple range test at $p = 0.05$.

Importance of internode position also varied among and sometimes within clones. For example, explants of K417 produced

Table 3. Effect of Internode Position on Mean Percentages of Cottonwood Internode Explants Forming (FS) and Elongating (ES) Shoots for Six Clones. Data were Collected at 63 (FS) and 91 (FS) After Days of Culture.

Clone No.	Variable FS(±SE) ES(±SE)	Clone Means	Internode Position A	B	C	D	E	F	G
			%						
K417	FS	77.1a[1]	57.5 (7.5)[2]	73.6 (15.9)	93.3 (4.1)	77.8 (11.9)	75.8 (15.8)	82.2 (9.1)	75.3 (12.5)
	ES	71.5a	45.0 (16.6)	67.5 (15.5)	90.0 (6.7)	69.4 (11.7)	71.7 (15.2)	84.2 (12.3)	67.2 (10.5)
C175	FS	77.1a	41.7 (8.3)	60.8 (7.9)	67.5 (15.5)	100.0 (0.0)	83.3 (16.7)	90.8 (5.4)	76.7 (19.5)
	ES	42.7b	8.3 (8.3)	27.9 (7.6)	55.8 (17.2)	63.9 (17.4)	31.9 (18.0)	37.5 (14.6)	53.3 (9.7)
ST66	FS	17.4bc	0.0 (0.0)	0.0 (0.0)	2.8 (2.8)	25.6 (9.3)	36.7 (16.5)	43.3 (8.0)	16.7 (7.5)
	ES	7.7c	0.0 (0.0)	0.0 (0.0)	2.8 (2.8)	5.6 (5.6)	26.7 (9.1)	25.8 (14.3)	0.0 (0.0)
ST70	FS	21.8b	14.6 (8.6)	20.0 (20.0)	8.3 (8.4)	12.5 (8.5)	20.0 (20.0)	54.2 (15.8)	26.4 (9.2)
	ES	7.9c	0.0 (0.0)	0.0 (0.0)	6.7 (6.7)	4.2 (4.2)	20.0 (13.3)	16.7 (11.4)	6.7 (6.7)
ST72	FS	10.3c	0.0 (0.0)	0.0 (0.0)	8.9 (5.9)	22.2 (8.0)	16.7 (16.7)	15.3 (11.1)	5.6 (5.6)
	ES	1.3c	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	8.3 (9.8)	0.0 (0.0)
ST75	FS	27.8b	22.2 (11.1)	0.0 (0.0)	15.0 (8.0)	33.9 (14.8)	27.5 (9.5)	47.2 (18.5)	46.1 (14.4)
	ES	9.0c	0.0 (0.0)	0.0 (0.0)	3.3 (3.3)	3.3 (3.3)	0.0 (0.0)	22.2 (16.5)	28.3 (15.2)
Internode Position Means	FS	38.5	21.8c[3]	25.3c	32.3bc	45.3ab	44.4ab	54.1a	40.8ab
	ES	23.4	8.9c	17.22bc	25.9ab	24.4ab	25.1ab	32.6a	24.9ab

harvestable shoots regardless of internode position (Table 3), and only minor differences occurred among internodes for this clone (Figure 2). For elite clones, however, elongated shoots were obtained only from the fifth through ninth internodes. Internodes 7 and 8 from St 66, for example, were more productive than others (Figure 2). Using the more responsive internodes may lead to improved efficiencies in future.

Some portion of clone and internode effects may be associated with explant size (diameter). Larger explants, a function of both clone and internode, could contain larger quantities of responsive tissues or perhaps natural growth regulators, and therefore form and elongate more shoots. Explants from elite clones and lower internodes typically were larger than others. While size may have had some influence, such effects seem minor in that C175, the second most responsive clone, consistently had the smallest explants. Elite clones, on the other hand, were always larger, but were least responsive.

SUMMARY, RECOMMENDATIONS, AND FUTURE WORK

In sum, shoot production from internode explants seems a workable method for establishing cultures to use in future IPST hardwood research. Cultures from several elite clones have been established, and are being expanded for use in other work.

The process of shoot production may well consist of two separate steps, induction and elongation. Until more is known about this aspect, however, the best tack seems refinement of CIM exposure lengths and adjustment of Z levels. The foregoing indicates that one protocol seldom is efficient for diverse genotypes, particularly elite clones. Model clones are useful, but elite clones are needed. Refined protocols are essential to overcome apparent recalcitrance of such materials and to ensure reproducible results and efficiency.

Thus, directed trials of additional 2,4-D exposure lengths, higher Z levels, and the most responsive internodes appear lucrative. Such refinements could increase production of harvestable shoots from aforementioned clones, and permit extension to a wider array of elite genotypes.

Probably the best combinations of elite clones and treatments for future work are St 66 and CIM 4, St 70 and CIM 1, and St 75 and CIM 1 or 4. Of the several opportunities, we favor additional work with St 75, because of its high volume productivity and above average alpha-cellulose content.

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STUDENT RESEARCH

STUDENT RESEARCH - COMPREHENSIVE LIST

Completed

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

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

Lois Forde - M.Sc., Phenylalanine ammonia lyase and lignin biosynthesis. Advisors, Conners and Dinus. Expected to return for Ph.D. in 1992.

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

IN PROGRESS

Teri Ard - M.Sc., Use of a nematode, Caenorhabditis elegans, for biomonitoring pulp and paper mill wastes. Advisor, Dinus

* Deborah Consani -M.Sc., Development of a polymer coating for encapsulation, storage, and handling of somatic embryos. Advisor, Dinus

Lois Ford - Ph.D., Biological amelioration of pitch problems in pulping and papermaking operations. Advisor, Dinus.

* Rene Kapik - Ph.D., A study of plant growth regulators during zygotic embryogenesis in loblolly pine. Advisor, Dinus.

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

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

Colleen Walker - Ph.D., Development of a biomimetic approach for pulping bleaching. Advisor, Dinus.

* Mike Wood - Ph.D., Examination of genetic structure in Pinus elliottii populations using the Polymerase Chain Reaction. Advisors, Dinus and Mathis.

* Specifically targeted at 3223-00

** Specifically targeted at 3223-03

RECENT PUBLICATIONS

STATUS OF RECENT PUBLICATIONS

PUBLISHED/IN PRESS:

Dinus, R.J. Developments in genetic manipulation of woody plant fiber and energy traits. in Proc. IEA/BA Task 5 Workshop on Woody Plant Feedstock. New Orleans, LA. December 7, 1991. (in press).

Nagmani, R.; Johnson, M.A.; and Dinus, R.J. Effect of explant and media on initiation, maintenance, and maturation of somatic embryos in **Pseudotsuga menziesii (Mirb.) FRANCO (DOUGLAS-FIR)**. Woody Plant Biotechnology, edited by M.R. Ahuja. Plenum Press, New York, (p 171 - 178), 1991.

Nagmani, R. and Dinus, R.J. Somatic embryo development and maturation in suspension cultures of Douglas-fir **Pseudotsuga menziesii (Mirb.) FRANCO**. in Proc. 21st Southern Forest Tree Improvement Conference. Knoxville, TN. June 17-20, 1991. (in press).

PRESENTATATIONS/ABSTRACTS:

Dinus, R.J. Developments in genetic manipulation of woody plant fiber and energy traits. in Proc. IEA/BA Task 5 Workshop on Woody Plant Feedstock. New Orleans, LA. December 7, 1991. (in press).

Dinus, R.J.; Johnson, S.M.; Ozturk, S.O.; and Stephens, C.J. Shoot induction from internodes of elite **Populus deltoides** clones. in Proc. 13th N. Am. For. Biol. Workshop. Sault Ste. Marie, Ontario, Canada. August 17-20, 1992. (submitted).

Nagmani, R. and Dinus, R.J. Maturation of Douglas-fir somatic embryos in suspension cultures. in Proc. 21st Southern Forest Tree Improvement Conference. Knoxville, TN. June 17-20, 1991. (in press).

OTHERS FOR INFORMATION:

PUBLISHED:

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GLOSSARY

GLOSSARY

Adventitious - Roots, shoots, embryos, or other organs or tissues developing in an abnormal position.

Agar - Polysaccharide complex extracted from algae. Used as gelling agent in tissue culture medium.

Agarose - A gelling agent derived from agar: the neutral (charge) fraction of agar.

Agrobacterium tumefaciens - Bacterial plant pathogen responsible for crown gall in plants. Harbors a tumor inducing (Ti) plasmid which can be used to transport a foreign gene into a plant cell.

Antibiotic resistance gene - A gene that codes for a protein, which imparts resistance to an antibiotic that allows cells to live in the presence of the drug that would normally kill them.

Archegonium - The flask-shaped container of the ovum (egg cell) of some gymnosperms. The swollen base (venter) contains the egg cell and is surrounded by the neck, with neck canal cells.

Aseptic culture - Surface sterilization of parental explants, free from pathogens, but not necessarily free of internal symbionts.

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.

Bacillus thuringiensis - Bacterium which produces a protein having a strong insecticidal activity. Depending upon the strain of the bacteria, the toxin may exhibit specificity toward Lepidopteran, Dipteran or Coleopteran insect groups.

Bacteriophage - A virus that attacks bacteria; also called a phage.

Base (nucleic acid) - A flat, ring compound that forms part of one of the nucleotide links of a nucleic acid chain. The bases are adenine, thymine, guanine, cytosine and uracil (commonly abbreviated A, T, G, C, U).

Base pair - Two bases, one in each strand of a double stranded DNA molecule, which are attracted to each other by weak chemical interactions. Only certain combinations of bases will pair: A-T, G-C and A-U.

Callus culture - Proliferation from a parental explant of many cells in protoplasmic continuity, but having no equivalence with any normal tissue. Same as tissue culture.

Cell differentiation - Internal chemical or ultrastructural changes preceding or accompanying specialization of function.

Cell suspension - Culture of single cells in moving liquid medium, often used to describe suspension cultures of cells and cell aggregates.

Chloroplast - A membrane-enclosed subcellular organelle containing chlorophyll. Chloroplasts are the sites of photosynthesis. They contain DNA and ribosomes and can replicate.

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.

Clone - 1. (verb) to undergo the process of creating a group of identical DNA molecules or genes derived from a single source. 2. (noun) a group of genetically identical cells (plants), all derived from a single ancestor.

Cloning vector - Small plasmid, phage or virus DNA molecules used to transfer a DNA fragment or gene from a test tube to a living cell. Some vectors are capable of multiplying inside living cells (bacteria) to result in the multiplication or cloning of the transferred DNA or gene.

Codon - A group of three nucleotides coding for an amino acid.

Conversion - Development of cotyledonary embryo to rooted plantlet.

Coumarins - A class of phenylpropanoid phenolic compounds of which coumarin itself typifies the structures.

Cotyledon - The leaf formed directly from the embryo of an angiosperm or gymnosperm. There may be one (in monocotyledons), two (in dicotyledons), or several (in gymnosperms). They act as storage organs in nonendospermous seeds and as the first photosynthetic organs in endospermous seeds.

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

cDNA (complementary DNA) - DNA synthesized from an RNA template in test tubes using the enzyme reverse transcriptase. The DNA sequence is thus complementary to that of the RNA. cDNA is usually made with radioactive nucleotides and is used as a hybridization probe to detect specific RNA or DNA molecules (genes).

Denature - In reference to DNA, denaturation means conversion of double stranded to single stranded DNA.

2D TLC - Two-dimensional thin-layer chromatography.

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

Electroinjection - Method of transporting naked DNA into a plant cell having a cell wall using a short duration DC electrical pulse (see electroporation).

Electroporation - Method of transporting naked DNA (gene) into a protoplast using a short duration DC electrical pulse.

E. coli (Escherichia coli) - A bacterium commonly found in the digestive tracts of many mammals, including humans.

EM- Electron microscope.

Embryo - The young plant developing in the megagametophyte from the fertilization of an egg cell, or without fertilization. In aseptic cultures, adventitious embryos show polarization followed by the growth of a shoot from one end and a root from the other end.

Embryogenesis - Initiation of embryoids or embryos from cultured cells.

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

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

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

Erosion zone - Zone in the gametophytic tissue below the archegonium that is degraded by the developing embryo.

Eucaryotic cells - Cells with true nuclei bounded by nuclear membranes and which undergo meiosis.

Excise - To cut or isolate callus tissue from its parental explant or to remove adventitious shoots from callus tissue for rooting.

Explant - A plant part excised and prepared for aseptic culture by surface sterilization followed by the exposure of live cells to a nutrient medium.

Fertilization - The normal union of two gametes during sexual reproduction.

Flavonoids - A class of phenolic compounds usually consisting of two hydroxylated aromatic rings joined by a three-carbon chain.

Gametophytic tissue - Haploid tissue of the seed that surrounds the developing embryo during the latter stages of embryogenesis.

Gel electrophoresis - A method for separating molecules based on their size and/or electrical charge. Molecules are forced to run through a gel (e.g., agarose or polyacrylamide) by placing them in an electric field. The speed at which they move depends on their size and/or charge.

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

Gene cloning - A way to use microorganisms to produce millions of identical copies of a specific region of DNA or gene.

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

Genetic engineering - The formation of new combinations of heritable material by the insertion of nucleic acid molecules into a vector system so as to allow their stable incorporation into a host organism in which they do not naturally occur.

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.

Genome - May refer to the full genetic complement in the haploid set of chromosomes of a species, but one may speak of nuclear, chloroplastid and mitochondrial genomes.

Genotype - The genetic makeup of an individual; carried in the chromosomes.

Grana - Association of thylakoids in a stack.

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.

Homologous - Describing regions of DNA molecules that have the same nucleotide sequence. Complementary base pairing can occur between homologous regions in two different DNA molecules.

Hormone - Any growth substance which is 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.

Hypocotyl - The part of a seedling axis between the radicle and the cotyledon(s).

Induction - To cause initiation of a plant structure, organ or process.

Inoculation density - "ID" is the volume of cells per unit of medium, i.e., μL/mL.

Inoculum - A small piece of tissue cut from callus, or a small amount of cell material from a suspension culture placed in contact with fresh medium for continued growth of the culture. Inocula (plural).

Interspecific hybrid - The progeny from matings between species.

Intraspecific hybrid - The progeny from matings within species.

Intron - A noncoding section of a gene that is spliced out of mRNA before translation into proteins.

<u>In vitro</u> - Outside the living organism.

<u>In vivo</u> - Within the living organism.

Isozymes - Multiple forms of a single enzyme.

Kanamycin - Antibiotic that disrupts protein synthesis in some bacteria and plants.

Lamda - The name of a particular bacteriophage (virus) used extensively in gene cloning.

Launch - (Induction), to cause the initiation of a process that will result in the development of a plant structure (shoots, roots, or embryos); sometimes used to describe the log phase of the growth cycle.

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

Maturation - Development of proembryo to cotyledonary embryo.

Milieu - The whole chemical and physical environment of a culture.

Meristem - A localized group of cells, actively dividing and undifferentiated but ultimately giving rise to permanent tissue such as shoots, roots, wood or bark.

Meristemoid - A localized group of cells in callus tissue, characterized by an accumulation of starch, RNA and protein, and giving rise to adventitious shoots or roots.

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

Morphogenesis - Initiation of organized tissue in callus or suspension cultures.

mRNA (messenger RNA) - RNA that is used by the ribosome to synthesize proteins.

Nick translation - A procedure for radiolabelling DNA <u>in</u> <u>vitro</u>. Used to make a radioactive probe.

Nuclease - A general term for an enzyme that cuts DNA or RNA.

Nucleic acid - DNA or RNA.

Nucleotide - One of the building blocks of nucleic acids. A nucleotide consists of three parts: a base, a sugar and a phosphate.

Nutrient medium - A solid or liquid combination of major and minor salts, and energy source (sucrose), vitamins, hormones, and occasionally other defined or undefined supplements. Usually made up from previously prepared stock solution, then sterilized by autoclaving or filtering through a micropore filter. Media (plural).

Organized tissue - Tissue composed of regularly differentiated cells.

Organelle - A complex cytoplasmic structure of characteristic morphology and function, such as a mitochondrion or plastid.

Organogenesis - Initiation of roots or shoots from callus meristemoids.

Packed cell volume - "pcv" is the volume of cells determined by centrifugation.

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

Passage - The duration of growth of callus or cell material from one subculture to another.

Photoperiod - Length of daily light cycle.

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.

Plasmid - A small circular DNA molecule found inside bacterial cells.Plasmids reproduce every time the bacterial cell reproduces. Once infected, the bacteria will always contain a plasmid. Some plasmids continue to replicate in a bacterial cell so that a single cell may contain 200 plasmids. Plasmids are thus used to clone a gene.

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

Procaryotic cells - Single-celled organisms and reproducing entities that lack a membrane-bound nucleus; they do not undergo meiosis; these include the viruses, bacteria, and blue-green algae.

Probe - A radioactive DNA or RNA molecule used to detect the presence of its complementary strand on an electrophoretic "gel" by hybridization and autoradiography.

Proembyro - Used here to mean the embryo in very early precotyledonary stages of development.

Prolamellar body - Semicrystalline structure from which thylakoid membranes arise during chloroplast development in dark grown seedlings.

Promotor - A short nucleotide sequence on DNA recognized by RNA polymerase to initiate transcription (synthesis of mRNA).

Proplastids - A group of plastids which are progenitors of chloroplasts.

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

Protoplast fusion - Union of two protoplasts into one cell.

Recombinant DNA (rDNA) - Chimeric DNA molecule formed by cutting and splicing of DNA (genes).

Restriction endonucleases - (Restriction enzymes) enzymes that cut DNA at specific nucleotide sequences yielding fragments of various sizes. These enzymes are isolated from a variety of bacteria, and are identified by a three letter abbreviation consisting of the first letter of the genus and the first two letters of the bacterial species name, followed by the strain number (e.g., a particular enzyme isolated from an E. coli strain is designated Eco R1).

Reverse transcriptase - An enzyme purified from tumor viruses that synthesizes DNA complementary to an RNA template.

Ribosomes - Organelles containing protein and RNA. They are seen as dense particles in electron micrographs. They are found in all types of cells in which protein is being synthesized.

RNA - Ribonucleic acid. RNA is usually single stranded.

RNA polymerase - The enzyme responsible for making RNA complementary to a DNA template. RNA polymerase binds at specific nucleotide sequences (promotors) in front of genes in DNA. It then moves through a gene and makes an RNA molecule that contains the information contained in the gene.

SEM - Scanning electron microscope.

Sequence - The order of the nucleotides in the DNA or RNA chain.

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

Somatic cell hybrid - The plant resulting from fusion of protoplasts from somatic cells of genetically different sources.

Splicing - Removal of introns from the "immature" form of eukaryotic mRNA. Carried out in the nucleus of the cell.

Subculture - Dividing agar grown callus or liquid cell suspensions for transfer to fresh medium.

Suspension culture - Cells or cell aggregates dispersed and growing in moving liquid medium.

Suspensor - Elongated, vacuolated cells subtending the embryonal cells in a developing zygotic embryo.

Tannins - A class of complex phenolic compounds known for their astringency and ability to tan the proteins of animal skins. There are two major types of tannins, the hydrolyzable and the condensed tannins.

TEM - Transmission electron microscope.

Template - A pattern of nucleotide sequences in DNA or RNA used by polymerases to specify the sequence in a new polymer by complementarity.

Tetracycline - An antibiotic that kills bacteria by blocking protein synthesis.

Thylakoids - Complex system of flattened membranes within a chloroplast; are often found in stacks to form grana.

Ti plasmid - The plasmid carried by the bacterium Agrobacter tumefaciens which is used to carry foreign genes into a plant cell.

Tissue culture - General term for callus and cell cultures.

Totipotency - A cell characteristic in which the cell retains the potential of forming all the cell types of the adult organism.

Transcription - The process of converting information in DNA into information in RNA. The copying of a gene into RNA. RNA polymerase is the enzyme that executes this conversion of information.

Transformation - The process whereby a cell takes up free DNA such that the free DNA (gene) becomes a permanent part of the cell's genome.

Translation - The process of converting the information in mRNA into protein. Also called protein synthesis.

Transposon - A short section of DNA capable of "jumping" to another region of a chromosome or to a different chromosome.

Transposon tagging - Method of using a transposon to locate a gene. When a transposon inserts into a chromosome, it causes a knockout mutation leading to a distinct mutant phenotype. A radioactive probe made from this transposon can then be used to identify the DNA sequence (gene) into which it has been inserted. The gene can then be localized on a gel and perhaps on a particular chromosome from the mutant plant. In short, the mutated gene is tagged or made identifiable by the transposon.

Ultrastructural - Sublight microscopic, intracellular structure.

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

Vegetative cells - 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.

STATISTICS

STATISTICS

Where statistics beyond means and standard deviations (S.D.) were used in the evaluation of results to be presented, the data were subjected to analysis of variance (ANOVA) followed by Duncan's New Multiple Range Test for multiple comparison of means. Values with a common superscript letter are not significantly different from each other ($P < 0.05$). The number of replications is indicated by N.

AMINO ACIDS

ABBREVIATIONS

AMINO ACIDS ABBREVIATIONS

ala	alanine
arg	arginine
asn	asparagine
asp	aspartic acid
cit	citrulline
cys	cysteine
γ-aba	aminobutyric acid
gln	glutamine
glu	glutamic acid
gly	glycine
his	histidine
hyp	hydroxyproline
ile	isoleucine
leu	leucine
lys	lysine
met	methionine
orn	ornithine
phe	phenylalanine
pro	proline
ser	serine
thr	threonine
trp	tryptophan
tyr	tyrosine
val	valine

CUMULATIVE LIST OF

ABBREVIATIONS

CUMULATIVE LIST OF ABBREVIATIONS

AA	Ascorbic acid
2,4-D	2,4-dichlorophenoxyacetic acid
ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
ADC	Arginine decarboxylase
ADP	5'-Adenosine diphosphate
AMP	5'-Adenosine monophosphate
ANOVA	Analysis of variance
AOA	Aminooxyacetic acid
AOAA	Aminooxyacetic acid
AOPP	α-Aminooxy-β-phenylpropionic acid
ATP	Adenosine triphosphate
AVG	Aminoethoxyvinylglycine
BA	Benzylaminopurine = benzyl adenine
BAP	Benzylaminopurine = benzyl adenine
BLG	Brown and Lawrence medium plus glutamine
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
C	Cotyledon
cAMP	3', 5'-Cyclic adenosine monophosphate
CIM	Callus inducing medium by Coleman and Ernst 1990
C/N	Carbon/nitrogen
D	Dark
DCHA	Dicyclohexylammonium sulfate
DCR	Durzan sugar pine medium by Gupta and Durzan 1985
DF	Douglas-fir
DFMA	α-difluoromethylarginine
DFMO	α-difluoromethylornithine
DHA	Dehydroascorbic acid
DKW	Driver and Kuniyuki medium 1984
DKW-C	McGranahan, Driver, and Tulecke medium modification 1987
dSAM	Decarboxylated SAM
DW	Dry weight
E	Embryogenic
EC or ec	Embryogenic callus
EDTA	Ethylenediaminotetraacetic acid
E_i	Embryonal initial
FAA	Free amino acid(s)
FTIR	Fourier transform infrared
FW or fr.wt.	Fresh weight
G-1-P	Glucose-1-phosphate
GA	Gibberellic acid (gibberellin)
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
GSH	Gluthathione (reduced)
GSSG	Gluthathione (oxidized)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HFBI	Heptafluorobutyrylimidazole
HFSE	High frequency somatic embryogenesis

HM	Hakman medium
HPLC	High performance liquid chromatography
IAA	Indoleacetic acid
IBA	Indolebutyric acid
IEF	Isoelectric focusing
IPA	Isopentenylaminopurine = 2iP
L	Light or liter
LA	Larch
LB	Luria - Bertani bacterial culture medium
LFSE	Low frequency somatic embryogenesis
LM	Litvay medium
LP	Loblolly pine
lx	Lux
MEOI	Methyleneoxindole
MES	Morpholinoethane sulfonic acid
MGBG	Methylglyoxal bis-guanyl hydrazone
MOI	Methyloxindole
MOPS	Morpholinopropane sulfonic acid
MS	Murashige and Skoog medium 1962
MSG	MS medium modification by Becwar 1988
MSCG	MS medium modification by Nagmani 1991
NAA	Naphthalene acetic acid
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NBT	Nitrobluetetrazolium
NE	Nonembryogenic
NOAA	Naphthoxyacetic acid
NS	Norway spruce
OBHA	o-benzylhydroxylamine
ODC	Ornithine decarboxylase
P	Putrescine or phosphate
PAL	Phenylalanine ammonia lyase
pcv	Packed cell volume
PEG	Polyethylene glycol
PEM or pem	Preembryonal mass
PO	Pond pine
PP	Pitch pine
PPi	Pyrophosphate
ProA	Proanthocyanidin
RP	Red pine or research plan
S	Suspensor
SAM	S-adenosylmethionine
Sd	Spermidine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE or se	Somatic embryo
S_i	Suspensor initial
SIM (GC/MS)	Selective ion monitoring
SIM	Shoot induction medium by Coleman and Ernst 1990
Sp	Spermine

TLC	Thin-layer chromatography
TrpAM	Tryptamine
2iP	Isopentenylaminopurine
UDP	Uridine diphosphate
UDPG	Uridine diphosphate glucose
UTP	Uridine triphosphate
WC	Wild carrot
WCM	Wild carrot medium
WH	White's medium
WNA	WPM medium modification by Coleman and Ernst 1989
WP	White pine
WPM	Woody plant medium by Lloyd and McCown 1980
WS	White spruce

APPENDIX

APPENDIX

BASAL MEDIA FORMULATIONS - COMPARISON

Components, mg/l	MS	MSG[a]	BLG	DCG	HM	WNA	DKWC
NH_4NO_3	1650	----	----	400	1200	1650	1416
K_2SO_4	----	----	----	---	----	990	1559
KNO_3	1900	100	100	340	1900	----	----
$MgSO_4$ $7H_2O$	370	370	320	370	370	370	740
KH_2PO_4	170	170	170	170	340	170	265
$CaCl_2$ $2H_2O$	440	440	440	85	180	96	149
$Ca(NO_3)_2$ $4H_2O$	----	----	----	556	----	2000	1968
KCl	----	745	745	----	----	----	----
KI	0.83	0.83	0.83	0.83	0.75	----	----
H_3BO_3	6.2	6.2	6.2	6.2	0.63	6.2	4.8
$MnSO_4$ H_2O	16.9	16.9	16.9	22.3	2.2	22.3	33.5
$Zn(NO_3)_2$ $6H_2O$	----	----	----	----	----	----	17.0
$ZnSO_4$ $7H_2O$	8.6	8.6	8.6	8.6	2.87	8.6	----
Na_2MoO_4 $2H_2O$	0.25	0.25	0.25	0.25	0.025	0.25	0.39
$CuSO_4$ $5H_2O$	0.025	0.025	0.025	0.25	0.0025	0.25	0.25
$CoCl_2$ $6H_2O$	0.025	0.025	0.025	0.025	0.0025	----	----
$NiCl_2$ $6H_2O$	----	----	----	0.025	----	----	----
$NiSO_4$ $6H_2O$	----	----	----	----	----	----	0.005
$FeSO_4$ $7H_2O$	27.8	27.8	27.8	27.8	13.9	27.8	33.8
Na_2EDTA $2H_2O$	37.3	37.3	37.3	37.3	18.65	37.3	45.4
myo-Inositol	100	100	100	200	100	100	100
Glycine	----	----	----	2	----	2	2
Nicotinic acid	0.5	0.5	0.5	0.5	2	0.5	1
Pyridoxine	0.1	0.1	0.1	0.5	1	0.5	----
Thiamine HCl	0.1	0.1	0.1	1.0	5	1.0	2
Sucrose	30000	30000	20000	30000	34200	20000	30000
Glucose	----	----	----	----	180	----	----
Xylose	----	----	----	----	150	----	----
Arabinose	----	----	----	----	150	----	----
Casein hydrolysate	----	---a	----	500	----	500	----

APPENDIX (Continued)

BASAL MEDIA FORMULATIONS - COMPARISON

Components, mg/l	MS	MSG[a]	BLG	DCG	HM	WNA	DKWC
Glutamine	----	1450[a]	1500	250	----	----	----
Asparagine	----	----	100	----	----	----	----
Agar	0.8%	0.8%	0.7%	0.8%	0.5%	----	----
Gelrite	----	----	----	----	----	0.25%	0.25%
pH	5.8	5.8	5.8	5.8	5.5	5.8	5.7

[a]MSCG = MSG + 1 g/L casein hydrolysate + 500 mg/L GLN.

LB Medium

ingredient	g/l
bacto-tryptone	10
bacto-yeast extract	5
NaCl	10
Difco-bacto agar	20

pH = 7.0