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AN ABSTRACT OF THE THESIS OF

Wolfgang D. Binder (Name) for the Ph.D. (Degree) in Forest Science (Major)

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Title: SURVIVAL AND SOME PHYSIOLOGICAL ASPECTS OF TISSUE CULTURED CELLS FROM DOUGLAS-FIR (PSEUDOTSUGA MENZIESII MIRB. (FRANCO)) AND A POPLAR HYBRID AFTER FREEZING TO LIQUID NITROGEN TEMPERATURE.

Abstract approved: ______________________
Dr. Joe B. Zaerr

Cell aggregate size in both Douglas-fir and poplar suspension cultures was reduced by the addition of the chelator compounds EDTA and CDTA at concentrations under 100 ppm. Reduced cell aggregate size increased growth efficiency of suspension cultures of both species. Cell aggregates 550 μ or smaller in size were used in the cryogenic studies.

At room temperature, the cryoprotectants dimethyl sulfoxide (DMSO) and glycerol at concentrations above 5% were toxic to cell survival; however when these were washed out with fresh media, the growth rate of both Douglas-fir and poplar suspension cultures was increased. The optimum cryoprotectant concentration was about 1.0%. At concentrations above 5% repeated washing with fresh media did not

reduce the cellular cryoprotectant concentration and cellular injury or death may have resulted solely from the presence of cryoprotectants, even in the absence of freezing. Cryoprotectant influences observed were thought to be due to cell membrane changes allowing increased nutrient flow in the case of cell death. Cold conditioning or chilling callus at +4°C allowed higher concentrations of cryoprotectants to be added without killing cells.

Suspension cultures showed that Douglas-fir entered the log phase of growth 7 days and poplar 3 days after innoculation. Cells were small and densely cytoplasmic during this time. For both species slow freezing of log-growth-phase cells at 1°C/min was better than fast freezing; a two-step freezing protocol, cooling first to -40°C and then immersion into liquid nitrogen was successful; cooling to -40°C was more effective than cooling to higher temperatures; and fast thawing to +40°C was more effective than slow thawing in air.

The cryoprotectants DMSO, glycerol and sucrose are effective alone and in combination. Most effective was a combination of all three; DMSO 5%/glycerol 1%/sucrose 4% for Douglas-fir and DMSO 5%/ glycerol 5%/sucrose 4% for poplar.

Douglas-fir and poplar cultures on thawing were tested for viability by assessing ability to reduce tetrazolium salts and ability to continue growth. Poplar cultures resumed growth after approximately 5 weeks post-thaw culture, whereas for Douglas-fir over 80 days was required. Thawed and recultured poplar callus differentiated into shoots and roots.

Cold conditioned callus of both Douglas-fir and poplar produced high TTC reduction values directly after thawing. Cold conditioned poplar callus frozen without cryoprotectants or liquid responded better on thawing than when frozen with cryoprotectants in liquid media.

Douglas-fir buds obtained from field trees in August or seedlings cold conditioned at +4°C for 8 weeks were frozen under various conditions, but no buds survived the freezing process.

The development of techniques for successful freeze preservation requires a detailed empirical examination of; prefreezing culture conditions, physiological state and size of cells, cryoprotectant type and concentration used, freezing rate and temperature frozen to, and thaw rate and post-thaw culture conditions.

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TABLE OF CONTENTS

I. INTRODUCTION 1

II. CHAPTER 1 - THE EFFECTS OF TWO CHELATING COMPOUNDS ON THE GROWTH OF DOUGLAS-FIR AND POPLAR SUSPENSION CULTURES

Introduction 5
Methods and Materials
1. Callus Initiation 7
2. Preparation of Suspension Cultures 9
3. Determination of Cell Dry Weight 12
4. Counting of Cells in Suspension Culture 12
Results 13
Discussion 25
Conclusions 32

III. CHAPTER 2 - CONTROLLED FREEZING OF SUSPENSION CULTURED CELLS OF DOUGLAS-FIR AND A POPLAR HYBRID: STORAGE AT AND RECOVERY FROM -196°C

Introduction 33
Methods and Materials
1. Growth of Douglas-fir and Poplar Callus 36
2. Preparation of Suspension Cultures of Douglas-fir and poplar 38
3. Determination of Cryoprotectant Toxicity 38
4. Production of Growth Curves 40
5. Methods for Freezing to -196°C and Recovery. . . 42
a. Preparation of Cultures for Freezing 43
b. Freezing Equipment 44
c. Freezing, Storage and Thawing 46
6. Estimation of Cell Survival 47
a. Triphenyl Tetrazolium Chloride (TTC) Test. . 47
b. Post-Thaw Culture and TTC Reduction 55
c. Determination of the Effects of Washing Cryoprotectants from Cell Aggregates on Cell Survival of Douglas-fir 55
d. Regeneration of Shoots with Thawed Poplar Material 56
7. Cold Conditioning of Douglas-fir Poplar Callus and Seedlings of Douglas-fir 56
8. Freezing to -196°C of Cold Conditioned Douglas-fir and Poplar Callus 57
9. Freezing Douglas-fir Shoot Apices to -196°C . . 58

Results
1. Growth Curves 59
2. Toxicity Studies 60
3. Cryoprotectant Washing Experiments 68
4. Freezing of Suspension Cultures of Douglas-fir and Poplar 80
a. Freezing in the Presence of Single Cryoprotectants 80
b. Freezing in the Presence of Cryoprotectants in Combination 98
5. Environmental Effects on the Survival of Douglas-fir Callus 150
6. Freezing Douglas-fir Buds to -196°C 157

Discussion
1. Introduction 162
2. Suspension Cultures and Growth Curves 164
3. Pre-Freezing Conditions of Cultures. 166
4. Colligative and Non-Colligative Properties of Cryoprotectants 168
5. Freezing Injury and Cryoprotective Action. 172
6. Physiological Effects of Cryoprotectants 179
a. Toxicity and Sub-cellular Effects 179
b. Beneficial Growth Effects of Cryoprotectants 187
c. Cryoprotectant Washing Experiments 194
d. Cryoprotectant Addition - The Effect of Temperature 196
7. Post-Thaw Culture Physiology: Cell Dry Weight Increase and TTC Testing 199
8. Cold Hardiness and Low-Temperature Survival. . . 207
9. Freezing Douglas-fir Buds to -196°C 219
10. Summary of Discussion. 226

IV. SUMMARY 231

V. BIBLIOGRAPHY 233

VI. APPENDICES 269

A. REVIEW OF LITERATURE 270
1. Survival of Plant Cell and Tissue Culture at -25°C or Lower. 271
a. Suspension Cultures 271
b. Callus Cultures 274
c. Shoot Tips 275
d. General Required Conditions for Freezing. . 275
e. Summary 276

2. Choice of Plant Tissue Material for Freezing and Regeneration of Plants 290
3. Low Temperature Injury to Plant Cells 292
a. Chilling Injury and Thermal Shock 292
b. Freezing Injury 293
c. Cryoprotectants 296
4. Estimation of Survival after Freezing and Thawing 300
5. Rationale for Study 307

B. THE REGENERATION AND DEVELOPMENTAL ANATOMY OF A POPLAR HYBRID FROM CELL SUSPENSION CULTURED CELLS IN LIQUID CULTURE 309

Introduction 309
Methods and Materials 313
Results 318
Discussion 331

B_1 Basal Medium after Murashige and Skoog 335

B_2 Basal Medium after Schenk and Hildebrandt 336

B_3 Poplar Media 337

C. ROTATION APPARATUS FOR SUSPENSION CULTURES 338

D. PROCEDURE FOR OBTAINING FREE CELLS 339

E. MATERIALS AND METHODS TO STAIN DNA IN DIVIDING CELLS 340

F. METHOD OF BACTERIA IDENTIFICATION 341

LIST OF FIGURES

Figure		Page No.
1.	Photograph showing: (1) Growth room, rotation apparatus with "T" tubes and nipple flasks; (2) Suspension cultures of Douglas-fir growing in nipple flask; (3) Douglas-fir EDTA test results in "T" tubes; (4) Poplar suspension culture; (5) 50 ppm EDTA treated poplar culture; (6) 50 ppm EDTA treated Douglas-fir culture.	10
2.	Photograph showing: Results of EDTA concentration on Douglas-fir after aspiration filtration.	15
3.	Photograph showing: Results of CDTA concentration on aspiration filtration.	15
4.	Effect of various concentration ppm EDTA on Douglas-fir culture growth after 70 days.	16
5.	Effect of various concentration ppm CDTA on Douglas-fir culture growth after 70 days.	17
6.	Effects of various concentration ppm EDTA on growth of soft, small callus clumps of poplar.	18
7.	Growth effects of various concentration ppm EDTA on hard clumps of poplar.	22
8.	Photograph showing: Effect of EDTA concentration on poplar. Different cell clump sizes are shown.	23
9.	Photograph showing: Effect of CDTA concentration on poplar after aspiration filtration.	23
10.	Growth effects of various concentration ppm CDTA on large clumps of poplar (dry weight increases).	24
11.	Photograph showing: The beginning and the end of a cryogenic storage program: a stand of Douglas-fir trees.	37
12.	Photograph showing: Douglas-fir trees reduced to their smallest whole elements - cells.	37
13.	Photograph showing: Douglas-fir callus and poplar callus growing in tubes.	39

(Continued)

LIST OF FIGURES (Continued)

Figure — Page No.

14. Photograph showing: Rotating apparatus for and containing nipple flasks and "T" tubes. 39

15. Photograph showing: Rotating apparatus for and containing 13 x 100 mm culture tubes for treatment testing of cell cultures. 42

16. Photograph showing: Douglas-fir cells after treatment with nitric/chromic acid. 42

17. Photograph showing: Cryotube ampules containing material to be frozen.. 45

18. Photograph showing: Freezing apparatus - shown are the low pressure liquid nitrogen tank, freezing chamber, control unit, and chart recorder.. 45

19. Photograph showing: Triphenyl tetrazolium chloride reduction by Douglas-fir and poplar cells. 49

20. Photograph showing: Spectrophotometer scan of formazan production color. Peak absorbance is shown at 485 nm.: 49

21. Douglas-fir TTC reduction study. 51

22. Poplar TTC reduction study. 52

23. TTC reduction procedure flow sheet. 53

24. Douglas-fir suspension culture growth curve (total dry weight basis and total cell number basis). 61

25. Poplar suspension culture growth curve (total dry weight basis and total cell number basis).. 62

26. Effects of DMSO on growth rate of old, stationary growth phase suspension cultures of Douglas-fir 64

27. Effects of glycerol on growth rate of old, stationary growth phase suspension cultures of Douglas-fir. . . . 65

28. Growth rate effects of DMSO and glycerol using Douglas-fir cells.. 66

(Continued)

LIST OF FIGURES (Continued)

Figure | Page No.

29. Photograph showing: Amont of Douglas-fir culture material produced with different percent concentrations of DMSO. 67

30. Photograph showing: Amount of Douglas-fir culture material produced with different percent concentrations of glycerol. 67

31. Effects of Hydroxy-free-L-Proline on growth of log phase growth suspension cultures of Douglas-fir. . . . 69

32. Effects of DMSO on growth of log phase suspension cultures of poplar. 70

33. Photograph showing: Effects of various percent concentrations of DMSO on the growth of poplar liquid cell cultures. 71

34. Effects of glycerol on growth of log phase suspension cultures of poplar. 72

35. Effects of cryoprotectant exposure and subsequent washing on Douglas-fir test cultures. 74

36. Photograph showing: Effects of cryoprotectant exposure and subsequent washing on TTC reduction of Douglas-fir test material. 75

37. Effects of exposure and washing of cryoprotectants on post treatment culture of Douglas-fir cultures (45 days). 76

38. Effects of exposure and washing of cryoprotectants on the post treatment culture of Douglas-fir. Total dry weight taken after 84 days. 77

39. Photograph showing: Color of Douglas-fir cell cultures after exposure to cryoprotectants, washing, and post treatment culture. 79

40. Photograph showing: Douglas-fir cell cultures of Figure 39 on filter paper. 79

41. Photograph showing: Effects of cryoprotectants in combination exposed to and washed from poplar callus on agar medium. 75

(Continued)

LIST OF FIGURES (Continued)

Figure | Page No.

42. TTC reduction of Douglas-fir suspension cultures immersed directly in liquid nitrogen. DMSO and glycerol used singly. Thawing in +40°C water bath to 0°C then room temperature in air. 81

43. Photograph showing: TTC reduction of Douglas-fir using single cryoprotectants and direct immersion into liquid nitrogen. Thawing was fast in a +40°C water bath. 82

44. Photograph showing: Cell damage in Douglas-fir cells after using DMSO, direct liquid nitrogen exposure and fast thawing. 82

45. TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min. from +20°C to -15°C then immersed in liquid nitrogen. Single cryoprotectants were used at 0.2% and 2%. 85

46. TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min. from +2-°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants were used at 0.2% and 2% sucrose 2.5M. 87

47. TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants were used at 0.2% and 2%, sucrose 2.5M. The TTC test made 97 days after thawing. 88

48. Photograph showing: TTC reduction color 97 days after being exposed to conditions given in Figure 70. 89

49. Photograph showing: Damage done to Douglas-fir cells by freezing using DMSO and placing in liquid nitrogen. 89

50. Total dry weight of Douglas-fir suspension cultures cooled at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants used at 0.2%, 2%, and sucrose 2.5M. Dry weight taken 97 days post-thaw culture. 91

(Continued)

LIST OF FIGURES (Continued)

Figure | Page No.

51. TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants were used at 5% and 10%. 92

52. Total dry weight of Douglas-fir suspension cultures cooled at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants were used at 5% and 10%. Dry weight was taken 40 days post-thaw culture. 94

53. Total dry weight of Douglas-fir suspension culture cooled at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants were used at 5%, 10% and 2.5M. Dry weight was taken 108 days post-thaw culture.. 95

54. Photograph showing: TTC reduction of Douglas-fir cultures after treatment given in Figure 77. The TTC test was made 118 days after thawing.. 96

55. Photograph showing: Douglas-fir cultures frozen at 1°C/min. to -40°C then immersed directly in liquid nitrogen. Cryoprotectant cultures were used at 5% and 10% and sucrose at 2.5M. Photo was taken 108 days after thawing. 96

56. TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants were used at 5% and 10% and sucrose at 2.5M. Test was made 118 days post-thaw culture. 97

57. Photograph showing: Three views of Douglas-fir suspension culture cooled at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen. DMSO 5% was used as cryoprotectant. Photo taken 145 days after thawing. 99

58. Photograph showing: Three views of Douglas-fir suspension culture shown in Figure 81 and magnified x280 and x400. 100

(Continued)

LIST OF FIGURES (Continued)

Figure		Page No.
59.	TTC reduction of Douglas-fir suspension cultures cooled at $1^{o}C/min.$ from $+20^{o}C$ to $-40^{o}C$ and immersed in liquid nitrogen. Cryoprotectants were used in combination. DMSO $\leq 5\% \geq 2.5\%$ and glycerol $\leq 5\% \geq 2.5\%$ and sucrose at 2.5M and water without 4% sucrose.	101
60.	Photograph showing: TTC reduction of post-thawed Douglas-fir cultures using cryoprotectants in combination.	104
61.	TTC reduction of Douglas-fir suspension cultures cooled at $1^{o}C/min.$ from $+20^{o}C$ to $-40^{o}C$ then immersed in liquid nitrogen. Cryoprotectants were used in combination DMSO $\leq 5\% \geq 0.5\%$ and glycerol $\leq 2.5\% \geq 0.5\%$ and water with 4% sucrose.	103
62.	Effects of cryoprotectants in combination on TTC reduction of Douglas-fir suspension cultures cooled at $1^{o}C/min.$ from $+20^{o}C$ to $-40^{o}C$ then immersed in liquid nitrogen. Cryoprotectants were used in combination DMSO $\leq 5\% \geq 0.5\%$ and glycerol $\leq 2.5\% \geq 0.5\%$ and in water only. The TTC test made 53 days after thawing.	105
63.	Total dry weight of Douglas-fir cultures cooled at $1^{o}C/min.$ from $+20^{o}C$ to $-40^{o}C$ then immersed in liquid nitrogen. Cryoprotectants were used in combination. Dry weight taken 73 days post-thaw culture.	106
64.	Photograph showing: TTC reduction of post-thawed Douglas-fir cultures using cryoprotectants in combination after 83 days post-thaw culture.	104
65.	Effects of cryoprotectants in combination on TTC reduction of Douglas-fir suspension cultures at $1^{o}C/min.$ cooled from $+20^{o}C$ to $-40^{o}C$ and immersed in liquid nitrogen. Cryoprotectants were used in combination or as 2.5M sucrose. The TTC test was made 83 days after post-thaw culture.	107

(Continued)

LIST OF FIGURES (Continued)

Figure		Page No.
66.	Total dry weight of Douglas-fir cultures cooled from +20°C to -40°C and immersed in liquid nitrogen. Cryoprotectants were used in combination or as 2.5M sucrose. Dry weight was taken at 83 days post-thaw culture.	109
67.	Photograph showing: Cultures of Douglas-fir on solid growth media 60 days post-thaw culture. Freezing was at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen. Cryoprotectants were used in combination or frozen in dry condition.. . . .	104
68.	Effects of single cryoprotectants on TTC reduction of poplar suspension cultures cooled at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen. Cryoprotectants were used at 0.2% and 2%. Test cultures not washed in water after TTC incubation. . .	111
69.	Photograph showing: Poplar cultures frozen at 1°C/min. from +20°C to -40°C and immersed in liquid nitrogen. Cultures not washed with water after TTC incubation..	12
70.	Effects of cryoprotectants in combination on TTC reduction of poplar suspension cultures cooled at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen. Cryoprotectants were used in combination at 5% and 10% or as 2.5M sucrose. Test cultures were not washed in water after TTC incubation.	113
71.	Photograph showing: Poplar callus which was TTC tested, followed by centrifugation for 20 mintues. . .	112
72.	Photograph showing: Poplar medium TTC color reduction due to bacterial contamination in TTC buffer. . .	115
73.	Photograph showing: TTC color reaction in Douglas-fir and poplar culture medium. Due to bacterial contamination in TTC buffer.	115

(Continued)

LIST OF FIGURES (Continued)

Figure | Page No.

74. Effects of single cryoprotectants on TTC reduction of poplar suspension cultures cooled at $1^{\circ}C$/min. from $+20^{\circ}C$ to $-40^{\circ}C$ then immersed in liquid nitrogen. Cryoprotectants were used at 5% and 10%. Shown are cultures not washed and washed with water after TTC incubation. 119

75. Effects of single cryoprotectants on TTC reduction of poplar suspension cultures immersed directly into liquid nitrogen. Cryoprotectants were used at 5% and 10%. Data based on new TTC buffer and post TTC incubation washing. 120

76. Total dry weights taken of poplar suspension cultures immersed directly in liquid nitrogen. Cryoprotectants were used at 5% and 10%. Dry weights were measured 122 days after thawing. 121

77. Effects of cryoprotectants in combination on TTC reduction of poplar suspension cultures pre-grown 5 days and cooled at $1^{\circ}C$/min. from $+20^{\circ}C$ to $-40^{\circ}C$ then immersed in liquid nitrogen. Cryoprotectants were used in combination. DMSO $\leq 5\% \geq 1\%$ and glycerol $\leq 5\% \geq 0.5\%$. Effects of 4% sucrose and 1% proline were also studied. Data based on post TTC incubation washing. 125

78. Effects of cryoprotectants in combination on TTC reduction of popler cultures pre-grown 5 days and frozen with proline at 1%. DMSO $\leq 5\% \geq 1\%$ and glycerol $\leq 5\% \geq 0.5\%$. Data based on TTC test made on green and non-green callus 66 days after thawing. . 127

79. Photograph showing: TTC reduction of poplar callus cultures frozen to $-40^{\circ}C$ and immersed in liquid nitrogen. Cryoprotectants were used in combination. The TTC test was made 66 days after thawing. 134

80. Total dry weights of poplar suspension cultures which were pre-grown then frozen with 1% proline. Cooling was at $1^{\circ}C$/min. then immersed in liquid nitrogen. Cryoprotectants used were DMSO at $\leq 5\% \geq 1\%$ and glycerol at $\leq 5\% \geq 0.5\%$. Dry weights were taken 66 days after thawing. 128

(Continued)

LIST OF FIGURES (Continued)

Figure | Page No.

81. Poplar callus TTC test after 8 weeks cold room conditioning or growth room callus immersed directly into liquid nitrogen in dry condition. Data was based on post TTC incubation washing with water. 130

82. Photograph showing: TTC test on poplar callus conditioned in cold room for 8 weeks and cooled at $1^{o}C$/min. from $+40^{o}C$ to $-40^{o}C$ and immersed in liquid nitrogen. Effects of freezing with DMSO 10%/glycerol 5%/sucrose 25% and freezing in dry condition. 131

83. Poplar callus TTC test after 8 weeks cold room conditioning or growth room callus cooled slowly at $1^{o}C$/min. to $-40^{o}C$ then immersed in liquid nitrogen either dry or in DMSO 10%/glycerol 5%/ sucrose 25% 132

84. Photograph showing: TTC reduction from various cold treatments. Poplar callus cooled at $1^{o}C$/ min. from $+20^{o}C$ to $-40^{o}C$ then immersed in liquid nitrogen 134

85. Photograph showing: Poplar callus cultures 50 days after being cooled slowly at $1^{o}C$/min. from $+20^{o}C$ to $-40^{o}C$ then immersed in liquid nitrogen. DMSO and glycerol were used at different concentrations 135

86. Photograph showing: Poplar callus cultures 50 days after being cooled slowly at $1^{o}C$/min. to $-40^{o}C$ then immersed in liquid nitrogen and thawed. DMSO and glycerol were used at different concentrations. . . 135

87. Photograph showing: Color differences between callus frozen in liquid nitrogen with 5% DMSO and callus grown at $+22^{o}C$. 135

88. Photograph showing: Poplar callus or cold conditioned callus growing shoots 55 days after being frozen to $-196^{o}C$ in dry condition with 5% DMSO. 139

89. Photograph showing: Paraffin section of poplar callus placed on callus medium and grown 55 days. . . . 139

(Continued)

LIST OF FIGURES (Continued)

Figure | Page No.

90. Photograph showing: Paraffin section of poplar callus showing meristemoids forming after being placed on solid agar shoot-forming media. 141

91. Photograph showing: Paraffin section of poplar callus formed from cell aggregates after being cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen in the presence of 5% DMSO 141

92. Photograph showing: Paraffin section of poplar callus cold conditioned before cooling at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Meristemoids are shown forming. 141

93. Photograph showing: Paraffin section of vascular development in poplar cultures shown in Figures 90 and 92 142

94. Photograph showing: Paraffin section of: (A) Root tip formed from poplar callus cooled at 1°C/min from +40°C to -40°C then immersed in liquid nitrogen in presence of 5% DMSO; (B) Shoot forming from culture shown in Figure 90; and (C) Shoot forming from cell aggregates shown in Figure 91. 143

95. Photograph showing: Paraffin sections of shoots forming from callus shown in Figure 92 144

96. Douglas-fir TTC test after 8 weeks cold room conditioning or growth room callus immersed directly into liquid nitrogen in dry condition 147

97. Douglas-fir callus TTC test after 8 weeks cold room conditioning, growth room callus and growth room callus held at +4°C for 24 hr before cooling in a 1°C/min from +20°C to -40°C then immersion in liquid nitrogen 149

98. Photograph showing: Douglas-fir and poplar callus growing after cold room conditioning for 8 weeks . . . 152

99. Photograph showing: TTC reduction after various treatments of Douglas-fir cultures for 24 hr. 152

(Continued)

LIST OF FIGURES (Continued)

Figure		Page No.
100.	Photograph showing: Douglas-fir cultures cold conditioned 8 weeks and treated in various ways before being frozen, thawed and placed on solid agar medium.	152
101.	Photograph showing: Douglas-fir callus from the warm growth room treated in various ways before being frozen, thawed and placed on solid agar medium.	152
102.	Photograph showing: TTC reduction of Douglas-fir callus from growth chamber or cold room immersed in liquid nitrogen and either thawed fast or slowly. . . .	152
103.	Response of Douglas-fir suspension cultures from growth room to various environmental treatments for 24 hrs.	155
104.	Photograph showing: Douglas-fir cell cultures from different sources, treated in various ways; frozen, thawed and placed on solid agar media for 70 days. . .	156
105.	Photograph showing: Enlargement of Figure 128-E . . .	156
106.	Photograph showing: Enlargement of Figure 128-D . . .	156
107.	Photograph showing: Douglas-fir buds obtained from young trees, either cold conditioned or from field-grown trees grown on agar medium for 6 weeks.	160
108.	Photograph showing: Douglas-fir buds cold conditioned for 8 weeks and frozen slowly (1°C/min to -40°C) then placed in liquid nitrogen for 8 weeks. . . .	160
109.	Photograph showing: Douglas-fir buds cold conditioned and immersed in liquid nitrogen.	160
110.	Photograph showing: Douglas-fir buds obtained from field trees in August and with bud scales removed were cooled at 1°C/min. to -40°C then immersed in liquid nitrogen.	161
111.	Photograph showing: Douglas-fir buds from cold conditioned trees or from field-grown trees exposed to liquid nitrogen thawed and placed in liquid growth medium.	161

(Continued)

LIST OF FIGURES (Continued)

APPENDIX B

Figure		Page No.
1.	Small clumps of poplar cells.	319
2.	Two poplar cells. One showing starch grains.	319
3.	Clumps of poplar cells with shoots.	320
4.	Longitudinal section of whole shoot growing from cell clump.	320
5.	Sectioned poplar cell clump showing internal meristemoids forming.	321
6.	Close-up view of early vascular formation in poplar callus.	321
7.	A differentiating meristemoid in poplar callus (x80).	322
8.	A meristemoid showing elongating side structure. . . .	322
9.	Meristematic cells of poplar at surface of callus becoming organized.	323
10.	Poplar shoot apex showing primary leaves.	323
11.	Poplar shoot apex showing apical initials, early tunica corpus growth zones and primary leaves.	325
12.	Primary leaves of poplar shoot showing vascular trace with apex in center.	325
13.	Apical meristem of poplar showing tunica corpus regions.	326
14.	Cross section of a young leaf of poplar showing vascular bundles.	326
15.	Section of leaf epidermis of a poplar leaf showing two stomates.	327
16.	Longitudinal section of a poplar leaf showing vascular bundle.	327

(Continued)

LIST OF FIGURES (Continued)

Figure		Page No.
17.	Young leaf of poplar showing branching vascular bundle.	328
18.	Poplar cell clumps with roots.	328
19.	Two lateral roots of poplar forming on side of vascular tissue within a root.	329
20.	Lateral root primordium of poplar showing vascular tissue, pericycle-like area, and endodermis-like area.	329
21.	Lateral section of a poplar root showing plerome, and calyptrogendermatogen.	330
22.	Longitudinal section of a vascular system of a poplar root showing metaxylem border pit pairs and cambium-like cells.	330

LIST OF TABLES

Table — Page No.

1. Poplar total cell number per culture using EDTA as chelator. 21

2. EDTA effect on poplar cell aggregate size and cell number, per aggregate. 21

3. Douglas-fir TTC reduction test showing effect of killing cells on amount of TTC reduction. 54

4. Effects of cryoprotectant exposure and washing on the growth of Douglas-fir suspension cultures. . . . 78

5. Douglas-fir single cryoprotectant exposure and direct liquid nitrogen dry weight after 41 days in two types of growth containers. 83

6. Douglas-fir dry weights on single cryoprotectant exposure cooling 59 -15°C at 1°C/min. and liquid nitrogen immersion experiment (DMSO and glycerol 0.2% and 2%). 84

7. The pH of post thaw Douglas-fir culture media tested 73 days after thawing. 108

8. Poplar suspension cultures frozen directly in liquid nitrogen in the presence of single cryoprotectants and total dry weights taken 21 days after thawing. . 122

9. Poplar cell culture dry weights taken after being cooled slowly at 1°C/min. from +20°C to -40°C then immersed in liquid nitrogen in presence of single cryoprotectants at 0.2% and 2%. Dry weights were taken 68 days after thawing. 122

10. Poplar suspension or callus culture dry weights taken after being cooled slowly at 1°C/min. from +20°C to -40°C and immersed in liquid nitrogen in presence of single cryoprotectants at 5% and 10%. Dry weights were measured 34 days after thawing. . . 124

11. Poplar callus TTC reduction tests made after cooling at 1°C/min. to -40°C and immersed in liquid nitrogen. The TTC test was made 50 days after thawing. 136

(Continued)

LIST OF TABLES (Continued)

Table		Page No.
12.	Summary of poplar post-thaw agar culture data after being treated as indicated at greater than 50 days after thawing.	145
13.	Douglas-fir post-thaw agar callus culture data after being treated as indicated at greater than 60 days after thawing.	153
14.	Douglas-fir buds taken from field-grown trees or from trees cold conditioned for 8 weeks and frozen in the dry condition slowly or fast to -196°C. Thawing was fast in a +40°C water bath. Freezing was carried out with and without bud scales.	158

APPENDIX A

1.	Summary of optimal conditions for the survival of plant cell cultures subjected to sub-zero temperatures.	277
2.	Summary of optimal freezing conditions for the survival of some marine unicellular algae cultures subjected to sub-zero temperatures.	288
3.	Summary of optimal cryoprotectant conditions for the survival of some marine unicellular algae cultures subjected to sub-zero temperatures.	289

APPENDIX B

1.	Broad-leaf tree species regenerated from somatic callus culture.	310
2.	Embedding schedule of poplar cell clumps.	316
3.	Staining schedule for poplar.	317

SURVIVAL AND SOME PHYSIOLOGICAL ASPECTS OF TISSUE CULTURED CELLS FROM DOUGLAS-FIR (*PSEUDOTSUGA MENZIESII* MIRB. (FRANCO)) AND A POPLAR HYBRID AFTER FREEZING TO LIQUID NITROGEN TEMPERATURE

I. INTRODUCTION

Freezing is lethal to most living systems, yet it can also preserve cells and their constituents, and it may someday permit the long-term storage of whole plants and animals. Freezing can slow or stop some biochemical reactions, but it accelerates others (Takehara and Rowe, 1971; and Meryman, 1974). It is used both to preserve the fine structure of cells and to disrupt cells. Freezing is a challenge that is successfully met by some organisms in nature but not by others.

In the past, interest in the cryobiology of plants was focused on the preservation of fruits, vegetables and various plant products (Dorsey, 1934). However, after the publication of "Life and Death at Low Temperatures" by Luyet and Geherico in 1949, there was an awakening of interest in the freeze-storage of biological material at super-low temperatures followed by an accumulation of literature in the field of cryobiology (Meryman, 1966; and Mazur, 1970). This information includes reports showing the revival of larvae and caterpillars (Scholander, *et al*., 1953), whole insects (Asahina and Aoki, 1958), and parts of plants and cells (Sakai and Yoshida, 1967;

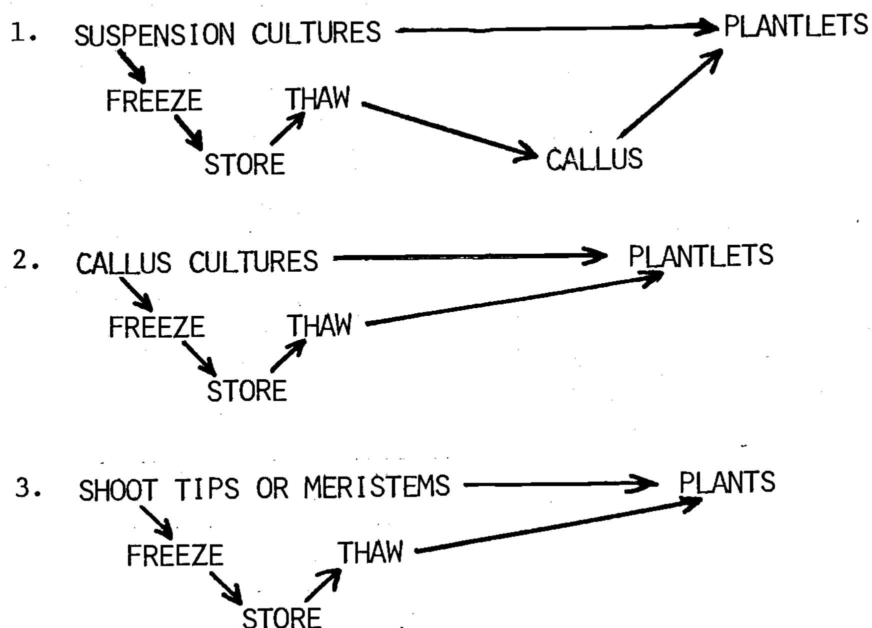
Sakai and Sugawara, 1973; Sugawara and Sakai, 1974; Street, 1975; Bajaj, 1976a, 1976b; and Bajaj and Reinert, 1977).

In recent years the investigation of biochemical processes and the formation of metabolites in solid and suspension cultures of plant cells has become increasingly popular (Grant and Fuller, 1968; Mehta, 1965; and Veliky, et al., 1969). Such cultures are usually maintained by methods involving repeated subculturing. However, repeated subculturing has some undesirable consequences; contamination, cell aggregation, loss of morphogenic potential, loss of flowering or fertility, mutation and selection, chromosomal aberrations, and changes in ploidy (Torrey, 1967; Myer-Teuter and Reinert, 1973; D'Amato, 1975; and Dix, 1979).

Liquid nitrogen freeze-storage procedures are becoming more often advocated in tissue culture and at present, it is probably the only feasible method for long-term storage of cultures in a completely non-dividing state.

The advantage of using freeze-preservation techniques for tissue cultured material storage is that once the material has been successfully frozen, little further attention is required except to ensure that the low temperature is maintained. Furthermore, evidence from work with animal cells suggests that there should be a high level of genetic stability, the limiting factor being the damage that occurs as a result of ionizing radiation. Such damage might only become significant after decades of storage (Whittingham, et al., 1977).

Three possible approaches to the storage of germ plasm of vegetatively propagated plants and plant cell lines are reported in the current literature of plant tissue culture.



Most of the reports of successful freeze-preservation of cultures have been concerned with callus or suspension cultures (Street, 1975; Bajaj and Reinert, 1977; and Withers, 1978). Also, there have now appeared a few reports of the successful freeze-storage of shoot meristems in carnation (Seibert and Wetherbee, 1977), tomato (Grout, et al., 1978), and potato (Grout and Henshaw, 1978; and Henshaw, 1979).

In addition, as a result of intensive genetic breeding there has been a displacement and loss of many local varieties of plants (Laselle, 1974). These plants being displaced are an essential source of genetic diversity and may be necessary for

future plant breeding programs (Wade, 1974). Specifically in forestry, preservation of genetic resources of trees can be achieved through seed collections and associated activities on a short-term basis usually. Storage of small amounts of tissue of present phenotypes would greatly reduce the amount of land required to maintain trees of different genetic type. When successful methods for the regeneration of coniferous trees from tissue culture become possible the freeze storage of tissue would provide a powerful tool for massive production of superior trees without genetic variation. In addition to cryogenic storage of superior tree material, other genotypes could also be maintained ensuring accessibility of a broad gene pool. Freezing of conifer culture tissue has immediate application in the study of cold acclimation and frost resistance mechanisms without interference of other tissues in the system.

The objective of this research is to study the physiology of, and to develop the methods needed to freeze to -196°C cultured cells of two diverse species of woody plants. These are Douglas-fir (*Pseudotsuga menziesii*), and a poplar hybrid (*Populus tristia* x *Populus balsmifera* cv. TRISTIS).

II. CHAPTER I

THE EFFECTS OF TWO CHELATING COMPOUNDS ON THE GROWTH OF DOUGLAS-FIR AND POPLAR SUSPENSION CULTURES

INTRODUCTION

The use of liquid suspension culture techniques for cryogenic studies have become increasingly important with the advance of plant cell biochemistry and plant tissue culture (Torrey, et al., 1961; Dougall and Fulton, 1967; Street, et al., 1970; Talmade, 1973; Siminovitch, et al., 1975; Withers, 1977; Spener, et al., 1978; Wang and Street, 1978; and Finkle and Ulrich, 1979). Unfortunately, the formation of cell aggregates in plant suspension cultures is one of the major technical problems still to be overcome (Jones, et al., 1973; El Hinnawy, 1973; Finkle,[1] 1979; Quantrano,[2] 1979; and Binder,[3] 1979). Some success has been obtained by selecting cell masses which are softer than other callus (Reinert, 1956), or by introducing cell wall digestion enzymes such as pectinase and cellulase

---

[1]Finkle, B.J., Research Scientist. U.S. Dept. of Agriculture. Berkeley, California. Personal communication, 1979.

[2]Quantrano, R.S., Professor of Botany. Oregon State University. Corvallis, Oregon. Personal communication, 1979.

[3]Binder, W.D., unpublished data, 1979.

to induce cell separation (Street, *et al.*, 1970; and Narayanaswamy, 1977) apparently without affecting the viability of the cells.

Few reports appear in the literature which deal specifically with chelators to reduce cell aggregate size and hence, enhance growth. Work with chelating agents ranges from maceration (Letham, 1960), growth regulation (Burström, 1963), separation of cells in root tips in plants (Ginzburg, 1958; and Cocking, 1960), to one case of dissociation of entire definitive primative-streak-stage chick embryos into suspension cells (Zwilling, 1954). Only one paper has appeared dealing with chelating agents and plant cell suspension cultures directly (El Hinnawy, 1973). That paper describes the growth effects of three chelating compounds on suspension cultures of *Melilotus alba* Desr. root tissue.

The present work reports the growth effects of two chelating compounds on two woody tree species, Douglas-fir and poplar. The purpose of the investigation is to produce a method which can reduce the cell aggregation size of the two species in suspension cultures so they may be used for cryogenic study.

METHODS AND MATERIALS

1. Callus Initiation:

Douglas-fir seeds were dipped twice in 70% ethanol, washed with sterile, distilled[4] water for two minutes, surface sterilized for 10 min in a 10% solution of commercial Clorox, and then rinsed twice for 5 min each in sterile distilled water. Both the Clorox solution and sterile water contained a small amount of commercial detergent to reduce water surface tension. Under sterile conditions, the seed coat and female gameophyte tissue were removed, and after a short rinse in sterile distilled water, the naked embryo was placed on a solid agar nutrient medium (Murashige and Skoog, 1962) containing 3% sucrose, 100 ppm myo-inositol, 2 ppm glycine, 0.5 ppm nicotinic acid, 0.5 ppm pyridoxine, 0.1 ppm thiamine, 20 ppm adenine and 10% coconut water.[5] The growth regulators added were 5 ppm NAA, 1 ppm 2ip, and 1 ppm kinetin.[6] The medium was adjusted to pH 5.65 with KOH and solidified with 0.8% Bacto-agar. The medium was heated to +121°C at 1.1 kg/cm^2 (15 lbs/sq inch) pressure for 7 min to ensure that the agar was completely melted before dispensing into tubes. Test tubes

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[4]For materials used in obtaining distilled water, see "Methods and Materials", Appendix B.

[5]Frozen coconut water was obtained from M.O. Mapes, Oregon State university.

[6]For a complete list of chemicals used, see Appendices B_1, B_2 and B_3.

(13 mm x 100 mm) were filled to about 1/3 their length, capped loosely with Bellco Kap-uts and autoclaved at +121°C at 1.1 kg/cm^{2} pressure for 20 minutes. Tubes were then capped tightly and placed at a 45° angle and were allowed to cool at room temperature. The embryo cultures, grown in a growth room, were exposed to a 16 hr day/8 hr night period at a constant +22°C. Light was provided by 6 Sylvania clear 60 watt incandescent bulbs and 6 General Electric F48T12 CW 1500 cool white fluorescent lamps. The light intensity at culture tube level was 820 foot candles, measured with a model 756 Weston Illumination Meter with a quartz filter.

Poplar callus was obtained from shoot tips of a poplar hybrid (Populus tristis x Populus balsamifera cv. TRISTIS). Shoot tips were excised from the shoot and bud scales were removed after a 70% ethanol dip, followed by a sterile distilled water rinse. The tips were surface sterilized in 5% commercial Clorox solution for 2 min, followed by two sterile distilled water rinses for 2 min each. The naked shoot tips were aseptically cultured on a solid nutrient medium consisting of, Murashige and Skoog (1962) salts and vitamins, 2% sucrose and 100 ppm myo-inositol. The growth regulators added were 10 ppm NAA and 0.5 ppm BAP. Bacto-agar was added at 1%. Melting of agar and sterilization of medium were as described above for Douglas-fir. Growth room and light conditions were

as with Douglas-fir, except that light intensity at culture tube level was 900 foot candles.

All cultures were subcultured at 30 day intervals. A total of 5 passages were made before callus was transferred to liquid media.

2. Preparation of Suspension Cultures:

Suspension cultures of both Douglas-fir and poplar were started by transferring several healthy, actively growing pieces of callus to medium-minus agar. The liquid medium was modified in the following manner: The basal medium salts, after Murashige and Skoog (1962), were reduced to half strength. For Douglas-fir, the adenine was reduced to 10 ppm the NAA reduced to 2.5 ppm, and the coconut water eliminated. All other components remained the same for both species.

Liquid cultures were started in Steward-type nipple flasks (Steward and Shantz, 1958) (Figure 1-2 and 1-3) containing 70 ml of medium each. The flasks were rotated on a special apparatus at 3 rpm (Figure 1-1; see also Appendix C).

Growth chamber temperature was a constant $+22^{\circ}C$. Light was provided by the same number and type of incandescent and fluorescent bulbs as for the callus (see above). The light was on a 16 hr day/8 hr night cycle. Light intensity was 850 foot candles at the center of the apparatus and ranges from a high of 1070 foot candles at the top of the wheel, to a low of 520 foot candles at the bottom of the wheel.

Figure 1. (1) Growth room, rotation apparatus with "T" tubes (T) and nipple flasks (N); (2) Suspension cultures of Douglas-fir growing in nipple flask; (3) Douglas-fir EDTA test results in "T" tubes: "A" - 25 ppm EDTA, "B" = 50 ppm EDTA, "C" = 100 ppm EDTA, and "D" = Control 0.0 ppm EDTA; (4) Poplar suspension culture; (5) 50 ppm EDTA treated poplar culture; and (6) 50 ppm EDTA treated Douglas-fir culture.

The chelating compounds used for the experiments were ethylene diamine tetraacetic acid (EDTA) obtained from the Aldrich Chemical Company, and cyclohexane -1, 2-diamine tetraacetic acid (CDTA) obtained from the Mallinckrodt Chemical Company. The EDTA was added to Douglas-fir medium at 25, 50, 100, and 200 ppm and to poplar medium at 50, 100, 200, 300, and 400 ppm. CDTA was added to Douglas-fir medium at 25, 50, 100, and 200 ppm. The pH was adjusted to 5.65 after addition of chelating compounds.

Innoculum used in the experiments were obtained from 15 day -old liquid cultures by filtering the suspensions through a 550 μ brass wire screen[7] for Douglas-fir, and through a 110.5 μ, 550 μ, and an 850 μ brass wire screen for the poplar in order to obtain "small" and "large" clumps of poplar callus. The screening insured that the cell aggregation size was the size of the screen or smaller at time zero of the experiment.

The experiments were carried out in Steward-type "T" tubes (Figure 1-1T and 1-3). The total test medium used per tube was 10 ml. Total test volume of cell suspension was 0.85 ml pipetted into each tube using a pasteur pipette. Growth room and light conditions were as for suspension culture initiation

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[7]Of the many materials tested as screens, a brass mesh worked best allowing repeated sterilization and therefore reuse. For screen size determination, see "Methods and Materials" - #4 Production of Growth Curves, Chapter 2.

described above. At least 4 replications of each treatment were made.

3. Determination of Cell Dry Weight:

At the conclusion of the experiments, the material was collected on Watman No. 5 (5.5 cm pre-weighed) filter paper, in a Buchner funnel (attached to a water-type aspirator). The material was washed twice with distilled water and dried in an oven at +60°C for 15 hours. The dried material was weighed on a Mettler Balance 3 hrs after cooling.

4. Counting of Cells in Suspension Culture:

For cell counts, 0.85 ml of the "T" tube culture were drawn from the experimental tube and macerated[8] for 2 hr in 2 volumes of a mixture of 10% nitric acid/10% chromium trioxide (v/wt) (see Appendix D). The sample was then shaken for 1 min at maximum speed on a test tube super-mixer and centrifuged in a Clay Adams Dynac-type, desk top, centrifuge at maximum speed for 5 minutes. The maceration solution was drawn off and the sample resuspended in a known amount of distilled water. One drop of the diluted sample was placed on a Levy Ultra-Plane improved Neubauer (1/400 sq mm, 1/10 mm deep) haemocytometer and counted at a magnification of 80 times (x80). For each

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[8]Maceration method suggested by Professor J.N. Owens, Dept. of Biology. University of Victoria. Victoria, B.C. 1978.

treatment tube, 5 counts were made on each of 6 samples, for a total of 30 counts per treatment.

Cell aggregation size determination was made on the same basis as above, but the samples were not macerated. Cell aggregate size was estimated[9] as aggregate size over 100 cells and aggregate size under 100 cells.

Data evaluation was made as paired student's "t" tests where N=4 is number of replications; H_o = means are equal (N.S. = Non-Significant), against H_1 = means are different at the 95% significance level (*).

RESULTS

Results indicate that, with one exception, the chelating compounds EDTA and CDTA at optimum concentrations would enhance the growth efficiency of cell suspension cultures of the two species studied.

For Douglas-fir, Figure 1-3 shows that as EDTA is added to the growth media, more cells were produced per unit time. Greatest turbitity per treatment could be seen at EDTA 100 ppm (C in Figure 1-3) when compared to control (D in Figure 1-3). Total dry weight (i.e., increase in cell numbers) was higher for any addition of EDTA, up to and including 200 ppm EDTA.

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[9]The estimation was practiced by doing actual cell counts of different aggregates.

Maximum growth increase was observed with 100 ppm EDTA and was reduced to less than half that amount at 200 ppm (Figures 2 and 4).

The results were not so dramatic when CDTA, rather than EDTA, was added to the medium of Douglas-fir. After 70 days in culture, there was an increase over control at 25 ppm, 50 ppm, and 100 ppm CDTA. Maximum growth effect was observed at 50 ppm CDTA (Figure 3). Tissue grown in CDTA at 200 ppm actually shows a smaller increase in total dry weight than the control, indicating that there was a retardation to growth at this concentration (Figures 3 and 5).

Results with lower EDTA concentrations using poplar cells as test material were similar to Douglas-fir. When soft callus screened through 110.5 μ mesh was used, 50 ppm gave the highest dry weight, while 100 ppm produced about 1/2 this amount. Concentrations of 200 and 400 ppm EDTA decreased growth substantially, with the actual total dry weights being not much above the initial dry weight. Chelator concentration at 200 ppm were however still significently higher than the initial weight. This would indicate that at these higher concentrations, little or no growth occurred (Figure 6).

When hard callus screened at 550 μ was used, maximum dry weight was recorded with 100 ppm EDTA, although the difference between 50 and 100 ppm EDTA was less than 4 mg (non-significent)

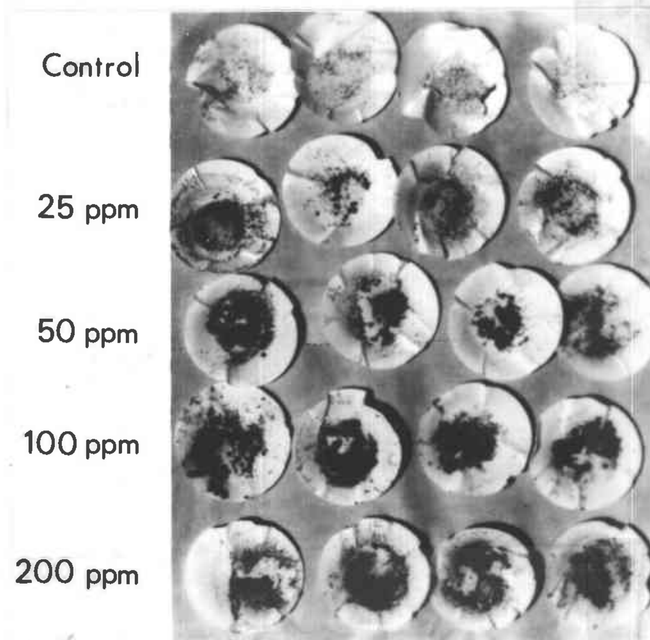


Figure 2. Results of EDTA concentration on Douglas-fir shown after aspiration filtration. Four out of six treatment replications are shown.

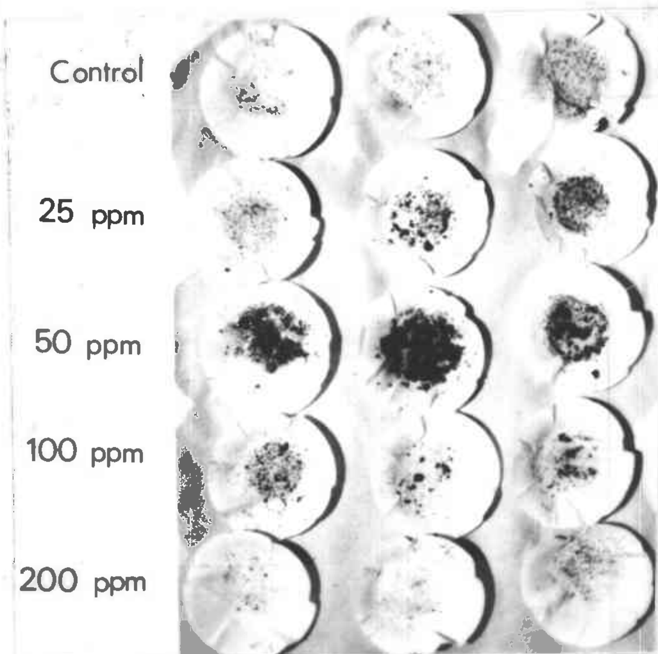


Figure 3. Results of CDTA concentration on Douglas-fir shown after aspiration filtration. Four out of six treatment replications are shown.

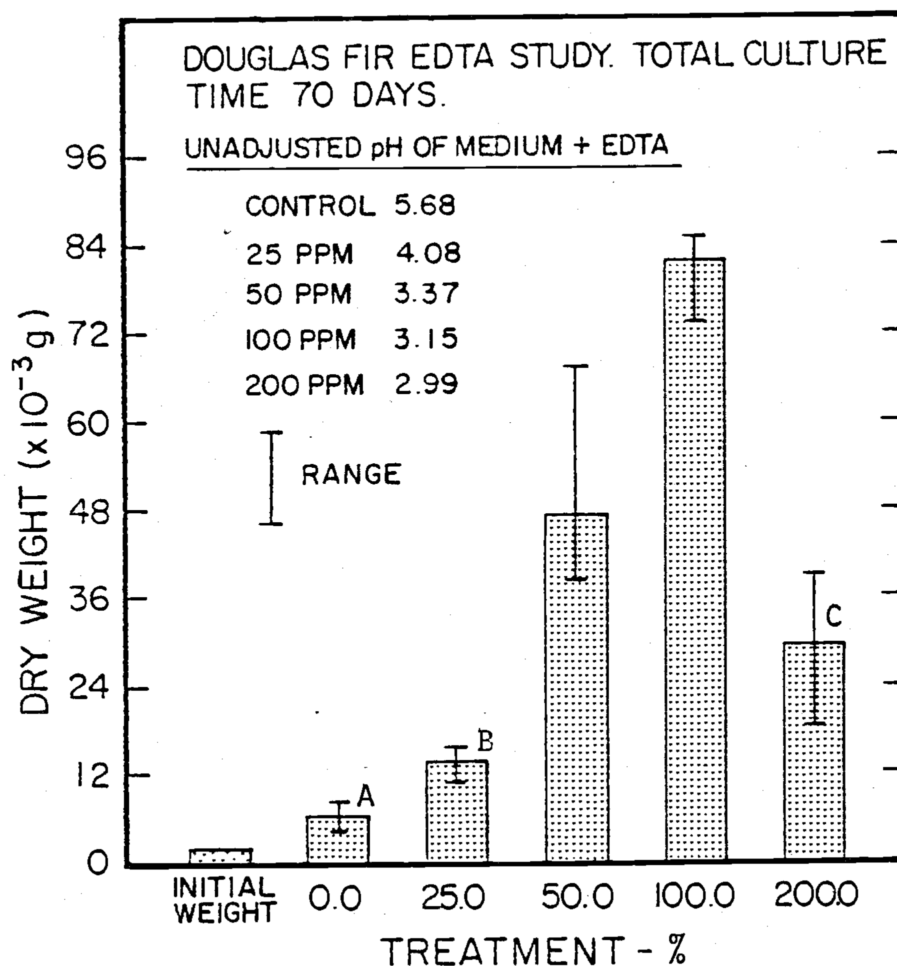


Figure 4. Effect of 25, 50, 100, and 200 ppm EDTA on Douglas -fir culture growth after 70 days. Experimental initial weight and control (0.0 ppm EDTA) are shown. Also given is the decrease in pH as EDTA concentration in media is increased. Final pH adjusted to 5.6. N=4 A:B=*; B:C=*

* Indicates a significant difference at the 95% confidence level.

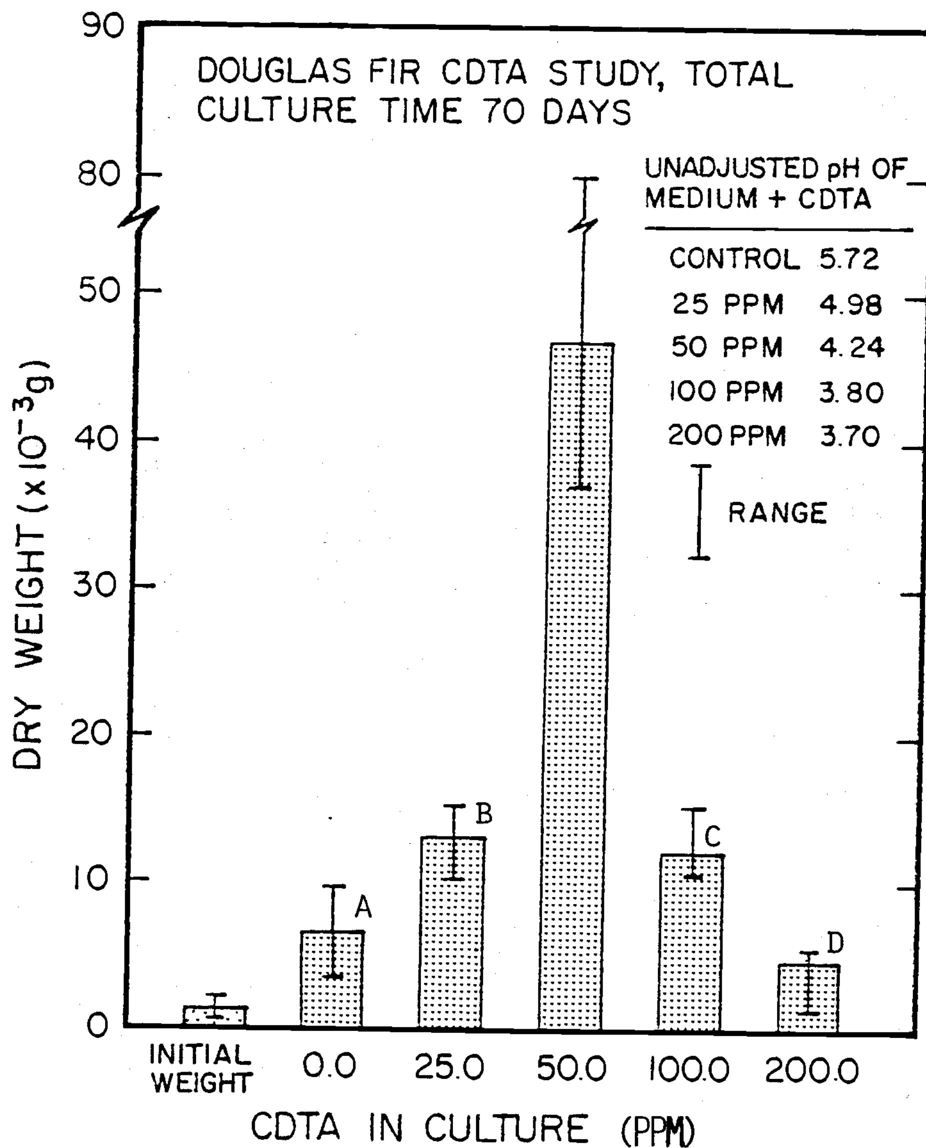


Figure 5. Effect of 25, 50, 100, and 200 ppm CDTA on Douglas -fir culture growth after 70 days. Experimental initial weight and control (0.0 ppm CDTA) are shown. Also given is the decrease in pH as CDTA concentration in media is increased. Final pH adjusted to 5.6. N=4 A:B=*; B:C=N.S.; A:D=*

* Indicates a significant difference at the 95% confidence level.

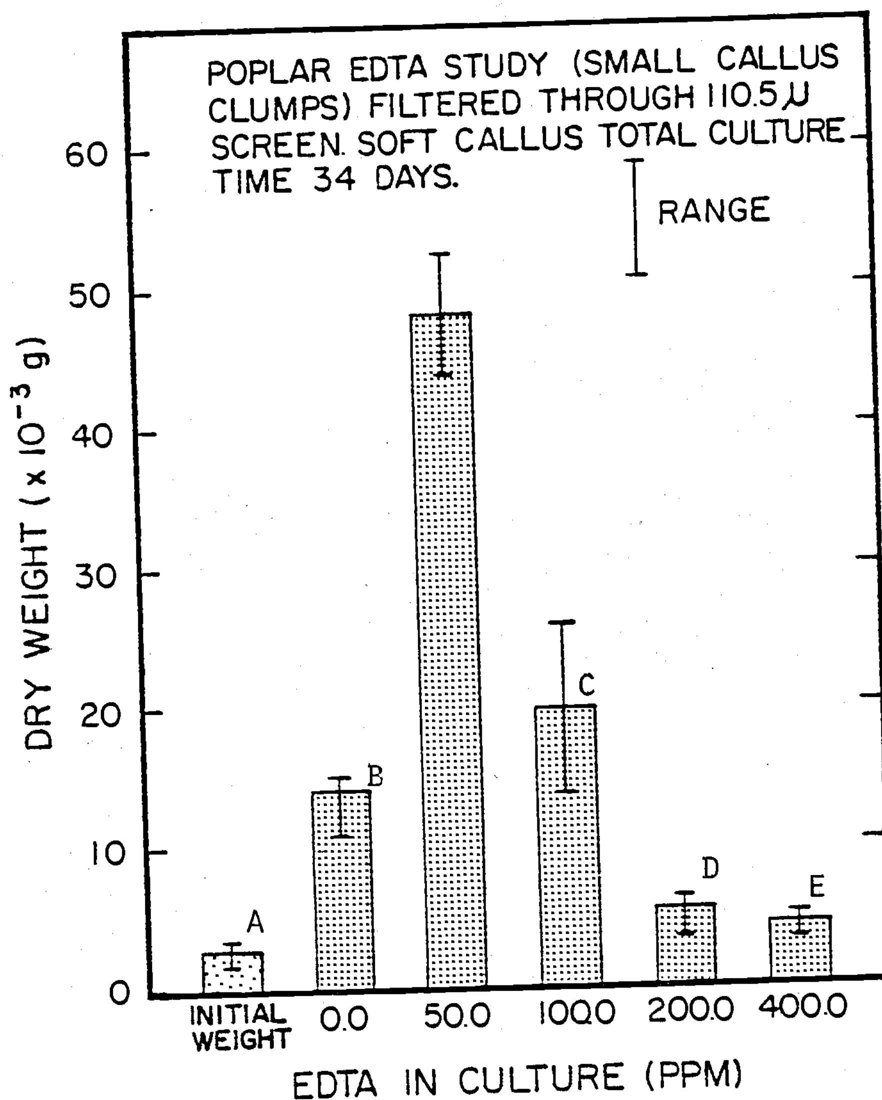


Figure 6. Effects of 50, 100, 200, and 400 ppm EDTA on the growth of soft, small callus clumps of poplar. Filter size is 110.5 μ. Total culture time 34 days. Experimental initial weight and control (0.0 ppm EDTA) are also shown. Final pH adjusted to 5.6. N=5 A:D=*; A:E=N.S.; B:C=*; D:E=N.S.

* Indicates a significant difference at the 95% confidence level.

on a total dry weight of 34 mg. At 300 ppm EDTA, no increase in weight over initial experimental weight was observed (Figure 7).

Results of the two EDTA studies given above (i.e., 110.5 μ screen and 550 μ screen) cannot be compared directly because a different batch of cells was used in each, the initial dry weight was different and total culture time was different. However, some general differences are evident. Material which was softer initially and in smaller clumps showed a greater increase in growth efficiency than does harder, more compact, larger clumps of cells.

The total dry weight and total cell number were related to the concentration of EDTA in the medium containing hard poplar aggregates filtered with the 550 μ screen (Table 1, Figure 7). The EDTA at 100 ppm showed maximum dry weight and maximum cell number. In addition, the decrease in total dry weight of the 300 ppm EDTA treatment compared to the initial dry weight suggests that cell lysis may actually take place at high concentrations of EDTA.

Medium containing 100 ppm concentration of EDTA produced the highest number of aggregates having $\leq$ 100 cells per aggregate using hard cell aggregates and screened with 550 μ screen (Table 2). Cell clump size differed between the control and EDTA added at 50 and 100 ppm. Large clumps formed in the

control, compared to much smaller cell clumps at 50 and 100 ppm EDTA (Figure 8-N, O).

The addition of CDTA to poplar growth media had a toxic effect after 55 days in culture (Figure 9). As the concentration of CDTA increases, the total dry weight decreased (Figure 10). Decrease in dry weight takes place even at 25 ppm CDTA and total growth inhibition takes place between 50 ppm and 100 ppm. A slight increase at 50 ppm and 100 ppm over initial dry weight was expected, as some cell division may occur before toxicity becomes apparent. The CDTA results with poplar differ from those obtained with Douglas-fir.

One very important aspect of the experiment was that regardless of chelating agent type or medium combination, the chelating agent reduced the pH of the growth medium drastically. Douglas -fir medium pH dropped from 5.6 to below 3.5 with addition of 50 ppm EDTA and decreased below 3.0 when 200 ppm EDTA was added (Figure 4). The addition of CDTA (Figure 4) to Douglas-fir medium was somewhat less severe than the addition of EDTA (Figure 5). The pH decrease in poplar medium resulting from the chelating agent CDTA was more severe from that with Douglas-fir (Figures 7 and 10). Data suggests that a very important part of the medium preparation when chelating agents are added is the adjustment of pH after chelating agent addition. This is apparently the case even at very low concentrations of the chelating agent.

Table 1. Poplar total cell number per culture using EDTA as chelator. Total culture time - 30 days. Poplar material is hard callus filtered through 550 μ screen. N=14

* Indicates a significant difference compared to control at the 95% confidence level.

TREATMENT	TOTAL CELL NUMBER IN 10 ML CULTURE
CONTROL (0.0 ppm)	9.80×10^4
50 ppm	3.96×10^5 *↑
100 ppm	7.32×10^5 *↑
300 ppm	3.61×10^4 *↓

Table 2. EDTA effect on poplar cell aggregate size and cell number, per aggregate. N=14

* Indicates a significant difference compared to control at the 95% confidence level.

TREATMENT	NUMBER OF AGGREGATES/FIELD	
	CELLS PER AGGREGATE $\geq$ 100	CELLS PER AGGREGATE $\leq$ 100
CONTROL	1.10	4.70
50 ppm	.40	17.50 *↑
100 ppm	.38	32.20 *↑
300 ppm	0.00	3.00

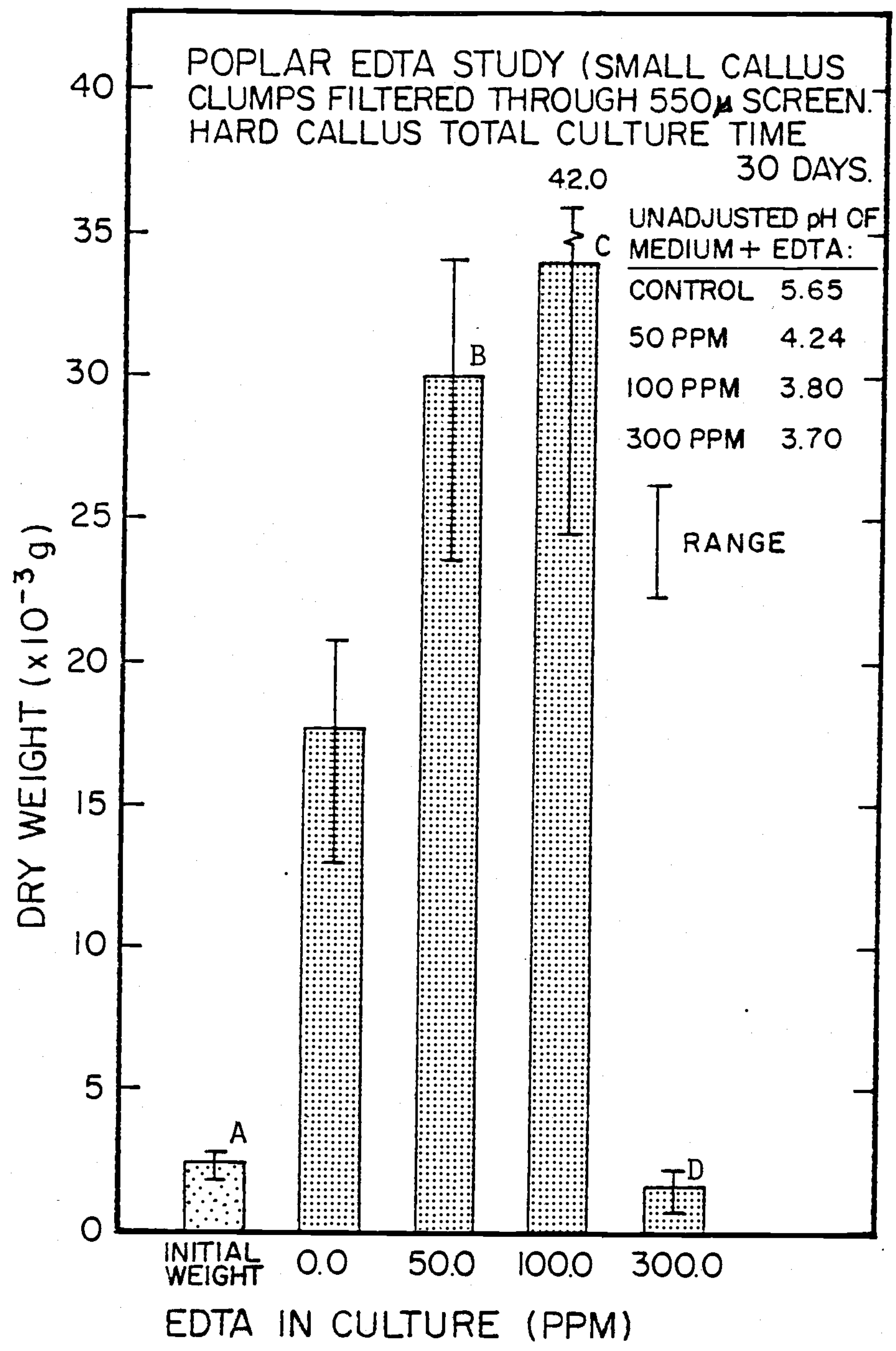


Figure 7. Growth effects of 50, 100, and 300 ppm EDTA on hard clumps of poplar. Filter size is 550 μ. Total culture time 30 days. Experimental initial weight and control (0.0 ppm EDTA) are shown. Also given is the decrease in pH as concentration of EDTA in media is increased. Final pH adjusted to 5.6. N=4 A:D=N.S.; B:C=N.S. * Indicates a significant difference at the 95% confidence level.

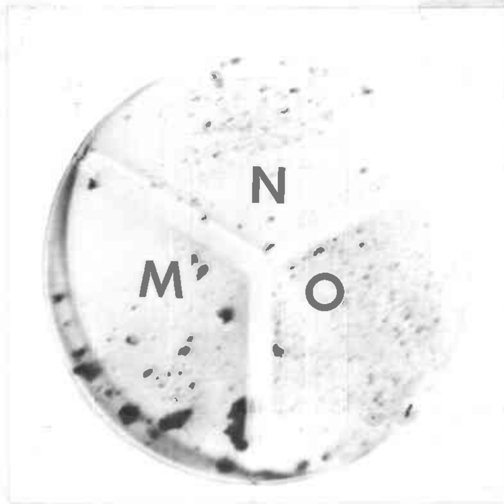


Figure 8. Effect of EDTA concentration on poplar. (M) Control 0.0 ppm EDTA; (N) 50 ppm EDTA; (O) 100 ppm EDTA. Amount shown is not quantitatively the same for all three treatments shown.

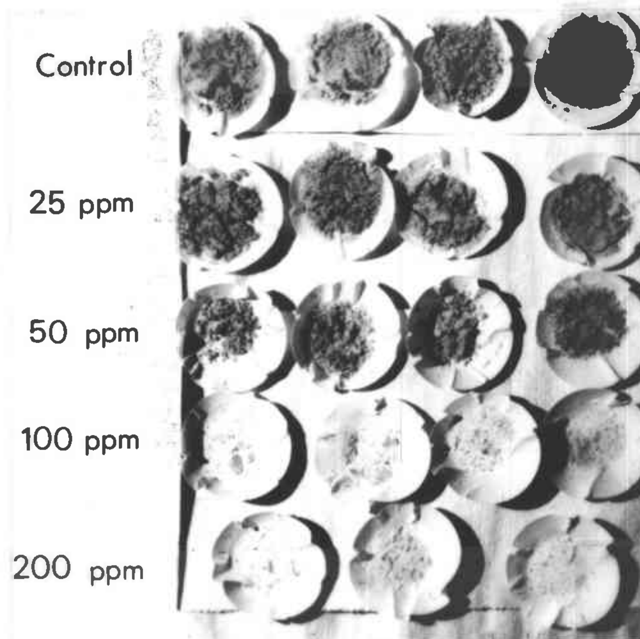


Figure 9. Effect of CDTA concentration on poplar shown after aspiration filtration. Four out of six treatment replications are shown.

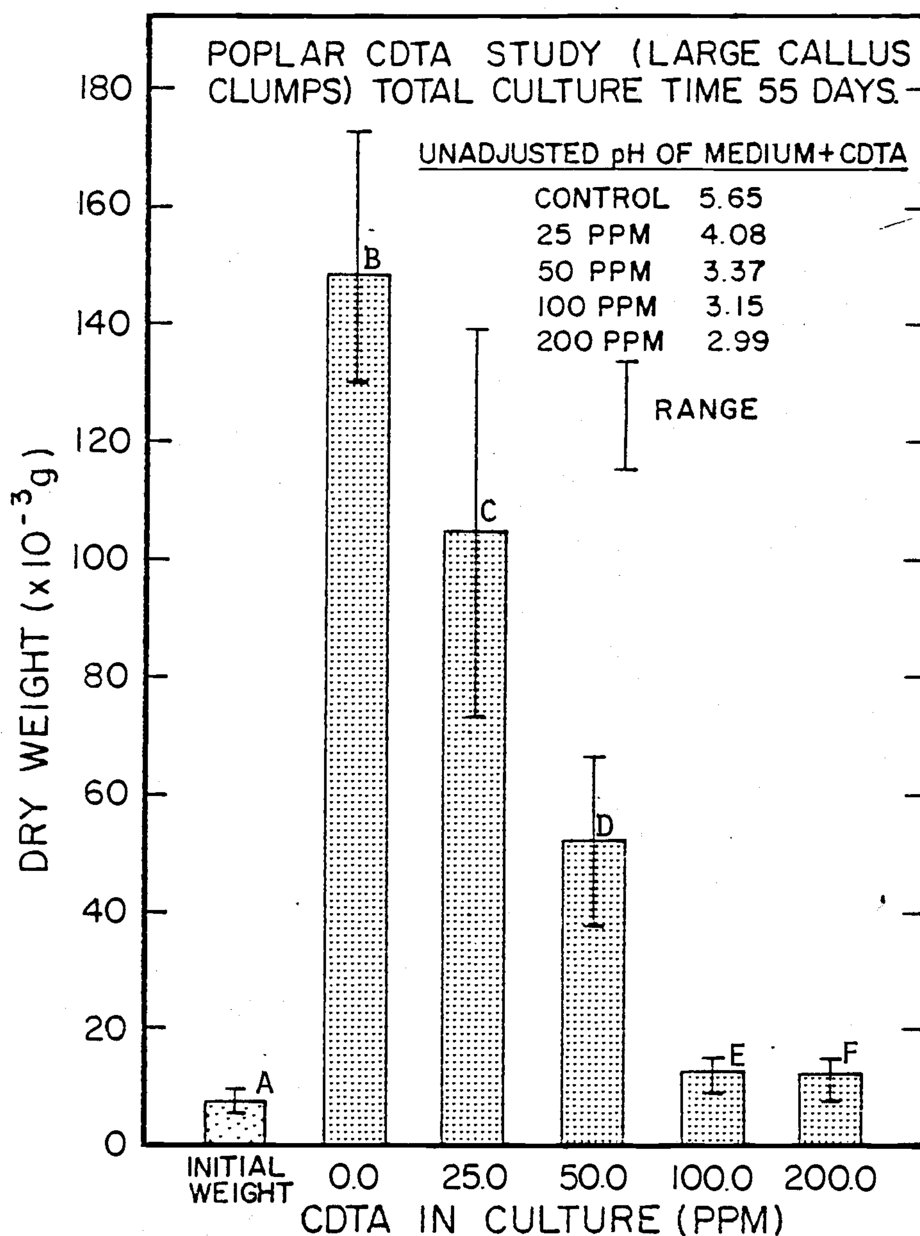


Figure 10. Growth effects of 25, 50, 100, and 200 ppm CDTA on large clumps of poplar. Filter size 850 μ. Total culture time 55 days. Experimental initial weight 5.11 x 10^4 cells/10 ml sample) and Control 0.0 ppm CDTA) are shown. Also given is the decrease in pH as concentration of CDTA in media is increased. Final pH adjusted to 5.6. N=8 A:E=*; B:C=*; C:D=*; E:F=N.S. * Indicates a significant difference at the 95% confidence level.

DISCUSSION

The fact that single cells in both plants (Torrey, *et al*., 1962) and animals (Zwilling, 1954) do not ordinarily produce other single cells, remains a problem to establishing small cell clump numbers in suspension cultures. Some attempts have been made to overcome this, however, success has been limited and reports on cell aggregate reduction vary between species. For example, Liau and Boll (1970) report that a high frequency of free cells of bush bean can be made, while Wallner and Nevins (1973) report extremely variable dimensions (40 to 400 cells per aggregate) of Paul's scarlet rose under the same conditions.

Generally, a free cell suspension devoid of cellular aggregates is possible only if there is a chemical change of the middle lamella so that dividing cells will separate before the next division (Narayanaswarny, 1977). Mineral nutrients in the medium can also influence the friability of the aggregates (Yoshida and Watanabe, 1971; Caldas and Caldas, 1976; Wetherell and Dougall, 1976; and Ojima and Ohira, 1978). Reinert and White (1956) obtained a softer tissue in *Picea glauca* by withdrawing folic acid and vitamin B_{12} from the culture media. Cell separation has been promoted by varying auxins, ctyokinins and coconut milk (Lamport, 1964; Davey, 1971, Wallner, 1973; Szweykowska, 1974; and Everett and Street, 1978). Incorporation of cell wall degrading enzymes, such as cellulase and pectinase,

also enhance cell separation apparently without affecting the viability of cells (Pelcher, et al., 1974). It has also been suggested that there may be a change in physiological characteristics (probably in the protein and carbohydrate metabolism) over a long time in culture and a selection of particularly fast growing cells, which may contribute to softer clumps of cells (Reinert, 1956).

Use of chelating compounds to separate cells began with Ginzburg's (1958) separation of Alaska pea root tip cells with EDTA. Letham (1960) using EDTA, separated a number of plant tissues into smaller fragments. These treatments macerated the cells and the cells were not alive at the conclusion of the procedure. Only El Hinnawy (1973) used the chelating agents EDTA, CDTA and diethylene triamine pentaacetic acid (DTPA) to cause wall loosening in living suspension culture, using *Malilotus alba*.

Results here agree with those of El Hinnawy in that both EDTA and CDTA promoted cell dissociation at different degrees according to their concentration in the suspension cultures. The data suggests that the chelating agents used may chelate a part of the calcium (Ca) present in the cultures, thereby stimulating the loosening of the cells from each other. It has been shown that Ca in the form of calcium pectates form intermolecular bridges in the middle lamella, thereby stabilizing the cell walls of plants (Bonner, 1950; Burström, 1963; and Northcots, 1963). In another

experiment, it was shown that low Ca levels in medium increased the percentage of free cells in culture and that EDTA, in the presence of low Ca levels, increased the percentage of free cells even further (El Hinnawy, 1973). The suggestion that decreased Ca increased free cell numbers is supported by recent pulse-chase experiments which show that the synthesis of pectic substances is more active in cells engaged in cell division that other cell phases (Takuchi and Komamine, 1980). It seems reasonable then, that a high free cell percentage can be expected from rapidly dividing cell populations when the suspension cultures are supplied with chelators. Further, it can be expected that the chelators may cause cells to be readily sloughed off from multicellular masses.

In this experiment the presence of some optimum concentrations of chelators caused an increase in dry weight over control, the exception being CDTA in the poplar medium. These results do not entirely agree with those of El Hinnawy (1973) who reported no increase or decrease in dry weight after 4 weeks of culture with added chelators. With some chelators (EDTA and DTPA) that author found a decrease of dry weight only at higher (100-200 mg/l) chelator concentrations. The data here generally agrees with these findings. Also some agreement was observed with the chelator CDTA. El Hinnawy (1973) found CDTA reduced total dry weight at all concentrations. In the present study, CDTA

reduced total dry weight of poplar cultures at all concentrations but this was not the case with Douglas-fir cultures.

The lack of growth at higher chelator concentrations here and elsewhere (El Hinnawy, 1973) may result from excessive removal of Ca and other ions from the culture medium, as well as the cells themselves, causing poor growth. Calcium is a requirement for cell growth in suspension cultures (Ojima and Ohira, 1978). Another possibility is that higher chelator concentrations may inhibit enzyme reactions because of excessive removal of metal ions (Burström, 1963). In addition, decline in activity may result from extraction of hemicellulose and related substances from the cell wall at high chelator concentrations (Letham, 1960).

The increased growth effect at some chelator concentrations is interesting when compared to El Hinnawy's 1973 results where no growth or decreased dry weight occurred at all chelator concentrations. However, in an experiment to determine growth efficiency of different aggregate sizes, it was found that total dry weight increased dramatically as aggregate size decreased (El Hinnawy, 1973). Henshaw, *et al*. (1966), however, reported that in *Rubus*, *Linus*, and sycamore cells during the early stage of growth, cells in large aggregates divided more rapidly than did cells in smaller aggregates. Conflicting results have been obtained regarding cell cluster size, chelating compounds and rate of growth. Kuboi and Yamada (1978) in an analysis of cell

growth dynamics, showed on the other hand that cells in small (less than 0.5 mm), medium (between 0.5 and 1 mm) and large clusters (more than 1 mm) all divided equally well. Further, these authors showed that small clusters produced larger medium sized aggregates. In light of those reports, it appears plausible to explain increased dry weight with some chelator concentrations because chelators reduce aggregation size which may, in turn, result in increased growth efficiency. The cause of this efficiency may be that, in addition to less growth pressure dynamically, smaller aggregates have more cells exposed to raw materials in the culture medium causing more effective cell cycling per unit time than larger volume aggregates. In addition, it has been suggested that even though a chelator may remove an essential metal from the tissue, the presence of another metal may release the essential metal again to the overall benefit of the tissue (Burström, 1963).

The mode of action of chelating agents as they affect growth has been approached in two different ways; 1) tracing a similarity between chelating agents and natural auxins, and 2) identifying the metals responsible for the growth actions when chelating compounds were added. Both of these methods may also be involved in the growth effects observed here. For example, EDTA has been shown to stimulate growth at low concentrations (Burström, 1963). Bennet-Clark (1956) first found that in a coleoptile section test for auxin, the addition of EDTA promoted growth in a manner

resembling that of an auxin, and showed an increase shoot and decrease root growth. However, a detailed study on roots has revealed that the mode of action of the two types of compounds is basically different and they are antagonistic to each other (Burström, 1963). The biochemistry may, in fact, be quite involved and there is a suggestion that EDTA inactivates some metal system destroying IAA (Burström, 1963). With soybean plants grown to maturity with EDTA at concentrations promoting growth, Burström (1963) observed a decrease in nitrogen content, increase in polyphenol oxidase and catalase activities, and decreases in cytochrome oxidase and peroxidase activities. The differences observed by El Hinnawy (1973) and the present study may be that El Hinnawy used root material, while here both Douglas-fir and poplar experimental material was from shoots.

The inhibitory effects of CDTA on poplar remains unclear at the present time. This chelator even at low concentrations may somehowcause inhibitions of enzyme reactions by removal of essential metals. Another explanation may be that CDTA causes an effective depression of the saturability of pectate, pectinate, and polysaccharide constituents of the cell wall. It is also possible that with certain tissues CDTA may have other modes of action compared to EDTA. Only further extensive testing can answer these questions.

The pH of the media is an important parameter in the growth of suspension cultures. It has been shown that if the pH varies

much from 5.8, cell growth is drastically reduced (El Hinnawy, 1973). In addition, Letham (1960) found that the maceration activity fell drastically below pH 5.0 and became near zero at pH 4.5. In the present study, early experiments where the pH of the media was unadjusted after chelator addition, the low pH produced by addition of chelators could have a detrimental effect both on cell physiology and the growth effect of the chelators.

The chelators used increased cell separation and total dry weight to different degrees, but the goal of producing a 100% free cell suspension culture was not realized. This result suggests that other components in the intercellular cement, in addition to Ca, are responsible for cell aggregation. Ginzburg (1958; and 1961) found that the intercellular cement contained calcium, and heavy metals, such as iron and copper. He suggested the presence of a protein complex in the middle lamella that strengthens the attachment of divided cells. Others have provided evidence for a covalent connection between the pectic polysaccharides and the structural protein of the cell wall using suspension cultured cells of sycamore (Bauer, *et al.*, 1973; and Talmadge, *et al.*, 1978).

Although a solution to the growth effects of the chelators observed here require more extensive study, cell aggregate size was controlled, which will allow the two plant species to be used

for cryogenic studies. Additionally, if chelators can be used to reduce cell aggregate size in other plant species, a major hurdle in plant suspension culture studies will have been overcome.

CONCLUSIONS

Suspension cultured cells of Douglas-fir and poplar may have their growth rates altered by the chelator compounds EDTA and CDTA. At some concentrations EDTA with both species and CDTA with Douglas-fir can increase growth efficiency. These chelators also reduce the cell aggregation size of both species studied.

The changes in growth rate observed when chelators are added to the growth medium are assumed to be due to calcium removal from both the growth medium and the cell walls of the cells themselves. High chelator concentrations would remove excessive calcium and heavy metals from the medium causing decreased enzyme activity and poor growth generally. Optimum chelator concentrations would remove some calcium causing loosing of the middle lamella between cells allowing them to dissociate easier. Smaller aggregates have more cells exposed to raw culture medium allowing more effective uptake of nutrients and increased growth.

III. CHAPTER 2

CONTROLLED FREEZING OF SUSPENSION CULTURED CELLS OF DOUGLAS-FIR AND A POPLAR HYBRID: STORAGE AT AND RECOVERY FROM -196°C

INTRODUCTION

Interest in plant tissue culture has increased in the recent years because many species belonging to diverse genera can now be vegetatively propagated through cell, tissue and organ culture (Murashige, 1974; and Winton, 1974). However, the routine techniques of tissue culture maintenance are expensive, and time and space consuming. There is also always the risk of irretrievable loss of the culture through genetic change, microbial contamination and human error (D'Amato, 1975; Bajaj, 1976a, 1976b; Withers and Street, 1977; and Henshaw, 1979).

The central probelm in using tissue culture techniques for genome storage is prevention of cytological change. A number of researchers have attempted to control such changes by reducing the growth rate of tissues by maintaining them at lower positive temperatures (Bannier and Steponkus, 1972; and Meyer-Teuter and Reinert, 1973), under mineral oil overlay (Caplin, 1959), in minimal media (Jones, 1974), or by freeze-drying and vacuum storage (Binder, et al., 1974). However, only storage at very low temperatures, produces total immobilization of metabolic activity and can prevent eventual change in the genome.

There is an added advantage in developing a metabolically inactive storage phase. The total suspension of biochemical activity is effectively a suspension of time and, therefore, would permit the worker to accumulate reference material in a chronological sequence, for subsequent assessment of change in a culture with time.

A successful method of freeze-preservation could accomplish this goal and particularly suspension cultures could be conveniently handled and provide innoculum material genetically defined, of predictable performance in culture and easily sorted and transported. Other advantages to freeze-storage are maintenance of disease-free material and study of cold acclimation and frost resistance. Perhaps the most important advantage of very cold storage, with respect to tissue culture, is the retention of morphogenetic potential of cold stored material. Long-term tissue cultures are known to lose their ability to undergo morphogenesis (Torrey, 1967; and Meyer-Teuter and Reinert, 1973).

Isolated reports of investigations into the freezing of biological materials have been made over the last 200 years (Meryman, 1966), however, significant progress has only been made since the middle of this century, when the beneficial effects of cryoprotectant additives were first reported (see Appendix A - "Review of Literature").

Our present understanding of the processes of freeze-preservation owes much to extensive studies carried out with bacteria, yeasts, and animal tissues including organs and cultured cells. Expertise has developed to such an extent that the reproducible storage of gametes, embryos, blood plasma and microorganisms can be numbered among the routine techniques of cyrobiology (Meryman, 1966; and Wolstenholme and O'Connor, 1970). Using material from the above sources, general observations can be made concerning the component stages of the freezing/thawing process, the value of cryoprotectant additives, and the cellular response to low temperature exposure.

The cryogenic storage of plants using a cryoprotectant began with the freezing to -196°C of flax cells by Quatrano in 1968. Others, such as Sakai and co-workers have added greatly to our knowledge of frost hardiness, low temperature survival, cooling and freezing regimes and as means of storage of callus and suspension cultures, shoot tips and embryos (Sakai and Sugawara, 1973; Sugawara and Sakai, 1974; and see Appendix A - "Review of Literature", Table 1, 2 and 3).

In exploring the possibility for freeze-preservation of plant material, it has become clear that the development of techniques for successful preservation requires a complete and detailed emperical examination of the specific system to be used. Plant cryobiology is today very much a subject in its own right.

No conifer cell culture has yet been frozen to liquid nitrogen temperature and recovered. One paper has appeared where poplar callus has been frozen to liquid nitrogen temperature and recovered (Sakai and Sugawara, 1973), but no cryoprotectants were used and survival was estimated only as callus growth. In addition, no attempt was made to generate shoots from recovered poplar callus.

The objectives of this research were to study the major physiological effects of freezing and thawing upon the viability of two woody plant species in suspension culture and to develop a protocol of optimum freezing rate, thawing rate, type and amount of cryoprotectants needed to freeze such cell cultures to super-low temperatures and recover them in a living state. Douglas-fir (*Pseudotsuga menziesii*) will be the main focus of the study with a hybrid poplar (*Populus tristia* x *Populus balsamifera* cv. TRISTIS) used for comparison. Ultimately, it is hoped trees of superior genetic quality (Figure 11) may be reduced to small clumps of cells, cryopreserved (Figure 12) and recovered to be regrown into trees of their former quality.

METHODS AND MATERIALS

1. Growth of Douglas-fir and Poplar Callus:

The origin of material for poplar and Douglas-fir callus was the same as given in Appendix B and Chapter 1 respectively, and should be consulted for material source, methods of isolation

Figure 11. The beginning and the end of a cryogenic storage program; a stand of Douglas-fir trees, the genetic qualities of which we would like to preserve.

Figure 12. Douglas-fir trees reduced to their smallest whole elements - cells, the basic material used in cryopreservation.

and content of culture media. Figure 13 shows Douglas-fir and poplar callus.

2. Preparation of Suspension Cultures of Douglas-fir and Poplar:

The methodology and liquid culture medium for preparing suspension and callus cultures of Douglas-fir are given in Chapter 1. Suspension cultures were grown in Steward-type nipple flasks (Figure 14) containing 70 ml of liquid culture medium. Since smaller cell aggregates could be obtained using chelators (see Chapter 1), 75 ppm EDTA was added to both Douglas-fir and poplar liquid media. After 4 passages, the EDTA was discontinued in the Douglas-fir media but continued throughout all experimental poplar media, both solid and liquid. The chelator was discontinued since apparently, through continued selection, a suspension culture containing only small clumps of cells was developed for Douglas-fir. Once obtained, this soft material could be propagated in both solid and liquid media. When placed in liquid medium and shaken, many of the cell clumps were easily filtered through a 550 μ screen. Poplar, on the other hand, became hard and large aggregates formed if EDTA was omitted from even one passage.

3. Determination of Cryoprotectant Toxicity:

Douglas-fir and poplar liquid media as given in Chapter 1 were added with DMSO (Crown Zellerbach, Chemical Products Division. Camas, Washington) or glycerol (Mallinckrodt Chemical) to

Figure 13. Douglas-fir callus growing in the three containers on the left and poplar callus growing in the two larger tubes on the right.

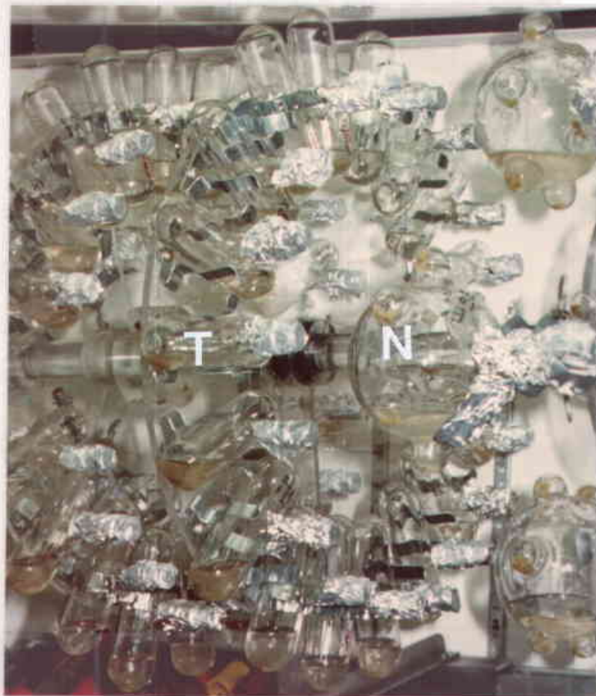


Figure 14. Rotating apparatus containing nipple flasks (N) and "T" tubes (T).

concentrations of 20%, 10%, 1%, 0.1%, 0.01%, 0.001% (v/v) in "T" tubes (Figure 14). The final volume was 10 ml. Suspension cultures of both species were grown in their respective liquid cultures for 1 week, then harvested and added to "T" tubes according to procedure 4 ("Production of Growth Curves") below. In addition, Douglas-fir suspensions also were grown under the same conditions as above, except that Hydroxy-free-L-proline (Sigma Chemical) was added at 20%, 10%, 5%, 1%, 0.1%, 0.01% to the growth media.

Cultures were harvested after at least 25 days growth and total dry weights determined as described in Chapter 1. At least 4 replications of each treatment were used.

4. Production of Growth Curves:

A beaker with a 550 μ screen wrapped in household aluminum foil was autoclaved at +121°C and 1 Kg/cm^2 pressure for 20 min and allowed to cool. Cultures of both species were grown in nipple flasks as described above for 7 days. Cell clumps from 5 or 6 nipple flasks were harvested at the end of this period by filtering through the screen. All operations described were carried out under asceptic conditions unless otherwise stated. Five different screen materials and their sizes were tested to determine which would lend itself best for cell clump filtering. The pore size of each screen was determined with a microscope eye piece micrometer. A screen of brass fitted over a 200 ml beaker was finally found to work best.

All filtered cell clumps were collected in one 500 ml erlenmyer flask. After allowing time for aggregates to settle, excess medium was drained off, thereby concentrating the cell volume.

While the flask was rotated to keep the aggregates suspended evenly, 0.85 ml of material was drawn out using a pre-sterilized pasteur pipette. (It was very important to fire-sterilize both pipette end and open end of flask after each withdrawal. If contaminated at this point the entire experiment was lost.) The cell clumps were withdrawn into 13 x 100 mm culture tubes (VWR Scientific) containing 6 ml of either poplar or Douglas-fir liquid culture media. The tubes were placed in a drum apparatus (Figure 15) rotating at 8 rpm. Continuous light intensity was at 200 foot candles supplied by General Electric cool white fulorescent lights. The growth room temperature was held at a constant +22°C.

Samples as indicated by preliminary studies, were removed at 0, 4, 7, 14, 19, 21, 26, 28, and 32 days for Douglas-fir and 0, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, and 18 days for poplar. Dry weights and cell counts were made. Dry weights were made by aspirating the entire contents of at least 4 sample tubes per date onto Watman No. 5 pre-weighed filter paper. The procedure for attaining dry weights is given in Methods and Materials of Chapter 1. Cell counts were made by treating each sample with 10% nitric/10% chromic acid (Figure 16), according to the method

Figure 15. Rotating apparatus containing 13 x 100 mm culture tubes for treatment testing of cell cultures. Note lights at "L".

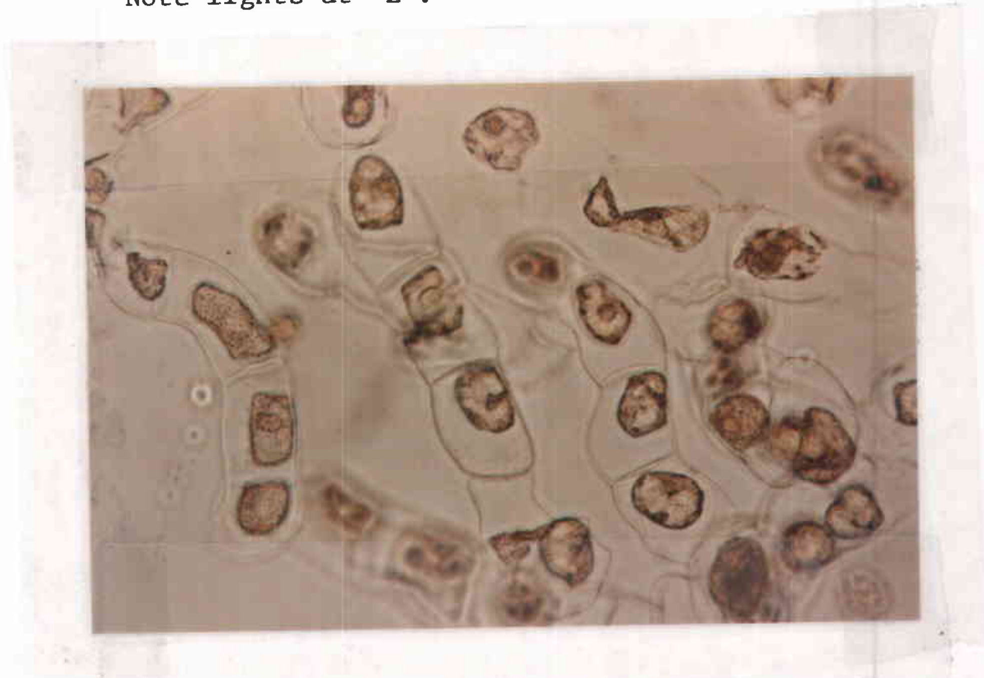


Figure 16. Douglas-fir cells after treatment with 10% nitric/ 10% chromic acid. The cytoplasm has contracted away from cell walls. The slight staining makes the cells easier to see and count.

given in Chapter 1. Five cell counts were made for each of two Douglas-fir cultures and three poplar cultures. Dry weights and cell counts were made under non-asceptic conditions.

5. Methods for Freezing to -196°C and Recovery:

a. Preparation of cultures for freezing

Poplar cultures were harvested in log phase of growth at 6 days and Douglas-fir in log phase at 10 days. Filtering and collection of cultures was as described under "Preparation of Suspension Cultures". Following concentration of cell material and while shaking to ensure even suspension, cell clumps were dispensed into 50 ml graduated cylinders. The cells were allowed to settle for 20 min and the culture medium decanted off. Fifty ml of cryogenic test solution was added to the graduated cylinder. After shaking to ensure suspension of cell clumps, the contents were washed into 100 ml flasks. The suspension was allowed to stand 1/2 hr before the next step (Figure 12).

Test solutions were made by adding distilled water and concentrations of either DMSO or glycerol at 10%, 5%, 2.5%, 1% and 0.5% (V/V); by combinations of DMSO and glycerol, respectively, at 5%/5%, 5%/2.5%, 5%/1%, 2.5%/2.5%, 2.5%/0.5%, 1%/1%, or by adding of DMSO and glycerol plus 0.5% proline for Douglas-fir and 1% proline in the poplar test solution. In the cases where medium was used as a test solution, the same medium in which

the suspension cultures were grown was used as freezing medium. With the exception of the case when whole medium was used as test solution, 4% sucrose was added to the test mixture and the pH adjusted to 5.6. The mixture was dispensed into 100 ml flasks and autoclaved at +121°C and 1 Kg/cm^2 for 20 minutes.

The suspension and test mixture were shaken continuously and added to 1.2 cc cryotubes using a pasteur pipette. (Cryotubes obtained from Vangard International; 1111A Green Grove; Neptune, New Jersey. Only pasteur pipettes from VWR Scientific, Order #1467220 5/3/4" L x 7.0 mm O.D. were used since the neck diameter of the pipette varies from supplier to supplier.) Each ampule was filled, the cell clumps allowed to settle and 1/2 of the medium pipetted off. The caps were replaced on the ampules and allowed to stand for 1.5 hr at room temperature (Figure 17). After 1.5 hr the ampules were placed in the freezing chamber and cooling began.

b. Freezing equipment

Freezing equipment was obtained from Union Carbide Cryogenic Equipment, Linde Division (2770 Lionis Blvd., Los Angeles, CA), and consisted of; a CRC-1 control rate console (to control cooling rates at 0.5°C to 35°C/min from +20°C to -100°C, with controls for a two-step cooling program and a CRFC-1 freezing chamber with space to freeze 64-1.0 cc ampules at one time. Specimens were cooled by rapid circulation of cold nitrogen

Figure 17. Cryotube ampules containing cell clumps to be frozen. Flasks on left contain test material. Asceptic conditions must be maintained.

Figure 18. Freezing apparatus showing low pressure liquid nitrogen tank (T), freezing chamber (F), control unit (C), and chart recorder (R). Container to lower and hold frozen samples in liquid nitrogen storage tank is shown at "e".

vapor in the freezing chamber. A differnetial thermocouple was used to maintain the desired cooling rate by controlling the flow of liquid nitrogen into the chamber. A second thermocouple was used to measure specimen temperature which was displayed and recorded on a 0-100 millivolts input temperature recorder. Chart paper speed was 1 cm/min and was calibrated for degrees Celsius from +20°C to -100°C. Liquid nitrogen was supplied from a low pressure (22 psi) 160 liter nitrogen tank. Samples were stored in a Linde LR 33 type biological freezer, filled 2/3 full with liquid nitrogen. The freezing apparatus is shown in Figure 18.

c. Freezing, storage and thawing

Samples were either frozen rapidly by immersing the ampules containing the cell clumps directly in liquid nitrogen or the ampules were frozen slowly in the freezing chamber. Slow freezing was at 1°C ± 0.5°C/min. In the case of slow cooling, cooling was started at +20°C and was continued to either -15°C or -40°C and held at that end temperature for 1/2 hr. The ampules were then quickly removed from the cooling chamber and immersed in the Linde liquid nitrogen storage tank for 5 days before they were removed and thawed.

Fast thawing was accomplished by immersing the ampules directly into the non-asceptic +40°C water bath. The thawing was allowed to continue until the frozen liquid in the ampules

became fluid; that is about zero degrees Celsius. At that point, the ampules were removed from the water bath, sprayed with 70% ETOH and placed upright at room temperature for 1 hr under asceptic conditions. Slow thawing was accomplished by removing the samples from liquid nitrogen and placing the ampules upright at room temperature until completely thawed.

Caps were removed from the ampules and fresh, sterile media of the appropriate type, was added at room temperature using a pasteur pipette. Total volume of medium added was 25 times the amount of aggregate test material. After 15, 30 or 45 minutes, the medium was removed from the sample and the next washing begun. Three such washings were made at 15 min intervals, 3 at 30 min intervals and 2 at 45 min intervals, in that order. Total number of washings of material with fresh medium was 8 times.

6. Estimation of Cell Survival:

a. Triphenyl tetrazolium chloride (TTC) test

Cell viability of thawed cells was modified according to the methods of Steponkus and Lamphear (1967). In this method, cell survival was estimated by the amount of formazan produced as a result of reduction of triphenyl tetrazolium chloride (TTC), which gives a red-orange color. The initial procedure used involved the following steps:

1) Buffer solution: 78% $Na_2HPO_4 \cdot 2H_2O$ solution: 0.05M (8.9 g/1), 22% KH_2PO_4 solution 0.05M (6.8 g/1).

2) TTC solution: 0.6% TTC dissolved in buffer solution.

3) About 150 mg of cell sample was put into 3 ml of TTC solution and incubated for 15 hr at +35°C.

4) The TTC solution was drained off and cells were centrifuged and extracted with 7 ml of ehtanol (95%) in a water bath at +80°C for 5 minutes.

5) The extract was cooled and made to 10 ml volume with 95% ethanol.

6) The absorbance (red color) was then recorded with spectrophotometer at 530 nm.

The amount of formazan produced by the frozen cells is expressed as a percentage (survival) of formazan produced by the control cell suspension. In the present study an absorbance scan from 900 nm to 250 nm showed the red reaction compound had its highest absorbance at 485 nm for both Douglas-fir and poplar (Figure 19 and 20). All papers to date with the exception of Withers (1978), who uses 485 nm, use a spectrophotometer reading at O.D. 530 nm. All TTC readings reported here were made at 485 nm (Figure 23).

Initial results showed some inconsistancy within species and between species, therefore a concentration study of TTC under different conditions was made. A Douglas-fir TTC concentration study was made at 0.001%, 0.025%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.4%, 1.7% and 2.5%, exposed to two different weights of material and one volume TTC.

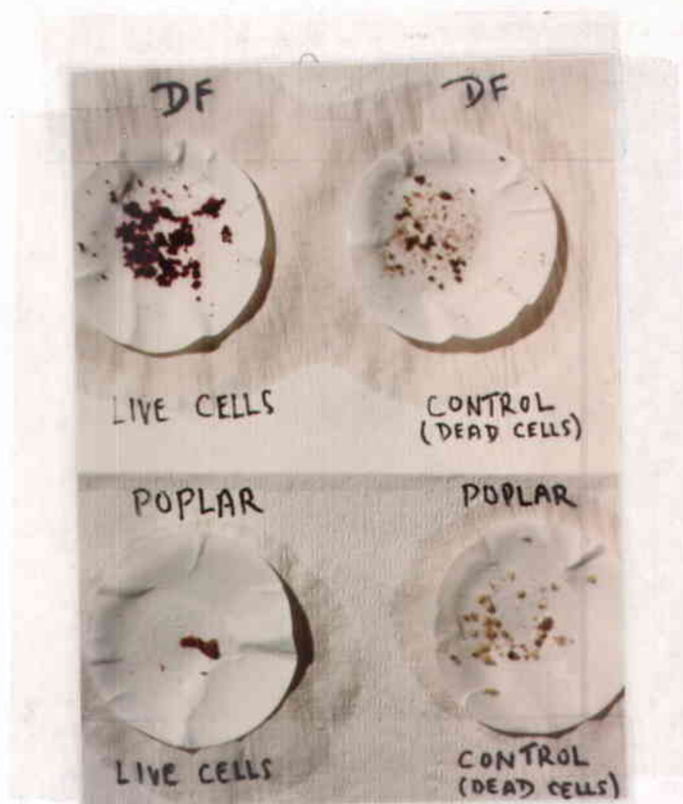


Figure 19. Triphenyl **tetrazolium chloride (TTC) reduction** by Douglas-fir and poplar cells. Live cells are shown on the left and boiled cells (dead cells) on the **right. Note red color of live cells.**

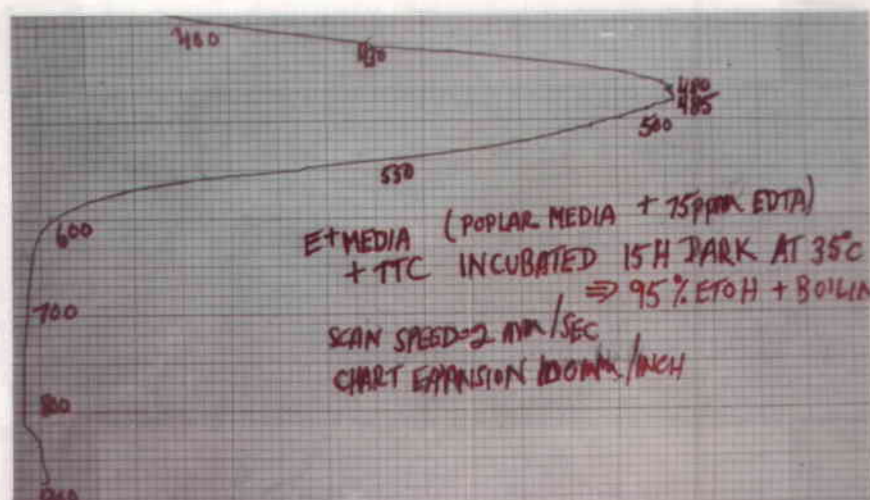


Figure **20**. **Spectrophotometer scan of formazan (TTC reduction product)** from 900 nm to 400 nm showing peak ab**sor**bance at 485 nm. Photo is result of contamination study found in 0.05% TTC used with poplar. (See "Results" section.)

Weight was determined as dry weight (see Chapter 1). In addition, samples of the lesser weight treatments were held at +4°C for 3 hr (tested with 0.6% TTC) and frozen to -10°C for 3 hr, thawed in air and tested at 0.6% TTC. Results are given in Figure 21.

A poplar TTC concentration study was made at 0.001%, 0.025%, 0.05%, 0.075%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.4%, and 2.5% and exposed to one weight (determined as in Chapter 1) and two different volumes (2 ml and 4 ml) of TTC. In addition, samples of the weight were held at +4°C for 3 hr (tested with 3 ml of 0.6% TTC). Samples which were heat killed, by boiling for 30 minutes, showed 7% of maximum reduction. Results given in Figure 22 are corrected for the boiled contamination value.

For both the Douglas-fir and poplar study, results per treatment are the result of 4 replications. Results show that maximum TTC reduction for Douglas-fir is achieved at 0.3% while this value was 0.05% for poplar. Since there was little difference between the value of TTC used in the poplar study (2 or 4 ml), the total volume set for both Douglas-fir and poplar was 3 ml.

In a test to determine increase of TTC reduction with increased amount of material, there was good correlation between amount of material present and formazan produced. When approximately equal amounts of fresh weights were taken and one heat

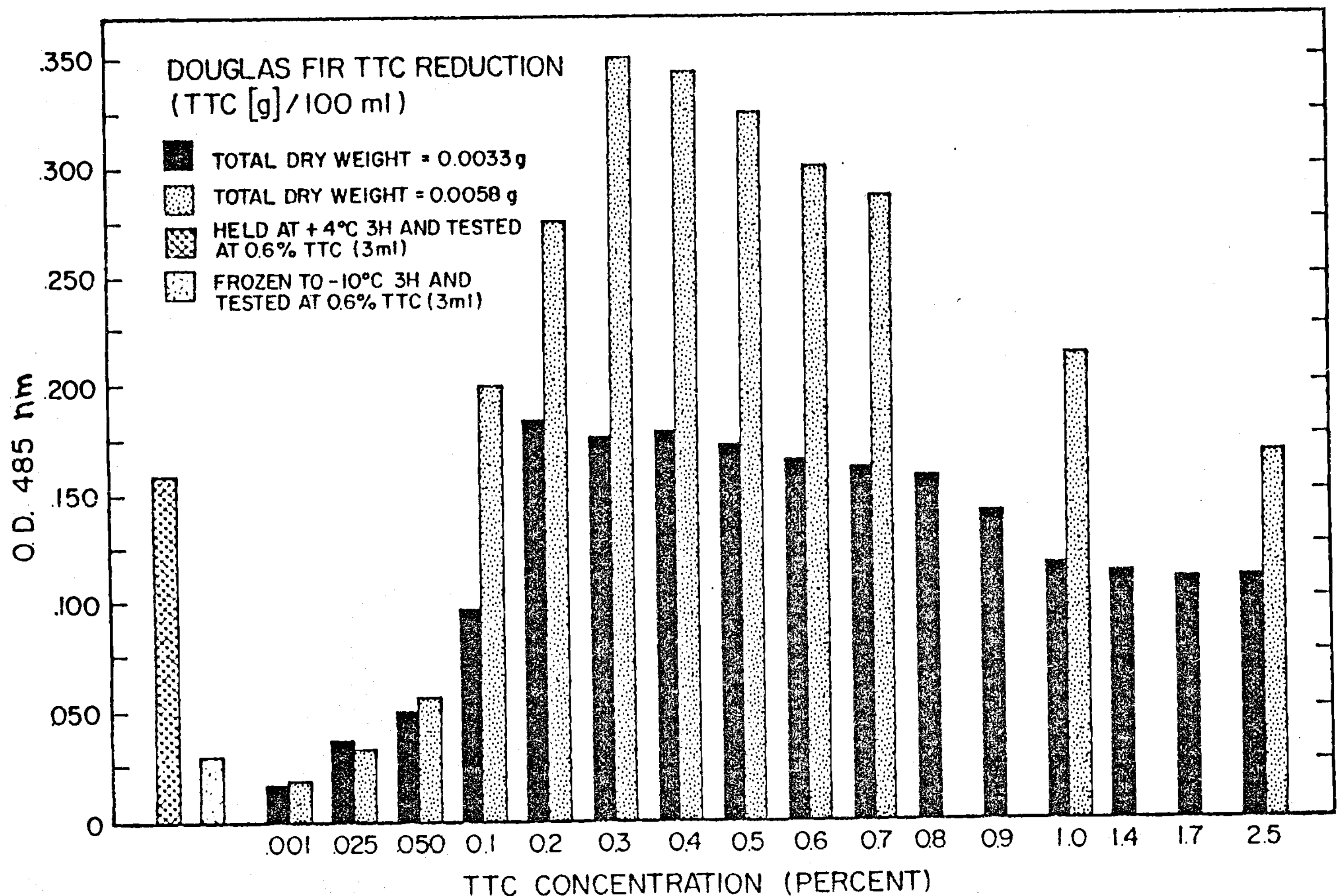


Figure 21. Douglas-fir TTC reduction study. TTC at 0.001, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.4, 1.7, 2.5% were exposed to two different weights of Douglas-fir material. In addition one sample of the lower weight was exposed at +40°C for 3 hr before testing and another frozen to -10°C for 3 hr before testing. O.D. was taken at 485 nm.

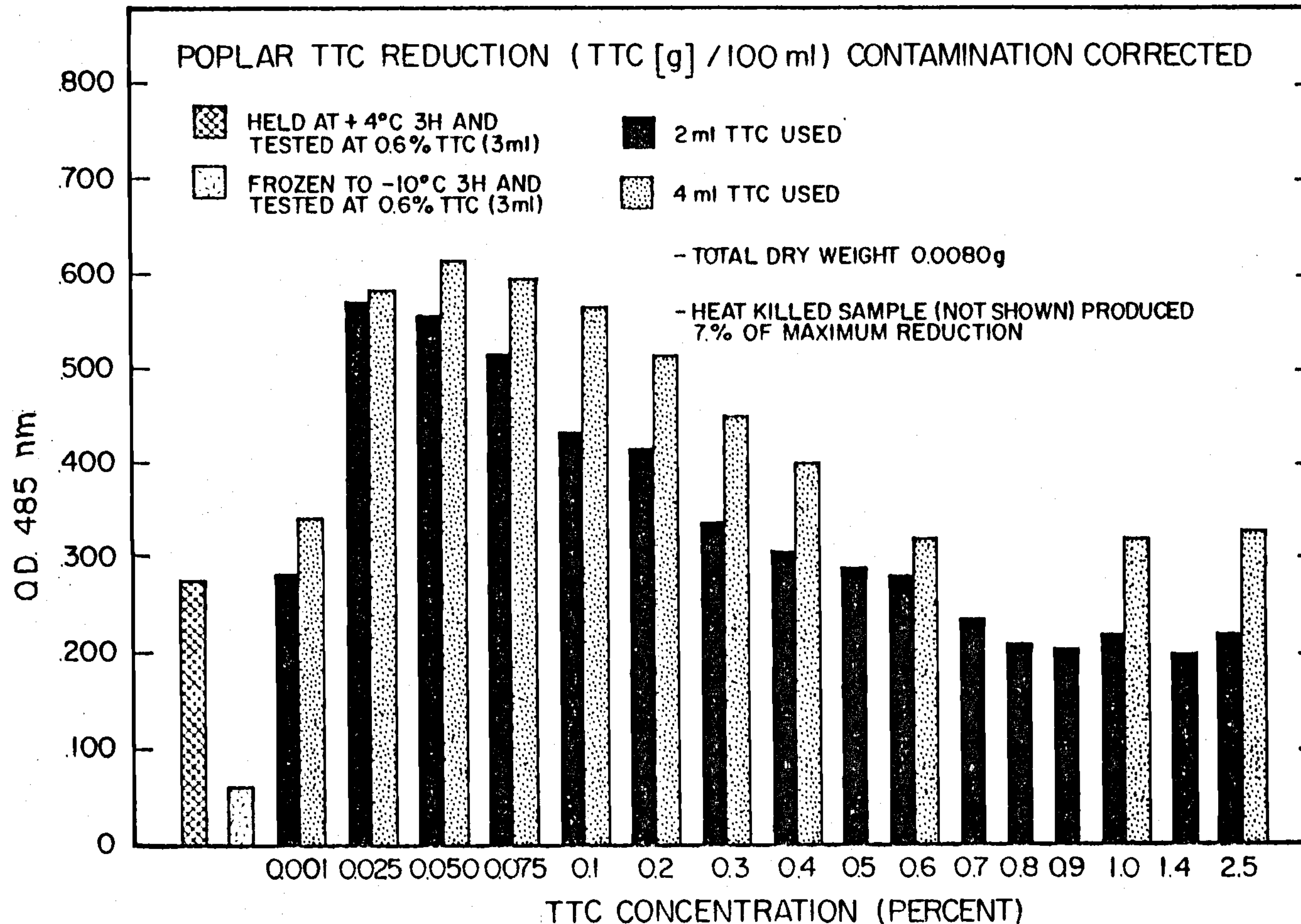


Figure 22. Poplar TTC reduction study. TTC at 0.001, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.4, and 2.5% were exposed to one weight of poplar material at two different volumes (2 ml and 4 ml). In addition cell samples were exposed for 3 hr at +4°C and frozen to -10°C before testing. O.D. was taken at 485 nm.

BUFFERED TTC * (78% Na HPO_4 +
22% KH_2 PO_4

↓

SAMPLE (x g)

↓

INCUBATE 15 hr at 35°C

↓

DRAIN OFF TTC

↓

WASH WITH DISTILLED H_2O

↓

CENTRIFUGE CELLS

↓

EXTRACT WITH HOT 95% ETOH
FOR 30 MIN IN HOT WATER BATH

↓

BRING TO VOLUME (x ml TOTAL) **

↓

READ IN SPECTROPHOTOMETER @ 485 nm

* Douglas-fir: Use 0.3% TTC
Poplar: Use 0.05% TTC

** Amount of volume 95% ETOH total used depends on amount of sample added per experiment.

Figure 23. Procedure for measuring reduction of TTC modified after Steponkus and Lamphear (1967)

killed by boiling 1/2 hr, the background was about 6% and is assumed to be contamination present in all sources (Table 3).

Table 3. Douglas-fir TTC reduction test (O.D. 485 nm/g fresh weight)

Fresh Weight (g)	O.D. Reading
.2110	2.029
.1190	1.534
.0500	.856
.0380	.778
.1250 (heat killed)	.094
% contamination - 6.0	

For both species, placing the test samples at +4°C for 3 hr did not effect the amount of TTC reduced, while freezing the samples to -10°C drastically reduced the TTC reaction amount.

Based on the results of these studies, all TTC reduction tests were conducted as shown on the flow sheet of TTC procedure (Figure 23). TTC reduction tests were begun between 2 and 4 hr after washing of the cell clumps was completed. For post-thaw TTC reduction, unless otherwise stated, the contents of at least 4 ampules were used per test. Each of the replications was read twice in the spectrophotometer.

b. Post-thaw culture and TTC reduction

The contents of two ampules were displaced, after thawing, into a 13 x 100 mm culture tube containing 4 ml of the respective medium and placed in the rotating apparatus described under "Growth Curves" (Figure 15).

After a variable (weeks) time period, the thawed and cultured samples were removed from the rotating apparatus and either dry weights (see "Methods and Materials" - Chapter 1) or TTC reductions (#5-a above) were measured. Data is the result of at least 4 replications (TTC reductions read twice per replication).

c. Determination of the effects of washing cryoprotectants from cell aggregates on cell survival of Douglas-fir

In order to determine the effects of washing with media following freezing to remove cryoprotectants, a washing experiment was carried out.

Cultures were carried through all steps given above for growing of suspension cultures and preparation of cultures for freezing. The DMSO and glycerol concentrations used were 0.2%, 2%, 5% and 10% with 4% sucrose. The cultures were exposed to cryoprotectants for 1.5 hr, as for freezing, but without freezing were washed with fresh media (see #5-c above). After 8 washings, the samples were either subjected to TTC reduction testing (#5-a above) or cultured according to #5-b. Where treated

cultures were grown, cultures were harvested and total dry weight per replication taken (see Chapter 1). Data is based on 4 replications per treatment.

d. Regeneration of shoots with thawed poplar material

Following thawing and washing, poplar material was either placed in "T" tubes containing 10 ml of liquid media or solid media, both containing ingredients specific for callus growth or placed in media specifically for organization into shoots (see Appendix B). Cultures initially placed on callus-forming media formed callus which was then placed on media for organogenesis after 2 subcultures (passages).

Ability to form shoots was examined by sectioning embedded material according to the methods used in Appendix B. Frozen material was compared to unfrozen treated controls forming shoots and roots.

7. Cold Conditioning of Douglas-fir and Poplar Callus and Seedlings of Douglas-fir:

Subcultured callus of Dougla-fir and poplar was grown on solid media under growth room conditions (see Chapter 1) for 3 weeks and transferred to a cold room to induce hardening. The temperature differential in the chamber was set at a high of +14°C and a low of +4°C for 7 and 17 hr respectively. Light was provided by General Electric cool white fluorescent and Sylvania clear 100 watt bulbs. Light intensity in the cold

room was 650 foot candles set at 7 hr light and 17 hr dark. Six Douglas-fir seedlings obtained locally were well-watered and placed in the cold room during the first week in May, 1980. Conditions in the chamber were as descrived above for callus. Potting soil was watered moderately at weekly intervals.

Callus material was cold conditioned at least 8 weeks before any experiments were made. Seedlings were conditioned at least 12 weeks before any testing.

8. Freezing to -196°C of Cold Conditioned Douglas-fir and Poplar Callus:

Eight week cold conditioned (#7 above) Douglas-fir and poplar callus was removed from the cold room and subjected to various treatments. Callus was either placed in freezing ampules and immersed in liquid nitrogen directly or was frozen slowly at 1°C/min to -40°C and then immersed in liquid nitrogen. These two rates of freezing were made with either dry callus (grown on solid agar medium and treated without liquid added to the ampules) or callus immersed in 10% DMSO, 5% glycerol and 25% sucrose at various times at room temperature for 1.5 hr before cooling was started.

In addition to having cryoprotectants added at room temperature, some cold conditioned cultures were held at +4°C for various times or frozen slowly to -10°C before further slow cooling to -40°C and immersion in liquid nitrogen or immersed

directly in liquid nitrogen from room temperature, +4°C or -10°C. For comparison, the same treatments were made on +22°C grown callus. After rapid thawing to 0°C in a +40°C water bath, air warming to room temperature and media washing of cells exposed to the various freezing treatments, TTC reduction tests were made accroding to #6-a above. Material of both species was also replaced on their respective medium and any growth observed.

A washing experiment as described in #6-c was made with Douglas-fir callus using 10% DMSO, 5% glycerol and 25% sucrose. Source of test material was from the cold room (8 weeks) or +22°C growth room. Material was exposed to the cryoprotectant solution either at room temperature or +4°C. Following washing, material was placed on solid medium for 2 months at room temperature under continuous low light (150 foot candles, fluorescent light) and growth observed. Results are based on at least 4 replications per treatment.

9. Freezing Douglas-fir Shoot Apices to -196°C:

Douglas-fir shoot apices were collected from 12 week cold conditioned seedlings (see #7 above) or from trees growing locally during August, 1980.

Buds with bud scales were dipped in 70% ethanol 2 to 3 times and washed with distilled, sterilized water for 5 min (see also "Poplar Bud Isolation", Appendix B). For those experiments requiring naked buds, bud scales were removed in a sterile

solution of 1% commercial Clorox, followed by sterile water washing for 5 minutes.

Buds were frozen quickly to liquid nitrogen temperature by immersing an ampule containing a bud directly in liquid nitrogen or more slowly at 1°C/min from +20°C to -40°C followed by immersion in liquid nitrogen. Either naked buds or buds containing bud scales were frozen dry or in a solution of 10% DMSO, 5% glycerol and 25% sucrose.

Samples were thawed for 5 min in a +40°C water bath and then left to warm in air for 2 hr.

Thawed apices were cultured either in "T" tubes containing 10 ml of Douglas-fir liquid media placed on the rotating apparatus or on solid media. Survival was measured as ability to grow. Results are based on at least 4 replications per treatment. Data evaluation was made as paired student's t tests as given at the end of "Methods and Materials", Chapter 1.

RESULTS

1. Growth Curves:

Douglas-fir dry weight increases was directly related to the increase in total cell number. After a short lag period, cell number increase became linear between 8 and 12 days. The rate became stationary after 12 days. After 22 days the cell number actually declined slightly while the dry weight rate continued to increase (Figure 24). It is suggested here that after some time

in stationary phase, some lysis of cells may take place while individual cells still increase volume and thereby account for any small increase in dry weight. Material used as freezing samples were grown in suspension culture and harvested after 10 days.

Poplar cultures produced a growth curve which became linear much faster than Douglas-fir, attaining its maximum cell number increase and weight increase per unit time after only 3 days lag. Stationary phase was fully established after 10 days. Material used as freezing samples were grown in suspension cultures in the presence of 75 ppm EDTA for 6 days and then harvested for testing (Figure 25).

With both species, material was filtered through a 550 μ screen to ensure some control over maximum aggregate size since apparently growth efficiency can change depending on aggregate size (see "Results", Chapter 1).

Since cultures of both species were harvested in the middle of their respective log phases of growth, the physiological state of the tissues was fairly uniform when used for the freezing tests. Cells in log phase of growth are assumed to be small, have dense cytoplasm and lower water content than those cells in stationary phase of growth.

2. Toxicity Studies:

It was found that the results which could be obtained in an experiment varied greatly as a function of the overall

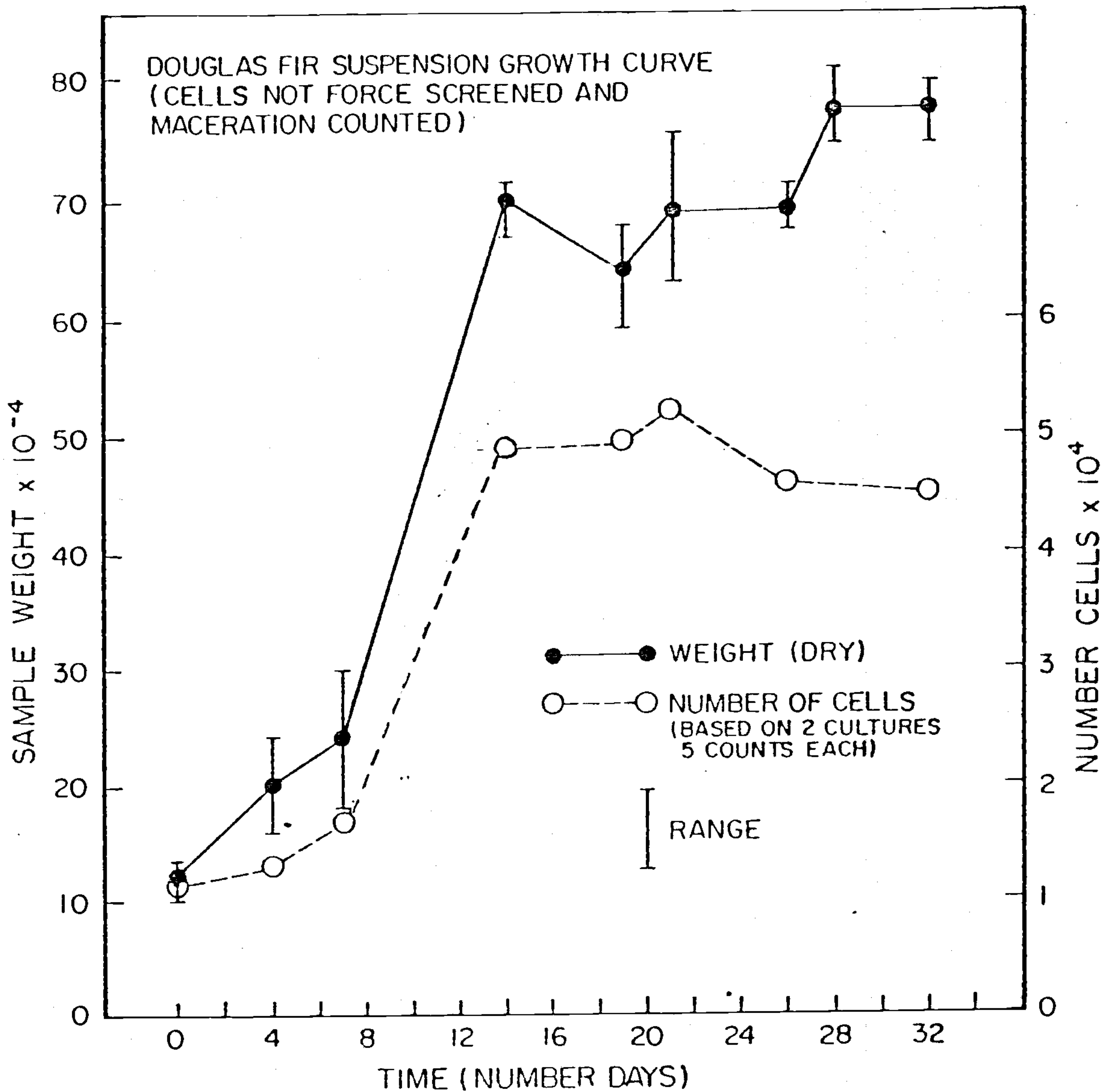


Figure 24. Douglas-fir suspension culture growth curve shown as both increase in total dry weight and cell number between 0 and 32 days. The greatest increase was observed at between 7 and 12 days. Material was filtered through a 550 µ screen at time zero. The filteration was passive, the cells not being forced through the screen. N=4

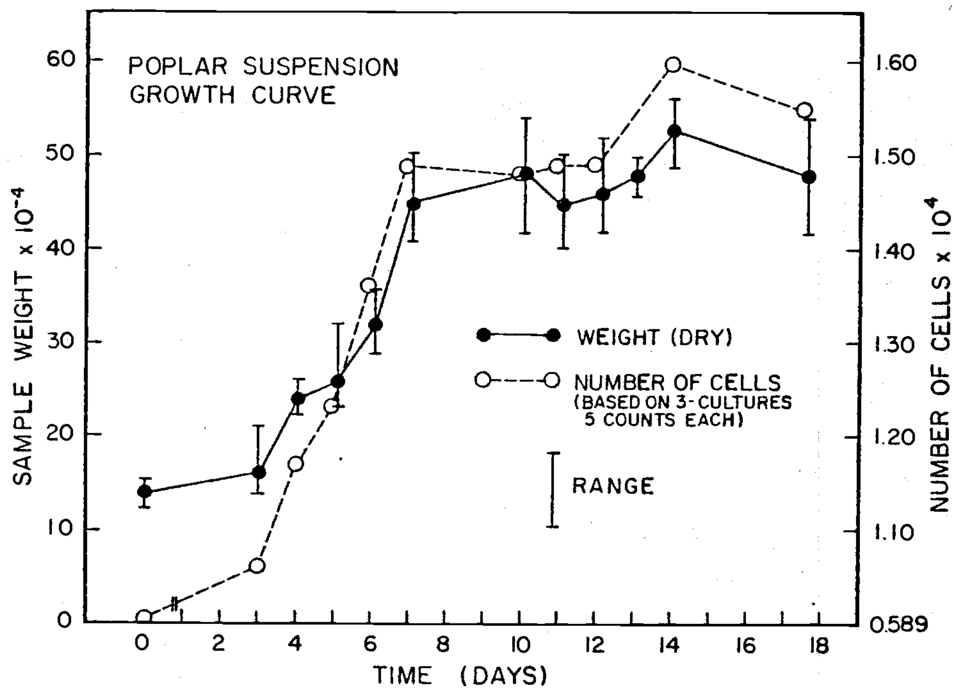


Figure 25. Poplar suspension culture growth curve shown as both increase in total dry weight and increase in total cell number between 0 and 18 days. The greatest increase was observed between 3 and 7 days. Cultures were filtered through a 550 μ screen at time zero. N=4

physiological condition and to some extent on the initial quantity of cells used. When older, stationary phase cultures were used as test material, DMSO, even at 20% produced more dry weight than did the control. Maximum dry weight was recorded at 1% DMSO (Figure 26). When glycerol was put in the growth medium of older cultures, maximum dry weight was found at 20% glycerol and minimum dry weight at 0.0% (control) glycerol (Figure 27).

The results obtained were dramatically different when the log phase growth suspension cultures were used as test material. With DMSO, treated test cell dry weight decreased significently at 20% DMSO compared to initial weight. Growth increased from initial value, but was still only 2/3 of the control value when 1% DMSO was used. Growth rate was doubled over control when 1% DMSO was used. Growth rate was doubled over control when 0.1% DMSO was included in the growth media (Figures 28 and 29).

With high glycerol concentrations (20% and 10% glycerol) a significent decrease in dry weight compared to initial dry weight was observed. With 1% glycerol in the media gorwth was about 1/3 the control value and 0.1% glycerol, dry weight was approximately equal to the control, the difference being nonsignificent (Figures 28 and 30).

When the toxicity of hydroxy-free-L-proline was tested on log phase growth suspension cultures of Douglas-fir, a slight, although nonsignificant, total dry weight decrease occurred even

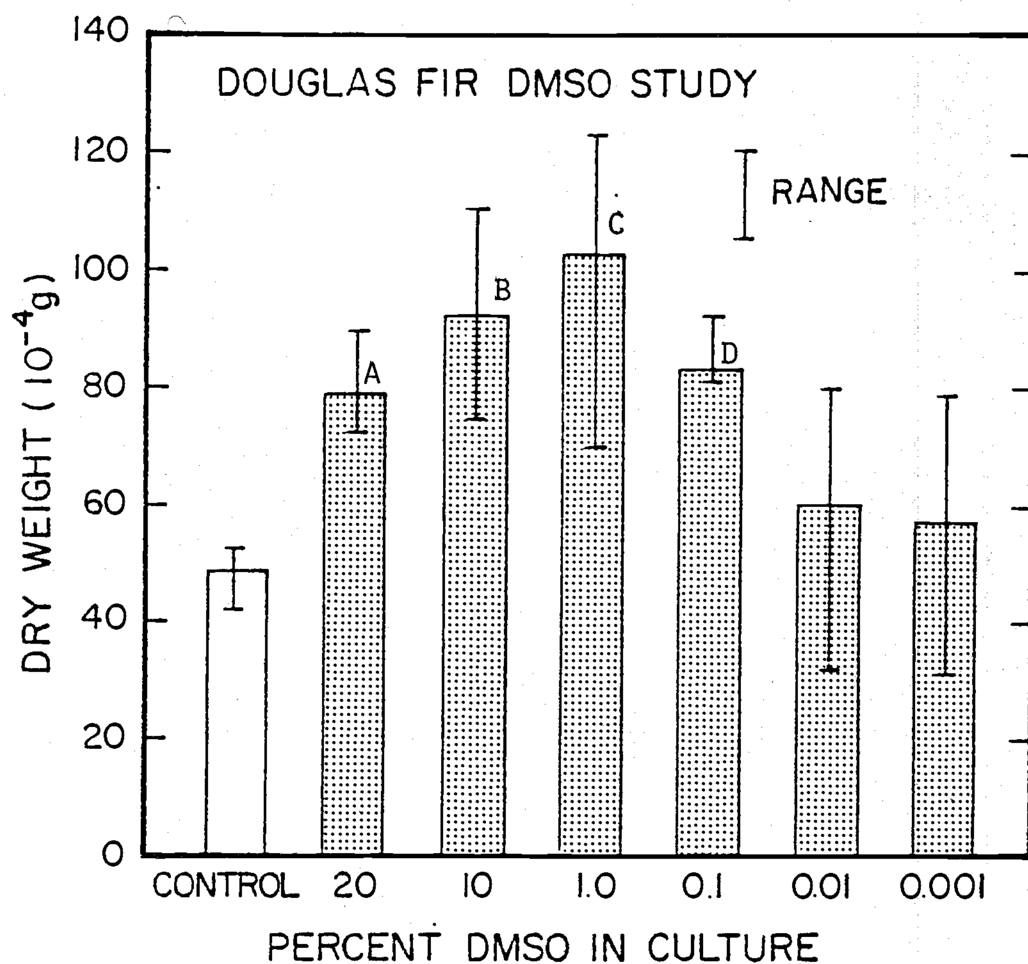


Figure 26. Effects of DMSO after 40 days on the growth rate of old, stationary growth phase suspension cultures of Douglas-fir. N=4 A:B=N.S.; A:C=*; A:D=N.S.

* Indicates a significant difference at the 95% confidence level.

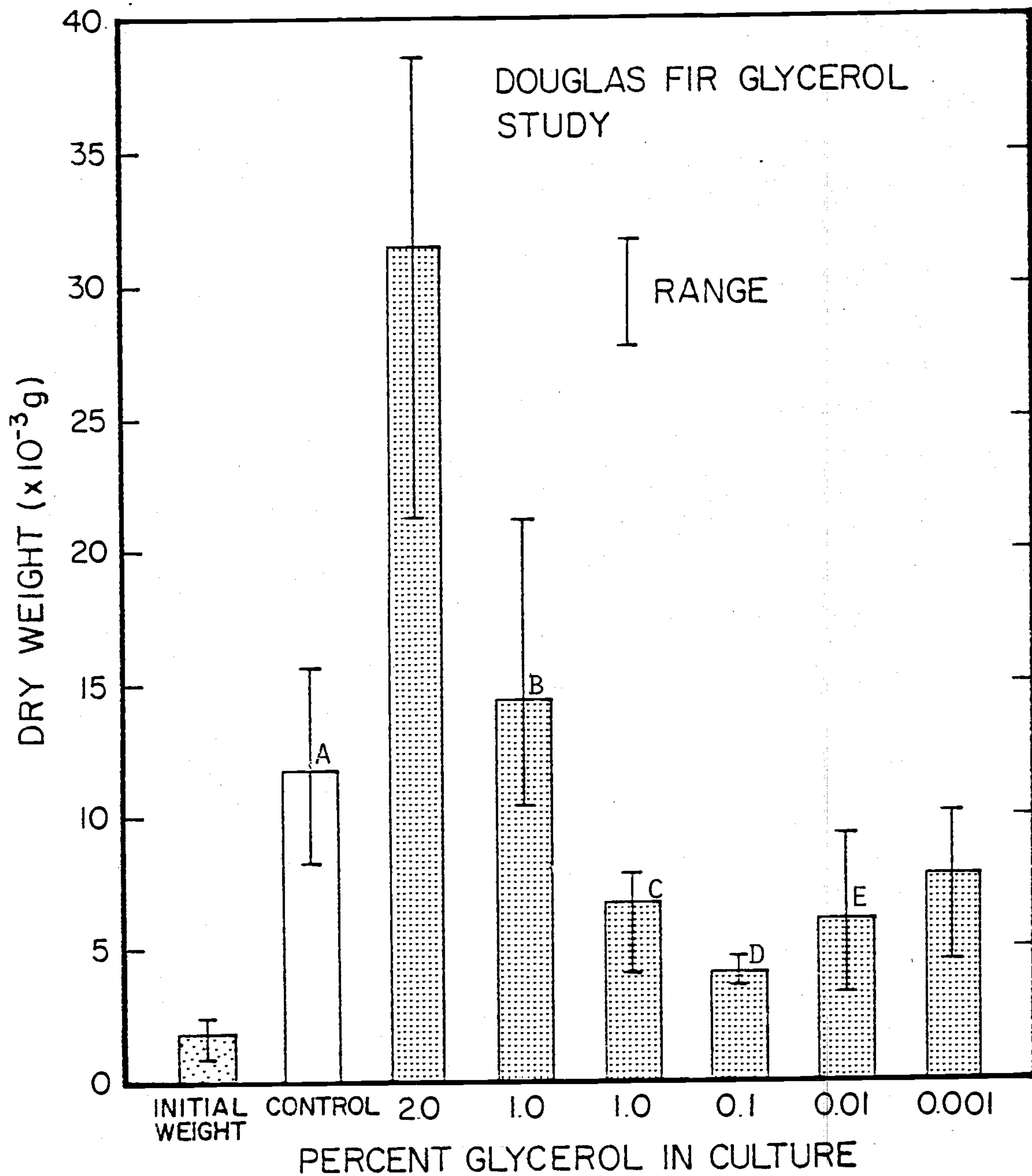


Figure 27. Effects of glycerol after 40 days on the growth rate of old, stationary growth phase suspension cultures of Douglas-fir. N=4 A:B=N.S.; C:D=N.S.; C:E=N.S.

* Indicates a significant difference at the 95% confidence level.

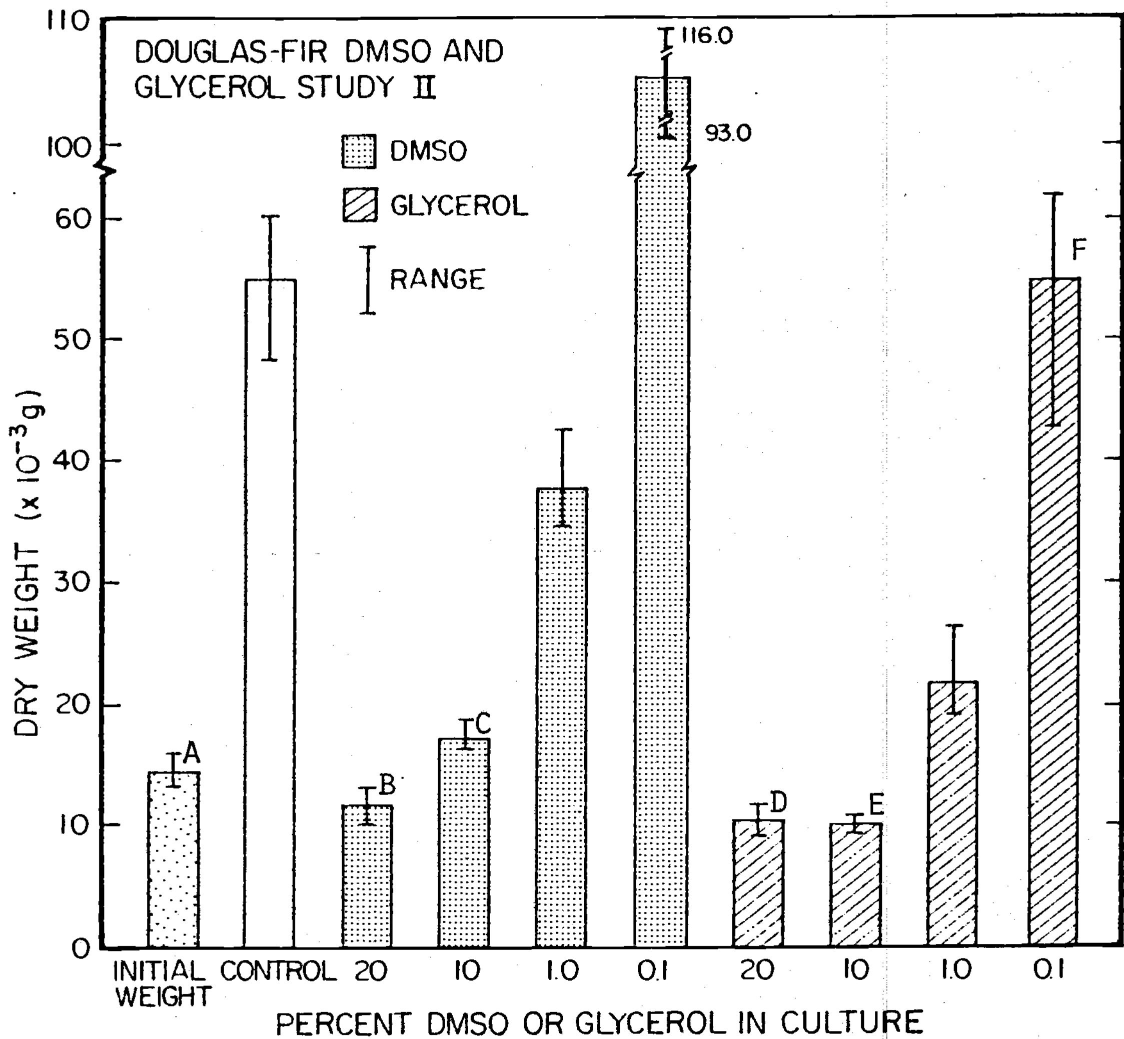


Figure 28. Effects of DMSO and glycerol 34 days after start of experiment on cells taken in log phase of growth. Initial experimental dry weight, control, percent amounts of DMSO or glycerol are shown. Total dry weight was taken 35 days after start of test. N=4 A:B=*; A:C=*; A:D=*; A:F=N.S.; B:C=*; D:E=N.S.

* Indicates a significant difference at the 95% confidence level.

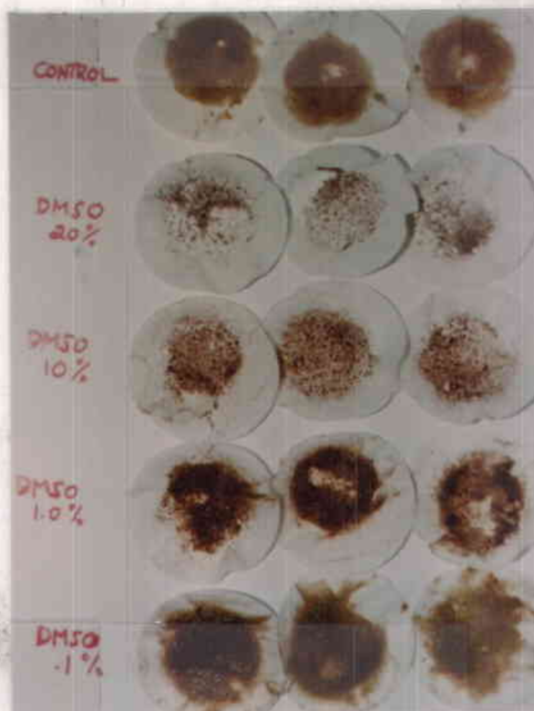


Figure 29. Amount of Douglas-fir culture material produced with different concentrations of DMSO in culture media. Three out of four replications are shown.

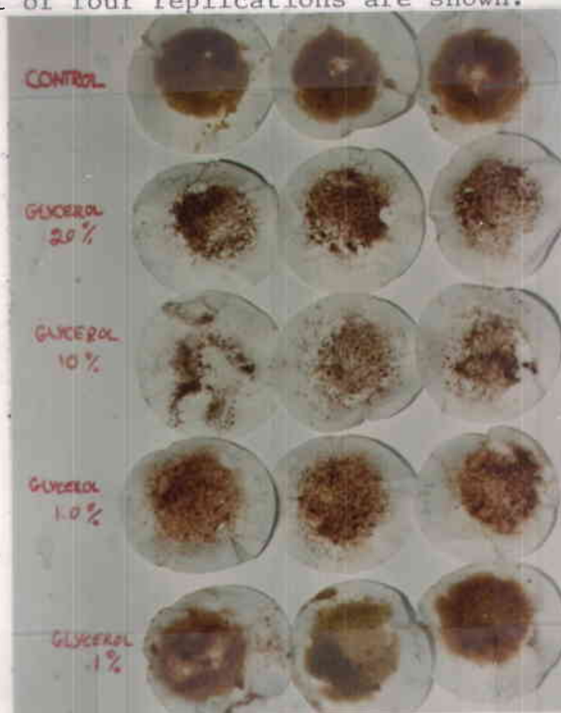


Figure 30. Amount of Douglas-fir culture material produced with different concentrations of glycerol in culture media. Three out of four replications are shown.

at 0.01% proline. Growth was reduced to half at 1% proline addition and to almost 1/10 the control at 20% (Figure 31).

The addition of DMSO had similar effects on poplar log phase growth culture as on Douglas-fir. High concentrations largely inhibited any dry weight increase. At 1% DMSO, total dry weight almost doubled and at 0.1% almost triple. Concentrations of 0.01% and 0.001% DMSO produced total dry weights nonsignificently different from the control value, suggesting little effect of the DMSO at these levels (Figure 32). Color and clump size changes are shown in Figure 33.

When glycerol was added to log phase, suspension cultures of poplar, results somewhat reflected those of Douglas-fir. All glycerol concentrations above 0.001% proved somewhat inhibitory to total dry weight increase. Glycerol at 10% in medium increased dry weight slightly more than 1/2 control value, while 0.001% glycerol resulted in total dry weight equal to control (Figure 34).

3. Cryoprotectant Washing Experiments:

To observe any differences that might occur from exposure to cryoprotectants and subsequent washing of test material, Douglas -fir cultures were exposed to cryoprotectant 1.5 hr then washed with 25 times (v/v) of test medium and either TTC tested or cultured.

Exposure to cryoprotectants at higher concentrations even with subsequent washing with media is detramental to the survival

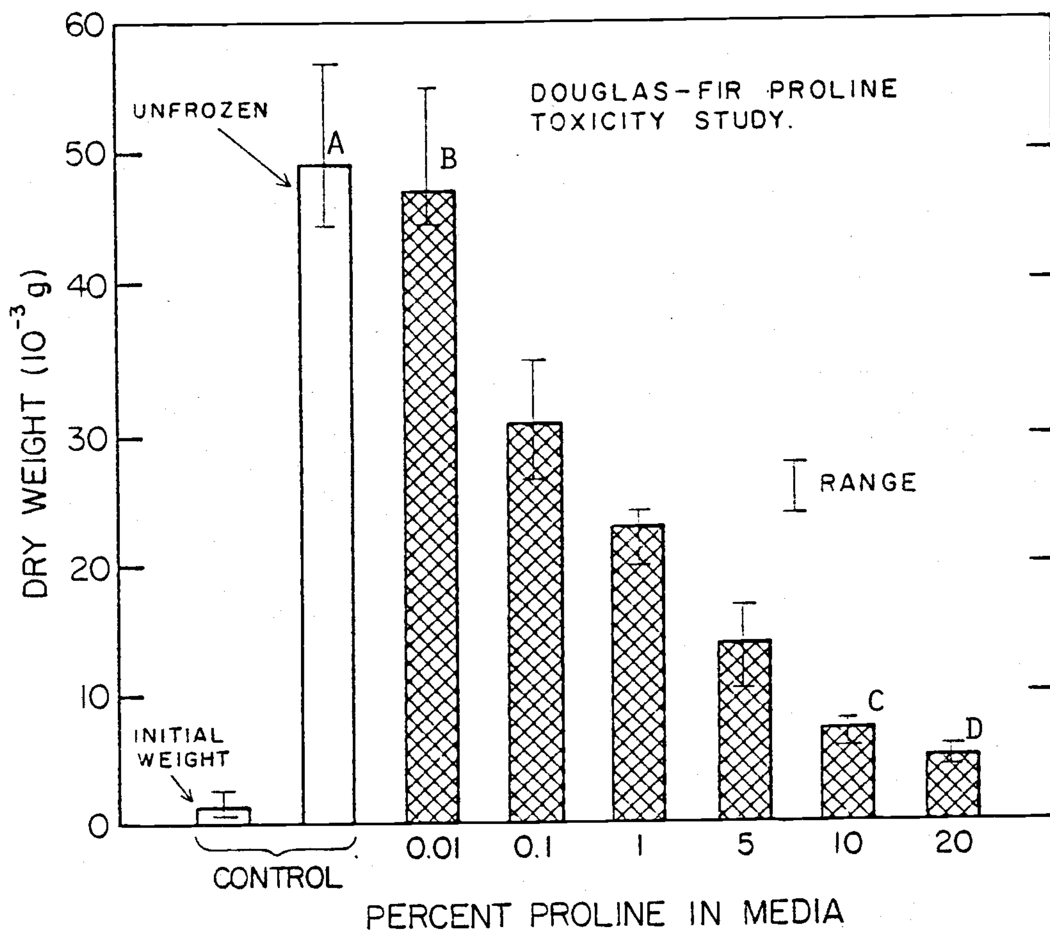


Figure 31. Effects of hydroxy-free-L-proline on the growth of log phase suspension cultures of Douglas-fir. Total dry weight was taken 35 days after start of test. N=4 A:B=N.S.; C:D=*

* Indicates a significant difference at the 95% confidence level.

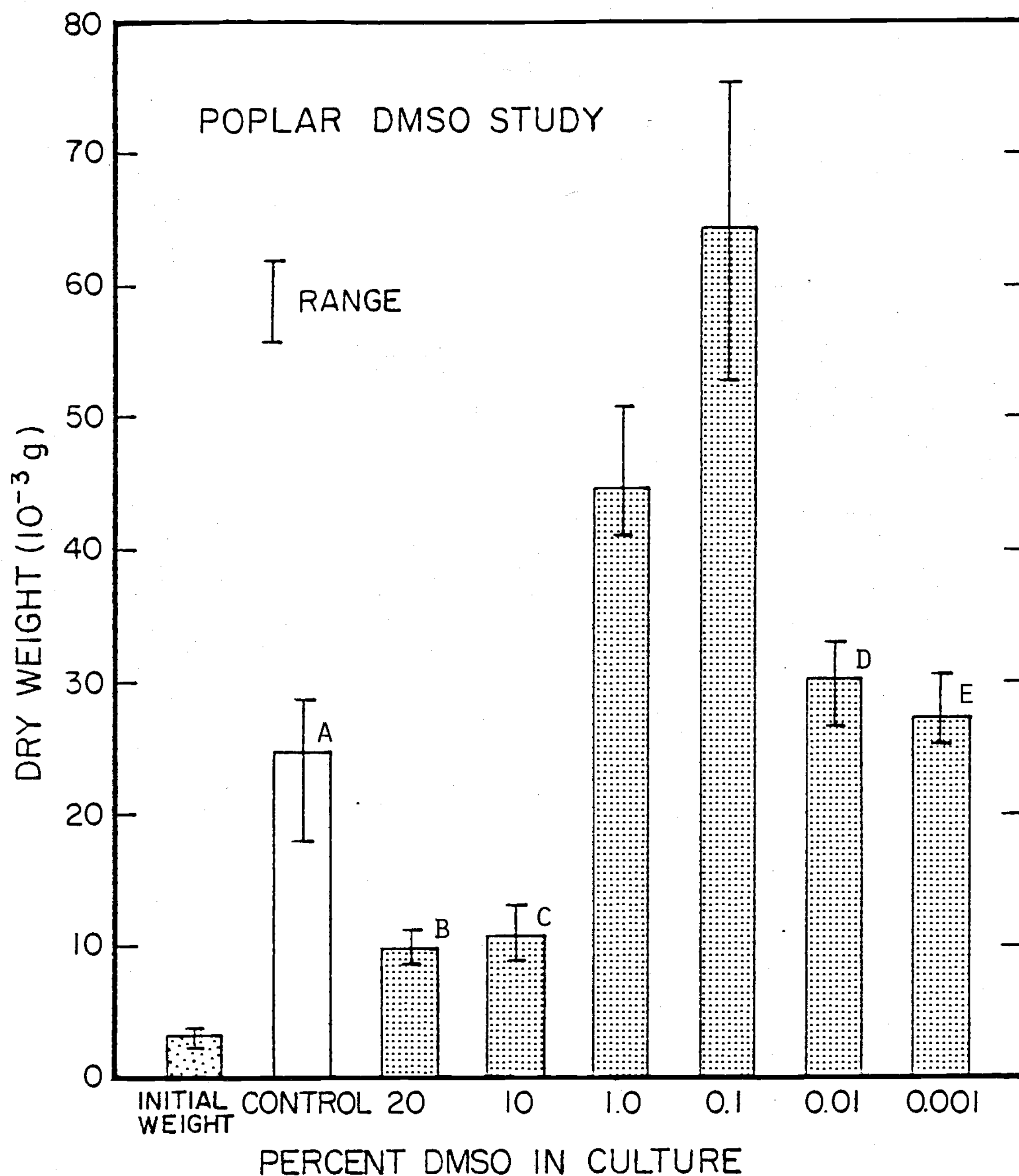


Figure 32. Effects of DMSO on the growth of log phase suspension cultures of poplar. Total dry weight taken 28 days after start of test. N=4 A:D=N.S.; B:C=N.S.; D:E=N.S.

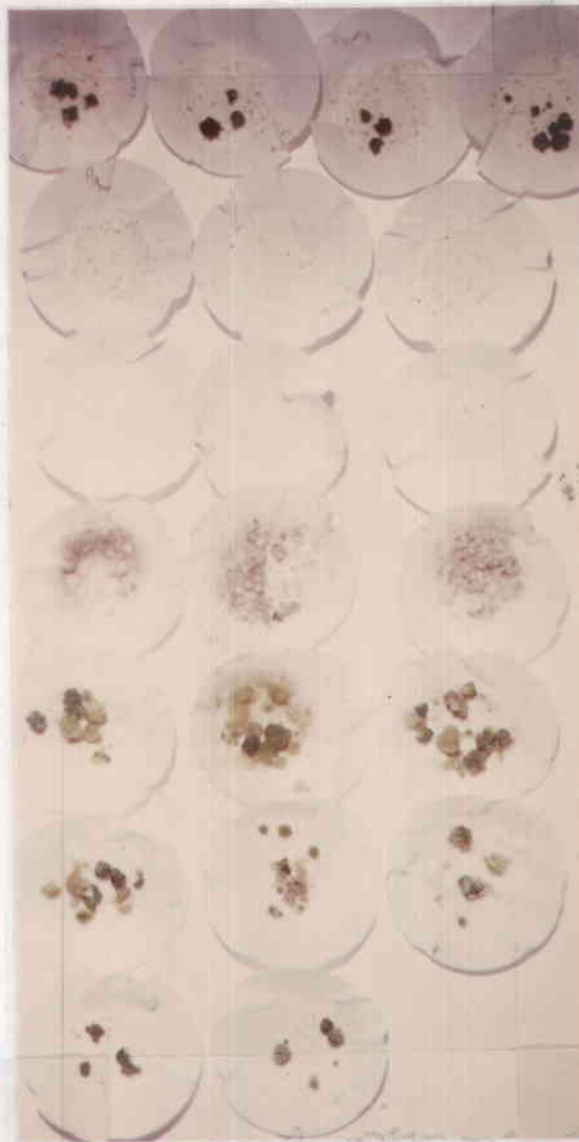


Figure 33. Effects of DMSO at various concentrations from 20% to 0.001% on the growth of poplar liquid cell cultures. Notice complete lack of growth at 20% and 10%; mass of growth (> over control) at 1%, 0.1% and 0.01% and return to approximately equal growth to control at 0.001%. Notice great changes in aggregate size and cell color between 0% and 0.1% DMSO. These results suggest that perhaps 0.5% DMSO in growth media may give small aggregates which may be green in color.

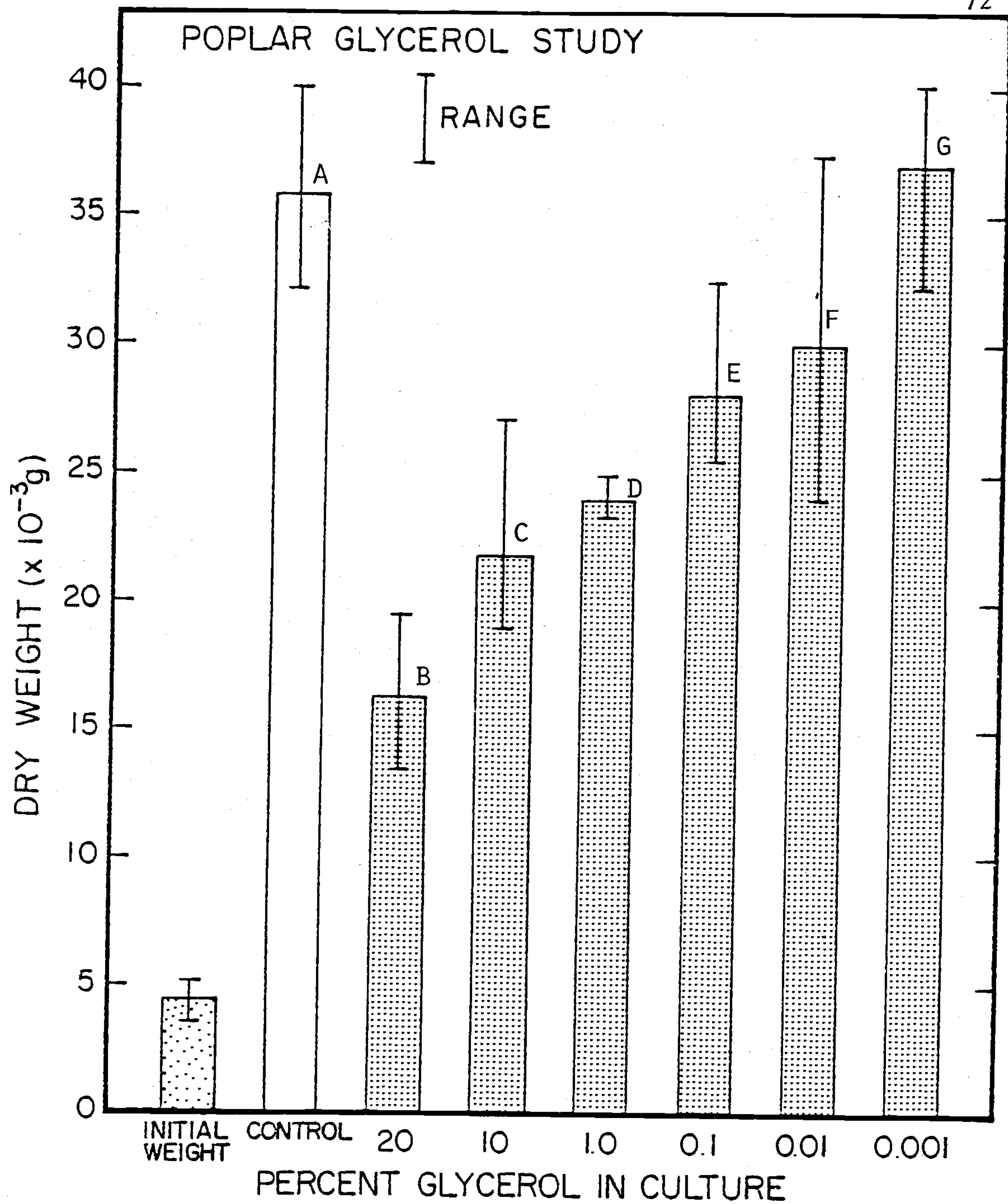


Figure 34. Effects of glycerol on the growth of log phase suspension cultures of poplar. Total dry weight taken 28 days after start of test. N=5 A:G=N.S.; A:F=*; B:C=*; C:D=N.S.; C:E=*; D:E=*; E:F=N.S.; F:G=*

* Indicates a significant difference at the 95% confidence level.

of the cell. DMSO at 5% or above caused an immediate 10% decrease in TTC reduction. At 10% DMSO there was a significant 30% decrease in TTC reduction compared to the control. At 2% DMSO or less, there was no significant effect on the reduction ability compared to the control.

Glycerol appears to be more detramental to Douglas-fir cells than does DMSO at comparable concentrations. Glycerol at 2% caused a nonsignificant 10% decrease in TTC reduction and at 5% caused a significant 40% decrease compared to the control (Figures 35 and 36).

Douglas-fir cells exposed to 2% and 5% DMSO then washed, showed an increase in growth efficiency. Exposure to 5% DMSO then washing tripled the total dry weight in some cases, and washing after 2% DMSO exposure resulted in a doubling of the dry weight. DMSO at 0.2% caused growth equal to the control, while 10% was inhibitory, causing nonsignificant growth over initial experimental dry weight (Table 4, Figures 37 and 38).

The effect of glycerol on washing was less dramatic than DMSO, but a significant increase of dry weight was observed at 2%, compared to control. Glycerol at 5% and 10% caused no increase in dry weight over initial experimental dry weight, indicating no growth (Table 4, Figures 37 and 38).

It should be noted that washing alone does not affect the growth rate in that the unwashed control and washed control values are similar, the differences being nonsignificant (Table 4, Figures 37 and 38).

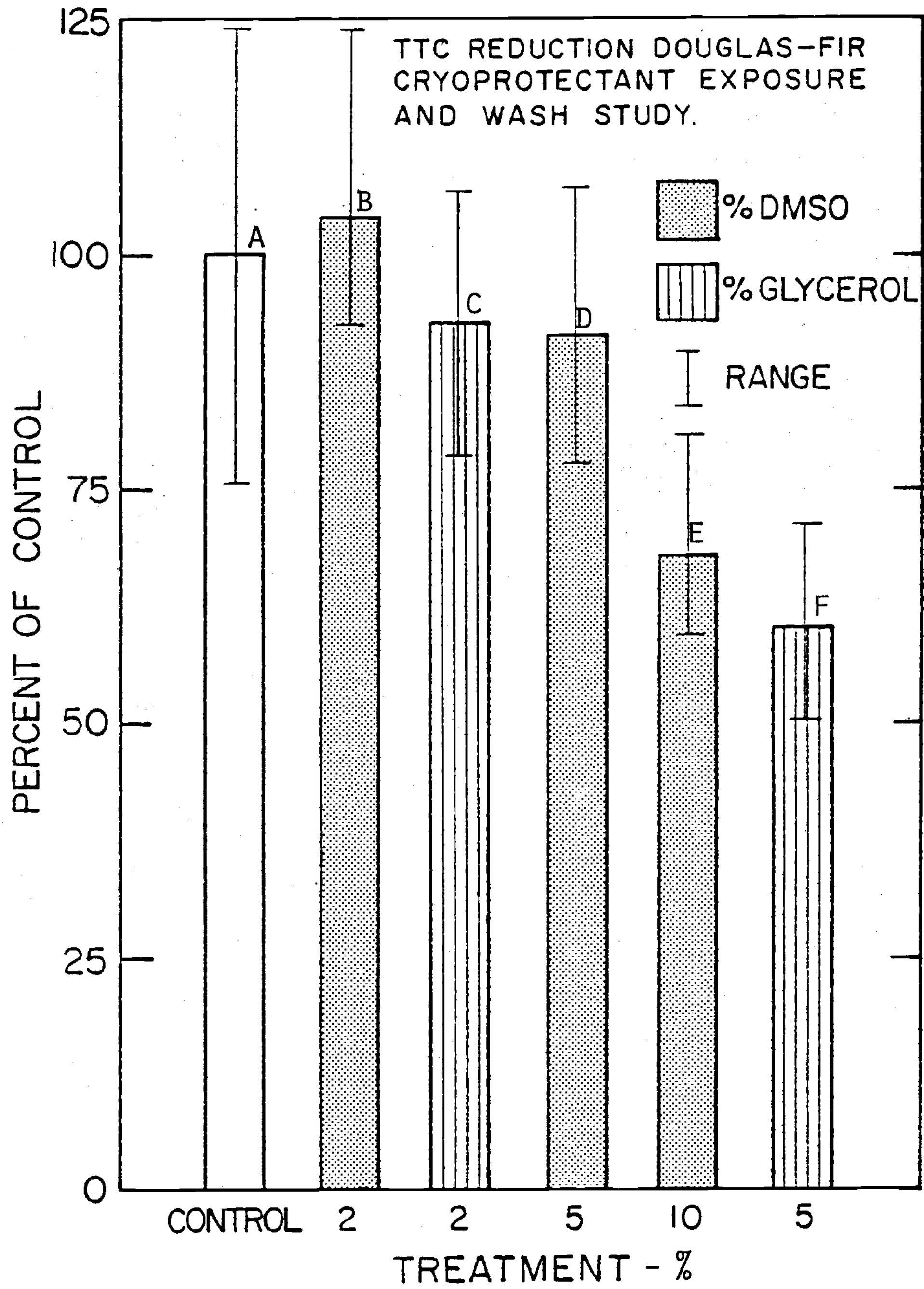


Figure 35. Effects of cryoprotectant (DMSO or glycerol), exposure for 1.5 hr and subsequent washing 8 times with 25 times fresh medium (v/v) on Douglas-fir test material. Shown are immediate TTC reduction amounts on washing, observed at 485 nm. N=4 A:B=N.S.; A:C=N.S.; A:E=*; B:C=N.S.; B:D=N.S.; D:E=*; E:F=N.S.

* Indicates a significant difference at the 95% confidence level.

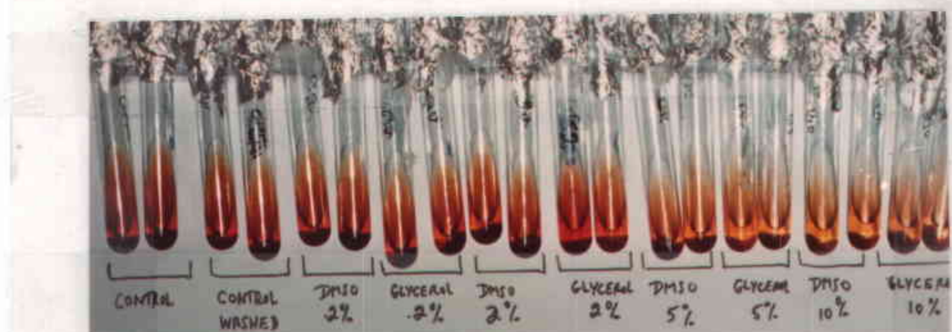


Figure 36. Effects of cryoprotectant exposure 1.5 hr and subsequent washing in TTC reduction of Douglas -fir test material. Note lighter color at higher concentrations of cryoprotectants.

Figure 41. Effects of cryoprotectants in combinations exposed to and washed from poplar in the same manner as for Douglas-fir. Material was cultured on solid agar medium. All cultures, with the possible exception of DMSO 5%/glycerol 5%, showed less growth than control.

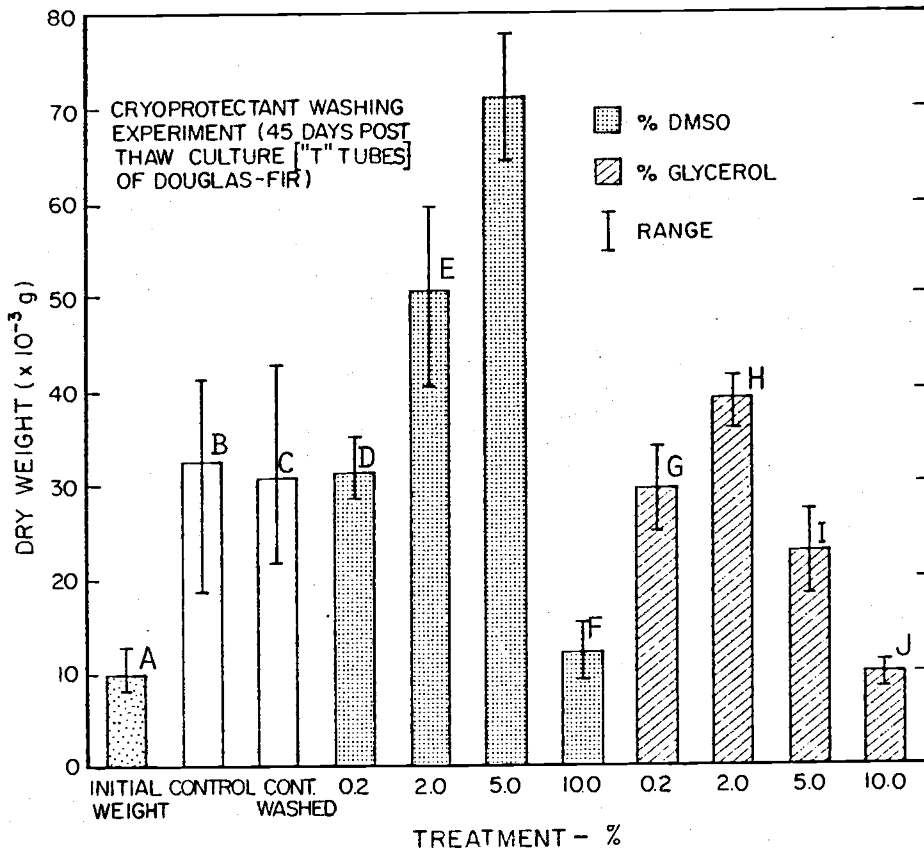


Figure 37. Effects of exposure and washing of cryoprotectants on the post treatment culture (45 days) of Douglas -fir cultures. Cultures were grown in "T" tubes. N=4 A:F=N.S.; A:J=N.S.; B:C=N.S.; B:D=N.S.; C:E=*; C:G=N.S.; C:H=*; G:H=*; G:I=*

* Indicates a significant difference at the 95% confidence level.

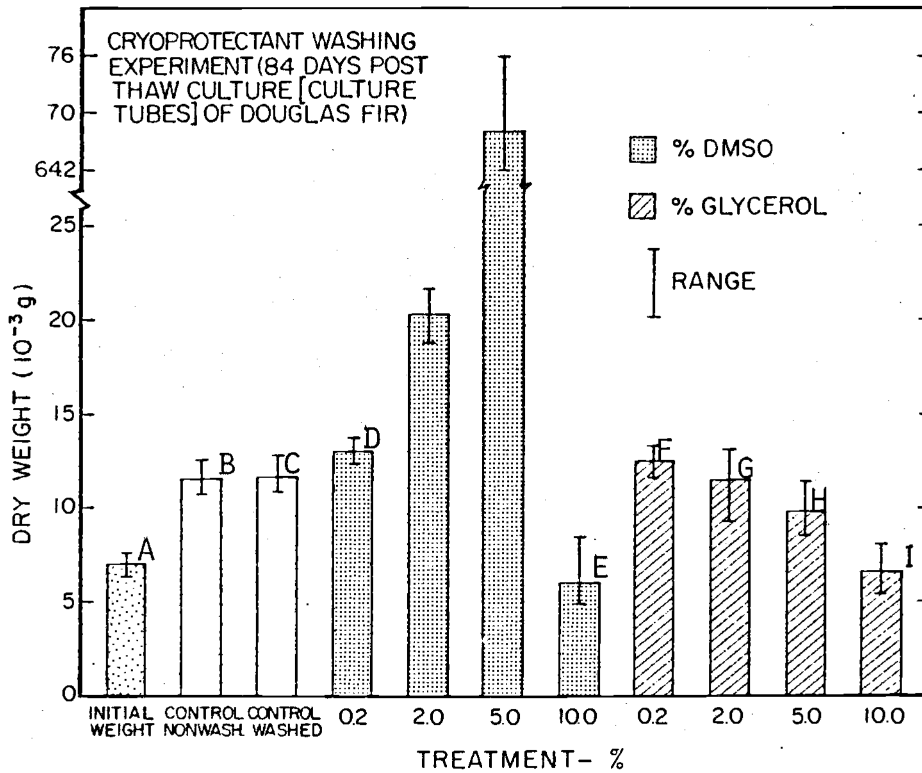


Figure 38. Effects of exposure and washing of cryoprotectants on the post treatment culture (84 days) of Douglas -fir cultures. Cultures were grown in 13 x 100 mm culture tubes. N=4 A:E=N.S.; A:I=N.S.; B:C=N.S.; C:D=N.S.; F:G=N.S.; F:H=*

* Indicates a significant difference at the 95% confidence level.

Table 4. Effects of cryoprotectant exposure for 1.5 hr and subsequent washing with 8 times, 25 times (v/v) Douglas-fir test material. Total culture time was 25 days in 13 x 100 mm culture tubes. N=4
* Indicates a significant difference at the 95% confidence level from initial weight.

TREATMENT	DRY WEIGHT (x 10^{-3}g)
Initial Weight	5.4
Control (unwashed)	9.8 *↑
Control (washed)	10.4 *↑
DMSO 10%	5.2
DMSO 5%	11.4 *↑
Glycerol 10%	5.8
Glycerol 5%	6.8

The Douglas-fir cell cultures after washing and culture were much greener at 2% and 5% DMSO than the controls (Figures 39 and 40). Glycerol exposure at 5% and washing produced slightly less total dry weight than the control (Figures 37 and 38) but cell cultures were light brown compared to the ash-gray observed when no growth took place at 10% glycerol.

When the type of vessels in which post treatment culture took place are compared, it appears that somewhat better separation of results are obtained with "T" than 13 x 100 mm culture tubes. The better aeration and medium mixing which can be obtained with the former could be at least part of the explanation.

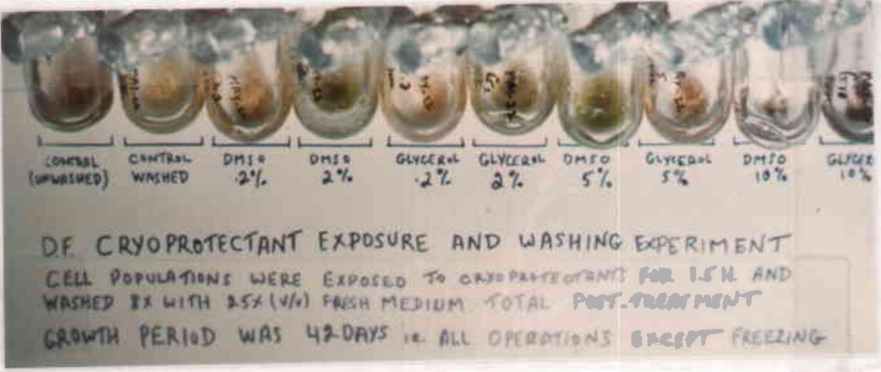
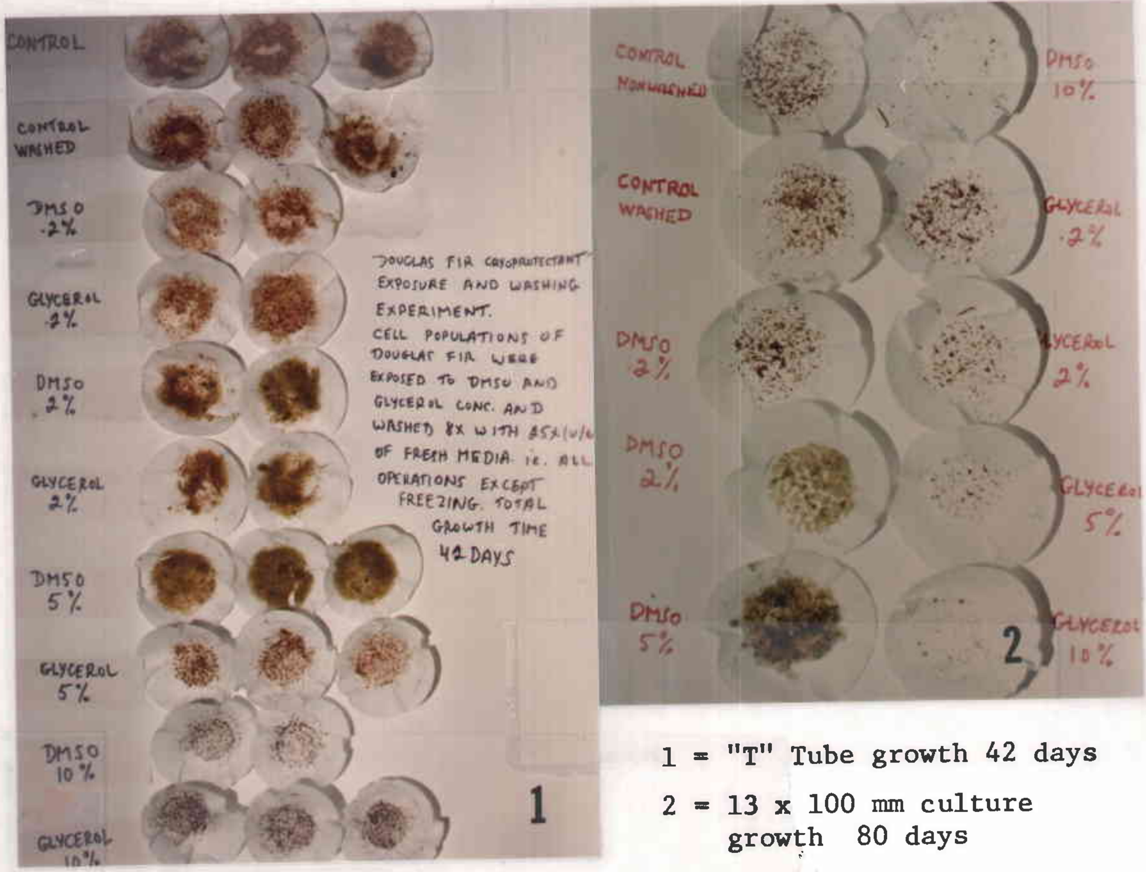


Figure 39. Douglas-fir cell clump amounts and color after exposure to cryoprotectants followed by washing. Photo taken after 42 days treatment culture. Notice green color at DMSO at 2% and 5%.



1 = "T" Tube growth 42 days

2 = 13 x 100 mm culture growth 80 days

Figure 40. Douglas-fir cell clumps shown in Figure 39, but on filter paper. Notice the color difference. Cultures washed in 5% glycerol, although not producing an increase in dry weight are less ash-gray than cells washed at 10% glycerol. Notice green color at 2% and 5% DMSO. Shown are the growth effects in "T" tubes (1) and in 13 x 100 mm culture tubes (2) at 42 days and 80 days post treatment growth respectively.

Exposure to and washing of cryoprotectants also affected the post treatment culture of poplar. When poplar callus was exposed to cryoprotectants and washed in a manner similar to Douglas-fir cultures, all the callus cultures with the possible exception of DMSO 5%/glycerol 5%, had reduced growth rate compared to the control when cultured on solid medium (Figure 41).

4. Freezing of Suspension Cultures of Douglas-fir and Poplar:

a. Freezing in the presence of single cryoprotectants

Highest survival rate was obtained with 5% DMSO when Douglas -fir suspension cultures were exposed to single cryoprotectants of DMSO and glycerol, then immersed directly in liquid nitrogen, thawed quickly in a +40°C water bath to 0°C and then in air to room temperature. This 28% survival rate, compared to control, was about 4% more than could be attained with 5% glycerol. However, the difference between 5% DMSO/5% glycerol and media alone was non-significant. Both cryoprotectants at 10% concentration showed less than 10% TTC redcution, with the amounts being signi-ficently less than that when frozen in media alone (Figure 42). A visual example of the TTC reduction can be seen in Figure 43. Protoplast shrinkage was quite severe even at 5% DMSO under these conditions (Figure 44).

Cultures treated as above grew little after 41 days post-thaw culture. The type of vessel used or the the addition of proline in the culture made little difference (Table 5).

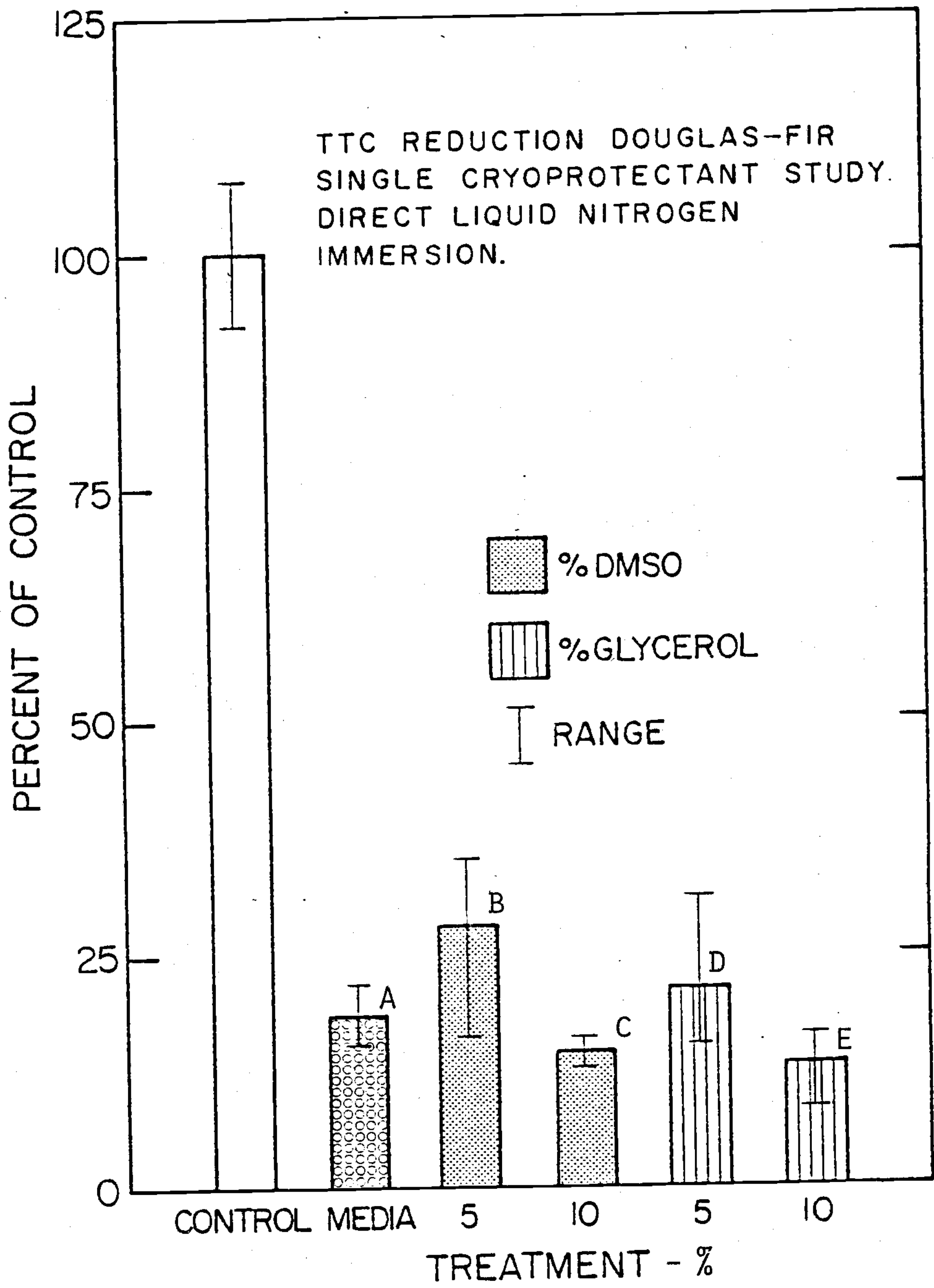


Figure 42. Effects of single cryoprotectants on TTC reduction of Douglas-fir suspension cultures immersed directly in liquid nitrogen. Cryoprotectants DMSO and glycerol were used singly. Thawing was fast in a +40°C water bath to 0°C then to room temperature in air. N=5 A:B=N.S.; A:C=*; A:D=N.S.; B:C=*; B:D=N.S.; D:E=*

* Indicates a significant difference at the 95% confidence level.

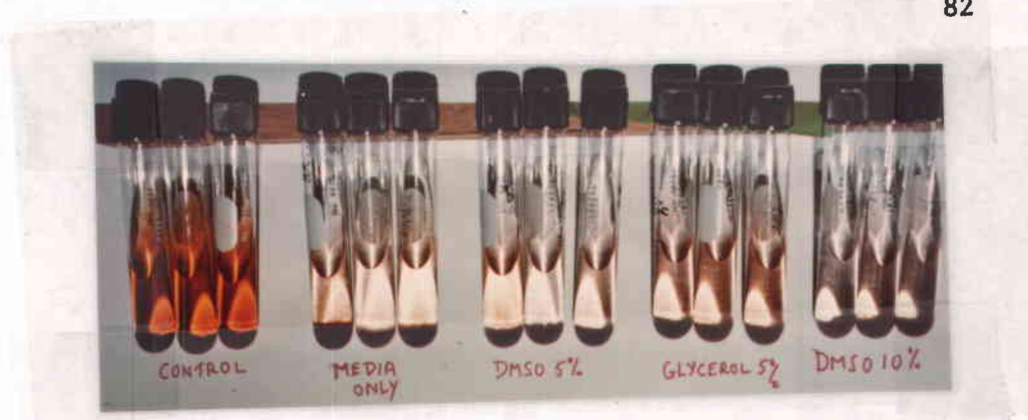


Figure 43. TTC reduction of Douglas-fir using single cryoprotectants and direct immersion into liquid nitrogen. DMSO at 5% was somewhat paler because of the camera flash.

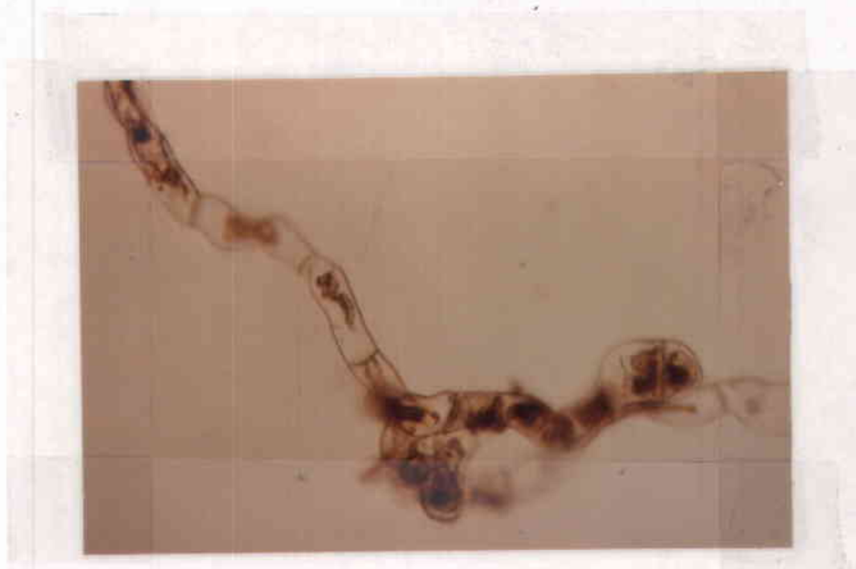


Figure 44. Cell damage in Douglas-fir culture when treated with DMSO at 5%, direct liquid nitrogen exposure, fast thawing to $0^{o}C$ in a $+40^{o}C$ water bath and then slowly to room temperature. Notice cell membranes have completely separated from the cell wall and the cell appears to be severely dehydrated in some cases; while in others there appears to be a complete cell lysis.

Table 5. Douglas-fir single cryoprotectant exposure and direct liquid nitrogen immersion experiment. Post-thaw culture with 5% proline was 41 days in two types of containers. N=4 * Indicates a significant difference compared to initial weight at the 95% confidence level.

TREATMENT	TOTAL DRY WEIGHT ($x 10^{-3}g$)	
	Test Tube ($x 10^{-3}g$)	"T" Tube ($x 10^{-3}g$)
Initial Weight	2.9	2.9
Control	19.2 *↑	19.6 *↑
DMSO 5%	3.6	4.7
DMSO 5% post-thaw growth in 5% proline	3.4	3.6
DMSO 10%	3.5	3.9
DMSO 10% post-thaw growth in 5% proline	2.6	4.0
Glycerol 5%	4.1	3.0
Glycerol 5% post-thaw culture in 5% proline	3.7	3.4
Media only	3.7	2.7

Douglas-fir cultures cooled slowly to -15°C at 1°C/min, then immersed into liquid nitrogen for 5 days and thawed, gave results parallel to direct liquid nitrogen immersion treatment when 0.2% and 2% cryoprotectants were used. Cryoprotectants at 0.2% proved non-significently different and 2% DMSO significently different (about 8%), better than media only (Figure 45).

Little difference existed between treatments when they were cultured for 24 days. Total dry weight paralleled the initial experimental dry weight although control growth was significantly greater (Table 6).

Table 6. Effect of DMSO or glycerol (0.2% and 2%) on dry weight of frozen Douglas-fir cultures. Douglas-fir cells were frozen slowly to -15°C, then to liquid nitrogen for 5 days, then thawed fast in +40°C water bath and cultured for 24 days. N=4 * Indicates a significant difference compared to initial weight at the 95% confidence level.

TREATMENT	DRY WEIGHT (x 10^{-3}g)
Initial weight	5.8
Control	22.5 *↑
Media only	6.0
DMSO 0.2%	6.6
DMSO 2%	6.4
Glycerol 0.2%	5.3
Glycerol 2%	6.1

Greater survival compared to slow freezing to -15°C, then -196°C occurred, according to the TTC reduction test, if cultures

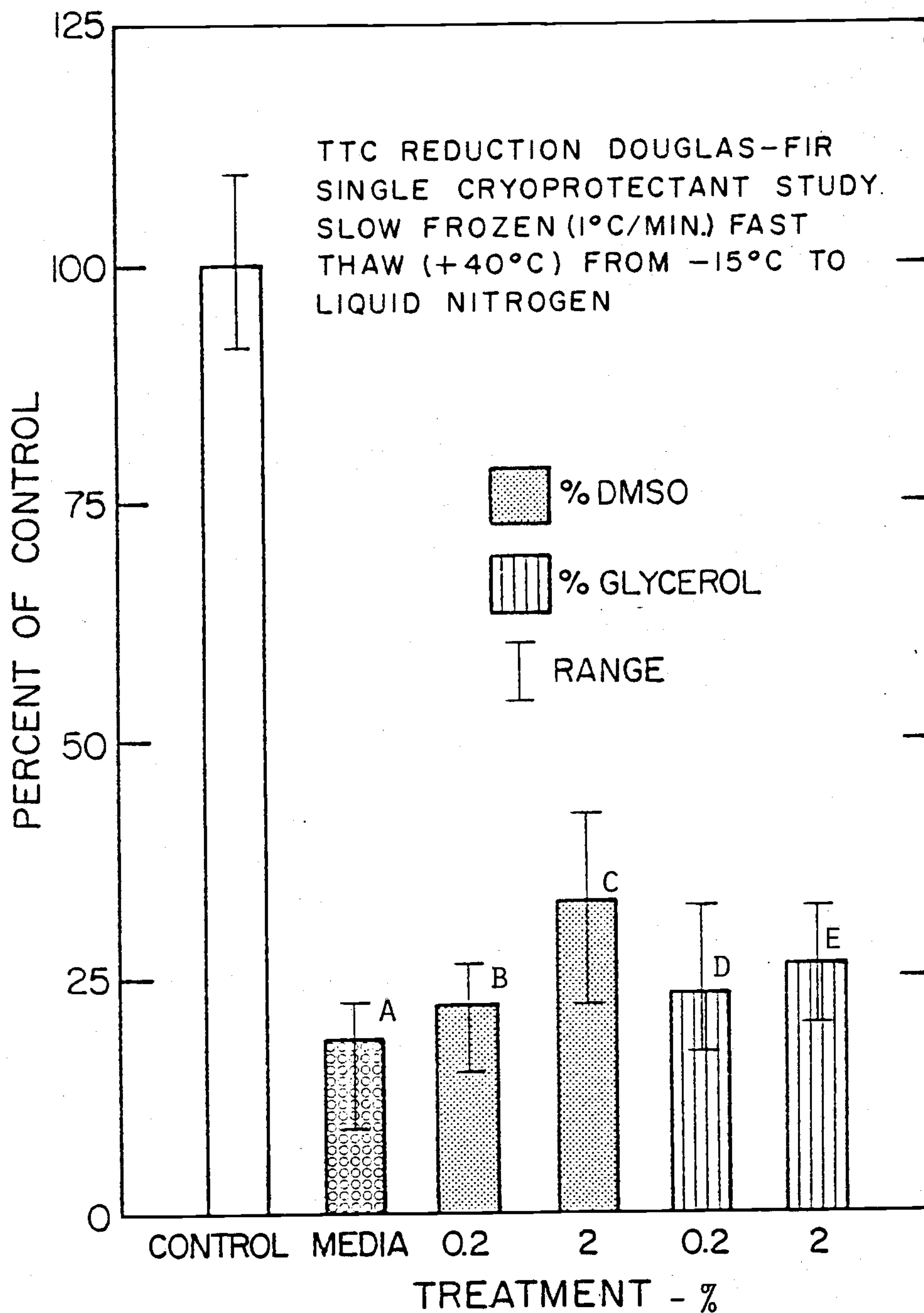


Figure 45. Effects of single cryoprotectants on TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min from +20°C to -15°C and immersed in liquid nitrogen. Thawing was fast in +40°C water bath to 0°C, to room temperature. N=8 A:B=N.S.; B:C=*; B:D=N.S.; C:E=N.S.; D:E=N.S. * Indicates a significant difference at the 95% confidence level.

were frozen at 1°C/min from +20°C to -40°C then held there 1/2 hr and immersed in liquid nitrogen. Thawing in both cases was fast in a +40°C water bath. The 2% DMSO treatment showed significently better TTC reduction under this treatment than when frozen only to -15°C before immersion in liquid nitrogen (Figures 45 and 46). The DMSO at a 2% concentration was significently (15%) better than at 0.2%, non-significently (6%) better than glycerol at the same concentration and 12% better than was 2.5M sucrose. Media alone produced only 17% of TTC reduction compared to the control value (Figure 46).

Bases on the TTC reduction test made after 97 days post-thaw culture began, cultures frozen in media only showed values about 10% of initial thawed control, while DMSO at 0.2% and sucrose at 2.5M were significently reduced (12% and 10%) of their initial thawed values. Glycerol at 0.2% was reduced 3% further compared to TTC reduction right after thaw (Figure 46). TTC reduction after 97 days post-thaw culture for DMSO at 2% and glycerol at 2% were increased 2% and 8% respectively when compared to TTC reduction right after thawing (Figure 47); these differences are however, not significent. TTC reduction after 97 days can be seen visually in Figure 48. The greatest treatment TTC reduction measured 97 days after thawing occurred with 2% DMSO followed by a somewhat lighter color at 2% glycerol. Glycerol at 0.2% was significently more effective than 0.2% DMSO.

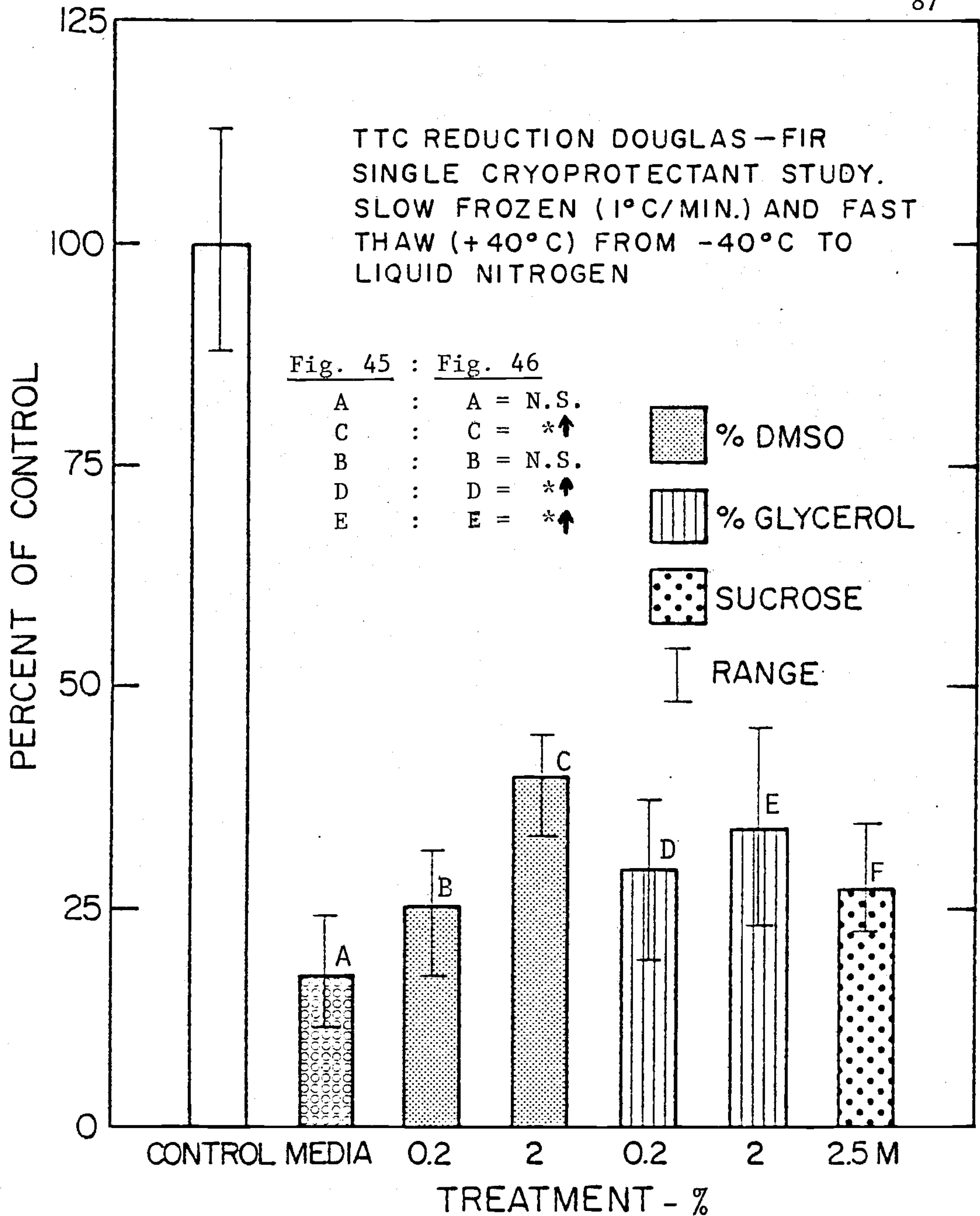


Figure 46. Effects of single cryoprotectants on TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Single cyroprotectants were used at 0.2% and 2% and sucrose at 2.5M. Thawing was fast in +40°C water bath. N=8 A:B=*; B:C=*; B:D=N.S.; B:F=N.S.; D:E=N.S.; C:E=N.S.; E:F=N.S. * Indicates a significant difference at the 95% confidence level.

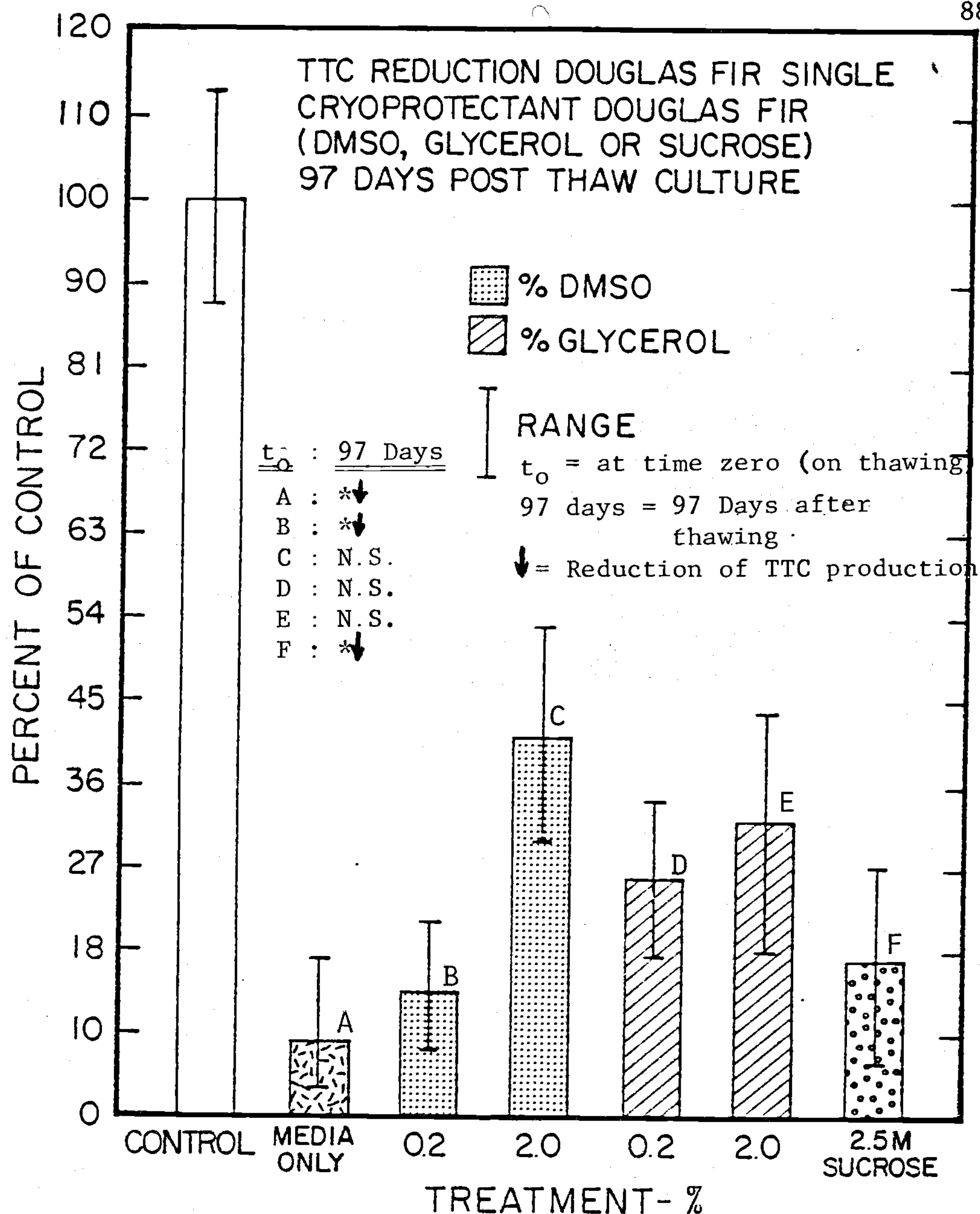


Figure 47. Effects of single cryoprotectants on TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min for +20°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants were used at 0.2%, 2% and sucrose at 2.5M. Thawing was fast in +40°C water bath. The TTC test was made 97 days after thawing. N=8
A:B=*; B:D=*; C:D=*; E:E=N.S.; C:E=N.S.; E:F=*
* Indicates a significant difference at the 95% confidence level.

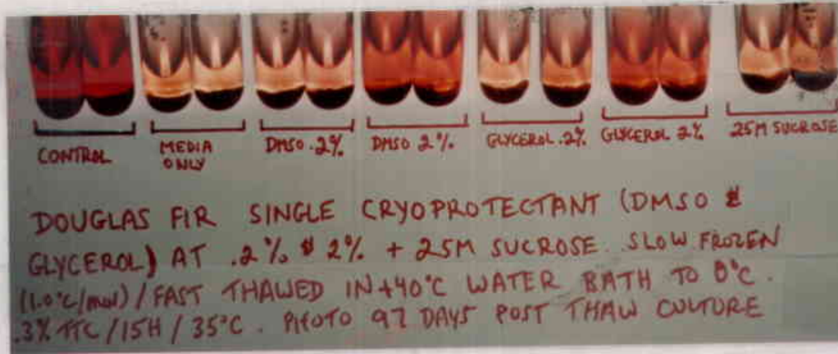


Figure 48. Douglas-fir cultures showing reduced TTC after 97 days post-thaw culture. Freezing and thawing treatments are shown on the photograph.

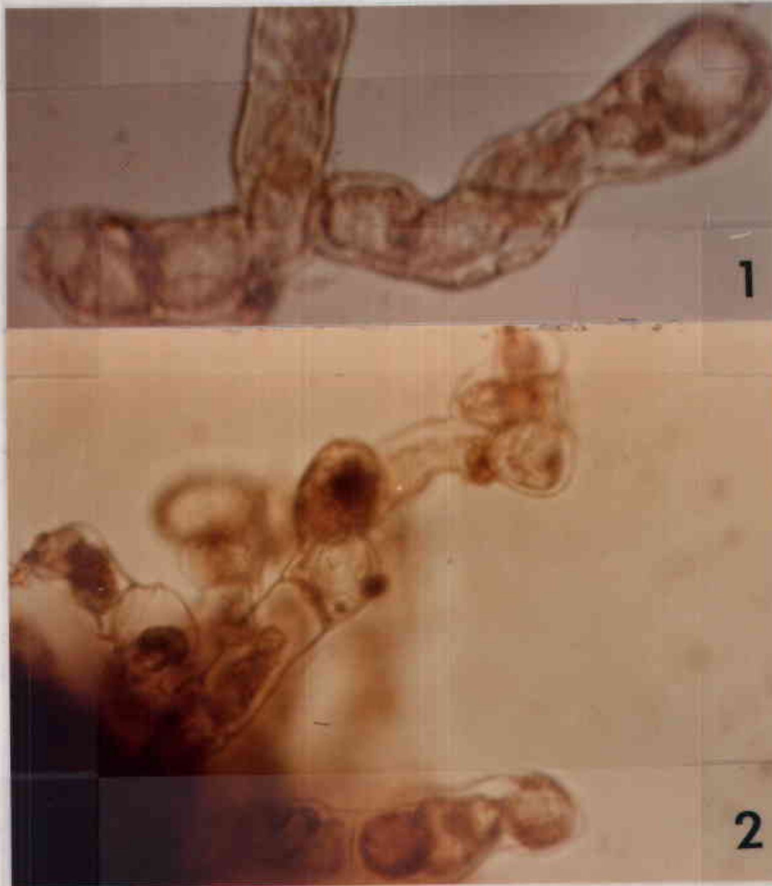


Figure 49. Damage done to Douglas-fir cells by freezing at 1°C/min to -40°C using 2% DMSO and placing in liquid nitrogen. Thawing is fast in +40°C water bath (2). "1" Shows turgid control cells for comparison. Notice cell membranes pulled away from cell walls in "2".

Damage occurred to cells when frozen slowly (1°C/min) to -40°C then immersed in liquid nitrogen. Using 2% DMSO cell protoplasm shrinkage still occurred under these conditions (Figure 49).

There was a slight increase in dry weight after 97 days post-thaw culture when 0.2% DMSO and 0.2% glycerol were used. Growth with 2% glycerol was about 4 times the initial dry weight, 2% DMSO was only slightly less, and 2.5M sucrose was double the initial weight (Figure 50). However, data was based only on one culture, so little confidence can be placed on that data.

Higher survival values, based on direct post-thaw TTC reduction could be obtained with cryoprotectant concentrations above 2%. DMSO at 5% concentration produced almost 49% TTC reduction based on nonfrozen control while for glycerol at 5% concentration this value was 35%. At 10% both cryoprotectants showed values at less than 1/2 that shown at 5% concentration.

Based on the initial non-frozen control (above), DMSO at 5% and glycerol at 5% were 30% and 19% respectively after 6 weeks post-thaw culture. This showed that after 6 weeks the initial TTC post-thaw reduction was reduced to about 1/2 for the two cryoprotectants under the conditions stated. Initial TTC reduction immediately on thawing was 21% for cultures frozen in media only, 22% for cultures frozen with 10% DMSO, and 19% for cultures frozen with 10% glycerol. Six weeks after thawing values were 12%, 11% and 8% respectively (Figure 51).

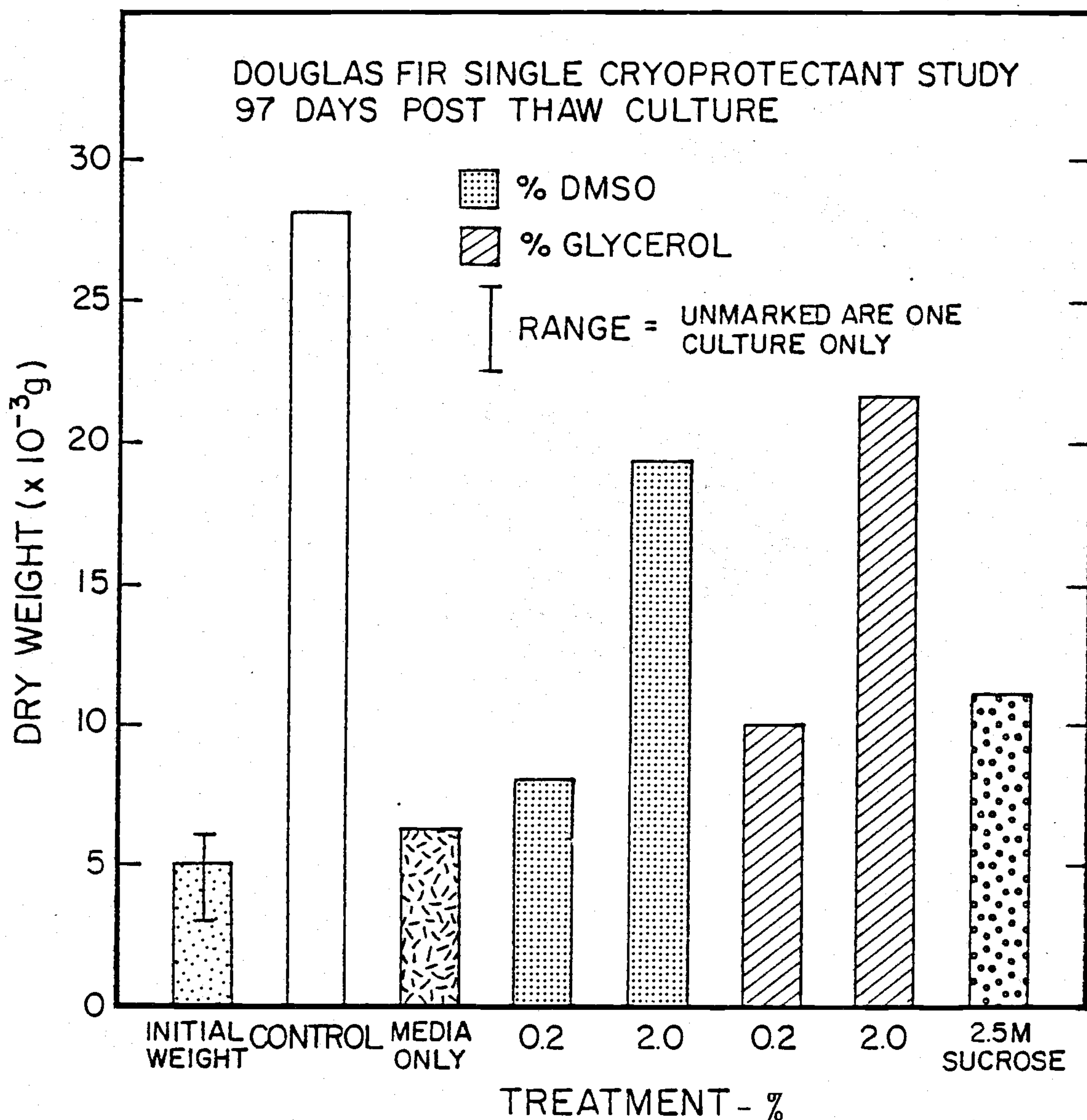


Figure 50. Effects of single cryoprotectants on total dry weight of Douglas-fir suspension cultures in liquid nitrogen. Single cryoprotectants were used at 0.2%, 2% and 2.5M Thawing was fast in +40°C water bath. Dry weight was measured 97 days after freezing post-thaw culture. Initial dry weight N=4.

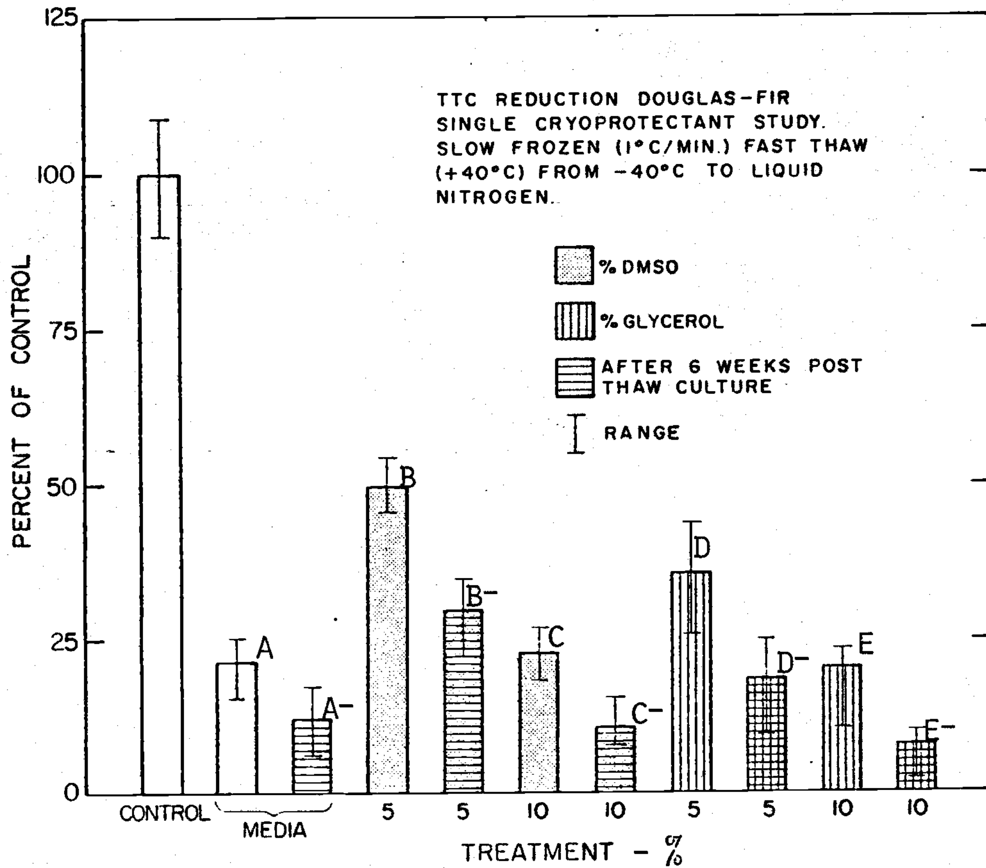


Figure 51. Effects of single cryoprotectants on TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants were used at 5% and 10%. Thawing was fast in +40°C water bath. TTC reduction was measured immediately after thawing and after 6 weeks post-thaw culture. N=5 A:A⁻=*; B:B⁻=*; C:C⁻=*; D:D⁻=*; E:E⁻=* * Indicates a significant difference at the 95% confidence level.

Measured 40 days after thawing, dry weights did not vary from initial test weight of cells more than 2×10^{-3}g regardless of treatment. However, the non-frozen control grew vigorously (Figure 52).

Dry weight measured 108 days after thawing showed greater differences between treatments than at 40 days. Dry weight of culture grown in DMSO at 5% was almost double the initial dry weight, but much less than the control. A slight increase in dry weight was observed with glycerol at 5% and sucrose at 2.5M. Cryoprotectants at 10% did not show dry weight values different from the initial experimental values (Figure 53). However, little confidence can be placed in this data since they are based only on one culture each. Differences can also be seen visually (Figure 55).

Greater dry weight occurred with 5% DMSO and 2.5M sucrose and these cultures were more tan-brown than cultures grown in the 10% concentrations.

Douglas-fir cultures frozen in media containing 5% DMSO then grown for 118 days after thawing had a TTC reduction value 62% of the control value which was 12% higher than the initial thawed value. Glycerol at 5% produced a TTC value of 33% of initial control after 118 days, almost double the 18% of control observed after six weeks. This 33% value was about equal to the vâlue shown directly after freezing (Figures 54 and 56).

Douglas-fir cultures frozen in media containing 2.5M sucrose treatment does not appear to increase in TTC reduction with time

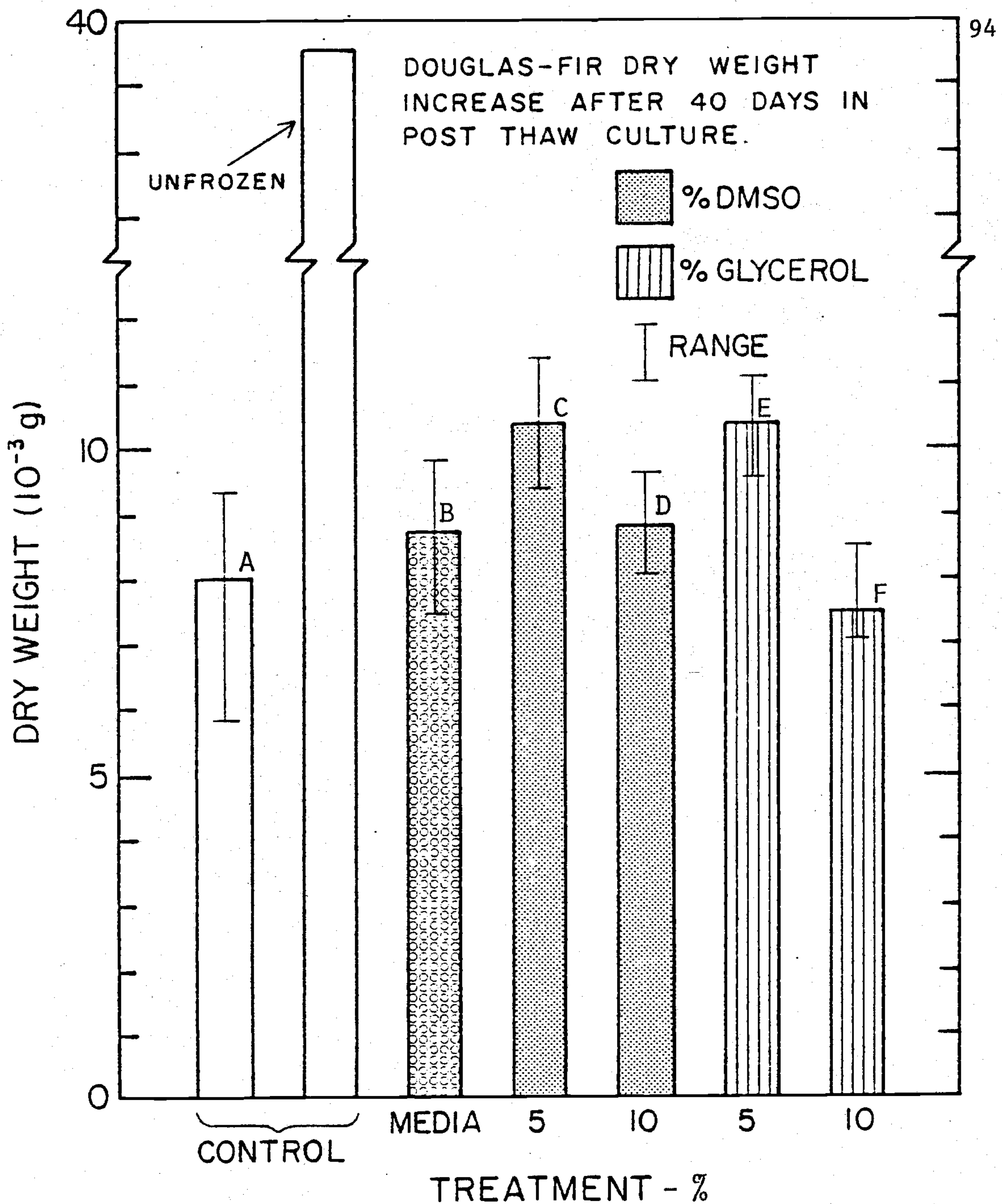


Figure 52. Effects of single cryoprotectants on total dry weight of Douglas-fir suspension cultures cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants were used at 5% and 10%. Thawing was fast in a +40°C water bath. Dry weight was measured 40 days after thawing. N=4 A:B=N.S.; A:C=*; B:C=*; B:D=N.S.; C:D=*; C:E=N.S.; E:F=* * Indicates a significant difference at the 95% confidence level.

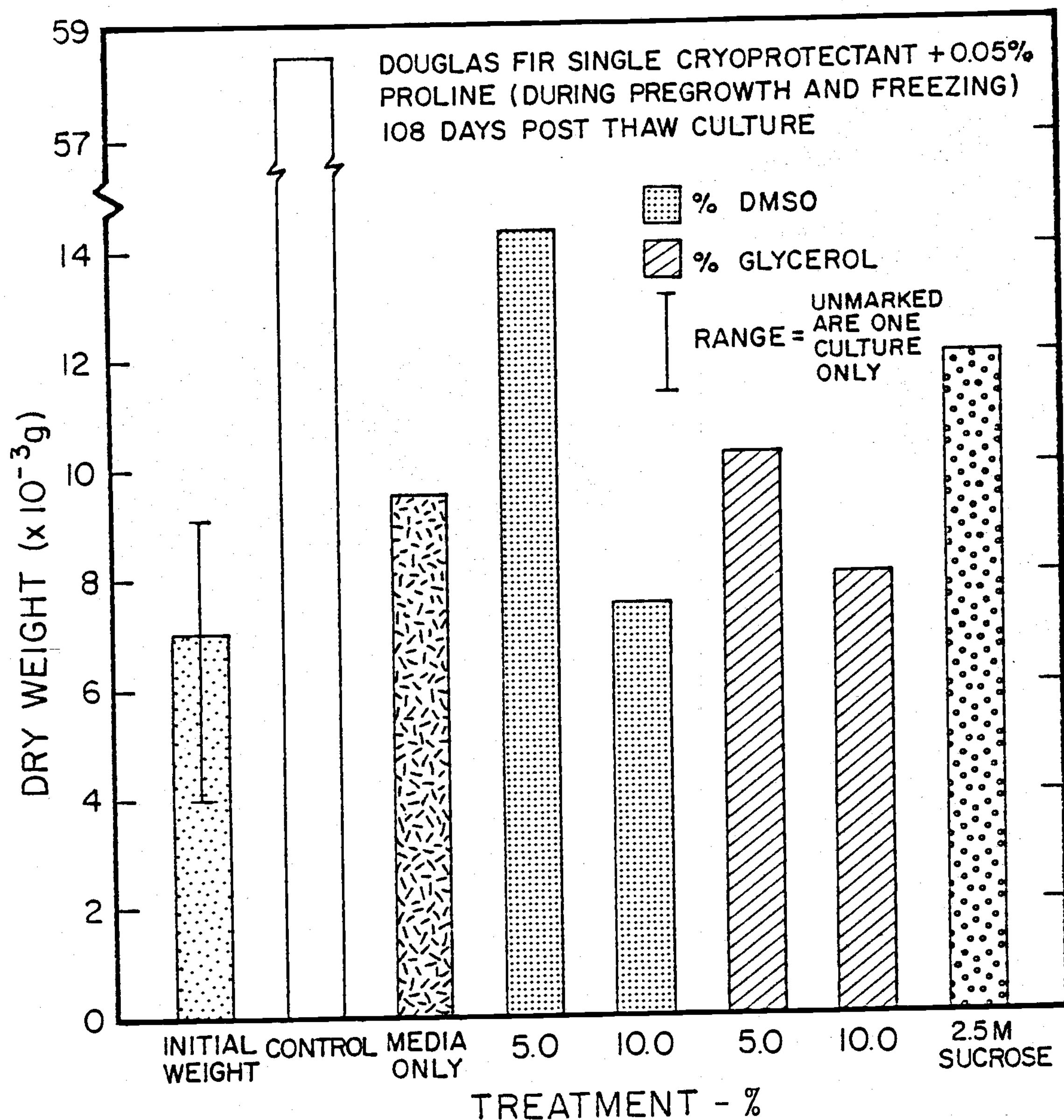


Figure 53. Effects of single cryoprotectants on total dry weight of Douglas-fir suspension culture cooled at 1°C/min from +20°C to -40°C and immersed in liquid nitrogen. Single cryoprotectants were used at 5%, 10% and 2.5M with 10 days pre-growth and freezing in 0.05% proline. Thawing was fast in a +40°C water bath. Dry weight was measured 108 days after thawing. Initial dry weight N=4.

Figure 54. Douglas-fir cultures showing reduced TTC after 118 days post-thaw culture. Freezing and thawing treatments are shown in Figure 53.

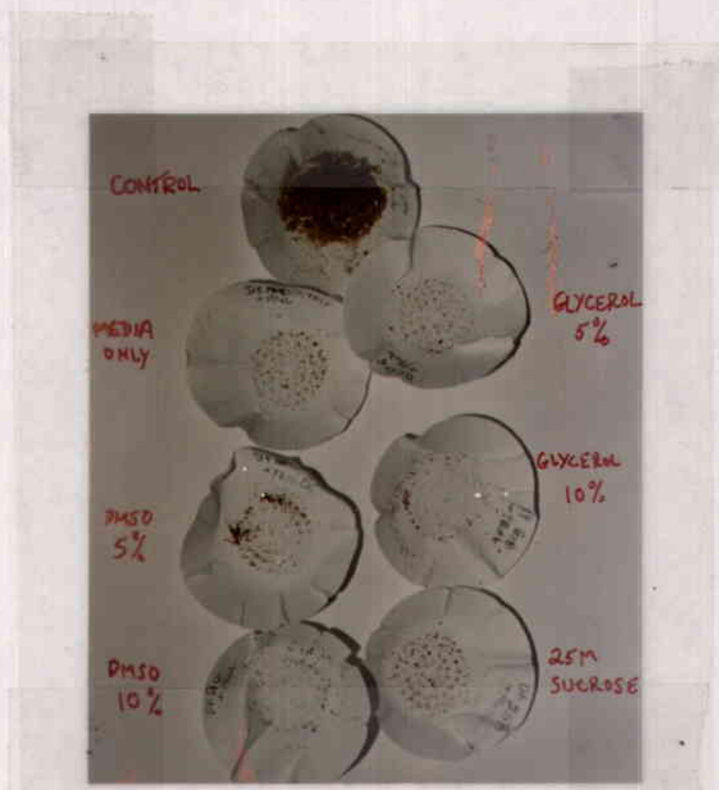


Figure 55. Douglas-fir cultures frozen $1^{o}C/min$ to $-40^{o}C$ then immersed directly in liquid nitrogen. Cultures were pre-grown 10 days with 0.05% proline before treatment. Single cryoprotectants used. Thawed fast in $+40^{o}C$ water bath. Photograph shows 108 days post-treatment culture. Notice more cell volume with 5% DMSO concentration.

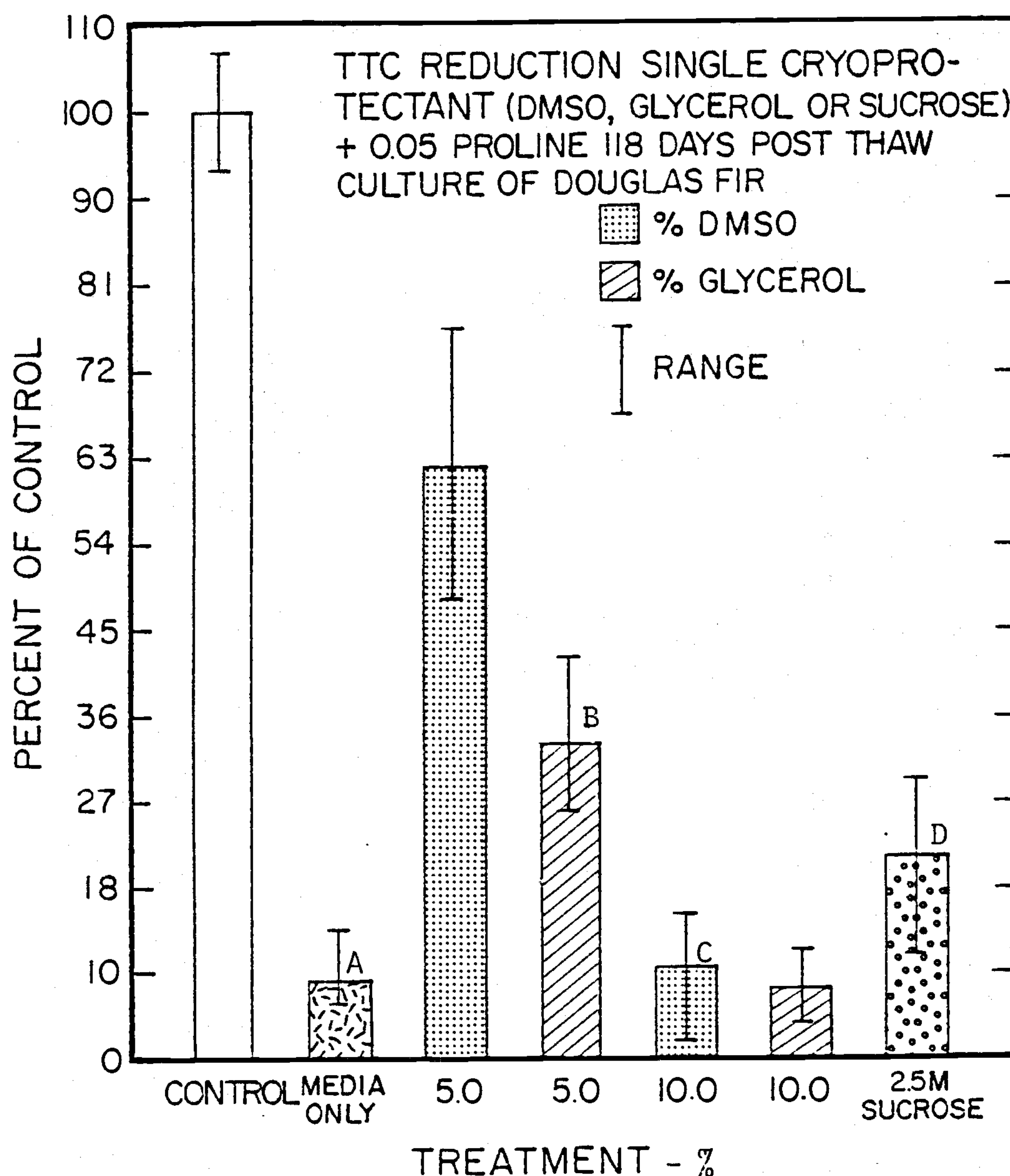


Figure 56. Effects of single cryoprotectants on TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Single cryoprotectants were used at 5%, 10% and sucrose at 2.5M with 10 days pre-growth and at freezing in 0.05% proline. Thawing was fast in a +40°C water bath. TTC test was made 118 days post-thaw culture. N=8 A:C=N.S.; A:D=*; B:D=*; C:D=* * Indicates a significant difference at the 95% confidence level.

in post-thaw culture. Cells frozen in media only and in 10% DMSO and glycerol had a TTC value of about 10% of the initial thaw control after 118 days indicating little activity considering unexplained residual TTC reduction contamination in this species is 6% (Figure 56).

The high TTC reduction values observed here for Douglas-fir cells frozen in DMSO at 5% concentration after 118 days post-thaw culture suggests that cells may be in the process of being repaired and cell division is taking place. This is suggested by a "T" tube culture left standing for 142 days by accident (Figures 57 and 58). Colorless cells, indicating a lack of chlorophyll, grew out of a dark mass of cells (Figures 57 and 58). Using a DNA staining technique (Appendix E) cells were seen in division Figure 58-f).

b. Freezing in the Presence of Cryoprotectants in Combination:

Better results were obtained, based on post-thaw TTC reduction, when cryoprotectants were used in combination compared to using cryoprotectants singly. The combination of DMSO 5%/glycerol 2.5% produced 63% survival directly after thawing compared to unfrozen control (Figure 59). This TTC reduction value was much better than the 40% survival produced at 5% DMSO or the 24% survival obtained with 2% glycerol after freezing. TTC reduction can be seen visually in Figure 60. This data indicates that a sub-optimal concentration of one of the cryoprotectants in the combination can greatly reduce TTC reduction.

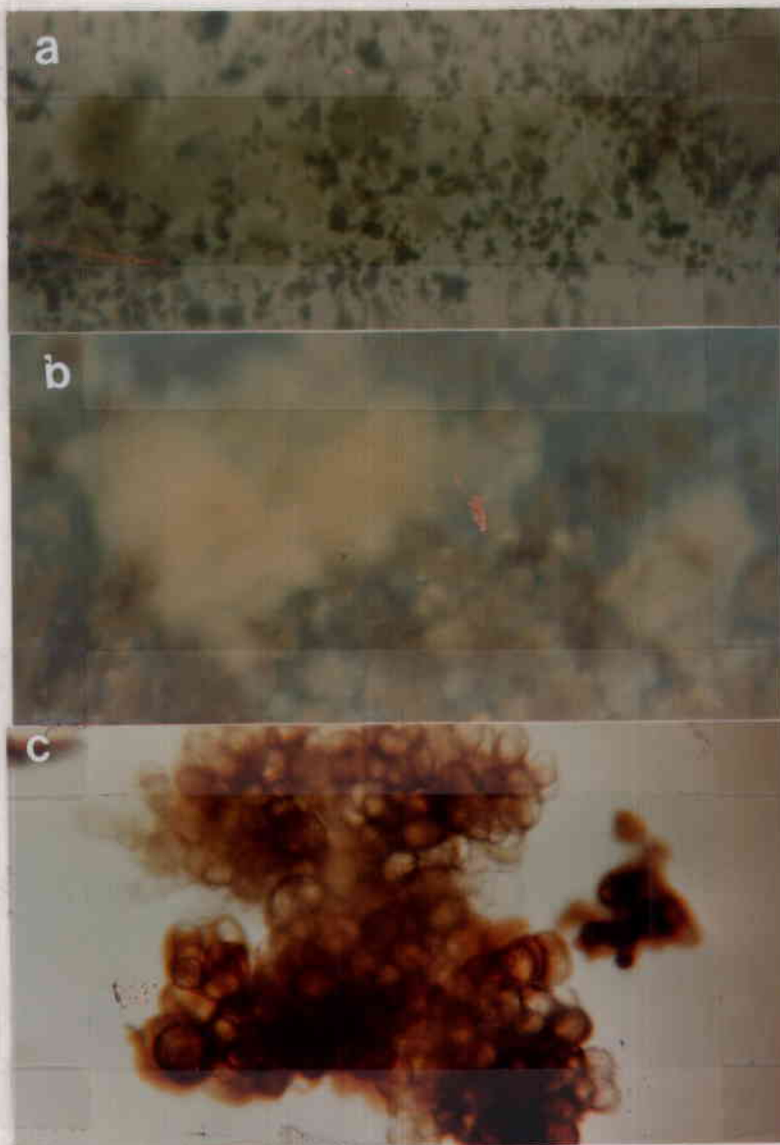


Figure 57. Three views of Douglas-fir suspension culture cooled at 1°C/min from +20°C to -40°C and immersed in liquid nitrogen, with 5% DMSO. Thawing was fast in a +40°C water bath. Photos 142 days post-thaw culture. a) x4, b) x40, c) x160. Note white cells growing out of dark brown cells. Photographs taken 145 days after thawing.

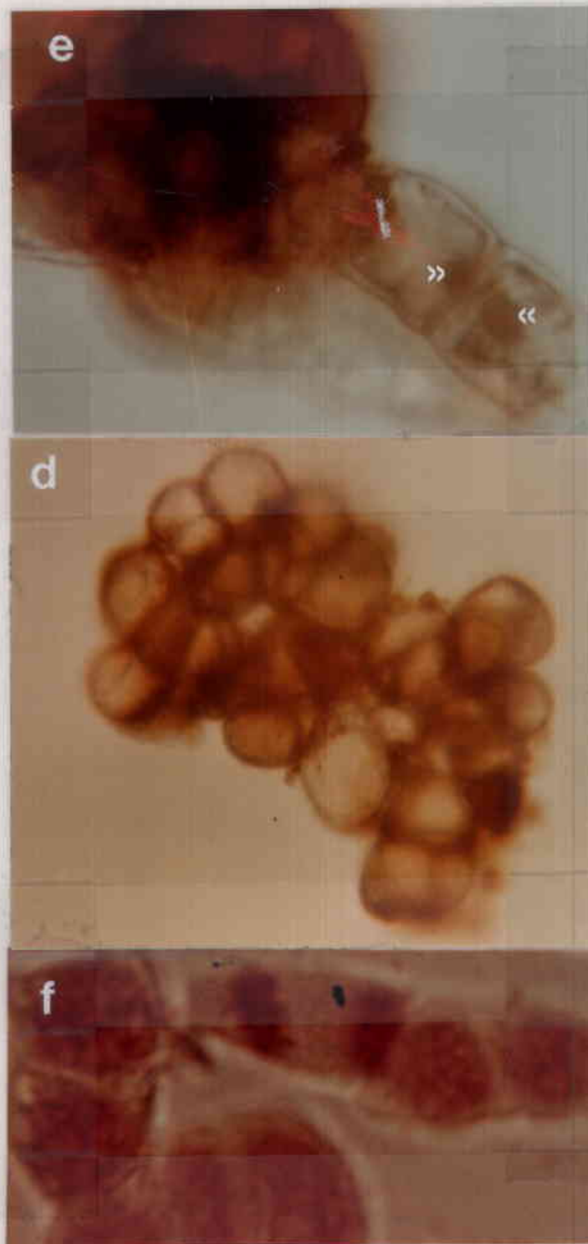


Figure 58. Same culture as in Figure 57. d) Showing white growing cells x280. e) 2 white living cells x400. Note dark nuclei (>>). f) Dividing cell in (center). Cells on either side have just divided x400.

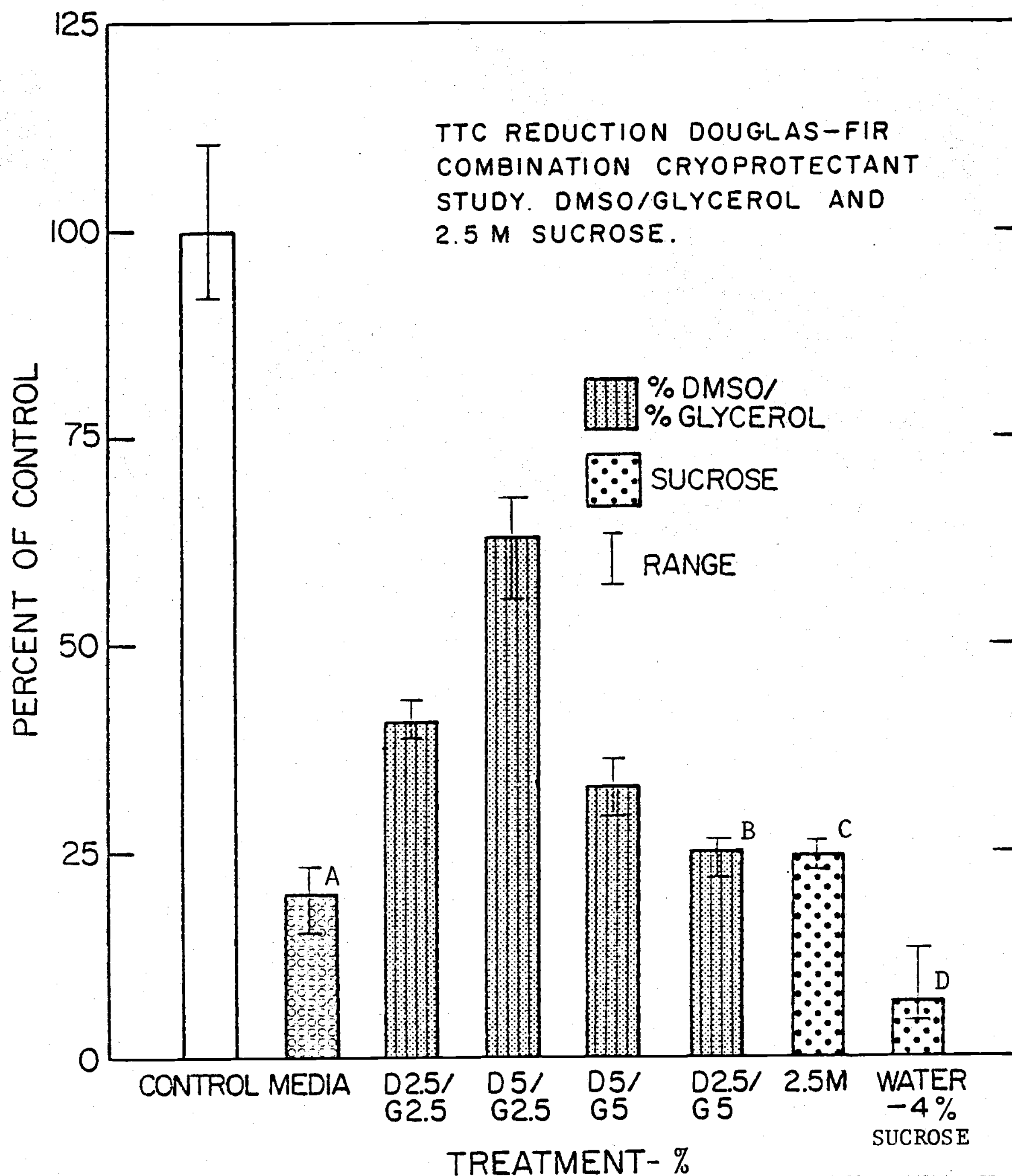


Figure 59. Effect of cryoprotectants in combination on TTC reduction of Douglas-fir suspension cultures cooled at 1°C/ min from +20°C to -40°C then immersed in liquid nitrogen. Cryoprotectants were used in combination from DMSO ≤ 5% ≥ 2.5% and glycerol ≤ 5% ≥ 2.5%. Thawing was fast in a +40°C water bath. N=8 A:B=*; A:D=*; B:C=N.S.

* Indicates a significant difference at the 95% confidence level.

The best cryoprotectant combination obtained was with DMSO 5%/ glycerol 1% which produced 66% TTC production compared to control and was significently (6%) better than DMSO 5%/glycerol 2.5%. Cryoprotectant combinations above or below 5% DMSO and above or below 1% glycerol decreased the amount of TTC reduction (Figure 61).

The effect of the 4% sucrose added to the cryoprotectant solution was additive to the cryoprotectant combination (Figures 59 and 61). The 4% sucrose increased TTC reduction 6% over no sucrose.

Thawing cultures at room temperature, instead of in a +40°C water bath significently decreased (8%) the TTC reduction (Figure 61).

TTC reduction after 53 days post-thaw culture was decreased 22% more than the value of the TTC test made immediately on thawing for cultures treated with DMSO 5%/glycerol 1%. After 53 days in post-thaw culture, for DMSO 5%/glycerol 2.5% this TTC reduction was 25% less, while for DMSO 1%/glycerol 0.5% and DMSO 0.5%/ glycerol 0.5% this was about 1/2 the TTC value observed for these treatments immediately after thawing (Figure 62).

No growth was observed after 73 days post-thaw culture. No treatments had total dry weight increases significently above experimental initial dry weight, with the exception of the unfrozen controls (Figure 63). The pH of cultures was well within that required for growth and varied little between treatments (Table 7).

Cell repair appeared to have started about 83 days after thawing (Figure 64 and 65). For example, TTC reduction compared to

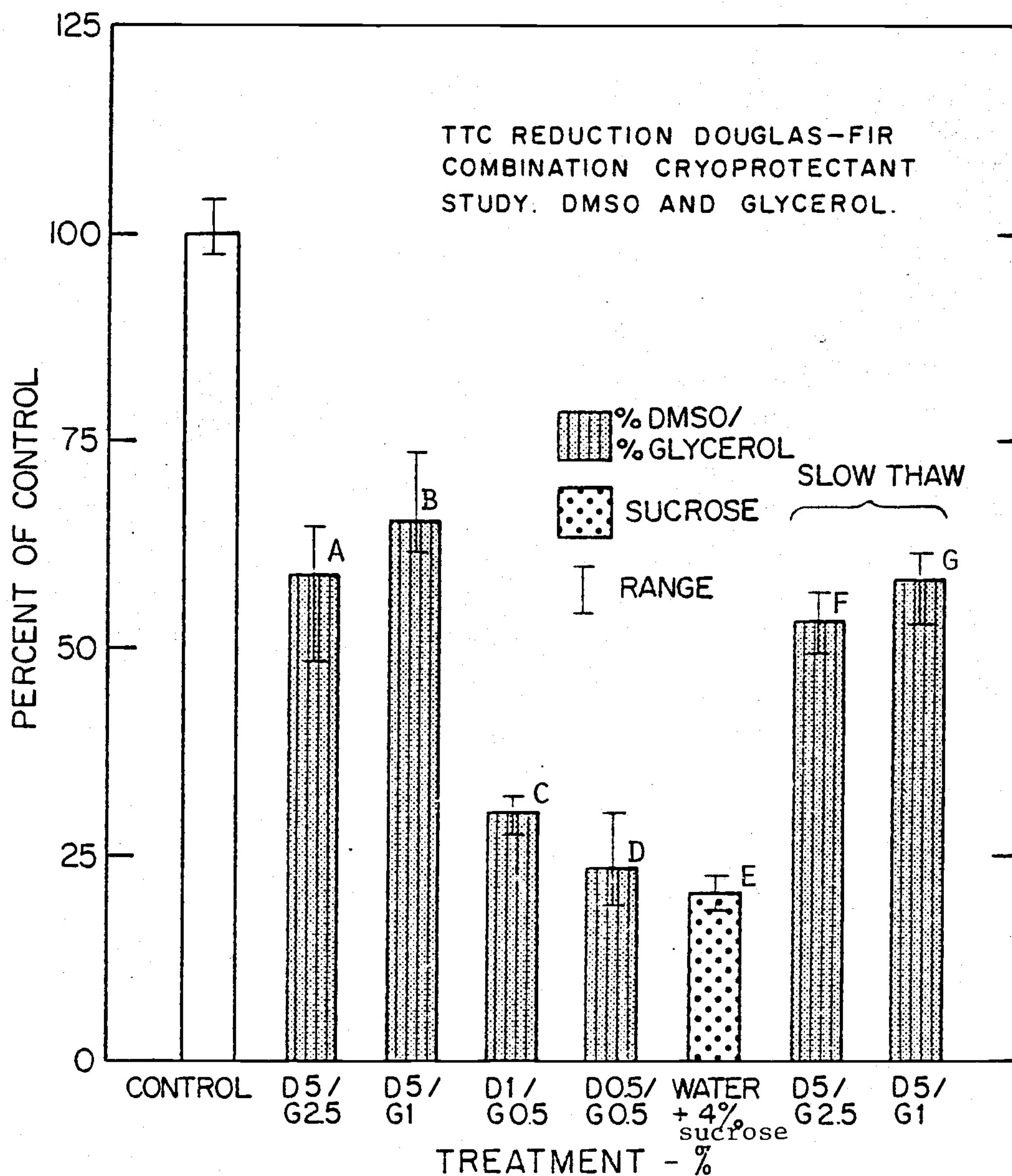


Figure 61. Effects of cryoprotectants in combination on TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Cryoprotectant used in combination from DMSO $\leq$ 5% $\geq$ 0.5% and glycerol $\leq$ 2.5% $\geq$ 0.5%. Thawing was fast in a +40°C water bath or slow in air to room temperature. N=8 A:B=*; A:F=*; B:G=* C:D=*; D:E=N.S. * Indicates a significant difference at the 95% confidence level.

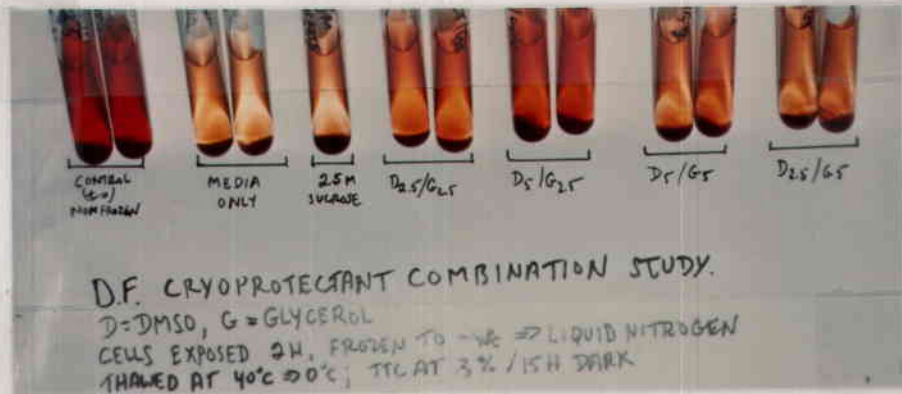


Figure 60. TTC reduction of post-thawed Douglas-fir cultures using cryoprotectants in combination.

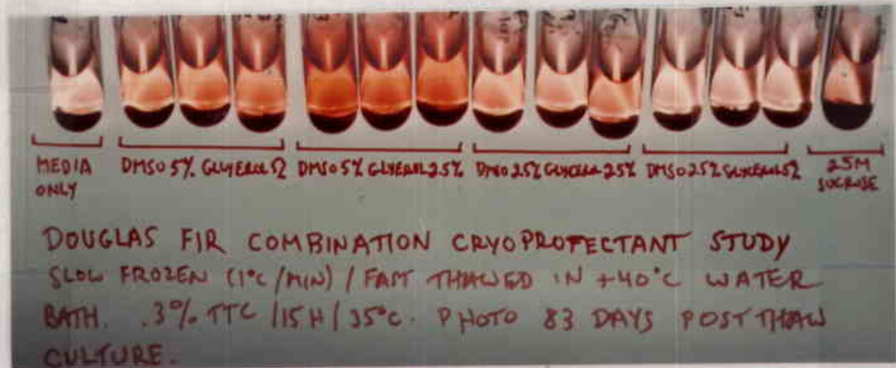


Figure 64. TTC reduction of post-thawed Douglas-fir cultures using cryoprotectants in combination after 83 days post-thaw culture.

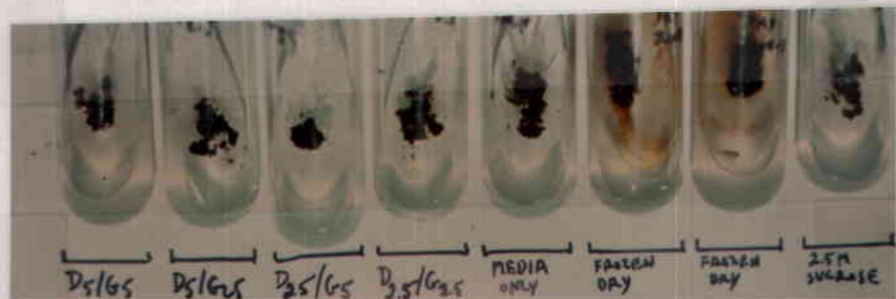


Figure 67. Culture of Douglas-fir on solid media 60 days post-thaw culture. Material was treated as indicated and cooled slowly $1^{o}C/min$ from $+20^{o}C$ to $-40^{o}C$ then immersed in liquid nitrogen. Thawing was fast in a $+40^{o}C$ water bath.

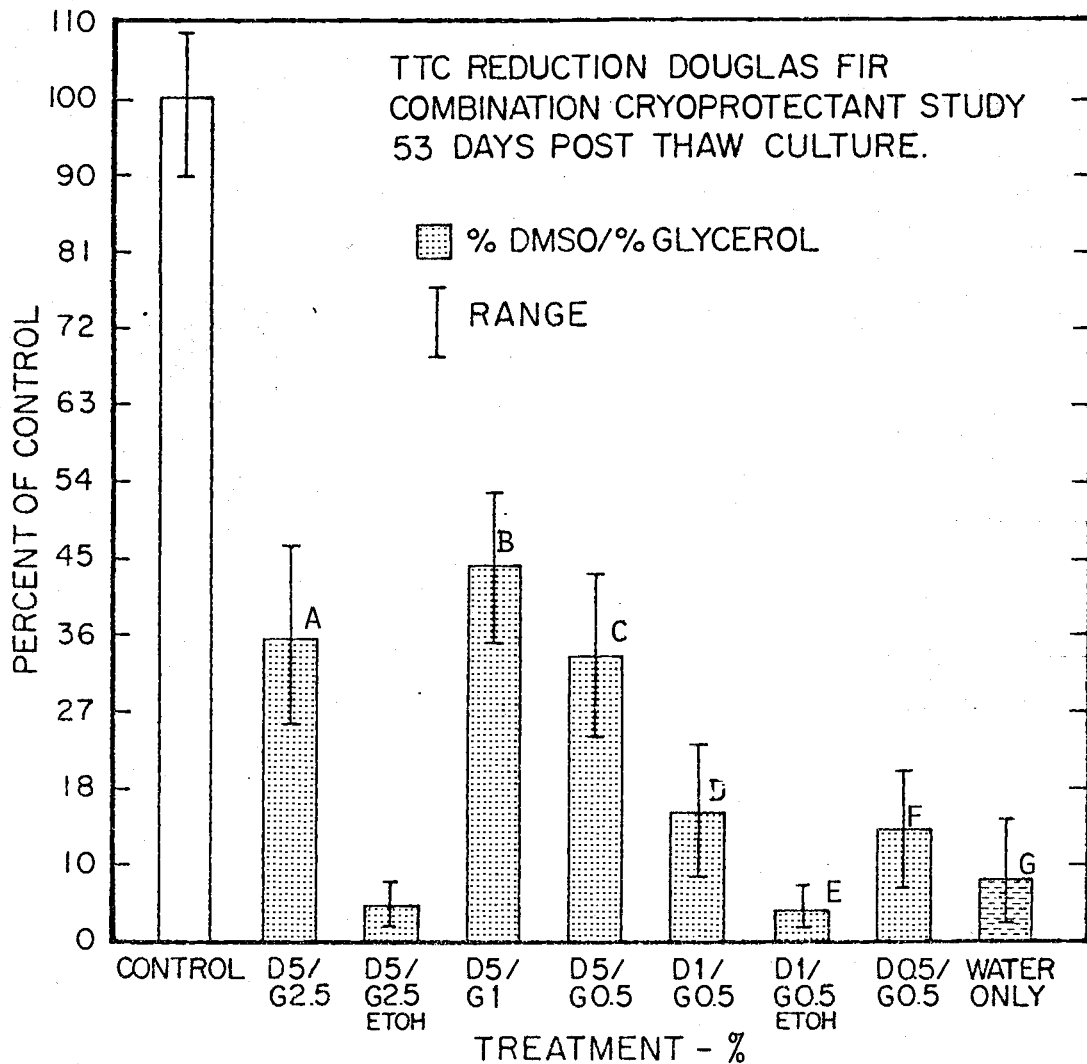


Figure 62. Effects of cryoprotectants in combination on TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Cryoprotectants were used in combination from DMSO $\leq$ 5% $\geq$ 0.5% and glycerol $\leq$ 2.5% $\geq$ 0.5% or water only. Thawing was fast in a +40°C water bath. (New TTC buffer and post TTC incubation washing used.) TTC test was made 53 days after thawing. Some cultures were treated with 70% ETOH before TTC testing. N=8 A:B=*; A:C=N.S.; B:C=*; D:F=N.S.; E:F=*; E:G=N.S. * Indicates a significant difference at the 95% confidence level.

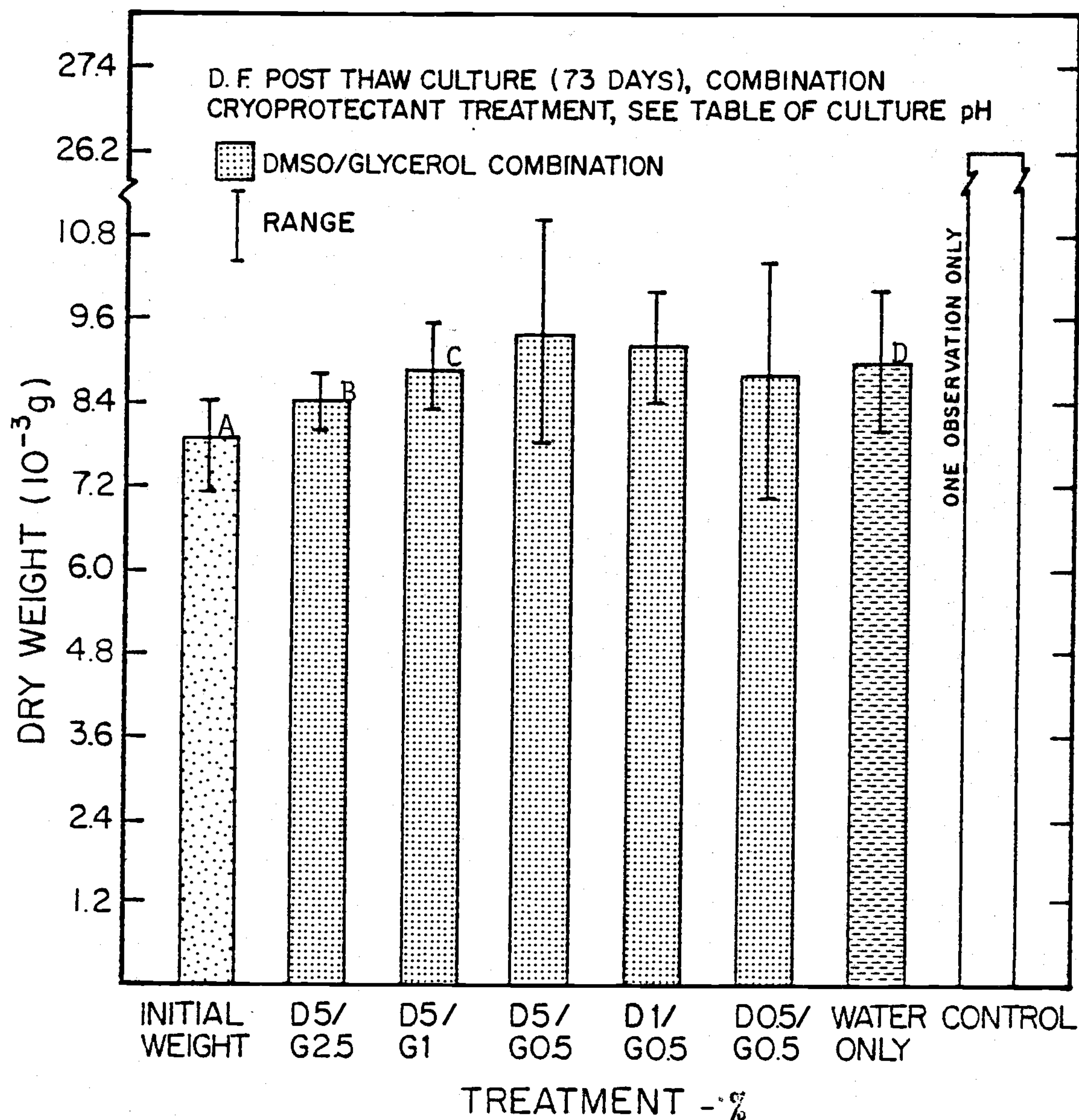


Figure 63. Effects of cryoprotectants in combination on total dry weight of Douglas-fir cultures cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Thawing was fast in +40°C water bath. Dry weight was measured 73 days after thawing. N=4 B:C=N.S.; All treatments N.S. with respect to A or D.

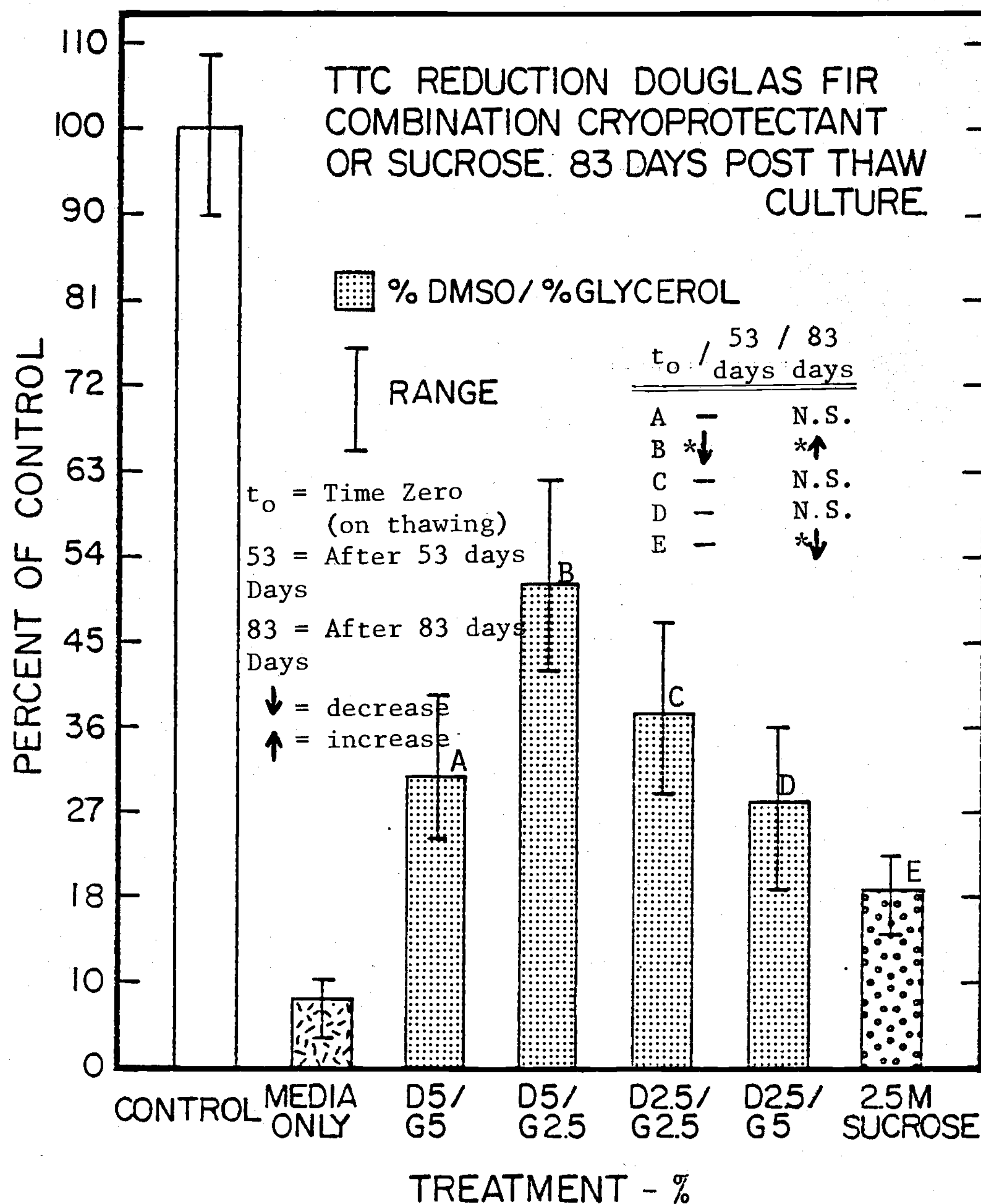


Figure 65. Effects of cryoprotectants in combination on TTC reduction of Douglas-fir suspension cultures cooled at 1°C/min from -20°C to -40°C then immersed in liquid nitrogen. Cryoprotectants were used in combination or as 2.5M sucrose. Thawing was fast in a +40°C water bath. The TTC test was made 83 days after post-thaw culture. N=8 A:B=*; A:C=N.S.; B:C=*; C:D=* * Indicates a significant difference at the 95% confidence level.

Table 7. The pH of Douglas-fir post-thaw culture media tested 73 days after culturing thawed cells. Conditions of treatment are those given in Figure 63. * The pH value is the result of 4 cultures of that media type mixed together.

TREATMENT	pH *
DMSO 5%/glycerol 2.5%	6.4
DMSO 5%/glycerol 1%	6.4
DMSO 5%/glycerol 0.5%	6.4
DMSO 1%/glycerol 0.5%	6.8
Water only	6.2
Control (media)	6.0

initial unfrozen control for the DMSO 5%/glycerol 2.5% combination after 83 days was 51% of unfrozen control, while after 53 days post-thaw culture (Table 7) this value was 34% and directly after thawing it was 59% (Figure 59). This result indicated that although recovery of the cells was apparent, the amount of TTC reduction was still less than that obtained directly after thawing. Some total dry weight increase also occurred 83 days after thawing at optimum cryoprotectant combinations (DMSO 5%/glycerol 2.5%; DMSO 2.5%/ glycerol 2.5%). For these two combinations there was about a 1/3 increase of total dry weight over the initial weight (Figure 66).

Post-thaw culture conditions appear to be very important to the success of the experiment. Douglas-fir cell clumps do not grow well on solid agar medium after thawing and cultures turned black within 24 hr (Figure 67). In Figure 67, notice a tan exudate occurred

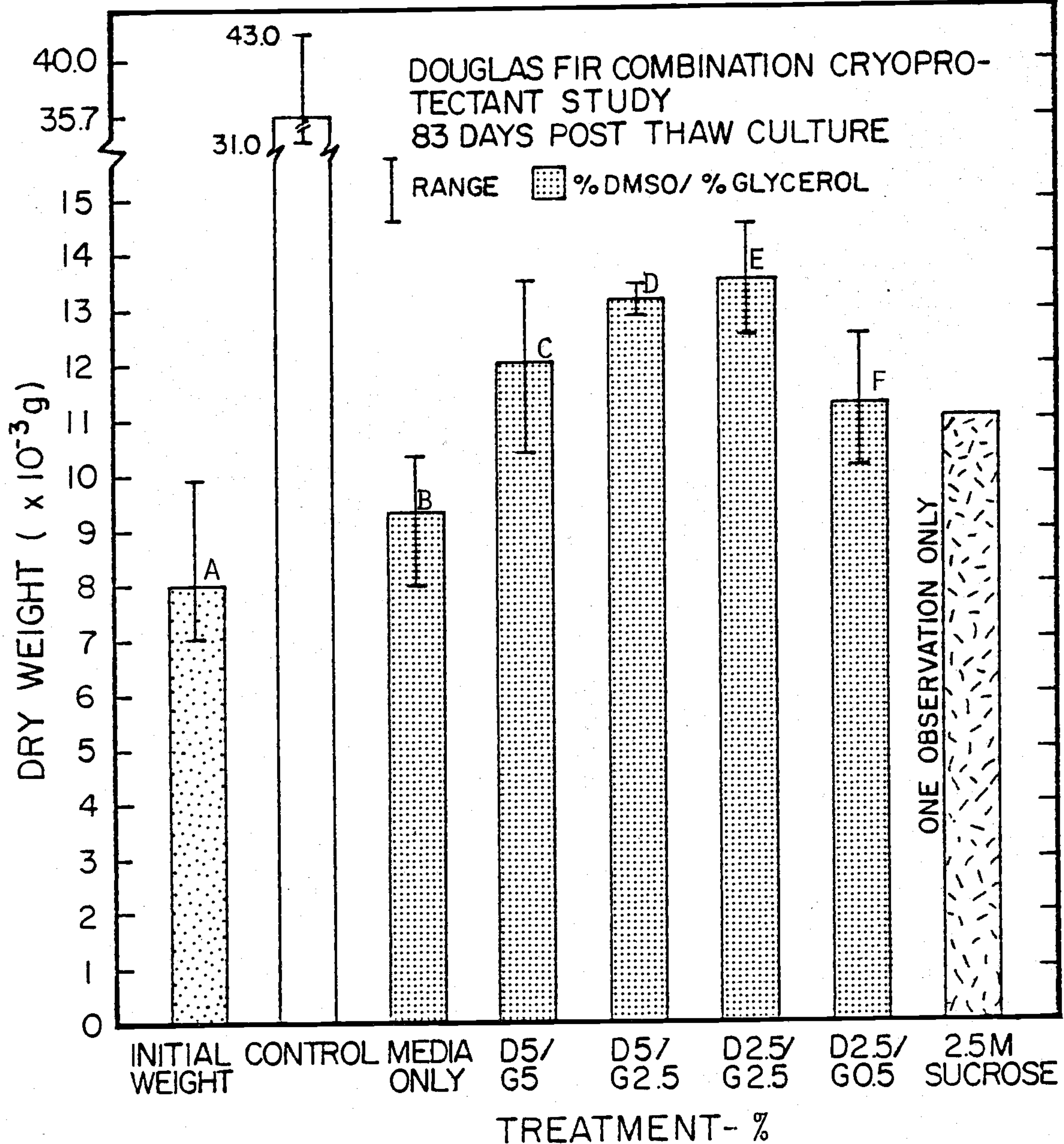


Figure 66. Effects of cryoprotectants in combination on total dry weight of Douglas-fir cultures cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Cryoprotectants were used in combination or as 2.5M sucrose. Thawing was fast in a +40°C water bath. Dry weight was taken at 83 days post-thaw culture. N=5 A:B=N.S.; B:C=*; C:D=N.S.≈*; C:E=*; D:F=*; C:F=N.S.
* Indicates a significant difference at the 95% confidence level.

when cultures were frozen without cryoprotectants. The reason for this is unclear, but may result in part from excessive phenol production on thawing or excessive membrane damage or a combination of both.

Overall, when using cryoprotectants, the initial thaw TTC reduction was less for poplar than for Douglas-fir under the same conditions. Early TTC reduction concentrations obtained with poplar however, were confusing. When the test cultures were not washed with water several times after the 15 hr of TTC incubation at +35°C in the dark, formazan production was high. DMSO at 2% and glycerol at 2% produced almost 49% and 52% TTC reduction respectively, compared to the control while at 1/10 of these concentrations, both cryoprotectants produced values only about 10% less TTC reduction. When growth media alone was used as cryoprotectant there was a 34% TTC reduction compared to the control value (Figures 68 and 69). When cryoprotectants in combination were tested, also without post TTC incubation washing, the TTC values were 67% and 60% for DMSO 10%/glycerol 5% and DMSO 10%/ glycerol 10%, respectively. For DMSO 5%/glycerol 5% and DMSO 5%/glycerol 10% the formazan production values were within 6% of the maximum color production observed in the control. Media only as cryoprotectant showed almost 31% TTC reduction compared to control, while with 2.5M sucrose this value was 44% (Figure 70). Data of Figure 70 was generated 5 weeks after data of Figure 68.

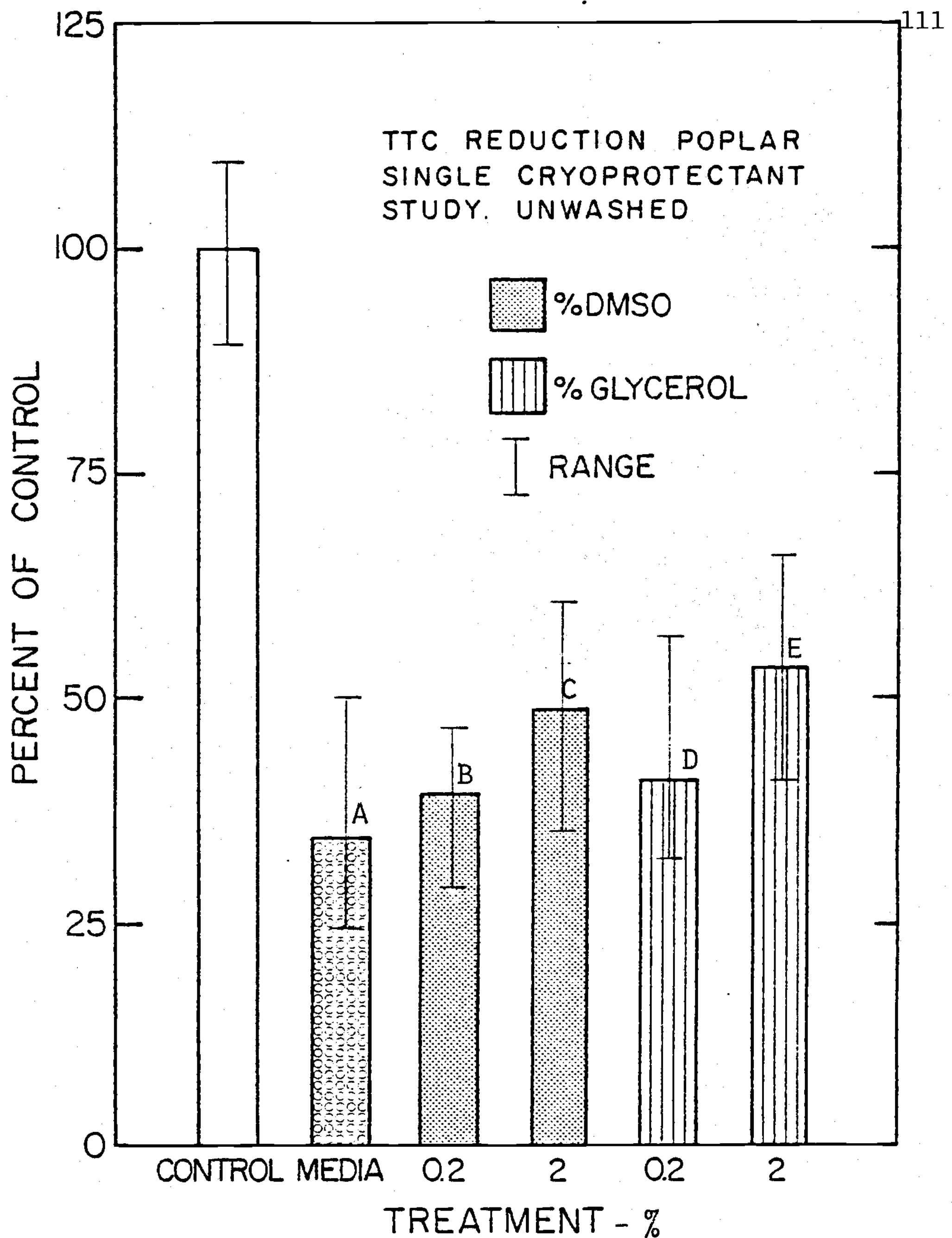


Figure 68. Effects of single cryoprotectants on TTC reduction of poplar suspension cultures cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Cryoprotectants were used at 0.2% and 2%. Thawing was fast in a +40°C water bath. Test cultures were not washed after TTC incubation. N=8 A:B=N.S.; B:C=N.S.; B:D=N.S.; C:E=N.S.; D:E=* * Indicates a significant difference at the 95% confidence level.

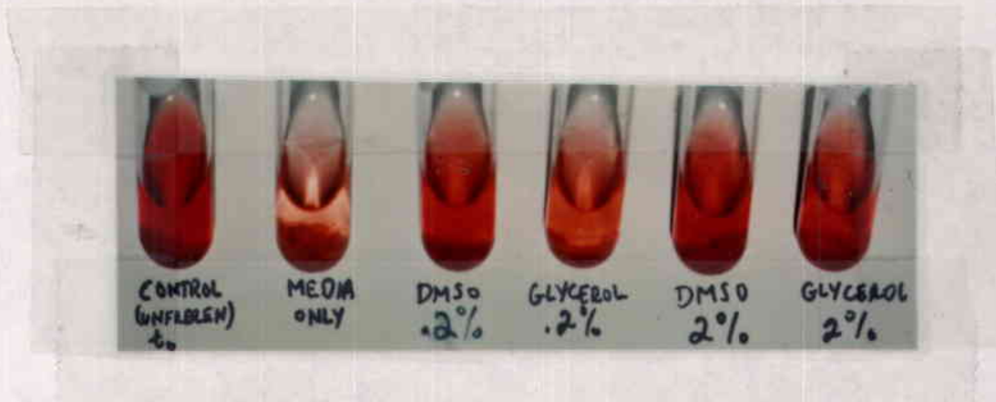


Figure 69. Poplar cultures frozen at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. The TTC test made using contaminated buffer and test material was not washed with water after TTC incubation.

Figure 71. Poplar callus TTC tested at 0.05% TTC/+35°C/15 hr/ dark. Photograph shows culture medium color before and after centrifugation for 20 minutes.

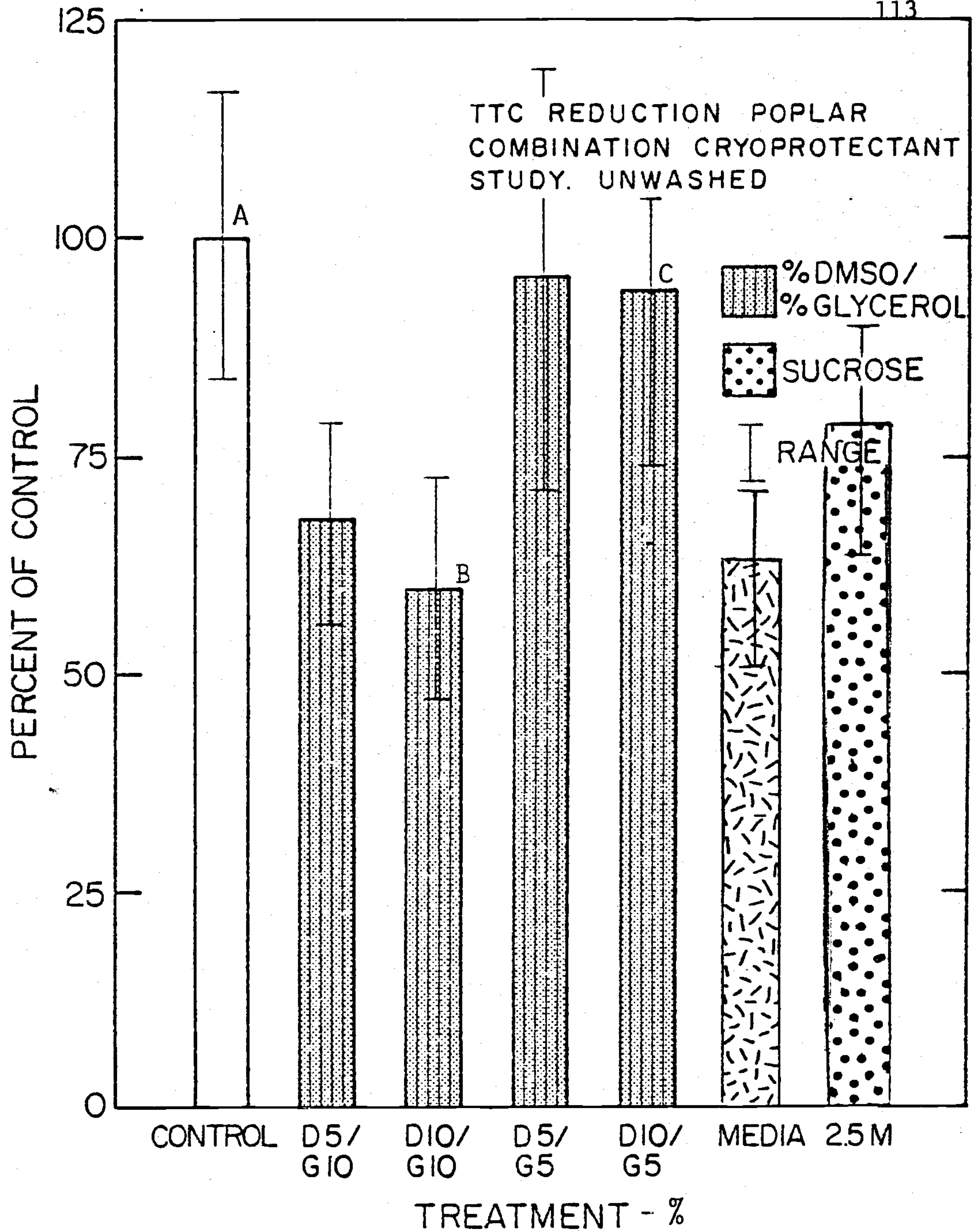


Figure 70. Effects of cryoprotectants in combination on TTC reduction of poplar suspension cultures cooled at 1°C/min from +20°C to -40°C and immersed in liquid nitrogen. Cryoprotectants were used in combination at 5% and 10% or 2.5M sucrose. Thawing was fast in a +40°C water bath. Test cultures were not washed in water after TTC incubation. N=4 A:B=*; A:C=N.S. * Indicates a significant difference at the 95% confidence level.

As the same buffer was used for all experiments there was a progressive increase in purple color over months on the outside of the test cell clumps during the TTC test. Eventually the entire TTC test solution was colored and could only be cleared by centrifugation for 20 min (Figure 71). The fact that the purple material could be centrifuged out of solution indicated considerable size and weight of the colored particles.

A study was initiated to identify the purple material. When TTC was added to poplar callus and left at room temperature for 4 hr in the dark then boiled, no color appeared (Figure 72-g); nor did any appear when TTC was replaced by 95% ethanol (Figure 72-h). The TTC solution plus culture media produced no color at room temperature for 4 hr but did when incubated with culture media for 15 hr at 35°C in the dark (Figure 72-i,m). Color was also produced with previously boiled (killed) callus plus poplar culture medium and TTC when incubated for 15 hr at 35°C in the dark (Figure 72-k), but not when this material was washed with water after incubation (Figure 72-s). When the purple material of Figure 72-k and 72-m were dissolved and boiled in 95% ethanol the red material showed a maximum absorbance at 485 nm, identical to formazan (Figure 20).

Since the poplar culture medium containing 75 ppm EDTA and TTC alone could produce the purple-red color it was initially thought that an inorganic reaction was occurring between EDTA and TTC under elevated temperatures. To test this hypothesis, Douglas -fir medium, without EDTA was compared to poplar medium with EDTA.

Figure 72. Poplar color reaction testing. Treatments are as indicated. Tubes g, h, s, k, and i contain callus. Callus was boiled 1 hr before testing. Tubes g, h, and i were not incubated and s, k, l, m and n were incubated 15 hr/+35°C in the dark. Tube m is shown peaking at 485 nm in Figure 20. Time of test was 15 hr at +35°C in the dark.

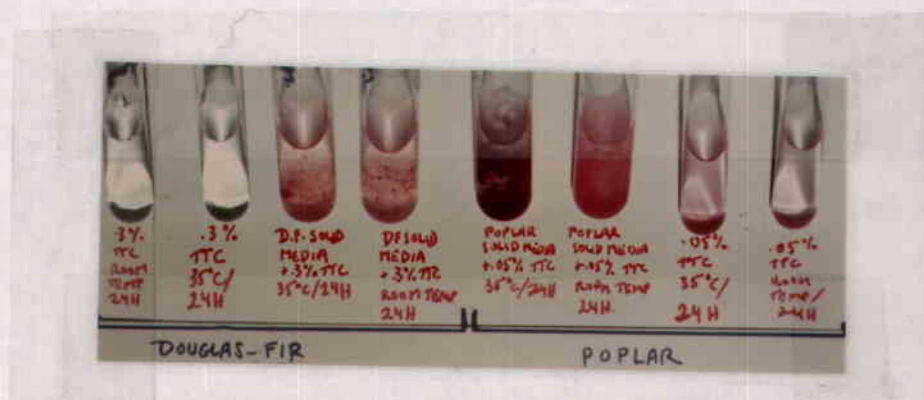


Figure 73. Color reaction of Douglas-fir and poplar after being treated as shown. Notice how much darker poplar color is than Douglas-fir under same condition. The tube second from right shows intense color of TTC alone without media when incubated at +35°C for 24 hr. Tube at far right also shows the same color. All incubations were in the dark as indicated.

Douglas-fir solid medium plus 0.3% TTC did produce formazan reduction both at +35°C and at room temperature after 24 hr, although at the lower temperature the amount produced was much less than at the higher temperature. The 0.3% TTC alone at either +35°C or room temperature produced no color. Poplar solid medium plus 0.05% TTC produced a red color many times in excess of that produced at +35°C or at room temperature for Douglas-fir. Poplar at +35°C produced much more formazan than at room temperature when incubated for 24 hr. More curious however, is the fact that TTC alone at 0.05% showed color both at room temperature and +35°C for 24 hr in the dark although at the higher temperature the amount of formazan produced was much greater than at the lower temperature (Figure 73). Extensive testing, using an API 20F scheme (Appendix F) indicated that the formazan produced in the TTC solution with medium was a gram negative interobacteria, *Klebsiella pneumoniae*.

Klebsiella pneumoniae is a non-motile, capsulated rod bacterium found widely distributed in nature (soil, water, grain, etc.) and is normally found in the intestinal canal of animals. Some stains of the bacterium are highly virulent for mice. *K. pneumoniae* requires no special growth requirements and most strains can use citrate and glucose as a sole carbon source and ammonia as nitrogen source. Optimal temperature for this bacteria's growth is +35°C-+37°C and optimal pH is about 7.2 (Buchanan and Gibbons, 1974). The incubation conditions required to carry out the TTC reduction test appear perfect for growth of the bacteria. Carbon and

nitrogen sources are provided by the residual growth medium while the pH of the TTC bathing buffer and the incubation temperature of +35°C is ideal for the bacteria's growth. The origin of the bacteria is not clear. It may be speculated that perhaps mice came in contact with the building distilled water supply. Support for such a hypothesis was gained when water used to mix new buffer was sterilized before using. Also curious was the fact that the same buffer was used for the Douglas-fir culture TTC test but much less contamination color resulted. The cause of this difference is not assumed to be due to media since the Douglas-fir media did not vary greatly from that used to grow poplar cultures. A possible answer may be the almost 10 times greater TTC concentration used to test the Douglas-fir cells than the poplar cells. The 0.3% TTC concentration used with the Douglas-fir may be inhibitory to the bacteria's metabolism or division. Alternately, perhaps the 0.05% TTC concentration used with the poplar may be advantageous to the bacterial growth. The first expanation seems most plausable but further testing is needed to answer the question.

On using newly mixed TTC with sterilized buffer and washing test material with water after TTC incubation, the bacterial formazan production was eliminated, greatly reducing the amount of color produced after thawing. The color in the poplar media alone when used as a cryoprotectant was reduced significently (about 75%) using this procedure, whereas when using DMSO and glycerol at 5% the color was reduced by about 1/2 and at 10%

by 2/3 (Figure 74). Using the cultures washed in 5% glycerol showed a survival rate of 35% of the control directly after slow cooling at $1^{o}C$/min to $-40^{o}C$ and immersion in liquid nitrogen followed by thawing in a $+40^{o}C$ water bath, whereas cultures washed in 5% DMSO had a survival of 32% of the control. At 10% both DMSO and glycerol showed a survival only of 20% and 22% of the control (Figure 74).

Cryoprotectants used singly followed by fast freezing of cultures directly in liquid nitrogen gave the following survival values after thawing at $+40^{o}C$; 5% glycerol = 22.8% and 5% DMSO = 22.2% (Figure 75). These values were significently (10%) lower than when test cells were frozen slowly at $1^{o}C$/min (Figure 74). Dry weights of test samples did not show any increase over initial weight after 21 days post-thaw liquid culture (Table 8). Control dry weight increase was significant over the same time period. Glycerol at 10% showed a decrease in dry weight compared to initial weight, indicating perhaps some lysis in cells. No increase in dry weight was observed with poplar under these conditions even after 122 days post-thaw culture (Figure 76).

When poplar cultures were cooled slowly at $1^{o}C$/min from $+20^{o}C$ to $-40^{o}C$ using DMSO and glycerol at 0.2% and 2% respectively there was a 33% increase over the initial weight with DMSO at 2% and a 44% increase with glycerol at the same concentration. There was no increase in weight when glycerol at 0.2% was used and only a 6% increase of initial experimental weight when DMSO was used at this concentration.

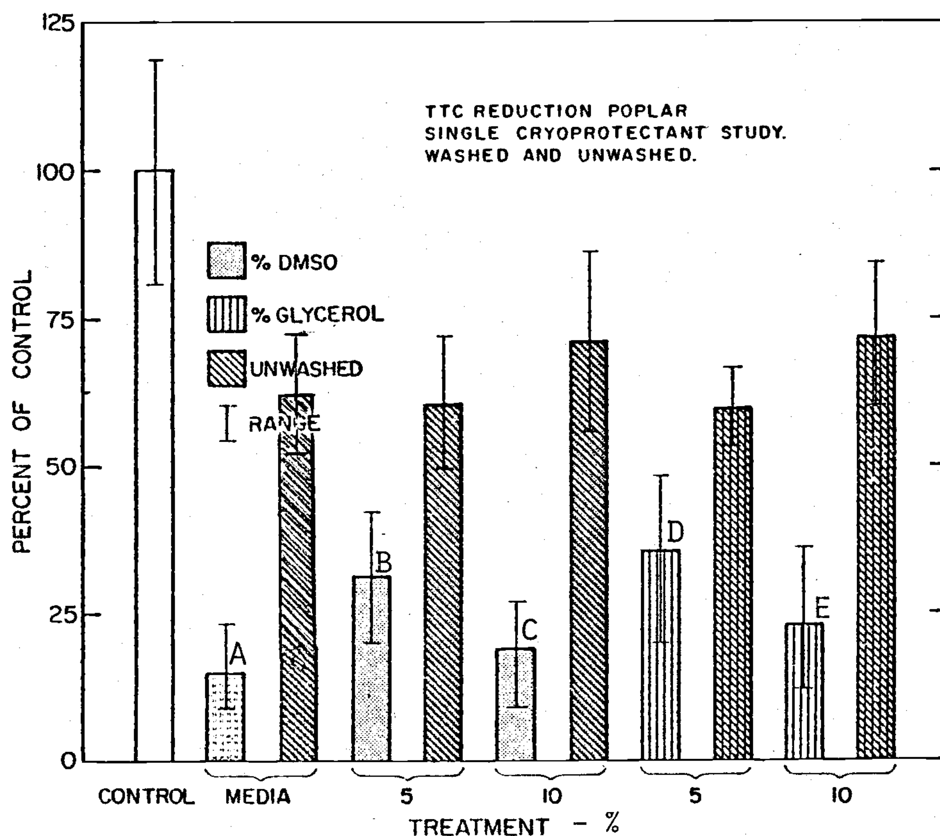


Figure 74. Effects of single cryoprotectants on TTC reduction of poplar suspension cultures cooled at 1°C/min from +20°C to -40°C and immersed in liquid nitrogen. Cryoprotectants were used at 5% and 10%. Thawing was fast in a +40°C water bath. Shown are cultures not washed with water after TTC incubation and also that after washing with water after TTC incubation. N=8
A:B=*; A:C=N.S.; A:E=N.S.; B:C=*; B:D=N.S.; D:E=*
* Indicates a significant difference at the 95% confidence level.

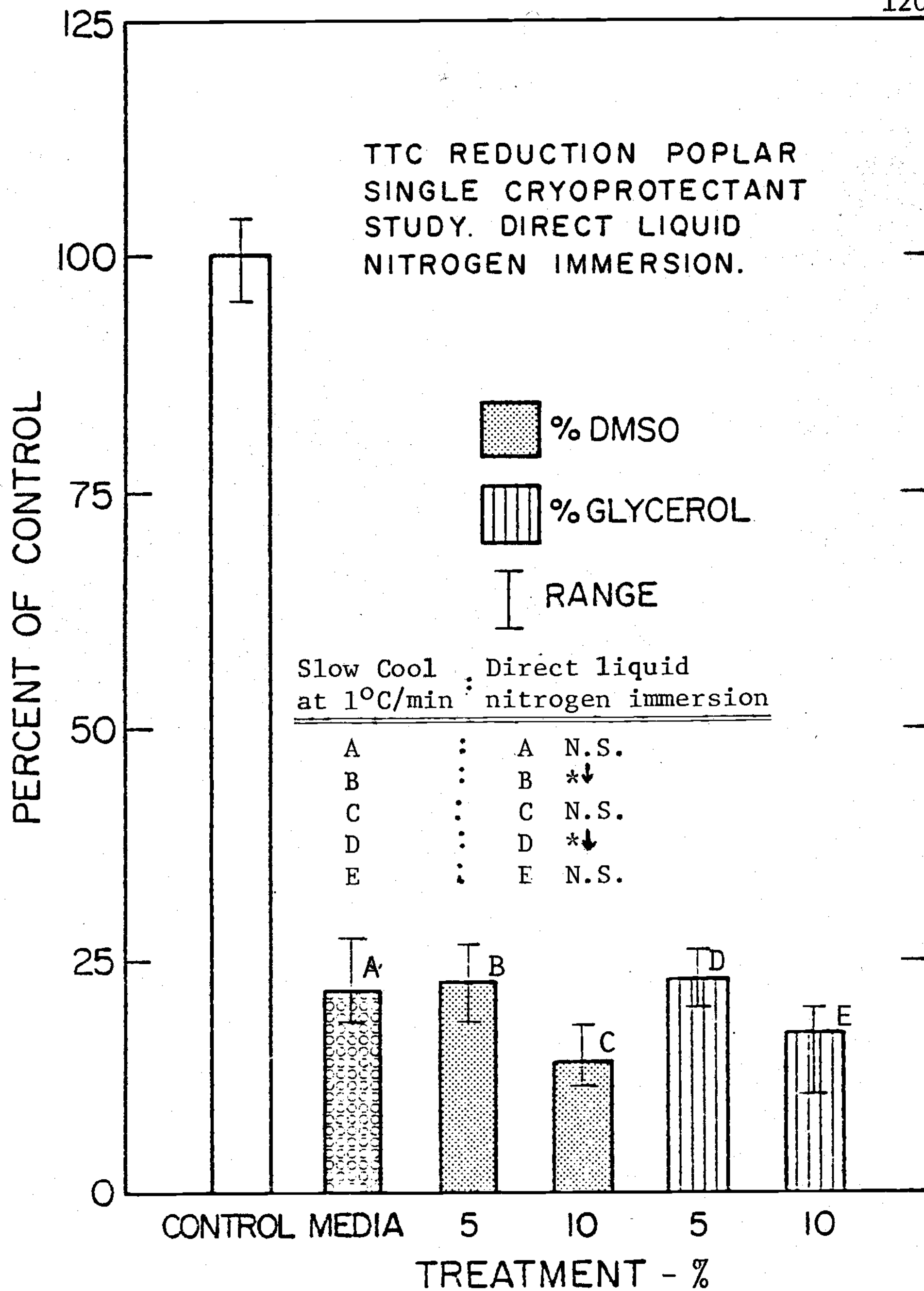


Figure 75. Effects of single cryoprotectants on TTC reduction of poplar suspension cultures immersed directly into liquid nitrogen. Cryoprotectants were used at 5% and 10%. Thawing was fast in a +40°C water bath. Data was based on new TTC buffer and post TTC incubation washing. N=8 A:B=N.S.; B:C=*; B:D=N.S.; D:E=*

* Indicates a significant difference at the 95% confidence level.

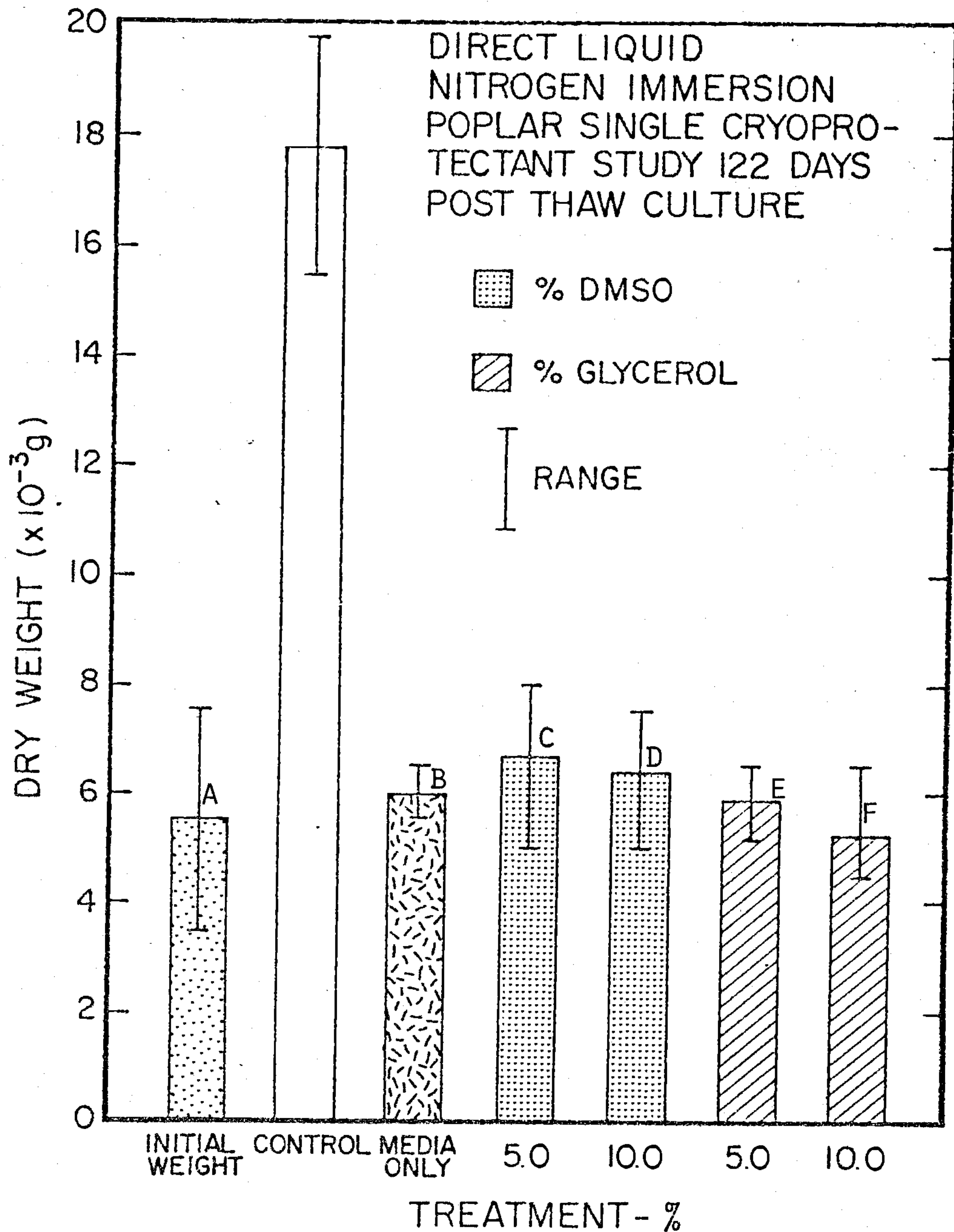


Figure 76. Effects of single cryoprotectants on total dry weight of poplar suspension cultures immersed directly in liquid nitrogen. Cryoprotectants were used at 5% and 10%. Thawing was fast in a +40°C water bath. N=4 A:B=N.S.; A:C=N.S.; A:D=N.S.; A:E=N.S.; A:F=N.S.; C:D=N.S.; E:F=N.S. * Indicates a significant differerence at the 95% confidence level.

Table 8. Poplar suspension cultures frozen directly in liquid nitrogen in the presence of single cryoprotectants at 5% and 10%. Dry weights taken 27 days after fast thawing at +40°C and culture in liquid media for 21 days. N=4 * Indicates a significant difference compared to initial weight at the 95% confidence level.

TREATMENT	DRY WEIGHT (x 10^{-3}g)	% INCREASE OF INITIAL WEIGHT
Initial weight	5.3	0.0
Control	10.2 *↑	+92.5
Media only	5.5	+3.8
DMSO 5%	5.7	+7.5
DMSO 10%	5.4	+1.9
Glycerol 5%	5.5	+3.8
Glycerol 10%	4.5 *↓	-15.1

Table 9. Poplar cell culture dry weights taken after being cooled slowly at 1°C/min from +20°C to -40°C then immersed into liquid nitrogen in the presence of single cryoprotectants at 0.2% and 2%. Thawing was rapid in at +40°C water bath. Post-thaw culture was in liquid medium for 68 days. Data is shown as direct weight increase and as % increase of initial weight (10^{-3}g). N=6 * Indicates a significant difference compared to initial weight at the 95% confidence level.

TREATMENT	DRY WEIGHT (x 10^{-3}g)	% INCREASE OF INITIAL WEIGHT
Initial weight	5.9	0.0
Control	21.2 *↑	+259.3
Media only	5.2	-11.9
DMSO 0.2%	6.3	+6.0
DMSO 2%	7.9 *↑	+33.9
Glycerol 0.2%	6.0	+1.6
Glycerol 2%	8.5 *↑	+44.1

Control value compared to initial control increased almost 260% during the same time period. When material was frozen in medium only, a 12% decrease occurred (Table 9).

A dry weight increase was also observed with poplar using DMSO and glycerol at 5% and 10%, even after 34 days post-thaw culture. The increases at 10% were however not significant. The increased values were 18% and 10% of initial control using 5% DMSO and 5% glycerol respectively. Medium only and glycerol at 10% both showed decreases in dry weights compared to the initial value, whereas DMSO at 10% was only slightly higher than the initial value (Table 10).

In order to distinguish between actual freezing damage and culture requirements, poplar cultures were frozen either as small aggregates (suspension) or large aggregates (callus) and post-thaw cultured in liquid or solid media. Post-thaw culture on solid medium produced a greater increase in weight than culture in liquid medium (Table 10). The implication here is that poplar survival is better on solid medium than in liquid medium after thawing. From the poor results obtained on solid medium, the reverse appears to be true for Douglas-fir.

Cryoprotectants added in combination, plus 1.0% proline were better than without proline, but the effect was not (Figure 77). Medium containing DMSO 5%/glycerol 5% provided the best post-thaw formazan production (40% of control value). On the average this was 8% and 5% better than for DMSO 5% and glycerol 5% alone, respectively.

Table 10. Poplar suspension or callus culture dry weights taken after being cooled slowly at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen in the presence of single cryoprotectants at 5% and 10%. Thawing was rapid in a +40°C water bath. Post-thaw culture was either in liquid medium or agar medium for 34 days. Data is shown as direct weight increases of suspension and callus culture after 34 days and as % increase of initial weight (10^{-3}g). N=4 * Indicates a significant difference compared to initial weight at the 95% confidence level.

TREATMENT	SUSPENSION ($x\ 10^{-3}$g)	% INCREASE OF INITIAL WEIGHT	CALLUS ($x\ 10^{-3}$g)	% INCREASE OF INITIAL WEIGHT
Initial weight	6.0	0.0	16.9	0.0
Control	14.3 *↑	+138.0	48.5 *↑	+187.0
Media only	5.9	-2.0	15.9	-5.9
DMSO 5%	7.1 *↑	+18.3	27.9 *↑	+65.2
DMSO 10%	6.4	+6.6	19.4	+14.9
Glycerol 5%	6.6	+10.0	24.2 *↑	+43.4
Glycerol 10%	5.5	-8.3	18.9	+11.6

Medium containing DMSO 5%/glycerol 2.5% was significantly less effective in medium containing DMSO 5%/glycerol 5% than rapid thawing in a +40°C water bath. The TTC reduction became non-significant for fast verses slow thawing when a combination of DMSO 1%/ glycerol 1% was used.

Formazan reduction was significantly higher when 4% sucrose was added to the cryoprotectant solution (Figure 77-f and g). Proline addition increased formazan production little; the difference being non-significant (Figure 77-e and f).

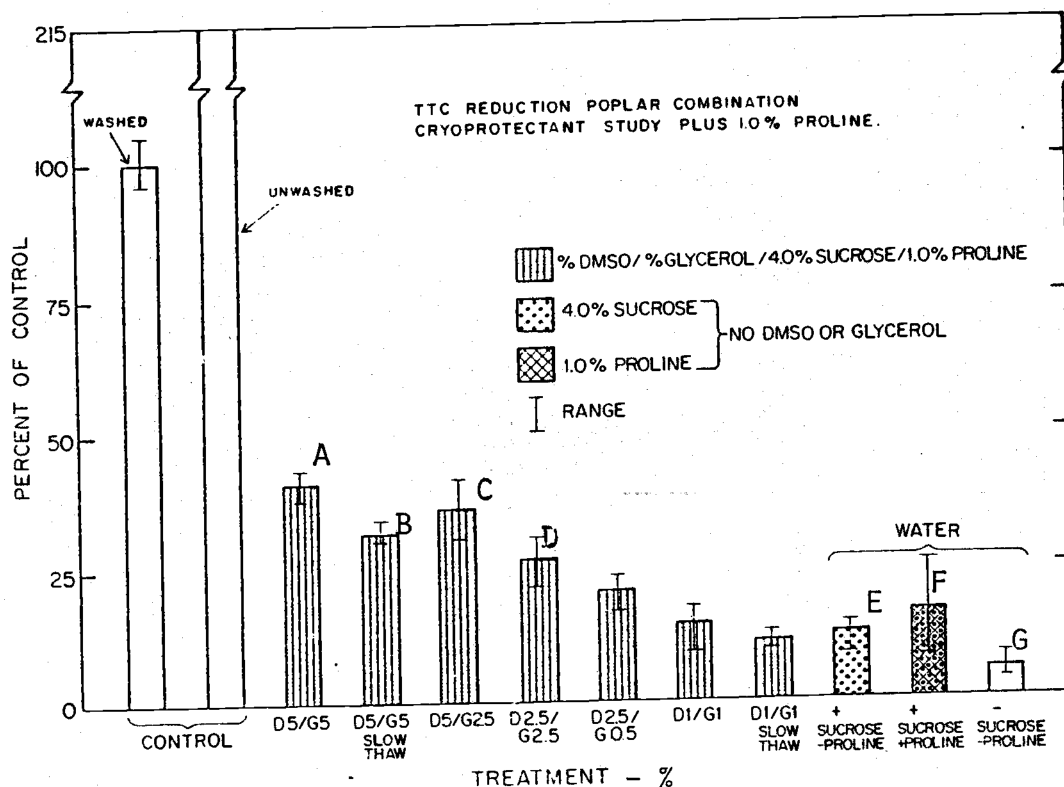


Figure 77. Effects of cryoprotectants in combination on TTC reduction of poplar suspension cultures pre-grown 5 days and cooled with 1% proline. Cultures were cooled at $1^{o}C$/min from $+20^{o}C$ to $-40^{o}C$ then immersed in liquid nitrogen. Cryoprotectants were used in combination: DMSO $\leq 5\% \geq 1\%$ and glycerol $\leq 5\% \geq 0.5\%$. Also the effect of proline with and without 4% sucrose was shown. Thawing was fast in a $+40^{o}C$ water bath or slowly at room temperature. Data was based on new TTC buffer and post TTC incubation washing. N=8 A:B=*; A:C=*; C:D=*; E:F=N.S.; E:G=*; F:G=* * Indicates a significant difference at the 95% confidence level.

When TTC reduction was tested after 66 days post-thaw culture, differences in amount of formazan produced was observed depending on whether callus was green or non-gren and depending upon the combination and amount of cryoprotectants originally used in the freezing process. Cultures which had become green produced about twice as much formazan as did the non-green cultures using DMSO 5%/glycerol 5% and DMSO 5%/glycerol 2.5% as cryoprotectants. Using both combinations of cryoprotectants, the non-green cultures produced reduction values close to the initial values obtained after freezing while the values of the green cultures doubled. At the most optimum cryoprotectant combination conditions (DMSO 5%/glycerol 5%), formazan production was only 20% below initial control value (Figure 78), and differences were visible in the cultures (Figure 79).

Dry weights taken at 66 days under the same conditions showed there was an apparent dry weight increase compared to initial dry weight amount even at DMSO 1%/glycerol 1% but this difference was not significant. Green callus dry weights were close to double the non-green dry weights (Figure 80).

The greatest TTC reduction occurred with poplar if callus was cold conditioned 8 weeks at +4°C/17 hr in the dark and +14°C/ 7 hr in the light before freeze testing. Cold conditioned callus, if frozen by direct immersion in liquid nitrogen in the dry condition (i.e., without any liquid in the freezing vial) showed 30%

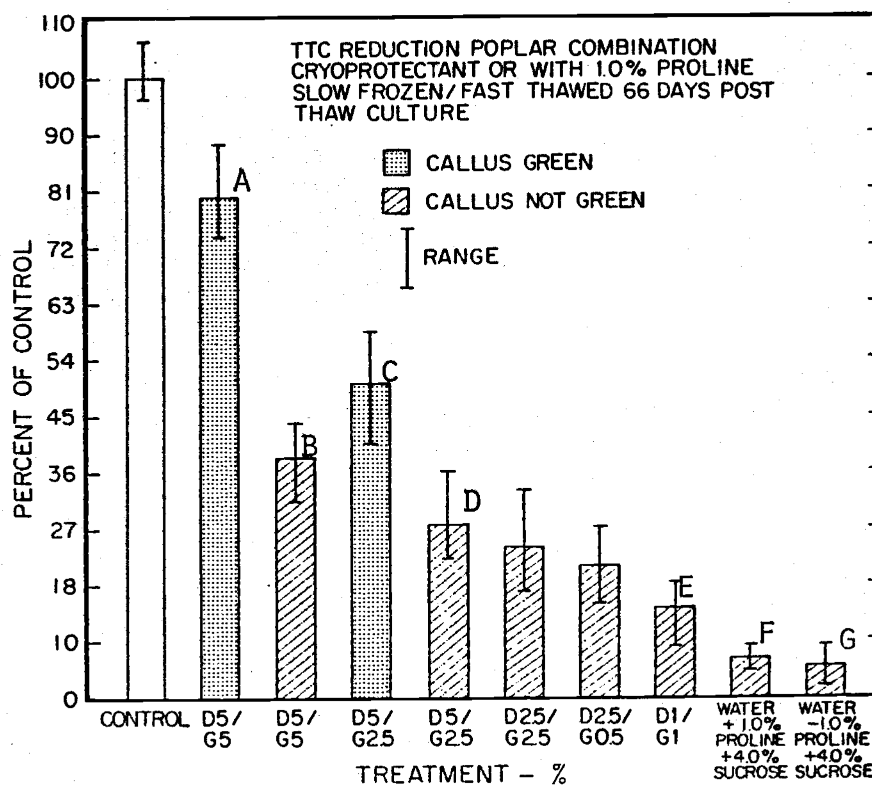


Figure 78. Effects of cryoprotectants in combination on TTC reduction of poplar cultures which were pre-grown 5 days then frozen with 1% proline. Cooling was at 1°C/min from +20°C to -40°C then immersed into liquid nitrogen. Cryoprotectants were used in combination at DMSO ≤ 5% ≥ 1% and glycerol ≤ 5% ≥ 0.5%. Also the effects of proline are shown. Thawing was fast in a +40°C water bath. Data was based on new TTC buffer and post-thaw non-green callus 66 days post-thaw culture. N=8 A:B=*; B:C=*; C:D=*; B:D=*; D:E=*; E:F=*; F:G=N.S.

* Indicates a significant difference at the 95% confidence level.

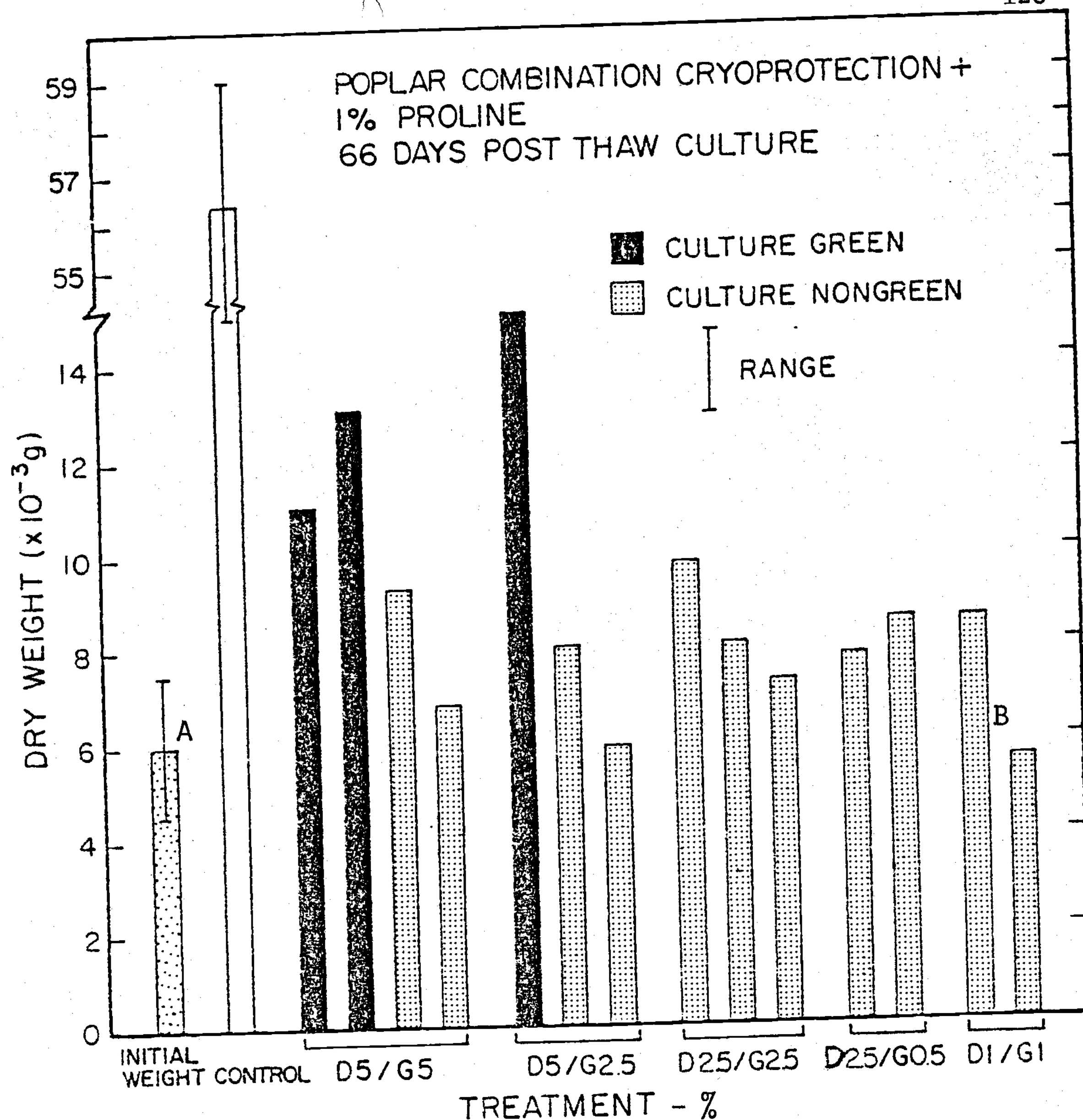


Figure 80. Total dry weights of poplar suspension cultures which were pre-grown then frozen with 1% proline. Cooling was at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Cryoprotectants were used in combination DMSO $\leq 5\% \geq 1\%$ and glycerol $\leq 5\% \geq 0.5\%$. Dry weights were taken at 66 days post-thaw culture. Shown are single observations of green and non-green cultures, initial weight and control weight are averages of 4 observations. N=4 A:B=N.S.

more formazan production whether thawed slowly in air or thawed rapidly at +40°C, compared to the same conditions using growth room callus. Cold conditioned callus and growth room callus thawed at +40°C gave initial thaw formazan production values of 61% and 28% respectively (Figure 81). About 6% less TTC reduction occurred when callus was directly immersed in liquid nitrogen and DMSO at 5% was used as cryoprotectant (Figure 83), than when callus was frozen dry under the same conditions (Figure 81).

TTC reduction occurred more rapidly even after 2 hr at +22°C if cold room conditioned callus was frozen dry rather than if this callus was frozen with DMSO 10%/glycerol 5%/sucrose 25% (Figure 82-A). Only after 11 hr at +35°C in the dark did cold room material frozen slowly at 1°C/min from +20°C to -40°C using DMSO 10%/glycerol 5%/sucrose 25% as cryoprotectants show formazan production in the callus (Figure 82-C). The TTC reduction was over 83% of the control for cold room, frozen dry callus, and over 53% of the control for callus frozen with DMSO 10%/glycerol 5%/sucrose 25%. Growth room callus cooled with DMSO 10%/glycerol 5%/sucrose 25% showed 12% higher TTC reduction than the same treated callus cooled dry (Figure 83), but 6% lower than the same treated callus cooled with DMSO 5%/glycerol 5%/sucrose 4% (Figure 77).

Callus grown for 50 days after thawing showed formazan production greater than that of the initial control for growth room callus frozen with DMSO 5%/glycerol 5%; with DMSO 5%; and with

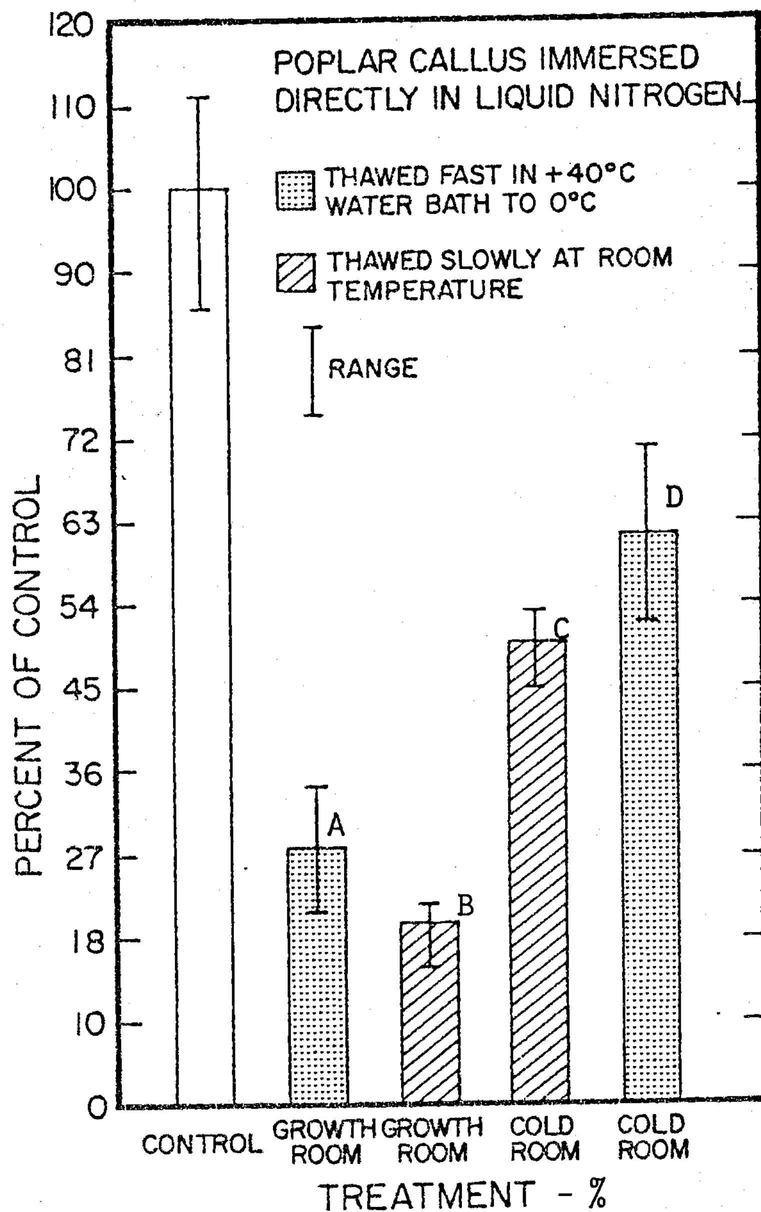


Figure 81. The TTC test of poplar callus after 8 weeks cold room conditioning or growth room callus immersed directly into liquid nitrogen in dry condition (without liquid). Thawing was fast in a +40°C water bath to 0°C, then warming at room temperature to +22°C or thawing from -196°C to +22°C slowly in air. Data was based on new TTC buffer and post TTC incubation washing. N=8
A:B=*; C:D=* * Indicates a significant difference at the 95% confidence level.

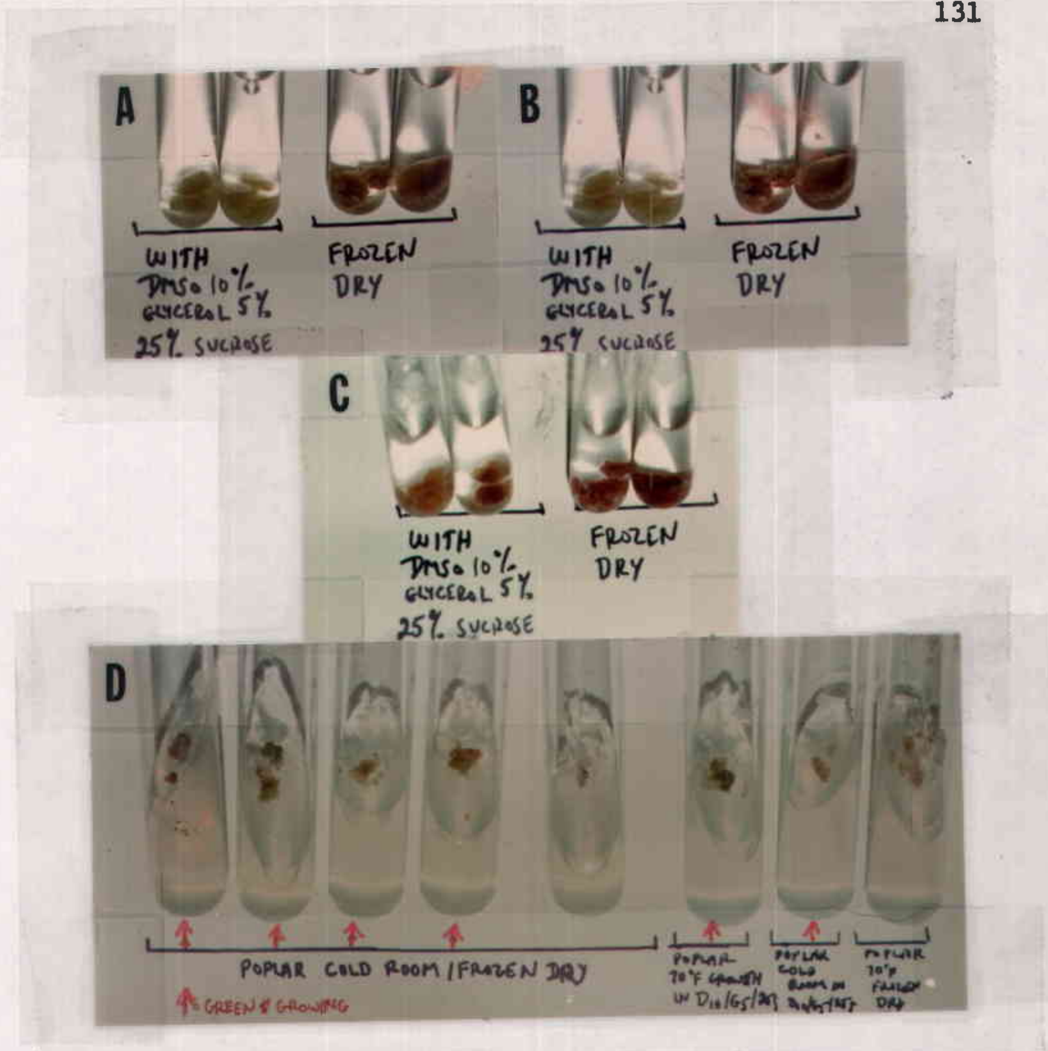


Figure 82. Poplar callus conditioned in cold room for 8 weeks then cooled at $1^{o}C/min$ form $+40^{o}C$ to $-40^{o}C$ and immersed in liquid nitrogen under conditions shown. Thawing was fast in a $+40^{o}C$ water bath. "A" shows TTC reduction after 2 hr, (B) TTC reduction after 4 hr, (C) TTC reduction after 15 hr. "A" and "B" were at $+25^{o}C$ and "C" after 4 hr at $+25^{o}C$ and 11 hr at $+35^{o}C$ in the dark. The TTC reduction quickly becomes noticable in the callus frozen in dry condition compared to callus frozen with cryoprotectants. "D" shows callus growing after 50 days post-thaw culture (red arrows indicate growing callus).

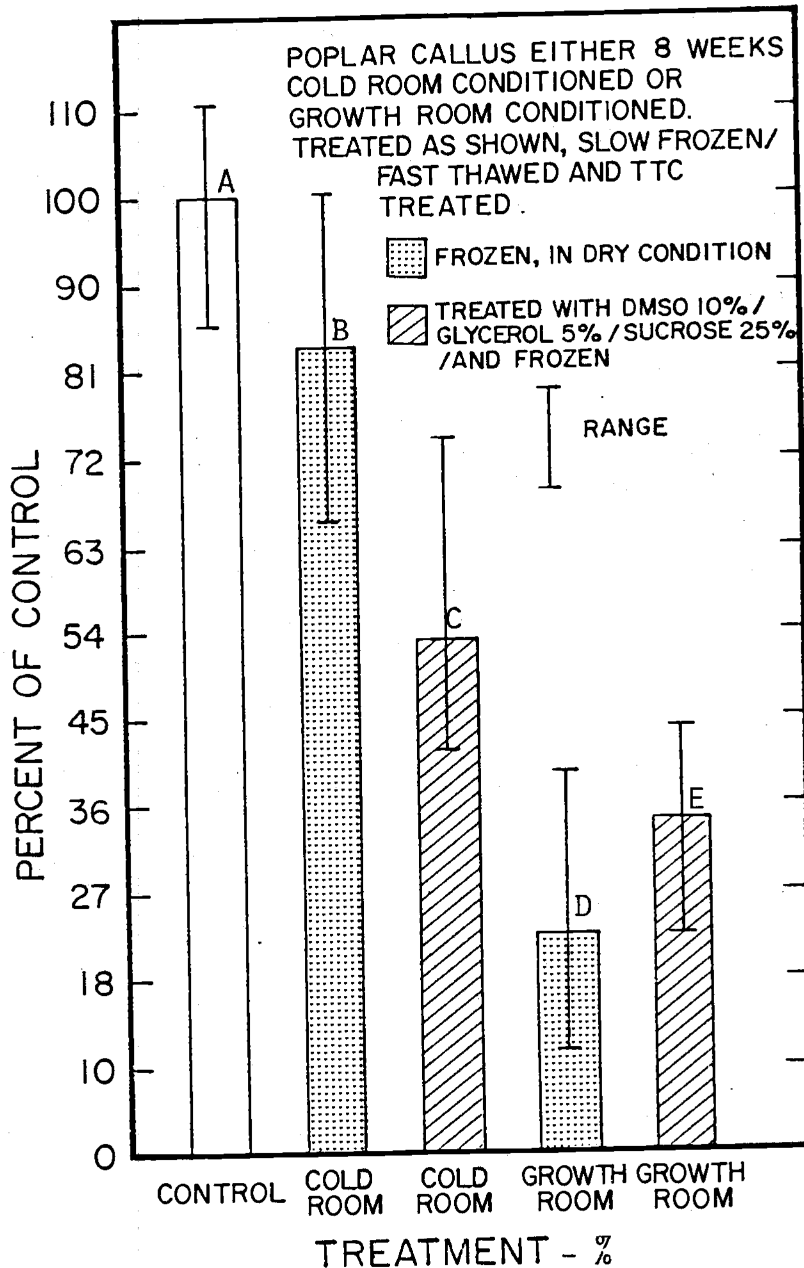


Figure 83. The TTC test of poplar callus after 8 weeks cold room conditioning or growth room callus cooled slowly at $1^{o}C$/min to $-40^{o}C$ them immersed in liquid nitrogen either dry (without liquid) or in the presence of DMSO 10%/glycerol 5%/sucrose 25%. Thawing was fast in a $+40^{o}C$ water bath. Data was based on new TTC buffer and post TTC incubation washings. N=8 A:B=*; B:C=*; C:E=*; D:E=* * Indicates a significant difference at the 95% confidence level.

cold room conditioned frozen dry. The latter showed values much greater than the former two (Table 11). Cold room conditioned callus gave a TTC reduction value 80% of control and growth room callus treated with DMSO 10%/glycerol 5%/sucrose 25% produced a TTC reduction value 43% of initial control. Cold conditioned callus TTC values were double and growth room callus TTC values were only 3% higher when compared to initial growth room callus TTC values using DMSO 5%/glycerol 5% as cryoprotectants (Figure 77). Cultures treated with DMSO 10%/glycerol 10%, DMSO 1%/ glycerol 1% and growth room callus frozen dry, all showed reduced TTC reduction values after 50 days compared to initial thaw values (Table 11 and Figure 84). To ensure no TTC values recorded resulted from contamination, a callus sample was heat-killed and TTC tested. Little color resulted (Figure 84-t and Table 11) indicating that the color seen in other test samples did result from the test sample reaction and not from contamination.

Thawed samples of callus treated with DMSO 5%, glycerol 5% (Figure 85) and DMSO 5%/glycerol 5%, DMSO 5%/glycerol 2.5% (Figure 86) frozen then thawed and grown on solid callus media for 50 days were green and growing. Poplar callus which had been frozen in dry condition after 8 weeks cold conditioning, growth room callus and cold room callus exposed to DMSO 10%/glycerol 5%/ sucrose 25% while freezing are also shown growing after 50 days post-thaw culture (Figure 82-D).

Figure 79. The TTC test of poplar callus cultures frozen at 1°C/min to -40°C then immersed in liquid nitrogen. Cryoprotectants were used in combination as shown. The TTC test was made 66 days after thawing. New buffer was used and callus was washed after TTC incubation.

Figure 84. The TTC test of poplar callus which had been cooled at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen. Treatments shown are (l) gorwth room callus treated with DMSO 10%/glycerol 10%; (m) growth room callus treated with DMSO 5%; (n and o) cold room callus frozen dry; (p) growth room callus treated with DMSO 10%/glycerol 5%/sucrose 25%; (q) cold room callus treated with DMSO 10%/glycerol 5%/sucrose 25%; (r) growth room callus treated with DMSO 1%/glycerol 1%; (s) growth room callus treated with DMSO 5%/glycerol 5%; (t) heat-killed callus. For all cultures, thawing was fast in a +40°C water bath. The TTC test was made after more than 50 days of growth on solid medium after thawing. The data was based on new TTC buffer and water washing after TTC incubation. Note difference between cold room callus frozen dry (o) and cold room callus frozen with DMSO 10%/glycerol 5%/sucrose 25% (q).

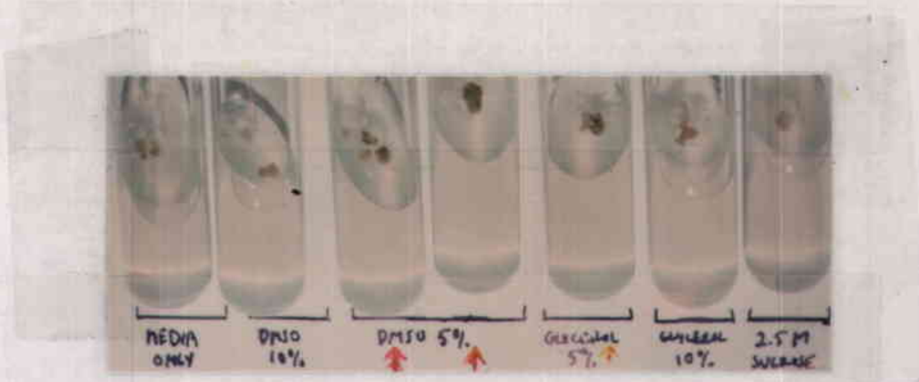


Figure 85. Poplar callus cultures 50 days after being cooled slowly at 1°C/min from +20°C to -40°C then immersed in liquid nitrogen and thawed fast in at +40°C water bath. Treatments are as indicated. Red arrows indicate cultures which are green and growing.

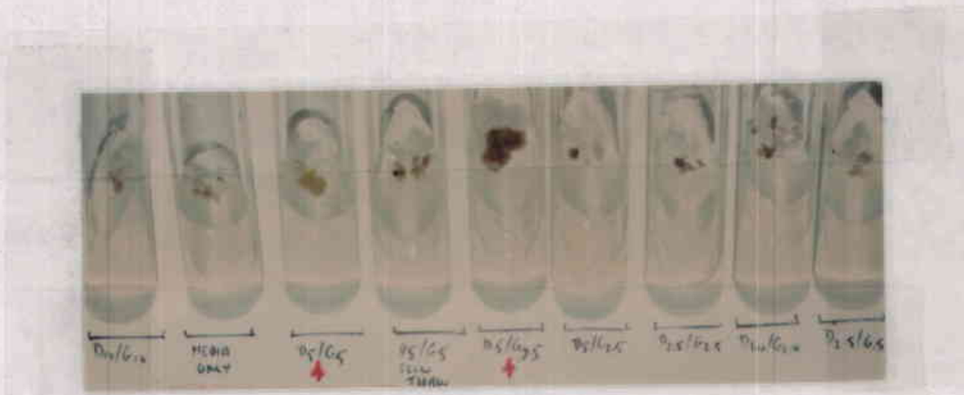


Figure 86. Poplar callus cultures 50 days after being cooled slowly at 1°C/min to -40°C then immersed in liquid nitrogen and thawed fast in +40°C or slowly in air. Treatments are as shown. Red arrows indicate green and growing cultures.

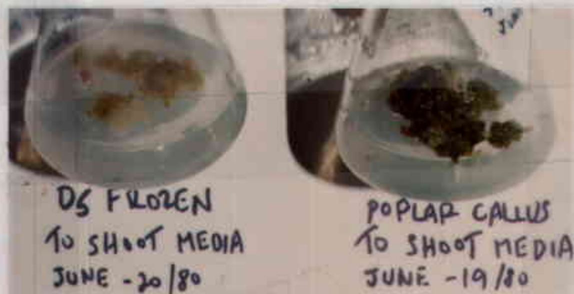


Figure 87. Color differences are shown between callus frozen to liquid nitrogen with 5% DMSO (left) and callus grown at +22°C (right). Both were placed on shoot media at this time.

Table 11. Poplar callus TTC reduction tests made after greater than 50 days post-thaw agar culture. Cultures were cooled slowly at 1°C/min to -40°C then immersed in liquid nitrogen under conditions shown. Thawing was fast in a +40°C water bath. Data was shown as % of control. (See also Figure 84.) N=4 * Indicates a significant difference from initial thaw time at the 95% level of confidence ↓ or ↑ indicates decrease or increase in TTC production.

TREATMENT	TTC REDUCTION (% OF CONTROL)	
DMSO 10%/glycerol 10%	15.39	N.S.
DMSO 5%/glycerol 5%	>120.00	*↑
DMSO 5%	>120.00	*↑
Cold room conditioned callus frozen with DMSO 10%/glycerol 5%/sucrose 25%	80.00	*↑
Cold conditioned callus frozen in dry condition	>>120.00	*↑
Growth room callus frozen with DMSO 10%/glycerol 5%/sucrose 25%	43.58	N.S.
DMSO 1%/glycerol 1%	13.20	*↓
Growth room callus frozen in dry condition	12.95	*↓
Heat-killed callus (not frozen)	6.38	*↓

Initial growth of callus was slow and callus culture color was pale or colorless compared to control cultures growing in the growth room. This suggested chloroplast damage resulting from freezing and thawing (Figure 87).

In order to determine whether post-thawed, growing poplar callus could regenerate shoots, an experiment was carried out where control (room grown callus), cold room conditioned callus frozen dry to -196°C then thawed fast at +40°C, and callus frozen to -196°C

using DMSO at 5% as a cryoprotectant then thawed fast were placed on poplar shoot forming medium. Shoots formed on all three test samples after 55 days on the shoot forming medium (Figure 88).

Paraffin sections were made of all frozen and control callus to determine if any gross anatomical differences occurred. Callus grown on callus medium for the same amount of time as the samples grown on shoot forming medium showed little organization, except for the occassional isolated cells with secondary cell wall, or distorted vessel. The majority were large vaculated parehymatous cells (Figure 89). Callus from the three test sample types given above showed extensive internal organization, including meristemoids containing vessels with well developed secondary cell walls (Figures 90, 91, and 92). Vessels had normal appearing secondary walls along their entire length (Figure 93-m) and had well developed bordered pit pairs between adjacent vessels (Figure 93-o). In some cases, roots formed, which anatomically were identical to those described in Appendix B, Figure 23 (Figure 94-A).

Shoot development also appeared essentually the same as described in Appendix B. Shoots with well organized apices and young leaves with vascular bundles extended out of the basic parenhymatous callus mass (Figures 94-C, D and E). Cold conditioned callus (Figure 95) which had not been placed on callus-forming medium prior to shoot-forming medium and callus from DMSO 5% treated, frozen and thawed callus first placed on callus forming medium (Figure 94-C) produced shoots which appeared very similar to

those described in Appendix B. With cold conditioned callus, shoots also extended beyond the initial callus (Figure 95-C) and showed developing vascular traces (Figure 95-D) and apices with a tunica-corpus zonation.

A summary of poplar material treated, frozen and thawed under different conditions as it appeared after 50 days, post -thaw culture is given in Table 12.

Douglas-fir results were encouraging for callus which had been cold conditioned for 8 weeks prior to freezing. When this callus was immersed directly in liquid nitrogen in the dry condition then thawed at +40°C and TTC tested, TTC activity was 40% of the control. This was almost 13% higher than growth room callus treated with DMSO at 5% then frozen. Thawing from -196°C at room temperature gave TTC activity 9% less than when callus was thawed in a +40°C water bath to 0°C and then warmed to room temperature in air.

Growth room Douglas-fir callus frozen dry in liquid nitrogen showed formazan production when thawed at +40°C that was 27% of the unfrozen control. When the callus was thawed in air from -196°C this value was reduced to 18% of the control (Figure 96). This difference was significant. What may be membrane and/or metabolic differences as indicated by the color differences are shown in Figure 102).

Figure 88. Poplar callus growing shoots 55 days after being placed on solid shoot media. (A) Callus from growth room (control); (B) callus cold conditioned for 8 weeks at +4°C; and (C) callus treated with 5% DMSO then frozen slowly at 1°C/min to -40°C then immersed in liquid nitrogen. Thawing was fast in a +40°C water bath.

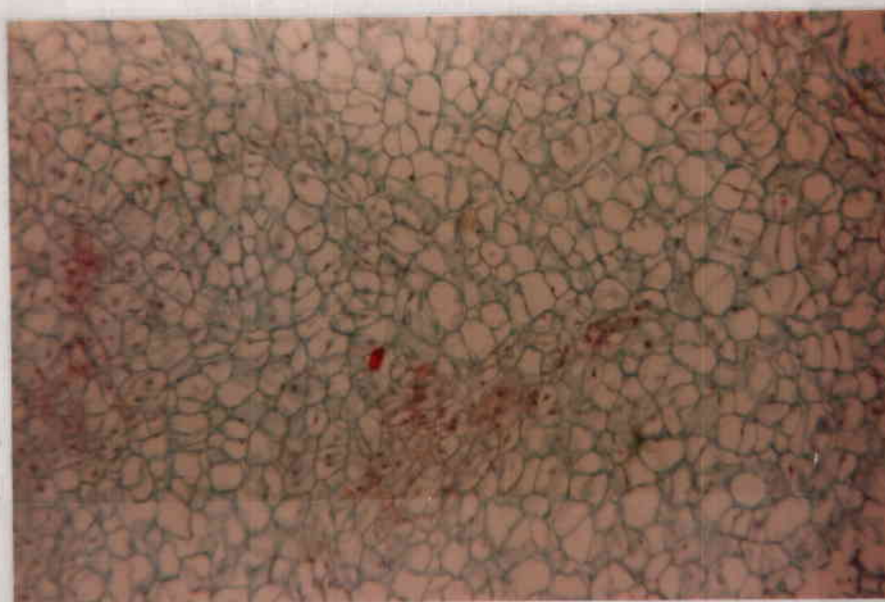
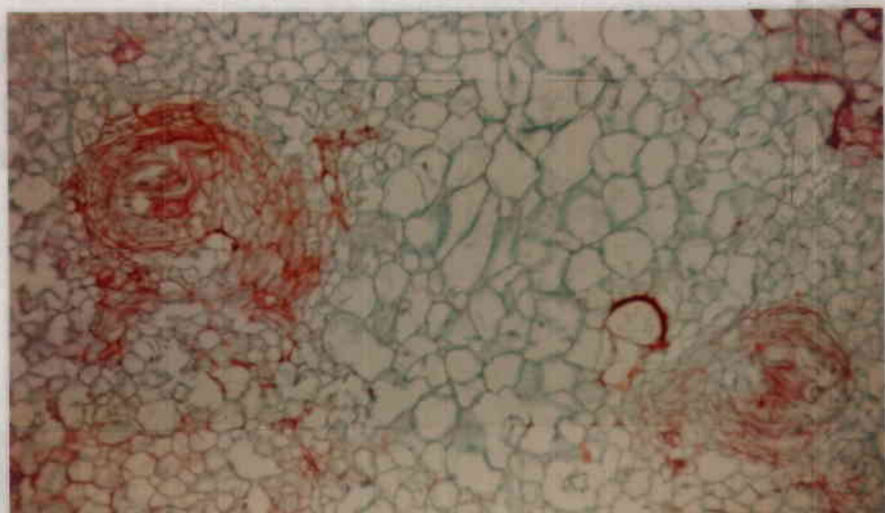


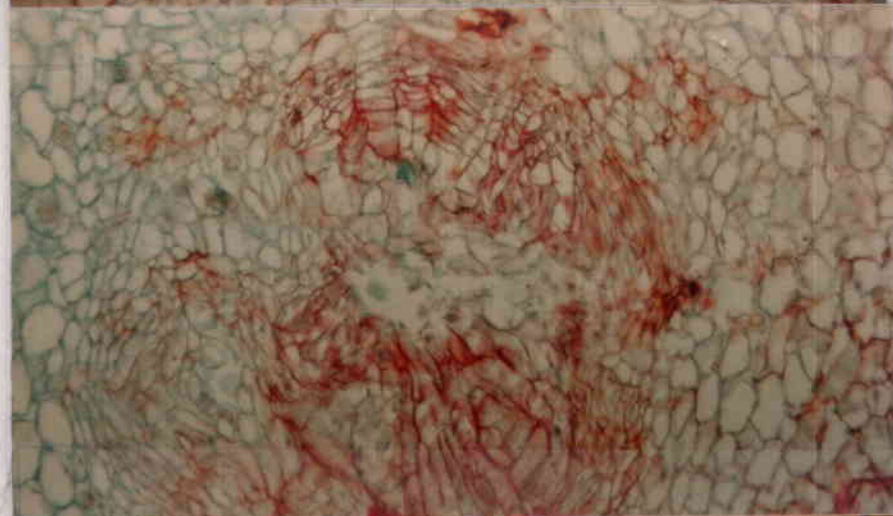
Figure 89. Paraffin section of poplar callus placed on callus medium and grown 55 days. Similar to cultures placed on shoot media. Notice the absence of meristemoids. (x160) (Compare to Figure 90.)

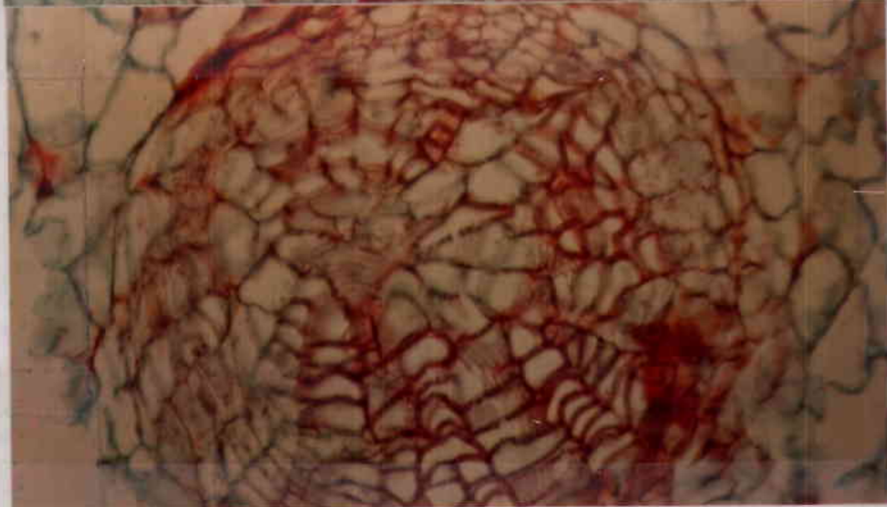
Figure 90. Paraffin section of poplar callus showing meristemoids (red areas) forming internally in callus initially grown at +22°C then placed on poplar shoot forming medium (x80). (Compare to Figure 89 which shows similar callus placed only on callus forming media).

Figure 91. Paraffin section of poplar callus formed from cell aggregates cooled slowly at 1°C/min from +20°C to -40°C in the presence of 5% DMSO and immersed in liquid nitrogen. After 5 days at -196°C the culture was thawed fast in a +40°C water bath to 0°C and then warmed slowly in air to room temperature. After fresh medium washing, the callus was placed on poplar callus-forming solid medium for 36 days and then transferred to poplar shoot-forming media for 61 days. Note internal meristemoid (red area) showing vascular development.(x160)

Figure 92. Paraffin section of poplar callus formed from callus cold conditioned at +4°C/17 hr, +14°C/7 hr for 8 weeks and cooled slowly dry at 1°C/min from +4°C to -40°C then immersed in liquid nitrogen. After 5 days at -196°C the callus was thawed fast in a +40°C water bath. The callus was placed directly on poplar shoot-forming medium for 55 days. Note internal meristemoid showing vascular development.(x400)







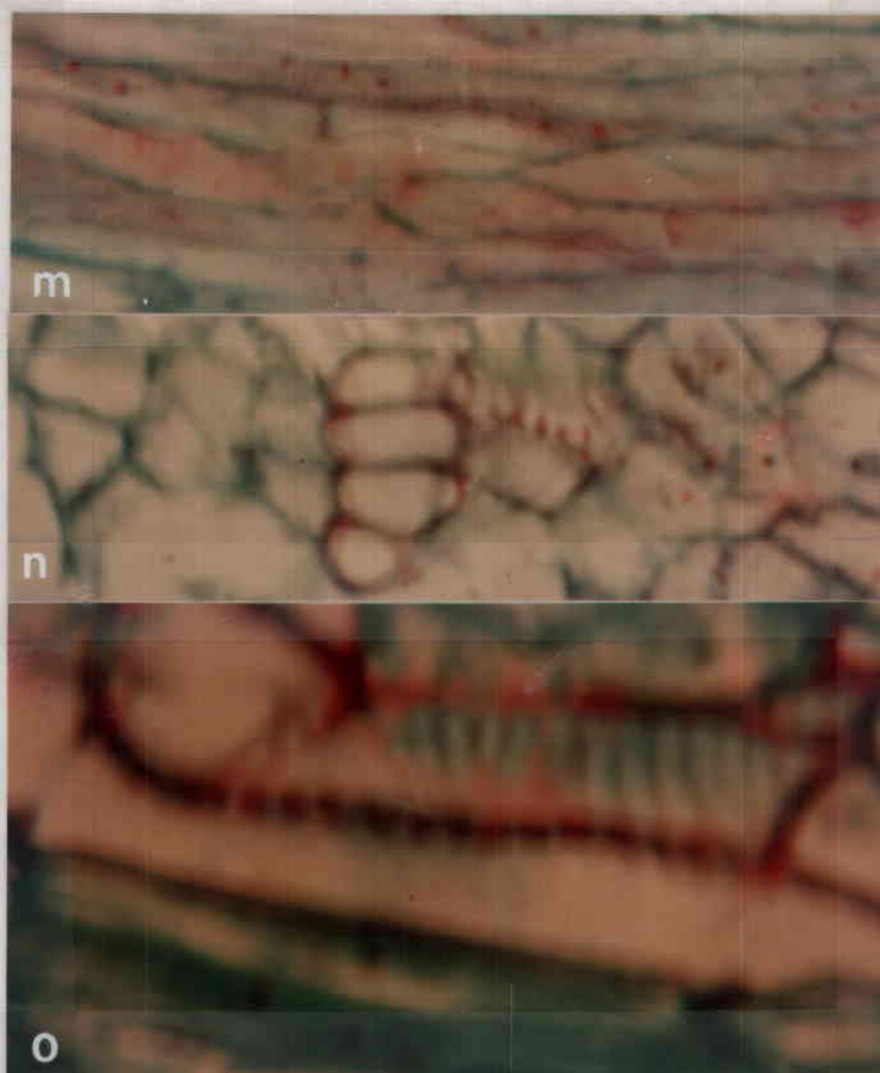


Figure 93. Vascular development in poplar callus previously frozen. Red stain shows the secondary cell wall. (M and N) callus as shown in Figure 90 (x500, x630 respectively); and (o) callus as shown in Figure 92. A large vessel is shown with secondary walls and well developed bordered pit pairs.

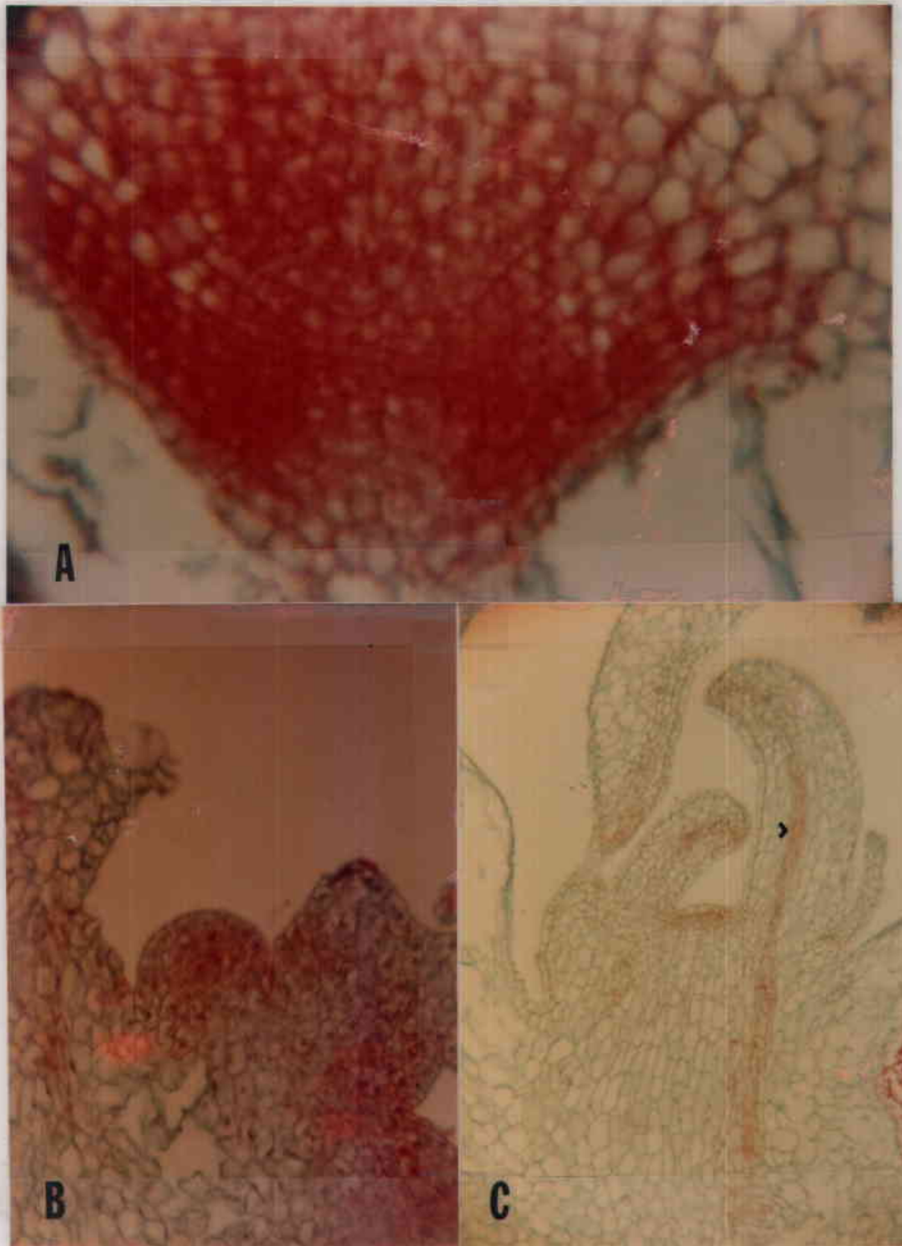


Figure 94. (A) Root tip formed from poplar callus cooled at 1°C/min from +40°C to -40°C then immersed in liquid nitrogen in the presence of 5% DMSO. Thawing was fast at +40°C. Callus was then placed for 36 days in callus-forming media then 61 days in shoot-forming media. Compare to Figure 23 in Appendix B (x200). (B) Shoot forming from treatment described in Figure 90 (x160). (C) Shoot forming from treatment described in Figure 91. Notice vascular trace (>) x125.

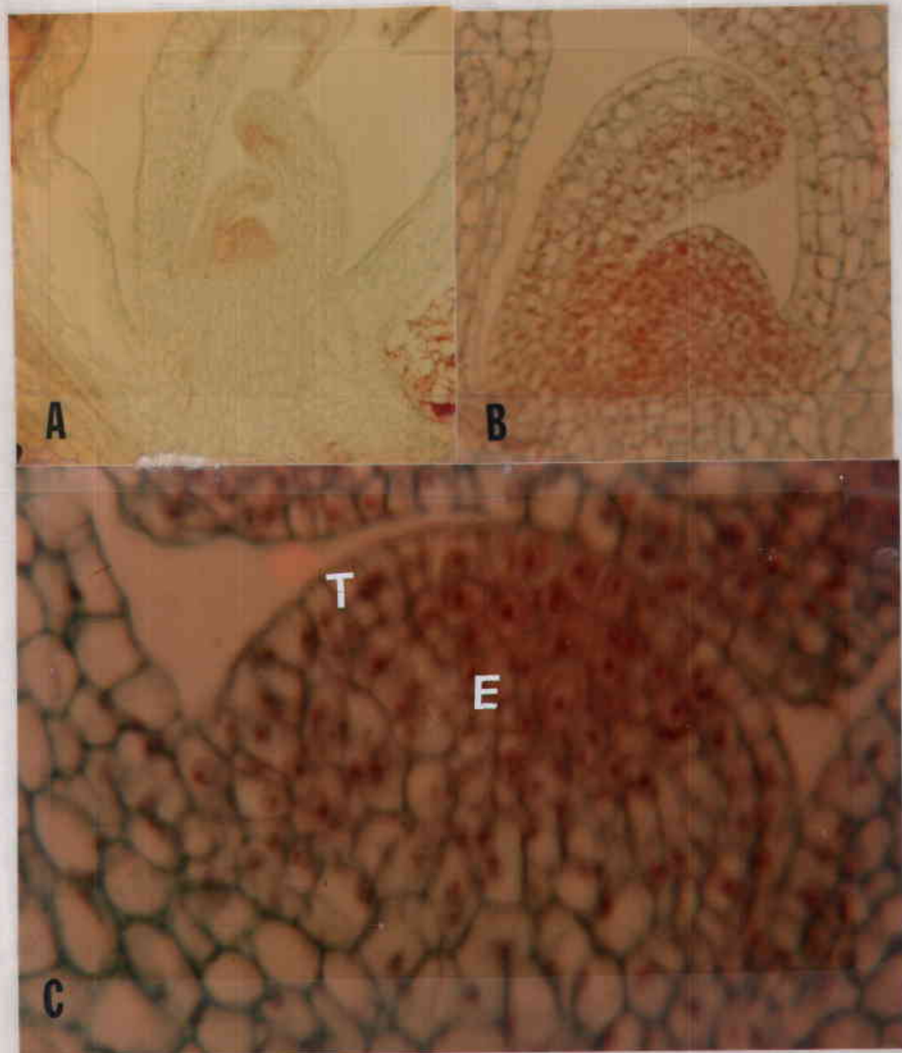


Figure 95. Shoot formed from callus treated as described in Figure 92. (>) Indicates developing vascular system; (A) x80 and (B) x160. (C) Shows close-up of an apex from poplar callus described in Figure 92; showing the tunica (T) and corpus (E) x400.

Table 12. A summary of poplar post-thaw agar culture data after being treated as indicated at greater than 50 days after thawing.

TREATMENT	RESULTS*	FIGURE #
A. Growth room source callus frozen slowly at $1^{o}C/min$ to $-40^{o}C$, then immersed in liquid nitrogen and fast thawed ($+40^{o}C$ water bath). Cell pre-growth and freezing were done in 1% proline. Callus was grown for 108 days after thawing.		
Media only	-	85
DMSO 10%	-	85
DMSO 5%	+	85
Glycerol 5%	+	85
Glycerol 10%	-	85
2.5M Sucrose	-	85
Thawed fast ($+40^{o}C$ water bath) or slowly (in air) at 82 days post-thaw culture.		
Media only	-	86
DMSO 10%/glycerol 10%	-	84 & 86
DMSO 5%/glycerol 5%	+	79, 84 & 86
DMSO 5%/glycerol 5% slow thaw	-	86
DMSO 5%/glycerol 2.5%	+	79 & 86
DMSO 2.5%/glycerol 2.5%	±	79 & 86
DMSO 1%/glycerol 1%	-	84 & 86
DMSO 2.5%/glycerol 0.5%	-	79 & 86

(Continued)

Table 12. (Continued)

TREATMENT	RESULTS*	FIGURE #
B. Frozen as "A" but without proline, at 106 days post-thaw culture.		
DMSO 10%	-	
DMSO 5%	+	84 & 88
Glycerol 5%	+	
Glycerol 10%	-	
C. Cold room callus (conditioned 8 weeks at +40°C/17 hr dark and +12°C/7 hr light at 800 foot candles) or growth room callus frozen as "A" but without proline, after 66 days post-thaw culture.		
Cold room callus frozen dry	++	82
Growth room callus exposed 1.5 hr at room temperature and frozen with DMSO 10%/glycerol 5%/ sucrose 25%	±	82
Cold room callus exposed 1.5 hr at room temperature and frozen with DMSO 10%/glycerol 5%/ sucrose 25%	+ to ++	82
Growth room callus frozen dry	-	82

*

Result Key

++ > 50% of cultures showed growth

\+ 50% of cultures showed growth

± < 50% of cultures showed growth

_ No cultures showed growth

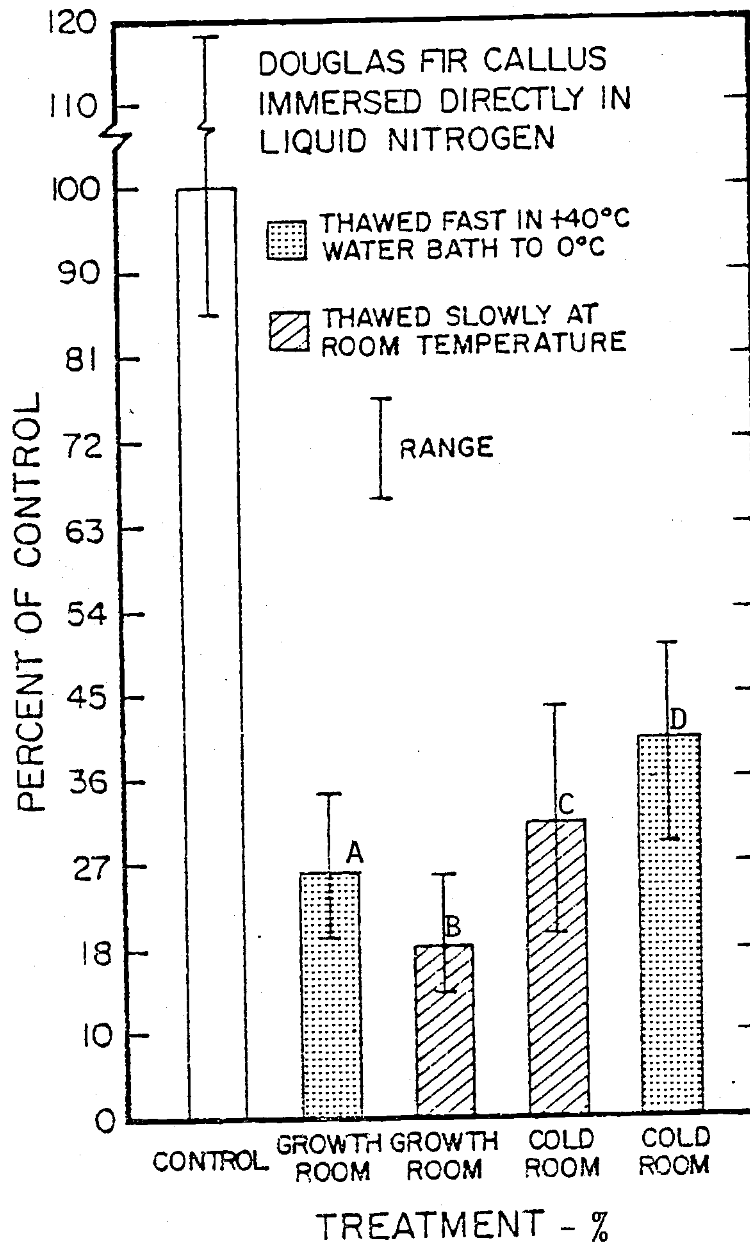


Figure 96. Dougla-fir TTC test after either 8 weeks cold room (+4°C) conditioning or growth room callus was immersed directly into liquid nitrogen in the dry condition. Thawing was fast in a +40°C water bath to 0°C and then warmed at room temperature to +22°C or thawed from -196°C to +22°C slowly in air. N=8 A:B=*; C:D=* * Indicates a significant difference at the 95% confidence level.

When cold room callus of Douglas-fir was cooled slowly at 1°C/min from +20°C to -40°C and thawed at +40°C, the initial TTC reduction was about equal to that achieved using the best cryoprotectant combination conditions (DMSO 5%/glycerol 1%) (Figure 96). If the same callus was treated with DMSO 10%/glycerol 5%/sucrose 25%, then frozen and thawed under the same conditions formazan production was decreased by 10% from the former to 59% of initial control value (Figure 97). This difference was however non-significant when growth room callus was cooled and thawed in the same way as the cold conditioned callus, TTC reduction values were severely reduced, compared to cold conditioned callus. In callus which was frozen dry, formazan production was only 17% of the initial control and adding DMSO 10%/glycerol 5%/sucrose 25% reduced this value to 12%. Growth room callus TTC values could be increased by placing the test material at +4°C for 24 hr either before freezing slowly in the dry condition or with DMSO 10%/glycerol 5%/sucrose 25%. The latter treatment increased TTC reduction by almost 14%. When the test material was allowed to cool below 0°C and freeze, TTC reduction values were again reduced. Callus frozen with DMSO 10%/glycerol 5%/sucrose 25% gave a TTC reduction of 6% better than did callus frozen dry (Figure 97). This value is however not-significant at the 95% level of confidence.

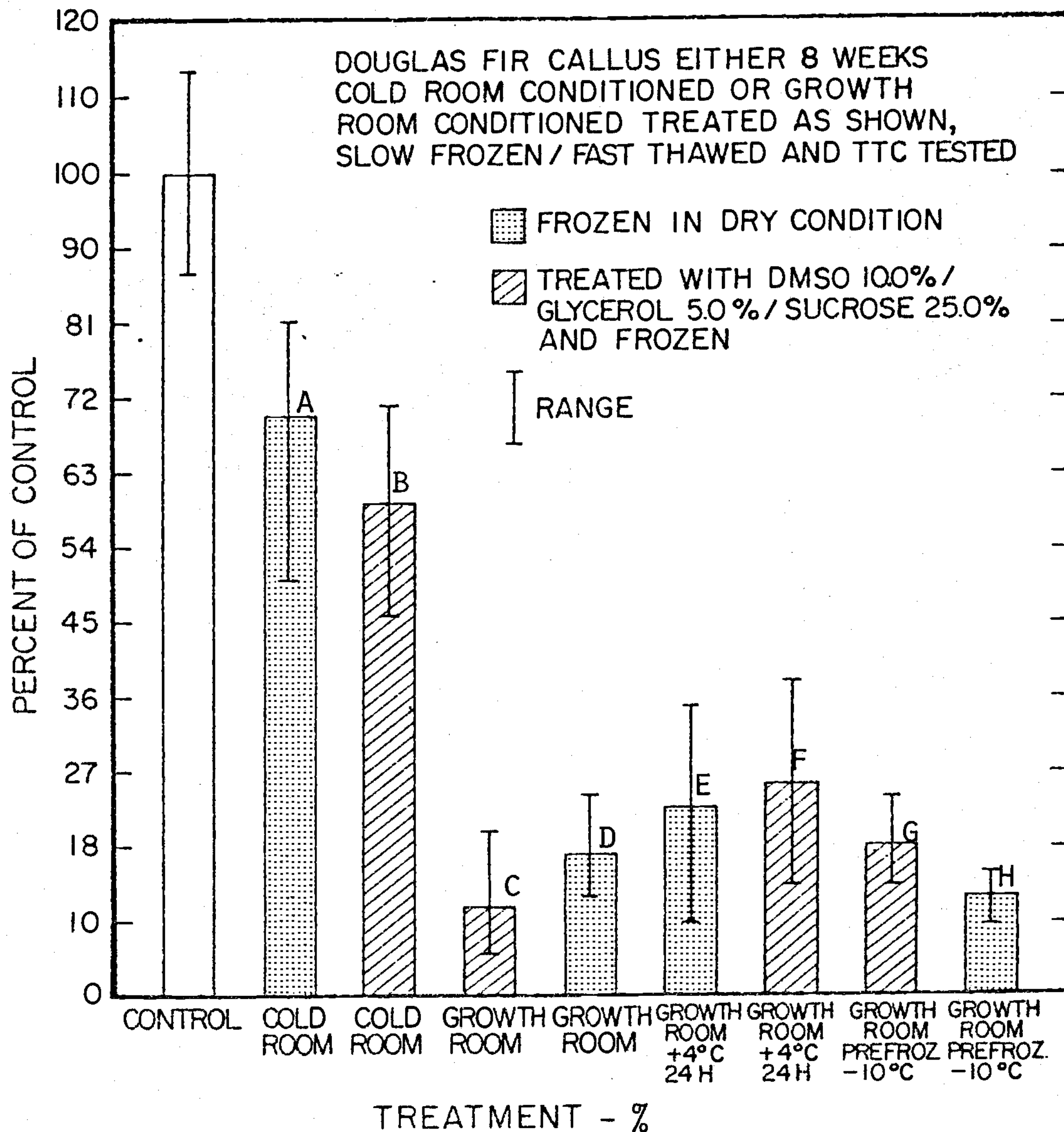


Figure 97. Douglas-fir callus TTC test with either 8 weeks cold room conditioned callus or callus from the growth room. Growth room callus held at +4°C for 24 hr, or pre-frozen to -10°C and cooled slowly at 1°C/min to -40°C then immersed in liquid nitrogen either in the dry condition or in the presence of DMSO 10%/ glycerol 5%/sucrose 25%. Thawing was fast in a +40°C water bath. N=8 A:B=N.S.; C:D=N.S.; C:E=*; D:E=N.S.; E:F=N.S.; C:F=*; G:H=* * Indicates a significant difference at the 95% confidence level.

Little success was obtained in growing previously frozen Douglas-fir callus on solid media using any pre-conditioning treatment (Figures 100 and 101). This is somewhat reversed to what was observed when compared with poplar cultures. Cold room conditioned and frozen Douglas-fir callus did not grow at +4°C but resumed growth when placed in the growth room at +22°C once again (Figure 98). Table 13 gives a summary of treatments given to Douglas-fir before freezing and were unsuccessful.

5. Environmental Effects on the Survival of Douglas-fir Callus

In order to study some of the environmental effects on treatments an experiment was done where Douglas-fir callus was exposed to various treatments for 24 hr and rather than freezing and thawing, was washed with fresh media and TTC reduction tested directly.

Callus left in media at +4°C for 24 hr gave TTC reduction significantly (16%) less than the control whereas callus left standing at +25°C gave a TTC reduction (non-significant; 10% less) from the control (Figure 103). Adding DMSO 10%/glycerol 5%/ sucrose 25% to both treatments severely reduced TTC reduction being 32% of control at +4°C and only 6% of control at +25°C. Freezing callus to -10°C in media produced only 8% as much formazan as the control.

The beneficial effect of exposing Douglas-fir callus to high concentrations of cryoprotectants at low positive temperatures is shown by the fact that this callus grew, whereas callus exposed

Figure 98. Douglas-fir and poplar callus which had been cold room conditioned and returned to +22°C temperature. Both continued to grow. Photo was taken 73 days after return to +22°C.

Figure 99. TTC reduction of Douglas-fir cultures after 24 hr at +35°C and treatment shown. Callus held at +22°C or at +4°C overnight showed high TTC reduction, whereas the same callus in DMSO 10%/glycerol 5%/sucrose 25% were apparently badly damaged according to the low TTC reduction color produced. Notice some reduction with the cryoprotectants given above held at +4°C overnight. Photo was taken 24 hr after conclusion of TTC test and cell breakdown may account for some of the faded color.

Figure 100. Douglas-fir callus cold conditioned for 8 weeks and treated as listed then slow cooled at 1°C/min to -40°C, immersed in liquid nitrogen then fast thawed to 0°C in a +40°C water bath. No growth was observed after 90 days culture on solid medium. All cultures were black in color indicating callus was no longer alive.

Figure 101. Douglas-fir callus grown in the warm growth room and treated as shown then slowly cooled at 1°C/min to -40°C, immersed in liquid nitrogen and thawed to 0°C in a +40°C water bath. No growth was observed after 70 days culture on solid medium.

Figure 102. TTC reduction of Douglas-fir callus grown in a growth chamber (GC) or cold room (CR) then immersed in liquid nitrogen and either thawed fast (at +40°C) or slowly (in air). (L) Control; (m) GC-fast thaw; (n) GC-slow thaw; (o) CR-slow thaw; (p) CR-fast thaw. Photographed 24 hr after start of TTC test. Cold room material was tan compared to growth room and control. Color differences may indicate metabolic or membrane alterations.

Figure 98.

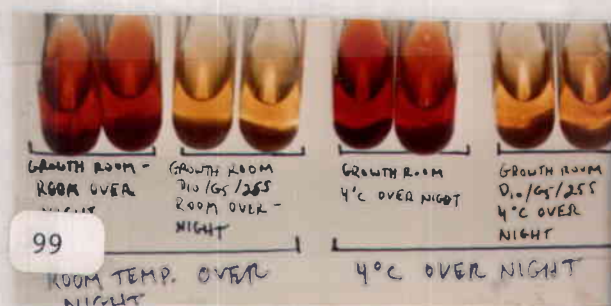


Figure 99.

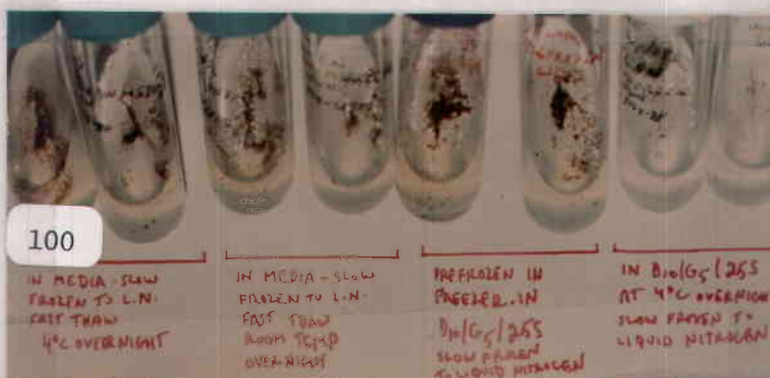


Figure 100.

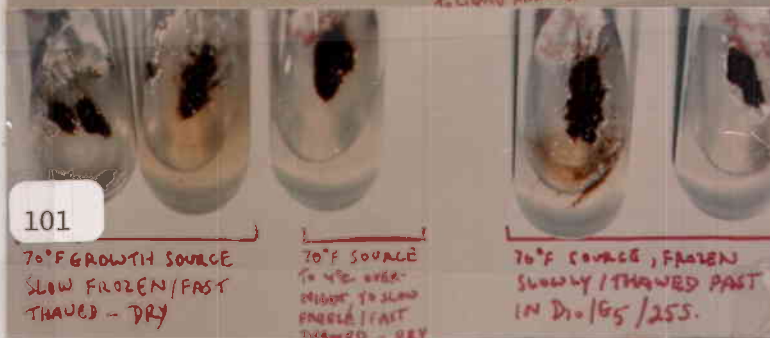


Figure 101.

Figure 102.

Table 13. Douglas-fir post-thaw agar callus culture results after being treated as indicated. Callus source was from the growth room (+22°C). Data was taken at least 60 days after thawing. No positive results were obtained.

TREATMENT	RESULT*	FIGURE #
A. Frozen slowly (1°C/min) to -40°C then to liquid nitrogen. 90 Days post-thaw culture.		
Frozen in media after standing at room temperature (+22°C) for 24 hr.	-	100
Pre-frozen to -10°C in freezer for 24 hr in DMSO 10%/glycerol 5%/ sucrose 25%.	-	100
Frozen in DMSO 10%/glycerol 5%/ sucrose 25% after standing at +4°C for 24 hr.	-	100
Frozen in DMSO 10%/glycerol 5%/ sucrose 25% after standing at room temperature (+22°C) for 24 hr.	-	100
B. Treatment frozen as "A" above but 70 days post-thaw culture.		
Frozen in DMSO 10%/glycerol 5% sucrose 25% after standing 1.5 hr at room temperature (+22°C).	-	101
Frozen in dry condition after standing 1.5 hr at room temperature (+22°C).	-	101
Frozen in dry condition after standing overnight at +4°C	-	101

(Continued)

Table 13. (Continued)

TREATMENT	RESULT*	FIGURE #
C. Frozen as "A" but 75 days post-thaw culture		
DMSO 5%/glycerol 5%	-	N/A
DMSO 2.5%/glycerol 5%	-	
Media only	-	
Frozen dry	-	
2.5M Sucrose	-	
D. Frozen by direct immersion in liquid nitrogen in 2.5M sucrose. 143 days post-thaw culture.		
After standing at room temperature (+22°C) for 6 hr.	-	N/A
After standing at +4°C for 6 hr.	-	

* (-) = No growth observed

at high temperature did not grow even after washing with fresh media (Figure 104). Cold treated callus either as long term, +4°C exposure before cryoprotectant treatment, or +4°C short term exposure during cryoprotectant exposure allows growth after washing, even when placed on solid agar medium (Figures 104, 105, and 106).

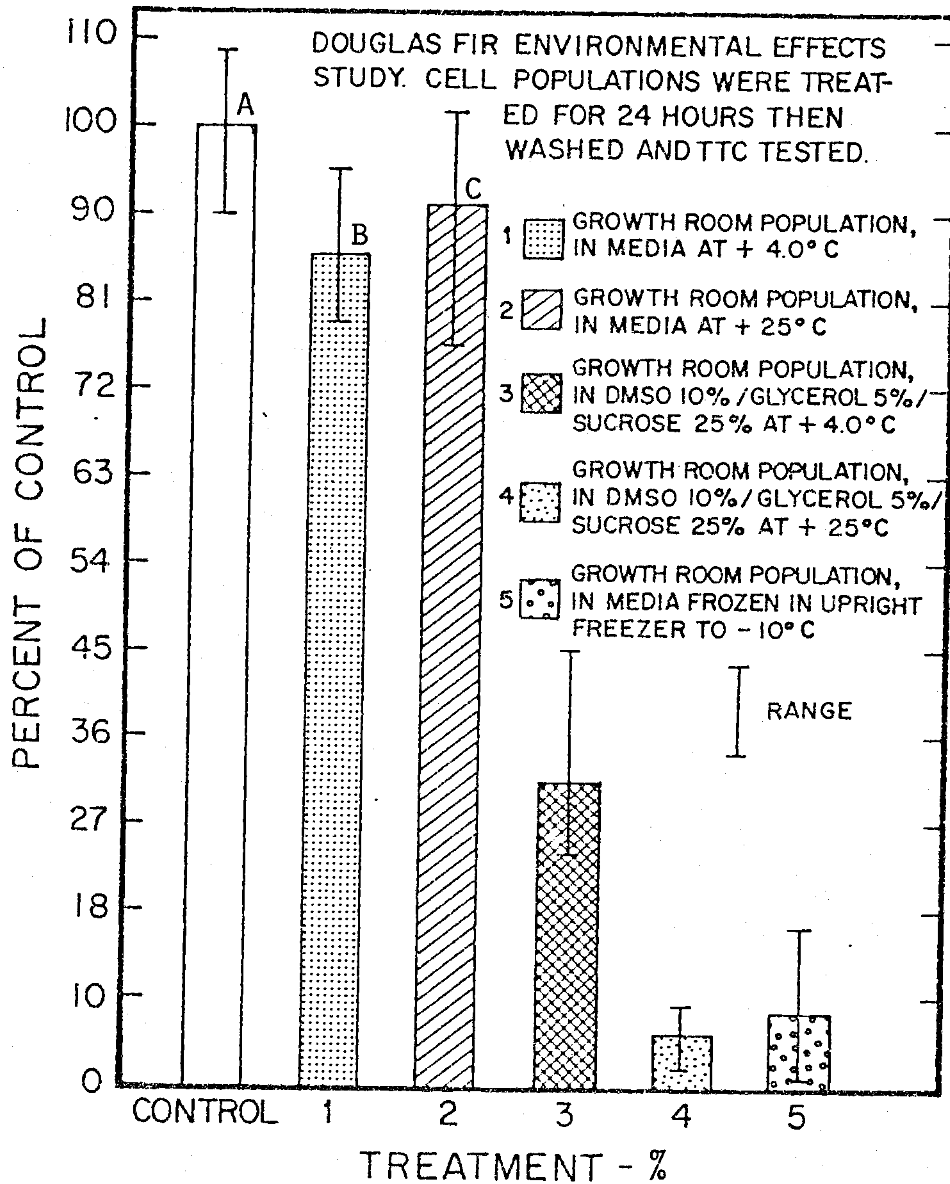


Figure 103. Response of Douglas-fir fir suspension cultures grown in the growth room then given various treatments as shown on the graph for 24 hr and washed with distilled water and TTC tested. N=8 A:B=*; A:C=N.S.; B:C=N.S. * Indicates a significant difference at the 95% confidence level.

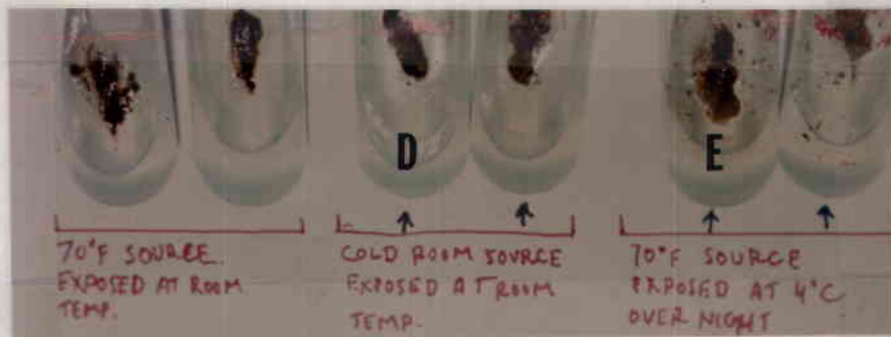


Figure 104. Douglas-fir cell populations from different sources (indicated) exposed to DMSO 10%/glycerol 5%/sucrose 25% at conditions shown for 1.5 hr then washed 8 times with 25 times (v/v) fresh media. Photographed after 70 days post-thaw culture (continuous low light). Blue arrows indicate green and growing cultures.

Figure 105. Enlargement of "E" in Figure 104. Note green callus growing out of black dead tissue.

Figure 106. Enlargement of "D" in Figure 104. Note green callus growing out of black dead tissue.

6. Freezing Douglas-fir Buds to -196°C

Douglas-fir buds did not grow when they were taken from trees which were cold conditioned for 8 weeks at +4°C or taken from field trees in mid-August and treated in various ways before direct immersion to -196°C or slow freezing at 1°C/min to -40°C and exposed to liquid nitrogen. Regardless of whether the buds were frozen fast or slow, with or without bud scales, with or without cryoprotectants, from field grown or cold conditioned trees, callus was formed (Figures 108, 109, 110, and 111). Buds used as controls and collected from cold conditioned or field grown trees produced callus on solid medium (Figure 107) or liquid medium (Figure 111).

Table 14 gives a summary of results obtained after various freezing and other treatments on Douglas-fir buds after 5 weeks post-thaw culture.

Table 14. Douglas-fir buds obtained from field grown trees in August or from cold conditioned trees (17 hr at +4°C dark and 7 hr at +12°C light at 800 foot candles). Buds had scales and needles removed or with scales and needles on were frozen dry slowly (1°C/min) or fast in liquid nitrogen, then thawed fast (+40°C water bath). Post-thaw culture in either agar or liquid medium for 5 weeks.

SOURCE OF BUDS AND TREATMENT	RESULTS		FIGURE #
	CONTROL	TREATED	
Cold room buds with young needles; slow frozen; fast thawed; agar medium	+	-	108
Cold room buds with young needles; direct liquid nitrogen immersion; fast thawed; agar medium	+	-	109
Field grown buds with bud scales removed; slow frozen; fast thawed; agar medium	+	-	110
Field grown buds with bud scales; slow frozen; fast thawed; agar medium	*	-	N/A
Field grown buds with bud scales; direct liquid nitrogen immersion; fast thawed; agar medium	*	-	N/A
Field grown buds with bud scales removed; direct liquid nitrogen; fast thawed; liquid culture medium	+	-	111
Cold room buds with young needles; direct liquid nitrogen; fast thawed; liquid culture medium	+	-	111

Results = (-) No growth observed after 5 weeks post-thaw culture
(+) Growth observed after 5 weeks
(*) No growth observed after 5 weeks - cultures contaminated.

Figure 107. Douglas-fir buds obtained either from young trees cold conditioned at +4°C/17 hr and +14°C/7 hr under 17 hr dark and 7 hr light or directly from field trees in mid-August. Buds were sterilized and grown on solid Douglas-fir media for 6 weeks. Callus formed from both.

Figure 108. Douglas-fir buds cold conditioned as given in Figure 92 and cooled slowly at 1°C/min from +4°C in the presence of DMSO 5%/glycerol 2.5% then immersed in liquid nitrogen. Thawing was fast in a +40°C water bath. Post-thaw culture was on solid agar.. Bud scales were left covering buds. Buds and bud scales were brown after 6 weeks in post-thaw culture.

Figure 109. Douglas-fir buds cold conditioned as given in Figure 92 in the presence of DMSO 5%/glycerol 2.5% then immersed directly in liquid nitrogen. Thawing was fast in a +40°C water bath. Post-thaw culture was on solid agar. Bud scales were left covering buds. Bud and bud scales were brown after 6 weeks in post-thaw culture.

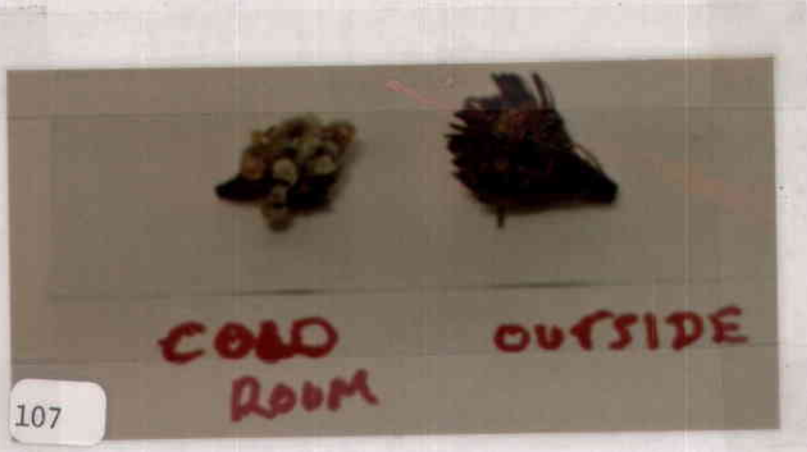


Figure 107.

Figure 108.

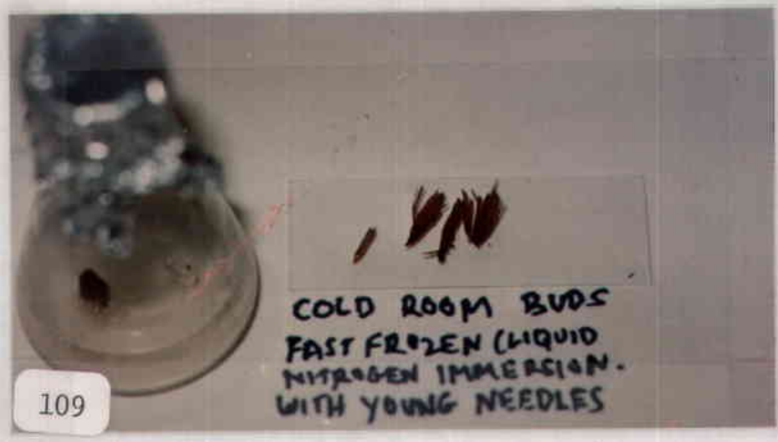


Figure 109.

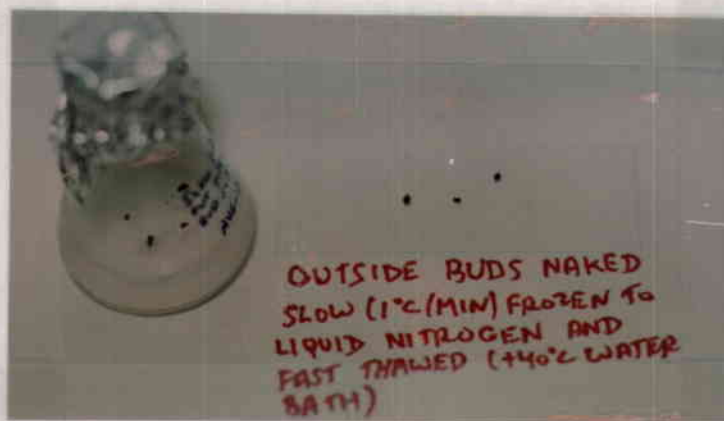


Figure 110. Douglas-fir buds collected in late August from field trees. Bud scales were removed and buds were cooled slowly at $1^{o}C/min$ to $-40^{o}C$ then immersed in liquid nitrogen. Buds were frozen in dry condition. Thawing was fast in a $+40^{o}C$ water bath. Post-thaw culture was 6 weeks on solid agar. Apices and needle primordia were black after 6 weeks in post -thaw culture.

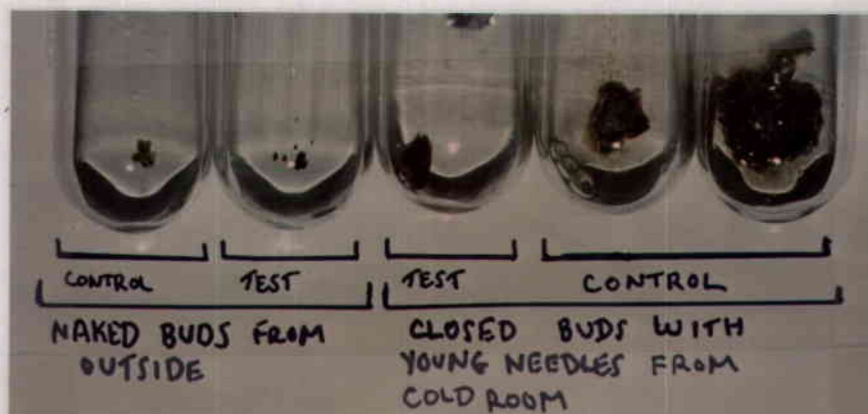


Figure 111. Douglas-fir buds collected from young trees conditioned at $+4^{o}C/17$ hr and $+14^{o}C/7$ hr under 17 hr dark and 7 hr light or collected in late August from field grown trees. Buds were exposed directly to liquid nitrogen. Thawing was fast in a $+40^{o}C$ water bath. All bud scales were removed and cold room source buds had young expanding needles. Post-thaw culture was in Douglas -fir liquid media for 5 weeks. Controls of all treatments were green, and callus formed whereas all test samples were black and not growing (dead).

DISCUSSION

1. Introduction:

It was found that the development of techniques for successful preservation requires a detailed emperical examination of the entire system in question. The pre-freezing culture conditions, physiological state, size and age of the cells, cryoprotectant type and concentration used, and post-thaw culture conditions; such as post-thaw washing and growth media type and conditions are all important.

Even more critical is the freezing rate and holding temperature. Slow freezing, normally in the presence of a cryoprotectant and at an "optimum" rate, specific to the material under investigation, followed by rapid thawing implies the involvement of three critical factors; 1) freezing at too slow a rate leads to excessive dehydration and electrolyte concentration effects, including enzyme denaturation; 2) too rapid a rate leads to intracellular ice formation by super-cooling; and 3) slow thawing results in damaging recrystallization of any intracellular ice.

A situation is obviously integrated and complex. Much information is required before a meaningful, controlled attempt can be made at cell preservation in liquid nitrogen. The problem is compounded in that biologists interested in freezing and its prevention are of two different groups; 1) those workers concerned with the use of low temperature for the long term preservation (mainly animal and microorganisms); and 2) those concerned with

the problem of how organisms (mainly plants) in nature survive sub-zero temperatures. Both groups have accumulated large masses of data, but have not studied the results obtained by the other. Those concerned with preservation have devoted most of their effort to improving survival by the addition of protective solutions, and manipulating the physical variables involved, such as cooling and thawing rates. This group generally use single cells or isolated pieces of animal tissue rather than intact organisms, paying little attention to the biochemical or physiological differences between species' susceptibility to freezing. On the other hand, the other group concerned with frost injury have been chiefly interested in the changes in sensitivity to freezing that occur on a seasonal basis and correlate these changes with biochemical and physiological alterations. These workers in turn have devoted little effort to uncovering the relationship between survival and changes in physical factors, such as cooling and warming velocity.

In addition, little attempt has been made by those working with plants to integrate biochemical studies on such subjects as cryoprotectant effects with studies made by workers using animal tissues.

It is the object of this discussion to define the conditions required to produce a successful freezing protocol for the two species used and at the same time provide some explanation of physical and biochemical changes brought about by added components,

such as cryoprotectants and natural changes such as cold hardening. Since such a discussion is involved and multifacetted subheadings are provided to ease subject orientation.

2. Suspension Cultures and Growth Curves:

A suspension containing free cells and small aggregates of cells may be produced by shaking or rotating liquid, rather than solid agar cutlure (which produces large clumps of callus). Such suspensions can be serially subcultured by pipetting aliquots of the suspension to new liquid culture medium. Serially propagated suspension cultures always contain a range of "cellular units" ranging from free cells to cell aggregates which may contain up to hundreds of cells (Street, et al., 1965). This heterogeneity of plant suspension cultures so far studies is a barrier to their more widespread use in physiological and biochemical studies (see Chapter 1).

If suspension culture aggregate size can be controlled, however, liquid cultures do have many advantages over callus culture (see Chapter 1). In his studies with carrot callus and cell suspensions, W. Halperin found that suspension cultures retained their high morphogenetic capacity for long periods, whereas this capacity was soon lost in callus cultures; other workers, however, report the opposity (see Appendix B). He observed also that carrot callus became entirely aneuploid, but the suspensions remained diploid. Plants regenerated from callus tissues sometimes show aberrations such as the sudden appearance of leaf

variegation, or changes in flower color (Thomas and Davey, 1975). Moreover, using callus cultures to study biochemical changes such as nutrient effects in terms of growth rates is meaningless. After reaching an optimum size, growth rate declines as a result of the depletion of nutrients or dehydration of the medium directly below the callus, the buildup of toxic metabolic products, or oxygen starvation of the central cells of the callus mass.

In suspension cultures it has been shown that the degree of cell separation changes during the progress of culture in a fixed volume of medium and the phase when cell expansion predominates over cell division seems to be the time when cell aggregates tend to break-up, yielding free cells (Henshaw, et al., 1966). This state is known as the stationary phase of growth (Thomas and Davey, 1975; Butcher and Ingram, 1976). Both of the growth curves generated here showed this phase and at least one showed a decline in cell number on reaching this phase. This phenomenon seems to be normal for suspension cultures. After entering the stationary phase there occurs a decline, sometimes very sharp, indicative of cell lysis. The cell suspension then stabilizes at a density well below the maximum reached at early stationary phase (Thomas and Davey, 1975). Sub-culture of quiescent, stationary phase cells to fresh medium shows a continued short quiescent state (the lag phase) followed by a resumption of cytoplasmic activity, and the appearance of prominent cytoplasmic strands. Some movement of organelles such as mitochondria and plastids can be observed in the streaming

cytoplasm of these strands by phase contrast light microscopy. Cell division results in the formation of hcains or tight aggregates of cells. The cells are small in size with minimum vaculation. This is the linear phase of growth, where cell number may actually double per unit time. The lower bounds of the linear phase constitute the exponential phase (where cell number is consistently increasing per unit time) and the upper bounds make up the progressive deceleration phase (where cell number increase is decelerated over unit time). Following the deceleration phase the cells continue to expand and separate as they again enter stationary phase. That cells continue to get larger during this phase (increase volume) can at least partly explain why there cen be a continued dry weight increase while cell number remains constant or decreases.

3. Pre-freezing Condition of Cultures:

The physiological state of the plant cell cultures considerably influences their reaction to cooling, and the extent of injury caused depends upon such things as; 1) whether or not the cells are meristematic; 2) the amount of water content; 3) if the cells are single vs. clumped; 4) the density of the cells; and last but not least, 5) the degree of cold hardiness of the cells (Bajaj and Reinert, 1977).

Small cytoplasmically rich cells generally have a greater capacity to survive than larger, more highly vaculated cells (Nag

and Street, 1975a; 1975b; Withers and Street, 1977; Withers and Davey, 1978; Withers, 1978; Withers and King, 1980; and Ben-Amotz and Gilboa, 1980). The latter has more freezable water, and a smaller surface area to volume ratio; both characteristics impede protective dehydration (Mazur, 1963; 1965b; 1970; 1977; Meryman, 1966; and Farrant, et al., 1977). Generally, relatively small celled species are thus at an advantage.

Within any one species, cells at a minimum size; eg., in exponential or linear growth (Withers and Street, 1977) or in a meristematic zone (Nag and Street, 1975b; Withers, 1978; and Grout, et al., 1978) are more likely to survive. The location of the cells within a multicellular structure can affect their survival, central cells being more susceptible to damage than superficial ones (Withers and Street, 1977; and Withers, 1978). A culture of small aggregate size is at a double advantage, since it has a higher proportion of superficial cells, and dehydration/rehydration stresses due to aggregate size are minimal. However, the amount of material placed in the freezing ampules is also important, those containing thick and packed suspensions often show higher cell survival compared with those of lower cell density (Brown, et al., 1974; Nag and Street, 1975b; and Bajaj, 1976a). In addition, the sensitivity of cells to cold also depend on the degree of physiological frost hardiness of a plant. For example, cells of a very frost resistant tree (eg. Douglas-fir), at its northern range may contain higher amounts of sugars and sugar alcohols (Sakai, et al., 1968: Bajaj, 1977; and Levitt, 1980).

From the above it is clear that proper pre-freeze handling is essential to optimum conditioning of cells to obtain reproducible results. Survival potential of frozen/thawed cells can be artificially enhanced by filtering out highly vacuolated cells and very large aggregates, selecting linear growth stage or more complex procedures such as growth medium supplementation with cryoprotectants, such as sugars or sugar alcohols. For example, in the case of a culture of Acer pseudoplatanus, partial synchrony of cell division and oscillations in survival after freezing and thawing were simulaneously induced by regular weekly passages (Withers, 1978). Experimentation with an intentionally synchronized culture indicated a positive correlation between mitotic index and survival. This correlation was not apparently due to survival of mitotic cells, but rather survival of those cells which had recently entered G_1 phase. These findings could not, therefore, be explained by cell size considerations alone. Although synchronous cultures may be advantageous for some purposes, it is unlikely that a degree of synchrony could be attained with most cutlure types which could justify the technical complexities of the system. Still, such avenues of investigation would increase understanding of freezing technology, and therefore should be undertaken in the future.

4. Colligative and Non-colligative Properties of Cryoprotectants:

The results obtained here and as well as by others (see Appendix A - "Review of Literature", Table 1), points out the

importance of choice of cryoprotectant(s) to enhance survival of frozen biological material. Generally cellular injury during freezing and thawing can be related to the amount of intracellular ice formed (Farrant and Woolgar, 1970; Bank, 1974; and McGann, 1978). In the case of slow cooling where ice is restricted to the extracellular regions, water is removed from the cell osmotically and thus intracellular freezing is avoided (Mazur, 1963; Sakai, 1965; Maryman, 1970; Nag and Street, 1975; Pushkar, et al., 1976; and Withers and Street, 1978). However, the cells may be damaged by the environment of the concentrated solutions during slow cooling (Nash, 1966; Meryman, et al., 1977; and McGann, 1978). The two-factor hypothesis of freezing injury (Mazur, et al., 1972), adequately describes this situation, where maximum recovery is obtained following cooling at a rate sufficiently slow to avoid intracellular freezing, yet sufficiently rapid to miminize damage due to the deleterious action of electrolytic concentration caused by dehydration during freezing (Nash, 1966; Duebbler, 1966; Mazur, 1969; Pushkar, et al., 1976; Meryman, et al., 1977; and Fahy, 1980).

The concept of cryoprotection through some biochemical action which renders cells less susceptible to freezing injury seems valid. This concept is supported by the observations made here. The cryoprotective agents used in the present work are three penetrating ones (DMSO, glycerol and sucrose) and are members of a class of solutions which has been shown to stabilize

macromolecular structures through the strengthening of both hydrophobic and hydrophilic forces (Rowe, 1966). Such a capability is consistent with the concept that cryoprotectants can stabilize the cell or its membranes against the adverse environment created by the freezing of water in a cell. For example, the action of DMSO, at least in part, reduces the amount of ice formed at any temperature during cooling, thereby postponing increased ionic strength conditions to lower temperatures where damage to the cell is retarded. Non-penetrating agents osmotically "squeeze" water from the cells primarily during the initial phases of freezing at temperatures between -10°C and -20°C where these additives become concentrated in extracellular regions (McGann, 1978).

For colligative cryoprotection to take place it is generally agreed that the agent must freely penetrate the cell, otherwise the cell may be injured by osmotic dehydration. The fact that solutes, such as sugars and other polymers, eg., polyvinyl/pyrrolidone (Doebbler, 1966) do not readily penetrate at least animal cells, can therefore be taken as evidence that those cryoprotective properties that they demonstrate are not primarily colligative.

Evidence now, however, also suggests that some classical colligative compounds have protective qualities beyond those that can be attributed solely to colligative factors (Mazur and Miller, 1976). The ultimate evidence of this suggestion is implied by the observations of Mironescu and Simpson (1975) that 5% and 10% DMSO can reduce or prevent the killing of Chinese hamster ovary

(CHO) cells which have been suspended at either +37°C or +4°C in tissue culture made hyperosmotic with sodium chloride. In the absence of freezing any protection must be on a non-colligative basis (Meryman, _et al_., 1977). These studies clearly suggest that DMSO can protect against the damaging effects of hyperosmolality such as occur during freezing.

Since 5% (0.7M) and 10% (1.4M) DMSO are sufficient concentrations to exert a significant colligative effect, Meryman, _et al_. (1977) did a series of experiments to discriminate between the colligative component of DMSO protection and what Mironescu and Simpson (1975) clearly indicated were additional non-colligative property. The conclusion of these experiments was that good evidence for a true biochemical stabilizing or repair mechanism conferred by cryoprotectants remained lacking. In addition to the colligative action (reducing the amount of ice formed) requiring penetration into the cell, however, cryoprotectants such as DMSO can increase the time required for water to leave the cell at lower temperatures and no penetration into the cell is required (Meryman, _et al_., 1977). Such non-colligative benefits of the DMSO cryoprotectant class are explained on the basis of effecting the diffusion rate of water in the unfrozen solution. As the cryoprotectant is concentrated by freezing, solution viscosity is further increased, which in turn increases the lag in cell dehydration permitting more cells to avoid injury.

5. Freezing Injury and Protective Action of Cryoprotectants:

While physical phenomena of energy removal and imput are often emphasized in cryobiology, chemical pehnomena, composition, phase equilbria and solute effects on crystallization or solute structure must be recognized as equally or more important in cryobiological injury and protection.

Almost without exception, biological systems must be modified in solute composition or concentration, artificially or naturally, to withstand the otherwise lethal consequences of freezing and thawing. These cryoprotectants, when added to biological tissues, increase the survival rate from zero to even 100% in a freeze-thaw cycle (Litvan, 1972). In dealing with tree sp. with the exception of one example (Sakai and Sugawara, 1973), the present work dealing with poplar cold conditioned material describes a successful preservation protocol without artificial cryoprotectant application.

The theory of cryoprotective action depends on the class of chemical, either penetrating or nonpenetrating and have been described elsewhere (Doebbler, 1966; Rowe, 1966; Andrews and Levitt, 1967; Litvan, 1972; Meryman, et al., 1977; McGann, 1978; and Withers, 1980). In some cases, singly cryoprotectants, usually dimethyl sulfoxide (Quatrano, 1968; Sherman, 1972; Dougall and Witherell, 1974; Sugawara and Sakai, 1974; Nag and Street, 1975; Baldini, 1975; Ashwood-Smith, 1975; and Foster, et al., 1976), glycerol (Lovelock, 1953b; Pyle, 1964; Samygin and Matveeva, 1967; Trunova, 1968; Sherman and Lui, 1973; and Mazur and Miller, 1976),

sugars (Heber and Santarius, 1964; Mazur and Miller, 1976; Steponkus, 1979; Dalgliesh, et al., 1980; and Lineberger and Steponkus, 1980), amino acids, such as proline (Heber, et al., 1971; and Withers and King, 1979), alcohols (Morris, et al., 1980), water soluble synthetic polymers such as hydroxy-ethyl starch and polyvinyl pyrrolidone (Morris, et al., 1979; and Franks, et al., 1980), colchicine (Law, et al., 1978; and Kikin, et al., 1979) and growth retardents such as absscisic acid (Kikin, et al., 1979) have been used successfully. In many cases a comparison between the protecting ability of two or more cryoprotectants has been made at one time (Narula and Jacob, 1965a; Narula, et al., 1965; Niwayama, et al., 1965; Carroll and Lin, 1968; Van der Berg and Soliman, 1969; Sherman, 1972; Ashwood-Smith, 1975; Mazur and Miller, 1976; Williams and Harris, 1977; and Rowe and Lenny, 1980). In other cases, mixtures of two or more cryoprotectants have been employed with an improvement in response (Narula and Jacob, 1965b; Ashwood -Smith, 1975; Finkle, et al., 1979; Finkle and Ulrich, 1979; Ulrich, et al., 1979; Gazeau, 1979; and Withers and King, 1980). This is the case here, both with Douglas-fir and poplar, where a combination of the cryoprotectants DMSO and glycerol proved to provide more protection on freezing than did either alone. Nobody to date has yet provided an explanation for the reason(s) for the increased survival and cryoprotectant combinations, but Finkle and Ulrich (1979) and Withers, (1980) attributed the improved survival to a reduction in toxicity by any one cryoprotectant while

Lionetti, et al. (1980) suggested that one may specifically reduce the toxicity of another. In any case, at least with animal tissues, combinations of dextrans of varying molecular weight with small, essentially undamaging (in an osmotic sense), amounts of either DMSO or glycerol can result in additive cryoprotection, and in some instances the effect is synergistic (Ashwood-Smith, 1975; and Jeyendran and Graham, 1980).

Various protective actions of these cryoprotectants have been suggested: Decreasing pH changes during freezing (by increasing amount of water remaining unfrozen (Van der Berg and Soliman, 1969; and Lineberger and Steponkus, 1980), stabilization of membranes (Litvan, 1972; Henderson, et al., 1975; Meryman, et al., 1977; Williams and Harris, 1977; and Steponkus, 1979), and stabilization of organelles such as mitochondria (Sherman, 1972; and Gazeau, 1979) (i.e., prevent breakdown of ATP synthesis by uncoupling (Damage is assumed to be uncoupling since electron transport is unaffected) (Hever and Santarius, 1964), plastids (Litvan, 1972; and Gazeau, 1979), golgi apparatus (Gazeau, 1979) and lysosomes (Rowe and Lenny, 1980). In addition, at least in the case of chilling injury, cold sensitive microtubules may be involved (Kikin, et al., 1979) and cells may be physically separated from the extracellular ice which forms during freezing as took place in a case with glycerol (Allen and Weatherbee, 1980). The mechanisms of injury during freezing have been treated in the "Review of Literature" (Appendix A).

Several theories have been advanced to explain the mechanism of cryo-injury, but the two chief ones deal with ice formation and production of high electrolyte concentration. Much literature has been accumulated in the last few years dealing with manipulating the physical variables involved in freezing, especially cooling velocity and addition of protective solutes and understanding the physics and biochemistry involved (Farrant, 1965; Jencks, 1965; Chilson, et al., 1965; Sakai, 1966; Sakai and Yoshida, 1967; Sakai, et al., 1968; Mazur, 1969; Sakai, 1970; Takehara and Rowe, 1971; Latta, 1971; Litvan, 1972; Sakai and Ötuska, 1972; Dougall and Wetherell, 1974; Sugawara and Sakai, 1974; Nag and Street, 1975a, b; Sanderson and Nath, 1975; Pushkar, et al., 1976; Bajaj, 1976; 1977; Mazur, 1977; Farrant, et al., 1977; Meryman, et al., 1977; Withers, 1978a, b, c; 1979; 1980; Ben-Amotz and Gilboa, 1980; and Rall, et al., 1980).

Ice can form inside the cell through intracellular ice formation either on cooling or warming. Whatever its course, injury from intracellular freezing can be eleminated by the simple expedient of cooling cells slowly enough to eliminate the formation of internal ice in the first place. The problem in this case is that cooling rates that are slow enough to eliminate intracellular ice may also be slow enough to injure cells by events that have nothing to do with the location of the ice. Such events are associated with the major alterations in extra and intracellular solutions produced by the removal of liquid water during

freezing, and which therefore, have been termed "solution effects" by established workers in this field.

Production of intracellular ice may also be prevented by freezing very rapidly, such as placing tissue directly in liquid nitrogen. Sakai and Otuska, (1967) and Sakai, et al. (1968) have demonstrated that no cavities from ice crystals were observed when freezing was very rapid. Based on ice growth on cooling as a source of injury, this suggests that survival should be high. In actual fact survival was near zero, frozen cells being killed within 1 min when rewarmed to -30°C. Electron micrographs indicate a very rapid growth of intracellular ice crystals approaching 1 μ in size within only 5 sec at -30°C (Sakai, 1970; Mazur, 1977; and Rall, et al., 1980).

Results obtained here both with the Douglas-fir and poplar suggest that direct liquid nitrogen immersion does not allow much survival. Whether this is due to damage on freezing or thawing is not known. The fact that slightly better results could be obtained when cryoprotectants were used, and even better results when cold conditioned material was fast frozen, suggests that perhaps both mechanisms are involved. The lower success rate found when freezing was fast compared to slow with cryoprotectants suggests that fast freezing does not allow the cryoprotectants to exert their effects, at least completely.

Present results indicate that certainly the slow, two-step method of freezing is better than fast freezing with the two species

used. The protocol of slow cooling and holding at a sub-zero temperature (often termed pre-freezing) permits cellular protective dehydration. Only one slow cooling rate was used in the present study because of time limitations. Review of the literature suggested 1°C/min was optimum for plant cells, and that a faster or slower rate may have proved better cannot be discounted.

The sub-zero holding temperature certainly makes a difference to the final results. Douglas-fir suspensions held at -15°C did not survive well at all compared to those held at -40°C. Such results can be explained, at least in part, by the production of ice crystals. Other workers have observed that at temperatures below -60°C the growth rate of fine intracellular ice crystals is so low that formation of ice crystals of a sufficient size to cause structural damage of the cells does not take place (Sakai, 1966; and 1967). Holding at a relatively low end temperature allows extra dehydration before immersion into liquid nitrogen. However, holding at some sub-zero temperature for too long may cause excessive dehydration and resultant injury.

During warming, temperatures above -40°C, apparently, the growth of fine crystals is great enough to cause immediate damage (Sakai, 1970). Present data shows that passing through this critical temperature area quickly on warming may be as important as the cooling rate. Cell populations thawed slowly always produced 10% less formazan than when thawed rapidly in a +40°C water bath.

Generally, experiments here as well as evidence from the literature, suggest that the cooling rate, holding temperature, length of time held at holding temperature and thawing rate need to be determined empirically for the system under investigation. Present results cannot be taken as the most optimum. Cell survival of the two species studied is possible under the conditions used but best conditions for survival require more extensive detailed analysis. For example, extended or shorter sub-freezing holding periods may be better than the 1/2 hr used. Extended periods of sub-freezing temperature has not been found successful in some studies (Bajaj, 1976). Possible reasons may include over-dehydration, temperature fluctuations and spontaneous intracellular freezing at that temperature (Withers, 1978).

A slow cooling step-wise freezing method has yielded excellent results with suspension cultures but have been found less satisfactory for organized structures. In the case of pollen embryos (Bajaj, 1977) or somatic embryos formed in suspension culture (Withers, 1979) only the early developmental stages survived. In these cases it has been suggested that either a rapid freezing method or a dry freezing method (lyophilization) may be successful (Withers, 1978). However, freeze-drying is generally not too successful unless the natural state of the test sample is a dehydrated one, eg., conifer pollens.[10]

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[10] Binder, W.D. unpublished results, M.Sc. Thesis, 1974.

6. Physiological Effects of Cryoprotectants:

a. Toxicity and Sub-cellular Effects:

It is clear that addition of cryoprotectants could enhance cell survival on freezing. While exploring the possibility for freeze-preservation of the two species used here, it became increasingly clear that a detailed empirical examination of the specific systems in question was needed.

Hypothesis of freezing injury generally assume that cryoprotective agents are biologically inert and affect the outcome of a freeze-thaw cycle only to the extent that they are able to reduce the amount of ice formed during freezing. A growing body of evidence now suggests, however, that this simplistic viewpoint is in need of modification and that cryoprotectants may not be exempt from the damaging effects usually attributed to all other solutes. It has been shown, for example, that red blood cells frozen in the presence of high concentrations of glycerol or DMSO usually suffer more damage than expected on the basis of classical colligative cryoprotection (Fahy and Karow, 1977). Additionally 30% DMSO did not affect the ability of kidney sections to accumulate potassium or to decrease their water content upon warming, but 50% DMSO negatively affected both these activities. The effects of 40% DMSO were intermediate between the effects of 30% and 40% DMSO, affecting potassium transport but not water content (Fahy, 1980).

The highest concentrations of potential cryoprotective additives which can be added and be non-cytotoxic must be determined.

Data obtained here while culturing cells with different concentrations of DMSO and glycerol suggest that different results can be obtained depending on the physiological condition of the cell population used. First results with Douglas-fir indicated that DMSO and glycerol at 20 ppm would produce growth in excess of control proved to be in conflict with later studies. The reason for this inconsistency most likely was the fact that early studies were made with cell populations in a generally poor physiological state. Aggregates were brownish in color and controls of experiments grew poorly. On this basis any gorwth observed in treated samples could be mistaken as effects of the cryoprotectant. In contrast, for experiments in which very green, vigorously growing (log phase) aggregates are used, cryoprotectant concentrations only of 1 ppm or lower showed any increases over initial experimental dry weights. With glycerol, both for Douglas-fir and poplar, control rates were equalled only when cryoprotectant concentration values were at 0.1% and 0.001% respectively. For DMSO, increases were generally observed between 1% and 0.1% for both species. These increases in fact exceeded the control values suggesting a beneficial effect of the cryoprotectants at these concentrations. Higher cryoprotectant concentrations reduced growth and lower concentrations showed little or not effect compared to controls. The molecular reasons for such observations are unclear, but some suggestions are indicated.

DMSO has physio-chemical properties such as its faculty to substitute for water molecules around macromolecules such as proteins (Rammler, 1967; 1971; Rammler and Zaffaroni, 1967; Karow, 1969; Szmant, 1971; Henderson and Henderson, 1975; and Nilsson, 1977). This subsistution may result in conformational changes which may affect the physiological function of these macromolecules. Furthermore, since this effect of the aprotic solvent is a general phonomenon it may, in part, explain the large variety of effects ascribed to DMSO (Nilsson, 1980).

Since it is a dipolar aprotic solvent, DMSO differs from protic solvents such as water and alcohols because of its tendency to accept rather than donate protons. In the polarized form (at +37°C) both the DMSO molecules, oxygen and sulfur, have unshared electron pairs so that the broad solvent characteristics are the result of its ability to form either stable solvents by dipole-dipole interactions or solvent-solute associations by hydrophobic interactions (Rammler and Zaffaroni, 1967).

In the present sutdies DMSO and glycerol at high concentrations proved to be potent inhibitors of growth, and even survival. Toxicity particularly with DMSO has been extensively studied in animals. The Annals of the New York Academy of Science devoted an entire volume (670 pages) to DMSO effects (Vol. 141, 1967) and other reivew have followed (Daivd, 1972). However, little data on cryoprotectant effects have been collected using plants.

Studies with animal cells show that damage by cryoprotectants on the cell may be severe. For example, the high concentrations of ethylene glycol encountered during freezing of rabbit hearts was found to be the main cause of damage (Armitage and Pegg, 1979). In another study it was concluded that 30% DMSO is toxic in rat heart preparations in contrast to previous reports of lack of toxicity of similar concentrations in these tissues (Karow, et al., 1967).

The molecular effects of DMSO are thought to result from the formation of hydrogen bonds with proton-donor groups which are stronger than those formed with water (Nilsson, 1977). Because DMSO also contains methyl groups, however, effects on hydrophobic bonding in proteins can be expected at higher DMSO concentrations. In one *in vitro* study, at a concentration of 20% or less, DMSO changed glutamate dehydrogenase into the inactive nonomer, and the effects were fully reversible with the activator (ADP) (Henderson, et al., 1975). Higher DMSO levels resulted in irreversible inactivation. DMSO also inhibited the clotting of fibrinogen by purified thrombin. The major effect appeared to be due to competition between thrombin and DMSO for binding sites on fibrinogen. These effects were interpreted as being largely due to interactions between DMSO and hydrophobic bonding groups in fibrinogen. DMSO also appeared to interfere with the aggregation of fibrin monomers through its effects on hydrophobic groups (Henderson, et al., 1975).

At DMSO concentrations of 12% a complete inhibition of protein synthesis occurred within 3 to 4 min in HeLa cells. This inhibition was reversible; on removal of DMSO, cells resume their normal rate of protein systhesis. Very interestingly, the inhibition of protein synthesis was accompanied by a complete breakdown of polyribosomes and release of nascent polypeptides (Saborio and Kock, 1973). The *in vivo* breakdown of polyribosomes was not, however, due merely to a physiochemical effect of dimethyl sulfoxide in these structures since it can be prevented by inhibiting translation or peptidyltransferase. In addition, it was found that when actimomycin D was added prior to DMSO, the transcription inhibitor did not interfere with either the reformation of polyribosomes or resumption of protein synthesis when DMSO was removed from the medium. According to the authors, these results suggested that the resumption of protein synthesis and reformation of polyribosomes was not dependent on *de novo* synthesis of mRNA. It was suggested that the mRNA released during degration of polyribosomes by DMSO was preserved and could be utilized when DMSO was removed from the medium.

In a study using Tetrahymena pyriformis it was found that DMSO suppresses protein synthesis and RNA synthesis in a dose dependent manner (2%, 3%, and 4%) whereas DMSO reduced the rate of leucine incorporation to about 30% in all cases (Nilsson, 1978). This result, together with the fact that DMSO has been shown to induce the formation of helical patterns of polyribosomes in this

ciliate, suggests interference with protein synthesis at the level of translation (Nilsson, 1976; 1977). In addition, a dose-dependent effect has been shown on the rate of turnover of ATP in cell extracts of Tetrahymena spp. the amount of ATP expended per unit time in 7.5% DMSO being only 60% of that expended by extracts of control cells. These findings are interpreted as in accord with a random interference of DMSO, presumably by inducing conformational changes in some macromolecules which affects their cellular function (Nilsson, 1980).

Ten percent DMSO proved inhibitory to ^{14}C-uracil and ^{14}C-methionine uptake and incorporation while uptake of ^{65}Zn was increased in bean (Phaseolus vulgaris) excised roots, leaf disks, callus tissue and enzymically isolated leaf cells (Bajaj, et al., 1970). That study also showed that oxygen uptake as measured with Warburg manometers was impaired; the inhibition showing a time and concentration dependency. The fact that the same concentration of DMSO inhibited respiration, RNA metabolism and protein metabolism but increased zinc uptake suggested that zinc uptake in beans was primarily a non-metabolic process. Such observations pointed out clearly the value of cultured cells in biochemical studies.

Using an *in vitro* enzymatic assay with a partially purified preparation of the enzyme trypotophan synthetase (catalysing the conversion of indoleglycerol phosphate and L-serine to L-tryptophane and glyceroldehyde 3-phosphate) 80% inhibition of enzyme

activity was observed in a 10% DMSO solution. Rammler (1967) has reported direct effects of DMSO on several dehydrogeneses and peroxidases obtained from various animal and plant sources. With HeLa cells, cell viability decreased rapidly and an increase in the number of smaller cells was observed after exposure to concentrations of DMSO greater than 1% for periods greater than 12 hr. There is a lag in the incorporation of the nucleosides uridine and thymidine at concentrations of 1%, 2% and 3% DMSO which is partially overcome at 24 hr. With 4% DMSO this lack of incorporation was irreversible (Hellman, et al., 1967).

The rate of vacuole formation in *Tetrahymena* decreased to about 50% of the control value in 5% DMSO (v/v) and to zero in 7.5%. At the latter concentration the inhibition was expressed immediately, but the effect of a 1 hr exposure was reversible after removal of DMSO by washing (Nilsson, 1974). *In vivo* observations revealed abnormal function of the contractile vacuole in 7.5% DMSO, while cell mortality and cell division appeared to be unaffected, although there was little or no increase in cell number. Feulgen preparations showed that nuclear division was inhibited and that cell division resulted in one anucleate and one nucleate daughter cell. Interestingly, the present study confirms Nilsson's (1974) observations on overall physiology of the cells. That author suggested, and the present data agreed, that the effect of DMSO was dependent not only on the concentration of the compound, but also on the physiological state of the cells.

In another study using cultured chick embryonic dorsal root ganglia, it was shown that a maximum cytotoxic response on neurite development could occur at concentrations above 5.5% DMSO. It was suggested that this inhibitory action on neurite maturation may be due to a generalized cytotoxic response to the chemical of membranous organelles and that DMSO at higher concentrations was responsible for an increased intracellular disorganization (Roisen, 1975).

Recently, chemical studies have shown that DMSO under some conditions may interact with other chemicals producing short-lived mutagenic derivatives. For example, a chemical reaction occurred when trichloroacetic acid (TCA) was dissolved in DMSO. The result was dimethyl-carbamylchloride (DMCC) which proved mutagenic in a Samonella/mammalian-microsome plate overlay assay (Nestmann, et al., 1980).

Specifically with plants, it has been shown that glycerol has extensive effects on the environment and components of the photosystem I reaction center. Apparently variations of potential of the primary electron acceptor of photosystem I measured by optical and EPR procedures arises from glycerol alterations (Evans and Heathcote, 1980).

With Datura spp. 10% DMSO was toxic to leaf growth which caused an immediate severe burning of the leaves and loss of leaves within 3 days of treatment. Plant metabolism was altered and there was a reduction in alkaloid production and the concentration of chlorophyll (Sciuchetti, 1967).

b. Beneficial Growth Effects of Cryoprotectants

The increase in dry weight of plant cellular material observed between 1% and 0.1% of the cryoprotectants used here has not been reported previously. Some general growth study effects have been carried out by others however. Suspension cultures of Daucus carota Ca68, Cannabis pativa 95c and Ipomeoa spp. were screened for their ability to utilize glycerol (2%) as a sole carbon source for growth. Using glycerol above 2% resulted in reduced final yield amount, based on a dry weight basis in all three cultures (Jones and Veliky, 1980). Such observations are not much different from those observed here and these authors did not use lower concentrations of glycerol than 2%. Further, these authors showed that the respiratory activities of the cultures were of the same general pattern with glycerol as when sucrose was used. The bio-transformation capabilities of the 3 cultures also remained the same. That is, qualitatively the same spectrum of products was formed based on a variety of substrates tested for irrespective of the nature of the carbon source.

On a whole plant basis DMSO enhanced the effect of the growth regulator, B995 in Datura innoxia. With D. ferox the effect of DMSO when combined with phosphon appeared to be potentiated at an early stage of plant growth but additive at a later stage. In the case of D. tatula, DMSO appeared to potentiate the effect of GA on alkaloid production, whereas the effect on growth appeared to be additive (Scinchetti, 1967).

It may be that the cryoprotectant molecule can combine with some metabolic components in a kind of detoxification process of simply allow them to escape into the medium. Alternately a combination of these processes may perhaps be more likely.

Such suggestions are not without foundation, particularly since Nilsson (1977) showed that with high DMSO concentrations changes such as those of the nuclear, mitochondrial and peroxisomal regions resemble those seen during starvation. Perhaps in addition to those insults to the cell listed above, high DMSO concentration increases the permeability of the membranes (cryosol and organelle) to such an extent that the cell lost its comconent integrity (Nilsson, 1980). Such action in addition to interaction with enzymes and other cell components with the cryoprotectant may explain cell death at higher concentrations and are not inconsistent with a beneficial effect at some lower optimum concentrations. This idea supported also by the fact that the *Tetrahymena* at least, 1 hr after removal of DMSO the rate of RNA synthesis was higher than that in cells before the exposure to DMSO (Nilsson, 1977). Moreover, that study showed that full nutrients were required for full recovery of DMSO treated *Tetrahymena*. In another study, thylakoids frozen in the presence of some amino acids such a proline, threonine or lysine - HCl stabilized both membranes and reduced the concentration of toxic substances (Heber, *et al*., 1971). In addition, DMSO has been found to be beneficial to potato tuber sprouting; the DMSO being

metabolized to dimethyl sulfoxide and dimethyl sulfone. The main site of the DMSO metabolism was in the growing sprouts and buds (Diamalla and Van Staden, 1980).

In the present study the increase in growth observed was well within the previously observed limits of cryoprotectant concentrations, if one assumes that washing cells exposed to 5% or 2% DMSO or glycerol will reduce the residual concentration to below 1%. Such an assumption, although imperically untested, is not unreasonable.

DMSO and glycerol have been proven to increase growth behavior of animal cell cultures and viruses (Narula, 1965a, b cited by Narula and Gill, 1967), increased weight of rats fed DMSO Purina Mouse Breeder Diet (Narula and Gill, 1967) and increased weight gain of rabbit kidneys when perfused with DMSO (Karow and Keske, 1976).

It was established here that DMSO and glycerol were favorable to cell growth at some concentration. The reason(s) for these results remains unclear however. It is postulated that at some concentration the cryoprotectants became beneficial to growth by opening optimal pores in the cell membrane thus allowing the cell to take up materials or chemicals that the membrane barrier would ordinarily block. Such pores could be produces either in the membrane proteins or by solubility of the membrane lipids. In addition, the polarity along with high permeability of the cryoprotectant molecule could increase transport across membranes of

other materials by acting as a carrier. Whether chemical action on the membrane and permeability of the cryoprotectant molecule are separate events, or the second is a consequence of the first is unknown. Moreover, the healthy appearance of the test cultures after long periods before subculture, compared to controls may suggest additional effects. It is well known that as cells age there is an accululation of potentially toxic cell components and metabolism may be involved. (See end of this section.)

With strawberry plants, grown in both soil and nutrient solutions with DMSO, there was a 21-fold increase in ^{32}P concentration in the leaf samples as compared to controls (Garren, 1967). In addition to enhanced movement of materials through roots, DMSO was able to penetrate the back of young pear trees and become systemic in the foliage. Moreover, labeled material was carried in with with DMSO. It was suggested by Garren, (1967) that bark penetration and transport offered an effective method of treatment with certain chemicals used as insecticides, fungicides, or for correcting nutrient element deficiencies.

DMSO has been shown generally to enhance the penetration of substances into both plant and animal tissue (Jacob, et al., 1964). In connection with enhanced penetration of antibiotics and fungicides, initial studies with plants revealed that DMSO exerted a profound effect on the biologic membrane, altering its natural selectivity. Membranes treated with DMSO were rendered porous to compounds generally considered to be non-dialyzable. In addition,

penetration of normally dialyzable ions and compounds were increased (Herschler, unpublished, cited by Jacob, *et al*., 1964).

Some reports, mainly with animal tissues, have suggested that the primary action of DMSO is on cell membrane structure and permeability. Increased permeability of red blood cells to sodium and potassium ions subsequent to treatment with DMSO has been observed (Berg, *et al*., 1965). A similar increase in the permeability of *Nicotiana tabacum* callus cells on treatment with DMSO has been reported (Delmer and Mills, 1969). A study of effects of DMSO on frog skin inferred that it increased the permeability of the membrane to passive diffusion of electrolytes. A flux ration of greater than 1 was observed with mannitol, sucrose, thiourea and urea (Franz and Van Bruggen, 1967; and Sams, 1967). It has been shown that the permeability of the rat liver lysosomal membrane to β-glycerophosphate is increased, reversibly and progressively in the presence of between 5% and 25% DMSO (Lee, 1971). Fowl spermatozoa in the presence of a high concentration of glycerol (15%) shows irreversible loss of ions and a certain enzyme (Lake, *et al*., 1980). Erythrocytes lost osmotic and mechanical resistance that was related to severe membrane injury (Hope and Saager, 1967). In addition, reports on redistribution of membrane interrelated particles (McIntyre, *et al*., 1974) and stabilization of lipid bi-layer inducing a decrease in membrane fluidity (Lyman, *et al*., 1976) have been observed after DMSO exposure. However, this last observation was in direct opposition to another

study using methanol as the cryoprotectant. In this case there were alterations to the relative proportions of all fatty acids, but the most significant effect was an increase in the values of 16:4 and 18:3 fatty acid concentrations, with compensatory alterations in the other C16 and C18 acids. Such increase in average value of fatty acid unsaturation would result in a membrane with greater fluidity (Morris, et al., 1980). Apparently these changes are analogous to the changes induced by a reduction in growth temperature (Morris and Clark, cited by Morris, et al., 1980). Interestingly, growth in the presence of non-inhibitory concentrations of methanol (0.125M and 0.25M) did not affect the phospholipid fatty acid composition (Morris, et al., 1980). Both Lyman, et al. (1976) seeing a decrease in membrane fluidity with DMSO and Morris, et al. (1980) showing an increase in fulidity with methanol, claim such changes stabilize the membrane at lower temperatures.

The postulation that DMSO opens pores in the cell membrane is further supported with the aid of electron micrographs when it was suggested that such pores actually open directly into channels that supposedly represent a part of a system in most cells (Sandborn, et al., 1975). These authors suggested that transport could take place in part by propulsion through such a tubular system. Such a suggestion is apparently not altogether without foundation since it has been calculated that the consumption of energy by the Na^{+}, Ca^{++} and Mg^{++} pumps alone would be far in

excess of the total energy available to the cell. A great deal more energy would be required to operate the membrane breakdown and reconstruction involved in a pinocytosis-exocytosis type phenomenon (Ling, 1969).

The exact molecular effects of cryoprotective agents on phospolipid membranes is still obscure but it is known that the effect is much more pronounced with acid than with neutral phospholipids, suggesting that ionic change interactions and perhaps hydrogen bonding may be involved (Lyman, et al., 1976). Further, it has been suggested that increases in permeability of lipoprotein membrane of cells and cell organelles probably are a consequence of lipid peroxidation. That is when an unsaturated fatty acid is autoxidated or peroxidated, a shift of double bonds takes place, together with a modification of the geometrical isomerism (Muset and Martin-Esteve, 1965). These authors showed that there are isomeric conformation changes in linoleic and oleic acids when exposed to DMSO. Considering the steriometric structure of DMSO and the known special bond between S and O in the sulphoxides, such changes seem reasonable. It has also been suggested that the remardable penetration abilities of dimethyl sulfoxide may be due to some alteration of protein structure as a result of dehydration at the membrane caused by DMSO (Weiner, et al., 1972). In this regard, phospholipids may exert a protective action on the protein when exposed to DMSO (Weiner, et al., 1972).

The kinetics of the DMSO effect on respiration, RNA and protein metabolism (all are decreased at 10% DMSO) show that DMSO can act as a metabolic inhibitor. Since the effect is more pronounced and appears earlier on oxygen uptake than on either protein or nucleic acid, it was concluded by Bajaj, et al. (1970) that the site of DMSO action is on some reactant of the respiratory pathway or a closely related reaction. These workers showed that with bean leaf disks DMSO at concentrations between 0.1% and 1% stimulated oxygen uptake. They suggested that certain respiratory enzymes were stimulated at these low DMSO concentrations. Stimulated metabolism could account, at least in part, for the increased growth at between 0.1% and 1% cryoprotectant concentrations observed in the present work.

c. Cryoprotectant Washing Experiments

In order to test the hypothesis that cryoprotectants play a significant role in the injury observed after thawing, the washing experiments were carried out with Douglas-fir. These experiments confirmed that high DMSO or glycerol could significantly reduce the amount of TTC reduced, suggesting cell damage. At 10% concentration cryoprotectants could not be washed out enough to cause recovery of the exposed cells even without freezing. At large it is generally assumed that cellular damage is due to freezing and thawing stresses entirely. Evidence obtained here

and by others[11] suggests that such an assumption is not justified.

Suggestions that considerable viability loss can result from cryoprotectant application are now appearing in the plant literature (Withers and Street, 1977; and Withers, 1978). It has been suggested that because of the cryotoxicity of conventional cryoprotectants, especially glycerol, other compounds should be found should be found to fill these cryoprotectant roles (Withers and King, 1979).

With rat heart cells in suspension, without freezing, increasing DMSO concentrations caused an increase of morphological damage, correlating with a decrease of the survival in culture. With 2.5% DMSO there was no difference with untreated cells. At higher DMSO concentrations, the ultrastructural damage increased from spaces between cell membrane and cytoplasm at 5% DMSO to interrupted cell membranes, swollen or destroyed mitochondria and nuclei with clumped chromation at 10% DMSO (Alink, _et al_., 1976).

In another study recovery was very low when red cells were exposed to cryoprotectants (no concentrations were given) at +20°C for 5 min. Dilution by washing was rapid and slower rates of dilution were not found to be beneficial. The non-penetrating, low molecular weight additives (glycerol, glucose and sucrose) were more damaging than were penetrating (ethanol, methanol and DMSO) or higher molecular weight (PVP) additives (Morris, _et al_., 1979).

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[11]Finkel, B.J., U.S. Department of Agriculture, Berkeley, California. Personal Communication, 1979.

Recently it has been shown that washing cryoprotectants out of both plant and animal cells at room temperature was much more beneficial to cell recovery than washing at $0^{o}C$ (Persidski, et al, 1980; and Binder, (plant cell only), unpublished observations, 1979). With HeLa cells it has been shown that the reformation of polyribosomes and recovery of protein synthesis after removal of DMSO are temperature dependent. When cell suspensions are washed with medium at $+37^{o}C$, the reformation of polyribosomes and recovery of protein synthesis after removal of DMSO are rapid and complete. On the other hand, if cell suspensions are washed in cold medium, the formation of polysomes and synthesis of proteins are retarded (Sabario and Kock, 1973).

d. Cryoprotectant Addition - The Effect of Temperature

Data obtained in the present study suggested that cold conditioned or cryoprotectants infiltrated at lower temperatures ($+4^{o}C$) could increase the survival level of cell populations exposed to cryoprotectants (Figures 127 and 128). In fact, chilling or cold conditioning allowed greater concentrations of cryoprotectants to be added than would normally have been tolerated. Such observations are being made with both plant and animal test species. Conover (1969) states that the reactions of oxidative phosphorylation were irreversibly inhibited if the concentration of DMSO exceeded 2.8M, but Baxter and Lathe (1970) reported that even twice this concentration was harmless to rat kidney slice respiration provided the temperature of exposure was $+4^{o}C$.

Bovine liver protein studies showed that reversible alterations in protein structures are the major effects of exposure of protein subunits to low DMSO levels at low temperatures. Irreversible denaturation of protein subunits may be an appreciable effect at higher temperatures combined with higher DMSO concentrations (Henderson, et al., 1975). It was shown that concentrations of DMSO required to effectively freeze-preserve granulocytes were destructive unless the temperature was quickly reduced to +4°C or below (Lionetti, et al., 1980). The cells at 5% DMSO and room temperature or higher exhibited unstable nuclei and fragmented rapidly. Interestingly, these workers demonstrated that the effects at high temperatures can be overcome if hydroxyethyl starch (HES) is incubated with the DMSO.

In another study, when mammalian cells were incubated with the cryoprotectants DMSO and glycerol, pre-treatment of the cells with colchicine significantly enhanced the survival of these cells. This protection was observed even if cells were frozen without cryoprotectants. Colchicine at the concentrations used is known to disrupt microtubules in cells, allowing surface proteins to move more freely, and with less damage being done (Law, et al., 1978). Such observations deserve close examination with plant material. Hanging drop preparations of fowl spermatozoa when helf at +37°C in 15% glycerol were much more severly damaged than when kept at +3°C (Lake, et al., 1980).

The limited amount of data accumulated with plants shows that considerable viability loss can result from cryoprotectant application but slow addition over a limited period of time (1 hr) at chilling temperatures minimized this loss (Nag and Street, 1975a; Withers and Street, 1977; Withers, 1977; Withers and King, 1979; 1980; and Withers, 1980). However, the low permeability of glycerol into many plant cell types may necessitate the use of an extended period of application (up to 24 hr) (Towill and Mazur, 1976; Withers and Street, 1977; Withers, 1977; and Morris, et al., 1979). Bajaj and Reinert (1977, unpublished, cited in Bajaj and Reinert, 1977) examined the effects of various concentrations (5%, 7% and 10%) of DMSO on survival of carrot cell suspension, pre-treated in ice (about +3°C) and at +22°C (room temperature). Survival was determined by FDA staining. It was found that at +3°C DMSO even at 10% reduced survival less than 20% after 60 min while during the same time period at +22°C, 10% DMSO reduced cell survival almost 80%; 60% reduction occurring after the first 15 min.

During the laboratory phase of the present research this worker was not completely aware of the observations listed above or did not fully understand their implications. In light of those observations, as well as the present findings, it must be concluded that had chilling temperatures during cryoprotectant infiltration been used prior to freezing, much better results would probably have been obtained on freezing and thawing. The fact that callus could grow from cold conditioned callus or tissue held at +4°C for 24 hr

on exposure to normally very toxic cryoprotectant conditions (DMSO 10%/glycerol 5%/sucrose 25%) is strong evidence for such a statement. Confirmed here is Wither's (1977) statement that chilling temperatures reduce cryoprotectant injury compared to addition at room temperature, and appear to fall within the limits observed by Bajaj and Reinert (1977, unpublished, cited in Rajaj and Reinert, 1977) who state that some loss of viability on exposure to chilling temperatures is expected, but that it should be minimal, at least when compared to the benefits derived from these conditions.

7. Post Thaw Culture Physiology: Cell Dry Weight Increase and TTC Testing:

Both frozen and thawed cells when returned to culture frequently enter a prolonged "lag phase" of growth (Quatrano, 1968; Nag and Street, 1975; and Withers and Street, 1977). Both Douglas-fir and poplar cell cultures displayed this "lag phase" after freezing though to different degrees. Poplar cultures began to increase dry weight after about 4-5 weeks after freezing while for the Douglas-fir this only took place at well over 80 days. Such lag time periods as observed here are not uncommon, however, and lag periods that span from a few weeks to 6 months have been reported (Bajaj, 1977; and Sala, et al., 1979). The fastest recovery results recorded to date with plants are those of Withers and King (1979; and 1980) with corn (Zea mays) and Sala, et al (1979) with rice (Oryza sativa L.). The former showed mitotic

figures from 2 to 3 days after thawing, while the latter entered linear expotentual growth phase 2-8 days after thawing.

Culutres which have unknown, long lag periods after freezing are at a disadvantage where time and culture space are limited. Many experimental cultures in the present study were discarded after considerable time because they did not appear to be growing and space was required for other experiments. In this respect the reduction of TTC is of value since its results are known within a day of testing. But TTC reduction tests, in themselves, are meaningless unless they can be correlated with growth rate. For instance, Daucus carota L. cells showed a 70% survival soon after thawing, but a lag phase of 20-30 days was necessary before cell growth began (Nag and Street, 1973; and Bajaj, 1977). Further, no growth was observed with Capsicum annuum L. cells despite a 30% survival value according to the TTC test soon after thawing (Withers and Street, 1977).

In agreement with results obtained here, Withers and Street (1977) and Sala (1979) have observed that TTC reduction was consistently reduced during the early stages of post-thaw culture. This data clearly emphasize the fact that viability values observed soon after thawing do not necessarily give a figure of the percentage of cells that can resume growth and undergo cell division after freeze treatment (Towill and Mazur, 1975). With rice it was observed that although TTC reduction dropped initially after freezing, it resumed reduction and approached the values

observed in untreated cells before onset of cell growth (Sala, _et al._, 1979). These results were interpereted as meaning that the drop in viability was not directly related to death of part of the cell population. While similar changes in TTC reduction were observed in present experiments, the reason for this is not so clearly defined. The possibility here that TTC increases are due to surviving cells dividing rather than subcellular repair cannot be discounted here.

Latency in resumption of growth and achievement of maximum growth rate may be partially due to toxic effects of dead and dying cells. Total faulure to enter growth may indicate that the viable cell density has fallen below the "minimum inoculum level", (Stuart and Street, 1969; 1971; and Thomas and Davey, 1975). Furhter, it must be considered that either the biability test used is deceptive in that it fails to identify cells which are lethally injured, or post-thaw treatments are failing to "rescue" or "nurse" cells through a recovery phase. In reality, it is likely that all of these factors are operative. No attempt was made here to study the cellular events that take place during the first hours after thawing. The further reduction in apparent viability after initial thawing may be of great interest if we wish to control this critical phase of the freeze-preservation of cultured plant cells.

Observations of cellular changes after freezing have been made by many (Quatrano, 1968; Li and Sakai, 1978; Lyons, _et al._, 1979; and Levitt, 1980). Physiology may be altered since it has been

noticed in some cases that when growth eventually resumes, the generation time is considerably increased as compared to that of unfrozen cells (Nag and Street, 1973; and Bajaj, 1976). Recently the exact cellular changes which occur on thawing have become a topic of intensified research. Extensive damage to membranes of the plasmalemma and organelles have been observed (Palta and Li, 1978; Niki, et al., 1979; Lineberger and Steponkus, 1980; and Pearce, 1980). It has been observed that some cells can reseal leaky membranes and become "osmotically active" (Law, *et al.*, 1980). In a very clever and interesting study, a cell wall-less mutant (CW15+)[2] of the unicellular green alga *Chlamydomonas reinhardii* was used to study selective permeability of the membrane to enzymes, changes in membrane phospholipid composition and altered electrophoretic mobility after freezing. All these parameters are altered in post-thaw culture (Grout, *et al.*, 1980).

Alterations in rat liver endoplasmic reticulum microsomes, terminals of the electron transport chain and/or the hydroxylation system including cytochrome P-450 have been observed (Kudokotseva, *et al.*, 1980). In a quantative study using Chinese hamster fibroplasts it was found that about 60% of unprotected cells which survived a freeze/thaw cycle were sub-lethally damaged, while addition of cryoprotectants could reduce this figure to 30%. Of interest is the fact that maintaining the cells at +37°C after the freeze/thaw cycle results in complete repair of sub-lethal

damage within 3 hr. Onabain, an inhibitor of the Na^+-K^+ ATPase of the plasma membrane, was able to reduce the amount of this repair. This result indicates that the repair process is dependent on energy production being facilitated by K_2HPO_4. In addition, it was found that the process of repair was not prevented by either protein synthesis inhibitors or by RNA synthesis inhibitors (Law, et al., 1980). These workers point out that this repair of sub-lethal damage is different from the resealing of leaky membranes. The latter is completed within 30 minutes after thawing while the former takes two to three hours to complete. Damage to the chloroplasts has been observed here with both Douglas -fir and poplar as evidenced by loss of color. Such damage is repaired after time, the cultures turning green again.

Evidence accumulated in the present work and by others (Towill and Mazur, 1976; and Withers, 1978) indicate that osmotic conditions and nutrient supplemented media (Withers and Street, 1977) are particularly critical in the early recovery phase. Douglas-fir cell populations became black in color within 48 hr after being frozen, thawed and placed on solid media. Much better results were obtained with liquid post-thaw culture. On the contrary, poplar cultures recovered much more quickly and in some cases only recovered on solid media, compared to liquid culture after a freeze-thaw cycle.

Support for the superiority of specific media for recovery growth of a certain species type has been made with Zea mays over

semisolid medium using electron microscopical examination of cells during recovery growth (Withers and Street, 1977; and Withers, 1980). Cells returned to liquid medium resume growth after a lag period, during which some of the cells continue to deteriorate, accumulating osmiophilic cytoplasmic deposits, masses of lipid/ lipoprotein globules, and excessive wall material. In a minority of the cells the dilation of cytoplasmic organelles noted immediately after thawing is reversed and resorption of the globules takes place with the regeneration of cell membranes. After a number of days mitosis occurs. In cells recovering over semi -solid medium, organelle deteriation is negligible.

The hormonal content of the recovery medium can also be critical. For example, in the case of recovering clonal plantlets of *Daucus carota* L., the presence or absence of 2,4-D determines whether the meristem proper recovers growth and whether organized growth ensues immediately or after a period of callus formation (Withers, 1978). Others report a requirement for gibberellin in the recovery medium to promote outgrowth of seedling meristems of L. esculentum (Grout, *et al.*, 1978).

Loss of viability caused by post-thaw washing or by too rapid dilution of the cryoprotectant containing medium has been observed with suspension culture cells, algal cells, and somatic embryos (Withers, 1978). Physical deplasmolysis injury per se and loss of vital solutes through leaky cell membranes may be involved (Towill and Mazur, 1976).

Withers (1980) now firmly believes that there is no categorical requirement to remove completely cryoprotectants after thawing. According to that author, in no case does it appear to be possitively beneficial. On the contrary, an indication of the deleterious effects of post-thaw washing is given in the response of somatic embryos of Daucus carota L. (Withers, 1978), and of suspension cultured cells of Zea mays (Withers and King, 1979). In the latter case, resuspension of the cells in liquid medium (in effect a partial washing) leads to a continued loss of viability and slow eventual recovery. Droplet culture, in which the cells remain in a liquid environment but are not exposed to dilutents, stabilizes viability but fresh medium must be added eventually to prevent senscence. Apparently washing with complete medium with or without added cryoprotectants is as deleterious as washing with water alone (Withers and King, 1979). The suspending medium in which cells are frozen and thawed uniquely provides a satisfactory environment for "convalescence" after cryopreservation having become modified by leakage of vital solutes from the cells (Withers, 1980). Resorption of these solutes and a balanced uptake of water, originally withdrawn to form extracellular ice during slow freezing, may be critical events in the early stages of recovery. Continued growth will then depend upon the availability of nutrients from the semi-solid medium.

In Addition to the actual make-up of the medium, perhaps other conditions also are important. In the only Douglas-fir culture

which following freezing actually continued to grow, the culture was left standing rather than being rotated. Whether this condition is important or not must remian speculation. Others have also observed that a period of reduced lighting aids the recovery of cryopreserved meristems of *Solanum goniocalyx* (Grout and Henshaw, 1978).

An important question which must be addressed is the likelyhood that the freeze-preservation protocol is acting to select freezing resistant mutant cells. Such a question takes on importance in light of altered growth characteristics on post-thaw culture as discussed above. Poplar shoots grown here, at least at the anatomical level, show no alterations. However, the fact that sub-cellular changes could have taken place on freezing and thawing can not be discounted. Withers (1978) points out that freeze selection on the basis of cell size and location within aggregate is not likely to be of genetic significance, except where cell size reflects genotypic differences, such as in a culture of mixed ploidy levels.

However, to date, whenever tested, preserved cultures have had characteristics identical to the original cultures, in terms of morphogenic potential, and after a recovery period, morphology and growth rate (Nag and Street, 1973; Dougall and Wetherell, 1974; Bajaj, 1976; and 1977; Withers, 1978; and Sala, *et al*., 1979). Additionally there is no evidence for genome alteration occurring as a direct result of cryopreservation procedures

(Withers, 1978). The question of selection is now being further investigated. For example, the consistently high recovery rates with species such as *Acer pseudoplatanus* and *Paul's Scarlet Rose*, for which a range of cell lines with differing hormone requirements or levels of resistance to antimetabolites and herbicides (Withers and King, 1980) are available, mean that it is now possible to test retention of specific characters in cryopreserved cell lines.

8. Cold Hardiness and Low Temperature Survival:

In order to survive a sub-freezing temperature a plant must adapt. As a result of these adaptations, all gradations of freezing resistance occur in plants, from none to an ability to survive the lowest temperature tested (Levitt, 1980). Unfortunately, the total number of factors involved in freezing tolerance in unknown. Genetic investigations have so far succeeded only in pointing to a multifactor relationship. In the case of *Brassica oleracea*, for instance, the genetic evidence suggested that two dominant, epistatic genes conditioned freezing tolerance (Bouwkamp and Homna, 1969). A seemingly unlimited number of factors has been investigated (Levitt, 1978; 1980; Li and Sakai, 1978; and Lyons, *et al*., 1979). Certainly total solutes, sugars and related substances, water (total and bound), amino acids, proteins, nucleic acids and simpler nucleotides, lipids, growth regulators as well as other miscellaneous substances are involved (Levitt, 1978; 1980).

Excellent general reviews on the subject of cold hardiness have been published in the last few years (Alden and Hermann, 1971; Levitt, 1972; 1980; Kacperska-Palacz, 1978; Li and Sakai, 1978; Mussell and Staples, 1979; and Underwood, et al., 1979). More specific articles dealing with membrane alterations (Siminovitch, et al., 1968; Litvan, 1972; De Yoe, 1977; Steponkus and Wiest, 1978; Palta and Li, 1978; Yoshida, 1978; 1979a, b, c; Sikorska and Kacperska-Palacz, 1979; Horvath, et al., 1979; and Priestley and Leopold, 1980), the role of water (Chen and Gusta, 1978; Parsons, 1978; Sakai, 1979; Burke, 1979; and Kedrowski, 1980), protein synthesis mechanisms and increases in protein, nucleic acids (Siminovitch, et al., 1968; De Yoe, 1977; Brown, 1978; Sobczyk and Kacperska-Palacz, 1978; and Pacperska-Palacz, et al., 1978), hormones (Hatano, 1978; and Spomer, 1979), sugars (Levitt, 1972; 1980; and Hatano, 1978), and changes in structure (Palta, et al., 1977; Steponkus, et al., 1977; and Pomeroy and Andrews, 1978) in regard to cold hardiness are also available.

In the present study, holding cultures of both Douglas-fir and poplar at low positive temperature and light for 8 weeks increased the survival when placed in liquid nitrogen. The greatest benefit was observed with poplar where the survival rate was within 20% of the unfrozen control values, based on TTC reduction. This value was double from the which could be obtained with cryoprotectants. TTC reduction on thawing was also slightly higher with cold acclimated Douglas-fir than with that which could be obtained using

cryoprotectants. However, post-thaw culture conditions with the confier appeared to allow possible recessive secondary metabolism and leakage into culture media from cells so that little or no survival was observed in this case.

In addition, the physiology and/or membranes appeared to be altered by cold conditioning in the Douglas-fir material. Exposure to low positive temperatures allowed greater concentrations of cryoprotectants to be added to the material without totally killing the test material. Material grown in the growth chamber was always killed 100% when exposed to high cryoprotectant concentrations. Apparently a low rate of metabolism is less harmful when cells are exposed to cryoprotectants, low temperature would provide these conditions. This is supported by the fact that cells appear to be most susceptable to cryoprotectant toxicity when they are in log phase of growth (see also Nilsson, 1980).

Cold hardening of course is not new but has only been demonstrated in a very few cases with culture material. Early studies using twigs showed that cold hardened woody plants could withstand lower freezing temperatures (Sakai, 1965). The first culture to survive liquid nitrogen temperature after cold conditioning at a day temperature of +12°C and a night temperature of 0°C for 25 days and subsequently acclimated at 0°C for an additional 20 days was Populus euramericana cv. GELRICA (Sakai and Sugawara, 1973). Callus cultures of this species continued to grow 14 days after thawing from -196°C. This is much faster than continued post-thaw

growth observed in the present experiment using a poplar. It is assumed that the difference observed is due to the species differences, initial day/night temperature conditions and the additional 20 days cold acclimation at $0^{o}C$ used by Sakai and Sugawara (1973).

Since the demonstration of Sakai and Sugawara (1973) of cold acclimation in plant cell cultures by low positive temperatures, others have demonstrated the same using carnation plants (Dianthus caryophyllus L.; Seibert and Wetherbee, 1977), Chlorella ellipsoidea (Hatano, et al., 1978; and Hatano, 1979), Rosen rye (Gregory and Poff, 1979), Kharkov winter wheat (Chen and Gusta, 1979), Jerusalem artichoke (Sugawara and Sakai, 1979), Rauwolfia serpentina var. Bentham (Yamada, et al., 1980), and 11 species of unicellular marine algae (Ben-Amotz and Gilboa, 1980b). The present study appears to be the first reporting survival of cold acclimated conifer cell cultures and actual regeneration of shoots and roots from tree cold acclimated cultures (poplar) after freezing to $-196^{o}C$.

The majority of other worker's reports suggest that the cell membrane, in particular phospholipids and proteins, is modified to increase resistance to freezing injury. Ultrastructural changes in artichoke callus suggests alterations take place in hydrophobic regions, especially in the half closest to the extracellular space of the plasma membrane, changing from a lipid depleted state to a lipid enriched state (Sugawara and Sakai, 1978; and Yoshida, 1979a, b, c).

It is of interest to know whether the state of the intramembrane particles within the cell membrane affects the ability of a cell to withstand the stresses of freeze-thaw. The relationship of these particles to their immediate environment (lipid bilayer) is of particular importance in light of the studies of Williams, *et al.*, (1975). These workers have shown that during plasmolysis of cells from a very cold hardy wheat, lipid is transferred from the membrane to cytoplasmic lipid vesicles. During deplasmolysis (and possibly hydration during thawing), the cytoplasmic vesicles decrease in volume and are believed to add lipid material back to the membrane. Less cold hardy wheats may show increases in cytoplasmic lipid vesicles on cooling and dehydration but these are not replaced into the membrane again on thawing. Such types are known to be killed by cell lysis on thawing (Williams, *et al.*, 1975).

The fulidity of the membrane is also important. Using cultured cells of *Kauwolfia* species, Yamada and co-workers (1980) showed that holding cells at low positive temperatures caused them to contain larger proportions of phospholipids of low phase transition point, (the 1, 2-dilinolemyl - type), than those cultured at normal temperature (+25°C). Avoidance of intracellular freezing required a sufficiently large specific surface of the cell combined with a high cell (plasma membrane) permeability to water, to permit a rapid efflux of the cell water to the extracellular ice loci. One method in increasing cell

permeability to water is to increase the unsaturation of membrane lipids. It has long been known that the unsaturation of fatty acids increases during the exposure of some plants to hardening low temperatures (Levitt, 1972). The phase transition of a lipid membrane from the liquid crystalline to the solid (gel) state has been shown to lower the permeability of a lipid membrane four-fold compared to its original value (Levitt, 1978). This change would markedly increase the danger of intracellular freezing, since the water would move more slowly through the plasma membrane to the extracellular ice loci.

Levitt (1978) explains hardiness on the above facts in the following fashion: If a phase transition of the membrane lipids at or above $-1^{o}C$, for example, took place, intracellular freezing and cell death would take place. Based on the assumption that the phase transition of the membrane lipids to the solid (gel) state decreases the water permeability of cell to 1/4 its rate in the absence of phase transition, the following is calculated and assumed to happen: In order for the cells to avoid intracellular freezing, 50% of their water must diffuse out of the cells within the first few hours. After that, half as much (25%) must leave within two hours and half as much again (12.5%) within the next four hours. According to Levitt (1978) this means that in order for extracellular freezing to continue, the exomosis during the cooling from $-4^{o}C$ to $-8^{o}C$ needs to occur at only 1/16 the rate of the exosmosis between $-1^{o}C$ and $-2^{o}C$.

However, if phase transition reduces exosmosis by four times, this is 4 times too low to support the rate of exosmosis required to remove the first 50% of the cell's water, but would be fully adequate to support the exosmosis of the subsequent 25% or any subsequent amount. Thus if hardening can lower the phase transition temperature from $-1^{o}C$ to $-2^{o}C$, (placing exosmosis values in the 25% water needed to be removed or a relative rate of 4 as opposed to 16 for the first 50% of water), no intracellular freezing would take place.

On this basis, Levitt (1978) concludes that a hypothetical unhardened plant such as the one above, with a phase transisition temperature of its membrane lipids above $-1^{o}C$, needs to accumulate only enough unsaturated fatty acids to lower this temperature to $-2^{o}C$ in order to have fully adequate intracellular freezing avoidance for the rates of cooling found in nature. However, phase transition in response to hardening temperatures is a general response and occurs to the same degree in less hardy as well as hardier varieties of a species (Wilson and Rinne, 1976; Willemot, _et al._, 1977). That is, there does not seen to be a direct correlation between linolenic-like acids, these being shown to increase equally in non-hardy and hardy wheat cultivars (Horváth, _et al._, 1979) and black locust (Yoshida, 1979a, b, c). In these studies exposure of sensitive species to low temperatures resulted in the loss of phosphatidyl-choline and accumulation of phosphatidic acid. The ratio of phosphatidic acid to phosphatidyl-

choline but not the level of polyunsaturated fatty acids is related to ability to survive at low temperatures.

In non-hardy living bark tissues of poplar as well as black locust the degradation of phosphatidyl-choline into phosphatidic acid is enzymatic, being regulated by phospholipase D. at sub -lethal temperatures. On thawing, this enzymatic reaction was drastically accelerated and resulted in degradation of the other phospholipids such as phosphatidylethanolamine and inositol (Yoshida, 1979a, b, c).

Phospholipase D. is bound tightly to the membrane (Yoshida, 1978). That worker, on experimental evidence, speculates that phospholipase D. consists both of catalytic and regulatory sub-units. Binding of Ca^{++} at the regulatory site may be a pre-requisite for the activity and a competitive binding of Ca^{++} and Mg^{++} a the regulatory subunit(s) may bring about a great change in the conformation of the enzyme molecule (Yoshida, 1978). The alteration of the regulatory properties as affected by freezing was more effectively protected by low concentration of sucrose in hardy microsomes than in less hardy microsomes. Accordingly, some qualitative changes in membranes affecting the regulatory properties of phospholipase D. are likely to be involved in the mechanism of cold hardiness of plant cells. Others conclude that plants, depending on their degree of resistance to cold, produce an unknown substance of lipidic nature upon exposure to cold, with the aid of which they adjust the transitional state of

their membranes to the prevailing temperature and, at the same time, facilitate the efflux of water from the cells (Horváth, et al., 1979).

Of course other factors are also involved (Brown, 1978; Kocperska-Palacz, 1978). RNA, protein and lipid changes are involved in the hardening process (Hatano, 1978). Organelles may also be involved. For example, in Chorella it was found that O_2 uptake activity in unhardened cells increased during hardening in the light, while the O_2 evolution activity decreased, when these activities were measured at $+25^oC$. Algal hardiness development in the dark was very limited. The addition of glucose during hardening in the dark, however, caused a remarkable development of frost hardiness. These results suggest that mitochondria and chloroplasts closely interact at low temperature and the former plays a principal role in the hardening process and the latter serves as substrate donor in the light (Hatano, et al., 1978). These workers further showed that O_2 evolution in cells which survived freezing was remarkably decreased by freeze-thawing while the O_2 uptake was hardly affected. Freeze-injured chloroplasts were repaired during the following incubation. Such observations are confirmed in the present study. Hatano, et al., (1978) suggested that their results show that mitochondria seem to change their membranes into a structure hardier than chloroplasts, and ATP synthesized by mitochondria seems to be essential for the repair of freeze-injured chloroplasts. Marked increases

in ATP content have also been observed in the first stage hardening of winter ripe plants (Sobczyk and Kacperska-Palacz, 1978).

In a recent study with unicellular marine algae, cold induction and freezing to liquid nitrogen was demonstrated (Ben-Amotz and Gilboa, 1980b). Resistance to freezing was induced and developed in cryosensitive algae during growth under limiting conditions of low temperature and nutrient deficiency. Algae grown at +4°C for 4 weeks developed high resistance to freezing. Deficiency of nutrients such as nitrate, phosphate and bicarbonate slowed the rate of growth and increased significantly the ability of the cell to survive freezing in liquid nitrogen. The workers interpreted these results as indicating low positive temperatures and deficiency of nutrients reduced metabolic growth of active cells causing augmentation or modification of the cell membrane which serves to increase the resistance to freezing injury.

Proline has been shown to occur naturally during environmental stresses (Stewart and Lee, 1974; Blum and Ebercon, 1976; Sotrey, et al., 1977; and Widholm, 1980) and is sometimes associated with increased cold hardiness in plants (Benko, 1968; Levitt, 1972; 1980; Kacperska-Palacz, et al., 1977; and Yelenosky, 1979). Possible roles suggested for free proline in plant cold hardiness range from protection of cellular membraned (Heber, et al., 1973) to regulation of enzymes (Stefl, et al., 1978).

Because it occurs naturally, has a very high solubility, and exerts a high osmotic pressure, proline has been successfully used as a hardener and natural cryoprotectant recently (Withers and Street, 1978; Withers and King, 1979; and 1980). It is suggested that when added as a cryoprotectant shortly before freezing, proline acts as a nontoxic intracellular (and possibly extracytoplasmic) solute, protecting the cell against the denaturing effects of hyperosmololity induced by dehydration during slow freezing (Withers and King, 1979).

The enhanced recovery potential of proline-treated cells suggests that either there is a reduced level of latent injury, a protection against post-thaw deplasmolysis effects, eg., by membrane stabilization (Heber, et al., 1971) or that proline has and active role in recovery metabolism as proposed by Blum and Ebercon (1976). The effect of pregrowth in proline may be due to enhanced uptake during prolonged period of exposure causing alterations in cell size and cytoplasm to vacuole ratio (Withers and King, 1979).

In the present study, addition of proline to the pre-freezing conditioning media proved unsuccessful for both Douglas-fir and poplar. It may have been that the proline effect is of little importance here but more likely toxicity and concentration are most important. Others report that proline is nontoxic at high concentrations (Stewart and Lee, 1974; Brown and Hellebust, 1978). Withers and King (1979) used proline at concentrations up to 25%

before loss of viability (25% - 30% mortality) due to excessive plasmolysis was observed. These workers routinely used 10% proline in growth media without seeing any loss of viability. In the present study with Douglas-fir suspension cultures, concentrations above 0.01% showed toxic effects on growth. No tests were made with poplar although the 1% used in the conditioning media may have been significant but cannot be interpreted as being beneficial at this point. Perhaps if used with poplar at higher percentages or at lower temperatures than the 1% used here, the amino acid could have been substantually beneficial in freeze survival. The toxicity expressed with the Douglas-fir at such low concentrations is, however, a problem.

Generally, it is safe to assume that cold hardiness is very complex and dependent upon a number of factors acting in concert to bring it about. Moreover, it has been shown that hardening is actually a three stage process (Kacperska-Palacz, 1978; Sikorska and Kacperska-Palacz, 1979). During the first stage, occurring at low but above freezing environment temperatures, phospholipid changes do not seem to be directly related to cold tolerance. This stage of hardening is possibly related to a metabolic shift caused by the cessation of growth. The achievement of the second level of tolerance depends on the occurrence of sub-freezing temperature and appears to be related to increase in phospholipid level and involves phospholipase D. The third stage of hardening is related to cold induced dehydration of the cells and may overlap

the second one. If such a system is indeed operational in cold hardening of callus it is suggested that cold hardiness induction could be much improved upon from the present results. It may well be that cold acclimation of callus at low positive temperature for some weeks, followed by a few weeks of exposure to sub-zero temperatures could provide very high survival after exposure to liquid nitrogen even without the use of cryoprotectants.

"Cold hardening" by pre-growth at low temperatures (Sakai and Sugawara, 1973; Seibert and Wetherbee, 1977; and Ben-Amotz and Gilboa, 1980a, b), pre-growth in medium of enhanced osmosity to reduce cell volume and increase the cytoplasm to vacuole ratio (Withers and Street, 1978; Siminovitch, 1979; and Withers, 1979) and desiccation (Withers, 1979) have led to marked improvements in recovery of some frozen plant tissue cultures. It appears that the goal of such cold hardening should be to produce membrane changes and internal changes such as sugar or proline accumulation, the former increasing membrane fulidity at low temperatures to facilitate dehydration, the latter protecting against the consequent solute concentration. Tissue culture offers a powerful tool to study in depth and at length the changes which occur during such cold hardening.

9. Freezing Douglas-fir Buds to -196°C

Meristem culture has become important as a tool in clonal propagation and elimination of viral pathogens, in particular in

crop plants (Kartha, et al., 1975). Since the constitutent cells of apical meristems are less differentiated and more uniformly diploid than those of mature tissues, plant regeneration by *in vitro* culture of meristems should result, as with suspension cultures, in the recovery of true-to-type progenies as opposed to callus tissue culutre propagation. Therefore, controlled freezing and low temperature storage of isolated meristems has the potential of providing a suitable and reliable means of germ plasm preservation. To date, meristems of only a very few species have been successfully revived following freezing and storage in low freezing or liquid nitrogen temperature (Seibert and Wetherbee, 1974; Seibert, 1976; Mullin and Schlegel, 1976; Grout and Henshaw, 1978; Grout, et al., 1978; Sakai, et al., 1978; Sakai and Nishiyama, 1978; Kartha and Leung, 1979; Kartha, et al., 1979; Anderson, 1979; Towill, 1979; Sakai, 1979; Haskins and Kartha, 1980; Uemura and Sakai, 1980; and O'Hara and Henshaw, 1980).

The methods used for cryoprotection of shoot tips have been varied as have the results. Seibert and Wetherbee (1974) reported that maximum survival occurred at cooling rates of about 50°C/min in carnation shoot apices. Sakai, et al., (1978) obtained little or no survival with shoot apices of carnation and strawberry runner. Kartha, et al., (1979) also reported that direct immersion in liquid nitrogen resulted in the death of all pea meristems in contrast to the results reported by Seibert and Wetherbee (1974).

Uemura and Sakai (1980) with carnation shoot tips and Kartha, et al., (1980) with strawberry meristems reported successful freezing to -196°C if cryoprotectants were used. The former used 10% DMSO and the latter 5% DMSO or glycerol. In the latter study cooling velocities of 0.5°C to 1°C/min to -40°C or rapidly and stored in liquid nitrogen were used. Maximum viability and plant regeneration (95%) was obtained when the meristems were pre-cultured on medium supplemented with 5% DMSO, and then frozen at a cooling velocity of 0.84°C/min. A viability of 35% was observed when meristems pre-cultured on 5% glycerol supplemented medium for 3 days were frozen at a cooling velocity of 0.94°C/min. Rapid freezing and rapid dry freezing using either DMSO or glycerol resulted in reduced viability. The effectiveness of rapid freezing as a means of cooling meristem tissue to liquid nitrogen temperatures is uncertain. Seibert and Wetherbee (1974); Bajaj (1977); Grout, et al., (1978); Grout and Henshaw, (1978); Withers (1978); and O'Hara and Henshaw (1980), all report success with rapid freezing; others (see above) report the opposite.

In pea meristems that remained viable after freezing at a cooling velocity of 0.6°C/min down to -40°C with 5% DMSO and then immersed in liquid nitrogen, most of the actively growing cells were located on primordial leaf tissues and in the auxillary bud and stipul meristemic areas. Growth generally resumed at many sites, some of these being lateral on the dome but not in the dome (Haskins and Kartha, 1980).

In the present study with Douglas-fir, buds collected from field-grown trees in August, or cold conditioned seedlings at +4°C/17 hr dark and +14°C/7 hr light for at least 8 weeks, frozen slowly (1°C/min to -40°C) or fast (direct liquid nitrogen immersion) with cryoprotectants or dry, naked or with bud scales proved unsuccessful. Buds under any of the above conditions did not survive freezing, turning brown within 48 hr after thawing and post-thaw culture. Part of the failure may have been proceeding with the experiment at the wrong time of year. Buds collected in August from the field appear to be dormant but much mitotic activity continues at this time (Carlson, et al., 1980). Seedlings brought into the cold room in late May never entered a true dormancy period, minor flushing (leaf elongation) taking place even after 8 weeks in the cold. Success in freezing tree species to very low temperatures or liquid nitrogen immersion has been accomplished only in cold hardened, dormant stage material (Sakai, 1960; Sakai and Weiser, 1973; Sakai and Hishiyama, 1978; Sakai, 1979; and Kedrowski, 1980). Conditions must be exact. Increases in cold hardiness in fall appears to be initiated by environmental factors, both day length or spectral distribution of light and air temperature are involved, either simultaneously or sequentially (Weiser, 1970). The onset of frost hardening appears at least to be initiated by day length (Smithberg and Weiser, 1968; and Siminovitch, et al., 1975).

Improper conditioning may lead to improper dehydration. Most hypothesis explaining the mechanism of freezing injury in plant cells assign a primary role to dehydration (Burke, *et al.*, 1976; Cowling and Kendrowski, 1979; and Kedrowski, 1980).

Anatomy appears to be of prime importance in the dehydration process of winter hardy buds. It has been suggested that one of the functions of the flower bud scales was to accomodate ice derived from the flower primordium and thus to prevent injury to the flower primordium caused by the spreading of freezing (Dorsey, 1934; Iskikawa and Sakai, 1978; and Quamme, 1978). It has also been reported that azalea flower scales served as an ice sink as temperature drops (Graham and Mullin, 1976; Iwaya and Kaku, 1980).

Sakai (1978; and 1979) has shown that ice segregation outside primordial shoots clearly takes place through the crown in some conifer winter buds. In the genera which belong to subfamily *Abietordeae* and *Laricoideae* of *Pinaceae*, the pith cavity located between the crown of the primordial shoot and the head of the bud axial pith is formed in early winter (Shibakusa and Kimata, 1976), which effectively prevents ice spreading from the bud axis into the primordial shoot. Low temperature exotherms (below about -20°C - indicating freezable water is still present) were exclusively observed in the genera which belong to *Abietordeae* and *Laricoideae*. If the terminal bud has been cut from the crown in low temperature, exotherms below -10°C could not be detected.

Other genera of conifers such as _Pinus_, _Sequoia_, _Metasequoia_, _Cryptomeria_, _Taxus_, _Podocarpus_ and _Tsujopsis_ do not show this low temperature exotherm, only a high exotherm above -5°C. These genera do not have a crown in the primordial shoot and bud axial tissues directly connected with the primordial shoots (Sakai, 1979). It is assumed that the crown of the primordial shoots plays a principle role in the freezing avoidance mechanism of some conifer buds. Based on this information, Sakai suggested that since Douglas-fir belongs to the group which does not have a crown, it could not be made to follow the ice blocking freezing avoidance mechanism.[12] The crown in part acts as an ice sink and the dry region caused by withdrawn water above the crown prevents the spread of an ice boundry (Quamme, 1978; cited by Sakai, 1979).

In a finely dispersed state, pure water can be super-cooled to as low as -40°C (Fletcher, 1979), which corresponds to the homogeneous nucleation temperature of water. Xylem ray praenchyma appears to be capable of super-cooling to the same degree as dispersed water systems (Quamme, _et al_., 1973; George and Burke, 1977; and George, _et al_., 1977). This is not, however, the case in primordial shoots of conifer buds. A freezing avoidance mechanism in which substantial decrease in water content exits,

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[12]Sakai, A., Institute of Low Temperature Science, Hokkaido University, Sapporo 060, Japan. Personal communication, 1980.

could reasonably explain why primordial shoots of conifers which are marginally hardy to -10°C or above naturally, can winter in Alaska and Siberia where the air temperature cools down to as low as -60°C (Sakai, 1978).

With this mechanism there would appear to be no low temperature limit, provided that the primordial shoots can withstand deminished quantities of liquid water. Sakai (1979) showed that the freezing point of Abies homolepis winter primordial shoots with a water content of 56% was around -5.5°C. Excised winter primoridal shoots when suspended in a small water drop did not survive freezing even to -7°C. The super-cooling ability of excised primordial shoots increased with decreasing water content and no exotherm could be detected in the excised primordial shoots with water contents below about 20%. It was further reported that air-dried primordial shoots of Abies hormolepis and A. balsamea with water contents as low as 26% remained alive but the critical water content which excised primordial shoots will tolerate remains unknown (Sakai, 1979).

The exotherm temperature of the primordial shoot in excised buds of Abies was greatly affected by the cooling rate (Sakai, 1979). Excised winter buds cooled at 5°C increments at daily intervals from -5°C to -20°C and then at 0.11°C/min from -20°C to -40°C survived much better than buds cooled continuously at 0.11°C/min. Little or no exotherm was detected even after cooling down to -40°C to -50°C, probably due to removal of freezable

water from the primordial shoots through the crown, to which the shoots are attached. Abies balsamea frozen in the above manner to -20°C for 30 days survived down to -60°C and even immersion into liquid nitrogen temperature from -30°C (Sakai, 1973). The differential survival rate at different cooling rates probably reflects rate of water removal from the primordial shoots during freezing. Sakai (1979) also noticed that in the very hardy firs, lateral buds were more hardy than terminal buds when cooled very slowly and that the exotherm temperature was not significantly altered whether the bud cooled was left intact or had all bud scales (except a few inner layers) stripped off.

It remains to bee seen, however, whether or not Douglas-fir and other apices in its genera can be exposed to very low temperatures and survive. Using winter buds and with proper slow cooling to perhaps -30°C or -40°C buds of these species may then survive temperature as low as -196°C. Perhaps mild, initial freeze-drying may aid in obtaining the dehydration needed.

10. Summary of Discussion

The development of techniques for successful preservation required a detailed emperical examination of the entire system in question. Both researchers interested in freezing as a means of preservation, (mainly animal and microorganism), and those concerned with the problem on how organisms (mainly plants) in nature seasonally survive sub-zero temperatures have

accumulated large masses of data. To date each, by in large, have not studied or integrated the results obtained by the others.

There are 5 stages to growth of plant cells in culture. These are; 1) lag phase (cells not dividing); 2) exponential phase (cell number constently increasing per unit time); 3) linear phase (cell number doubles per unit time); 4) deceleration phase (cell number increase is decelerating per unit time); and 5) stationary phase (cells not dividing, cell lysis may be taking place). Plant cell cultures in the linear phase of growth are best for freezing since they are small in volume, have little excess water and are densely cytoplasmic. Plant cell cultures in this state are also most susceptable to cryoprotectant effects.

On cooling below freezing, a cell is subjected to growing ice crystals, rising salt concentration, and a dehydrative environment. Protective compounds, called cryoprotectant compounds, through both colligative and non-colligative properties, by forming hydrogen bonds with solvent water, would modify these conditions. Cryoprotective compounds, either of the cell penetrating or non -cell penetrating-type are thought to limit ice formation, retard ice growth rate and reduce salt concentration. In addition, these compounds may interact directly or indirectly with the cell membranes to stabilize the water-lipid-protein complex tertiary structure. Some other beneficial effects of cryoprotectants are;

stabilization of organelles (mitochondria - i.e., prevent breakdown of ATP synthesis by uncoupline), stabilization of golgi apparatus, lysosomes, and cold sensitive microtubules as well as detoxifying by nutralizing damaging free radicals and acting as a membrane carrier.

Maximum recovery, on a two-factor hypothesis basis, is obtained following cooling at a rate sufficiently slow to avoid intracellular freezing, yet sufficiently rapid to minimize damage due to deleterious action of electrolytic concentration caused by dehydration during freezing. Beneficial effects of holding at some sub-zero temperature before exposing to $-196^{o}C$ in a two-step freezing protocol is attributed to the osmotic removal of water from the cells at that holding temperature. This minimizes or avoids intracellular freezing during subsequent cooling to $-196^{o}C$. On the other hand, damage at the holding temperature may be the result of a direct effect of the high ionic strength conditions on the cell, excessive dehydration or an indirect effect of these conditions rendering the cells susceptible to the stress of dilution during thawing.

The popular and convenient tendency to use compounds such as DMSO without regard for their potentually harmful physical, chemical and biochemical effects is now beginning to be altered. Cryoprotectant compounds, used at freezing concentrations may change enzyme structure, breakdown polyribosomes, inhibit protein and RNA synthesis, inhibit nuclear division, retard respiration

and metabolism generally, and induce isomerization of cis/trans double bonds in membrane unsaturated fatty acids.

Exposing cells to cryoprotectants at low positive temperatures, since these compounds can be potent metabolic inhibitors, having the cells in a low metabolic state may be beneficial. At certain concentrations, cryoprotectants may also be beneficial to cell growth. This effect may be due to, at least in part, cell membrane alterations or metabolic changes, or both of these processes.

Suspension cultures frozen, thawed, and cultured may show a lag in growth between 2 days and 6 months. This lag may be due to toxic effects of dead and dying cells or to sub-lethal cell damage to part or all of the cell population. Injury of this latter type may be a result of altered physiology or damage to cell membranes and organelles. The lag in growth may be due to a need to repair such sub-lethal damage. Cultures may fail to grow altogether if the minimum inoculum level is not exceeded with live cells. Important also to the lag period of post-thawed cultures are osmotic and nutrient conditions, speed of cryoprotectant washing, as well as physical factors such as culture shaking speed and light.

Callus cultures may become cold acclimated by long exposure to low positive and slightly below negative combinations of temperatures. Such acclimation can alter sugars, total solutes, water amino acids, proteins, and nucleic acids, etc. Membranes are also changes, the phospholipids and proteins being altered.

Natural substances such as proline may act as non-toxic intra-cellular and extracellular protectants during freezing. To be effective such substances should have high solubility (form hydrogen bonds) and excert high osmotic pressure. The goals of natural acclimation to cold should be to decrease growth, make membranes more fluid, increase proteins and sugars and facilitate dehydration. With proper acclimation and dehydration it may be possible to freeze conifer callus and buds to liquid nitrogen temperature and effect successful recovery.

IV. SUMMARY

1. The chelating compounds EDTA and CDTA can be used to reduce cell aggregate size in suspension cultures of Douglas-fir and poplar.

2. The chelating compounds EDTA and CDTA can cause an increase in growth efficiency in both species studied.

3. Douglas-fir suspension cultures grew in log phase between 7 and 14 days, while for poplar this was between 3 and 7 days. During this time cells were most susceptible to cryoprotectants.

4. At concentrations above 1% the cryoprotectants DMSO and glycerol were toxic to cell growth, while at 0.1% they stimulate growth.

5. At room temperature cryoprotectants above 5% are toxic to viability of Douglas-fir cells after 1.5 hr of exposure. Higher than usual concentrations could be tolerated by both Douglas-fir and poplar if exposed to cold acclimated cells or at $+4^{o}C$.

6. Washing cryoprotectants out of cells improves survival but above 5% cryoprotectant concentration cells cannot survive, even after washing up to 8 times. At exposure to concentrations between 2% and 5%, washing causes an increase in growth rates.

7. Using a two-step freezing protocol with cryoprotectants, cultures of both Douglas-fir and poplar could be frozen to $-196^{o}C$. Warming cultures from liquid nitrogen temperature at $+40^{o}C$ in a water bath was significently better than warming at $0^{o}C$ in air.

8. Douglas-fir cells recovered from $-196^{o}C$ continued growth in liquid media after 80 days. No regeneration of shoots or roots was attempted.

9. Poplar cells recovered from $-196^{o}C$ continued growth on solid media after 40 days. Anatomically normal shoots and roots could be regenerated from these recovered cells.

10. Cold acclimation of both Douglas-fir and poplar callus enhanced survival when frozen to $-196^{o}C$. This was the case even without added cryoprotectants.

11. Buds of Douglas-fir taken from field-grown trees in August or from cold conditioned trees for 12 weeks did not survive freezing to $-196^{o}C$.

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VI. APPENDICES

APPENDIX A

REVIEW OF LITERATURE

Two groups of biologists are chiefly interested in freezing injury and its prevention: Those concerned with the use of low temperature for long-term preservation (Huggins, 1965; Mazur, 1970; Street, 1975; Dougall, 1975; Bajaj, 1976b; Withers and Street, 1977; Bajaj, 1977; Brochu, 1978; Withers, 1978a; Withers and Davey, 1978; Withers, 1978b; and Henshaw, 1979), and those concerned with the problem of how plants in nature survive sub -zero temperatures (Mazur, 1969; Weiser, 1970; Alden and Hermann, 1971; Sakai and Weiser, 1973; Burke, *et al*., 1976; and Miller, *et al*., 1979). Both groups have amassed large volumes of data, but not until recently (Li and Sakai, 1978; Mussell and Staples, 1979; and Levitt, 1980) neither made much effort to study results obtained by the other or to incorporate the results into concepts of freezing injury.

With regard to the first group, isolated reports of investigations into the freezing of biological materials have been made for over the last 200 years (Meryman, 1966). However, significant progress has only been made since the middle of this century when the beneficial effects of cryoprotectant additives were discovered.

In 1949, Polge and co-workers demonstrated the successful freezing of spermatozoa utilizing added glycerol. Frozen spermatozoa is now a valuable technique for the transportation of sperm in the dairy and cattle industry. Cultured mammalian cells were

similarly frozen by Scherer and Hoogasian in 1954, and ten years later a frozen cell culture bank was established in the United States (Registry, 1964). The freeze-preservation of rat ovarian tissue was reported in 1957 (Deansley), and mammalian embryos in 1972 (Whittingham, et al.).

The first report of the successful use of dimethyl sulfoxide (DMSO) as a cryoprotectant was made in 1959 (Lovelock and Bishop). However, it was not until nine years later that its use as a cryoprotectant in the freezing of plant tissue culture cells was demonstrated (Quatrano, 1968).

Because of the mass of literature in the general field, this review will be confined to the preservation of germ-plasm of plants, and storage mainly in liquid nitrogen or its vapor is considered (-196°C). Temperature above -196°C will allow changes to occur in the cells leading to loss of viability and thus shortening the life of the tissue in storage (Bajaj, 1977).

1. SURVIVAL OF PLANT CELL AND TISSUE CULTURES AT -25°C OR LOWER

a. Suspension Cultures:

The freezing and thawing of plant cell cultures began with Quantrano's (1968) successful freezing to -50°C of cells from suspension culture of flax (*Linum usitatissimum* L.). Flax cells were suspended in fresh medium plus 10% dimethyl sulfoxide (DMSO) and frozen at rates of -5°C to -10°C/min to -50°C. After approximately one month, the cells were rapidly thawed and resuspended in fresh media. Fourteen percent of the cells survived, based on

triphenyl tetrazolium chloride (TTC) reduction. A lag of 10 to 14 days occurred before cell growth was observed. No difference in the growth curves or chlorophyll abosrption spectra of frozen and non-frozen cells was detected. However, comparisons of proteins by disc electrophoresis showed several quantitative differences after freezing and thawing.

Latta (1971) recovered wild carot (*Daucus carota*) cultures after freezing to -40°C at -2°C to -4°C/min in the presence of glycerol and/or DMSO. Morning glory (*Ipomoea*) cells were also recovered but only when grown in media containing high levels of sucrose before freezing. Latta (1971) suggested that membrane permeability was the critical difference between the two cell lines because 5% glycerol rapidly plasmolyzed morning glory cells but not wild carrot cells.

Nag and Street (1973), using liquid cultures of both domestic and wild carrot, investigated the influence of a range of freezing rates (-0.5°C/min to -120°C/min), different concentrations and combinations of cryoprotective agents (DMSO, glycerol and sucrose) and thawing rates on survival of the cells. Cells frozen in media plus 5% DMSO at a rate of -1.8°C/min, stored at -196°C and thawed at a rate of 120°C/min gave the highest survival based on the use of fluorescein diacetate as a vital stain. Using these conditions, provided survival exceeded 35%, cultures would grow after freezing and thawing. Recovered cells on culture could yield embryos and plants. No alteration of

chromosome number was observed. There was a 20-30 day lag during the first culture passage regardless of cell survival. This lag indicated either latent freezing injury which was being repaired by the cell before growth began or that the number of surviving cells in the frozen-thawed sample was low enough to prevent immediate logarithmic growth in suspension culture. It was also noted that storage of frozen cells at -20°C was ineffective and storage at -78°C resulted in a progressive loss of cell viability indicating that intracellular ice crystals were growing and damaging the cells or that some metabolic pathways were not stabilized at these temperatures.

Dougall and Wetherell (1974) have reported the cryogenic storage of wild carrot proembryonic masses. Pieces of tissue, 60-120 microns in size, were frozen in media plus 5% or 10% DMSO at a rate of -1°C or -2°C/min and thawed both rapidly and slowly after storage in liquid nitrogen vapor. These workers did not note any increased lag phase after freezing nor any apparent advantage for rapid thawing of frozen cells. The embryogenic capacity of the frozen and thawed cultures was also demonstrated. There was no loss of growth potential over 3 years' storage under these conditions (Dougall and Wetherell, 1974).

Sugawara and Sakai (1974) have shown the viability of suspension cultures of sycamore (_Acer pseudoplatanus_ L.) and common rue (_Ruta graveolens_ L.) after freezing and storage in

liquid nitrogen. These workers used an equal volume of 24% DMSO and 10% glucose added to packed cells and media. Samples after ice nucleation, were held for 3 minute intervals at -15°C, -23°C, -30°C, -40°C, -50°C and -70°C. After each temperature, samples were transferred to liquid nitrogen. Cells survived when rapidly thawed and only when samples were held at -30°C, -40°C or -50°C before transfer to liquid nitrogen. Sugawara and Sakai (1974) found that sycamore cells in the 5th or 6th day of culture corresponding to early or the late log phase gave the maximum triphenyl tetrazolium chloride (TTC) reduction following freezing and thawing.

b. Callus Cultures:

Steponkus and Bannier (1971) showed that callus cultures of *Chrysanthemum morifolium* L. and *Hedera helix* would survive lower freezing temperatures (-17°C and -13°C respectively) after conditioning at +4.5°C.

Sakai and Sugawara (1973) have shown that poplar (*Poplus euramericana* cv. GELRICA) callus will survive freezing to -196°C and thawing after acclimatization. Callus grown in alternating temperatures (8 hr at +10°C and 16 hr at 0°C) survived lower temperatures than callus maintained at +20°C. After an additional period at 0°C, the tissue could withstand freezing to -196°C followed by thawing. These studies show that the ability of plant cells to tolerate low temperatures can be modified by the conditions of growth of the cultures.

c. Shoot Tips:

Carnation shoot tips (meristem and two-leaf primordia) have been shown to survive freezing to $-196^{o}C$ (Seibert and Wetherbee, 1974). Plants grown for a minimum of 7 weeks under short days have shoot tips which would grow after freezing to $-196^{o}C$ and thawing. Isolated shoot tips were frozen in growth medium containing 5% DMSO. The highest recovery of viable shoot tips was achieved when thawing was rapid regardless of the freezing rate. A delay period of 2-4 weeks after thawing was noted before any growth was apparent.

d. General Required Conditions for Freezing:

Recently, Ben-Amotz and Gilboa (1980a) have closely examined the ability of about a dozen species of marine unicellular algae to survive freezing in liquid nitrogen. All species survived poorly the uncontrolled direct freezing in liquid nitrogen. However, addition of DMSO at 5% concentration and the application of a two-step cooling procedure (cells suspended in 5% DMSO at $+20^{o}C$ for 15 min, then cooled at $-30^{o}C$ for 15 min and finally frozen in liquid nitrogen) enhanced the freezing resistance of most of the algae. Optimal conditions for two-step cooling were, initial cooling in 5% DMSO to $-30^{o}C$, then freezing to liquid nitrogen and finally thawing at $+30^{o}C$. Freezing tolerance of viable algae was not related to culture age, photosynthetic activity or chlorophyll to cell ration, but rather was specifically related to the algae species and in a

few cases to the algae size and to the specific growth rate. In addition, algae grown at +4°C for 4 weeks developed high resistance to freezing. Deficiency of nutrients such as nitrate, phosphate and bicarbonate, showed reduced rate of growth and increased significantly the ability of the cell to survive freezing in liquid nitrogen. The observations are interpreted as indicating that reduced metabolic growth of active cells causes augmentation or modification of the cell membrane which serves to increase the resistance to freezing (Ben-Amotz and Gilboa, 1980b). Conditioning has also been successful with poplar. Callus derived from the cambial area of poplar twigs survived freezing down to -120°C and -196°C after cold acclimation (+12°C day temperature/0°C night temperature for 20 days) (Sakai and Sugawara, 1973).

Table 1 shows some higher plant species and the green algae _Chlamydomonas_ under the conditions which they were frozen and thawed. Tables 2 and 3 show similar data for other algal species.

e. Summary:

The available data shows that cultures from diverse species will survive freezing to -140°C or -196°C. Suspension cultures seem to be the condition of choice for cryogenic storage. The difficulty of freezing callus cultures or shoot tips may be more apparent than real though theories of freezing and thawing injury suggest that increased size leads to increased injury.

Table 1. Summary of the optimal conditions for the survival of cell cultures of some higher plants and a green algae when subjected to sub-zero temperatures.

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Acer pseudoplantanus	DMSO 24% + glucose 10%	Pre-freezing $-30^{o}C$ to $-50^{o}C$ and then at $-196^{o}C$	$-196^{o}C$	$+40^{o}C$	Cells showed 20%-30% TTC reduction	SUGAWARA and SAKAI (1974)
"	Glycerol 10%	at $1^{o}C$/min.	$-196^{o}C$	$+37^{o}C$	28% cell survival	NAG and STREET (1975b)
"	DMSO 15%	at $1-3^{o}C$/min.	$-196^{o}C$	$+30^{o}C$	20% cell survival	HENSHAW (1975)
"	Glycerol 10% + DMSO 5%	at $2^{o}C$/min. to $-100^{o}C$, transfer to $-196^{o}C$	$-196^{o}C$	$+37^{o}C$	22% FDA	NAG and STREET (1975b)
"	Glycerol 10% + DMSO 5%	$1^{o}C$/min. to $-100^{o}C$, transfer to $-196^{o}C$	$-196^{o}C$	$+40^{o}C$	30% FDA	WITHERS and STREET (1977)
"	Pregrowth in medium + 3.3% with Mannitol + Glycerol 10% + DMSO 5%	$1^{o}C$/min. to $-100^{o}C$, transfer $-196^{o}C$	$-196^{o}C$	$+40^{o}C$		WITHERS and STREET (1977)
"	Synchronized Suspension Glycerol 10% + DMSO 5%	$1^{o}C$/min. to $-100^{o}C$, transfer $-196^{o}C$	$-196^{o}C$	$+40^{o}C$	25% FDA 45% TTC	WITHERS and STREET (1977)

Table 1. Continued

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Acer pseudoplantanus	Glycerol 1M DMSO 1M 2M Proline or 2M Sucrose	1°C/min. to -35°C, transfer after 30 min. to -196°C	-196°C	+40C	70%-90% at once & 100% after 14 days	WITHERS and KING (1980)
Atropa belladonna	DMSO 5%	at 2°C/min.	-196°C	+37°C	40% cell survival	NAG and STREET (1975a,b)
Atropa belladonna pollen embryo	DMSO 5%-7%	at 1-3°C/min.	-196°C	+37°C	31% globular embryos 9% early heart shaped embryos	BAJAJ (1977) BAJAJ (1979)
Capsicum annuum	Glycerol 10% + DMSO 5%	1°C/min. to -100°C transfer to LN	-196°C	+40°C	<1% FDA	WITHERS and STREET (1977)
"	"	Chilled overnight then frozen as above	-196°C	+40°C	25% FDA	WITHERS and STREET (1977)
"	"	Chilled overnight then frozen at 1°C/min. to -30°C	-196°C	+40°C	50% FDA+	WITHERS and STREET (1977)
Chlamydomonas reinhardii	DMSO 10%	1°C/min. to -30°C to -70°C directly -196°C	-196°C	+35°C	--	HWANG and HUDOCK (1971)
"	Methanol 2.5M	.25°C to -196°C	-196°C	LT$_{50}$-14.4		MORRIS, *et al.* (1979)

Table 1. Continued

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Chlamydomonas reinhardii - (Cont.)	DMSO 1.5M	.25°C to -196°C	-196°C	LT_{50}-4.9		MORRIS, et al. (1979)
"	Glycerol .48M	"	-196°C	LT_{50}-2.1		MORRIS, et al. (1979)
"	Sucrose .42M	"	-196°C	LT_{50}-2.5		MORRIS, et al. (1979)
Chrysanthemum morifolium	DMSO 5% or Sucrose 10% (callus)	-3.5°C -5.5°C	-196°C	+27°C	Low survival	BANNIER and STEPONKUS (1972)
Datura stramonium	DMSO 5%	Cooling at 1°-2°C/min. till -100°C and then at -196°C	-196°C	+37°C	40% Cell survival	BAJAJ (1976)
Daucus carota	DMSO 10% or Glycerol 5%	at 2°-4°C/min. stored at -40°C	-40°C	+37°C	Cells survived 2 months storage (no value recorded)	LATTA (1971)
"	DMSO 5%	at 2°-4°C/min. to -196°C	-196°C	+37°C	Cells survived (no value recorded)	LATTA (1971)
"	DMSO 5%	1.8°C/min. to -196°C	-196°C	+37°C	65% FDA	NAG and STREET (1973)

Table 1. Continued

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Daucus carota - (Cont.)	Glycerol 5% + DMSO 5%	1.8°C/min. to -196°C	-196°C	+37°C	48% FDA	NAG and STREET (1973)
"	DMSO 5% or DMSO 10%	1°-2°C/min. to -70°C, transfer to -196°C	-196°C	+30°C or air	+ embryogenesis (no value recorded)	DOUGALL and WETHERELL (1974)
"	DMSO 5%	2°C/min. to -100°C transfer to -196°C	-196°C	+40°C	65% FDA + embryogenesis	WITHERS and STREET (1977)
"	DMSO 5% (somatic embryos)	"	-196°C	+40°C	Viability < with > embryo size	WITHERS and STREET (1977)
"	DMSO 5% (somatic embryos)	30 min. vacuum dessication then	-196°C	+40°C	Meristems and callus regrowth	WITHERS and STREET (1977)
"	DMSO 5% (protoplasts)	2°C/min. to -100°C, transfer to -196°C	-196°C	+40°C	>90%	WITHERS and STREET (1977)
"	4 % Glucose + DMSO 3% + Ethylene Blycol 2.5%	Stepwise to -15°C	--	Rapid	40% TTC	FINKLE, *et al.* (1975)
"	"	Stepwise to -23°C	--	Rapid	20% TTC	FINKLE, *et al.* (1975)

Table 1. Continued

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Daucus carota - (Cont.)	DMSO 5%-7%	Pre-freezing at -20°C and -70°C, or cooling at 20°C till -100°C and stored at -196°C	-20°C -196°C	+37°C	70% survival plants regenerated maximum	BAJAJ and REINERT (1975)
"	DMSO 2.5%-20% (somatic embryos)	1°C and 5°C/min. to -196°C	-100°C	+40°C	Embryo growth on semisolid media-no growth in liquid media	WITHERS (1979)
Dianthus caryophyllus	DMSO 5% (cultured shoot)	Direct immersion -196°C (1000°C/ min.	-196°C	+37°C	Little success (no value recorded) 15%-33% callus regrowth	SEIBERT (1976)
"	DMSO 10% + Glucose 5% (cultured shoot)	0.5°C/min. to -50°C to -196°C	-196°C	+37°C or in air	80% of apices survived	UEMURA and SAKAI (1980)
"	DMSO + Glucose (no % given)	Direct immersion to -196°C or 0.5°C/min.	-196°C	+40°C	Slowly frozen shoots were white to yellowish turning green 60%-70% survival	ANDERSON (1979)
Fragaria spp.	-- (runner tips)	--		+4°C	Maintained 10 years in dark (periodic addition of fresh media)	MULLIN and SCHLEGEL (1976)

Table 1. Continued

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Fragaria spp. - (Cont.)	DMSO 5% or Glycerol 5% (runner tips)	0.5°-1.0°C/min. to -40°C to -196°C or direct immersion to -196°C	-196°C	+37°C	95% meristem regeneration (direct immersion reduced regeneration	KARTHA, LEUNG, and PAHL (1980)
"	DMSO 5% (cultured w/DMSO 5% for 48 hr. before freezing runner tips)	0.25°-1.0°C/min to -40°C to -196°C	-196°C	+37°C for 90 sec.	95% survival of tips	KARTHA and LEUNG (1979)
Glycine max *Ipomoea sp.*	DMSO 7%	1°-2°C/min. to -196°C	-196°C	+40°C	52% survival	BAJAJ (1976)
"	DMSO 2.5% + Glycerol 2.5% + Sucrose 6.5%	at 2°-4°C/min. to -196°C	-196°C	+40°C	Heavy growth in 10 days	LATTA (1971)
Haplopappus ravenii	DMSO 10%	1.7°C/min. to -20°C	--	not given	3 x control TTC	HOLLEN and BLAKELY (1975)
"	DMSO 10%	1.7°C/min to -20°C	-20°C	not given	not given	HOLLEN and BLAKELY (1975)
Ipomea sp.	Glycerol 2.5% + + DMSO 2.5% (pre-grown w/6.5% sucrose)	2°-4°C/min. to -40°C	--	+37°C	Growth	LATTA (1971)
Linum usitatissimum	DMSO 10%	at 5°-10°C/min. to -50°C	-196°C	+40°C	14% cell survival	QUATRANO (1968)

Table 1. Continued

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Lycopersicon esculentum	DMSO 5 % (seeds)	changing from 20°C 55°C/min. between 0 & -120°C	-160°C	+40°C for 15 minutes	no embryo growth but wound callus formation	GROUT, *et al.* (1978)
"	DMSO 10 % - 25 % (seeds)	direct immersion to -196°C	-196°C	+40°C	seed water content $\leq$25% seed weight use $<$10% DMSO & for $\geq$25% seed weight use 10%-20% DMSO	GROUT (1980)
Mabis domestica	Shoot pieces artificial hardening regine of -3°C for 14 days, -5°C for 3 days and -10°C for 1 day	Prefrozen from -30°C to -50°C to -196°C	-196°C	Slow at 0°C	When buds grafted most grew Longest storage 23 months	SAKAI and SISHIYAMA
Medecago sativa L.	Polyethylene glycol, glucose and DMSO (10 % - 8 % - 10 %) callus	1°C/min. to -30°C to -196°C	-196°C	+40°C	Callus grew rapidly-little or no freeze damage	FINKLE, *et al.* (1976b)
Nicotiana sylvestris	Glycerol 15 % in media w/reduced (10 %) salts	1°C/min. to -100°C to -196°C	-196°C	+40°C	15% FDA	SHILLITO (Reported by WITHERS and STREET) (1977)
Nicotiana tabacum	Glucose 4 % + 3 % DMSO & 2.5% Ethylene Glycol	Stepwise to -23°C	--	Rapid	20% TTC	FINKLE, *et al.* (1975)

Table 1. Continued

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Nicotiana tabacum (haploid)	DMSO 5%	Pre-freezing at $-20^{o}C$, $-70^{o}C$ or at $1^{o}-2^{o}C/min.$ to $-196^{o}C$	$-196^{o}C$	$+37^{o}C$	Long log phase 5%-10% survival or plants regenerated	BAJAJ and REINERT (1975) BAJAJ (1976)
Nicotiana tabacum	DMSO 5%-7% pollen embryo	$1^{o}-3^{o}C/min.$ to $-196^{o}C$	$-196^{o}C$	$+37^{o}C$	31% globular embryos 9% early heart-shaped embryos 2% last heart-shaped embryos	BAJAJ (1977)
"	pollen embryo (cryoprotectant not given)	$1^{o}-3^{o}C/min.$ to $-196^{o}C$	$-196^{o}C$	$+37^{o}C$	Optimal viability from 3-4 wk. old anthers after a log phase 2-4 wk.	BAJAJ (1979)
Oryza sativa	Polyethylene glycol Glucose & DMSO	$1^{o}C/min.$ to $-30^{o}C$ to $-196^{o}C$	$-196^{o}C$	$+40^{o}C$	Callus grew rapidly no freezing effect	FINKLE, et al. (1979)
Oryza sativa L.	DMSO 5%	in a thermos flask at $-70^{o}C$ for 18 hr. to $-196^{o}C$	$-196^{o}C$	$+30^{o}C$	65% TTC of control	SALA, CELLA, and ROLLO (1979)
Pisum sativum	DMSO 5% (pre-cultured w/5% DMSO for 48 hr.) (meristems)	$0.5^{o}-1.0^{o}C/min.$ to $-40^{o}C$ to $-196^{o}C$	$-196^{o}C$	$+37^{o}C$	73% regrowth to plants	KARTHA, LEUNG, & GAMBORG (1979) & HASKINS and KARTHA (1980)

Table 1. Continued

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Phoenix dactylifera	Polyethylene Glycol, Glucose, DMSO (10% - 8% - 10%) (callus)	1°C/min. to -30°C to -196°C	-196°C	+40°C	Callus grew	FINKLE, et al. (1979)
Polystichum retroso-paleaceum	Mature sporophytes innoculated w/snow at -5°C for 24 hr.	from -5°C to -70°C at 2hr. intervals	-196°C	Slowly at 0°C	Marginal cells remained alive	SATO and SAKAI (1980)
Porphyra yezonesis	DMSO 1.5M	Prefrozen to -5°C, -10°C, -15°C -20°C, -30°C at 170°C/sec. to -196°C	-196°C	Fast to 30°C or or slowly at 0°C	100% survival from -10°C to -30°C fast thawing. 15%-60% from -10°C -30°C slow thawing	SAKA and OTSUKA (1972)
Populus euramericana	Callus	Pre-freezing -30°C, -70°C, -120°C and then at -196°C	-196°C	Slow warming in air	Callus masses survived	SAKAI and SUGAWARA (1973)
Prunus cerasus	10% - 20% Sucrose medium	Hardened at 2°C, and subjected to subfreezing temp.	--	--	Callus showed resistance to -30°C	TUMANOV, et al. (1968)
Robinia psudoacacia L.	Sucrose 1M (protoplasts)	2°C/H to -40°C to 10°C/H to -196°C	-196°C	Slow thawing	100% (Neutral red stain plasmolysis de-plasmolysis)	SIMINOVITCH (1978; 1979)

Table 1. Continued

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Saccharum sp.	Polyethylene, Glycol, Glucose, DMSO (10% - 8% - 10%)	Stepwise to -10°C, -15°C, -23°C, -34°C, at 4 min. ea. temp. or -2°C/min. to -40°C or -5°C/min. to -80°C	--	+40°C	50% of control	FINKLE and ULRICH (1979) & ULRICH, *et al.* (1979)
Solanum sp.	DMSO 10% (shoot tips)	0.3-0.5°C/min. to -40°C to -196°C	-196°C	--	10%-50% survived	TOWILL (1979)
"	DMSO 10% (shoot tips)	Direct immersion to -196°C	-196°C	--	20% survived, 60% shoots; 40% callus	GROUT and HENSHAW (1978) & O'HARA and HENSHAW (1980)
Sorghum bicolor	Pregrowth in media + 10% Proline DMSO 1M, Glycerol 1M and L-Proline 2M	1.0°C/min. to -35°C to -196°C	-196°C	+40°C	70%-90% viability FDA	WITHERS and KING (1980)
Tetrahymena pyriformis	DMSO 10% (ciliated protozoa)	1°-3°C/min. to -20°C to -196°C	-196°C	+35°C	Motility and ability to reproduce +	HWANG, *et al.* (1964)
Triticum aestivum L.	Glycerol 2.7M, DMSO 1.4M and Sucrose 0.5M (24 hr. at 20°C) (seedlings)	1°C/min. to -30°C	-30°C	+37°C	+	GAZEAU (1979)

Table 1. Continued

PLANT SPECIES	CRYOPROTECTANT	COOLING	STORAGE	THAWING	RESULTS	REFERENCE
Zea mays L.	Proline 5% - 10% Pregrowth and freezing	$1^{\circ}C$/min. to $-30^{\circ}C$ hold 40 min. to $-196^{\circ}C$	$-196^{\circ}C$	$+40^{\circ}C$	Post freezing growth on solid media	WITHERS and KING (1979)
"	Pregrowth in media + 10% Proline, DMSO 1M, Glycerol 1M, and L-Proline 2M	$1.0^{\circ}C$/min. to $-35^{\circ}C$ to $-196^{\circ}C$	$-196^{\circ}C$	$+40^{\circ}C$	Resume growth on semisolid media after 2 days	WITHERS and KING (1980)

- Unless otherwise stated, culture state is suspension culture.

- TTC = Triphenyl tetrazolium chloride test.

- FDA = Fluorescein diacetate test.

- DMSO = Dimethyl sulfoxide.

Table 2. Summary of optimal freezing conditions for the survival of some marine unicellular algae cultures subjected to sub-zero temperatures. (After Saks, 1978 and Ben-Amotz and Gilboa, 1980.)

SPECIES	Viability[a] (% of Control)		
	[b] DIRECT FREEZING IN LIQUID N_2	[c] ONE-STEP FREEZING TECHNIQUE	[d] TWO-STEP FREEZING TECHNIQUE
Chlorelia marina	8	52.2	82.2
" ovalis	6	64.4	86.1
" salina	4	53.2	87.1
" spaerckii a	3	64.3	72.2
" spaerckii b	2	62.8	72.1
Dunaliella salina	0	8.2	27.2
Nannochloris atomus	3	25.5	54.2
" ovilata	8	52.1	63.1
" sarniensis	2	0	34.3
" sp.	0	51.5	31.2
Phaeodactylum tricornutum	0	5.0	29.1
Platymonas sueaca	0	8.6	20.0

Cells from the late logarithmic phase of culture (8-16d at 20°C) were frozen by one of the following techniques:

[a] Measured as oxygen evolution.

[b] Cells frozen directly in liquid nitrogen.

[c] Cells resuspended in 5.0% DMSO at 20°C for 15 min. and then frozen in liquid nitrogen.

[d] Cells resuspended in 5.0% DMSO at 20°C for 15 min. and then cooled at -30°C for 15 min., and finally frozen in liquid nitrogen.

Table 3. Summary of optimal cryoprotectant conditions for the survival of some marine unicellular algae cultures subjected to sub-zero temperatures. (After Saks, 1978 and Ben-Amotz and Gilboa, 1980.)

SPECIES	Viability[a] (% of Control) ALGAE SURVIVAL[b] AFTER STORAGE AT -196°C	
	1 DAY	12 MONTHS
Nitzchia acicularis		
Control	2	0
Glycerol	84	42
DMSO	93	54
Cylindrotheca chosterium		
Control	3	0
Glycerol	87	38
DMSO	86	47
Phaeodactylum tricornutum		
Control	57	42
Glycerol	95	68
DMSO	94	84
Nannochloris adamsii		
Control	72	67
Glycerol	92	73
DMSO	81	54
Dunaliella quartolecta		
Control	47	37
Glycerol	93	79
DMSO	100	64

[a] Measured as oxygen evaluation.

[b] Measured as cell growth curves.

Glycerol = 10.0% DMSO = 10.0%

Freezing - from ambient to +5°C at 1°C/min. to -5°C in 2 min., 1°C/min. to -50°C to 5°C/min. to -100°C to -196°C. Thawing at +32°C to 0°C.

The reports available further suggest that the physiological state of the cell cultures or tissues may predispose tissue to survival after freezing to -196°C. This predisposition to survival through freezing and thawing was found in early log phase cells in one case, after a short day regime in another case, and after cold acclimation by diurnal temperature cycles in a third.

2. CHOICE OF PLANT TISSUE MATERIAL FOR FREEZING AND REGENERATION OF PLANTS

The literature dealing with plant tissue culture provides ample evidence for the feneral feasibility of regeneration of plants from culture and the nurturing of shoot tips into plants. Murashige (1974) has listed over 150 plants with demonstrated potential for clonal multiplication through tissue culture. In the majority of cases, regeneration has been from callus cultures.

The use of callus cultures does not seem to be an obligatory requirement for regeneration because plants have been regenerated from suspension cultures of endive (Vasil and Hildebrandt, 1966), asparagus (Wilmar and Hellendoorn, 1968), carrot (Nag and Street, 1973), sugar cane (Nickell and Heinz, 1973), ginseng (Jhang, et al., 1974), and koa (Skolmen, 1977). Further, plants can be regenerated from single cell clones of a number of plant species (Nag and Street, 1973; Dougall and Wetherell, 1974; and Withers and Street, 1977). It may be that

specific conditions of suspension culture select against the capacity for regeneration more heavily than do the specific conditions of callus culture, but it should be possible to find the conditions leading to the retention of the capacity for regeneration in suspension cultures of any species. Although the capacity to regenerate plants is retained in suspension cultures, the expression of this capacity may occur preferentially in callus cultures (Nag and Street, 1973).

While suspension cultures seem preferable for cryogenic storage on the basis of the number that can be frozen successfully, the storage of tissue or callus cultures should not be discarded as an alternative particularly if difficulty is experienced in regenerating plants from suspension cultures. In some cases suspension cultures frozen to $-196^{\circ}C$ cannot be grown in liquid media and must be regrown on semi-solid or solid media (Withers and King, 1979). The conversion of suspension to callus cultures can be made and involves only growing cells from suspension culture on agar.

The cryogenic storage of isolated shoot tips or embryos is another possibility which has the advantage that the tissues in addition to being embryonic would be small, thus avoiding size problems during freezing and thawing (Seibert, 1976; Uemura and Sakai, 1980). However, the isolation and nurture of shoot tips or somatic embryos is much more

demanding than the manipulation of suspension or callus cultures. (See also Table 1.)

3. LOW TEMPERATURE INJURY TO PLANT CELLS:

The mechanisms of low temperature injury in plants remain to be fully explained. There are two kinds of freezing injury: 1) That occurring above the freezing point of the cellular contents (chilling injury and thermal shock), and 2) That occurring during freezing and thawing of the cellular contents.

a. Chilling Injury and Thermal Shock:

Many organisms are injured when held at sub-optimal temperature for several hours or days. In some cases, chilling injury is reversible when the temperature is raised. If chilling is rapid, it may result in immediate death to the cell (thermal shock). The mechanisms of chilling injury and thermal shock are unknown. Brandts (1967) and Brandts, *et al*. (1970) had suggested that chilling injury is the result of disruption of hydrophobic interactions which lead to protein denaturation. Susceptibility to chilling injury or thermal shock appear to be related to the physiological state of the organism. For example, thermal shock occurs with *E. coli* in log phase, but not when stationary phase cells are cooled rapidly (Meryman, 1966, 1970; Sakai and Sugawara, 1973; and Nag and Street, 1975a). Steponkus and Bannier (1971); Seibert and Wetherbee (1974, 1977); Bajaj (1976a, 1976b, 1977); and Withers and Street (1977) have

observed that the conditions under which plant cells or cell cultures are grown before freezing appears to predispose the cell to survive cryogenic stress.

b. Freezing Injury:

The freezing and thawing process results in several well studied physiochemical events, one or more of which may lead to injury. Due to interactions of the biochemical events, effects of freezing of plant cells is complex (Levitt, 1980).

The two main physiochemical events which are associated with freezing and thawing of cells are dehydration and ice crystal growth.

Dehydration occurs as a result of water diffusing out of a cell when extracellular ice is present. The rate of movement of water to the outside of the cell is limited by the membrane, the diffusion constant of water, and the free energy difference between the inside and the outside of the cell. As the dehydration of the cell progresses, changes in cellular pH, solute concentration, and cell volume occur. Initially these changes are reversible. At some point the effects of dehydration will become irreversible depending on the extent to which dehydration has progressed.

If cooling is continued when extracellular ice formation is initiated, then two further possibilities for change exist in addition to dehydration. These are nonequilibrium situations,

which if allowed to reach equilibrium, result in complete dehydration of the cell or intracellular ice formation. The events occurring in these two situations are:

1. The cells may super-cool in the presence of extracellular ice. Mazur (1966) has suggested that this is dependent on the ability of the plasma membrane to prevent initiation of ice crystallization within the cells. This situation will result in the continued diffusion of water from the cells with the formation of extracellular ice and result in complete dehydration of the cells.

2. Solidification of the cell contents may occur if the cooling is rapid and the holding temperature is low enough to restrict the initiation and growth of ice crystals. If the cooling is slow or the holding temperature is elevated, intracellular ice formation may occur.

Intracellular ice formation is damaging to the cell (Mazur, 1963, 1965b, 1966, 1977; Mazur, *et al*., 1972; Bank, 1973; Meryman, 1966, 1968, 1970, 1974; Shimada and Asakina, 1975; Sherman, 1962; Sherman and Kan, 1967; Sherman and Lin, 1976; Farrant, 1970; Farrant, *et al*., 1977; McGann, 1978: and Levitt, 1980). The extent to which intracellular ice forms and disrupts a cell is largely dependent on the cooling and warming velocities employed and the membrane permeability of the particular cell (Mazur, 1969).

The targets of cryoinjury by either ice formation or dehydration are largely unknown. It is generally considered that nucleic acids are not damaged by freezing and thawing (Shikama, 1965); that cryogenic storage of cells is not mutagenic (Mazur, 1969); and that soluble enzymes are relatively unaffected by freezing and thawing (Anderson and Nei, 1973; Anderson and Nath, 1975; Brandts, 1967; Brandts, et al., 1970; Chilson, et al., 1965; Hanafusa, 1972; and Heber, 1964, 1967, 1968). However, Siminovitch (1968) showed phospholipids, proteins and nucleic acids increased in cold hardiness and Levitt (1980) listed at least 16 enzymes that do change during hardening. Lehmann (1965) showed that in blood, aldolase activity decreased more than 50% after 3 years of storage at -196°C. It is generally agreed that cryo-freezing is too fast for any newly manufactured qualitative protein changes to take place and injury must be due to structural changes.

Some membrane-bound enzymes are inactivated during freezing and thawing (Chilson, 1965; and Anderson and Nath, 1975). It appears that the membranes are one of the primary sites of cryoinjury (Mazur, 1969; Krasnuk, et al., 1975; Siminovitch, et al., 1975; Steponkus, et al., 1977; and Lyons, et al., 1979). Cellular damage from intracellular ice formation may be due to the direct interaction of ice crystals with the membranes and fine structure of the cell. This was thought to occur by ice crystals causing alteration of the "ordered" hydration shell surrounding

proteins and leading to their denaturation (Hanafusa, 1972). Damage may also occur through penetration of the cell membrane system by large intracellular ice crystals.

c. Cryoprotectants:

A number of compounds (cryoprotectants) have been observed to provide a degree of protection to cells during freezing and thawing. Observational and experimental studies in biology at low temperatures have occurred over several hundred years; but the recognition and use of cryoprotective agents represents a relatively recent innovation. Before the direct specific cryoprotective action of any compounds were appreciated, biologists employed treatment of cell, tissues, and organisms with concentrated solutions of glycerol or sugars, during or prior to freezing to enhance resistance to low temperature injury.

In 1913, Keith reported the improved survival of microorganisms frozen to -120°C in milk glucose, sucrose, or glycerol solutions over those frozen in water or saline. Luyet and Hodapp in 1938 used sucrose treatment to improve the survival of frog spermatozoa subjected to freezing. In 1941, Luyet and Harting used ethylene glycol treatment to protect Anguillula aceti organisms during rapid cooling.

In all of the investigations prior to 1949, cryoprotection was not ascribed to a direct protective action of the solutes used, but rather to nonspecific effects. Some examples are;

osmotic withdrawal of water to render more likely the possibility of vitrification, or to provide channels between ice crystals in which cells might escape crushing action.

In 1949. Polge, Smith, and Parkes became the first investigators to recognize and appreciate that specific compounds provided direct protection from some injurious parameter of a freezing system. Following their studies of the protection of spermatozoa by glycerol, Smith found similar protection of erythrocytes by the same compound. From these discoveries have come the current rapid advances in cryobiology leading to numerous practical preservation procedures for many cell types and to renewed interest in the mechanisms of freezing injury.

During the process of slow cooling where ice was restricted to extracellular regions, water was removed from the cell osmotically, and thus intracellular freezing was avoided. However, the cells may be damaged by the environment of concentrated solutes during slow cooling. The two-factor hypothesis of freezing injury (Mazur, et al., 1972) adequately describes this situation. Maximum recovery was obtained following cooling at a rate sufficiently slow to avoid intracellular freezing, yet sufficiently rapid to minimize damage due to the deleterious effect of the environment on the cell.

Most cell types yielded high recovery only if a cryoprotective chemical was present in the suspending solution during cooling. The mechanisms by which these chemicals protect form

the basis for some fundamental questions in the area of cryobiology.

Protective additives fall clearly into two groups; 1) those which penetrate cells; and 2) those which do not. Different mechanisms of action have been proposed for these two groups of additives (Meryman, 1971, 1974) based primarily on the conclusion that penetration of some was required for full protective action to have been exerted, while others did not have to enter the cell.

Glycerol, low molecular weight glycols, several amides and substituted amides, some sugars (pentoses and hexoses), and dimethyl sulfoxide (discovered by Lovelock and Bishop, 1959), are all classed as penetrating cryoprotectants. Polyvinylpyrrolidone, ethylene glycol, and polyoxyethylene are examples of non-penetrating cryoprotectants (Doebbler, 1966; and Meryman, 1971).

From an examination of the molecular structures for known cryoprotective solutions it was apparent that all were capable of some degree of hydrogen bonding (Doebbler, 1966). Formation of few or weak hydrogen bonds (i.e., as with ketones) failed to provide cryoprotective activity. As more numerous or stronger hydrogen bonds became possible in a molecular structure, excess enthalpy of mixing for water-solute became increasingly negative. Thus, the more numerous and stronger the solute hydrogen bonds

were with water, the more soluble the compound and the more stable the solution was at any temperature.

The literature was consistent with the concepts that cryoprotection is bases on the avoidance or minimization of intracellular freezing and the minimization of damage to the cell from the environment of concentrated solutions during cooling. The colligative action of both penetrating and non-penetrating agents allowed the cells to survive a reduction of cell water content during cooling, thereby reducing the amount of intracellular freezing. Penetrating and non-penetrating agents accomplished this in different ways. Penetrating agents created an environment for a reduction of cell water content at temperatures sufficiently low to reduce the damaging effect of the concentrated solutions on the cells. For example, DMSO reduced the amount of ice found at any temperature during cooling, thereby postponing increased ionic strength conditions to lower temperatures where damage to the cell was reduced. Since DMSO permeated the cell, it was unlikely to contribute to osmotic gradients across the cell membrane during cooling (McGann, 1978). Non-penetrating agents osmotically "squeezed" water from the cells primarily during the initial phases of freezing at temperatures between -10°C and -20°C when these additives became concentrated in the extracellular regions (Meryman, 1971; Farrant, _et al_., 1977).

It has also been suggested (McGann, 1978) that penetrating substances may act similarly to non-penetrating substances if they did not enter the cell. When glycerol was allowed to enter the cell, it acted very much like DMSO; whereas if it were not allowed to enter the cell before freezing, the response was similar to hydroxyethyl starch (HES), a non-penetrating cryoprotective agent.

The list of cryoprotectants and their actions was a long one. Some of the more noteworthy articles were; Lovelock, 1953a, 1953b, 1954; Doebbler, 1965, 1966; Doebbler, et al., 1965; Rowe, 1966, Andrews and Levitt, 1967; Rapatz and Luyett, 1969; Farrant, 1969; Farrant and Woolgar, 1970; Heber, 1970; Meryman, 1971; Connor and Ashwood-Smith, 1973; Farrant, et al., 1977; Meryman, et al., 1977; McGann, 1978; Dalgliesh, et al., 1980; Lineberger, 1980; and Rowe and Lenny, 1980. Some of the more recent papers delt with newly discovered classes of protectants, such as proline (Withers and King, 1979; Yelenosky, 1979; and Widholm, 1980).

4. ESTIMATION OF SURVIVAL AFTER FREEZING AND THAWING

A prerequisite to conducting research in the area of stress physiology, generally is a need to have a reliable method to determine tissue viability (Drawert, 1968; Binder, et al., 1974; and Levitt, 1980). Ideally, the method should; 1) eliminate bias associated with visual observations; 2) be based on a quantitative system that could be analyzed statistically; 3) utilize

small quantities of tissue; 4) be relatively quick; and 5) be capable of predicting the future performance of the material.

Some of the easier visual tests included protoplasm streaming or cyclosis (Kamiya, 1959) and plasmolysis (Siminovitch and Briggs, 1953; and Collander, 1959).

The release of amino acids and other ninhydrin reacting substances were used by Siminovitch, et al., (1962) as a sensitive measure of freezing injury. Direct current conductivity tests have been used to establish the 50% killing point (Aronsson and Eliasson, 1970). Alternating current impedance decreases with increasing freezing injury (Glerum, 1973; Van den Driessche, 1973).

Correlation between tissue ATP level or adenylate energy charge and survival have also been made. The level of ATP and the adenylate energy charge of the tissue can be measured before and after freezing and during thawing. ATP levels can be measured from hot water extracts using the luciferin-luciferase method (Ching and Ching, 1972). For determination of energy charge, the AMP and ADP can be measured as described by Ching and Ching (1972, 1975, and 1976) and the energy charge value calculated according to Atkinson (1968, and 1971). Energy charge reflects the metabolic state of the tissue and values have been reported for a wide range of organisms and plants (Atkinson, 1968; Chapman, et al., 1971; Ching and Ching, 1972; Moreland, et al., 1972; Obendorf and Marcus, 1974).

Cell ATP has been used to predict the germination rate of conifer pollen (Ching, _et al_., 1975), radish (Moreland, _et al_., 1974), and wheat seeds (Obendorf and Marcus, 1974). Although there appeared to be a general trend to correlation between Douglas-fir pollen viability and ATP amount (based on controlled pollinations), this trend was not consistent (Binder, 1974). The measurements of ATP and adenylate energy charge would have to have been compared with the estimates of survival from growth data to determine whether or not there was a correlation.

If there is a positive relationship, perhaps the amount of ATP present could be a representative test of early biochemical changes which take place in suspended cells or callus cultures during and after freezing.

A rapid and consistent test of viability (based on controlled pollination/x-ray of seed/germination of seed using Douglas -fir pollen) is cellular respiration using a oxygen electrode (Binder, 1974; and Binder and Ballantyne, 1975). Measuring the utilization of oxygen (respiration) or the release of oxygen (photosynthesis), survival could also be measured using the oxygen electrode and would be a good test of survival of cells after freezing. The uptake and release of oxygen gave some clues about the physiological state of the mitochondria and chloroplasts after freezing when compared to control values. Hatano, _et al_. (1978) measured the oxygen evolution of cells which survived freezing and found that it was remarkably decreased by

freeze-thawing, while the oxygen uptake was hardly affected. The freeze-injury chloroplasts were repaired during the following incubation. Oligomycin inhibited the repair of freeze-injured chloroplasts. The results suggested that mitochondria change their membranes into a structure hardier than chloroplasts, and ATP synthesis by mitochondria is essential for the repair of freeze-injured chloroplasts.

Another tool to measure cell recovery from freeze-thaw cycles is through use of radiochemical markers (Griffiths and Beldon, 1978). Radiochromate binds nonspecifically cell protein and it is released in a nonspecific manner depending upon the degree of protein denaturation. Its release occurs as a result of lethal and nonlethal (repairable) stress. Other radiochemical procursors for the cell membrane (fucose, cholesterol, and palmitic acid), cytoplasm (uridine, proline), intracellular pool (rubidium), and structural protein (amino acids, zinc) are also beginning to be used. It was suggested that the release of these radiochemicals from cells after cooling from $0^{o}C$ to $-196^{o}C$ and thawing could show whether different parts of the cells were affected at different phases of the freeze-thaw cycle, or at different rates of cooling, and link these results with the death or recovery of the cells.

In the case where plants contain both cyanogenic glycosides and degradative enzymes which could hydrolyze the glycosides and release hydrogen cyanide (HCN), it was suggested that these

compounds may have been used as a measure of membrane damage (Reaney, 1978; and Stout, et al., 1980). The reasoning here was that the glycoside and degradative enzymes were isolated from each other by membranes.

Currently, the most convincing criterion of overall survival after freezing and thawing was the ability of the cells or tissue to grow. This method may however, not qualitatively reflect the immediate post-thawing status of a preparation and may, in addition, take considerable time to show results. Some physiological and cytological examinations and testing of morphogenetic potential were also dependent upon regrowth (Quantrano, 1968; Nag and Street, 1973; Dougall and Wetherell, 1974; Seibert, 1976; Withers and Street, 1977; Grout, et al., 1978; Finkle, et al., 1979; O'Hara and Henshaw, 1980; and Eumura and Sakai, 1980). An immediate post-thawing test, while generally useful was essential in cases where very low or ephemeral viability was attained.

Vital staining represented a fast, easy test to facilitate the screening of large numbers of thawed specimens. Live cells had the ability to reduce triphenyl tetrazolium chloride salts (TTC), by mitochondrial activity, to formazan, a water insoluble red compound (Steponkus and Lampnear, 1967; Withers and Street, 1977; and Bajaj and Reinert, 1977). A reduction of formazan production after cold injury does not result from inactivation of dehydrogenases, but from co-factor and

substrate limitations (Steponkus and Lamphear, 1967). These limitations could have been caused by inactivation of enzymes other than dehydrogenases, required for the continued synthesis of substrates and co-factors. The irreversible inactivation of oxidative phosphorylation by freezing (Heber and Santarius, 1964) represented such a key-step, leading to a depletion of substrates and co-factors and subsequent decrease in the amount of TTC reduction, in a manner similar to the inhibition of TTC reduction by 2,6-dinitrophenol (Roberts, 1951). Substrate and co-factor limitations may also arise due to diffusion or dilution of the substrates. Since freezing disrupted lipoprotein complexes and affected membrane permeability, intracellular localization and concentration of substrates at enzyme sites may have been greatly diminished (Lovelock, 1957; Levitt, 1962, 1965, 1966, 1970, 1972, and 1980).

Another vital stain test was the intracellular breakdown of fluorescein diacetate by esterase activity to yield the fluorescent compound fluorescein. The fluorescein diacetate nonpolar molecule enters the living cells where esterase cleaves off the acetate residues leaving fluorescein which then accumulates. These fluorescein molecules could fluoresce in the cells (Hesolp-Harrison and Heslop-Harrison, 1970; and Widholm, 1972).

Recently an extract method to determine total fluorescein diacetate has been developed (Persidsky and Baillie, 1977). In this method it was demonstrated that the amount of fluorescein

extracted from the labelled cells was directly proportional to the number of intact cells and that its relationship was not affected by the presence of dead cells. However, the experimental conditions required for any accuracy with this test were exacting. During the incubation with FDA, the number of cells should not exceed 3×10^6 cells/ml. With fluorescein concentrations of 2µg/ml the incubation temperature should be within $+20^{\circ}C$ to $+30^{\circ}C$ for 10 to 20 minutes. To prevent the loss of fluorescein by labelled cells during washing, the cells must be maintained at a temperature near $0^{\circ}C$. The authors state however, that provided the controls are appropriately adjusted, the accuracy of the test should not be affected by the presence of medium and/or DMSO.

A useful alternative method was the use of Evans' Blue staining, in which lethally damaged cells were stained blue, leaving surviving cells unstained and therefore available for other specific histochemical reactions (Gaff and Okong 'O-ogola, 1971; and Withers and Street, 1978).

There was one overall disadvantage to vital staining, that was that the stain generally could not distinguish between viable and genetically dead cells. Some survivors die immediately upon thawing, whereas genetically dead cells may survive and metabolize for some time but were too damaged, dying at some later time (Binder, 1974; and Frim, *et al.*, 1978). In addition, there must generally be some critical number of cells present

in order for the culture to continue growth. Nag and Street (1973) reported that growth of frozen and thawed wild carrot cells could not be achieved unless 35% or more of the cells stained with fluorescein diacetate. There has also been some indication in the literature that an intact cell membrane was to some extent impermeable to these stains (Melick, 1973).

5. RATIONALE FOR STUDY

The background review clearly illustrates several points that are directly related to this study:

a. There is a growing need for methods by which the germ plasm of vegetatively propagated plants may be conserved.

b. The methodology needed to regenerate plantlets from cells in culture is available in some species.

c. Recent developments in the areas of plant tissue culture and cryobiology offer a possible solution to this problem. Cryogenic storage has been successfully applied to several plant cells in suspension culture, one callus culture, and shoot tips of one species.

d. Cold acclimation of plant cell cultures leading to greater survival at low temperatures is possible in some cases.

e. The mechanisms of cryoinjury are understood to some extent and some methods are available to minimize this injury.

f. Methods of estimating survival after freezing and thawing are available. These include the ability of the cells to grow in culture, measurement of ATP content and adenylate energy charge and other methods such as staining by fluorescein diacetate or triphenyl tetrazolium chloride.

g. Interpretation of experimental results may suggest the ability of a particular tissue or cell type to withstand freezing and thawing when examined over a wide range of conditions. Moreover, it may be possible to infer which physiochemical processes are involved in the cryoinjury. For example, if a freezing rate of $200^{o}C/min$ gives lower survival than a rate of $1^{o}C/min$, then intracellular freezing may be significant. If rapid thawing gives significantly higher viability than slow thawing, it is likely that intracellular ice crystal growth is leading to cell damage. If freezing at $0.1^{o}C/min$ gives lower survival than $5^{o}C/min$, then cellular dehydration may be the main source of damage.

Based on this background, it is the purpose of the present study to develop a protocol to freeze to $-196^{o}C$ two tree species; Douglas-fir and poplar. To produce a successful freezing protocol consideration must be made of pre-freezing culture conditions, cooling rate, type and amount of cryoprotectants used, thawing rate, and post-thaw culture conditions.

APPENDIX B

THE REGENERATION AND DEVELOPMENTAL ANATOMY OF A POPLAR HYBRID FROM CELL SUSPENSION CULTURED CELLS IN LIQUID CULTURE

INTRODUCTION

The induction of complete plants from cell and tissue cultures of herbaceous angiosperms has been reviewed by Tulecke (1964); Murashige and Nakano (1966); Freson and Vanseveren (1968); Nishi (1968); Pillai and Hildebrandt (1969); Murashige (1977 - organ initiation); Halperin (1978); and Green (1980 - shoot apex organogenesis).

Work on differentiation from callus cultures of tree species began with Jacquiot (1951) using *Ulmus campestris*. Since then the importance of the tissue culture technique for clonal propagation of tree species has been amply stressed by many (e.g., Geissbuhler and Skoog, 1957; Haissig, 1965; Brown, 1967; Winton, 1970; Bonga, 1974; Konar and Nagmani, 1974; Winton and Huhtinen, 1976; Thompson, 1977; Hall, 1977; Mott, *et al*., 1977; Skolmen, 1977; and Gupta, *et al*., 1980). A list of broad leaf tree species regenerated from somatic callus cultures are given in Table 1. A rather complete list for tree species to that date is given by Winton (1974), and Durzan and Campbell (1974), and a list for all calsses of plants by Murashige (1978).

Table 1. Broad leaf tree species regenerated from somatic callus culture. (Modified after Winton and Huhtinen, 1976)

SPECIES	COMMON NAME	REFERENCE
Acacia koa gray	Koa	SKOLMEN and MAPES (1976); SKOLMEN (1977)
Betula pendula	European Birch	HUHTINEN and YAHYAOGLU (1974)
" "	"	HUHTINEN (1978)
" verrucosa	Birch	JACQUIOT (1966)
Broussonetia kazinoki	Paper Mulberry	OKA and OHYAMA (1972)
Larica papaya L.	Papaya	AL-MENDI and HOGAN (1979)
Citris maxima (grandis)	Shaddock	CHATURVEDI and MITRA (1974); Burger and BANKS (1979)
" sinensis	Sweet Orange	CHATURVEDI and MITRA (1974)
Elaeis guineensis	Oil Palm	JONES (1974)
" sp.	Oil Palm	EEUWENS and ALAKE (1978)
Eucalyptus citriodora	Lemon-scented Gum	ANEJA and ATAL (1969)
Kalanchoe sp.	Coconut Palm	EEUWENS and ALAKE (1978)
Musa sp.	Banana	EEUWENS and ALAKE (1978)
Phoenix dactylifera	Date Palm	EEUWENS and ALAKE (1978); AL-MEHDI and HOGAN (1979)
Populus canescens	Gray Poplar	CHALUPA (1974)

Table 1. (Continued)

SPECIES	COMMON NAME	REFERENCE
Populus (continued)		
" *deltoides*	Eastern Cottonwood	BERBEE and HILDEBRANDT (1972)
" *euroamericana*	Carolina Poplar	BERBEE and HILDEBRANDT (1972)
" *nigra*	Black Poplar	BERBEE and HILDEBRANDT (1972); CHALUPA (1974)
" *var. typica*	Black Poplar	CHALUPA (1974)
" *var. italica*	Lombardy Poplar	VENVERLOO (1973)
" *tristis* (hybrid)	Poplar	THOMPSON (1977)
" *tremula*	European Aspen	CHALUPA (1974); JACQUIOT (1964)
" " (tetraploid)	Euorpean Aspen	WINTON (1971)
" *tremuloides*	Quaking Aspen	WOLTER (1968)
" " (triploid)	Quaking Aspen	MATHES (1964a, 1964b); WINTON (1968, 1970)
" " (hybrid)	Quaking Aspen	WINTON (1972)
" *trichocarpa*	Black Cottonwood	BAWA and STETTLER (1972)
Prunus amygdalus	Almond	MEHRA and MEHRA (1974)
Tectona grandis L.	Teak	GUPTA, *et al*., (1980)
Ulmus americana	American Elm	DURZAN and LOPUSHANSKI (1975)
" *campestris*	English Elm	JACQUIOT (1951)
" *sp.*	Elm	REDENBAUGH (1980)

While plant regeneration from callus tissue has been reported in increasing numbers, regeneration of plants from tissue originating from suspension culture cells has been limited to only a few species, such as tobacco (Hildebrant, 1973; and Street, 1973), carrot (Steward, *et al.*, 1967, 1969, 1975; Earle and Langhans, 1975; and see also Table 1), and alfalfa (McCoy and Bingham, 1977). Only one report is available on the regeneration of a tree species (*Acacia koa* gray) from suspension cultures directly (Skolmen, 1977), but there have been a few cases of regeneration from callus grown on solid media which had been obtained from suspension culture, e.g., American elm (Durzan and Lopushanski, 1975). Poplar also has been grown in suspension culture then transferred to solid media where callus formed (Fukuda, 1976) and shoots were generated (Gautheret, *et al.*, 1975).

One of the reasons there has been little or no attempt, for most plant species, to induce regeneration from suspension cultures is that cell clumping occurs in most and therefore is of primary concern (see Chapter 1). Poplar is somewhat unusual in that it forms a very hard callus and does not form even small clumps of cells easily. This makes it a somewhat poor plant type to use for regeneration from suspension cultures. Since the ultimate test of any successful cryogenic preservation is a vigorously healthy growing plant after freezing, and when the freezing protocol calls for the use of small clumps of cells,

root regeneration is desirable. The present study was undertaken to produce shoots and roots completely in liquid media from small clumps of poplar cells. In this way post-thaw organogenesis can also be compared to normal organogenesis. For this reason, the developmental anatomy of organogenesis in cultured cell clumps was studied.

METHODS AND MATERIALS

All solutions were mixed using distilled water which had been passed through Barnstead Sybron Corporation cartridges #D8904 (organic removal), #D8901 (high capacity ion removal), and D#8902 (ultra-pure ion removal).

Media was made according to Murashige and Skoog (1962), (see Appendix B_1), or 4.3 g/l Murashige and Skoog plant salt mix obtained from Flow Laboratories; McLean, Virginia. Sucrose was added at 2% (20 g/l). The growth regulator additive was naphthaleneacetic acid (Sigma Chemical Company) at 10 mg/l (see Appendix B_3). Agar was added at 1% (10 g/l) (Bacto-agar, Difco Laboratories; Detroit, Michigan). The medium was heated in a medical sterilizer (autoclave) at 121°C, 15 psi for 7 min to melt the agar. After thorough mixing, the medium was dispensed into test tubes or small flasks and the ends stoppered loosely. The tubes and flasks were then sterilized in an autoclave at +121°C, 15 psi for 20 min. On removal of the tubes and flasks from the autoclave, the stoppers were then

securely set in the necks and either set upright or at a slight angle to cool at room temperature.

Liquid medium was made the same as solid media except no agar was added and the medium was placed in "T" tubes (Steward, et al., 1968; see also Figure 1-3).

Large shoot tips of the poplar clone Tristis #1 were obtained from an experimental plot north of Ames, Iowa at the beginning of September, 1978.[1] The shoots were wrapped in wet paper towelling and stored at +4°C until used. The shoots were dipped in 70% ethanol for 1 sec, then dissected to expose about 2 mm of the shoot tipe bearing the shoot apex. The shoot tips were then dipped in 10% clorox solution several times then washed with distilled water and placed on solid media. Callus formed in about 2 weeks. The cultures were subcultured every 30 days.

After 5 passages (subcultures) on solid agar, small amounts of callus were placed in "T" tubes containing 10 ml of either Schenk and Hildebrandt's (1972) medium (see Appendix B_2) containing 20 ppm adenine (Jacquiot, 1951; and Mapes 1975), 10% (v/v) coconut water,[2] 2.5 ppm indole-3-acetic acid (IAA), and 1% mg/1 benzylaminopurine (BAP), or one-half strength basal

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[1] Thanks to D.G. Thompson, Graduate Research Assistant, O.S.U. Forest Science Dept. for obtaining the shoots.

[2] Frozen coconut water from green coconuts was obtained from M.O. Mapes, Oregon State University.

medium[3] of Murashige and Skoog (1962) with 20 ppm adenine (Mapes, 1975), 10 ppm NAA and 0.5 ppm BAP. After 30 days, aliquots of the above suspenison culture were transferred to "T" tubes containing full strength Murashige and Skoog salts nd vitamins, 2.5 ppm NAA, 2.5 BAP and 1 ppm zeatin or Schenk and Hildebrandt's medium with 1000 ppm myo-inositol, 20 ppm adenine, 5% (v/v) coconut water, 5 ppm IAA and 1 ppm 2-isopentenyl adenine (2ip).

"T" tubes were rotated at 3 rpm on an apparatus similar to the one designed by Steward and Shantz (1958) (Figure 1-3; and Appendix C). Growth chamber temperature was for callus and suspension cultures. Light was supplied by six General Electric F48T12 CW 1500 cool white fluorescent lights and six Sylvania clear 60 watt incandescent bulbs. Callus cultures received a total of 900 foot candles and the rotation apparatus an average of 850 foot candles (high 1070 and low 520 foot candles), measured by a Model 756 Weston Illumination Meter with quartz filter.

After six weeks in culture, the cell clumps were removed from "T" tubes and fixed for 12 hr in formalin-aceto-alcohol (FAA), according to Johnson (1949), and then embedded (Table 2). Sections were then cut at 10 μ thickness and stained (Table 3).

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[3]Binder, W.D., unpublished results. University of Hawaii. Honolulu, Hawaii. 1976.

Table 2. Embedding schedule of poplar cell clumps. (Modified after Johanson, 1940.)

STEP NO.	TREATMENT	TIME (HR)
1	50% Ethanol	1
2	50% Ethanol	1
3	Johanson #1	2
4	Johanson #2	overnight
5	Johanson #3	2
6	Johanson #4	2
7	Johanson #5	2
8	t-Butyl Alc.	1
9	t-Butyl Alc.	1
10	t-Butyl Alc.	overnight
11	t-Butyl-paraffin oil	1
12	paraffin *	1
13	paraffin	2
14	paraffin	3
15	paraplast	2
16	paraplast **	3
17	embed.	-

* Obtained from Arco Oil Company

** Tissue embedding medium. Sherwood Medical Industries; St. Louis, Missouri.

Table 3. Staining schedule for poplar. (Modified after Johanson, 1940.)

STEP NO.	TREATMENT	TIME (MIN)
1	Mount sections on microscope slide treated with Haupt's adhesive (Johanson, 1940 p. 20). Float section on slide with 3% formalin.	-
2	100% xylene	5
3	100% xylene (drip bottle)	Rinse
4	100% ethanol (drip bottle)	Rinse
5	95% ethanol	5
6	distilled water	5
7	1% safranine stain	30-45
8	distilled water	5
9	50% ethanol	5
10	1% picric acid	Rinse
11	100% ethanol (drip bottle)	Rinse
12	fast green (0.5g/33 ml 100% ethanol, dissolve and add 66 ml clove oil, and filter	2-4
13	clove oil (eye drop bottle) *	2-3 Rinse
14	clove oil/100% ethanol/100% xylene (2:1:1) (eye drop bottle) *	Rinse
15	100% xylene (drip bottle)	Rinse
16	100% xylene	5
17	Add cover slip adhesive and cover slip.	-

* Solution was put directly onto slide. After several washings over material, solution was drained back into original container and may be reused.

RESULTS

Shoots could be grown after 6 weeks in full Murashige and Skoog media, 2.5 ppm NAA and 2.5 ppm BAP (previous culture - 1/2 Murashige and Skoog with 20 ppm adenine, 10 ppm NAA and 0.5 ppm BAP) or Schenk and Hildebrandt's medium with 1000 ppm myo-inositol, 20 ppm adenine, 5% coconut water, 5 ppm IAA and 1 ppm 2ip (previous culture - Schenk and Hildebrandt 20 ppm adenine, 2.5 ppm NAA, 2.5 ppm IAA and 1 ppm BAP).

Cell divisions occurred within the small clumps of cells (Figure 1) and the clumps enlarged. The first signs of organization appeared as starch grains accumulated within some of the cells (Figure 2).

New shoots formed from within small cell aggregates Figure 3). Figure 4 shows one of these shoots after embedding and sectioning. Early was embedded sections of these shoots showed that meristemoids formed within the cell clumps (Figure 5) and xylem differentiation began within the meristemoid. Oblique sections through elongated, branched meristemoids showed that differentiated vascular tissue and a cambial zone (Figure 7). These meristemoids extended from their internal positions in the cell clumps to the cell surface and proliferated a clump of surface meristematic cells (Figure 8). The peripheral meristematic cells at the surface appear to become organized into two embryonic "leaves" (E) and the apical meristem (A) in Figure 9.

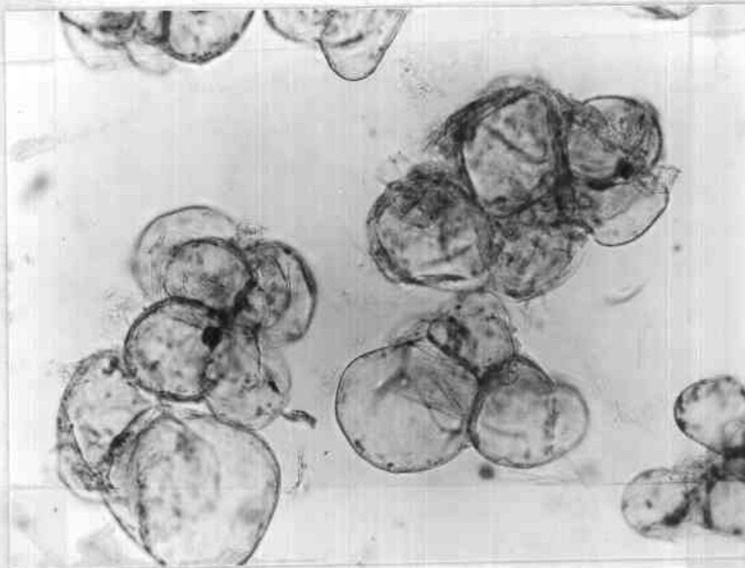

Figure 1. Small clumps of poplar cells.

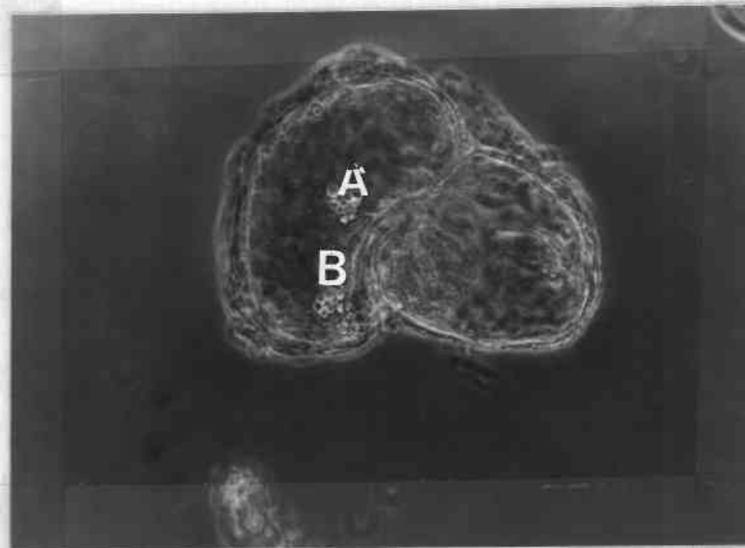

Figure 2. Two poplar cells. One showing
starch grains at "A" and "B".

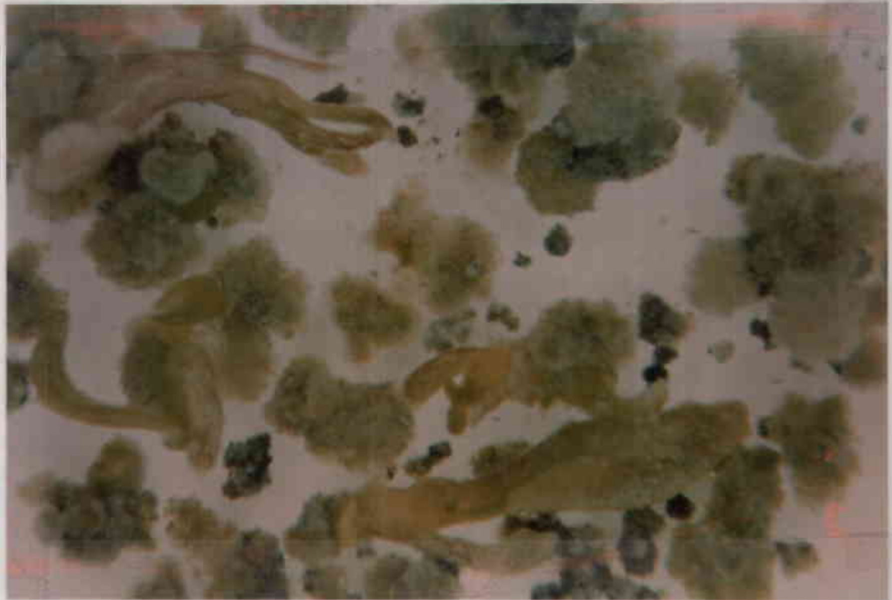

Figure 3. Clumps of poplar cells with shoots.

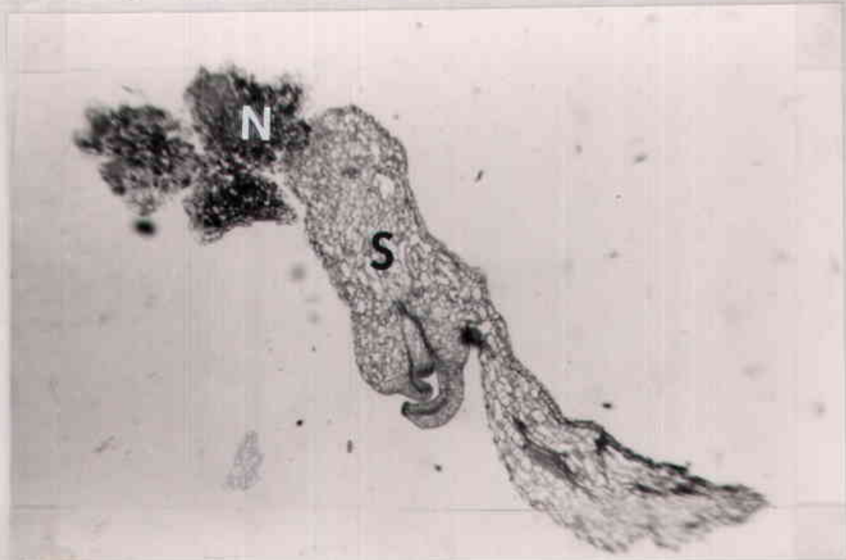

Figure 4. Longitudinal section of whole shoot (S) growing from cell clump (N).

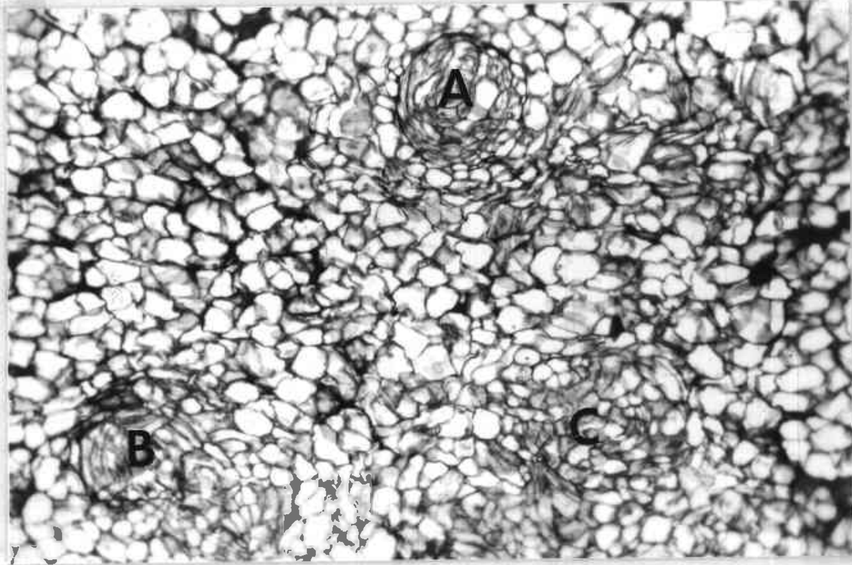

Figure 5. Sectioned poplar cell clump showing internal meristemoids forming at "A", "B" and "C".

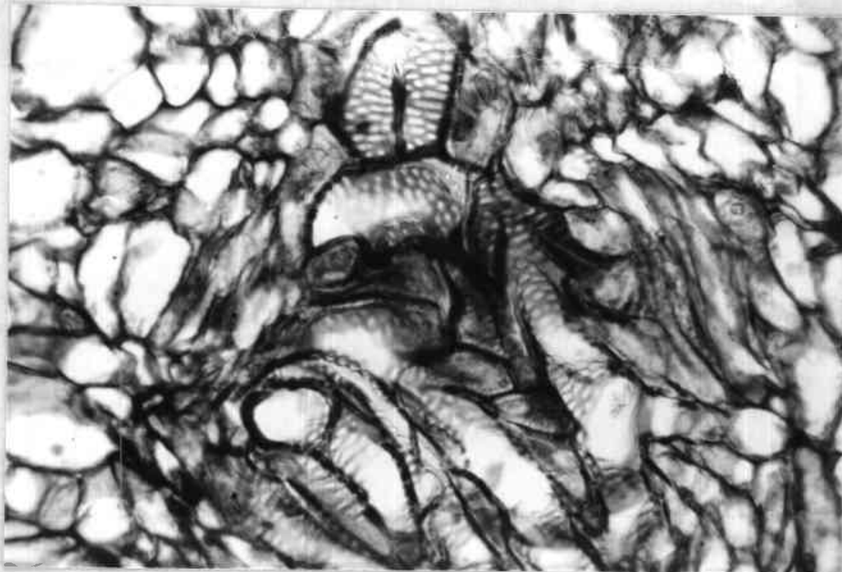

Figure 6. Close-up of area "B" of Figure 5 showing early vascular formation in poplar callus.

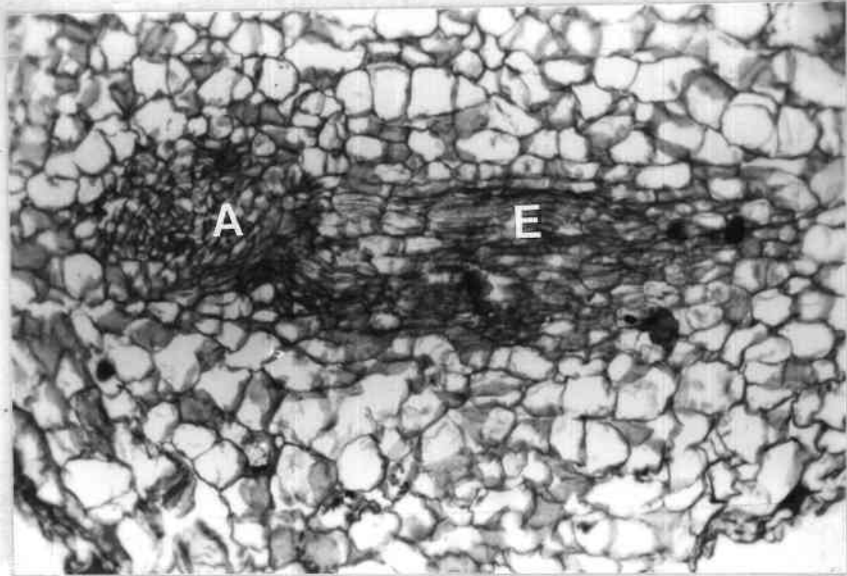

Figure 7. A differentiating meristemoid of poplar showing internal meristemoid (A) and elongating side structure (E). x80

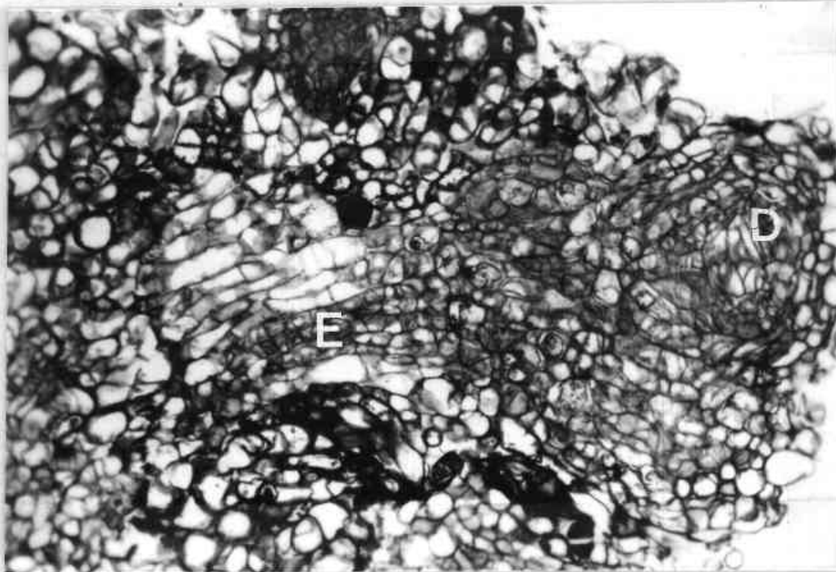

Figure 8. A meristemoid of poplar showing elongating side structure (E) with developing "head" (D) at cell clump surface.

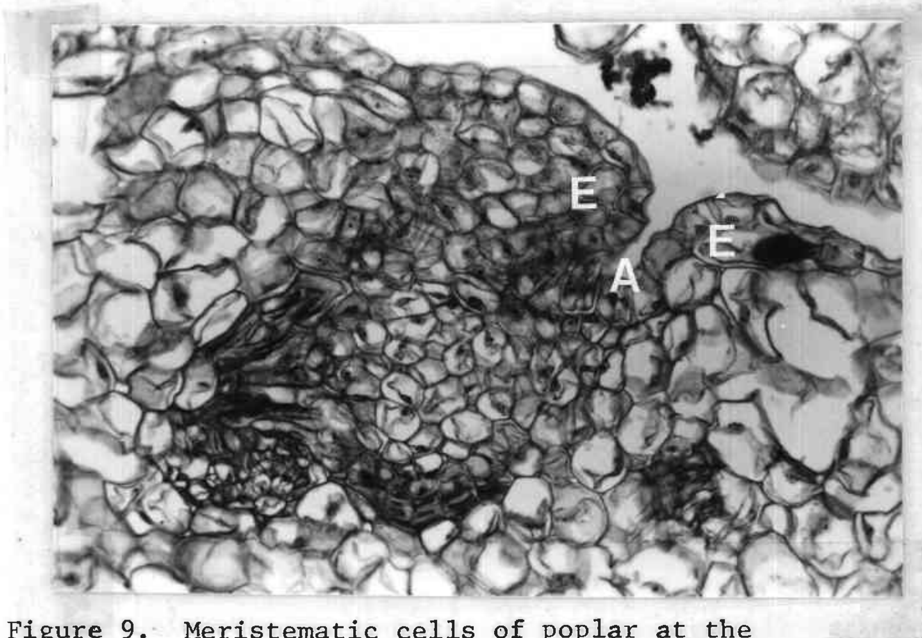

Figure 9. Meristematic cells of poplar at the surface of the callus appearing to become organized into two embryonic "leaves" (E) and apical meristem (A).

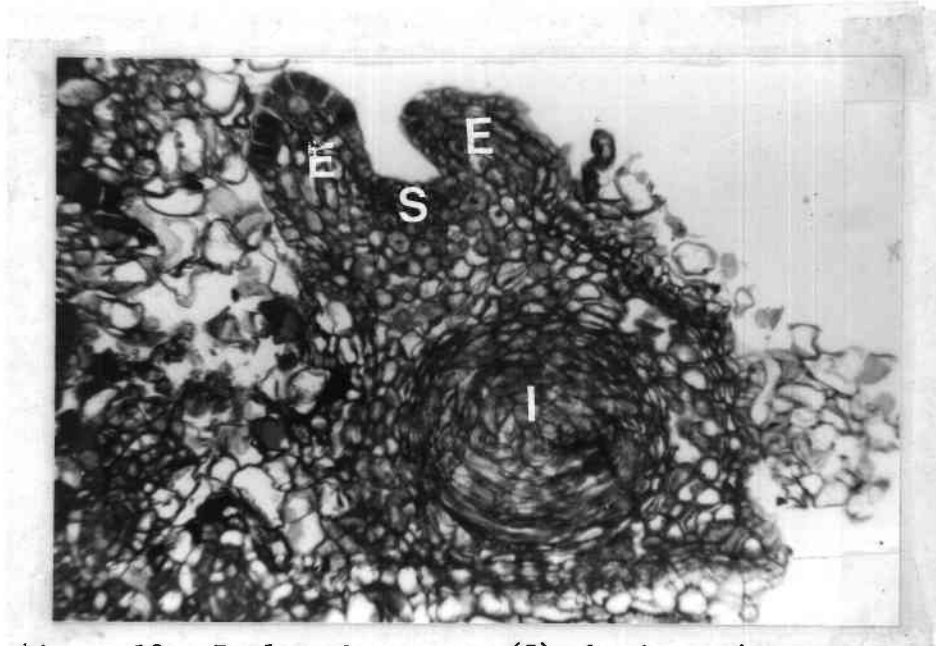

Figure 10. Poplar shoot apex (S) showing primary leaves (E) and internal meristem (I).

These "leaves" appear about the same as the first two prophylls that develop at the flanks of a newly initiated auxiliary bud. This suggests that prophylls may appear first and may lead to apical development. The primary leaves become elongated and the shoot apex forms a dome showing a tunica layer of cells with apical initial cells and a lower corpus growth zone (Figures 10, 11, 12, and 13). Cross sections of the leaves showed good vascular bundle development (Figure 14) internally while the surface epidermal layer contained apparently functional stomata (Figure 15).

Close examination of longatudinal sections of vascular bundles displayed considerable xylem differentiation enclosed by a parenchymatous sheath (Figure 16) which sometimes branched (Figure 17).

Roots could be produced from clumps of callus in suspension culture. Roots formed from 1/2 strength Murashige and Skoog media without glycine and with 10 ppm adenine and 5 ppm NAA. No cytokinins were added. (Previous culture was Schenk and Hildebrandt with 1000 ppm myo-inositol, 20 ppm adenine, 2.5 ppm NAA and 0.05 ppm zeatin.) Roots formed from clumps of cells which seemed to have many cell proliferations at the surface (Figure 18). Longitudinal sections through the cell clumps showed long roots with many lateral roots (Figure 19). Developing lateral root primordia displayed pericycle and endodermal-like areas beneath which cell division at these regions was prolific (Figure 20).

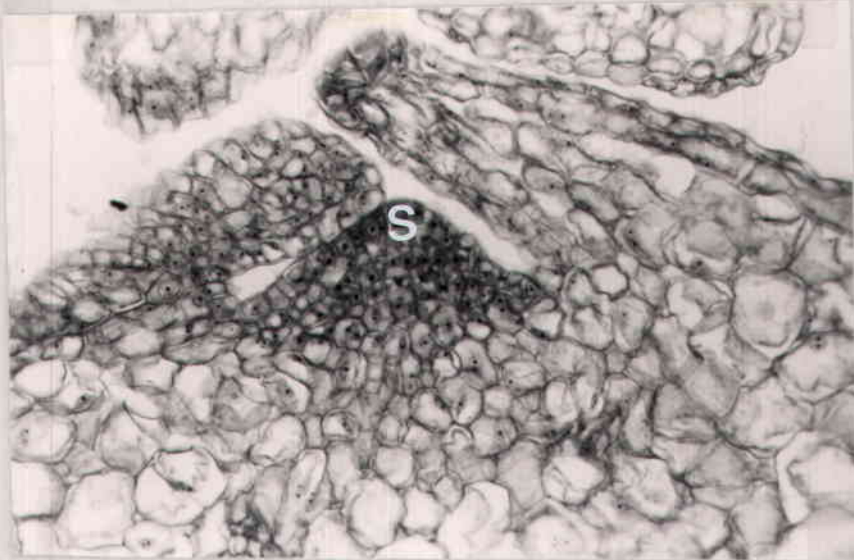

Figure 11. Poplar shoot apex (S) showing apical initials, early tunica and corpus growth zones with primary leaves.

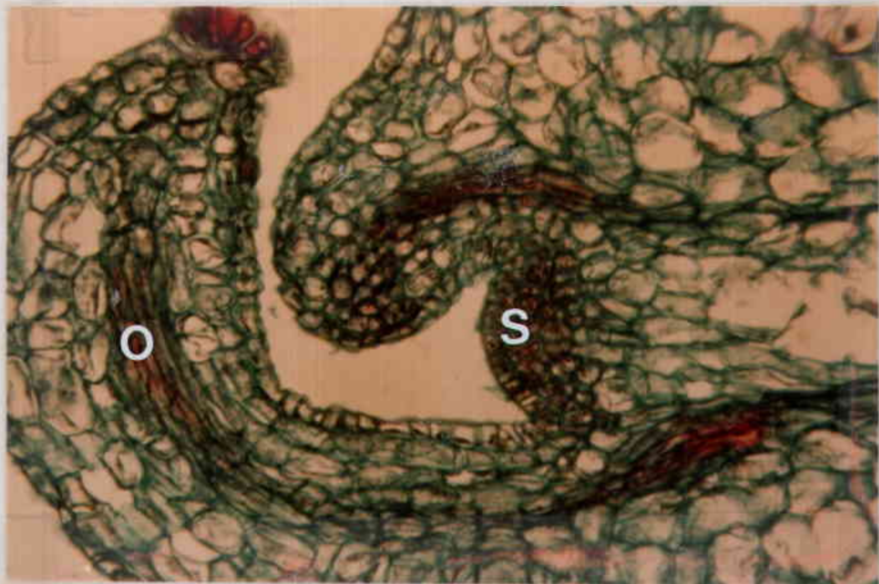

Figure 12. Primary leaves of poplar shoot showing vascular trace (O) with apex in center (S)

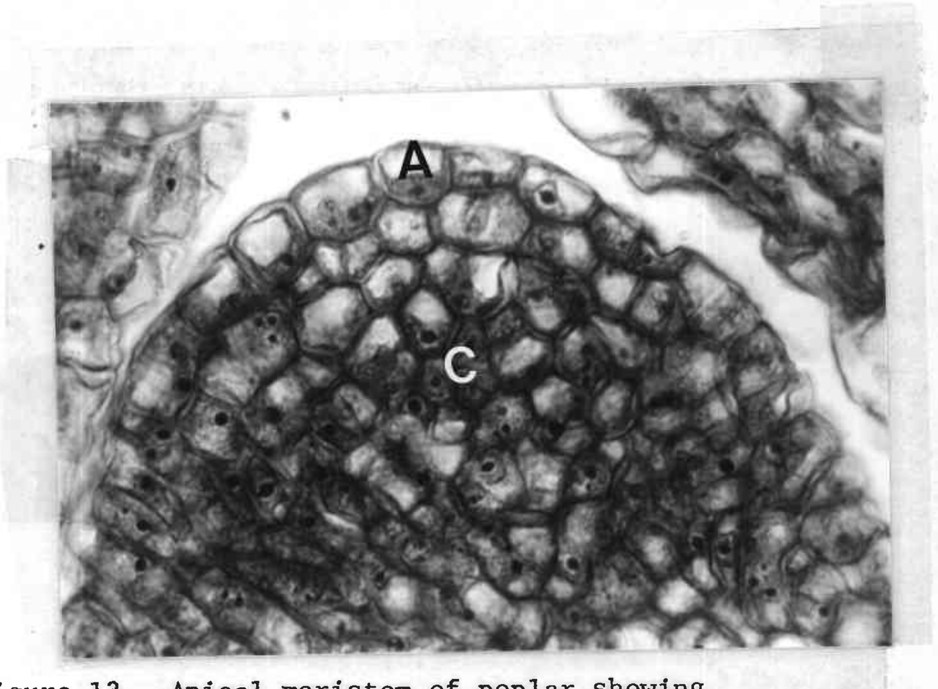

Figure 13. Apical meristem of poplar showing tunica (A) and corpus (C) regions. x400

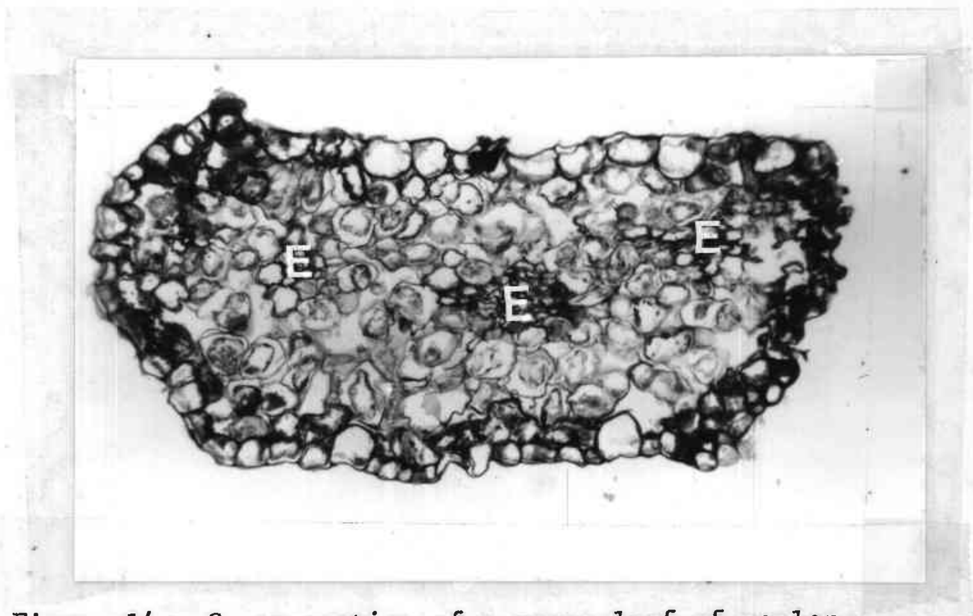

Figure 14. Cross section of a young leaf of poplar showing vascular bundles (E).

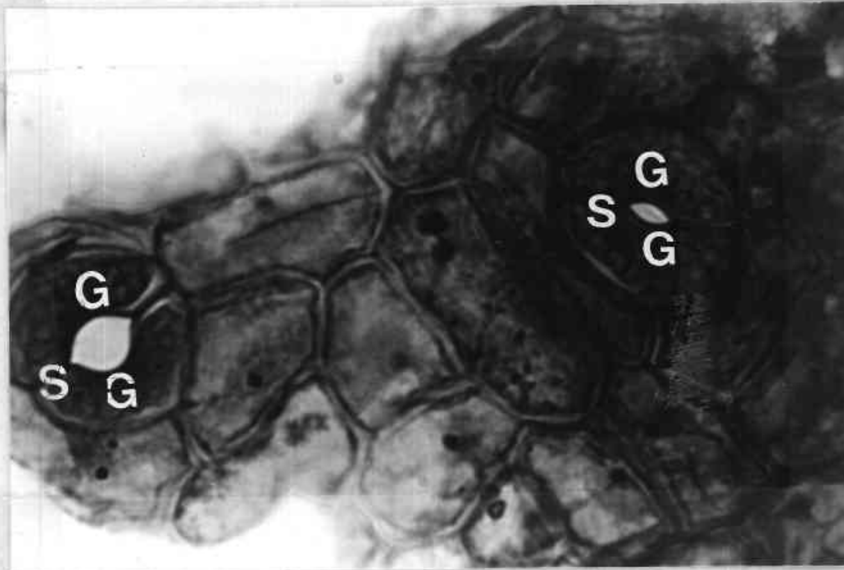

Figure 15. Section of leaf epidermis of a poplar leaf showing two stomata (S). Guard cells (G) are shown.

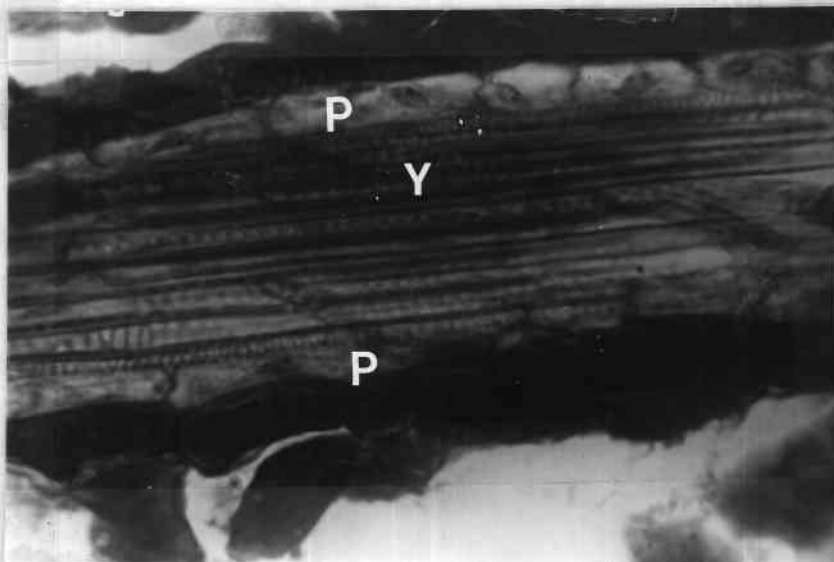

Figure 16. Longitudinal section of a leaf of poplar showing a vascular bundle containing xylem (Y) enclosed by a parenchymatous sheath (P).

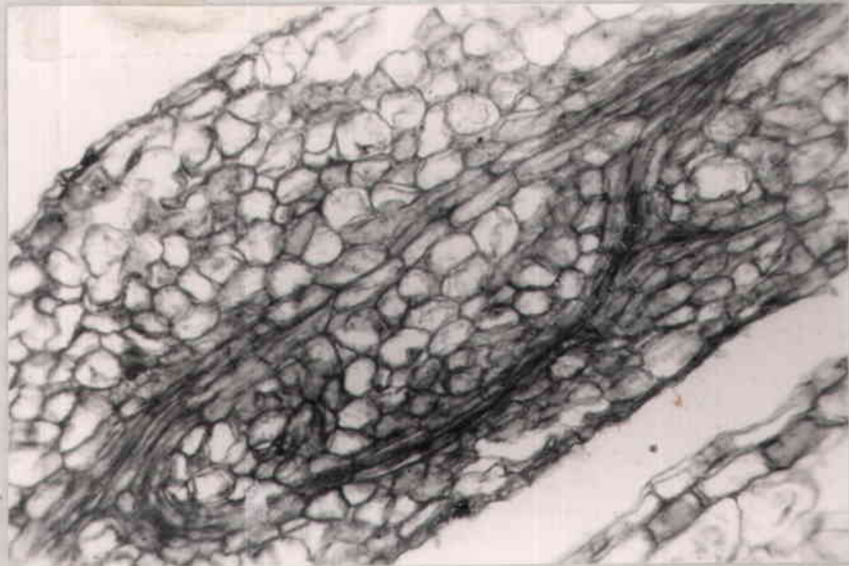

Figure 17. Young leaf of poplar showing branching vascular trace.

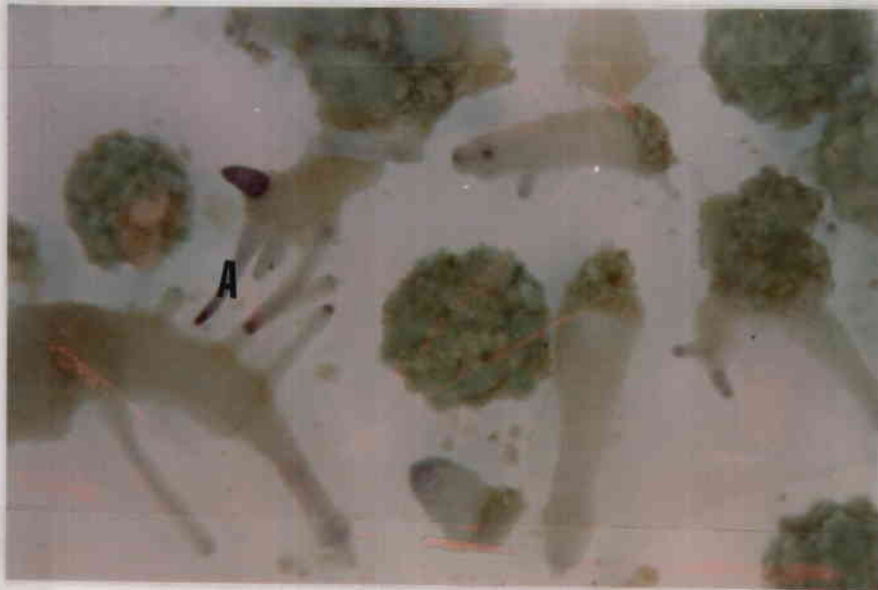

Figure 18. Poplar cell clumps with root at "A". Notice proliferated cells at the callus surface.

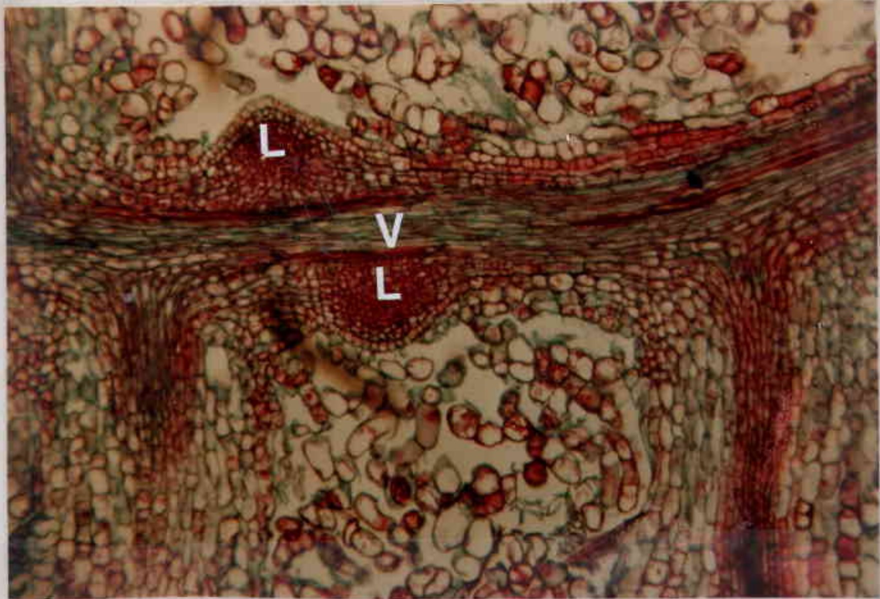

Figure 19. Two lateral roots of poplar (L) forming on either side of vascular tissue (V) within a root.

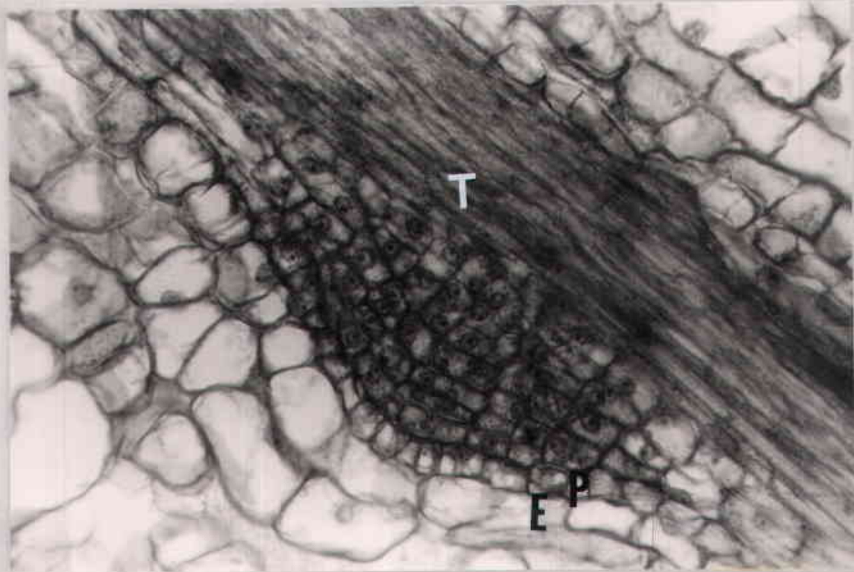

Figure 20. Lateral root primordium of poplar showing vascular tissue (T), pericycle-like area (P), and endodermis-like area (E).

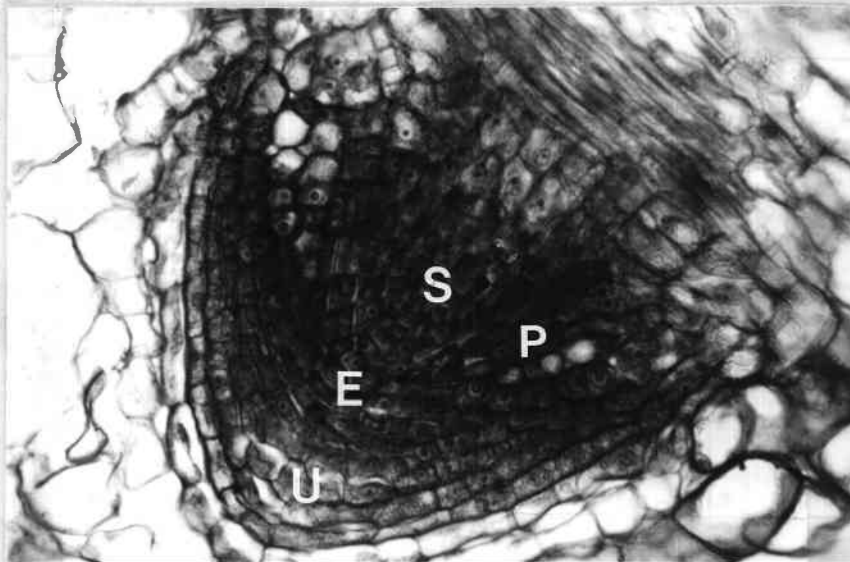

Figure 21. Longitudinal section of a poplar lateral root apex showing plerome (S), periblem (P), and calyptrogen-dermatogen (U). "E" shows the initial cells.

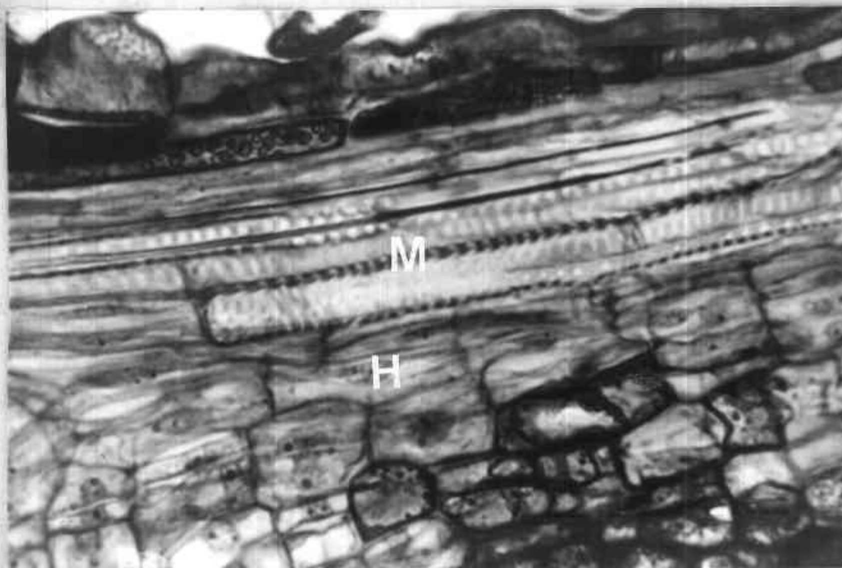

Figure 22. Longitudinal section of a vascular system of a poplar root showing metaxylem border pit pairs (M) and cambial-like cells (H).

As these lateral roots elongated classical root apical organization could be detected (Figure 21). Longitudinal root sections of vascular regions displayed well developed metaxylem border pits and cambial-like cells (Figure 22).

DISCUSSION

Although poplar callus has been grown and shoots regenerated on solid culture media since Jacquiot (1964) and Mathes 1964a, and 1964b), only two reports of poplar cells growing in suspension culture are available (Gautheret, *et al.*, 1975; and Fukuda, 1976). To date, no reports are available on poplar shoot or root regeneration in liquid media. With tree species, only Skolmen (1977) using *Acacia koa* has successfully regenerated shoots in liquid media and in fact observed embroid formation from cell suspensions.

This study substantiates the view of Steward, *et al.* (1969), that somatic cells are totipotent. The developmental stability of callus is retained when transferred to liquid culture. Although shoots did appear after 6 weeks, this time might have been reduced if other cytokinins had been used. That is, the nutrients and growth regulators used may not have been in the optimum concentrations or combinations to cause optimum production of shoots and roots.

The general view that emerges from the anatomical study is that the changes leading to organogenesis are a long and complex series of presumed biochemical and physiological changes.

Torrey (1966) suggested that organization begins with changes in a single vacuolated parenchyma cell within a mass of cells. This cell somehow becomes activated and undergoes a series of rapid cell divisions; i.e., it becomes mitosis-determined (Thorpe, 1978). It could not be determined here if only one or more than one cell was involved initially in organogenesis. However, the developmental pattern looks very similar to that described by Skolmen (1977). As described by Thorpe and Murashige (1968), starch does seem to form early (Figure 2) during organization.

Continuing cell division lead to the formation of a meristem-like aggregation of relatively small isodiametric, thinner-walled, micro-vacuolated cells having densely staining nuclei and cytoplasm (Thorpe and Murashige, 1970; Skolmen, 1977; and see also color photo of lateral root, Figure 19). These meristemoids are initially plastic in a morphogenetic sense and capable of giving rise to either a shoot or root primordia (Thorpe, 1978). These in turn give rise to respective organs. The type of primordium produced depends on the ratio of auxin and cytokinin in the medium (Moore, 1979). However, for balances of growth regulators to be effective in organ initiation, the critical balance must be within the tissue, and at the primordium forming loci. The osmoregulation and generation ability or such balances during non-shoot and shoot-forming is now beginning to be studied by others

(Thorpe, 1978), as are the cytodifferentiation changes within cells (Bornman, 1974).

As shown here and elsewhere (Halperin, 1973; Bornman, 1974; and Skolmen, 1977), one of the first differentiation stages within the meristemoid is xylogenesis (Figure 6), complete with secondary thickening. Later, several layers of xylem containing bordered pits and phloem from cambial initials (Figure 22). All this growth occurs completely within the callus. The bud apex, presumable initiated from the cambial area eventually reaches the surface by growing through the callus parenchyma (Figures 7-10). Eventually a shoot forms, complete with a typical apex, clearly showing a tunica and corpus arrangement (Figure 13). Normal-looking leaves complete with epidermal stomata are then formed Figure 14 and 15).

Root formation shows typical histogen (primary meristem) areas. Well-developed plerome (stele histogen), periblem (cortex histogen) and a combination calyptrogen-dermatogen histogen (epidermis-root tip) can be observed in Figure 21 (Popham, 1966; and Owens,[4] 1979).

This study has described for the first time, poplar organogenesis completely in liquid culture. The anatomical

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[4]Owens, J.N., Professor of Biology. University of Victoria. Victoria, B.C. Personal communication, 1979.

study provides a means to study the stages of differentiation in liquid culture. The two achievements outlined above provide a valuable tool to study regeneration of poplar after cryogenic storage.

APPENDIX B_1

Basal medium after Murashige and Skoog*

Stock Solution I	Inorganic salts (10x)--10 liters:		
	NH_4NO_3	165 g	
	KNO_3	190 g	
	$CaCl_2 \cdot 2H_2O$	44 g	
	KH_2PO_4	17 g	
	H_3BO_3	0.62 g	
	$MnSO_4 \cdot H_2O$	1.69 g	
	$ZnSO_4 \cdot H_2O$	0.614 g	
	KI	0.083 g	
	$Na_2MoO_4 \cdot 2H_2O$	0.025 g	
	$CuSO_4 \cdot 5H_2O$	0.00250 g	
	$CoCl_2 \cdot 6H_2O$	0.00250 g	
Stock Solution II	Magnesium sulphate (100x)--1 liter:		
	$MgSO_4 \cdot 7H_2O$	44 g	
Stock Solution III	Chelated iron (200x)--1 liter:		-- 500 ml:
	Sodium EDTA	7.45 g	3.725 g
	$FeSO_4 \cdot 7H_2O$	5.57 g	2.785 g
Stock Solution IV	Vitamins (1000x)--100 Milliliters:		
	Glycine	0.200 g	
	Nicotinic acid	0.050 g	
	Pyridoxine	0.050 g	
	Thiamine	0.010 g	

Preparation of 1 liter of basal medium (M):

Start with 500 ml of distilled H_2O, then add:

Stock I	100 ml
Stock II	10 ml
Stock III	5 ml
Stock IV	1 ml
myo-inositol	0.1 g
sucrose	30 g

For the M medium, make up the volume to 1000 ml. Adjust pH, 5.6-5.8.

If coconut milk (CM) were to be added, add 100 ml for a 10% concentration (v/v) prior to making up the volume to 1000 ml. (MCM medium) pH 5.6-5.8.

* Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

APPENDIX B_2

Basal medium after Schenk & Hildebrandt*

Stock I Major and minor elements (10x)--1 liter: -- 2 liters:

	1 liter	2 liters
KNO_3	25.0 g	50.0 g
$MgSO_4 \cdot 7H_2O$	4.0 g	8.0 g
$NH_4H_2PO_4$	3.0 g	6.0 g
$CaCl_2 \cdot H_2O$	2.0 g	4.0 g
$MnSO_4 \cdot H_2O$	0.1 g	0.2 g
H_3BO_3	0.05 g	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.01 g	0.02 g
KI	0.01 g	0.02 g
$CuSO_4 \cdot 5H_2O$	0.002 g	0.004 g
$NaMoO_4 \cdot 2H_2O$	0.001 g	0.002 g
$CoCl_2 \cdot 6H_2O$	0.001 g	0.002 g

Stock II Iron compounds (1000x) --final volume 200 ml

$FeSO_4 \cdot 7H_2O$	3.0 g heat
Sodium EDTA	4.0 g heat

Stock III Organics (1000x) --final volume 100 ml

Thiamine . HCL	0.5 g
Nicotinic acid	0.5 g
Pyridoxine . HCL	0.05 g

Preparation of 1 liter of basal medium (SH):

Stock I	100.0 ml
Stock II	1.0 ml
Stock III	1.0 ml
Sucrose 3.0%	30.0 g
myo-Inositol---100 ppm	0.1 g for SH medium
1000 ppm	1.0 g for SH^+ medium
2,4-D 0.2 ppm (instead of .5 ppm)	0.0002 g (SH .2 or SH^+ .2)
** Kinetin 0.1 ppm	0.0001 g
pCPA 2.0 ppm	0.002 g

pH adjusted to 5.6-5.8
Autoclave at 15 lb pressure for 30 min.

* Schenk, R. and A. Hildebrandt. 1972. Medium and technique for inducting and growth of monocotyledonous dicotyledonous plant cell cultures. Can. J. Bot. 50: 199-204.

** Coconut water, 10.0% by volume, may be used instead of kinetin.

APPENDIX B_3

Poplar Media

To produce multiple buds from shoots:

Murashige and Skoog inorganic salts
+ Murashige and Skoog vitamins at 10 ml/1
+ Myo-Inositol at 100 mg/1
+ 2.0% Sucrose
+ 0.4 ppm IAA
+ 1.2 ppm BAP

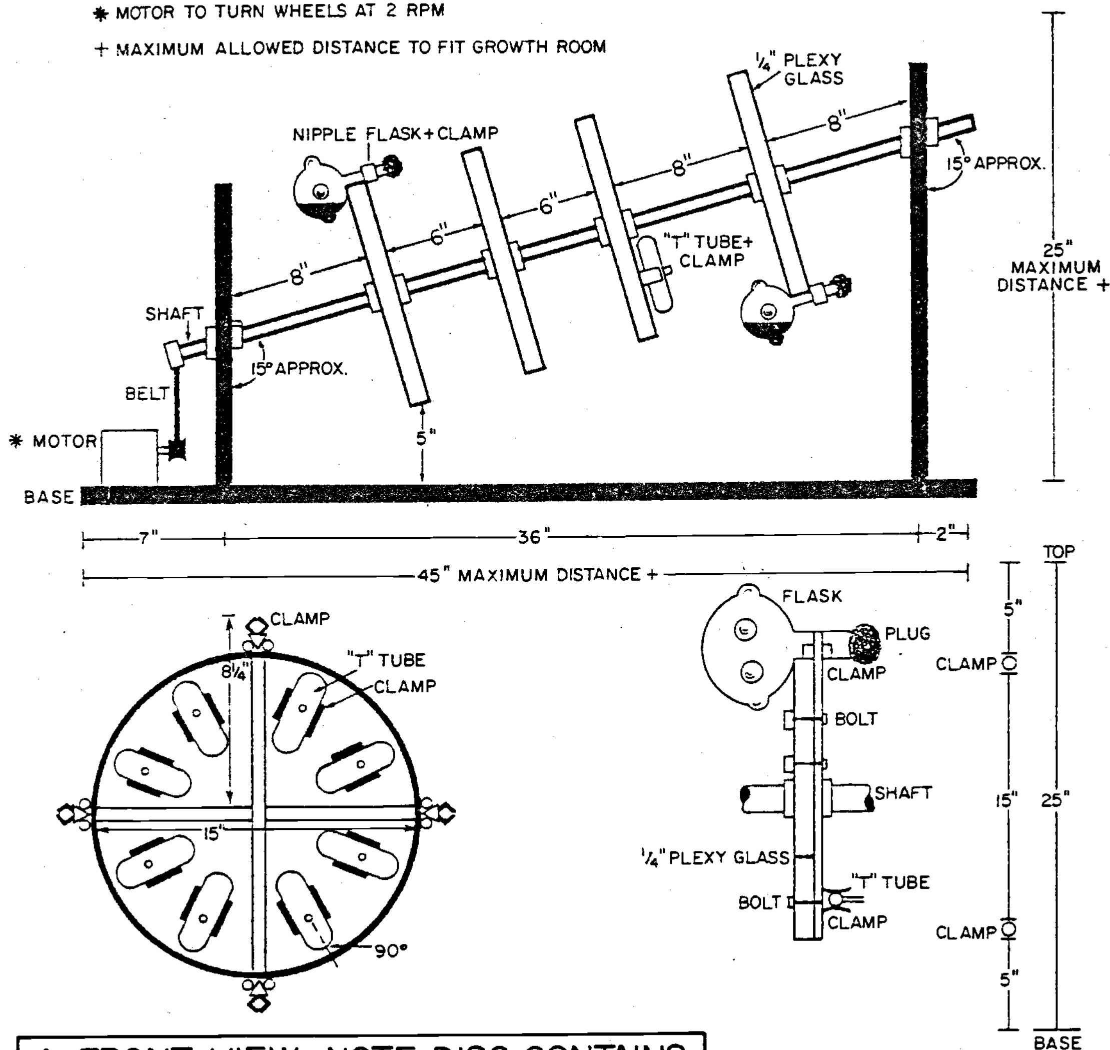
To produce callus from bud or leaf tissue:

Murashige and Skoog inorganic salts
+ Murashige and Skoog vitamins at 10 ml/1
+ Myo-Inositol at 100 mg/1
+ 2.0% Sucrose
+ 10.0 ppm NAA
+ .005 ppm or .05 ppm BAP

To produce shoots from callus:

Murashige and Skoog inorganic salts
+ Murashige and Skoog vitamins at 10 ml/1
+ Myo-Inositol at 100 mg/1
+ 2.0% Sucrose
+ .5 ppm BAP
+ .01 or .1 ppm NAA

ROTATION APPARATUS FOR SUSPENSION CULTURES



A FRONT VIEW. NOTE DISC CONTAINS EITHER "T" TUBES OR NIPPLE FLASKS.

A SIDE VIEW

Procedure for obtaining free cells

In order to obtain reliable information on cell growth under various experimental conditions it is necessary to obtain single or near single cells so they can be counted reproducibly. The cells under natural conditions tend to form aggregates and divide end on end to form "radiating spokes on a wheel". Such clumping causes uneven distributions when pipetted out. The result of clumping is that the true number of cells present in the sample will either be grossly overcalculated (in the event that a large clump is pipetted out) or underestimated (in the event a clump is missed in pipetting). Not only may the count be in error due to physical absence or presence of clumps, but also due to difficulty in counting large numbers of cells closely packed together.

A severe treatment for lignin digestion allows cell clumps to be reduced. Callus can be macerated successfully in a mixture of nitric acid and chromic acid (Chromium Trioxide (CrO_3)) 10.0%/10.0% vol/wt. Best results obtained after extensive investigation was obtained by using 2 times the initial volume of the cell sample plus growth medium of the digestion solution. The mixture was allowed to stand for 2 hr with periodic shaking. Cells were counted after 2 spins in the centrifuge, 15 min each time. Before each spin the digestion solution was washed with distilled water and pipetted off after the spin. Cell numbers were counted as given in "Methods and Materials" of Chapter 1.

APPENDIX E

Materials & Methods to stain DNA in dividing cells

Solutions required:

McClintock's Solution: Acetic Acid (concentrated) : 100.0% Ethanol 1:3 v/v

Warmke's Solution: 95.0% Ethanol : HCL (concentrated) 1:1 V/V

Carnoy's Solution: 100.0% Ethanol : Acetic Acid : Chloroform 6:1:3 v/v

Orcein or Aceto-Carmin: 0.5% in 45.0% acetic acid. Heat acid solution to boiling point, remove from heat, add dye, stir and cool. After solution has cooled, pour off the liquid or filter

Procedure:

- Fix sample in McClintock's solution at least 12 hr.
- Place in Warmke's solution 10-20 min.
- Place in Carnoy's solution 5-15 min.
- Stain 5-20 min., place on slide, add cover slip, squash and heat slightly over open flame.

APPENDIX F

Method of Bacteria Identification

1) Day 1: Gram stain, catalase, oxidase, motility, of glucose, triple sugar iron agar (TSI), colony color

2) Day 5:

Gram + Organisms

Cocci
a. Catalase +, oxidase -, of glucose + ⟶ Staphylococcus
b. Catalase +, oxidase -, of glucose - ⟶ Micrococcus

Rods
a. Spores present ⟶ Bacillus
b. Spores not present, rods to cocci transformation ⟶ Arthrobacter
c. Spores not present, snapping division ⟶ Corynebacterium

Branching Filaments
a. No spores, not acid fast, colonies rubbery and firmly attached to agar medium ⟶ Actinomycete

Gram - Organisms

1. Of Glucose + Rods
   a. Oxidase + ⟶ Place into OF Mannitol to confirm as Aeromonas
   b. Oxidase - ⟶ Do IMViC's (Indole, Methyl Red, Voges-Proskauer, Simmon's Citrate), Arginine decarboxylase, Lysine decarboxylase, Orinthine decarboxylase, Urease, or run API 20E scheme* ⟶ Citrobacter, Escherichia, Enterobacter, Klebsiella, Hafnia or Serratia

II. Of Glucose - Rods
   a. Motile ⟶ Pseudomonas/Alcaligenes group
   b. Non-motile ⟶ Run Indole
      1. Indole + ⟶ Flavobacterium
      2. Indole - ⟶ Moraxella-like Group M

III. Of Glucose - Cocci or Coccobacillus
   a. Oxidase + ⟶ Moraxella
   b. Oxidase - ⟶ Acinetobacter

* API 20E scheme used here. The API 20 Enterobacteriaceae (API 20E) procedure and materials are obtained from Anaytab Products, 200 Express Street, Planiview, N.Y. 11803.