INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

SOMATIC EMBRYO DEVELOPMENT AND PHENOTYPIC VARIATION IN AN ABSCISIC ACID-INDEPENDENT LINE OF *LARIX X EUROLEPIS*

by

Elizabeth Irene Hay
B.Sc. , Universitiy of Guelph, 1985
M.Sc. University of Guelph, 1987

A Dissertation Submitted in Partial Fulfillment of the requirements for the Degree of
DOCTOR OF PHILOSOPHY
in the Department of Biology

We accept this dissertation as conforming to the required standard

Dr. P. von Aderkas. Supervisor (Department of Biology)

Dr. N. Livingston, Departmental Member (Department of Biology)

Dr. J. Owens. Departmental Member (Department of Biology)

Dr. P. Charest. Additional Member (Ministry of Natural Resources)

Dr. D. James, Additional Member (Agriculture Canada)

Dr. J. Webber, Additional Member (B.C. Ministry of Forests)

Dr. J. Bonga, External Examiner (Ministry of Natural Resources)

University of Victoria

Supervisor: Dr. P. von Aderkas

ABSTRACT

The objectives of this thesis were to trace the developmental pathways of somatic embryos of an abscisic-acid independent line of *Larix x eurolepis*, to catalogue the phenotypes of mature embryos, to determine critical stages of development and to attempt to increase the number of maturing somatic embryos. The low rate of maturation could not be entirely explained by differences in phenotypes of early embryos, critical stages of development, or the lack of plant growth regulators in the medium. In addition, the shape and epidermal type of the mature embryo did not always determine the type of epicotyl produced, nor did it affect the rooting and mortality rates. Six types of embryonal structures were evident in the aggregates of line 2086: (1) a smooth (SEMLS) or (2) rough (REMLS) embryonal mass subtended by a cylindrical, compact, long suspensor, (3) a rough embryonal mass subtended by a long, loose suspensor (REMLLS), (4) a rough embryonal mass subtended by a suspensor arising from greater than one quarter of the surface area of the embryonal mass (REMST), (5) a rough embryonal mass subtended by a short, compact, cylindrical suspensor (REMSS), and (6) a cluster of meristematic cells which may or may not have single suspensor cells attached (MC). For isolated embryonal structures of all types, to continue development into a nodule or a mature embryo was the least common fate, while proliferation and developmental arrest were more common. In general, the more organized embryonal strucutre types (SEMLS and REMLS) had higher rates of maturation compared to the other 4 types but the most common fate was still developmental arrest (74% SEMLS, 62% REMLS), followed by proliferation (10% SEMLS, 30 % REMLS), and nodule or embryo development (16% SEMLS, 9% REMLS). REMLLS and REMST embryonal structures became developmentally arrested or proliferated (43-47%) while the rate of nodules/mature embryos production was 9-11%. Neither individual REMSS nor MC structures produced any nodules or mature embryos, but REMSS had a lower rate of developmental arrest (81%) and a higher rate of proliferation (19%) than MC (89% and 11% respectively). Embryos at more advanced stages of development were less likely to die, become developmentally arrested or become nodules, but more likely to become mature embryos than embryos at less advanced stages of development. A critical stage of development appeared to be the focal zone stage at the formation of a complete polyphenol band around the basal end of the embryonal mass. At this stage, the majority of immature embryos became mature embryos (61%) while only 3% of the embryos died, 10% became developmentally arrested, and 20% became nodules. The majority of mature somatic embryos were normally proportioned with a smooth epidermis (43%) rather than vitrified (12%), normal with a rough epidermis (12%) or misshapen (smooth or rough, 33%). The shape of the mature embryo was associated with the type of epidermis, with mature somatic embryos with normal proportions more likely to have smooth epidermis (78%) than a rough epidermis (22%) while mature embryos with abnormal proportions were as likely to have a smooth epidermis as a rough epidermis. The shape of the mature embryo was associated with the shape of the epicotyl produced. Normal-smooth, mature embryos were more likely to produce normal-smooth epicotyls (73%) than twin epicotyls (21%), vitrified epicotyls (2%) or misshapen epicotyls (5%) compared to vitrifed mature embryos (42% normal-smooth epicotyls, 34% twin epicotyls, 23% vitrified epicotyls, 1% misshapen epicotyls) or misshapen mature embryos (22% normal-smooth epicotyls, 47% twin epicotyls, 7% vitrifed epicotyls, 24% misshapen smooth/rough embryos). The number of mature embryos which germinated or died was not associated with either the epidermal quality or the shape of the mature embryo. Few SEMLS or REMLS embryonal structures responded to auxin and cytokinin treatments. There appeared to be a trend towards less developmental arrest and proliferation and more nodules/mature embryos produced on media with no auxin compared to media with 2,4-D and a trend towards more developmental arrest and fewer nodules/mature embryos on media without BA compared to media with BA. Only nodules on media without plant growth regulators produced roots or cotyledons. There was no effect of embryonal structure type (SEMLS or REMLS), or sucrose concentration (58 μM or 174 μM) on the maturation of immature embryos, but on media without ABA, fewer immature embryos proliferated or became developmently arrested and more embryos became nodules or mature embryos than on medium with 6-24 μM ABA.

Examiners:

Dr. P. von Aderkas, Supervisor (Department of Biology)

Dr. N. Livingston, Departmental Member (Department of Biology)

Dr. J. Owens, Departmental Member (Department of Biology)

Dr. P. Charest, Additional Member (Natural Resources)

Dr. D. James, Additional Member (Agriculture Canada)

Dr. J. Webber, Additional Member (B.C. Ministry of Forests)

Dr. J. Bonga, External Examiner (Natural Resources)

TABLE OF CONTENTS

Abstract ii

Table of Contents iv

List of Tables ix

List of Figures xi

Definitions and Abbreviations xv

Acknowledgements xvi

Chapter I: Introduction

1.1 The potential of somatic embryogenesis 1
1.2 The importance of efficient *Larix* somatic embryogenesis systems 3
1.3 The problem of poor conversion rates and abnormal phenotypes in conifer somatic embryogenesis systems 3
1.4 Research objectives 4

Chapter II: Literature Review

2.3 Somatic embryogenesis in conifers

- **2.3.1** Introduction 6
- **2.3.2** Non-media factors affecting induction of embryogenic tissue 7
  - **2.3.2.1** Choice of explant 7
  - **2.3.2.2** Organization of initiated tissue 9
  - **2.3.2.3** Embryogenic potential 10
- **2.3.3** Initiation and maintenance of embryogenic cultures 11
  - **2.3.3.1** Media requirements 11
  - **2.3.3.2** Proliferation and ultrastructure of established cultures 12

- **2.3.4** Maturation of somatic embryos 14
- **2.3.5** Protein deposition in developing somatic embryos 18
- **2.3.6** Germination rates of somatic embryos 20
- **2.3.7** Phenotypic variation in early stage somatic embryos 22
- **2.3.8** Phenotypic variation in mature somatic embryos 25
- **2.3.9** Protoplast cultures 26
- **2.3.10** Somaclonal variation 28

2.4 Larch embryogenesis 29
- **2.4.1** Zygotic embryo development 29
- **2.4.2** Somatic embryo development 31
  - **2.4.2.1** Initiation of embryogenic tissue 31
    - **2.4.2.1.1** Effect of medium and plant growth regulators on the rate of initiation 31
  - **2.4.2.2** Maintenance of embryogenic tissue 32
    - **2.4.2.2.1** Variation in embryonal mass organization 32
  - **2.4.2.3** Effect of abscisic acid and osmoticum on yield and quality of somatic embryos 34
  - **2.4.2.4** Protoplast cultures of *Larix* 37
  - **2.4.2.5** Somaclonal variation in *Larix* cultures 38

Chapter III: Organization of tissue aggregates and phenotypic variation in embryonal masses

3.1 Introduction 39

3.2 Materials and Methods 41
- **3.2.1** Culture maintenance 41
- **3.2.2** Resin-embedding and sectioning 41
- **3.2.3** Determining the internal composition of aggregates 42
- **3.2.4** Developmental fate of embryonal structures 43

3.3 Results 45
- **3.3.1** Description of the six types of embryonal structures 45
  - **3.3.1.1** smooth embryonal mass plus long compact suspensor (SEMLS) 45
  - **3.3.1.2** rough embryonal mass plus long compact suspensor (REMLS) 48

3.3.1.3 rough embryonal mass plus long, loose suspensor (REMLLS) 48
3.3.1.4 rough embryonal mass plus short compact suspensor (REMSS) 49
3.3.1.5 rough embryonal mass plus short loose suspensor (REMST) 52
3.3.1.6 microcluster (MC) 52
3.3.2 Relationship between external appearance and internal organization 55
3.3.3 Relationship between internal organization and productivity 60
3.3.4 Developmental fate of the six types of embryonal structures 61
3.4 Discussion 67
3.4.1 The organization of line 2086 of *Larix x eurolepis* 67
3.4.2 The relationship between internal organization and mature embryo production 68
3.4.3 The association between embryonal structure and developmental fate 70
3.4.4 Conclusion 74

Chapter IV: Pathways of development

4.1 Introduction 75
4.2 Materials and methods 76
4.2.1 Culture maintenance 76
4.2.2 Resin-embedding and sectioning 76
4.2.3 Scanning electron microscopy 77
4.2.4 Developmental fates of embryos at different stages of development 77
4.3 Results 80
4.3.1 Stages of development from embryonal mass to final morphology 80
4.3.1.1 Description of stages of development 80
4.3.1.1.1 Embryonal mass 80
4.3.1.1.2 Focal zone 80
4.3.1.1.3 Focal zone plus anthocyanin band 83
4.3.1.1.4 Initiation of cotyledons 86

            4.3.1.1.5 Mature somatic embryos 86
        4.3.1.2 Developmental pathways 89
            4.3.1.2.1 Proliferation 89
            4.3.1.2.2 Nodule formation 92
                4.3.1.2.2.1 External appearance of nodule 92
                4.3.1.2.2.2 Internal organization of nodule 92
                4.3.1.2.2.3 Meristem development in nodules 93
    4.3.2 Critical stages of development 93
    4.3.3 Pathways of development chart 98
4.4 Discussion 99
    4.4.1 Histological studies of embryos of line 2086 99
    4.4.2 Alternative developmental pathways 101
    4.4.3 The relationship between stage of development and developmental fate 102
    4.4.4 Conclusion 103

Chapter V: Phenotypic variation in mature and germinating somatic embryos

5.1 Introduction 104
5.2 Materials and methods 106
    5.2.1 Culture maintenance 106
    5.2.2 Determination of mature embryo morphologies and epicotyl characteristics 106
    5.2.3 Resin-embedding and sectioning 107
    5.2.4 Transplanting success 108
5.3 Results 109
    5.3.1 Types of final morphologies 109
    5.3.2 Rates of reversion to normal shoot growth and percent rooting 118
    5.3.3 Outplanting success 126
5.4 Discussion 127
    5.4.1 The relationship between proportion and epidermal

quality of mature embryos and epicotyls....127
5.4.2 Rooting and mortality rates....131
5.4.3 Conclusion....132

Chapter VI: Manipulation of embryo development with media supplements

6.1 Introduction....134
6.2 Materials and methods....135
    6.2.1 Culture maintenance....135
    6.2.2 Effects of auxins and cytokinins on immature embryo development....135
    6.2.3 Effects of auxins and cytokinins on the development of nodules....136
    6.2.4 Effects of ABA and sucrose concentrations on the development of immature embryos....137
6.3 Results....139
    6.3.1 Effects of auxins and cytokinins on developmental fate....139
    6.3.2 Effects of abscisic acid and sucrose concentrations on developmental fate....144
6.4 Discussion....146
    6.4.1 Effects of auxin and cytokinin on embryo development....146
    6.4.2 Effects of ABA and sucrose on embryo development....148
        6.4.2.1 Effects of ABA on embryo development....148
        6.4.2.2 Effects of sucrose on embryo development....151
    6.4.3. Conclusion....153

Chapter VII: General Discussion....154

Chapter VIII: Summary and Conclusions....162

Literature Cited....164

Appendix A....189

Appendix B....198

List of Tables

Table 1: The production of somatic embryos over 12 weeks from embryogenic aggregates classified according to the most prevalent type of embryonal structure present 62

Table 2: The production of somatic embryos over 12 weeks from embryogenic aggregates classified according to the most advanced type of embryonal structure present 63

Table 3: The developmental fate of the six types of embryonal structures after isolation 64

Table 4: The types of embryonal structures found in proliferating tissue derived from individual embryonal structures of six different types........ 66

Table 5: The effect of stage of development at plating out on the developmental fate of individual somatic embryos........ 97

Table 6: The root and shoot production of individual somatic embryos 119

Table 7: Epidermal qualities of epicotyls produced by somatic embryos 120

Table 8: Relationship between embryo shape and epidermal quality of mature somatic embryos 121

Table 9: Relationship between embryo shape and epicotyl shape 122

Table 10: Relationship between embryo type and epicotyl shape 123

Table 11: The percent rooting and the percent mortality for all embryo types........ 124

Table 12: Final morphologies of individual embryonal masses subjected to 2,4-D and BA treatments........ 140

Table 13: Main effects of 2,4-D on SEMLS embryos 141

Table 14: Main effects of BA on SEMLS embryos...................................... 142

Table 15: Final morphologies of nodules subjected to 2,4-D and BA treatments.... 143

Table 16: Final morphologies of individual embryonal masses on media with varying sucrose and ABA levels.. 145

Table 17: Effect of time of bombardment and vector construction on transient expression of the GUS gene in conifer pollen 206

Table 18: Comparison of histological and fluorescent assays for transient gene expression of the GUS gene in conifer pollen..........................207

Table 19: Neomycin phosphotransferase II as a reporter gene in conifer pollen.....212

List of Figures

Figure 1: Smooth embryonal mass plus long compact suspensor (SEMLS): squashed specimen ... 47

Figure 2: Smooth embryonal mass plus long compact suspensor (SEMLS): median sectioned specimen ... 47

Figure 3: Rough embryonal mass plus long compact suspensor (REMLS): squashed specimen ... 47

Figure 4: Rough embryonal mass plus long compact suspensor (REMLS): cross sectioned specimen ... 47

Figure 5: Rough embryonal mass plus long loose suspensor (REMLLS): squashed specimen ... 51

Figure 6: Rough embryonal mass plus long loose suspensor (REMLLS): median sectioned specimen ... 51

Figure 7: Rough embryonal mass plus short suspensor (REMSS): squashed specimen ... 51

Figure 8: Rough embryonal mass plus short suspensor (REMSS): median sectioned specimen ... 51

Figure 9: Rough embryonal mass plus short loose specimen (REMST): squashed specimen ... 54

Figure 10: Rough embryonal mass plus short loose suspensor (REMST): median sectioned specimen ... 54

Figure 11: Microcluster (MC): squashed specimen ... 54

Figure 12: Microcluster (MC): median sectioned specimen ... 54

Figure 13: Squash of a highly organized aggregate showing embryonal mass types 57

Figure 14: Squash of a moderately organized aggregate showing embryonal mass types 57

Figure 15: Squash of a poorly organized aggregate showing embryonal mass types 57

Figure 16: Normal rooted somatic embling 57

Figure 17: Abnormal rooted somatic embling 59

Figure 18: Embryogenic aggregate with a filamentous appearance. showing maturing somatic embryos 59

Figure 19: Embryogenic aggregate showing little organization 59

Figure 20: Unrooted red nodule 59

Figure 21: Fresh specimen of an SEMLS embryonal structure 82

Figure 22: Scanning electron micrograph of an SEMLS embryonal structure 82

Figure 23: REMLS embryonal structure and immature somatic embryo at the focal zone stage 82

Figure 24: A median sectioned immature somatic embryo at the focal zone stage 82

Figure 25: Fresh specimens of immature somatic embryos at the focal zone plus polyphenol band stage and the indentation stage 85

Figure 26: A cross-sectioned immature somatic embryo at the focal zone plus polyphenol band stage showing the beginning of a root meristematic region 85

Figure 27: A median sectioned immature somatic embryo at the focal zone plus polyphenol band stage showing the development of a root meristem......85

Figure 28: A scanning electron micrograph of an immature somatic embryo at the focal zone plus polyphenol band stage 85

Figure 29: Fresh specimens of immature somatic embryos at the indentation stage and the beginning of cotyledonary primordia development..................88

Figure 30: Scanning electron micrograph of an immature somatic embryo at the beginning of cotyledonary primordia development 88

Figure 31: Resin-embedded median section of an somatic embryo at the beginning of cotyledon development..88

Figure 32: Scanning electron micrograph of a mature somatic embryo..................88

Figure 33: Resin-embedded median section of a mature somatic embryo..............91

Figure 34: Squashed sample of cleaving embryonal masses.............................91

Figure 35: Fresh specimen of a nodule..91

Figure 36: A median section of a nodule 91

Figure 37: A nodule with cotyledons...95

Figure 38: Median section of a nodule with cotyledons 95

Figure 39: A nodule with an extended root...95

Figure 40: Abnormal smooth embryo.. 111

Figure 41: Abnormal smooth embryo.. 111

Figure 42: Normally proportioned mature somatic embryo with rough epidermis 111

Figure 43: Abnormally proportioned mature somatic embryo with a rough epidermis 113

Figure 44: Vitrified abnormal embling with multiple epicotyls 113

Figure 45: Mature embling with twin epicotyls 113

Figure 46: Mature somatic embryos with twin epicotyls exhibiting different growth patterns 113

Figure 47: Normal embling with normal epicotyl and root 115

Figure 48: Abnormally proportioned somatic embryo with normal epicotyl 115

Figure 49: Median section of a normal mature somatic embryo with a smooth epidermis 117

Figure 50: Median section of an abnormal mature somatic embryo with a smooth epidermis 117

Figure 51: Median section of a normally proportioned mature somatic embryo with a rough epidermis 117

Figure 52: Median section of a vitrified mature somatic embryo 117

Figure 53: Squash of callus cells of *Chamaecyparis nootkatensis* showing a haploid chromosome set 196

Figure 54: Detection of ß-glucuronidase gene activity in black spruce pollen using the X-glu histochemical assay 209

Definitions and Abbreviations

Definitions

Abnormal somatic embryo: a mature somatic embryo which does not resemble a zygotic embryo in terms of the proportions of the hypocotyl and cotyledons.

Aggregate: a subcultured mass of embryogenic tissue

Embling: a germinated somatic embryo.

Normal somatic embryo: a mature somatic embryo whose hypocotyl and cotyledons have the same proportions as those of zygotic embryos.

Precocious germination: the greening and radicle elongation of somatic embryos without a period of quiescence after maturation.

Somaclonal variation : an inherited change in genes occurring in somatic cells.

Somatic embryogenesis: the development from diploid somatic cells *in vitro* of polar embryos with shoot and root meristems connected by vascular tissue

Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid

BA: benzylaminopurine

IBA: indolebutyric acid

NAA: naphthaleneacetic acid

PEG: polyethylene glycol

Acknowledgements

I would like to thank my supervisor. Dr. Patrick von Aderkas. for his guidance and financial support during this project. In addition. I would like to thank my co-supervisor. Dr. Pierre Charest for both his financial support and his encouragement. Dr. Delano James Dr. J. Owens and Dr. Joe Webber have also provided me with timely advice and assistance for which I am very grateful. I would also like to thank the Science Council of British Columbia. Canadian Pacific Forest Products. Fletcher-Challenge and the Canadian Forestry Service for their financial support and provision of material. I would like to thank Claude Moffet. Dr. Danny Rioux and Michelle Bernier-Cardou for their assistance at CFL.

I would like to thank my lab-mates Rungnapar Pattinavibool and Dr. Nicole Dumont-BéBoux. for their forebearance and companionship. I could not have completed this project without the support of the best group of friends anyone could ask for - Steeve Pepin and Suzanne Renaud. Rob Myers. Robin Percy. Alicia Mazari. Margaret Dawkins. Francis Dearman. John Runions. Edgar Fuchs. Erin Linger. Susan Skaalid. David Parker and Bethan Chancey. Don Skinner. Kim Rensing. the Goldies. Derek Harrison and the Friday afternoon Grad Lounge crowd. I would like to thank especially Diane Gray. Glenda Catalano and Tokushiro Takaso for allowing me to interrupt them at random for various kinds of help.

Chapter 1: Introduction

1.1 The potential of somatic embryogenesis

Somatic embryogenesis is the development of bipolar embryos with shoot and root meristems connected by vascular tissue from diploid somatic cells *in vitro* (Cheliak and Rogers 1990). This process involves the initiation of embryogenic tissue from an explant (usually a zygotic embryo), maturation of the somatic embryos produced, germination of the mature somatic embryos and acclimatization to the greenhouse followed by outplanting in the field (Adams *et al.* 1994).

Immature somatic embryos can proliferate by a process involving the growth of daughter embryonal masses followed by independent embryonal tube production, resulting in new independent immature embryos. The ability of embryogenic tissue to continually cleave under certain culture conditions, and then to stop proliferating and allow somatic embryos to mature when the culture conditions are changed, allow the production of somatic embryos with potentially less manual manipulation than rooted cuttings or adventitious budding *in vitro*. Production of mature somatic embryos varies from 23 to 700 mature embryos per gram of embryogenic tissue (Krogstrup *et al.* 1988, Kartha *et al.* 1988, Becwar *et al.* 1991, Jalonen and von Arnold 1991). This makes somatic embryogenesis potentially more productive than conventional rooted cuttings for some species, where 400-500 propagules per year per seedling can be produced (Bonga *et al.* 1995), compared to 40 plantlets per seedling over a six month period for *Picea glauca* organogenic systems (Tremblay 1990).

The initiation of adventitious buds, shoot elongation, rooting and acclimatization are often rate-limiting steps for organogenic systems, though organogenesis has worked well for some species such as *Pinus radiata* (Lu and Thorpe 1987, Cheliak and Rogers 1990). In addition, adventitious shoots can display abnormalities common to *in vitro* systems such as reduced wax deposition and other undesirable traits such as plagiotropism (Mohammed and Vidaver 1990, Park and Bonga 1992). In contrast to organogenesis, where adventitious buds are induced directly from an explant or indirectly from caulogenic callus, and then rooted, somatic embryogenesis results in a complete somatic embryo with both a root and a shoot meristem. Somatic embryogenesis systems can proliferate mature somatic embryos for the greenhouse and in addition, can provide embryogenic protoplasts for genetic engineering (Lu and Thorpe 1987).

Tissue culture in general is most suited for species where: 1. conventional planting material is scarce or expensive; 2. there is great genetic variability in yield and/or resistance to disease or pests and; 3. there is a potential for genetic engineering and a subsequent need for sterile material (Pachauri and Dhawan 1988, Tautorus *et al.* 1991). Eighty percent of the cost of propagule production is in labour costs, thus somatic embryogenesis would likely be more economical if embryogenic cultures could be quickly bulked up in bioreactors (Locy 1988) and the embryogenic tissue could be cryopreserved until field assessment of the parent material were complete without compromising the long-term field performance of the embryos derived from the cryopreserved material (Adams *et al.* 1994). At present, 120 clones of *Larix* and *Picea* have been cryopreserved, with 100% regrowth after thawing (Charest *et al.* 1993). However, these costs would only be justified for elite stock.

The potential increase in production from the selection and propagation of elite trees has been demonstrated in other species. The selection of elite trees in plantations of *Eucalyptus* raised the average yield from 35 m^3/ha/yr harvest to 100m^3/ha/yr (Srivastava 1988). Elite trees of West Coast Tall coconut (*Cocos nucifera*) have fruit yields 5-10 times that of average trees (Bhaskaran and Prubhudesai 1988). The establishment of seed orchards of several forest species in British Columbia has made improved genotypes available for somatic embryogenesis (Adams *et al.* 1994). In addition, there are ongoing efforts in other provinces such as *Picea mariana* in the Maritimes where most reforestation stock comes from first generation seed orchards (Adams *et al.* 1994).

As with other clonal systems, somatic embryogenesis can capture both additive and non-additive genetic variances (Bonga 1991, Mullin and Park 1992, Adams *et al.* 1994). Though somatic embryogenesis has the potential to outproduce organogenesis, in most cases only organogenesis has been induced from tissue from mature trees (Laliberté and Lalonde 1988, Chesick *et al.* 1990, Bonga and Pond 1991, Westcott 1994).

Effective utilization of genetic engineering depends on the ability to produce not just aseptic cells which can be transformed in the lab, but also on the production of stably transformed plants successfully established in the field. Several conifer species have already been genetically transformed, including *Larix* (Charest *et al.* 1991, Huang *et al.* 1991, Duchesne and Charest 1992, Duchesne *et al.* 1993), *Picea mariana* and *Picea glauca* (Ellis *et al.* 1991, Bomminemi *et al.* 1993, Li *et al.* 1994). The economic potential of producing high quality, genetically engineered somatic embryos rests on the genes and vectors available. In addition to disease and insect resistance, other economically important traits could be manipulated. For example, the production of lignin in plants is largely under genetic control. If conifer embryos could be genetically altered to produce angiosperm-type lignin rather than conifer-type lignin, the cost of pulp processing could be reduced (estimated savings $6 billion U.S. in 1988 dollars), with further savings if the amount of lignin could be controlled as well (Timmis and Trotter 1988). Improving the rate of production and quality of mature somatic embryos per gram of embryogenic tissue would also increase the productivity and the cost efficiency of genetically engineered tissue culture systems.

1.2 The importance of efficient *Larix* somatic embryogenesis systems

Larix is an ideal genus for tissue culture because many of its species are important forestry species. it is widespread across temperate northern climates and its hybrids often exhibit heterosis (Owens and Molder 1979a. Park and Fowler 1987. McLaughlin and Karnosky 1989, Gower and Richards 1990. Lelu *et al.* 1994). Larch is especially cold and desiccation tolerant due to its deciduous habit. yet can be superior to other conifers in trapping carbon dioxide and growth (Gower and Richards 1990). Hybrid larch (*Larix x eurolepis*). the hybrid of Japanese larch (*Larix leptolepis*) and european larch (*Larix decidua*). grows faster than its parent species over a wide range of site conditions and this has generated interest from the forestry industry (Klimaszewska 1989a. McLaughlin and Karnosky 1989). On better sites. *Larix decidua* and *Larix leptolepis* and their hybrid can outperform pine and spruce by 200-300%. have good wood quality. and are resistant to spruce budworm and scleroderris canker (Park and Fowler 1987. Bonga *et al.* 1995).

However. the poor seed production capacity of larch makes fulfilling demands for seedlings very difficult (Owens and Molder 1979b. Park and Fowler 1987. Klimaszewska 1989a. McLaughlin and Karnosky 1989). In all species of larch. seed production is low. *Larix occidentalis* trees rarely produce cones before 25 years of age. good cone crops occur every 5 years on average. and seed set is poor (Owens and Molder 1979b). Hence. the production of multitudes of high-quality plantlets of larch via somatic embryogenesis would be of great interest to commercial forestry.

1.3 The problem of poor conversion rates and abnormal phenotypes in conifer somatic embryogenesis systems

Tissue culture systems have the potential to produce amounts of plant material impossible to obtain using other vegetative propagation or seed production systems. This is of great importance in two areas: the propagation of species with low natural seed production. including many conifer species (particularly *Larix* spp.) and the production of clonal material from genetically transformed plant cells (Attree and Fowke 1993). In both cases. the greatest benefits would come from high rates of conversion of immature embryos into plantlets suitable for outplanting. The plantlets produced must be of high quality, and must possess both good vascular systems connecting the root and shoot regions, and meristematic regions capable of producing normal functioning roots and shoots.

Since 1985 when the production of somatic embryos derived from somatic cells of zygotic conifer embryos was first reported (Chalupa 1985, Hakman *et al.* 1985), many conifer species have demonstrated embryogenic capability (Attree and Fowke 1993, Tautorus *et al.* 1991). The differences in embryogenic potential found in these species are likely genetic in origin, as are the differences in productivity between different lines in each species (Park *et al.* 1994). Nevertheless, in aggregates of each line the number of potential somatic embryos is always greater than the actual number produced (Pitel *et al.* 1992). Aggregates are masses of embryogenic tissue subcultured at regular intervals, consisting of embryonal masses at various stages of development as well as single cells and cell clusters. Even in aggregates of the most productive lines of the most productive species, hundreds of embryonal masses can be found which are either dying or are continuing to cleave rather than maturing into somatic embryos.

Since each embryogenic line is derived from a single zygotic embryo and the culture environment is generally uniform, individual embryonal masses would be expected to respond to culture conditions in a similar fashion. Though microclimates and media gradients could exist in culture, somatic embryos are able to mature both in contact with the medium on the margins of tissue aggregates as well as isolated from the medium on top of aggregates by hundreds of embryonal masses and suspensors. Therefore, even when the plant material is genetically uniform, and culture conditions do not explain differences in behaviour, why do some embryonal masses mature while others continuously cleave or die? In addition, why does the morphology of mature somatic embryos produced vary under uniform conditions from normal (resembling zygotic embryos) to vitrified (glassy) or misshapen structures?

1.4 Research objectives

The potential use for a somatic embryogenesis system for larch is great, but the efficiency of the present system must be improved. Researchers using other species have observed a lack of uniformity in embryogenic tissue, poorly developed somatic embryos and low rooting and transfer rates (Jalonen and von Arnold 1991, Kristensen *et al.* 1994, Bercetche and Pacques 1995, David *et al.* 1995). Differences in somatic embryo production in *Picea abies* have been traced to two distinct types of organization of embryonal mass and suspensors that could be manipulated to a limited extent by changing the growing conditions (Jalonen and von Arnold 1991). Similar differences in embryo structure have been noticed in *Larix* (Thompson and von Aderkas 1992). Theoretically, differences at the outset of embryonal development which affect differentiation of tissues such as the epidermis or meristems could be a fundamental reason for later differences in both somatic embryo production and phenotype. In order to explain the low conversion rate of embryonal masses to mature phenotypically normal somatic embryos in *Larix* embryogenic tissue, a series of experiments were conducted in order to systematically examine the origins and development of one *Larix x eurolepis* line.

The purpose of this thesis was three-fold:

a) to trace the developmental pathways from embryonal mass to mature somatic embryo and to catalogue the different phenotypes of the mature somatic embryos,

b) evaluate the relationship between developmental arrest, embryo phenotype and media composition,

c) to manipulate critical stages of development both to increase the number of embryos that mature and to improve the proportion of mature somatic embryos with the normal phenotype that resembles a mature zygotic embryo.

The plant material chosen for this work was line 2086 of *Larix x eurolepis* (von Aderkas *et al.* 1990). The tissue aggregates of line #2086 display considerable variety of phenotypes at all stages despite their origin from a single zygotic embryo and the uniformity of the growing conditions *in vitro*. This line is unusual in that it requires neither plant growth regulators nor osmotic treatments to proliferate embryonal masses and to mature somatic embryos which develop into normal plantlets. By using this line, the complications inherent in tracing the development of an embryo while manipulating it with exogenous plant growth regulators are avoided, ensuring that the observed differences in embryo development are due to embryo development and not to the release from or application of plant growth regulators (Thorpe 1988), which has complicated the work of other groups (Eastman *et al.* 1991). In this way, the developmental pathways can be traced and manipulated, with the knowledge that embryonal masses have the potential to complete development without plant growth regulators, though relatively few do. The hypothesis of this thesis is that differences in morphologies of immature embryos are linked to differences in productivity and mature embryo phenotypes in *Larix x eurolepis* , and that identifying and manipulating critical stages of development will improve both the number and quality of mature somatic embryos produced.

2.3 Somatic embryogenesis in conifers

2.3.1 Introduction

In 1985, two groups of researchers, Hakman and coworkers, and Chalupa, cultured immature and mature zygotic embryos of *Picea abies* on modified Murashige and Skoog medium (Murashige and Skoog, 1962) with benzylaminopurine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D). White, translucent, mucilaginous tissue composed of embryonal masses and suspensors was produced, and these were the first instances of somatic embryogensis in conifers. Concurrently, megagametophytic tissue of *Larix decidua* yielded haploid embryogenic tissue (Nagmani and Bonga 1985). Over 25 conifer species have yielded embryogenic tissue (Tautorus *et al.* 1991, Attree and Fowke 1993). However, not all genotypes of these species can produce embryogenic tissue. In some species, such as *Picea glauca*, initiation of embryogenetic tissue was under strong additive genetic control (Park *et al.* 1993a).

Difficulties in initiating tissue from all desired genotypes is only the start of the production processs. Embryogenic tissue formed on 50 % of the *Picea sitchensis* zygotic embryos cultured on induction medium, but only 20% of the zygotic embryos produced tissue that continued to develop (von Arnold and Woodward 1988). Up to 93% of zygotic explants of *Larix occidentalis* initiated embryogenic tissue but only 3% produced sustainable embryogenic lines (Thompson and von Aderkas 1992). In *Picea mariana* and *Picea glauca* , all families produced embryogenic tissue but up to 26% of the families failed to produce mature embryos (Adams *et al*. 1994, Park *et al.* 1993, Park *et al.* 1994). The genetic variances for maturation and germination were largely non-additive (Park *et al.* 1994).

These production barriers are detrimental to the ability of breeders to preserve genetic diversity and to maximize genetic gain. In propagating desirable genotypes, within-family initiation rates for embryogenic tissue (largely under additive genetic control) are not important since as long as some explants of each family can initiate embryogenic tissue, the number of excised explants can be increased to give the desired number of initiated lines to preserve genetic diversity (Adams *et al.* 1994). Genetic improvement programs require the use of many families to ensure that genetic diversity is maintained and that genetic gain by selection is made possoble (Adams *et al.* 1994). Clonal propagation through somatic embryogenesis can be a valuable tool for preserving genetic gain in improvement programs since up to 30-50% of total genetic variation can be due to non-additive variance, which would be lost in the sexual propagation process (Cheliak and Rogers 1990).

2.3.2 Non-media factors affecting the initiation of embryogenic tissue

2.3.2.1 Choice of explant

Successful initiation of embryogenic tissue has proven to be extremely sensitive to explant choice in almost every conifer species, with cone collection dates differing by only a few days having significantly different initiation rates. Different species have different optimal stages for initiation.

Only immature zygotic embryos have yielded embryogenic tissue from *Abies alba* (Lang and Kohlenbach 1989, Schuller and Reuther 1993), *Abies nordmandiana* (Norgaard and Krogstrup 1995), *Larix decidua* (Cornu and Geoffrion 1991), *Larix occidentalis* (Thompson and von Aderkas 1992), *Larix x eurolepis* (Klimaszewska 1989a,b, von Aderkas *et al.* 1990), *Picea glauca - engelmannii* (Roberts *et al.* 1990a,b, Webster *et al.* 1990, Flinn *et al.* 1991b, Roberts 1991), *Pinus caribaea* (Laine and David 1990), *Pinus nigra* (Salajova *et al.* 1995), *Pinus strobus* (Finer *et al.* 1989), *Pinus taeda* (Gupta and Durzan 1987a, Becwar *et al.* 1990, 1991) and *Pseudotsuga menziesii* (Gupta and Durzan 1987b).

Both mature and immature zygotic embryos have yielded embryogenic tissue in *Picea abies* (Chalupa 1985, Hakman *et al.* 1985, Hakman and von Arnold 1985, Gupta and Durzan 1986, Becwar *et al.* 1989, Boulay *et al.* 1988, von Arnold and Hakman 1988, Jain *et al.* 1989, Feirer *et al.* 1989, Verhagen and Wann 1989, Hakman *et al* 1990, Jalonen and von Arnold 1991, Bozhkov *et al.* 1992), *Picea glauca* (Hakman and Fowke 1987, Lu and Thorpe 1987, Hakman and von Arnold 1988, Kartha *et al.* 1988, Dunstan *et al.* 1988, Attree *et al.* 1989b, Webb *et al.* 1989, Tremblay 1990, Attree *et al.* 1989a, 1991, 1992, Dunstan *et al.* 1991, Joy *et al.* 1991, Park *et al.* 1993), *Picea mariana* (Hakman and Fowke 1987, Tautorus *et al.* 1990a, Cheliak and Klimaszewska 1991, Tautorus *et al.* 1992, Adams *et al.* 1994), *Picea sitchensis* (Krogstrup *et al.* 1988, von Arnold and Woodward 1988, Krogstrup 1990), and *Picea engelmannii* (Webb *et al.* 1989). Mature zygotic embryos have yielded embryogenic tissue from *Picea pungens* (Afele and Saxena 1992), *Picea rubens* (Harry and Thorpe 1991), *Pinus lambertiana* (Gupta and Durzan 1986), and *Sequoia sempervirens* (Bourkgard and Favre 1988).

As embryos develop there could be a shift in genetic expression from an embryogenic program to a germination program and hence a possible loss of embryogenic potential in mature embryos compared to immature embryos (Lelu *et al.* 1994). The competence of *Picea glauca* zygotic embryos to produce embryogenic tissue was closely related to the absence of storage proteins (Roberts *et al.* 1989). As zygotic seeds develop, storage products such as proteins and lipids accumulate. Protein content can comprise 8-25% of the total dry weight of the seed (Gifford 1988, Gifford and Tolley 1989), are highly conserved (Gifford and Tolley 1989, Roberts *et al.* 1989, Hakman *et al.* 1990) and their accumulation is likely influenced by abscisic acid (ABA) (Tautorus *et al.* 1991, Misra 1995).

For species which, to date, depend upon immature zygotic embryos at specific stages of development for the initiation of embryogenic tissue, the ability of stored immature zygotic embryos to produce embryogenic tissue increases the availability of explants. Storing seeds at low temperatures (usually 4 °C) prolongs the availability of initiation explants, and may improve initiation rates providing the storage period is not long enough to cause desiccation or further maturation of the embryo (Hakman and Fowke 1987). Immature zygotic embryos of *Picea mariana* were successfully stored at 4 °C for two months before being excised. As with fresh seeds, stages of development made a difference in the ability of the embryos to produce embryogenic tissue. Embryos stored 8 weeks after pollination had a 62% induction rate, while embryos stored 10 weeks after pollination had a 47 % induction rate (Adams *et al.* 1994). Embryogenic tissue which produced normal somatic embryos was initiated from 20 year-old mature seeds of *Picea rubens* (Harry and Thorpe 1991), 5 year-old mature seeds of *Pinus lambertiana* (Gupta and Durzan 1986) and 3-11 year-old mature seeds of *Picea glauca* (Tremblay 1990).

In some species, later stages of development have yielded embryogenic tissue. Seedlings have yielded embryogenic tissue from *Picea abies* (Krogstrup 1986), *Picea mariana* and *Picea glauca* (Attree *et al.* 1990a), *Picea omorika* (Budimir and Vujicic 1992) and *Sequoia sempervirens* (Bourgkard and Favre 1988). Embryogenic tissue arose from nonembryogenic callus initiated from needle and bud explants from 26 year-old *Picea abies* trees in a nurse culture system (Westcott 1994). These are very promising developments because they not only eliminate the need for tedious, labour-intensive embryo excisions, they are also a step towards using tissue from mature trees to allow for selection of desirable genotypes.

The stage-specificity of embryogenic tissue induction is not the only evidence of differences in the expression of cell totipotency in explants. Spatial differences also exist. Usually excised embryos of *Picea sitchensis* initiated embryogenic tissue from the junction between the hypocotyl and the cotyledons while nonembryogenic tissue was initiated from all regions of the embryo (von Arnold and Woodward 1988). The nonembryogenic callus always died after 1 month, and only the embryogenic callus continued to grow. However, reinitiation of secondary somatic embryos from somatic embryos came from both the suspensor region near the base of the embryo and the embryo region (von Arnold and Woodward 1988). Thus, not only do different stages of development and different regions of the zygotic embryo show differences in embryogenic potential, somatic embryos do not demonstrate the regional differences in embryogenic potential as zygotic embryos do.

2.3.2.2 Organization of initiated tissue

Despite the uniform white, mucilaginous appearance of embryogenic tissue initiated by conifer species, there are differences in embryogenic tissue organization between species and between freshly-initiated tissue and long-established cultures. The earliest embryogenic stage found in proliferating *Picea sitchensis* tissue were small clusters of meristematic cells intermingled with single elongated vacuolate cells (von Arnold and Woodward 1988). These clusters were only found in the first month of culture before the tissue became composed of embryonal masses plus suspensors that characterize embryogenic tissue (von Arnold and Woodward 1988).

Not all species have an intermediate cluster stage before embryonal mass development. In Douglas-fir (*Pseudotsuga menziesii*), initiated cell suspensions produced somatic embryos without an intermediate cluster stage (von Arnold and Woodward 1988). Somatic embryos continued to proliferate from cleavage of these embryonal masses and from meristematic areas in suspensors, a pattern of development also found in *Picea glauca* and *Picea abies* cultures (von Arnold and Woodward 1988). In *Abies* spp., initiated embryogenic tissue was white and mucilaginous, consisting of small embryonal masses with long multicellular suspensors. Many single, elongated, vacuolate cells are present in the cultures too (Norgaard and Krogstrup 1995). The earliest stages of embryo development found in *Picea omorika* are filamentous embryonal masses with 2 rows of cells extending to suspensor-like cells (Vujicic and Budimir 1995). Initial divisions in *Picea abies* cell suspensions of embryonal tube cells were asymmetrical, in a process which involves division of the nucleus and the degeneration of one of the resulting daughter nuclei (Durzan *et al.* 1994, Havel and Durzan 1996).

Several papers report the origin of somatic embryos as the unequal division and subsequent unequal cytoplasmic distribution of a single embryogenic cell. In *Pinus nigra* cultures, after the unequal division of a single elongated cell, the vacuolated suspensor cell senesced (Jasik *et al.* 1995). The smaller, densely cytoplasmic cell and its daughter cells divided transversely until a file of single cells arose. The cells then began to divide randomly until an embryonal mass formed. Cells at the proximal end of the embryo gradually became vacuolated and differentiated into suspensor initial cells (Jasik *et al.* 1995). Thus, while some species have an intermediate cell cluster stage, others immediately show organized growth of embryonal masses, which may originate from the uneven division of a single embryogenic cell.

2.3.2.3 Embryogenic potential

The ability to initiate embryogenic tissue appears to vary with genotype, number of years the seed has been stored, and the imbibition period. Initiation rates are also affected by light regimes, nutrient content of the initiation medium (especially sucrose levels), types and concentration of nitrogen, as well as the pH of the medium, the gelling agent used and the types and concentrations of plant growth regulators (Tautorus *et al.* 1990a, Verhagen and Wann 1989).

The discovery of markers capable of identifying potentially embryogenic genotypes would greatly improve the efficiency of conifer tissue culture systems. Several markers for embryogenic potential have been identified in angiosperms, including specific esterase isozyme patterns in barley, maize and carrot (Pitel *et al.* 1992). Other markers include isoenzyme patterns for glutamate dehydrogenase, peroxidase, and malate dehydrogenase. Protein patterns specific to embryogenic cultures have been identified in carrot, rice and pea (Pitel *et al.* 1992). If these phenomenon apply to conifers as well, much time and effort in identifiying embryogenic genotypes will be saved.

In conifers, the presence of pre-prophase bands has been linked with embryogenic potential (Roberts *et al.* 1989). Once tissue has been initiated, there are differences in ethylene evolution rates, glutathione concentrations and total reductants between embryogenic and nonembryogenic tissue, with nonembryogenic lines having a reduced enzymatic activity (Salajova *et al.* 1995). The enriched enzyme activity of the embryogenic cultures may be due to increased metabolic potential. Peroxidase seems to be the best marker so far for identifying embryogenic vs. nonembryogenic lines of *Pinus nigra* (Salajova *et al.* 1995).

Klimaszewska (1989a) reported that no nonembryogenic tissue had produced embryogenic. No reversion of mucilaginous embryogenic lines into non-embryogenic lines occurred in *Pinus nigra* (Salajova *et al.* 1995). However, nonembryogenic green callus has formed embryogenic white, mucilaginous tissue after several months of subculture in *Picea mariana* , *Picea glauca* and *Larix occidentalis* (Tautorus *et al.* 1990a, Tremblay 1990, Thompson and von Aderkas 1992). Embryogenic tissue arose from nonembryogenic callus initiated from needle and bud explants from 26 year-old *Picea abies* trees in a nurse culture system (Westcott 1994). The conversion of nonembryogenic tissue to embryogenic tissue may have been due to a change in the tissue itself, either genetic or physiological. Determining the origin of the change could provide important information regarding the nature of embryogenesis.

2.3.3 Initiation and maintenance medium of embryogenic cultures

2.3.3.1 Media requirements

In general, the same medium is used for initiation and proliferation of embryogenic tissue, containing an auxin, a cytokinin and low sucrose levels (Attree and Fowke 1993). However, some species can successfully proliferate without plant growth regulators.

In *Abies* species, embryogenic tissue is induced with 5 µM BA but no plant growth regulators are required for proliferation (Lang and Kohlenbach 1989, Schuller and Reuther 1993, Norgaard *et al.* 1995, Norgaard and Krogstrup 1995), and in some instances the addition of BA has reduced proliferation (Schuller and Reuther 1993). In other studies, the addition of 5 µM 2,4-D with or without a cytokinin increased the rate of proliferation of *Abies nordmannia* (Krogstrup 1991) and the addition of 10.7 µM NAA (naphthalene acetic acid) plus 4.5 µM BA increased proliferation of *Abies balsamea* (Norgaard and Krogstrup 1995).

In *Pinus* species, the proliferation medium is largely the same as the induction medium, as for *Pinus pinaster* (Bercetche and Pacques 1995), and *Pinus strobus* (Finer *et al.* 1989, Kaul 1995). Unlike *Abies*, *Pinus* species appear to require auxins and cytokinins for proliferation. The initiation and proliferation media for *Pinus caribaea* includes 5-50 µM 2,4-D and 2-20 µM BA and 0-20 µM kinetin (Laine and David 1990, David *et al.* 1995), for *Pinus nigra* 9.05 µM 2,4-D plus 2.22 µM BA or 9.05 µM 2,4-D plus 8.87 µM BA (Salajova *et al.* 1995), for *Pinus sylvestris* 4.52 µM 2,4-D, 1.78 µM BA and 1.86 µM kinetin (Hohtola 1995), for *Pinus lambertiana* 13.5 µM 2,4-D (Gupta and Durzan 1986), for *Pinus taeda* either no plant growth regulators or 11.3 µM 2,4-D and 0.32 µM BA for both initiation and proliferation (Becwar *et al.* 1990, 1991) or 2 µM BA and 2 µM NAA (Franklin *et al.* 1989) or 50 µM 2,4-D, 20 µM kinetin and 20 µM

BA with proliferation medium containing 10% of initiation levels of plant growth regulators (Gupta *et al.* 1988).

For *Picea* species, the initiation medium and the proliferation medium are often the same. In *Picea glauca*, the initiation and proliferation media both contain 1 µM 2,4-D and 5 µM BA (Hakman *et al.* 1987), though other papers report proliferation media containing 10 µM 2,4-D and 2-5 µM BA (Lu and Thorpe 1987, Hakman and von Arnold 1988, Kartha *et al.* 1988, Attree *et al.* 1989a,b, Tremblay 1990, Joy *et al.* 1991). *Picea abies* requires an auxin (1-10 µM NAA or 2,4-D) and a cytokinin (1-5 µM BA) for initiation and proliferation of embryogenic tissue (Hakman and von Arnold 1985, Hakman *et al.* 1985, Krogstrup 1986, Becwar *et al.* 1989, Chalupa 1989, Verhagen and Wann 1989, Hakman *et al.* 1990, Jalonen and von Arnold 1991, Egertsdotter *et al.* 1993, Bozhkov *et al.* 1992) as does *Picea mariana* (Hakman and Fowke 1987, Isabel *et al.* 1993, Adams *et al.* 1994). *Picea nigra* requires 9 µM 2,4-D and 2.22 µM BA for both initiation and proliferation (Jasik *et al.* 1995). *Picea omorika* requires 22.5 µM BA for initiation then 9 µM 2,4-D and 4.5 µM BA for proliferation (Budimir and Vujicic 1992).

The concentration of sucrose in the initiation medium was critical to the yield of embryogenic tissue from mature zygotic embryos of *Picea abies* ; the yield was significantly higher at 29 µM sucrose compared to other concentrations ranging from 3 µM to 145 µM(von Arnold and Hakman 1985). Sucrose concentrations of 29 µM were also optimal (from a range of 3 µM to 145 µM) for initiation rates of embryogenic callus of *Picea glauca* and *Picea engelmannii* from different collection dates (Webb *et al.* 1989) and immature embryos of *Picea mariana* (Hakman and Fowke 1987). However, initiation rates for *Picea rubens* were best on 58 µM sucrose (Harry and Thorpe 1991). The use of an organic nitrogen source tripled the number of embryogenic lines initiated from mature zygotic embryos of *Picea abies* compared to inorganic nitrogen sources (Verhagen and Wann 1989).

2.3.3.2 Proliferation and ultrastrucutre of established cultures

Embryogenic tissue may proliferate in one of three ways: 1. single cells or small clusters can undergo asymmetric division, 2. division of small meristematic cells, from either asymmetrically dividing suspensor cells or meristematic cells which failed to elongate into suspensor cells, can occur in the suspensor, 3. cleavage polyembryony from the embryonal mass (observed in *Abies alba, Larix decidua, Pinus* species, *Picea* species and *Pseudotsuga menziesii*)(Tautorus *et al.* 1991). In *Abies, Pseudotsuga, Picea* and *Pseudolarix*, cleavage polyembryony rarely occurs in zygotic embryogenesis, therefore, this third pattern of proliferation is a departure from zygotic embryo development (Singh 1978). Continued cleavage polyembryony allows for growth of the tissue in culture and subsequent subculturing. Though interest in embryogenic tissue has focused on the production of somatic embryos, several studies have attempted to characterize proliferating embryogenic tissue.

In several species, the ultrastructure of embryonal masses and other stages have been examined. Cells in the embryonal masses of *Picea abies* somatic embryos had high nucleus:cytoplasm ratios and small vacuoles (von Arnold *et al.* 1995). The nuclei often had more than one nucleolus (von Arnold *et al.* 1995). Embryonal masses of *Picea glauca* consisted of small, densely cytoplasmic cells with small vacuoles and abundant organelles, indicating rapid growth (Hakman *et al.* 1987). As the embryonal mass developed, the plastids and mitochondria gradually became differentiated as cellular volume decreased. The embryonal mass was subtended by long, thin highly vacuolated suspensor cells with thin layers of peripheral cytoplasm with undifferentiated plastids (Hakman *et al.* 1987, Hakman and von Arnold 1988, Fowke *et al.* 1990). This pattern of plastid differentiation also occured in *Larix* (Rohr *et al.* 1989).

Embryonal masses of embryogenic lines of *Pinus taeda, Pinus strobus, Pinus nigra* and *Picea glauca* appeared similar in organization and closely resemble those of zygotic embryos (Salajova *et al.* 1995). The embryonal masses had suspensors with a thin parietal layer of cytoplasm, a cluster of small isodiametric embryonal mass cells with dense cytoplasm and a centrally located nucleus. In *Pinus nigra,* the nuclei had several nucleoli, and the cytoplasm was dense with organelles. Cell walls to the interior of the embryonal mass typically had thin walls, whereas cells on the outside had thicker walls (Salajova *et al.* 1995).

Microtubule development was studied in two nonembryogenic *Picea banksiana* lines and an embryogenic *Picea mariana* line (Tautorus *et al.* 1992). The microtubules in suspensor cells of embryogenic *Picea mariana* tissue were fewer and rougher than those in embryonal mass cells, possibly due to degradation as the cells began to senesce. Microtubules in nonembryogenic *Picea banksiana* cultures were fewer and thicker than those in the embryogenic *Picea mariana* line. The development of preprophase bands was similar in both lines, though they were broader and less organized in the nonembryogenic *Picea banksiana* line. In the embryogenic *Picea mariana* line adjacent cells often had preprophase bands, indicating synchronicity (Tautorus *et al.* 1992).

2.3.4 Maturation of somatic embryos

The desired result of somatic embryogenesis is a healthy, thriving tree established in the field. Despite the successes in generating embryogenic tissue, obtaining plantlets from these tissues has proven problematic in many conifer species. A number of factors are important to maturation, such as the reduction of plant growth regulator concentrations, the use of ABA and high osmoticum concentrations and manipulation of the culture atmosphere.

In some species, lowering the concentrations of plant growth regulators is sufficient to allow some somatic embryos to mature (*Picea abies, Picea glauca, Picea mariana, Pinus taeda, Pinus caribaea,* and *Sequoia sempervirens*) but plantlet production is infrequent (Hakman and von Arnold 1985, Gupta and Durzan 1987a, Hakman and Fowke 1987, Bourgkard and Favre 1988, Hakman and Fowke 1987, Lu and Thorpe 1987, Laine and David 1990, Tremblay 1990). With embryogenic lines of *Pinus caribaea*, lines comprised of well developed stage 1 somatic embryos matured on proliferation medium, but poorly-organized lines required 7.5-15 µM ABA. Early work using no or reduced plant growth regulators resulted in few mature embryos of *Picea abies*, and those were abnormal (Attree and Fowke 1993). Using no plant growth regulators and 29-87 µM sucrose, mature embryos were obtained from *Abies alba* (Schuller *et al.* 1989), haploid *Larix decidua* (Nagmani and Bonga 1985, von Aderkas and Bonga 1988), *Picea abies* (Hakman *et al.* 1985), *Picea glauca* (Hakman and Fowke 1987, Lu and Thorpe 1987, Kartha *et al.* 1988), *Pinus lambertiana* (Gupta and Durzan 86), and *Sequoia sempervirens* (Bourkgard and Favre 1988). *Larix decidua* somatic embryos were matured with 87 µM sucrose, 60-120 µM PEG 6000 and no plant growth regulators (Cornu and Geoffrion 1990).

Other species required auxins and/or cytokinins to mature in the absence of ABA, including *Picea abies* (0.1-5 µM BA, 2ip, kinetin or zeatin, 1-2.3 µM 2,4-D and 29-116 µM sucrose) (Chalupa 1985, Hakman and von Arnold 1985, Gupta and Durzan 1986), *P. glauca* (5 µM Kin, 1 µM 2,4-D, 174 µM sucrose)(Tremblay 1990), *Picea mariana* (5 µM BA, 0.5 µM 2,4-D)(Hakman and Fowke 1987), and *Pinus taeda* (2 µMKin, 2 µM BA, 5 µM 2,4-D, 87 µM sucrose)(Gupta and Durzan 1987a).

In three *Pinus* species, a reduction in plant growth regulator concentration in the proliferation medium is not sufficient to stimulate maturity in somatic embryos. In one study of *Pinus caribaea*, 2,4-D in the media promoted proliferation, but the embryos had to be transferred to plant growth regulator-free media to mature (David *et al.* 1995) but in another study, embryos matured spontaneously on proliferation medium (Laine and David

1990). However, in *Pinus lambertiana* and *Pinus taeda*, after transfer to plant growth regulator-free medium with activated charcoal and 58 µM sucrose, only 1-2% of the embryos germinated. All embryos failed to establish in soil, possibly due to poor quality (Gupta 1995). In *Pinus taeda* cultures, after being maintained on BA and kinetin medium, reducing plant growth regulators by a factor of ten resulted in stage 2 embryo formation. If high osmoticum was used as well, the early stage embryos grew from 10 cells or less to approximately 100 cells. These larger embryos matured under ABA, while the smaller ones did not. Development of cotyledonary embryos improved if maltose was used instead of sucrose (Becwar and Pullman 1995).

However, in most cases, a treatment of ABA alone or in conjunction with high osmoticum is required to maximize mature embryo production and reduce precocious germination. Due to its presumed involvement in zygotic embryo maturation and previous research involving angiosperm somatic embryos, ABA has been used extensively in the maturation process of conifer somatic embryos. ABA appears to be involved in both the suppression of cleavage polyembryony, allowing individual somatic embryos to mature, and the suppression of precocious germination, allowing storage products to accumulate (Tautorus *et al.* 1991). In cell suspensions of embryonal suspensor masses of *Picea abies*, ABA inhibited cleavage and stimulated the further development of embryonal masses (Boulay *et al.* 1988).

Optimal ABA concentrations depend on species, genotype, stage of embryo development, use of other plant growth regulators, and likely other undiscovered factors. The concentration of ABA, the length of time of exposure to ABA, concentrations of sugars and various osmotica (sugar alcohols and PEG), and charcoal concentrations have been varied in many studies in order to determine the most effective way to mature somatic embryos. Concentrations of ABA between 7.6-16 µM for 4-5 weeks were effective for *Picea abies*, *Picea mariana*, *Pinus taeda* and *Pinus strobus*, while *Picea glauca-engelmannii*, *Picea rubens*, *Picea glauca* and *Picea sitchensis* responded to 40-60 µM ABA and partial desiccation (von Arnold and Hakman 1988, Finer *et al.* 1989, Attree *et al.* 1991, Becwar *et al.* 1990, Hakman *et al.* 1990, Roberts *et al.* 1990a, Webster *et al.* 1990, Dunstan *et al.* 1991, Harry and Thorpe 1991, Jalonen and von Arnold 1991, Roberts *et al.* 1991, Adams *et al.* 1994). A treatment of 90 µM sucrose and 7.6 µM ABA yielded the highest number of *Picea abies* plantlets (von Arnold and Hakman 1988). In contrast, somatic embryos of *Pinus nigra* matured on low levels of ABA (1.89-3 µM) (Salajova *et al.* 1995).

Other plant growth regulators can react either negatively or synergistically with ABA. Different analogues of ABA had different effects on maturation rates of *Picea glauca* somatic embryos, but none could promote maturation in the presence of 2,4-D and benzyladenine (Dunstan *et al.* 1988). However, the use of indole-butyric acid (IBA) in conjunction with ABA increased the yield of mature somatic embryos of *Picea glauca x engelmannii* (Roberts *et al.* 1991b, Roberts 1991, Amarasingh *et al.* 1996) . Somatic embryos of *Picea abies* could not mature on media without plant growth regulators, but would mature on medium containing 1 µM ABA and 1 µM IBA (Chalupa 1989, Verhagen and Wann 1989). The combination of ABA and BA increased by 10-fold the number of mature somatic embryos recovered per gram of tissue of *Picea abies* if 0.5-10 µM BA was used in conjunction with ABA compared to the use of ABA alone (Bozhkov *et al.* 1992).

The use of ABA alone or with other plant growth regulators can affect the quality of somatic embryo production, as well as the yield. The further maturation of *Picea sitchensis* somatic embryos was enhanced by removing the individual embryos from the tissue aggregate and the addition of ABA alone or in combination with 2,4-D, Kin, and BA to the media (Krogstrup *et al.* 1988). Using ABA alone gave the somatic embryos with the best morphologies. ABA synchronized maturation and promoted it; without ABA there was limited growth and development. On media without ABA, the few *Picea sitchensis* somatic embryos that did mature became green and showed rapid development without any growth arrest, and were often abnormal in morphology (Krogstrup *et al.* 1988).

The addition of charcoal to the medium, usually as a one week treatment before transfer to ABA maturation medium, is often beneficial (Harry and Thorpe 1991, Roberts 1991). If activated charcoal was used in addition to ABA, the yield of *Pinus taeda* cotyledonary embryos (stage 3) increased and these embryos exhibited improved apical dome regions, had greater desiccation tolerance and the embryos were more vigourous after germination (Becwar and Pullman 1995). *Picea abies* and *Pseudotsuga menziesii* respond to the ABA-charcoal medium in the same way. The improved somatic embryo production when both ABA and charcoal are used may be due to the reduced availability of the ABA when charcoal is added (Becwar and Pullman 1995). Putting somatic embryos of *Picea pungens* on activated charcoal after 4 weeks on ABA increases embryo conversion two-fold but prolonged culture on charcoal led to vitrification, possibly as a result of unavailability of various nutrients (Afele and Saxena 1995).

In some instances, the addition of ABA did not improve the production of mature somatic embryos. After being placed on ABA medium at 4-30 µM, then onto plant growth regulator-free medium, cultures of *Pinus sylvestris* gradually died 3-4 weeks later without producing any embryos (Hohtola 1995). In *Abies nordmanniana*, proliferation continued on medium containing ABA, and this often resulted in the developing embryos being overrun by proliferating tissue. As a result embryo growth ceased, and the ones that formed were often abnormal (Norgaard and Krogstrup 1995). The use of ABA medium with somatic embryos of *Picea glauca* did not increase the yield of mature embryos over that of maturation medium without ABA, but increasing the sucrose content from 87 µM to 261 µM did improve maturation yields (Lu and Thorpe 1987).

In one study of *Pinus strobus* , stage 2 embryos were obtained on medium with 174 µM sucrose and 37.84 µM ABA (only stage 1 was reached on the maintenance medium)(Kaul 1995). Cotyledonary embryos were produced on medium with 95 µM ABA, PEG and 0.125% charcoal. However, of the ten stage 4 embryos grown further, only 1 germinated (root and hypocotyl elongation). The rest did not develop roots or showed poor hypocotyl elongation. In *Pinus strobus* as in *Picea abies, Pseudotsuga menziesii* and *Pinus taeda* , ABA alone could not halt precocious germination without the addition of high osmoticum (Kaul 1995).

In most species, maturation of somatic embryos improves on media with high osmotic levels regardless of the plant growth regulators used. Early stage somatic embryos of *Abies alba* matured better on high sucrose (Bercetche and Pacques 1995). Early stage somatic embryos of *Picea abies* produced better formed mature embryos on high sucrose. Cultures of *Pinus pinaster* grown on 174 µM sucrose yielded the highest number of stage 2 embryos . However, poorly organized lines of *Pinus caribaea* and *Picea abies* could not be matured, indicating that only somatic embryos in a certain physiological or differentiated state could respond to the maturation media (Bercetche and Pacques 1995). In *Picea glauca x engelmanni* cultures, PEG increased the number of mature somatic embryos 300% and improved the conversion rate (Norgaard and Krogstrup 1995). High osmoticum in addition to ABA and activated charcoal are required to obtain good *Pinus taeda* somatic embryos (Gupta 1995). Pulse treatments with mannitol improved the maturation of *Picea glauca-engelmannii* somatic embryos (Roberts 1991).

Organic nitrogen, various gases such as ethylene and CO_2/O_2 ratios, and some unidentified substances can also have an effect on maturation of somatic embryos. Adding organic nitrogen has varying effects. Glutamine at low concentrations stimulated stage 2 embryo formation in *Abies* species, while higher concentrations increased the number of stage 3 somatic embryos significantly (Norgaard and Krogstrup 1995). Unfortunately, they were often vitrified. Casein hydrolysate had a negative effect on stage 2 embryos but not on stage 3 embryos (Norgaard and Krogstrup 1995). The addition of 1.89 mM of asparagine doubled the number of plantlets produced from embryogenic tissue of *Picea pungens* (Afele and Saxena 1995).

The levels of various gases in the culture vessel can affect the growth of the embryogenic tissues. Ethylene tends to promote the growth of embryogenic cultures while reducing the ability of the embryonal masses to mature. Ethylene promoted growth in embryogenic tissue of *Pinus radiata* (Kumar and Thorpe 1988). Adding silver nitrate (a competitive inhibitor of ethylene) to the medium of *Pinus pinaster* cultures containing ABA and 174 µM sucrose reduced the number of stage 2 embryos produced, and at high enough concentrations (58.8 and 117.6 µM), stopped stage 2 production altogether (Berceteche and Pacques 1995). Production of ethylene and carbon dioxide inhibited the growth and embryo production of *Picea glauca* (Kumar and Thorpe 1988), in agreement with several angiosperm species (Pius *et al.* 1993). Low partial pressure of O_2 and elevated levels of CO_2 with low levels of ABA aided maturation in *Picea abies* (Attree and Fowke 1993).

Recently, various unidentified substances have been found to have an effect upon embryo maturation. A higher number of stage 2 embryos formed in *Pinus pinaster* when 4 or 6 tissue aggregates were in the dish rather than just one, indicating that maturing embryos may be secreting a substance which promotes maturation in other somatic embryos (Bercetche and Pacques 1995). Proteins excreted by type A lines (with well-formed embryonal masses capable of developing into mature somatic embryos) of *Picea abies* induced embryonal masses in type B lines (disorganized and incapable of forming somatic embryos) to develop further (Egertsdotter *et al.* 1993). Further experiments to clarify the specific effects of these substances and their identification will likely prove to be very interesting.

2.3.5 Protein deposition in developing somatic embryos

A few studies that have compared the effects of ABA on storage product accumulation and utilization have shown both parallels and differences between somatic and zygotic conifer embryos (Tautorus *et al.* 1991). Though most of the major storage proteins in seeds are found in the megagametophyte and are hydrolysed after germination (Gifford and Tolley 1989), the deposition of storage proteins is considered to be necessary for vigourous germination of somatic embryos especially if the pattern of deposition was similar to that of zygotic embryos (Gifford 1988). Somatic embryos of *Picea abies, Picea glauca-engelmannii* and *Picea sitchensis*, when matured with ABA on low or high low osmoticum, had the same storage protein profile as zygotic embryos (Hakman *et al.* 1990, Attree and Fowke 1993). Somatic embryos of *Picea glauca* began to produce polysaccharides and deposit lipids within 1-2 weeks after being placed on medium containing ABA (Joy *et al.* 1991). Storage product deposition began in basal regions near the suspensor and continued peripherally before proceeding inward to the cortex. Somatic embryos had less lipid and protein but more starch than zygotic embryos at comparable stages of development (Joy *et al.* 1991).

Somatic embryos of *Picea abies* matured on medium with 7.6 µM ABA and 90 µM sucrose were similarily organised though less expanded than zygotic embryos at maturity (Hakman 1993a). Lipid bodies and protein bodies comprised the majority of storage organelles, with prominent starch grains present early in the maturation period though less so in somatic embryos as compared to zygotic embryos (Hakman 1993a). Somatic embryos accumulated storage proteins in both the hypocotyl-shoot axis and the cotyledons (Hakman 1993b).

Somatic embryos of *Picea glauca -engelmanni* matured on 40 µM ABA and low osmoticum contained the same storage proteins as zygotic embryos though total protein levels differed between somatic embryo genotypes (Flinn *et al.* 1991a,b), with twice as much protein deposited at 20 µM ABA compared to 40 µM ABA (Roberts *et al.* 1991a). The rates of accumulation differed between somatic and zygotic embryos, with somatic embryos having a slower and steadier rate of accumulation in the period of cotyledon maturation (Flinn *et al.* 1991a, Flinn *et al.* 1993). However, the proteins of somatic embryos of *Picea glauca -engelmanni* matured on low ABA (10-16 µM) and low osmoticum had a profile similar to immature zygotic embryos, with several major storage proteins absent or at low levels, and a diffuse protein deposition pattern, while somatic embryos matured on high osmoticum had normal storage protein patterns (Roberts *et al.* 1991a, Joy *et al.* 1991, Misra and Green 1991, Misra *et al.* 1993, Misra 1994). The use of mannitol as an osmoticum increased storage protein deposition in *Picea glauca -engelmanni* but not as much as the use of ABA (Roberts 1991), indicating that both high osmotic levels as well as the use of ABA are necessary for good protein deposition.

Without elevated levels of ABA and osmoticum, somatic embryos of *Picea glauca* failed to deposit the same levels of storage proteins and triacyglycerols as zygotic embryos (Feirer *et al.* 1989, Joy *et al.* 1991, Attree *et al.* 1992 , Misra *et al.* 1993), though their crystalloid protein mRNA profiles were comparable, whether the somatic embryos were matured on high ABA (32 µM) alone or 16 µM ABA plus PEG (Misra *et al.* 1993, Misra 1994).

2.3.6 Germination rates of somatic embryos

Little information is available relating to the establishment of somatic embryos in soil (Tautorus *et al.* 1991, Adams *et al.* 1994), but many papers cite the percentage of mature somatic embryos which rooted after transfer to plant growth regulator-free germination medium. In conifers, root development occurs *in vivo* before shoot development (Singh 1978), and proper root meristem development is likely crucial to outplanting success. Plantlets of *Picea, Pinus, Abies* and *Larix* have been established in soil, as well as haploid plantlets of *Larix decidua* (Chalupa 1985, von Arnold and Hakman 1988, Becwar *et al.* 1989, Klimaszewska 1989a).

Though comparisons between papers can be difficult, there appears to be a marked decrease in the number of embryos progressing to every stage in the maturation process, from maturation of stage 1 embryos to stage 3 embryos, and from stage 3 embryos to emblings established in the field. Though one gram of embryogenic tissue (fresh weight) of *Picea glauca* yielded 14.2 x 10^3 stage 1 embryos (morphologically at the embryonal mass stage), only 23 mature embryos were produced (Kartha *et al.* 1988). Somatic embryos of *Abies* species grown on medium with PEG were cream coloured and germinated at rates similar to green embryos. On average, 101 stage 3 embryos were formed per gram of embryogenic tissue fresh weight (Norgaard and Krogstrup 1995) compared to *Picea sitchensis* with 60 mature somatic embryos per gram of embryogenic tissue (fresh weight) (Krogstrup *et al.* 1988). Mature embryo yield for *Picea abies* could be as high as 120 mature embryos per gram of embryogenic tissue (fresh weight) (Jalonen and von Arnold 1991) but 25 plantlets per gram of embryogenic tissue (fresh weight) were obtained (Boulay *et al.* 1988). However, *Picea abies* embryogenic tissue matured on ABA plus IBA yielded 700 mature somatic embryos per gram of embryogenic tissue (Becwar *et al.* 1991), which indicates that there may be either genetic or cultural differences which can enhance maturation.

Plantlets derived from mature somatic embryos have been established in the soil for several *Picea* species. Apical shoot development in *Picea sitchensis* was enhanced by root development (Krogstrup *et al.* 1988) with soil transfer rates of 90% (Vujicic and Budimir 1995). Soil transfer rates of 38% have been reported for *Picea omorika* (Vujicic and Budimir 1995). Fifty-six percent of mature *Picea abies* somatic embryos rooted, and 29% of these were successfully established in soil (Vujicic and Budimir 1995). In *Picea pungens* less than 20% of plantlets develop roots and these single roots stop elongating at 2-3 cm (Afele and Saxena 1995). Using IBA (indole butyric acid) and NAA (napthalene acetic acid) to increase rooting was successful. A rooting rate of 27% was achieved using 0.5 µM NAA and 100 µM PG (phloroglucinol) (Afele and Saxena 1995).

In an extensive study, 80% of the 1200 plantlets of *Picea glauca-engelmannii* survived the first growing season in the field, and the plants resembled plants from zygotic embryos in terms of growth rates, final height, shoot and root morphology and frost hardiness (Becwar *et al.* 1989). However, somatic emblings of *Picea abies* had fewer lateral branches than zygotic seedlings in other studies (Becwar *et al.* 1989).

Sixty-one percent of *Picea mariana* and 18% of *Picea glauca* somatic embryos were successfully established in soil, and the success rate rose to 52% for *Picea glauca* if a misting bed was used for acclimatization (Vujicic and Budimir 1995). Proper fertilizer regimes and the use of misting beds were critical for the survival of *Picea mariana* somatic embryos in the greenhouse (Adams *et al.* 1994). If transfer to soil was delayed until the hypocotyl and root were elongating and true needles were forming, the proper acclimatization regime increased greenhouse survival from 11% to 45% with excellent subsequent survival in the field (Adams *et al.* 1994).

As in *Picea* species, germination of *Pinus* somatic embryos is generally good. Seventy-one percent of *Pinus caribaea* somatic embryos transferred to plant growth regulator -free medium turned green and elongated, and 69% of these rooted. Occasionally, in a few lines, root development occurred in the maturation medium prior to shoot elongation and was associated with polyphenol synthesis in the hypocotyl (David *et al*. 1995). Somatic embryos of *Pinus taeda* (583 in total) have been established in the field, with survival similar to zygotic embryos at 98% after approximately 3-4 years (Becwar and Pullman 1995).

In *Abies* species, germination rates vary, with *Abies balsamaea* and *Abies alba* responding better to somatic embryogenesis systems than *A. nordmannia* (Norgaard and Krogstrup 1995). *Abies nordmannia* had very low rates of rooting and hypocotyl elongation, and very poor soil establishment rates. In contrast, somatic embryos of *Abies alba* have a germination rate of 81%, a 98% hypocotyl elongation rate and a 71% epicotyl development rate. Somatic embryos of *Abies balsamea* have a 87% germination rate (Norgaard and Krogstrup 1995).

In determining the germination rate, care must be taken that the roots produced are healthy and will aid the plant during establishment in soil. In *Sequoia sempervirens*, only 2 out of 21 mature somatic embryos developed root tips, while the other 19 developed lateral roots after root tip necrosis (Bourgkard and Favre 1988). This prevalence of root tip necrosis indicates that the culture environment may have been missing some crucial element for good root development, and therefore, opportunities exist for improving the efficiency of many somatic embryogenesis systems.

2.3.7 Phenotypic variation in early stage somatic embryos

Most studies of somatic embryo production in conifers report the production of somatic embryos from embryogenic tissue in response to various stimuli (plant growth regulators, sucrose levels) without commenting on the organization of the embryogenic lines. However, in a few papers the authors have examined the embryogenic tissue microscopically in an effort to understand the ways in which the tissues respond to the culture environment.

The best studied species is *Picea abies* , likely because it was one of the first conifer species to be established in a somatic embryogenesis system (Hakman and von Arnold 1985). In 1991, Jalonen and von Arnold identified three types of embryogenic cultures of *Picea abies* : type A1 - polar (an embryonal mass with a suspensor), type A2 - solar (an embryonal mass surrounded by elongate, vacuolate cells), and type B - undeveloped. While A1 and A2 could transmute into each other, B type was never found mixed with either A type in an embryogenic line. Slow growth favoured the development of polar embryonal masses, while cell suspension culture fostered development of solar embryonal masses. Only the A type lines could produce mature somatic embryos when exposed to ABA. Most of these mature embryos developed further into plantlets (Jalonen and von Arnold 1991). Approximately 20 different proteins were secreted by culture lines of *Picea abies*, and though some of the proteins were secreted by all the lines, some proteins were specific to groups of cell lines or individual cell lines (Egertsdotter *et al.* 1993). Somatic embryos of group B lines developed further and more closely resembled embryos from group A lines if proteins specific to group A lines were added to group B lines, indicating that specific proteins are essential to somatic embryo formation (Egertsdotter *et al.* 1993).

In another study of *Picea abies*, the formation of two types of embryogenic lines was independent of the genotype of the initial explant, but very dependent on the medium composition (Bozkov 1995). The 2 types of lines described did not correspond to those described by Jalonen and von Arnold (1991). One type of embryogenic line was composed entirely of cleaving embryonal masses plus suspensors (homogenous). The other type of line was a mixture of embryonal masses plus suspensors and disorganized callus, described as globular and opaque (heterogenous). The heterogenous lines grew 2-4 times faster than the homogenous lines, but were more likely to become brown and necrotic. These characteristics remained stable for up to 2 years. However, by changing the medium, a heterogenous line could be transformed into a homogenous line, and *vice versa*. Somatic embryos matured from homogenous lines were normal, but somatic embryos from heterogenous lines were all abnormal, with short hypocotyls and distorted cotyledon formation (Bozkov 1995).

In contrast to established embryogenic lines of *Picea abies* , subcultured lines of *Picea mariana* were a mixture of embryonal structures and loose disorganized nonembryogenic cell clusters (Klimaszewska 1995). The ratio of these two types of structures could change with selection. The somatic embryos produced resembled zygotic embryos morphologically except for the polyphenol-rich region around the radicle which developed if the somatic embryos were matured in the light (Klimaszewska 1995).

In the *Pinus* species, different lines exhibited different characteristics. Embryogenic *Pinus caribaea* lines fell into 5 groups, each having different types of embryonal masses plus suspensors (David *et al.* 1995). The faster the embryogenic tissue of *Pinus caribaea* grew, the fewer the number of mature embryos produced. Embryonal mass (stage 1) types of lines (E4 and E5) were 100-150 µm, consisting of only a few cells loosely attached to the suspensor cells. Line E4 had very small embryonal masses (10 or so cells) with a suspensor of single suspensors while line E5 had slightly larger embryonal masses with loosely attached suspensors. Line E6 consisted of well-formed polarized embryonal masses associated with a bundle of suspensors and line E7 consisted of very large embryonal masses undergoing cleavage partitioning almost completely surrounded by bundles of long vacuolated cells (David *et al.* 1995). The different phenotypes of embryogenic lines could be characterized by slight qualitative and quantitiative variations in a few extracellular polypeptides but whether the differences came from the embryonal mass region or the suspensor could not be determined. Lines E7 and E9 produced mature embryos on proliferation medium in the presence of auxins and cytokinins, while poorly organized lines consisting of cell clusters and single elongate cells yielded low numbers of mature embryos only on ABA medium (Laine and David 1990).

Once on the maturation medium, lowering the sucrose level from 580 µM to 145 µM resulted in the disappearance of embryogenic capacity of all lines. Doubling the macronutrient level (from 1/2 to full) and the ABA concentration promoted the differentiation of embryos into cotyledonary stage 3 embryos in all the lines, but the best length of exposure to ABA differed between the lines (David *et al.* 1995).

Unlike *Pinus caribaea* cultures, embryogenic lines of *Pinus pinaster* were of only three distinct types. Lines in group 1 were mixtures of poorly organized small densely cytoplasmic cells with a diameter of 10-20 µm and suspensor-like cells 600 µm long and 120 µm wide. Lines in group 2 were composed of polarized somatic embryos with distinct embryo heads consisting of densely cytoplasmic cells diameter 10-30 µm and well developed suspensors 800 µm long and 150 µm wide (likely stage 1 embryos). Lines in group 3 were composed of clusters of small densely packed cells completely surrounded by elongated vacuolated suspensor cells. Group 2 corresponded to A1 (polar type) lines, group 3 corresponded to A2 (solar type) and group 1 corresponded to group B of *Picea abies* as described by Jalonen and von Arnold (1991) (Bercetche and Pacques 1995).

Group 2 embryos of *Pinus pinaster* could develop to stage 3 (embryos with unexpanded cotyledons and a meristem) but group 1 lines could rarely get to stage 3 possibly because they were not at a stage that could respond to ABA. *Pinus pinaster* cultures needed 1 week on activated charcoal before they would respond to 15 µM ABA and 174 µM sucrose. After 2 weeks on ABA the stage 2 embryos had embryonal masses 5 mm in diameter with a smooth glossy surface and a long translucent suspensor. Few embryos developed beyond this stage. Some embryos produced 5-7 cotyledons but no embryos developed beyond the cotyledon stage (Berceteche and Pacques 1995).

Growth rates differed between the different embryogenic lines. Group 1 lines (disorganized) of *Pinus pinaster* grew 44% faster than group 2 lines (polar), and group 3 lines (solar) grew the slowest (Bercetche and Pacques 1995). In *Pinus caribaea*, the faster the embryogenic tissue grew, the fewer the number of embryos produced, but growth rates between the lines did not differ greatly (David *et al.* 1995). In *Picea abies*, group A lines (embryogenic) grew faster than group B lines (nonembryogenic) (Jalonen and von Arnold 1991).

Two types of *Pinus taeda* embryogenic tissue were identified and both could produce precotyledonary somatic embryos on medium with 10 µM ABA (Becwar *et al.* 1990). Type 1 was composed mostly of embryonal masses plus suspensors while the type 2 was composed of single elongated cells interspersed with small clusters of meristematic cells with a few subtending suspensor cells (Becwar *et al.* 1990). Thus, the three *Pinus* species studied each had distinctive homogenous embryogenic lines, which ranged from cultures of readily identifiable embryonal masses plus suspensors to meristematic clusters of cells.

Mixed results have been obtained with *Sequoia sempervirens*. In one study, the embryogenic potential of initiated tissue differed depending on the level of 2,4-D in the initiation media (Bourgkard and Favre 1988). Eighty percent of the mature zygotic embryos cultured on 2.5 µM 2,4-D and 87 µM sucrose produced embryogenic callus capable of producing mature somatic embryos, while initiation medium containing higher concentrations of 2,4-D favoured the development of non-productive callus. The embryogenic tissue was not proliferating embryonal masses plus suspensors but compact, firm callus which concurrently produced adventitious buds and somatic embryos. The adventitious buds were indistinguishable from somatic embryos until the somatic embryos produced root tips or cotyledons. Unfortunately, squashes of the tissues were not provided. Mature, normal, rooted somatic embryos were obtained without using ABA or high osmoticum (Bourgkard and Favre 1988).

However, in another study, initiated tissue of *Sequoia sempervirens* was composed of elongate, vacuolate cells and clustered small meristematic cells in unpolarized aggregates. Within a month after initiation, very small embryonal masses with narrow suspensors (3-5 cells in width) and small star-like embryonal masses surrounded by attached single suspensors (called MC for meristematic cells) were observed, similar to the solar type of *Picea abies* (Jalonen and von Arnold 1991). None of these structures could be matured any further (Favre *et al.* 1995).

In conclusion, differences in the organization of embryogenic lines of *Picea, Pinus, Abies* and *Sequoia* have been documented, but in some cases the differences are between types of homogenous lines (*Pinus caribaea, Pinus pinaster*), while in other cases, embryogenic lines were composites of different cell and embryonal mass type (*Picea sitchensis, Picea mariana, Abies* spp., *Sequoia sempervirens*). In some instances, transmutation of one embryonal mass type into another was observed (*Picea abies*).

2.3.8 Phenotypic variation in mature somatic embryos

Evidence suggests that the *in vitro* environment affects the development of somatic embryos compared to the development of zygotic embryos, though many papers emphasize the similarities in appearance between somatic and zygotic embryos. In *Picea abies*, the differences in composition of embryogenic lines persisted as differences in mature embryo morphology (Jalonen and von Arnold 1991). Type A lines of *Picea abies* produced normal somatic embryos but type B lines of *Picea abies* never produced normal somatic embryos, but only nodules which develop several shoot meristems. These nodules either degenerated, regenerated callus tissue or rooted (Jalonen and von Arnold 1991). Somatic embryos matured from homogenous lines of *Picea abies* were normal, but somatic embryos from heterogenous lines were all abnormal, with short hypocotyls and distorted cotyledon formation (Bozkov 1995). Sectioned *Picea abies* somatic embryos may (Attree *et al.* 1993) or may not have intercellular spaces (Hakman and von Arnold 1985) in the region of the shoot meristem.

Somatic embryos of *Picea glauca* had less organized embryonal mass and suspensor regions compared to zygotic embryos (Wilson and Thorpe 1995). Unlike zygotic embryos, somatic embryos had intercellular air spaces. As the number and /or size of the air spaces increases, there was a decrease in shoot apical meristem development. In addition, the embryonal mass of the somatic embryos were broad and flat compared to the more conical zygotic embryos (Wilson and Thorpe 1995). However, scanning electron micrographs of hydrated and desiccated somatic and zygotic embryos of *Picea glauca* at different stages of development showed similar morphologies (Fowke *et al.* 1994).

Two potential effects of *in vitro* culture are vitrification (a glassy green, wet-soaked appearance) and callusing of the somatic embryo. The salt composition of the medium made a difference in both cuticular development and the number of vitrified somatic embryos of *Picea sitchensis* (Krogstrup *et al.* 1988). Increased concentrations of ABA decreased the incidence of vitrification and precocious germination in somatic embryos of *Picea rubens* (Harry and Thorpe 1991). Often germinated somatic embryos of *Picea pungens* had swollen hypocotyls or callusing of the stem base which interfered with root elongation. As with vitrification, these two effects have been attributed to the effects of plant growth regulators in the medium (Afele and Saxena 1995).

Other, less quantifiable effects of *in vitro* culture have been noted in somatic embryos. Growth arrest and failure to elongate roots are common traits in somatic embryos of *Pinus caribaea*, and several adjustments have been tried (a decrease in ABA treatment time or concentration, a decrease in the macronutrient levels, manipulation of light and use of cheese cloth bridges and liquid medium), but with little success (David *et al.* 1995). Most of the *Picea sitchensis* emblings survived transfer to the greenhouse but tended to show slower growth than seedlings (Krogstrup *et al.* 1988). This agrees with findings with *Picea mariana*, where emblings required different fertilizer and misting bed regimes than seedlings to survive in the greenhouse (Adams *et al.* 1994). Somatic embryos of *Pinus taeda* which were established in the field tended to exhibit diminished vigour and growth compared to seedlings (Becwar and Pullman 1995). Apparently, good morphology alone does not ensure outplanting success, though ranking media treatments by visually assessing morphology is more straightforward than attempting to quantify vigour when transferring somatic embryos to germination medium.

2.3.9 Protoplast cultures

Protoplast cultures result in the development of somatic embryos from individual protoplasts, either directly or through an intermediate free-growth stage. In this way, they most closely parallel the development of zygotic embryos from the single egg cell. For this reason, protoplast culture as it pertains to early somatic embryo development and differences in embryogenic lines will be discussed here.

Though only embryogenic cell lines have yielded plantlets from protoplasts, in some species the most embryogenic lines gave the lowest embryo recovery after protoplasting (Attree *et al.* 1989a, Egertsdotter and von Arnold 1993). In *Picea abies* embryogenic cultures, only group A lines (polar and solar types of embryonal masses) could produce mature somatic embryos in response to ABA while group B lines could not (Jalonen and von Arnold 1991). The two types of lines responded differently to the protoplast process as well. Protoplasts from both types A and B could form cell clusters but only in liquid medium on TCI (tissue culture inserts) supports rather than suspended in liquid medium or embedded in alginate. Although viability was 40% for protoplasts from tissue grown on solid media and 80% for tissue from lines grown in liquid media, less than 1% of viable protoplasts formed cell colonies. No colonies formed from protoplasts from solid-medium-grown type B lines. Most colonies in group A and B started from symmetrical divisions of cells, but type B lines produced bigger colonies, and the only embryogenic line recovered was from a type B line (in liquid culture on TCI's). This embryogenic culture eventually adopted the characteristics of a type B line and could not produce mature somatic embryos when treated with ABA (Egertsdotter and von Arnold 1993). However, Nagmani *et al.* (1987) report the first division of *Picea abies* protoplasts as asymmetrical, which resulted in a polar structure rather than a cell cluster.

Picea glauca protoplast cultures had the same tendencies as *Picea abies* - the lines which were the most embryogenic tended to be the least successful for protoplast work (Attree *et al.* 1989a) Though protoplast viability was 75-85%, plating efficiencies were only 5-9% (plating efficiency equals 100 times the number of viable colonies over the intial plating density). After 8 days, the original type of cell (small, densely cytoplasmic) had divided into a cell cluster with elongated vacuolate suspensor-type cells attached. Only normal-looking stage 3 embryos matured to stage 4. Stage 2 embryos that did not mature to stage 3 embryos converted into nonembryogenic callus or produced root primordia. Somatic embryos developed from protoplasts directly, with protoplasts first developing cell walls, then dividing symmetrically until a colony formed. Suspensor cells emerged from the colonies and enlarged until a proper suspensor was formed (Attree *et al.* 1989a). However, like *Picea abies*, other researchers have reported the first division of *Picea glauca* protoplasts to be unequal (Hakman and Fowke 1987).

In another experiment using 10 lines of *Picea glauca*, Attree *et al.* (1989b) found that all cell lines regenerated somatic embryos from protoplasts. The somatic embryos that developed began as cell colonies from which several suspensor cells arose on 99% of the colonies 6-8 days after isolation (from newly established cultures) or 12 days after isolation (for older cultures). Thus, individual cell colonies developed into individual somatic embryos after the initial asymmetrical division (Attree *et al.* 1989b, Fowke *et al.* 1990, Tautorus *et al.* 1990b).

As found in the previous two species, the most embryogenic lines of *Picea mariana* yielded the fewest protoplasts (Tautorus *et al.* 1990b). In some lines, protoplasts developed cell walls, then began symmetrical divisions. Cell colonies could either begin producing suspensor cells or continue disorganized growth before forming embryos. Over 99 % of the colonies from these lines produced embryonal mass-suspensor structures. In other lines, less than 1% of the protoplasts produced colonies and somatic embryos. These colonies and embryos were small, composed of only a few cells, and did not survive more than 2 weeks. A low percentage of protoplasts from one of the poorly responding lines produced a green, non-embryogenic callus (Tautorus *et al.* 1990b).

In contrast to the species mentioned above, *Pseudotsuga menziesii* protoplasts appeared to follow a different route to somatic embryogenesis. Protoplasts formed cell walls and underwent symmetrical division to form colonies, with proembryos and globular embryos developing after the growing colonies were transferred to media containing ABA (Gupta *et al.* 1988). However, proembryo formation appeared to result from an asymmetrical division of a single cell of a colony rather than by suspensor cell emergence from a cell cluster (Gupta *et al.* 1988).

Protoplasts of *Pinus banksiana* and *Abies alba* divided symmetrically, giving rise to cell clusters which produced suspensor cells within 2 weeks (Lang and Kohlenbach 1989, Tautorus *et al.* 1990b). However, the first division of protoplasts from another *Pinus* species, *Pinus caribaea*, was asymmetrical and produced a small, densely cytoplasmic cell atop an elongate, more vacuolate cell (Laine and David 1990).

In each of the three *Picea* species described, the most embryogenic lines yielded the fewest protoplast. In addition, differences in development were apparent, with both direct and indirect development of somatic embryos from individual protoplasts, depending on the nature of the first division.

2.3.10 Somaclonal variation

Because only elite genotypes are cost-effective for somatic embryogenesis systems, and the rotation age of conifer trees is so long, genotypic fidelity of cloned trees is important. Somaclonal variation is the inherited change in the genes of somatic cells. Three different methods of determining somaclonal variation were performed on embryogenic cultures of three different species of *Picea*. Nuclear DNA content of embryogenic cultures and plantlets regenerated from embryogenic cultures of *Picea abies* were the same as that isolated from seedlings, as determined by flow cytometry (Mo *et al.* 1989). Isozyme analysis of the parent lines and subclone embryogenic cultures as well as cotyledonary somatic embryos of *Picea glauca-engelmannii* demonstrated a high degree of genetic stability (Eastman *et al.* 1991). Random amplified polymorphic DNA (RAPD) analysis of somatic embryos of *Picea mariana* showed no genetic variation within three embryogenic cell lines (Isabel *et al.* 1993). However, in a later study using *Picea glauca*, RAPD analysis detected somaclonal variation in variagated plants (Isabel *et al.* 1996).

Though disorganized callus cultures are generally considered to be less stable than the more organized embryogenic cultures, long-term callus cultures of *Pinus taeda* were relatively stable (Franklin *et al.* 1989). Testing of nuclear DNA content by Feulgen cytophotometry indicated that ploidy levels rose from predominantly diploid cells in the initial stem explant to cells with higher ploidy levels during callus initiation. However, established callus cultures of over 18 weeks showed a reduction in ploidy levels to a diploid nuclear DNA distribution (Franklin *et al.* 1989).

2.4 Larch embryogenesis

2.4.1 Zygotic embryo development

Zygotic embryo development in *Larix* follows the normal pattern of development for conifers (Schopf 1943, Chowdhury 1962, Singh 1978, Owens and Molder 1979c, von Aderkas *et al.* 1991). After fertilization of the female egg cell with the male pollen nucleus (syngamy), free nuclear division results in four nuclei which migrate to the base of the archegonium, where another division occurs. The resulting eight nuclei form two tiers of four nuclei: a primary upper tier (which remains open to the egg cell cytoplasm) and a primary embryonal tier (which becomes enclosed). The primary embryonal tier divides into two embryonal tiers, forming a total of three tiers. The middle tier divides, giving rise to a 16 cell proembryo about one week after fertilization (usually during the first two weeks of June) (Owens and Molder 1979c, von Aderkas *et al.* 1991).

The open tier and the tier immediately above it (the rosette tier) do not divide or develop any further. The next tier, the primary suspensor, elongates, pushing the embryonal mass into the corrosion cavity and ending the proembryo stage (von Aderkas *et al.* 1991). The uppermost tier, the embryonal tier, divides to give rise to both the embryonal tube cells, which elongate and form the secondary suspensor, and the embryonal mass cells . While in some species of *Pinaceae* dysfunctional suspensor cells can become meristematic and give rise to rosette embryos . this does not happen in *Larix* (von Aderkas *et al.* 1991). Simple polyembryony is common, but true cleavage polyembryony does not occur (Owens and Molder 1979c). One cell in the embryonal layer typically outgrows the others and functions like an apical cell until it is subsumed into the enlarging embryonal mass in a type of delayed cleavage (Schopf 1943, von Aderkas *et al.* 1991). Schopf (1943) considered this to be an advanced character in conifers.

In the embryonal mass, cell division occurs in all planes to form a club-shaped embryo by the end of June. Division is predominantly transverse at the distal end near the suspensor (von Aderkas *et al.* 1991). By mid-July, divisions become less random in the embryonal mass and distinct meristematic regions appear. The interior cells become the focal zone once division ceases to be completely random (von Aderkas *et al.* 1991). In late embryogeny, the intercalary region of transverse division develops into the root cap, and just distal to that, a root meristem begins to form. A procambium and a stele promeristem develop (von Aderkas *et al.* 1991). Surface cells on the shoulders of the embryo divide to form the cotyledons (Owens and Molder 1979c). Cotyledons arise, an epidermis forms and a small cluster of relatively inactive cells is evident between the cotyledons where the shoot meristem is to form (von Aderkas *et al.* 1991). The number of cotyledons formed can vary from three to eight, with an average of six (Chowdhury 1962). The embryos enlarge for another month and are mature by mid-August (Owens and Molder 1979c).

A comparison of the stages of development of zygotic and somatic embryos is briefly presented here. There are three stages of development in zygotic embryos: 1. proembryo - all stages before the elongation of the suspensor (no corresponding stage in somatic embryogenesis), 2. early embryo - after suspensor elongation and before the formation of the root meristem (stages 1 and 2 in somatic embryogenesis), and 3. late embryo - root and shoot meristem formation and subsequent events leading to a mature embryo with cotyledons (stage 3 in somatic embryogenesis) (Singh 1978, Hakman and von Arnold 1988, von Aderkas *et al.* 1991). The stages of development for somatic embryos, as outlined by Hakman and von Arnold (1988) are:

stage 1 - embryonal masses plus translucent suspensors,
stage 2 - a prominent, opaque, enlarged embryonal mass with a smooth surface subtended by a suspensor,
stage 3 - somatic embryos with cotyledonary primordia clustered around a central meristem.

Other researchers have extended the stages of development for somatic embryos to stage 4 (Dunstan *et al.* 1988):

stage 4a - distinct, partly elongate cotyledons, surrounding a central meristem.
stage 4b - green appearance, elongating cotyledons and hypocotyl, rudimentary radicle development.

Other authors have defined exceedlingly precise stages of development, including 4 substages of stage 3 and nine substages of stage 4 (Flinn *et al.* 1991), but these are not in general use.

2.4.2 Somatic embryo development

2.4.2.1 Initiation of embryogenic tissue

In larch, embryogenic tissue has been initiated from immature zygotic embryos in *Larix decidua, Larix leptolepis, Larix x eurolepis, Larix x leptoeuropaea* and *Larix occidentalis,* with small numbers of somatic embryos produced, and a few established in soil (von Aderkas *et al.* 1991, Thompson and von Aderkas 1992, Lelu *et al.* 1993a,b). Haploid cultures (gynogenic) exist of *Larix decidua, Larix leptolepis, and Larix x eurolepis* (von Aderkas *et al*. 1991) and a few haploid plants have been produced (von Aderkas and Bonga 1993). Once an embryogenic line is initiated, proliferation comes from cleavage of a meristematic centre (the embryonal mass) which expands differentially and produces two sets of embryonal tubes in a kind of budding process (von Aderkas *et al.* 1991). Hence, there is no similarity between zygotic proembryo development and the early stages of somatic embryogenesis.

The optimal stage of development for zygotic embryo explants for embryogenic tissue induction varies. Precotyledonary zygotic embryos of *Larix x leptoeuropaea* were the best for initiation (Lelu *et al.* 1993a). However, later stages of development do yield embryogenic tissue, such as mature zygotic embryos of *Larix decidua* and cotyledons and needles from plantlets derived from somatic embryos of *Larix x leptoeuropaea* (Lelu *et al.* 1993b).

2.4.2.1.1 Effect of medium and plant growth regulators on the rate of initiation

For *Larix* species, the proliferation media is often modified from the initiation medium. *Larix decidua* , *Larix leptolepis, Larix x eurolepis* and *Larix x leptoeuropaea* require 9.05 µM 2,4-D and 1.78-3.55 µM BA for proliferation, the same as initiation (Klimaszewska 1989a, Becwar *et al.* 1990, von Aderkas *et al.* 1990, Lelu *et al.* 1993a) though other authors have reported reducing or eliminating plant growth regulators from the initiation media for the proliferation media (Cornu and Geoffrion 1990). For *Larix occidentalis* and *Larix x leptoeuropaea*, initiation requires 9 µM 2,4-D and 2.2 µM BA, but proliferation requires only 9 µM 2,4-D (Thompson and von Aderkas 1992, Lelu *et al.* 1993a).

In some instances, the type of media used had an effect on initiation rates. *Larix leptolepis* and *Larix x eurolepis* had the same intiation rate on MSG (Murashige and Skoog medium plus glutamine) but *Larix leptolepis* had a lower initiation rate on 1/2 LM (Bonga *et al.* 1995). Ammonium nitrate concentrations greater than 20 mM had a negative effect on initiation but the addition of glutamine had no effect on intiation rates, which ranged from 60-93% (Thompson and von Aderkas 1992).

In *Larix* species, initiation can occur from the upper portion of the suspensor, the base of the embryo and the mid-portion of pre-cotyledonary embryos, and from the hypocotyl and cotyledons of mature embryos (Bonga *et al.* 1995). Suspensor cells of the zygotic embryo explant are often the origin of embryogenic tissue of *Larix* (von Aderkas *et al.* 1991). Apparently, large isolated elongate vacuolate cells could divide asymmetrically and give rise to embryonal masses plus suspensors. Further proliferation was by cleavage polyembryony (Bonga *et al.* 1995). Small, densely cytoplasmic cells and single, elongate, vacuolate cells were initiated from the explants of *Larix decidua* (Cornu and Geoffrion 1991). Isolated elongate cells formed multiple cross walls to form a file of cells. These cells disassociated to form the single, small cells (Cornu and Geoffrion 1991).

2.4.2.2 Maintenance of embryogenic tissue

2.4.2.2.1 Variation in embryonal mass organization

The focus of most of the research into *Larix* somatic embryogenesis systems has been the yield of somatic embryos from embryogenic tissue. The embryogenic tissue itself has rarely been examined microscopically. However, several papers have reported differences within embryogenic lines of larch.

Embryogenic cultures of *Larix* were described in detail by Bonga *et al.* (1995). The cultures varied in colour, from white to yellow, and often included anthocyanins. Embryogenic lines were composed of embryonal masses with suspensors (composed of embryonal tube cells) which proliferated by cleavage polyembryony. Two types of embryonal masses were found: 1. smooth epidermis and subtending suspensor (polar) or, 2. irregular embryonal mass surrounded by single suspensor cells (solar) (Bonga *et al.* 1995), similar to solar type of embryonal mass in *Picea abies* (Jalonen and von Arnold 1991).

In *Larix occidentalis* embryogenic tissue, solar types of embryonal masses appeared in cultures which previously contained the polar types (Thompson and von Aderkas 1992). Putting the cultures in liquid suspension favoured the proliferation of polar embryonal masses. Cultures could lose their embryogenicity without changing their outward appearance, but if examined microscopically showed either long thin polarized structures with no embryonal mass (no small densely cytoplasmic cells) or mixtures of single elongate vacuolate cells and small-cell clusters. The outward appearance of this second type of tissue aggregate was smooth, with no protrusions, in contrast to the filamentous appearance of embryogenic tissue composed of embryonal masses and suspensors. All types of embryonal masses could be found in an embryogenic line, even in the same Petri dish (Thompson and von Aderkas 1992). Other anomalies were found, such as cells that divide internally (Bonga *et al.* 1995).

Two lines of *Larix x. leptoeurolepis* were slow-growing and formed globular structures that either died or proliferated nonembryogenic callus. Another line slowly degenerated into clusters of spherical cells, loose suspensor cells and a few somatic embryos (Klimaszewska 1989a). Unfortunately, none of these lines were examined microscopically.

Thirteen lines of *Larix x eurolepis* were examined and found to consist of stage 1 somatic embryos, single elongate cells and cell clusters (Klimaszewska 1989b). Ten of the lines were fast-growing while two were slower growing and produced globular structures. One line gradually degenerated and became mostly single cells, both isodiametric and elongate, and a few stage 1 embryos (Klimaszewska 1989b).

In an attempt to discern the fundamental differences between embryogenic and nonembryogenic lines of *Larix*, the differences in microtubule arrangement in protoplasts of embryogenic and nonembryogenic lines of *Larix x eurolepis* were studied (Staxen *et al.* 1994). Within 3 days of isolation, protoplasts from embryogenic lines had parallel microtubule arrays and started to elongate. Protoplasts from nonembryogenic lines had a random arrangement of microtubules for a longer period, but protoplasts from both embryogenic and nonembryogenic lines formed preprophase bands. However, at telophase, protoplast-derived cells from nonembryogenic lines had normal phragmoplasts, while protoplast-derived cells from embryogenic lines had Y-shaped phragmoplasts which resulted in 2 nucleated cells and one enucleate cell (Staxen *et al.* 1994).

The fact that solar types of *Larix occidentalis* can suddenly appear in polar type lines (Thompson and von Aderkas 1992), and that type A and type B lines of *Picea abies* can transmute (Jalonen and von Arnold 1991) indicate that the different types of embryonal masses found in different lines may not be the result of somaclonal variation. The discovery of proteins which can trigger embryogenesis in nonembryogenic lines (Egertsdotter *et al.* 1993) suggest that physiological adaption and modification of the culture environment by the cultures themselves may partly explain the differences in behaviour amongst embryogenic lines.

2.4.2.3 Effects of abscisic acid and osmoticum on yield and quality of somatic embryos

As with other conifer species, adjustments to abscisic acid levels, sucrose concentrations, carbohydrate type, gelling agents and type of nitrogen have improved the number of maturing somatic embryos of *Larix* and their morphology (Lelu *et al.* 1993b).

ABA was not an absolute requirement for maturation of some somatic embryos of *Larix x eurolepis* matured on media without ABA (Klimaszewska 1989a). Embryogenic tissue of *Larix decidua* was transferred to medium with plant growth regulators at 10% the initial levels. This produced embryogenic tissue which needed PEG but no ABA to mature the embryos. More normal mature embryos were produced if the cultures were kept in the dark for 3 weeks (Bonga *et al.* 1995). Though somatic embryos of some lines of *Larix x eurolepis* mature continuously without ABA or any treatment (Bonga *et al.* 1995), the use of ABA is normally required to obtain mature *Larix* somatic embryos.

In general, ABA is a requirement for maturation (ranging from 0.025 μM to 60 μM) and increasing the osmoticum (sugars, sugar alcohols, or neutral polymers (PEG)) enhances the effectiveness of the ABA (Bonga *et al.* 1995). The combination of ABA and osmoticum often improves the maturation of somatic embryos, but in some cases the high osmotic level of the media has more of an effect than the ABA content.

Though some somatic embryos of *Larix x eurolepis* did mature on medium without plant growth regulators, the yield of mature embryos increased greatly on medium with 0.38 μM ABA and 0.93 μM kinetin. Thirty five plantlets from these mature embryos were established in the soil (Klimaszewska 1989b).

Embryogenic lines of *Larix occidentalis* on media with 10, 60, or 80 μM ABA did not produce embryos (von Aderkas *et al.* 1995). Some lines did not produce embryos on any media while others produced the most embryos on 0.025 μM ABA or on 40 μM ABA. Other lines produced embryos only in the abscence of ABA, with or without 0.9 μM 2,4-

D. The use of charcoal plus or minus ABA did not give conclusive results. The embryos matured on 0.025 µM ABA produced 1.1 - 3.2 mature embryos per gram of embryogenic tissue (fresh weight), which in one line converted into plantlets at a rate of 71% (Thompson and von Aderkas 1992). Apart from this one productive line, all other lines produced embryos which failed to elongate a root (Thompson and von Aderkas 1992). Raising the osmotic level with PEG or melibiose along with 20 µM ABA increased the quality rather than the quantity of embryos produced (von Aderkas *et al.* 1995).

Haploid and diploid lines of *Larix decidua, Larix leptolepis, Larix x eurolepis* and *Larix x leptoeuropaea* matured after transfer to plant growth regulator-free medium with charcoal and 99 µM sucrose for one week followed by transfer to media with 40 µM ABA, 1 µM BA and 99 µM sucrose (Duchesne *et al.* 1993). This treatment yielded 0-49 mature embryos per 100 mg of embryogenic tissue (fresh weight) (Duchesne *et al.* 1993). Alternatively, the tissue was transferred onto 0.44 µM BA and 1.89 µM ABA (Bonga *et al.* 1995). Somatic embryos of *Larix decidua* never showed advanced development without the use of plant growth regulators (Cornu and Geoffrion 1990). In addition, the use of light exposure, gelrite rather than agar, and PEG increased the number of maturing somatic embryos (Cornu and Geoffrion 1990).

In *Larix x leptoeuropaea* cultures, sucrose at 200 µM was superior to 100 µM in improving maturation of somatic embryos, but ABA concentrations did not affect the quantity of the germinated somatic embryos. The maturation treatment had an effect on the suitability of the plantlets for transfer to soil, based on visual assessment. The 4 weeks on ABA followed by 1 week on ABA-free media treatment resulted in the lowest number of plants transferred. The most vigourous plants came from the 3 week treatment on 20, 40 or 60 µM ABA. Approximately 92% of the embryos germinated, and 74-80% of these converted into plantlets (Lelu *et al.* 1993b).

The result of the interaction of ABA and osmoticum on the maturation of somatic embryos is complex. At 100 µM sucrose, increasing ABA levels resulted in higher numbers of somatic embryos of *Larix x leptoeuropaea* while at 200 µM sucrose, increasing the ABA from 40 to 60 µM lowered the number of embryos produced. In highly embryogenic lines, embryogenic potential varied from 40 to 403 embryos produced per gram of embryogenic tissue, while in a poorly reproducing line it varied from 0 to 17. This variation in production between embryogenic lines has also been noted in *Picea abies* and *Picea glauca-engelmannii* (Lelu *et al.* 1993a).

Embryogenic tissue of *Larix x leptoeuropaea*, when transferred to maturation media continued proliferation at 100 µM sucrose but slowed growth at 200 µM sucrose. Cleavage of embryonal masses stopped on 400 µM sucrose. The addition of ABA to the medium did not change the rate of cleavage and proliferation (Lelu *et al.* 1993a). Of the somatic embryos of *Larix x leptoeuropaea* produced, precocious germination occurred on 100 µM sucrose, and on 200 µM sucrose but on 200 µM sucrose hypocotyl elongation did not occur. On 400 µM sucrose the embryos arrested development at the precotyledonary stage. ABA at any concentration resulted in embryos of good appearance and inhibited precocious germination. However, at 400 µM sucrose plus any concentration of ABA the embryos produced failed to mature beyond the precotyledonary stage(Lelu *et al.* 1993a).

As with other conifer species, ABA can act synergistically with other plant growth regulators. The highest yield of mature embryos of *Larix x leptoeuropa* was obtained after 3 weeks on MSG with 0.38 µM ABA and 0.93 µM kinetin, followed by 6 weeks on MSG without plant growth regulators. Of the seven lines investigated 4 lines produced embryos with roots and shoots only at low ABA concentrations (best at 0.025 µM) or no ABA. Transferring to charcoal medium for one week prior to ABA had no effect, neither did using PEG 1450 or melibiose along with the ABA treatment though using ABA+PEG promoted germination. Embryogenic lines produced 30 mature embryos maximum per gram fresh weight. Cleavage polyembryony continued on the maturation medium, meaning synchronization of maturation will be difficult (Bonga *et al*. 1995).

The most mature embryos from *Larix x eurolepis* were produced on MSG with 0.38 µM ABA, 0.93 µM kinetin and 58 µM sucrose for three weeks then 2 subcultures onto media with no plant growth regulators. Eighty percent of *Larix x eurolepis* embryos produced roots and secondary needles, though some plantlets were abnormal, with fused cotyledons, or suppressed root and or shoot meristems. Adjusting the media had no effect on the number of normal/abnormal plantlets produced (Klimaszewska 1989a).

The length of exposure to ABA can affect somatic embryo quality . Leaving the somatic embryos of *Larix x leptoeuropaea* on the maturation media for four weeks rather than three weeks resulted in lowered germination frequency from 92-96% down to 70-75%, lowered plantlet frequency and slower epicotyl elongation. Radicles growing beneath the surface of the medium had reduced elongation. Leaving larch embryos on ABA medium too long could raise the level of ABA inside the embryos so high that release into germination was impossible or reluctant, with adverse effects on plantlet development (Lelu *et al.* 1993b, Lelu *et al.* 1995). Not only were few mature *Larix .x leptoeuropaea* somatic embryos produced, their morphology was usually abnormal, displaying precocious germination, as well as fused cotyledons and lack of shoot and root meristems (Lelu *et al.* 1993b).

The addition of ABA to the maturation medium affects the quality of somatic embryos produced as well as the number of somatic embryos produced. Cultures of *Larix x* *leptoeuropaea* on maturation medium containing either 0 or 60 µM ABA began to show differences after 2-3 weeks. On media without ABA, somatic embryos remained stubby and were largely made up of expanded highly vacuolate cells. This indicated that growth was likely due to cell expansion rather than cell division. After 4 weeks the hypocotyls began to elongate and precocious germination was common. These embryos yielded lower amounts of total protein compared to somatic embryos on medium with 60 µM ABA. Somatic embryos matured on media with ABA were composed of densely cytoplasmic cells with a high rate of cell division. Storage proteins were only found in embryos on ABA (Gutmann *et al.*, in press).

A few papers have investigated the effects of desiccation of the mature somatic embryos on subsequent germination rates (Livingston *et al.* 1992, Dumont-BéBoux *et al.* 1996). Somatic embryos of *Larix x leptoeuropaea*, after one week on charcoal medium followed by three or four weeks on medium with 1 µM IBA and 40 or 60 µM ABA had germination rates of 92-96% and 72% conversion to plantlets (3 week treatment) and 70-75% germination (4 week treatment), while mature embryos which had the same ABA treatments followed by desiccation under high relative humidity conditions had germination rates of 73-100% and conversion rates of 69% (3-week ABA treatment) or 89-92% conversion rates (4-week ABA treatment)(Lelu *et al.* 1995). The water relations of a somatic embryo changed with development (Livingston *et al.* 1992, BéBoux*et al.* 1996). For both somatic and zygotic embryos, the hypocotyls could resist dehydration better than the roots, and these differences were more pronounced in zygotic embryos as compared to somatic embryos (Livingston *et al.* 1992).

2.4.2.4 Protoplast cultures of *Larix*

Protoplast isolation of *Larix x eurolepis* resulted in the formation of embryonal mass and suspensor cells either from the first division, which was asymmetrical (Klimaszewska 1989a, Staxen *et al*. 1994) as in protoplasts of haploid *Larix decidua* (von Aderkas 1992) or in the formation of cell colonies from a symmetrical first division (Klimaszewska 1989a, Charest and Klimaszewska 1994). When examining the microtubular organization in protoplasts, Staxen *et al.* (1994) noted 3-cell structures consisting of small cells and elongate cells after 3 days of culture. These developed into embryonal masses plus suspensors. Protoplasts from nonembryogenic cultures had symmetrical first divisions (Staxen *et al.* 1994). In the study by Klimaszewska (1989a), embryogenic cultures of two lines of *Larix x eurolepis* were used as sources of protoplasts. Line 1 produced few somatic embryos and was a heterogenous mix of cell clusters, single elongate cells and early somatic embryos. Line 2 was very embryogenic and was a mixture of early somatic embryos, and a few elongate cells and cell clusters. All protoplasts of Line 1 divided symmetrically and gave rise to microclusters which occasionally produced elongate cells, and a few somatic embryos. Half the divisions of Line 2 protoplasts were asymmetrical, resulting in a small cell subtended by an elongate cell. Within 3-4 weeks these 2-cell structures had developed into somatic embryos. The cells which would divide asymmetrically were identified prior to division as elongate and containing 1-3 nuclei (Klimaszewska 1989a).

Protoplasts of hybrid *Larix* lines exhibited direct development of somatic embryos, and the more embryogenic the line, the more likely its protoplasts would divide asymmetrically. This indicates that, despite the effects of communal extracellular embryogenic proteins (Egertsdotter *et al.* 1993), embryogenic potential resides in the individual cell, rather than in the aggregate as a whole.

2.4.2.5 Somaclonal variation in *Larix* cultures

In *Larix* specifically, there have been a number of studies on the incidence of somaclonal variation and changes in ploidy level *in vitro*, with mixed findings. Two papers which studied the ploidy levels of *Larix* species *in vitro* found stability in ploidy levels, while two papers which studied ploidy levels and mitochondrial DNA variation found incidences of genetic instability. Ploidy levels in adventitious shoots, callus, and shoots regenerated from callus of *Larix x eurolepis* kept *in vitro* for over a year showed no change in ploidy levels compared to mother-plant tissue, as determined by flow cytometry analysis (Wyman *et al.* 1992). Aneuploid lines from spontaneously doubled haploid cultures of *Larix decidua* remained stable for 6 months, as determined by repeated squashes (von Aderkas *et al.* , in press).

However, in earlier studies of haploid lines of *Larix decidua*, polyploidism ranging from haploid to tetraploid counts were found, as well as aneuploidy (von Aderkas and Anderson 1993, Pattinavibool *et al.* 1995). In addition, using Southern hybridization analysis, differences in mitochondrial DNA hybridization patterns were found between embryogenic cell cultures and seedlings or seed source trees of *Larix leptolepis, L. decidua,* their reciprocal hybrids and *Larix x eurolepis* (DeVerno *et al.* 1994). Hence, the possibility of somaclonal variation in embryogenic cultures of *Larix* species cannot be discounted.

Chapter III: Organization of tissue aggregates and phenotypic variation in embryonal masses

3.1 Introduction

Somatic embryogenesis of larch species provides the possibility of clonally propagating improved plantlets from selected elite trees, preserving the non-additive genetic variance which would be lost by sexual propagation. Many other conifer species have responded to somatic embryogenesis (Tautorus *et al.* 1991, Attree and Fowke 1993) and plantlets have been established in the field. However, despite the continued improvements in protocol and outplanting successes, there is a sharp decline in the numbers of somatic embryos which progress through each stage of development in every somatic embryogenesis system. For instance although the number of stage 1 (Hakman and von Arnold 1985) somatic embryos per gram of embryogenic tissue was estimated at 14.2 x 10^3 for *Picea glauca* (Kartha *et al.* 1988), the number of embryos which stop cleaving and mature to stage 3 was estimated on a per gram of fresh weight basis at 23 for *Picea glauca* (Kartha *et al.* 1988), 80 for *Picea sitchensis* (Krogstrup *et al.* 1988) between 120 (Jalonen and von Arnold 1991) and 700 (Becwar *et al.* 1991) for *Picea abies* . The rest of the somatic embryos on maturation medium died and degenerated. Discovering why so few somatic embryos mature could lead to improvements in the maturation protocols and improve the efficiency of the tissue culture systems, making it more cost effective.

Embryogenic lines of several species have been examined to determine the types of somatic embryos present. Several papers (Nagmani and Bonga 1985, Gupta and Durzan 1986, Gupta and Durzan 1987a, Lu and Thorpe 1987, Rohr *et al.* 1989) characterized typical stage 1 embryos, with long, translucent suspensors and smooth embryonal masses with the underlying assumption that their lines were uniformly composed of this one type of somatic embryos. However, other researchers have discovered that embryogenic lines of different species such as *Picea abies, Picea sitchensis,* and *Picea mariana* were either homogenous lines of stage 1 embryos, heterogenous lines composed of stage 1 embryos plus cell clusters and single cells, or both (Jalonen and von Arnold 1991, Bozkov 1995, Klimaszewska 1995). In *Picea abies,* three types of lines were identified: polar somatic embryos (an embryonal mass subtended by a suspensor), solar embryos (an embryonal mass surrounded by single elongate cells), or disorganized clusters and cells (Jalonen and von Arnold 1991). The polar and solar lines could produce mature somatic embryos but the disorganized lines could not. These differences in morphology and embryogenic capacity suggested that the ability of somatic embryos to respond to the maturation protocols may be determined by their initial morphology.

This section investigates the effect of early embryonal morphology on embryo maturation. The purpose of these experiments was to determine why the majority of somatic embryos fail to mature, either due to death or continued cleavage. The line 2086, an embryogenic line of *Larix x eurolepis* (von Aderkas *et al.* 1990) which required neither osmotic nor ABA treatments to mature somatic embryos presented differences in the external appearances of embryogenic tissue aggregates . Some of the aggregates appeared filamentous and full of stage 1 embryos,while other aggregates appeared smooth with only a few or no embryos apparent on the surface. This suggested that different types of aggregates existed. This chapter describes the internal composition of the aggregates of line 2086 and the types of embryos found, the relationship between the internal composition of an aggregate and its ability to produce mature somatic embryos, and the developmental fates of the different types of immature somatic embryos.

3.2 Materials and methods

3.2.1 Culture maintenance

The *Larix x eurolepis* line 2086 originated from an immature zygotic embryo of a hybrid between *Larix leptolepis* (maternal parent) and *Larix decidua* (paternal parent)(von Aderkas *et al.* 1990). The embryogenic tissue was initiated on 1/2 Litvay's medium (Litvay *et al.* 1981) supplemented with glutamine and 0.45 µM 2,4-D. Line 2086 was routinely cultured every 2-3 weeks onto 1/2 strength Litvay's medium supplemented with 59 µM sucrose, 4 g/l gelrite, 1 g/l casein hydrolysate, 3422 µM glutamine without plant growth regulators. The medium was adjusted to pH 5.6-5.8 before autoclaving. The glutamine was filter sterilized using a 22 µm filter and was added after the medium had been autoclaved in an Agarmatic (New Brunswick Scientific Co. Inc.). The medium was dispensed by 20 ml aliquots into 100 x 15 Petri dishes (Fisher) and allowed to cool to room temperature before storage at 4 ^{o}C. Four aggregates of embryogenic tissue were cultured in each Petri dish and were individually marked. The plates were kept in a growth chamber at 22 ^{o}C with a 16 hour light/8hour dark photoperiod at 36 µmol/m^2/s photosynthetically active radiation. In the absence of plant growth regulators, mature somatic embryos of line 2086 germinated precociously and asynchronously.

3.2.2 Resin-embedding and sectioning

Individual structures, either embryonal masses or more advanced structures, were separated from the tissue aggregates before fixation. Usually, 10-25 embryonal masses or 5-10 more advanced structures were fixed at a time in each vial. At least 10 specimens of each type of embryonal mass and stage of development were embedded, sectioned and examined. The tissue was fixed for 2-3 hours in 2.5% glutaraldehyde in 0.075M phosphate buffer. Post fixation, the tissue was washed in 0.075M phosphate buffer three times, for 10 minutes each. The tissue was then fixed for 2-3 hours in 1% osmium tetroxide in 0.075M phosphate buffer followed by a short buffer wash. The tissue was then dehydrated for 10 minutes in 20, 40, 60 and 80 % acetone followed by 3 changes of 100% acetone for 10 minutes each. The tissue was then infiltrated by 30% Spurr's resin in acetone for 10-12 hours and this was followed by infiltration by 75% then 100% Spurr's resin in acetone for 2 hours each. The tissue was finally embedded in 100% Spurr's resin overnight at 70 ^{o}C (Karnovsky 1965, Glauret 1974, Roland and Vian 1991). Spurr's resin (Spurr 1969) was prepared from ERL 4206 (vinyl cyclohexene dioxide), D.E.R. 736

(diglycidyl ether of polypropylene glycol), NSA (nonenyl succinic anhydride, and S-1 (DMAE - dimethyl aminoethanol), (Marivac Ltd, Halifax N.S.).

The resin blocks were sectioned at 0.3 - 0. 8 µm using glass knives on either a Reichert-Jung Ultracut E microtome or a Reichert Om U2 microtome, both from American Optical Instrument Company, Buffalo, N.Y. The sections were floated on a drop of water on a slide and placed on a hot plate until the water evaporated. The sections were stained with Richardson's stain (1 g borax plus 1.5 g methylene blue in 100 ml water added to 0.5 g azure B in 100 ml, mixed and filtered) and the slide was placed on the hot plate for 25 seconds. The slide was rinsed with water and allowed to air dry before being mounted in Permount (Fisher). The sections were examined under a light microscope. Photographs were taken using Tmax 100 film or Kodak technical pan film (100 ASA).

3.2.3 Determining the internal composition of aggregates

Individual aggregates (subcultured masses of embryogenic tissue) were assigned a Petri dish number and a position marker to identify their position in the Petri dish. A portion of each aggregate was removed, placed on a microscope slide, mixed with a drop of water and examined under a light microscope. The entire tissue sample on the microscope slide was examined and the relative abundance of the 6 different types of embryonal structures was determined for each aggregate. For a period of 12 weeks at each subculture interval (6 subculture intervals), all green structures were removed individually from each aggregate and identified as normal (closely resembling mature zygotic embryos) or misshapen (having proportions different to that of zygotic embryos). At the end of the experimental period, the total number of normal and misshapen embryos were tallied for each aggregate.

Using the same embryo production data, the aggregates were classifed according to the most abundant type of embryonal structure present (table 1) and then reclassified according to the most advanced type of embryonal structure present (table 2). The most advanced type of embryonal structure was considered to be SEMLS, followed by REMLS, REMLLS, REMSS, REMST and the least organized to be MC, based on how closely the embryonal mass and the suspensor resembled those of zygotic embryos. These were termed embryonal structures since microclusters did not have a suspensor and therefore could not be considered an immature embryo.

For both Tables 1 and 2, the 12 week total embryo prodution was analysed using the nonparametric Kruskal-Wallis procedure for Statview 512$^+$ (Abacus Concepts, USA) due to nonnormality in the data and uneven aggregate numbers. The percentage of normal somatic embryos was analysed by transforming with the angular transformation (arc sine (square root(x)) for proportions (Snedecor and Cochran 1980) and using the ANOVA procedure of Staview 512+, with a significance level of p= 0.001. Data entries which recorded 0 normal embryos were disregarded when calculating the percentage of normal embryos.

3.2.4 Developmental fate of embryonal structures

Each aggregate of line 2086 was pulled apart with forceps under a binocular microscope (Wild). Every individual embryonal structure was removed from the aggregates, identified as either SEMLS, REMSL, REMLLS, REMSS, REMST, or MC and placed onto medium in a corresponding fresh Petri dish. Each fresh Petri dish was gridded by affixing a 5 x 5 paper grid onto the bottom. A single embryonal structure was placed in each square of a grid. Every aggregate (120 in total) was examined and all recognizable embryonal structures were utilised. Therefore, the total number of embryonal structures in each of the six categories differs and reflects the relative abundance of each of the 6 types of embryonal structures in the entire line. This procedure was repeated twice for a total of three replications.

In preliminary experiments, isolated embryonal structures did not develop any further after five weeks following isolation; therefore, the developmental fate of the embryonal structures in this experiment were assessed five weeks after isolation. The embryonal structures were examined and assigned to one of five developmental fates. The five developmental fates were:

a. developmentally arrested (stalled): no apparent growth or development,
b. proliferation: a proliferation of immature embryonal masses,
c. nodule: a roughly spherical red structure with no epidermis, no cylindrical hypocotyl or cotyledons,
d. abnormal: a mature or germinating somatic embryo which did not resemble a normal zygotic embryo in terms of general proportions of hypocotyl and cotyledons or epidermis,
e. normal: a mature or germinating somatic embryo which resembled a zygotic embryo in terms of the general proportions of hypocotyl and cotyledons.

The tabulated data (Table 3) were analysed as a 6 x 3 contingency table using Statview 512+ with a significance level of p=0.001. To ensure no expected frequencies below 5, the number of normal and abnormal embryos and the number of nodules produced had to be pooled into a total structure category . All internal associations between different classes and categories were significant, therefore, no further pooling could be done, with the exception of REMLLS and REMST, which could be pooled. Deviations, which were the observed frequencies minus the expected frequencies, indicated whether a specific observed frequency was higher than expected if the two factors of the contingency table were independent. Thus, embryonal structures could be reported as being more likely than would be expected to become nodules or mature embryos.

To determine if the embryonal structures which proliferated produced homogenous aggregates of the original embryo type, all proliferations were squashed to ascertain if the original type of embryonal structure gave rise to different types of cleavage products (Table 4). Two types of observations were made using squashed specimens and resin-embedded sections to determine if differences in external morphologies indicated internal differences as well.

3.3 Results

3.3.1 Description of the six types of embryonal structures

3.3.1.1 Smooth embryonal mass plus long compact suspensor (SEMLS)

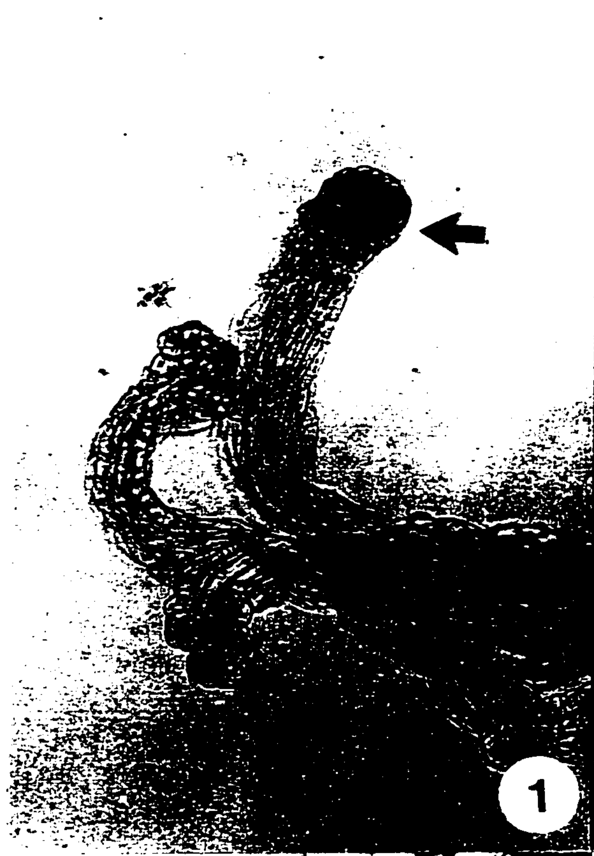
Squashed specimen (Figure 1): The smooth nature of the embryonal mass and the compactness of the suspensor was evident in squashed samples. The small, isodiametric cells of the embryonal mass did not form a sphere, but a lenticular mass; the transition between the embryonal mass and the embryonal tube cells was abrupt and linear. The nuclei of the embryonal tube cells were present in the cells immediately behind the embryonal mass and usually for a distance of 6-8 cell lengths.

Resin-embedded specimens (Figure 2): The surface of the embryonal mass was smooth, with all surface cells adhering tightly together. The first two to three surface cell layers of the embryonal mass had small isodiametric cells which were densely cytoplasmic. The nuclei were very prominent and there appeared to be no vacuole. These small densely cytoplasmic cells formed a dome over the embryonal mass. The surface cells divided both periclinally and anticlinally, and thus there was no true epidermis.

The cells forming the centre of the embryonal mass were larger than the surface cells and had both prominent nuclei and vacuoles. The cells contained multiple medium-sized vacuoles. These cells were roughly isodiametric and appeared to divide in random planes.

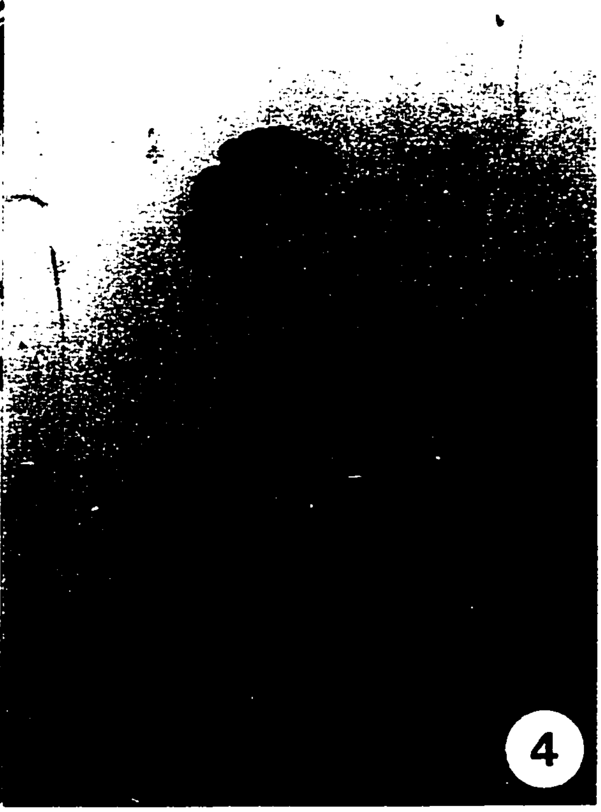
The shift in cell type from embryonal mass to suspensor was abrupt, indicating the presence of rib meristem. In line with the cessation of the dome of densely cytoplasmic surface cells, the cell type of the core of the embryonal mass switched to embryonal tube cells. The embryonal tube cells contained nuclei, though the further back from the embryonal mass the suspensor cell was, the less apparent the nucleus was, likely due to increased vacuolation of the cells. The embryonal tube cells were elongated, with the cells just behind the embryonal mass being in various stages of elongation. Intercellular spaces were not apparent in the suspensor, at least in the first 6-7 layers.

Figures 1-4: Figure 1:Smooth embryonal mass plus long compact suspensor (SEMLS): squashed specimen (88 x). Figure 2: Smooth embryonal mass plus long compact suspensor (SEMLS): median sectioned specimen (120 x). Figure 3: Rough embryonal mass plus long compact suspensor (REMLS): squashed specimen (44 x). Figure 4: Rough embryonal mass plus long compact suspensor (REMLS): cross-sectioned specimen (80 x).


1


2


3


4

3.3.1.2 Rough embryonal mass plus long compact suspensor (REMLS)

Squashed specimen (Figure 3): These embryonal masses closely resembled SEMLS embryonal masses except for the difference in appearance of the surface of the embryonal mass. The surface of the embryonal mass of a REMLS appeared rough and somewhat jagged, compared to the smooth surface of a SEMLS. The suspensor was long and cylindrical. The suspensor directly behind the embryonal mass was cylindrical and stiff, while further behind the embryonal mass the suspensor lost turgor.

Resin embedded specimen (Figure 4): The surface of the embryonal mass was irregular, often with one or two prominent multicellular protrusions. In comparison to a SEMLS, the uneven surface of the REMLS may have been due to less organized divisions in the embryonal mass of the REMLS. Unlike the SEMLS, there was no distinct dome of densely cytoplasmic cells. Rather, there was a layer (4-5 cells deep) of densely cytoplasmic, isodiametric cells which formed most of the embryonal mass. The cells of the embryonal mass became increasingly vacuolated towards the basal end of the embryonal mass. The nucleus was prominent in the cells and there appeared to be no central vacuole, but rather several small vacuoles, if there were any. These cells divided in random planes. The shift from the small densely cytoplasmic cells to embryonal tube cells was abrupt, indicating the presence of a rib meristem which produced the embryonal tube cells. The two layers of small non-elongated cells in the embryonal mass adjacent to the suspensor were larger than the cells inside the embryonal mass, had several medium-sized vacuoles and were presumed to be the rib meristem.

The embryonal tube cells were elongated and highly vacuolated. The further away the embryonal tube cell was from the embryonal mass the longer it was. Nuclei could be seen in the first 3-4 layers of suspensor cells. There appeared to be intercellular spaces between the embryonal tube cells, though the first 2-3 layers of embryonal tube cells adjacent to the embryonal mass typically adhered tightly together.

3.3.1.3 Rough embryonal mass plus long, loose suspensor (REMLLS)

Squashed specimen (Figure 5): The uneven surface of the embryonal mass and the loosely associated nature of the embryonal tube cells was evident when compared to squashed specimens of SEMLS and REMLS. The cells of the embryonal mass were small and appeared to be organized into lobes. The embryonal tube cells were long and vacuolated. Nuclei were clearly seen in the embryonal tube cells directly behind the embryonal mass. Unlike the suspensors of the SEMLS and REMLS, the embryonal tube cells on the periphery of the suspensor of the REMLLS did not appear to be fully integrated into the suspensor and were loosely connected to the rest of the suspensor.

Resin-embedded sections (Figure 6): The surface of the embryonal mass was irregular, typically with one or two protrusions. As with the REMLS, there was no dome of densely cytoplasmic cells, but rather a flattened cluster of 4-5 layers of small densely cytoplasmic cells which divided at random. The two layers of cells between the embryonal mass and the suspensor were approximately twice as big as the cells in the embryonal mass and have multiple vacuoles. These transitional cells appeared to be actively dividing, and likely gave rise to the embryonal tube cells and were considered to be the rib meristem. The embryonal tube cells were elongate. Nuclei were commonly found in cells of the first 4-5 layers. Nuclei were found less commonly in embryonal tube cells further back from the embryonal mass. The embryonal tube cells did not divide.

3.3.1.4 Rough embryonal mass plus short compact suspensor (REMSS)

Squashed specimen (Figure 7): The embryonal mass of the REMSS appeared to be similar to the embryonal masses of REMLS and REMLLS embryonal masses. The cells of the embryonal mass were small and isodiametric and tightly associated into a lobed cluster. The transition zone between the embryonal mass and the suspensor was abrupt. The dividing, elongating and elongated embryonal tube cells appeared turgid and had a nucleus.

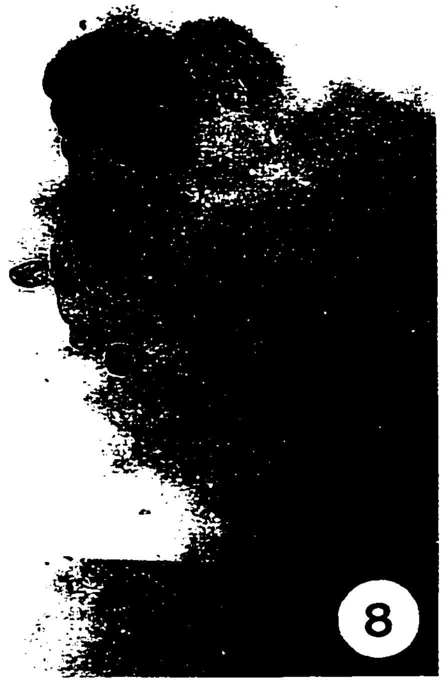
Resin-embedded sections (Figure 8): The cells of the embryonal mass were small with numerous small vacuoles and a prominent nucleus containing one or two nucleoli. The cells were tightly associated with no intercellular spaces. On the surface of the embryonal mass, small clusters of cells formed lobes or protrusions. On the basal surface of the embryonal mass was the transition zone between the embryonal mass and the suspensor. The cells of this region were larger than the cells of the embryonal mass and had larger vacuoles. The embryonal tube cells distal to the transition zone were elongated and vacuolated. Nuclei were visible in some of the embryonal tube cells, indicating that they were still alive though there appeared to be little cytoplasm in them. The embryonal tube cells distal to the transition zone were tightly associated to each other but further away from the embryonal mass the embryonal tube cells became more loosely associated.

Figures 5-8: Figure 5:Rough embryonal mass plus long loose suspensor (REMLLS): squashed specimen (44 x). Figure 6: Rough embryonal mass plus long loose suspensor (REMLLS): median sectioned specimen (120 x). Figure 7: Rough embryonal mass plus short suspensor (REMSS): squashed specimen (44 x). Figure 8: Rough embryonal mass plus short suspensor (REMSS): median sectioned specimen (110 x).

5

6

7



8

3.3.1.5 Rough embryonal mass plus short loose suspensor (REMST)

Squashed specimen (Figure 9): The embryonal mass was approximately the same size as that of the other embryonal mass types described. The embryonal mass appeared to be lobed. In some cases the embryonal mass appeared to be completely surrounded by elongated embryonal tube cells. In other instances, a portion of the embryonal mass was free of embryonal tube cells and there was a suspensor, but it did not arise from a discrete transition zone as in REMLS, REMLLS and REMSS embryonal mass types. The embryonal tube cells appeared to have little cytoplasm but nuclei were present.

Resin-embedded sections (Figure 10): These structures were varied. The distinguishing feature of the REMST was a group of densely cytoplasmic cells surrounded by suspensor cells on an area greater than 90 degrees of the circumference of the embryonal mass. The surface of the embryonal mass could be smooth or irregular. The cluster of small densely cytoplasmic cells comprising the embryonal mass could be larger than the embryonal mass of the REMLLS, REMLS and SEMLS, occasionally having twice the number of cells. There could be little transition zone between the embryonal mass and the suspensor. In at least one instance, a cluster of highly vacuolate, nonelongate cells was attached to the embryonal mass. The suspensor was typically short, 4-5 cells long, but it could be longer. The embryonal tube cells were elongate and highly vacuolate. As in the other embryonal mass suspensors, near to the embryonal mass, the suspensors had nuclei, whereas further away from the embryonal mass the presence of nuclei were found less commonly but the cells were still turgid. If an organized suspensor was present, it ended after a length of 4-5 cells. REMSTs were found growing free from the mass of suspensors that typically make up a tissue aggregate since their suspensors are not long enough to entangle in other suspensors.

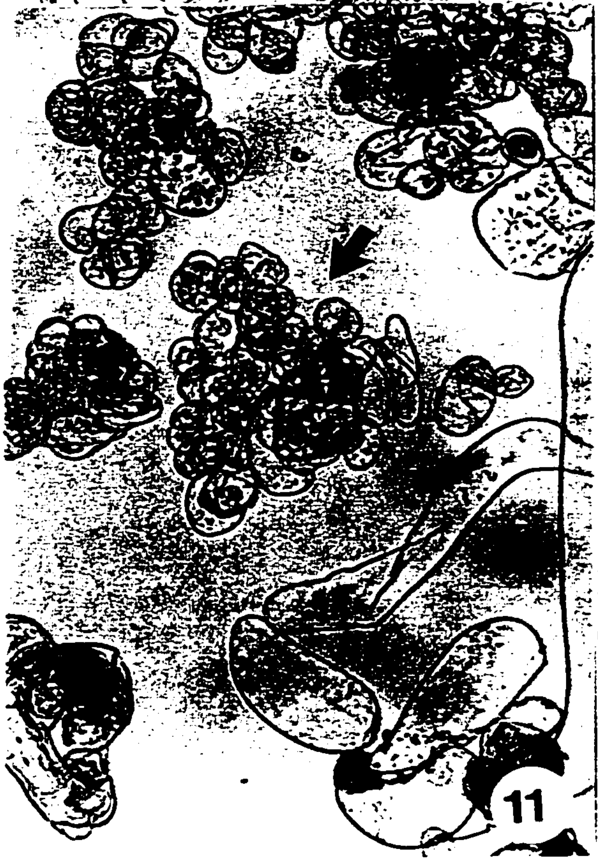
3.3.1.6 Microcluster (MC)

Squashed specimen (Figure 11): The cells of the microcluster were small, isodiametric and highly cytoplasmic. The clusters resembled an embryonal mass without a suspensor. The clusters were often lobed. Occasionally, microcluster had one or two embryonal tube cells associated with it. If present, the embryonal tube cells were not associated with each other.

Figures 9-12: Figure 9: Rough embryonal mass plus short loose specimen (REMST): squashed specimen (120 x). Figure 10: Rough embryonal mass plus short loose suspensor (REMST): median sectioned specimen (110 x). Figure 11: Microcluster (MC): squashed specimen (120 x). Figure 12: Microcluster (MC): median sectioned specimen (110 x).

9

10



11

12

Resin-embedded sections (Figure 12): The microcluster appeared to be a collection of smaller clusters joined together by a few intercellular connections. Therefore, though a microcluster may have appeared to be spherical under low magnification, it was an amorphous structure with a very irregular surface and, often, with no central core. The cells of the microclusters were mostly homogenous and typically small and densely cytoplasmic, with prominent nuclei and several small vacuoles. Though some cells appeared to have more vacuoles than others, these cells were randomly dispersed throughout the microcluster.

3.3.2 Relationship between external appearance and internal organization

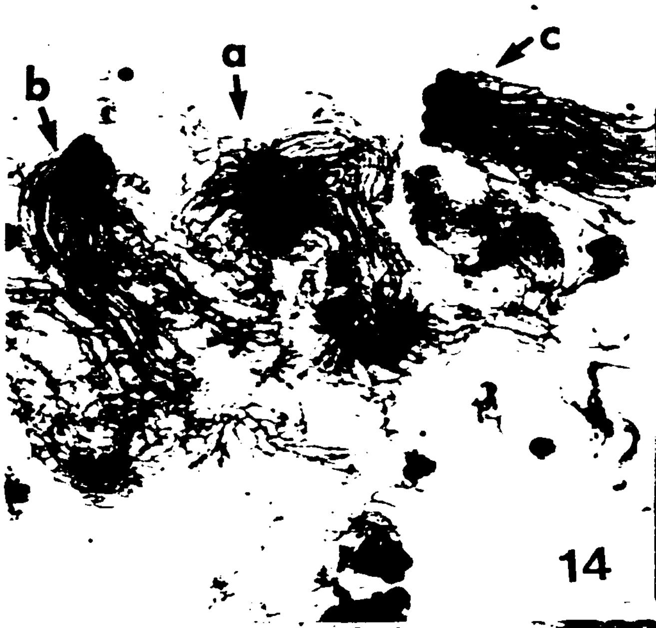
The organization of a tissue aggregate could vary from highly organized aggregate (Figure 13), to moderately organized aggregate (Figure 14), to poorly organized aggregate (Figure 15). In highly organized tissue aggregates (figure 13), nearly all the embryo types were SEMLS or REMLS. With few single cells or short-suspensor structures (REMST, REMSS, MC), the aggregates had a filamentous appearance due to the long suspensors present. In a typical aggregate, embryos at all stages of development were present.

In moderately organized aggregates (Figure 14), all the different types of embryonal masses could be present. These aggregates usually did not have a filamentous appearance. The presence of microclusters, REMST and REMSS structures gave a less filamentous appearance to the aggregate. Embryonal masses could be seen extending from the surface of the aggregate but they did not have a uniform appearance since they might have been SEMLS, REMLS or REMLLS.

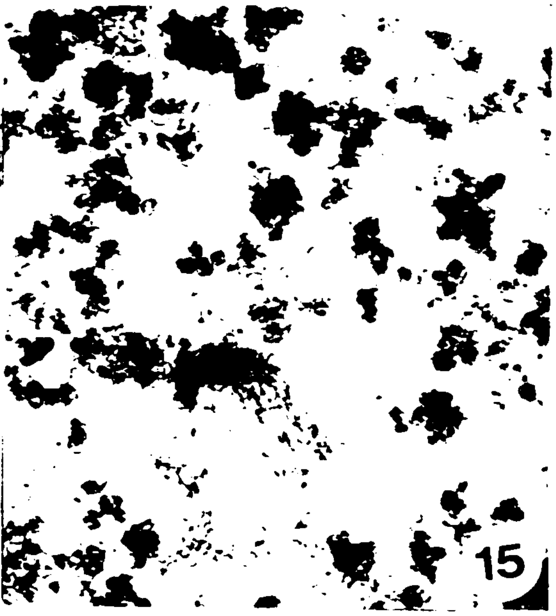
In poorly organized tissue aggregates (Figure 15), usually only microclusters, REMST and REMSS embryonal types were present, though a few specimens of SEMLS, REMLS or REMLLS embryonal masses were be found. These aggregates had an unstructured, smooth appearance. Few or no embryonal masses extended from the surface. The aggregate was easily pried apart by forceps since there were few long suspensors to bind together.

Most aggregates were mixtures of three or more of the embryonal mass types (Figure 14). Therefore, besides differences in colour, there was often little difference in the external appearance of the aggregates despite differences in the types of embryonal masses prominent in the aggregate. Without the aid of a microscope, aggregates composed mostly of SEMLS, REMLS and REMLLS could be identified by their highly

Figures 13-16: Figure 13: Squash of a highly organized aggregate showing embryonal structures (60 x). Figure 14: Squash of a moderately organized aggregate showing embryonal structures (60 x).a. REMST. b. REMLLS. c. REMLS. Figure 15: Squash of a poorly organized aggregate showing embryonal mass types (60 x). Figure 16: Normal rooted somatic embling (20 x).



14



15

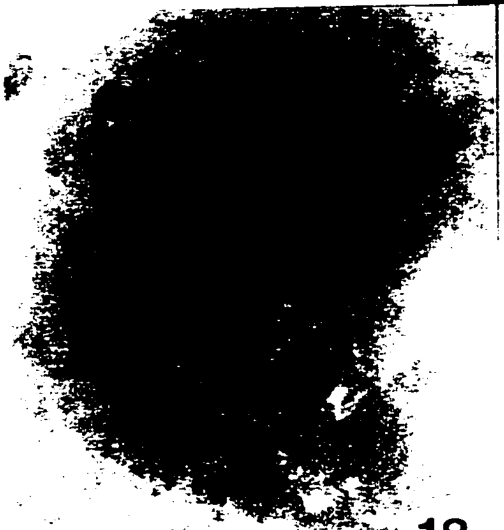
13

16

Figures 17-20: Figure 17: Abnormal rooted embling (20 x). Figure 18: Embryogenic aggregate with a filamentous appearance, showing maturing somatic embryos (20 x). Figure 19: Embryogenic aggregate showing little external organization (20 x). Figure 20: Unrooted red nodule (20 x).

17

19



18

20

organized filamentous appearance (Figure 18). In these aggregates, the scarcity of embryos with short suspensors made the presence of the prominent embryonal masses and suspensors of the SEMLS and REMLS easy to observe. Without a microscope, the differences between SEMLS and REMLS were not easy to detect since the suspensors of both types were identical and the main differences was the relative smoothness of the embryonal masses.

Aggregates that were composed mostly of microclusters and REMSS appeared similar (Figure 19). These aggregates did not look filamentous since their most prominent embryo types had short or no suspensors. The aggregates appeared structureless and homogenous, with few protruding embryonal masses. The aggregates were easily divided by forceps, since there were no entangling suspensors.

Though there were few aggregates that were composed mostly of REMST, these structures could occasionally be noticed in aggregates by their compact, rounded appearance. Lacking an organized suspensor, they could not be mistaken for SEMLS or REMLS, yet they were significantly larger than REMSS or MC. If they were held in a pair of forceps and pressure was applied, they would not disintegrate like a group of microclusters would.

The colour of the aggregate was not as important as the structure. Only the homogenous-appearing aggregates were colours other than white/translucent. In aggregates which were stippled with small areas of anthocyanin, the red pigment appeared to be in large nonembryogenic cells.

3.3.3 Relationship between internal organization and productivity

The number of normal, misshapen and total somatic embryos produced by the aggregates are outlined in Tables 1 and 2. Normal somatic embryos and emblings (Figure 16) had a smooth epidermis and proportions similar to zygotic embryos and seedlings. Abnormal embryos and emblings had a rough epidermis or abnormal proportions (Figure 17). The different types of mature embryo morphoplogies are described in Chapters 4 and 5. In Table 1, each aggregate was categorized according to the embryonal structure type found in the greatest abundance. In Table 2, each aggregate was categorized according to the most advanced embryonal structure type in the internal organization.

Whether the data were analysed with the aggregates classified according to most prevalent embryonal structure or most advanced embryonal structure, both the total embryo production and the percentage of embryos that were normal did not significantly differ between the six different aggregate types. A greater abundance of SEMLS and REMLS embryos did not result in greater embryos production, nor did the disorganized growth of the MC aggregates result in fewer embryos. The percentage of normal embryos produced did not differ between the predominantly SEMLS aggregates and the aggregates composed mostly of embryonal structures with rough, uneven embryonal masses.

In addition, though the most advanced type of embryonal structure found in an aggregate may be expected to be the type most likely to form an embryo, aggregates which contained some SEMLS embryo types did not produce more embryos than aggregates which had only MC types. The percentage of normal embryos produced was the same for aggregates which had some SEMLS embryo types and aggregates which had embryo types with rough, uneven embryonal masses but no SEMLS types.

3.3.4 The developmental fate of the six types of embryonal structures

An immature somatic embryo could either become developmentally arrested, continue to cleave, mature or develop into a nodule. Nodules were red, spherical structures (Figure 20). They are described further in Chapter 4. In this experiment, which followed the development of individual embryonal structures rather than aggregates, the most common fate was developmental arrest (Table 3). For all six types of embryonal structures, 41% or more became developmentally arrested, though the percentages varied: SEMLS 74%, REMLS 62%, REMLLS 47%, REMSS 71%, REMST 46%, and MC 89%. With the exception of SEMLS, the second most common fate was proliferation: SEMLS 10%, REMLS 29%, REMLLS 43%, REMSS 29%, REMST 49% and MC 11%. REMSS and MC embryonal types formed neither nodules nor mature embryos. Of the remaining four types of embryonal structures which formed nodules or mature embryos, over three times as many nodules formed than mature embryos: SEMLS 18% nodules vs. 4% embryos, REMLS 7% vs. 2%, REMLLS 9% vs 1%, and REMST 8% vs. 1%.

Table 1: The production of somatic embryos over 12 weeks from embryogenic aggregates classified according to the most prevalent type of embryonal structure present

Type of Aggregate	#Aggregates per Category (A.#)	Number of Normal SE (N)	Number of Misshapen SE (M)	Total (N+M) (T)	Ave. (T/A.#)	N/M
SEMLS	30	586	360	946	32	1.63
REMLS	26	380	173	553	21	2.20
REMLLS	18	183	82	265	15	2.23
REMSS	23	375	168	543	24	2.23
REMST	4	20	9	29	7	2.22
MC	19	203	100	303	16	2.03

Average T/A# : total number of mature somatic embryos (SE) produced divided by the number of aggregates.

Statistics:

Results of Kruskal -Wallis test for total number of embryos produced by aggregate type:

Degrees of freedom	5
H statistic	7.424
H corrected for ties	7.504
Number of tied groups	18

Probability of a greater value: $0.250 < p > 0.100$, no significant difference in embryo production between the different aggregate types

Results of ANOVA test for percentage normal embryos:

Test percentage: arc sine (square root[(number of normal embryos)/ (total number of embryos)]

Source	Degree of freedom	Sum of squares	Mean squares	F-test
Between groups	5	1.168	0.234	1.446
Within groups	89	14.382	0.162	
Total	94	15.55		

Probability of a greater value: $p=0.2157$, no signifiant difference in the percentage of normal embryos between the different aggregate types.

Table 2: The production of somatic embryos over 12 weeks from embryogenic aggregates classified according to the most advanced type of embryonal structure present

Type of Aggregate	#Aggregates per Category (A.#)	Number of Normal SE (N)	Number of Misshapen SE (M)	Total (N+M) (T)	Ave. (T/A.#)	N/M
SEMLS	37	673	395	1068	29	1.70
REMLS	20	316	130	446	22	2.43
REMLLS	17	185	134	319	19	1.38
REMSS	23	379	167	546	24	2.27
REMST	9	52	38	90	10	1.37
MC	14	173	72	245	18	2.40

Average T/A# : total number of embryos produced (N+M) divided by the number of aggregates.

Statistics:

Results of Kruskal -Wallis test for total number of embryos produced:

Degrees of freedom	5
H statistic	4.791
H corrected for ties	4.842
Number of tied groups	19

Probability of a greater value: $0.50 < p > 0.25$, no significant difference in embryo production between the different aggregate types

Results of ANOVA test for percentage normal embryos:

Test percentage: arc sine (square root[(number of normal embryos)/ (total number of embryos)]

Source	Degree of freedom	Sum of squares	Mean squares	F-test
Between groups	5	1.191	0.238	1.377
Within groups	89	15.396	0.173	
Total	94	16.587		

Probability of a greater value: p=0.2405, no signifiant difference in the percentage of normal embryos between the different aggregate types.

Table 3: The developmental fate of the six types of embryonal structures after isolation

			embryos					
Em type	total	stalled	normal	miss.	total	nod.	tot.s	prolif.
SEMLS	314	233 (74)	3 (1)	9 (3)	12 (4)	37 (18)	49 (16)	32 (10)
REMLS	241	149 (62)	2 (1)	2 (1)	4 (2)	18 (7)	22 (9)	70 (29)
REMLLS	190	89 (47)	0	2 (1)	2 (1)	17 (8)	19 (10)	82 (43)
REMSS	183	148 (81)	0	0	0	0	0	35 (19)
REMST	274	126 (46)	0	3 (1)	3 (1)	22 (9)	25 (9)	123 (49)
MC	201	179 (89)	0	0	0	0	0	22 (11)

Note: the number in brackets is the percentage

Em type: embryonal structure type

total: total number of embryonal structures plated out in each classification

stalled: number of embryonal structures which showed no development after transfer

embryos normal: number of somatic embryos producedwith a phenotype similar to zygotic embryos

embryos miss.: number of misshapen embryos having abnormal proportions or epidermis

embryos total: total number of embryos produced, normal and misshapen

nod.: number of nodules produced

prolif.: number of embryonal masses which commenced cleavage after transfer

tot.s: total number of embryos and nodules produced

Statistics: 3 x 6 contingrency table - 3 classes of fates (stalled, proliferation, total structures) and 6 classes of embryo types

Degrees of freedom	10
Total chi-square	208.144
Contingency coefficiant	0.357

Probability: p=0.0001

Deviations (observed-expected)

Fate	Type of embryo					
	SEMLS	REMLS	REMLLS	REMSS	REMST	MC
stalled	29.68	-7.05	-34.03	13.96	-51.42	48.85
proliferation	-53.38	4.47	30.34	2.72	48.5	-32.65
total structures	23.7	2.58	3.69	-16.68	2.92	-16.2

There was an association between the type of embryonal structure and its fate (p=0.0001). Though the pattern of deviations (calculated by observed frequencies - expected frequencies) was complex, in general, the more organized embryonal structures (SEMLS, REMLS) were more likely than expected to become embryos or nodules while the least organized embryonal structures (REMSS, MC) were more likely than expected to become developmentally arrested. SEMLS embryonal structures were more likely to become developmentally arrested (74%) or become a nodule (18%) or mature embryo (4%) more often than expected, but proliferated less often than expected (10%). Both REMLLS and REMST embryonal structures were less likely to become developmentally arrested than expected (47-46%), and more likely to proliferate than expected (43-49%), but became nodules (8-9%) or mature embryos (1%) only slightly more often than expected. REMLS embryonal structures contributed the least to the chi-square total, with the lowest magnitude of deviations. REMLS embryonal structures became developmentally arrested less likely than expected (62%) and proliferated (29%) became nodules (7%) or mature embryos (2%) more often than expected. REMSS embryos became developmentally arrested more often than expected (81%) and became nodules (0%) or mature embryos (0%) less often than expected. MC embryos were more likely to become developmentally arrested than expected (89%), and less likely to proliferate (11%) or become nodules/mature embryos (0%) than expected. Though REMSS and MC embryos both produced no structures, neither nodules nor mature embryos, their distributions over the 3 fates was significantly different (p=0.0001) and could not be pooled. However, REMLLS and REMST embryos behaved in a similar fashion, and their data were pooled (p=0.9117).

Isolated embryonal structures which proliferated produced aggregates which were composed of several types of embryonal structres, not just the original type (Table 4). SEMLS, REMLS, REMLLS, REMSS, and REMST embryonal masses produced aggregates which were predominantly REMLS. Only MC embryonal masses proliferated into aggregates which were predominantly microclusters.

In aggregates proliferating from SEMLS, REMLS, REMLLS and REMST embryonal strucutres, the most common secondary embryonal structures found were SEMLS, REMSS and MC. In aggregates proliferating from REMSS embryonal structures, the secondary embryonal structures found were microclusters. In aggregates proliferating from microclusters, the secondary embryonal structures found were REMLS.

Table 4: The types of embryonal structures found in proliferating tissue derived from individual embryonal structures of six different types

Original embryonal structure	Predominant embryonal structure found in proliferating tissue	Also common
SEMLS	REMLS	SEMLS. REMSS. MC
REMLS	REMLS	REMSS. SEMLS. MC
REMLLS	REMLS	REMSS. MC. SEMLS
REMSS	REMLS	MC
REMST	REMLS	REMSS. MC. SEMLS
MC	MC	REMLS

3.4 Discussion

3.4.1 The organization of line 2086 of *Larix x eurolepis*

Six different types of embryonal structures were found in aggregates of embryogenic tissue of line 2086 of hybrid larch (*Larix x eurolepis*). The six types were mainly distinguishable by the type of suspensor present, though one type had a smooth embryonal mass (SEMLS) while the other five types had rough embryonal masses. Apart from the surface cell layer of the SEMLS, the cluster of small, isodiametric cells which make up the embryonal mass were almost identical in embryonal structures of REMLS, REMLLS, REMSS, REMST and MC. Since anticlinal divisions could be seen in the surface layer of all the embryo types, this layer could not be considered an epidermis. Therefore, the smooth appearance of the SEMLS embryos was not due to the presence of an epidermis which would indicate a change in division patterns related to a cessation of cleavage polyembryony. The incidence of proliferation in SEMLS embryonal structures indicates that the smooth external appearance of the embryonal mass was not due to a change in the behaviour of the embryo or an advancement in development which precludes cleavage. Thus, very few embryos matured, and the embryos which were destined to mature could not be identified by external appearance.

Though aggregates consisting of only one type of embryonal structure (usually SEMLS) could be found, most of the aggregates were heterogenous mixtures of two or more types of embryonal structures. Therefore, line 2086 is similar to embryogenic lines of *Picea abies* , *Picea mariana* , *Pinus taeda* , *Larix occidentalis, Larix x eurolepis* and *Sequoia sempervirens* (Klimaszewska 1989a, Becwar *et al.* 1990, Jalonen and von Arnold 1991, Thompson and von Aderkas 1992, Bozkov 1995, Favre *et al.* 1995, Klimaszewska 1995). The embryogenic lines of these species were mixtures of polar (SEMLS) and solar (REMST, MC with several embryonal tube cells) embryos, with transmutation between the types. Transmutation of types was found in line 2086 when proliferating tissue derived from individual embryonal structures was examined and found to consist of several different embryonal structure types (Table 4). In contrast, individual lines of *Pinus caribaea* and *Pinus pinaster* (Bercetche and Pacques 1995, David *et al.* 1995) were homogenous though differences between the lines existed within a species.

The types of embryonal structures found in line 2086 are not unique and have been found in other species. Several papers reported typical stage 1 embryos, similar to SEMLS embryonal structures, with long, translucent suspensors and smooth embryonal masses, including *Pinus lambertiana* , *Pinus taeda, Picea glauca,* and haploid *Larix decidua*

(Nagmani and Bonga 1985, Gupta and Durzan 1986, Gupta and Durzan 1987a, Lu and Thorpe 1987, Rohr *et al.* 1989). Somatic embryos resembling REMLS and REMSS were commonly found in embryogenic lines of other species, often together in the same line, including *Picea glauca*, *Picea abies*, *Picea rubens*, *Picea sitchensis*, *Pinus caribaea*, *Pinus nigra* and *Pinus taeda* (Becwar *et al.* 1988, Hakman and Fowke 1987, Boulay *et al.* 1988, Hakman and von Arnold 1988,Gupta *et al.* 1988, Kartha *et al.* 1988, von Arnold and Hakman 1988, Attree *et al.* 1989b, Becwar *et al.* 1990, Cornu and Geoffrion 1990, Laine and David 1990, Tremblay 1990, Harry and Thorpe 1991, Joy *et al.* 1991, von Aderkas *et al.* 1991, Attree and Fowke 1993, Charest *et al.* 1993, Kristensen *et al.* 1994, Jasik *et al.* 1995). Several papers have found cell clusters resembling MC embryonal masses and solar embryos resembling REMST embryonal structures (Jalonen and von Arnold 1991, Thompson and von Aderkas 1992). In most papers, the types of embryonal structures were not discussed but could be observed in photograghs, but not as sectioned tissue.

3.4.2 The relationship between internal organization and mature embryo production

The results of the experiments relating to internal organization of an aggregate with mature embryo production indicated that there was no significant difference in the total number of mature embryos produced and the percentage of normal mature embryos between the least organized (MC) and the more organized (SEMLS, REMLS) aggregates. Whether the aggregates were classified according to the most prevalent embryonal structure type or the most advanced type did not change the conclusion that the 6 types of aggregates had similar rates of somatic embryo production and percentage of normal mature embryos.

This result was unexpected in light of findings in other species. In *Picea abies*, the organized types of lines containing solar and polar embryos (group A) (SEMLS, MC) were embryogenic whereas the disorganized lines containing cell clusters (MC) did not produced embryos (Jalonen and von Arnold 1991). In another study, somatic embryos matured from lines of *Picea abies* composed entirely of cleaving embryonal masses (SEMLS) were all normal, while embryos matured from heterogenous lines composed of callus (MC) and stage 1 embryos (SEMLS), were all abnormal (Bozkov 1995). However, both types of *Pinus taeda* lines could produce embryos, even though one type was composed mostly of stage 1 embryos (SEMSL) while the other was composed mostly of clusters of meristmatic cells (MC, REMST) with an few subtending suspensor cells (Becwar *et al.* 1990). The embryo production results for *Larix x eurolepis* presented in this thesis are similar to the *Pinus taeda* findings in that all types of aggregates could produce embryo. Unlike lines of

Picea abies, no type of aggregate produced a greater percentage of normal embryos than another.

Other authors have identified different types of embryonal masses in their embryogenic cultures and have indicated that the different types have different embryogenic potential. In *Pinus pinaster* cultures, group 1 lines are a mix of microclusters and long, single cells which are incapable of responding to ABA to form somatic embryos, while group 2 lines (polarized somatic embryos) and group 3 lines (non-polarized solar-shaped somatic embryos) could both develop to stage 2 , but only group 2 could continue to develop to stage 3 (Bercetche and Pacques 1995). In *Picea abies* , the lines were either undeveloped (B type), or composed of solar (nonpolarized, A2 type) somatic embryos or polar somatic embryos (A1 type). The B type was never found intermingled with the A types nor could mature somatic embryos be produced from them, though the two A types could transmute from one to the other and produce mature somatic embryos (Jalonen and von Arnold 1991). In *Pinus caribaea*, two general groupings of lines were found: those with recognizable embryonal masses plus suspensors and those with microclusters. The lines with embryonal masses could be further subdivided into 4 types: E4 (very small embryonal masses and suspensors with single cells), E5 (like E4 but bigger), E6 (polarized embryonal masses and suspensors like SEMLS and REMLS) and E7 (nonpolarized solar type embryonal masses surrounded by single suspensor cells, REMST). However, these embryonal mass types are described as lines, where the line was composed of one embryonal mass type. All the lines, including the MC type, were reported to be embryogenic (David *et al.* 1995). In the above-cited papers, the different types of embryonal masses were found in different embryogenic lines. However, in *Picea abies* and *Larix occidentalis* the solar and polar types of embryonal masses can transmute into each other if the culture conditions are manipulated (Jalonen and von Arnold 1991, Thompson and von Aderkas 1992), which implies that during the shifting phase, the two types of embryonal masses could be found in the same aggregate. Nevertheless, differences in somatic embryo production are not reported for the different types of embryogenic lines. Variation in morphology can exist at a cellular level, but not affect productivity, such as in *Daucus carota* cultures where all five of the types of single cells in carrot cultures could develop into somatic embryos with frequencies between 19 and 100 somatic embryos per 10,000 cells (Toonen *et al.* 1994). The behaviour of embryonal masses of line 2086 of *L. x eurolepis* indicates that the different types of embryonal masses comingle and do not correspond to different lines, and that their productivity does not differ.

However, the aggregate itself does not produce the mature somatic embryos, the individual embryonal masses do. Few aggregates of line 2086 were homogenous for any embryo type, which agrees with the results for *Larix occidentalis* cultures (Thompson and von Aderkas 1992, Bonga *et al.* 1995), and makes it difficult to assess which embryonal structures in an aggregate are the ones maturing. Possibly, the fact that most aggregates were mixtures of embryonal mass types would affect the rate of production of somatic embryos. In *Picea abies* cultures, proteins secreted by group A lines (SEMLS and REMST type embryos) affected the types of embryos produced by group B lines (MC type structures) (Egertsdotter *et al.* 1993). Though aggregates of line 2086 composed mostly of SEMLS and REMLS embryo types did not produce more mature embryos than aggregates composed mostly of MC embryo types, the possibility remains that in heterogenous aggregates, the more advanced embryo types could be secreting substances which affected the behaviour of the other embryo types in the aggregate. Only by following the development of individiual somatic embryos could the differences in behaviour between the six types of embryos be determined. Accordingly, the rest of the experiments outlined in this thesis used individual embryos rather than aggregates as the experimental units.

3.4.3 The association between embryonal structure and developmental fate

Though there was an association between embryonal structure type and the final fate of the individual embryo, the relationship was a complicated one. For every embryonal structure type, the most common fate was developmental arrest, while the least common fate was maturation into an embryo. The deviations (observed frequency - expected frequency) showed no readily identifiable pattern. Only REMLLS and REMST categories could be pooled since their distribution between the different fates was similar. The two embryo types which seemed the most advanced and similar in structure (SEMLS and REMLS), had very different deviations and chi-square contributions over the five developmental fates. Though it was anticipated that the SEMLS embryos would be more likely to form nodules or mature embryos, and that MC embryos would be more likely to become developmentally arrested or proliferate than become a nodule or a mature embryos, the behaviour of the intermediate embryo types (REMLS, REMLLS, REMST and REMSS) was not anticipated. Though REMSS and MC embryos both produced no structures, neither nodules nor mature embryos, their distributions over the 3 fates were significantly different ($p=0.0001$) and could not be pooled. However, REMLLS and REMST embryos behaved in a similar fashion, and their data could be pooled ($p=0.9117$), though the

REMLLS embryonal masses produce a recognizable suspensor, and the REMST embryos did not. These results seem to contradict the findings of the previous experiments, where aggregates composed largely of REMSS and MC produced as many somatic embryos as aggregates composed of the other types of embryonal structures. This apparent contradiction affirms the necessity of isolating individual embryonal structures to determine their fate, rather than using aggregate productivity. Since most aggregates are mixtures of different embryonal structures, reliably identifying which embryonal strucutres are maturing is impossible. Since individual REMSS and MC embryonal structures did not mature into embryos in this experiment, the somatic embryo production from the REMSS and MC aggregates was likely due to the maturation of the scarcer SEMLS, REMLS, REMLLS and REMST embryonal structures in those aggregates.

Though the type of embryo is associated with its fate, the ranking of the embryo types in terms of the type of suspensor produced was not reflected by a clear trend in the number of embryos which ceased cleaving and become nodules or embryos. The fact that aggregates composed mainly of SEMLS or REMLS produced as many somatic embryos as aggregates of microclusters, and the fact that fewer than 5% of every embryonal structure type became embryos, indicated that the similarities between the 6 types of embryonal masses were more important in terms of development potential than the differences. In carrot suspension cultures, all 5 of the different single cell types could form embryos, indicating that the morphology of a cell does not determine embryogenic capacity. But, the developmental pathway leading to the formation of a somatic embryo is determined by the morphology of the initial cell (Toonen *et al.* 1994). Oval and elongated cells form irregular cell clusters which develop into embryos, while spherical cells form symmetrical cell clusters which develop into embryos (Toonen *et al.* 1994). However, the types of somatic embryos produced (total number and percent normal) was not affected by the initial embryonal mass plus suspensor phenotype, though the percentage of embryonal structures which became nodules varied from 18% (SEMLS) to 0% (REMSS and MC).

When the percentage of embryonal structures in each class of fate are examined, the ranking of embryonal structures appears to be SEMLS, REMLS, REMLLS, REMST, REMSS and MC in terms of productivity. This contrasts with the original ranking of the embryonal structures at the beginning of the experiments, which rated REMSS embryonal structures, with their organized rib meristems and compact suspensors, to be better organized than REMST embryonal structures with their disorganized suspensors. Apparently, the level of organization of the rib meristem is not a sound criteria for judging the likelihood of an embryonal structure maturing or not.

The differences in appearance of the types of embryos is mainly due to the organization of the suspensor. However, the embryonal mass produces the suspensor. Thus, though the embryonal masses of the REMLS, REMLLS, REMST and REMSS embryos appeared similar, the differences in suspensor types indicated differences in the organization of the rib meristem of the embryos. When proembryos of *Phaseolus vulgaris* and *Phaseolus coccineus* were cultured, detaching the suspensor did not lessen the survival rate if the suspensor was placed in contact with the developing proembryo (Steeves and Sussex 1989). In *Larix x eurolepis* , the type of suspensor did influence the fate of the embryo, but not in a definitive way. The purpose of the suspensor of a developing somatic embryo is unclear. Because many somatic embryos form on the surface of the aggregate, the suspensor is unlikely to be in contact with the medium and unable to transport nutrients from the medium to the embryonal mass. Possibly the role of the suspensor is to produce hormones, as in the suspensors of *Phaseolis coccineus* (Steeves and Sussex 1989). In the case of embryos of *Larix x eurolepis*, the organization of the embryonal tube cells did not seem as important as their presence. However, only REMLLS and REMST embryonal types behaved the same despite the differences in their organization. Since no individual microclusters produced mature somatic embryos, the suspensor may be required for embryogenesis. Therefore, an asymmetrical first division may be necessary to establish polarity.

The failure of such a high percentage of embryonal structures to develop any further after transfer (46-89% developmental arrest) was observed previously. Even in highly embryogenic lines, underneath the maturing somatic embryos are many embryonal masses which fail to respond to the culture conditions. It is assumed that these unresponsive embryonal masses eventually die and form the darkened core of the aggregate. Unexpectedly, the best organized embryonal structures (SEMLS) were almost as likely to become developmentally arrested (74%) as microclusters (89%). Theoretically, the more refined the selection process for experimental material , the more uniform the response of that material should be. That some embryonal masses in a heterogenous aggregate should fail to develop and eventually die is understandable, but that the carefully chosen and categorized embryonal masses in this experiment should show the same rate of failure to develop is puzzling. In addition, the careful selection and categorization of embryonal structures into each of the 6 types, the physiological state of the different embryonal structures must differ enough to give rise to developmentally arrested embryonal masses, nodules, proliferation and somatic embryos of varying phenotypes.

Theoretically, if the transition from random division in the embryonal mass to polarized growth in the early embryo is a significant development, then the more polarized the early growth the more likely the embryonal mass will form a normal somatic embryo. However, though REMST types can display a variety of morphologies, ranging from unpolarized (solar type embryonal masses) to marginally polarized (embryonal masses with embryonal tube cells present on more than 1/4 of the surface), it was as productive as the more polarized REMLLS type (7-8% became mature embryos) and more productive than REMSS (0% became mature embryos). A more careful tracking of the development of the individual types of embryonal masses from isolation to final morphology may explain this discrepancy.

The results for proliferation were also unexpected. As expected, the SEMLS type appeared to cleave and proliferate less than the other 5 types (10% vs 29-49%), but the MC type had almost the same rate of proliferation (11%). The lower rate of proliferation of the microclusters compared to the other types of embryonal masses may indicate a decrease in vitality or a change in the physiology of the small densely cytoplasmic cells which do not (or no longer) differentiate into embryonal tube cells. The solar and polar types of embryonal masses of *Picea abies* showed differences in proliferation rates though both could produce somatic embryos. Slowing the rate of growth by manipulating the culture conditions could prompt one type to transform into the other type and *vice versa* (Jalonen and von Arnold 1991).

Transformation was also found in line 2086. None of the proliferating embryonal masses gave rise to uniform aggregates. All the resulting proliferating aggregates were mixtures of the original embryonal mass type and others. The most common product of proliferation was REMLS, possibly indicating that the lobes on the embryonal mass of REMLS types were the beginning of cleavage into daughter embryonal masses and the suspensor characteristics of REMST, REMLLS and REMSS arise after cleavage. Alternatively, because REMST, REMLL and REMSS types also had lobed embryonal masses, the prevalence of REMLS might have been due to a faster growth rate and subsequent faster cleavage rate for this type. The two embryonal masses which seemed to diverge both in their relatively lower rate of somatic embryo production and nodule production and their proliferation products were microclusters and REMSS. REMSS embryonal masses behaved like microclusters which appeared to have lost their ability to produce suspensors. This may indicate that the peculiar configuration of REMST suspensors is not as important as the brevity of the REMSS suspensors. However, microclusters were common proliferation products of the more organized embryonal mass types (SEMLS, REMLS, REMLLS). The types of proliferation products found indicated that the 6 types of embryonal masses were interchangeable, i.e. that finding them together in one aggregate does not mean that the aggregate is a mixture of self-proliferating embryonal mass types, but that a single embryonal mass of any type can give rise to daughter embryonal masses of any type.

3.4.4 Conclusion

In conclusion, the six types of embryonal structures differed mainly in terms of embryonal tube differentiation and organization. Tissue aggregates were usually mixtures of more than one type of embryonal structure and aggregates containing mostly well-organized embryonal structures could be visually separated from aggregates composed of mostly MC and REMSS. The rate of production of normal and misshapen somatic embryos was the same between well-organized and poorly organized aggregates though the final developmental fate was associated with embryonal structure type, with developmental arrest being the most common fate, and maturation being the least common fate.

Chapter IV: Pathways of development

4.1 Introduction

The embryology of *Larix* has been studied extensively (Schopf 1943. Chowdhury 1962. Owens and Molder 1979c). Within the confines of the megagametophyte and seed coat, the development of zygotic embryos is consistent. with few abnormalities (Schopf 1943. Owens and Molder 1979c). as has been found in other species (Mikkola 1969). Seed production from *Larix* species is low. good cone crops can occur up to 12 years apart and seed set is poor (Owens and Molder 1979c). This makes the development of somatic embryogenesis systems for *Larix* species particularily valuable to reforestation programs. Though the development of somatic embryos of *Larix* has been less extensively studied. studies have indicated that the development of somatic embryos is analogous. within limits. to zygotic embryo development (von Aderkas *et al.* 1991).

However, in somatic embryogenesis, the number of somatic embryos which continue development from the earliest stages to maturation drops off rapidly. At each successive stage of development. the number of somatic embryos per gram of embryogenic tissue which continue developing further decreases. from 14.2 x 10^3 stage 1 embryos for *Picea abies* (Kartha *et al.* 1988). to 23-126 stage 3 embryos of *Picea abies. Picea glauca*, *Picea rubens* and *Picea sitchensis* (Kartha *et al.* 1988. Harry and Thorpe 1991. Jalonen and von Arnold 1991). Since the majority of somatic embryos either die or become developmentally arrested, critical stages must occur between stage 1 (embryonal mass stage). stage 2 (focal zone stage) and stage 3 (mature somatic embryos with discernable hypocotyls and cotyledons). Because the rate of failure-to-mature appears to be higher in somatic embryos as compared to zygotic embryos. a study of the critical stages could help identify sensitive steps in development that could be manipulated to improve the efficiency of the process.

This chapter investigates the stages of development of somatic embryos and the efficiency of somatic embryogenesis in hybrid larch (*Larix x eurolepis*). This was accomplished by using resin-embedded sections to examine embryo development and by isolating embryos at different stages of development to record both the number of embryos that progressed to later stages of development and the final developmental fate of each embryo.

4.2 Materials and methods

4.2.1 Culture maintenance

Line 2086 originated from an immature zygotic embryo of a hybrid between *Larix leptolepis* (maternal parent) and *Larix decidua* (paternal parent)(von Aderkas *et al.* 1990). The embryogenic tissue was initiated on 1/2 Litvay's medium supplemented with glutamine and 0.45 µM 2,4-D. Line 2086 was routinely cultured every 2-3 weeks onto 1/2 strength Litvay's medium (Litvay *et al.* 1981) supplemented with 56 µM sucrose, 4 g/l gelrite, 1 g/l casein hydrolysate, 3422 µM glutamine without plant growth regulators. The medium was adjusted to pH 5.6-5.8 before autoclaving. The glutamine and all plant growth regulators were filter sterilised using a 22 µm filter and were added after the medium had been autoclaved in an Agarmatic (New Brunswick Scientific Co. Inc.). The medium was dispensed by 20 ml aliquots into 100 x 15 Petri dishes (Fisher) and allowed to cool to room temperature before storage at 4 °C. Four aggregates of embryogenic tissue were cultured in each Petri dish and were individually marked. The plates were kept in a growth chamber at 22 °C with a 16 hour light/8hour dark photoperiod at 36 umol/m^2/s photosynthetically active radiation. In the absence of plant growth regulators, mature somatic embryos of line 2086 germinated precociously and asynchronously.

4.2.2 Resin-embedding and sectioning

Somatic embryos at various stages of development were separated from the tissue aggregates before fixation. Usually, 10-25 embryonal masses or 5-10 more advanced structures.were fixed at a time in each vial. The tissue was fixed for 2-3 hours in 2.5% glutaraldehyde in 0.075M phosphate buffer. Post fixation, the tissue was washed in 0.075M phosphate buffer three times, for 10 minutes each. The tissue was then fixed for 2-3 hours in 1% osmium tetroxide in 0.075Mphosphate buffer followed by a short buffer wash. The tissue was then dehydrated for 10 minutes in 20, 40, 60 and 80 % acetone followed by 3 changes of 100% acetone for 10 minutes each. The tissue was infiltrated by 30% Spurr's resin in acetone for 10-12 hoursfollowed by infiltration by 75% and 100% Spurr's resin in acetone for 2 hours each. The tissue was finally embedded in 100% Spurr's resin overnight at 70°C (Karnovsky 1965, Glauret 1974, Roland and Vian 1991). Spurr's resin (Spurr 1969) was prepared from ERL 4206 (vinyl cyclohexene dioxide), D.E.R. 736 (diglycidyl ether of polypropylene glycol), NSA (nonenyl succinic anhydride, and S-1 (DMAE - dimethyl aminoethanol), (Marivac Ltd, Halifax N.S.).

The resin blocks were sectioned at 0.3 - 0. 8 µm using glass knives on either a Reichert-Jung Ultracut E microtome or a Reichert Om U2 microtome, both from American Optical Instrument Company, Buffalo, N.Y. The sections were floated on a drop of water on a slide and placed on a hot plate until the water evaporated. The sections were stained with Richardson's stain (1 g borax plus 1.5 g methylene blue in 100 ml water added to 0.5 g azure B in 100 ml, mixed and filtered) and the slide was placed on the hot plate for 25 seconds. The slide was rinsed with water and allowed to air dry before being mounted in Permount (Fisher). The sections were examined under a light microscope. Photographs were taken using Tmax 100 film or Kodak technical pan film (100 ASA).

4.2.3 Scanning electron microscopy

Somatic embryos at various stages of development were fixed for 2-3 hours in 2.5% glutaraldehyde in 0.075M phosphate buffer. Post fixation, the tissue was washed in 0.075M phosphate buffer 3 times, for 10 minutes each. The tissue was then fixed for 2-3 hours in 1% osmium tetroxide in 0.075M phosphate buffer followed by a short buffer wash. The tissue was then dehydrated for 10 minutes in 20, 40, 60 and 80 % acetone followed by 3 changes of 100% acetone for 10 minutes each. The tissue was then run through a critical point dryer (SPC-1500, Bomar Co., Tacoma WA.) for 20 minutes between 1100 and 1500 psi pressure before being mounted on stubs with nail polish (Cutex, "Opaline") and sputter-coated with gold for 3-4 minutes (S150B, Edwards). The prepared specimens were loaded into the scanning electron microscope (JEOL JSM-35) and scanned at 60 kV.

4.2.4 Developmental fates of embryos at different stages of development

Each aggregate of line 2086 was individually pulled apart with forceps under a binocular microscope (Wild). Each immature or mature somatic embryo was individually removed from the aggregate and its stage of development determined. The embryo was then placed onto medium in a corrresponding fresh Petri dish. Each fresh Petri dish was gridded by affixing a 5 x 5 paper grid onto the bottom. A single somatic embryo was placed in each square of a grid. Every aggregate (120 in total) was examined and all recognizable somatic embryos utilised. Therefore, the total number of somatic embryos in each of the five stage of development categories differed. This procedure was done in three replicates. Preliminary experiments using isolated immature somatic embryos indicated that development was complete in five weeks, and therefore after this period, the developmental fates of the isolated immature embryos in this experiment were assessed.

The five stages of development chosen for this experiment were:

a. SEMLS: smooth embryonal mass with long suspensor.

b. focal zone: immature somatic embryos exhibiting a swollen, opaque embryonal mass region.

c. focal zone plus streak: immature somatic embryos exhibiting a swollen, opaque embryonal mass region with some streaks of red pigmentation around the basal end of the embryonal mass.

d. focal zone plus band: immature somatic embryos exhibiting a swollen opaque embryonal mass regions with a band of red pigmentation around the basal end of the embryonal mass.

e. cotyledons initiated: immature somatic embryos exhibiting cotyledonary primordia.

The stages of development were chosen for their ease of identification. The SEMLS stage of development was chosen to represent the earliest stage of embryo development because they were abundant. The three successive focal zone stages were chosen because it was believed that they would span the development of the root meristem. At the focal zone stage in zygotic embryos, cell division had ceased to be random and therefore somatic embryos at this stage were considered to have developed beyond the stage of cleavage polyembryony but not to have developed a root meristem. The focal zone plus polyphenol band (FZ+PB) stage was chosen because it is known that the localized production of polyphenols at the basal end of the focal zone where the root meristem would arise indicated the beginning of tissue differentiation and root meristem formation (Gutmann *et al.* 1996). As the polyphenol band develops, it appeared first as a red streak which then intensified into a solid band. Therefore, the focal zone plus polyphenol streak (FZ+STR) was chosen as an intermediate stage between the focal zone stage and the polyphenol band stage.

The six developmental fates were:

a. dead: all tissue necrotic.
b. developmentally arrested (stalled): some growth or development beyond the initial, plated-out stage of development evident but development ended before reaching the mature embryo stage; tissue not necrotic.
c. proliferation: a proliferation of immature embryonal masses.
d. nodule: a roughly spherical red structure with no cylindrical hypocotyl or cotyledons.
e. embryo: a mature somatic embryos with a hypocotyl, and cotyledons.

This experiment was analysed as a 5 x 5 contingency table, using the Statview 512^+ program. As all but one expected frequency was over 5, no pooling was done prior to analysis. All 2 x 5 subset comparisons were significant at $p=0.001$, therefore no pooling of categories could be done.

4.3 Results

4.3.1 Stages of development

4.3.1.1 Description of stages of development

Due to the small size and bent or twisted orientation of many of the samples, longitudinal sections of all the stages of development were difficult to obtain. However, in general, the samples were uniform in organization at each stage of development, indicating strong genetic control.

4.3.1.1.1 Embryonal mass

Fresh specimen (Figure 21): The smooth nature of the embryonal mass and the compactness of the suspensor was evident in fresh samples. The embryos were translucent and smooth in appearance. The suspensor had a striated appearance. The suspensor was stiff for several cell lengths behind the embryonal mass. The transition zone between the embryonal masss and the suspensor was continuous, with no constriction in the embryo's diameter.

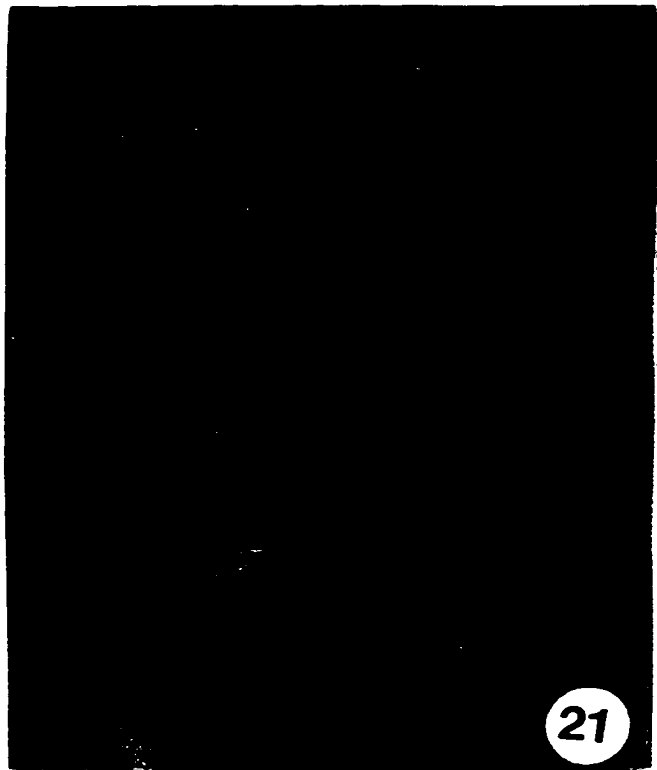
Scanning electron micrograph specimens (Figure 22): The surface showed the surface cells adhering tightly together. The difference in size between the small embryonal mass cells and the long embryonal tube cells was evident, as was the difference between the live embryonal tube cells just behind the embryonal mass and the dead embryonal tube cells further along the suspensor.

The histology of embryonal masses is described in section 3.31.

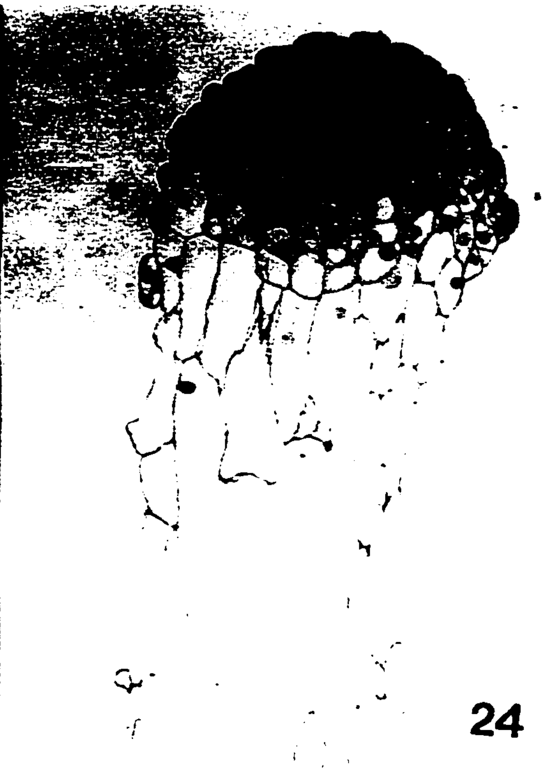
4.3.1.1.2 Focal zone

Fresh specimen (Figure 23): the focal zone stage was distinct in appearance from the early embryo stage. Typically, the suspensor was compact and striated. Every focal zone stage somatic embryo had a long compact suspensor. The embryonal mass was enlarged compared to the embryonal mass stage, and was wider in diameter than the suspensor, giving the embryonal mass a prominent, bulbous appearance. In some cases there appeared to be a protrusion of cells which surround the middle section of the

Figures 21-24: Figure 21: Fresh specimen of an SEMLS embryonal structure (60 x). Figure 22: Scanning electron micrograph of an SEMLS embryonal structure (80 x). Figure 23: REMLS embryonal structure and immature somatic embryo at the focal zone stage (60 x). a. REMLS, b. somatic embryo at the focal zone stage. Figure 24: A median sectioned immature somatic embryo at the focal zone stage (92 x).

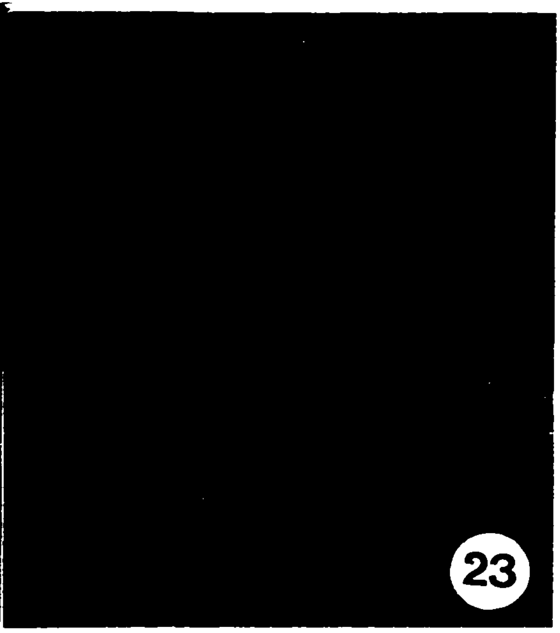


21



24

22



23

embryonal mass and appear to be part of the suspensor. Though the suspensor remains white and translucent, the embryonal mass was opaque and a creamy white colour.

Resin-embedded section (Figure 24): A constriction was apparent in the suspensor just below the focal zone. The embryonal mass was larger than the earlier embryonal-mass-plus-suspensor types described in Chapter 3, commonly 16-20 cells in width (compared to the 7-8 cell width embryonal mass stage) and approximately 11-13 cells deep. The surface was relatively smooth, though periclinal divisions seen in the surface cells indicated that there was no true epidermis. Though the cells of the focal zone were isodiametric and mostly of the same size and appearance, the cells on the surface tended to be slightly smaller and more densely cytoplasmic, with no or few small vacuoles. The cells in the interior of the focal zone varied from densely cytoplasmic with small vacoules to highly vacuolate. There did not appear to be any organized planes of division at either end of the focal zone. The transition between cells of the focal zone and embryonal tube cells of the suspensor was abrupt, indicating the presence of a rib meristem.

4.3.1.1.3 Focal zone plus polyphenol band

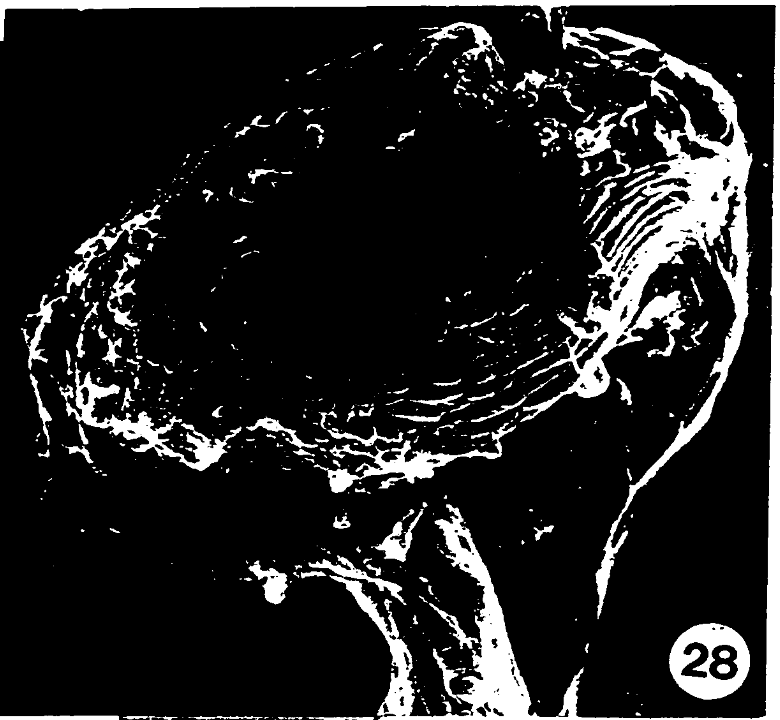
Fresh specimen (Figure 25): The acropetal surface was smooth and creamy white in colour. A red band which encircled the focal zone was just visible under the surface layers of the suspensor. The suspensor was still turgid for several cell lengths behind the developing embryo. In later stages, the surface began to turn red, often with a darker spot in the middle of the acropetal surface.

Resin-embedded (Figures 26, 27): The acropetal surface of the structure was typically rough, often with small protrusions. The cells in the first 2-3 layers of the apical surface were small and noticeably more densely cytoplasmic than the other cells in the structure. Periclinal divisions in the surface layer indicated that there was no true epidermis. The layers of dense cells formed a dome across the top of the structure. Under this dome, in the interior of the structure, the cells were larger and appear to be highly vacoulated in many cases. A transition zone between the cells of the interior of the sphere and the suspensor no longer existed.

In some sections, an area of small, densely cytoplasmic cells was apparent in the basal portion of the embryo . This area appeared where the root meristem would be expected to appear. In some cases, the cells of the basal meristematic region and the apical surface cells in that the cells were slightly smaller and did not appear to be dividing randomly, compared to the cells of the interior of the embryonal mass.

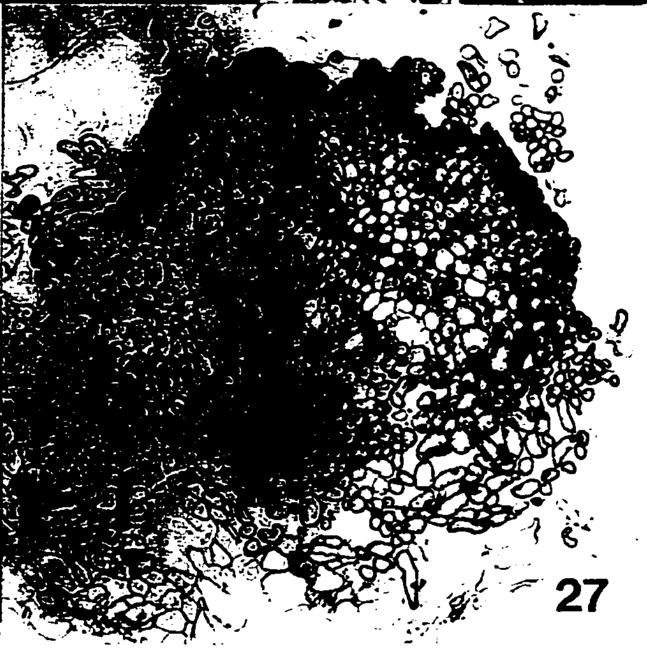
Figures 25-28: Figure 25: Fresh specimens of immature somatic embryos at the focal zone plus polyphenol band stage and the indentation stage (20 x). a. somatic embryos at the focal zone plus polyphenol band stage. b. somatic embryo showing beginning of indentations which indicate start of cotyledonary primordia formation. Figure 26: A cross-sectioned immature somatic embryo at the focal zone plus polyphenol band stage showing the development of a root meristem (88 x). a. developing root meristrem. Figure 27: A cross-sectioned immature somatic embryo at the focal zone plus polyphenol band stage showing the development of a root meristem (88 x). Figure 28: A scanning electron micrograph of an immature somatic embryo at the focal zone plus polypheol band stage (88 x).

25



28

26



27

Scanning electron micrograph (Figure 25): The apical surface of the structure was smooth, with no prominent protrusions. On the sides of the structure, the cells were elongated and adhered tightly together, creating a relatively smooth surface. The overall appearance retained the delineation between embryonal mass and suspensor cells though the embryonal mass had enlarged substantially as observed from its increased width as compared to the diameter of the suspensor. The 4 cell lengths back from the embryonal mass the embryonal tube cells began to loose their tight adhesion and began to appear disassociated. This likely marked where the suspensor cells were dying. The indentations which marked the beginning of the cotyledonary primordia were present. The cotyledons arose on the periphery of the smooth surface.

4.3.1.1.4 Initiation of cotyledons

Cotyledons arose from the apical margins (Figures 29,30). The first indication of cotyledon growth was slight indentations in the acropetal surface. The indentations were normally linear, dividing the surface into several equal sections. In resin-embedded sections, the cotyledons began as small protrusions on the surface, consisting of a small group of densely cytoplasmic, meristematic cells in the area between the smooth apical surface and basal region (Figure 31). The surface of the emerging cotyledons was smooth. Periclinal divisions in the surface cells indicated that no true epidermis had formed in the emerging cotyledons.

4.3.1.1.5 Mature somatic embryos

Mature somatic embryos had a ring of cotyledons (Figures 16, 32). Typically, the hypocotyl was extended and cylindrical, with a smooth epidermis. The number of cotyledons present ranged from 1 to 8, but was usually 6-7. The cotyledons and hypocotyl were green, but the base of the hypocotyl was red. A remnant of the suspensor occasionally remained attached to the base of the hypocotyl. As the cotyledons expanded, they occasionally reflexed slightly. The shoot meristem was not usually apparent for several weeks after the cotyledons expanded. There was quite a variation in the appearance of the hypocotyls and the cotyledons in immature somatic embryos. This will be discussed in Chapter 5.

The first indication of the development of an epidermis was found when the cotyledons began to elongate (i.e. when they were longer than they were wider)

Figures 29-32: Figure 29: Fresh specimens of immature somatic embryos at the indentation stage and the beginning of cotyledonary primordia development (20 x). a. somatic embryo showing indentation, b. somatic embryo with cotyledonary primordia. Figure 30: Scanning electron micrograph of an immature somatic embryo at the beginning of cotyledonary primordia development (188 x). Figure 31: Resin-embedded median section of an somatic embryo at the beginning of cotyledon development (70 x). a. cotyledonary primordia, b. meristematic zone. Figure 32: Scanning electron micrograph of a mature somatic embryo (30 x).

29

30

32

31

(Figure 33). At this stage, the surface cells did not appear to be dividing periclinally. The epidermis extended down the hypocotyl to the red cells which still encircled the basal portion of the immature embryo. No shoot apical meristem was obvious. Intercellular spaces could be present beneath the top 2-3 surface cell layers between the cotyledons. Intercellular spaces did not seem to be common in the cotyledons nor in any area other than just below the surface where the shoot meristem was expected to originate between the cotyledons. The cells of the hypocotyl were becoming elongate. Since the cells were not densely cytoplasmic, the increase in hypocotyl length was likely due to cell expansion rather than cell division.

Even when the cotyledons were fully extended, there appeared to be no shoot apical meristem. A root meristem could not always be found in the sectioned material. If a root meristem was not present in the section, then the basal portion of the hypocotyl appeared to end with several layers of very loosely adhering isodiametric cells. If a root meristem was present in the section, the hypocotyl had begun differentiation into an epidermis, cortex and stele. Cells in the stele elongated and began to take on the appearance of provascular tissue. While the stele was developing and the cotyledons were fully expanded, the root meristem was still rudimentary.

Scanning electron micrographs show normal mature somatic embryos with smooth hypocotyls and numerous cotyledons (Figure 32). The hypocotyl was cylindrical. The cotyledons were in close contact until they reflexed, likely when the shoot meristem began to form. The shoot meristem formed inside the ring of cotyledons.

4.3.1.2 Developmental pathways

4.3.1.2.1 Proliferation

Proliferation began with the enlarging of one of the lobes of an embryonal mass (Figure 34). As the subsidiary embryonal mass approached the size of the main embryonal mass, the entire embryonal mass complex became ellipsoidal in cross section. The rib meristems of the two embryonal masses divided apace to continue the common

Figures 33-36: Figure 33: Resin-embedded median section of a mature somatic embryo (75 x). Figure 34: Squashed sample of cleaving embryonal masses (120 x). Figure 35: Fresh specimen of a nodule (20 x). Figure 36: Median section of a nodule (70 x). a. root meristematic zone.


33


34


36


35

suspensor. Eventually, the rib meristems of the two embryos and their suspensors started to separate. Cleavage was then complete and the suspensors continued to elongate. Eventually, the embryonal tube cells which the embryos shared died and the embryos plus their respective suspensors disassociated from each other.

Commonly, an embryonal mass would continue to cleave rather than to develop to the focal zone stage. The rough surface of the REMLS and REMLLS types of embryonal masses were considered to be the results of ongoing cleavage, where cell divisions in the embryonal mass gave rise to two or more lobes. Each lobe then differentiated their own embryonal tube cells which elongated into a suspensor. The new daughter embryonal masses continued to proliferate or to develop into the focal stage. No meristematic region was noticed within the embryonal tube cells of a suspensor, indicating that proliferation arose from division of the meristematic embryonal mass cells. However, infrequently, the lobes of a cleaving embryonal mass failed to separate completely. In this case, a compound embryonal mass formed. The compound embryonal mass typically had the multiple embryonal masses in a linear arrangement with a common suspensor .

4.3.1.2.2 Nodule formation

4.3.1.2.2.1 External appearance of nodule

In some cases, the cells of the white, acropetal surface of a developing embryo at the focal zone with polyphenol band stage developed polyphenols. The embryo then became a rough spherical red nodule (Figure 35). The nodule was isodiametric and was typically uniformly deep red in colour.

4.3.1.2.2.2 Internal organization of nodule

In cross sections the red nodules were roughly spherical and closely resembled an embryo at the focal zone plus polyphenol band stage (Figure 36). No epidermis was apparent since the surface cells divided periclinally. The interior cells were typically highly vacuolate and isodiametric. In some cases, intercellular spaces were evident. The acropetal 2-3 layers of cells on the surface opposite the suspensor were typically small, isodiametric and densely cytoplasmic. The cells in the interior were slightly larger and more vacuolated. As the suspensor degenerated and detached from the structure the basipetal cells of the nodule appeared disorganized with large intercellular spaces.

In some cases, cotyledons arose from the acropetal region of the nodule, followed by an epicotyl (Figures 37,38). In some cases, a meristematic region in the basipetal region was also seen, indicating the emergence of a root meristem. In the case of the cotyledons, a small group of interior cells below the acropetal surface became meristematic. The development of the cotyledons resembled the emergence of cotyledons in normally developing embryos, with a meristematic group of cells giving rise to a cotyledonary primordium on the periphery of the acropetal area of the nodule . The internal cells of the nodule showed no change after the cotyledons begin to form, but remained undifferentiated.

4.3.1.2.2.3 Meristem development in nodules

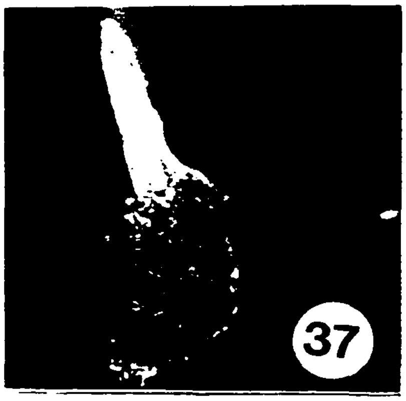
A root meristem could arise within a nodule . In this case, the acropetal layers of surface cells remained small and densely cytoplasmic. A group of cells in the basipetal area became small and densely cytoplasmic, indicating the origin of the root meristem. The roots of several nodules elongated (Figure 39). The roots appeared normal in shape and colour. In some cases, no cotyledons formed on the germinating nodules.

A shoot meristem could form on a nodule (Figure 38). A shoot meristem could form inside the ring of cotyledons and give rise to an epicotyl and true needles, though the epicotyl was not always normal.

4.3.2 Critical stages of development

Embryos isolated at different stages of development displayed different developmental fates (p=0.0001)(Table 5). Proliferation did not occur in more that 5% of the embryos in any one stage of development. The combined percentages for dead and developmentally arrested was greater than 50% for the SEMLS, focal zone, and focal zone plus polyphenol streak stages, but dropped to 13% for the focal zone plus polyphenol band stage, then rose to 25% for the cotyledonary primordia stage. The percentage of immature embryos which developed into mature embyos rose steadily from 7% for the SEMLS stage to 69% for the cotyledonary primordia stage. The percentage of immature embryos which developed into nodules was less consistent, but in general rose from 10% for the SEMLS stage to 20% for the focal zone plus polyphenol stage before dropping to 2% for the cotyledonary primordia stage.

Figures 37-39: Figure 37: A nodule with cotyledons (20 x) a. unextended cotyledons. b. extended cotyledons. Figure 38: Median section of a nodule with cotyledons (70 x). Figure 39: A nodule with an extended root (20 x).



37

38

39

None of the initial stages of development could be pooled, indicating that they had independent associations with the final stages of development. By examining the deviations (observed frequencies - expected frequencies), a trend for later stages of development to be less likely to die, stall or proliferate and more likely than expected to mature into embryos becomes apparent. Both SEMLS and embryos with cotyledons started were less likely than expected to become nodules. In terms of deviations from expected frequencies, the focal zone stage appeared to be intermediate between the stages of development which were more likely than expected to stall and proliferate (SEMLS and FZ) and the stages of development which were more likely than expected to become embryos (FZ+PB and cotyledons started).

Table 5: The effect of stage of development at plating out on the developmental fate of individual somatic embryos

Stage of Develop.	# plated	Embryos	Nodules	Prolif.	Stalled	Dead
SEMLS	303	21 (7)	31 (10)	5 (2)	116 (38)	130 (43)
Focal zone	192	36 (19)	35 (18)	2 (1)	87 (45)	32 (17)
FZ+STR	218	62 (28)	32 (15)	1 (0.5)	103 (47)	20 (9)
FZ+PB	346	211 (61)	70 (20)	19 (5)	34 (10)	12 (3)
Start of cotyl.	150	103 (69)	3 (2)	7 (5)	15 (10)	22 (15)

Percentages are indicated in brackets () after the frequencies
dead: embryonal masses which did not develop after transfer
stalled: embryonal masses did develop to a stage further but then became developmentally arrested and matured no further.

Statistics: analysed as a 5 x 5 contingency table with 5 classes of stages of development and 5 classes of developmental fate. All subset contingency tables showed significant differences, therefore no classes could be pooled

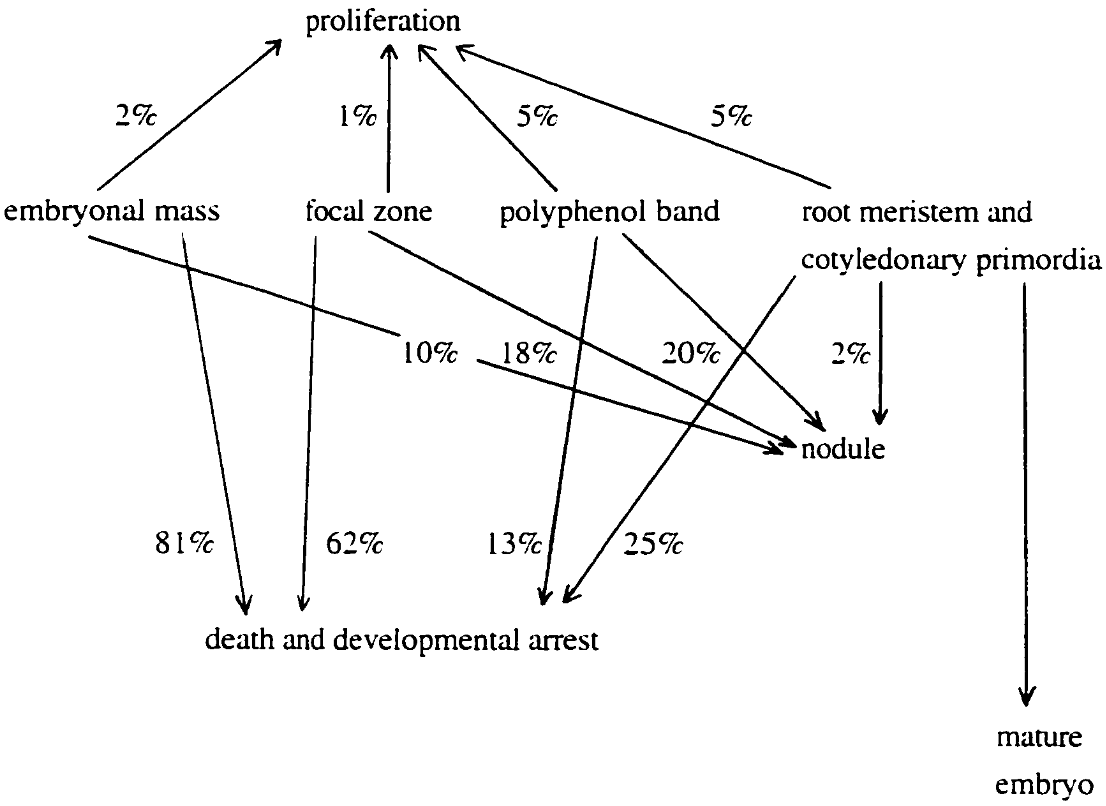
Degrees of freedom	16
Total chi-square	514.169
Contingency coefficient	0.546
Probability =	0.0001

Deviations

Fate	Stage of development				
	SEMLS	FZ	FZ+STR	FZ+PB	Cotyl. started
Dead	75.87	-2.30	-18.95	-49.82	-4.80
Stalled	27.03	30.62	38.99	-67.60	-29.04
Prolifertion	-3.52	-3.40	-5.13	9.27	2.78
Embryo	-87.52	-32.76	-16.08	87.08	49.28
Nodules	-11.86	7.84	1.17	21.06	-18.22

4.3.3 Pathways of development chart

The following chart outlines the different pathways of development possible for an embryonal mass. Though not included in the chart, there are several different types of somatic embryos that can form. These will be discussed in Chapter 5.



4.4 Discussion

4.4.1 Histological studies of line 2086

Larch somatic embryos at the embryonal mass and focal zone stages correspond to stage 1 and stage 2 somatic embryos, respectively, according to the classification of Hakman and von Arnold (1988). In their development, as shown by resin-embedded sections, they resemble zygotic embryos at the same stages of development (Owens and Molder 1979c). The organization of *Larix occidentalis* buds showing true needle primordia and a shoot meristem is similar to that found for *Larix x eurolepis* somatic embryos in this study (Owens and Molder 1979 a,b,c).

However, unlike *Picea glauca*, proliferation from meristematic suspensor cells was never observed (Hakman *et al.* 1987). Sections of stage 1 embryos of *Picea glauca* resemble the organization of *Larix x eurolepis* embryos, especially at the focal zone stage (Hakman *et al.* 1987), though Joy *et al.* (1991) observed a smooth embryonal mass with a disorganized suspensor. Similarily, sections of *Picea abies* embryos at stage 1, stage 2 and stage 3 closely resemble those of *Larix* (Hakman and von Arnold 1985).

Unlike somatic embryos, zygotic embryos do not form a polyphenol band in the basipetal region of the focal zone, and this may be due to exposure of somatic embryos to light (Klimaszewska 1989a). Similar pigmentation was noted on immature somatic embryos of *Larix x leptoeuropaea* (Lelu and Label 1994). The purpose of the formation of the polyphenol band in somatic embryos is not clear. The band forms in the area where the root meristem will arise and clusters of meristematic cells arise in the basal region of the embryo when the polyphenol band forms, though not all sections showed the presence of a root meristem.

Root meristems could not be found in all sectioned specimens of mature *Larix x eurolepis* embryos, and this was considered an artifact of sectioning through asymmetrical embryos looking for a small cluster of meristematic cells in the basal region. Sectioned mature embryos of *Picea abies* and *Sequoia sempervirens* had root meristems (Hakman and von Arnold 1985, Boulay *et al.* 1988, Bourgkard and Favre 1988). In somatic embryos of *Picea sitchensis*, root development usually occurred before shoot development, and apical shoot development was enhanced by root development (Krogstrup *et al.* 1988). However, in the median sections of *Picea sitchensis* immature embryos, there was cotyledon development but no root or shoot meristems (Krogstrup *et al.* 1988).

The hypocotyl of mature *Larix x eurolepis* somatic embryos was cylindrical, with parallel sides and often showed the begining of stele differentiation. In contrast, the hypocotyl of mature *Sequoia sempervirens* somatic embryos was irregular, with a narrowing of its width just below the cotyledons (Bourgkard and Favre 1988).

Schopf (1943) declared that no true epidermis formed on zygotic embryos of *Larix* until the cotyledons were fully formed. The epidermis appeared first over the cotyledons and the apical meristem. This appears to be true for somatic embryos of *Larix x eurolepis*. There is clearly no epidermis at the focal zone plus polyphenol band stage (Figures 26,27). However, a distinct cell layer had formed over the cotyledonary primordia, with few anticlinal divisions observed (Figures 31, 33, cross sections of cotyledons). Schopf (1943) also noted the presence of secretory elements in the hypocotyl, similar to the findings of Owens and Molder (1979c), but it was not found in somatic embryos of hybrid larch in this study. The presence of ABA in the maturation medium resulted in the formation of secretory elements in somatic embryos of *Larix x eurolepis* (Gutmann *et al.* 1996); therefore, a comparison of sectioned material from somatic embryos of line 2086 grown on medium with ABA and medium without ABA is needed before any definite conclusions can be drawn.

The linear depressions which mark the beginning of cotyledon formation are likely the result of the initiation of meristematic activity below the surface of the embryonal mass forming protrusions and consequent depressions on the surface. Chowdhury (1962) found the number of cotyledons in *Larix* zygotic embryos to be 3-8, and usually 8, which is similar to the mature somatic embryos in this study. Infrequently, only one cotyledon forms on a somatic embryo, with no abrupt distinction with the hypocotyl. These embryos are capable of forming epicotyls (P. von Aderkas, personal communication) but none of these types of somatic embryos were monitored to determine if a shoot meristem formed. Unlike the other structures found in the plant embryo, the cotyledon serves as a photosynthetic source and a storage organ for food reserves for the developing embryo during germination. These structures usually senesce soon after the seedling emerges from the soil, which may permit greater variation in cotyledon development (Goldberg *et al.* 1994). The internal organization of the cotyledons in *Larix x eurolepis* somatic embryos resemble those of *Picea glauca* somatic embryos (Attree *et al.* 1992).

One difference noted between somatic and zygotic embryos of larch was the presence of intercellular spaces in the somatic embryos in the area where the shoot meristem will arise. These intercellular spaces are absent in zygotic embryos (Schopf 1943, von Aderkas *et al.* 1995). Though some mature somatic embryos of *Larix x eurolepis* did not have intercellular spaces (Figure 31, 33), some did. The intercellular spaces may be a result of the fast and unconfined growth of the somatic embryos but intercellular spaces only formed in the area of the shoot meristem, not throughout the embryo. Median sections of *Picea* *glauca* shoot meristems had no intercellular spaces (Attree *et al.* 1992). However, in a later study, intercellular spaces directly behind the meristematic zone were found in *Picea glauca* somatic embryos which were not subjected to a desiccation treament. Often, these embryos could not form functional shoot meristems (Attree and Fowke 1993). No intercellular spaces were observed in the apical meristems of *Picea abies* (Hakman and von Arnold 1985). Somatic embryos of *Pinus taeda* also have intercellular spaces and less organized shoot meristem regions than zygotic embryos. As the number and/or size of the intercellular spaces increases, there is a decrease in shoot meristem development (Becwar and Pullman 1995). Since detection of intercellular spaces only occurs after fixing and sectioning, comparing the epicotyl development of somatic embryos with intercellular spaces to those without is impossible.

4.4.2 Alternative developmental pathways

The alternative pathway involving the formation of the red nodules correspond to results found in other cultures. Proembryoids formed in habituated callus derived from unfertilized ovules of *Citrus sinensis* grown on plant growth regulator-free medium can develop into pseudo-bulbils which resemble the nodules found in line 2086 in sections, or can develop into mature embryos (Button *et al.* 1974). Both pathways occur simultaneously in the same culture conditions. In addition, nodules were found in cultures of *Picea abies* (Hakman and von Arnold 1985). Hakman and von Arnold (1985) noted that all the somatic embryos of *Picea abies* developed into nodules from which needles arose. Buds which could be easily removed and roots were also formed by these nodules. In cross section, these nodules closely resemble the nodules found in *Larix x eurolepis*. Though the nodules of *Larix x eurolepis* were capable of forming cotlyedons and roots, no multiple, easily-removed buds were formed.

In sections of *Larix x eurolepis* nodules, the central area had intercellular spaces and isodiametric cells which appeared to be undifferentiated. The root primordia formed in the same area the suspensor was attached. The root primordia arose from a localized zone of cells in the periphery (but not the surface) of the nodule. In embryogenic carrot cultures, clumps of cells could form with tracheid-like cells in the interior. Root primordia could develop from these clumps of tracheids, and these structures could form plantlets if transferred to semi-solid media. No sectioning was done on these structures to determine where the shoot apex originate in relation to the central tracheid cells and the root primordium (Halperin 1966).

The two most distinctive features of the nodules were their red appearance and the formation of cotyledons on the apical surface in the apparent absence of a hypocotyl. Though all developing somatic embryos form a band of polyphenols around the basal area, the apical surface remains free of polyphenol deposition. For some reason, polyphenol production does not remain localized in the embryos which become nodules. Since nodules and mature somatic embryos develop concurrently on the same aggregates, the absence of ABA in the medium would not explain this difference. The effects of ABA on developing somatic embryos of line 2086 is discussed in Chapter 6.

Though there is no chlorophyll development in nodules, there is still a hypocotyl. By comparing Figures 31 and 38, an undifferentiated area between the root meristem and the cotyledons is evident in both nodules and stage 3 embryos. Developmentally then, nodules can be said to be somatic embryos with unextended hypocotyls and a lack of chlorophyll synthesis.

The reasons why nodules occur in larch embryogenic cultures is not known, especially since normal somatic embryos formed on the same aggregate at the same time as the nodules. Carrot somatic embryos developed into giant spherical structures if auxin transport is inhibited (Goldberg *et al.* 1994), indicating that disruption of polarity may be involved. Possibly, in some embryonal masses, the proper synthesis and/or transport of endogenous auxins was lacking. The reasons why the nodules were entirely covered by cells containing polyphenols while focal zones destined to become somatic embryos confined anthocyanin production to a band encircling the basipetal area is also unclear. Further study is necessary to determine if the smooth white surface of the focal zone was covered by red cells as the nodule forms or if the smooth surface cells themselves were converted into pigment-producing cells.

4.4.3 The relationship between stage of development and developmental fate

As expected, there was an association between the stage of development of isolated *Larix x eurolepis* somatic embryos and their subsequent developmental fate. Embryos isolated at more advanced stages of development were less likely to stop maturing than less advanced stages. Proliferation was the least common fate for all the stages of development, though more than 2% of the SEMLS were expected to proliferate, based on the experiments in Chapter 3. Eastman *et al.* (1991) isolated 45 embryos of *Picea glauca-engelmannii* at each of the following stages of development: SEMLS, focal zone, cotyledonary primordia and mature somatic embryo. Though 100% of the *Picea* SEMLS embryos proliferated, the proliferation percentage declined to 30% for mature embryos, but the differences were not significant. Since the SEMLS embryos were removed from embryogenic tissue on maintenance medium containing auxins and cytokinins while the other stages were removed from tissue on maturation medium, there may have been a carry-over effect of the plant growth regulators which induced 100% proliferation in the SEMLS (Eastman *et al.* 1991). This result supports the use of line 2086 of *Larix x eurolepis* for studying embryo development because the ability of this line to proliferate and mature on plant growth regulator-free medium removes the confounding effect of exogenously applied growth regulators.

Embryos isolated at earlier stages of development were more likely to die or become developmentally arrested than embryos at later stages of development. In addition, embryos isolated at later stages of development were more likely to become mature embryos, though the number of immature embryos which became mature embryos rather than a nodule did not greatly increase until after cotyledons had formed. The percentage of immature embryos that halted development dropped off abruptly once the polyphenol band began to develop. Further studies may indicate that the formation of the polyphenol band is a significant stage of development. Therefore, using embryos at the focal zone or focal zone plus polyphenol band stage for experimental manipulation will likely be more efficient than selecting embryonal masses, in terms of the number of embryos which finish development, whether it be a nodule or a mature embryo. An embryo was less likely to become a nodule only after the cotyledons had started to form.

4.4.4 Conclusion

In conclusion, the stages of development of *Larix x eurolepis* line 2086 closely resembled those of *Larix* zygotic embryos and somatic embryos of other species. Alternative pathways of development included continual cleavage and the formation of nodules. Since nodules could form roots, cotyledons and epicotyls, they were likely a temporary disorganization of embryo formation rather than a separate pathway of development. The reasons why some somatic embryos became nodules is unclear, but embryos could develop into nodules at any stage of development before the cotyledons started to form. As somatic embryos developed, they were less likely to proliferate and become developmentally arrested, and more likely to develop into mature somatic embryos. A critical stage of development was the formation of the polyphenol band at the basal end of the focal zone, because the rates of proliferation and developmental arrest declined after this stage.

Chapter V: Phenotypic variation in mature and germinating somatic embryos

5.1 Introduction

The goal of somatic embryogenesis is to produce a vigourous plant in the field. The criteria by which somatic embryos are judged are their similarity to zygotic embryos, both in morphology, and storage protein deposition. Normally, only somatic embryos which resemble zygotic embryos are selected from the maturation medium to be placed on germination medium. For instance, only *Picea mariana* somatic embryos resembling zygotic embryos (1.5-2 mm long, 3-6 cotyledons) were placed on maturation medium, and this resulted in an increased percentage of mature embryos which rooted (Adams *et al.* 1994). Though several papers do mention the formation of abnormal somatic embryos on maturation medium, few mention the percentage of mature somatic embryos that are abnormal and whether these embryos represent a complete loss in terms of inability to form normal root and shoot meristems.

Depending on the maturation treatment, the yield of somatic embryos which are capable of germination and of surviving acclimatization in the field can vary greatly. Under optimum maturation conditions followed by transfer to germination media, germination rates can range from 20% for *Picea pungens* (Afele and Saxena 1995) to 87% for *Abies balsamea* (Norgaard and Krogstrup 1995). Transplant survival rates vary from 45% for *Picea mariana* (Adams *et al.* 1994) to 98% survival after 3 years for *Pinus taeda* (Becwar and Pullman 1995).

The tissues of the immature embryo arise from the organized divisions of the embryonal mass as it progresses through the focal zone stage (stage 2) to produce a hypocotyl, cotyledons, a root meristm and a shoot meristem. However, all subsequent tissues - the elongated root and the epicotyl - arise from the apical meristems. Because embryos display modular development (Goldberg *et al.* 1994), abnormalities in the hypocotyl and cotyledons may not affect the development and growth of the apical meristems. If a proper vascular connection forms between the root and shoot meristem, then the morphology of the hypocotyl and cotyledons may be an irrelevant criterium when assessing the acclimatization success of any particular embryo.

The hypocotyl and cotyledons form as a result of changes in planes of division in the apparently disorganized embryonal mass, whereas the epicotyl and root form from orderly divisions of cells in meristems in already polarized embryos. Theoretically, the culture environment should have a greater effect upon the more plastic early stages of hypocotyl and cotyledon formation as opposed to root and epicotyl development from meristems. Therefore, the purpose of this section was to determine the relationship between mature embryo morphology, epicotyl morphology and root production. If normal roots and shoots can be produced by abnormal somatic embryo then the abnormal embryos need not be discarded, resulting in increased production efficiency.

This chapter describes the different types of morphology displayed by mature somatic embryos of a hybrid larch species (*Larix x eurolepis*) both in fresh specimens and in resin-embedded sections. The effects of final morphology on rooting percentages, mortality rates and the types of epicotyls produced are also presented.

5.2 Materials and methods

5.2.1 Culture maintenance

Line 2086 originated from an immature zygotic embryo of a hybrid between *Larix leptolepis* (maternal parent) and *Larix decidua* (paternal parent)(von Aderkas *et al.* 1990). The embryogenic tissue was initiated on 1/2 Litvay's medium supplemented with glutamine and 0.45 µM 2,4-D. Line 2086 was routinely cultured every 2-3 weeks onto 1/2 strength Litvay's medium (Litvay *et al.* 1981) supplemented with 59 µM sucrose, 4 g/l gelrite, 1 g/l casein hydrolysate, 3422 µM glutamine without plant growth regulators. The medium was adjusted to pH 5.6-5.8 before autoclaving. The glutamine and all plant growth regulators were filter sterilised using a 22 µm filter and were added after the medium had been autoclaved in an Agarmatic (New Brunswick Scientific Co. Inc.). The medium was dispensed by 20 ml aliquots into 100 x 15 Petri dishes (Fisher) and allowed to cool to room temperature before storage at 4 °C. Four aggregates of embryogenic tissue were cultured on each Petri dish and were individually marked. The plates were kept in a growth chamber at 22 °C with a 16 hour light/8hour dark photoperiod at 36 µmol/m^2/s photosynthetically active radiation. In the absence of plant growth regulators, mature somatic embryos of line 2086 germinated precociously and asynchronously.

5.2.2 Determination of mature embryo morphologies and epicotyl characteristics

Over a two month period, all mature embryos produced by line 2086 were removed from the aggregates and placed onto fresh media. Each structure was classified according to their proportions (normal or misshapen), vitrification, and epidermal qualities (smooth or rough). Every vitrified embryos had a vitrified epidermis, therefore there was only one category for vitrified embryos. The embryos were subcultured every 4 weeks. After 8 weeks, the embryos were examined to determine root or epicotyl formation. All roots produced were normal and resembled the roots of germinating zygotic embryos in terms of colour and form and therefore the embryos were simply noted as having rooted or not. This experiment was repeated twice with three replications. Because every embryo produced was utilised, the number of embryos in each replicate and each category varied.

The types of epicotyls produced were classifed as twins, normal or misshapen, and according to their epidermal qualities (rough, smooth or vitrified). The number of dead embyros was recorded for replications 2 and 3, but not for replication 1. For replications 2 and 3, the number of dead embryos that produced a root before death was noted also. The number of dead embryos was not recorded for replicate 1, therefore the number of dead was calculated by summing the number of dead in replicate 2 and replicate 3. If an embryo rooted before it died, it was counted as rooted, because in this experiment the development of meristems was considered more important than survival. If twin epicotyls were different, the embryo was categorized according to the most abnormal epicotyl, i.e a normal smooth /abnormal smooth twin would be classified as abnormal smooth.

Table 6 gives the root and epicotyl characteristics of 1332 mature embryos. Table 7 gives a further break-down of the types of epicotyls produced.

The experiment was analysed as a series of subset contingency tables. The first was a 2 x 2 contingency table, with 2 categories of embryo proportions (normal and misshapen) and 2 categories of epidermal types (smooth and rough). This contingency table determined if an abnormally shaped embryo was more likely to have a rough epidermis than a normal embryo.

The second was a 2 x 2 contingency table, with 2 epidermal types (smooth and rough) of somatic embryos and 2 epidermal types (smooth and rough) of epicotyls to determine whether the epidermal type of the embryo affected the epidermal type of the epicotyl. The third was a 3 x 4 contingency table, with 3 categories of proportions of somatic embryo (normal, vitrified and misshapen) and 4 categories of epicotyl proportions (twin, normal, misshapen and vitrified). When tallying the totals for the normal category, only embryos with a smooth epidermis and normal proportions (normal-smooth) were placed in this category. Embryos with normal proportions and rough epidermis were placed in the misshapen category. This contingency table determined if the type of embryo affected the type of epicotyl. For this 3 x 4 contingency table, no categories could be pooled.

The fourth contingency table tested was a 2 x 2 contingency table, with two categories of embryo type (normal and abnormal (misshapen plus vitrified)) and two categories of epicotyl type (normal and abnormal (misshapen plus vitrified)). This contingency table tested whether an abnormal embryo type was more likely to produce an abnormal epicotyl type compared to a normal embryo.

5.2.3 Resin-embedding and sectioning

Individual mature embryos were separated from the tissue aggregates before fixation. The tissue was fixed for 2-3 hours in 2.5% glutaraldehyde in 0.075M phosphate buffer. Post fixation, the tissue was washed in 0.075Mphosphate buffer 3 times, for 10 minutes each. The tissue was then fixed for 2-3 hours in 1% osmium tetroxide in 0.075M phosphate buffer followed by a short buffer wash. The tissue was then dehydrated for 10 minutes in 20, 40, 60 and 80 % acetone followed by 3 changes of 100% acetone for 10 minutes each. The tissue was infiltrated by 30% Spurr's resin in acetone for 10-12 hours, followed by infiltration by 75% then 100% Spurr's resin in acetone for 2 hours each. The tissue was finally embedded in 100% Spurr's resin overnight at 70°C (Karnovsky 1965, Glauret 1974 Roland and Vian 1991). Spurr's resin (Spurr 1969) was prepared from ERL 4206 (vinyl cyclohexene dioxide), D.E.R. 736 (diglycidyl ether of polypropylene glycol), NSA (nonenyl succinic anhydride, and S-1 (DMAE - dimethyl aminoethanol). (Marivac Ltd, Halifax N.S.).

The resin blocks were sectioned at 0.3 - 0. 8 µm using glass knives on either a Reichert-Jung Ultracut E microtome or a Reichert Om U2 microtome, both from American Optical Instrument Company, Buffalo, N.Y. The sections were floated on a drop of water on a slide and placed on a hot plate until the water evaporated. The sections were stained with Richardson's stain (1 g borax plus 1.5 g methylene blue in 100 ml water added to 0.5 g azure B in 100 ml, mixed and filtered) and the slide was placed on the hot plate for 25 seconds. The slide was rinsed with water and allowed to air dry before being mounted in Permount (Fisher). The sections were examined under a light microscope. Photographs were taken using Tmax 100 film or Kodak technical pan film (100 ASA).

5.2.4 Transplanting success

Fifty-seven mature somatic embryos, 42 with roots and true needles, 15 with roots and only a ring of cotyledons, were planted in soilless mix (3:2:1 peat moss:vermiculite: perlite) in the greenhouse on bottom heat in a mist bed covered with a white muslin cloth in early autumn.

5.3 Results

5.3.1 Types of final morphologies

Several types of final morphologies were found in line 2086. The epidermis could be smooth (Figures 16, 32, 40, 41) or rough (Figures 17, 42, 43). The embryo could be vitrified (Figure 44). The proportions of the hypocotyls and cotyledons could be normal (resembling zygotic embryos) (Figures 16, 32, 40, 41) or abnormal (Figures 40, 41, 43). Somatic embryos could have cotyledons ranging from 1 to 7. Multiple epicotyls formed (Figures 44, 45, 46), and some did not share the same characteristics; one epicotyl could be normal while its twin could be vitrified. (Figure 46). However, usually, normal mature somatic embryos produced single, normal epicotyls (Figure 47). An abnormal embryo could produce a normal epicotyl (figure 48) or an abnormal epicotyl.

Resin-embedded sections were taken of normally proportioned embryos and emblings with a normal epidermis (Figure 49), abnormal embryos with a smooth epidermis (Figure 50), normal embryos with a rough epidermis (Figure 51) and vitrified embryos (figure 52). On smooth embryos (Figure 49), the epidermis is a continuous layer, one cell thick, though in abnormal embryos with a smooth epidermis, the epidermis may not extend down the hypocotyl (Figure 50). In rough embryos (Figure 51), the outermost cells were only loosely associated and the inner tissues of the embryo seemed less organized. In some rough embryos, the surface of the shoot meristem was rough as well, indicating that the formation of the shoot meristem did not convert the surface cells to organized growth as an epidermis. Vitrified embryos (Figure 52) seemed to have an internal organization similar to normal embryos with a smooth epidermis, but in vitrified embryos the epidermis was discontinuous in places.

Not all mature embryos rooted. The roots themselves, once formed, appeared normal in every instance, whether the root was from a nodule (Figure 39), from a normal embryo (Figure 16) or from an abnormal embryo (Figure 17). The roots were uniformly white, cylindrical, with a rough but continuous epidermis, and a red tip.

Figures 40-42: Figure 40: Abnormal smooth embryo (20 x). Figure 41: Abnormal smooth embryo (20 x). Figure 42: Normally proportioned mature somatic embryo with rough epidermis (20 x).

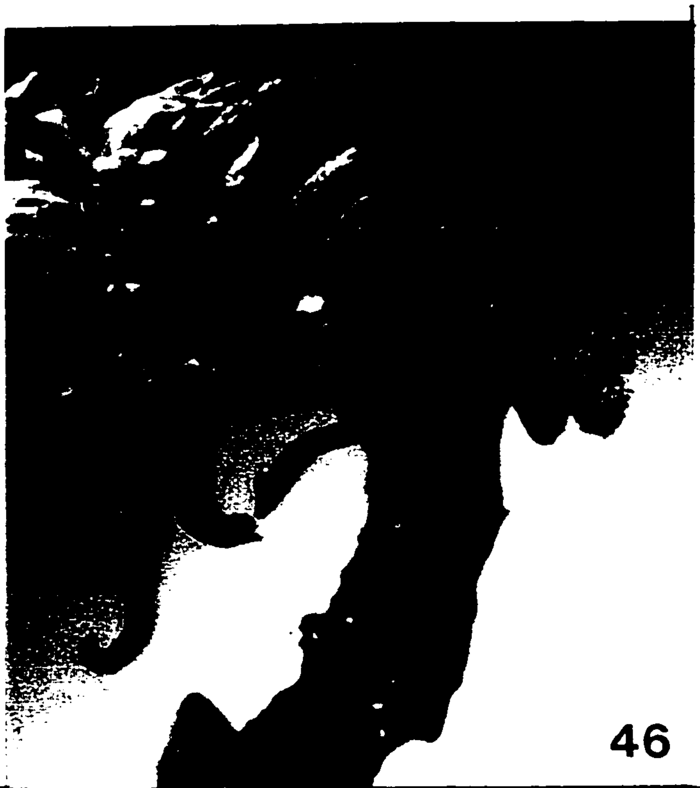

40


41


42

Figures 43-46: Figure 43: Abnormally proportioned mature somatic embryo with a rough epidermis (20 x). Figure 44: Vitrified abnormal embling with multiple epicotyls (20 x). Figure 45: Embling with twin epicotyls (20 x). Figure 46: Mature somatic embryos with twin epicotyls exhibiting different growth patterns (20 x). a. epicotyl exhibiting normal growth. b. epicotyl exhibiting vitrified growth.

43



46

44

45

Figures 47-48: Figure 47: Normal embling with normal epicotyl (20 x). Figure 48: Abnormally proportioned somatic embryo with normal epicotyl (20 x).

47

48

Figures 49-52: Figure 49: Median section of a normal mature somatic embryo with a smooth epidermis (70 x). Figure 50: Median section of an abnormal mature somatic embryo with a smooth epidermis (70 x). Figure 51: Median section of an normally proportioned mature somatic embryo with a rough epidermis (70 x). Figure 52: Median section of a vitrified mature somatic embryo (70 x).


49


50


51


52

5.3.2 Rates of reversion to normal shoot growth and percent rooting

The complete data sets are presented in Tables 6 and 7. Tables 8-11 are subsets of Tables 6 and 7 and were analysed to answer specific questions concerning the relationship between embryo morphology and meristematic growth. Every root produced was normal in appearance - cylindrical, white with a red tip. Few twin roots were produced. With the exception of vitrified embryos, the number of embryos which rooted was greater than the number of embryos which produced normal epicotyls (Table 6). The proportions of the embryo were strongly associated with the epidermal qualities of the embryo (p=0.0001) (Table 8). An embryo with normal proportions was more likely to have a smooth epidermis than expected, and less likely to have a rough epidermis. The converse was true for abnormal embryos.

There was an association between the shape of the embryo and the shape of the epicotyl (p=0.0001) (Table 9). Normal embryos produced normal epicotyls much more frequently than expected, and twin epicotyls, abnormal and vitrified epicotyls much less frequently than expected. Vitrified embryos produced vitrified epicotyls more often than expected, and normal and abnormal epicotyls less frequently than expected. Abnormal embryos produced more twin and abnormal epicotyls than expected, and fewer normal epicotyls than expected.

There is an association between the type of embryo and the type of epicotyl it produces (p=0.0001)(Table 10). Though not all normal embryos with smooth epidermis' produce normal smooth epicotyls, a normal smooth embryo is more likely to produce a normal smooth epicotyl than would be expected if embryo type had no effect on epicotyl type. In addition, though abnormal embryos are more likely to produce an abnormal epicotyl than a normal epicotyl, of the 526 abnormal embryos examined, 145 produced normal smooth epicotyls.

Neither embryo shape nor epidermal quality had an effect on the percent dead embryos, the percent live rooted or the percent total rooted (Table 11). The overall rooting percentage was 58 % (852 rooted somatic embryos out of 1332). The percentage of rooted abnormal somatic embryos (smooth and rough epidermis combined) was 57%.

Table 6: The epicotyl production of individual somatic embryos

				epicotyl characteristics (shape)		
Embryo type	Epidermis type	Total#	rooted	twins	normal	misshapen
normal	smooth	572	431	110	375	28
	rough	158	104	61	33	30
vitrified	vitrified	159	65	51	96	5
abnormal	smooth	229	81	65	65	18
	rough	214	119	53	22	32

Table 7: Epidermal qualities of epicotyls produced by somatic embryos.

epicotyl characteristics

original

Embryo	Epidermis	twins			normal			misshapen		
type		norm.	rough	vit.	norm.	rough	vit.	norm.	rough	vit.
normal	smooth	102	0	8	352	12	11	16	12	0
	rough	18	1	42	27	5	1	11	19	0
vitrified	vitrified	30	1	20	64	0	32	0	2	3
misshapen	smooth	28	1	36	37	12	16	2	16	0
	rough	9	2	42	17	2	3	3	21	8

Table 8: Relationship between embryo shape and epidermal quality of mature somatic embyros

Observed Frequencies

Embryo shape	Epidermal characteristics	
	smooth	rough
normal	513	124
misshapen	148	107

Degrees of freedom	1
Total chi-square	48.016
Chi-square with continuity correction	46.851
Probability	p=0.0001

Table 9: Relationship between embryo shape and epicotyl shape

Observed frequencies

Shape of embryo	Shape of epicotyl			
	twin	normal	abnormal	vitrified
normal	110	352	40	11
vitrified	50	64	2	35
abnormal	175	81	91	28

Degrees of freedom	6
Total chi-square	280.654
G statistic	278.549
Probability	p=0.0001

Deviations (observed frequencies - expected frequencies)

Shape of embryo	Shape of epicotyl			
	twin	normal	abnormal	vitrified
normal	-55	107	-26	-26
vitrified	1	-8	-17	24
abnormal	54	-98	43	0

Table 10: Relationship between embryo type and epicotyl shape

	Observed frequency Epicotyl shape	
	normal/smooth	twin+abn+vit
Embryo type		
normal/smooth	352	161
abn+vit	145	381

Degrees of freedom	1
Chi-square with continuity correction	173.738
Contingency coefficient	0.38
Probability	p=0.0001

Table 11: The percent rooting and percent mortality rates for all embryo types.

Embryo shape	Epidermal	% dead	% rooted
normal	smooth	20	67
normal	rough	30	55
vitrified		29	54
abnormal	smooth	43	51
abnormal	rough	74	63

For percentage live rooted, reps 1, 2 and 3 could be used. However, due to missing values for percent dead and percent total rooted, only reps 2 and 3 could be used.

Table 11 continued: Statistics – the sums of squares and mean squares reported are for the transformed data (arc sine(sqrt(x)))

Effects of epidermal quality on percent live rooted, percent total rooted and percent dead.

		Sum of squares		Mean square		F-test		probability	
Source	DF	%live rooted	%total rooted	%live rooted	%total rooted	%live rooted	%total rooted	%live rooted	%total rooted
Between gr.	2	0.092	0.06	0.046	0.03	2.172	2.827	**0.1952**	**0.1365**
Within gr.	6	0.127	0.064	0.021	0.011				
Total	8	0.22	0.124						

Effects of epidermal quality on percent dead

Source	DF	Sums of squares	Mean squares	F-test	Probability
Between groups	2	0.143	0.072	1.062	**0.4029**
Within groups	6	0.405	0.067		
Total	8	0.548			

Effects of embryo shape on percent live rooted and percent total rooted and percent dead.

		Sum of squares		Mean square		F-test		probability	
Source	DF	%live rooted	%total rooted	%live rooted	%total rooted	%live rooted	%total rooted	%live rooted	%total rooted
Between gr.	2	0.081	0.061	0.041	0.03	1.77	2.884	**0.2487**	**0.1326**
Within gr.	6	0.138	0.063	0.023	0.011				
Total	8	0.22	0.124						

Effects of embryo shape on percent dead

Source	DF	Sums of squares	Mean squares	F-test	Probability
Between groups	2	0.25	0.125	2.52	**0.1605**
Within groups	6	0.298	0.05		
Total	8	0.548			

5.3.3 Outplanting success

Fifty-seven mature somatic embryos (42 with roots and true needles. 15 with roots and only a ring of cotyledons) were planted in soilless mix in the greenhouse with bottom heat in a mist bed covered with a white muslin cloth in late September. Four weeks later. 4 emblings had died but the rest were healthy. Therefore. the emblings were removed from the mist bed. Six weeks after transplanting to the greenhouse. 21 of the 57 planted emblings were still alive. all of them well rooted. The emblings were kept inside the greenhouse until spring. when they were moved to a shade house. The emblings remained outside and went dormant and flushed twice. The appearance of the emblings resemble that of seedlings the same age. and the setting of dormant buds and the flushing shoots in the spring are concurrent and resemble those of mature *Larix* species on campus.

5.4 Discussion

5.4.1 The relationship between proportion and epidermal quality of mature embryos and epicotyls

A high degree of uniformity in somatic embryo development is expected, given the single explant origin of a line and the uniform environmental growth conditions (Chalupa 1989). However, mature somatic embryos of *Larix x eurolepis* line 2086 had a variety of morphologies, including normal and abnormal proportions, vitrification, and smooth or rough epidermis.

The embryo shape was associated with the type of epidermis. The majority of embryos had normal proportions, and if an embryo had normal proportions, it was more likely to have a smooth epidermis than a rough epidermis. However, only 43% of the mature embryos produced were normal with a smooth epidermis. The percentage of embryos which did not have normal proportions/ smooth epidermis was 47%, which represents a substantial loss of mature embryos if these must be discarded. As discussed in the previous chapters, this loss is in addition to the failure of the majority of stage 1 embryos to mature, which, in *Picea glauca*, results in a decrease from 14.2 x 10^3 stage 1 embryos per gram of embryogenic tissue to 126 mature embryos per gram (Kartha *et al.* 1988). Since line 2086 matures embryos asynchronously, the total production of mature somatic embryos per gram of embryogenic tissue cannot be compared with that of other species but a visual assessment of the embryogenic aggregates indicates that a similar failure rate occurs for line 2086, since only 15-20 embryos maximum are maturing at any time on each aggregate. If the abnormal embryos can produce normal roots and epicotyls, and develop proper vascular connections between the two meristems, then the abnormal embryos can be utilised for outplanting.

Though most epicotyls produced were normal (48%), there was a high incidence of abnormal (13%), vitrified (7%), and twin (32%) epicotyls. The association between embryo proportions and epidermial qualities was significant ($P=0.0001$), indicating that whatever affects the proportional development of the embryos is likely to affect epidermal development. Most normally proportioned embryos had a smooth epidermis (81%), while only a slim majority of the abnormally proportioned embryos had a smooth epidermis (58%). Though the largest category of embryos produced were normal with a smooth epidermis, the incidence of abnormal embryos was higher than commonly reported in the literature and represents a substantial loss of suitable plant material before transfer to germination medium.

The association between the shape of the embryo and the shape of the epicotyl was significant (p=0.0001). The majority of normal-smooth embryos gave rise to normal-smooth epicotyls (68% of live embryos), 8% abnormal, 2% vitrifed and 21% multiple epicotyls. However, the majority of abnormal embryos formed multiple epicotyls (47% of live embryos), 24% abnormal, 22% twin and 7% vitrified embryos. The majority of vitrified embryos gave rise to normal-smooth epicotyls (42%), indicating that the physiological condition of vitrification did not affect the formation of the shoot meristem in all affected embryos.

Studies using mutants of *Arabidopsis* indicated that different regions of the embryo develop independently therefore abnormalities in one region would not affect development in another (Goldberg *et al.* 1994). Mutants of *Arabidopsis* without cotyledons produced normal epicotyls, therefore the type of epicotyl produced by a *Larix x eurolepis* somatic embryos may not have the same characteristics as the hypocotyl and cotyledons. However, in *Arabidopsis* mutants, abnormal cell differentiation patterns resulted in abnormal plant phenotypes (Goldberg *et al.* 1994).

Accordingly, the different types of somatic embryos produced in embryogenic lines should be sectioned and examined for cell differentiation patterns. The ability of an abnormal somatic embyo to produce a normal root and shoot may not necessarily guarantee that the vascular connections between the root and shoot are normal, or that later procambial development will be normal. In embryogenic carrot cultures, abnormally formed somatic embryos could produce normal roots but could not form cotyledons or epicotyls, and so could never be established in the field (Krikorian and Smith 1992, Smith and Krikorian 1990).

The reasons for abnormal development are not clear. Abnormal development in other species such as *Picea abies* and *Pinus strobus* were recorded (Hakman and von Arnold 1985). *Picea sitchensis* seeds germinated and grown *in vitro* develop into normal seedlings (von Arnold and Woodward 1988), therefore the cause of abnormal somatic embryo development may be related to the absence of the seed coat or megagametophyte rather than the culture envrionment. However, the effects of the *in vitro* environment on plant development can be extensive, both at the cellular and tissue level, though there are indications that lowering the relative humidity of the culture vessels using bottom-cooling, improving the lighting in growth chambers and transferring the somatic embryos to medium free of sucrose prior to transfer to soil may eliminate some of the adverse effects (Bhojwani and Dhawan 1988, Maene 1988). Germinated somatic embryos of *Picea pungens* often had swollen hypocotyls or callusing of the stem base which interfered with stem elongation, but these effects were considered to be caused by the residual effects of plant growth regulators in the medium (Afele and Saxena 1995). *Picea mariana* hypocotyls recallused if left on germination medium (no plant growth regulators) for over 4 weeks (Adams *et al.* 1994).

Environmental conditions could be another reason for abnormal growth. Thirty-five percent of stage 3 *Picea abies* somatic embryos in the dark produced epicotyls, compared to only 25% of those grown in the light (Budimir and Vujicic 1992). Though every live embryo produced an epicotyl, experiments with changing the environmental conditions for line 2086 could reduce the rate of mortality.

The issue is further confused by the phenomenon of dissimilar twin epicotyls. In the case of twin epicotyls, the epicotyl characteristics of the two (or more) epicotyls could differ, i.e. one could be vitrified and the other one normal, or one could be misshapen and rough and the other one smooth and normal. Each shoot meristem developed independently of the other, as well as independently of the root meristem. The microenvironment for each of the twin meristems must have been identical since they were adjacent to each other on the same embryo. The hormonal and nutritional effect of the embryo is likely the same on the two adjacent meristems. Therefore, the stimuli which fosters abnormal growth of a meristem is unlikely to come from either the environment or the rest of the embryo.

This was especially puzzling if one epicotyl was normal while its twin was vitrified. The prevailing notion is that vitrification is a physiological phenomenon related to the *in vitro* environment which can be corrected by changing the medium (Gaspar *et al.* 1987, McLaughlin and Karnosky 1989). Vitrified plantlets of *Gypsophila paniculata* had discontinuous epidermis with holes appearing in a regular pattern at each end of raised, nonfunctional stomata (Gribble *et al.* 1996). The increased water loss associated with vitrification was considered to be caused by the discontinuous epidermis, which also allowed leaching of nutrients from the leaves, rather than the nonfunctional stomata. These translucent, thick, brittle plantlets had higher fresh weights but lower dry weights than normal plantlets due to increased water content, likely in intercellular spaces. Less wax deposition was found on the leaves compared to seedlings (Gribble *et al.* 1996). Fewer *Picea rubens* somatic embryos were vitrified on maturation medium with higher ABA concentrations. On 20 µM ABA or less, most embryos were vitreous and germinated precociously (Harry and Thorpe 1991).

In *Larix* species, vitrification of organogenic cultures has been recorded. Caulogenesis on callus tissue of *Larix x eurolepis* produced shoots, of which less than 2% were vitrified (Laliberté and Lalonde 1988), which is nevertheless lower than the 14% of mature somatic embryos and 7% of epicotyls that were vitrified in line 2086. Manipulation of the media has often resulted in reduced vitrification for organogenic and embryogenic cultures. Vitrification rates for adventitious buds of *Larix decidua* were reduced by lowering cytokinin levels (decreased vitrification 48%) or replacing glutamine with $Ca(NO_3)_2$ (decreased vitrification 66%), but also bud production rates were lowered (from 61 down to 17 per plant for the cytokinin effect, 75 down to 42 per plant for the nitrogen source effect) and the mortality rate increased (30% increase for cytokinin effect) (McLaughlin and Karnovsky 1989).

Vitrification has also been a problem in synthetic seed technology. The use of charcoal in the encapsulation medium reduced vitrification of *Picea glauca-engelmannii* synthetic seeds and increased germination to 50%, when compared to the addition of charcoal to the germination medium (Lulsdorf *et al.* 1993). Adding macronutrients and sucrose to the capsule increased germination to 62%, compared to the 98% germination rate of unencapsulated somatic embryos. Somatic embryos of *Picea mariana* responded in the same way; embryos in a charcoal-macronutrient-sucrose capsule germinated at a rate of 40%, compared to nonencapsulated embryos at 88% germintaion (Lulsdorf *et al.* 1993).

Whatever the cause or cure for vitrification, reversion of growth from vitrified to normal did occur. Reversion to normal growth occurred in 81% of the adventitious buds of *Larix decidua* (McLaughlin and Karnovsky 1989). Reversion was also found in this study with line 2086. The growth of these reverted plants in the soil would have to be followed to determine if the formerly-vitrified plantlets had lower survival rates and were therefore a reduction in productivity.

Considering that somatic embryos develop outside the physical restraints and hormonal influence of the megagametophytic tissue and surrounding maternal tissue, it is remarkable that they resemble zygotic embryos (Zimmerman 1993). Therefore, each somatic cell must contain a genetic program that can function in the absence of maternal stimuli and is not controlled by environmental influence or spatial considerations (Zimmerman 1993).

Other researchers have found variation in somatic embryo morphology of different species. Often, either the absence or the presence of plant growth regulators is considered to be the cause. When embryogenic tissue of *Picea omorika* was matured on 12 µM ABA and left for 4 weeks, embryos formed normally, but if they were subcultued every week the embryos were abnormal (no elaboration) (Budimir and Vujicic 1992). Abnormal embryos of *Picea glauca* with asymmetrical arrangements of cotyledons (fused in extreme cases) formed on all concentrations of ABA but occurred most frequently at higher concentrations, and never developed any further (Dunstan *et al.* 1988). On plant-growth regulator-free medium, several embryos of *Picea glauca* matured, and only 2 were abnormal (one had arrested root development and the other had 2 cotyledons rather than the usual 4-5) (Kartha *et al.* 1988). Occasionally *Pinus caribaea* embryos formed with abnormal numbers of cotyledons, and somatic embryos of one line (the embryos with anthocyanin synthesis in the hypocotyl) germinated precociously on the ABA maturation medium (Laine and David 1990). *Picea glauca-engelmannii* somatic embryos of some lines were mostly abnormal with basal callus, elongated cotyledons and poor hypocotyl development if matured on 1-10 µM ABA, but not on 30 µM ABA (Roberts *et al.* 1990a). Some *Picea glauca* somatic embryos matured on medium with 174 µM sucrose, 2,4-D and kinetin were fasciated and had asymmetrical cotyledon development (Tremblay 1990). These embryos did not develop any further and their root apices became necrotic (Tremblay 1990). Some abnormal embryos of *Larix x eurolepis*, with fused cotyledons or suppressed root and/or shoot meristem development were produced, though there was no correlation between maturation treatment and morphology (Klimaszewska 1989a).

5.4.2 Rooting and mortality rates

Neither embryos proportions nor embryo epidermal qualities had a significant effect on rooting or mortality rates. The metabolism of dying embryos may give rise to abnormal growth. Alternatively, abnormal growth may interfere with proper translocation of nutrients and plant growth regulators or a discontinuous rough epidermis interferes with proper water relations. However, not all abnormal embryos die. In addition, all rooted embryos produced normal roots, whether they died or not. Why the root meristem should be less sensitive to adverse stimuli compared to the shoot meristem is unclear. The consistency of root morphology aids in transplanting success, because the ability of *Psuedotsuga menssziesii* plantlets grown *in vitro* to survive transfer to the greenhouse appears to be related to the quality of the roots produced (Mohammed and Vidaver 1991).

The overall germination rate of line 2086 was 58%, with a 67% germination rate for normal-smooth embryos and a 52% rooting rate for abnormal embryos. This is slightly lower than the percent rooting found in another study with *Larix x eurolepis*, where 80% of mature embryos rooted and produced epicotyls, with 30% of plantlets producing adventitious roots after planting (Klimaszewska 1989a). However, the use of plant growth regulators may increase the percent rooting of somatic embryos of line 2086 if applied at critical stages of root meristem development. In addition, germination rates of *Picea glauca-engelmannii* somatic embryos were improved by a high-relative-humidity desiccation treatment, with a 80% one-year-in-nursery survival rate for most genotypes, compared to the 29% survival for embryos not given a desiccation treatment (Becwar *et al.*

1989, Webb *et al.* 1989). Plantlets from partially dried *Picea glauca-engelmannii* somatic embyros had soil survival rates of 47% compared to 3% for undesiccated controls (Roberts *et al.* 1990b). Both the application of plant growth regualtors and desiccation treatments should be tried on somatic embryos of line 2086.

Only those embryos which mature are capable of germination. Seven genotypes of *Picea glauca-engelmannii* had production rates varying from 1 to 440 mature embryos per gram of embryogenic tissue (fresh weight), with germination rates varying from 16 to 100%, depending on the genotype (Webb *et al.* 1989). Because the labour is the same, lines which produced less than 10 mature embryos per gram were considered inefficient. In addition, poor quality of somatic embryos produced may reduce efficiency even further.

Picea mariana somatic embryos carefully selected for resemblance to zygotic embryos had a higher germination rate than nonselected somatic embryos, indicating that poorly formed somatic embryos did not produce roots as often as normal somatic embryos (Adams *et al.* 1994). However, in this work, normal somatic embryos had a rooting rate of 67%, while abnormal somatic embryos had a rooting rate of 52%. This difference was not significant. In addition, the number of abnormal somatic embryos which produced normal epicotyls was not significantly different from the number of normal somatic embryos which produced normal epicotyls. Normal somatic embryos were as likely to produce abnormal epicotyls (8%) as abnormal somatic embryos (11%). The morphology of a somatic embryo did not affect its ability to root or to produce a normal epicotyl.

The setting of dormant buds in the fall, renewed vegetative growth in the spring, and expansion of the root collar diameter by 21 transplanted emblings suggests that the proper flow of water, nutrients and plant growth regulators was occuring in the emblings. This is similar to findings with emblings of *Picea abies* which survived overwintering to -5 ^{o}C and produced new shoots at the same time as control seedlings (Becwar *et al.* 1988). Height, root and shoot morphology of *Picea glauca-engelmannii* emblings were similar to seedlings after 1 growing season, and the emblings had good frost hardiness (Webb *et al.* 1989). The successful establishment of emblings outside indicated that normal mature somatic embryos of *Larix x eurolepis* do have potential for commercial reforestation purposes.

5.4.3 Conclusion

In some species, abnormal leaves form on somatic embryos undergoing stress, such as during rooting and establishment in soil. After the plantlets were established, the new leaves produced were normal (Dhawan 1988). In one sugar palm species (*Borassus* *flabellifer*). the tendency for bulbil shoot production in field-established plants from somatic embryos was seen to revert to normal flowering after some time (Krikorian 1988). indicating that reversion to normal growth may not be immediate. Because some abnormal somatic embryos of line 2086 do produce normal epicotyls. embryos should not be discarded until they have produced true needles. Further research should investigate whether the organization of the internal tissues of the abnormal embryos resemble normal embryos. and whether survival in the field is affected.

The final morphologies of mature and germinating somatic embryos of line 2086 varied in proportion and epidermal qualities. Almost half of the embryos produced had either abnormal proportions or a rough epidermis and this represents a substantial loss on productivity because misshapen embryos were more likely to produce abnormal epicotyls than normal embryos. Though normal and abnormal embryos arose on the same aggregates under the same conditions. further studies may be able to identify certain culture conditions which cause abnormal development in somatic embryos. By increasing the percentage of normal embryos and emblings produced. somatic embryogenesis systems can become more efficient and economical.

Chapter VI: Manipulation of embryo development with media supplements

6.1 Introduction

Though somatic embryogenesis systems have the potential to outproduce both rooted cuttings and organogenesis, the costs incurred are high, especially in terms of skilled labour, which makes clonal propagation by somatic embryogenesis suitable only for elite trees. Improvements in the efficiency of somatic embryogenesis systems would lower the cost to produce each plantlet and would make clonal propagation through embryogenesis a more practical tool for tree improvement programs.

For embryogenic tissue of most conifer species, proliferation medium requires auxins and cytokinins to promote cleavage polyembryony while maturation media requires ABA and elevated osmotic levels to halt cleavage polyembryony and to promote development of the somatic embryos. Though the mechanisms of how the plant growth regulators work is unknown, combinations of an auxin, usually 1- 10 µM 2,4-D or NAA, with a cytokinin, usually 2-5 µM BA, inhibits maturation of somatic embryos and fosters continued cleavage polyembryony (Tautorus *et al.* 1991, Attree and Fowke 1993). Maturation occurs on medium with high concentrations of sucrose, usually 90 µM and 5-60 µM ABA, depending on the species. Occasionally, embryogenic lines arise which proliferated and/or mature without plant growth regulators.

Line 2086 is an embryogenic line of hybrid larch (*Larix x eurolepis*) which proliferates and produces mature somatic embryos on media without plant growth regulators (von Aderkas *et al.* 1990). Maturation occurs continuously, and therefore asynchronously, sporadically over the entire aggregate of embryogenic tissue, both in embryos close to the semi-solid medium surface and in those separated from the medium by hundreds of other somatic embryos. Despite the genetic uniformity of the tissue, and the uniformity of the culture conditions, the majority of somatic embryos either die or continue to cleave indefinitely while only a small percentage of the embryos mature. In an effort to understand why so few somatic embryos mature out of so many, this chapter describes the effects of auxins, cytokinins, sucrose and ABA to determine if their application would influence the development of isolated somatic embryos of line 2086. There was the possibility that somatic embryos of line 2086 would show improved embryogenic potential if subjected to the same conditions as other embryogenic lines.

6.2 Materials and methods

6.2.1 Culture maintenance

Line 2086 originated from an immature zygotic embryo of a hybrid between *Larix leptolepis* (maternal parent) and *Larix decidua* (paternal parent)(von Aderkas *et al.* 1990). The embryogenic tissue was initiated on 1/2 Litvay's medium supplemented with glutamine and 0.45 µM 2,4-D. Line 2086 was routinely cultured every 2-3 weeks onto 1/2 strength Litvay's medium (Litvay *et al.* 1981) supplemented with 56 µM sucrose, 4 g/l gelrite, 1 g/l casein hydrolysate, 3422 µM glutamine without plant growth regulators. The medium was adjusted to pH 5.6-5.8 before autoclaving. The glutamine and all plant growth regulators were filter sterilised using a 22 µm filter and were added after the medium had been autoclaved in an Agarmatic (New Brunswick Scientific Co. Inc.). The medium was dispensed by 20 ml aliquots into 100 x 15 Petri dishes (Fisher) and allowed to cool to room temperature before storage at 4 °C. Four aggregates of embryogenic tissue were cultured on each Petri dish and were individually marked. The plates were kept in a growth chamber at 22 °C with a 16 hour light/8hour dark photoperiod at 36 umol/m^2/s photosynthetically active radiation. In the absence of plant growth regulators, mature somatic embryos of line 2086 germinated precociously and asynchronously.

6.2.2 Effects of auxins and cytokinins on immature embryo development

Each aggregate of line 2086 was individually pulled apart with forceps under a binocular microscope (Wild). Every SEMLS was removed and placed onto one of nine different media. Each media contained either 0 µM, 2.26 µM, or 4.52 µM of 2,4-D, and 0 µM, 2.22 µM, or 4.44 µM of BA, for a total of nine combinations of auxin (2,4-D) and cytokinin (BA). Each petri dish was gridded by affixing a 5 x 5 paper grid onto the bottom of the petri dish. A single somatic embryo was placed in each square of a grid, for a total of 25 immature somatic embryos per petri dish. Preliminary experiments had indicated that embryos did not develop any further five weeks after isolation; therefore, five weeks after isolation the embryos were examined to determine their developmental fate. This procedure was repeated twice for a total of three replications.

The six developmental fates were:

a. developmentally arrested: no apparent growth or development.

b. callus: a proliferation of unstructured tissue with no apparent immature embryonal masses.

c. proliferation: a proliferation of immature embryonal masses.

d. nodule: a roughly spherical structure with no epidermis, no cylindrical hypocotyl and no cotyledons

e. abnormal: a mature or germinating somatic embryo which did not resemble a normal zygotic embryo in terms of general proportions of hypocotyl and cotyledons.

f. normal: a mature of germinating somatic embryo which resembled a zygotic embryo in terms of general proportions of hypocotyl and cotyledons.

The data were analysed as a 3 x 3 x 5 contingency table. The expected frequencies of the entire table were too low to permit testing, therefore the data were presented in a summation table. The data were pooled to determine first level effects for both auxin and cytokinin level effects in two 3 x 5 contingency tables (3 levels of plant growth regulator x 5 embryonal mass fates). In both cases, the total number of embryos and nodules had to be pooled to raise the expected frequencies above 5. The test for association between the plant growth regulator and the fate of the embryonal mass was significant, and no 2 x 5 comparisons were insignificant. Therefore, no pooling of categories could be done.

6.2.3 Effects of auxins and cytokinins on the development of nodules

Each aggregate of line 2086 was examined for the presence of nodules. Every nodule was removed and placed onto one of nine different media. The media contained one of three levesl of 2,4-D and one of three elves of BA in a 3 x 3 factorial experiment. Each media contained either 0 µM, 2.26 µM or 4.52 µM of 2,4-D, and 0 µM, 2.22 µM or 4.44 µM of BA, for a total of nine combinations of auxin (2,4-D) and cytokinin (BA). Each Petri dish was gridded by affixing a 5 x 5 paper grid onto the bottom of the Petri dish. A single nodule was placed in each square of a grid. Five weeks later the nodules were examined to determine their developmental fate and whether or not they produced callus (disorganized tissue) or proliferated embryonal masses. This procedure was repeated twice for a total of three replications.

The four developmental fates were:

a. dead: nodule fully necrotic.
b. shoot only: a hypocotyl and cotyledons present but no root.
c. root only: a root present but no hypocotyl or cotyledons.
d. shoot+root: a hypocotyl, cotyledons and a root present.

Due to the low numbers of shoots and roots produced, this experiment could not be analysed statistically. Therefore, the results are summed over the three replications and presented in a summation table.

6.2.4 Effects of ABA and sucrose concentrations on the development of immature embryos

The purpose of this experiment was to determine if ABA and a high sucrose level would stimulate the further development of individual embryonal masses. Eight different media were prepared, with two levels of sucrose, 58 µM and 174 µM sucrose, and 4 levels of (+-) cis, trans ABA (Sigma) (0, 6, 12, 20 µM) for a 2 x 4 factorial experiment. Two types of embryonal masses were plated onto each of the media, SEMLS and REMLS. The experiment was repeated twice, for a total of 3 replications. For SEMLS, the total number of embryonal masses per treatment was approximately 44. For REMLS, the total number of embryonal masses per treatment was approximately 58.

Each aggregate of line 2086 was individually teased apart with forceps under a binocular microscope (Wild). Every SEMLS and REMLS was removed and placed onto one of eight different media. Each media contained either 58 or 174 µM of sucrose, and 0, 6, 12 or 20 µM of ABA for a total of eight combinations of sucrose and ABA. Each Petri dish was gridded by affixing a 5 x 5 paper grid onto the bottom of the Petri dish. A single immature somatic embryo (SEMLS or REMLS) was placed in each square of a grid, for a total of 25 immature somatic embryos per petri dish. Five weeks later the embryos were examined to determine their developmental fate. This procedure was repeated twice for a total of three replications.

The four developmental fates were:

a. stalled: no apparent growth or development,

b. proliferation: a proliferation of immature embryonal masses,

c. nodule: a roughly spherical structure with no epidermis, no cylindrical hypocotyl or cotyledons,

d. embryo: a mature somatic embryo with a cylindrical hypocotyl and cotyledons (no abnormal embryos were produced in this experiment therefore only normal embryos could be tallied).

The data were analysed as a 2 x 4 x 2 x 4 contingency table, with 2 types of embryonal masses (SEMLS and REMLS), 4 levels of ABA (0, 6, 12, and 20 μM), 2 levels of sucrose (58 and 174 μM) and 4 fates (stalled, proliferated, nodule and embryo). When pooled for embryonal mass type and fate, the resulting 2 x 4 contingency table was insignificant (p=0.8627). When pooled for sucrose level and fate, the resulting 2 x 4 contingency table was insignificant. Consequently, embryonal mass type and sucrose levels were collapsed. Therefore, an analysis was done on a 4 x 4 contingency table, with the 4 levels of ABA and the 4 fates. Since less than 50% of the expected frequencies were below 5 and the G statistic could not be computed, the levels of fate were collapsed into 2 combined levels: stalled+proliferated and nodule+embryos. The final analysis was done on the resulting 4 x 2 contingency table, with 4 levels of ABA and 2 fates.

6.3 Results

6.3.1 Effects of auxins and cytokinins on developmental fate

The only normal mature embryos were produced on the plant growth regulator-free medium. In addition, the only mature embryos produced were on the medium free of BAP. In contrast, nodules were formed on all the media. Table 12 gives a summary of the main effects of the plant growth regulators.

There is an association between the level of auxin and the developmental fate of an SEMLS embryo ($p=0.0001$)(Table 13). SEMLS embryos are more likely to become developmentally arrested as the level of 2,4-D increases. SEMLS embryos were more likely to become nodules in the absence of 2,4-D. There is no pattern for proliferation across the levels of auxin.

There is an association between the level of cytokinin and the developmental fate of an SEMLS embryo ($p=0.0001$)(Table 14). SEMLS embryos were more likely to become developmentally arrested on medium with no BA. SEMLS embryos were more likely to become nodules or embryos if there was some BA in the medium.

Nodules formed roots and shoots only on medium which contained no hormones (Table 15). In addition, the only nodules which died, died on medium containing no hormones. The presence and level of BA may have had a slight effect on the formation of roots and cotyledons since the greatest number of cotyledons and roots occurred on medium with no cytokinins. However, the presence of cytokinins did not preclude the formation of cotyledons and roots. Only 6 nodules had both shoots and roots, 5 in treatment 0 μM 2,4-D + 0 μM BA, and 1 in treatment 0 μM 2,4-D + 4.44 μM BA.

Table 12: Final morphology of individual embryonal masses subjected to 2.4-D and BA treatments

Hormone treatment				embryos				
2.4-D (µM)	BA (µM)	total	stalled	t.emb.	n.emb	m.emb	nod	prolif
0	0	52	41	5	3	2	5	1
0	2.22	54	17	3	0	3	34	0
0	4.44	52	14	3	0	3	32	3
2.26	0	52	42	0	0	0	5	5
2.26	2.22	53	28	0	0	0	21	4
2.26	4.44	52	18	1	0	1	20	13
4.52	0	51	41	0	0	0	6	4
4.52	2.22	52	34	0	0	0	13	5
4.52	4.44	53	30	0	0	0	17	6

total: total number of embryonal masses in each treatment over 3 replications
stalled: number of embryonal masses which did not continue development after transfer
t.emb.: total number of embryos produced
n.emb: number of normal embryos produced which had a phenotype similar to zygotic embryos
m.emb: number of misshapen embryos produced. with abnormal proportions of hypocotyls and cotyledons
nod. : number of nodules produced
prolif.: number of embryonal masses which proliferated into aggregates

Table 13: Main effects of 2.4-D on SEMLS embryos

Auxin level (µM)	Developmental fate					
	total	developmentally arrested	proliferation	nodules	abnormal embryos	normal embryos
0	158	72	4	71	8	3
2.26	157	88	22	46	1	0
4.52	156	105	15	36	0	0

If a 3 x 3 contingency table is prepared from Table 9 without the data for mature embryos, the results are as follows:

Degrees of freedom	4
Total chi-squared	31.067
G statistic	32.157
Probability	p=0.0001

Deviations (observed – expected freq.)

Fate	Auxin level (µM)		
	0	2.22	4.44
Stalled	-12.87	-2	14.93
Prolif.	-9.13	8.07	1.07
Nodules	22	-6	-16

Table 14: Main effects of BA on SEMLS embryos

Cytokinin level (µM)	Developmental fate					
	total	developmentally arrested	proliferation	nodules	abnormal embryos	normal embryos
0	155	124	10	16	2	3
2.22	159	79	9	68	3	0
4.44	157	62	22	69	4	0

If a 3 x 3 contingency table is prepared from Table 10 without the data for mature embryos, the results are as follows:

Degrees of freedom	4
Total chi-squared	67.447
G staistic	73.382
Probability	p=0.0001

Deviations (observed – expected freq.)

Fate	Cytokinin level (µM)		
	0	2.22	4.44
Stalled	37.4	-11.07	-26.33
Prolif.	-3.4	-4.93	8.33
Nodules	-34	16	18

Table 15: Final morphologies of nodules subjected to 2,4-D and BA treatments

Treatment 2,4-D (µM)	BA (µM)	total#	dead	cotyl.	root	anyprolif	pr.em	callus
0	0	19	4	9	6	5	4	1
0	2.22	18	4	1	0	2	1	1
0	4.44	18	2	3	1	2	0	2
2.26	0	17	0	0	0	6	0	6
2.26	2.22	18	0	0	0	8	1	7
2.26	4.44	20	0	0	0	10	2	8
4.52	0	19	0	0	0	2	1	1
4.52	2.22	18	0	0	0	3	0	3
4.52	4.44	18	0	0	0	3	0	3

Cotyl. : the number of embryos that had cotyledons.

Root : the number of nodules that had roots.

Anyprolif.: the number of nodules which produced callus or embryogenic tissue

Pr.em.: the number of nodules which produced embryogenic tissue

Callus: the number of nodules which produced unorganized tissue

6.3.2 Effects of abscisic acid concentrations on developmental fate

Levels of ABA and developmental fate were associated (p=0.0001)(Table 16). If not exposed to ABA, immature embryos became developmentally arrested less frequently than expected and became structures more frequently than expected. If exposed to any level of ABA, immature embryos were more likely to become developmentally arrested and less likely to become a structure than expected.

No vitrified or misshapen embryos were produced by any of the treatments, not even the controls. Tissue aggregates were also plated onto the eight different media. ABA apparently had no effect on growth but increased sucrose levels halted development of the embryonal masses which matured.

Table 16: Final morphologies of individual embryonal masses on media with varying ABA levels

ABA (µM)	total#	stalled	norm.	nod.	prolif.
0	205	176	1	28	0
6	206	199	0	5	1
12	208	196	0	11	1
20	208	188	2	12	6

EM type: type of embryonal mass used
total#: total number of embryonal masses in each treatment
stalled: number of immature embryos which became developmentally arrested
norm.: number of immature embryos which became normal mature somatic embryos
nod.: number of immature embryos which became nodules

6.4 Discussion

6.4.1 Effects of auxin and cytokinin on embryo development

Reducing or eliminating the levels of 2,4-D and BA in the proliferation medium induced the maturation of *Picea glauca* and *Picea mariana* somatic embryos (Hakman and Fowke 1987), but the effects of 2,4-D and BA on line 2086 were not as clearcut. The effects of 2,4-D appear to be the same on both embryonal masses and nodules, since embryonal masses only produced somatic embryos when 2,4-D was not present, and nodules only produced shoots and roots in the same conditions. However, dead nodules occurred on medium without 2,4-D. Therefore, though the addition of 2,4-D to the medium stimulated the vitality of nodules, the absence of 2,4-D stimulated the establishment of meristems. Differences in sensitivity to 2,4-D are exhibited in protoplasts of embryogenic vs. nonembryogenic lines from the same genotype of *Medicago* (Dudits *et al.* 1991), which indicates that the interaction between plant growth regulators and genotype may also involve particular patterns of gene expression, possibly the differences in gene expression found in stage 1 somatic embryos and nodules.

Not all cells respond to auxins and only a fraction of cells are capable of an embryogenic response, possibly due to differences in sensitivity to auxin, but cells can respond in a organized manner (embryogenic tissue) or an unorganized manner (callus) (Dudits *et al.* 1991). When auxins are bound to their receptors, hyperpolarization of the membrane results, which is mediated by a plasmamembrane H^+ - ATPase. These receptors are found on the plasmalemma and endoplasmic reticulum. Relative abundances of receptors could be linked with sensistivity to auxins. No studies have been conducted yet on the relative abundance of receptors in embryogenic vs. nonembryogenic cultures but using *Agrobacterium rhizogenes* to transfer rol B and rol C genes (which can cause an increase in auxin sensitivity in transgenic plants) into *Medicago sativum* tissue resulted in embryogenic production from a previously nonembryogenic line. In carrot, an increase in pH in the medium caused embryo development in the absence of plant growth regulators (Smith and Krikorian 1990), possibly because it mimics the effects of auxin on cytoplasmic and cell wall pH's.

Proper polar transport of auxins is essential for normal morphology. Inhibitors of polar transport affect somatic embryogenesis of carrot by halting development (though size may continue to increase) while it affects *Brassica juncea* zygotic embryo development by fusing their cotyledons possibly because zygotic embryos are influenced by their own metabolism and polarity plus maternal influences (Zimmerman 1993). Possibly nodules are the result of an anomaly in the polar transport of auxins.

Typically, 2,4-D fosters the development of *Daucus carota* somatic embryos up to the globular stage but inhibits further development of the somatic embryos (De Vries *et al.* 1988). The type of auxin used to induce embryogenic tissue on pecan (*Carya illinoinensis*) affected both initiation rates and plantlet morphology (Rodriguez and Wetzstein 1994). Tissue initiated using naphthaleneacetic acid (NAA) was less prolific but the somatic embryos produced more closely resemble zygotic embryos and had higher conversion rates due to better developed apices than tissue induced with 2,4-D. Possibly, embryonal masses of *Larix x eurolepis* may respond better to different auxins.

In addition, auxins may interact with other elements of the medium. In embryogenic lines of *Daucus carota* , the use of 2,4-D interacts with the use of particular forms of nitrogen. 2,4-D plus NH_4NO_3 plus KNO_3 gave profuse nonembryogenic callus, while 2,4-D plus NH_4^+ gave rise to embryogenic globules (Smith and Krikorian 1990). Further experiments should include modifications in media composition to determine if the type of nitrogen has an effect on embryonal mass or nodule development.

The fact that increasing concentrations of both 2,4-D and BAP increased the number of embryonal masses that form nodules indicate that differences in endogenous plant growth regulator concentrations may be the reason why some embryonal masses become nodules on plant growth regulator-free medium. In embryogenic lines of *Stylosanthes scabra* (a perennial legume), the use of BA induced organogenesis, while the use of 2,4-D induced embryogenesis (Dornelas *et al.* 1992). This indicates that the two types of plant growth regulators may work antagonistically in certain situations, resulting in anomalies such as nodules.

The frequency of developmental arrest in embryonal masses appeared to be related to levels of cytokinins, since increasing the levels of BA reduced the percentage of stalled embryonal masses. However, the reduction in arrested embryonal masses appeared to be matched by an increase in the number of embryonal masses which became nodules. Therefore, though BA appears to induce embryonal masses to do something, it does not induce maturation. Increasing the level of BA had a significant effect on the root and cotyledon production of nodules, but the results were varied and showed no clear relationship between increased BA and increased polarity of the nodules. Therefore, the effects of cytokins and auxins on somatic embryo development are still unclear. *Picea glauca* stage 1 embryos could not be matured in the presence of auxins and cytokinins, even if ABA or one of 4 analogues was present (Dunstan *et al.* 1988), but line 2086 produced mature embryos whether BAP or ABA were present or not.

6.4.2 Effects of ABA and sucrose on embryo development

6.4.2.1 Effects of ABA on embryo development

For line 2086, increasing sucrose levels had no effect on the developmental fate of somatic embryos. The presence of ABA in the medium increased the rate of developmental arrest, but higher levels of ABA had no further effect. In many conifer species, ABA is required to halt cleavage and to promote normal maturation of somatic embryos. In cell suspensions of embryonal masses of *Picea abies*, ABA inhibited cleavage and stimulated further development of embryonal masses (Boulay *et al.* 1988). However, in another study, maturation of *Picea abies* embryos could occur on medium without plant growth regulators, and was not increased or decreased by 1-10 μM ABA (Hakman and von Arnold 1985). After transfer to ABA medium, the generation of new stage 1, 2 and 3 embryos of *Picea glauca* somatic embryos was repressed. On plant growth regulator-free medium with 29 μM sucrose, occasionally an embryo would mature to stage 3 but these never developed further to stage 4 (Dunstan *et al.* 1988). However, higher concentrations of ABA (40-60 μM) increased the incidence of abnormal embryos (Dunstan *et al.* 1991). Without the use of ABA for maturation, fewer numbers of *Picea sitchensis* embryos matured, and many of these were abnormal and the normal ones germinated precociously (Krogstrup *et al.* 1988).

Most species require ABA for maturation. Somatic embryos of *Picea glauca-engelmannii* were sensitive to levels of ABA (Roberts *et al.* 1990a). Embryos did not mature in the absence of ABA. On low levels of ABA (1-10 μM), embryos matured into abnormal embryos with basal callus, elongated cotyledons and no hypocotyl elongation on ABA. On 10-20 μM ABA, embryos matured normally but exhibited precocious germination and matured normally with root primordia but no precocious germination on 30+ μM ABA. Storage protein levels were low on 10 μM ABA, similar to zygotic embryos at 30 μM, and twice as high at 40 μM ABA than at 20 μM ABA (Roberts *et al.* 1990a). Somatic embryos of *Picea abies, Picea glauca-engelmannii* and *Picea sitchensis*, when matured with ABA and low osmoticum, had the same storage protein profile as zygotic embryos (Attree and Fowke 1993). In angiosperms, good homology in storage protein composition exists across the species, likely because of the demanding criteria which call for proteins with specific recognition sequences, tolerance to desiccation and imbibition, and resistance to hydrolysis during development but susceptibility after germination (Higgins 1984). Therefore, it is no surprise that storage proteins are similar across species, and between somatic and zygotic embryos (Higgins 1984).

The higher the ABA level the more vigorous the shoot growth of the stage 4b plantlets of *Picea glauca* (Dunstan *et al.* 1991). Best vigour and therefore survivability and field performance would come from somatic embryos with the highest reserves (lipids, proteins, carbohydrates) because somatic embryos do not have the reserves of a megagametophyte to draw on (Attree and Fowke 1993). Further studies with line 2086 should include protein analysis to investigate whether the embryos that mature on the various media have different levels and kinds of proteins despite the similarity in their morphology.

The expectation was that the low level of embryo maturation in line 2086 was due to the ability of a few random somatic embryos to produce enough endogenous ABA to mature, and that the addition of ABA to the medium would provide sufficient ABA for more somatic embryos to cease proliferation and to mature. However, though the presence of ABA increased the level of developmental arrest in line 2086, it had no effect on embryo maturation and the highest level of proliferation occurred on the medium with the highest level of ABA. In addition, in this study, all the embryos produced were normal. Therefore, ABA neither encouraged maturation, inhibited proliferation, nor did its abscence affect the quality of the mature embryos produced. However, none of the embryos produced by line 2086, either on medium with ABA or without, were tested to determine if ABA affected stroage protein accumulation and desiccation tolerance

In other studies, maturation of *Larix x leptoeuropaea* somatic embryos to the late cotyledonary stage took place on media without ABA, but embryos matured on ABA were of higher quality (Lelu and Label 1994). Embryos matured on ABA resembled zygotic embryos and had hypocotyls, elongated cotyledons surrounding an apical meristem, red root collars but no root elongation. Embryos matured without ABA did not resemble zygotic embryos. These abnormal embryos were dark pink, had very short or no hypocotyls or cotyledons, a red basal area, never developed past the cotyledonary stage and about 12% exhibited precocious germination (Lelu and Label 1994). Possibly, these abnormal embryos resemble the red nodules produced by line 2086. The level of nodule production in line 2086, though it did occur on media with 6-20 µM ABA, was highest on media without ABA, in agreement with the results of Lelu and Label (1994).

The abnormal embryos of *Larix x leptoeuropaea* , after transfer to germination medium, had germination rates of 46% and conversion rates of 23%, compared to 90 % germination and 70% conversion of embryos matured on ABA for 2-3 weeks (Lelu and Label 1994). The highest yields of mature embryos was 579-688 mature somatic embryos per gram of embryogenic tissue (fresh weight) on medium with 60 µM ABA. ABA content for high quality embryos matured on ABA medium was 15 times higher than that for poor quality embryos matured on medium without ABA. However, ABA was present in embryos on media with no ABA, indicating that ABA synthesis does occur in embryos. Therefore, line 2086 is not unique in its ability to mature somatic embryos in the absence of ABA. However, its ability to produce both nodules and normal embryos on medium without ABA, is unusual.

Possibly, the use of high osmoticum and ABA has no direct effect but only prompts the synthesis of substances in the embryos which then foster maturation (Krueger and van Holst 1993). Somatic embryos of *Picea glauca* could be matured on medium with either ABA or one of 4 ABA analogues (different from the analogues used by Dunstan *et al.* (1988)), but ABA was both more effective at promoting maturation than either analogue, and produced better quality plantlets than 2 of the analogues (Dunstan *et al.* 1991). *Picea glauca* embryogenic cultures only responded to and metabolized (+) ABA, and neither responded to nor metabolized (-) ABA. Embryogenic cultures of *Picea glauca* completely metabolized initial concentrations of 15 µM (+) ABA to phaseic acid within 7 days, which may indicate that phaseic acid is the active form (Dunstan *et al.* 1992). In suspension cultures of *Picea glauca* , 15 µM of the natural enantiomer (+) ABA was metabolized to phaseic acid within 7 days , whereas the unnatural enantiomer (-) ABA was not metabolized (Dunstan *et al.* 1992). Embryo size increased (indicating further development) on medium with (+) ABA but not on medium with (-) ABA, which suggests that phaseic acid is the active form which promoted maturation (Dunstan *et al.* 1992).

However, the ability of line 2086 to mature somatic embryos appears to be truly independent of exogenous levels of ABA, since adding ABA to the medium, in conjunction with high sucrose levels, had no effect on the rate of maturation. ABA-deficient (*aba*) and ABA-insensitive (*abi*) mutants of *Arabidopsis* showed normal seed development whereas double mutants (*aba/abi*) exhibited the expected precocious germination, desiccation intolerance and lack of storage protein accumulation (Koornneef *et al.* 1982, 1984, 1989). This indicates that both levels of ABA and sensitivity to ABA are vital components for response to ABA, though sensitivity to ABA can be heightened by increased osmoticum (Hetherington and Quatrano 1991).

Both the level of ABA and the embryo's sensitivity to ABA determine the embryos inhibition of precocious germination, accumulation of storage protein, and stress tolerance (Hetherington and Quatrano 1991). In normal plants, ABA and high osmoticum act in tandem, possibly by having increased osmotic levels lower the threshold level of ABA that elicits a response, a shift in the dose response curve for ABA (Hetherington and Quatrano 1991). In *Medicago stativa*, ABA plus high osmoticum prevented precocious germination of somatic embryos but only osmoticum maintained the synthesis of developmental proteins, indicating that the effects of ABA and osmoticum are distinct (Xu *et al.* 1990).

Possibly, the embryonal masses of line 2086 were at too early a stage to respond to the maturation stimuli. In *Pinus taeda*, early stage embryos of approximately 100 cells mature under ABA treatments, while smaller early stage embryos do not (Becwar and Pullman 1995). Further experiments should investigate the effects of plant growth regulators and osmotic treatments on later stages of development, ideally at the focal zone or anthocyanin band stage. However, once the embryonal masses reached these later stages they have already demonstrated an end to continued cleavage and a beginning of maturation.

6.4.2.2. Effects of sucrose on embryo development

Possibly the levels of sucrose used were not high enough to foster development. Essentially, the embryonal masses used in the experiments were analogous to the proembryos of angiosperms used in embryo rescue work. In general, the earlier the stage of development when the embryos is removed from the developing seed (embryos rescue), the higher the osmotic level of the medium has to be to ensure survival. Development of carrot somatic embryos more closely resembles zygotic embryo development if the osmotic concentration of the medium closely resembles that of fluids of the embryo sac, which keeps the developing embryos small and the growth slower and more organized (Krikorian and Smith 1992). *Picea glauca* embryos on 30 µM sucrose proliferated rather than matured, but at elevated levels of sucrose (120-150 µM) embryos at first developed but then were repressed (Hakman and von Arnold 1988). A wider range of sucrose levels may reveal that different sucrose levels allow proliferation while others inhibit it and promote maturation.

In ovulo cultures of *Pinus resinosa* zygotic embryos were unsuccessful in completing development (Gates and Greenwood 1991). Embryos degenerated on medium with 87-174 µM sucrose, and stopped development on medium with 261-1827 µM sucrose, though sucrose levels of 348 µM provided an osmotic environment similar to that of a seed (Gates and Greenwood 1991). Most media with 87 µM sucrose levels have an osmotic potential higher than *in vivo* *Pinus resinosa* seeds. A more negative osmotic environment may slow growth sufficiently to permit proper development and differentiation (Raghavan 1976, Gates and Greenwood 1991).

High osmoticum improved the development of *Abies alba* somatic embryos, but for *Pinus pinaster* a medium level of sucrose (174 µM) yielded the highest number of stage

2 embryos (Bercetche and Pacques 1995). *Picea mariana* somatic embryos matured on 58 µM sucrose developed true needles, and 60% formed roots, while embryos on 174 µM sucrose had stunted shoots, less root elongation, and a pronounced accumulation of anthocyanins in the hypocotyl and root meristem region. The embryos matured on 174 µM sucrose did not develop normally even if transferred to 58 µM (Cheliak and Klimaszewska 1991).

As osmotic treatments, sucrose and PEG are not similar (Attree *et al.* 1991). PEG cannot enter the cell and its application imparts a nonplasmolysing water stress similar to drought stress while the use of sucrose at similar osmotic potentials was detrimental to the maturation of *Picea glauca* embryos due to plasmolysis. The use of PEG increased the maturation rate by 300% and the embryos had normal morphologies and greater storage reserves than controls. A gradual desiccation after ABA treatment was critical to the survival of embryos during desiccation . Embryos matured without PEG germinated precociously during the desiccation treatment and were irregular in appearance, whereas embryos matured with PEG had water contents already reduced to 45% before the desiccation treatment, ensuring that desiccation would be uniform and subsequent plant quality would be high. High levels of ABA prepared embryos for desiccation treatments but accumulating storage reserves was greatest when both ABA and osmoticum treatments were combined. This resulted in triacylglycerol (TAG) biosynthesis levels at 5 times that of zygotic embryos (Attree *et al.* 1992). Further research on the maturation of line 2086 should include the effects of PEG and higher sucrose levels to determine if osmotic treatments work synergistically with ABA to increase maturation, though these treatments would not explain why some embryos mature spontaneously while others in the same aggregate do not.

One aspect of the culture environment which is usually overlooked is the gaseous environment. Cell suspension cultures of white spruce cultures grew better in gas permeable flasks, while radiata pine grew better in gas impermeable flasks. For both species, the high levels of carbon dioxide and ethylene in the gas impermeable flasks appeared to promote bud differentiation but caused some dedifferentiation at excessive levels (Kumar and Thorpe 1988). Adding silver nitrate (a competitive inhibitor of ethylene) to the medium of *Pinus pinaster* cultures containing ABA and 174 µM sucrose reduced the number of stage 2 embryos produced (Bercetche and Pacques 1995). Low partial pressures of O_2 and elevated levels of CO_2 with low levels of ABA aided maturation in *Picea abies*. (Attree and Fowke 1993). In this thesis, only 25 embryonal masses were placed in each Petri plate, which is a small amount of plant material. Increasing the number of embryonal masses per Petri dish may alter the number of maturing somatic embryos.

6.4.3 Conclusion

Is it possible that the maturing embryonal masses were different than the embryonal masses which continued to cleave? The low rate of maturation in this experiment, regardless of embryo type, sucrose level or ABA level, may suggest that the few embryos that did mature were different from the embryos that did not mature. When subculturing line 2086, several aggregates appeared to be different than the rest; very little cleavage was evident in these aggregates and most of the embryonal masses became a structure, either a nodule or a somatic embryo. These aggregates did not survive long because eventually the last embryonal mass developed into a structure rather than daughter embryonal masses. The structures produced from these aggregates were the same as those of other aggregates. The different behaviour exhibited by these short-lived aggregates may indicate that a certain physiological priming, which is carried on in daughter embryonal masses after cleavage, is necessary to begin maturation. However, secondary embryogenesis is common in line 2086, indicating that even cells which have differentiated into an embryo are still capable of reverting to the continuously cleaving state. Thus, the nature of this primed state is not clear.

In conclusion, despite the use of auxins, cytokinins, abscisic acid and elevated sucrose levels, the most common fate of an embryonal mass was developmental arrest. The least common fate was development into a somatic embryo. Cleavage into proliferating embryonal masses or development into a nodule (which may develop a root and/or cotyledons) were more common fates than normal development into a somatic embryo. Why an embryonal mass would be more likely to develop into a nodule than an embryo, when zygotic embryos never develop into nodules, is not clear. Why supposedly uniform embryonal masses would take different pathways of development, even when subjected to the same concentrations of powerful growth regulators, is also a mystery. Though the use of an abscisic-acid independent line such as line 2086 can help eliminate some possible causes for maturation, there is still much more work to be done.

Chapter VII: General Discussion

The ultimate goal of any somatic embryogenesis sytem is the establishment of healthy, vigorous elite plants in the soil. Increased demand for wood products as the world's population increases, climate changes and increased pressure to preserve old growth forests make the need for intensively managed plantations of elite trees imperative. Oncoming climate changes due to global warming will likely be too fast for tree populations to adapt naturally, therefore time constraints (rotation age, selection age, maturation age) will have to be reduced (Fowler and Loo-Dinkins 1992) and genotypes with widespread adaptability have to be identified and propagated. Germplasm storage will be essential if populations start to die out due to predicted changes in temperature and rainfall patterns and increased logging. Improved somatic embryogenesis systems will greatly increase the flexibility of mass propagation, especially if cultures can be induced from mature trees, since conventional seed orchards restrict genetic gain to parental genotypes but do produce hybrids which can have good growth and are easier to propagate than obtained by repeated difficult crosses (Fowler and Loo-Dinkins 1992).

With few exceptions, such as the early selection for height in *Picea sitchensis* at 2-3 years old, many traits of conifers require one-half the rotation age for selection (Park and Bonga 1992). This, combined with the long time period to sexual maturity for many conifers and the number of lethal recessives, makes inbred lines impossible to obtain, necessitating the use of interspecific hybrids to get heterosis. Rapid cloning can aid in tree improvement programs, as long as the propagules are true to type, because it captures total genetic variance, including non-additive variance, which can be a third to a half of the total variance in *Larix larcina* and may be larger in other species. The use of genetic engineering could reduce breeding time and introduce new genes while retaining non-additive variance. The combination of somatic embryogenesis and cryopreservation would ensure preservation until field tests were over, and rapid propagation once elite trees were identified (Park and Bonga 1992). By investigating the histological nature of embryogenic tissue, the reasons why so few embryos mature may be discovered, and the efficiency of embryogenic systems could be improved.

The need for efficient embryogenic systems is increasing constantly as the ability to genetically engineered conifer cells improves. Genetic transformation has been achieved for *Picea glauca* (Bommineni *et al.* 1993, Charest *et al.* 1993, Li *et al.* 1994), *Larix x eurolepis* , *Larix decidua*, *Larix leptolepis* and *Larix x leptoeuropae* (Charest *et al.* 1991, Charest *et al.* 1993, Duchesne and Charest 1992, Duchesne *et al.* 1993), *Larix decidua* (Huang *et al.* 1991, Huang *et al.* 1993 , Diner and Karnosky 1987), *Picea rubens* and

Picea mariana (Charest *et al.* 1993). These developments anticipate the introduction of useful foreign genes such as disease and insect resistance into *Larix spp.* through *in vitro* culture, which will make the recovery of large numbers of viable somatic embryos from small samples of successfully transformed tissue of great importance.

Advancements in our knowledge of *Larix* genetics through the use of karyotyping (Nkonogolo and Klimaszewska 1995), nucleotide sequencing (Hutchinson *et al.* 1990, Kumar *et al.* 1995), and restriction fragment length polymorphisms (Cheliak and Rogers 1990) can aid breeders in identifying lines with desirable traits, detecting genetic diversity in populations for germplasm preservation and phylogenetic analysis and developing linkage maps for phenotypic traits. Once linkage maps have been constructed, trees can be selected for genotype, rather than phenotype. The gains from selection and breeding are realized at the propagation phase, and because the major restraint of tree improvement is time (rotation age, selection age, maturity age), the faster the propagation system the better (Cheliak and Rogers 1990).

Both cryopreservation and synthetic seeds have been used to successfully to store either embryogenic tissue or mature embryos. The successful cryopreservation and regeneration of embryogenic cultures of *Larix x eurolepis* will enable researchers to field test elite and genetically transformed material and return to promising lines for more plantlets (Klimaszewska *et al.* 1992). Previously cryopreserved *Picea glauca* , *Picea mariana* and *Larix x eurolepis* embryogenic tissues have yielded normal plantlets and did not suffer any loss of embryogenic potential (Kartha *et al.* 1988, Klimaszewska *et al.* 1992).

Synthetic seeds of *Picea glauca-engelmannii* and *Picea mariana* germinated even after storage at 4°C for one month (Lulsdorf *et al.* 1993), which is comparable to synthetic seed technology in angiosperms (Onay *et al.* 96). These advances indicate that once embryonal masses can be converted to immature somatic embryos, the technology needed to effectively integrate them into commercial reforestation programs will exist.

While initiation of embryogenic tissue of *Picea glauca* is under strong additive genetic control, variance in maturation and germination rates are largely due to non-additive genetic control, indicating that amenability to somatic embryogenesis systems may be difficult to breed into conifer families (Park *et al.* 1994). This makes the propagation of desirable genotypes even more important. One obstacle has been the difficulty in initiating embryogenic tissue from mature trees, though there have been successes in initiating organogenesis from 30 -year-old *Larix decidua, L. x eurolepis* and *L. leptolepis* trees (Bonga and Pond 1991, Bonga 1991), 12-year-old *Larix x eurolepis* trees (Laliberté and Lalonde 1988), and 20-year-old *Larix occidentalis* trees (Chesick *et al.* 1990). Results in organogenesis have been even more encouraging in other species using older plant material such as 60-year-old *Pseudotsuga mensiezii* trees (Gupta and Durzan 1987) and 600-year-old *Sequoia sempervirens* trees (Bourgkard and Favre 1988). These results with organogenesis in mature tissues encourage research into the initiation of embryogenic tissue from mature trees.

The incidence of vitrification in the organogenesis system using 12 year old *Larix x eurolepis* trees was less than that of mature somatic embryos of line 2086 of *Larix x eurolepis* (2% *vs.* 12%) though rooting was lower at only 4-22% (depending on the plant growth regulators used) (Laliberté and Lalonde 1988) compared to 67% for the somatic embryos of line 2086. This indicates that, unlike organogenesis, the nature of somatic embryogenesis ensures bipolar development and a root meristem, but possibly leaves embryogenic cultures more susceptible to the effects of the culture environment.

Line 2086 of *Larix x eurolepis* was capable of proliferating and maturing somatic embryos in the absence of plant growth regulators or osmotic treatments. This characteristic is rare but not unique amongst somatic embryogenic systems of angiosperms or conifers. In angiosperm species, embryogenic tissue of *Ilex aquifolium* (Hu *et al.* 1978), various tropical *Citrus* trees (*Citrus, Eriobotrya, Eugenia, Mangifera, Myciaria*) (Litz 1988), *Petroselinum crispum* (Masuda *et al.* 1977) and *Daucus carota* (Smith and Krikorian 1990) produced mature somatic embryos without plant growth regulators. In conifer species, the maintenance of embryogenic tissue without plant growth regulators is possible with *Larix decidua* (Cornu and Geoffrion 1990), as well as several *Abies* species (Schuller and Reuther 1993, Norgaard and Krogstrup 1995). Somatic embryos could be matured without plant growth regulators in *Abies alba* (Schuller *et al.* 1989), haploid *Larix decidua* (Nagmani and Bonga 1985, von Aderkas and Bonga 1988), *Picea abies* (Hakman *et al.* 1985), *Picea glauca* (Hakman and Fowke 1987, Lu and Thorpe 1987, Kartha *et al.* 1988), *Pinus lambertiana* (Gupta and Durzan 1986b) and *Sequoia sempervirens* (Bourkgard and Favre 1988). However, none of these species have been examined as precisely as line 2086, with individual embryonal structures isolated and studied.

Though the number of isolated embryonal structures which failed to develop in the experiments outlined in this study seemed high, this is in keeping with other studies, both of embryo rescue and somatic embryo maturation. Though isolation of embryonal structures has been attempted before for embryogenic tissue (Eastman *et al.* 1991), somatic embryo isolation more closely resembles embryo rescue techniques, where zygotic embryos at various stages of development are removed from the ovule to either examine the development of the embryo or to overcome embryo-endosperm incompatability in angiosperm hybrid crosses.

In embryo rescue, the less developed the embryo is upon excision, the more plastic it is and the more sensitive it is to induced morphogenic abnormalities (Sebanek *et al.* 1991). The nutritional requirements are more exacting for earlier stages of development, though the stage at which angiosperm embryo becomes heterotropic varies from plumule initiation to globular proembryos (Narayanaswami and Norstog 1964, Raghavan 1986). In line 2086, embryonal structures at a very early stage consisting of an embryonal mass and a suspensor were isolated, and though most of them failed to develop, several did mature. In angiosperms, excised proembryos of *Capsella* (Raghavan and Torrey 1963), *Brassica junica* (Liu *et al.* 1993), *Helianthus* (Chandler and Beard 1983), *Hordeum vulgare* (Narayanaswami and Norstog 1964), and *Datura stramonium* (Van Overbeek *et al.* 1942) continued development, but globular embryos of *Portulaca oleracea* (Narayanaswami and Norstog 1964) and *Triticum aestivum* (Comeau *et al* 1992) could not. In conifer species, zygotic embryos of *Pinus yunnamensis* and *Keteleeria davidiana* consisting of only several cells grew to 100 cells on medium supplemented with thiamine and indole acetic acid (Loo and Wang 1943), but zygotic embryos of *Pinus caribaea* consisting of 2-5 cells and embryos of *Larix decidua* up to 24 cells could not be cultured *ex ovulo* (Sterling 1949, David *et al.* 1995). Mature zygotic embryos of *Pinus lambertiana*, *Pinus lambertiana x Pinus armandi* and *Pinus lambertiana x Pinus koraiensis* have been successfully excised and germinated in culture, but immature embryos were not tried (Stone and Duffield 1950, Haddock 1954, Sacher 1956).

The difficulty in culturing excised zygotic proembryos may be due to damage to the embryo during excision or inadequate medium supplements, but this does not explain the failure of most somatic embryos in established cultures to grow and mature *in vitro*. Embryogenic tissue would not be as traumatised by subculture as a zygotic embryo would be by excision. In addition, unlike the single zygotic embryo, embryogenic tissue contains hundreds of somatic embryos which have successfully survived and proliferated under culture conditions for many subculture intervals. Therefore, isolated somatic embryos at early stages of development should be able to continue development just as they do on aggregates, yet in this study, only a few isolated somatic embryos matured, just as only a few somatic embryos mature on aggregates.

The composition of an embryogenic aggregate of line 2086 does not affect the number of embryos which mature on it. Six different types of embryonal structures were found in embryogenic aggregates of line 2086 and though the proportions of these six embryonal structures types differed in each aggregate, the rate of production of somatic embryos and the phenotypes of these embryos did not differ significantly between the aggregates. The differences between the phenotypes of embryonal structures largely related to the characteristics of the subtending suspensor, because the embryonal masses of five of the types (REMLS, REMLLS, REMSS, REMST, MC) were roughly the same size and morphology.

When the different types of embryonal structures were isolated, there was an association between the type of embryonal structure and its fate, but embryo production was low for all six types. Each type of embryonal structure could either become developmentally arrested (the most common fate for all types of embryonal structures) or proliferate. The four embryonal structure types with the most developed suspensors (SEMLS, REMLS, REMLLS, REMST) could also form nodules and embryos, though embryonal structures with either no suspensor (MC) or a short suspensor (REMSS) could not. Thus, the ability of the developing embryonal mass to produce a long compact suspensor or a smooth embryonal mass (SEMLS), analogous to the suspensor and embryonal mass produced by a developing zygotic embryo, does not indicate its ability to continue development into a normal somatic embryo only. Embryonal structures were more likely to become developmentally arrested, to cleave and to proliferate or to develop into a nodule than to develop into a somatic embryo.

If an embryonal mass cleaved and proliferated, the most common product of proliferation was REMLS type embryonal masses, though SEMLS, REMSS and microclusters were also common. Transformation from one type to another has been found in other species (Thompson and von Aderkas 1992, Egertsdotter *et al.* 1993). Differences in culture conditions can prompt the transformation of one type to another in *Picea abies* (Egertsdotter *et al.* 1993). This indicates that the different types of embryonal masses are not genetically distinct and can give rise to any of the other types. Though none of the six embryonal mass types of line 2086 produced many mature embryos (the most productive being SEMLS with 3 normal embryos and 9 misshapen embryos out of 314 embryonal structures), discovering which culture conditions encourages the proliferation of different types of embryonal structures could explain why the six types arise in the same aggregate.

The pathways of development for embryonal masses varied. An embryonal mass could continue to cleave, to die or to develop to the focal zone stage where cell divisions ceased to be random. An anthocyanin band developed in the basal portion of the focal zone. The embryo could then develop a hypocotyl and cotyledons or it could remain unpolarized and enlarge into a red nodule. If the embryo developed a hypocotyl and cotyledons, these structures could have normal or abnormal proportions, a smooth or rough epidermis, or the embryo could be vitrified. A root meristem may or may not have elongated into a root. If the focal zone enlarged into a red nodule, a root meristem and/or cotyledons may have formed. The cotyledons on a red nodule could be normally or abnormally proportioned.

with a smooth or rough epidermis, or the structure could have been vitrified. Any roots produced, by embryos or red nodules, appeared normal, though in some cases two root meristems formed on the same embryo/nodule, giving rise to a double root.

The embryo or red nodule could die or continue development. If a shoot meristem formed, the resulting epicotyl did not necessarily have the same characteristics as the cotyledons and hypocotyl. An abnormal embryo with a rough epidermis could produce a normal epicotyl with a smooth epidermis. Conversely, a normal embryo with a smooth epidermis could produce an abnormal epicotyl with a rough epidermis. Epicotyls formed from red nodules shared the same characteristics as epicotyls from somatic embryos. A vitrified embryo could have had a normal epicotyl, and a normal embryo could have had a vitrified epicotyl. Twin epicotyls were common and arose when two shoot meristems formed on the same embryo. The characteristics of the twin epicotyls were not always the same: one twin could be normal with a smooth epidermis and the other could be vitrified or abnormal with a rough epidermis.

This variation in both developmental pathways and final morphologies is a detriment to the efficiency of the somatic embryogenesis system. Not only do few somatic embryos mature, but the ones that do mature are not always normal in appearance. Because the selection criteria for outplanting is similarity to zygotic embryos, the large numbers of somatic embryos which are misshapen represent a loss in efficiency. It is commonly assumed that the closer somatic embryos are to zygotic embryos in terms of biochemistry, physiology and morphology, the more vigorous the plantlets will be (Flinn *et al.* 1993). An embryo with an abnormal epidermis likely represents a true loss, because an embryo with a vitrified or rough, discontinuous epidermis could not maintain proper water relations and would not survive transplanting. However, further studies with misshapen embryos may show a transition to normal growth once the embling is out of culture conditions. As long as the stele is normal and functioning, the root meristem (which always produces a normal root) should be able to supply the shoot meristem. Further studies using abnormal embryos may show that they are not a complete loss to the embryogenesis system, because they did not behave differently from normal embryos in terms of rooting and longevity.

The germination and mortality rates varied amongst the different mature embryo types. The overall rooting rate for *Larix x eurolepis* somatic embryos was 58%. However, normal-smooth embryos had a rooting rate of 67% while normal-rough embryos had a rooting rate of 55%. Vitrified embryos had a rooting rate of 63% and abnormal embryos had a rooting rate of 53%. The death rates were as follows: normal smooth embryos 20%, normal rough embryos 30% , vitrified embryos 29% , abnormal smooth embryos 43% , abnormal rough embryos 74%. Therefore, selection of normal smooth embryos which resemble zygotic embryos does select for embryos which are more likely to root and to survive in culture.

The addition of plant growth regulators negatively affected the growth of embryonal masses. Medium with no 2,4-D produced more embryos and nodules than medium with 2,4-D, and had a lower rate of developmental arrest and proliferation. Medium with no BA produced more embryos, fewer nodules and had a higher rate of developmental arrest. The level of sucrose in the medium had no effect on maturation, nor did the type of embryonal mass (SEMLS or REMLS), but the addition of any level of ABA to the medium resulted in more developmental arrest, more proliferation and fewer nodules but no change in the numbers of embryos produced. The only nodules that died, or produced roots or cotyledons did so on medium without plant growth regulators. BA also affected the number of nodules which produced roots and cotyledons but the trend was not linear with increasing concentrations of BA. In all of the treatments, whether it was increased sucrose, 2,4-D, BA or ABA, embryonal masses either became developmentally arrested (most common fate), proliferated, became nodules or matured into embryos. All four developmental fates were represented in each treatment, indicating that though the embryonal masses were carefully selected to be only SEMLS (2,4-D and BAP experiments) or SEMLS and REMLS (sucrose and ABA experiments), they still reacted differently when exposed to a variety of media.

This demonstrates that uniform morphology does not indicate uniform physiology. The triggering mechanism which prompts an embryonal mass to mature is still elusive. Other researchers have discovered extracellular proteins which affect the maturation of somatic embryos (Egertsdotter *et al.* 1993, Domon *et al.* 1994). Further experiments with line 2086 should include an examination of the effects of extracellular proteins on maturation, possibly by placing isolated embryonal masses on unconditioned medium, medium conditioned by non-productive aggregates, and medium conditioned by productive aggregates.

In conclusion, unlike zygotic embryos, somatic embryos can have many pathways of development, though the majority stall and do not develop beyond the embryonal mass stage. The low survival rates of embryonal masses is not explained by the initial phenotype of the embryonal mass, or the lack of auxins, ABA or low sucrose levels in the medium. The addition of benzylaminopurine to the medium did decrease the number of embryonal masses which became developmentally arrested, and further work should focus on finding the appropriate concentration. Because red nodules can form a normal root meristem, normal cotyledons and normal epicotyls, the enlarging of a focal zone stage embryo into a red nodule may be a delay in embryo development, rather than a departure from it. The internal organization of embryos formed from red nodules and the survival success of such embryos in soil should be further investigated to determine if the tissue of the nodule can properly differentiate into vascular and procambial tissue.

The diversity of morphologies of somatic embryos developed *in vitro* may be related to the effect of the culture environment on the division and differentiation of cells in the embryonal masses, but the fact that red nodules and normal and abnormal somatic embryos, with smooth and rough epidermal qualities, can arise in the same aggregate under the same conditions simultaneously, indicate that the forces which determine morphology are random. The morphology of the structures that arise from the organized growth of the embryonal mass (hypocotyl, cotyledons) are affected by different phenomenon than structures arising from meristems (roots, epicotyls) because the characteristics of the mature somatic embryo may be different from that of the epicotyl it produces, yet root formation is always normal. In addition, the characteristics of twin epicotyls may be different, though they arise from the same embryo and are in close proximity.

Though the reasons why some embryos mature and others don't was not identified, much information into the nature of somatic embryogenesis has been gained. Further reasearch should focus on molecular aspects of embryogenesis as well as the success rate of mature embryos of different phenotypes in the greenhouse. Long-term testing of *Picea glauca* emblings are already underway (Adams *et al.* 1994) and emblings of *Picea glauca-engenlmannii* met stock quality testing standards in British Columbia (Adams *et al.* 1994). Therefore, the potential is there for somatic embryos to provide multitudes of high-quality emblings of elite genotypes to the forestry industry. The continued use of different types of embryogenic tissue, such as line 2086 of *Larix x eurolepis* , will aid in the identification of culture conditions required to induce the maturation of vigorous, normal somatic embryos from embryogenic tissue of conifer species.

Chapter VIII: Summary and conclusions

Several conclusions were drawn from this study with respect to aggregate composition, early embryo structure, developmental pathways, the relationship between embryo morphology and apical development and the manipulation of maturation. These are summarized as follows:

1. Embryogenic aggregates of *Larix x eurolepis* line 2086 were usually mixtures of six types of embryonal structures. Aggregates composed of SEMLS and REMLS types were filamentous while aggregates composed of MC and REMSS types appeared smooth.

2. Embryonal structures consisted of a cluster of small, meristematic cells possibly subtended by embryonal tube cells. The embryonal mass could be smooth or rough, and the suspensor varied from nonexistent, to short and loosely associated to long and compact. The embryonal structures could transform into the different types: a single embryonal structure could proliferate into an aggregate composed of all six types. This indicates that the differences in morphology are not due to somaclonal variation.

3. The composition of the embryogenic aggregates had no effect on mature embryo production and the ratio of normal to abnormal mature embryos produced, whether the aggregates were classified according to the most prevalent or the most organized type present.

4. For all types of embryonal structures, the most common fate was developmental arrest, followed by proliferation, with the least common fate being maturation into a nodule or a mature somatic embryo. The type of embryonal structure had an effect on developmental fate: the more organized the suspensor (the more closely it resembled a suspensor of a zygotic embryo), the more likely it was to mature. This indicates that the organization of the rib meristem may affect the ability of an embryo to mature.

5. The immature embryo developed from a cluster of randomly dividing cells subtended by embryonal tube cells to an embryonal mass dividing in organized planes (focal zone stage), followed by the formation of a polyphenol band around the basal region. Cotyledonary primordia were intiated on the periphery of the embryonal mass followed by cotyledonary and hypocotyl expansion.

6. Alternative pathways of development included: 1. continuous random division of the embryonal mass cells and subsequent cleavage into daughter embryos, or, 2. the formation of nodules which were spherical structures capable of forming cotyledons, roots and epicotyls. Proliferation by cleavage indicates a lack of maturation, but the formation of nodules which could then revert into embryos with cotyledons and apices is an alternative pathway to maturation.

7. There were two critical stages of development: embryos that formed a polyphenol band in the basal region of the embryo usually matured, and once cotyledons formed an embryo was less likely to become a nodule rather than a mature somatic embryo.

8. Mature somatic embryos could be classified according to their proportions (normal or abnormal) and epidermal qualities (smooth or rough). Embryo shape and epidermal qualities were associated: normally proportioned embryos were more likely to have a smooth epidermis than a rough epidermis. Any type of embryo was capable of producing an epicotyl and a root, though germination rates were slightly higher for normal, smooth mature embryos compared to abnormal embryos. Normal smooth embryos tended to produce normal smooth epicotyls, though any combination of embryo and epicotyl morphologies were possible. There was a high incidence of multiple epicotyl production and occasionally two epicotyls on the same embryo would have different characteristics, i.e. one would be normal and the other one vitrified. These results indicate that apical formation is largely independent of embryo formation.

9. Abnormal and vitrifed embryos had a higher incidence of abnormal epicotyl growth but were capable of forming normal epicotyls. The relative success of these abnormal embryos in the greenhouse was not tested, but sectioned material showed that embryos with a rough epidermis had a discontinous epidermis which would negatively affect plant water relations.

10. Attempts to manipulate maturation by using media supplements were not very successful. Though few embryos responded to auxin and cytokinin treatments, there was a trend towards a higher production of nodules and mature somatic embryos on medium with BA and without 2,4-D. Sucrose concentrations had no effect on maturation, but ABA had a negative effect on embryo maturation. Therfore, a lack of plant growth regulators is not the reason why so few embryos mature.

Literature Cited

Adams. G.W.. Doiron. M.G.. Park. Y.S.. Bonga. J.M.. and Charest. P.J. 1994. Commercialization potential of somatic embryogenesis in black spruce tree improvement. For. Chron. 70 (5): 593-598.

Afele. J.C., and Saxena. P.K. 1995. Somatic embryogenesis in blue spruce (*Picea pungens* Engelmann). *In* Somatic embryogenesis in woody plants. *Edited by* S. Mohan Jain. P. K. Gupta. and R.J. Newton. Kluwer Academic Publishers. Dordrecht. pp. 99-110.

Amarasinghe. V.. Dhami. R.. and Carlson. J.E. 1996. Polyamine biosynthesis during somatic embryogenesis in interior spruce (*Picea glauca x Picea engelmannii* complex). Plant Cell Reports 15: 495-499.

Ammirato. P.V. . Embryogenesis. In: Handbook of Plant Cell Culture. Vol. 1. edited by: Evans. D.A.. Sharp. W.R.. Ammirato. P.V.. and Yamada. Y.. Macmillan. New York. pp. 82-123.

Attree. S.M.. Budimir. S.. and Fowke. L.C. 1990a. Somatic embryogenesis and plantlet regeneration from cultured shoots and cotyledons of seedlings germinated from stored seed of black and white spruce (*Picea mariana* and *Picea glauca*). Can. J Bot. 68: 30-34.

Attree. S.M.. Dunstan. D.I.. and Fowke. L.C. 1989a. Initiation of embryogenic callus and suspension cultures. and improved embryo regeneration from protoplasts. of white spruce (*Picea glauca*). Can. J. Bot. 67: 1790-1795.

Attree. S.M.. Dunstan. D.I.. and Fowke. L.C. 1989b. Plantlet regeneration from embryogenic protoplasts of white spruce (*Picea glauca*). Bio/Tech. 7: 1060-1062

Attree, S.M.. and Fowke. L.C. 1993. Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell. Tiss. Org. Cult. 35: 1-35.

Attree. S.M.. Moore. D.. Sawhney, V.K.. and Fowke. L.C. 1991. Enhanced maturation and desiccation tolerance of white spruce [*Picea glauca* (Moench) Voss] somatic embryos: Effects of a non-plasmolysising water stress and abscissic acid. Ann. Bot. 68: 519-525.

Attree, S.M., Pomeroy, M.K., and Fowke,L.C. 1992. Manipulation of conditions for the culture of somatic embryos of white spruce for improved triacylglycerol biosynthesis and desiccation tolerance. Planta 187: 395-404.

Attree, S.M., Pomeroy, M.K., and Fowke, L.C. 1994. Production of vigorous, desiccation tolerant white spruce (*Picea glauca* [Moench.] Voss.) synthetic seeds in a bioreactor. Plant Cell Rep. 13: 601-606.

Becwar, M.R., Blush, T.D., Brown, D.W., and Chesick, E.E. 1991. Multiple paternal genotypes in embryogenic tissue derived from individual loblolly pine seeds. Plant Cell, Tiss. Org. Cult. 26: 37-44.

Becwar, M.R., Nagmani, R., and Wann, S.R. 1990. Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Can. J. For. Res. 20: 810-817.

Becwar, M.R., Noland, T.L., and Wyckoff, J.L. 1989. Maturation, germination, and conversion of Norway spruce (*Picea abies* L.) somatic embryos to plants. In Vitro Cell. Dev. Bio. (25)6: 575-560.

Becwar, M.R., and Pullman, G.S. 1995. Somatic embryogenesis in loblolly pine (*Pinus taeda* L.). *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta, and R.J. Newton. Kluwer Academic Press, Dordrecht. pp. 287-302.

Becwar, M.R., Wann, S.R., Johnson, M.A., Verhagen, S.A., Feirer, R.P., and Nagmani, R. 1988. Development and characterization of *in vitro* embryogenic systems in conifers. *In* Somatic cell genetics of woody plants. *Edited by* M.R. Ahuja. Kluwer Academic Press, Dordrecht. pp 1-18.

Bercetche, J., and Pacques, M. 1995. Somatic embryogenesis in maritime pine (*Pinus pinaster*). *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta, and R.J. Newton. Kluwer Academic Press, Dordrecht. pp. 221-242.

Bhaskaran. S.. and Prabhudesai. V.R. 1988. Tissue culture of plantation crops. *In* Applications of biotechnology in forestry and horticulture. *Edited by* V. Dhawan. Plenum Press. New York. pp. 87-108.

Bhojwani. S.S.. and Dhawan. V.. 1988. Acclimatization of tissue culture-raised plants for transplantation to the field. In: *Applications of Biotechnology in Forestry and Horticulture* (V. Dhawan. ed.). pp. 249-256. Plenum Press. New York.

Bommineni. V.R.. Chibbar. R.N.. Datla. R.S.S.. and Tsang. E.W.T. 1993. Transformation of white spruce (*Picea glauca*) somatic embryos by microprojectile bombardment. Plant Cell Rep. 13: 17-23.

Bonga. J.M. 1991. *In vitro* propagation of conifers: fidelity of the clonal offspring. In: Woody Plant Biotechnology. ed: M.R. Ahuja. Plenum Press. New York. pp. 13-21.

Bonga. J.M.. Klimaszewska. K.. Lelu. M.-A.. and von Aderkas. P. 1995. Somatic embryogenesis in *Larix*.. *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain. P.K. Gupta. and R.J. Newton. Kluwer Academic Press. Dordrecht. pp. 315-340.

Bonga. J.M.. and Pond. S.E. 1991. Adventitious shoot formation in cultures of 30-year-old *Larix decidua*. *L. leptolepis*. *L. eurolepis*. and *L. laricina* trees. Plant Cell. Tiss. Org. Cult. 26: 45-51.

Bonga. J.M.. and von Aderkas. P. . 1988. Attempts to micropropagate mature *Larix decidua* Mill.. In: Somatic Cell Genetics of Woody Plants. Ed: Ahuja. M.R.. Kluwer Academic Publishers. Dordrecht. pp. 155-168.

Boulay. M.P.. Gupta. P.K.. Krogstrup. K.. and Durzan. D.J. 1988. Development of somatic embryos from cell suspension cultures of Norway spruce (*Picea abies* Karst.). Plant Cell Rep. 7: 134-137.

Bourgkard. F.. and Favre. J.M. 1988. Somatic embryos from callus of *Sequoia sempervirens* . Plant Cell Rep. 7: 445-448.

Bozhkov, P.V., Lebedenko, L.A., and Shiryaeva, G.A. 1992. A pronounced synergistic effect of abscisic acid and 6-benzyladenine on Norway spruce (*Picea abies* L. Karst) somatic embryo maturation. Plant Cell Rep. 11: 386-389.

Bozkov, P.V. 1995. Genotypic and media factors affecting stabilization of polyembryogenic cultures in norway spruce (*Picea abies* (L.) *Karst.*). Plant Cell Rep. 14: 389-392.

Budimir, S., and Vujicic, R. 1992. Benzyladenine induction of buds and somatic embryogenesis in *Picea omorika* (Pancic) Purk. Plant Cell, Tiss. Org. Cult. 31: 89-94.

Button, J., Kochba, J., and Bornman, C.H. 1974. Fine structure of and embryoid development from embryogenic ovular callus of "Shamouti" orange (*Citrus sinensis* Osb.). J. Exp. Bot. 25(85): 446-457.

Chalupa, V. 1985. Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (l.) Karst. Comm. Inst. For. Cech. 14: 57-63.

Chalupa, V. 1989. Plant regeneration by somatic embryogenesis in Norway spruce (*Picea abies* / L./Karst.) and sessile oak (*Quercus petraea* /Matt./Liebl.). Comm. Inst. For. Cech., Vol. 16: 135-143.

Chandler, J.M., and Beard, B.H. 1983. Embryo culture of *Helianthus* hybrids. Crop Science 23: 1004-1007.

Charest, P.J., Calero, N., Lachance, D., Datla, R.S.S., Duchesne, L.C., and Tsang, E.W.T. 1993. Microprojectile-DNA delivery in conifer species: factors affecting assessment of transient gene expression using the ß-glucuronidase reporter gene. Plant Cell Rep. 12: 189-193.

Charest, P.J., Devantier, Y., Ward, C., Jones, C., Schaffer, U., and Klimaszewska, K.K. 1991. Transient expression of foreign chimeric genes in the gymnosperm hybrid larch following electroporation. Can. J. Bot. 69: 1731-1736.

Charest. P.J.. and Klimaszewska. K.K.. 1994. Regeneration of plants from protoplasts of *Larix* species (Larch). In: Biotechnology in Agriculture and Forestry. Vol. 29. . edited by: Y.P.S. Bajaj. Springer-Verlasg Berlin. pp. 59-69.

Cheliak. W.M.. and Klimaszewska. K. 1991. Genetic variation in somatic embryogenic response in open-pollinated families of black spruce. Theor. Appl. Genet. 82: 185-190.

Cheliak. W.M.. and Rogers. D.L. 1990. Integrating biotechnology into tree improvement programs. Can. J. For. Res. 20: 452-463.

Chesick. E.E.. Bilderback. D.E.. and Blake. G.M. 1990. *In vitro* multiple bud formation by 20-year-old Western larch buds and stems. HortScience 25(1): 114-116.

Chowdhury. C.R. 1962. The embryogeny of conifers: a review. Phytomorphology 12: 313-338.

Comeau. A.. Nadeau. P.. Plourde. A.. Simard. R.. Maes. O.. Kelly. S.. Harper. L.. Lettre. J.. Landry. B.. and St-Pierre. C. -A. 1992. Media for the in ovulo culture of proembryos of wheat and wheat-derived interspecific hybrids of haploids. Plant Science 81: 117- 125.

Cornu. D.. and Geoffrion. C. 1990. Aspects de l'embryogenèse somatique chez le mélèze. Bull. Soc. bot. Fr.. 137. Actual. bot. (3/4): 25-34.

Cornu. D. and Geoffrion. C. 1991. Somatic embryogenesis in *Larix decidua*. In: Woody Plant Biotechnology. edited by: M.R. Ahuja. Plenum Press. New York. pp. 347-348.

David. A.. Laine. E.. and David. H. 1995. Somatic embryogenesis in *Pinus caribaea*. *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain. P.K. Gupta. and R.J. Newton. Kluwer Academic Press. Dordrecht. pp. 145-182.

DeVerno. L.L.. Charest. P.J.. Bonen. L. 1994. Mitochondrial DNA variation in somatic embryogenic cultures of *Larix*. Theor. Appl. Genet. 88: 727-732.

De Vries, S., Booij, H., Meyerink, P., Hulsman, G., Wilde, D., Thomas, T., and van Kammen, A. 1988. Acquisition of embryogenic potential in carrot cell-suspension cultures. Planta 176: 196-204.

Dhawan, V. , 1988. Micropropagation and nodulation of tree legumes. In: *Applications of Biotechnology in Forestry and Horticulture* (V. Dhawan, ed.), pp. 285-296, Plenum Press, New York.

Diner, A.M., and Karnovsky, D.F. 1987. Differential responses of two conifers to *in vitro* inoculation with *Agrobacterium rhizogenes* . Eur. J. For. Path. 17: 211-216.

Domon , J.M., Meyer, Y., Faye, L., David, A., and David, H. 1994. Extracellular (glyco) proteins in embryogenic and non-embryogenic cell lines of Caribbean pine. Comparison between phenotypes of stage one somatic embryo. Plant Physiol. Biochem. 32: 137-147.

Dornelas, M.C., Vieira, M.L.C., and Appezzato-da-Gloria, B. 1992. Histological analysis of organogenesis and somatic embryogenesis induced in immature cultures of *Stylossanthes scabra.* Ann. Bot. 70: 477-482.

Duchesne, L.C., and Charest, P.J. 1992. Effect of promoter sequence on transient expression of the ß-glucuronidase gene in embryogenic calli of *Larix x eurolepis* and *Picea mariana* following microprojection. Can. J. Bot. 70: 175-180.

Duchesne, L.C., Lelu, M.-A., von Aderkas, P., and Charest, P.J. 1993. Microprojectile-mediated DNA delivery in haploid and diploid embryogenic cells of *Larix* spp. Can. J. For. Res. 23: 312-316.

Dudits, D., Bogre, L., and Gyorgyey, J. 1991. Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro.* J. of Cell Sci. 99:475-484.

Dunstan, D.L., Bekkaoui, F., Pilon, M., Fowke, L.C. and Abrams, S.R. 1988. Effects of abscisic acid and analogues on the maturation of white spruce (*Picea glauca*) somatic embryos. Plant Science 58: 77-84.

Dunstan, D.I., Bethune, T.D., and Abrams, S.R. 1991. Racemic abscisic acid and abscisyl alcohol promote maturation of white spruce (*Picea glauca*) somatic embryos. Plant Science 76: 219-228.

Dunstan, D.I., Bock, C.A., Abrams, G.D., and Abrams, S.R. 1992. Metabolism of (+)- and (-)- abscisic acid by somatic embryo suspension cultures of white spruce. Phytochemistry 31(5): 1451-1454.

Durzan, D.J., Jokinen, K., Guerra, M.P., Santerre, A., Chalupa, V., and Havel, L. 1994. Latent diploid parthenogenesis and parthenote cleavage in egg-equivalents of Norway spruce. Int. J. Plant Sci. 155(6): 677-688.

Eastmann, P. A.K., Webster, F.B., Pitel, J.A., and Roberts, D.R. 1991. Evaluation of somaclonal variation during somatic embryogenesis of interior spruce (*Picea glauca engelmannii* complex) using culture morphology and isozyme analysis. Plant Cell Rep. 10: 425-430.

Egertsdotter, U., Mo, L.H., and von Arnold, S. 1993. Extracellular proteins in embryogenic suspension cultures of Norway spruce (*Picea abies*). Physiol. Plant. 88: 315-321.

Egertsdotter, U., and von Arnold, S. 1993. Classification of embryogenic cell-lines of *Picea abies* as regards protoplast isolation and culture. J. Plant Physiol. 141: 222-229.

Ellis, D.D., McCabe, D.E., McInnis, S., Ramachandran, R., Russell, D.R., Wallace, K.M., Martinell, B.J., Roberts, D.R., Raffa, K.F., and McCown, B.H. 1991. Stable transformation of *Picea glauca* by particle acceleration. Bio/Tech. 11: 84-89.

Favre, J.M., Tranvan, H., Arnaud, Y., and F. Bourgkard. 1995. Present state of somatic embryogenesis in *Sequoia sempervirens* (Lamb.) Endl. *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta, and R.J. Newton. Kluwer Academic Press, Dordrecht. pp. 357-370.

Feirer, R.P., Conkey, J.H., and Verhagen, S.A. 1989. Triglycerides in embryogenic conifer calli: a comparison with zygotic embryos. Plant Cell. Rep. 8: 207-209.

Finer. J.J.. Kriebel. H.B.. and Becwar. M.R. 1989. Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.). Plant Cell Rep. 8: 203-206.

Flinn. B.S.. Roberts. D.R.. Newton. C.H.. Cyr. D.R.. Webster. F.B.. and Taylor. I.E.P. 1993. Storage protein gene expression in zygotic and somatic embryos of interior spruce. Physiol. Plant. 89: 719-730.

Flinn. B.S.. Roberts. D.R.. and Taylor. I.E.P. 1991a. Evaluation of somatic embryos of interior spruce. Characterization and developmental regulation of storage proteins. Physiol. Plant. 82: 624-632.

Flinn. B.S.. Roberts. D.R.. Webb. D.T.. and Sutton. B.C.S. 1991b. Storage protein changes during zygotic embryogenesis in interior spruce. Tree Physiology 8: 71-81.

Fowke. L.C.. Attree. S.M.. and Rennie. P.J. 1994. Scanning electron microscopy of hydrated and desiccated mature somatic embryos and zygotic embryos of white spruce (*Picea glauca* [Moench] Voss.). Plant Cell Rep. 13: 612-618.

Fowke. L.C.. Attree. S.M.. Wang. H.. and Dunstan. D.I. 1990. Microtubule organization and cell division in embryogenic protoplast cultures of white spruce (*Picea glauca*). Protoplasma 158: 86-94.

Fowler. D.P.. and Loo-Dinkins. J.A. 1992. Breeding strategies in a changing climate and implications for biodiversity. For. Chron. 68(4): 472-475.

Franklin. C.I.. Mott. R.L.. and Vuke. T.M. 1989. Stable ploidy levels in long-term callus cultures of loblolly pine. Plant Cell Rep. 8: 101-104.

Gaspar. Th.. Kevers. C.. DeBergh. P.. Maene. L.. Paques. M.. and Ph. Boxus. 1987. Vitrification: Morphological. physiological. and ecological aspects. *In* Cell and tissue culture in forestry , Vol. 1 General principles and biotechnology. Martinus Nijhoff Publishers. Dordrecht. pp. 152-166.

Gates. J.C.. and Greenwood. M.S. 1991. The physical and chemical environment of the developing embryo of *Pinus resinosa* . Amer. J. Bot. 78(7): 1002-1009.

Gifford. D.J. 1988. An electrophoretic analysis of the seed proteins from *Pinus monticola* and eight other species of pine. Can. J. Bot. 66: 1808-1812.

Gifford, D.J., and Tolley, M.C. 1989. The seed proteins of white spruce and their mobilization following germination. Physiol. Plant. 77: 254-261.

Glauert. A.M. 1974. Fixation. dehydration and embedding of biological specimens. *In:* Practical methods in electron microscopy. edited by A.M.Glauert. North-Holland Publishing Company. Amsterdam.

Goldberg, R., de Paiva, G., and Yadegari. R. 1994. Plant embryogenesis: zygote to seed. Science 266:605-613.

Gower. S.T., and Richards. J.H. 1990. Larches: Deciduous conifers in an evergreen world. BioScience 40(11): 818-826.

Gribble. K.. Sarafis. V.. Nailon. J.. Holfird. P.. and Uwins. P. 1996. Environmental scanning electron microscope of the surface of normal and vitrified leaves of *Gypsophila paniculata* (Babies Breath) cultured *in vitro*. Plant Cell Rep. 15: 771-776.

Gupta. P.K. 1995. Somatic embryogenesis in sugar pine (*Pinus lambertiana*). *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain. P.K. Gupta. and R.J. Newton. Kluwer Academic Press. Dordrecht. pp. 197-206.

Gupta. P.K., Dandekar, A.M., and Durzan. D.J. 1988. Somatic proembryo formation and transient expression of a luciferase gene in Douglas-fir and loblolly pine protoplasts. Plant Science 58: 85-92.

Gupta. P.K., and Durzan. D.J. 1986. Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Tech. 4: 643-645.

Gupta. P.K., and Durzan. D.J. 1987a. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Bio/Tech. 5: 147-151.

Gutmann. M., von Aderkas. P., Label. P., and Lelu. M.-A. Effects of abscisic acid on somatic embryo maturation of hybrid larch: histological studies. In press.

Haddock, P.G. 1954. Sapling sugar pine grown from excised mature embryos. J. Forestry 52: 434-437.

Hakman, I. 1993a. Embryology in Norway spruce (*Picea abies*). Immunochemical studies on transport of a seed protein. Physiol. Plant. 88: 427-433.

Hakman, I. 1993b. Embryology in Norway spruce (*Picea abies*). An analysis of the composition of seed storage proteins and deposition of storage reserves during seed development and somatic embryogenesis. Physiol. Plant. 87: 148-159.

Hakman, I., and Fowke, L.C. 1987. Somatic embryogenesis in *Picea glauca* (white spruce) and *Picea mariana* (black spruce). Can. J. Bot. 65: 656-659.

Hakman, I., Fowke, L.C., von Arnold, S. and Eriksson, T. 1985. The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). Plant Science 38: 53-59.

Hakman, I., Rennie, P., and Fowke, L. 1987. A light and electron microscope study of *Picea glauca* (white spruce) somatic embryos. Protoplasma 140: 100-109.

Hakman, I., Stabel, P., Engstrom, P., and Eriksson, T. 1990. Storage protein accumulation during zygotic and somatic embryo development in *Picea abies* (Norway spruce). Physiol. Plant. 80: 441-445.

Hakman, I., and von Arnold, S. 1985. Plantlet regeneration through somatic embryogenesis in *Picea abies* (Norway spruce). J. Plant Physiol. 121: 149-158.

Hakman, I., and von Arnold, S. 1988. Somatic embryogenesis and plant regeneration from suspension cultures of *Picea glauca* (white spruce). Physiol. Plant. 72: 579-587.

Halperin, W. 1966. Alternative morphogenic events in cell suspensions. Amer. J. Bot. 53(5): 443-453.

Harry, I.S., and Thorpe, T.A. 1991. Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. Bot. Gaz. 152(4): 446-452.

Havel, L., and Durzan, D.J. 1996. Apoptosis during diploid parthenogenesis and early somatic embryogenesis of Norway spruce. Int. J. Plant Sci. 157(1): 8-16.

Hetherington, A.M., and Quatrano, R.S. 1991. Mechanisms of action of abscisic acid at the cellular level. Tansley Review 31. New Phytol. 119: 9-32.

Higgins, T.J.V. 1984. Synthesis and regulation of major proteins in seeds. Ann. Rev. Plant Physiol. 35: 191-221.

Hohtola, A. 1995. Somatic embryogenesis in Scots pine (*Pinus sylvestris* L.). *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta, and R.J. Newton. Kluwer Academic Press, Dordrecht. pp. 269-286.

Hu, C.Y., Ochs, J.D., and Mancini, F.M. 1978. Further observations on *Ilex* embryoid formation. Z. Pflanzenphysiol. 89: 41-49.

Huang, Y., Diner, A.M., and Karnovsky, D.F. 1991. *Agrobacterium rhizogenes*-mediated genetic transformation and regeneration of a conifer: *Larix decidua*. In Vitro Cell. Dev. Biol. 27P: 201-207.

Huang, Y., Stokke, D.D., Diner, A.M., Barnes, W.M. and Karnovsky, D.F. 1993. Virulence of *Agrobacterium* on *Larix decidua* and their cellular interactions as depicted by scanning electron microscopy. J. Exp. Bot. 44(264): 1191-1201.

Hutchinson, K.W., Harvie, P.D., Singer, P.D., Brunner, A.F., and Greenwood, M.S. 1990. Nucleotide sequence of the small subunit of ribulose-1,5-bisphosphate carboxylase from the conifer *Larix laricina*. Plant Molecular Biology 14: 281-284.

Isabel, N., Boivin, R., Levasseur, C., Charest, P.-M., Bousquet, J., and Tremblay, F. M. 1996. Occurrence of somaclonal variation among somatic embryo-derived white spruces (*Picea glauca*, Pinaceae). Am. J. Bot. 83(9): 1121-1130.

Isabel, N., Tremblay, L., Michaud, M., Tremblay, F.M., and Bousquet, J. 1993. RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill.) B.S.P. Theor. Appl. Genet. 86: 81-87.

Jalonen, P., and von Arnold, S. 1991. Characterization of embryogenic lines of *Picea abies* in relation to their competence for maturation. Plant Cell Rep. 10: 384-387.

Jain, S.M., Dong, N., and Newton, R.J. 1989. Somatic embryogenesis in slash pine (*Pinus elliottii*) from immature embryos cultured *in vitro*. Plant Science 65: 233-241.

Jasik, J., Salajova, T., and Salaj, J. 1995. Developmental anatomy and ultrastructure of early somatic embryos in European black pine (*Pinus nigra* Arn.). Protoplasma 185: 205-211.

Joy, R.W., Yeung, E.C., Kong, L., and Thorpe, T.A. 1991. Development of white spruce somatic embryos: I. Storage product deposition. In Vitro Cell Dev. Biol. 27P: 32-41.

Karnovsky, M.J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biology 27: 137a-138a.

Kartha, K.K., Fowke, L.C., Leung, N.L., Caswell, K.L., and Hakman, I. 1988. Induction of somatic embryos and plantlets from cryopreserved cell cultures of white spruce (*Picea glauca*). J. Plant Physiol. 132: 529-539.

Kaul, K. 1995. Somatic embryogenesis in white pine (*Pinus strobus* L.). *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta, and R.J. Newton. Kluwer Academic Press, Dordrecht. pp. 257-268.

Klimaszewska, K. 1989a. Plantlet development from immature zygotic embryos of hybrid larch through somatic embryogensis. Plant Science 63: 95-103.

Klimaszewska, K. 1989b. Recovery of somatic embryos and plantlets from protoplast cultures of *Larix x eurolepis*. Plant Cell Rep. 8: 440-444.

Klimaszewska, K. 1995. Somatic embryogenesis in *Picea mariana* (Mill.). *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta, and R.J. Newton. Kluwer Academic Press, Dordrecht. pp. 67-80.

Klimaszewska. K.. Ward. C., and Cheliak. W.M. 1992. Cryopreservation and plant regeneration from embryogenic cultures of larch (*Larix x eurolepis*) and black spruce (*Picea mariana*). J. Exp. Bot. 43(246): 73-79.

Koornneef. M.. Hanhart. C.J.. Hilhorst. H.W.M.. and Karssen. C.M. 1989. In vivo inhibition of seed development and reserve proteion accumulation in recombinants of abscisic acid biosynthesis and responsiveness in *Arabidopsis thaliana*. Plant Physiol. 90: 463-469.

Koornneef. M.. Jorna. M.L.. Brinkhorst-van der Swan. D.L.C.. and Karssen. C.M. 1982. The isolation of abscisic acid (ABA) deficient mutations by selection of induced revertants in non-germination gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heyn. Theor. Appl. Genet. 61: 385-393.

Koornneef. M., Reuling. G.. and Karssen. C.M. 1984. The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. Physiol. Plant. 61: 377-383.

Kreuger. M.. and van Holst. G.-J. 1993. Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta 189: 243-248.

Krikorian. A.D.. 1988. The context and strategies for tissue culture of date. african oil and coconut palms. In: *Applications of Biotechnology in Forestry and Horticulture* (V. Dhawan. ed.). pp. 119-144. Plenum Press. New York.

Krikorian. A.D.. and Smith D.L. 1992. Somatic embryogenesis in carrot (*Daucus carota*). *In* Plant tissue culture manual. *Edited by* K. Lindsey. Kluwer Academic Publishers. Dordrecht. pp. 1-32.

Kristensen. M.M.H.. Find. J.I.. Floto. F.. Moller. J.D.. Norgaard. J.V.. and Krogstrup. P. 1994. The origin and development of somatic embryos following cryopreservation of an embryogenic suspension culture of *Picea sitchensis*. Protoplasma 182: 65-70.

Krogstrup. P. 1986. Embryolike structures from cotyledons and ripe embryos of Norway spruce (*Picea abies*). Can. J. For. Res. 16: 664-668.

Krogstrup, P. 1990. Effect of culture densities on cell proliferation and regeneration from embryogenic cell suspensions of *Picea sitchensis* Plant Sci. 72: 115-123.

Krogstrup, P., Eriksen, E.N., Moller, J.D. and Roulund, H. 1988. Somatic embryogenesis in Sitka spruce (*Picea sitchensis* (Bong.) Carr.) Plant Cell Rep. 7: 594-597.

Kumar, R., Lelu, M.-A., and Small, I. 1995. Purification of mitochondria and mitochondrial nucleic acids from embryogenic suspension cultures of a gymnosperm, *Larix x leptoeuropaea.* Plant Cell Rep. 14: 534-538.

Kumar, P.P., and Thorpe, T.A. , 1988, Alteration of growth and morphogenesis by endogenous ethylene and carbon dioxide in conifer tissues. In: *Applications of Biotechnology in Forestry and Horticulture* (V. Dhawan, ed.), pp. 205-214, Plenum Press, New York.

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

Laliberté, S., and Lalonde, M. 1988. Sustained caulogenesis in callus cultures of *Larix x eurolepis* initiated from short shoot buds of a 12-year-old tree. Amer. J. Bot. 75(6): 767-777.

Lang, H., and Kohlenbach, H. 1989. Cell differentiation in protoplast cultures from embryogenic callus of *Abies alba* L. Plant Cell Rep. 8: 120-123.

Lelu, M.A., Bastien, C., Klimaszewska, K., Ward, C., and Charest, P.J. 1993a. An improved method for somatic plantlet production in hybrid larch (*Larix x leptoeuropaea*) : Part 1. Somatic embryo maturation. Plant Cell , Tiss. Org. Cult. 36: 107-115.

Lelu, M.A., Bastien, C., Klimaszewska, K., and Charest, P.J. 1993b. An improved method for somatic plantlet production in hybrid larch (*Larix x leptoeuropaea*) : Part 2. Control of germination and plantlet development. Plant Cell, Tiss. Org. Cult..

Lelu, M.A., Klimaszewska, K. and Charest, P.J. 1994. Somatic embryogensis from immature and mature zygotic embryos and from cotyledons and needles of somatic plantlets of *Larix*. Can. J. For. Res. 24: 100-106.

Lelu, M..A., Klimaszewska, K., Pflaum, G., and Bastien, C. 1995. Effect of maturation duration on desiccation tolerance in hybrid larch (*Larix x leptoeuropaea* Dengler). In Vitro Cell. Dev. Biol. 31: 15-20.

Lelu, M.-A., and Label, Phillipe. 1994. Changes in the levels of abscisic acid and its glucose ester conjugate during maturation of hybrid larch (*Larix x leptoeuropaea*) somatic embryos, in relation to germination and plantlet recovery. Physiol. Plant. 92: 53-60.

Li, Y.-h., Tremblay, F.M., and Sequin, A. 1994. Transient transformation of pollen and embryogenic tissues of white spruce (*Picea glauca* (Moench.) Voss) resulting from microprojectile bombardment. Plant Cell Rep. 13: 661-665.

Litvay, J.D., Johnson, M.A., Verma, D., Einspahr, D. and Weyrauch, K. 1981. Conifer suspension culture medium development using analytical data from developing seeds. Insti. Paper Chem. Tech. Paper Series No. 115, Appleton, WI.

Litz, R.E. , 1988, *In vitro* strategies for tropical fruit tree improvement. In: *Applications of Biotechnology in Forestry and Horticulture* (V. Dhawan, ed.), pp. 109-118, Plenum Press, New York.

Liu, C.-m., Xu, Z.-h., and Chua, N.-H. 1993. Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. The Plant Cell 5: 621-630.

Livingston, N.J., von Aderkas, P., Fuchs, E.E., and Reaney, M.J.T. 1992. Water relation parameters of embryogenic cultures and seedlings of larch. Plant Physiol. 100: 1304-1309.

Locy, R.D., 1988, Genetic variation in tissue culture as a consequence of the morphogenic process. In: *Applications of Biotechnology in Forestry and Horticulture* (V. Dhawan, ed.), pp. 165-180, Plenum Press, New York.

Loo, S.W., and Wang, F.H. 1943. The culture of young conifer embryos *in vitro*. Science 98(2555): 544-545.

Lu, C.-Y., and Thorpe, T.A. 1987. Somatic embryogenesis and plantlet regeneration in cultured immature embryos of *Picea glauca*. J. Plant Physiol. 128: 297-302.

Lulsdorf, M.M., Tautorus, T.E., Kikcio, S.I., Bethune, T.D., and Dunstan, D.I. 1993. Germination of encapsulated embryos of interior spruce (*Picea glauca engelmannii* complex) and black spruce (*Picea mariana* Mill.). Plant Cell Rep. 12: 385-389.

Maene, L.J. , 1988, Tissue culture of ornamental plants. In: *Applications of Biotechnology in Forestry and Horticulture* (V. Dhawan, ed.), pp. 231-244, Plenum Press, New York.

Masuda, K., Koda, Y., and Okazawa, Y. 1977. Callus formation and embryogenesis of endosperm tissue of parsley seed cultured on hormone-free medium. Physiol. Plant. 41: 135-138.

McLaughlin, J., and Karnosky, D.F. 1989. Controlling vitrification in *Larix decidua* via culture media manipulation. Can. J. For. Res. 19: 1334-1337.

Mikkola, L. 1969. Observations on interspecific sterility in *Picea*. Ann. Bot. Fenn. 6: 285-339.

Misra, S. 1994. Conifer zygotic embryogenesis, somatic embryogenesis, and seed germination: Biochemical and molecular advances. Seed Sci. Res. 4: 357-384.

Misra, S., Tranberger, T.J., Leal, I., and Chattai, M. 1993. Effect of abscisic acid, osmoticum, and desiccation on synthesis of storage proteins during the development of white spruce somatic embryos. Ann. Bot. 71: 11-22.

Misra, S., and Green, M.J. 1991. Developmental gene expression in conifer embryogenesis and germination. II. Crystalloid protein synthesis in the developing embryo and megagametophyte of white spruce (*Picea glauca* [Moench] Voss.). Plant Science 78: 61-71.

Mo. L.H., von Arnold. S., and Lagercrantz. U. 1989. Morphogenic and genetic stability in longterm embryogenic cultures and somatic embryos of Norway spruce (*Picea abies* {L.} Karst. Plant Cell Rep. 8: 375-378.

Mohammed. G.H., and Vidaver. W.E. 1990. The influence of acclimatization treatment and plantlet morphology on early greenhouse-performance of tissue-cultured Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco]. Plant Cell. Tiss. Org. Cult. 21: 111-117.

Mohammed. G.H., and Vidaver. W.E. 1991. Plantlet morphology and the regulation of net water loss in tissue-cultured Douglas-fir. Physiol. Plant. 83: 117-121.

Mullin. T.J., and Park. Y.S. 1992. Estimating genetic gains from alternative breeding strategies for clonal forestry. Can. J. For. Res. 22: 14-23.

Murashige. T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nagmani. R., and Bonga. J.M. 1985. Embryogenesis in subcultured callus of *Larix decidua*. Can. J. For. Res. 15: 1088-1091.

Nagmani. R., Becwar. M.R., and Wann. S.R. 1987. Single-cell origin and development of somatic embryos in *Picea abies* (L.) Karst. (Norway spruce) and *Picea glauca* (Moench) Voss (white spruce). Plant Cell Rep. 6: 157-159.

Narayanaswami. S. and Norstog. K. 1964.. Plant embryo culture. Bot. Rev. 30:587-628.

Nkongolo. K.K., and Klimaszewska. K. 1995. Cytological and molecular relationships between *Larix decidua*. *L. leptolepis* and *Larix x eurolepis*: identification of species-specific chromosomes and synchronization of mitotic cells. Theor. Appl. Genet. 90: 827-834.

Norgaard. J.V., and Krogstrup. P. 1995. Somatic embryogenesis in *Abies* spp. *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta. and R.J. Newton. Kluwer Academic Press. Dordrecht. pp. 341-356.

Onay . A., Jeffree, C.E., Yeoman, M.M. 1996. Plant regenration from encapsulated embryoids and an embryogenic mass of pistachio, *Pistacia vera* L. Plant Cell Rep. 15: 723-726.

Owens, J.N., and Molder, M. 1979a. Bud development in *Larix occidentalis*. I. Growth and development of vegetative long shoot and vegetative short shoot buds. Can. J. Bot. 57(7): 687-700.

Owens, J.N., and Molder, M. 1979b. Bud development in *Larix occidentalis* . II. Cone differentiation and early development. Can. J. Bot. 57: 1557-1572.

Owens, J.N. and Molder, M. 1979c. Sexual reproduction of *Larix occidentalis*. Can. J. Bot. 57: 2673-2690.

Pachauri, R.K., and Dhawan, V., 1988, Cost analysis of micropropagated plants. In: *Applications of Biotechnology in Forestry and Horticulture* (V. Dhawan, ed.), pp. 275-282, Plenum Press, New York.

Park, Y.S., and Bonga, J.M. 1992, Conifer micropropagation: its function in tree improvement programs. In: Micropropagation of Woody Plants, edited by: M.R. Ahuja. Kluwer academic Publishers, Netherlands. pp. 457-470.

Park, Y.S., and Fowler, D.P. 1987. Genetic variances among clonally propagated populations of tamarack and the implications for clonal forestry. Can. J. For. Res. 17: 1175-1180.

Park, Y.S., Pond, S.E., and Bonga, J.M. 1993. Initiation of somatic embryogensis in white spruce (*Picea glauca*): genetic control, culture treatment effects, and implications for tree breeding. Theor. Appl. Gent. 86: 427-436.

Park, Y.S., Pond, S.E., Bonga, J.M. 1994. Somatic embryogenesis in white spruce (*Picea glauca*): genetic control in somatic embryos exposed to storage, maturation treatments, germination, and cryopreservation. Theor. Appl. Genet. 89: 742-750.

Pennell, R.I., Janniche, L., Scofield, G.N., Booij, H., de Vries, S., and Roberts, K. 1992. Identification of a transitional cell state in the developmental pathway to carrot somatic embryogenesis. J. Cell Biol. 119: 1371-1380.

Pitel, J.A., Yoo, B.Y., Klimaszewska, K. and Charest, P.J. 1992. Changes in enzyme activity and protein patterns during the maturation phase of somatic embryogenesis in hybrid larch (*Larix x eurolepis*). Can. J. For. Res. 22: 553-560.

Pius, J., George, L., Eapen, S., and Rao, P.S. 1993. Enhanced plant regeneration in pearl millet (*Pennisetum americanum*) by ethylene inhibitors and cefotaxime. Plant Cell, Tiss. Org., Cult. 32: 91-96.

Raghavan, V. 1976. Experimental embryogenesis in vascular plants. Academic Press, London.

Raghavan, V. 1986. Embryogenesis in angiosperms: a developmental and experimental study. Cambridge University Press, New York.

Raghavan, V., and Torrey, J.G. 1963. Growth and morphogenesis of globular and older embryos of *Capsella* in culture. Amer. J. Bot. 50(6): 540-551.

Ranga Swarmy, N.S. 1958. Culture of nucellar tissue of *Citrus in vitro*. Experientia 14: 111-112.

Roberts, D.R. 1991. Abscisic acid and mannitol promote early development, maturation and storage protein accumulation in somatic embryos of interior spruce. Physiol. Plant. 83: 247-254.

Roberts, D.R., Flinn, B.S., Webb, D.T., Webster, F.B., and Sutton, B.C.S. 1989. Characterization of immature embryos of interior spruce by SDS-PAGE and microscopy in relation to their competence for somatic embryogenesis. Plant Cell Rep. 8: 285-288.

Roberts, D.R., Flinn, B.S., Webb, D.T., Webster, F.B., and Sutton, B.C.S. 1990a. Abscisic acid and indole-3-butryic acid regulation of amturation and accumulation of storage proteins in somatic embryos of interior spruce. Physiol. Plant. 78: 355-360.

Roberts, D.R., Lazaroff, W.R., and Webster, F.B. 1991. Interaction between maturation and high relative humidity treatments and their effects on germination of Sitka spruce somatic embryos. J. Plant Physiol. 138: 1-6.

Roberts, D.R., Sutton, B.C.S., Flinn, B.S. 1990b. Synchronous and high frequency germination of interior spruce somatic embryos following partial drying at high relative humidity. Can. J. Bot. 68: 1086-1090.

Rodriguez, A.P.M., and Wetzstein, H.Y. 1994. The effect of auxin type and concentration on pecan (*Carya illinoinensis*) somatic embryo morphology and subsequent conversion into plants. Plant Cell Rep. 13: 607-611.

Rohr, R., von Aderkas, P., and Bonga, J.M. 1989. Ultrastructural changes in haploid embryoids of *Larix decidua* during early embryogenesis. Amer. J. Bot. 76(10): 1460-1467.

Roland, J.C., and Vian, B. , 1991, General reparation and staining of thin sections, In: Electron Microscopy of Plant Cells, Ed.: Hall, J.L., and Hawes, C. Academic Press Ltd., San Diego, pp. 1-66.

Sacher, J.A. 1956. Observations on pine embryos grown *in vitro*. Bot. Gaz. 117: 206-214.

Salajova, T., Salaj, J., Jasik, J., and Kormutak, A. 1995. Somatic embryogenesis in *Pinus nigra* Arn. *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta, and R.J. Newton. Kluwer Academic Press, Dordrecht. pp. 207-220.

Schopf, J.M. 1943. The embryology of *Larix*. Illinois Biological Monographs 19: 1-97.

Schuller, A., Reuther, G., and Geier, T. 1989. Somatic embryogenesis from seed explants of *Abies alba*. Plant Cell Tiss. Org. Cult. 17: 53-58.

Schuller, A., and Reuther, G. 1993. Response of *Abies alba* embryonal suspensor mass to various carbohydrate treatments. Plant Cell Rep. 12: 199-202.

Sebanek, J., Sladky, Z., Prochazka, S., Kutacek, M., Luxova, M., and Erdelska, O. Developments in Crop Science 18. Experimental morphogenesis and integration of plants. *Edited by* M. Penka. Elsevier Press, Amsterdam, 1991.

Singh, H. , 1978. Embryology of gymnosperms, Encyclopedia of plant anatomy, Vol. 10. Germany, Gebruder Borntraeger, Berlin.

Smith, D.L., and Krikorian, A.D. 1990. Low external pH replaces 2,4-D in maintaining and multiplying 2,4-D-initiated embryogenic cells of carrot. Physiol. Plant. 80: 329-336.

Snedecor, G.W., and Cochran, W.G. , 1980, Statistical Methods, Iowa State Press, Iowa.

Srivastava, H.K., 1988, Genetic engineering of tree species. In: *Applications of Biotechnology in Forestry and Horticulture* (V. Dhawan, ed.), pp. 323-330, Plenum Press, New York.

Staxen, I., Klimaszewska, K., and Bornman, C.H. 1994. Mictotubular organization in protoplasts and cells of somatic embryo-regenerating and non-regenerating cultures of *Larix*. Physiol. Plant. 91: 680-686.

Steeves, T.A., and Sussex, I.M. Patterns in plant development. Cambridge University Press, Cambridge1989.

Sterling, C. 1949. Preliminary attempts in larch embryo culture. Bot. Gaz. 111: 90-94

Stone, E.C., and Duffield, J.W. 1950. Hybrids of sugar pine by embryo culture. J. Forestry 48: 200-201.

Strickland, S.G., Nichol, J.W., McCall, C.M., and Stuart, D.A. 1987. Effect of carbohydrate source on alfalfa somatic embryogenesis. Plant Science 48: 113-121.

Tautorus, T.E., Attree, S.M., Fowke, L.C., and Dunstan, D.I. 1990a. Somatic embryogenesis from immature and mature zygotic embryos, and embryo regeneration from protoplasts in black spruce (*Picea mariana* Mill.). Plant Science 67: 115-124.

Tautorus, T.E., Fowke, L.C., and Dunstan, D.I. 1990b. Comparative studies of protoplast development in jack pine (*Picea banksiana*). Can. J. Bot. 68: 1774-1779.

Tautorus, T.E., Fowke, L.C., and Dunstan, D.I. 1991. Somatic embryogenesis in conifers. Can. J. Bot. 69: 1873-1899.

Tautorus, T.E., Wang, H., Fowke, L.C., and Dunstan, D.I. 1992. Microtubule pattern and the occurence of pre-prophase bands in embryogenic cultures of black spruce (*Picea mariana* Mill.) and non-embryogenic cultures of jack pine (*Pinus banksiana* Lamb.). Plant Cell Rep. 11: 419-423.

Thompson, R.G., and von Aderkas, P. 1992. Somatic embryogenesis and plant regeneration from immature embryos of western larch. Plant Cell Rep. 11: 379-385.

Thorpe, T. 1988. *In vitro* somatic embryogenesis. ISI Atlas of Science: Animal and Plant Sciences, pp. 81-88.

Timmis, R., and Trotter, P.C., 1988, Biological and economic feasibility of genetically engineered trees for lignin properties and carbon allocation. In: *Applications of Biotechnology in Forestry and Horticulture* (V. Dhawan, ed.), pp. 349-368, Plenum Press, New York.

Toonen, M.A.J., Hendriks, T., Schmidt, E.D.L., Verhoeven, H.A., van Kammen, A., and de Vries, S.C. 1994. Description of soamtic-embryo-forming single cells in carrot suspension cultures employing video cell tracking. Planta 194: 565-572.

Tremblay, F.M. 1990. Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. Can. J. Bot. 68: 236-242.

van Overbeek, J., Conklin, M.E., and Blakeslee, A.F. 1942. Cultivation *in vitro* of small *Datura* embryos. Am. J. Bot. 29: 472-477.

Verhagen, S. A., and Wann, S.R. 1989. Norway spruce somatic embryogenesis: high frequency initiation from light-cultured mature embryos. Plant Cell, Tiss. Org. Cult. 16: 103-111.

von Aderkas, P. 1992. Embryogenesis from protoplasts of haploid European larch. Can. J. For. Res. 22: 397-402.

von Aderkas, P., and Anderson, P. 1993. Aneuploidy and polyploidization in haploid tissue cultures of *Larix decidua*. Physiol. Plant. 88: 73-77.

von Aderkas, P., and Bonga, J.M. 1988. Formation of haploid embryoids of *Larix decidua* : early embryogenesis. Amer. J. Bot. 75(5): 690-700.

von Aderkas, P., and Bonga, J.M. 1993. Plants from haploid tissue culture of *Larix decidua*. Theor. Appl. Genet. 87: 225-228.

von Aderkas, P., Bonga, J., Klimaszewska, K., and Owens, J. 1991. Comparison of larch embryogeny *in vivo* and *in vitro*. *In* Woody Plant Biotechnology. *Edited by* M.R. Ahuja. Plenum Press, New York. pp 139-155.

von Aderkas, P., Klimaszewska, K., and Bonga, J.M. 1990. Diploid and haploid embryogenesis in *Larix leptolepis*, *L. decidua*, and their recipricol hybrids. Can. J. For. Res. 20: 9-14.

von Aderkas, P., Pattanavibool, R., and Colangeli, A. Stable aneuploidy in embryogenic tissue culture of a conifer, *Larix decidua* Mill. in press.

von Aderkas, P., Thompson, R.G., Zaki, M., and Benkrima, L. 1995. Somatic embryogenesis of western larch (*Larix occidentalis*). *In* Biotechnology in Agriculture and Forestry Vol. 30, Somatic embryogensis and synthetic seed. *Edited by* Y.P.S. Bajaj. Springer-Verlag, Berlin. pp.

von Arnold, S., Egertsdotter, U., Ekberg, I., Gupta, P., Mo, H., and Norgaard, J. 1995. Somatic embryogenesis in Norway spruce (*Picea abies*). *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta, and R.J. Newton. Kluwer Academic Press, Dordrecht. pp. 17-36.

von Arnold, S., and Hakman, I. 1985. Effect of sucrose on initiation of embryogenic callus cultures from mature zygotic embryos of *Picea abies* (L.) Karst. (Norway spruce). J. Plant Physiol. 122: 261-265.

von Arnold, S., and Hakman, I. 1988. Regulation of somatic embryo development in *Picea abies* by abscissic acid (ABA). J. Plant Physiol. 132: 164-169.

von Arnold, S. and Woodward, S. 1988. Organogenesis and embryogenesis in mature zygotic embryos of *Picea sitchensis* Tree Physiology 4: 291-300.

Vujicic, R., and Budimir, S. 1995. Somatic embryogenesis and plant regeneration in *Picea omorika*. *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta, and R.J. Newton. Kluwer Academic Press, Dordrecht. pp. 81-98.

Webb, D.T., Webster, F., Flinn, B.S., Roberts, D.R., and Ellis, D.D. 1989. Factors influencing the induction of embryogenic and caulogenic callus from embryos of *Picea glauca* and *P. engelmannii*. Can. J. For. Res. 19: 1303-1308.

Webster, F.B., Roberts, D.R., McInnis, S.M., and Sutton, B.C.S. 1990. Propagation of interior spruce by somatic embryogenesis. Can. J. For. Res. 20: 1759-1765.

Westcott, R.J. 1994. Production of embryogenic callus from nonembryogenic explants of Norway spruce *Picea abies* (L.) Karst. Plant Cell Reports 14: 47-49.

Williams, E.G., and Maheswaran, G. 1986. Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Ann. Bot. 57: 443-462.

Wilson, S.M., and Thorpe, T.A. 1995. Somatic embryogenesis in *Picea glauca* (white spruce), *P. engelmannii* (Engelman spruce) and *P. glauca engelmannii* complex (interior spruce). *In* Somatic embryogenesis in woody plants. *Edited by* S. M. Jain, P.K. Gupta, and R.J. Newton. Kluwer Academic Press, Dordrecht. pp. 37-54.

Wyman, J., Brassard, N., Flipo, D., Laliberté, S. 1992. Ploidy level stability of callus tissue, axillary and adventitious shoots of *Larix x eurolepis* Henry regenerated *in vitro*. Plant Science 85: 189-196.

Xu, N., Coulter, K.M., and Bewley, J.D. 1990. Abscisic acid and osmoticum prevent germination of developing alfalfa embryos, but only osmoticum maintains the synthesis of developmental proteins. Planta 182: 382-390.

Zimmerman, J.L. 1993. Somatic embryogenesis: a model for early plant development in higher plants. The Plant Cell 5: 1411-1423.

Appendix A: Experiments in androgenesis of *Chamaecyparis nootkatensis*

1 Introduction

1.1 Induction of haploid plants

The induction of haploid plants from gametophytic tissue is a significant advancement in tissue culture for the study of plant morphogenesis because it provides a chance to study plant development under the control of a minimized genetic complement free of dominant and recessive genes. The disruption of normal pollen grain development from pollen tube production into haploid plant induction provides an opportunity to study determination and competence in conifer pollen grains. In addition, doubling the chromosome number of haploid tissue would result in homozygous plants useful for breeding, especially in species not amenable to inbreeding such as conifers. All recessive genes are revealed in a haploid cell, and cryptic genetic charateristics are unmasked, making selection for desirable traits easier. In addition to its usefulness in conventional breeding, androgenesis would provide embryogenic haploid cells for somatic hybridization.

Haploid cultures can be induced in cereals (Lorz *et al.* 1988), gymnosperms (Rohr 1987), and woody angiosperms (Chen *et al.* 1988). Since the first formation of haploid embryos in *Datura innoxia* (Guha and Maheshwari 1966), haploid plantlets have been induced in over 247 species of angiosperms. Haploid monocots and woody angiosperms plantlets have been induced from male and/or female gametophytic tissue. The first doubled-haploid variety of *Triticum aestivum* was licensed in 1985 (De Buyser *et al.* 1987). If successful, this project would have been the first instance of androgenesis in a conifer species.

This appendix outlines the work done on androgenesis of yellow cypress (the induction of haploid plantlets from immature microspores). One of the biggest problems in androgenesis is the failure to induce division in microspores which have developed past the tetrad or uninucleate stage of development. Indirect androgenesis involves a three step procedure: dedifferentiation, differentiation and maturation. Direct androgenesis is preferable since it bypasses the genetically unstable callus stage, but since the genetic component of each microspore is the result of recombination during meiosis, direct androgenesis would result in plants of untested genotypes different from that of the parent plant. An established androgenesis system would have created novel genotypes of yellow cypress.

2. Gibberellic sprays to induce cone production

There is a limit to the effectiveness of gibberellic acid applications . The flowering period of yellow cypress can only be extended by a few months. The potted trees can only be induced if they are actively growing after their dormancy requirement is fulfilled.

3. External cone appearance as an indicator of microspore development

Dissection of microstrobili at various stages of development showed that the outside appearance of the pollen cone is a good indication of the inside stage of development of the microspores. When the pollen cones are almost full size but the microsporangia are not exposed. the microspores are at the tetrad stage.

4. Sterilization and media trials

The first attempt to induce division of haploid microspores was begun by culturing entire microstrobili in an approach similar to anther culture. Originally the microstrobili were sterilized by dipping them in ethanol and then flaming them but this caused extensive damage to the tissue. Subsequent experiments in sterilization showed that minimal sterilization treatments were adequate. Sterilization treatments from that point on were 10 minutes in 10% Javex. Contamination rates were low throughout all subsequent experiments on yellow cypress even on material taken from the field.

The microsporangia with subtending microsporophylls were cultured on six media - full strength 1) Litvays, 2) Schenk and Hildebrandt and 3) Whites media: and half strength 4) Litvays, 5) Schenk and Hildebrandt and 6) Whites media . The Petri plates were either wrapped in tin foil or exposed to the light in a growth chamber. By arranging the plates on different shelves in the growth chamber the plates were exposed to different temperatures.

Results: Callus arose from most of the microsporophylls and microsporangia but did not appear to arise from the microspores. All chromosome squashes showed diploid counts.

5. Centrifugation and storage trials

Centrifugation of the microstrobili did not result in proliferation of the microspores. Microstrobili kept in the fridge for up to 3 months produced diploid callus when cultured. The best media for diploid callus culture appeared to be 1/2 strength Schenk and Hildebrandt media; therefore, this was the medium used in subsequent experiments. No haploid callus was recovered whether the microstrobili were from trees grown in the greenhouse, the field or the growth chamber.

6. Plant growth regulators

Experiments using different combinations of the hormones 2,4-D, BAP, NAA, kinetin and GA3, to induce androgenesis did not result in cell division of the microspores. The hormone combinations did affect diploid callus growth. In this set of experiments, the culture of individual microsporangia was begun in order to remove the microsporophylls as a source of diploid callus. Callus proliferated easily from the microsporangia but though it appeared that the callus came bursting out of the microsporangia, all the callus tested was diploid.

The experiment using different hormone treatments was repeated but instead of placing isolated microsporangia on the solid media the microsporangia were crushed by forceps over the Petri plate, allowing the microspores to fall onto the medium Though there was some callus originating from this, chromosome squashes showed diploid chromosome numbers. Possibly the callus arose from tapetal cells or from fragments of the microsporagia that fell onto the plate.

7. Culture of isolated microspores

In order to remove all sources of diploid callus, experiments were begun using microspores isolated according to the technique developed at the University of Guelph for isolating *Brassica* microspores. The microstrobili are ground in a mortar and pestle in chilled liquid medium then filtered through 75 µm and 53 µm Nitex nylon meshes. The liquid is then centrifuged and washed with chilled medium four times. The microspores were kept at one of three temperatures (4 °C, 25 °C, 35 °C) after aliquoting into liquid medium (1/2 Schenck and Hildebrandt media, 319 µM sucrose, 6.78 µM 2,4-D and 3.48 µM kinetin) in small Petri dishes (35 x 10 mm). After one week, viability of the cells kept at the various temperatures was 24 % at 25 °C, 62% at 35 °C, 77% at 4 °C. After two weeks, the microspores kept at 4 °C were larger and the starch grains in the microspores were all to one side of the microspore rather than concentrated in the centre of the cell.

8. Media and temperature shock trials

Genotypes of yellow cypress from both Mount Newton seed orchard and Canadian Pacific Forest Products seed orchard were used in a 3 x 4 factorial experiment with three temperature treatments (4 C, 25 C, 35 C) and four hormone treatments : 1) 0 µM 2,4- D + 0 µM kinetin, 2) 0 µM 2,4-D + 4.65 µM kinetin, 3) 9.05 µM 2,4-D + 0 µM kinetin, 4) 9.05 µM 2,4-D + 4.65 µM kinetin. Isolated microspores were added to the media to a final density of 75,000 - 100000 microspores/ ml. Four ml of the media plus microspores was aliquoted into each cell of a 24 well cell plate (Falcon). Contamination in this experiment was higher than in experiments using intact microsporangia on solid media. Intact somatic cells could occasionally be seen in cultures. Some callus was recovered from treatments cultured in small Petri plates rather than the cell plates, indicating that aeration might be important for cell survival and division. Not enough callus was recovered for chromosome counting. In another experiment the pH of the media (containing 319 µM sucrose, 6.78 µM 2,4-D and 3.48 µM kinetin) was modified from pH 5.7 to pH 4.8, pH 5.8, pH 6.25 and pH 7.21 but none of these treatments induced division.

9. Liquid medium

In order to increase aeration to the cells, isolated microspores were placed in Erlenmyer flasks on a shaker. Mannitol was added to the medium to improve buoyancy . The medium contained 319 µM sucrose, 6.78 µM 2,4-D and 3.48 µM kinetin . The microspores did not divide.

10. Lysozyme trials

In other experiments, lysozyme at varying concentrations (0 mg/l, 0.5 mg/l, 1 mg/l, 2 mg/l) was added to isolated microspores in 1/2 strength Schenck and Hildebrandt medium containing 319 µM sucrose, 6.78 µM 2,4-D and 3.48 µM kinetin. The cell plate was kept on a shaker at 22 °C. Though lysozyme stimulates division in wheat microspores it did not induce division of yellow cypress microspores. The antibiotics cefotaxime and pyopen were added in a 2 x 2 x 2 factorial experiment The two antibiotics were added separately at two concentrations (0 mg/l and 500 mg/l) to media which either had no growth hormones added or had 6.78 µM 2,4-D and 3.48 µM kinetin The cell plates were kept at 4 °C for one week then kept at 22 °C. There was no division of the microspores.

11. Cryopreservation

Cryopreservation of immature microspores was investigated. Isolated microspores were suspended in liquid medium (1/2 strength Schenck and Hildebrandt, 319 µM sucrose, 6.78 µM 2,4-D and 3.48 µM kinetin, 3 % mannitol) in an Erlenmyer flask on a shaker at 25 °C. After two days, on the shaker, the flask was placed on ice before adding 0.603 µl DMSO and 0.626 µl glycerol three times over 30 minutes. The microspores were then pipetted into cryovials and left on dry ice for 24 hours. The cryovials were then placed in a cryofreezer at 80 °C. After 8 days, the microspores were quickly thawed in a 40 °C water bath and immediately washed with fresh liquid medium. The viability of the microspores at this point was 17 %. The microspores were then either plated onto solid medium or resuspended in fresh media. Six weeks later the viability of the microspores in the liquid medium was 10%. None of the microspores divided whether they were on solid or in liquid media.

12. Androgenesis trials with western hemlock

Experiments on androgenesis of western hemlock from the Lost Lake Seed Orchard and *Taxus brevifolia* from trees located on campus commenced. Cuttings from Western Hemlock were stored in the fridge or given heat treatments (33 °C) for 24 hours but this pretreatment of the microsporangia did not result in division of the microspores. Media containing different sugars (dextrose, sucrose and glucose) at different concentrations did not induce microspores to divide. Liquid cultures with different support systems (folded Kimwipe, either alone or with whatman filter paper or cellulose acetate paper) did not induce division in microspores. The use of alternative gelling agents such as rice starch, wheat starch and corn starch did not result in division of microspores.

Callus arose in some of the treatments but did not appear to come from haploid tissue nor did the callus proliferate enough to enable chromosome testing

Androgenesis work on western hemlock continued with MB 13, a clone kept in the clonal orchard at the Provincial Research Station at Mesachie Lake. A factorial experiment with hormone treatments, temperature treatments and sucrose concentration treatments was conducted using isolated MB13 microspores. The hormone treatments were: 1) 0 µM 2,4-D + 0 µM kinetin, 2) 0 µM 2,4-D + 4.65 µM kinetin, 3) 9.05 µM 2,4-D + 0 µM kinetin, 4) 9.05 µM 2,4-D + 4.65 µM kinetin , 5) 0 µM NAA + 0 µM BAP, 6) 0 µM NAA + 4.44 µM BAP, 7) 10.74 µM NAA + 0 µM BAP, 8) 10.74 µM NAA + 4.44 µM BAP. The temperature treatments were 20 °C, 4 °C and 35 °C. The sucrose concentrations were 58 µM, 232 µM and 435 µM . No division occured in any treatment. Microspores isolated at the tetrad stage degenerated without being released from the microspore mother cell wall. Microspores isolated at the immature microspore stage continued development and produced pollen tubes in most treatments. Both fungal contamination and bacterial contamination were a problem.

13. Conclusions

Though one anueploid count was obtained (figure 53), no haploid tissue was established in culture from microspores of either yellow cypress or western hemlock. The reasons why conifer microspores are are so recalcitrant to androgenesis is not known. The success with gynogenesis in some conifer species (Rohr 1987) indicates that isolation of haploid cells from ovules is more likely to produce haploid cultures than miocrospores, and gynogenesis should be the focus of all further research into conifer haploid culture.

Figure 53: Squash of callus cells of *Chamaecyparis nootkatensis* showing a haploid chromosome set (100 x).


53

REFERENCES

Chen, Z., 1987, Induction of androgenesis in hardwood trees. In: JM Bonga and DJ Durzan (eds)Cell and Tissue Culture in Forestry 1897, Martinus Nijhoff Publishers, Dordrecht, 230-246.

De Buyser J, Henry Y, Lonnet P, Hertzog R, Hespel A, 1987. 'Florin': A doubled haploid wheat variety developed by the anther culture method. Plant Breeding 98:53-56.

Guha S, Maheshwari SC. 1966. Cell division and differentiation of embryos in the pollen grains of *Datura* in vitro. Nature 212: 97-8.

Lorz H, Gobel E, Brown P. 1988. Advances in tissue culture and progress towards genetic transformation of cereals. Plant Breeding 100:1-25.

Rohr R. Haploids (Gymnosperms) In: JM Bonga and DJ Durzan (eds)Cell and Tissue Culture in Forestry 1897, Martinus Nijhoff Publishers, Dordrecht, 230-246.

Schenk, R.U., and Hildebrandt, A.C. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50: 199-204.

White, P.R., 1954, The cultivation of animal and plant cells, 2nd edition, Ronald Press, New York.

Appendix B: Transient chimeric gene expression in pollen of five conifer species following microparticle bombardment

Irene Hay[1], Denis Lachance[2], Patrick von Aderkas[1], and Pierre J. Charest[2]

1- Dept. of Biology
University of Victoria
Victoria, BC, Canada
V8W 2Y2

2-Molecular Genetics and Tissue Culture Group
Petawawa National Forestry Institute
Natural Resources Canada
Chalk River, Ontario, K0J 1J0
Tel: 613-589-2880
Fax: 613-589-2275

Can. J. For. Res. 24(12): 2417-2423.

Note: This paper resulted from a project pursued at the end of the androgenesis work outlined in Appendix A. Though it has little to do with the rest of this thesis, the genetic transformation of conifer pollen would be used in conjunction with *in vitro* fertilization methods, likely followed by somatic embryogenesis. The difficulty in efficiently transforming pollen and using it to fertilized ovules *in vitro*, would make the efficient recovery of transformed, normal, mature somatic embryos of considerable economic importance. The greatest potential of somatic embryogenic systems lies in the recovery of transformed plants, and in this light, the careful analysis of the development and manipulation of conifer somatic embryos reported in this thesis is necessary preparation for the use of *in vitro* fertilization to gain transformed somatic embryos.

Abstract

Mature pollen of lodgepole pine (*Pinus concorta*), yellow cypress (*Chamaecyparis nootkatensis*), western hemlock (*Tsuga heterophylla*), jack pine (*Pinus banksiana*), and black spruce (*Picea mariana*) was bombarded with gold particles coated with four different plasmid constructions, pRT99GUS, pBM113Kp, pAct1-D, and pGA984, using the Biolistic PDS-1000/He device. A protocol was devised for efficient gene transfer and gene expression assay in pollen. False positive results for expression of the b-glucuronidase gene (GUS) assayed with the substrate X-glucuronide were observed with pollen of yellow cypress, western hemlock, and lodgepole pine. The highest levels of transient GUS gene expression were obtained with plasmid pBM113Kp which carried the b-glucuronidase gene under the control of the wheat ABA-inducible EM promoter. The plasmids pRT99GUS (35S promoter) and pAct1-D (rice actin promoter) yielded similar intermediate levels of transient GUS gene expression. The pollen-specific promoter of the a-tubulin gene from *Arabidopsis thaliana* (pGA984) yielded the lowest levels of gene expression in pollen. Of the four species, yellow cypress showed the lowest levels of transient GUS gene expression and black spruce yielded the highest levels. The neomycin phosphotransferase II (NPT II) gene was also tested as a reporter gene for pollen transformation and was easily assayed via ELISA. The NPT II::GUS fusion protein was detected at a lower level than the non-fusion NPT II protein when under the control of the same 35S promoter. The method devised here could be used for the study of tissue-specific gene expression in conifer pollen.

Resumé

Du pollen mature de pin tordu (*Pinus concorta*), de cyprès jaune (*Chamaecyparis nootkatensis*), de pruche occidentale (*Tsuga heterophylla*), de pin gris (*Pinus banksiana*) et d'épinette noire (*Picea mariana*) a été bombardé avec des particules d'or enrobées avec quatre constructions plasmidiques différentes; pRT99GUS, pBM113Kp, pAct1-D et pGA984 en utilisant le canon à particule de type Biolistic PDS-1000/He. Un protocole a été établi pour le transfert efficace des gènes et l'évaluation de leurs niveaux d'expression dans du pollen. De fausses réactions positives pour le gène b-glucuronidase (GUS) testé avec le substrat X-glucuronide ont été observées pour le pollen de cyprès jaune, pruche occidentale et pin tordu. Les niveaux les plus élevés d'expression transitoire du gène GUS ont été obtenus avec le plasmide pBM113Kp qui porte le gène de la b-glucuronidase sous le contrôle du promoteur EM induisible à l'ABA du blé. Les plasmides pRT99GUS (promoteur 35S) et pAct1-D (promoteur d'un gène de l'actine du riz) ont donné des niveaux équivalents intermédiaires d'expression transitoire du gène GUS. Le promoteur spécifique au pollen du gène de l'a-tubuline d'Arabidospsis thaliana (pGA984) a donne les niveaux d'expression les moins éléves dans le pollen. En ce qui a trait aux espèces, le cyprès jaune a donné les niveaux les plus bas pour l'expression transitoire du gène GUS et l'épinette noire, les niveaux les plus hauts. Le gène rapporteur néomycine phosphotransférase (NPRT II) a été utilisé pour la transformation du pollen et son enzyme a été facilement détectée par ELISA. La protéine de fusion NPT II::GUS a été detectee à des niveaux inférieurs à la protein native NPT II lorsque soumises au contrôle du même promoteur 35S. La méthode établie ici pourrait être utilisée pour l'étude de gènes spécifiques à certains tissus dans le pollen de conifères.

1. Introduction

Microprojectile DNA delivery has been used to study pollen-specific gene expression in several angiosperm species such as *Lilium longiflorum* (Nishihara et al. 1993, van der Leede-Plegt et al. 1992), *Nicotiana tabacum* (Nishihara et al. 1993, Stoger et al. 1991, Twell et al. 1989), *Nicotiana rustica* and *Paeonia lactiflora* (Nishihara et al. 1993), *Nicotiana glutinosa* (van der Leede-Plegt et al. 1992), and *Zea mays* and *Tradescantia palludosa* (Hamilton et al. 1992). Consistency in gene expression patterns between transient gene expression using particle bombardment and using transgenic plants was observed (Twell 1992, Twell et al. 1991). Tissue-specific gene expression of genes such as the LAT52 gene of *Lycopersicon esculentum* and the chiA-PA2 gene of *Petunia hybrida* has been demonstrated (Twell et al. 1991, Stoger et al. 1991).

Microprojectile-mediated DNA delivery is a valuable tool, particularly because it circumvents the tedious and lengthy process otherwise necessary to obtain transgenic plants of tree species. For instance, microprojectile DNA delivery has been used in conifers to study transient gene expression in embryonal cell masses (Duchesne et al. 1993, Newton et al. 1992, Ellis et al. 1991, Charest et al. 1993a, Duchesne and Charest 1991, 1992), mature somatic embryos (Ellis et al. 1993, Charest et al. 1994a), cell suspensions (Campbell et al. 1992), zygotic embryos (Newton et al. 1992, Ellis et al. 1991), seedling tissues (Ellis et al. 1991, Charest et al. 1994a, Stomp et al. 1991, Goldfarb et al. 1991), developing wood (Loopstra et al. 1992), and flower parts (Charest et al. 1994a). Furthermore, this has been the only successful method to regenerate transgenic trees of white spruce *Picea glauca* (Ellis et al. 1993) and black spruce *Picea mariana* (Charest et al. 1993b, Charest et al. 1994b).

In this study, mature pollen of five conifer species, *Pinus contorta* Dougl. (lodgepole pine), *Chamaecyparis nootkatensis* (D.Don) Spach (yellow cypress), *Tsuga heterophylla* (Raf.) Sarg. (western hemlock), *Pinus banksiana* Lamb. (jack pine), and *Picea mariana* (Mill.) B.S.P. (black spruce) were bombarded with four plasmid vectors all having the GUS gene cassette as a reporter gene; (1) pRT99GUS carrying the GUS gene under the control of the 35S promoter of cauliflower mosaic virus, (2) pBM113Kp carrying the GUS gene with the wheat ABA inducible EM promoter, (3) pAct-1 carrying the GUS gene under the control of the rice actin promoter, and (4) pGA984 carrying the GUS gene under *Arabidopsis thaliana* a-tubulin promoter. This work aimed at developing an efficient protocol of gene delivery in conifer pollen.

2. Materials and Methods

2.1 Plant material

Stored mature pollen of lodgepole pine (*Pinus contorta* Dougl.), yellow cypress (*Chamaecyparis nootkatensis* (D. Don) Spach), and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) were obtained from the B.C. Ministry of Forests. The pollen was kept in sealed bags at 4°C until the experiments commenced within 2 months of the collection date. Mature pollen of jack pine (*Pinus banksiana* Lamb.) and black spruce (*Picea mariana* (Mill.) B.S.P.) was collected at the Petawawa National Forestry Institute and stored at 4°C in a dessicator and used within 3 months of the collection date. One pollen lot was used per species for all the experiments.

2.2 Microparticle Bombardment:

Pollen of each species was weighed out and suspended in sterile distilled water. This suspension was pipetted and vacuum filtered into a sintered glass filter leaving the pollen uniformly covering a nylon membrane (Biotrans, ICN, Ca). The size of the pollen lawn was of 3 cm. The membrane was then removed from the filter and placed in a 60 mm petri dish containing 20 mL Brewbakers' solution (1 g/L H_3BO_3, 3 g/L $Ca(NO_3).H_2O$, 2 g/L $MgSO_4.H_2O$, 1 g/L KNO_3) supplemented with 10% sucrose and solidified with 0.5% Gellan Gum (Chemical Dynamics Corp., NJ) for pollen of lodgepole pine, yellow cypress, and western hemlock and containing 20 mL of a solution of 5% sucrose and 0.6% agar for jack pine and black spruce (Copis 1990). The plates were bombarded i) within 30 min of plating, ii) after being kept at room temperature for 4 hours in the case of lodgepole pine, or iii) after being kept 4.5 hours at room temperature in the case of western hemlock. In the case of yellow cypress, the plates were bombarded 24 hours after plating and the plates were kept at room temperature. For jack pine and black spruce, the pollen on the nylon membrane was bombarded within 30 min of plating.

DNA transfers were carried out using the Biolistic particle-delivery system PDS-1000/He (DuPont, Wilmington, DE). Plasmid DNAs were precipitated onto gold particles (1.6 mm, Dupont) using the $CaCl_2$ spermidine method (Klein et al. 1987). A total of 1 mg of DNA was delivered per bombardment. Bombardment conditions were performed with a rupture disk pressure of 1100 psi, a gap distance of 1/4 inch, an internal nest settings of 6 mm, and a sample distance of 9.5 cm.

2.3 Plasmids

Plasmid pRT99GUS (6.7 kb) contains the b-glucuronidase (GUS) gene and the neomycin phosphotransferase II (NPT II) gene under the control of 35S promoter sequence of the cauliflower mosaic virus (CaMV; Topfer et al. 1988). Plasmid pBM113kp (5.4 kb) contains the GUS gene under the control of the abscisic acid-inducible promoter sequence of the EM (early methionine) protein gene of wheat (Marcotte et al. 1988, Litts et al. 1987). Plasmid pAct1-D (4.2 kb) contains the GUS gene under the control of the rice actin 1 gene (McElroy et al. 1990). Plasmid pGA984 (4.2 kb) contains the GUS gene under the control of the a-tubulin gene of *Arabidopsis thaliana* (Kim and An 1992, Carpenter et al. 1992, Kopczak et al. 1992, Snustad et al. 1992). Plasmids pBI426 and pBI404 carried a translational fusion between the NPT II gene and the GUS gene under the control of a double 35S promoter with an alfalfa translational enhancer and a single 35S promoter, respectively (Datla et al. 1991). The plasmids were isolated using the method of Tartof and Hobbs (1987).

The pollen of the five species were bombarded with DNAs of the four plasmids and control (gold particles precipitated with CaCl2 and spermidine but no DNA). For lodgepole pine, yellow cypress, and western hemlock the experiment was replicated three times with three repetitions per replicate and for black spruce and jack pine the experiment was replicated three times with five repetitions.

2.4 b-glucuronidase histochemical assays

After bombardment, the Petri dishes were sealed with parafilm and placed in the dark in a growth chamber at 24oC for 24 hours. The membrane with pollen was then transferred onto another Petri dish containing a piece of filter paper saturated with 600 mL modified GUS reaction buffer (20 mM Na_2HPO_4 + NaH_2PO_4 (pH 7.0), 10 mM $K_3[Fe(CN)_6]$, 10 mM $K_4Fe(CN)_6.3H_2O$, 10 mM Na_2EDTA, 0.1% Triton X-100, 1.5 mM X-Glucuronide; modified from Jefferson et al. 1987) and incubated at 37^oC in the dark overnight before examination. Assessment of GUS activity was based on the number of "expression units" (islands of cells showing blue colouration) per Petri dish.

2.5 b-glucuronidase fluorescent assay

Bombarded pollen was collected by washing off the membrane with 250 mL GUS extraction buffer modified from Jefferson (1987: 50 mM Na_2HPO_4 + NaH_2PO_4, 10 mM b-mercaptoethanol, 10 mM Na_2 EDTA, 0.1% Triton X-100) in an Eppendorf tube with a pinch of washed sand. The mixture was ground on ice and centrifuged at 10000 xg for 10 min. To 50 mL of supernatant was added 50 mL of substrate buffer (2 mM methyl umbelliferyl glucuronide) in an extraction buffer with 40% methanol. The final mixture was vortexed and incubated at 37°C. Forty mL samples were removed at 1 and 3 hours and the reaction was stopped with 0.2 M Na_2CO_3. For jack pine and black spruce bombarded with the vector pBM113Kp, the sampling was done at 10 and 30 min. The fluorescence of the sample in a final volume of 2 mL of 0.2 M Na_2CO_3 was determined using a TKO 100 fluorometer (Hoeffer Scientific Instruments, San Francisco, CA). The data were expressed in pmole 4-MU released/min/mg of extracted protein as determined by the Bradford assay (Bio-Rad, Mississauga, Ont).

2.7 Neomycin phosphotransferase assay:

The neomycin phosphotransferase enzyme was detected using an NPT II ELISA kit (5 Prime ---> 3 Prime Inc., CO). The pollen was washed off the membrane with 0.25 M Tris-HCl pH 7.8 containing 1.0 mM PMSF (phenylmethylsulfonylfluoride) and ground on ice. The extract was centrifuged at 10 000 xg at 4°C for 30 min. The protein content was determined in a manner similar to the fluorescent MUG assay: the sample was frozen at -70°C until assayed. The ELISA was conducted as recommended by the manufacturer. Data were recorded as ng of NPT II protein/ mg total extracted protein.

2.8 Statistical analysis:

The statistical analyses were performed using Sigma-Stat (Jandel Scientific, CA). A Kruskal-Wallis two-way anova was performed to analyze the main effects of the time of bombardement and the species with $P<0.001$. The pairwise comparisons were done using Dunn's test for non-paramatric data with $P<0.001$.

3. Results

To develop a reliable gene transfer protocol for conifer pollen, five species from four different genera (*Pinus contorta, Tsuga heterophylla, Chamaecyparis nootkatensis, Pinus banksiana,* and *Picea mariana*) were tested using the Biolistic PDS-1000/He delivery system with 4 different plasmid constructions (pRT99GUS, pBM113Kp, pAct1-D, pGA984) all carrying the GUS reporter gene. pRT99GUS carried the NPT II and GUS genes under the control of 35S promoters of CaMV, pBM113Kp carried the GUS gene under the control of the EM (early methioneine) promoter from wheat, pAct1-D carried the promoter for a rice actin gene and pGA984 carried the promoter for the pollen specific a-tubulin gene from *Arabidopsis thaliana*. The experiments were done in two groups. The first group included pollen of lodgepole pine, yellow cypress and western hemlock and aimed at devising a general protocol to be used for gene delivery. Evaluation of the effect of the time of bombardment after placing pollen on germination media and the effect of promoter sequence on levels of transient GUS gene expression using the histochemical X-glu assay was determined (Table 17) by calculating the number of GUS expression units per mg of pollen bombarded. The second group included pollen of jack pine and black spruce, and was conducted to reduce the variation of the method and to compare the histochemical X-glu and fluorescent MUG assay for evaluating levels of transient gene expression in pollen (Table 18). Gene delivery was demonstrated by transient GUS gene expression as evaluated by the histochemical assay in all species and vectors (Figure 54, Tables 17 and 18) except for yellow cypress, with the vector pGA984 where only background levels of X-glu were detected. X-glu reaction was observed with no specific distribution pattern in the germinated pollen grains (Figure 54). Bombarded control pollen yielded positive X-glu reaction at a low frequency for lodgepole pine, yellow cypress and western hemlock; however, this was not observed with jack pine and black spruce. The vector pGA984 was tested in embryonal cell masses of black spruce and yielded low levels of transient GUS gene expression (data not shown).

With lodgepole pine, yellow cypress, and western hemlock, differences between species and vectors were observed but the high level of variation did not yield many statistically

Table 17. Effect of time of bombardment and vector construction on transient expression of the GUS gene in conifer pollen (number of GUS expression unit / mg of bombarded pollen).

Species:	Lodgepole pine		Yellow cypress		Western hemlock	
Time of bombardment*	30 min	4 hrs	30 min	24 hrs	30 min	4.5 hrs
Vector						
Control	1.0±0.9f	1.0±1.1fg	2.7±3.1fg	1.1±1.1fg	0.3±0.3fg	0.2±0.2g
pRT99GUS	211.8±25.0a	144.8±62.0abce	50.7±25.6abcde	16.6±18.6defg	31.9±1.1bcdef	34.3±16.1abcdef
pBM113Kp	236.9±47.0a	193.6±22.1abc	170.0±214.0abcde	15.1±15.7dfg	35.3±9.1abcdef	96.8±29.0abcde
pAct1-D	193.0±23.8ab	215.0±47.4ab	52.8±52.0.8abcdef	27.0±42.7cdefg	109.3±15.1abcde	104.1±48.6abcde
pGA984	178.1±53.0ab	102.3±23.4abcde	2.1±2.6fg	1.4±2.1fg	15.2±5.4efg	14.1±9.9efg
Germination:	+	+	–	–	+	+

Average # of pollen grains/mg: 2967±523 (lodgepole pine), 12783±1973 (yellow cypress), 1700±198 (western hemlock)

Average size of pollen grain (diameter) 67.1μm x 50.5μm winged shape (lodgepole pine), 29.0μm round shape, (yellow cypress), 64.4μm round shape (western hemlock)

Data is given ± standard error of the mean. Data with the same letters are not significantly different (Dunn's test, $P < 0.001$).

For this experiment, 4.4 mg of pollen were used for each bombardment.

The time of bombardment indicates the approximate time after plating when the actual delivery of microparticles occurred (± 30 min).

The average number of pollen grains and the average size were calculated from 10 different samples per species.

Table 18. Comparison of histological and fluorescent assays for transient gene expression of the GUS gene in conifer pollen

Jack pine/Black spruce

	X-glu	MUG	X-glu	MUG
Control	0	—	0	—
pRT99GUS	316.2 ± 103.02	7.9 ± 4.1b	721.0 ± 186.63	4.8 ± 1.7b
pBM113Kp	591.4 ± 214.03	39.1 ± 11.6a	1085.6 ± 192.81	256.4 ± 63.9a
pAct1-D	516.4 ± 57.23	12.8 ± 7.3b	986.6 ± 224.43	8.6 ± 2.1b
pGA984	638.6 ± 183.83	4.8 ± 1.6b	819.8 ± 154.43	4.9 ± 1.6b

Average # of pollen grains/mg: 50 730 ± 4063 (Jack pine). 20 710 ± 2058 (Black spruce)

Average size of pollen grain (diameter): 44µm x 41µm winged shape (Jack pine). 70µm x 58µm winged shape (Black spruce)

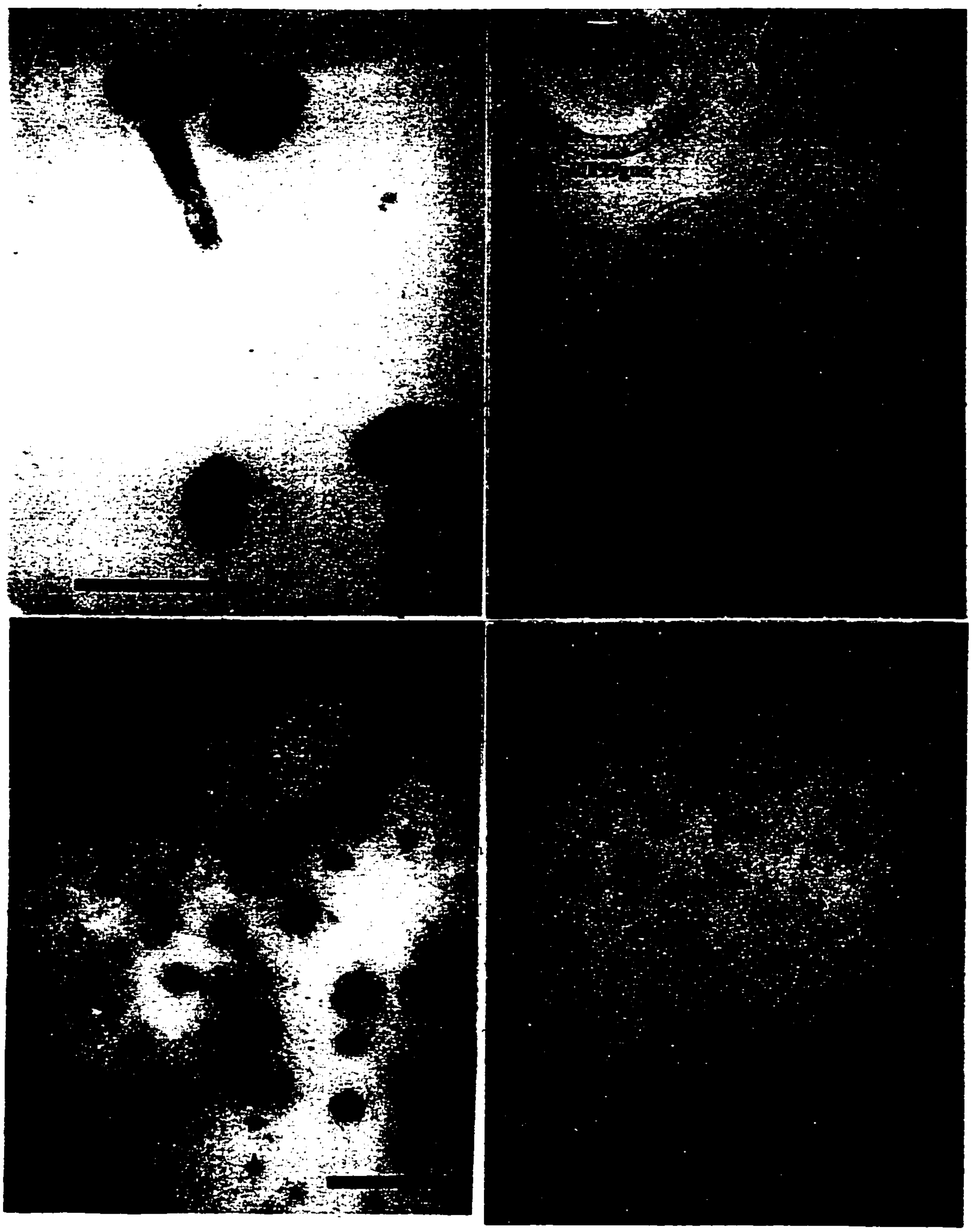
Germination: both jack pine and black spruce pollen germinated

MUG= nmoles of MU/min/mg protein

X-glu= number of expression units/mg of bombarded pollen. data ± standard error of the mean.

Data with the same upper case number for X-glu and the same upper case letter for MUG are not statistically different (Dunn's test with $P<0.001$). For the X-glu assay. 0.5 mg of pollen per bombardment was used and for the MUG assay. 1 mg of pollen per bombardment. The average number of pollen grains and the average size were calculated from 10 samples per species.

Figure 54: Detection of b-glucuronidase gene activity in black spruce pollen using the X-glu histochemical assay. A) Germinated and non-germinated pollen grains showing positive X-glu reaction, bar = 100 µm; B) Filter disks after bombardment with four different vectors (pRT99GUS, pBM113Kp, pAct1-D, pGA984) and without DNA, bar = 3.9 cm; C) and D) Difference in transient GUS expression yielded by vectors pBM113Kp (c) and pAct-1D (d) as observed with bombarded pollen on filter disk, bar = 0.23 cm.



significant differences among the treatments. There was no statistical difference as detected by the two-way ANOVA between the times of bombardment tested for the three species. Whether the pollen was shot within 30 minutes of being removed from cold storage or 4 hours later, for western hemlock and lodgepole pine (4.5 hours), there was no difference in the yield. The same was true for yellow cypress pollen bombarded either 30 minutes or 24 hrs after plating. The bombardment was delayed with this species because it is slow to germinate and in order to make sure that the pollen was metabollically active. In general, lodgepole pine yielded the highest numbers of GUS expression units per mg of pollen bombarded and the two other species gave lesser numbers. The lowest numbers of GUS expression units were obtained with the vector pGA984 which carries an a-tubulin pollen specific promoter. The other three plasmids gave similar numbers. Pollen germination was observed for lodgepole pine and western hemlock.

Pollen from species of the second group (jack pine and black spruce) yielded higher numbers of expression units per mg of pollen than pollen from the first group and the variation was reduced considerably by using less pollen per bombardement (0.5 mg versus 4.4 mg). This was determined by testing different amounts of pollen for bombardment (5, 1.7, 1, 0.7, 0.5 mg of pollen per bombardment) and visually evaluating which amount gave the most consistent results. The variation with the first set of experiments with lodgepole pine, yellow cypress, and western hemlock ranged from 3.5 to 125% (standard error of the mean/average). In the second set, including jack pine and black spruce, the variation ranged from 18 to 36%.

The apparent expression levels for the different plasmids varied depending on the assay used in Jack pine and black spruce. The levels of transient GUS expression as detected by the histochemical assay for vector pGA984 were as high as the expression from the other three vectors. In contrast, the fluorescent assay detected differences among the different vectors. pBM113Kp gave the highest levels (with significant difference) of transient gene expression followed by pAct1-D, pRT99GUS and pGA984. The results of the fluorescent assay were standardized for the amount of protein extracted.

The number of transformed pollen grains per mg of bombarded pollen varied with the species used (Tables 17 and 18); jack pine and black spruce had the highest numbers (50730 pollen grains/mg and 20710 pollen grains/mg respectively), followed by yellow cypress (12783), lodgepole pine (2967) and western hemlock (1700). The weights of the pollen grains did not correlate with their sizes indicating that the pollen from each species has its own specific density as shown in Tables 17 and 18.

The neomycin phosphotransferase gene was also evaluated as a potential screenable marker for gene expression studies with jack pine and black spruce pollen (Table 19).

NPT II expression, which was monitored using ELISA and enzyme detection, was lower in jack pine than in black spruce. The fusion protein between GUS and NPTII (pBI404, pBI426) was detected at lower levels than with the native NPTII gene (pRT99GUS). The double 35S promoter with the alfalfa mosaic virus translational enhancer (pBI426) yielded higher levels of transient gene expression than the single 35S promoter (pBI404).

Table 19. Neomycin phosphotransferase II as a reporter gene in conifer pollen

Vector	Promoter-Coding region	Jack Pine	Black spruce
pBI404*	35S-GUS::NPTII	562.8 ± 155.3	646.7 ± 294.2
pBI426*	35S-35S-AMVE-GUS::NPTII	1681.0 ± 345.3	2120.3 ± 634.9
pRT99GUS**	35S-NPTII	3070.0 ± 1150.0	4590.0 ± 1410.0

Data expressed in pg of NPT II enzyme/mg of total protein. *from 10 samples and **from 3 samples. Data given ± standard deviation.

4. Discussion

Microprojectile DNA delivery is an efficient method for gene transfer in conifer pollen as reflected by the high frequencies of X-glu expression units detected in all the five species tested. The transformation protocol was improved by using less pollen per bombardment, which yielded relatively low levels of variation (18-36%) that could be further reduced by improving the protein extraction procedure from pollen and the method to spread the pollen on the filter. However, the non-uniform spread of the bombarded gold particles will be a harder problem to resolve. A low frequency of false positive results using the X-glu histochemical assay were observed with lodgepole pine, yellow cypress, and western hemlock but not with jack pine and black spruce. This could be due to a different intracellular pH in the first three species that caused cleavage of the X-glu molecule. It did not really represent a problem because of the very low numbers observed (< 3 expression units/mg bombarded pollen). False positives have also been observed in the pollen of other species (Plegt and Bino 1989, Sood 1980, Gorska-Brylass 1965). While the X-glu histochemical assay seemed to indicate frequencies of gene delivery and not levels of gene expression in these experiments (Tables 17 and 18), the MUG fluorescent assay was quantitative and reflected levels of gene expression of the GUS gene (Table 18). This is consistent with one of our earlier studies where we found that the histochemical X-glu assay is quantitative only at low levels of gene expression (Charest et al. 1993a). The NPT II gene can also be used as a reporter gene for transient gene expression in conifer pollen and is assayed conveniently by ELISA.

With the first set of experiments that included yellow cypress, western hemlock, and lodgepole pine, the frequencies of gene delivery expressed as expression units per mg of bombarded pollen were similar with all vectors in all species except for vector pGA984 which did not yield significant levels of gene expression in yellow cypress and very low levels in western hemlock. The *A. thaliana* a-tubulin promoter is strongest in mature pollen and its strength decreases when the pollen germinates (Kim and An 1992). This could explain the low level of GUS activity obtained in the species with germinating pollen (logepole pine, western hemlock) but does not explain why there was no expression of GUS in yellow cypress where there was no pollen germination. One possibility is that the regulatory sequences of the *Arabidopsis* a-tubulin promoter were not recognized properly in yellow cypress. The lack of GUS gene expression is not likely due to metabolically inactive yellow cypress pollen because transient gene expression was obtained with the other vectors. The number of expression units found with the three species did not correlate to the average number of pollen grains bombarded. For instance, yellow cypress had c.a. 5 times more pollen grains per mg and did not yield higher levels of expression than the other two species. This indicates that other factors could be involved.

The results with jack pine and black spruce yielded more significant differences because of the use of the quantitative MUG fluorescent assay. The histochemical assay did not show any differences except for lower frequencies with pRT99GUS, which could probably be related to a lower level of gene expression with the 35S promoter as shown by the MUG fluorescent assay. Very low levels of expression were also observed with the fluorescent assay with the plasmid carrying the a-tubulin promoter (pGA984) in germinating pollen, which is in agreement with the decrease in expression of the GUS reporter gene found by others (Kim and An 1992). It cannot be concluded that the a-tubulin promoter gives tissue specificity in jack pine and black spruce because low levels of GUS gene expression were also detected after bombardment of black spruce embryonal cell masses (data not shown). The wheat ABA-inducible EM promoter yielded the highest levels of transient gene expression in pollen which seems consistent with the high levels of expression given by this promoter in several conifer species and tissues (Duchesne and Charest 1991, Charest *et al.* 1993a, Duchesne and Charest 1992) and could be useful for transgenic tree recovery. Higher numbers of expression units were obtained in black spruce and jack pine than with the 3 other species (lodgepole pine, yellow cypress and western hemlock). This could be due to the higher numbers of pollen grains per mg bombarded with black spruce and jack pine but as indicated earlier other factors could influence these results such as different physiological state or a difference in the penetration of the pollen wall.

With the NPT II ELISA assay, the fusion gene between NPT II and GUS genes yielded lower levels of detected enzyme than with the non-fused NPT II gene. This is similar to what was observed in embryonal cell mass bombardment of black spruce and other spruce and larch species (Charest *et al.* 1993a). The double 35S promoter with the alfalfa mosaic virus translational enhancer yielded higher levels of transient gene expression than the single 35S promoter which, again, is in agreement with previous results for embryogenic cell mass bombardment (Charest *et al.* 1993a).

With the species yielding the highest number of expression units per mg of pollen bombarded (black spruce), the frequency of gene delivery could be estimated at 8.7%, as calculated by the average of X-glu positive pollen grains per bombardment with all vectors combined (903.3) divided by the number of pollen grains per bombardment (10355). This frequency is relatively low if the goal is fertilization with transformed pollen; however, this could be increased by changing bombardment conditions.

Microparticle bombardment is an useful tool for tree molecular genetics because it permits the study of gene expression without the regeneration of transgenic trees. The present study showed that it can be used for gene delivery in pollen and that quantitative results of transient gene expression can be obtained. Moreover, gene delivery in pollen could open the way to the use of bombarded pollen for fertilization and subsequent recovery of transgenic seeds.

5. Acknowledgments

The authors are thankful to Dr. Joe Webber and Peter Copis for supplying the pollen, SCBC for financial assistance through the GREAT Award program, and Cathy Jones for her technical assistance. Acknowledgements are due to Drs. Bao Xue and Sharon Regan for critical reading of the manuscript. S. Moreau deserves thanks for the final formatting and correction of the document.

6. References

Alwen, A., R.M.B. Moreno, O. Vicente and E. Heberle-Bors. 1991. Plant endogenous b-glucuronidase activity: How to avoid interference with the use of the E. coli b-glucuronidase as a reporter gene in transgenic plants. Transgenic Res. 1: 63-70.

Campbell, M.A., C.S. Kinlaw and D.B. Neale. 1992. Expression of luciferase and b-glucuronidase in Pinus radiata suspension cells using electroporation and particle bombardment. Can. J. For. Res. 22:2014-2018.

Carpenter, J.L., S.E. Ploense, D.P. Snustad and C.D. Silflow. 1992. Preferential expression of an a-tubulin gene of Arabidopsis in pollen. Plant Cell 4: 557-571.

Charest, P.J., N. Calero, D. Lachance, M. Mitsumune and B.Y. Yoo. 1994a. The use of microprojectile DNA delivery to bypass the long life cycle of tree species in gene expression studies. Curr. Topics Bot. Res. (in press).

Charest, P.J., N. Calero, D. Lachance, R.S.S. Datla, L.C. Duchesne and E.W.T. Tsang. 1993a. Microprojectile-DNA delivery in conifer species: factors affecting assessment of transient gene expression using the b-glucuronidase reporter gene. Plant Cell Rep. 12:189-193.

Charest,P.J., D. Lachance, Y. Devantier and K. Klimaszewska. 1994b. Transient gene expression and stable genetic transformation in Picea mariana (black spruce) and Larix laricina (tamarack). Rev. Invest. Agraria: Serie Syst. Rec. Forest. (in press).

Charest, P.J., D. Lachance, C. Jones and Y. Devantier. 1993b. Microprojectile and silicon carbide mediated DNA delivery in conifers and recovery of transgenic black spruce. In Vitro Cell. & Dev. Biol. 29A:87.

Copis, P.L. 1990. Pollination techniques II. Pollen extraction and storage. PNFI Technical Rep. #5. 4pp.

Datla, R.S.S., J.K. Hammerlindl, L.E. Pelcher, W.L. Crosby and G. Selvaraj. 1991. A bifunctional fusion between b-glucuronidase and neomycin phosphotransferase: a broad-spectrum marker enzyme for plants. Gene 101:239-246.

Duchesne.L. and P.J. Charest. 1991. Transient expression of the b-glucuronidase gene in embryogenic callus of Picea mariana following microprojection. Plant Cell Rep. 10:191-194.

Duchesne. L.C. and P.J. Charest. 1992. Effect of promoter sequence on transient expression of the b-glucuronidase gene in embryogenic calli of Larix x eurolepis and Picea mariana following microprojection. Can. J. Bot. 70:175-180.

Duchesne. L.C., M.A. Lelu. P. von Aderkas and P.J. Charest. 1993. Microprojectile-mediated DNA delivery in haploid and diploid cell lines of Larix spp. Can. J. For. Res. 23:312-316.

Ellis. D.D., D.E. McCabe. S. McInnis. R. Ramachandran. D.R. Russell. K.M. Wallace. B.J. Martinell. D.R. Roberts. K.F. Raffa and B.H. McCown. 1993. Stable transformation of Picea glauca by particle acceleration. BioTechnol. 11:84-89.

Ellis. D.D., D.E. McCabe. D. Russell. B. Martinell. and B.H. McCown. 1991. Expression of inducible angiosperm promoters in a gymnosperm. Picea glauca (white spruce). Plant Mol. Biol. 17:19-27.

Goldfarb. B., S.H. Strauss. G.T. Howe and J.B. Zaerr. 1991. Transient gene expression of microprojectile-introduced DNA in Douglas-fir cotyledons. Plant Cell Rep. 10: 517-521.

Gorska-Brylass. A. 1965. Hydrolases in pollen grains and pollen tubes. Acta Soc. Bot. Pol. 34:589-604.

Hamilton. D.A., M. Roy. J. Rueda. R.K. Sindhu. J. Sanford and J.P. Mascarenhas. 1992. Dissection of a pollen-specific promoter from maize by transient transformation assays. Plant Mol. Biol. 18:211-218.

Jefferson. R.A., T.A. Kavanagh and M.W. Bevan. 1987. GUS fusions: b-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Kim, Y. and G. An. 1992. Pollen-specific expression of the Arabidopsis thaliana a-tubulin promoter assayed by b-glucuronidase, chloramphenicol acetyltransferase and diphtheria toxin reporter genes. Transgenic Res. 1: 188-194.

Klein, T.M., L. Kornstein, J.C. Sanford and M.E. Fromm. 1989. Genetic transformation of maize cells by particle bombardment. Plant Physiol. 91: 440-444.

Kopczak, S.D., N.A. Haas, P.J. Husey, C.D. Silflow and D.P. Snustad. 1992. The small genome of Arabidopsis contains at least six expressed a-tubulin genes. Plant Cell 4:539-547.

Litts, J.C., G.W. Colwell, R.L. Chakerian, and R.S. Quattro. 1987. The nucleotide sequence of a cDNA clone encoding the wheat EM protein. Nucleic Acids Res. 15 (8): 3607-3618.

Loopstra, C.A., Weissinger, A.K. and R.R. Sederoff. 1992. Transient gene expression in differentiating pine wood using microprojectile bombardment. Can J. For. Res. 22:993-996.

Marcotte, W.R. Jr., C.C. Bayley and R.S. Quattro. 1988. Regulation of a wheat promoter by abscissic acid in rice protoplasts. Nature 335: 454-457.

McElroy, D., W. Zhang, J. Cao and R. Wu. 1990. Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2:163-171.

Mendel, R.R., B. Muller, J. Schulze, V. Kolesnikov and A. Zelenin. 1989. Delivery of foreign genes to intact barley cells by high-velocity microprojectiles. Theor. Appl. Genet. 78: 31-34.

Morikawa, H., A. Iida and Y. Yamada. 1989. Transient expression of foreign genes in plant cells and tissues obtained by a simple biolistic device (particle-gun). Appl. Microbiol. Biotechnol. 31: 320-322.

Newton, R.J., H.S. Yibrah, N. Dong, D.H. Clapham and S. von Arnold. 1992. Expression of an abscisic acid responsive promoter in Picea abies (L.) Karst. following bombardment from an electric discharge particle accelerator. Plant Cell Rep. 11:188-191.

Nishihara, M., M. Ito, I. Tanaka, M. Kyo, K. Ono, K. Irifune, and H. Morikawa. 1993. Expression of the b-glucuronidase gene in pollen of lily (Lilium longiflorum), tobacco (Nicotiana tabacum), Nicotiana rustica, and peony (Paeonia lactiflora) by particle bombardment. Plant Physiol. 102:357-361.

Plegt, L. and R.J. Bino. 1989. b-glucuronidase activity during development of the male gametophyte from transgenic and non-transgenic plants. Mol. Gen. Genet. 216:321-327.

Snustad, D.P., N.A. Haas, S.D. Kopczak, and C.D. Silflow. 1992. The small genome of Arabidopsis contains at least nine expressed b-tubulin genes. Plant Cell 4: 549-556.

Sood, P.P. 1980. Histoenzymological compartmentation of b-glucuronidase in the germinating pollen grains of Portulaca grandiflora. Biol. Plant (Praha) 22:124-127.

Stoger, E., R.M. Benito-Moreno, B. Ylstra, O. Vicente and E. Heberle-Bors. 1992. Comparison of different techniques for gene transfer into mature and immature tobacco pollen. Transgenic Res. 1:71-78.

Stomp, A.-M., A. Weissinger and R.R. Sederoff. 1991. Transient expression from microprojectile-mediated DNA transfer in Pinus taeda. Plant Cell Rep. 10: 187-190.

Tartof, K.D. and C.A. Hobbs 1987. Improved media for growing plasmid and cosmid clones. Focus (Idaho) 9:2

Topfer, R., J. Schell and H.-H. Steinbiss. 1988. Versatile cloning vectors for transient gene expression and direct gene transfer in plant cells. Nucleic Acid Res. 16: 8725.

Twell, D., T.M. Klein, M.E. Fromm and S. McCormick 1989. Transient expression of chimeric genes delivered into pollen by microprojectile bombardment. Plant Physiol. 91:1270-1274.

Twell, D., J. Yamaguchi and S. McCormick 1990. Pollen-specific gene expression in transgenic plants: co-ordinate regulation of two different tomato gene promoters during microsporogenesis. Devel. 109:705-713.

Twell, D., J. Yamaguchi, R.A. Wing, J. Ushiba and S. McCormick 1991. Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. Genes Dev. 5:496-507.

Twell, D. 1992. Use of a nuclear-targeted b-glucuronidase fusion protein to demonstrate vegetative cell-specific gene expression in developing pollen. Plant J. 2:887-892.

van der Leede-Plegt, L.M., B.C.E. van der Ven, R.J. Bino, P.M. van der Salm, and A.J. van Tunen 1992. Introduction and differential use of various promoters in pollen grains of Nicotiana glutinosa and Lilium longiflorum. Plant Cell Rep. 11:20-24.