GROWTH AND MORPHOGENESIS OF TISSUE

CULTURES OF

PINUS CONTORTA AND PICEA SITCHENSIS.

by

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Thesis submitted to the

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TO MY PARENTS

AND TO THE MEMORY OF PROFESSOR H. E. STREET.

This thesis is my own work unless otherwise acknowledged and has at no time been submitted for another degree.

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R. Wanger

I certify that this statement is correct.

Some of this work has been published: Street H.E. and Webb K.J. (1975) Tree Physiology. Applications of plant tissue culture in forestry. Forestry Commission Report on Forest Research 58-59. Street H.E. and Webb K.J. (1976) Ibid. 58-59. Street H.E. and Webb K.J. (1977) Ibid. 53-54. Webb K.J. and Street H.E. (1977) Morphogenesis <u>in</u> <u>vitro of Pinus</u> and <u>Picea</u>. Acta Horticulture (78) 259-270. CONTENTS

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ABBREVIATIONS

BAP	Benzvl amino purine (benzvl adenine)
2iP	$N^{6} - (\Delta^{2}-Isopentenyl)$ adenine ($6\gamma\gamma$ -Dimethylallyl amino purine)
IAA	Indole acetic acid
IBA	Indolebutyric acid
NAA	Naphthalene acetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
3,5-D	3,5-Dichlorophenoxyacetic acid
GA	Gibberellic acid
FDA	Fluorescein diacetate

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"Applications of tissue and cell cultures to propagation are at present confined mainly to herbaceous plants and to species which can alternatively be propagated vegetatively by traditional methods"..."Interesting current work is directed to achieving somatic embryogenesis with a number of important tree crops such as the oil and coconut palms and coniferous forest trees".

Street, 1976.

Plant regenerated from an excised embryo of <u>Picea</u> <u>sitchensis</u> (see facing page).



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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

In recent years the public and private wood-producing sectors in Britain have been capable of producing about 8% of the country's total requirement for hard-wood (angiosperm) and soft-wood (gymnosperm) timber (British Forestry, 1973). This degree of self-sufficiency should increase with the introduction of new tree-improvement*programmes. One of the most important aspects of such programmes is the development of techniques allowing the mass propagation of genetically superior populations. At the present time, however, softwood forests are generally planted from nursery-grown seedlings, the vegetative propagation of improved stock plants having proved to be relatively unsuccessful on a commercial scale.

In 1973 the composition of British softwood plantations was as follows: <u>Picea sitchensis</u> (32%), <u>Pinus sylvestris</u> (23%), <u>Larix spp. (14%), <u>Picea abies</u> (11%), <u>Pinus contorta</u> (8%) and other species (12%). The vegetative propagation of these species by traditional methods of grafting and</u> rooting of cuttings have been studied intensively but the problems encountered have not yet been completely overcome. In the formation of grafts, incompatibility between the root stock and the scion is a severe problem in many species, including Pinus contorta (see Dormling et al, 1976). This incompatibility may be revealed immediately or several years after grafting. In the latter case, the use of clonal root stocks tested for their future compatibility may prove to be the solution. The rooting of cuttings of difficult-toroot species, for instance, Pinus sp., may be promoted by auxin treatments, However, in all species, the physiological condition, ontogenetic age, genotype of the cuttings, and the position of the shoot on the tree, influence the rooting response. In both grafts and rooted shoots the plagiotropic growth of the shoot may continue for some time in Picea spp., Larix spp., and Pseudotsuga sp. Such growth has not been observed in shoots arising from adventitious buds induced by hard shearing of the parent plants (Dormling, 1976).

In recent years, attention has been turned to the possibility of using a third alternative method of vegetative propagation, i.e. the regeneration of plants via plant tissue culture. Whilst successful commercial application of tissue culture techniques has been achieved for the propagation of some species e.g. orchid (Holdgate and Aynsley, 1977) success with the economically important tree crops has been limited (see Bonga, 1977). Clonal propagation via tissue culture may be achieved by several methods, the approach used generally depending on the type of explant material available. Embryogenesis has been induced in callus and suspension cultures of several species (Halperin and

Wetherell, 1964; Radojević <u>et al</u>, 1975) but has not been universally applicable. Morphogenesis via callus cultures and the proliferation of meristem and shoot tip cultures have proved to have a greater potential for immediate application. All of these techniques involve three main steps: the isolation of contaminant-free explants, the induction of a morphogenetic response followed by the production of plantlets capable of autotrophic growth, and eventual planting out.

In general, trees which may be easily proagated by cuttings have proved relatively easy to regenerate in culture e.g. Populus spp. (Winton, 1971; Chalupa, 1974; Venverloo, 1973), Citrus spp. (Button and Bornmann, 1971; Kochba et al, 1972) and Corylus avellana (Radojević et al, 1975) whilst those species which are considered to be difficult-to-root such as the gymnosperms and the monocotyledons e.g. the oil, date and coconut palms (Rabechault and Martin, 1976; Ammar and Benbadis, 1977; Eeuwens and Blake, 1977) have proved more difficult. The choice of the source of the explant may be very important in determining the subsequent regeneration of the cultures. Callus has been successfully initiated from a variety of explant sources in gymnosperms, including embryos, seedlings, stems and shoot tips of mature trees and from both male and female gametophytes (see Brown and Sommer, 1975). In general, however, explants of juvenile material and young actively growing stems are the best callus producers. The controlled induction of adventitious buds has been achieved only in juvenile tissues (embryos and seedlings up to 2 years old). Although these shoots do not show plagiotropic growth, rooting

them has proved difficult with few plantlets being regenerated (Campbell and Durzan, 1975; Brown and Sommer, 1977). This inability to produce roots either on extended shoots or on callus cultures contrasts sharply with most other species cultures when root production is the most common form of organogenesis observed (see Reinert et al, 1977).

In the study to be described below, tissue culture techniques were applied to the economically important gymnosperms <u>Pinus contorta</u> and <u>Picea sitchensis</u> with the objective of regenerating plantlets. Initially attempts were made to define the conditions of growth of callus from various explant sources of both species (Chapter 3). The problems encountered in initiating and establishing cultures are discussed. Later the possibilities of regenerating plantlets via somatic embryogenesis (<u>P. contorta</u>) and adventitious bud production from juvenile tissues of both species were explored.

CHAPTER 2

MATERIALS & METHODS

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CHAPTER 2

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MATERIALS & METHODS.

2.1 GENERAL

Cleansing of the silica-borate glassware and general sterile techniques were as described by Street (1973). 'Analar' grade chemicals and double distilled water were used in the preparation of culture media, the desired pH (5.8 ± 0.2) being achieved by the addition of 1.0/0.1N HCl or 1.0/0.1N NaOH, as appropriate, prior to sterilisation. Agar was added after the correct pH had been attained for semi-solid media. Culture vessels and media were autoclaved at 121° C (approximate pressure 15 p.s.i.) for 15 minutes. Thermolabile solutions were sterilised by passage through appropriate 'Millipore' filters. Acid washed sand for the germination of sterile seeds was sterilised by dry heat $(160^{\circ}$ C for 5 hours).

2.2 PLANT MATERIAL

All plant material was provided by the British Forestry

Commission (Northern Research Station, Roslin, Edinburgh). Vegetative shoots and shoots bearing male and female cones were excised from the mother plant and immediately packed into polythene bags and sealed. Transportation of these shoots was by postal service.

Shoots regenerated from excised tissues in culture were conveyed from Leicester to Edinburgh by the private carrier service, Securicor. The shoots were packed in a plastic box containing moistened peat and sealed to maintain high humidity. Rooting of the shoots at the Northern Research Station, Roslin, was under a mist propagator with the air and bed temperature at 20° C. No hormones were applied to the shoots which were maintained under 18 hour days at 5-6000 lux (ca. 7-8 ergs cm⁻² sec⁻¹). Rooted shoots were potted on and maintained in greenhouses under long days.

2.2.1 EXCISED EMBRYOS AND GERMINATED SEEDLINGS

Sterilisation of the seeds was achieved by immersing the dry seed in a 30% solution of the commercial hypochlorite, 'Domestos', (Unilever Bros., U.K.) for 30 minutes. After 3 x 5 minute washes in sterile distilled water the seeds were left to imbibe water either at 5° C for a minimum of 3 weeks (stratification) or at 25° C for 40 hours (non-stratified seed). Prior to excision of the embryos, the entire seeds were individually dipped in alcohol and then 'flamed'. This ensured total sterility of the seed coat and facilitated embryo excision.

Sterile seedlings were obtained by germinating the sterilised and stratified seeds on agar (<u>Pinus contorta</u>) or sterile sand (<u>Picea sitchensis</u>) under long days at 25^oC.

2.2.2 SHOOTS

Terminal and lateral shoots, either actively growing or dormant (i.e. before chilling) were used in callus initiation experiments. The clonal origin (when known) is specified for each experiment.

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Preliminary sterilisation was achieved by washing the shoots in running tap water for 5 minutes followed by a soak (1-2 minutes) in alcohol. The sterilisation time required by the two species varied, actively growing shoots of both species requiring a shorter sterilisation time than those in seasonal dormancy. Approximately 5-10 minutes sterilisation in 20% 'Domestos' was required to disinfest actively growing shoots, the time being extended to 10-20 minutes for dormant shoots. Endogenous contamination of the shoots was frequently observed in segments of dormant shoots.

Shoot tips (2-3mm) and leaves were excised using slivers of fractured razor blade mounted in a metal chuck and tungsten wire needles; stem explants (approximately 15mm) were cut transversely with a scalpel blade.

2.2.3 OVULATE & MICROSPORANGIATE STROBILI

The strobili were stored in brown paper bags at $2-4^{\circ}C$ at 70-100% relative humidity until required. Surface sterilisation of both ovulate and microsporangiate strobili was achieved by immersion in 20% 'Domestos' for 5 minutes, followed by 3 x 5 minute washes in sterile water; microsporangiate strobili were also sterilised by dipping the entire strobilus in 95% methanol which was then removed by 'flaming'. Individual microsporophylls were dissected and transferred to the culture medium.

2.3 CULTURE MEDIA

2.3.1 SEMI-SOLID MEDIUM

Callus cultures were initiated and maintained on 0.7% w/v agar. The source of the agar changed during the course of experiments. Ionagar No 2 was used in all the original callus initiation experiments, later cultures were initiated and maintained on Oxoid technical agar grade 3.

100ml Erlenmeyer flasks (25ml medium), McCartney bottles (28ml total volume), specimen tubes (1 x 2ins) with metal caps (Oxoid) or sterile plastic petri dishes (50mm diam) with 10ml medium were used for the initiation and maintenance of callus cultures.

2.3.2 LIQUID MEDIUM

Routine maintenance of the cell suspension cultures involved the inoculation of aliquots of stock culture into fresh medium using automatic syringes (A R Horwell Ltd., London). The volume of the inoculum was dependent on the cell density of the stock culture. Both 100ml and 250ml Erlenmeyer flasks were used, with 25ml or 55ml medium respectively. Agitation was achieved using Gallenkamp orbital shakers or 2 tier modified platform shakers (L H Engineering Co Ltd., Stoke Poges, U.K.) set at 120 r.p.m. under constant illumination (ca. 4.0 x $10^3 \text{ ergs cm}^{-2} \text{ sec}^{-1}$).

The flasks for both liquid and semi-solid medium were sealed with sterile squares of aluminium foil moulded to the neck of the flask after inoculation.

2.4 CONDITIONS OF CULTURE

Subcultured callus cultures described in Chapter 3 were maintained either in total darkness or under constant illumination (ca. 4.0×10^3 ergs cm⁻² sec⁻¹) at 25°C.

The cultures described in Chapter 5 were incubated in either

- a) 25° C 16 hour photoperiod at light intensities of I 1.24 x 10^4 ergs cm⁻² sec⁻¹ or
 - II $4.5 \times 10^3 \text{ ergs cm}^{-2} \text{ sec}^{-1}$

or b) 20° C 13.5 hour light, 7° C 10.5 hour dark at a light intensity of 5 x 10^{3} ergs cm⁻² sec⁻¹.

2.5 MEASUREMENT OF CULTURE GROWTH

2.5.1 CALLUS CULTURES

Growth of the callus was monitored by measuring fresh and dry weight. The fresh weight of the inoculum was obtained by sterile weighing of the callus in a pre-weighed sterile petri dish. At the termination of an experiment the callus was sacrificed and weighed under non-sterile conditions. The dry weight was determined by drying the callus in an 80^oC oven until constant weight was reached.

2.5.2 CELL SUSPENSION CULTURES

a) <u>Packed cell volume (p.c.v.)</u> - 10ml of the suspension culture was centrifuged in a graduated glass centrifuge tube at about 1,600g for 10 minutes. The volume of the pellet was expressed as a percentage of the sample volume.
b) <u>Dry weight</u> - 2 x 5ml samples of the suspension culture

were filtered onto separate pre-weighed dry Whatman GF/C

filter pads (25mm diameter) and washed with 3 x 5 aliquots of distilled water prior to drying to constant weight in an 80° C oven. The average of the weights was taken for each sample.

c) <u>Cell number</u> - 5ml 30% (w/v) chromium trioxide were added to a 5ml sample of the suspension culture in a screw-capped vial (28ml). The vial was then immersed in a 70° C water bath for ca. 5 minutes and then shaken violently on a flask shaker (Baird and Tatlock Ltd) for approximately 5 minutes. The exact timing of the chromium trioxide and shaker treatments depended on the degree of lignification of the cells and the phase in the growth cycle.

Special cell counting slides were prepared as described by Henshaw et al (1966), with the depth of the chambers accurately ground down to lmm. The field volume (as calculated from the depth of the slide and the diameter of the field of view at a magnification x 100) was calculated to be 0.8µl. The macerated cell suspension was diluted with tap water to give 15 - 20 cells per field when samples were placed on the counting slide and viewed at x 100 magnifica-The cells in 10 random fields in each of 10 chambers tion. were counted using a Watson Microsystem 70 microscope. An estimation of the number of cells ml^{-1} was made from the mean number of cells per field by multiplying by the following correction $(\frac{10,000}{8})$ to give an expansion of the volume counted (0.8 μ l) to lml, and then multiplying by the dilution from the culture. The mean number of total cells and tracheids from the 10 sets of 10 fields were counted and the standard error of the mean was calculated for both cell populations. Standard error of the mean values greater than

10% were considered unsatisfactory for the total cell and the tracheid populations, and the counts were repeated.

2.6 ASSAY PROCEDURES

2.6.1 VIABILITY

The viability of cells of callus and suspension cultures and of pollen grains was estimated using the technique of Widholm (1972) modified by Nag and Street (1975). A stock solution of fluorescein diacetate (National Biochemicals Coroporation) was prepared (5mg 1^{-1} in 100% acetone) and stored at 20° C. 0.5ml of the stock solution was added to either culture medium or water (25ml) in ice. This solution (0.01% w/v) should be freshly made up every 30 minutes. The addition of one or two drops to a few drops of cell suspension culture or macerated callus pieces results in the green fluorescence of viable cells when viewed with UV illumination. The deposition of lignin in the cell walls may also be observed by this technique, the lignin fluorescing yellow.

2.6.2 DETECTION OF LIGNIN

Cells from callus or suspension cultures were stained with a few drops of phloroglucinol (0.63g in 5ml ethanol) followed by the addition of a drop of conc.HCl (the Wiesner reaction).

Unsuccessful attempts to quantify the lignin content of suspension culture cells are described in Appendix II.

2.6.3 ESTIMATION OF TOTAL SOLUBLE PHENOLICS

The technique used depends on the reaction of methanol soluble phenolic compounds with Folin-Ciocalteu reagent (B D H Ltd).

METHOD

Collect cells onto Whatman GF/C disc 5m1 Wash with distilled water 3 x 5ml Extract for 10 minutes 85% v/v methanol 10m1at 70°C Wash with hot 85% methanol $2 \times 5m1$ Dry down collected filtrate with compressed air Take residue up in 95% v/v methanol 0.5ml Make up with water 4.Oml Sample (volume depending on phenol concentration) 0.1-0.5ml Make up with water to final volume 2.5ml Add Folin-Ciocalteu reagent 0.2mlMix and incubate at 25[°]C for exactly 3 minutes. Add saturated Na_2CO_3 solution 0.5ml Mix and incubate at $25^{\circ}C$ for 1 hour

Read optical density against a reagent blank at 725nm. Use Gallic acid (40ppm) as a standard.

2.6.4 ESTIMATION OF TOTAL CELL PROTEIN

The protein content of cells was estimated by the method of Lowry <u>et al</u> (1951). The reaction between Folin-Ciocalteu reagent and any peptide bond will yield some colour, but certain amino acid sequences, especially aromatic residues, are more chromogenic than others and largely account for the colour yield of the protein. Thus many substances interfere with this test.

METHOD

Alkaline copper reagent: lml copper sulphate (1.0% w/v)lml sodium potassium tartrate (2.0% w/v)100ml sodium carbonate (2.0% w/v) in 0.1M Sodium hydroxide Collect cells onto GF/C (25mm diameter) 5m1 Wash with distilled water $3 \times 5ml$ Extract with 85% v/v methanol at $70^{\circ}C$ for 10 minutes 10mlWash with ether $2 \times 5ml$ Incubate dried discs in 1.OM NaOH at 100°C for 30 minutes 10m1Mix contents and filter Filtrate or standard (up to 300µg protein) 0.2mlAdd alkaline copper reagent 3.Oml Incubate for 10 minutes at 25°C Add Folin-Ciocalteu reagent (diluted x2) 0.2mlMix and leave for 30 minutes at 25^oC

The optical density was read at 750nm against a reagent blank. Bovine serum albumen (BSA) is used as a standard (O D 1.0 = $300\mu g$ BSA).

Attempts to use this method to estimate the total protein content of <u>P. contorta</u> cells were unsuccessful. Extraction of the protein by alkaline hydrolysis resulted in the development of a red colouration in the filtrate, presumably due to the extraction of flavonoids from the cells (King,1976). This extraction method would also extract lignin, the phenol content of which would interfere with the Folin-Ciocalteu reaction, thereby giving an inaccurate estimation of the total protein content of the cells.

2.6.5 AMINO-ACID NITROGEN ESTIMATION

Amino-nitrogen was estimated by a modification of the ninhydrin method of Yemm and Cocking (1955). METHOD

Collect cells onto GF/C disc 5m1 $3 \times 5ml$ Wash with distilled water Transfer to 60% v/v ethanol 5m1 Incubate at 50°C for 15 minutes Collect filtrate Filtrate or standard (up to 0.21 µmoles NH₂) 1.Oml Add citrate buffer (0.2M pH 4.75) 0.5ml Ninhydrin reagent (1.9 x 10^{-3} M ascorbic acid) 1.2ml and 5.2 x 10^{-2} M ninhydrin in 2-methoxyethanol) Incubate 20 minutes at 100[°]C Cool in iced water 5 minutes Add 60% v/v ethanol 3.Om1 Mix and leave to stand 15 minutes at 25°C Read optical density at 570nm against water, use leucine as a standard.

2.7 HISTOLOGICAL TECHNIQUES

2.7.1 WAX EMBEDDING AND STAINING

The cells were fixed overnight in F.A.A. (70% alcohol (90% v/v), formalin (5% v/v) and glacial acetic acid (5% v/v)).

After 2 washes in 70% alcohol the samples were infiltrated with 100% ethanol and taken through an alcohol:xylene series to 100% xylene. Molten fibrowax (R A Lamb, London; melting point 57[°]C) was gradually added to the sample maintained at ca. 60-65[°]C. When the xylene had been completely removed (over a period of 8 hours) and the sample was totally infiltrated with the wax, the samples were solidified into small wax blocks. Sections (ca. 20µm) were cut using a rocking microtome(Cambridge Instrument Co Ltd U.K.) and wax ribbons dried onto glass slides pre-treated with Haupt's adhesive (lg gelatin in 100ml distilled water plus 15ml glycerin and 2 crystals phenol). After staining with Paragon Stain (G T Gurr Ltd) the excess stain was removed with water and the slides dried. Immersion in xylene removed the supportive wax. The sections were mounted in Xam (G T Gurr Ltd).

2.7.2 PLASTIC EMBEDDING

Cells were fixed for 3 hours to overnight in 6% (w/v) glutaraldehyde (B.D.H., E.M. grade) in 0.025M phosphate buffer, pH7 at 4° C. The samples were washed in 0.1M phosphate buffer pH7 (3 x 10 minutes) prior to being post-fixed in 1% (w/v) osmium tetroxide for 30 minutes, dehydrated through a series of alcohols and stained with alcoholic uranyl acetate (1% w/v). The tissues were embedded in a methacrylate styrene medium, and hardened in dried gelatin capsules at 56° C over 40 hours.

EMBEDDING MEDIUM:

7 parts methacrylate (3.5 : 3.5 butyl to methyl)

3 parts styrene

1% w/v benzoyl peroxide

CaSO, (anhydrous) to dehydrate

The protocol for fixation and embedding was modified from Mohr and Cocking (1968) by Withers and Cocking (1972).

Sections were cut on a Tesla ultramicrotome with a glass knife and were post-stained with lead citrate for 5 minutes and then washed in distilled water (Reynolds 1963). The material was examined in a A E I - E M 802 operating at 80ky.

For light microscope studies the post-fixation with osmium tetroxide and staining with uranyl acetate were omitted. Thick sections (approximately 2µm) were cut using a glass knife on a Reichert OM-D microtome. A 'Millipore' filtered solution of 1% w/v toluidine blue in absolute ethanol was added to the sections mounted on glass slides. This was allowed to dry down on a hot plate, excess stain being removed in hot water. When dry the preparations were permanently mounted in Euparal (G B I Laboratories Ltd).

2.8 CYTOLOGICAL TECHNIQUES

2.8.1 FEULGEN MICRODENSITOMETRY

The tissues were fixed in 50%(v/v) formic acid for 7 - 14 days at 3 - 4^OC to ensure the complete dispersal of starch grains. The hydrolysis and staining of the samples was modified from that described by Darlington and La Cour (1962). Fixed cells were washed x 3 with distilled water and N HCl (pre-heated to 60° C) was added to the samples. Hydrolysis at 60° C was for 20 minutes, an extra 2 minutes being allowed for removal of the acid by centrifugation when handling suspension cultures. The samples were treated with basic fuchsin (Magenta, B D H Ltd., U.K.) and incubated at 25° C for 2 hours. After washing in three changes in sulphite water (5ml 10% (w/v) ag. Na₂S₂O₅ + 5ml 1N NCl + 100ml distilled water) each for 10 minutes, the preparation was washed in distilled water and transferred to 25% aqueous acetic acid prior to the preparation of squashes.

To make permanent preparations of the squashes the slide was placed on a block of dry ice, the cover slip prised off the frozen slide and the preparation dehydrated through a series of alcohols to xylene prior to mounting in Xam (G T Gurr Ltd).

The Vickers M85 Scanning Microdensitometer was used to assess the nuclear DNA content of the cells. The light absorption by 200 - 300 random interphase nuclei was measured for each tissue. Comparisons of the DNA content of the cells were made with metaphase nuclei of root tips of <u>P. contorta</u> stained simultaneously.

The microdensitometer was used under the following conditions:- Objective x 20 or x 40, slit width 30, spot aperture 2, wavelength 55.

2.8.2 CHROMOSOME COUNTS

Samples (suspensions or plant material) were pretreated with a 0.05% colchicine solution for 2 hours before fixation. The protocol was as above for Feulgen staining, but if the cells were not required for densitometry further

staining with 2.0% orcein in 45% propionic acid provided better contrast.

2.8.3 ACETO-CARMINE STAIN

Rapid nuclear staining of cultured cells and pollen grains was achieved using aceto-carmine (2.0g carmine heated to 100° C in water (55ml) and 45ml glacial acetic acid added prior to cooling and filtering). A few drops of the stain were added to the sample and heated over a flame to aid penetration of the nuclear stain.

2.9 GAS-LIQUID CHROMATOGRAPHY ANALYSIS OF GAS PHASE ABOVE CULTURES

A Pye 104 Model 64 twin F.I.D. G.L.C. was used with a standard flame ionisation detector. The column was a 5ft glass coil packed with Poropak Q (Waters Associates, Massachusetts, U.S.A.). The column was heated to 50° C with a detector temperature of 150° C. The total flow rate of the gas was 60ml minute⁻¹. The unit was fitted with a Catalytic Conversion Cell (part no. 707100) which allowed low levels of carbon monoxide and carbon dioxide to be detected on the flame ionisation detector by reducing the oxides of carbon to methane and water. To measure CO₂ levels 10μ l of the gas phase above the culture was injected into the injection port, for ethylene measurements 0.5ml were used.

CHAPTER 3

CALLUS INITIATION & MAINTENANCE

CHAPTER 3

CALLUS INITIATION AND MAINTENANCE

3.1 INTRODUCTION

Very few gymnosperm cultures have been successfully initiated on the relatively simple media of Knop (1865), Heller (1953), White (1943) and Gautheret (1942). For instance, callus cultures, isolated from epicormic shoots produced on a burr of <u>Sequoia sempervirens</u>, have been maintained on a diluted Knop's solution with 3% sucrose (Ball 1950). However this tissue has very simple requirements compared with most gymnosperm cultures.

Both normal and tumerous tissues of <u>Picea glauca</u> have been cultured by Reinert and White (1956) on a defined medium which contained, in addition to White's basal medium, a supplement of 15 amino acids and 10 other organic compounds including sucrose as a carbon source. This complex medium was later considerably simplified by Risser and White (1964) who found that 5% sucrose and 250mg 1^{-1} glutamine could replace the supplement completely.

The complex and undefined organic supplements casein

hydrolysate, yeast and malt extracts and coconut milk have been incorporated into the basic nutrient media with varying degrees of success. Hotson and Cutter (1951) and Constabel (1957) were unable to maintain growth of <u>Juniperis virginiana</u> cultures for longer than 6 months, even when coconut milk or yeast extract was added to the medium. Loewenberg and Skoog (1952) reported that vigorous growth of callus cultures of <u>Pinus banksiana</u> and <u>Pinus strobus</u> seedlings could only be achieved when Gautheret's medium was supplemented with either an aqueous extract of pine seeds or malt. Further investigations by Steinhart <u>et al</u> (1961) using <u>Picea abies</u> callus initiated on the same medium, suggested the importance of the nitrogen source for the continued proliferation of these callus cultures.

Harvey and Grasham (1969) obtained callus from <u>Pinus contorta</u> plants. The callus was maintained on a simple inorganic medium developed by Harvey (1967) supplemented with the hormones NAA (0.5mg 1^{-1}) and kinetin (0.5-2.0mg 1^{-1}), and the compounds ascorbic acid (0.05mg 1^{-1}), inositol (0.05mg 1^{-1}), urea (0.1mg 1^{-1}), arginine (0.2mg 1^{-1}), tyrosine (0.02mg 1^{-1}), and glucose (1.0%).

The development of the salt solution of Murashige and Skoog (1962), higher in ionic strength and containing NH_4^+ and NO_3^- as the source of nitrogen, resulted in the successful initiation and maintenance of many more gymnosperm cultures. The superiority of Murashige and Skoog's medium, as compared to the earlier media, has been reported for several <u>Pinus</u> and <u>Picea</u> species (Brown and Lawrence, 1968; Chalupa and Durzan, 1973a; Durzan <u>et al</u>, 1976).

Reviewing these earlier studies makes it clear that they

have not resulted in the development of a general medium widely successful in culturing gymnosperm tissues, nor have they identified any specific requirement of gymnosperm tissues distinguishing them from angiosperm tissues. In considering the culture of any gymnosperm tissue, it is, therefore, necessary to approach the problem empirically, testing such media as that of Murashige and Skoog or other formulae which have been successful with the genera concerned. It may also be important, in relation to culture initiation, to consider the explant source (e.g.juvenile versus mature tissues and diploid sporophytic versus haploid gametophytic tissues) and to realise that the conditions promoting callus initiation in each instance may not be optimal for serial subculture.

Tissues derived from somatic explants of mature plants (i.e. over 2 years old) have generally been more difficult to maintain <u>in vitro</u> than juvenile tissues, although there are numerous reports of callus initiation from mature gymnosperm material (Reinert and White, 1956; Bogdanovic, 1968; Winton and Verhagen, 1977). In a number of cases continued proliferation of the derived callus has not been achieved. For example, explants of 3-5 year old stems of <u>Pseudotsuga menziesii</u> while proliferating initially failed to give tissue capable of survival on subculture (Blakely, 1958). By contrast callus initiated from juvenile tissues of gymnosperms has been repeatedly subcultured (Brown and Lawrence, 1968) and used in biochemical and nutritional studies (Higuchi, 1962; Chalupa <u>et al</u>, 1976; Durzan and Chalupa, 1976b).

Successful initiation of haploid callus from female

gametophyte tissue has been reported for several gymnosperm species. The proliferating haploid cells of three <u>Pinus</u> species (Bonga and Fowler, 1970; Bonga, 1974) have been reported to retain their haploid state through prolonged subculture whilst spontaneous doubling has been reported for haploid cultures of <u>Pinus lambertiana</u> (Borchert, 1968) and Taxus baccata (Zenkteler and Guzowska, 1970).

Haploid callus initiation has also been reported from pollen grains from some gymnosperms, including several <u>Pinus</u> species (Bonga and Fowler, 1970; Brown and Sommer, 1974). The maintenance of callus initiated from pollen grains, in a stable haploid state has been reported (Tulecke, 1960), but mixed haploid-diploid or polyploid callus often results (Tulecke, 1953; Bonga, 1974; Bonga and McInnis, 1975). Bonga and McInnis (1975) claimed an enhancement of callus production from immature pollen in microsporophyll cultures of <u>Pinus resinosa</u> by dry centrifugation prior to inoculation. Prolonged cold storage of the microsporophylls also appeared to affect callus initiation, as did the stage of development of the pollen grains (Bonga, 1974).

The development of a defined medium capable of supporting both the initiation and continuous growth of <u>P. contorta</u> and <u>P. sitchensis</u>, from a variety of explant sources, was required as a basis for studies on the possible morphogenetic responses of cultures, and studies directed to this end are now described.

3.2 CALLUS INITIATION FROM SEEDLINGS

The formulae for all media employed are set out in Appendix I. All the cultures described in this section were maintained on medium solidified with 0.7% agar. The first
experiment was designed to test the effectiveness of various basal media and hormonal combinations for the induction and growth of callus from <u>Pinus contorta</u> and Picea sitchensis seedlings.

3.2.1 CALLUS INITIATION FROM P. SITCHENSIS & P. CONTORTA SEEDLINGS

3-4 week old sterile germinated seedlings of both species were cultured either whole or minus the root system (Plate 1A),or were cut transversely into three or four segments comprising the root (A), hypocotyl (either 1 or 2 segments depending on its length) (B) and the cotyledons plus the enclosed meristem (C) (Fig.3.1). All the segments from the same seedling were placed horizontally on medium in each 100ml Erlenmeyer flask and cultured at 25°C in the dark.

The basal media of Murashige and Skoog (1962) (MS), Linsmaier and Skoog (1965) (LS), Reinert and White (1956) (RW) and Heller (1953) with the combinations of 2,4-D, NAA and kinetin shown in Table 3.1 were used in attempts to initiate callus. Ten seedlings were used per treatment.

Picea sitchensis

None of the media supplemented with 2,4-D initiated callus production. A small quantity of white, slow growing callus was formed from seedlings cultured on medium containing NAA. Transfer of this callus, with the original explant, resulted in the continued but slow proliferation of the callus. Other combinations of NAA and kinetin (not shown in Table 3.1) with MS basal salts proved unsuccessful



Diagram of a gymnosperm seedling.

Table 3.1 The effectiveness of various basal media and

hormonal combinations for callus initiation from seedling material of <u>Pinus contorta</u> and Picea sitchensis.

MEDIUM	HORMONE	S mg 1 ⁻¹	PINUS CONTORTA	PICEA SITCHENSIS
	2,4-D	Kinetin		
HELLER	1.0	0.5	+	-
	1.0	1.0	+	-
R₩	1.0	0.5	+	-
	1.0	1.0	+	-
LS	0.4	0.3	++	
	0.4	0.03	-	-
	1.0	0.5	· ++	-
	1.0	1.0	+++	-
MS	1.0	1.0	++	-
	1.0	5.0*	+++	-
	5.0	1.0	++	-
	5.0	5.0	++	
	NAA	Kinetin		
MS	1.0	1.0	+	+

KEY TO TABLES 3.1 and 3.2.

- No callus initiated

+ Callus not transferable

++ Callus capable of subculture

+++ Prolific callus production

* Best callus production from P. contorta

Plate 1

<u>Pinus contorta</u> cultures incubated at $25^{\circ}C$ in the dark on MS basal medium with lmg 1⁻¹ 2,4-D and 5mg 1⁻¹ kinetin.

A. Callus production from the hypocotyl (h) of three seedlings (minus roots); the cotyledons (c) did not respond.
B. Callus, originating from seedling hypocotyl,

30 days after the first subculture. x 4



in stimulating prolific callus production from this material source.

Pinus contorta

Explants from both the same and different seedlings, cultured on the same initiation medium showed variable amounts of callus production. Some segments produced subculturable callus whilst others produced a small quantity of callus which did not survive transfer.

The basal media of MS and LS supported the most prolific callus growth, the media of RW and Heller induced callus initiation but the cultures did not survive transfer. Quantitative analysis was not possible at this time due to the low yield of callus material and the possibility of contamination during sterile weighing. Visually, the callus produced on MS medium supplemented with $lmg 1^{-1}$ 2,4-D and $5mg 1^{-1}$ kinetin was both the most abundant and the healthiestlooking, being creamy-white in colour with no necrotic regions (Plate 1B).

MS and LS are very similar media, differing in the addition of $\lg l^{-1}$ casein hydrolysate to LS and the higher concentrations of vitamins of MS medium (see Appendix I for details). Since MS is a defined medium it is preferable to that of LS on the basis that the cultural requirements of the tissues can be precisely defined.

Thus, the media of MS supplemented with $lmg 1^{-1} 2,4-D$ and $5mg 1^{-1}$ kinetin was routinely used to initiate callus production from <u>Pinus contorta</u> seedlings, the kinetin concentration being lowered to $lmg 1^{-1}$ during serial subculture.

3.2.2 CALLUS PRODUCTION BY VARIOUS EXPLANT SOURCES

The initiation medium MS with $lmg l^{-1}$ 2,4-D and 5mg l⁻¹ kinetin was used to initiate callus production from various parts of P. contorta seedlings. Approximately 10 segments 4-5mm long were cut from each of ten sterile seedlings, utilising successive segments from the root tip through to the cotyledons (Fig. 3.1). The segments were incubated (one per flask) in separate 100ml Erlenmeyer flasks at 25[°]C in the dark. After 54 days incubation, the callus formation was scored (Table 3.2). The callus produced was consistently creamy-white in colour. Maximal proliferation occurred from the hypocotyl region, the callus often originating along the whole length of the segments. The root tip produced a small quantity of non-subculturable callus, whilst the cotyledons swelled prior to browning.

<u>Table 3.2</u> Callus initiation from segments of <u>P</u>, contorta seedlings after 54 days at 25° C in the dark on MS with lmg 1⁻¹ 2,4-D and 5mg 1⁻¹ kinetin.

SECTION	NUMBER	RESPONSE OF EXPLANT
Root tip	1	+
Root	2	·
	. 3	- -
	4	+
Hypocotyl	5	++
	6	++
Plumular meristem	7	+++
Cotyledons	8	-
	9	-
	10	_

3.2.3 THE EFFECT OF GENETIC VARIATION ON CALLUS PRODUCTION

The degree of proliferation of similar explants varied considerably within and between seedlings. This marked variation in the response of seedlings in culture raises the question whether or not callus production is genetically controlled. Since the various regions of the seedlings have been shown to have different capabilities for callus production, only the hypocotyl segments were used for this experiment.

The hypocotyls of 10 sterile seedlings (A-J) of P. contorta were divided into 5mm segments. These segments were numbered 1-4 from the cotyledon to the root and placed in separate flasks. After 40 days incubation in the dark (on MS medium with $lmg 1^{-1} 2, 4-D$ and $5mg 1^{-1}$ kinetin) the callus plus explant was weighed under sterile conditions, and weighed callus pieces (200-300mg fresh weight) were used to inoculate fresh medium (passage 1). After 30 days the procedure was repeated (passage 2) and the total fresh weight of the callus culture was calculated for each culture The data were expressed as the average yield (gm gm⁻¹ line. fresh weight) of the cultures (Fig.3.2). The maximum and minimum yield or standard error of the mean for each callus line is indicated by the vertical lines superimposed on the histogram. In passages 1 and 2 the number of callus pieces per histogram varies, in some lines the callus produced only sufficient tissue for the inoculation of one or two flasks, therefore standard errors could not be calculated for all of the samples.

Interpretation of these data is problematical. Of the

Fig. 3.2 Callus initiation and growth from explants from the same and different seedlings of P. contorta.

Hypocotyl segments (numbered 1-4) from seedlings (designated A-J) were inoculated onto MS medium containing $lmg l^{-1}$ 2,4-D and 5mg l^{-1} kinetin. Each explant plus callus was weighed under sterile conditions after 40 days dark incubation at $25^{\circ}C$ (Passage O). Weighed callus pieces (200-300mg fresh weight) were either subcultured or transferred onto fresh medium, depending on the amount of callus produced (Passage 1). This was repeated after a further 30 days dark incubation (Passage 2). The final yields (gm/gm fresh weight) were calculated for each culture line. The vertical lines superimposed on the histogram represent the standard errors for those culture lines comprising at least 4 callus pieces (indicated by * in Fig.) or the maximum and minimum yields for culture lines with 2-4 callus pieces.



ten original seedlings, three showed no callus production (D, G, J), five exhibited cell proliferation from all the cultured explants (B, C, E, F, H) and the remaining two showed a variable response between the explants (A,I). These data suggest that certain seedlings may have been more responsive to culture conditions than others, resulting in callus production. The physiological state of the seedlings is probably very important when considering callus initiation and makes a decision regarding genetic factors difficult.

Those seedlings producing callus were either transferred or subcultured (depending on the quantity of callus produced) and monitored through two more passages. The yield of callus per cell line was not consistent, lines such as II & 2H which initially produced high callus yields did not continue to do so over the subsequent passages, whilst lines 1F, 2F and 3F which initially produced small quantities of callus were more prolific in later passages.

In addition to the variability in growth between consecutive passages, there was also high variability between replicates within each line (as indicated by the lines representing the range of yield or standard error of the mean). One possible explanation for these observations could be related to the time at which the callus was subcultured. Inoculation of stationary phase callus could result in an increased lag phase of the culture when compared with callus subcultured whilst in exponential growth. Such a situation would markedly affect the relative final yields of the replicate cultures.

The viability of several regions of random callus pieces

(as determined by the FDA test) revealed a mosaic of viable and non-viable cells. Despite precautions to take only apparently healthy callus, this variation must cause considerable discrepancy between the apparent and real inoculum densities in each experiment. The large variations in yield observed in replicate samples may be due, in part, to this factor.

3.3 ASSESSMENT OF P. CONTORTA HYPOCOTYL CALLUS GROWTH

Several of the lines of callus initiated in section 3.2 proved to be capable of regular 30 day subculture on MS medium with $\text{lmg } 1^{-1}$ 2,4-D and $\text{lmg } 1^{-1}$ kinetin. The line designated 1A proved to be capable of reproducible subculture, and was, therefore, used in all experiments. Stock callus cultures were incubated at 25° C in the light.

Growth curves of the callus line 1A were constructed (Fig.3.3) using the fresh and dry weights of 5 callus pieces for each point on the graphs. Approximately 200mg fresh weight (20mg dry weight) of apparently healthy callus was used to inoculate the cultures. Similar trends in the growth curves for both the fresh and dry weights of the cultures were observed, the dry weight comprising between 5 and 10% of the fresh weight. After 40 days in culture the yield (gm gm⁻¹ fresh weight) of the cultures was $4.8^+_{-}1.03$. The yields from previous subcultures (Fig. 3.2) were approximately 5.0 and 2.6. The variation between replicate samples is high; at day 18 the standard error of the mean was about 40% of the average callus weight; some callus pieces had grown well whilst others had not grown at all.



Fresh (2) and dry (1) weight accumulation by hypocotyl callus of <u>P. contorta</u>(line 1A) maintained on MS with $lmg 1^{-1}$ 2,4-D and $lmg 1^{-1}$ kinetin, incubated in the light at $25^{\circ}C$ for 40 days.

Preliminary work indicated that MS and LS basal media with $\log 1^{-1}$ 2,4-D and $\log 1^{-1}$ kinetin supported both callus initiation and subsequent growth of the cultures. Since the callus subcultured on MS medium showed marked variations in the growth of replicate samples, it seemed possible that the basal medium did not completely satisfy the nutritional requirements of the tissue. A comparison of growth of the tissue on MS basal medium with different organic supplements was made.

3.3.1 THE EFFECT OF ORGANIC SUPPLEMENTS ON P. CONTORTA CALLUS GROWTH

(LINE 1A)

The cultures were inoculated (approximately 250mg fresh weight) on to the experimental media and subcultured through two passages of 30 days prior to sampling. Two hormonal treatments in both the light and the dark were employed. The organic supplements were coconut milk (CM), yeast extract (YE) and casein hydrolysate (CH) (Table 3.3a). The effect of the addition of amino acids, singly or in combination, (as described by Durzan <u>et al</u>, 1973) on the callus growth was also investigated (Table 3.3b). These cultures were maintained in the dark on MS with lmg 1⁻¹ 2,4-D and lmg 1⁻¹ kinetin.

The addition of yeast extract to the medium caused the rapid death of the cultures, browning occurring within 4 days of transfer. Further transfer of the necrotic looking callus to the same experimental medium did not result in any resumption of growth. None of the other organic supplements significantly affected the growth of the cultures (except

perhaps glycine) nor was the variability between replicates diminished.

Since glycine was the only supplement which appeared to enhance growth of the callus, and this amino acid has been reported as being toxic to growth of pine callus (Brown and Lawrence, 1968), the control medium was used routinely. However, stocks were also maintained on medium supplemented with casein hydrolysate at $\lg 1^{-1}$ as a precaution.

The growth of the callus was not consistently better on either level of kinetin employed (Table 3.3a), therefore the lower concentration was used for maintenance of the stocks. The yields of the callus were not significantly different from callus incubated in the light or the dark, nor was there any difference in the appearance of the callus over the time course used. However, if the calluses were left longer than 30 days, the callus grown in the light showed browning earlier than those maintained in the dark.

It should be noted that the yields of callus after 30 days incubation on MS medium with $lmg l^{-1}$ 2,4-D and either $lmg l^{-1}$ or $5mg l^{-1}$ kinetin are higher than those from previous passages. In Figs. 3.2 and 3.3, yields of approximately 5.0, 2.6 and 4.8 were noted, whereas in Table 3.3 the yields of the callus are 5.70, 8.89, 8.06, 10.17 and 8.91. It seems that the callus growth has improved with the time in culture. The lower callus yields observed in the light grown control as compared to dark grown control (Table 3.3a) may reflect a real difference but this is by no means conclusive.

At this time supply difficulties made the agar

(Ionagar No.2) hitherto used for maintenance of the cultures unavailable. The alternatives were Oxoid technical agar, grade 3 and the impure Lab M. The latter caused the rapid death of the cultures whereas Oxoid technical agar, grade 3 appeared to support callus growth equivalent to that observed on Ionagar No.2. No direct comparison could be made between the callus growth in the two brands of agar which supported callus growth.

Table 3.3The effect of various organic supplements with MSbasal salts on the growth of the P. contortahypocotyl callus (line 1A).Inoculum size approximately 250mg fresh weight;yields expressed as gm gm⁻¹ fresh weight after 2subcultures of 30 days at 25° C.

Expt. a 10 replicates per treatment. All media contain $lmg l^{-1}$ 2,4-D, kinetin

SUPPLEMENTS	YIELDS						
·	LIGHT	[DARK				
KINETIN (mg 1 ⁻¹)	1.0	5.0	1.0	5.0			
Coconut milk (10% w/v)	$7.75^{+}1.89$	8.70 ⁺ 1.50	$8.85^{+}1.92$	8.70 ⁺ 1.43			
Casein hydrolysate (l g l ⁻¹)	10.05_1.70	8.25 ⁺ 1.69	9.50_1.74	10.35 ⁺ 2.01			
Yeast estract (2 g 1 ⁻¹)	0	0	0	0			
No supplements	5.70 <mark>+</mark> 1.93	8.89 ⁺ 1.12	8.06 ⁺ 1.63	10.17 ⁺ 1.89			

concentrations are as stated.

Expt. b 5 replicates per treatment.

All media contain $lmg 1^{-1}$ 2,4-D and $lmg 1^{-1}$

kinetin. The cultures were incubated in the dark.

SUPPLEMENTS (mg 1 ⁻¹)	YIELDS
L - glycine 300	12.90_1.85
L arginine 50	7.40 ⁺ 1.65
L – glutamine 600	8.34 ⁺ 1.43
L - methionine 30	8.12 2.95
L – asparagine 200	8.56 1.64
All 5 amino acids	9.74 ⁺ 1.69
No supplements	8.91_1.18

3.3.2 HORMONE EFFECTS ON THE GROWTH OF P. CONTORTA CALLUS

The availability of subculturable callus allowed the investigation of the morphogenetic potential of the cultures. The problems involved in the use of callus stocks in experiments designed to stimulate morphogenesis by alteration of the hormonal milieu includes the effect of carry-over of hormones from the previous passage. In an attempt to deplete the endogenous levels of hormones, the callus was maintained on a hormone-free medium for 14 days in the dark prior to inoculation through 2 passages on the experimental media.

(1) NAA and IBA

The auxins NAA and IBA stimulate the rooting of gymnosperm mature shoot cuttings (Bowen <u>et al</u>, 1975). Combinations of these two auxins were used in an attempt to induce root formation from the callus (Table 3.4). In the light the callus was green and compact, whilst in the dark the callus was creamy-white and more friable in appearance. None of the cultures showed any evidence of root or shoot production.

Table 3.4 Growth of <u>P. contorta</u> hypocotyl callus (line 1A) on MS basal medium supplemented with NAA and IBA. Yields (gm gm⁻¹ fresh weight) are presented after 2 subcultures of 30 days at 25° C (standard errors not available).

HORMONES (mg 1 ⁻¹)		YIELDS (gm g	m ⁻¹ fresh weight)
IBA	NAA	LIGHT	DARK
2.0	0	10.45	5.32
1.0	0	3.16	7.11
0.1	0	0.84	2.93
1.0	0.1	3.47	7.88
1.0	0.5	9.09	10.71
1.0	1.0	8.34	11.05

(2) 2,4-D and kinetin

The effect of a range of 2,4-D and kinetin concentrations (Table 3.5 (a) and (b)) on the growth of callus line 1A, were investigated. Although these cultures showed no gross morphological differentiation, cytodifferentiation in the form of xylogenesis was frequently observed in the cultures (Plate 2). Therefore an attempt was made to relate the tracheid formation to the hormonal supply to the callus. Quantification of the tracheary elements in the callus was attempted using plastic embedded sections stained with toluidine blue (Table 3.5a). Each result is the average of the % tracheary elements in the total cell population for

Plate 2

Thick sections (ca. $2\mu m$, stained with toliudine blue)of callus cultures of <u>P. contortá</u> showing tracheids (t) and parenchymatous cells (p). The cultures were incubated in the light at $25^{\circ}C$. x 400

A. Callus maintained on MS medium with $lmg l^{-1}$ 2,4-D and $lmg l^{-1}$ kinetin. Note that the tracheids tend to be centrally located in the callus.

B. Callus maintained on MS medium with $lmg l^{-1} 2, 4-D$ and $3mg l^{-1}$ kinetin.



at least four sections from two callus pieces.

No relationship can be seen between the hormonal supply and the tracheid production, nor between the amount of growth of the callus and tracheid production.

Withdrawal of the hormones 2,4-D and kinetin from the cultures for 2 passages resulted in a marked decrease in the yield of the cultures incubated in the dark, and a total cessation of growth in the light. The presence of 2,4-D $(lmg l^{-1})$ alone improved the growth but further enhancement resulted from the addition of 0.25mg l^{-1} kinetin (Table 3.5a). Further increases in the kinetin level in this experiment did not reveal any correlation between callus growth and kinetin concentration. Callus incubated in the dark at concentrations of 0.1, 0.5 and 1.0mg 1^{-1} kinetin, at all 2,4-D levels used, showed an increasing yield with the increasing cytokinin level (Table 3.5b). The yields obtained from callus grown on 0.5mg 1^{-1} 2,4-D and lmg 1^{-1} kinetin appeared to be optimal whilst cultures maintained in the light produced lower yields and grew more erratically. These latter cultures were observed to have localised brown necrotic regions.

Experiments 3.5a and b were set up at different times from the same cell line. A comparison between treatments reveals a decreased yield in experiment (b) as compared with experiment (a). Over a number of passages the cultures became more difficult to maintain until finally the line died. In addition to the decreased viability of the cells (as detected by the FDA test), it was noticed that a high proportion of the cells of the callus samples fluoresced yellow under UV illumination. This fluorescence was due to the random lignification of parenchymatous cells; this was confirmed by the Wiesner reaction for lignin.

It was noteworthy that suspension cultures initiated from this callus line remained viable for some months after the failure of the callus line (which survived approximately 13 months).

<u>Table 3.5</u> The effects of 2,4-D and kinetin on the growth and cytodifferentiation of <u>P. contorta</u> hypocotyl callus (line 1A) after 2 subcultures of 30 days each.

Yields expressed as gm gm⁻¹ fresh weight (standard errors not available).

% tracheids = % total cell population differentiated into tracheary elements.

Experiments a and b were inoculated at different times.

HORMONE	S (mg 1^{-1})	DARK		TI	GHT
2,4-D	kinetin	Yield	% tracheids	Yield	% tracheids
0	0	2.67	-	0	-
1.0	0	5.89	0.8	4.34	3.9
1.0	0.25	12.15	3.7	7.78	4.3
1.0	0.50	8.13	0	11.04	1.0
1.0	1.0	-	-	11.36	2.3
1.0	3.0	9.37	2.8	6.35	4.2
1.0	4.0	3.47	5.5	7.87	2.2
1.0	5.0	8.22	6.1	10.78	0.7

<u>Expt. a</u>

kinetin mg l ⁻¹	2,4-D mg 1 ⁻¹					
	DARK LIGH			GHT		
	0.1 0.5 1.0 0			0.1	0.5	1.0
0.1	0,80	2.93	2.18	0.41	1.29	0.26
0.5	3.74	4.39	4.51	3.33	2.69	4.77
1.0	5.07	9.54	7.06	2.12	5.73	4.70

Expt. b Yields of callus (line 1A)

· KEY

- Samples lost.

3.4 CALLUS INITIATION FROM MATURE TREES

Shoots of <u>P. contorta</u> and <u>P. sitchensis</u> from clones of known and unknown origin were used to determine whether the media conducive to callus initiation from seedling material would cause cell proliferation from mature tissues.

Endogenous bacterial contaminations were major problems when using dormant shoots (i.e. shoots which have not been chilled). Actively growing shoots proved easier to disinfest.

3.4.1 PINUS CONTORTA

The initiation medium (MS with $lmg 1^{-1} 2,4-D$ and $5mg 1^{-1}$ kinetin) developed for callus initiation from seedling hypocotyl caused callus formation in 21/100 actively growing shoot segments from unknown clones. The non-responsive shoots frequently appeared healthy although no callus was produced even at the wounded regions. The callus was often formed at the point where the dwarf shoots bearing the needle pairs had been attached, as well as at the cut ends (Plate 3A).

Plate 3

Callus (c) initiation from tissues from mature trees:

A. <u>P. contorta</u> stem segment maintained on MS with $lmg l^{-1} 2,4-D$ and $5mg l^{-1}$ kinetin. Callus production occurred at the wounded regions i.e. the cut end and where the dwarf shoot bearing the needle pairs had been attached. x 5

B. <u>P. sitchensis</u> stem segment maintained on MS with 2.5mg 1^{-1} NAA and lmg 1^{-1} kinetin. Callus production occurred along the length of the segment. x 8 C. <u>P. sitchensis</u> needles, excised from the stem and maintained on the medium above (B). The cambial cells at the base of the needles proliferated but the callus was not capable of subculture. x 8



The callus initiation from shoots of 5 clones was monitored (Table 3.6). The results indicate that the ease of callus formation is related to the age (and hence probably the physiological state) of the plant material, which also appears to affect the rooting success of cuttings (Howarth, personal communication). The healthier cultures survived through 3 passages of 40 days before finally dying. The cultures showed a mosaic of brown and creamy-yellow areas on the callus surface and although only apparently healthy areas were subcultured, the browning finally overtook the growth of new cells.

<u>Table 3.6</u> Callus initiation from mature shoot material of <u>P. contorta</u> from known clonal material.

10 explants were used per treatment, contaminated

ORIGINAL SEED PROVENANCE	UK SOURCE	CLONE	AGE OF: TREE(YRS)	ROOTING [†] PERFORMANCE	CALLUS PRODUCTION
Sushwap Lake,					
Southern Interior	Loch Ard	10 3	11	good	7/7***
British Columbia.					
Shelton,	Culbin	27	50	moderate	0/5
Peugeot Sound.					
Coastal Washington.	Rendle- sham	111	43	very poor	5/5*
Shelton,	Culbin	22	50	poor	2/5**
Peugeot Sound.					
East Washington.	Elgin	353	13	moderate	9/9***

cultures were discarded.

KEY

* Not capable of subculture .*** Good growth

** Very poor growth

*** Good growth f Information pr

Information provided by the British Forestry Commission

3.4.2 PICEA SITCHENSIS

Shoot segments, apices and needles of known clones were introduced to culture. The medium of MS supplemented with either 2,4-D ($lmg l^{-1}$), or NAA (2.5mg l^{-1}) and kinetin ($lmg l^{-1}$) supported callus initiation and its subsequent slow growth. Alterations of the hormone levels did not provide consistent results, but NAA generally enhanced growth as compared to 2,4-D. No quantative measurements could be made on these slow growing calluses.

Callus was produced from the shoot tip, stem and the ⁿeedle base of the clones (Plate 3). The apices of actively growing shoots showed a greater tendency to callus than did dormant apices. The latter occasionally elongated but growth did not continue.

The callus formed from the stem segments (following the removal of the epidermis) was compact and slow growing, adhering very strongly to the original explant. Removal of this tissue from the explant was often impossible and further transfer of the explant plus callus did not always result in further callus growth.

The base of young needles pulled away from the stem proliferated on the initiation medium. The white and compact callus only formed if the needle remained green and healthy. These calluses did not survive transfer if excised from the explant. Callus initiated from stem and shoot apices was used to test the effect of various organic supplements. After removal from the explant, the cultures were maintained on MS medium with 2.5mg l^{-1} NAA and lmg l^{-1} kinetin through 2 subcultures of 60 days in the dark. Only qualitative scores were made owing to the small quantity of

the material available. None of the amino acids, either alone or in combination or the casein hydrolysate and coconut milk significantly affected growth of the cultures. However, yeast extract caused the rapid death of the callus.

A difference was observed in the callus growth between the two clones used. Clone 8000, whether actively growing or dormant shoots were used, responded by greater callus production that did clone 8012.

These callus lines were maintained through four more 60 day transfers before the growth stopped.

Table 3.7Growth of P. sitchensis callus (initiated from
shoot apices) on MS basal media containing
 $2.5 \text{mg } 1^{-1}$ NAA and $1 \text{mg } 1^{-1}$, kinetin with various
organic supplements. Qualitative scores were
made after the 2nd 60 day subculture.

SUPPLEMENTS	CLONAL MATERIAL		
	8000 Active	8000 Dormant	8012 Dormant
Casein hydrolysate lg 1^{-1}	++	++	+
Coconut milk 10% v/v	++	++	+
Yeast extract 2g 1 ⁻¹	-	-	-
Glycine 300mg 1 ⁻¹	++	++	+
Arginine 50mg l ⁻¹	++	++	+
Glutamine 600mg 1 ⁻¹	++	++	. +
Methionine 30mg 1 ⁻¹	++	++	+
Asparagine 200mg 1^{-1}	++	++	+ .
All the above amino acids	++	++	+
Control (no supplements)	++	++	+

KEY

No growth ++ Moderate growth Clones 8000 10-15 years
+ Poor Growth 8012 from seed

3,5 CALLUS INITIATION FROM MALE AND FEMALE GAMETOPHYTE TISSUE OF PINUS CONTORTA

All cultures were maintained on MS medium with the added hormones as specified.

3.5.1 METHODS OF STORAGE

Ovulate strobili (containing the female gametophyte) were used fresh, although the effect of storage on the tissues appeared to be negligible.

Three alternative methods of storage were investigated for the pollen-containing microsporangiate strobili. The entire shoots bearing the male cones were maintained with their morphological basal end in water at

- a) $25^{\circ}C$
- b) $2-4^{\circ}C$
- or c) the male cones were removed from the shoots and stored in brown paper bags at 2-4^OC under 70-100% relative humidity (as described by Bonga, 1974)

Maintenance of the cones at $25^{\circ}C$ allowed further development of the pollen grains (if the developing pollen had passed through the tetrad stage). Pollen liberation occurred within a week of storage. Storage of the cones, either on the shoot or alone, at 2-4°C retarded pollen development and thereby made material available over a longer period of time. No loss of viability was observed during this method of storage.

3.5.2 METHODS OF DISINFESTATION

The entire strobilus (male or female - Figs. 3.4.1 and 3.4.5) was immersed in 'Domestos' (20% v/v) for 5 minutes and thoroughly rinsed with sterile distilled water. The male strobili were also freed from contamination by dipping in 95% (v/v) alcohol and 'flaming' (Bonga and Fowler, 1970). The initial and final viability (FDA test) of the pollen grains (Table 3.8) female and gametophyte tissue was checked and a record of the contamination of the cultures was kept.

Sterilisation by 'Domestos' was effective for the female gametophyte tissue, the viability not being adversely affected; however, alcohol flaming of the male strobilus was the most effective method with respect to both the retention of pollen grain viability and the removal of fungal and bacterial contaminants. This method was used in all future experiments.

<u>Table 3.8</u> Disinfestation methods and their effect on the viability of the pollen grains of <u>P. contorta</u> and the % contamination of the cultures.

METHODS OF DISINFESTATION	CONE	VIABILI INITIAL	TY (%) FINAL	CONTAMINATION %
5 minutes in 'Domestos' (20% v/v)	A	32.60	14.54	10.0
	В	48.46	11.38	14.0
'Flaming' in alcohol	А	32.60	28.87	1.0
	В	48.46	34.16	0

A total of 500 pollen grains from several microsporophylls were counted to calculate the % viability (as estimated by the FDA test).

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Fig. 3.4

Details of microsporangiate and ovulate strobili of Pinus

1. L.S. very young ovulate (female) strobilus.

2,3. Underside and upperside views of ovuliferous scales.

4. Ovule at fertilisation. The female prothallus is made up of haploid tissue.

5. L.S. microsporangiate (male) strobilus.

6. Underside and lateral views of sporophylls.

(Taken from Vine and Rees, 1968).

Fig. 3.4

DETAILS OF MICROSPORANGIATE AND OVULATE STROBILI OF PINUS















3.5.3 DEVELOPMENTAL STAGE OF MALE AND FEMALE GAMETOPHYTE AT INOCULATION

Female gametophytes from strobili collected in June and July were introduced to culture. After surface sterilisation the female gametophyte (prothallus) (Fig.3.4.4) was dissected out by removing the integuments of the ovule, the diploid nucellar tissue, and the fertilised ovule. 10 female gametophytes (female prothalli) were inoculated onto media, supplemented with either Img 1^{-1} 2,4-D, 5mg 1^{-1} 2,4-D or Img 1^{-1} 2,4-D and Img 1^{-1} kinetin. None of the cultures showed proliferation after incubation for 100 days at 25° C under continuous light although they appeared healthy.

In 1975, microsporangiate strobili were collected at intervals from the end of April, through until the middle of June when all the pollen had been shed. The sequence of pollen grain development of Pinus species has been described by Sterling (1963). Fig. 3.5 summarises the main stages in the development of Pinus pollen grains. After its liberation from the wall of the pollen mother cell (Fig.3.5.2) the pollen grain enlarges and commences a programme of cell division (Fig. 3.5.4). The cell divides to produce the vegetative gametophyte generation (prothallial cells) and the tube cell. The prothallial cells are short-lived, disintegrating very quickly. Further division of the tube cell results in two cells with unequal sized nuclei, the antheridial and tube cells (Fig. 3.5.5). The pollen is shed at this stage of development, further cellular divisions occurring in the following Spring (Figs. 3.5.6 - 3.5.8).

Material collected in April was in the tetrad stage, no further development of the pollen grains was observed

Fig. 3.5

Diagram of the stages in development of a pollen grain of Pinus

1. Pollen mother cells.

2. Tetrad of microspores (pollen grains).

3. Prior to shedding.

4. After shedding.

5. Prior to pollination.

6. Growth into nucellus.

7. Continued growth during the following spring.

8. Before fertilisation.

(Adapted from Vines and Rees, 1968)

Fig. 3.5

DIAGRAM OF THE STAGES IN DEVELOPMENT OF A POLLEN GRAIN OF PINUS









either during culture or storage. Material collected later in May and June was in the uninucleate and bicellular stages; storage at 25[°]C for 7 days resulted in pollen liberation. The effect of cold storage on the viability of the pollen grains predominantly in the bicellular stage was compared with pollen grains predominantly in the uninucleate stage. The results presented in Table 3.9 show that the immature pollen grains (uninucleate stage) survived cold storage (16 days) better than the mature pollen.

<u>Table 3.9</u> <u>P. contorta</u> pollen grain survival after cold storage (2.4^oC) at 70-100% relative humidity for 16 days.

APPEARANCE OF	STAGE OF POLLEN	VIABILITY (%)*		
MALE STROBILUS	DEVELOPMENT	INITIAL	FINAL	
Brown, firm	Uninucleate- bicellular	47.21	36.78	
Yellow, separated microsporophylls	Bicellular	44 94	4 70	
microsporophyris	DICEILUIAI	11.04	· · · · · ·	

* The results are taken from the average of 5 different cones for each stage.

3.5.4 CULTURE OF INDIVIDUAL MICROSPOROPHYLLS PLUS MICROSPORANGIA

Stage of pollen_development	:	tetrad - uninucleate
Storage	:	25 ⁰ C for 3 days
Viability	:	41.38%
Hormones	:	$lmg 1^{-1}_{-1} 2, 4-D and lmg 1^{-1} kinetin$

A total of 100 microsporophylls were inoculated into McCartney bottles and incubated at 25⁰C in continuous light.
After 16 days in culture, green callus was produced from 22% of the explants. The callus appeared to originate from the microsporophyll or microsporangia and not from the pollen grains. The cells exhibited a high degree of wall lignification which prevented microdensitometric investigations of the ploidy level of these cells. No reliable chromosome counts were made, but the apparent origin of the callus from the microsporophyll or microsporangia and the failure to detect any divisions in the pollen grains suggests strongly that the callus was of diploid origin.

This experiment was repeated using three kinetin concentrations in combination with 2,4-D ($lmg l^{-1}$) and strobili from a later harvest.

Since the developmental stage of pollen with each cone proved to be very similar (pollen in the basal microsporophylls being slightly in advance of those at the apex), microsporophylls from the same cone were used.

Cultures were sacrificed at intervals in order to determine the % viability of the pollen grains (average of 10 microsporophylls) and to observe any cytological events occurring during the culture period. After only 7 days, the microsporangia had expanded and become yellow- brown in colour. Microscopical examinations revealed that some of the pollen grains had produced pollen tubes (Plate 4). After 19 days in culture, the viability of the 'germinating' and'non-germinating'pollen grains had decreased to less than 5% (Table 3.10). The cultures were discarded after a further 80 days.

Plate 4

1

Abnormal growth of pollen tubes (t) from pollen grains (g) of <u>P. contorta</u> cultured on MS with $lmg 1^{-1}$ 2,4-D and $lmg 1^{-1}$ kinetin, 19 days from inoculation. Note the high starch content of the grains.

Α. x 200 B, C, D. x 600



PLATE 4

Table 3.10 The effect of kinetin and incubation time on the

viability and germination of <u>P. contorta</u> pollen grains.

Initial viability = 91% Developmental stage = Uninucleate - bicellular Storage = 25⁰C for 3 days

HORMONES lmg 1 ⁻¹ 2,4-D	VIABILI	TY (%) A	AFTER	POLLEN TUBE EMERGENCE (%) AFTER		
plus kinetin at	7 days	12 days	19 days	7 days	12 days	19 days
0.5mg 1 ⁻¹	22.03	7.71	4.11	40.00	49.32	51.35
1.Omg 1 ⁻¹	20.39	0.98	0	25.00	31.69	36.74
3.Omg 1 ⁻¹	24.46	0.60	0.88	17.12	22.74	40.19

3.5.5 THE EFFECT OF COLD STORAGE AND CENTRIFUGATION ON HAPLOID CALLUS INITIATION

Haploid callus initiation from gymnosperm pollen grains has been reported to be enhanced by subjecting the gametophyte tissue to cold storage (Tulecke, 1960; Bonga, 1974) and dry centrifugation at 2000 revs min⁻¹ for 30 minutes (Bonga and McInnis, 1975). No additional information was provided to indicate the gravitational pull exerted on these centrifuged pine pollen grains, however, centrifugation of <u>Nicotiana</u> anthers at 500 g for 60 minutes has been reported to stimulate haploid plant production in culture (Nitsch, 1977). In the experiment described here microsporangiate strobili of <u>Pinus contorta</u> were centrifuged at 500 g for 30 minutes, after a period of cold storage, prior to inoculation.

The microsporophylls from one cone of <u>P. contorta</u> were divided into a control and a centrifugation treatment. After dry centrifuging the strobili, the microsporophylls were inoculated onto the three media (100 per treatment). Samples were taken at intervals and the pollen viability monitored. After 15 days the pollen viability had decreased to 10% (Table 3.11) but no pollen emergence was observed. The cultures were stained with acetocarmine but no unusual cytological events could be seen to be occurring in the pollen at this time.

The cultures were examined at day 35 from inoculation; some of the control microsporophylls had burst open revealing a mass of creamy-white callus. The inocula which had been centrifuged showed no visible signs of callus initiation until 85 days in culture (Table 3.11).

<u>Table 3.11</u> Microsporophyll culture of <u>P. contorta</u> with and without prior centrifugation.

Initial viability : 46.15% Final viability (day 15) : 10.02% Stage of pollen development: Uninucleate-bicellular Storage : 2-4^oC for 24 days

(Centrifugatio	ņ	treatment	:	500g	for	30	minute	s
	·····								_

HORMONĘS	TREATMENT	%MICROSPOROPHYLLS	PRODUCING CALLUS*
$(mg 1^{-1})$		36 days	85 days
2,4-D (1.0)	Control	14.0	21.0
	Centrifuged	0	1.0
2,4-D (5.0)	Control	· 14.0	20.0
	Centrifuged	0	3.0
2,4-D (1.0)	Control	10.0	12.0
kinetin(3.0)	Centrifuged	0	4.0

KEY

* data based on the appearance of callus from the microsporophyll (100 treatment $^{-1}$)

Plate 5

P. contorta pollen grains stained with aceto-carmine.

A. Fresh pollen at the uninucleate-bicellular stages. Note the unequal sized nuclei and the position of the prothallial cells (arrowed), the nuclei of which do not stain.
B. Uninucleate pollen grains.

C,D. Pollen grains with two equal sized nuclei. x 900
E,F. Pollen grains with three nuclei. x 900
G. Pollen grains with four nuclei. x 900
H. Release of pollen grain protoplast containing two nuclei. x 900
I,J. Dividing cells of haploid origin after release from pollen grains. x 500



Plate 6

A - D Callus initiated from pollen grains of <u>P. contorta</u>. Note the pollen grains (g) interspersed in the callus mass and the tracheids (t) present in the callus in Plate 6C. \times 400



Microscopic examinations were made on microsporophylls which showed no visible signs of callus production in order to determine the origin of the callus. Normally developing pollen grains in the bicellular stage exhibit unequally staining nuclei sited along an axis with the prothallial cells (Plate 5.1), the prothallial nuclei rarely staining at all. However, in cultured microsporophylls, the pollen was observed to contain abnormally dividing nuclei. Numerous pollen grains contained two equally staining nuclei in random positions, in others three or even occasionally four nuclei were present (Plate 5). Whether the grains were multinucleate or comprised several cells at this stage was difficult to determine, cell walls rarely being evident. The contents of the pollen grain were released (Plate 5.8) and further cell divisions occurred to give rise to callus masses (Plate 6). These cells divided until the callus eventually caused the microsporangia to burst open, releasing the creamy-white callus. The data presented in Table 3.11 are observations of the appearance of callus from the cultures and therefore not the actual % of microsporophylls in which pollen division was occurring.

The origin of these calluses was undoubtedly from the pollen grains; however, to confirm this, stationary phase callus samples were taken and prepared for Feulgen microdensitometry. Small calluses evidently arising from different pollen grains were frequently of different ploidy levels, which complicated the data obtained. In addition to taking readings for totally random nuclei, areas of callus were selected which appeared to have arisen from the same source. Figs 3.6.1 and 2 present readings taken from

Fig. 3.6

DNA content of cells from callus initiated from pollen grains of Pinus contorta.

- Histogram 1. Readings taken from a restricted area of the slide, where the one pollen grain probably gave rise to the callus (see Plate 6). The callus was predominantly haploid.
- Histogram 2. As histogram 1 but the callus was predominantly diploid.
- Histogram 3. Readings taken from 3 separate slides from the same experimental treatment show a range of ploidy levels.



different regions of the same slide, whereas Fig. 3.6.3 comprises totally random readings. Thus the total callus mass produced from the pollen grains is a haploid-diploid mixture, with calluses originating from different pollen grains apparently being predominantly haploid or diploid.

Transfer of the callus with the microsporophyll to fresh medium resulted in the necrosis of the callus in all instances, therefore no investigation of the stability of the ploidy level of the callus pieces through subsequent passages could be made.

It is interesting that the callus produced from the pollen grains occasionally produced tracheids (Plate 6.4) within very few divisions from initiation.

3.5.6 THE EFFECT OF COLD STORAGE TIME ON CALLUS INITIATION FROM P. CONTORTA POLLEN

Microsporangiate strobili of <u>P. contorta</u> were collected on May 25th <u>1976</u>. The object of this experiment was to determine the effect of cold storage on callus initiation from pollen under the conditions found to be optimal in the previous year (Section 3.5.5).

100 microsporophylls from cone A and cone B were inoculated on to media containing $lmg 1^{-1}$ 2,4-D after 16, 24 and 27 days cold storage. Whole strobili were used for inoculation of media after 35 days cold storage. Table 3.12 shows that pollen stored for up to 27 days prior to culture produced germ tubes, whereas some of the intact strobili (after 35 days cold storage) showed callus production.

It would appear that the cold storage time of 27 days was not sufficient to stimulate callus production from the the dissected microsporophylls, the intact strobili had been stored for a longer time. Whether the different type of inocula (i.e. dissected microsporophylls versus intact strobili) had an effect or not is open to debate. Microdensitometric studies of the callus produced from 3 separate strobili revealed a range of ploidy levels, but no haploid cells (Fig. 3.7). However microscopic examinations revealed that the callus had probably originated from the pollen, many pollen grains containing 3 or 4 nuclei.

Table 3.12 Cold storage effect on callus initiation of

P. contorta pollen.

Stage of pollen development : uninucleate-bicellular

:

Viability

cone	A –	34.17%
cone	в –	56.67%
2-4 ⁰	C fo	r various

times

Storage

Hormones

 $: lmg 1^{-1} 2, 4-D$

CONE	TIME OF STORAGE (DAYS)	POLLEN TUBE EMERGENCE %	% MICROSPOROPHYLLS PRODUCING CALLUS
A	16	12.0	0
В		6.4	0
A	24	70-80	0
В		70-80	0
A	27	70-80	0
В		70-80	· 0
A	35	0	4/11*
·B		0	1/9*

KEY

* Intact strobili

Fig. 3.7

The range of DNA content of cells from callus initiated from pollen grains of <u>Pinus contorta</u>.

Histograms 1, 2 & 3. Readings from several slides from three strobili. No peak was observed for haploid cells.

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3.5.7 GENERAL POINTS TO BE CONSIDERED

There are inherent problems in the development of haploid callus cultures of trees. The relatively short season when male strobili are produced, and the female strobili are at the optimal developmental stage for callus induction from the gametophyte, severely restricts experimental work. Prolonged cold storage extends the period during which pollen is available, in addition to stimulating haploid callus production, but the pollen deteriorates after a few months in store.

A comparison between the data obtained from male strobili collected in 1975 and 1976 reveals a difference in the percentage of the microsporophylls producing callus from pollen (Tables 3.11 and 3.12). The viability and developmental stage of the pollen was similar in both experiments and the medium employed in Table 3.12 had previously supported callus initiation from pollen. It appears that the material collected in 1976 required longer cold storage to induce callus initiation.

Since the strobili were harvested in Edinburgh and introduced to culture in Leicester, the time and conditions in transit could have adversely affected the material. Ideally this work should be carried out within easy access to the mother plants in order to minimise damage to the pollen prior to inoculation into culture.

3.6 DISCUSSION

Callus cultures have been initiated from seedlings and mature shoots of both <u>Pinus contorta</u> and <u>Picea sitchensis</u>.

<u>P. contorta</u> seedling callus proved to be capable of continued subculture over a period of 18 months, whilst the mature tissues of both species and seedling tissues of P. sitchensis only survived a limited number of passages.

Although the basal media of Heller (1953), Reinert and White (1956), Linsmaier and Skoog (1965)(LS) and Murashige and Skoog (1962) (MS) all supported callus initiation from <u>P. contorta</u> seedlings, only the media MS and LS allowed further subculture. The relatively simple basal media of Gautheret (1942), Heller (1953) or White (1943) have generally been found to be incapable of supporting the continued growth of gymnosperm cultures. The addition of an organic nitrogen source (Reinert and White, 1956; Steinhart <u>et al</u>, 1961) or ammonium ions (David, 1972; Steinhart <u>et al</u>, 1961) to the simple media has been reported to enhance callus initiation and growth.

Neither <u>Picea sitchensis</u> stem callus nor <u>Pinus contorta</u> seedling callus, maintained on MS media, was significantly affected by the addition of the undefined organic supplements casein hydrolysate and coconut milk, nor the amino acid supplement of Durzan <u>et al</u> (1973) (with the possible exception of glycine) alone or in combination. Only yeast extract altered the growth response of the cultures, causing their rapid death.

In the absence of the high levels of ammonium ions characteristic of both LS and MS media, the organic supplements markedly affect the growth of gymnosperm cultures. Steinhart <u>et al</u> (1961) reported the effects of the range of amino acids found in malt extract on the growth of <u>Picea</u> <u>abies</u> callus cultures maintained on Gautheret's medium

(as adapted by Loewenberg and Skoog, 1952). The amino acids asparagine, glutamine, arginine and aspartic acid (2-4 mM) markedly stimulated growth whilst the remainder, including glycine, methionine, glutamic acid and tyrosine (0.5 mM) were inhibitory.

Callus cultures of Pinus banksiana and Picea abies have since been successfully established by Durzan et al (1976) and Chalupa and Druzan (1973a) on LS medium supplemented with arginine (400 and 100 mg 1^{-1} respectively). In neither system studied here was arginine found to enhance growth significantly. Glycine has been reported as inhibitory at low levels $(1.0 \text{mg s}^{-1} \text{ or } 1.33 \text{ x } 10^{-5} \text{M})$ to pine root growth (Barnes and Naylor, 1959) and Pinus palustris callus (Brown and Lawrence, 1968). Both glycine and methionine (37.5mg l^{-1}) were inhibitory to Picea abies callus cultures (Steinhart et al, 1961). However, in the present study no adverse effect of glycine (300mg 1^{-1}) or methionine (30mg 1^{-1}) was observed on callus initiat_{ed} from Pinus contorta seedling or Picea sitchensis stem tissue, in fact glycine appeared to stimulate growth of P. contorta callus. Callus initiated from embryos of Welwitschia mirabilis (Button et al, 1971) has been successfully maintained on MS medium with 5mg l^{-1} glycine, whilst concentrations up to 7.5 g 1^{-1} glycine did not inhibit the growth of Acer pseudoplatanus suspension cultures (AMline) maintained on Heller's medium supplemented with 8 mM urea (480mg l^{-1}) (Everett, unpublished).

Arginine, glutamine, asparagine, coconut milk and casein hydrolysate have all been reported to be stimulatory to callus when included in simple basal medium (Steinhart

<u>et al</u>, 1961; Risser and White, 1964; Brown and Lawrence, 1968). However, in the present work, none of these compounds improved callus growth of the <u>P. contorta</u> or <u>P. sitchensis</u> cultures maintained on MS medium. Similarly Chalupa <u>et al</u>, (1976) have reported that callus initiation from <u>Pinus</u> <u>banksiana</u> seedling hypocotyls was optimal on their standard medium containing MS inorganic salts. The addition of NH₄Cl or an organic nitrogen source such as asparagine, glutamine or arginine (all at 2.3 mM) did not significantly enhance callus production.

These results suggest that the presence of high levels of ammonium ions in LS and MS media negate, or substantially reduce, the stimulatory or inhibitory effects of the amino acid supplements in the simpler basal media. Nitrate reduction and assimilation may, therefore, be a limiting factor to callus growth in the absence of ammonium ions or amino acids. Studies on nitrate uptake and nitrate reduction in gymnosperm tissues should be undertaken. Such studies may indicate that a high concentration of molybdenum is required by the culture in order to achieve a non-limiting state of nitrate reduction.

Callus cultures from sporophytic tissues of <u>Pseudotsuga</u> <u>menziesii</u>(Winton, 1972a) and female gametophyte tissue of <u>Pinus palustris</u> (Sommer <u>et al</u>, 1974) exhibited a difference in the growth of callus versus suspension cultures. In both systems, the tissue grew for a limited number of subcultures. The cultures maintained on semi-solid medium ceased growth before those growing in liquid medium. A similar effect has been observed in <u>P. contorta</u> seedling callus, where cultures maintained on semi-solid medium

survived fewer subcultures than the suspension cultures initiated from the same cell line (Chapter 4).

Romberger and Tabor (1971) reported the adverse effects of various brands of agar on the <u>in vitro</u> growth of <u>Picea abies</u> shoot tips. One of the brands mentioned as being detrimental to the growth of the cultures was Ionagar No.2, the brand used in the present callus initiation experiments on <u>P. contorta</u> and <u>P. sitchensis</u>. It is therefore possible that certain impurities in the agar used to maintain the callus stocks could have adversely affected both the callus initiation of <u>P. sitchensis</u> and the growth of <u>P. contorta</u> callus. Although any agar effect might contribute towards the final death of the cell line, it probably is not determinative. Maintenance of the callus supported on filter paper bridges over stationary liquid medium would by-pass the possible toxic effects of agar contaminants.

The limited growth of the callus cultures of <u>P. contorta</u>, and more especially <u>P. sitchensis</u>, cannot be attributed to the basal medium (MS) employed in the initiation experiments alone since media of similar inorganic composition have successfully supported the continued growth of callus cultures of <u>Pinus</u> and <u>Picea</u> species (Chalupa and Durzan, 1973a; Durzan <u>et al</u>, 1976). The problems experienced here in the maintenance of <u>P. contorta</u> and <u>P. sitchensis</u> callus over a prolonged period of time also seem to have been encountered and possibly overcome by Winton (1972a) with <u>Pseudotsuga menziesii</u> callus cultures. Alterations in the hormonal and environmental conditions allowed the continued growth of the <u>P. menziesii</u> cultures over a minimum of 12

months, as compared to 1-2 months previously. The substitution of 1AA and kinetin by NAA and BAP respectively. in combination with incubation of the cultures under long days with a diurnal range of temperature markedly improved the growth of these cultures. It is possible that growth of gymnosperm cultures is affected by the light regime. Durzan et al,(1973) reported differences in the cytodifferentiation of suspension cultures of Picea glauca when maintained under different environments. Incubation of the cultures under continuous light at 22.5°C resulted in cells at the centre of aggregates developing secondary walls with bordered pits, typical of tracheary elements, whilst vacuolated, but undifferentiated, cells exhibiting high levels of tannin and phenol accumulation were formed under a 14 hour photoperiod with a diurnal range of temperature of 22.5/12°C. The light activated enzyme, phenylalanine ammonia lyase (PAL) is a key enzyme in the secondary metabolism of higher plants, being involved in the regulation of phenyl propanoid metabolism (see Camm and Towers, 1973). Forrest (1969) observed that the activation of polyphenol synthesis in tissue cultures of higher plants most frequently occurred under high illumination. In the present study P. contorta callus cultures were generally more prone to localised browning when maintained in the light rather than in the dark. The effect of different light intensities on callus growth should be investigated to determine whether the failure of the callus and suspension culture line could be attributed to the physical environment, although White and Risser (1964) reported little difference in Picea glauca callus growth in the dark or under

various photoperiods at different light intensities.

The hormones most frequently employed in the early studies on gymnosperm cultures have been 2,4-D, NAA, IAA and kinetin. NAA was claimed to be the most effective callus inducer in Picea abies (Steinhart et al, 1961; Chalupa and Durzan, 1973a). 2,4-D also supported callus proliferation. In the present study, NAA was more effective than 2,4-D in callus initiation from P. sitchensis, but P. contorta responded to both auxins, 2,4-D producing the better callus growth. Throughout the culture period, <u>P. contorta</u> callus was maintained on $1 \text{ mg } 1^{-1}$ 2,4-D. Although this concentration of auxin supported callus initiation and its subsequent growth, it is possible that during the culture time the requirement of the callus for an exogenous hormonal supply may have altered. Supraoptimal auxin levels would result in the inhibition of growth whilst suboptimal levels could result in an increased production of secondry metabolites (i.e. phenols, tannins, lignins) as was reported in Acer turbidostat culture (King 1976), rose (Davies, 1972b) and Haplopappus (Constabel et al, 1971).

No chemical analysis or electron microscopy studies were made on the cells of the <u>P. contorta</u> callus cultures described here. It was noted, however, that the inability of the calluses to survive subculture was associated with a general decrease in cell viability and the overall lignification of both differentiated (tracheid) and nondifferentiated (parenchymatous) cells. A relationship between phenolics accumulation and growth has been observed in intact plants (Kefeli and Kutacek, 1977) and in

suspension cultures of Paul's Scarlet Rose (Nash and Davies, 1972). In general, phenolics accumulation occurs when growth slows down eg. during the late and post exponential growth phases in Rosa suspension cultures. The toxicity of exogenously supplied phenols to Paul's Scarlet Rose suspension cultures has been reported by Danks et al (1975). Certain phenols, cinnamic, p-coumaric and ferulic acids proved to be very inhibitory to the growth of the rose suspension cultures, whilst others (chlorogenic acid and scopoletin) were effective as inhibitors only at high concentrations. Thus, the overproduction of specific phenolics (accumulating when growth ceases) could result in the build-up of toxic levels of phenols or tannins in the cells. The failure of the cells to channel these phenolics into wall lignin production or into vacuoles could perhaps account for the decrease in viability observed in callus cultures of P. contorta described in this study.

Tannin accumulation has been studied in callus cultures of <u>Pinus elliottii</u> (Baur and Walkinshaw, 1974; Croley <u>et al</u>, 1973). Tannin deposition in membrane-bound structures or in the cell vacuole did not necessarily result in cell degradation, but it was suggested that the formation of free tannin molecules in the cytoplasm would precipitate proteins, causing cell death. It therefore seems feasible that the major factor causing the progressive decline in growth of the callus cultures of <u>P. contorta</u> was the uncontrolled activation of the secondry metabolite pathway, resulting in the overproduction of phenols, tannins and lignin. Suppression of phenol synthesis may possibly be achieved by incubating the cultures under lower light

intensities or in the dark. Maintenance of the cultures in the presence of higher intial levels of 2,4-D and more frequent subculture of the callus might maintain the exogenous auxin at an optimal concentration, so preventing phenol accumulation in the cells due to 2,4-D depletion.

Although Picea sitchensis tissues of both juvenile and mature origin showed poor callus initiation and growth, . callus initiated from Pinus contorta showed marked differences in the ease of callus productions from juvenile and mature explants under the conditions employed here. Juvenile tissues proliferated more rapidly to produce callus and the cultures were capable of subculture over a longer period of time. The potential of the tissues to produce callus appeared to be related to the age and possibly the physiological status of the mother plant. In P. contorta, shoots from 11 and 13 year old trees rooted well, and in culture produced more callus than did shoots from older trees (43 and 50 years old). Stoutemyer and Britt (1963) have established callus cultures of Hedera helix (ivy) from both juvenile and mature material. A comparison of the growth rates of the ivy cultures initiated from the juvenile or mature explants revealed that callus from juvenile tissue maintained a significantly higher growth rate through several subcultures. The ability of the callus to produce roots was also a characteristic of the cultures initiated from the juvenile material. Thus, juvenile and mature characteristics appear to be maintained in culture, suggesting a genotypic change or an alteration in gene expression in the mature plant (caused by irreversible gene repression during the course of maturation) leading to an altered stable

physiological phenotype.

The relative ease with which juvenile tissues proliferate in culture may be due to the cellular origin of the callus. Stimulation of cell division in both cambial and differentiated tissues may occur during callus initiation, the ability of the latter cells to divide possibly diminishing as differentiation proceeds (Yeoman, 1973). Thus the ease of dedifferentiation of cells may also affect the proliferation of the tissues, juvenile explants being relatively undifferentiated as compared to mature material.

Many of the early studies on gymnosperm cultures utilised explants from mature trees (Reinert and White, 1956; Harvey and Grasham, 1969) which may, in combination with the relatively simple media used to initiate callus cultures, explain the low success rate in continued maintenance of these early cultures.

The ability of haploid tissues (from both male and female gametophyte generations) to produce callus has been reported for several gymnosperm species. In the present study, immature pollen grains of <u>Pinus contorta</u> could be induced either to produce germ tubes or to give rise to callus, whereas the female gametophyte failed to proliferate in culture. It is possible that the stage of development of the female gametophyte material was not optimal for callus production. The megagametophytes of <u>Pinus resinosa</u> (Bonga and Fowler, 1970) and <u>Picea abies</u> (Huhtinen, 1976) showed optimal callus production if collected at a specific developmental stage - approximately 1 month after the fertilisation of the embryo.

Callus initiation from the immature pollen grains within cultured strobili or separated microsporophylls appeared to be dependent on several factors. Callus cultures were formed from pollen grains which were in the uninucleate-bicellular stage of development and which had been subjected to prolonged cold storage. The results obtained indicated that the omission of the cold storage treatment of pollen in this developmental stage resulted in pollen germination and not callus production. These results agree with the findings of Bonga (1974) and Tulecke (1960) who reported that cold storage enhanced callus production from gymnosperm pollen. A cold shock treatment (from $20^{\circ}C$ to $3-5^{\circ}C$) enhanced embryoid production from Datura and Nicotiana (Nitsch and Norreel, 1972). Thus it would appear that a cold treatment alters the normal developmental pattern of the pollen grains, perhaps by the disruption of the microtubules during the first haploid mitosis. The duration of the cold treatment required may vary according to the species studied since the retention of pollen grain viability differs between spieces. The application of this technique to anther cultures of species which have not responded by either haploid embryogenesis or callus production may be advantageous.

In addition to the stimulation of abnormal divisions of pollen grains by a cold treatment, centrifugation of the pollen also enhances this response in culture. Embryoid production from anthers of <u>Nicotiana</u> (Nitsch, 1977) and callus production from <u>Pinus resinosa</u> (Bonga and McInnis, 1975) were stimulated by centriguation prior to culture. The mechanism by which the pollen is forced to alter its

normal developmental sequence is open to speculation. However, these two treatments, cold and centrifugation may affect the inherent polarity governing the division of the single nucleus within the pollen grain, thereby giving rise to two equally instead of unequally - staining nuclei within the grain. This production of pollen grains containing two randomly positioned nuclei of equal size appears to be the first major event in the differentiation of these grains. Further studies are obviously required in order to understand the nature of the control of the divisions which may determine whether the dividing pollen produces callus or embryoids.

It has been suggested that the development of a technique whereby homozygous diploid plantlets could be obtained from gymnosperms would be useful in tree breeding (Bonga, 1977). However, many conifers contain deleterious recessive genes which may result in the depression of growth of the plants obtained (Winton & Stettler, 1974). Therefore, the potential of haploid callus for homozygous diploid regenerants may be limited, although it could be important for the expression of advantageous recessive mutants.

Limited differentiation has been observed in haploid tissues of several gymnosperms (La Rue, 1954; Radforth and Bonga, 1960; Bonga, 1974) and small plantlets have been regenerated from megagametophyte cultures of <u>Zamia integrifolia</u> (Norstog, 1965) and <u>Ginkgo biloba</u> (Tulecke, 1965). Although the haploid callus cultures of <u>P. contorta</u> described here did not exhibit plantlet regeneration, cytodifferentiation was observed in the form of tracheid production. Cell differentiation (tracheids and resin cells) has previously

been reported in female gametophyte cultures of <u>Ginkgo</u> <u>biloba</u> (Tulecke, 1967) and in <u>Picea abies</u> Huhtinen (1976). Cytodifferentiation, as xylogenesis, has also been observed in gymnosperm cultures from sporophytic tissues. Tracheid production in <u>Cupressus lusitanica</u> (Borchert, 1968) and <u>Pinus palustris</u> (Perry, 1972) could not be controlled by alterations in the hormonal milieu, an observation also made here for <u>Pinus contorta</u> callus. White and Gilbey (1966) reported the differentiation of tracheids in <u>Picea glauca</u> callus cultures when incubated in the light and on an ammonium ion containing medium. The development of suspension cultures capable of continued subculture from these <u>P, contorta</u> callus cultures would possibly allow further investigation into this phenomenon.

The immediate objective of the work described in this chapter was to initiate and maintain callus proliferation on a defined medium. Callus initiation has been successful from diploid (juvenile and mature) tissues of Pinus contorta and Picea sitchensis, and from haploid (male gametophyte) material of P. contorta. Maximal callus initiation from P. sitchensis explants was achieved when NAA substituted 2,4-D as the auxin source, whilst P. contorta explants responded to the presence of 2,4-D by prolific callus production. The limited survival of the callus cultures from all sources might not necessarily interfere with studies on the morphogenetic capability of the cultures. The decline of embryogenic expression during serial subculture has been reported for cultures of several species, including Daucus carota (Smith and Street, 1974). Studies directed towards the expression of the morphogenetic

potential of explants of <u>P. contorta</u> and <u>P. sitchensis</u> both with and without a preceding callus phase, are described in Chapter 5. Investigations of the phenomenon of cytodifferentiation in suspension cultures initiated from <u>P. contorta</u> callus and seedlings are presented in Chapter 4.

CHAPTER 4

SUSPENSION CULTURE INITIATION & ESTABLISHMENT

CHAPTER 4

4.1 INTRODUCTION

The many possible applications of plant cell suspension cultures have been fully discussed by Street (1977). The expression of totipotency by such cultured cells must be of high interest when considering the possibilities of clonal propagation of economically important species. Previous workers have initiated suspension cultures from juvenile somatic tissue of several gymnosperms, but the continued proliferation of these suspensions on subculture has been reported for only some of these species : <u>Picea glauca</u> and <u>Pinus banksiana</u> (Durzan and Steward, 1970), <u>Pinus gerardiana</u> (Konar, 1963) and Pseudotsuga menziesii (Winton, 1972a).

The embryogenic potential (capacity to produce embryolike structures from somatic cells) of suspension cultures initiated from <u>Daucus carota</u> (carrot) and other Umbelliferae has been well-documented. The withdrawal of an exogenous auxin supply from the cells results in the induction of numerous somatic embryos (embryoids) which are capable of continued growth to form apparently normal plants (Halperin and Wetherell, 1964; Steward et al, 1964; Williams and

Collin, 1976). The embryogenic potential of the carrot suspension cultures declines with increased time in culture. The factors controlling this loss of embryogenic potential have not yet been unequivocally determined (Reinert <u>et al</u>, 1971; Meyer-Teuter and Reinert, 1973; Smith and Street, 1974).

The ability to produce embryoids from cultures initiated from somatic cells is not restricted to the Umbelliferae but has been shown to occur in cultures initiated from a wide taxonomic range of angiosperms (see Reinert et al, 1977). However, very few species which are generally referred to as 'woody' (i.e. trees and shrubs) have been reported to produce embroids in vitro. Callus cultures from immature embryos of Corylus avellana (Radojević et al, 1975), nucellar and somatic, stem-derived tissues of Vitis (Mullins and Srinivasan, 1976; Krul and Worley, 1977) and nucellar tissue of Citrus spp. (Button and Bornman, 1971; Kochba et al, 1972) have, on appropriate media, yielded embryoids. In unpublished work (cited by Winton, 1974), Winton claimed the induction of embryoids from callus and suspension cultures of several gymnosperm species - Pinus taeda, Pseudotsuga menziesii, Tsuga heterophylla and Pinus echinata and from callus cultures of Pinus contorta. The suspension cultures initiated from P. contorta callus were very nodular in appearance yet did not form embryoids. In suspension cultures of Pice glauca and Pinus banksiana, Durzan and Steward (1970) described patterns of growth which showed some resemblance to the development of the zygotic embryo of these species.

Whilst the embryogenic potential of cultured gymnosperm tissues may be limited, many cultures exhibit continued

xylogenesis, e.g. callus cultures of Pinus cembra (Salmia, 1975) and both callus and suspension cultures of Picea glauca (White and Gilbey, 1966; Durzan et al, 1973). Both of these species retained the capacity for such cytodifferentiation through several successive passages, indicating that the production of tracheids was not due to any carry-over of explant cells destined to become tracheids. The physical and nutritional environment appeared to be an important factor in determining the cytodifferentiation response of Picea glauca cultures. Thus cells with the reticulate and lignified cell walls typical of tracheary elements were observed only in callus which had been incubated at $25^{\circ}C$ in the light, on a medium containing NH_ACl (White and Gilbey, 1966) and in suspension cultures which were subjected to continuous light at 25[°]C (Durzan <u>et al</u>, 1973). Maintenance of these suspension cultures under a 14 hour photoperiod with a diurnal temperature oscillation of 23.5° C and 12.0° C inhibited tracheid production.

The ability of <u>P. contorta</u> callus cultures to produce tracheids after many subcultures (Chapter 3) suggested that in addition to studies to determine any morphogenetic potential of the cells, the cultures might prove to be useful in investigations into the general phenomenon of xylogenesis (i.e. cytodifferentiation).

Cell differentiation producing tracheary elements has been extensively studied in several systems, including suspension cultures of <u>Centaurea cyanus</u> (Torrey, 1975) and explants of pea roots (Phillips and Torrey, 1973), lettuce pith (Dalesandro and Roberts, 1971), <u>Coleus</u> sp. (Fosket, 1968) and Jerusalem artichoke tuber (Minocha and Halperin,

1974). The latter tissue produced tracheary elements up to 30% of the total cell population. In a survey of the literature, no example was found to exceed this percentage of tracheary element formation. Whilst the study of xylogenesis in explant and suspension cultures is not without difficulties, the two systems studied in conjunction may yield progress in understanding the cellular regulation of cytodifferentiation.

The study described below is mainly concerned with the establishment and maintenance of suspension cultures of <u>Pinus contorta</u>. The potential of these cells to develop into specialised cell types i.e. tracheary or phloem elements, or more complex structures such as roots, shoots or embryoids will be explored.

During these investigations the two culture lines (1A and 721) which were studied most intensively showed erratic growth, especially after a prolonged time in culture; this was particularly noticeable in culture line 1A. The variable growth of these cultures made the repetition and confirmation of results very difficult. The cells inoculated into experimental media frequently failed to divide or if they did proliferate the data obtained did not confirm previous results. Such data which have been considered to be relevant to the main text have been included in Appendices and referred to in the text.

EXPERIMENTAL

4.2 THE INITIATION OF SUSPENSION CULTURES

Throughout the following experiments, the medium of Murashige and Skoog (1962) supplemented with lmg 1^{-1} 2,4-D and lmg 1^{-1} kinetin, and 6.2mg 1⁻¹ boric acid will be referred to as MS1. Media with O and 124mg 1⁻¹ boric acid will be designated MSO and MS2 respectively.

The following two methods were employed to initiate suspension cultures of Pinus contorta and Picea sitchensis :

A. Friable pieces of callus (approximately 0.5g fresh weight) were transferred from semi-solid to agitated liquid medium (MS1). <u>P. contorta</u> callus quickly dispersed into smaller cell aggregates whereas <u>P. sitchensis</u> callus remained compact.

B. Proliferation of sterile hypocotyl explants was induced by introduction into agitated liquid initiation medium (MS) supplemented with auxins and cytokinins at a range of levels (Tables 4.1 and 4.2).

The <u>P. contorta</u> suspension cultures produced by either of these methods continued to proliferate on transfer to larger vessels (with an additional 30ml fresh medium). The cultures of <u>P. sitchensis</u> failed to continue growth on any of the media employed.

When the cell density of the <u>P. contorta</u> cultures was judged to be sufficiently high, the cells were subcultured into fresh medium using an automatic syringe fitted with a wide bore canula (2.5mm diameter). By the techniques described, several suspension culture lines have been
successfully initiated, either directly (method B) or indirectly (method A) from <u>P. contorta</u> seedling hypocotyl.

<u>Table 4.1</u> Suspension culture initiation from seedling hypocotyls of <u>P. sitchensis</u> and <u>P. contorta</u> using MSl medium with different hormone combinations.

AUXIN (lmg l ⁻¹)	CYTOKININ (lmg 1 ⁻¹)	P.CONTORTA	P. SITCHENSIS
IBA	BAP	O/5	0/4
IBA	kinetin	0/5	0/5
NAA	BAP	1/3 ^a	3/4
NAA	kinetin	9/9 ^a	4/6
2,4-D	BAP	5/6 ^b	1/6
2,4-D	kinetin	7/7 ^c	0/5

KEY

a Callus, browned quickly

b Callus mainly, creamy-yellow

c Fine suspensions, creamy-yellow

Table 4.2 Suspension culture initiation from seedlings of

P. contorta on MS1 medium.

SEED BATCH	GEOGRAPHICAL EXE SOURCE TO	PECTED TOLERANCE WATERLOGGING	NO. SUSPENSION a CULTURES INITIATED
72 (7986)1	Outer Island, Alaska.	Tolerant	5/5
65 (7113)1	Fort Fraser	Sensitive	5/6
64 (79)	South coastal.	Unknown	4/6

KEY

a number of suspensions initiated per number of seedlings

cultured.

Only one suspension culture line was initiated from callus (line 1A - Chapter 3). These cultures were made up of small cell aggregates which were creamy-yellow in colour. The culture lines initiated from the three seed batches with different expected tolerances to waterlogging (Table 4.2) showed considerable morphological differences. Cultures established from batch 72 (7986)1 were characteristically creamy-yellow in colour comprising small cell aggregates up to 3mm in diameter. By contrast, the cultures initiated from seed batches 65 (7113)1 and 64 (79) were more brown and exhibited a higher degree of cell aggregation (up to 10mm in diameter).

The cultures initiated from each seed batch will subsequently be designated according to their source and the number of lines established per seed batch. The first two figures indicate the original seed batch i.e. 72, 65 or 64 from the coding above, whilst the third number indicates the line number within each of the three original batches. Thus lines 720, 721, 722, 723 and 724 are five separate lines initiated from the seedlings with expected waterlogging tolerance.

These <u>P. contorta</u> suspension cultures were routinely maintained on either MS1 or MS2 media. The cell lines used in these investigations were initiated at different times, the survival time of each culture proving to be limited. Of the 37 <u>P. contorta</u> culture lines initiated 7 were subcultured through several passages. The culture lines designated 1A and 721 survived longest - 15 and 16 months respectively.

4.3 THE ESTABLISHMENT AND CHARACTERISATION OF SUSPENSION CULTURES

4.3.1 AN INVESTIGATION OF THE TOLERANCE OF SUSPENSION CULTURE LINES 720, 721 AND 650 TO ANAEROBIOSIS

As stated previously, the morphology of these culture lines differed considerably. The cultures initiated from the seedlings with expected sensitivity to waterlogging (S) did not survive subculture as well as those from the 'tolerant' seedlings (T). Since waterlogging results in a rapid decline in the dissolved oxygen content of the soil water (Jackson and Campbell, 1976) the effect of anaerobiosis on the cell viability of 'T' and 'S' culture lines was investigated.

Suspension lines 720, 721 (T) and 650 (S) were chosen as experimental systems because the viability of the cells was the highest from each source. Two ml aliquots of cells, four days from inoculation into the second passage from initiation, were dispensed into 4ml screw-capped vials. Nitrogen was bubbled through the samples for 3 minutes, after which time no oxygen was found to be in the medium. The vials were immediately sealed with isoprene discs and screw caps and strapped to a tumble-shaker to prevent the cells Samples were taken at intervals over 43 from settling. hours (2580 minutes) and the viability of the cells was estimated using the FDA test (Table 4.3). Accurate estimation of the viability of these cells was difficult due to the aggregation of the cells, only the outermost cells being observed. The UV fluorescence of FDA treated cells of the 'S' line 650 was generally weaker than that

observed in the 'T' lines 720 and 721. In the former case, the yellow fluorescence of lignin interfered with the detection of the typical green fluorescence of the viable cells.

Table 4.3	Survival of culture lines 720 and 721 (T	') and
• •	650 (S) under anaerobic conditions.	

TIME (Mins)	% VI 720	ABILITY OF THE 721	CELLS 650
0	90	85	70 ·
3*	86	81	70
65	65	70	35
120	55	70	35
300	40	_a	20
1320	40	50	20
2580 (43hrs)	35	37	15
% cells remaining viable after 43 hours	39	44	21

KEY

* anaerobic

a reading not taken

The similar initial viabilities of lines 720, 721 and 650 allow a comparison between their responses to anaerobiosis. The viability of the 'S' line dropped more quickly within the first hour of anaerobiosis that did that of the 'T' lines. After a further 42 hours anaerobiosis a higher percentage of cells of the 'T' lines had retained their viability (Table 4.3).

Regrowth of the cells after 'plating out' would have

provided a more reliable estimate of the viability of the cells after a period of anaerobiosis. However, this experiment was not repeated under sterile conditions, as was originally intended, because the 'S' line 650 survived for only one more passage.

4.3.2 THE CONSTRUCTION OF GROWTH CURVES FOR SUSPENSION LINES 1A AND 721

Two culture lines initiated from <u>P</u>, contorta seedling hypocotyl by methods A and B proved capable of continued subculture. Suspension culture line 1A was derived from callus (Chapter 3) whilst 721 was initiated directly from the seedling. Both culture lines were successfully subcultured through several passages of 21 days before any attempt was made to characterise their growth. The three parameters of growth cell number, dry weight and packed cell colume (p.c.v.) were determined as described in Materials and Methods, Chapter 2.

Culture line 1A was inoculated into fresh medium at a density of 3 x 10^4 cells ml⁻¹ (Fig. 4.1). After a 4-5 day lag phase the cells divided rapidly reaching stationary phase after 12-14 days. Dry weight accumulation showed a growth curve similar to that of cell number, while p.c.v. (reflecting cell expansion as well as cell division) increased more slowly. During the lag phase the medium became more acid (pH 4.0), presumably due to NH⁺₄ ion uptake. The pH rose again during the growth of the cells reaching pH 5.0-6.0 in stationary phase. Although these growth parameters were measured for each subsequent experiment, they will only be presented if they differ from those of



Changes in the parameters of growth (p.c.v., dry weight and total cell number) and pH of the medium plus cells during one passage of suspension culture of <u>P. contorta</u> (line IA).

the control.

It was observed that both culture lines contained two distinct cell populations, thin-walled parenchymatous cells and thick-walled cells with sculpturing typical of tracheary elements (Plate 7). The pitted walls of the latter cells stained positively with phloroglucinol-HCl, indicating the presence of lignin. Although the shape of these lignified cells varied and generally lacked the elongate form typical of tracheids, they undoubtedly were comparable with cultured cells conventionally described as tracheary elements or tracheids. Accordingly, the term tracheids will be used in subsequent discussions.

Growth curves were constructed for cells of lines 1A (MS1 medium $\frac{+}{-}$ lg 1⁻¹ casein hydrolysate) and 721 (MS2 medium); total cell counts and tracheid counts are presented in Figs. 4.2 and 4.3 respectively. In culture line 1A tracheids accounted for 6-15% of the total population, regardless of the casein hydrolysate supplement. However, in culture line 721 which was inoculated into MS2 medium at three different cell densities (Figs. 4.3.1, 4.3.2 and 4.3.3), between 35 and 50% of the total cell population had differentiated into tracheids. The duration of the lag phase was directly related to the inoculum density in these cultures, inoculation at low cell densities resulting in an extended lag phase.

The standard errors have been inserted on Fig. 4.3.1 only; the accepted level was 5% for total cell number and 10% for tracheid number. In future experiments the standard errors will be presented for the last counts only.

Plate 7

Cells of a suspension culture of <u>P. contorta</u> (line IA; 2 months after initiation) maintained on MSl medium.

A - A cell aggregate showing thin-walled parenchymatous
(p) cells and thicker sculptured walls of tracheary
elements (t). The aggregate has been stained with
phloroglucinol-HCl to indicate the presence of lignin
(darkly stained walls) x 50
B - 'Chromed' cells showing tracheids with characteristic
sculpturing. x 150





Total cell and tracheid numbers of suspension cultures of <u>P. contorta</u> (line 1A) maintained on MS1 (l) and MS1 plus lg 1^{-1} casein hydrolysate (2).



Total cell and tracheid numbers of suspension cultures of <u>P. contorta</u> (line 721) maintained on MS2, inoculated at three different cell densities.

4.3.3 THE LOCALISATION OF TRACHEIDS WITHIN THE CULTURES

Examination of both cultures under the light microscope after staining the cells with phloroglucinol-HCl revealed that the tracheids were generally localised centrally in the larger cell aggregates (ca. 2mm). This was examined quantitatively in line 1A by the following technique:

Cells were harvested from a 21 day old stock suspension culture and poured through an 'Endecotts' sieve tower, with the metal mesh sizes as stated in Table 4.4. The cells were washed through the towers with medium to which 1 drop of 'Photoflo' (Kodak Ltd.) had been added to reduce the surface tension at the mesh interface. The cells settling on each mesh were collected and resuspended in 50ml of medium. Samples of the suspensions of different sized aggregates were taken for cell counts (Table 4.4). A comparison of the total cell count was made with a control flask to determine the recovery of the cells after filtration.

Approximately 70% of the tracheids were located in the aggregates of 2.00-3.35mm diameter, 20% in the 1.0-2.0mm aggregates and only 6% in the smaller aggregates. It would appear that the degree of aggregation and tracheid production are positively correlated in these cultures.

Table 4.4 Numbers of cells and tracheids within different

The stock suspensions were maintained on MS1 medium; samples were taken after 21 days growth.

sized aggregates of suspension line 1A.

AGGREGATE (mm)	SIZE NO.CELLS ml ⁻¹ (x 10 ³)	NO. TRACHEIDS ml ⁻¹ (x 10 ³)	% TRACHEIDS
3.35	0	0	0
2.00	851 <mark>+</mark> 30	108.0 ⁺ 7.0	12.69
1.00	363_18	32.0 ⁺ 3.0	8.82
0.50	$132^{+}_{-}6$	8.9_0.9	6.74
0.30	32 2	1.0 ⁺ 0.3	3.16
0.11	$40^{+}_{-}2$	2.0 ⁺ 2.0	5.00
Filtrate	$10^{+}_{-}5$	0.8_0.1	8.00
Total	1428	152.7	10.69
Control	1457	162.0	11.12

4.3.4 FLUCTUATIONS IN THE XYLOGENIC RESPONSE OF SUSPENSION CULTURES 1A AND 721

A close scrutiny of the growth and cytodifferentiation of stock suspension cultures of both lines 1A and 721 revealed a background pattern of cytodifferentiation during the culture time. The percentage of tracheids produced by the stock cultures was estimated at each subculture (Figs. 4.4 and 4.5). It is clear that there was a general peak of ability of the cells to differentiate soon after introduction into suspension culture, and that this progressively declined with continued subculture.

In line 721 shortening the subculture time from 3 weeks



of <u>P. contorta</u> (line 1A) maintained on MS1 over 20 x 3 weekly passages.



Percentage tracheids produced by the stock suspension cultures of <u>P. contorta</u> (line 1A) maintained on MS2 over 9 x 3 weekly and 19 x 2 weekly passages.

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to 2 weeks appeared to alter the general pattern of xylogenic response which dropped sharply before rising again to its former level. The marked variation in the percentage tracheids produced by the suspension cultures at different times obviously influenced the number of viable cells in the inoculum for the following passage. Therefore, at each subculture the viable cell number (i.e. total cell number minus tracheid number) was calculated.

Prior to the death of the culture lines 1A and 721 the growth of these cultures was erratic, preventing the meaningful replication of experiments. The variable growth of these cultures coincided with the decline in the xylogenic response of the cells. It would therefore appear that the production of tracheids by the cultures did not cause the variability and eventual death of the cell lines. At this time the culture line 1A was shown to have an increased commitment to secondary metabolism, the parenchymatous cells containing high levels of soluble and condensed phenolic compounds. The general loss of viability of this culture may have been associated with this deposition of polyphenolics in the cells.

4.3.5 ESTIMATION OF THE DNA CONTENTS OF LINES 1A AND 721 BY FEULGEN MICRODENSITOMETRY

Changes in the ploidy levels of cells are often associated with prolonged culture time (Bayliss and Gould, 1974; Smith and Street, 1974). Gene amplification has also been suggested to be important in the induction of tracheary elements in plants (see Roberts, 1976) but in some culture systems (Phillips and Torrey, 1974; Dodds and Phillips, 1977)

endopolyploidy has been shown to be absent. In both lines of suspension cultures described here tracheids were produced during culture.

The nuclear DNA contents of lines 1A and 721 were measured by Feulgen microdensitometry after 6 and 8 months in culture respectively. The diploid levels of the suspension culture samples were determined by comparison with metaphase plates of <u>P. contorta</u> seedling root tips processed simultaneously. The levels were confirmed by chromosome counts (2n = 24) but very few reliable counts could be made since the chromosomes are very long and do not give good separation even after colchicine treatment.

In both culture lines the samples were taken from stock suspension cultures 8 days after inoculation into fresh medium, in order to include dividing cells. Both lines proved to be predominantly diploid with a low incidence of cells in the higher ploidy range (see Fig. 4.6).

In neither culture line was the ploidy level of the cells determined after further subculture. Line 721 died before samples were prepared for examination and the increased general lignification of the cells in line 1A after 12-15 months in culture rendered it unsuitable for nuclear microdensitometric analysis. The aldehyde groups of lignin reacted with the Feulgen stain, interfering with the DNA measurements.

4.4 THE GROWTH AND CYTODIFERRENTIATION OF SUSPENSION LINE 1A

In addition to the differences in the xylogenic response of the stock suspension cultures observed with time in

Fig. 4.6

Range of DNA contents of suspension cultures of <u>Pinus contorta</u> 8 days from inoculation.

Histogram 1. Line 1A maintained on MS1, 6 months from initiation.

Histogram 2. Line 721 maintained on MS2, 8 months from initiation.

Arrows indicate G_1 and G_2 levels of diploid root cells of <u>P. contorta</u> seedlings processed simultaneously.



culture (Fig. 4.4), experimental media did not always induce the same percentage of tracheids in subsequent confirmatory passages.

Xylogenesis in cultured plant tissues requires the presence of an exogenous source of carbohydrates (Wetmore and Rier, 1963; Fosket and Roberts, 1964). For the effects of altered levels of the carbohydrate sucrose and the substitution of glucose on cell growth and tracheid induction see Appendix III. The preliminary data suggested that the optimal xylogenic response was achieved with 1-2% sucrose, glucose (2%) also inducing tracheid production. Conflicting results were obtained in the two subsequent experiments using 1, 2 and 4% sucrose, the inoculum of one of the experimental treatments failing to grow. Sucrose at 2% was used to support the growth of the stock suspension cultures.

The stock suspension cultures were routinely maintained on MSl medium, containing 2,4-D and kinetin at $lmg 1^{-1}$. The effect of altered levels of these hormones on the growth and cytodifferentiations of the cultures was investigated (for details see Appendix IV). Lowering the 2,4-D concentration from 1.0 to 0.5mg 1^{-1} both shortened the lag phase and induced earlier tracheid production in the cultures. The presence of low levels of kinetin appeared to be essential for the growth of the cultures. Two attempts were made to repeat this experiment but on both occasions the inocula failed to grow.

A light microscopical examination revealed that at this time many of the parenchymatous cells were heavily lignified but not differentiated into tracheids. These non-

differentiated but lignified cells were generally not viable (determined by FDA test followed by the addition of phloroglucinol-HCl), therefore the inocula densities were lower than the calculated values. Samples of this stock were taken and prepared for electron microscopical examination (see section 4.4.2 and Plates 8 and 9.

Attempts were made to quantify both the soluble . phenolic and lignin contents of the cells and the 'spent' It is well known that estimations of the endmedium. products of phenolics metabolism are difficult. Soluble phenolics may be used as a measure of the phenolics metabolism of cells as any change in the rate of synthesis or condensation into alcohol insoluble products should result in altered levels of soluble phenolics. It proved impossible to measure the phenolic levels in the medium due to the interference of Mn^{2+} ions with the Folin-Ciocalteu reagent. No reliable method of estimation of the lignin content of the cells could be developed (Appendix II). The addition of phloroglucinol-HCl to the 'spent' medium (as described by Carcellar et al, 1971) did not indicate the presence of coniferaldehyde in any of the cultures tested.

4.4.1 THE GROWTH AND CYTODIFFERENTIATIONS OF CULTURE LINE 1A IN LIGHT AND DARKNESS

Phenylalanine ammonia lyase (PAL) is a key enzyme in the secondary metabolism of plants, possibly channelling the amino acid phenylalanine into phenolics (secondary metabolism) rather than proteins (primary metabolism). Numerous factors, including light, influence the levels of PAL, and therefore possibly also the phenolic concentrations within the tissues.

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The effect of incubating the cultures in the light or in total darkness was therefore investigated.

Cultures were maintained either in the light or in total darkness. Since red light is known to stimulate the activity of PAL, the cultures were transferred in low intensity green light and maintained wrapped in black polythene. The number of cells and tracheids and the concentrations of soluble phenolics were determined for three successive 21 day passages (Table 4.5). In passages 1 and 2 the dark-grown cultures produced a much higher percentage of tracheids than those incubated in the light. Further subculture of the dark-grown cells was impossible due to the low cell density after 21 days growth in passage 3.

Over the three passages in the dark there was no decrease in the final soluble phenolic concentration of the cells, nor was there any decrease as compared to the lightgrown cultures. There appeared to be no relationship between the percentage tracheids produced and the phenolic concentration of the cells.

4.4.2 LIGHT AND ELECTRON MICROSCOPICAL STUDIES ON SUSPENSION CULTURE LINE 1A, 15 MONTHS FROM INITIATION AS A SUSPENSION CULTURE

The suspension line grew erratically at the time of sampling, the viability of the cells was low and browning of the cultures was prevalent. Within the cell aggregates four distinct cell types were observed:

A. Apparently healthy cells containing nuclei, mitochondria, starch grains and numerous vacuoles. The walls

Table 4.5 Total cell and tracheary elements (T.E) numbers $(x \ 10^3 \ ml^{-1})$ and soluble phenolic concentrations (ng. e.g. gallic acid x $10^3 \ cells^{-1}$) at day 21 for light and dark grown suspension culture line IA maintained on MS1 through three consecutive passages.

LIGHT				I	DARK				
	PASSAGE NO.	CELL NO.	T.E. NO.	% T.E.	PHENOLICS	CELL NO.	T.E. NO.	% T.E.	PHENOLICS
-	1	1543	19	1.24	176.3	1089	269	24.7	356.3
	2	1398	23	1.65	439.2	1375	311	22.6	215.6
	3	1041	64	6.15	349.1	556	89	16.0	640.3

tended to be thickened and possibly lignified (Plates 8C and 9A).

B. Cells with dense vacuolar inclusions. This material varied in texture (as revealed by sectioning properties) from hard and granular to soft and homogeneous. In such cells and their neighbours the plastids were frequently in the form of starch-filled amyloplasts (Plates 8A, 8B and 9B).

C. Cells containing little or no cytoplasm but with thickened and sculptured cell walls. These secondary walls were perforated by bordered pits linking adjacent cells. The original middle lamella could still be seen in the centre of the secondarily thickened wall and sometimes also extending into the bordered pits (Plates 8C and 8D). These cells are interpreted as being tracheary elements.

D. Cells characterised by the thickenings of the cell wall marking the position of groups of plasmodesmata. Cells of this type with living cytoplasmic contents were rich in mitochondria (Plate 9C). Others with degenerating

Plate 8

into the pits.

Light and electron micrographs of cells of a suspension culture of <u>P. contorta</u> (line 1A) maintained on MS1.

- Α. Light micrograph of an aggregate containing healthy cells and cells with high levels of densely-staining material interpreted as tannin or polyphenolic material This material is difficult to section, frequently (p). breaking and leaving 'holes' in the cells. X 50 В. Enlarged region of A. Note the starch-filled amyloplasts (s) and the thick cell walls. X 130 с. Electron micrograph showing healthy cells containing nuclei (n) and starch grains (s), highly vacuolated (v) cells with dense cytoplasm and tracheids (t) with characteristic bordered pits. X 1,000 D. Bordered pits of tracheids with the middle lamella (ml) in the secondarily thickened cell wall (w) extending
 - X 7,000



Plate 9

Electron micrographs of cells of a suspension culture of $\underline{P. \text{ contorta}}$ line 1A maintained on MS1.

- A. Cell with dense cytoplasm containing mitochondria (m) and numerous vacuoles (v). Note the thickened cell wall (w). X 3,000
- B. Cell with vacuoles containing high levels of electrondense polyphenolic material (p). Note the starch grains (s) within amyloplasts.
 X 4,000
- C. Cells with mitochondria (m) and unusual thickenings of the cell wall with groups of plasmodesmata (pl). X 6,000
- D. Cell with degenerating cytoplasm. Note the lamellate cell wall (lw) and the irregular ingrowths from the wall, also the group of plasmodesmata (pl) present at one thickened region. X 2,500



Fig. 4.7

Percentage tracheids and phenolic content of suspension cultures of <u>P. contorta</u> lines 721, 723 and 640 maintained on MS2 with three concentrations of boric acid :

 $\begin{array}{c} \text{Omg } 1^{-1} \\ \text{6.2mg } 1^{-1} \\ 124.\text{Omg } 1^{-1} \end{array}$

Samples were taken at intervals during 4×3 weekly passages (arrowed).



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Fig 4.7

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tracheids produced by these lines maintained in the medium with no added boron was consistently lower than in either of the other two media. Line 723 showed tracheid production when maintained on media containing the lowest concentrations of boron, high levels of which appeared to be inhibitory to the growth of the culture line.

The production of soluble phenolics by the cells was not related to the concentration of boric acid in the medium in any of the culture lines, but the presence of low levels of boron (MSO) did appear to inhibit xylogenesis in lines 640 and 721. This phenomenon was studied further using culture line 721.

4.5.1 THE EFFECT OF BORIC ACID CONCENTRATION ON THE GROWTH AND CYTODIFFERENTIATION OF SUSPENSION CULTURE LINE 721

At this time, the cultures showed variable growth after inoculation, some cultures failing to grow. In order to avoid the problems associated with loss of cell viability during a prolonged stationary phase, the cultures were maintained on a 14 day subculture regime instead of the 21 day regime used previously.

Stock cultures were inoculated into MS2 or MSO media. The data presented in Table 4.6 are the 14 day samples from each of three consecutive passages. Both media supported tracheid production during the first passage, although fewer were formed in the low boron medium. This MSO medium inhibited xylogenesis in the second and third passages although similar final total cell densities were achieved in both media.

<u>Table 4.6</u> Cell and tracheid counts of suspension line 721 after 3 passages of 14 days in either MSO or MS2 media.

BORIC ACID	PASSAGE NO.	$\begin{array}{c} \text{CELL NO.} \\ \text{x10}^3 \text{ m1}^{-1} \end{array}$	TRACHEID NO. x10 ³ m1 ⁻¹	% TRACHEIDS
124 (MS2)	1	679	155	22.8
	2	809	152	18.8
	3	723	283	39.1
O (MSO)	1	605	85	14.1
	2	775	35	4.5
	3	745	10	1.3

This experiment was repeated using a wider range of boric acid concentrations. Since the effect of withdrawing boron on the xylogenic response was not observed until the second passage, data from the cell and tracheid counts are presented for this passage only (Table 4.7). In all treatments the shapes of the growth curves for total cell number are very similar. Since the data are so similar, detailed results are presented for only three representative concentrations of boric acid - 0, 6.2 and $124mg 1^{-1}$ (Fig. 4.8). There is no significant difference in the phenolic or amino acid accumulation by the cells maintained on these three treatments.

The withdrawal of boron from the cells resulted in a decrease in the xylogenic response of the cultures and in the synthesis of lignin (as detected by phloroglucinol-HCl and by toluidine blue which reacts to become yellow-green with soluble phenolics and dark-blue with lignin). The lack

Fig 4.8

Dry weights, p.c.v., total cell and tracheid numbers, phenolic and amino nitrogen contents of suspension cultures of <u>P. contorta</u> (line 721) maintained on MSO with three concentrations of boric acid.

 $\begin{array}{c} \text{Omg } 1^{-1} \\ \text{6.2mg } 1^{-1} \\ \text{124.Omg } 1^{-1} \end{array}$

(S.E.M. given for the last total cell and tracheid counts only).



of lignification of the cells was also reflected in their dry weight accumulation which was greater in the xylogenic cultures. The average size of the non-xylogenic culture was smaller (measured by (p.c.v.). Samples from these treatments were prepared for electron microscopical examination.

The non-xylogenic cultures were inoculated back into MS2 medium after varying numbers of passages in MSO. One passage in this boron-deficient medium did not impair the tracheid production on inoculation back into MS2 but after two or more passages in MSO, subculture through five or six passages in MS2 medium was necessary to obtain xylogenesis.

Light and electron microscopical examination of cells of line 721 subjected to two different concentrations of boric acid

The cultures maintained on the MS2 and MSO media (Plates 10A and 10B respectively) comprised three cell-types at different frequencies. These cells were characterised by the following features:

A. Cells which were apparently healthy with nonlignified cell walls and dense cytoplasm containing small rounded mitochondria and amyloplasts with starch grains (Plates 10C and 10D)

B. Cells with heavily lignified cell walls which had either bordered pits (as described previously in section 4.4.2) or irregular ingrowths resembling imperfectly formed bordered pits (Plate 11A). The cytoplasm of these cells was in various stages of degeneration. C. Cells with thickened regions of the cell wall traversed by groups of plasmodesmata which were frequently branched at either or both sides of the middle lamella (Plates 11A (arrowed), 11B, 11C and 11D). The cytoplasm of these cells frequently contained elongated mitochondria (Plates12A), large aggregates of smooth endoplasmic reticulum (Plate 12B), and unusual plastids (Plates 12B, 12C and 12D). These plastids contained internal membranes in parallel arrays but apparently not organised into grana and intergrana lamellae. Some of the plastids contained small starch grains.

In both the presence and absence of boric acid celltype A occurred most frequently but the proportion of celltypes B and C varied. The withdrawal of boron resulted mainly in the production of cell-type C whereas in the presence of boron cell-type B predominated.

<u>Table 4.7</u> Cell and tracheid counts of suspension line 721 maintained on MSO with a range of boric acid concentrations.

Samples were taken after 15 days incubation in the 2nd passage.

BORIC ACID mg 1 ⁻¹	CELL NO x10 ³ m1 ⁻¹	TRACHEID NO x10 ³ m1 ⁻¹	% TRACHEIDS
0	747	36	4.8
6.2	799	278	36.6
62.0	676	223	33.0
124.0	732	289	39.5
186.0	717	284	39.6
248.0	652	283	43.4

Plate 10

Light (A and B) and electron (C and D) micrographs of cells of a suspension culture of <u>P. contorta</u> (line 721) maintained on MSO and MS2.

- A. Cells maintained on MS2 showing parenchymatous cells and tracheids (t). X 50
- B. Cells maintained on MSO showing parenchymatous cellsonly. X 50
- C. and D. Healthy cells with a nucleus (n), small rounded mitochondria (m), starch-filled amyloplasts (s), vacuoles (v) and cell wall (w). D. X 10,000


PLATE 10

Plate 11

Electron micrographs of cells of a suspension culture of <u>P. contorta</u> (line 721).

 A. Cells maintained on MS2 showing irregular ingrowth of the cell walls resembling imperfectly formed bordered pits. Groups of plasmodesmata (arrowed) are also present in thickened regions of the cell wall. These cells show imperfect features generally considered to be characteristic of tracheary and phloem elements. X 1,500
 B, C, D. Thickened regions of cell walls of cells maintained on MSO with plasmodesmatal (pl) connections between adjacent cells. X 3,000



PLATE 11

Plate 12

Electron micrograph of cells of suspension cultures of <u>P. contorta</u> (line 721) maintained on MSO showing the following features:

A. Elongate mitochondria (m) X 10,000
B. Unusual plastids (p), elongate mitochondria (m) and a large aggregate of smooth endoplasmic reticulum (er). X 3,000
C. Unusual plastids (p) with starch grains and elongate mitochondria (m). X 4,000

D. Enlarged plastid showing matrix or grana (g) with parallel arrays of membranes (l). X 12,000



4.5.2 THE INFLUENCE OF ALTERATIONS OF THE SUCROSE AND NITROGEN CONCENTRATIONS ON THE GROWTH AND CYTODIFFERENTIATION LINE 721

The regulation of secondary metabolism and the induction of xylogenesis are independent processes although they may be temporally related during tracheary element formation. The possible relationship between the availability of carbohydrate and nitrogen and the regulation of the secondary metabolite pathway has been investigated in several systems (Szweykowska, 1959; Davies, 1972a; Phillips and Henshaw, 1977). Previous data obtained in investigations of the effect of carbohydrate source and concentration on suspension line 1A are presented in Appendix III. The effects of alterations in the relative concentrations of the carbohydrate sucrose and the total nitrogen supply to suspension line 721 was investigated biochemically and quantitatively.

Maintaining the cultures on MS2 medium with altered levels of sucrose (2, 4 and 6%) or nitrogen (at 0.5, 0.25 or 0.1 of the full nitrogen complement of the medium or with 0.25 times the normal nitrogen complement of MS medium substituted by glutamate) did not significantly alter the phenolic acid or amino adid nitrogen accumulation by the cells (Figs. 4.9 and 4.10). Differences were observed in the percentage tracheids produced and the dry weight accumulated by the cultures with higher sucrose levels. Increased sucrose levels (especially at 6%) resulted in lower tracheid production and higher dry weight accumulation (Fig. 4.9). Optimal tracheid production was achieved on the medium containing 0.5 times total nitrogen complement (Fig. 4.10). Only those cells maintained on the full or Fig. 4.9

Total cell and tracheid numbers, dry weights, phenolic and amino nitrogen contents of suspension cultures of <u>P. contorta</u> (line 721) maintained on MS2 with three concentrations of sucrose:

 2%

 4%

 6%

(S.E.M. given for the last total cell and tracheid counts only).



Total cell and tracheid numbers, amino nitrogen and phenolic contents of suspension cultures of <u>P. contorta</u> (line 721) maintained on MS2 with the following alterations in the supplied nitrogen:

Control, full nitrogen complement MS medium * 0.50 times the normal MS complement nitrogen 0.25 times the normal MS complement nitrogen 0.10 times the normal MS complement nitrogen 0.25 normal MS complement nitrogen substituted by glutamate.

(S.E.M. given for the last total cell and tracheid counts only).



half of the normal nitrogen complement of the medium survived further subculture.

The low percentage of tracheids produced on inoculation into medium with 0.25 of the nitrogen level as compared to 0.5, could prove to be advantageous. It could possibly be used as a basis for investigations of factors (such as gases) which may induce xylogenesis in cultures.

4.5.3 THE ANALYSIS OF THE GAS PHASE ABOVE XYLOGENIC AND NON-XYLOGENIC CULTURES (LINE 721)

The possible roles played by ethylene and carbon dioxide in the regulation of xylogenesis have been reviewed by Roberts (1976). Several experiments have indicated that in whole plants ethylene may be important in tracheary element production. Experimental systems involving the use of excised plant tissues are complicated by the production of wound-induced ethylene by the cells. The use of suspension cultures in which the xylogenic response can be either suppressed or induced could overcome this problem.

Stock suspension cultures were inoculated into MS2 medium containing either 0.5 or 0.25 times the normal nitrogen concentration of MS medium. In addition to sampling the cells for cell counts, estimations of soluble phenolics and amino acid nitrogen, gas samples were taken from the head space of the foil-sealed culture vessels. These gas samples were analysed for carbon dioxide and ethylene content by GLC (Materials and Methods - Chapter 2). The percentage CO_2 present in the atmosphere of the flask increased during the growth of the cells, decreasing again when stationary phase was reached (Fig. 4.11). Two ethylene Phenolic content, total cell and tracheid numbers and the CO_2 and ethylene content of the gas phase above the suspension cultures of <u>P. contorta</u> (line 721) maintained on MS2 with 0.5 times (1) and 0.25 times (2) the normal nitrogen complement of MS medium.

(S.E.M. given for the last total cell and tracheid counts only).



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peaks were observed in both experimental cultures, one at inoculation, the other smaller peak was produced when exponential growth ceased. This latter peak is probably comparable to that observed in Acer cultures when the cells bein to dissociate in stationary phase (MacKenzie and Street, 1970). This peak did not coincide with tracheid production in either culture. The first peak produced at inoculation was possibly trauma-induced, the ethylene production being about 10 times higher in the cells subjected to the greatest change in the medium. It is possible that this early ethylene production could affect the future xylogenic response of the cultures. No further investigations into this possible effect were made since the cells failed to grow after inoculation. In order to eliminate variations between flasks, such gas phase analyses should be made using larger 4 litre batch cultures which would allow regular sampling from the same cell population. This work should ideally be repeated using newly initiated suspension cultures producing optimal numbers of tracheids in order to determine whether a) the nitrogen level or the sucrose : nitrogen ratio controls the xylogenic response of the cultures as it appeared to here and whether b) the gases ethylene and carbon dioxide regulate xylogenesis.

4.6 SOME GENERAL OBSERVATIONS ON THE GROWTH AND CYTODIFFERENTIATION OF CULTURE LINES 1A AND 721

The variability in the growth of the total cell population and the degree of cytodifferentiation in these culture lines was possibly related to the viable cell density of the inoculum. In both culture lines a proportion of the cells differentiated into either tracheids or phloem elements, so decreasing the number of viable cells capable of further growth. Both culture lines showed a similar pattern of cytodifferentiation with time in culture, the death of both lines occurring when tracheid production was lowest; line 1A also exhibited the uncontrolled production of phenolic materials at this time. It is possible that the maintenance of line 721 on a 14 day subculture regime and on a medium with a higher boron content inhibited the overproduction of phenolic compounds observed in line 1A. The possibility that the death of line 721 may even have resulted from boron toxicity cannot be ignored.

The loss of cell viability, by whatever method, decreases the viable cell inoculum which has been shown to determine the shape of the growth curve of the cultures (Fig. 4.3) This may influence the production of gaseous factors such as carbon dioxide and ethylene which may in turn affect the xylogenic response of the cultures (see Roberts, 1976). Similarly the rate of metabolism of 2,4-D added to the cultures may depend on the physiological state and cell density of the initial inoculum. The presence of sub-or supra-optimal concentrations of auxin can be expected to alter the xylogenic response of the cultures.

Although both tracheids and phloem elements were observed in both of these culture lines, no organisation of these cell-types into more complex structures such as roots, shoots or embryoids was observed. Further investigations of the morphogenetic potential of such cultures should involve newly initiated cultures at their peak of cytodifferentiation.

4.7 DISCUSSION

Suspension cultures from somatic tissues of at least four species of gymnosperm have been reported to be capable of prolonged subculture. The paucity of information in this area of study suggests that either insurmountable problems have been encountered in determining optimal conditions for the growth of gymnosperm suspension cultures or that there has been a lack of concentrated work on this subject. The differences in response to the hormonal milieu noted here for Pinus contorta and Picea sitchensis tissues suggest interspecies variations in the cultural requirements, so that optimal conditions for growth have to be determined for each species under consideration. The process of suspension culture initiation from P. sitchensis appeared to have specific hormonal requirements: optimal response was achieved with the auxin NAA, both IBA and 2,4-D being relatively ineffective as auxins. The cytokinin employed was less critical, both kinetin and BAP supporting cell growth when in combination with NAA. However, none of the hormone combinations tested supported further growth of the cultures on transfer to fresh medium. Pinus contorta tissues responded equally to 2,4-D and NAA, whilst IBA failed to initiate cultures from the seedlings. There was no specific cytokinin requirement by these cultures although the presence of low levels of kinetin appeared to be essential for the continued maintenance of suspension culture line 1A (for details see Appendix IV). This cytokinin requirement had been observed previously for P. contorta callus cultures, line 1A (Chapter 3). The auxins NAA and IAA have been most commonly used for the

initiation and maintenance of gymnosperm callus and suspension cultures, although 2,4-D has occasionaly been substituted in both <u>Pinus</u> spp. and <u>Picea</u> sp. cultures (Reinert and White, 1956; Brown and Lawrence, 1968). Alterations of the source and concentration of auxin supplied to <u>P. contorta</u> suspension line 721 described here revealed that both NAA and 2,4-D at 1 and 5mg 1⁻¹ supported similar growth and xylogenic response in the cultures whereas these auxins at 0.1mg 1⁻¹ and IAA at concentrations up to 10mg 1⁻¹ failed to maintain the cultures (see Appendix IV).

Of the four gymnosperm cultures reported to show continued growth as suspension cultures, only two publications mention the number and duration of the passages through which the cultures were maintained. Suspension cultures of Pinus gerardiana (Konar, 1963) and Pseudotsuga menziesii (Winton, 1972a) survived 20 fourweekly and 20 weekly transfers respectively. The survival of the Pinus contorta suspension culture lines initiated during the course of this study was also found to be limited. Only two lines were maintained for any significant length of time : line 1A (20 x 3 weekly subcultures) and line 721 (9 x 3 weekly and 19 x 2 weekly subcultures). The genotype of the seedling may determine the ease of initiation and maintenance of suspension cultures. The survival of the cultures initiated from the potentially waterloggingsensitive seedlings was poor as compared to those from 'tolerant' seedlings as was their tolerance of anaerobic conditions. This suggests that there may be a critical physiological difference between these 'T' and 'S' cells

related to their origin and expressed as a difference in sensitivity to anaerobiosis. Considering this apparent sensitivity of the 'S' lines to anaerobiosis in the context of subculturing routines, it is conceivable that the time during which these cultures remain static is critical.

Initially, attention was concentrated upon the maintenance of the P. contorta culture lines and not on any regulation of the xylogenic response observed in the cultures. In both culture lines 1A and 721 successive passages of the stock cells yielded variable growth patterns, in terms of both the final cell number of the cultures and the percentage tracheids formed. Suspension cultures have a critical initial cell density defined as 'the smallest inoculum from which a new suspension culture can be reproducibly grown' (Street, 1973). Although the total cell density and the viable cell density (total cell number minus the tracheid number) of the P. contorta suspension cultures was estimated at each subculture, a further discrepancy between the real and apparent viable inocula was revealed owing to the inclusion of other non-viable cells. Cultures inoculated at levels below the critical cell density showed erratic growth (see Fig. 4.3.3.), the lag phase often extending to more than 21 days. Such cultures could not be used for further experiments owing to the possible selection of cells from the original population.

The loss of cell viability by the two <u>P. contorta</u> culture lines 1A and 721 was probably due to different reasons. The xylogenic response of the cultures when the lines died was very low, approximately 10%, and the presence of the tracheids was taken into account on subculture. Line 721 was maintained on a shorter subculture regime and on a higher boron concentration than line 1A. The eventual death of the latter line was associated with the accumulation of high concentrations of phenolic compounds. The accumulation of such compounds frequently occurs when the growth rate of tissues decreases (Kefeli and Kutacek, 1977; Nash and Davies, 1972) and when tissues are boron deficient (see Hewitt and Smith, 1975). Not enough samples were taken during the 21 day growth phase of culture line 1A to determine the kinetics of accumulation of the soluble phenolic compounds by the cells. In line 721 the concentrations of soluble phenolics were very much lower than those measured in line 1A, with some accumulation occurring towards the end of the growth phase.

The toxicity of certain exogenously supplied phenolic acids to Rosa sp. suspension cultures has been reported by Danks et al, (1975). The inhibitory effects of the phenolic acids varied, the concentration required to effect inhibition being very high. Thus the presence of unidentified soluble phenolics within the cells cannot be assumed to be detrimental to the growth of the cultures. Under normal conditions plant cells may produce phenolic material which is diverted from the cytoplasm into either the vacuole or the cell wall. Such compartmentation has been suggested to be essential for the retention of cell viability (Baur and Walkinshaw, 1974). By contrast the uncontrolled production of increased levels of phenolics by the cells, and the failure to localise these compounds in an inert form either in the vacuole or in the cell wall may lead to the death of the cells (Withers, 1976).

In addition to the lignified tracheids present in the P. contorta line 1A, the parenchymatous cells were also heavily lignified. Examinations of the cultures by electron microscopic techniques revealed the presence of electrondense inclusions within the cell vacuoles. The occurrence of such deposits has been reported in connection with a variety of cellular events. Electron-dense deposits have been observed in cells during the cytodifferentiation of xylen parenchyma (Wardrop, 1965; Barnett, 1974) and phloem parenchyma (Esau, 1973) and during fungal invasion of callus cultures of Pinus monticola (Robb et al, 1975). Nondifferentiating tissue cultures have also exhibited these electron-dense deposits (Chafe and Durzan, 1973; Baur and Walkinshaw, 1974; Withers, 1976). Interpretations as to the nature of this material have varied, although it has generally been referred to as 'polyphenolic' or 'tannin' and less often as 'proteinaceous' (Shumway et al, 1972; Smith, 1974). The ability of polyphenolics to precipitate protein may account for these alternative interpretations. The presence of this 'tannin' is often associated with the death of the cells.

To summarise, it would appear that the erratic growth and eventual death of lines 1A and 721 could probably be attributed to the inadvertent inoculation of cultures at sub-critical viable cell density levels. The loss of viability may be caused by differentiation into cells no longer capable of division, the accumulation and deposition of potentially toxic polyphenolics within the cells (line 1A) or a possible sensitivity to anaerobiosis. Considering line 721, none of these possibilities seems likely to have

caused its death. Other factors such as an altered cell physiology, e.g. the loss of the ability to synthesise essential vitamins not supplied in the medium, could have influenced the survival of the cells. Alternatively the cells may have suffered from boron toxicity.

The ability of a proportion of the cell populations studied here to differentiate into tracheary elements was of very great interest since xylogenesis is one of the easiest forms of cytodifferentiation to identify and consequently has been the subject of many detailed investigations. Most of these studies have utilised the ability of explants of various tissues to form tracheary elements under defined culture conditions (see Torrey, 1976) although suspension cultures of Centaurea cyanus have also been studied (Torrey, 1975). One major disadvantage in using explant material is the inherent pre-existing gradient In Helianthus tuberosus (Jerusalem within the tissues. artichoke), at least three concentric zones of tissues may be observed in the inoculum after 14 days growth, the middle zone containing most of the dividing and differentiating cells (Minocha and Halperin, 1973). Such gradients do not exist in single cell suspension cultures which exhibit a high degree of uniformity in response to cultural conditions. However it should be noted that the degree of uniformity of aggregated suspension cultures may be inversely proportional to the extent of cellular aggregation, with highly aggregated cells displaying a range of morphologically and physiologically distinct cell types. Pinus contorta suspension cultures which exhibited tracheid

production were highly aggregated with most tracheids being located centrally in the larger cell aggregates. These observations correlate with reports of the location of tracheids in suspension cultures of <u>Picea glauca</u> (Durzan <u>et</u> <u>al</u>, 1973) and imperfectly formed xylogenic elements in turbidostat cultures of <u>Acer pseudoplatanus</u> subjected to withdrawal of 2,4-D (Withers, 1976).

Studies on the induction of tracheary elements in suspension cultures ideally require that non-xylogenic stocks be maintained , so that a consistent xylogenic response may be induced only following introduction into conducive media. Despite the variations in the growth and cytodifferentiation observed in these <u>Pinus contorta</u> suspension cultures some preliminary investigations into the regulation of tracheid induction and the concomitant secondary metabolite biosynthesis were made.

The absolute and relative concentrations of plant hormones and sucrose are generally considered to be the critical variables in the initiation and normal development of tracheary elements in culture. Other factors such as the gas phase, the environment and inorganic and organic nutrients may also exert some limited control over these processes (see Roberts, 1976). In this study tracheid induction in <u>Pinus contorta</u> culture lines was not consistently influenced by alterations in sucrose concentrations although sucrose at concentrations of ca. 2.0% appeared to be optimal (see Appendix III and Fig. 4.9). Decreasing the concentration of 2,4-D supplied to line IA caused the tracheids to be induced earlier whilst increasing the 2,4-D level in culture line 721 resulted in no significant

difference in the xylogenic response observed (see Appendix IV). Auxin requirements of cultures frequently are observed to decrease with time, which may explain the enhanced growth and earlier cytodifferentiation observed in line 1A on decreasing the 2,4-D supply. It is possible that the exogenously supplied 2,4-D does not itself induce the xylogenic response of the cultures but influences the cellular production of endogenous auxins which control tracheid induction. The autolysis of the contents of differentiating tracheary elements has been suggested produce tryptophan for IAA synthesis (Sheldrake and Northcote, 1968) so resulting in an autocatalytic process of xylogenesis. The enhancement of tracheid production in the dark above that observed in the light in line 1A (Table 4.5) supports the possibility that the endogenous levels of auxins are critical; the rate of degradation of the photolabile natural auxins would be lower in the dark than in the light. Phillips and Dodds (1977) reported that the incubation of Jerusalem artichoke tuber explants in the dark resulted in a 50% increase in cell and tracheary element number over light-incubated cultures.

In <u>P. contorta</u> suspension culture line 721, tracheid production was enhanced when the total nitrogen concentration of the MS medium was reduced by half, whilst further reductions or the substitution by glutamate failed to support xylogenesis (Fig. 4.10). A similar observation has been made using Jerusalem artichoke explants (Phillips and Dodds, 1977). The auxin content of shoot tips of <u>Helianus</u> sp. and <u>Nicotiana</u> sp. as measured by the Went <u>Avena</u> sp. technique (Avery et al, 1937) and leaves of Brassica oleracea var

capitata (Avery and Pottorf, 1945) has been shown to be related to the nitrogen (supplied as nitrate) available to the plants. In some tissue culture systems, the availability of nitrogen has been implicated in the regulation of phenolics biosynthesis (Phillips and Henshaw, 1977) and in the expression of totipotency by cells (Reinert et al, 1971). Nitrogen depletion coincided with increased activity of the enzyme phenylalanine ammonia lyase in sycamore cells (Westcott and Henshaw, 1976) and soybean cells (Hahlbrock The addition of a nitrogen source to nitrogenet al, 1974). depleted stationary-phase cultures of sycamore inhibited phenolics biosynthesis and stimulated protein synthesis (Phillips and Henshaw, 1977). These workers have suggested that protein synthesis may be involved in the regulation of secondary metabolism by competition for common precursors. Similarly the availability of the amino acid tryptophan would affect the synthesis of auxin.

In the <u>P.contorta</u> cultures described here there was no significant difference in phenolic acid or amino nitrogen accumulation between the xylogenic and non-xylogenic cultures. The total protein content may however have decreased in the non-xylogenic cells, but it was not estimated. Thus, the influence of critical levels of inorganic nitrogen on the xylogenic response of these <u>P. contorta</u> cultures, on Jerusalem artichoke explants (Phillips and Dodds, 1977) and on the embryogenic response of <u>Daucus carota</u> cultures (Reinert <u>et al</u>, 1971), may all be related to the production of endogenous auxin by the cells.

Attempts were made to use these 'inductive' and 'noninductive media to monitor differences in ethylene and

carbon dioxide production by xylogenic and non-xylogenic cultures (Fig. 4.11). The possible involvement of ethylene in xylogenesis and the suggested antagonistic effect of carbon dioxide on ethylene-mediated responses have been reviewed by Roberts (1976). The data presented here are inconclusive since the proportion of cells undergoing xylogenesis was low and the experiment was not repeated. However, such a system could be developed to allow investigations into the kinetics of ethylene production by these cultures. The use of larger (4 litre) batch cultures would allow the regulation of the gas supply of the cultures and the effect of exogenously supplied ethylene on the cultures to be studied. Such a stystem would also allow regular samples to be taken from one cell population, eliminating variations between flasks.

The death of culture line 1A, described previously, was associated with the following changes in the biochemical composition and ultrastructure of the cells : the accumulation of high levels of soluble phenolics, the deposition of tannin in the vacuoles and a diminished capacity to differentiate into tracheids whilst the walls of the 'parenchymatous' cells were thickened, sometimes with a roughened irregular inner surface. These features are symptomatic of boron deficiency, a condition known to occur in <u>Pinus</u> spp. (Hewitt and Smith, 1975). The addition of boric acid to cuttings of <u>Pinus contorta</u> has been shown to enhance rooting when applied in combination with auxins although variations in response existed between clones of different genetic origins (Bowen et al, 1975). Preliminary experiments on three newly initiated suspension culture lines of <u>P. contorta</u> revealed inter-line variations in response to different levels of boric acid in the medium.

Trace quantities of boron are required for the normal growth and development of all higher plants; the physiological rôle of the element is still the subject of discussion. Boron nutrition has been implicated in several areas of plant metabolism (see Hewitt and Smith, 1975) including sugar translocation (Gauch and Dugger, 1953) and phenolics production (Lee and Arnoff, 1967). These latter workers presented evidence that borate regulates phenolics synthesis by the complexing of borate ions with various polyhydroxy compounds, particularly carbohydrates and phenolics. The mechanism proposed involves the partitioning of metabolism between the glycolytic and pentose phosphate pathways by boron. The complex formed between borate and 6phosphogluconic acid inhibits the action of 6-phosphogluconate dehydrogenase, a key enzyme in the pentose phosphate shunt pathway. In the absence of boron, inhibition of this enzyme is released and excess phenolics are formed. The complexing of these phenolic acids with borate further depletes the boron available, so an autocatalytic system results, generating excess phenolic compounds. The predicted result of withdrawing boron from cells, by this theory, could be the over-production of phenolic compounds. In fact when P. contorta suspension line 721 was inoculated into media containing a wide range (O-124mg 1^{-1}) or boric acid concentrations, no difference in the accumulation of soluble phenolics was observed. Although it is possible that there was sufficient boron present in the medium both

from the inoculum carry-over and as a contaminant released by silica-borate glassware, ultrastructural differences observed by electron microscopy suggest that there was an cytological effect of boron deficiency on the cultures.

A comparison of the growth parameters of the cultures maintained on either boron sufficient (B+) or deficient (B-) media revealed that whilst the apparent growth rates of the cultures were similar, a maximum of 50% of the cells in the B+ medium differentiated into tracheids and were therefore incapable of further division. The cells maintained on the B- medium were smaller, with a lower biomass, non-lignified walls and ca. 90% cell viability. Either the real growth rates of the cultures differed or a proportion of the cell population in B- medium lost the capacity to divide, but did not visibly differentiate into tracheids, at the same rate as tracheid production occured in B+ medium.

Electron microscopic examination revealed that differentiated cells were present in both treatments. Cells with wall sculpturing and bordered pits (tracheids) had previously been observed in stock cultures of both lines IA and 721. The other cell-type was detected mainly in cells incubated in B- medium, although certain of the diagnostic features were also observed in cells in B+ medium and the senescing culture line IA. These cells showed ultrastructural features characteristic of phloem elements in <u>Pinus</u> spp. (Neuberger and Evert, 1974, 1975, 1976; Sauter and Ulrich, 1977). The identification of these phloem elements as either sieve cells or albuminous cells (alternatively known as Strasburger cells) was not always possible. The latter cells are analagous in function to the companion cells in angiosperms. The characteristic features of these cells include the presence of dense cytoplasm, large nuclei, numerous mitochondria, conspicuous connections with sieve cells and the absence of starch The identification of these cells relies on (Esau, 1969). the observation of the connections with sieve cells. These connections differ on the sive-cell side of the wall from the albuminous-cell side, being similar to sieve area pores in the first case and plasmodesmata in the second (Neuberger and Evert, 1975). In virtually all cases, the connections between cells were located on thickened wall regions, and the plasmodesmata radiated out from the middle lamella, a feature more characteristic of albuminous-cells than sieve-However, the latter cell-type could be identified cells. in the various stages of maturation by the increased endoplasmic reticulum, increased number of mitochondria and unusual plastids, and later by the degenerating cytoplasm and thickened lamellate walls

Thus, withdrawal of boron from <u>P. contorta</u> suspension cultures appeared to diminish the xylogenic response whilst enhancing phloem-type cytodifferentiation. Phloem of borondeficient plants have been observed to show increased wall thickening (Spurr, 1957) suggesting that perhaps, in these pine cultures, the withdrawal of boron from the medium facilitated recognition of incipient phloem cells.

Boron appears not to be required for cell division; in fact, under certain circumstances its absence may promote rapid cell proliferation (Skok, 1957; Birnbaum <u>et al</u>, 1974). These workers also presented evidence that boron-deficiency lowered metabolic activity and retarded maturation of cells. The IAA metabolism of boron-deficient plants is also affected (see Gauch, 1972) although the exact nature of this relationship is controversial. Pollard et al, (1977) have demonstrated that boron plays an important role in the regulation of the function of membranes of higher plants. The complexing of borate ions with polyhydroxy compounds (such as carbohydrates) alters the properties of the parent compounds, possibly increasing the permeability of membranes to them (Hewitt and Smith, 1975; Gauch and Dugger, 1953). The presence of growing points which may be regarded as physiologically active sinks with respect to sugar has complicated the interpretation of data indicating a role of boron in sugar translocation in whole plants (Sisler et al, 1956; Nelson and Gorham, 1957; Lee et al, 1966). However, evidence obtained from tissues lacking active growing points has indicated that boron may enhance the uptake and translocation of sugar (Turnowska-Starck, 1960) and therefore sucrose-mediated translocation of growth regulators (Mitchell et al, 1953a, b). The data presented here for boron-deficient pine cells are consistent with carbohydrate limitation. When compared to the control boron-deficient cultures, the cells had a lower biomass, contained fewer starch grains, were less lignified and produced unusual plastids resembling chloroplasts. The production of chloroplasts is generally associated with low carbohydrate in cultured tissues (Dalton and Street, 1977). The limited availability of carbohydrate for metabolism could be expected to result in the chanelling of the compounds into primary rather than secondary metabolism, resulting in

decreased lignin biosynthesis.

The observed effects of boron on sugar uptake and translocation have been interpreted both in terms of the actively-growing meristems of plants being physiological 'sinks' (Skok, 1957) and the formation of a sugar-borate complex facilitating sugar transport across membranes (Gauch and Dugger, 1953, 1954). The data presented here suggest that boron-deficiency limits carbohydrate uptake by cells and induces differentiation of phloem. In the growing points of intact plants, especially the root metstems, the differentiation of protophloem precedes that of protoxylem (Esau, 1953). Considering the active menstems as physiological 'sinks' which may cause deficiencies in both organic and inorganic nutrients in the procambial region, transient boron deficiency may induce protophloem production (as has been observed here in Pinus cultures). The resultant increased sugar translocation by the protophloem and the concomitant increase in auxin translocation would result in conditions ideal for subsequent xylogenesis The manifestation of boron-deficiency in this P. contorta suspension culture could provide a suitable system for the study of the mode of action of this trace element.

The variation in xylogenic response of both culture lines of <u>P. contorta</u> suspension cultures was monitored throughout their culture periods (Figs. 4.4. and 4.5). In both lines the overall ability to express their xylogenic capability reached a maximum within a few passages from initiation with up to 50% of the total cell population differentiating into tracheids. This tracheid production diminished with increased time in culture until less than

10% of the cell population regularly showed such differentiation. A similar loss of xylogenic response has been observed in Populus nigra L. cv. 'Italica' callus cultures when maintained on 2,4-D containing medium (Venverloo, 1969). Such Populus cultures maintained on IAA retained their xylogenic potential over a longer period of time. The embryogenic expression of Daucus carota suspension cultures exhibit a similar pattern of response, such that the induction of embryoids from these cultures on withdrawing 2,4-D occurs most frequently in newly initiated suspensions or calluses. This capability dimishes with prolonged culture (Reinert et al, 1971; Smith and Street, 1974) and possibly relates to the number of cell generations of the culture (Meyer-Teuter and Reinert, 1973). The mechanism involved in this progressive decline in the embryogenic potential of the cultures has not yet been fully elucidated. Polyploidisation and alterations in the cell genome are common features in plant tissue cultures (see Sunderland, 1977) and have been implicated in the loss of embryogenic potential of the carrot cultures (Smith and Street, 1974). Physiological factors may also be important in determining the embryogenic response of cultures, e.g. increasing the nitrogen level in the medium allows the reintroduction of embryoid production in cultures from which this capacity has been lost (Reinert et al, 1971).

Withdrawing 2,4-D from continuous cultures of <u>Acer pseudoplatanus</u> (maintained in culture for many years) resulted in increased secondary metabolism and ultimately cell death (King, 1976). Associated with these changes was imperfect xylogenesis (Withers, 1976) and cells with

ultrastructural features characteristic of phloem (Withers, unpublished). In the present study, <u>P. contorta</u> suspension cultures indicated a capacity for both tracheid and phloem element production under appropriate conditions, although withdrawing 2,4-D resulted in increased browning and eventual death of the cultures. Thus both 'woody' species, <u>Acer</u> <u>pseudoplatanus</u> and <u>Pinus contorta</u>, indicated residual capacity to respond to conditions inductive of vascular development. However, in neither case was there at any time the suggestion of the typical pattern of segmentation indicating embryogenesis.

The growth patterns of suspension cultures of <u>Picea</u> <u>glauca</u> and <u>Pinus banksiana</u> have been closely observed by Durzan and Steward (1970). Attention has been drawn to certain similarities in the orientation of cell divisions in these cultures with those observed in zygotic embryos of the species. However the claim of Winton (unpublished, cited by Winton, 1974) that callus and suspension cultures of five gymnosperm species, including <u>Pinus contorta</u>, have exhibited embryoid production has not been substantiated by photographs or by further information concerning the culture conditions employed.

The ability of many 'herbaceous' (see Reinert <u>et al</u>, 1977) and several 'woody' species (Button and Bornman, 1971; Radojević <u>et al</u>, 1975; Krul and Worley, 1977) to exhibit somatic embryogenesis reinforces the idea that the totipotency of cells is not restriced to any particular group of plants. The expression of that totipotency may, however, depend on the physiological state of the explant cells, the time they have been maintained in culture and the fortuitous choice of inductive media.

CHAPTER 5

PLANTLET REGENERATION FROM JUVENILE TISSUES

CHAPTER 5

5 5.1 INTRODUCTION

Somatic tissues of at least 50 gymnosperms have been successfully cultured since Gautheret (1934) induced the \mathbf{s} f first gymnosperms (Pinus pinaster and Abies pectinata) to proliferate in culture (Winton, 1974). Sporadic organogenр esis has been observed in such cultures (Ball, 1950; Konar е а and Oberoi, 1965; Norstog, 1965; Sankhla et al, 1967). Whilst the initiation of roots, shoots or both in Nicotiana sp. t callus (Skoog and Miller, 1957) and in Populus sp. callus с (Chalupa, 1974) can be induced by alterations in the auxin : (cytokinin ratio, such controlled regeneration has not с generally been achieved from established gymnosperm callus g cultures. However, successful stimulation of adventitious с b buds has been achieved directly from explants of juvenile t tissues of several gymnosperm species. Excised mature embryos have been used frequently as the explant source е (Cheng, 1975; Sommer et al, 1975; Winton and Verhagen, 1977). (А Adventitious bud production also has been achieved from the hypocotyls and cotyledons of seedlings (Isikawa, 1974; h С Campbell and Durzan, 1975; Cheng, 1976). Although the

formation of adventitious buds has been reported from stem and needle explants from 2 year old seedlings (Cheng, 1977) and stem explants from 15-20 year old trees (Winton and Verhagen, 1977), more mature tissues usually have proved less responsive in culture.

The critical hormonal factor influencing the direct formation of adventitious buds from cultured gymnosperm explants is the auxin: cytokinin ratio (Cheng, 1976). However, a survey of the literature has indicated that many species may have specific cultural requirements: enhanced adventitious bud induction from explants of some species has been achieved by alterations in the inorganic salts in the medium (Sommer, 1975; Sommer <u>et al</u>, 1975; Brown and Sommer, 1977) and in the light regime (Cheng, 1977).

The outgrowth of such adventitious buds has been achieved by transferring the excised tissue to a hormonefree, diluted basal medium (Cheng, 1975) or to a medium containing different inorganic salts (Sommer <u>et al</u>, 1975). These regenerated shoots occasionally have been stimulated to root in culture (Sommer and Brown, 1974: Brown and Sommer, 1977; Winton and Verhagen, 1977).

In this study, explants of juvenile tissues of <u>Pinus</u> <u>contorta</u> and <u>Picea sitchensis</u> have been used in an attempt to determine optimal conditions for the induction and subsequent outgrowth of adventitious buds and eventual plantlet formation.

EXPERIMENTAL

5.2 THE INDUCTION OF CALLUS AND ADVENTITIOUS BUDS FROM EMBRYOS OF PINUS CONTORTA AND PICEA SITCHENSIS

5.2.1 PRELIMINARY INVESTIGATIONS USING MEDIA KNOWN TO BE CONDUCIVE TO EITHER CALLUS OR ADVENITIOUS BUD PRODUCTION IN GYMNOSPERMS

Initially MS medium supplemented with combinations of 2,4-D and kinetin (as previously used to initiate callus cultures from <u>P. contorta</u> and <u>P. sitchensis</u> seedlings, see Chapter 3) was employed to induce the proliferation of excised embryos of these two species. The embryos of <u>P. contorta</u> were more responsive than those of <u>P. sitchensis</u> (Table 5.1). Callus initiated from the former embryos was creamy-white, friable and unorganised, and was capable of limited subculture, whereas <u>P. sitchensis</u> embryos showed little proliferation, the tissues rapidly becoming brown and necrotic.

<u>Table 5.1</u> Number of <u>P. contorta and <u>P. sitchensis</u> embryos producing callus and adventitous buds when incubated on MS medium with the combinations of 2,4-D and kinetin below.</u>

Cultures incubated under continuous light at $25^{\circ}C$ for 60 days; 10 embryos treatment⁻¹.

a) Pinus contorta

2,4-D	 KINETIN (mg 1 ⁻¹)		
$(mg l^{-1})$	5.0	1.0 .	0.5
5.0	3	5	1
1.0	 8	6	2
0.5	2	O	0
b) Picea sitchensis

2,4-D	KII	NETIN (mg	1 ⁻¹)
$(mg 1^{-1})$	5.0	1.0	0.5
5.0	1	0	0
1.0	2	1	0
0.5	0	0	. 0

Using media 1 and 2 of Sommer <u>et al</u>, (1975) (see Appendix I) some of the embryos swelled slightly and browned, but there was no evidence of either callus or adventitious bud production as had been reported for <u>Pinus palustris</u> embryos. Since Sommer <u>et al</u>, (1975) did not state the incubation conditions employed during their experiments it is possible that the conditions chosen here $(25^{\circ}C, continuous$ light) may not have been comparable.

The incubation of embryo explants on 'MD' medium modified from Durzan <u>et al</u>,(1973) (see Appendix I) under environment <u>b</u> $(20^{\circ}C, 13.5 \text{ hours light}; 7^{\circ}C, 10.5 \text{ hours dark})$ was more successful (Table 5.2). Adventitious buds were produced along the hypocotyl and cotyledons of <u>Pinus contorta</u> (Plates 13A, 13B, 13C and 13D) and <u>Picea sitchensis</u> (Plates 13E and 13F). The latter species appeared to require an exogenous auxin source for both callus and adventitious bud production, the presence of BAP being required for morphogenesis in both species.

- A D. Excised embryos of <u>P. contorta</u> showing adventitious bud (b) initiation along the colyledons (c) and hypocohyl (h). The original embryo meristem (m) occasionally grows out, but the root (r) rarely does. X 25
- E, F. Excised embryos of <u>P. sitchensis</u> showing adventitious bud (b) production.



<u>Table 5.2</u> Number of <u>P. contorta</u> and <u>P. sitchensis</u> embryos producing callus and adventitious buds when incubated on MD medium with the combinations of NAA and BAP below under environment <u>b</u>.

Results scored after 70 days incubation; 10 embryos treatment⁻¹.

a) Pinus contorta

NAA(M)	BAP(M)										
	0		10 ⁻⁷		10 ⁻⁵		2×10^{-5}				
	Callus	Buds	Callus	Buds	Callus	Buds	Callus	Buds			
0	Ο	0	0	0	1	3	5	2			
10 ⁻⁷	5	0	5	0	7	3	0	0			
10 ⁻⁵	8	0	10	0	8	0	0	0			

b) Picea sitchensis

NAA(M)	BAP(M)									
	0 10 ⁻⁷			10	-5	2×10^{-5}				
	Callus	Buds	Callus	Buds	Callus	Buds	Callus	Buds		
0	0	0	0	0	0	0	0	0		
10 ⁻⁷	0	0	3 ·	0	3	0	0	0		
10 ⁻⁵	5	0	5	1	2	1	0	0		

The addition of the following combinations of hormones to the 'MD' basal medium (IAA, IBA, 2iP and BAP all at 5 μ M, the 'CI' medium of Cheng, 1975) as well as a 7 day dark pretreatment of the excised embryos before incubation under environment <u>b</u> enhanced the morphogenetic response of <u>P. sitchensis</u> embryos (Table 5.3). The recognition of the earliest stages of development, the appearance of primordia, was possible after 28 and 56 days incubation in <u>P. sitchensis</u> and <u>P. contorta</u> respectively. <u>Table 5.3</u> Number of <u>P. contorta</u> and <u>P. sitchensis</u> embryos producing callus and adventitious buds when incubated on CI medium under environment <u>b</u>, after a 7 day dark pretreatment at 25^oC.

,	P.	contort	a	P. sitchensis			
Time (days)	Viable	Callus	Buds	Viable	Callus	Buds	
0	a	0	0	_a	0	0	
14	46	46	0	43	43	0	
28	27	27	0	39	35	4	
42	12	12	0	39	26	13	
56	12	9	3	39	20	19	
95	6	1	5	33	3	30	

50 embryos treatment⁻¹.

KEY

-^a Number unknown

The viability of these embryos was assessed according to their response in culture : embryos which neither produced callus nor adventitious buds were scored as dead; only calluses which continued to proliferate were scored at each subsequent examination. However, in all future experiments, data will be expressed in relation to the original viability, all the embryos producing callus being scored as such regardless of whether they continued to proliferate

In <u>P. contorta</u>, the embryos did not callus, but adventitious buds were produced on either the hypocotyl or cotyledonary regions as had previously been observed. By contrast the embryos of <u>P. sitchensis</u> callused, primordia being produced on the proliferating explant. The distribution of these primordia was either apparently random or in a regular phyllotactic arrangement. This latter situation was interpreted as indicating the development of an organised bud primordium. Several stages of development of the bud primordia could be observed on each callusing embryo (Plates 14A and 14B). These primordia slowly developed into typical buds, but some leaves remained which apparently were not associated with a bud (Plates 14C and 14D).

Although excised embryos of both <u>P. contorta</u> and <u>P. sitchensis</u> have been shown to be capable of expressing their morphogenetic potential, the proportion of <u>P. contorta</u> embryos responding was consistently low. In an attempt to improve the number of these embryos showing adventitious bud production the influence of cultural and environmental factors was investigated.

Throughout these experiments, the same seed batch of each species was used to minimise any genetic variation and difference in seed storage time. Stratified <u>P. sitchensis</u> embryos have been demonstrated to contain increased levels of endogenous hormones, especially cytokinins (Taylor, 1976). Since the stratification time was not necessarily the same for each lot of seeds used, a comparison was made between stratified and non-stratified seeds. It was generally found that the non-stratified seed was less contaminated than the stratified, especially in <u>P. contorta</u>. Several experiments (using different media) were set up and the data collated. The application of the X^2 test to these data (see Appendix VI) revealed that there was no significant difference in the percentage of embryos producing adventitious buds. Thus, in

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A - D. Callus and leaf and bud primordia (p) development on excised embryos of <u>P. sitchensis</u>. The primordia may be observed to develop into buds (b) which continue to extend. Isolated leaves (l) apparently not associated with a bud may also be observed. X 15



PLATE 14

all future experiments the data presented are collated either from stratified and non-stratified seeds or from non-stratified seed only.

5.2.2 THE INFLUENCE OF THE INORGANIC SALTS AND ORGANIC SUPPLEMENTS ON THE MORPHOGENETIC RESPONSE OF P. CONTORTA AND P. SITCHENSIS

A comparison was made between MD and MS basal inorganic media supplemented with the levels of sucrose, thiamine-HCl and mesoinositol described by Cheng (1975) and with 10^{-7} M NAA and 10^{-5} M BAP (Table 5.4a). The cultures were incubated in the dark at 25° C prior to exposure to environment <u>b</u>. There was no significant difference in either the survival or the adventitious bud production of the embryos of either species on the two media.

Table 5.4Number of P. contorta and P. sitchensis embryosproducing callus and adventitious buds whenincubated under environment \underline{b} after a 7 day darkpretreatment.

a) Using basal media of MD and MS modified by the addition of the levels of sucrose, thiamine-HCl and mesoinositol (Cheng, 1975) and 10^{-7} M NAA and 10^{-5} M BAP.

	P.	CONTORT	A	P. 5	SITCHENSI	S
BASAL MEDIUM	VIABLE	CALLUS	BUDS	VIABLE	CALLUS	BUDS
MS	86	82	4	77	43	34
MD	89	81	8	71	40	31

Results scored after 60 days : 100 embryos treatment⁻¹.

b) Using	basal	MD	medium ⁺	amino	aci	id s	upple	ement	Durz	zan	<u>et</u>
<u>al</u> , (1973	3) with	h a	constant	level	of	NAA	(10	⁻⁹ м),	BAP	as	
shown.	•										

Results score	ed afte:	r 56 d	days : 4	40 em	oryos ta	reatme	ent ⁻¹ .	
P. contorta		<u></u>		BA	AP(M)			
AMINO ACID		Э	10	-7	10	-5	2 x	10 ⁻⁵
SUPPLEMENT	CALLUS	BUDS	CALLUS	BUDS	CALLUS	BUDS	CALLUS	BUDS
+	0	0	4	0	2	0	0	0
	4	0	25	0	24	6	8	1
P. sitchensis	<u>5</u>	····		B	AP(M)			
AMINO ACID	(0	10	-7	10	-5	2 x	10 ⁻⁵
SUPPLEMENT	CALLUS	BUDS	CALLUS	BUDS	CALLUS	BUDS	CALLUS	BUDS
+	0	0	0	0	0	0	0	0
-	0	0	0	24	6	18	2	4

Using the MD basal medium, embryos were incubated with a constant level of NAA $(10^{-9}M)$ and varying BAP concentrations $(0-2x10^{-5}M)$ under the same conditions as above. The addition of the amino acid supplement of Durzan <u>et al</u>, (1973) (see Appendix I) proved to be detrimental to the embryos of both species (Table 5.4b).

In later experiments, <u>P. contorta</u> embryos were incubated under different environmental conditions (environment <u>a</u> I – see section 5.2.4) which proved more conducive to adventitious bud production from this species. Under these conditions and on MD medium supplemented with either 10^{-5} or 10^{-6} M BAP, the addition of lg 1^{-1} casein hydrolysate was found to inhibit the morphogenetic response of the cultures (Table 5.5). <u>Table 5.5</u> Number of <u>P. contorta</u> embryos producing callus and adventitious buds under environment <u>a</u> I after a 12 day dark pretreatment. The cultures were incubated on MD medium with 10^{-5} and 10^{-6} M BAP ⁺______ lg 1⁻¹ casein hydrolysate.

Results scored after 60 days incubation : 50 embryos treatment⁻¹.

	10-5	⁵ M BAP		10 ⁻⁶ M BAP			
CASEIN HYDROLYSATE	VIABLE	CALLUS	BUDS	VIABLE	CALLUS	BUDS	
+	40	30	10	34	31	3	
· _ .	31	9 -	22	29	5	24	

5.2.3 THE INFLUENCE OF PLANT HORMONES ON THE MORPHOGENETIC RESPONSE OF P. CONTORTA AND P. SITCHENSIS

Since alterations in the basal medium and different levels of NAA and BAP did not enhance adventitious bud production from <u>P. contorta</u> embryos, the effects of different **auxins** and cytokinins were studied.

Both <u>P. contorta and P. sitchensis</u> embryos respond to culture in a similar manner, the root rarely growing out even when cultured on a hormone-free medium. Sommer <u>et al</u>, (1975) have observed that the embryonic root of <u>Pinus</u> <u>palustris</u> also failed to grow in culture; the root cap either died or callused and browned. During the experiments on the effects of different auxins and cytokinins it was noticed that certain hormonal combinations stimulated the outgrowth of these pre-existing root meristems. This was frequently associated with healthy shoot growth (Plate 15A).

Whilst none of the hormones employed enhanced the adventitious bud production from P, contorta above 6%, kinetin,

2iP and especially zeatin in combinations with IAA and IBA allowed normal root growth from both species (Table 5.6).

<u>Table 5.6</u> Number of embryos of <u>P</u>, <u>contorta</u> and <u>P</u>. <u>sitchensis</u> producing callus and adventitious buds when incubated under environment <u>b</u> after a 7 day dark pretreatment. Either the auxins (IAA and IBA) or cytokinins (2iP BAP) of the CI medium were substituted or altered as stated below.

Results scored after 60 days : 50 embryos treatment⁻¹.

a) <u>P, contorta</u>

HORMONES	VIABLE	CALLUS	BUDS	ROOTS
Cytokinins			<u></u>	
5µM BAP + 5µM 2iP*	47	44	3	0
lOµM kinetin	48	46	2	1
10µM 21P	42	41	1	5
lOµM BAP	46	43	3	0
2.5µM BAP	39	38	1	0
1.0µM zeatin	49	48	0	5 .
Auxins				
2.5µM 2,4-D	42	39	3	Ο
5.0µM 2,4-D	42	42	0	0
10.0µM 2,4-D	40	40	Ο	0
0.5µM 3,5-D	<u>4</u> 6	44	2	0
5.0µM 3,5-D	47	46	1	0

A. Root and shoot growth from an embryo of <u>P. contorta</u> after 60 days incubation on 'CI' medium with the cytokinins substituted by lµM zeatin. The cultures were maintained under environment <u>b</u> after a 7 day dark pretreatment. x 5
B. Shoot initiation from an embryo of P. sitchensis after 100 days incubation on the same medium and under the same conditions as A. Note the shoot (s) arising from the tip of a cotyledon (c) of the embryo. The root (r) of this embryo has failed to grow. x 5



b) P. sitchensis

HORMONES	VIABLE	CALLUS	BUDS	ROOTS
Cytokinins				
5µM BAP + 5µM 2iP*	43	22	21	0
10µM kinetin	42	23	19	0
lOμM 2iP	41	35	6	1
10µМ ВАР	35	21	14	. 0
2.5µM BAP	35	22	13	0
lµM zeatin	41	0	0	10
Auxins				
2.5µM 2,4-D	32	19	13	0
5.0µM 2,4-D	29	28	1	0
10.0µM 2,4-D	20	7	13	0
0.5µM 3,5-D	43	39	4	3
5.OµM 3,5-D	35	34	1	0

KEY

* CI control medium.

In neither <u>P. contorta nor P. sitchensis</u> did the latter cytokinin induce adventitious bud formation during the time presented here (60 days); however, after a further 40 days in culture occasional isolated buds were formed. These buds tended to be located at the tips of the stunted cotyledons or along the hypocotyl (Plate 15B). Occasionally the original shoot meristem produced two instead of one actively-growing shoots.

In <u>P. sitchensis</u> the cytokinin 2iP was the least effective bud inducer, giving both a low number of responsive explants and few buds per explant. Whilst BAP and kinetin appeared to induce a similar response from the embryos, ca. 50% producing buds, the number and the growth of the buds differed. Numerous small buds were produced by BAP treatment whilst the kinetin-induced buds were generally fewer in number, appeared to be etiolated, and started to grow out whilst still on the initiation medium. The ability of the cultures to continue to proliferate on subculture and the rooting performance of these BAP and kinetin derived shoots also differed and will be discussed further in section 5.5.

In combination with IAA and IBA, BAP alone or with 2iP induced a high proportion of <u>P. sitchensis</u> embryos to produce adventitious buds. The substitution of the synthetic auxin 2,4-D for the natural auxins did not suppress this response at either 2.5 or 10.0 μ M, but did appear to do so at 5 μ M. The numbers of embryos responding in culture by either callus or adventitious bud production when incubated on 2,4-D or high levels of the anti-auxin 3,5-D were lower than on the control medium. The latter hormone also inhibited the expression of the morphogenetic response of the cultures.

5.2.4 THE INFLUENCE OF THE ENVIRONMENT ON THE MORPHOGENETIC RESPONSE OF P. CONTORTA AND P. SITCHENSIS

Incubation of the embryos of <u>P. contorta</u> and <u>P. sitchensis</u> under either environment <u>b</u> or under continuous light at $25^{\circ}C$ following a 7 day dark pretreatment revealed a difference in response of the two species studied. Whilst <u>P. sitchensis</u> embryos showed better adventitious bud production, the survival of <u>P. contorta</u> embryos appeared to be lower in continuous light at $25^{\circ}C$ than under environment b (Table 5.7). <u>Table 5.7</u> Number of <u>P. contorta</u> and <u>P. sitchensis</u> embryos producing callus and adventitious buds when incubated on MD medium with the levels of NAA and BAP below under two light regimes after a 7 day dark pretreatment.

Results	scored	after	60	days	:	10	embryos	treatment	· · .

			P. CONT	P	. SITCH	ÈNSIS				
HORMO	NES (M)	 ENVIRON	MENT <u>b</u>	CONT. L	LIGHT 250	C ENVIRO	NMENT <u>b</u>	CONT.	LIGHT	25 ⁰ C
NAA	BAP	CALLUS	BUDS	CALLUS	BUD	S CALLUS	BUDS	CALLUS	5	BUDS
0	10 ⁻⁵	0	1	0	0	2	2	0		3
0	2x10 ⁻⁵	ο	1	0	2	· 2	5	1		6
10 ⁻⁹	10-5	8	2	7	. 1	6	2	3		2
10 ⁻⁹	2×10^{-5}	9	1	4	1	1	4	2		1
10 ⁻⁷	10 ⁻⁵	10	0	3	. 1	3	6	1		7
10 ⁻⁷	2x10 ⁻⁵	6	. O	0	0	0	4	1		4

A comparison of the morphogenetic response of the embryos of both species under various environmental conditions was made.

The alternative conditions available were:

environment \underline{a} at either high light intensity (I) or low light intensity

(II) or total darkness (III) all at 25⁰C with a 16 hour photoperiod.

environment <u>b</u> at a low light intensity

20^oC, 13.5 hour light and

 $7^{\circ}C$, 10.5 hour dark.

Using embryos of <u>P. contorta</u> and <u>P. sitchensis</u> on CI medium (with the cytokinins BAP nnd 2iP substituted by $10\mu M$ kinetin) a comparison was made between environment <u>a</u> II and <u>b</u> (Table 5.8).

Table 5.8Number of P. contorta and P. sitchensis embryosproducing callus and adventitious buds whenincubated on CI medium with kinetin ($10\mu M$)substituting for BAP and 2iP under environmentsa II and b.

50 embryos treatment⁻¹.

a) P. contorta

- -	ENVI	RONMENT	<u>a</u> II	ENVIRONMENT b			
TIME (DAYS)	VIABLE	CALLUS	BUDS	VIABLE	CALLUS	BUDS	
0	a	0	0	_a	0	0	
7	49	49	0	48	48	0	
28	49	49	0	48	48	0	
42	49	48	1	48	47	1	
49	49	48	. 1	48	47	1	
60	49 [·]	48	1	48	47	2	

b) P. sitchensis

	ENVI	RONMENT	<u>a</u> II	ENVIRONMENT b			
TIME (DAYS)	VIABLE	CALLUS	BUDS	VIABLE	CALLUS	BUDS	
0	_a	0	0	_a	0	0	
7	39	0	0	42	0	0	
28	39	39	0	42	42	0	
42	39	20	19	42	38	4	
49	39	17	22	42	30	12	
60	39	12	27	42	23	19	

KEY

^a No data.

<u>P. contorta</u> showed no difference in the number of embryos responding by bud induction. However, environment <u>a</u> stimulated a higher proportion of <u>P. sitchensis</u> embryos to produce adventitious buds. This effect may be due to the constant temperature of this environment, as the low night temperature ($7^{\circ}C$) of environment <u>b</u> might slow the growth and metabolism of the cultures. Therefore environment <u>a</u> was adopted for the subsequent experiments.

The effects of a 12 day dark pretreatment at $25^{\circ}C$ and incubation under three light intensities were investigated (Table 5.9).

<u>Table 5.9</u> Number of <u>P. contorta</u> and <u>P. sitchensis</u> embryos producing callus and adventitious buds under different light intensities, with and without a l2 day dark pretreatment.

Results scored after 60 days

a) En	nbryos	were	incubat	ed	on	CI	medium	und	ler e	nvi	ronment	<u>a</u> ,
with	light	inten	sities	Ι,	ΪI,	or	III.	15 e	embry	os	treatmer	nt ⁻¹

			12 DAY	DARK PRETREA	TMENT	NO DARK	PRETREA	TMENT
SPI	ECIES	LIGHT	VIABLE	CALLUS	BUDS	VIABLE	CALLUS	BUDS
<u>P.</u>	cont- orta	I	13	5	8	12	10	2
		II	11	- 9	2	13	11	2
		III	12	12	0	12	11	1
<u>P.</u>	sitch- ensis	I	13	4	9	11	7	4
		II	14	6	8	14	7	7
_		III	11	5	6	12	8	4

b) <u>P. contorta</u> embryos incubated on MD medium with BAP under environment <u>a</u> with light intensities I and II following a 12 day dark pretreatment. 50 embryos treatment⁻¹.

BAP (M)	DTT	LIGHT	VIABLE	CALLUS	BUDS
10 ⁻⁶		II	44	38	6
	-	I	34	10	24
	-	I	29	5	24
	+	I	34	2	32
10 ⁻⁵	_	II	45	36	9
	. –	I	35	13	22
	-	I	31	9	22
	+	I	42	8	34

There was a marked enhancement of bud induction from the dark-pretreated embryos of <u>P. contorta</u> under the highest light intensity (I), with 62% of the embryos producing buds as compared to 0 and 18% under the other conditions (Table 5.9a). The morphogenetic response of <u>P. sitchensis</u> was not significantly affected by alterations in the light intensities employed although the dark pretreatment did appear to be of benefit in all the conditions, particularly at the highest light intensity (I).

The enhanced morphogenetic response of <u>P. contorta</u> embryos under these conditions was confirmed by a second experiment in which the cytokinin BAP alone was used (Table 5.9b). The effect of the addition of dithiothreitol (DTT), an anti-oxidant which has been shown to be capable of protecting IAA from oxidation <u>in vitro</u>, was also investigated under the same conditions. The use of this compound possibly

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Callus and adventitious bud induction from excised embryos of <u>P. contorta</u> incubated under environment <u>a</u> I after a 12 day dark pretreatment. Photographs taken after 70 days in culture.

x 15

- A. Bud primordia (b) were initiated on the apical meristem and cotyledons of the embryo. The hypocotyl (h) has no bud and the root (r) has not grown out.
- B,C. Numerous leaf or bud primordia (p) formed on the callus arising from the embryos. Buds (b) were also observed.
- D. Elongating leaves probably enclosing buds (b).



enhanced the absolute number of embryos surviving and producing adventitious buds.

The embryos of <u>P. contorta</u> initiated callus production before bud induction (Plates 16A and 16B) under these conditions (compared with Plates 13A-D). Since in experiment 5.9b there was no exogenous auxin supply, it would appear that the protection of the endogenous auxin by a dark pretreatment and the addition of an antioxidant may have been very important.

Thus <u>P. contorta</u> embryos show a marked response to light immediately following excision, and also require a high light intensity to initiate adventitious bud production. <u>P. sitchensis</u> embryos do not appear to have such strict requirements.

5.3 THE INDUCTION OF CALLUS AND ADVENTITIOUS BUD PRODUCTION FROM SEEDLING EXPLANTS OF P. CONTORTA AND P. SITCHENSIS

5.3.1 2 - 8 WEEK OLD SEEDLINGS

Use of the basal MD medium with the combination of NAA and BAP shown in Table 5.10 resulted in successful callus but not adventitious bud production from hypocotyl segments of <u>P. contorta</u> and <u>P. sitchensis</u>. An exogenous source of auxin was required for callus induction by both species, explants of <u>P. contorta</u> showing better callus proliferation. The higher cytokinin levels appeared to inhibit callus production from <u>P. sitchensis</u> whereas <u>P. contorta</u> explants were apparently unaffected.

The introduction of hypocotyl and cotyledon explants of <u>P. contorta</u> and <u>P. sitchensis</u> into media containing a variety of hormonal combinations has consistently resulted

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in callus production from the hypocotyl but not from cotyledon explants. Alterations in the orientation of these segments (i.e. horizontal or vertical with the morphologically basal or apical ends in the medium) failed to induce adventitious bud production.

Table 5.10	Number of hypocotyl segments of P. contorta and
	<u>P. sitchensis</u> producing callus after 49 days
	incubation on medium MD under environment <u>b</u> .
	20 segments treatment ^{-1} .

NAA (M)		BAP (M)									
		PINUS CONTORTA PICEA SITCHENSIS									
	0	10 ⁻⁷	10 ⁻⁵	2×10^{-5}	0	10-7	· 10 ⁻⁵	2×10^{-5}			
0	0	0	0	0	0	0	0	0			
10 ⁻⁷	17	20	14	_ ^a	0	9	3	0			
10 ⁻⁵	20	17	_ ^a	17	11	11	4	1			

KEY

-^a Contaminated cultures.

However, it was found that culturing the hypocotyl segments in close proximity to the original seedling shoot meristem with its associated cotyledons occasionally resulted in adventitious bud production from these detached explants. Similarly the small section of hypocotyl remaining attached to this seedling meristem was also capable of adventitious bud production. No quantitative data are available to support these observations, but generally it was found that BAP concentrations up to 10^{-6} M tended to cause the extension growth of the original shoot meristem. Occasionally the meristem proliferated to produce two or more shoots.

- A. Callus and bud or leaf primordia (p) initiation from hypocotyl attached to the cotyledons (c) and extending shoot (s) of a 2-8 week old seedling of <u>P. sitchensis</u>. x 3
- B. Callus and bud or leaf primordia (p) initiation from the shoot meristem enclosed in the cotyledons (c) of a 2-8 week old seedling of <u>P. sitchensis</u>. x 20
- C,D. Callus and leaf primordia (p) initiation from the shoot apical meristems of 2 year old seedlings of <u>P. sitchensis</u>. x 20



Higher BAP levels increased the degree of proliferation, inducing the formation of callus and leaf primordia both at the original meristem and along the length of the attached hypocotyl (Plates 17A and 17B).

Thus the hypocotyl segments retain their morphogenetic potential but it seems that the meristem provides some essential factor lacking in the medium. This may also apply to the embryo cultures.

The successful initiation of green callus capable of subculture from hypocotyl segments of both P. contorta and P. sitchensis seedlings has allowed the multiplication of tissue originating from one source. Cheng (1977) reported the induction of advenitious buds from such cultures by either directly applying drops of a cytokinin solution onto the callus or transferring the callus to a high-cytokinin medium. Both of these techniques have been tried with limited success. The first method frequently resulted in the death of the callus although on some buds were produced. The alternative method proved less damaging to the tissue but very few cultures produced adventitious buds. Further work may yield more reproducible results but the advantages of the establishment of rapidly proliferating callus cultures are dubious. Under such conditions genetic aberrations may occur, possibly resulting in an altered genotype of any plantlets regenerated from the cultures.

5.3.2 2 YEAR OLD SEEDLINGS

Shoot tips, stems and needles of <u>P. contorta</u> and and <u>P. sitchensis</u> seedlings transported from Edinburgh were used as explant sources. The cultures were all incubated under environment <u>a</u> II, <u>b</u> or in continuous light $(7 \times 10^3 \text{ ergs cm}^{-2} \text{ sec}^{-1})$ at 25° C.

Preliminary experiments using excised actively-growing shoot tips of both species resulted in callus production from 130 explants of <u>P. sitchensis</u> and from 10 out of the 40 explants of <u>P. contorta</u> when incubated on the CI medium. The environment did not affect this response in either species. The <u>P. contorta</u> explants were heavily contaminated, a problem which was diminished but not completely overcome. Transfer of these cultures to media with high cytokinin levels did not result in adventitious bud production.

Later occasional 'de novo' leaf and bud primordia were obtained on cultured stem and shoot tip explants (ca. 1%) excised from seedlings grown in Leicester. These primordia did not develop further. These results suggested that the time involved in the transportation of the shoots may have adversely affected their response in culture. Therefore, cultures of excised shoot tips of both species and needle pairs of <u>P. contorta</u> were inoculated at the Northern Research Station, Roslin.

A range of concentrations of BAP and zeatin were used in combination with NAA at 10^{-7} M. The CI medium was also used. All cultures were subjected to a 7 day dark pretreatment prior to being exposed to continuous light at 25° C. None of the <u>P. contorta</u> cultures, shoot tips or needle pairs responded to any of the media by adventitious bud production. Creamyyellow callus was produced from ca. 20% of the shoot tips and ca. 38% of the needle pairs.

Adventitious bud production was stimulated from the

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callused shoot tips of <u>P. sitchensis</u> when cultured on BAP containing medium (Plates 17C and 17D). The most effective concentration of BAP was 10^{-6} M (Table 5.11). The medium containing zeatin supported the extension growth of the pre-existing leaf primordia of the shoot tip but neither adventitious buds nor roots were produced.

Table 5.11Number of shoot tips of 2 year old seedlings ofP. sitchensis producing callus and adventitiousbuds when incubated on MD medium with a constantlevel of NAA $(10^{-7}M)$ and the cytokinin levelsas shown or on the control CI medium. Thecultures were incubated under continuous lightat $25^{\circ}C$.

Results were scored after 84 days including 7 day dark pretreatment : 25 shoot tips treatment⁻¹.

	10 ⁻⁵ M		10	6 _M	10 ⁻⁷ M		
CYTOKININ	CALLUS	BUDS	CALLUS	BUDS	CALLUS	BUDS	
BAP	0	4	7	13	0	3	
zeatin	0	0	0	0	0	0	
control medium (CI)	14	3					

Further transfer of these cultures to the same initiation medium resulted in further growth and leaf and bud production. Many of the cultures fell naturally into several smaller pieces which have continued to proliferate. No attempt has been made to stimulate these buds to extend or root.

Wax embedded sections (stained with toliudine blue) of embryos of <u>P. contorta</u> showing adventitious bud initiation.

A. Small protruberance (p) from the embryo tissue (e) which may be an early stage of the development of a primordium.

x 50

B,C. Bud primordia (b) with small dense cells developing from the degenerating embryo tissues (e). Note the bud apex (a) and well-developed leaves (l). x 50
D. Shoot apex (a) and leaf (l) with the position of vascular tissue (v) shown. x 50



PLATE 18

5.4 MORPHOLOGY AND ANATOMY OF THE ADVENTITIOUS BUDS PRODUCED FROM EMBRYOS OF P. CONTORTA AND P. SITCHENSIS

The morphology of the adventitious buds of the two species differed considerably. Whilst <u>P. sitchensis</u> buds were clearly defined and protruded from the callus mass (see Plate 14D) those produced by <u>P. contorta</u> tended to form in whorls of extending leaf primordia (see Plates 15C and 15D) and were therefore difficult to identify. The extension growth of these primordia was often due to leaf not shoot growth.

The formation of adventitious buds from the embryo cultures of <u>P. contorta</u> was studied. Wax embedded samples after 70 days incubation on BAP containing medium were sectioned and stained (Plate 18). The embryos had degenerated, the cells were large and dissociated whilst the epidermis remained intact. The buds appeared to be formed from the epidermal cells : small protuberances were observed which may have been early stages of primordium development (Plate 18A). The later stages of bud production have clearly defined opical domes and leaf primordia (Plates 18B and 18C), the vascular tissue being developed later (Plate 18D). The developing primordia had small dense cells which contrasted with the degenerating tissue of the explant. No root initials were observed.

5.5. MULTIPLICATION AND FURTHER GROWTH AND ROOTING OF REGENERATED SHOOTS FROM EMBRYOS OF P. CONTORTA AND P. SITCHENSIS

Up to 40 buds have been counted from one embryo of both P. contorta and P. sitchensis. Further multiplication of these shoots has been achieved by either subculturing or transferring these explants plus buds onto the same initiation medium. The hormones employed in the initiation medium appear to be important in governing the response of these cultures. The CI medium, containing the auxins IAA and IBA and the cytokinins 2iP and BAP initiated further callus but not bud production from the organised explants. By contrast, the maintenance of the cultures on NAA and BAP allowed the continued production of buds even on subculture.

<u>P. sitchensis</u> cultures initiated on medium containing BAP were capable of continued growth and bud production on transfer to fresh medium. However, the use of kinetin and 2iP as the **c**ytokinin source did not result in further callus or bud production and some explants became brown and necrotic. These kinetin-or 2iP-induced buds grew out into shoots whilst still on the initiation medium. Thus the judicious choice of auxins and cytokinins may allow controlled and continued proliferation of callus plus buds.

<u>P. sitchensis</u> cultures (callus plus buds) were also placed in liquid medium in rotating Steward flasks. Although they produced several pieces of callus each with buds, their growth was not enhanced by this treatment and with time they became brown and necrotic.

In both species the slow outgrowth and occasional rooting of the adventitious buds initiated on BAP containing medium was achieved on transfer of the callus plus buds to a half strength, hormone-free medium (BG) (Plates 19A and 19B). Initially several of the shoots started to grow out but only one or two continued to extend, possibly due to an apical

- A. Outgrowth of shoots (s) regenerated from an excised embryo of <u>P. contorta</u>. Shoots plus callus (c) maintained on 'BG' medium. x 4
- B. Root production (r) from shoot (s) regenerated from an excised embryo of P. contorta.
 x 2
- C. Extension growth of shoots regenerated from an excised embryo of <u>P. sitchensis</u>, whilst still on initiation medium which contained kinetin. x 2
- D. Root production (r) from excised regenerated shoot of P. sitchensis. x 4
- E. Continued bud induction (arrowed) from excised regenerated shoot of <u>P. sitchensis</u>, re-introduced to a BAP-containing medium.
 x 5



dominance effect, as seen in intact plants. This was supported by the observation that the excision of these extended shoots resulted in one or more of the inhibited shoots growing out. In <u>P. sitchensis</u>, at least, buds initiated on kinetin medium did not show this pattern of response. All of these buds elongated simultaneously (Plate 19C) both on the initiation medium and on the 'BG' medium, growing at a faster rate than was observed for buds initiated on BAP medium (Table 5.12). Approximately 5% of these shoots procuced roots (Plate 19D). The inclusion of 2iP or kinetin in the BG medium used for the outgrowth of BAP initiated buds appeared to enhance the outgrowth of one or two shoots.

To improve their growth and rooting performances in culture excised shoots, at least 10mm long, were transferred to BG medium alone or with various hormones. The shoots were cultured both on semi-solid medium and on filter paper bridges over stationary liquid medium, and incubated under an 18 hour photoperiod at 25^oC. No difference was observed in the response of the shoots when maintained by these two different methods.

The addition of GA_3 to the medium did not increase the growth rate or induce rooting of the shoots (Table 5.12). BAP in the medium inhibited the growth of the shoots and prevented root induction at all except the lowest concentrations $(10^{-8}M)$. Some of the shoots responded to the higher levels of BAP by the production of adventitious buds along the stem and needles (Plate 19E). No attempt was made to grow these buds into shoots.

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<u>Table 5.12</u> Growth, rooting and bud induction of excised regenerated shoots of <u>P. sitchensis</u> incubated under 18 hour photoperiod at 25^oC for 7 weeks. Values expressed as percentages : 10 shoots treatment⁻¹ unless otherwise stated.

		AVERAGE RATE		
MEDIU	M CYTOKININ IN	OF GROWTH	% SHOOTS	% SHOOTS
	ORIGINAL MEDIUM	$(mm week^{-1})$	ROOTING	WITH BUDS
BG	BAP + 2iP	0.50	1.05 ^a	Q
BG	kinetin	2.00	4.60 ^b	0
BG + I	АР			
10	⁸ _c	0	10.0	0
10	7 _	0	0	20
10	6 _	0	0	40
10	5 _	0	0	50
BG + (A ₃	· • • • • • • • • • • • • • • • • • • •		
10	7 –	0.20	0	0
10	6 _	0.30	0	0
10	5 _	0.25	0	0
BG + 1	AA			
and II	A -	0	40.0 ^d	0

KEY

a Out of 95 shoots.

b Out of 87 shoots.

c Not known.

d Not necessarily attached to shoot.

In an attempt to induce rooting, the auxins NAA and IBA were applied to the basal end of the excised shoots by a 7 day transfer into auxin media followed by transfer to BG

Plate 20

A. Regenerated plant from excised embryo of Pinus contorta.

B. Regenerated plant from excised embryo of <u>Picea</u>

sitchensis.



medium. This treatment resulted in the production of callus from the base of the shoot; although roots were frequently produced from this callus, they did not always connect with the shoot (Table 5.12).

Shoots of both <u>P. contorta</u> and <u>P. sitchensis</u> were transported to Edinburgh for non-sterile rooting, where they were maintained under a mist propagator. About 20% of these shoots from both species have been rooted. Some have been 'hardened off' and successfully maintained in the greenhouse (Plates 20A and 20B). Four such regenerated plants known to originate from one embryo of <u>P. contorta</u> show similar growth and morphology implying genetic uniformity.

5.6 DISCUSSION

It is generally considered that the expression of the totipotency of cells is most readily achieved in cultures which have been recently initiated from embryonic material. Such tissues are highly meristematic and relatively uncommitted to a pathway of differentiation. In mature trees the equivalent state is found only in cambial cells and root and shoot apical meristems, hence their use for the establishment of tissue cultures in the above experiments. In some gymnosperm genera, including the Pinaceae there are buds present on the dwarf shoots which bear the leaves. These buds are capable of further development and outgrowth after stimulation by removal of the dominant apical meristem or by spraying the intact plant with BAP. Haines and de Fossard (1974) have successfully introduced such needle fascicles of <u>Araucaria cunninghamii</u> into culture and induced the shoot to grow out; in some cases, these buds have proliferated and some of these shoots have rooted.

In the present study, excised embryos of P. contorta and P. sitchensis were found to be the most responsive source of explants. In contrast to the results obtained by Cheng (1977) neither P. contorta nor P. sitchensis cotyledon explants produced either callus or adventitious buds. The morphogenetic potential of the hypocotyl segments of these two species was only expressed while either still attached to or immediately adjacent to the original seedling meristem with its associated cotyledons. This implies that the seedling meristem and cotyledons supply some essential factors for bud differentiation which leach out into the Preliminary experiments using more mature tissues medium. (2 year old seedlings) of both species indicated that under appropriate conditions P. sitchensis shoot tip and stem cultures were able to express their morphogenetic potential. Incubation under optimal conditions may therefore allow the excised tissues of P. contorta to respond similarly.

The major part of this study involved the use of the more responsive embryo cultures in an attempt to define conditions conducive to adventitious bud production and ultimately plantlet formation.

The ratio between auxin and cytokinin has been found to be important in regulating root and shoot production from callus cultures of tobacco (Skoog and Miller, 1957) and poplar (Chalupa, 1974). In gymnosperm cultures the response of the explants is also dependent upon the hormonal balance of the medium. Shoot initiation results from high cytokinin: auxin ratios in callus cultures of tobacco, poplar and gymnosperms. However in gymnosperm cultures, callus but no root initiation is induced in the presence of a high auxin : cytokinin ratio.In fact,gymnosperm root development appears to be generally inhibited in culture; the pre-formed roots of the <u>P. contorta and P. sitchensis</u> embryos studied here rarely developed even when cultured on a hormone-free basal medium. It is surprising that in contrast to these general findings, callus cultures of <u>Pinus radiata</u> have been used as a bioassay to detect chemicals which stimulate root formation (Anon., Report of Forest Research Institute, 1976).

The hormones used influence both the form of the response and the ability of the cultures to continue callus and bud proliferation on subculture. Combinations of NAA and BAP have been commonly used in adventitious bud initiation from explants of gymnosperms (Isikawa, 1974; Campbell and Durzan, 1975; Sommer et al, 1975; Cheng, 1977). The effects of the auxins IAA, IBA and 2,4-D (Cheng, 1975,1977; Winton and Verhagen, 1977) the anti-auxin NOA (Winton and Verhagen, 1977) and the cytokinins 2iP and kinetin (Cheng, 1975, 1977) have been less extensively studied. From the results obtained in this study <u>P. contorta</u> and <u>P. sitchensis</u> embryos, it would appear that incubating the explants on the CI medium (containing 5µM IAA and IBA) resulted in callus initiation followed by bud differentiation. The degradation of these auxins by photo-oxidation and the IAA oxidase and peroxidase activities of the tissues would result in the increased cytokinin : auxin ratio required for bud induction. However further subculture of these explants plus buds into the same initiation medium resulted in a reversion of the organised

primordia into callus. In Pseudotsuga menziesii cotyledon cultures the substitution of the more stable synthetic auxin NAA and 2,4-D for IAA and IBA decreased variations in the response of the explants due to physiological conditions or genetic complement of the tissues (Cheng, 1977). These tissues showed maximal morphogenetic response at concentrations of NAA and 2,4-D in the 0.5-50.0nM range. Similarly Sommer .(1975) reported the enhancement of adventitious bud formation in embryo explants of Pseudotsuga menziesii at low exogenous auxin concentrations. NAA (at 10^{-7} M) was also found to be an effective substitute for the natural auxins in the P. sitchensis embryo cultures studied here, allowing more controlled callus and bud proliferation of the explants on subculture. The response of P. sitchensis to 2,4-D differs from that observed in most other species (excepting monocotyledons); 2,4-D was ineffective in suppressing adventitious bud formation and stimulating callus production even at high concentrations. Winton and Verhagen (1977) reported that the anti-auxin NoA supported adventitious bud production from Pseudotsuga menziesii cultures. However, in the present Picea sitchensis embryo cultures adventitious bud production was suppressed by the anti-auxin 3,5-D. Since auxins and anti-auxins compete for the same sites, this suggests that endogenous auxin, as well as cytokinin, is required to initiate bud formation.

Alterations in the cytokinin source for bud induction resulted in different morphogenetic responses of the cultures of <u>P. sitchensis</u>. BAP resulted in the production of numerous leaf and bud primordia whilst 2iP or kinetin at the same concentration initiated the development of fewer buds. When incubated with zeatin, the preformed roots of the excised embryo were able to grow and develop further, the shoot meristem often growing out. This was also observed in <u>P. contorta</u> cultures. Occasional buds were formed in cultures of both species when incubated for a longer time. The location of these isolated buds was similar to that observed in embryos of <u>Pseudotsuga menziesii</u> cultured on low BAP concentrations (Winton and Verhagen, 1977). In contrast to 2iP and kinetin, BAP inhibited shoot extension growth in <u>P. sitchensis</u> cultures, similar observations have been made for cultures of <u>Populus nigra</u> (Venverloo, 1973) and <u>Pseudotsuga menziesii</u> (Cheng, 1977).

In addition to the influence of hormones on the differentiation of buds from cultures of gymnosperms, the physical environment (i.e. basal media and light regime) has also been shown to be important. Maximal adventitious bud production from <u>Pseudotsuga menziesii</u> and <u>Pinus palustris</u> has been shown to be achieved on different basal media (Brown and Sommer, 1977). No difference was observed in cultures of <u>P. contorta</u> or <u>P. sitchensis</u> maintained on the basal media of MS and MD.

Although successful adventitious bud production has been induced from embryos of <u>Pseudotsuga menziesii</u> under a variety of photoperiods (Cheng, 1975, 1977: Winton and Verhagen, 1977) 12 hours was more effective than 18 hours. The environment was also found to affect morphogenesis in the cultures of <u>Pinus contorta</u> embryos studied here; these embryos were found to have a marked sensitivity to light immediately after excision and after a dark pretreatment. Although the medium was capable of supporting both callus

and adventitious bud production this potential was suppressed until the environmental conditions were suitable.

As previously mentioned the shoot growth of P. sitchensis was influenced by the cytokinin employed to initiate the adventitious buds. These hormones also appeared to affect the rooting performance of the extended shoots. On transfer to the hormone-free 'BG' medium, BAP-induced shoots grew slowly and only ca. 1% of them rooted. On the same medium, kinetin-induced shoots grew more rapidly and ca.5% rooted. Application of either NAA or IBA to the basal end of the shoot resulted in callus formation followed by root The shoots and roots were often separated by initiation. the callus. Poor root initiation has also been found in shoots regenerated from other gymnosperm species (Campbell and Durzan, 1975; Brown and Sommer, 1977; Winton and Verhagen, 1977). Cytokinins have been shown to inhibit rooting in cuttings of several species (Humphries, 1960; Eriksen, 1974; Smith and Thorpe, 1975) and in callus of Populus sp. (Venverloo, 1973). An endogenous carry-over of cytokinins in the shoots of P. contorta and P. sitchensis described here could have had an inhibitory effect on their rooting. Lowering the cytokinin levels could result in rooting, the rate of depletion determining the timing of root production. Kinetin is generally considered to be less effective as a cytokinin than BAP, and this could explain the difference in response of the P. sitchensis cultures to these hormones. A combination of BAP and kinetin (as suggested by Cheng, 1977) in the medium may prove advantageous in obtaining prolific bud induction with the buds capable of more rapid extension growth and better rooting. If the rooting of these shoots,

preferably in culture, cannot be increased this technique offers no advantage over the traditional method of the rooting of cuttings.

The high percentage (20%) of regenerated shoots of both species under non-sterile growth conditions may have resulted from the selection of the healthier shoots. The conveyance of plant material to Edinburgh possibly damaged the less vigorous shoots. Ideally further work on propagation of plants via tissue culture techniques should be undertaken where there is easy access to the parent plants and to facilities for rooting the regenerated shoots under mist.

The full potential of the application of tissue culture techniques to the mass propagation of gymnosperms has not been realised. The factors known to influence the rooting of cuttings appear also to affect the response of the tissues in culture. Further investigations into the cultural and environmental requirements of cultures initiated from mature trees (as opposed to juvenile plants) may allow the expression of the totipotency of these cells.

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CHAPTER 6

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GENERAL DISCUSSION

CHAPTER 6

GENERAL DISCUSSION

The main points which have arisen from each aspect of the work reported here have been discussed at the end of the relevant chapter. It thus remains only to consider the progress made during this investigation and to suggest the direction of future work.

The callus and adventitious bud induction in both species <u>Pinus contorta</u> and <u>Picea sitchensis</u> was found to be influenced by three main factors: the explant source, the environmental conditions, and the media and hormones employed. Although callus was initiated from a variety of sources of both species it was found possible to establish cultures only from juvenile explants; the survival of the callus and suspension cultures of <u>P. contorta</u>, at least, proved to be limited. Optimal induction of adventitious buds from explants of both species was also achieved in juvenile tissues, the morphogenetic response of the cultures diminishing with increasing age of the explant.

For the application of tissue culture techniques to the

problem of clonal propagation of a species, it is not necessary to develop a fully defined medium. Thus if the undefined organic supplements casein hydrolysate, coconut milk, and yeast or malt extracts improve the growth or morphogenetic response of the cultures they should be used. However, if a study of the control of differentiation (either on the tissue or cellular levels) is to be made, it is necessary that the medium should be completely defined.

The nitrogen source and concentration in the medium was found to be important to cell division and to both cell and tissue differentiation. The use of media containing NH_{A}^{+} ions allowed the continued proliferation of callus and suspension cultures of P. contorta. In the presence of NO_{2} or glutamic acid alone growth could not be maintained through successive passages. The addition of casein hydrolysate to the $\operatorname{NH}_{4}^{+}$ ion containing media inhibited the morphogenetic response of the embryo cultures of P. contorta but not the xylogenic response of the suspension cultures. Yeast extract caused the immediate death of both P. contorta and P sitchensis callus cultures. Steinhart et al, (1961) reported a similar affect of malt extract on Picea abies cultures. The addition of other amino acids alone or in combination had little effect on the callus cultures of P. contorta but the combination of amino acids killed the embryo cultures of both species.

The availability of nitrogen (supplied as NH_4^+ and NO_3^-) has been implicated in the regulation of embryogenesis (Reinert <u>et al</u>, 1971), xylogenesis (White and Gilbey, 1966; Phillips and Dodds, 1977), in secondary metabolism (Phillips and Henshaw, 1977), and also in the biosynthesis of auxin

(Avery et al, 1937; Avery and Pottorf, 1945). This is obviously a very important aspect of the control of differentiation (possibly mediated by auxin production) and should be investigated further, particularly with respect to the possibility of inducing embryogenesis in suspension cultures of <u>P. contorta</u>. The cells of these cultures did not show the typical segmentation observed during embryogenesis although they were capable of differentiating into tracheary and phloem elements. Whilst preliminary investigations into factors controlling the regulation of cytodifferentiation were hampered by the change in the response of the cultures with time, further work may allow critical investigations to be made.

In the P. contorta suspension line 721 described here, the frequency of the cell-type observed could be altered by the manipulation of the concentration of boric acid. Whilst high levels (124mg l^{-1}) increased tracheid production, low levels (Omg 1^{-1}) reduced tracheid but enhanced phloem element production. In culture line 1A, maintained on a constant level of boric acid $(6.2 \text{mg } 1^{-1})$, ultrastructural features of both cell-types were observed although tracheid production predominated. The increased lignification and final death of this culture line may have been related to this lower level of boric acid supplied in the medium, whereas the death of line 721 may have been due to toxic effects of the high level of boron or the loss of ability to synthesise some essential vitamin. Thus, in addition to studying the effects of various organic and major inorganic components of the medium, it is very important to study the effects of the trace elements and possibly vitamins on the differentiation

of the cells.

Embryogenesis has a great potential as a means of mass propagation of gymnosperm species (Winton and Huhtinen, 1976) and would circumvent the problem of the rooting of shoots regenerated via tissue culture. The pattern of tracheid production during the culture time of both lines 1A and 721 was reminiscent of the time course of embryogenesis in <u>Daucus carota</u> suspension cultures (Meyer-Teuter and Reinert, 1973; Smith and Street, 1974). Genetic variation has been suggested to influence the embryogenic response of suspension cultures (Smith and Street, 1974) and is known to occur in suspension cultures of other species e.g. <u>Acer pseudoplatanus</u> (Bayliss and Gould, 1974). An investigation of the ploidy levels of suspension cultures of <u>P. contorta</u> revealed that after 6 and 8 months in culture, they remained predominantly diploid.

Although general principles may be applied, the optimal combination of hormones for callus and adventitious bud production must be determined for each species considered. Callus initiation from <u>P. contorta</u> was achieved easily on both NAA and 2,4-D whereas <u>P. sitchensis</u> explants were relatively unaffected by this synthetic auxin. Incubation of embryos of the latter species on high levels of 2,4-D with BAP and 2iP did not prevent morphogenesis as had been reported in <u>Populus</u> sp. (Venverloo, 1973). The effectiveness of 2,4-D as an auxin is known to vary between species, possibly due to the rate of metabolism of the compound by the tissues (Feung <u>et al</u>, 1975). The presence of endogenous auxin appeared to be essential for the induction of callus and adventitious buds from embryos of P. sitchensis, **as** the presence of the

anti-auxin 3,5-D inhibited morphogenesis. The addition of an anti-oxidant such as DTT may protect the endogenous auxin (Stonier, 1970) and may prove advantageous for cultures arising from more mature explant material. In juvenile explants of <u>P. sitchensis</u>, it was found that the use of the more stable auxin NAA was better than IAA or IBA since controlled callus and adventitious bud proliferation occurred simultaneously. This could be important in retaining the genetic uniformity of the propagated shoots since organised cultures are considered to be more stable than disorganised callus.

The influence of cytokinins on the morphogenetic response of the embryos of <u>P. sitchensis</u> was studied. Their marked effect on the multiplication of cultures, shoot growth and should be investigated more closely. Certain hormonal combinations may prove ideal for the multiplication of organised cultures without inhibiting later shoot growth and rooting. Although non-sterile rooting has proved more successful than sterile rooting in culture, the advantages of such a method are not great since ideally the extended shoots should be able to regenerate into complete (rooted) plantlets whilst still in culture, thus facilitating the 'hardened off' procedure and eventual planting out.

In addition to the use of cambial tissue and apical shoot tips, the possibilities of utilising other sources of explants from mature trees have also been investigated. The introduction of male and female gametophyte tissues of <u>P. contorta</u> into culture resulted in callus initiated from the pollen grains only. Whilst in two species in the family Solanaceae (Nicotiana tabacum and Datura innoxia) haploid

plantlets have been produced by pollen grains undergoing embryogenesis in culture (see Sunderland, 1971, 1973) this is by no means a universally applicable technique. If the <u>P. contorta</u> pollen grains or callus initiated from them proved to be morphogenetic the production of haploid or homozygous diploid plants may greatly benefit tree-breeding programmes (Winton and Huhtinen, 1976), however as Winton and Stettler (1974) have stated many tree species have deleterious recessive genes which may depress the growth and fertility of these progeny.

Mature trees of <u>P. contorta</u> bear dwarf shoots on which the needle pairs are produced. These shoots have a partially developed bud. Although attempts to induce this bud to develop further in culture were unsuccessful, it is a readily available source of meristematic cells and may prove useful either for callus initiation or direct bud production and ultimately plantlet formation as described for <u>Araucaria</u> <u>cunninghamii</u> (Haines and de Fossard, 1977).

The initiation of callus and suspension cultures from seedlings of <u>P. contorta</u> and from mature explants of both <u>P. contorta</u> and <u>P. sitchensis</u> was found to be influenced by the genotype and/or the physiological condition of the source material. In <u>P. contorta</u> the rooting of these mature shoots also depended upon the clonal origin and possibly the age of the parent trees. The variations observed in the morphogenetic response of the embryos of both species also may reflect differences in the genotype or the physiological condition of the explants. These factors are involved in the control of embryogenesis in suspension cultures of <u>Daucus carota</u> (Smith and Street, 1974) and morphogenesis in

cotyledon explants of Pseudotsuga menziesii (Cheng, 1977). Although the genotype of the explants is important in determining the response of the cultures, the influence of physiological age is of major concern if the techniques described are to be extended to mature trees. In the present study juvenile explants proved more responsive in culture, although adventitious bud induction was also achieved from shoot tip cultures initiated from 2 year old seedlings of P. sitchensis. Stem explants from this latter source and hypocotyl segments of both species have produced occasional buds in culture. Cheng (1976) has reported bud induction from explants of seedlings up to 2 years old of several gymnosperm species whilst Winton and Verhagen (1977) have reported the induction of bud-like structures on callus from mature (15-20 year old) trees. The development of such structures is very encouraging since it indicates the retention of totipotency by the cells of these explants. Α similarly low frequency of bud induction was observed in the P. contorta embryos studied here until the optimal environmental conditions for morphogenesis were defined. The culture of the explants from mature trees under conditions which satisfy all their requirements may allow the full expression of their morphogenetic capability.

Thus, although cultures of juvenile tissues of <u>P. contorta</u> and <u>P. sitchensis</u> have been successfully induced to regenerate into plantlets, the potential for the use of such regenerants is limited since propagation by traditional methods is also feasible from juvenile material. It is, however, possible that plants regenerated via tissue culture could be used for clonal root stocks for grafts, once tested and approved for

compatibility. This method of propagation has one advantage over grafting and rooting of cuttings, i.e. their growth is orthotropic. Otherwise, the same factors which influence the rooting of cuttings appear also to affect callus induction and possibly the morphogenetic response of the cultures. One alternative to attempting plantlet regeneration directly from the mature trees would be to 'rejuvenate' the material by serial grafting or rooting of cuttings. This is obviously both time consuming and costly but may be necessary to obtain explant material in a physiological state permissive of morphogenesis.

Thus, two possible approaches to clonal propagation of gymnosperm species have been considered: the induction of embryogenesis and the production of adventitious buds followed by the growth and rooting of the extended shoots. The latter method only has proved to have limited success, its application being restricted to juvenile tissues. A closer examination of the influence of the environment and the availability of hormones and nutrients to the cultured explants may eventually result in the definition of conditions optimal for morphogenesis, not only from explants from various species but also of different physiological ages.

APPENDIX I

CONSTITUENTS	OF BASAL MEDI	[<u>A</u> (all value	s as mg 1^{-1}))
А		MEDIA		
Constituents	Murashige & Skoog (1962)(MS)	Linsmaier & Skoog (1965)(LS)	Reinert* & White (1956)(RW)	Heller (1953)
Macronutrient	ts:			
NH4NO3	1650	1650	50	-
KNO3	1900	1900	170	-
Ca(NO ₃) ₂	-	-	425	-
NaNO ₃	-	-	-	600 ⁻
CaC12.2H20	440	440	-	75
KH ₂ PO ₄	170	170	-	-
NaH ₂ PO ₄ .H ₂ O	-	_	35	125
KC1	-	_	140	750
$MgSO_4.7H_2O$	370	370	764	250
$^{\text{Na}_2\text{SO}_4}$	-	_	425	-
Micronutrien	ts:			
$MaSO_4.4H_2O$	22.3	22.3	9.0	0.01
KI	0.83	0.83	1.6	0.01
$CoCl_2.6H_2O$	0.025	0.025	-	-
$ZnSO_4.7H_2O$	8.6	8.6	3.2	1.0
$CuSO_4.5H_2O$	0.025	0.025	-	0.03
H ₃ BO ₃	6.2	6.2	3.2	1.0
NiCl ₂ .6H ₂ O		-	-	0.03
$Na_2MoO_4.2H_2O$	0.25	0.25	-	-
AlCl	-	-	-	0.03
FeSO4	_	_	2.5	-
FeCl ₃ .6H ₂ O	-	_	-	1.0
$FeSO_4.7H_2O$	27.8	27.8	27.8	-
na_2^{EDTA}	37.25	37.25	37.25	-

* as modified by Wolter and Skoog (1966)

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	Murashige	Linsmaier	Reinert	
Constituents	& Skoog (1962)	& Skoog (1965)	& White (1956)	Heller (1953)
Organic:	******	- <u>100 - 717 - 100 - 717 - 100 - 100 - 100 - 100 - 100 - 100 - 100 - 100 - 100 - 100 - 100 - 100 - 100 - 100 - 100</u>	······································	
Sucrose	20000	20000	20000	20000
Meso-inositol	100	100	100	100
Casein hydrol	ysate -	1000	-	-
Glycine	3.0	3.0	_	
Thiamine-HCl	0.1	0.1	0.1	1.0
Pyridoxine-HC	1 0.1	0.1	0.1	-
Nicotinic aci	d 0.5	0.5	-	-

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B. MEDIA

	Somme (1	r <u>et al</u> 975)	Durz		
Constituents	1	2	Original	Modified*	'BG'
Macronutrients:			· · · · · · · · · · · · · · · · · · ·		
NH4NO3		1000	1600	800	400
$(NH_4)_2 SO_4$	200	- .	-	-	-
KNO3	1000	1000	170	340	170
$Ca(NO_3)_2 \cdot 4H_2O$	_	347	980	980	490
CaCl ₂ .2H ₂ O	150	_	-	_	-
KH ₂ PO ₄	_	300	170	170	85
NaH ₂ PO ₄ .H ₂ O	90	-	· <u>-</u>	-	-
Na ₂ HPO ₄	30	-	-	-	-
KCI	300	65	. –	65	32.5
$MgSO_4.7H_2O$	250	35	370	370	185
<u>Micronutrients</u> :					
MnSO ₄ .H ₂ O	10.0	10.0	16.9	16.9	16.9
KI	0.75	0.8	0.83	0.83	0.83
CoCl ₂ .6H ₂ O	0.25	0.25	0.025	0.025	0.025
$2nSo_4.7H_2O$	3.0	3.0	8.6	8.6	8.6
$CuSO_4.5H_2O$	0.25	0.25	0.025	0.025	0.025
II ₃ BO ₃	3.0	3.0	6.2	6.2	6.2
$Na_2MOO_4.2H_2O$	0.25	0.25	0.25	0.25	0.25
FeSO4.7H2O	27.8	27.8	27.8	27.8	27.8
Na ₂ EDTA	37.3	[•] 37.3	37.3	37.3	37.3

*Salts of Campbell and Durzan (1975) and ** sucrose, mesoinositol and thiamine-HCl levels of Cheng (1975).

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	Sommer (19	et <u>al</u> 75)	Durz		
Constituents	1	2	Original	Modified	* 'BG'
Organic:	······································				
Sucrose	20000	20000	35000	30000**	15000
Meso-inositol	10	10	100	500**	50
Thiamine-HCl	1.0	1.0	0.4	5.0**	0.05
Nicotinic acid	0.1	0.1	-	-	-
Pyridoxine-HCl	0.1	0.1	· <u> </u>	-	-
L-glutamine	-	_	600	. –	-
L-glycine	-	_	300	-	_
L-asparagine	· _	-	200	-	
L-arginine	-	-	50	-	-
L-methionine	-	- .	30	-	-

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APPENDIX II

MEASUREMENT OF THE LIGNIN CONTENT OF SUSPENSION CULTURES.

The properties of lignin (Brauns, 1952; Brauns and Brauns, 1960) have been utilised in attempts to develop methods for the estimation of the lignin content of tissues (see Venverloo, 1969).

The lignin content of vascular bundles of celery (Siegel et al, 1960) and of Acer cell-suspension cultures (Carcellar et al, 1971; King, 1976) has been estimated by the quantification of the colour reaction between the soluble products of ethanolysis of lignin and phloroglucinol-HCl. However, since only certain aldehyde groups of the lignin react with the stain, and these groups do not always occur at a constant frequency (Spurný and Sladký, 1955), the intensity of the resultant colour is not necessarily a measure of the degree of lignification of tissues. Bearing in mind this major consideration, this method of lignin estimation was chosen as the basis for the studies described here. An outline of the procedure developed using Pinus contorta suspension cultures and both Acer pseudoplatanus and P. contorta wood is given here.

Acer and Pinus Wood

Non-compressed branches from trees of both species were chosen, debarked, milled, boiled in water (2 x 6 hours), dried and stored in a desiccator for use throughout the experiments. Initially the wood was pretreated by continuous extraction (24 hours) with ethanol : benzene (1 : 2) (Roadhouse and MacDougall, 1956) but this was found not to alter the readings obtained significantly and was later discontinued.

Samples of the stored wood were weighed into separate McCartney bottles with 5ml of acidified ethanol and were left to infiltrate for 30 minutes at 25°C prior to heating. After 20 minutes ethanolysis at 70°C the filtered supernatant gave no colour reaction with phloroglucinol-HCl, whilst the wood residue remained positive. Extending the ethanolysis treatment to 24 hours resulted in the phloroglucinolpositive response of both the filtrate and the residue. Autoclaving the residues (121[°]C for 15 minutes) was found to substitute satisfactorily for the prolonged ethanolysis at 70°C. In both Acer and Pinus woods, the peak of colour development was reached 2 minutes after the addition of conc.HCl. A similar pattern of colour development has also been reported for alcohol-extracted residues from celery (Siegel et al, 1960) where the maximum density was reached after 1 minute, fading after 3 minutes.

Calibration curves were constructed for both <u>Acer</u> and Pinus wood (Fig.II.1). There was good agreement between replicate samples of both species but the slopes of the lines for <u>Acer</u> wood were much more consistent than those for Pinus wood.

Since angiosperm and gymnosperm lignins differ considerably in their composition (Venverloo, 1969), it was decided that only the gymnosperm wood should be used as a standard.



Calibration curves for estimation of lignin content of <u>Acer</u> and <u>Pinus</u> wood.

Pinus contorta suspension cultures

The technique described above was applied to cells harvested from stock suspension cultures of P. contorta. The cells were collected, washed with distilled water and then hot methanol before being "acetone-dried". Weighed samples of cells were autoclaved (121°C for 15 minutes) in sealed vials containing the acid-ethanol. During the ethanolysis treatment, the cells and the supernatant became red (absorbance peak between 539-554nm). This red colouration was assumed to be due to the hydrolysis of procyanidins to anthocyanidins, some of which exhibit a phloroglucinolaldehyde type of reaction in the presence of acid. Since anthocyanidins are soluble in aqueous NaOH, these compounds were extracted by alkaline hydrolysis prior to the ethanolysis treatment. One possible disadvantage of this pretreatment is that hot alkali also solublises lignin (Venverloo, 1969), therefore the extraction time was minimised.

Cells thus pretreated were autoclaved (121°C for 15 minutes as for milled wood samples but no phloroglucinolpositive compounds were detected in the filtrate. The ethanolysis was repeated twice more using the same cells with fresh acid-ethanol each time. The third autoclaving extracted ca. 60% of the total phloroglucinol-positive compounds. The autoclaving times were accordingly extended from 15 minutes to 30 minutes and were repeated twice more (Table II.1). The filtrate from each ethanolysis treatment was tested for its reactivity with phloroglucinol-HCl and the values summed. Using the extended autoclaving times, over 70% of the phloroglucinol-positive component of the cells was solublised by the 1st autoclaving. Whilst the replicate samples showed consistency, the values obtained from confirmatory extractions differed markedly (Table II.2) a characteristic also observed in pine wood samples.

Table II.1Optical density values for the phloroglucinol-
positive coniferaldehyde extracted by ethanolysis
from <u>P. contorta</u> stock suspension cultures.
The samples were autoclaved for 3 x 30 minutes
 $(121^{\circ}C)$ in acidified ethanol. The optical
density was read at 540nm.

WEIGHT SAMPLE	ETHANOLYSIS TIME				
(mg)	lst x 30min	2nd x 30min	3rd x 30m i n	TOTAL	
50	0.13	0.040	0.00	0.170	
50	0.15	0.035	0.01	0.195	
100	0.32	0.080	0.00	0.400	
100	0.30	0.060	0.00	0.360	

<u>Table II.2</u>. Optical density values for the phloroglucinolpositive coniferaldehyde extracted by ethanolysis from <u>P. contorta</u> stock suspension cells. Extraction was by 1 x 30 minutes autoclaving in acid-ethanol.

> Optical density values from repeated single ethanolysis treatments using the same stock sample of cells

WEIGHT SAMPLE (mg)	OPTICAL DENSITY						
50	0.220	0.778	0.525	0.537	0.525		
100	0.400	1.415	1.000	1.120	1.000		

METHOD

Pretreatment for suspension cu	ltures of Pinus contort	a
Filter cells onto GF/C pa	1	
Wash with distilled water	2 x	5ml
Wash with methanol (80 ⁰ C)	3 x	5m1
Wash with acetone	2 x	5ml
Dry and store in a desicc	ator	·
Weigh out 25mg dried samp	le	
Add 1.OM NaOH		5m1
Leave at 100 ⁰ C for 30 min	ates	
Wash with cold 1.OM NaOH	(by centrifugation) 5 x	5m1
Wash with distilled water	3 x	5m1
Wash with acetone	l x	5ml
Stimation of lignin content o	f cultures and wood	
Place sample (dried cells	or wood) in McCartney bottle	nmg
Add ethanol – 3% (v/v) HC	1	5m1
Seal tightly and leave fo	r 30 minutes at 25 ⁰ C	
Ethanolysis treatment:	· · · ·	
for wood 15 minutes at	121 ⁰ C (15 p.s.i.)	
for cells 30 minutes at	121 ⁰ C (15 p.s.i.)	
Filter and wash with acid	ified ethanol 2 x	lml
Make filtrate up to 5ml		
Add phloroglucinol (625mg	in 10ml ethanol)	O.4ml
Leave 1 minute at 25 ⁰ C, a	dd conc.HCl	0.2m1
Allow colour to develop,	record at peak	·
Read optical density at :	540nm for coniferalde	nyde
465nm for vanillin - agai	nst a reagent blank.	

In the <u>P. contorta</u> suspension culture samples, the colour reaction between phloroglucinol-HCl and the coniferaldehyde groups reached the maximum intensity after varying times in the range of 2-15 minutes.

General observations for both P. contorta suspension cultures and wood.

A repeating scan of the visible region of the spectrum during the development of the colour reaction revealed variation in the two main absorption peaks with λ max at 540nm (coniferaldehyde) and 465nm (vanillin). In some samples both of these peaks increased with time, although not equally, whilst in others the decay of the 540nm peak coincided with an increase in the 465nm peak.

Thus it would appear that the compounds extracted from the lignified tissues are unstable, the rate of interconversion of these compounds affecting the readings obtained. A method for stabilising the indicator compounds (coniferaldehyde or vanillin) would therefore be desirable.

APPENDIX III

THE EFFECT OF CARBOHYDRATE SOURCE AND CONCENTRATION ON THE GROWTH AND CYTODIFFERENTIATION OF SUSPENSION CULTURE LINE 1A

A range of sucrose concentrations (0-10%) and glucose (2%) were added to MS1 medium. Two flasks were inoculated into each sucrose concentration. Data from only one of the flasks are presented (Table III.1) since the response was consistent between replicates. All but the lowest sucrose concentrations (0 and 0.5%) supported good growth of the cultures. Concentrations of sucrose at 1.0, 1.5 and 2.0% and glucose at 2.0% supported similar growth and tracheid production. At levels above 2.0% sucrose both the percentage and absolute numbers of tracheids present in the cultures decreased (excepting at 3.0%), despite higher final cell numbers being achieved. The increased starch deposition at the higher sucrose concentrations caused problems in the early identification of tracheids. At 10% sucrose the cells disintegrated during preparation for cell counting.

The sucrose concentration influenced the duration of growth and the final cell numbers achieved. Therefore growth curves were constructed for cells inoculated into 1.0, 2.0 and 4.0% sucrose (Fig. III.1). Increased sucrose levels resulted in higher final cell number and decreased tracheid production.

This experiment was repeated (Fig. III.2) using stock cells from a subsequent passage. Cells inoculated into 1.0 and 2.0% sucrose divided, producing ca. 37% tracheids whilst those in 4.0% sucrose failed to grow after 24 days incubation. Table III.1 The effect of the carbohydrate concentration

and source on the growth and cytodifferentiation

of <u>P. contorta</u> suspension culture line 1A.

Initial inoculum density = 31.0×10^3 total cells ml⁻¹ 3.7 x 10^3 tracheids ml⁻¹ (ca.12%)

Samples were taken after 21 days growth on MS1 supplemented with the following concentrations of carbohydrate.

CARBOHYDRATE CONCENTRATION (%)	CELL NO x10 ³ m1 ⁻¹	TRACHEID NO x10 ³ m1 ⁻¹	% TRACHEID
Sucrose			
O	$21^{+}_{-}1$	3 ⁺ 0.1	14.29
0.5	277 ⁺ 12	22 ⁺ 2 .3	7.94
1.0	910 ⁺ 45	$143^{+}_{-}14.0$	15.65
1.5	894 [±] 51	$126^{+}_{-}16.0$	14.13
2.0	1199 <mark>*</mark> 67	$164^{+}_{-}22.0$	13.71
2.5	954 ⁺ 57	64_10.0	6.74
3.0	1321 ⁺ 64	104 ⁺ 13.0	7.91
4.0	1394 ⁺ 59	80 [±] 15.0	5.76
5.0	1453_61	$23^{+}_{-}16.0$	1.58
10.0	_ ^a	_ ^a	_ ^a
	· · · · · · · · · · · · · · · · · · ·		
Glucose			
2.0	1179 ⁺ 61	107_16	9.08

KEY

-^a Uncountable sample



Total cell and tracheid numbers of suspension cultures of <u>P. contorta</u> (line 1A) maintained on MS1 with sucrose at 1% (graph 1), 2% (graph 2) and 4% (graph 3). (S.E.M. given for last total cell and tracheid counts only).



Total cell and tracheid numbers of suspension cultures of <u>P. contorta</u> (line 1A) maintained on MS1 with sucrose at 1% (graph 1) and 2% (graph 2). (S.E.M. given for the last total cell and tracheid counts only).

APPENDIX IV

PLANT HORMONE EFFECTS ON THE GROWTH AND CYTODIFFERENTIATION OF SUSPENSION CULTURE LINE 1A AND 721

At intervals during the culture time of both of these lines, the effect of withdrawing 2,4-D on the cultures was investigated. One passage in the absence of exogenous 2,4-D did not significantly affect the growth or tracheid production of the cultures but a second passage in this 2,4-D free medium resulted in the death of the cells. None of the cultures subjected to this treatment showed embryoid production.

Line 1A

Stock suspension cultures were subcultured through one passage in media containing 2,4-D at 0.5 or 1.0mg 1^{-1} , with no kinetin. These cultures were inoculated into a second passage at the same 2,4-D concentrations with a range of kinetin levels as indicated in Table IV.1 in which the tracheid levels at days 24 and 28 are given. Growth curves were constructed for these cultures (Fig. IV.1). The lower 2,4-D concentration shortened the lag phase of the cultures with the exception of the lowest kinetin concentration (0.0lmg 1^{-1}). The initial growth rate of the cells is similar within each 2,4-D treatment but the cell number reached at day 28 varies. It is apparent that low levels of kinetin may limit the growth of the cultures.

In the lower 2,4-D concentration at all kinetin levels, tracheid production appeared to have reached its maximum level by day 24, whereas in the higher level some variation Fig. IV. 1.

Cell numbers of suspension cultures of <u>P. contorta</u> (line 1A) maintained on MS1 with either 0.5mg 1^{-1} 2,4-D (graph 1) or 1.0mg 1^{-1} 2,4-D (graph 2) with the following kinetin levels:

Table	IV.	1	Number	of	tracheids	(x	10^{-3} ml	.1)	after	24	and
	. <u></u>	<u> </u>	28 davs	s ir	ncubation.						

	0.5mg 1	⁻¹ 2,4-D	1.Omg 1 ⁻¹ 2,4-1		
KINETIN mg 1 ⁻¹	24 days	28 days	24 days	28 days	
0.01	232	255	3	0	
0.1	333	257	259	118 ·	
0.5	304	357	94	242	
1.0	298	312	151	417	


existed between samples at day 24 and 28 (Table IV.1). This increase is probably real, similar increases at the end of the culture time have been observed in other cultures.

This experiment was not repeated since the cells failed to divide on inoculation in the experimental media.

Line 721

A range of concentrations of the auxins IAA, NAA and 2,4-D were tested to observe their effect on cell growth and differentiation over 3 fourteen day passages. The cells were subcultured through 1 passage in the absence of 2,4-D prior to inoculation into the experimental media (Table IV.2). IAA at both concentrations and both NAA and 2,4-D at 0.lmg 1^{-1} failed to support cell growth, the cultures rapidly becoming brown and necrotic. Both NAA and 2,4-D at 1.0 and 5.0mg 1^{-1} supported good cell growth, in all instances tracheid production in these cultures was low (less than 7.0%). Only in the third passage in lmg 1^{-1} NAA was there a significant increase in tracheid production (13.6% - * in table).

The viability and form of the cells maintained in 2,4-D and NAA media differed considerably at the end of the second passage; the shape of the tracheids also reflected this difference (Plate IV.1). <u>Table IV.2</u> Effects of auxins on growth and cytodifferentiations of suspension culture line 721 during 3 fourteen day passages.

The cells were maintained in MS2 medium with half the normal nitrogen concentration and with the standard auxin level substituted by the following:

AUXIN (mg 1 ⁻¹)	PASSAGE NO.	CELL NO x10 ³ m1 ⁻¹	TRACHEID NO x10 ³ m1 ⁻¹	% TRACHEID ELEMENT
IAA	·			
1.0	1	119 3	$17^{+}_{-}2$	14.3
10.0	1	148 6	33 4	22.3
NAA				
0.1	1	211 5	35 <mark>+</mark> 3	16.6
1.0	1	860_27	98 <mark>+</mark> 7	11.4
	2	933 <mark>+</mark> 32	43 <mark>+</mark> 7	4.6
	3	1002 ⁺ 36	*137_13	13.6
5.0	1	689 ⁺ 22	29 <mark>+</mark> 10	4.2
	2	1143 <mark>-</mark> 36	29 <mark>+</mark> 8	2.5
	3	953 ⁺ 24	45 ⁺ 3	4.7
2,4-D				
0.1	1	$278^{+}_{-}12$	32 ⁺ 2	11.5
**1.0	1	921 <mark>+</mark> 58 [·]	59 <mark>1</mark> 13	6.4
	2	1033 <mark>*</mark> 43	31 <u>+</u> 5	3.0
	3	1122 ⁺ 40	39 <mark>+</mark> 5	3.5
5.0	1	870 ⁺ 27	43 <mark>+</mark> 8	4.9
	2	1130_84	53 ⁺ 11	4.7
	3	877 ⁺ 33	25 ⁺ 7	2.8

KEY

****** Control medium

Plate IV.1

Viability (as detected by FDA test) and shape of parenchymatous cells (p) and tracheids (t) in suspension cultures of <u>P.contorta</u> (line 721) maintained on MS2.

A,B. Rounded cells which are highly aggregated were seen in cultures maintained on $lmg l^{-1} 2, 4-D$. x 40 C,D. Elongated cells and tracheids were typical of cultures maintained on $lmg l^{-1}$ NAA. x 40



PLATE TV. I.

APPENDIX V

CELL AND TRACHEID NUMBERS AND ESTIMATIONS OF THE SOLUBLE PHENOLICS OF THREE SUSPENSION CULTURES OF PINUS CONTORTA MAINTAINED ON THREE LEVELS OF BORIC ACID THROUGH 4 CONSECUTIVE PASSAGES. (REPRESENTATIVE SAMPLES FOR DAY 21 FOR EACH PASSAGE).

Table V.1 Suspension culture line 640

Total cell number of initial inoculum : 110×10^3 cells ml⁻¹ (5 x 10³ tracheids ml⁻¹)

mg 1 ⁻¹ BORIC ACID	PASSAGE NUMBER	CELL NO x10 ³ m1 ⁻¹	TRACHEID NO ×10 ³ m1 ⁻¹	% TRACHEID ELEMENT	ng. eq GALLIC ACID x10 ³ cells ⁻¹
124 ⁻	1	370_13	10 3	2.7	195.2
	2	895 <mark>*</mark> 51	99 <mark>-</mark> 17	11.1	96.0
	3	764 ⁺ 13	160_11	20.9	95.9
	4	786 <mark>-</mark> 18	37_10	4.7	391.8
6.2	1	228 ⁺ 4	3_ 1	1.3	326.3
	2	890 <mark>+</mark> 42	38 7	4.3	117.5
	3	779 <mark>*</mark> 33	101 9	13.0	146.0
	4	851 <mark>-</mark> 34	68 <mark>+</mark> 6	8.0	559.3
None added	1	114_11	4_ 1	3.5	561.4
	2	301 <mark>*</mark> 23	6_ 2	2.0	199.0
	3	622 <mark>*</mark> 34	38 <mark>*</mark> 6	6.1	65.3
	4	1097_23	23 * 8	2.1	353.7

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Table V.2 Suspension culture line 721

Total cell number of initial inoculum : 118×10^3 cells ml⁻¹ (16 x 10³ tracheids ml⁻¹)

mg 1 ⁻¹ BORIC ACID	PASSAGE NUMBER	CELL NO x10 ³ m1 ⁻¹	TRACHEID NO x10 ³ m1 ⁻¹	% TRACHEID ELEMENT	ng. eq GALLIC ACID x10 ³ cells ⁻¹
124	1		210 ⁺ 35	13.8	161.7
	2	1192 <mark>+</mark> 60	235 ⁺ 30	19.7	125.8
	3	805 ± 35	221 ⁺ 17	27.5	120.7
	4	975 <mark>-</mark> 37	183 <mark>-</mark> 13	18.77	1010.2
6.2	1	1430 <mark>*</mark> 76	176_18	12.3	258.7
	2	1340_68	153 <mark>*</mark> 30	11.4	67.2
	3	1276 <mark>*</mark> 51	337 <mark>*</mark> 26	26.4	32.0
	4	1005 ⁺ 40	215_16	21.4	419.4
None added	1	1499 <mark>+</mark> 55	98 _ 18	6.5	124.2
	2	1317 <mark>*</mark> 70	95_19	7.2	305.2
	3	1646 <mark>*</mark> 60	150 _ 13	9.1	89.3
	4	1310 <u>+</u> 60	191_22	14.6	226.0

Table V.3 Suspension culture line 723

Total cell number of initial inoculum : 107×10^3 cells ml⁻¹ (4 x 10³ tracheids ml⁻¹)

mg 1 ⁻¹ BORIC ACID	PASSAGE NUMBER	CELL NO x10 ³ m1 ⁻¹	TRACHEID NO x10 ³ m1 ⁻¹	% TRACHEID ELEMENT	ng. eq GALLIC ACID x10 ³ cells ⁻¹
124	1	221_12	6 3	2.7	810.9
	2	227_17	6_ 2	2.6	308.4
	3	153 <mark>+</mark> 8	20 <mark>+</mark> 3	13.1	27.9
	4	_ —	-	· –	· _
6.2	1	273_10	10 <mark>+</mark> 3	3.7	498.2
•	2	464 <mark>+</mark> 43	2_0.3	0.4	319.0
	3	1086_69	402 ⁺ 25	37.0	19.3
	4	939 ⁺ 45	313 <mark>-</mark> 37	33.3	221.5
None added	1	84 <mark>+</mark> 6	4_0.8	4.8	238.1
	2	951 <mark>*</mark> 71	17 <mark>+</mark> 9	17.9	235.5
	3	1223_52	320 ⁺ 23	26.2	5.1
	4	1038_59	248_16	23.9	96.3

KEY

- No growth

APPENDIX VI

<u>Table VI.1</u> Numbers of embryos from stratified and nonstratified seed of <u>P. sitchensis</u> incubated on MD medium with the hormones as stated, under environment <u>b</u>.

05		· · · · ·	- T
25	embryos	treatment	

		ST	STRATIFIED			STRATIFI	ED
HORMONES	μM	VIABLE	CALLUS	BUDS	VIABLE	CALLUS	BUDS
IAA, IBA	5	19	7	12	24	6	18
2iP, BAP	5						
IAA, IBA	5	19	11	8	22	8	14
2iP	10						
2,4-D		19	6	13	24	13	11
2iP, BAP	5						
2,4-D		13	8	5	21	10	11
2iP, BAP	5						
TOTAL		70	32	.38	91	37	54

Sum of stratified and non-stratified seed: Viable 161 Callus 69 Buds 92

$$X^2 = \sum \left(\frac{\text{observed} - \text{expected}}{\text{expected}}\right)^2$$

There was no significant difference at the 5% level.

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21 March 1978

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Dear Dr Jones,

With reference to my letter of 14 March 1977 I am now enclosing a copy of Miss K J Webb's thesis. Also enclosed are a copy of the Higher Degree Regulations and an Examiners' Report form. Dr Wangermann, the internal examiner, will be getting in touch with you about the oral examination.

Would you be good enough to return the thesis in due course, together with a note of your expenses on the enclosed form.

Yours sincerely, PR Williams Senior Administrative Assistant

MORPHOGENESIS IN VITRO OF PINUS AND, PICEA

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Abstract

Cultures have been initiated from embryos and seedlings and from shoot meristems and needles of 2 year old seedlings of *Pinus contorta* and *Picea sitchensis*. Excised embryos of *P. contorta* and *P. sitchensis* and 2 year old shoot meristems of *P. sitchensis* form adventitious buds under defined cultural conditions. Alterations in the hormonal milieu result in either the direct formation of buds on the original explant or the initiation of a callus with associated buds. Subculture of the callus with buds onto the same initiation medium results in further bud formation, whereas transfer to a low salt, hormone-free medium promotes the outgrowth of shoots from the preformed buds. As the shoots extend, they may be individually. dissected out and rooted by traditional non-sterile sylvicultural techniques.

Introduction

Successful stimulation of adventitious bud production from juvenile tissue has now been reported for several species of gymnosperms. Excised embryos have frequently been used as the explant source (Sommer et al., 1975; Cheng 1976; Winton and Verhagen, 1976; Street and Webb, 1976, 1977). Adventitious buds have also been produced from seedling hypocotyl segments of *Picea glauca*, *Pseudostuga mensiesii* and *Cryptomeria japonica* (Campbell and Durzan, 1975; Cheng, 1976; Isikawa, 1974) and cotyledon explants of *Tsuga heterophylla* and *P. mensiesii* (Cheng, 1976, 1977).

There are few reports of adventitious bud production from more mature tissues. Two year old seedlings of *P. menziesii* and *T. heterophylla* have been shown to retain their morphogenic potential in culture (Cheng, 1976). Winton and Verhagen (1976) reported the initiation of callus from stems of 15-20 year old trees of several conifers, but although bud-like structures were produced on the callus of four of these species they showed no further development.

These studies on adventitious bud formation in culture have involved considerable variation in the physical cultural environment adopted. For instance embryos and cotyledon explants of *P. menziesii* have been successfully

Acta Horticulturae 78, 1977 Tissue culture for horticultural purposes maintained at $25^{\circ}C \pm 2^{\circ}C$ under continuous light (Cheng, 1975) and under photoperiods of 16 (Winton and Verhagen, 1976), 18 and 12 h (Cheng, 1977). The shorter photoperiod (12h) was more effective in the promotion of bud production than the longer photoperiod (18h) when the cotyledon cultures were maintained on 5nM Naphthalene acetic acid (NAA) and 5 μ M kinetin.

In the present study, embryos of *P. contorta* and *P. sitchensis* have been used to determined the hormonal and environmental conditions promoting initiation and subsequent outgrowth of buds. Successful bud induction has also been achieved from 2 year old seedling shoot meristems of *P. sitchensis*.

Materials and Methods

Seeds of *P. contorta* and *P. sitchensis* were provided by the British Forestry Commission. Preliminary sterilization of the seeds of both species was achieved by immersing the dry seed in a 30% solution of the commercial hypochlorite Domestos (Unilever Bros. U.K.) for 30 min. After 3 x 5min. washes in sterile distilled water the seeds were left to imbibe sterile water either at 5°C for a minimum of 3weeks (stratification) or at 25°C for 40 hours (non-stratified seed). Prior to the excision of the embryos, the entire seeds were individually immersed in alcohol for several mins. and then 'flamed'. This served the dual purpose of ensuring total sterility of the seed coat and facilitated embryo excision.

Five excised embryos (I-2mm long) were placed in each 50 x 50mm sterile plastic petri dish containing semi-solid medium (IOmI). After sealing the dishes with Nescofilm (Nippon Shoji Kaisha Limited, Osaka, Japan) they were maintained at 25°C in the dark for either 7 or 12 days prior to subjecting them to either a) 25°C 16 hour light 8 hour dark at light intensities of

 1.25×10^4 ergs lcm^2/sec (1) or 4.5×10^3 ergs lcm^2/sec (11) or b) $20^{\circ}C$ 13.5 hour light, $7^{\circ}C$ 10.5 hour dark at a light intensity of 5 x 10^3 ergs/cm² sec.

Sterile seedlings were obtained by germinating the sterilised seeds on agar (*P. contorta*) or sterile sand (*P. sitchensis*) under long days at 25°C. Hypocotyl segments (ca 5mm long) and the intact cotyledons (enclosing the shoot meristem) of these seedlings were placed with their morphological basal end embedded in the medium.

Sterilization of terminal and lateral shoots of 2 year old seedlings of *P. contorta* and *P. sitchensis* was by a soak (I-2min) in alcohol followed by a 5 min. immersion in 20% 'Domestos'. The 'Domestos' was removed by 3 x 5 min washes in sterile water. The shoot meristems and leaves were excised using slivers of fractured razor blade mounted in a chuck, and tungsten wire needles. The meristems (ca 2-3mm) were placed with the cut basal end in direct contact with the medium. The explants were maintained in LOOmm sterile square dishes with 25 compartments (ca 2ml semi solid medium per compartment) each containing a single explant. After 7 days in the dark the cultures were maintained at 25°C under continuous light (7 x 10^3 ergs /cm^2 / sec).

The basal medium comprised the salts of Campbell and Durzan(1975), with 3% w/vsucrose 500mg meso-inositol and 5mg I⁻¹ thiamine HCI (Cheng, 1975). The standard CI medium is this medium with 5μ M each of Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), N₆-(2-isopentenyl) amino purine (2iP) and benzyl amino purine (BAP) (Cheng, 1975). The medium used for bud outgrowth was half-strength basal salts, I.5% v/v sucrose, IOOmg mesoinositol and 0.4mg I⁻¹ thiamine HCI and no added hormones. Any experimental variations in the hormones or their concentrations are specified for each experiment. All chemicals used were Analar grade, the agar was Oxoid technical agar grade 3.

Non-sterile rooting of the extended shoots was attempted by the British Forestry Commission, Northern Research Station, Roslin, using a mist propagator, air and bed temperature 20° C (without prior auxin treatment the shoots were maintained under 18 hours days at 5-6000 lux (approximately 7-8 x 10^4 ergs/cm²/sec).

Results

3. 1. Cultures from excised embryos

The viability of the excised embryos was assessed according to their response in culture. Embryos which showed no visible change were scored as dead. None of the treatments proved to significantly affect the viability of *P. sitchensis* embryos. Stratification of *P. contorta* embryos resulted in a higher incidence of contamination and a lower viability than in non-stratified material. Since the stratification process is known to raise hormone levels (specifically cytokinins) in *P. sitchensis* embryos (Taylor 1976) a comparison was made between the stratified and nonstratified embryos. There was no significant difference in either species in the proportion of the viable embryos which produced adventitious buds. The data presented therefore includes results from embryos from stratified and non-stratified seeds.

'De novo' leaf primordia can be recognised at an early stage of development. The distribution of those primordia may be random or in a regular phyllotactic arrangement, the latter situation being interpreted as indicating bud primordia (Fig. 1, A, D, E). The CI medium supported the continuous production of those primordia so that each explant showed several stages of bud development. Repeated subculture of these callused explants, with their associated buds, into the same initiation medium resulted in further callus and bud production. Transfer of the callus with buds to a half strength hormone-free medium promoted the outgrowth of the preformed buds (Fig. IF).

Initiation of primordia showed variable delay (even upto 100 days of incubation) and additional primordia continued to arise over an extended period. The data presented are scores on day 60 of the initiation passage.

3. 11. The effect of light intensity on the morphogenic response of excised embryos

The stimulation of bud induction in P. contorta embryos is dependent on both the environment and the hormonal complement of the initiation medium. The P. contorta embryos exhibit a marked response to light immediately following excision and after a dark pretreatment. On CI medium (table IA) and the basal medium with 10-6M or 10"5M BAP (table IB) a 12 day dark pretreatment followed by long days under a high light intensity enhanced the morphogenic response of the embryos as compared with the lower light intensities and the higher light intensity without the dark pretreatment. Bud production occurred in in 53-71% of the embryos under these optimal conditions described (Fig. IA, B, C). P. sitchensis embryos do not have a requirement for either high light intensity or the dark pretreatment for bud induction, although the dark pretreatment was of some benefit when the embryos were subsequently subjected to the higher light intensity (Table IA).

3. III. The influence of cytokinins on bud induction from embryos of P. sitchensis

The data presented (Table 2) shows that with constant level of auxin, BAP either alone or in combination with 2iP causes maximal expression of bud induction in *P. sitchensis*. A comparison between BAP and kinetin reveals that both induce ca 50% of the cultured explants to undergo bud production, but the number of buds per explant differs. BAP causes the production of numerous small buds which show slow extension growth when transferred to a dilute, hormone free medium. The buds initiated in the presence of kinetin commence outgrowth into shoots (upto lcm long) on the initiation medium (Fig. IH). Individual transfer of these extending shoots to the hormone-free medium results in ca 5% spontaneously rooting. 2iP proved to be less effective as a bud inducer, giving both a low percentage of explants showing bud induction and very few buds per explant. Neither callus growth nor bud induction was initiated by 1μ M zeatin but occasional outgrowth of the embryonic shoot and root meristem occurred, and seedlings were produced.

3. IV. Hypocotyl segments from germinated seedlings

Regular bud induction on hypocotyl segments of 2 week to 2 month old seedlings has not been achieved with both species on any of the combinations of hormones used. However, production of leaf and bud primordia was observed in segments of hypocotyl which were placed immediately adjacent in the culture medium to seedling meristems with associated cotyledons. In both species, the small section of the hypocotyl remaining attached to the original seedling meristem was also capable of adventitious bud production on a variety of media. Levels of cytokinin lower than 10^{-6} M BAP tended to cause extension growth of the original shoot meristems.

3. V. Excised meristems of two year old seedlings

Preliminary work indicated that CI medium was capable of supporting callus production from apical meristems of main and lateral shoots of *P. sitchensis*. Occasional buds were observed but they did not develop further.

A range of concentrations of the synthetic cytokinin BAP and the natural cytokinin zeatin were used as additions to the basal medium containing a constant level of NAA (10^{-7} M) (Table 3). The most effective BAP concentration for bud induction was 10^{-6} M. BAP at all the concentrations tested resulted in a percentage of the meristems responding by either callus or bud initiation (Fig. IG). The media with zeatin supported some extension growth of the leaf primordia of the original meristems at all concentrations but no 'de novo' bud primordia were observed.

Transfer of the proliferating cultures to the same medium with NAA and BAP resulted in continued callus and leaf and bud production. Many of the cultures fell naturally into several smaller pieces, which have continued to proliferate. Outgrowth and rooting of the buds formed has not been attempted.

Meristems and needle fascicles of 2 year old seedlings of *P. contorta* have been cultured. Neither bud initiation nor extension growth of the meristem was observed. Creamy yellow callus was produced from some of the meristems and needle fascicles.

3. VI. Rooting of extended shoots

About 20% of the shoots produced from embryo cultures of *P. sitchensis* and *P. contorta* have been rooted under non-sterile conditions. Four rooted shoots of *P. contorta* initiated from the same original embryo show remarkably similar growth and morphology, implying genetic uniformity. There are problems involved in the 'hardening off' of shoots without roots, ideally the shoots should root whilst still in sterile culture. Shoots initiated on 2iP and kinetin have a higher frequence of spontaneous root initiation in culture than those produced on BAP containing medium.

DISCUSSION

Adventitious bud initiation has been successful from (1) embryos and segments of hypocotyl associated with the shoot apex (and occasionally from isolated hypocotyl segments) of *P. contorta* and *P. sitchensis* and (2) from 2 year old seedling meristems of *P. sitchensis*. Outgrowth and rooting of buds initiated from embryo cultures has been accomplished for both species.

In the presence of a suitable auxin: cytokinin combination, viable embryos were stimulated to produce leaf and bud primordia or only bud primordia. The cytokinins BAP, 2iP, kinetin and zeatin elicited different morphogenic responses from the cultured embryos of *P. sitchensis*. The incorporation of BAP into the medium resulted in the production of numerous leaf and bud primordia, whilst kinetin and 2iP caused the formation of a smaller number of bud primordia and few isolated leaves. BAP in contrast to kinetin or 2iP inhibited shoot elongation. A similar pattern of response to these cyotkinins has been reported in *Populus nigra var Italica* callus cultures (Venverloo 1973) and *Pseudostuga menziesii* cotyledon explants (Cheng, 1977).

The origin of isolated leaves and separate organised bud primordia in the same callus strongly suggests that a single initial form of primordium is arising and which depending upon nutrition and 'available space' can give rise either to a shoot or to a single leaf. This would be in line with the indeterminate nature of young fern primordia as seen in experimental work with Osmunda (Steeves, 1971. Kuenhert and Steeves 1962).

Under defined environmental conditions, the CI medium initiates the production of callus with buds from embryos. This callus with its associated buds has proved to be capable of continued subculture and bud proliferation. Buds initiated on this medium however, show slow extension growth after transfer to a diluted hormone-free medium. Rooting of these shoots in culture is rare. Cheng (1977) suggested that a combined use of BAP and kinetin may be advantageous in overcoming this problem of slow outgrowth of initiated buds. Alterations in the relative proportions of BAP and 2iP in the initiation medium or the substitution of 2iP by kinetin or zeatin may well be advantageous in developing a medium which will support continued callus and adventitious bud production and more rapid shoot outgrowth.

Successful bud initiation has been reported for hypocotyl explants of *P. menziesii* and *P. glauca* (Cheng, 1976, Campbell and Durzan, 1974) and cotyledon explants for *P. menziesii*, *Tsuga heterophylla* and *Pinus taeda* (Cheng, 1976). Isolated seedling hypocotyl explants of *P. contorta* and *P. sitchensis* did not reliably produce adventitious buds under the culture conditions employed. Bud induction occurred in hypocotyl segments which remained attached or adjacent to the original seedling meristem in the culture medium. The expression of the morphogenic potential of the hypocotyl segments under these specific conditions suggests that the explants retain the ability to produce adventitious buds but depended upon substances leachingout from the cotyledons or associated meristem.

Adventitious bud production from *P. contorta* embryos was enhanced by a dark pretreatment prior to exposure to high light intensity. The media on which these embryos were cultured proved to be capable of supporting callus and adventitious bud production, but this potential was suppressed whilst the environmental requirements of the embryo were not satisfied. Embryos of *P. sitehensis* were less critical of the physical environment of the culture.

The lack of morphogenic response from the 2 year old seedling shoot meristems of *P. contorta* could have been due to suboptimal conditions of culture. The CI medium which had supported maximal expression of morphogenesis in embryos of *P. sitehensis* was not the optimal medium for bud induction in 2 year old seedling shoot meristems of this species. The initiation of subculturable callus which can produce adventitious buds from 2 year old seedlings is of obvious importance when considering possible uses of tissue culture in clonal propagation of conifers.

The data presented here suggests the importance of the light regime for production of adventitious buds from explants from some gymnosperms. Appropriate cytokinin levels promoted bud formation, the bud frequency and degree of bud extension varying according to the cytokinin used.

Acknowledgements

Grateful acknowledgement is made to M. Jackson for technical assistance, to Dr. Alan John of Northern Research Station, Forestry Commission, Roslin, Scotland, for propagation of plants from the shoots arising in culture. One of us was able to undertake this work by the award of a Research Studentship of the British Forestry Commission.

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Venverloo, C.J., 1973. The formation of adventitious organs 1 Cytokinin induced formation of leaves and roots in callus cultures of *Populus vigra var Italica*. Acta. Bot. Neerl. 22 (4) 390-398. Table I - The effect of light intensity on the morphogenic response of excised embryos of *P. contorta* and *P. sitchensis*, with or without a 12 day dark pretreatment. A) CI medium. The 15 embryos cultured per treatment were maintained under either a 16 hour photoperiod with the light regimes - I and II or in total darkness (III).

	1.2_day	<u>dark</u>	pretrea	t <u>men</u> t	No_dark	pretrea	tment
Species	Light	Viable	No. fo Callus	rming Buds	Viable	No. fo Callus	rming Buds
P. sitchensis		3 4 	4 6 5	9 8 6	 4 2	7 7 8	4 . 7 4.
P. contorta	 	13 11 12	5 9 12	8 2 0	2 3 2	10 11 11	2 2 1

B) Basal medium with BAP. Fifty *P. contorta* embryos were cultured per treatment. After 12 day dark, the embryos were maintained under a 16 hour photoperiod at light intensities I and II (as in table IA).

			No. f	orming
BAP(M)	Light	Viable	Callus	Buds
10-6		.34	10	24
	U.	44	38	- 6
- 5				
10		35	13	22
	11.	45	36	9

Table 2 - The influence of cytokinins on the initiation and development of buds from excised embryos of *P. sitchensis*. The auxins were those of the CI medium, the cytokinins were as shown. Incubation of the embryos (50 per treatment) was at 20°C, 13.5 hr. light 7°C 10.5 hr. dark, following an initial 7 days in the dark.

		No	. forming	1
Cytokinins	Viable	Callus	Buds	Roots
E-MOAD E-Marp	4.7		· · ·	
5μMBAP + 3JM 21P	4.5	22	41	0
10µM Kinetin	42	23	19	0
10µM 2 i P	41	35	6	1
IOHM BAP	35	21	14	0
2.5µM BAP	35	22	13	0
IµM zeatin	41	0	0	10
IµM zeatin	4 1	0	0	10

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Legend to Fig. 1.

A-C. Excised embryo cultures of *Pinus contorta* after 70 days in culture. A. B. show leaf (1) and bud (b) primordia production in the callused explant. C. shows numerous leaves, some of which may enclose buds.

D-F. Excised embryos of *Picea sitchensis* showing: D.E. showing recently initiated bud (b) and leaf (1) primordia D = after 96 days, E = after 150 days in culture; F. commencement of outgrowth of buds after transfer of culture to diluted hormone-free medium (total incubation time of 240 days).

G. Culture of meristem excised from a 2 year old seedling shoot of *P. sitchensis* showing several 'de novo' leaves (1).

H. Extension, whilst still on the initiation medium of kinetin-initiated buds arising on an excised embryo culture of *P. sitchensis*.

Table 3 - Callus and adventitious bud production from excised meristems (25 pertreatment) of 2 year old seedlings of *P. sitchensis* using basal medium with cytokinins as shown with constant auxin (NAA 10^{-7} M). After 7 days in the dark, the meristems were incubated at 25°C under continuous light (7 x 10^3 ergs/cm²/sec).

Cytokinin 10		го-б _м				
C	allus E	Buds	Callus	Buds	Callus	Buds
BAP	0	4	7	13	0	3
Zeatin	0	0	0	0	0	0

Callus Buds Control (Cl medium) 14 3
. .

BY

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APPLICATIONS OF PLANT TISSUE CULTURE IN FORESTRY

By H. E. STREET and JUDITH WEBB Botanical Laboratories, University of Leicester

Plant tissue cultures could possibly be used for the clonal propagation of coniferous forest trees; they might also provide a valuable experimental system for the study of factors controlling the differentiation of xylem cells in conifers. A callus culture of Sequoia was established by Ball in 1950. More recently evidence has been obtained that plantlets can be regenerated from tissue cultures of Ginkgo biloba (Hackett, 1964) and Ephedra foliata (Sankhla, Sankhla and Chatterji, 1967). Nevertheless the state of the art of conifer tissue culture has developed very slowly. Greater success with hardwood trees (Winton, 1973) has led to a recent renewal of interest in a number of laboratories in conifer tissue culture and in a recent review 47 species of Gymnosperms were listed as having been used for callus initiation, 27 species of which had yielded callus capable of subculture (Winton, 1972). A group working in this country enables work to be directed to species where a new method of clonal propagation would be of value to British forestry and will permit application to such species without delay of any technical advances as they are reported by the overseas laboratories working in this field.

Tissue cultures have been established from Pinus contorta and Picea sitchensis. In the case of *Picea*, callus can be initiated from the hypocotyl tissue of seedlings but little experimental work with this species has so far been possible because of the very slow growth rate of the cultured tissue. In the case of Pinus, cultures have been established from seedling hypocotyl tissue, excised embryos and actively growing shoots. The callus grows as a healthy cream-yellow tissue at a rate permitting repeated subculture at monthly intervals in a fully defined medium. Liquid cell suspension cultures can be established from the callus and these can be repeatedly subcultured every 21 days. The growth of the suspension cultures can be accurately assessed by cell counting and determinations of cell dry weight yield. These cultures are now being used to examine systematically the effects of the nutrient and growth factor composition of the culture medium, and of physical factors (aeration, temperature, light) upon growth rate and the development of organised structures. The cytological stability of the cultured cells and differences in behaviour of cultures derived from different genotypes and from different explants taken at different seasons from within the same genotype are also being studied.

Neither the callus nor the suspension cultures of *Pinus* have yet shown any evidence of root or shoot bud formation or of somatic embryogenesis. Experimental treatment based upon those reported to induce such morphogenesis in *Ginkgo* and *Ephedra* cultures are being tested.

Both the callus and suspension culture of *Pinus contorta* show the presence of living dividing cells and dead tracheary-like elements with heavily lignified walls. The proportion of these two cell types can be changed by changing the cultural conditions. Work is proceeding to define more precisely the factors promotive of

the differentiation of the tracheary elements, and determining their lignin content.

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changes in soil water must have increased the permeability to air, thus other factors obviously outweighed them. The likely explanation is the increased respiratory demand for oxygen by the roots as temperatures increased above $5-6^{\circ}$ C. This is supported by the experimental evidence from thermistor readings.

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TREE PHYSIOLOGY

APPLICATIONS OF PLANT TISSUE CULTURE IN FORESTRY

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The potential of tissue culture as a means of clonal propagation of coniferous trees is under scrutiny. As indicated in a previous *Report* (1975), work has been mainly (see Plate 4) confined to *Pinus contorta*; experiments with *Picea sitchensis* are still restricted by the poor growth of cultures of this species.

Callus cultures may be initiated from seedling hypocotyls and active shoot meristems of P. contorta. Initially healthy cream-yellow callus is obtained which may be capable of sub-culture at monthly intervals. However, these callus cultures and particularly the suspension cultures (derived either from callus or directly from the explant) show varied responses to culture conditions, their growth rates and the periods over which successful serial subculture is possible varying substantially. The cultures, after a variable period in culture, turn brown and become necrotic. The browning is associated with a high content of phenolic acids and their oxidation products in the cells. One particular line, which has been maintained in suspension culture for about two years, has been used in an attempt to study the production of tracheids which are regularly observed in the suspensions. A progressive change has been observed in these cells. The newly initiated suspension exhibited two distinct populations, dead lignified tracheary elements and viable thin walled unlignified "parenchyma" cells. In the older suspension there is a lesser distinction between the two cell types; virtually all the cells have very thick lignified cell walls, though not the characteristic sculpturing typical of tracheids, and the vacuoles of the parenchyma cells often contain osmophilic particles, probably polyphenolic-protein conjugates. The cells in which such lignin production occurs may lose their viability and this can occur to such an extent as to lead to failure of the cultures. We cannot, at present, regulate this lignin production in a reproducible way by changes in culture medium composition. The serially propagated cultures considered above cannot be induced to embark upon morphogenesis.

Although the ultimate objective must be propagation from mature trees, it has been suggested that expression of totipotency in culture is most readily achieved in cultures recently initiated from embryonic material. There is evidence from current work with other gymnosperm species that morphogenesis in culture can be achieved by using explants derived from seedlings (Campbell and Durzan, 1975) or embryos (Sommer and Brown, 1974; Sommer, Brown and Kormanik, 1975). Our exploration of this approach with Pinus contorta and Picea sitchensis has produced negative results when using hypocotyl segments but work with excised embryos is more encouraging. In a proportion of our cultures, meristematic nodules (up to 30 per explant) have developed from the hypocotyl and cotyledonary regions of the embryos and these have given rise to leafy shoots on transference to hormone-free medium. Current work is directed to increasing the frequency of this shoot bud initiation and to finding conditions conducive to root induction from such shoots. The culture medium used in these studies (as devised by Campbell and Durzan, 1975) has also permitted active growth of shoot tips of P. contorta seedlings, and attempts are now being made to induce proliferation of apical meristems of *Picea sitchensis* in similar cultures (by initiation of new growth centres) and to root the growing shoots.

The demonstration that callus can be obtained from mega- and microgametophytes of *Pinus resinosa* (Bonga and Fowler, 1970; Bonga, 1974; Bonga and McInnis, 1975) has led us to explore this approach with *P. contorta* in an attempt to obtain haploid cultures. The callus initiated from megagametophyte material of *P. contorta* is of mixed ploidy; attempts to isolate a haploid cell line from such callus have been unsuccessful. However the callus obtained from microsporangia contains a high proportion of haploid cells and hence was probably of microspore origin. Division of the microspores was enhanced by pre-storage of the material for 14 days at 4°C. The callus obtained could not be serially subcultured. Work with microsporangia and free microspores will be further developed as soon as appropriate material is available once more.

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TREE PHYSIOLOGY

APPLICATIONS OF PLANT TISSUE CULTURE IN FORESTRY

By H. E. STREET and K. JUDITH WEBB Botanical Laboratories, University of Leicester

The initiation of adventitious buds on the hypocotyl and cotyledonary regions of sterilised and excised embryos of *Pinus contorta* has previously been reported (*Report* 1976). Studies have since been extended successfully to cultures of *Picea sitchensis* embryos and seedling hypocotyls. Combinations of various concentrations of cytokinins and auxins have been employed in attempts to optimise the culture conditions for bud induction in both species under consideration. A high cytokinin to auxin ratio is required for the initiation of

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"de novo" buds in both species. Results obtained indicate that *P. sitchensis* is less critical in its requirement for specific hormones than is *P. contorta*. The *P. contorta* embryos also exhibit a marked sensitivity to light immediately following excision.

The feasibility of using tissue culture for clonal propagation is supported by our findings that numerous "de novo" bud primordia may be formed from each explant under suitable culture conditions. Current work is being directed towards increasing the frequency and overall yield of shoot production. In *P. sitchensis*, the hormone additions to the medium can be adjusted to promote either direct formation of bud primordia from the original explant material, or initiation of a callus phase. Subculture of the explant, with its associated buds, into the same medium results in further bud formation. Bud induction may also be achieved from the undifferentiated callus by its transfer to a high cytokinin medium or by the application of a concentrated cytokinin solution directly onto the callus surface. This latter method has been reported for other gymnosperm cultures—specifically *Pseudotsuga menziesii* (Cheng, 1975). Direct application of the concentrated cytokinin, however, has the disadvantage that it frequently causes browning and subsequent death of the callus, although in some cases buds have been produced from the apparently necrotic callus.

Transfer of the cultures bearing buds to a low salt, hormone-free medium results in the extension growth of the buds. These may then be individually dissected out and further growth promoted prior to rooting the shoots by traditional sylvicultural techniques (carried out by the Northern Research Station, Roslin). Preliminary investigations indicate that the chromosome number of *P. contorta* plantlets produced by this method is at the normal diploid level.

Although complete plantlet regeneration is presently restricted to juvenile tissues in these species, the development of successful bud initiation media for these tissues may provide a basis for work using mature tissues, such as shoot segments, needle fascicle buds (in *P. contorta*) and meristems. A low percentage of mature tissues introduced into culture have survived to produce callus or axial extension but have, so far, not given rise to new shoot buds.

Studies on the control of xylogenesis in a new suspension culture of *P. contorta* are continuing. The conditions for the maintenance of the cultures have we believe, been successfully optimised. Regular fourteen day subculture has maintained the original rate of growth and tracheid production over twelve months without encountering the problems exhibited by the previous cell lines (*Report* 1976). It has also proved possible to exert a greater degree of control over the tracheid production of these cultures. The stock lines routinely contain 10-20 per cent tracheids. Manipulation of the mineral nutrition of the cultures can control tracheid production, decreasing the percentage of tracheary elements so formed to 0.5-5.0 or increasing it to 40-50.

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REGULATIONS FOR HIGHER DEGREES



UNIVERSITY OF LEICESTER

Attention is also drawn to Regulations for Matriculation number (4). "A student whose native tongue is not English must have passed in English Language at G.C.E. Ordinary Level or in an examination accepted by the Senate as equivalent thereto. Other evidence of proficiency in English may be accepted in lieu, in respect of candidates who have been ordinarily resident in the United Kingdom for at least three years immediately prior to the commencement of the course."

This booklet is issued on the express condition that it shall not form part of any contract between the University and any student. All matters covered in this booklet are subject to change at any time before or after a candidate's admission. Admission to the University is subject to the requirement that the candidate will comply with the University's registration procedure and will duly observe the Charter, Statutes, Ordinances, and regulations of the University from time to time in force.

REGULATIONS FOR HIGHER DEGREES

[Note. The qualifications for admission to read for a higher degree as prescribed by the Regulations which follow, represent only the minimum requirements for acceptance, and satisfaction of these requirements does not entitle a candidate to admission. This applies particularly to a candidate who wishes to pursue a course of study for a higher degree in a field different from that of his first degree].

THE DEGREE OF MASTER

(other than by course of instruction)

Faculty of Arts (M.A., M.Phil., M.Ed.)

 To be admitted to the degree of Master of Arts a candidate shall

 (a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners in a written examination, except that part of the written examination shall be omitted if the candidate submits a dissertation or, with the special permission of the Senate the whole of the written examination may be omitted if part of the work has already been published and the remainder of the work has been undertaken during the period of registration and is considered by the examiners to be worthy of publication. An oral examination may be held at the Examiners' discretion.

(2) To be admitted to the degree of Master of Philosophy a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners by means of a thesis and an oral examination. A candidate may submit in support of his thesis any of his published work in the general field of his approved study.

(3) To be admitted to the degree of Master of Education a candidate shall

(a) either hold the Diploma in Education of this University or a qualification recognised by the Senate as equivalent thereto, or hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose, or satisfy the Senate that he is by virtue of his previous training and experience qualified to pursue an advanced course of study;

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners by means of thesis, dissertation, or written papers, with an oral examination; a candidate who submits a thesis or dissertation may be required to take a written examination.

- (4) In special cases the Senate may waive the requirement that a candidate shall have obtained First or Second Class Honours or shall be a graduate of a University, but candidates without such qualifications or their equivalent shall normally be required to satisfy the examiners in a qualifying examination.
- (5) The period of registration shall be effective from the earliest of the prescribed dates (1 January, 1 April, 1 July, 1 October) following the approval of the application for registration, and the registration will lapse after the expiration of twice the period stated in (1) (b), (2) (b) and (3) (b) above.
- (6) A full-time student may be granted dispensation by the Senate, for such period as the Senate may decide, from the normal requirement of residence.

Faculty of Science (M.Sc., M.Phil.) and Faculty of Medicine (M.Phil.)

(7) To be admitted to the degree of Master of Science a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year, or, if a part-time student, for not less than two calendar years, an approved course of study under a supervsior appointed by the Senate; and

(c) have satisfied the examiners in a written examination or by means of written papers and a dissertation. An oral and/or practical examination may be held at the examiners' discretion. Where appropriate, candidates in Geology reading for the degree of M.Sc. will be required to submit for approval by the examiners representative suites from the geological materials used and other relevant data, including computer programmes.

(8) To be admitted to the degree of Master of Philosophy in the Faculties of Science and Medicine a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners by means of a thesis and an oral examination. A candidate may submit in support of his thesis any of his published work in the general field of his approved study. Where appropriate, candidates in Geology reading for the degree of M.Phil. will be required to submit for approval by the examiners representative suites from the geological materials used and other relevant data, including computer programmes.

- (9) In special cases the Senate may waive the requirement that a candidate shall have obtained First or Second Class Honours or shall be a graduate of a University, but candidates without such qualifications or their equivalent shall normally be required to satisfy the examiners in a qualifying examination.
- (10) The period of registration shall be effective from the earliest of the prescribed dates (1 January, 1 April, 1 July, 1 October) following the approval of the application for registration, and the registration will lapse after the expiration of twice the period stated in (7) (b) or (8) (b) above.
- (11) A full-time student may be granted dispensation by the Senate, for such period as the Senate may decide, from the normal requirement of residence.

Faculty of the Social Sciences (M.A., M.Phil.) and the Faculty of Law (LL.M., M.Phil.)

(12) To be admitted to the degree of Master of Arts in the Faculty of the Social Sciences a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners in a written examination. A candidate may be required to submit a dissertation. An oral examination may be held at the discretion of the examiners.

(13) To be admitted to the degree of Master of Philosophy in the Faculty of the Social Sciences a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than two academic nor more than three calendar years or, if a part-time student, for not less than three academic nor more than five calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners by means of a thesis and an oral examination. A candidate may submit in support of his thesis any of his published work in the general field of his approved study.

(14) To be admitted to the degree of Master of Laws in the Faculty of Law a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year, or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners by means of a dissertation and written papers; the candidate will also be required to take an oral examination.

(15) To be admitted to the degree of Master of Philosophy in the Faculty of Law a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners by means of a thesis and an oral examination. A candidate may submit in support of his thesis any of his published work in the general field of his approved study.

(16) In special cases the Senate may waive the requirement that a candidate shall have obtained First or Second Class Honours or shall be a graduate of a University, but candidates without such

qualifications or their equivalent shall normally be required to satisfy the examiners in a qualifying examination.

- (17) The period of registration shall be effective from the earliest of the prescribed dates (1 January, 1 April, 1 July, 1 October) following the approval of the application for registration, and the registration will lapse after the expiration of twice the period stated in (12) (b), (14) (b), and (15) (b) above, or the maximum periods stated in (13) (b).
- (18) A full-time student may be granted dispensation by the Senate, up to one-half of the period of his course of study, from the normal requirement of residence.

Centre for Mass Communication Research (M.A., M.Phil.)

 (19) To be admitted to the degree of Master of Arts a candidate shall
 (a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners in a written examination. A candidate may be required to submit a dissertation. An oral examination may be held at the discretion of the examiners.

.(20) To be admitted to the degree of Master of Philosophy a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners by means of a thesis and an oral examination. A candidate may submit in support of his thesis any of his published work in the general field of his approved study.

- (21) In special cases the Senate may waive the requirement that a candidate shall have obtained First or Second Class Honours or shall be a graduate of a University, but candidates without such qualifications or their equivalent shall normally be required to satisfy the examiners in a qualifying examination.
- (22) The period of registration shall be effective from the earliest of the prescribed dates (1 January, 1 April, 1 July, 1 October) following the approval of the application for registration, and

the registration will lapse after the expiration of twice the period stated in (19) (b) and (2) (b) above.

(23) A full-time student may be granted dispensation by the Senate for such period as the Senate may decide, from the normal requirement of residence.

School of Social Work (M.A., M.Phil.)

(24) To be admitted to the degree of Master of Arts a candidate shall(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners in a written examination. A candidate may be required to submit a dissertation. An oral examination may be held at the discretion of the examiners.

(25) To be admitted to the degree of Master of Philosophy a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners by means of a thesis and an oral examination. A candidate may submit in support of his thesis any of his published work in the general field of his approved study.

- (26) In special cases the Senate may waive the requirement that a candidate shall have obtained First or Second Class Honours or shall be a graduate of a University, but candidates without such qualifications or their equivalent shall normally be required to satisfy the examiners in a qualifying examination.
- (27) The period of registration shall be effective from the earliest of the prescribed dates (1 January, 1 April, 1 July, 1 October) following the approval of the application for registration, and the registration will lapse after the expiration of twice the period stated in (24) (b) and (25) (b) above.
- (28) A full-time student may be granted dispensation by the Senate, for such period as the Senate may decide, from the normal requirement of residence.

Department of Museum Studies (M.Phil.)

(29) To be admitted to the degree of Master of Philosophy a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, if a full-time student, for not less than one calendar year or, if a part-time student, for not less than two calendar years, an approved course of study under a supervisor appointed by the Senate; and

(c) have satisfied the examiners by means of a thesis and an oral examination. A candidate may submit in support of his thesis any of his published work in the general field of his approved study.

- (30) In special cases the Senate may waive the requirement that a candidate shall have obtained First or Second Class Honours or shall be a graduate of a University, but candidates without such qualifications or their equivalent shall normally be required to satisfy the examiners in a qualifying examination.
- (31) The period of registration shall be effective from the earliest of the prescribed dates (1 January, 1 April, 1 July, 1 October) following the approval of the application for registration, and the registration will lapse after the expiration of twice the period stated in (29) (b) above.
- (32) A full-time student may be granted dispensation by the Senate, for such period as the Senate may decide, from the normal requirement of residence.

THE DEGREE OF DOCTOR OF PHILOSOPHY

(33) To be admitted to the degree of Doctor of Philosophy in any Faculty a candidate shall

(a) hold a degree with First or Second Class Honours or a higher degree of this University or of another University recognised by the Senate for this purpose, or hold a qualification recognised by the Senate as equivalent thereto;

(b) have pursued, if a full-time student, for not less than two calendar years or, if a part-time student, for not less than three calendar years, an approved course of study under a supervisor appointed by the Senate;

(c) have presented a thesis on his advanced study and research which has satisfied the examiners and contains original work and is deemed worthy of publication; and

(d) have satisfied the examiners in an oral examination.

A candidate may submit in support of his thesis any of his published work in the general field of his approved study. Where appropriate, candidates in Geology reading for the degree of Ph.D. will be required to submit for approval by the examiners representative suites from the geological materials used and other relevant data, including computer programmes.

A candidate wishing to be admitted to the degree of Doctor of Philosophy (Musical Composition) shall submit a folio of compositions in place of a thesis. The proposed contents of the folio must first be approved by the Director of the School of Music, and the works then composed under the direction of a supervisor appointed by the Senate. In addition, a dissertation shall be required, relating directly both to the contents of the folio and to its relationship with contemporary music.

- (34) In special cases the Senate may waive the requirement that a candidate, who is a graduate, shall have obtained First or Second Class Honours or shall hold a higher degree.
- (35) The period of registration shall be effective from the earliest of the prescribed dates (1 January, 1 April, 1 July, 1 October) following the approval of the application for registration, and the registration will lapse after the expiration of twice the period stated in (33) (b) above.
- (36) A full-time student may be granted dispensation by the Senate, for such period as the Senate may decide, from the normal requirement of residence for two calendar years.

SUPPLEMENTARY REGULATIONS

- (37) A candidate for a Master's degree or for the degree of Doctor of Philosophy must state in his application the general field of the work which he proposes to undertake for the degree.
- (38) No candidate shall be allowed to present himself for any part of his examination for a higher degree before the expiration of the minimum period of registration as prescribed for the degree.
- (39) A candidate must give to the Registrar not less than three months' notice in writing of the date on which he intends to submit his dissertation, thesis, or published work, such date to fall within the maximum period of registration as prescribed for the degree; and, when giving notice, must state the title of his dissertation or thesis.
- (40) A candidate for a Master's Degree must give to the Registrar not less than three months' notice in writing of his intention to present himself for a written examination which will normally take place in April, June, or September. A candidate who is proceeding to the Degree by means of a written examination and a dissertation must submit the dissertation at the time that he sits the written examination.

- (41) A candidate must submit two copies of his dissertation or thesis together with three copies of a summary or abstract of the work, not exceeding 300 words in length. The dissertation or thesis must be in English unless special permission is given for it to be in another language. The copies must be typewritten or printed, and bound in the prescribed manner. The copies, if approved for the degree, will be retained by and become the property of the University. Any additional material submitted with a dissertation or thesis will be retained by and become the property of the University.
- (42) The dissertation or thesis must be accompanied by a statement signed by the candidate giving consent to its being made available for consultation, photocopying and for use through other libraries either directly or via the British Lending Library. A candidate may withold this consent to the dissertation or thesis being made available for photocopying and for use through other libraries for a period of not more than three years. The dissertation or thesis is required to be made available for consultation within the University Library, unless the express permission of the Higher Degrees Board is obtained by the author to restrict access to it. Any such dispensation must be sought before the work is undertaken.
- (43) The dissertation or thesis must be accompanied by a statement signed by the candidate certifying that it is the result of work done mainly during the period of registration and that, in the case of conjoint work, a substantial part is the original work of the candidate. Where the dissertation or thesis incorporates material submitted for another degree the extent of that material and the degree, if any, awarded, must be indicated.

THE DEGREE OF DOCTOR OF MEDICINE

(44) The Degree of Doctor of Medicine may be awarded

(a) to a graduate in medicine of the University of at least three years' standing; or

(b) to a graduate in medicine of another University recognised by the Senate for this purpose, provided that the qualification was obtained at least three years previously, is recognised for full registration by the General Medical Council of the United Kingdom, and the intending candidate is, at the time of his application, a member of the academic staff of the University or is otherwise engaged in appropriate clinical or scientific work within the Leicestershire Area.

(45) To be admitted to the Degree of Doctor of Medicine a candidate shall have satisfied the examiners either

(a) by means of a thesis, in any branch of medicine, surgery, or medical science, which has been specially composed for the

purpose, includes a review of the relevant literature, contains a critical account of original work carried out personally by the candidate, constitutes a significant contribution to knowledge, and is deemed worthy of publication; or

(b) with the approval of the Board of the Faculty of Medicine, by the submission of published work or inter-related works, embodying original work of which a substantial amount has been carried out personally by the candidate, and constituting a significant contribution to knowledge.

In either case an oral examination may be held at the discretion of the examiners. A candidate may submit in support of his thesis any of his published work in the general field of his approved study.

- (46) A candidate intending to submit a thesis shall first provide, for the approval of the Board of the Faculty of Medicine, an outline of his proposed field of study, its scope, and the methods of investigation to be employed. The thesis must normally be presented within five years of the date on which the outline is approved, and a candidate must give to the Registrar not less than three months' notice in writing of the date on which he intends to submit his thesis, stating, when he gives such notice, the title of the thesis.
- (47) Two copies of the thesis must be submitted in a form suitable for publication, together with three copies of a summary or abstract of the work not exceeding 300 words in length. The thesis must be in English unless special permission is given for it to be in another language. The copies must be typewritten or printed, and bound in the prescribed manner. The copies of the summary must be typewritten but not bound in with the thesis. The copies of the thesis and summary will, if approved for the degree, be retained by and become the property of the University.
- (48) A candidate intending to submit published work in accordance with regulation (45) (b) shall first provide, for the approval of the Board of the Faculty of Medicine, a brief synopsis of the publications to be presented; if his application is accepted, he must furnish two copies of all published work which he wishes to submit, together with two copies of a critical assessment of the current state of knowledge in his chosen field and the way in which his studies relate to it. Where the submission consists of a number of publications other than books, the whole must be bound together, with the critical assessment, in the form prescribed for the binding of theses. One copy of the work or works approved for the award of the degree will be retained by and become the property of the University.
- (49) The thesis or published work must be accompanied by a statement signed by the candidate declaring that the work is the result of his personal observations or original research or,

in the case of conjoint work, clearly defining the extent to which he was responsible for the initiation and conduct of the work; the statement must also certify that the thesis or published work has not been submitted for a degree of another University or must specify, if material submitted for another degree is incorporated, the extent of that material and the degree, if any, awarded.

(50) Examiners may recommend that a candidate shall pass, shall fail, or shall be referred.

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It shall not be recommended that a candidate shall pass unless his submission, which may be satisfactory in other respects, reaches an approved standard in such matters as methodical exposition and demonstration, relevance and coherence of argument, and effectiveness of style.

The period of reference shall be not less than three months and not more than two further years.

THE DEGREES OF DOCTOR OF LETTERS DOCTOR OF SCIENCE AND DOCTOR OF LAWS

- (51) The degree of Doctor of Letters, Doctor of Science, or Doctor of Laws may be awarded to a graduate of the University, or to a graduate of the University of London who took his degree as a registered student of the University College of Leicester, or to a graduate member of the full-time staff of the University, who shall be deemed by the Senate, after considering a report from the Board of Examiners, to have produced published work constituting a sustained, original, and distinguished contribution to knowledge.
- (52) To be admitted to the degree of Doctor of Letters, Doctor of Science, or Doctor of Laws a candidate must have been admitted to a Bachelor's degree not less than nine years previously or to a Master's degree not less than eight years previously or to the degree of Doctor of Philosophy not less than six years previously.
- (53) An application for the degree of Doctor of Letters, Doctor of Science, or Doctor of Laws may be submitted at any time, and the Senate, if it accepts the application, shall appoint a Board of three examiners, two of whom shall be external to the University, except that, where the application is made by a member of the staff of the University, the Senate may decide not to appoint an internal examiner.
- (54) The application must be accompanied by two copies of all published work which the candidate wishes to submit. A substantial amount of the work submitted must be independent work published in the candidate's own name, and in the case of conjoint work the candidate must produce satisfactory evidence

of the extent to which he was responsible for the initiation and conduct or direction of the work. The candidate must also indicate what part, if any, of the work has been submitted for a degree of this or any other University by himself or, in the case of conjoint work, by any of his collaborators.

(55) One copy of the work or works approved for the award of the degree may be retained in the University Library.

GENERAL REQUIREMENTS

FOR THE DEGREES OF MASTER AND Ph.D.

Part-time Students

Every part-time student shall be required, before he is registered, to provide an assurance that he will be able to consult his supervisor regularly (normally at least three times each term with ensuing vacation), and that adequate facilities are available to him for pursuing his proposed course of study. The responsibility for arranging regular consultations with his supervisor rests on the candidate and failure to comply with this important requirement may result in the termination of a candidate's registration.

Associate Supervisor

In the Faculties of Arts and Science and the Department of Museum Studies a person deemed suitable by the Senate may be accorded the status of "associate supervisor". The position of associate supervisor is an official but honorary appointment. The associate supervisor is not eligible for appointment as the External Examiner of any student whom he supervises. The associate supervisor assists the supervisor in overseeing the research work of the student and, in appropriate circumstances, undertakes direct personal supervision. Final responsibility for ensuring the provision of adequate supervision rests with the supervisor.

Probationary Period of Study

Faculty of Arts, Faculty of Law, Faculty of the Social Sciences, Centre for Mass Communication Research, Department of Museum. Studies, and School of Social Work

Every candidate for a higher degree shall be required to submit himself to a probationary period of study of six months from the date of registration. Upon special request from a candidate's supervisor, the period may be extended by a further three months.

Faculty of Science and Faculty of Medicine

A candidate who wishes to read for the degree of M.Phil. or Ph.D. in the Faculties of Science and Medicine shall normally register as an advanced postgraduate student and be given a period of twelve months before the expiry of which he shall be required to register for a specific degree. Such a candidate shall, for the duration of his registration as an advanced postgraduate student, be regarded as undergoing the requisite period of probationary study for the degree to which he aspires, and the period of probation shall terminate on registration for a specific degree.

A candidate who in exceptional circumstances is registered initially for a specific degree shall be required to submit himself to a probationary period of study of six months from the date of registration. Upon special request from a candidate's supervisor, this period may be extended by a further three months.

The prescribed minimum and maximum periods of registration for the degrees of M.Phil. and Ph.D. (See Regulations (8) (10) (33) and (35) above) shall be calculated by reference to the date of a candidate's original registration either as an advanced postgraduate student or as a student reading for a specific degree.

Form of Examination for the M.A., LL.M. and M.Sc. Degrees

M.A. (Arts). A candidate proceeding to the degree wholly by written examination shall be required to sit four papers. For a candidate proceeding partly by written examination and partly by dissertation, the written examination shall consist of two papers. In all cases the written papers shall include an adequate test of the candidate's knowledge of his general field of study.

LL.M. Degree. A candidate proceeding to the degree by means of a dissertation with a written examination shall be required to sit two papers on a prescribed area of study including the specific field of the dissertation.

M.A. (*Social Sciences*). Normally four written papers, or three papers if a dissertation is required.

M.A. (School of Social Work). For a candidate proceeding partly by written papers and partly by dissertation, the written examination shall consist of two papers on a prescribed area of study including the specific field of the dissertation.

M.A. (Centre for Mass Communication Research). Normally four written papers or two papers if a dissertation is required.

M.Sc. Degree. A candidate proceeding to the degree wholly by written examination shall be required to sit four or five papers with a practical examination in appropriate cases. For a candidate proceeding partly by written papers and partly by dissertation, the written examination shall consist of one or two papers on a prescribed area of study including the specific field of the dissertation.

Qualifying Examinations. A qualifying examination shall consist of at least two written papers of final honours degree examination standard. An external examiner shall be involved in the examinations.

Lengths of Dissertations and Theses

The Faculty of Arts. The lengths of dissertations and theses (including appendices and footnotes) should not normally exceed the following limits: M.A. (dissertation) 35,000 words; M.Phil. (thesis) 50,000 words; M.Ed. (thesis) 65,000 words; Ph.D. 100,000 words; but in appropriate cases candidates may be allowed, in consultation with their supervisor and with the approval of the Dean of the Faculty (the Senior Tutor for higher degrees in education), to exceed these limits. Candidates will be required to state the approximate number of words in their thesis when submitting it for examination.

The Faculty of Science and Faculty of Medicine. There is no prescribed length for dissertations and theses, but in general it should not be necessary to exceed 30,000 words of text (excluding tabulated data and diagrams) for a Ph.D. thesis, or 20,000 words of text (excluding tabulated data and diagrams) for an M.Phil. thesis.

The Faculty of the Social Sciences, Faculty of Law, Centre for Mass Communication Research, Department of Museum Studies, and School of Social Work. No prescription.

Format and Binding

Dissertations and theses must be typewritten or printed. If typewritten, the following requirements must be strictly observed:

Size: A4 (210 x 297 mm) (unless special permission is otherwise given).

One side of the paper only to be used.

Spacing: double.

Inner margin: 35 mm minimum.

Head, foot and outer margins: 15 mm minimum.

Footnotes: to stand at the foot of the relevant page.

Bibliography: to follow text and any appendices.

Title page: to include, as well as the full title of the dissertation or thesis, the degree for which the work is submitted, the year of submission, and the candidate's name. Where necessary, the binder should be supplied with a short title to show on the spine of the bound copies.

The candidate shall have his dissertation or thesis bound by the University binders, whose names may be obtained from the Registrar.

Abstracts

Abstracts of theses and dissertations are deemed to be an integral part of the work to be examined, and must be produced in strict accordance with the following requirements:

Three loose copies of the abstract are to be submitted at the same time as the thesis or dissertation;

the abstract must not exceed 300 words, must be produced with single-spacing on one side of A4 paper and must be suitable for photographic reproduction;

the abstract must show the author and title of thesis in the form of a heading.

Results

Except in the examinations for the degree of Doctor of Philosophy (for which see below), examiners may recommend that a candidate shall pass, shall fail, or shall be referred. The degrees of Master of Arts, Master of Education, Master of Laws, Master of Philosophy (in the Faculty of the Social Sciences), and Master of Science may be awarded with distinction in cases of exceptional merit.

Examiners for the degree of Doctor of Philosophy in the Faculties of Arts and of Law may recommend that a candidate shall pass either for the degree of Doctor of Philosophy or for the degree of Master of Philosophy, or shall fail, or shall be referred either with a view to resubmission for the degree of Doctor of Philosophy or with a view to resubmission for the degree of Master of Philosophy.

Examiners for the degree of Doctor of Philosophy in the Faculties of Science, the Social Sciences, and Medicine, may recommend that a candidate shall pass for the degree of Doctor of Philosophy, shall fail, or shall be referred with a view to resubmission for either the degree of Doctor of Philosophy or the degree of Master of Philosophy.

Examiners for the degree of Doctor of Philosophy in the School of Education may recommend that a candidate shall pass either for the degree of Doctor of Philosophy or for the degree of Master of Education, or shall fail, or shall be referred either with a view to resubmission for the degree of Doctor of Philosophy or with a view to resubmission for the degree of Master of Education.

A candidate shall fail or be referred if his written papers, dissertation, or thesis, although satisfactory in other respects, do not reach an approved standard in such matters as methodical exposition and demonstration, relevance and coherence of argument, and effectiveness of style.

The period of reference shall not be less than three months in any case and not more than one further year of full-time or two further years of part-time study.

COURSES FOR THE DEGREE OF MASTER

[Note. Courses of instruction, normally of one year's duration, leading to the degree of Master are offered in the following subjects or fields: Classics, English (English Literature and its Background 1830-75; Modern English and American Literature); English Local History; Victorian Studies; Museum Studies; Education (Education and Mass Communication; History of Education; Philosophy of Education; Psychology of Education; Educational Studies); Geology (Mining Geology; Mineral Exploration); Physics (Experimental Space Physics); Psychology (Psychopathology); Economics (Economic Development; Economics of Public Policy); Politics (European Political Studies); Social Work; Sociology; Law (Welfare Law)].

Faculty of Arts (M.A., M.A.(Ed.), M.Ed.Stud.)

(1) To be admitted to the degree of Master of Arts by means of an advanced course of instruction a candidate shall

(a) hold a degree with First or Second Class Honours of this University, or of another University recognised by the Senate for this purpose;

(b) have pursued, as a full-time student of this University, an approved course of instruction for not less than one academic year; and

(c) have satisfied the examiners in the examinations approved for the course.

- (2) In special cases the Senate may waive the requirement that a candidate shall have obtained First or Second Class Honours or shall be a graduate of a University, but candidates without such qualifications or their equivalent shall normally be required to satisfy the examiners in a qualifying examination.
- (3) To be admitted to the degree of Master of Arts (Education) by means of an advanced course of instruction a candidate shall

(a) either hold a Diploma in Education of this University or a qualification recognised by the Senate as equivalent thereto, or hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose, or satisfy the Senate that he is by virtue of his previous training and experience qualified to pursue the advanced course of study;

(b) have pursued as a full-time student of this University an approved course of instruction for not less than one academic year;

(c) have satisfied the examiners in the examinations approved for the course.

(4) To be admitted to the degree of Master of Educational Studies by means of an advanced course of instruction, a candidate shall

(a) either hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose, or hold a Diploma in Education of this University or a qualification recognised by the Senate as equivalent thereto, or satisfy the Senate that he is, by virtue of his previous training and experience, qualified to pursue the advanced course of study;

(b) have pursued, if a full-time student, for not less than one academic year or, if a part-time student, for not less than two academic years, the approved advanced course of instruction;

(c) have satisfied the examiners in the examinations approved for the course.

(5) The Degrees of Master of Arts, Master of Arts (Education), and Master of Educational Studies may be awarded with distinction in cases of exceptional merit.

Faculty of the Social Sciences (M.A.)

(6) To be admitted to the degree of Master of Arts by means of an advanced course of instruction a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued as a full-time student of this University, an approved course of instruction for not less than one academic year; and

(c) have satisfied the examiners in the examinations approved for the course.

- (7) In special cases the Senate may waive the requirements that a candidate shall have obtained First or Second Class Honours or shall be a graduate of a University, but candidates without such qualifications or their equivalent shall normally be required to satisfy the examiners in a qualifying examination.
- (8) The degree of Master of Arts may be awarded with distinction in cases of exceptional merit.

Faculty of Science (M.Sc.)

(9) To be admitted to the degree of Master of Science by means of an advanced course of instruction a candidate shall

(a) hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose;

(b) have pursued, as a full-time student of this University, an approved course of instruction for not less than one academic year; and

(c) have satisfied the examiners in the examinations approved for the course.

- (10) In special cases the Senate may waive the requirement that a candidate shall have obtained first or second class honours or shall be a graduate of a University, but candidates without such qualifications or their equivalent shall normally be required to satisfy the examiners in a qualifying examination.
- (11) The degree of Master of Science may be awarded with distinction in cases of exceptional merit.

Faculty of Law (LL.M.)

(12) To be admitted to the degree of Master of Laws by means of an advanced course of instruction a candidate shall

(a) either hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose or satisfy the Senate that he is by virtue of his previous training and experience qualified to pursue the advanced course of study.

(b) have pursued as a full-time student of this University, an approved course of instruction for not less than one academic year; and

(c) have satisfied the examiners in the examinations approved for the course.

(13) The degree of Master of Laws may be awarded with distinction in cases of exceptional merit.

Centre for Mass Communication Research (M.A.)

(14) To be admitted to the degree of Master of Arts by means of an advanced course of instruction a candidate shall

(a) either hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose or satisfy the Senate that he is by virtue of his previous training and experience qualified to pursue the advanced course of study;

(b) have pursued as a full-time student of this University, an approved course of instruction for not less than one academic year; and

(c) have satisfied the examiners in the examinations approved for the course.

(15) The degree of Master of Arts may be awarded with distinction in cases of exceptional merit.

School of Social Work (M.A.)

(16) To be admitted to the degree of Master of Arts by means of an advanced course of instruction a candidate shall

(a) either hold a degree with First or Second Class Honours of this University or of another University recognised by the Senate for this purpose or satisfy the Senate that he is by virtue of his previous training and experience qualified to pursue the advanced course of study;

(b) have pursued, as a full-time student of this University, an approved course of instruction for not less than two academic years;

(c) have satisfied the examiners in the examinations approved for the course.

(17) The degree of Master of Arts may be awarded with distinction in cases of exceptional merit.

Department of Museum Studies (M.A. and M.Sc.)

(18) To be admitted to the degree of Master by means of an advanced course of instruction a candidate shall

(a) hold a degree with First or Second class honours of this University or of another University recognized by the Senate for this purpose;

(b) have pursued, as a full-time student of this University, an approved course of instruction for not less than one academic year; and

(c) have satisfied the examiners in the examinations approved for the course.

- (19) In special cases the Senate may waive the requirement that a candidate shall have obtained first or second class honours or shall be a graduate of a university, but candidates without such qualifications or their equivalent shall normally be required to satisfy the examiners in a qualifying examination.
- (20) The degree of Master of Arts or Master of Science may be awarded with distinction in cases of exceptional merit.

DIPLOMA IN SOCIAL WORK

The School of Social Work provides a full-time course of combined academic study and supervised field training which qualifies successful students as professional social workers.

Qualifications for Admission

Applicants must be graduates in any subject(s) or hold a University Diploma or Certificate in Social Science, but applications may be considered from those with other qualifications and experience.

Duration

1. Students with an appropriate qualification in the Social Sciences may undertake the course in one session. For other students the course will normally extend over two sessions.

2. Students will be required to undertake some field training during University vacations.

Academic Syllabus

- (1) Professional Studies:
 - The role of the professional social worker. Social casework; social groupwork; community organisation. Principles and values in social work. Techniques and processes of social work diagnosis and treatment. Social work in specific agencies.

Objectives and principles of social policy. The historical development, structure and functions of social services. Contemporary policy issues. Analysis of social work agencies as organisations: communication, conflict, authority, goals, change. Administrative processes in specific agencies. The nature, sources and branches of English Law. The Court system and administration of law. Court procedure and the law of evidence. Family law and adoption. Legal and administrative provisions related to specific social work agencies.

(2) Behavioural Science

The study of society. Small groups, communities, organisations, population. Social institutions: family economic and occupational, political, legal, religious, educational, welfare. Social processes: stratification, socialization, control, deviance, change.

Assessment of people: interpersonal perception, clinical judgment, psychological measurement. Development: behaviour genetics, social learning, stages of development. Dynamics: motivation and emotion, cognitive controls, stress and stress reactions. Behaviour modification: social influence and attitude change, psychotherapeutic systems. Psychopathology: definition and classification, concepts and models, subnormality, neurosis, nature, issues, approaches. Physical change throughout life. Physiology of the main systems of the body. The nature, causation and prevention of disease. Some common pathological conditions and their personal and social implications.

The nature and process of research in the behavioural sciences. Design of surveys and experiments. Data collection techniques. The processing, analysis and interpretation of data.

Field Training

All students are required to undertake field training under appointed supervisors.

Examination and Assessment

1. For students undertaking the course in one session the written examination shall consist of two written assignments, one long essay on a subject to be approved by the Director of the School, and a case analysis. For students undertaking the course in two sessions the written examination shall consist of four written assignments, one long essay on a subject to be approved by the Director of the School, and a case analysis.

2. Performance in field training throughout the course is assessed.

3. Only students who satisfy the Examiners in each element of the written examination (viz. the written assignments, the long essay and the case analysis) and in field training are eligible for the award of the Diploma in Social Work.

4. A candidate of special merit may be awarded a mark of distinction.