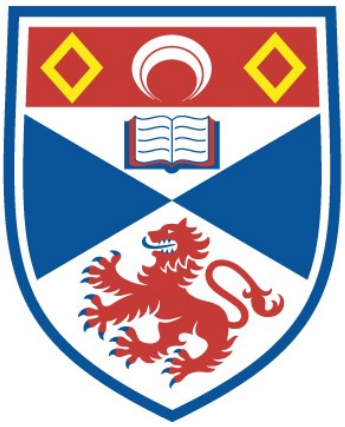
THE ROOT IN WINTER

Lisa C. Smith

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1994

University of St Andrews
School of Biological and Medical Sciences
Sir Harold Mitchell Building
St Andrews
Scotland

THE ROOT IN WINTER

by

Lisa C. Smith

A thesis submitted to the University of St Andrews in application for the degree of Doctor of Philosophy

April 1994

Declarations

I Lisa Smith hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Date 11/4/94

I was admitted to the Faculty of Science of the University of St Andrews as a candidate for the degree of Ph.D. on 1st October 1990.

Signed Date 11/4/94

I hereby certify that the candidate has fulfilled the conditions of the resolution and regulations appropriate to the degree of Ph.D.

Signed Date 21/6/94

Copyright

ACKNOWLEDGEMENTS

Many thanks go to Professor Crawford for all his help and encouragement over the last few years and especially during the writing of this thesis. Like a great many of my predecessors I am deeply indebted to Mr Harry Hodge for all his technical expertise and kindness.

Thanks also to my friends and lab-mates; Fabio, Brigitte, Rupert, Andrea, Andrew and Adrian who made the lab an enjoyable as well as educational establishment, and to Sandra Dunn for all her help in the last few weeks. My special thanks go to Miles for all his support and constant enthusiasm.

I would like to thank Dr Jeff Graves, Andy Lowe and Professor Cormack for their statistical advice.

The trees tortured in this thesis were mainly supplied by the Forestry Commission Northern Research Station. Thanks must go to Dr M. Coutts, Keith Clifford, Dr H. McKay and Bill Mason for all their help and advice.

During the three years of my PhD I have had the financial support of a NERC studentship.

Finally, I would like to dedicate this thesis to my Mother and Father and my sister, Lindsey, for their constant support and encouragement.

TABLE OF CONTENTS

ABSTRACT

Factors affecting the survival of over-wintering tree roots during waterlogging were investigated. Die-back of roots covered by high Winter watertables results in shallow rooted trees susceptible to wind-throw. Such is the scale of the problem in Britain, this research is considered to be of practical relevance. Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Lodgepole pine (*Pinus contorta* Douglas ex Loudon) were chosen, being the most predominant species planted and showing differing susceptibility to flooding. Sitka spruce is considered flood-intolerant (Crawford 1982) and Lodgepole pine highly-tolerant (Minore 1968).

In both species, waterlogging the whole root system for 3 months severely reduced the carbohydrate content of the distal 15cm root, reflecting almost total depletion of starch reserve and varying degrees of glucose depletion. In Sitka spruce, depletion depended on the date of flood-initiation, being most severe after flooding from October when root respiration rate was higher, rather than November as the roots became dormant. Flooding injury was reflected in decreased tri-phenyl tetrazolium chloride reduction and loss of respiratory capacity. Greater loss of aerobic (as compared to anaerobic) respiration capacity after flooding suggested damage to the aerobic pathway, either directly through anoxia or indirectly due to depletion of sugars important in cell maintenance. Injury appeared to be greater when respiratory activity at flood-initiation was high.

Aeration from stem lenticels ameliorated flood-injury and carbohydrate depletion in Lodgepole pine, although the roots appeared to have no metabolic adaptation to anoxia.

Increased soil temperature during Winter flooding increased carbohydrate depletion in the distal root and reduced viability relative to seedlings flooded at ambient temperature.

Carbohydrate depletion during cold storage and its effect on survival of soil waterlogging at out-planting was determined in Sitka spruce. Cold storage leads to increased root growth and slightly superior flood-tolerance when compared to nursery over-wintered seedlings, presumably due to the more dormant state of stored seedlings.

CHAPTER 1

General introduction

The introduction describes firstly the aims of the thesis in brief, followed by a more detailed summary of the background knowledge and practical relevance of this area of research.

The physiology of the over-wintering root is a relatively neglected field but one of increasing commercial importance. Most research investigates the root during the growing season when growth, carbon allocation and survival have important implications for agricultural yield, however the condition of the root system following Winter is a primary factor in determining the species' future performance. Stress during this period may have severe consequences during the growing season.

This thesis examines the effect of Winter waterlogging on the roots of two tree species of prime importance in Scottish forestry. The effects of soil waterlogging are manyfold and fall into two categories: direct effects of anoxia and post-anoxia on root metabolism; and indirect effects through products of anaerobic soil conditions. Survival of roots of forest trees during Winter waterlogging has already been observed (Coutts and Nicoll 1990b). This thesis concentrates on the effect of waterlogging on the carbohydrate metabolism of the root, and examines how variations in energy supply and demand affect survival. Supply and demand vary due to seasonality of root growth and through the effects of climate change and forest practice.

1.1 BACKGROUND INFORMATION

1.1.1 Implications of current forest practice on stress of over-wintering root

Sitka spruce is the most common species in Scottish forests. In recent years over 70% of the trees planted annually in Scotland were

Sitka spruce, which in 1980 made up 364,000ha. This represents almost 50% of Scotland's coniferous high forest area (Forestry Commission census 1983 - See Low 1987). Lodgepole pine is the next most planted species.

Low (1987) has reviewed Sitka spruce silviculture in Scotland. Sitka spruce is most extensively planted because of its fast growth rate in most parts of Scotland. It is one of the highest yielding tree species available for forestry in Britain, performing well over a wide range of site conditions (Malcolm 1987). Despite their lower net assimilation rates and shorter period of shoot extension than broadleaved trees (Jarvis and Jarvis 1964), conifers have greater annual production due to their evergreen habit and ability to photosynthesise at low temperature. Sitka spruce is suited to an oceanic climate and tolerates the poor nutrient levels and acidic soils in the upland planting areas. Its wood is used for paper and board manufacture and production of structural sawn timber. However, the species is intolerant to flooding.

Current forestry practice amplifies this problem. There is an ever decreasing availability of land suitable for forestry plantation, and much of the already afforested land in Britain is far from ideal. In 1974, Toleman and Pyatt reported 50% of Forestry Commission land to be periodically or permanently waterlogged and 2 years later Coutts and Armstrong suggested that almost all current planting sites fit into this category. In recent years planting has been stretched up to and above the climatic tree line and extensively in the waterlogged flow country of North East Scotland (Nature Conservancy Council 1986).

In Winter, many Scottish forestry plantations suffer soil anoxia/hypoxia as a result of rising water tables which may inundate the root system from Autumn to Spring (Pyatt and Smith 1983, King *et al.* 1986; See Coutts and Nicoll 1990b). As a result, roots which have grown

in freely drained soil during the Summer die-back, producing shallow rooted forests susceptible to windthrow (Armstrong *et al.* 1976, Sanderson and Armstrong 1978). An examination of the root systems of poorly growing seedlings showed that 84% of roots lay in the litter layer. Tree growth and stability increased with soil aeration, especially in young seedlings (Adams *et al.* 1972).

The risk of windthrow on many sites prevents thinning (see McKay and Coutts 1989) and often necessitates premature felling. In the most susceptible areas, McKay and Coutts report reduction in harvested timber to 45 and 25% of the potential yield in unthinned and thinned stands respectively due to premature felling. Wind speed increases Northwards and Westwards and with altitude, thus many existing and projected plantations are in the most susceptible areas. In these locations trees are being felled at 30-35 years rather than the optimum 55-60 (Nature Conservancy Council 1986). Such is the scale of the problem, the Forestry Industry suffers large yearly economic losses.

Although land is drained in preparation for planting, sites often remain waterlogged due to the heavy nature of the soil. The ground is ploughed to produce furrows and ridges of turf specified distances apart. This promotes drainage and nutrient mineralisation and the raised soil is an improved planting site as it is weed free and well aerated (Taylor 1970). However, spaced-furrow planting results in the biased development of roots in the ridges, leading to an asymmetrical root system, further pre-disposing the root to windthrow (Savill 1976, Miller, 1987). Other methods of ground preparation to reduce this problem are being developed, but their effectiveness requires long-term testing (see Low 1987). Sitka spruce can produce deep fibrous roots in free drained soil, but on peaty gley soils and deep peat, root penetration was only 42 and 63.5cm respectively (Fraser and Gardiner 1967).

Trees for plantation are grown in nurseries from seed and transplanted in late Winter and Spring before bud-break (Low 1973). Seeds of specific origin which give the best performance under field conditions are selected. In Britain, seedlings are usually grown from seed of Queen Charlotte Islands (British Columbia) origin collected from existing plantations in Britain. Genetically improved seed is also being developed. Cold storage of seedlings lifted when fully dormant is used to increase quality of planting stock on delivery and extend the planting season into early Summer (Low 1987).

Although the choice of seed of certain origin may reduce frost damage or other traits, there is a danger of selecting seedlings which do not respond well to stress conditions often arising in the field. McKay and Coutts (1989) suggest that the selection of young progeny and clones with fast growing shoots will indirectly select for trees with a small coarse root biomass. This assumes the genetic control of C allocation to the course roots and stem. Such selection would be unwise considering the importance of a large root system in anchorage (Coutts 1983). Roots are more tolerant to flooding when dormant, therefore selection of seedlings which grow deep into the soil during the Summer and become dormant before the water table rises in Winter would improve anchorage and may be useful for wet exposed sites (Coutts and Nicoll 1990b).

1.1.2 Effects of waterlogging on plant survival

While it is impossible to investigate each aspect of injury in one experiment, it is important to recognise that the causes and effects of anoxia and post anoxia may be complex. Not only is anoxia dangerous to the plant, but also the post-anoxic period when the water table subsides and the anaerobic tissues are re-exposed to oxygen.

a) Effects of anoxia

Roots soon cease growth in waterlogged soil but must continue to respire to produce sufficient energy to meet their maintenance requirements. Some species can supply oxygen to the roots from the shoot to some extent, but in most cases the supply of oxygen, if any, is insufficient. Anaerobic respiration proceeds, but is very inefficient when compared to that using oxygen. For every mole of glucose respired, 38 moles of ATP are generated in oxygen compared to only 2 under anoxia. To maintain energy levels, glycolysis would need to proceed at a very fast rate, such that carbohydrate reserves would be depleted very quickly and large quantities of CO_2, ethanol or alternative end-products may accumulate. There is no evidence that plants increase their respiration rate to such an extent, but species show varying responses in their glycolytic rates when conditions become anaerobic (see Crawford 1989). Adenylate energy charge often falls, due to this inability to produce sufficient ATP, and as a result cellular dysfunction ensues. The lack of sufficient energy for general cell maintenance can lead to degradation of cell ultrastructure and a reduction in protein synthesis (Crawford 1989). The plasma membrane may depolarise when ATP levels are low, resulting in the efflux of K^+ ions (Buwalda *et al.* 1988). All ATP requiring processes slow down ; e.g. uptake of nutrients from the soil (Kozlowski and Pallardy 1984). Transport of H^+ from the cytoplasm to the vacuole is inhibited (Bennett and Spanswick 1984) and leads to cytoplasmic acidosis (Roberts *et al.* 1984). Membrane permeability may also decrease, reducing water uptake and leading to water stress (Slatyer 1967). Water stress leads to stomatal closure, although closure may occur before turgor loss and wilting and may be controlled by abscissic acid (see Coutts 1981).

Flooding reduces the rate of translocation of photosynthates from leaves to growing and storage tissue. Saglio (1985) found that anoxia

inhibited the phloem transport of a labelled glucose analogue in maize roots, and similar inhibition was noted when stolons of *Saxifraga sarmentosa* were confined to anoxia. This inhibition was found to be reversible (Qureshi and Spanner 1973). The mechanism preventing translocation is not well understood but is not thought to involve ATP or energy deficiency (Kozlowski and Pallardy 1984). It is possible that the accumulation of toxic end products of anaerobic respiration may block translocation (Geiger and Savonick 1975).

Although the shoots are in air, photosynthesis is impaired, first by the closure of stomata and secondly by the changes in carboxylation enzymes and reduced chlorophyll content. In some species, leaf abscission or reduced leaf formation and expansion heighten the problem (Kozlowski 1982).

b) Effects of post-anoxia

This period is often considered more dangerous than that without oxygen. Super-oxide and hydroxyl radicals are generated leading to lipid peroxidation and membrane damage; breaking-up of carbohydrates; enzyme denaturation and DNA mutation (e.g. Hendry and Brocklebank 1985, Monk *et al.* 1989). Anaerobically accumulated metabolites are oxidised into more toxic products e.g. ethanol into acetaldehyde and ACC into ethylene (Studer and Braendle 1987).

c) Anaerobic soil conditions

Roots and soil organisms rapidly deplete oxygen from the soil leading to its degradation. Reduced ions are produced, many of which are phytotoxic and taken up in the transpiration stream, e.g. manganous and ferrous ions, Mn^{2+} and Fe^{2+}. Anaerobic organisms, especially bacteria, proliferate in the soil producing gases, hydrocarbons, alcohols, volatile and non-volatile fatty acids and sulphur compounds (Ponnamperuma

1984). pH changes ensue as a result of Fe^{2+} and CO_2 accumulation and the nutrient content of the soil falls reflecting decreased rates of decomposition. Soil nitrate is reduced in content by conversion to nitrous oxide or nitrogen by denitrification. As a result, and due to impaired uptake by the energy deficient root, plants in flooded soil suffer nitrogen deficiency.

1.1.3 Survival under anoxia

Species may survive anoxia by a number of strategies. As is the pattern in adapting to most stress situations, species may *avoid* or *tolerate* the stress period.

a) Avoidance

Plants undergo morphological changes to maintain the oxygen supply to roots, e.g. development of aerenchyma, proliferation of bark and lenticels, growth of adventitious roots above the water table. Radial diffusion of oxygen from the roots may also protect the roots by oxidising the rhizosphere and protecting the tissues from reducing soil conditions. Oxygen diffusing from the roots oxidises both organic and inorganic soil compounds (e.g. Fe^{2+} and sulphide), and prevents them from entering and damaging the roots (Green and Etherington 1977, Sanderson and Armstrong 1978).

b) Tolerance

Plants must endure the period of anoxia, maintaining enough reserves to re-establish themselves as the water table falls. They must also protect themselves from free radicals generated under post-anoxia. Possession of protective enzymes such as super-oxide dismutase, peroxidase and catalase and various natural anti-oxidants seems to aid survival (see Crawford 1992). Large carbohydrate reserves in underground organs support inefficient anaerobic respiration for longer

and are characteristic of wetland plants (Crawford 1978), e.g. *Phragmites australis* and *Typha latifolia*.. The reserves present in the root at the time of flooding are important as translocation is impaired under anoxia. Reserves must therefore be sufficient to meet energy requirements of the plant through the period of anoxia. Anoxic tissues must also either tolerate the accumulation of end-products of anaerobic respiration within the cells or they must be removed by diffusion or transport in the transpiration stream (Crawford 1982, 1992). Whether the levels of ethanol which accumulate under anoxia are directly toxic is still a much debated issue. Evidence that its removal promotes survival has been given (e.g. Crawford *et al.* 1987), although application of 'natural' concentrations of ethanol to plant tissue are often harmless (Jackson *et al.* 1982). The role of alternative end-products is also controversial. Malic, shikimic, lactic, oxalic, and succinic acid and certain amino-acids often increase in concentration on flooding and may help to accumulate the oxygen debt and maintain cellular pH (see Crawford 1992).

The optimum strategy to survive anoxia should, in theory, depend on its duration and the environmental conditions. When a mature stand of flood-tolerant *Quercus palustris* was flooded during the dormant period, Black (1984) found no apparent effects on either phenology or physiology in the following growing season. In contrast continuous flooding (<2 years) lead to reduced reproductive fitness and caused premature Autumn coloration and leaf abscission. Under short term anoxia acceleration of glycolysis to maintain high enough ATP levels would seem beneficial but this strategy could prove fatal if tissues were deprived of oxygen in the long term. Injury depends on the physiological state of the root at the time of flooding which in turn is governed by a number of environmental factors:

climatic conditions, i.e. temperature, soil moisture before flooding,

soil type and nutrient availability,

type of flood water, static or moving and depth of flooding,

handling and storage conditions before planting (in the case of forest seedlings).

1.1.4 Flooding during Winter

In Winter soil temperature is generally lower and solubility of oxygen in soil water higher. Coutts and Philipson (1978a) measured oxygen flux 10cm below the water table in experimentally flooded tubes of peat. Oxygen declined to approximately zero within three days at 15°C while at 0°C oxygen was detected for approximately six days. Soil oxygen content has been recorded and related to the stability and growth of Sitka spruce in upland peaty gleys (Armstrong *et al.* 1976). During Winter and Spring oxygen was never detected below 20cm and in most deficient soils could only be detected at 9cm.

Flooding during Winter when roots are dormant is less harmful than that during the growing season (Broardfoot 1967). However when it is prolonged it can result in the death of a large number of roots and should be an important consideration (Armstrong *et al.* 1976). Under experimental flooding survival of Sitka spruce and Lodgepole pine roots increases with decreasing temperature (Coutts and Philipson 1978a), and was greater when roots were in a dormant condition (Coutts and Philipson 1978a, Coutts and Nicoll 1990b). Dieback of flooded Lodgepole pine and Sitka spruce roots was decreased by reduced temperature and duration of flooding, root tips being more sensitive than the rest of the root system. At a fixed temperature of 6°C only 60% of Lodgepole pine and 10% of Sitka spruce roots survived 28 days flooding. Dormant roots (including the tips) of both species under the same conditions survived intact. Coutts and Philipson (1978a) suggest that the pattern of dieback reflects the oxygen demand of the root, apical regions and actively

growing roots being the most oxygen demanding tissues, although respiration rates were not measured. Respiration is temperature dependant. In a study of taiga species, Lawrence and Oechel (1983) found total and maintenance respiration of roots increased as an exponential function of soil temperature in *Alnus crispa, Populus balsamifera, Populus tremuloides* and *Betula papyrifera*. It also depends on the phenology of the seedling. At a fixed temperature root respiration rates of *Pinus taeda, Pinus sylvestris* and *Picea abies* decrease during the Winter to between 0.3 and 0.6 times the rates in the growing season (Boyer, Romancier and Ralston 1971, Lahde 1966 - See Coutts and Philipson 1978a).

Coutts and Philipson (1978a) suggest that the phenology of root growth and fluctuations in the water table are important factors in the survival of roots of Sitka spruce seedlings in Scottish forests. Recently Coutts and Nicoll (1990b) flooded clones of Sitka spruce from October when roots were growing, and from November when growth had ceased. Flooding during the dormant period caused little root die-back whereas that initiated in October while roots were still growing slowly lead to severe die-back. Seedlings flooded when dormant thus had roots surviving to greater depth than those flooded only one month before. Coutts and Nicoll suggest that the ability to select and plant seedlings whose roots grow to substantial depths during the Summer and become dormant before the water table rises in the Winter would produce the greatest depth of survival. Although variation was found in the clones they analysed in the phenology of growth and, most importantly, the dates of initiation of root Winter dormancy (Coutts and Nicoll 1990a), the differences in flood tolerance associated with these variants were too small to be of practical relevance. However they concluded that the study

of further provenances/genetically improved varieties may yield seedlings with greater flood tolerance (Coutts and Nicoll 1990b).

The physiology underlying the observations of Coutts and co-workers have not been fully investigated. Indeed surprisingly little is known about the control of the phenology of root growth in trees.

Unlike shoots, roots do not have a chilling requirement for growth and despite the obvious practical advantages in understanding phenology of root growth and provenance/species variations (see Coutts 1981, Coutts and Nicoll 1990a), little research has been done in this field. Roots have a minimum temperature requirement for growth, and so long as soil conditions and temperature are favourable, both active and dormant roots can be found at all times of the year (Coutts and Philipson 1987). In conifers, dormant roots are recognised by the browning of the cortex which proceeds towards the tip and the formation of the metacutization layer when elongation has ceased (see Philipson and Coutts 1979). There is evidence however for a degree of control by the shoot. Coutts and Nicoll (1990a) reported a reduced tendency for root growth during Winter in Sitka spruce. Root growth was initiated in March under lower soil temperature than that at which roots remained dormant during the Winter. The authors suggest that short days and low light intensity experienced by the shoot in Autumn and Winter may reduce the tendency for root growth.

Dormancy of the root was suggested to be advantageous in that:

a) their oxygen demand is lower (Coutts and Philipson 1978a);

b) the metacutization layers which form around root tips (Wilcox 1954) may protect the tissues from soil toxins (Coutts and Philipson 1987);

c) the fall in transpiration rate characteristic of dormant roots under flooding may protect the root and shoot from uptake of soil toxins

which would normally enter the plant in the transpiration stream. In Sitka spruce transpiration rate slowly decreases under waterlogging, due to reduced root membrane permeability and stomatal closure and does not increase until the soil is drained and root growth has been initiated. Although seedlings flooded when actively growing rapidly reduce transpiration rate, it then increases during waterlogging. Coutts (1981) suggested that this response may be a factor in the superior survival of dormant seedlings.

Gaining an understanding of the metabolism of the overwintering tree root under soil waterlogging may be advantageous. Firstly it would allow selection of provenances suitable for sites prone to high water tables during Winter. Secondly measurement of the physiological response of roots to flooding may help to predict how plants may respond to variations in Winter stress.

1.2 STRUCTURE OF THE THESIS.

The thesis first aims to fill in some of the gaps in flood tolerance of Lodgepole pine and Sitka spruce. It is split into four experimental chapters and concludes with a general discussion. The techniques common to a number of experiments are detailed in Chapter 2 with more specific methods given in the appropriate chapter.

The thesis concentrates on the carbohydrate metabolism of the root. Anoxia is costly in terms of carbohydrate consumption, and root carbohydrate content must supply demand until the tissues are re-oxygenated and phloem transport resumes. Thus carbohydrate may be a limiting factor in root survival. In Coutts and his colleagues studies, oxygen demand at the time of flooding was suggested to be the likely cause of greater injury of actively growing roots and root tips to anoxia, when compared to dormant roots and more proximal regions. However measurements of the respiration rate of Sitka spruce and Lodgepole pine

roots and the accumulation of metabolic end products are lacking. In each experiment, these aspects of anoxia were investigated. Root survival was quantified by measuring the reduction of tri-phenyl tetrazolium chloride solution. The degree of reduction has correlated with tissue survival in a number of species (e.g. Steponkus and Lanphear 1967, Lindstrom and Nystrom 1987) and was considered useful as an immediate test of root viability after flooding.

In each experiment, except that in Chapter 6, distal 15cm lengths of root were analysed. Obviously, measurement of the whole seedling or root system would have been most informative, but was not possible in each experiment due to constraints of 'manpower' and time. The distal portion was selected because of the observed die-back within this region during Winter waterlogging (Coutts and Nicoll 1990b). Therefore, by measuring the response to anoxia, the cause of injury may be elucidated. In chapter 6, the whole root system was assessed in three groups, the main tap root, the fibrous roots and 5 cm distal sections.

Chapter 3 investigated the ability of Lodgepole pine roots to transport oxygen from the shoot to the root. This mechanism is thought to confer superior flood tolerance in comparison to Sitka spruce (Coutts and Philipson 1978b). These authors suggested that Lodgepole pine may also have a metabolic adaptation to anoxia (see Crawford 1976) which enables them to survive below the measured depths of oxygen transport. The ability of the shoots to supply the proximal roots with sufficient oxygen for Winter metabolism and the possibility of metabolic adaptation were investigated.

Chapter 4 largely repeated Coutts and Nicoll's 1990b investigation to measure the metabolic changes underlying the survival of the root system following early and late Winter flooding.

Chapters 5 and 6 conclude by investigating 'exaggerated' Winter stress on the root system. If root respiration and carbohydrate depletion are major determinants of Winter flood survival, then increasing temperatures and greater rainfall, as predicted by a number of models of the Greenhouse Effect, may increase the stress on roots. This was tested in chapter 5.

Possession of a large carbohydrate reserve is often associated with flood tolerance. Reduction of root carbohydrate reserve before waterlogging, as occurs during the cold storage of forest seedlings (e.g. Ritchie 1982), may adversely affect their survival when flooded in Spring. This was tested in the work described in chapter 6.

A number of the experimental chapters end in an appendix, containing tables of data presented in the text as graphs. These tables are for reference and are numbered separately from tables within the main text of the chapter. They are prefixed by a letter 'A' to distinguish them from main tables.

CHAPTER 2

Experimental Techniques

This chapter contains detailed descriptions of techniques used throughout the thesis and common to a number of experiments. Any additions or variations to these will be detailed with the appropriate experiment, each chapter having its own methods section.

2.1 SOURCE OF SEEDLINGS AND GROWTH CONDITIONS

1.1 Source

Seedlings of *Picea sitchensis* and *Pinus contorta* were supplied by the Forestry Commission and Alba trees p.l.c. respectively. Provenance numbers and seedling history are given in the relevant chapter. All seedlings were grown in peat and supplied with 8/9 month Osmocote plus™ slow release fertiliser as detailed. This supplies 15%N, 11%P_2O_5, 13%K_2O and 2%MgO with trace elements.

2.1.2 Planting and maintenance

In all experiments except Chapter 6 where the effects of cold storage were examined, seedlings were grown in 1 or 2 metre acrylic tubes. Seedlings used to determine the importance of the date of flood initiation (Chapter 3) were supplied already in 2m perspex tubes, whereas the spruce and pine seedlings supplied for Chapters 4 and 5 were transferred from storage or small pots respectively. The acrylic tubes were sealed with a rubber bung and filled with gravel to a depth of 12cm to cover the drainage hole (≈0.8cm diameter) and allow easy percolation of water through the tube. The rest of the tube was filled with dry peat, ensuring packing by constantly tapping the tube, and when 2/3 full, filled to the top with water which was allowed to drain through the tube to saturate the peat before planting. The seedling was then placed in the top of the tube and packed with more peat before watering. This method of planting prevented the seedling sinking by gradual settling of peat.

PLATE 2.1

Polystyrene-insulated root box containing Alaskan and Washington provenances of Sitka spruce seedlings. Black plastic wrap removed from two seedlings to show extent of root growth.

The acrylic tubes were placed at random in root boxes either supplied by the Forestry Commission or constructed in St. Andrews (Plate 2.1). The wooden boxes maintained the roots in darkness and were insulated with polystyrene sheets to reduce the daily fluctuation of root temperature. The shoots projected above the boxes through holes cut in the top which fitted tightly around the tube. Doors on the boxes allowed root growth to be monitored. The advantage of growing seedlings in the acrylic tubes is that it provides long straight roots, easily harvested and measured without damage. Seedlings were watered with tap water as required.

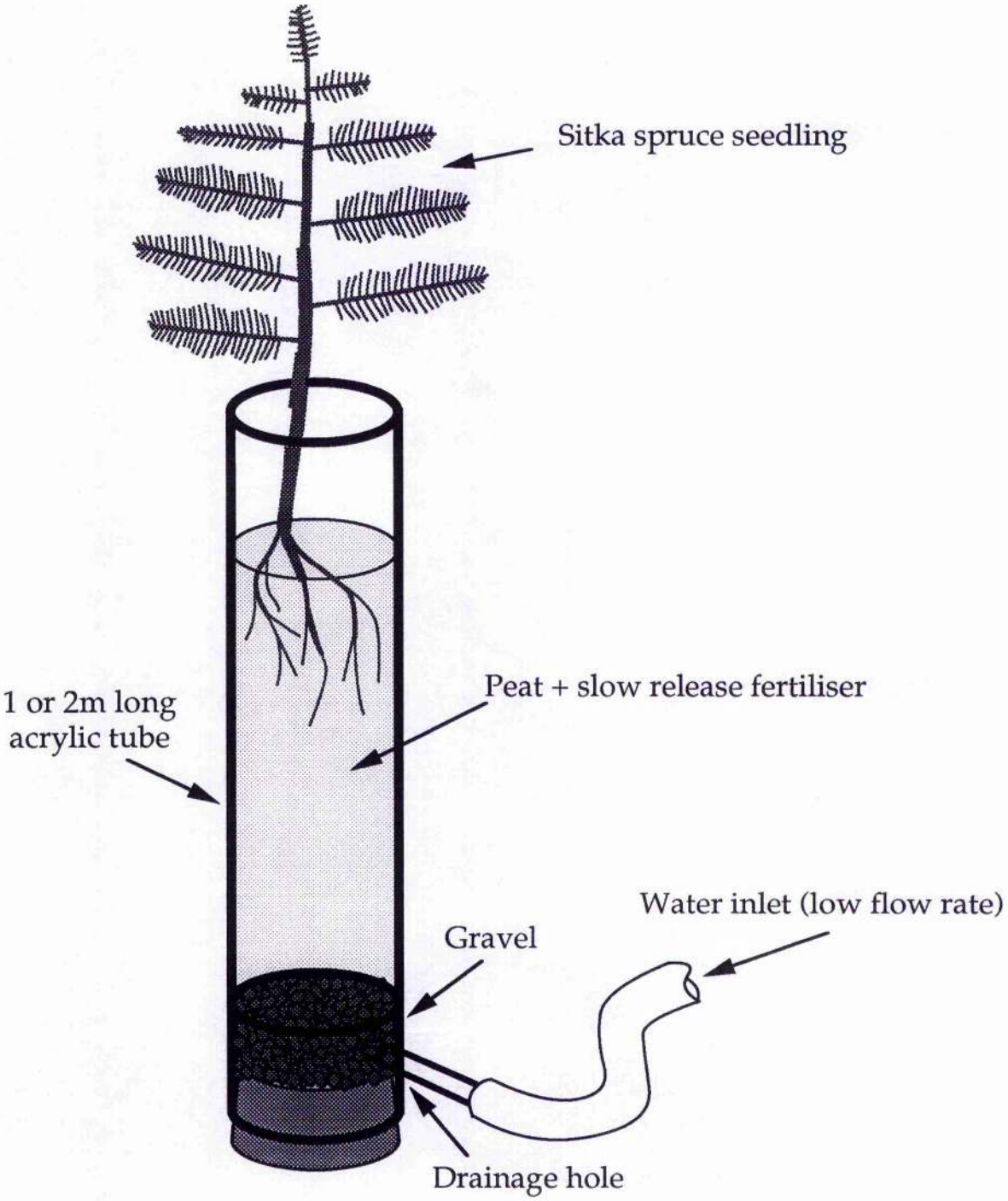
2.1.3 Flooding Treatment

Attempts were made to flood the tubes from the top, but in some cases water 'sat' at the top of the tube without draining through. The acrylic tubes were therefore flooded by slowly filling with tap water from the base. Attaching a hose to the drainage hole and allowing a very slow flow rate, water gradually rose up the tube displacing air from the peat. Tubes were filled to just above the soil surface such that it was easy to monitor the water table height. In some cases, due to the tight packing of the peat, considerable pressure was needed to fill the tubes with water, forcing water up the tube through the drainage hole often pushed out the large bung at the base of the tube. In these cases the acrylic tube was placed inside a specially constructed Dexian™ frame which held the bung in place (Fig 2.1). Flooding such acrylic tubes creates anaerobic conditions in the peat. Only the surface 15mm below the water table remains aerobic due to the diffusion of oxygen from above (Coutts and Nicoll 1990b).

2.2 HARVESTING OF ROOTS

For quantification of ethanol and malate accumulation in root tissue, the tubes were gently tapped to remove the peat core without

Figure 2.1: Controlled Flooding of seedlings in acrylic tube



washing. Otherwise roots were carefully harvested by flushing the peat away with tap water. In all experiments excised roots were analysed as quickly as possible, being kept on moist tissue paper between excision and measurement.

2.3 PHYSIOLOGICAL MEASUREMENTS

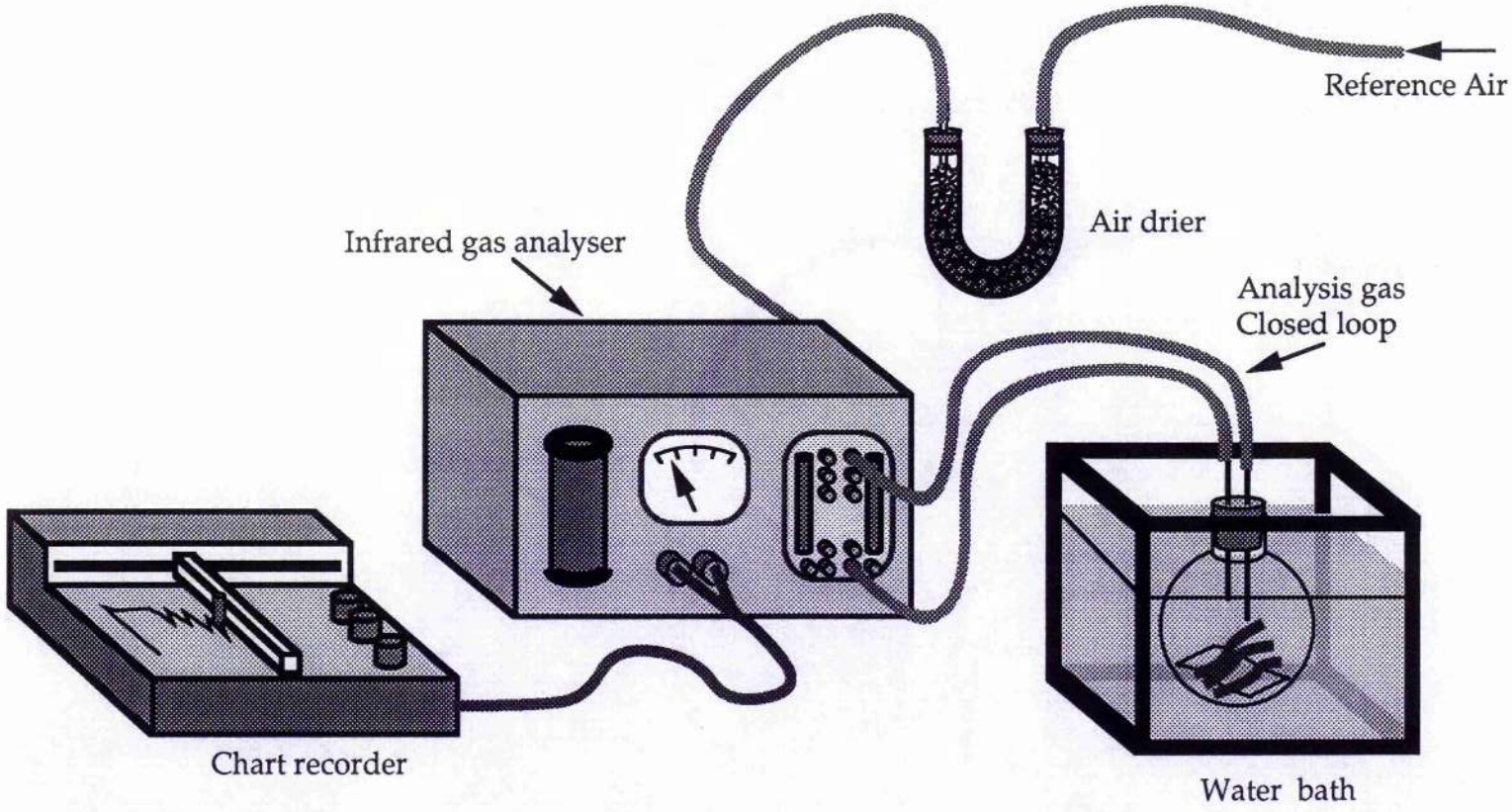
2.3.1 Infra-red gas analysis to determine CO_2 production

Root CO_2 production was determined using an Infra-red gas analyser (ADC 225*mk*3, Hoddesdon, Hertfordshire) calibrated regularly using air passed through soda lime[1] to produce gas of zero CO_2 content, and at 419ppm with standard gas[2]. For measurement in oxygen, outside air was passed in Portex™ tubing through coarse silica gel (BDH) before entering the IRGA, preventing condensation of water in the analysis cell. For analysis of anaerobic respiration measurement, oxygen-free nitrogen (BOC) was circulated through the IRGA, maintaining the flow rate in both the analysis and reference cell at approx. 200ml/min.

Once washed, roots were sectioned into 5cm lengths as detailed in each experimental chapter and weighed following gentle drying on tissue paper. When a number of different sections were analysed, the chronological order in which they were measured was varied between replicates. This cancelled any variation produced by excision or the varying length of time between excision and measurement. Roots awaiting analysis were placed back on moist filter paper and incubated at the measurement temperature. Approximately 3x5cm root sections were placed in a 50ml round-bottomed (RB) flask containing tissue paper moistened with 2ml distilled water (see Fig 2.2). The flask was sealed with a rubber septum stopper (Suba seal) and placed in a water bath at either 10 or 20°C. Two syringe needles were immediately pushed through

[1] Carbosorb non-deliquescent self-indicating granules (10-16mesh) BDH
[2] Cryoservice, Worcester.

Figure 2.2 Infra-red gas analysis of aerobic or anaerobic respiration rate - basic experimental set-up



the seal and air pumped through the IRGA passed around the roots at approximately 200ml min^{-1}. Air was flushed over the roots for 3 minutes before measurement of respiration rate to reduce the effects of handling. CO_2 production was then determined by connecting the outflow tube from the root flask to the IRGA to make a closed loop. The increase in CO_2 concentration was monitored on a chart recorder *(Gallenkamp)* over approximately 25 minutes. Following measurement of aerobic respiration rate, roots were removed from the RB flask and placed on moist filter paper in a glass specimen tube, transferred to an anaerobic chamber[3] and incubated for one hour. The roots were then replaced into the RB flask inside the anaerobic system and reconnected to a stream of nitrogen flowing through the IRGA at 200ml min^{-1}. Roots were flushed for 3min before the closed loop was re-connected. This procedure ensured that the roots did not have contact with air before measurement. The increase in CO_2 production was again monitored on the chart recorder and the rate of production calculated from the gradient of the resulting graph. Respiration rate was usually measured over a 20-30 minute period, on a range of 0-500 or 0-1000ppm. The chart recorder was set at 0.01V sensitivity and chart speed was typically 0.25cm min^{-1}.

When measuring anaerobic respiration rate, with N_2 flowing through the closed loop, a very small amount of CO_2 appeared to leak into the system, presumably due to the large diffusion gradient between the lab air and that inside the tubing. The rate of leakage was determined by sealing the closed loop without a root sample, and recording the change in CO_2 concentration over a 30 minute period. This was then subtracted from each measurement of root anaerobic respiration rate.

---

[3] Forma Scientific Anaerobic System , model 1024, Marietta, Ohio.
Contains atmosphere of 90%N_2, 10%H_2 with palladium catalyst to remove any traces of O_2.

For determination of anaerobic respiration rate at 10°C, root samples were incubated in RB flasks in a waterbath under a stream of water-saturated nitrogen (flow rate ~130ml min^{-1}), for one hour. This was necessary as the temperature of the anaerobic workbench was not controllable and remained at approximately 20°C.

Calculation of root respiration rate.

Respiration rate was determined in ppm of CO_2 produced per gram fresh weight of tissue per hour. To convert this into a molar concentration, the IRGA was calibrated by injecting a known volume of 100% CO_2. 30µl samples of CO_2 were injected into the closed loop and the concentration within the closed loop (in ppm) recorded. The number of moles of CO_2 in the injected sample could be calculated, (1µmole gas at N.T.P. occupies 22.4µl), and a multiplication factor to convert ppm into moles of CO_2 determined.

2.3.2 Determination of root ethanol production and accumulation using gas liquid chromatography

Root ethanol content was measured either after incubation in anoxia or in freshly harvested roots. The extraction procedure differed for the 2 determinations.

a) Ethanol production under anoxia

A minimum of three 5cm root sections were washed, tissue-paper dried and weighed as above. The root sample was then placed in a 30ml glass specimen tube on Whatman No.3 filter paper, moistened with 2ml distilled water and transferred to the anaerobic chamber. Inside, the tubes were sealed with rubber septum stoppers and placed in an anaerobe jar[4] containing an oxygen removing catalyst and methylene blue oxygen

[4] Gaspak, USA

indicator strip[5]. The jars were tightly sealed and placed in an incubator at 10 or 20°C for 24 hours. Use of the anaerobe jars allowed determination of ethanol production at controlled temperatures. Ethanol produced in the tissues may diffuse into the water in the glass tube and a very small percentage (0.129% at 20°C) equilibrates in the head space. The latter is negligible, but to determine total ethanol production the ethanol content of both the tissue and the water in the tube was determined.

After 24 hours, the roots were removed, very quickly wiped with a tissue to remove surface water and placed in a mortar with either 2 or 3ml 6% perchloric acid (see Methods section in appropriate chapter). The glass specimen tubes were immediately re-sealed and stored on ice for determination of ethanol in the water around the roots. The tissue ethanol extraction was performed on ice using freezer-stored pestle and mortar and refrigerated reagents, thus minimising evaporative loss of ethanol. Roots were quickly ground and the extract centrifuged[6] in Eppendorf tubes at 10,000 rpm (8832g) for 5min. The supernatant was decanted and 6M K_2CO_3 added dropwise until neutral *(indicator 1 drop methyl orange)*. Samples were stored at 0-5°C to allow the precipitate to settle.

The specimen tube was placed in a centrifuge to collect the water from the filter paper. 1-10µl samples were injected into the GLC to determine the leakage of ethanol from the roots.

b) Ethanol accumulation in root tissue

The root core was harvested from the acrylic tubing without washing and distal 15cm lengths of root excised and transferred to the cold room (0-5°C) where they were quickly wiped clean of peat. Roots were divided into 3x5cm sections, plunged into liquid nitrogen in a

[5]Gaspak, USA
[6] Heraeus sepatech centrifuge, Biofuge A.

mortar and ground to a fine powder. The root tissue was deproteinised using a method based on Bergmeyer (1963) involving a double extraction of metabolites from the root tissue. Samples of powdered, frozen root were transferred to Eppendorf tubes, 0.1-0.2g per tube, and the ethanol extracted in 0.75ml 6%perchloric acid by repeated mixing in a vortexer (Whirlimix™). After centrifuging at 3,000g for 5 minutes, the supernatant was decanted into a clean Eppendorf tube and stored on ice. A repeated extraction of the root tissue was made in 0.150ml perchloric acid and 0.150ml distilled water, and after mixing and centrifugation the supernatants were combined. 2.5µl methyl orange indicator was added to each extract and the samples neutralised dropwise with 5M K_2CO_3. After precipitation and centrifugation, the supernatant was collected and weighed. Mass and volume appeared to be equal, but determination of mass proved more accurate due to CO_2 bubbles in the extracts. The volume of extract was approximately 1ml. If a smaller sample of root was sampled, the volume of reagents was adjusted proportionately. 1µl samples of extract were injected into the GLC to determine ethanol content. The extract was then frozen and stored at -20°C for enzymatic determination of root malic acid content. Where possible, a number of replicate extracts were made from each sample of frozen and powdered root. In these cases the mean standard error was ±8.8% of the mean ethanol content.

c) Gas liquid chromatography

Samples of root extract or water from around anaerobically incubated roots were injected into a Pye Unicam series 104 model 64 Gas Liquid Chromatograph using a 1 or 10µl liquid syringe. Samples were flushed through a 1.75m glass column filled with Porapak Q in a stream of nitrogen. The degree of ionisation as the ethanol burned in the detector was recorded and the area of the ionisation peak produced

calculated by a Hewlett Packard Integrator. Details of operation of the GLC are given below:

Column: glass, 1.75m long, 4mm i.d.

Packing: porapak Q, 100-120mesh.

Carrier gas: nitrogen, flow rate 40ml min^{-1}.

Air and hydrogen at 15lb $sq.in^{-1}$.

Operating temperatures: detector 160°C and oven 150°C

Injections were repeated until the standard error of the mean ethanol content in each extract was less than ±5%. The volume of extract injected was between 1 and 10µl, and the attenuation 20-200, each adjusted to maximise the accuracy of ethanol content determination.

Calibration.

The GLC was calibrated daily by injection of 1µl samples of known ethanol content (0.05, 0.1 and 0.3% ethanol - Sigma Diagnostics). Ethanol concentration was plotted against ionisation peak area producing a regression equation. This was then used for calculating the ethanol content of root extracts from their mean peak areas. Where ethanol content of the extracts was low, standard ethanol solutions were diluted to increase accuracy of calibration. Using the regression equation, the percentage ethanol content of the samples was determined. The molar content of ethanol in either the root tissue or water bathing the roots was then calculated as described in Appendix 2.1.

2.3.3 Quantification of Tissue Viability

Damage to tissues is often assessed by measuring root growth in controlled conditions following stress treatments. These root growth potential tests are however long term and of no use in determining immediate damage in dormant roots during Winter. Thus the biochemical test of tri-phenyl tetrazolium chloride reduction was used.

Active dehydrogenase enzymes in healthy tissues reduce colourless tri-phenyl tetrazolium chloride to a red formazan derivative. Steponkus and Lanphear (1967) first developed a method of quantifying tissue viability by extracting the red dye in boiling ethanol and determining its concentration in a spectrophotometer. The degree of reduction correlated well with stem and leaf survival. Since then this test has been successfully used to determine the viability of roots of a number of conifers (Lindstrom and Nystrom 1987, Lindstrom and Mattsson 1989).

The viability of both tap roots and primary roots was determined by quantifying the reduction of tri-phenyl tetrazolium chloride solution using a method based on that of Steponkus and Lanphear (1967).

All roots were washed gently in tap water. Distal root samples of 5cm length were then tissue-dried and weighed before cutting into 1cm lengths. Transverse sections of tap roots approximately 1mm thick were sampled from various distances along the tap root, tissue dried and weighed. The roots were then placed in a 0.06M phosphate buffer solution at pH 7.4[7] containing 0.9%w/v tri-phenyl tetrazolium chloride and 0.05%w/v Tween 80. (Root samples of approx. 150mg were placed in 6ml reagent. If the mass of root was greater, the volume of reagent was increased proportionately such that the TTC was always in excess.) Root samples were infiltrated with reagent in a vacuum desiccator for 30 minutes and subsequently incubated in darkness at 30°C for 20 hours. The samples were then poured through a scinta-glass™ filter attached to a vacuum pump and rinsed with 20 ml distilled water. The washed roots were then collected from the filter, placed in a test-tube with 10ml 96% ethanol and heated in a water bath at 90°C for 10 minutes. The red formazan derivative was extracted and the extracts from successive boilings combined and made up to a known volume with ethanol.

[7] Phosphate buffer contains 0.06M KH_2PO_4 and 0.06M Na_2HPO_4

Repeated extraction:

boiled for 10 min in 10ml ethanol, extract rinsed with 3ml ethanol

boiled for 10 min in 10ml ethanol, extract rinsed with 2ml ethanol

boiled for 10 min in 5ml ethanol, extract rinsed with 1ml ethanol

boiled for 5 min in 5ml ethanol, extract rinsed with 1ml ethanol

The absorption of the final extract was determined at 520nm in a Unicam SP1800 Ultraviolet spectrophotometer[8]. The samples were placed in plastic cuvettes and read against ethanol. The 'viability index' of the root was then calculated:

$$\text{VIABILITY INDEX} = \frac{\text{A520 X V}}{\text{f. wt.}}$$

where V = volume ethanol extract (ml)
A520 = absorption at 520nm
and f. wt. = Fresh weight (g)

The viability index gives a measure of the enzyme activity but must be related to the viability of a comparable tissue sample from a control seedling to measure tissue health. Thus the relative viability is calculated:

$$\text{Relative viability} = \frac{\text{viability index stressed root x 100}}{\text{viability index untreated root}}$$

Relative viability therefore quantifies the damage to a root sample by a specific treatment.

2.3.4 Analysis of root carbohydrate content

Root samples, either primary, fibrous or tap roots were washed in tap water and plunged into liquid nitrogen before drying in an Edwards Modulyo freeze drier. Roots were stored at -20°C until analysis. When in liquid nitrogen or following freeze-drying, roots were ground to a fine

[8] Slit width 0.08mm, band width 0.3nm

powder. Root tip sections and those 5-10 and 10-15cm from the tip were ground using a pestle and mortar whilst the fibrous roots and tap roots analysed in Chapter 6 were ground to a fine powder in a Glen Creston Mill. Prior to analysis, stored roots were re-dried in the freeze drier to remove any water from storage. Starch and sugar content were then determined enzymatically using a starch test combination (Boehringer, Mannheim). Starch was initially solubilised using the method recommended in the test kit, but adjusting the volumes of reagents used as described below. This allowed the determination of root carbohydrate in a small sample of tissue, enabling replication of samples to check accuracy.

Solubilisation

Starch was initially treated with dimethylsulphoxide (DMSO) to convert it into a soluble form. Samples of dried root were weighed into Eppendorf tubes on a precision balance (±0.0001g, BDH), to which 0.075ml 8M hydrochloric acid and 0.300ml DMSO added. The tubes were sealed and thoroughly mixed using a vortexer then incubated in a water bath at 60°C for 1 hour. During solubilisation, the tubes were removed from the waterbath at 15 minute intervals and mixed thoroughly. After 1 hour, the tubes were cooled quickly to room temperature, neutralised with 0.075ml 8M NaOH and 1.05ml citrate buffer (0.112M, pH4) was added. The tubes were again thoroughly mixed on a vortexer. Under these conditions, no D-glucose is released from starch.

Enzymatic analysis of extract

After thorough mixing, the extract was centrifuged at 13,000rpm for 5 minutes to remove root material. Enzymatic analysis was carried out in glass cuvettes, all glassware being thoroughly cleaned in 2%EDTA and 2%nitric acid between use.

Up to 0.2ml of supernatant was incubated in a waterbath at 55-60°C for 15 minutes in a pH 4.6 buffer containing amyloglucosidase (AGS). This process hydrolyses starch to D-glucose according to equation 1:

$$\text{STARCH} + (n\text{-}1)\ H_2O \xrightarrow{\text{AGS}} n\ \text{D-GLUCOSE} \qquad (1)$$

1ml triethanolamine buffer (pH 7.6) containing NADP and ATP was added to the cuvette and the absorbence (A_1) read after 3 minutes. Absorbence was measured at 340nm, (the absorption maxima for NADPH) using a Pye Unicam Ultraviolet spectrophotometer operating with a double beam. Samples were read against air. On addition of 20µl enzyme suspension containing hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6P-DH), the cuvettes were mixed well and incubated at 20-25°C. After 10-15 minutes, the absorbence at 340nm (A2) was recorded. During this time glucose from starch hydrolysis is phosphorylated to glucose-6-phosphate by ATP in the presence of hexokinase (Eqn. 2).

$$\text{D-GLUCOSE} + \text{ATP} \xrightarrow{\text{HK}} \text{GLUCOSE-6-PHOSPHATE} + \text{ADP} \qquad (2)$$

The G-6-P formed is oxidised by NADP to gluconate -6-phosphate in the presence of G6P-DH, to form NADPH (Eqn.3)

$$\text{GLUCOSE-6-PHOSPHATE} + NADP^+ \xrightarrow{\text{G6P-DH}} \text{GLUCONATE-6-PHOSPHATE} + \text{NADPH} + H^+ \qquad (3)$$

The amount of NADPH formed in the reaction is stoichiometric with the amount of D-glucose formed by hydrolysis of the starch. Thus measuring the change in absorbence on adding the enzymes indicates the amount of NADPH produced and thus the starch content of the extract.

When analysing a tissue extract, a cuvette containing all reagents but the sample was analysed to correct for changes in NADPH concentration not produced by glucose phosphorylation. This was the reagent blank. The root extract also contained free D-glucose, present naturally in the root and from the acid hydrolysis of oligosaccharides in the solubilisation procedure. Free D-glucose content was determined by repeating the enzymatic analysis without the addition of amyloglucosidase. The procedure is detailed in Table 2.1.

For most accuracy, the starch present in the cuvette should be 3-40μg, therefore a sample solution of 0.03 to 0.4g/L was prepared. 0.1 or 0.2ml sample was used in the enzyme assay, adjusting the volume of distilled water added to each cuvette accordingly, such that the final cuvette volume remained at 2.32ml. To obtain a sample solution of the correct concentration and maximise the performance of the assay, the mass of root typically used per extract is shown in Table 2.2.

Table 2.1. Summary of enzymatic analysis

Based on table taken from Starch, UV method, Biochemical analysis test kit (Boehringer Mannheim 1989).

(Volumes /ml)

PIPETTE INTO CUVETTES	REAGENT BLANK	SAMPLE	SAMPLE BLANK
Solution 1	0.20	0.20	-
Sample solution	-	0.10*	0.10*
Milli-Q water	0.10	-	-
Mix by gentle swirling, cover and incubate at 55-60°C for 15 minutes.			
Solution 2	1.00	1.00	1.00
Milli-Q water	1.00	1.00*	1.20*
Mix with glass stirrer, After 3 minutes read absorbences of each cuvette, (A_1)			
Suspension 3	0.02	0.02	0.02
Mix as above, after completion of the reaction (15 min), read absorbence A_2. Calculate A2-A1 for each cuvette.			
A_2-A_1	**ΔRB**	**ΔS**	**ΔSB**

Solution 1 = amyloglucosidase in a citrate buffer at pH 4.6
Solution 2 = NADP and ATP in triethanolamine buffer at pH7.6
Suspension 3 = hexokinase and glucose-6-phosphate dehydrogenase
* The volume of sample in the cuvettes was increased to 0.2ml if the starch content was low. The volume of water added was then adjusted to give the same final volume.

Table 2.2. Mass of root per Eppendorf tube for starch extraction.

ROOT TYPE	TREATMENT	MASS ROOT/EPPENDORF(g)
Primary root - distal 15cm	*drained* *flooded*	0.005 0.015
Fibrous roots	*drained* *flooded*	0.005 0.008-0.015
Tap root	*drained* *flooded*	0.005 0.008-0.015

Calculation of results.

The sample cuvette, containing both the root extract and amyloglucosidase, generates NADPH stoichiometric with:

- glucose released by starch hydrolysis with amyloglucosidase,
- glucose released from oligosaccharides during the solubilisation procedure,
- glucose already present in the root tissue

The sample blank only contains glucose from the latter 2 sources. The absorbence differences were calculated for each cuvette as detailed in table 2.1.

Starch content

Due to the presence of free D-glucose in the sample blank, the absorbence difference due to starch hydrolysis was calculated:-

$$\Delta A_{STARCH} = \Delta A_S - \Delta A_{SB}$$

Concentration of starch in the root tissue was then calculated from the equation:-

$$\text{STARCH } (mg\ g^{-1} \text{ d.wt.root}) = \frac{V \times MW \times \Delta A_{STARCH} \times V_{ext}}{\varepsilon \times d \times v \times 1000 \times \text{d.wt.root}} \qquad (4)$$

where,
V= final volume cuvette
MW=molecular weight starch, (MW glucose - MW water =162.1)
Vext= volume extract = 1.5ml
ε = absorption coefficient of NADPH at 340nm
=6.3 [l x $mmol^{-1}$ x cm^{-1}]
d = path length=1cm
v = sample volume (ml)
d.wt.root = dry weight root (g)

Sugar content

The process of solubilising starch from the root tissue hydrolyses oligosaccharides present. Carbohydrates likely to be present in the roots would be hydrolysed as follows:-

Sucrose-——→ glucose + fructose
Maltose-——→ glucose
Raffinose-——→ glucose + fructose + galactose

Thus the sample blank determines the glucose released from the above reactions and that already present in the roots.

$$\Delta A_{SUGARS}=\Delta A_{SB} - \Delta A_{RB}$$

The sugar content was calculated according to equation 4, substituting ΔA_{SUGARS} for ΔA_{STARCH}.

This data is obviously needed for calculating starch content, but in itself gives a useful indication of the amount of sugar in the root. Fructose and galactose present could not be determined by the method above, but the results are presented throughout the thesis as they allow a comparison of the sugar reserve in different seedlings and during different treatments. The term 'sugars' in the thesis therefore refers to glucose present in the root and that formed by the hydrolysis of sucrose, maltose and raffinose.

As the source of the glucose in the extract was unknown, (i.e. whether free or from hydrolysis), the sugar content was calculated in terms of glycosyl (MW 162.1) content, and expressed in terms of g per g dry weight of root tissue. The correction factor for converting this value into glucose content (MW 180) is therefore 'x1.1'. In all experiments, starch and sugar content (both measured in terms of glycosyl content) were totalled and termed 'total non-structural carbohydrate' (TNC). This obviously excludes fructose and galactose present, but represents most of the total non-structural carbohydrate content of the roots and is a useful figure when comparing the effects of flooding treatments.

Accuracy of results

The suitability of this method for analysing tissue samples was checked by performing the determinations:

a) with two different sample volumes, (0.1 and 0.2ml)

b) with two extracts made with different root mass, (e.g. 0.005 and 0.010g).

The accuracy was also checked by solubilising a known quantity of starch and enzymatically examining the extract.

Analysis of starch standards proved to be 97.6 ±4.5% accurate, (n=6).

2.3.5 Analysis of root malate content

The extract prepared in 3.2.2 for analysis of ethanol accumulation was also used to assay the malic acid present in root tissue. Malate was determined enzymatically using a L-malic acid test kit (Boehringer Mannheim). Malic acid is oxidised by NAD to oxaloacetate in the presence of L-malate dehydrogenase (L-MDH). The oxaloacetate is then converted to L-aspartate by addition of L-glutamate and glutamate-oxaloacetate transaminase (GOT). This stage is necessary to drive the oxidation of malate to completion. The amount of NADH formed by malate oxidation is stoichiometric with the concentration of malate, and can be determined spectrophotometrically as for NADPH production when analysing starch content above.

$$\text{L-MALATE} + \text{NAD}^+ \xleftrightarrow{\text{L-MDH}} \text{OXALOACETATE} + \text{NADH} + \text{H}^+ \qquad (5)$$

$$\text{OXALOACETATE} + \text{L-GLUTAMATE} \xleftrightarrow{\text{GOT}} \text{L-ASPARTATE} + \text{2-OXOGLUTARATE} \qquad (6)$$

Root extracts were centrifuged at 10,000g and samples of supernatant incubated with the enzymes and buffers detailed in Table 2.3. The assay was conducted in glass cuvettes at 25°C and the absorbence of sample cuvettes read against the blanks in the double beam spectrophotometer.

Table 2.3. Method of enzymatic analysis
(From L-malic acid test combination, instruction leaflet 1989).

Volume (mls).

Pipette into cuvettes	BLANK	SAMPLE
Solution 1	1.00	1.00
Solution 2	0.20	0.20
Milli-Q water	1.00	0.90
Suspension 3	0.01	0.01
Sample solution	-	0.10*
Mix using glass stirrer, read absorbence of sample against that of blank = A1		
Solution 4	0.01	0.01
Mix, after 10 min, read absorbence of sample against that of blank = A2		

*Increased upto 0.6ml for samples of low malate content, adjusting the volume of water in the cuvette to maintain the same final volume.
Solution 1=glutamic acid in glycylglycine buffer, pH10.
Solution 2=NAD lyophilisate
Suspension 3=glutamate-oxaloacetate transaminase suspension
Solution 4=malate dehydrogenase

To maximise accuracy, the cuvette should contain 2-20μg of malate. The volume of extract added to the sample cuvette was therefore adjusted. Between 0.1 and 0.6ml were assayed depending on the malate content. Root samples of approximately 0.14g were used to provide ~1.00ml of extract.

As in the enzymatic analysis of starch, tests were conducted to check accuracy. Measurement of a malic acid standard of 0.198g/L proved to be 97.5% ± 1.5% accurate, (n=12). When replicate samples from one root were extracted, the standard error of the mean malate content was <5%.

Calculation

Root malate content (μmoles g^{-1}f.wt.root) was calculated according to equation 7:

$$\text{Malate content } (\mu\text{moles}/g^{-1}\text{f.wt.root}) = \frac{V \times \Delta A \times V_{ext}}{\varepsilon \times d \times v \times \text{f.wt.}} \qquad (7)$$

where V= final volume cuvette
$\Delta A = A2 - A1$
Vext= volume extract (ml)

ε = absorption coefficient of NADH at 340nm
=6.3 [l x $mmol^{-1}$ x cm^{-1}]
d = path length=1cm
v = sample volume (ml)
f.wt. = fresh weight root (g)

2.3APPENDIX

Calculating the molar concentration of ethanol from the percentage concentration in the injected sample

%ethanol=g ethanol/100g water.

Multiply by 10 to give g ethanol/Lwater.

1 mole ethanol weighs 46g, therefore 1g ethanol contains 1/46 moles.

Multiply the g/L concentration by 1/46 to give moles/L concentration.

Calculate the total number of moles of ethanol in the extract or water around the root.

Multiply the molar concentration by the volume of extract or water.

Correct for differing weights of root, to give molar ethanol content per g fresh weight of root.

Divide by the fresh weight of root tissue, or weight of frozen root powder.

Summarising

For calculating the ethanol content of root extracts:

$$\text{Ethanol content} = \frac{\%\text{ethanol} \times 10 \times V}{46 \times \text{fresh weight root(g)} \times 1000}$$

where V = volume extract/ml*

For calculating the ethanol content of water around the roots:

$$\text{Ethanol content} = \frac{\%\text{ethanol} \times 10 \times V}{46 \times \text{fresh weight root(g)} \times 1000}$$

where V = volume water = 2ml

---

* For the determination of ethanol accumulation during 24 hours anoxia, (Chapter 3,4 and 5)

V= 2.0ml or 3.0ml as detailed in each chapter.

The volume of methyl orange and potassium carbonate for neutalisation of the extract was negligable.

For determination of ethanol accumulation *in vivo*, (Chapter 3 and 5)

V=mass of final neutralised extract after removal of precipitate (g).

(= volume in ml).

In each case the units *μmoles ethanol g $^{-1}$fresh weight root* were used.

The sum of the above equations gives the total ethanol production, although the results are important in themselves as they indicate both the accumulation of ethanol and the leakage from the roots.

i.e. Total ethanol production = Ethanol accumulation in root tissue
+ ethanol content of water around root.

CHAPTER 3

Importance of Aeration Through Stem Lenticels in Supplying Oxygen to Flooded Distal Roots of Lodgepole Pine

3.1 INTRODUCTION

This chapter has two aims. Firstly, it investigates the mechanisms underlying the superior flood tolerance of Lodgepole pine over Sitka spruce. Coutts and Philipson (1978b) have attributed this to well developed internal aeration in Lodgepole pine, but recognised that metabolic adaptation to anaerobiosis may also play a part. The carbohydrate content and metabolic activity of each species were determined at flood initiation in late November in order to investigate the possibility that differences in these properties may pre-dispose the species to their respective flood-tolerances. The main purpose of the investigation, however, was to quantify the ability of internal aeration in Lodgepole pine to ameliorate anoxia in the distal roots. This was done by measuring the depletion of carbohydrates and accumulation of ethanol in seedlings where aeration was prevented. The introduction first covers current knowledge of internal gas transport mechanisms then discusses the aims of the experiment in more detail.

Underground organs may survive waterlogging by morphological and /or metabolic adaptation. Although surrounded by anoxic mud or water, roots may still opt for an 'avoidance' or 'tolerance' strategy common to most adverse conditions. To avoid anoxia, movement of air from the shoot to the submerged root is necessary. This may be facilitated by a number of different morphological adaptations and is an important strategy often observed in species which suffer long-term oxygen deprivation. Supply of oxygen to the roots may support aerobic respiration (fully or partially) and may allow oxidation of the rhizosphere, therefore protecting the root from toxic ions in the soil. Although the aeration of underground organs is a generally accepted phenomenon, the mode of gas movement from root to shoot and the

potential of this supply to meet respiratory demand in roots far beneath the water table are not fully understood.

Air may enter the shoot through the leaves and stem or the root system above the water table and diffuse through intercellular spaces to the submerged roots. On flooding, flood tolerant species often develop thicker, more porous roots aiding the downward diffusion of oxygen. This aerenchymatous tissue is a common feature of many wetland plants, though there are few recorded instances in tree roots (see Coutts and Armstrong 1976). Large gas filled spaces in the distal regions of roots suggest that their function may not only be in supplying the tissue with oxygen but also in the dilution of the toxic end products of anaerobic respiration and their removal up the root system to the shoot. This may readily remove acetaldehyde and CO_2, but is unlikely to be effective in the removal of ethanol whose partition coefficient favours its retention in the liquid phase (Crawford 1992, Crawford and Finegan 1989).

Some species have evolved special roots to provide the buried root system with an extra connection to the air. The pneumatophores of the black mangrove *Avicennia nitida* are air roots extending vertically upwards from the roots in anaerobic soil; one tree may produce several thousand air roots, usually 20-30cm high and approx. 1cm thick. *Rhizophora mangle* has arched stilt roots projecting from the stem, through the air. In each species the submerged roots arising from these structures are spongy and air filled.

Both types of root are covered in lenticels, and removal of the pneumatophores in *Avicennia* or blockage of the lenticels of *Rhizophora* with grease has been shown to reduce the oxygen content of the buried roots substantially (Scholander *et al.* 1955), proving that they are important ventilation structures.

In recent years, much evidence has suggested that the movement of air to the underground organs is not by diffusion alone. When a gas lies on each side of a porous partition in which the pore diameters are less than the mean free path length of the gas molecules, (<0.1μm), if one side of the partition is warmed, gas will move through the partition from the colder to the warmer side. This thermal transpiration continues if a pathway of less resistance allows gas movement in the opposite direction (see Crawford 1992). This phenomenon is likely to occur in species with similar physical properties. *Nuphar lutea*, the water lily, appears to meet these requirements. The possession of very small pores (0.7 to 1.2μm diameter) between the palisade tissue and spongy parenchyma (Schroder *et al.* 1986) and the temperature gradient of the leaf leads to an increase in internal gas pressure. Armstrong and Armstrong (1990) suggest that the evaporation of water inside the leaf may also cause such pressure increases. This results in the transport of substantial volumes of gas through the petioles (e.g. 22L of air per day - Dacey 1980). Such solar-powered gas transport has also been measured in *Phragmites australis* rhizomes where a linear flow of 1.7mm/sec. was recorded (Armstrong and Armstrong 1990).

Recent work by Grosse and his colleagues on this phenomenon in trees has shown that light-induced warming of the lower stem relative to air results in a positive pressure in the stems intercellular system and a down flow of air to the roots. The air moves through tiny pores in the phellogen layer of the lenticellular meristem. Buchel and Grosse (1990) observed pores of an average size of 0.014μm in black alder. Since the initial investigations of gas transport in alder, this phenomenon has been demonstrated in seedlings of other wetland tree species; *Taxodium distichum, Betula pubescens, Populus tremula* and in *Acer saccharinum* and *Acer rubrum* from wet sites (Grosse *et al.* 1992). As yet no

investigations of such pressurised gas transport in gymnosperms have been made.

Movement of air down the root system may also be aided by the reduction in internal pressure as oxygen is respired and the CO_2 produced dissolves into solution. Scholander *et al.* (1955) suggest that a partial vacuum is produced when the lenticels of *Avicennia* are blocked at high tide. This then draws in air as the tide falls.

Measurement of the rate and mode of movement of air through trees such as occur on wet sites in Britain is therefore in its early stages. Coutts and co-workers have examined the importance of aeration from the shoot in terms of survival of roots in two species of contrasting flood tolerance. Lodgepole pine is well known to have superior flooding tolerance when compared to Sitka spruce, its roots were observed at twice the depth on peaty gley soil (Everard *et al.* 1970). It is often planted before Sitka spruce to 'soak-up' soil moisture on wet sites. They have concluded that the superior flood tolerance of Lodgepole pine roots to waterlogging reflects a better developed internal aeration system, without precluding possible metabolic adaptation of the root (Coutts and Philipson 1978b).

When the supply of oxygen to the shoots of Lodgepole pine and Sitka spruce is prevented, the oxygen flux from the roots, detected by polarographic electrode, decreases rapidly (Armstrong and Read 1972). Philipson and Coutts (1978) showed the needles and lenticels on the woody roots in aerated surface soil to be important sites of oxygen entry in Lodgepole pine. Often the water table will rise to cover the primary root and young roots with secondary thickening while most woody roots remain in the upper aerated soil horizon. Lenticels in this region are thought to be the main site of air entry in older trees on wet sites (Philipson and Coutts 1978). Similarly most oxygen supplied to the submerged roots of pond and loblolly pine seedlings enters through stem

and root collar lenticels (Topa and McLeod 1986). If covered with water when the water table rises, being hydrophobic, the lenticels function immediately it subsides (Coutts and Armstrong 1976). In both Sitka spruce and Lodgepole pine, oxygen moves down the woody roots in the bark, the former species having greater capacity for transport in this tissue (Philipson and Coutts 1980). In contrast, considerable amounts of oxygen are transported in the xylem of Lodgepole pine, any movement in this portion of the root being undetectable in Sitka spruce. This difference appears to be due to the Lodgepole pine roots having a larger proportion of embolised xylem and gas in the roots. Not only is the air space larger, but most importantly it is longitudinally continuous in the pine in contrast to the discontinuous tracts observed in Sitka spruce (Philipson and Coutts 1980). It is not known if woody roots can increase their porosity under waterlogging, but Coutts and Armstrong (1976) suggested that anaerobic products may cause physiological damage and induce embolism in the xylem leading to enhanced gas pathways. This needs further testing.

Roots of primary structure in Lodgepole pine and Sitka spruce reflect the same pattern, the former species having greater capacity for oxygen transport. Waterlogging increases the porosity of the primary root such that oxygen diffusion can be detected from the tips of excised roots when the basal region is exposed to oxygen. Oxygen transport of previously drained roots is less extensive (Philipson and Coutts 1978). The main pathway for gas transport in the primary root of Lodgepole pine is the stele. In this species, roots which penetrate the water table develop large gas-filled cavities in the stele, these communicating with lenticels above the water table (Coutts and Armstrong 1976, Coutts and Philipson 1978b). This seems an important factor in the greater penetration of the water table by primary roots of Lodgepole pine in

comparison to Sitka spruce which do not develop such air spaces. Supply of oxygen to the root tips of Lodgepole pine allows them to penetrate the water table to depths of 20cm at 10°C, penetration being greater at 10° than 20°C presumably due to reduced respiratory demand at low temperature. Their growth is presumably dependant on the supply of oxygen to the root tip and declines with depth as the transported oxygen is depleted by respiration as it proceeds down the root (Coutts and Philipson 1978b). The permeability of roots can increase quickly on waterlogging. After only 15 days in anaerobic hydroponic culture, flood-tolerant loblolly and pond pine had increased root porosities which continued to increase as flooding progressed. During this time, secondary tissues in the roots formed intercellular air spaces (Topa and Mcleod 1986).

The depth to which oxygen may be supplied to Lodgepole pine root tissue in the field is uncertain. At 4°C radial diffusion of oxygen (seen by the oxidation of reduced indigo-carmine dye) was detected from root tissue 36cm from the oxygen source (Philipson and Coutts 1978). Using an ensheathing Pt electrode, Armstrong and Read (1972) demonstrated a maximum distance of only 6cm oxygen transport in a conifer seedling . It is likely that oxygen may be transported further than these estimates, since each method relies on the radial diffusion of oxygen in excess of the roots respiratory requirements being detected (Philipson and Coutts 1978). Coutts and Philipson (1978b) suggest that root growth in excess of distances over which O_2 transport has been demonstrated may be explained by metabolic adaptation of the root. A comparison of the modes of aeration and the physiology of the 2 species is given in Table 3.1.

This experiment is designed to measure the extent to which the downward movement of air from the shoot of Lodgepole pine seedlings

can supply the primary roots with oxygen when the whole root system is waterlogged during Winter dormancy. Winter waterlogging is less damaging than that in Summer due to the lower temperature and dormancy of the root reducing the respiratory demand for oxygen. Thus in Winter it is possible that aeration from the stem may meet the respiratory demands of the root. Cooling the root medium around conifer seedlings increases the oxygen flux from the root due to reduced respiratory demand (Armstrong and Read 1972). Crawford (1982) suggested that the accumulation of ethanol under flooding in Lodgepole pine roots indicates that the aeration system cannot satisfy the demand for oxygen. Tripepi and Mitchell (1984b) found that excluding gas exchange through the stem lenticels of flooded red maple and river birch had no effect on root respiratory capacity or flood-tolerance. Both results suggest that simply the presence and hypertrophy of stem lenticels cannot be presumed to supply *adequate* oxygen to the roots and increase flood tolerance. Their ability to do so should be tested.

The experiment entailed blocking the stem lenticels of a sample of flooded seedlings with petroleum jelly. Lenticels have been shown to be present in the lower stem by the release of small but detectable quantities of ethanol emanating here (Crawford and Finegan 1989). Another group of seedlings was flooded without covering the stems. Thus in the first group, air could only enter the plant via the needles, whereas in the latter, both the needles and lenticels on the stem could serve as ports of air entry. After 90 days of flooding, 15cm distal sections of root were analysed to measure the depletion of carbohydrate reserve, accumulation of ethanol and to quantify tissue viability. By measuring these physiological components rather than radial O_2 flux from the root, we could assess the ability of gas transport through the stem lenticels to supply the primary root with oxygen. Respiration rate under air and

anoxia and the root carbohydrate content at the initiation of flooding were also assessed and compared to Sitka spruce seedlings analysed concurrently. These physiological characteristics may also play a role in the superior flood tolerance of Lodgepole pine.

Table 3.1. Comparison of the 2 species and characteristics thought to explain their differing flood tolerance

LODGEPOLE PINE *flood tolerant*[9]	SITKA SPRUCE *flood sensitive*
greater proliferation of cork tissue and lenticels when flooded[10]	small increase in cork and lenticels
development of aerenchyma in waterlogged primary root aiding O_2 diffusion[11]	no aerenchyma in primary root, much lower oxygen transport
O_2 diffusion in late wood [12]	no O_2 diffusion in xylem of woody roots, (although O_2 diffusion aided by proliferation of outer bark).
greater proliferation of periderm of woody roots [13]	
Reduced O_2 leakage out of upper root and resulting greater depth of diffusion[14]	
Superior tolerance to soil toxins accumulating under anoxia ($Iron^{2+}$ and volatile fatty acids)[15]	less tolerant to toxins
lower ethanol accumulation under waterlogging [16,17]	10x ethanol accumulation of Lodgepole pine
formation of adventitious roots above water table[18]	formation of adventitious roots above watertable

[9] Minore (1968), Armstrong and Read (1972) and Boggie (1974).
[10] Coutts (1982).
[11] Coutts and Philipson (1978b)
[12] Philipson and Coutts (1978, 1980)
[13] Coutts and Philipson (1987)
[14] Crawford (1982)
[15] Sanderson and Armstrong (1980a,b)
[16] Crawford (1976)
[17] Crawford and Baines (1977)
[18] Gill (1970)

3.2 METHODS

3.2.1 Seedling origin and growth conditions

In late March 1992 Lodgepole pine seedlings[19] were transplanted into peat in 2m perspex tubing and immediately top-dressed with 2g Osmocote™ plus slow-release fertiliser, supplemented with a further 3g in June. Tubes were maintained in insulated root boxes, as detailed in Chapter 2, until Autumn when roots had grown up to half way down the tubes.

3.2.2 Experimental design

In early December, seedlings were randomly selected for 4 treatments; Figure 3.1.

Group 1: Free-drained seedlings, analysed immediately to indicate physiological status of the root at the time of flood initiation, in terms of carbohydrate depletion by respiration. Sitka spruce seedlings of Washington origin were analysed concurrently for a separate experiment, assessing the effect of temperature on survival of Winter flooding (see chapter 5). The results are presented together in this chapter as the two species were of a similar age and grown under the same conditions, therefore allow a comparison of root physiology during Winter.

Group 2: seedlings remained free-drained to be analysed as a control in March.

---

[19] Sown from seed by Alba Trees plc, Lower Winton, Gladsmuir, East-Lothian. At 10-20cm height, transfered to St. Andrews and transplanted from root trainers to peat. Maintained outdoors for 1 year. Provenence number PICO12B80X.

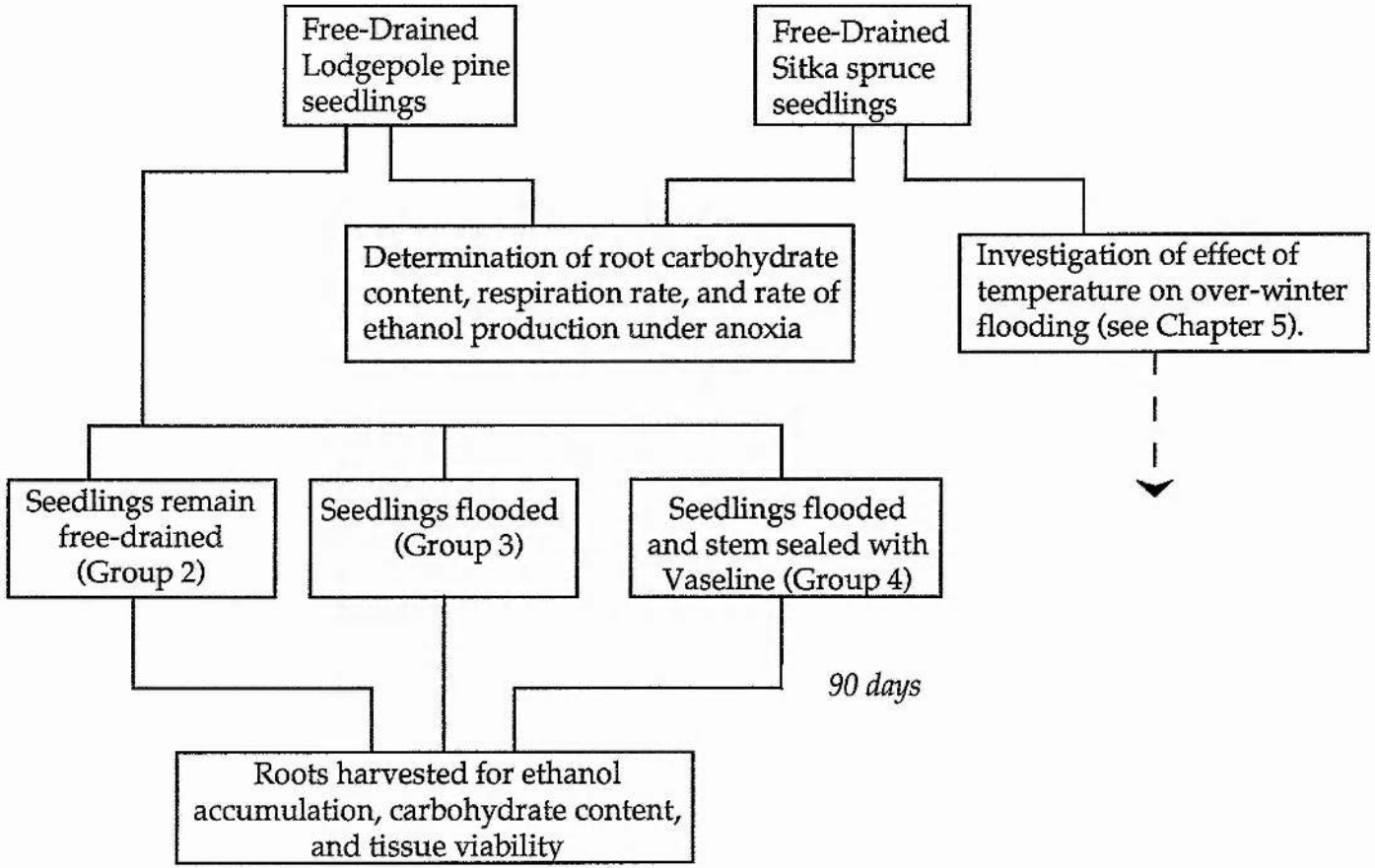


Figure 3.1 Experimental design flowchart

Group 3 and 4: seedlings flooded from the base with tap water at the beginning of December. The water level was maintained just above the soil such that it could easily be observed. Excess water was removed if rainfall increased the height of the water level. A thick layer of petroleum jelly was applied to the stem of Group 4 seedlings from the water level to the node of the first needle-bearing branch. This would block any gas exchange through the lower stem, (Figure 3.2).

3.2.3 Determination of carbohydrate metabolism of free-drained seedlings concurrent with flood initiation

The root core was gently removed from each tube whilst flushing with water to prevent root damage. 15cm distal sections of primary root[20], 1-2mm diameter were selected, washed in tap water and placed into 3 groups for the three physiological measurements. Each root was further sectioned into 3 x 5cm lengths; 0-5, 5-10 and 10-15cm from the tip.

For each class of root, aerobic and anaerobic respiration rate, starch and sugar content and ethanol production under anoxia were determined.

Three Lodgepole pine and four Sitka spruce seedlings were analysed to give a mean value for each parameter.

a) Carbohydrate content

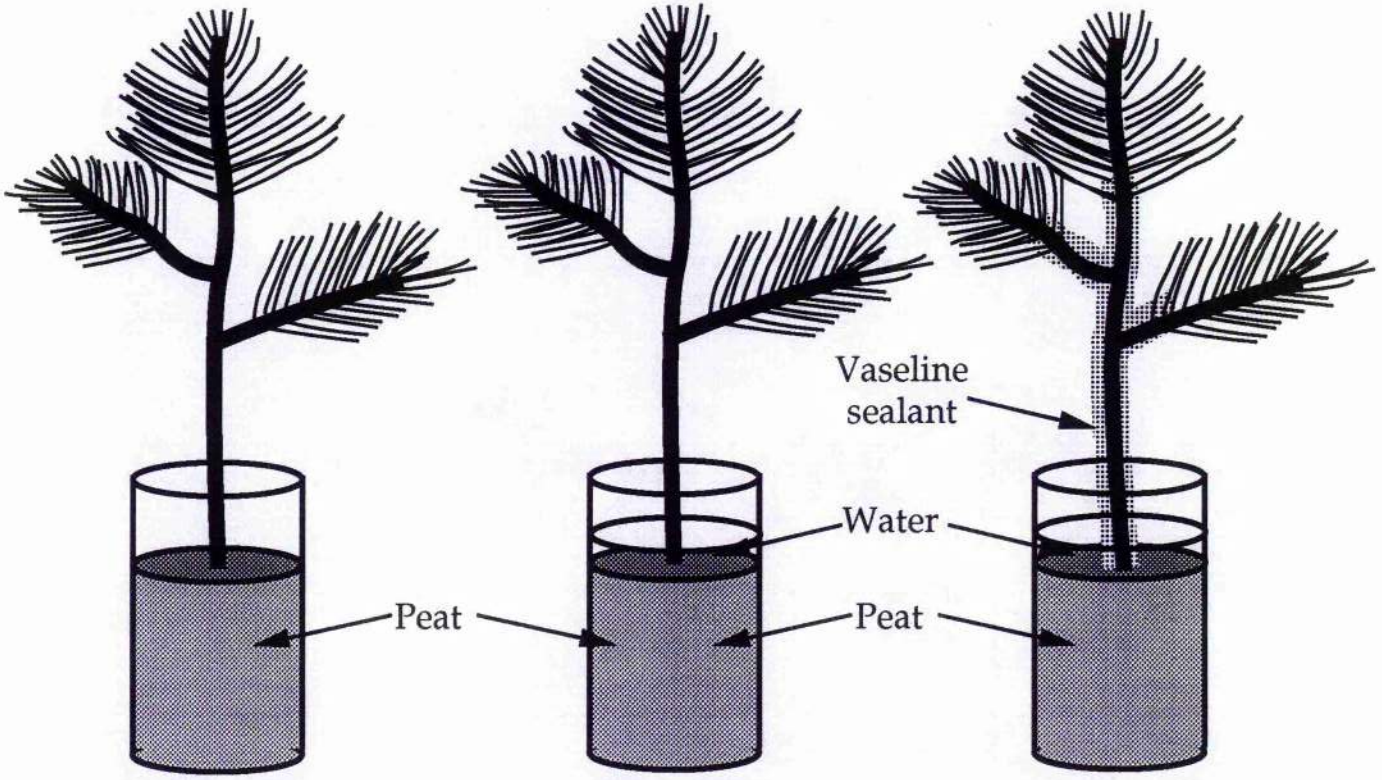
see experimental techniques.

b) Respiration rate

Root sections were immediately incubated at 10°C in round bottomed flasks containing moist tissue paper to prevent desiccation. CO_2 production of each section was determined by Infra-red Gas Analysis, initially in air then under nitrogen

[20] Described as 'long roots' of 0.5-3.0mm diameter by Coutts and Philipson (1987), vigourous and with well developed root cap.

Figure 3.2. Over-winter treatments of Lodgepole Pine seedlings.



Group 2:
Free-draining control

Group 3:
Flooded seedlings

Group 4:
Flooded, stem lenticels sealed with petroleum jelly

following one hours incubation in a stream of water-saturated Nitrogen. (see experimental techniques). All measurements were taken at 10°C.

c) Ethanol production

Root sections were incubated under anoxia in sealed vials at 10°C for 24hrs and *in vitro* ethanol production analysed. A perchloric acid extract of the root tissue and a sample of water from the sealed vial were injected into the GLC and ethanol content determined. This gives a measure of tissue ethanol accumulation and leakage into the root environment (see experimental techniques).

3.2.4 Analysis of the effect of flooding on root metabolism and survival

In early March, free-drained seedlings (group 2) and all flooded seedlings were harvested from the perspex tubing without flushing with water.

15cm distal lengths of root were sampled and placed into 3 groups as before. From each seedling, one sample of roots was immediately wiped clean of peat (without washing), and plunged into liquid nitrogen to determine the actual accumulation of ethanol in the tissues *in vivo*. The other roots were washed clean as before and analysed for tissue viability, (TTC assay) and carbohydrate content (detailed in chapter 2).

3.2.5 Sample Replication

Lodgepole pine group 1: 3 seedlings
groups 2-4: 4 seedlings.

Ideally a second control consisting of freely drained seedlings having stems sealed with petroleum jelly should have been assessed. This was impossible due to a limited number of seedlings.

3.3 RESULTS

3.3.1 Carbohydrate metabolism of free-draining seedlings concurrent with flood initiation

a) Root Carbohydrate Reserve

Having confirmed that the samples showed homogeneity of variance, the carbohydrate content of like sections of root were compared between the two species using Student's t-test. In each root section Lodgepole pine seedlings contained higher concentrations of both starch and sugars than Sitka spruce (Figure 3.3). Means significantly different at the 5% level are indicated by an asterisk in table 3.2.

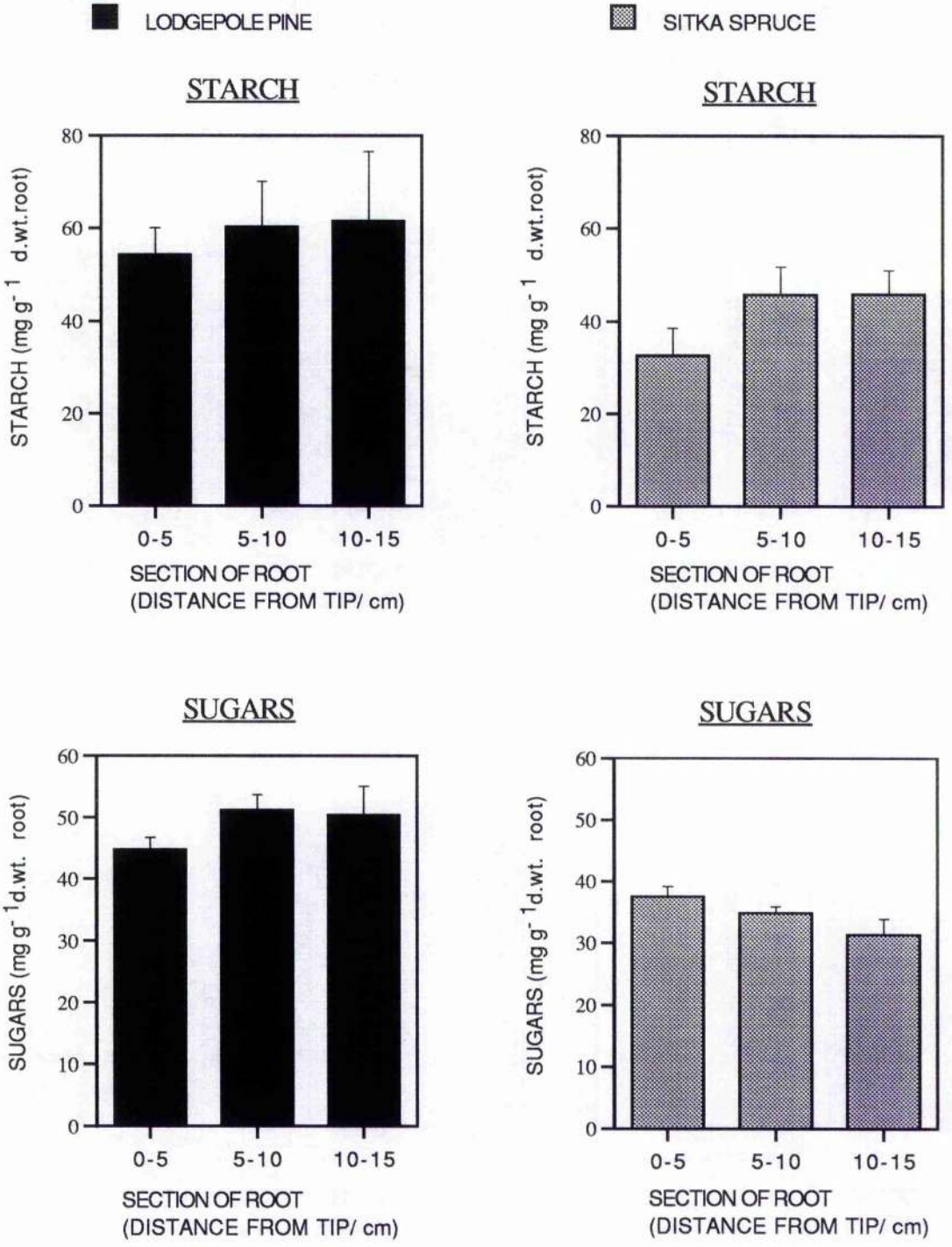
Table 3.2. Carbohydrate content of the distal 15cm of Sitka spruce and Lodgepole pine roots in late Autumn (mg g^{-1} d. wt. root)

	SITKA SPRUCE			LODGEPOLE PINE		
	0-5cm	5-10cm	10-15cm	0-5cm	5-10cm	10-15cm
STARCH	32.52	45.76	45.88	54.13	60.17	61.5
	±6.00	±6.05	±5.15	±5.92	±9.93	±15.1
SUGARS	37.50*	34.82*	31.32*	44.74*	51.14*	50.36*
	±1.73	±1.08	±2.62	±2.04	±2.58	±4.76
STARCH	70.02*	80.58	77.2	98.87*	111.3	111.8
+ SUGARS	±4.83	±6.19	±6.15	±7.93	±12.1	±19.1

Washington Sitka spruce, n=4; Lodgepole pine, n=3
Comparing like sections of root between the two species, non-asterisked values not significantly different at 5% level. Asterisked values show significantly different mean carbohydrate content.
Sugars: 0-5cm, p=0.042; 5-10cm p=0.0013; 10-15cm p=0.013.
TNC: 0-5cm, p=0.022

In each section of Lodgepole pine, the starch and sugar content was greater than that of equivalent roots of Sitka spruce, though only the

Figure 3.3
Root carbohydrate content in freely drained seedlings concurrent with initiation of flooding treatments in late November



Lodgepole pine, n=3, Sitka spruce, n=4

mean sugar content of each section and the TNC content of the tip section differed significantly.

Variation in total non-structural carbohydrate content between the different root sections was assessed using one-way ANOVA for each species. Although carbohydrate content was slightly lower in the root tip section, differences were non-significant at the 5% level.

b) Respiratory activity

Within each species the mean respiration rates of the three root sections were compared by one-way ANOVA. Under both air and nitrogen, both species showed a pattern of higher respiration rate in the root tip section (Figure 3.4). However differences between sections were only significant in Sitka spruce, ($F=7.15$, $df=2$, $p=0.043$ in air; $F=4.56$, $df=2$, $p=0.014$ in nitrogen). The mean respiration rate of each section of Sitka spruce was then compared using Tukey's test at the 5% level of significance. The aerobic respiration rate of the tip section was significantly greater than that of the 5-10cm section and under nitrogen the tip's anaerobic respiration rate was significantly greater than each proximal section.

In comparing like roots of the two species using Student's t-test, the respiration rates under air or nitrogen did not differ significantly between the two species.

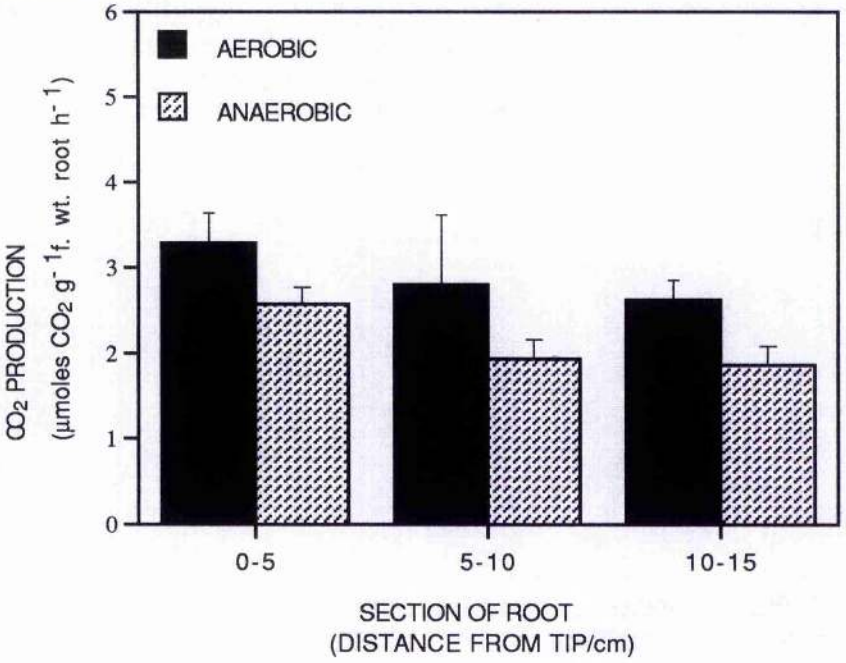
When placed under anoxia, the carbon dioxide production of each section indicated acceleration of glycolysis. If glycolytic rate remained constant during anaerobic respiration, CO_2 output would fall to one third its aerobic value as glucose is not fully oxidised:-

$$C_6H_{12}O_6 \xrightarrow{O_2} 6CO2 + 6H_2O \qquad 38 \text{ Molecules ATP} \qquad (1)$$

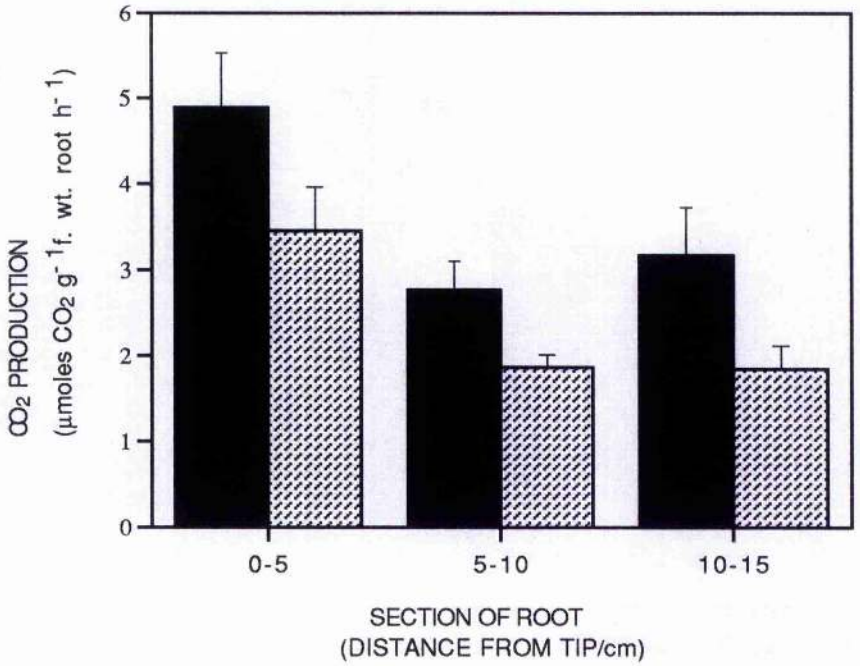
$$C_6H_{12}O_6 \xrightarrow{N_2} 2CH_3CH_2OH + 2CO_2 \qquad 2 \text{ Molecules ATP} \qquad (2)$$

Figure 3.4 Aerobic and anaerobic respiration rate of free-drained Lodgepole pine and Sitka spruce roots concurrent with flood initiation.

LODGEPOLE PINE



WASHINGTON SITKA SPRUCE



n = 4 Washington sitka spruce, n = 3 Lodgepole pine roots

Following one hours incubation under nitrogen, excised roots incurred approximately two fold increases in the rate of glycolysis. The degree of acceleration in glycolysis was similar in the pine and spruce roots (Table 3.3).

Table 3.3. Carbon dioxide production under anoxia as percentage of that in air

	0-5cm	5-10cm	10-15cm
Sitka spruce	70.7%	67.3%	58.1%
Lodgepole pine	78.2%	69.3%	71.2%

According to equation (1) and (2) above, and assuming all CO_2 output to be respiratory, the rate of glucose consumption under air and nitrogen was calculated. Aerobic respiration of one mole of glucose produces 6 moles of CO_2 whereas only 2 are evolved under anoxia. From the rate of glucose consumption and the measured total carbohydrate content, the time for which the measured carbohydrate reserve could sustain respiratory activity was calculated. This is the theoretical 'supply time' and though it gives a very false indication of the effect of anoxia under natural conditions[21] it does provide a useful comparison of the combination of energy supply and demand in different root sections and in the two species under air and anoxia.

Table 3.4 shows how costly anaerobic respiration is in terms of glucose consumption. In both species, approximately twice as much glucose was respired under nitrogen, yet according to the equations above, this would yield only ≈12% of the ATP molecules produced in air.

[21] Under long term soil waterlogging the respiratory rate is likely to vary, and therefore not be sustained at the rate measured initially. Also the total non-structural carbohydrate content used in the calculation represents most of but not *all* the carbohydrate content of the root. Small amounts of fructose and galactose from the breakdown of sucrose and raffinose in the extraction procedure may also be present.

Table 3.4. Glucose consumption and calculated supply time in air and anoxia

		AIR		NITROGEN		
	SECTION	Glucose respired	Supply time (hours)	Glucose respired	Supply time (hours)	N_2/O_2 *
Sitka spruce	0-5cm	5.52 ±0.09	87 ±6	12.47 ±3.06	41 ±12	2.25
	5-10	3.28 ±0.35	168 ±26	7.00 ±0.43	78 ±9	2.15
	10-15	3.10 ±0.12	147 ±4	6.02 ±0.30	76 ±5	1.95
Lodgepole pine	0-5	4.04 ±0.61	163 ±38	9.46 ±1.24	68 ±14	2.4
	5-10	3.90 ±1.06	205 ±61	8.13 ±0.93	87 ±13	2.3
	10-15	3.62 ±0.24	195 ±45	7.68 ±0.49	89 ±12	2.2

Glucose respired (µmoles g^{-1} d.wt.root hr^{-1})
Lodgepole pine, n=3; Sitka spruce, n=2; means and standard error of means shown.
* ratio of glucose respired under nitrogen to that under air.

Using three-way analysis of variance the supply time was compared under air and nitrogen and between the two species and three sections. (Samples showed homogeneity of variance and therefore satisfied the requirements for ANOVA.) Calculations assume that root reserves were not augmented by phloem transport from the rest of the seedling, as seems to be the case in anoxia. Results suggested that anaerobiosis may reduce root longevity to approximately half that in air. Supply time was significantly reduced by anoxia ($F=20.67$, $df=1$, $p<0.001$). In both the spruce and pine, survival time was lowest in the tip 5cm section which depletes its slightly smaller pool of carbohydrate (see Table 3.2) more quickly. The calculated supply time of each Lodgepole pine root section was higher than comparable sections of Washington Sitka spruce seedlings under both air and nitrogen, though differences were not significant at the 5% level. In theory, if root sections were to maintain a constant respiration rate, the glucose reserves present initially would support the tissues for 1.7 to 3.7 days under N_2 and 3.6 to 8.5 days under

air. The time depends on the section of root and the species. In each species, the root tip sections would deplete their reserves first.

c) Ethanol production under 24Hrs anoxia

CO_2 and ethanol production under nitrogen should be equi-molar. However when CO_2 production over 24Hrs was calculated from the output immediately after excision, the estimated level was between 2 and 5 times higher than the production of ethanol, (tables A3.1 and A3.2). Thus either the measured respiration rate was not maintained over the 24 hour period or ethanol was re-metabolised in the root tissue to another compound. The latter is unlikely under anoxic conditions.

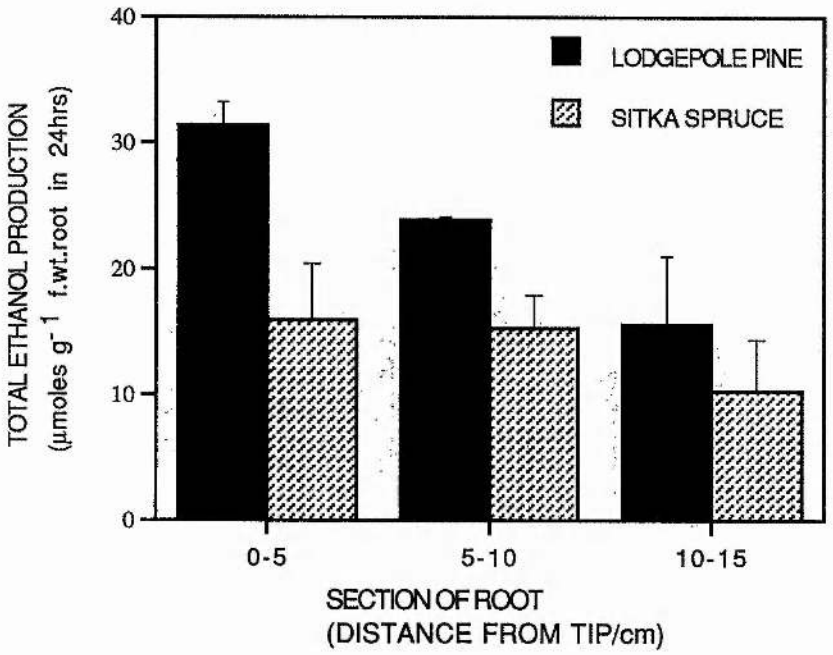
This anomaly may be because of the time scale of the measurements, respiration rates of all sections being measured during the first 3 hours after excision, whereas ethanol production was analysed after 24 hours under anoxia. It is likely that the initial respiration rate measured is not maintained over 24 hours. This may be higher initially due to injury by handling and excision from the root system. Saglio and Pradet (1980) found a decline in oxygen uptake immediately after excision when measuring the respiration rate of excised maize root tips (distal 0.5cm only). After 8 and 24 hours the respiration rate had fallen to 50 and 30% of the initial value respectively, though initial levels could be maintained by application of glucose. Respiration rate was therefore limited by the depletion of root reserves. It is unlikely that respiration rate would decline to such a degree in the pine and spruce roots measured here. Each have much higher carbohydrate content than the maize tips and the total non-structural carbohydrate content measured in the tissues would certainly support the initial rates of respiration over a 24hr period (see supply time, Table 3.4). It would be useful in further analyses to follow accumulation of both ethanol and CO_2 over a 24 hour period in anoxia and assay the remaining carbohydrate reserve.

Both species showed the highest rate of total ethanol production in the tip, correlating with highest CO_2 production, and a decreasing level moving up the root (see Figure 3.5). More than half of the ethanol produced leaked out of the roots into the water around them, approximately the same percentage leaking out in both species. Comparing ethanol leakage from Sitka spruce roots under anoxia at 10°C and that measured at 20°C in Chapter 4, anoxic roots at lower temperatures retained a greater proportion of ethanol in their tissues. At 10°C, 65.1% of ethanol produced leaked into the surrounding water compared to 85.5% at 20°C (mean leakage of three sections). This may be explained by a decrease in membrane porosity and/or reduced diffusion at lower temperature.

Most interesting is the unexpectedly high level of ethanol production in Lodgepole pine roots. CO_2 production was considerably lower in the tip and approximately equal higher up the root when compared to the spruce, In contrast, ethanol production is in each case much higher than in spruce, almost by a factor of 2 in the tip. This resulted in greater ethanol accumulation in the pine roots, (e.g. the mean level of tissue ethanol in Lodgepole pine root tips was greater than 3 times that in Sitka spruce).

The results of respiration analysis and ethanol production therefore do not coincide, Lodgepole pine root tips, with a lower anaerobic respiration rate had the highest rate of ethanol production. This discrepancy may be because of the time scale of the measurements. The high level of respiration seen in Sitka spruce may decrease within a few hours of excision to a steady level lower than that sustained by Lodgepole pine over the 24 hr period. The effect of CO_2 accumulation and glucose depletion on respiration rates in the 2 species is not known.

Figure 3.5 Ethanol production under 24 hours anoxia at 10°C in roots of free-drained Lodgepole pine and Sitka spruce roots.



Replication: Lodgepole pine, n=3;
Sitka spruce n=4

3.3.2 Effect of 90 days flooding on Lodgepole pine roots and effect of blockage of air movement through stem lenticels

a) Root Observations

By early March some freely drained seedlings had produced a substantial quantity of new roots. Flooding prevented new growth, roots appearing healthy in colour though some lacked the progressive browning towards the root tip normal in over-wintering roots (see Philipson and Coutts 1979). This response was also seen when Sitka spruce roots were flooded (Chapter 4).

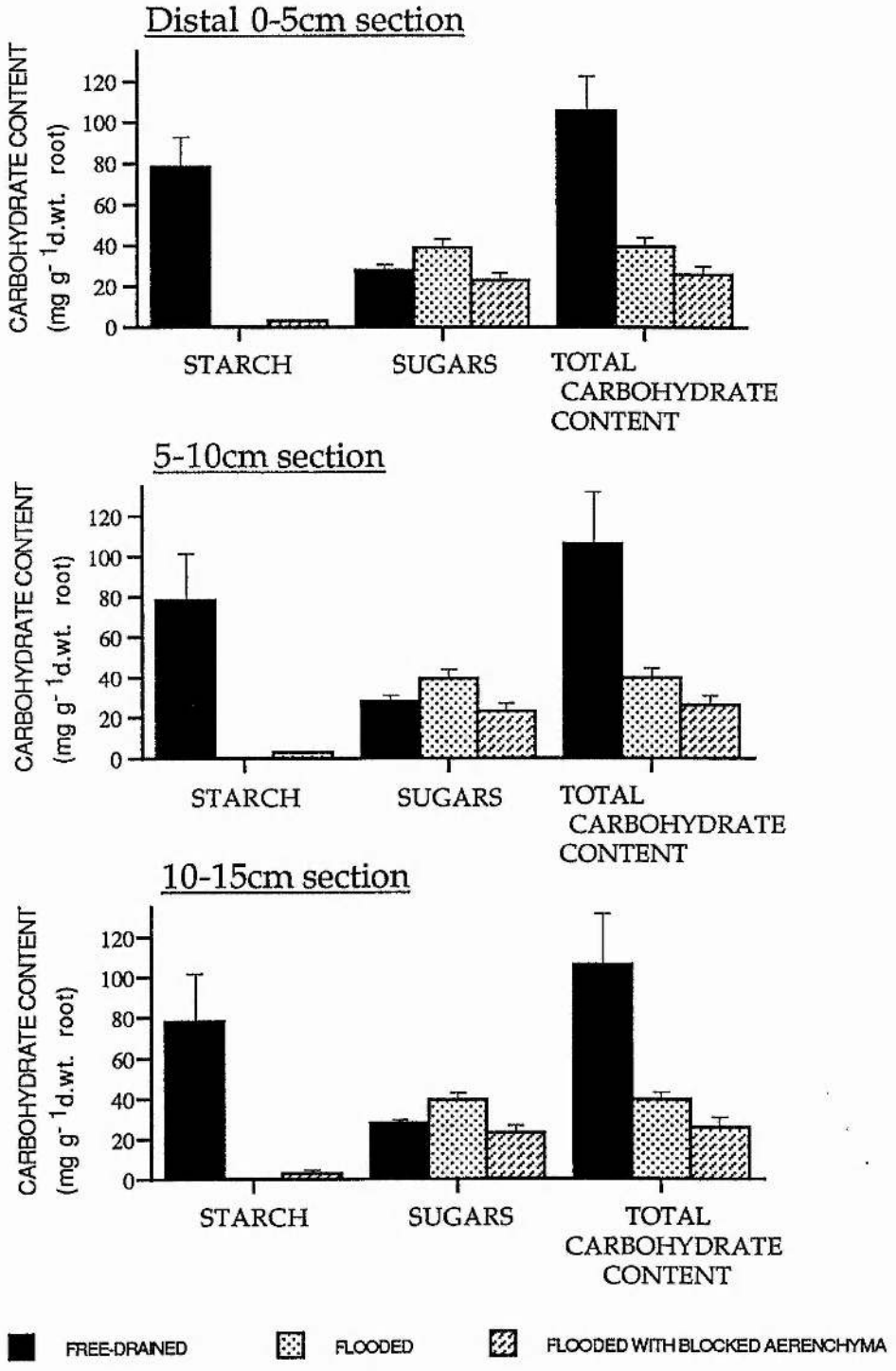
b) Carbohydrate Reserve

Tables A3.3 a-c.

90 days of flooding caused almost total depletion of root starch reserve in each section, whether with blocked or free lenticels, (Figure 3.6). Root sugar content in contrast was similar to that in the free-drained seedlings analysed concurrently. The effect of flooding with or without stem aeration was compared using 2 way ANOVA to determine the effect on sugar and TNC content. Samples showed homogeneity of variance for mean sugar and TNC content, but heterogeneity for starch content due to total depletion in some root samples.

Blockage of stem lenticels during flooding lead to greater reduction in root sugar and TNC content. Comparing the 2 flood regimes, the distal 15cm of root from seedlings with blocked stem lenticels contained significantly less TNC and sugars than those where movement of air into or out of the stem was possible (TNC: $F=12.6$, $df=1$, $p=0.003$; Sugars: $F=19.80$, $df=1$, $p<0.001$). Carbohydrate content of the three root sections did not differ significantly.

Figure 3.6 Carbohydrate content of distal 15cm of root after three months flooding with functional or blocked lenticels



n=4, each treatment and section

When compared to the carbohydrate reserves at the initiation of flooding in December, the degree of depletion was greater in roots from stem-sealed seedlings (Table 3.5):

Table 3.5. Total carbohydrate remaining in root as percentage of initial concentration.

	STARCH	SUGAR	TOTAL
Flooded	0.8%	74.5%	34.3%
Flooded with sealed stems	4.0%	44.2%	22.3%

Values calculated from mean carbohydrate content of each section initially and after flooding, and averaged to give the mean for the 3 sections.

c) Ethanol accumulation in flooded root tissue

Table A3.4.

Ethanol was detected *in vivo* in roots of both flooded and freely drained seedlings (Figure 3.7). Flooding caused approximately 2-3 fold increases in mean ethanol content. Sealing the stems resulted in lower mean ethanol content in each root section when compared to flooding alone, though differences were not statistically significant when compared using Student's t-test.

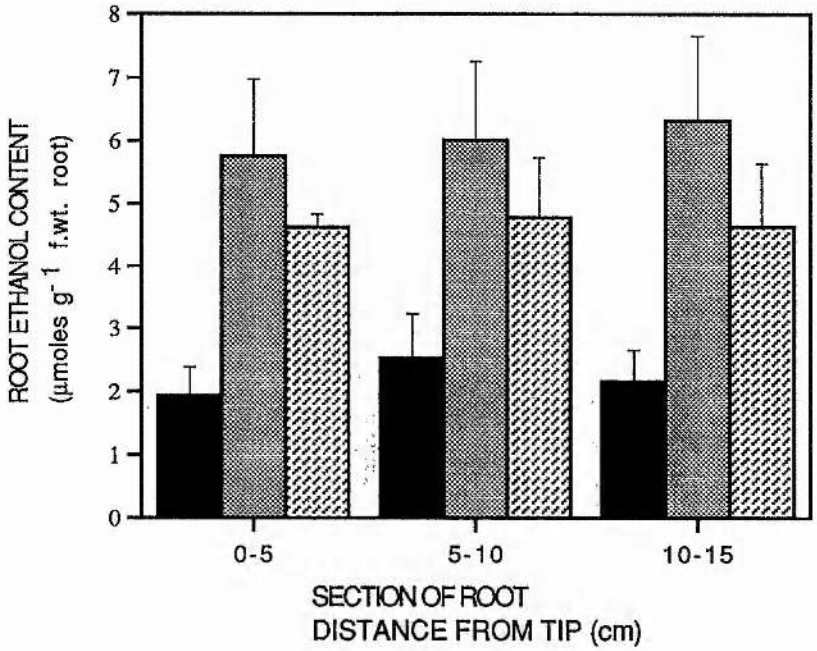
d) Tissue viability

Table A3.5, Figure 3.8.

In general, flooding reduced root viability relative to that of free-drained seedlings. Heterogeneous variance of the arcsin transformed samples prevented analysis of variance. However the means of arcsin transformed data were compared between like sections of root using t-tests, allowing for equal and unequal variance as appropriate[22]. The mean relative viability of each root section of seedlings with blocked

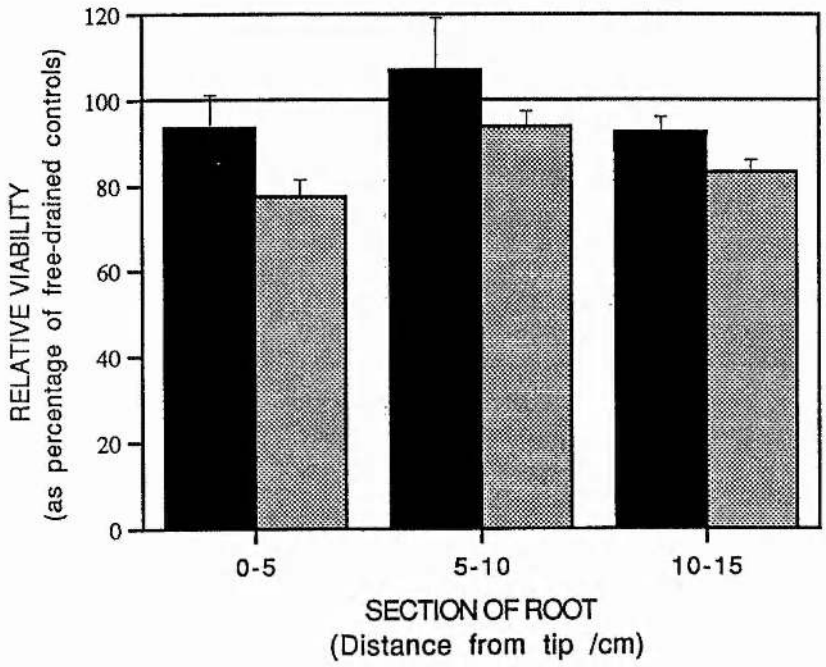
[22] Means with equal sample variances were compared using Student's t-test. When sample variances were unequal a Welsch test was used to compare the sample means. This type of testing is used throughout the thesis when t-tests are specified. The variances of samples were always tested for homogeneity of variance using the Fmax test before comparing means.

Figure 3.7 Ethanol accumulation in the distal 15cm root of Lodgepole pine seedlings after 90 days flooding with or without blockage of stem lenticels. Content is compared to that of free-drained seedlings.



Replicates: each value mean of 4 seedlings, showing standard error bars

Figure 3.8 Root relative viability after winter waterlogging with and without blockage of stem lenticels.



Replicates: 4 seedlings for each sample,
Standard error bars shown

FLOODED

FLOODED + BLOCKED STEM LENTICELS

stem lenticels was lower than that of equivalent roots where aeration from the stem was not impeded, although means were not statistically significant.

3.4 DISCUSSION

The physiological condition of Lodgepole pine and Sitka spruce roots at the time of flooding is known to be critical for survival (Coutts and Philipson 1978a, Coutts and Nicoll 1990b). Oxygen demand has been suggested to be the determining factor as dormancy and the presumed concurrent fall in respiration rate which accompanies it has been shown to be associated with decreased dieback (Coutts and Philipson 1978a). The metabolic rate and carbohydrate content of Sitka spruce and Lodgepole pine were measured to see if their physiological differences at flood initiation may lead to the observed superior flood tolerance of the latter.

Lodgepole pine roots contained higher mean carbohydrate levels in comparison to Sitka spruce. This may be advantageous under anoxia, especially if coupled with low respiration rate. Under waterlogging, phloem transport, an energy requiring process, ceases (see General introduction, Chapter 1). Root reserves at the time of flooding must therefore meet minimum energy requirements for cell survival until oxygen is again available.

As discussed above, anaerobic respiration is costly in terms of carbohydrate depletion and inefficient in terms of energy production. Survival of many wetland species appears to be aided by their large carbohydrate reserves in rhizomatous tissue and their ability to conserve them by reducing glycolysis under anoxia (e.g. Barclay and Crawford 1983).

Root respiratory activity at the initiation of waterlogging gives an indication of the relative oxygen demand. Measurement of respiration rate in excised roots is however not ideal:-

1) handling may cause damage to the root system and increase in glycolysis;

2) flooding of intercellular air spaces can increase the critical oxygen pressure considerably, causing a higher degree of hypoxia than that in intact roots (Armstrong and Gaynard 1976);
3) the root is detached from the rest of the plant, and therefore from the supply of air and respiratory substrate.

Higher carbon dioxide output in the root tip does not necessarily imply higher respiratory rates as they were expressed on a fresh weight basis. Root tip sections may contain many small cells and a higher density of mitochondria in comparison to more proximal sections. The measurements show which part of the root was most oxygen demanding on a gram fresh weight basis.

The results of respiration analysis allowed a comparison of the relative demand for oxygen in different sections and the two species, and their response to anoxia. Use of excised roots was useful in this experiment as it allows purely metabolic response to anoxia to be investigated without the possible supply of oxygen from the shoot. The respiration rate of Lodgepole pine and Sitka spruce were similar and each showed approximately a 2 fold increase in glycolysis on transferring the roots from air to nitrogen. Controversy exists as to merits of this response. Due to the inefficiency of anaerobic respiration, energy charge falls under anoxia (Crawford 1982). e.g. In *Solanum tuberosum* adenylate energy charge falls to only 0.3 after 6 hours anoxia (Sieber and Braendle 1991). Many flood tolerant species partially replace their depleted ATP by acceleration of glycolysis (Crawford 1989) e.g. *Acorus calamus*, the Sweet Flag can survive three months total anoxia and regenerate on return to air. The rhizomes actively ferment and release ethanol which may confer anoxia tolerance by maintaining ATP levels (Sieber and Braendle 1991). Other species seem survive by control of glycolysis, reducing carbohydrate depletion and build up of toxic end products (e.g. Crawford

1976, Crawford and Baines 1977). *Schoenoplectrus lacustris* only depletes its TNC reserve by 2% in experimental anoxia in contrast to in-tolerant *Glyceria maxima* in which 46% are depleted (Barclay and Crawford 1983). The optimum survival strategy depends on the duration of flooding and the tissue type. In short term anoxia acceleration of glycolysis will supply ATP without complete depletion of carbohydrate reserves and accumulation of toxins. However under long term anoxia in thickened roots from which ethanol cannot readily diffuse, reduction of glycolysis and energy expenditure would be advantageous (see Crawford and Baines 1977).

If the observed Pasteur effect was maintained in the intact root under soil waterlogging, the distal 15cm of root would dieback within a number of days (Table 3.4). Carbohydrates would very quickly be depleted and tissue ethanol increase. Respiration rate of intact roots obviously varies over time with fluctuation in soil temperature and energy supply/demand. Assuming that roots are devoid of oxygen, maintenance of high relative viability and glucose reserves in seedlings flooded with sealed stems suggests that the respiration rate of intact roots is much lower under flooding.

The calculated 'supply time' gives a useful but strictly theoretical comparison of the effect of anoxia on root survival, being dependant on both respiration rate and carbohydrate reserve. Differences were not statistically significant, but a pattern can be observed. The supply time of Lodgepole pine roots was greater than that of Sitka spruce roots under both air and nitrogen. Supply time in each species was lowest in the distal section due to a combination of higher mean respiration rate and lower carbohydrate content. Although Lodgepole pine has a larger carbohydrate reserve at flood initiation, if the measured anaerobic respiration rates reflect those in the field, the distal root did not appear to

have a metabolic adaptation to anoxia in terms of carbohydrate respiration.

Effect of flooding and blockage of stem lenticels.

Starch content of each root section decreased to almost zero over 90 days flooding with or without the blockage of stem lenticels, while root sugar levels generally remained similar to those of free-drained seedlings, (Table A3.3, Figure 3.6).

Prevention of root aeration from lenticels on the stem resulted in greater depletion of carbohydrate reserves. Significantly more total carbohydrate remained in roots of seedlings where stem lenticels were in contact with air, suggesting that aeration from this region was effective in supplying oxygen to the roots and ameliorating anaerobiosis. Supply of oxygen may reduce the magnitude of the Pasteur effect and appears to reduce the depletion of carbohydrate reserves.

All roots contained ethanol, indicating a degree of hypoxia even in freely drained soil. Flooding increased accumulation to a degree in agreement with Crawford and Baines (1977) who measured a 3 fold increase in the same species under 24hrs of flooding at 20°C. This suggests that any gas movement from the shoot, either from the leaves or stem lenticels in non-sealed seedlings, cannot fully meet the respiratory demands of the distal 15cm of root.

Sealing the stems of flooded seedlings is associated with slightly lower (not statistically significant) mean ethanol accumulation. This could be explained by a number of factors:-

-reduced glycolysis in the slightly damaged cells, reflecting either damage *per se* or the lower remaining carbohydrate content.

-increased leakage of ethanol through injured membrane resulting from fall in energy charge or damage by ethanol itself (Crawford 1978).

Although lenticels may have a function in ethanol removal from roots, the amount removed through Lodgepole pine roots has been shown to be insignificant. The transpiration stream removes most of the ethanol (Crawford and Finegan 1989). Thus blocking the stem lenticels would not be expected to lead to greater accumulation of ethanol in the roots.

The results suggest that Lodgepole pine roots are tolerant to long term inundation initiated in Winter when roots are dormant. Root viability was not reduced significantly and carbohydrate reserves were not fully depleted. Root viability estimates need to be supported by root growth observations. New growth may continue at the old root tips or lateral roots may appear more proximally depending on the viability of the apical meristem. Such observations would be a very useful addition to this data.

In assessing the importance of lenticels in the stem, most physiological comparisons between those with functional and blocked aerenchyma were not significantly different. The low number of replicates available creates problems in proving statistical differences. However in each section, blocking the lenticels with Vaseline was associated with a pattern of reduced viability, increased carbohydrate depletion and reduced ethanol accumulation. Most importantly the roots of seedlings flooded with blocked stem lenticels had *significantly* less glucose and total carbohydrate than those flooded with free stem aeration. This applies to roots approximately 20-60cm below the water table. Results therefore imply that gas entering through stem lenticels and passing down the whole root system can slightly ameliorate the

effects of anoxia. However, it cannot supply enough oxygen for aerobic respiration even though the root is dormant and in mid-Winter.

3.5 APPENDIX

Table A3.1. Activity of free-drained Lodgepole pine roots at initiation of flooding.

	SECTION OF ROOT		
	0-5cm	5-10cm	10-15cm
AEROBIC RESPIRATION RATE (μmoles CO_2 g^{-1} f.wt. h^{-1})	3.283 ±0.357	2.794 ±0.821	2.621 ±0.235
ANAEROBIC RESPIRATION RATE (μmoles CO_2 g^{-1} f.wt. h^{-1})	2.5656 ±0.205	1.9374 ±0.222	1.8658 ±0.218
TOTAL ETHANOL PRODUCTION (μmoles g^{-1} f.wt. in 24 hr)	31.345 ±1.893	23.865 ±0.264	15.548 ±5.449
TISSUE ETHANOL ACCUMULATION (μmoles g^{-1} f.wt. in 24 hr)	10.510 ±2.312	10.464 ±1.704	5.623 ±1.337
ETHANOL IN WATER AROUND ROOT (μmoles g^{-1} f.wt. in 24 hr)	20.835 ±4.128	13.401 ±1.750	9.925 ±4.243
% TISSUE ETHANOL*	34.635 ±9.726	43.872 ±7.270	41.365 ±7.459
% WATER ETHANOL*	65.366 ±9.726	56.128 ±7.270	58.636 ±7.459

Each value represents a **mean** and the standard error of the mean ($SD/\sqrt{n}$) of three replicate root samples, each from a different seedling.
*Amount of ethanol remaining in tissue or water around root as a percentage of total produced.

Table A3.2. Activity of free-drained Sitka spruce roots at initiation of flooding

(WASHINGTON PROVENANCE)

	SECTION OF ROOT		
	0-5cm	5-10cm	10-15cm
AEROBIC RESPIRATION RATE (μmoles CO_2 g^{-1} f.wt. h^{-1})	4.886 ±0.639	2.774 ±0.329	3.169 ±0.560
ANAEROBIC RESPIRATION RATE (μmoles CO_2 g^{-1} f.wt. h^{-1})	3.456 ±0.508	1.866 ±0.149	1.840 ±0.282
TOTAL ETHANOL PRODUCTION (μmoles g^{-1} f.wt. in 24 hr)	15.898 ±4.507	15.252 ±2.645	10.304 ±4.104
TISSUE ETHANOL ACCUMULATION (μmoles g^{-1} f.wt. in 24 hr)	3.342 ±0.553	4.347 ±0.504	3.378 ±0.835
ETHANOL IN WATER AROUND ROOT (μmoles g^{-1} f.wt. in 24 hr)	12.556 ±3.981	10.905 ±2.233	6.925 ±3.353
% TISSUE ETHANOL*	31.623 ±12.863	29.525 ±2.861	43.704 ±11.162
% WATER ETHANOL*	68.377 ±12.863	70.475 ±2.861	56.296 ±11.162

Each value represents a mean and standard error of four replicate root samples, each from a different seedling.

Tables A3.3. a-c. Carbohydrate remaining in root after 90 days waterlogging in comparison to pre-flood content and that of control. (Mean and standard error of n replicates shown)

a)Root starch content (mg g^{-1} d. wt. root)

	SECTION OF ROOT		
	0-5cm	5-10cm	10-15cm
Free draining, November n=3	54.13 ±5.92	60.17 ±9.93	61.5 ±15.1
Free draining, March n=4	41.7 ±14.9	69.3 ±23.4	78.1 ±23.7
Flooded 90 days n=4	0.578 ±0.578	0.470 ±0.376	0.361 ±0.208
Flooded 90 days + blocked aerenchyma (n=4)	1.921 ±0.748	2.094 ±0.978	3.04 ±1.77

b)Root sugar content (mg g^{-1} d. wt. root)

	SECTION OF ROOT		
	0-5cm	5-10cm	10-15cm
Free draining, November n=3	44.74 ±2.04	51.14 ±2.58	50.36 ±4.76
Free draining, March n=4	34.32 ±3.02	29.69 ±2.86	27.86 ±1.73
Flooded 90 days n=4	32.62 ±4.31	37.07 ±4.78	39.31 ±3.43
Flooded 90 days + blocked aerenchyma (n=4)	18.88 ±3.73	22.90 ±4.19	22.93 ±3.79

c)Total carbohydrate content (mg g^{-1} d. wt. root)

	SECTION OF ROOT		
	0-5cm	5-10cm	10-15cm
Free draining, November n=3	98.87 ±7.93	111.3 ±12.1	111.8 ±19.1
Free draining, March n=4	76.2 ±16.9	99.0 ±26.2	106.0 ±25.3
Flooded 90 days n=4	33.20 ±4.69	37.55 ±4.75	39.67 ±3.56
Flooded 90 days + blocked aerenchyma (n=4)	20.80 ±4.17	25.00 ±4.78	25.97 ±5.03

Table A3.4.Mean ethanol accumulation in root tissue (μmoles g^{-1} f.wt.) harvested after 90 days under free-drainage or flooding with and without stem seal

	SECTION OF ROOT (cm)		
TREATMENT	0-5cm	5-10cm	10-15cm
Free drained	1.93 ±0.46	2.53 ±0.71	2.16 ±0.50
flooded	5.75 ±1.23	6.01 ±1.25	6.32 ±1.34
flooded and blocked lenticels	4.62 ±0.21	4.77 ±0.97	4.63 ±1.01

Mean values shown ± standard error
n=4, each treatment, each section.

Table A3.5. Relative viability of roots after 90 days flooding with blocked or free lenticels.

	SECTION OF ROOT (cm)		
TREATMENT	0-5cm	5-10cm	10-15cm
flooded	93.7 ±7.7	107.1 ±12.2	92.6 ±3.7
flooded and blocked lenticels	77.4 ±4.2	93.9 ±3.7	83.2 ±2.8

Mean values shown ± standard error
n=4, each treatment, each section.

CHAPTER 4

Early and Late Winter Flooding of Sitka Spruce - Changes in Respiration Rate, Ethanol Production and Carbohydrate Depletion and Consequent Survival of the Root

4.1 INTRODUCTION

Coutts and Nicoll (1990b) observed reduced die-back and greater depth of survival when Sitka spruce roots were flooded from November as compared to October, and greater susceptibility of the root tip to flooding. This response was suggested to reflect the state of activity of the roots at the time of flooding. In October roots were slowly extending whereas in November most extension had ceased and some roots were dormant. The greater tolerance of dormant roots to waterlogging may also be associated with the formation of the protective metacutization layer (Coutts and Philipson 1987), and/or the reduced transpiration consequently limiting the uptake of soil toxins (Coutts 1981). This was discussed in more detail in Chapter 1.

An experiment was designed to investigate the differences in survival observed by Coutts and Nicoll in terms of the metabolic activity of roots in October and November. Sitka spruce seedlings were flooded for three months beginning in mid to late October or one month later in November. Such long term flooding is a common Winter stress in Scottish forestry plantations. Two provenances, Alaskan and Washington, were compared, in order to examine any variation in physiology and possible differences in survival. Although experiments on this scale cannot conclude with any statistical certainty that seedlings from one origin may perform better on wet sites, they may show variation in some metabolic aspects correlating with improved flood-tolerance. If such metabolic traits did correlate well with performance, this type of screening may be useful in identifying provenances where the more time consuming process of large scale determination of flood survival may prove fruitful. Coutts and Nicoll (1990b) have shown that flood tolerance does vary significantly between clones of Sitka spruce, and although these differences were suggested not to be of practical

importance, further screening may be very useful for future forestry. Screening for metabolic traits has also been suggested by Hook and Denslow (1987) as a means of selecting flood-tolerant genotypes of Loblolly pine. Their research showed that physiological responses could be detected before growth responses and therefore speeds up the screening process, although more research is needed to confirm the relationship between metabolism and growth responses and a number of physiological responses must be screened.

Any oxygen transport from shoot to root is unlikely to reach the root tip. Unlike Lodgepole pine, Sitka spruce has no aerenchyma in the primary root and no oxygen diffusion through the xylem of the woody root. However, proliferation of the outer bark aids oxygen diffusion and secondary roots can transport oxygen in their outer tissues (Philipson and Coutts 1980). It is suggested that this may be associated with the depth of survival of Sitka spruce roots flooded in the Winter, laterals regenerating from roots which may have had some secondary growth at the time of flooding (Coutts and Nicoll 1990b).

Consequently, when the soil is waterlogged, the metabolic response of the root to the subsequent depletion of oxygen may be a very important factor in its survival. Carbohydrate content and respiratory activity of the root were measured under standard conditions in the laboratory both at the initiation of flooding treatments and after 3 months. Long roots 1-2mm in diameter were assessed. Coutts and Nicoll (1990b) consider these roots to be important in anchorage as they undergo secondary thickening and form the woody root system. Their survival in Winter flooding is therefore necessary for tree stability.

Under anoxia, phloem transport is slowed down or ceases, (as detailed in Chapter 1), cutting the root off from sugar supply from the shoots. Carbohydrate reserve in the root at flood initiation must

therefore meet demand until the soil is re-oxygenated and phloem transport resumes. Root respiration must ideally proceed at a rate to maintain the minimum energy charge for survival without leading to the accumulation of toxic levels of respiratory end-product and depletion of carbohydrate reserve. While acceleration of glycolysis may be a good adaptive mechanism to maintain energy levels during short term anoxia, such a response may be dangerous under long term flooding as is the case here. Storage of large carbohydrate reserves and an ability to conserve them under flooding is a feature of anoxia tolerant rhizomes.

Root carbohydrate content, respiration rate (under both air and anoxia) and ethanol production were determined at the date of flooding to test Coutts and Nicoll's observation that root activity at the time of flooding is an important determinant of root survival. After 3 months, the glycolytic activity and quantity of remaining carbohydrate were assessed and related to the root viability.

The experiment aimed to test the following hypothesis:
Carbohydrate reserve at flood initiation and the metabolic activity of the root are important factors in determining survival of Winter waterlogging in Sitka spruce roots.

4.2 METHODS

4.2.1 Experimental design

Seedlings of 2 provenances, Alaska and Washington[23], were planted in 2m perpsex tubes in peat in April 1991 at the Forestry Commission Northern Research Station, Roslin, Midlothian. These seedlings had been grown from seed, maintained for one season in a seedbed and a further season as transplants. Each tube was top dressed with 2.0g Osmocote plus™ slow release fertiliser[24], and maintained in insulated root boxes at this location. In late September after one season of growth, the perpsex tubes were transferred to St. Andrews, wrapped in black polythene tubing and placed in polystyrene-insulated root boxes, (see experimental techniques). The plastic wrap ensured the roots were maintained in darkness and acted as an extra layer of insulation. The seedlings were then maintained for 4 weeks before initiating the treatments.

Within each provenance seedlings were randomly selected into six groups, each harvested at different intervals through the Winter for physiological measurements. For each provenance, one group of seedlings was harvested between mid-October and 25th October, and physiological measurements recorded (see below). Concurrently a second group of seedlings was waterlogged to just above the soil surface and maintained under flooded conditions for 94 days, after which the seedlings were harvested and physiological measurements repeated. Flooding was initiated over a two week period (Table 4.1) to allow harvesting at intervals for physiological measurements without varying the number of days of waterlogging. (The duration of waterlogging is a

---

[23] JUNEAU, ALASKA PROVENANCE, No. 81(7987)1
HOQUIAM, WASHINGTON PROVENANCE, No. 71(7972)5
[24] 15%N, 11%P_2O_5, 13%K_2O, 2%MGO + Trace elements

major factor determining the survival of roots). A third group of seedlings was analysed in late November, approximately one month later, concurrently flooding a fourth group as before. The latter were also maintained under flooding for 94 days and analysed during late February and early March. A further 2 groups of seedlings were maintained under free drained conditions during the Winter and analysed alongside the flooded seedlings as controls. Seedlings from Alaska and Washington were analysed and flooded together such that the results are comparable. The experimental details are summarised in Fig 4.1.

Table 4.1. Exact dates of measurement of root metabolic activity and initiation of flooding treatments

DATES OF ANALYSIS OF FREE-DRAINED ROOTS AND FLOOD INITIATION	ANALYSIS OF FLOODED SEEDLINGS AND *FREE-DRAINED CONTROLS*
Oct. 14-25 Free drained seedlings analysed	
Oct. 16-31 Flood initiation * →	Harvested 16 Jan. -5 Feb. for physiological analysis *29 Jan. -6 Feb., free drained seedlings analysed*
Nov. 20-30 free drained seedlings analysed	
Nov. 25-Dec. 2, Flood initiation** →	Harvested 24 Feb.-10 Mar. for physiological analysis *27 Feb.-3 Mar., free drained seedlings analysed*

* Referred to as 'flooded October to January'
**Referred to as flooded 'November to February'.

4.2.2 Measurements of root carbohydrate metabolism and viability

Physiological measurements were made on seedlings both free-drained at the period of flood initiation and after 94 days waterlogging at which time the free drained controls were also assessed. Seedlings cannot easily be replanted in the tubes and attempting to excise a sample of roots and replanting the seedling would have caused damage. Different seedlings were therefore analysed at each time, analysis being destructive, as many

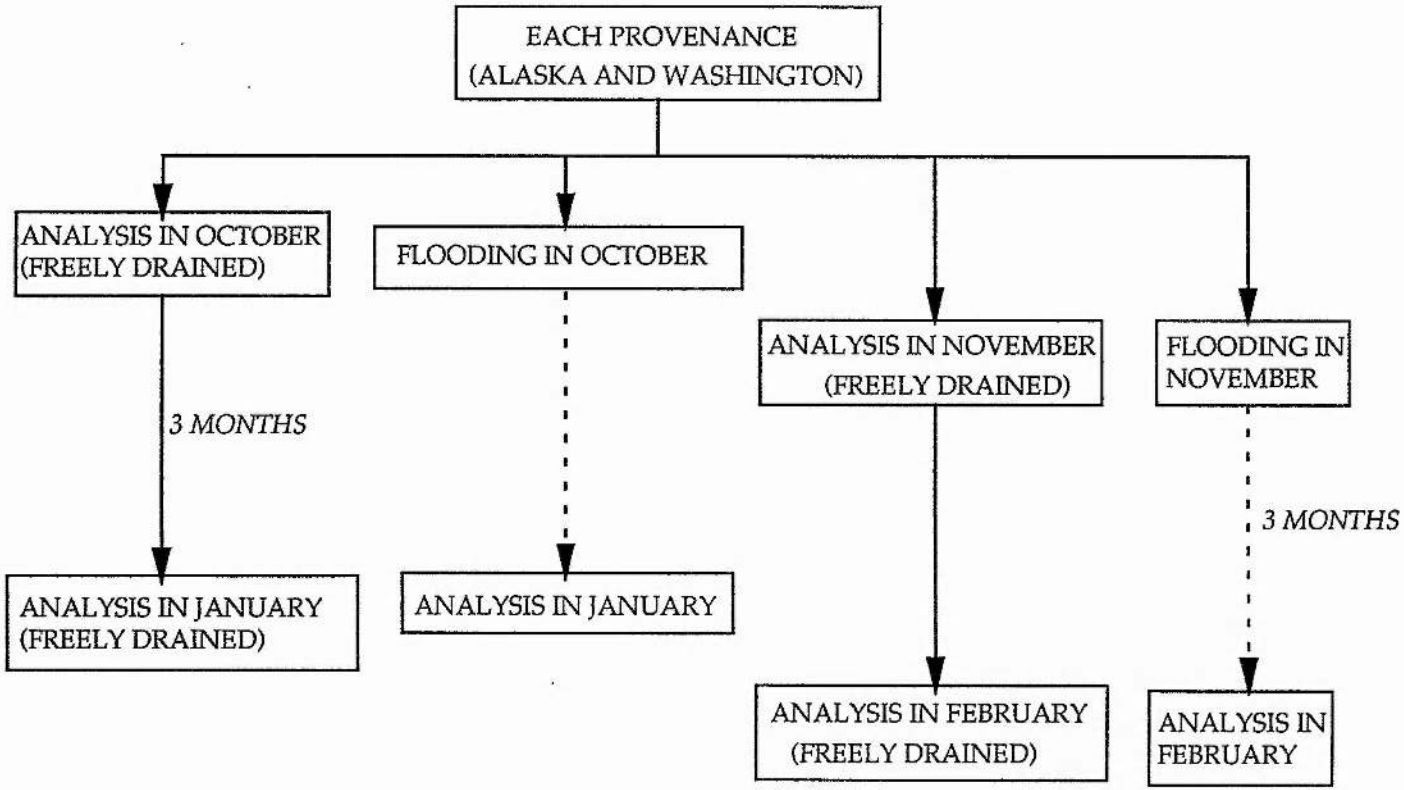


Figure 4.1. Experimental design.
Each provenance is analysed before and after three months flooding initiated either in October or November. Root metabolism is examined at each time

roots as possible were removed and measured to give a good indication of root physiology and the seedling was then discarded.

Seedlings were carefully harvested from the tubes by rinsing with tap water and 15cm distal lengths of root excised. Roots were approximately 1-2mm diameter and were carefully washed and separated into 3 or 4 groups (3 in October and November, 4 in January and February). Each group was used for a different physiological measurement, (Fig 4.2). Each root was then sectioned into 3x5cm lengths, 0-5, 5-10 and 10-15cm from the root tip. The root sections were kept on moist tissue paper during the experiment until analysis.

a) Respiration rate

One group of roots was analysed to determine the aerobic and anaerobic respiration rate at 20°C. A minimum of 3x5cm root sections were placed in a closed loop in an infra-red gas analyser and the rate of CO_2 production over a 20-30 minute period determined. For replicate seedlings, the different root sections, (0-5, 5-10 and 10-15cm from the root tip) were analysed in varying order to cancel possible effects of excision or the time delay between excision and measurement on respiration rate. After measurement in air, roots were incubated at 20°C in an anaerobic chamber under 90%N_2 and 10%H_2. Roots were kept in darkness and wrapped in moist tissue paper to prevent drying. After one hour, roots were transferred to a round bottomed flask inside the anaerobic environment, sealed with a serum stopper and reconnected to the IRGA through which oxygen-free nitrogen was flowing. Thus the roots were not re-exposed to oxygen during the transfer. After 2-3 minutes of nitrogen flow-through, the closed loop was quickly re-connected and the respiration rate under nitrogen recorded. Respiration rate was calculated in terms of CO_2 production (μmoles) per g fresh weight

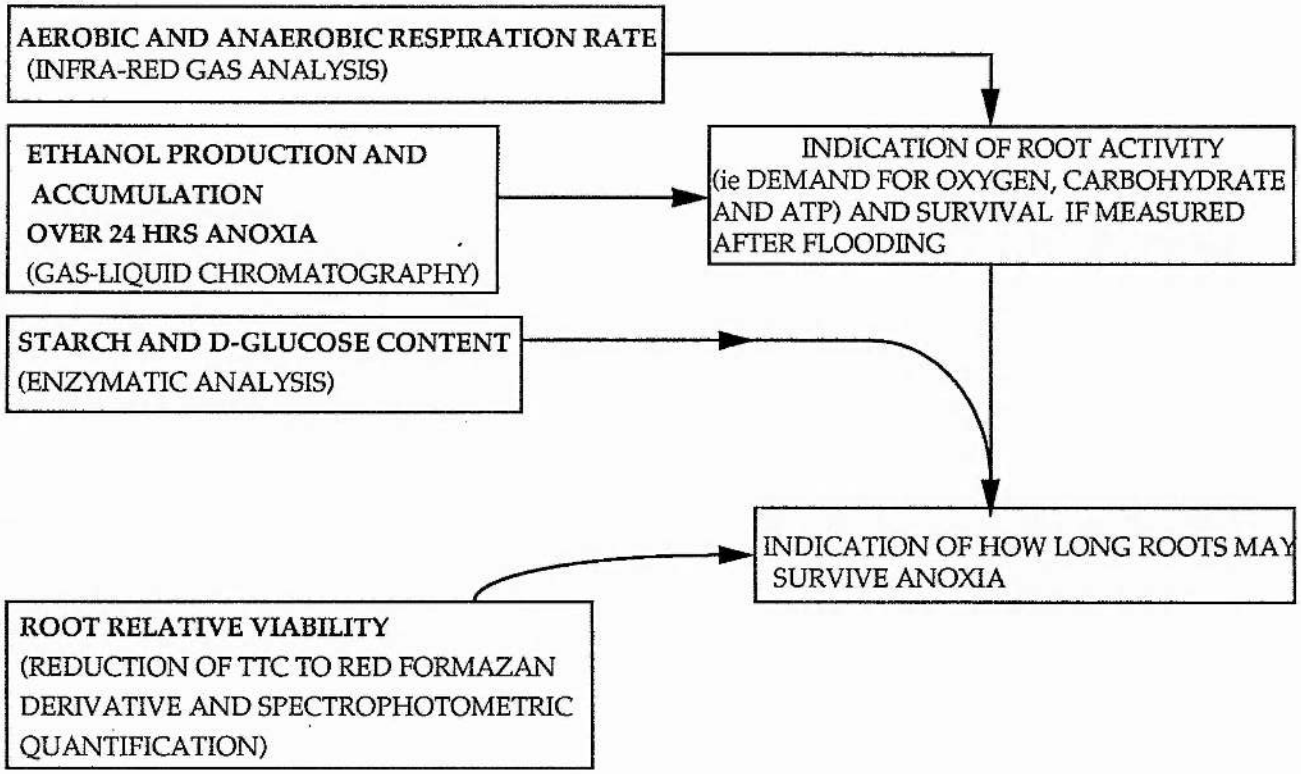


Figure 4.2. Analysis of root metabolic activity and use of results in determining their survival.

root tissue per hour. (For details, see Experimental techniques chapter.)

b) Ethanol production

A second sample of roots was used to determine ethanol production and accumulation in the root tissue under 24hr anoxia at 20°C. Again a minimum of 3 root sections were analysed, each weighed sample placed in moist tissue paper in a glass specimen tube and sealed with a serum stopper in the anaerobic incubator. The three root sections were then placed inside an anaerobe jar containing oxygen-removing catalyst pellets and a methylene blue oxygen-indicator strip. This jar was sealed inside the anaerobic system, then removed and incubated at 20°C for 24 hours. Maintaining the glass tubes inside the anaerobe jar removes the large diffusion gradient present if they were kept in air. After 24 hours, the roots were quickly removed and ethanol extracted in 3 ml ice-cold perchloric acid. The tissue extract and a sample of water from around the roots[25] were then injected into the GLC to determine ethanol concentration. (For details, see Experimental techniques chapter.) Total ethanol production is the sum of the molar concentration in the tissues and that in the water, head space ethanol being negligible at this temperature, (only 0.129% of total ethanol produced).

c) Carbohydrate content

The third root sample (as many root sections remaining after other physiological measurements) was quickly frozen in liquid nitrogen and stored at -20°C until analysis. Root starch and sugar (in terms of glycosyl content) were determined enzymatically (see Experimental techniques chapter).

---

[25] each root sample was kept moist with an equal volume of distilled water

d) Tissue viability

January and February controls and post - waterlogging treatments only.

Root tissue viability was determined using the Tri-phenyl tetrazolium chloride test (see Experimental techniques chapter).

4.3 RESULTS

4.3.1 Analysis of metabolic activity of free-drained roots at initiation of flooding in October and November

a) Respiration rate

CO_2 production of each type of root sample (0-5, 5-10, and 10-15cm) was recorded under air and subsequently nitrogen after one hour's incubation in the anaerobic incubator. Aerobic and anaerobic respiration rates of free drained roots at the time of flood initiation are displayed in Tables A 4.1 a and A 4.1b.

The respiration rate of each provenance was compared under both air and nitrogen, and between October and November (Figure 4.3 a-d). The data allowed comparison of root activity in the two consecutive months as well as possible variation between provenances and root sections. The samples showed heterogeneity of variance for both aerobic and anaerobic respiration rate. Log transformations failed to stabilise the variance, therefore comparisons were made between the means using t-tests with equal/unequal variances as appropriate.

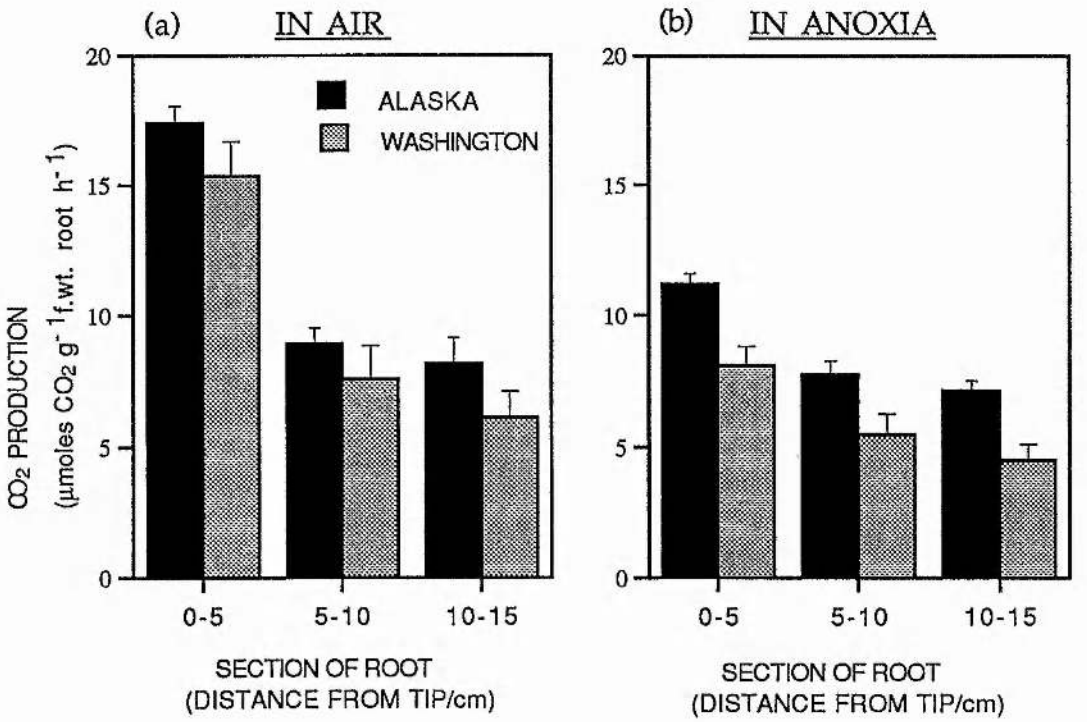
i) Respiration rate in different root sections

Root sections generally showed a pattern of highest respiration rate in the tip decreasing up the root whether measured under air or nitrogen. The tip was therefore the most oxygen demanding part of the root. In October and November, in each provenance, the aerobic respiration rate was significantly higher in the distal 5cm of root than that 5-10cm from the tip, (except the Washington provenance when measured in November, the decrease moving up the root was not statistically significant at $p \leq 0.05$)[26]. The respiration rate of the 5-10cm root

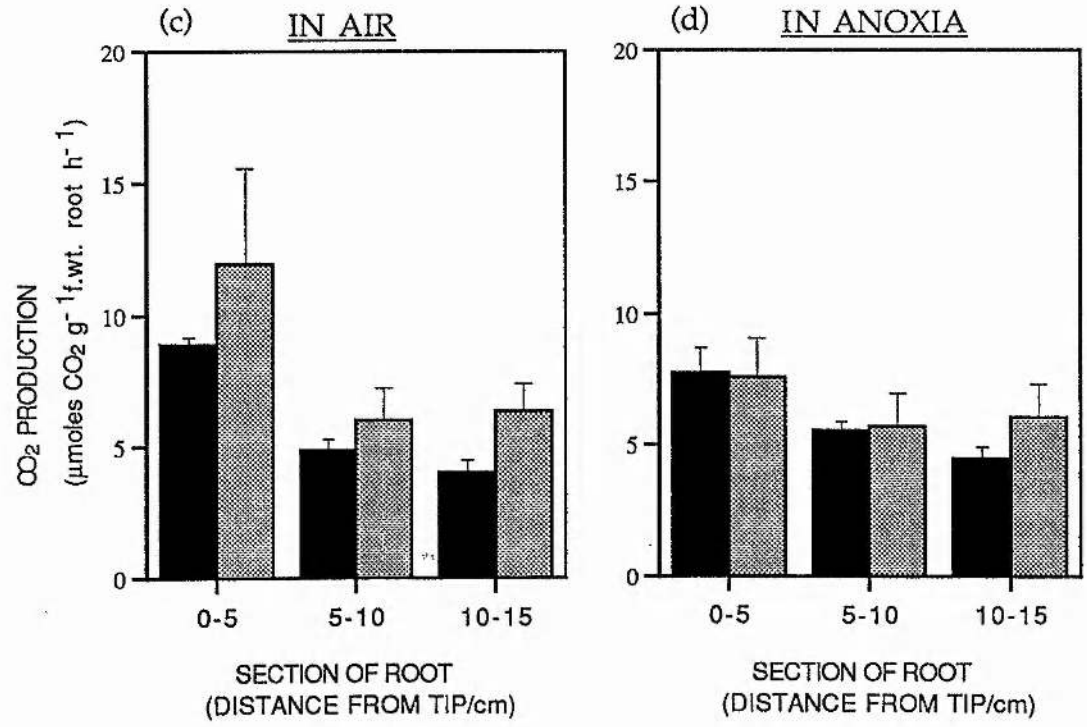
[26] Alaska: October p=0.0001; November p=0.0002.

Figure 4.3. Respiration rate of roots of Alaskan and Washington provenances of Sitka spruce under air or nitrogen.

Measurement in October



Measurement in November



Replicate seedlings, n=4 except Washington provenance in October, n=3 .

was higher than the more proximal section although means were not statistically significant, (again the Washington provenance measured in November is the exception, the proximal section having a slightly *though not significantly* higher respiration rate).

Anaerobic respiration rate decreased from the tip up the root[27], the only statistically significant decrease in respiration rate being that between the tip and middle section of the Alaskan provenance in October ($p=0.0019$).

ii) Effect of anoxia on respiration rate

The effect of anoxia on the glycolytic rate can be compared by calculating the anaerobic respiration rate as a percentage of that in air. This percentage was calculated for each root sample and the means and standard errors displayed in table 4.2.

Table 4.2. Anaerobic respiration rate of three sections of root as percentage of that in air.

	ALASKA		WASHINGTON	
	OCTOBER	NOVEMBER	OCTOBER	NOVEMBER
0-5cm	64.4 ±2.7	86.8 ±9.6	52.4 ±0.5	78.5 ±20.4
5-10cm	86.9 ±3.2	115.4 ±5.0	73.4 ±7.0	93.7±10.0
10-15cm	89.7 ±6.7	121.0 ±28.8	74.6 ±3.0	99.6 ±22.6

Figures show mean ±se of mean.

n=4 for all means, except Washington provenance in October, n=3.

If the glycolytic rate remains constant when tissues are transferred from air to anoxia, the CO_2 output falls to one third its aerobic value due to the incomplete breakdown of glucose, as explained in Chapter 3.

Washington: October $p=0.014$, November, NS.

[27] except the Washington provenance in November where the respiration rate of the 10-15cm dection is slightly >5-10cm section.

During the period of measurement, a maximum of two to three hours after excision, the root tissues respond to anoxia by increasing their rate of glycolysis (in terms of CO_2 output) approximately 1.6 to 3.6 fold. This response is not necessarily indicative of the long term response to anoxia under field waterlogging, as the root's metabolism may adjust to the lack of oxygen over a longer time period and may be affected as carbohydrate levels fall. It does however give an indication of the immediate response in the field, which may be when the root is very susceptible to injury. Such a response will rapidly deplete carbohydrate reserves and may lead to significant accumulation of anaerobic end products within the cell.

The degree of acceleration of glycolysis on transfer from air to anoxia (Pasteur effect), appeared to differ depending on measurement date, section of root and provenance. At each time and in each provenance, acceleration of glycolysis increased moving up the root, away from the tip. The increase in glycolysis on subjection to anoxia was greater in the Alaskan than the Washington provenance, and increased in each provenance in November as the roots become dormant.

iii) Respiratory activity in October and November

Aerobic:

In October, the aerobic respiration rate was higher in Alaskan Sitka spruce than those of Washington origin, though the means did not differ significantly at the 5% level. In general, aerobic respiration rate decreased between October and November in each provenance[28], the reduction most marked in the Alaskan provenance. In seedlings from this origin, November CO_2 production was approximately half that in October in

[28] Except the 10-15cm root section in the Washington provenance which shows a slight, but not statistically significant increase.

each root section[29]. The Washington provenance also showed a reduction in respiration rate in the distal 10cm of root, though the decrease in mean respiration rate in October and November was smaller and not statistically significant.

The greater decrease in activity in the Alaskan provenance between the two successive months resulted in it having higher aerobic respiration rate in October, but lower in November when compared to the Washington provenance (pattern repeated in each root section, but not statistically significant).

Anaerobic:

Anaerobic respiration rate showed a similar pattern, roots of the Alaskan provenance had a higher rate in October than those from Washington[30], although when measured in November the respiration rates did not differ significantly. Between October and November the rate of CO_2 evolution under N_2 decreased significantly in the Alaskan provenance[31], although not to the same extent as the aerobic values at this time. In contrast, respiratory activity of roots of Washington origin did not change significantly between October and November.

Although respiration was measured at 20°C, a significantly higher temperature than that outdoors, comparison with respiration measurements made for the same Provenance at the same time of the year show that doubling the measurement temperature leads to an approximate doubling of respiration rate (Table 4.3).

---

[29] Mean aerobic respiration rate is significantly higher in October than November in the Alaskan provenance.
(0-5cm $p<0.0001$; 5-10cm $p=0.0015$;10-15cm $p=0.0092$)
[30] (0-5cm $p=0.010$; 5-10cm NS;10-15cm $p=0.014$)
[31] (0-5cm $p=0.017$; 5-10cm 0.012;10-15cm $p=0.0043$)

Table 4.3. Comparison of root aerobic respiration rate for seedlings of Washington origin at 10 and 20°C, (μmoles CO_2 g^{-1} f.wt. root h^{-1}).

ROOT SECTION (cm from tip)	10°C	20°C
0-5	4.9	11.9
5-10	2.8	6.0
10-15	3.2	6.3

Replicates: 4 seedlings at each temperature.

b) Ethanol production under experimental anoxia

Changes in ethanol production under anoxia mirror those of anaerobic respiration rate (Tables A4.2+A4.3; Figure 4.4)

In each provenance, the ethanol production in October and November was highest in the tip section. In general, ethanol production decreased between October and November in the Alaskan provenance whereas it increased slightly in Washington seedlings (Table 4.4). Ethanol production was similar in the two provenances in November, but higher in the Alaskan provenance in October.

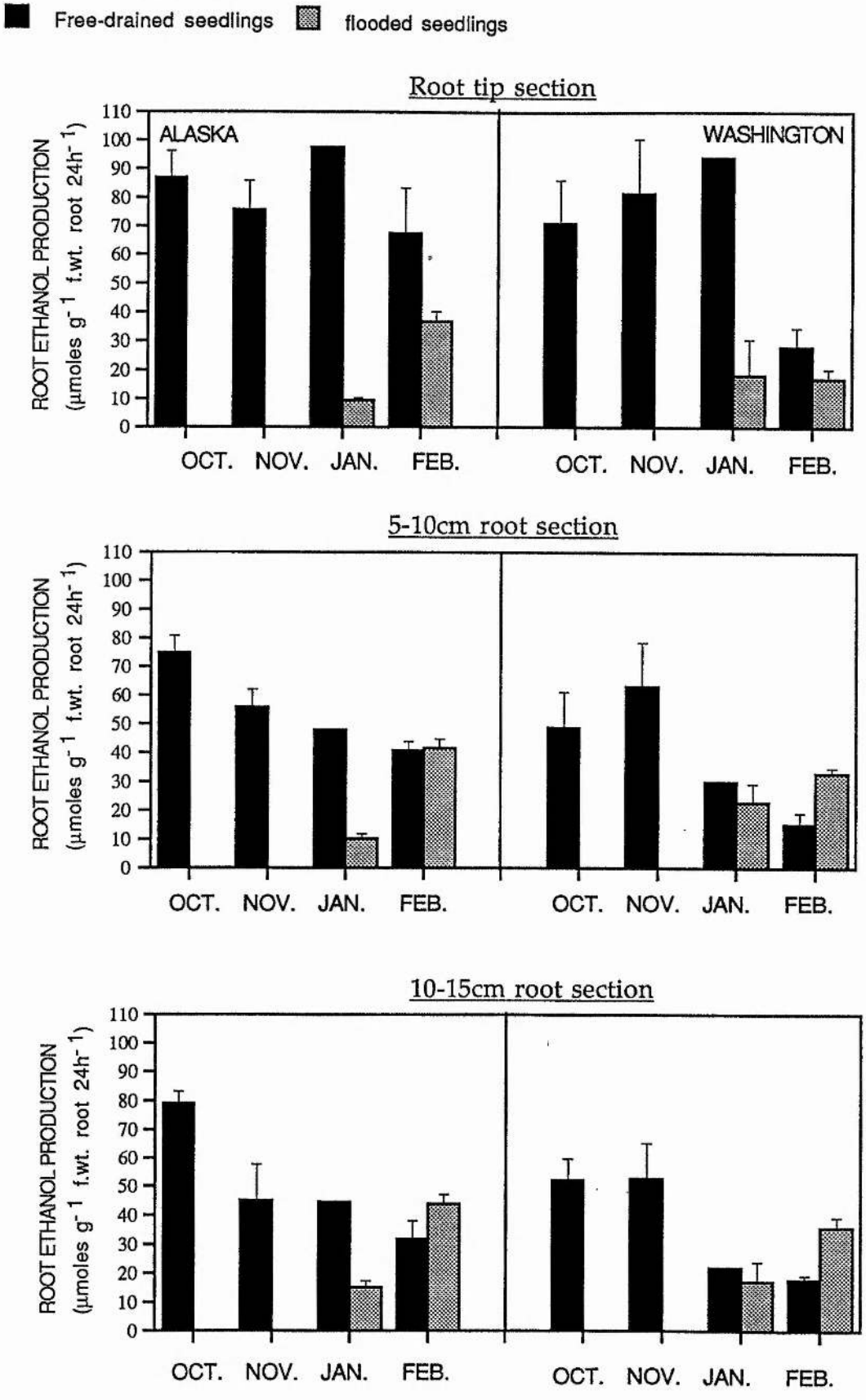
Table 4.4. Mean total ethanol production of each root section in November as a percentage of that in October

SECTION OF ROOT	ALASKA	WASHINGTON
0-5 cm	87.1	114.8
5-10 cm	74.5	129.6
10-15 cm	56.8	101.2

Replicates: 4 seedlings of each provenance in October and November.

In experimental anoxia, when the root sections were sealed in a glass tube containing moistened filter paper, most (between 66 and 88%) of the ethanol produced diffused out of the root tissue into the surrounding water. In October, slightly more ethanol diffused from the more active Alaskan roots, such that accumulation in the tissues of the two

Figure 4.4. Total ethanol production under 24 hours anoxia in excised root sections from seedlings following either free-drainage or flooding for three months.



Number of replicates, see tables A4.2-A4.5

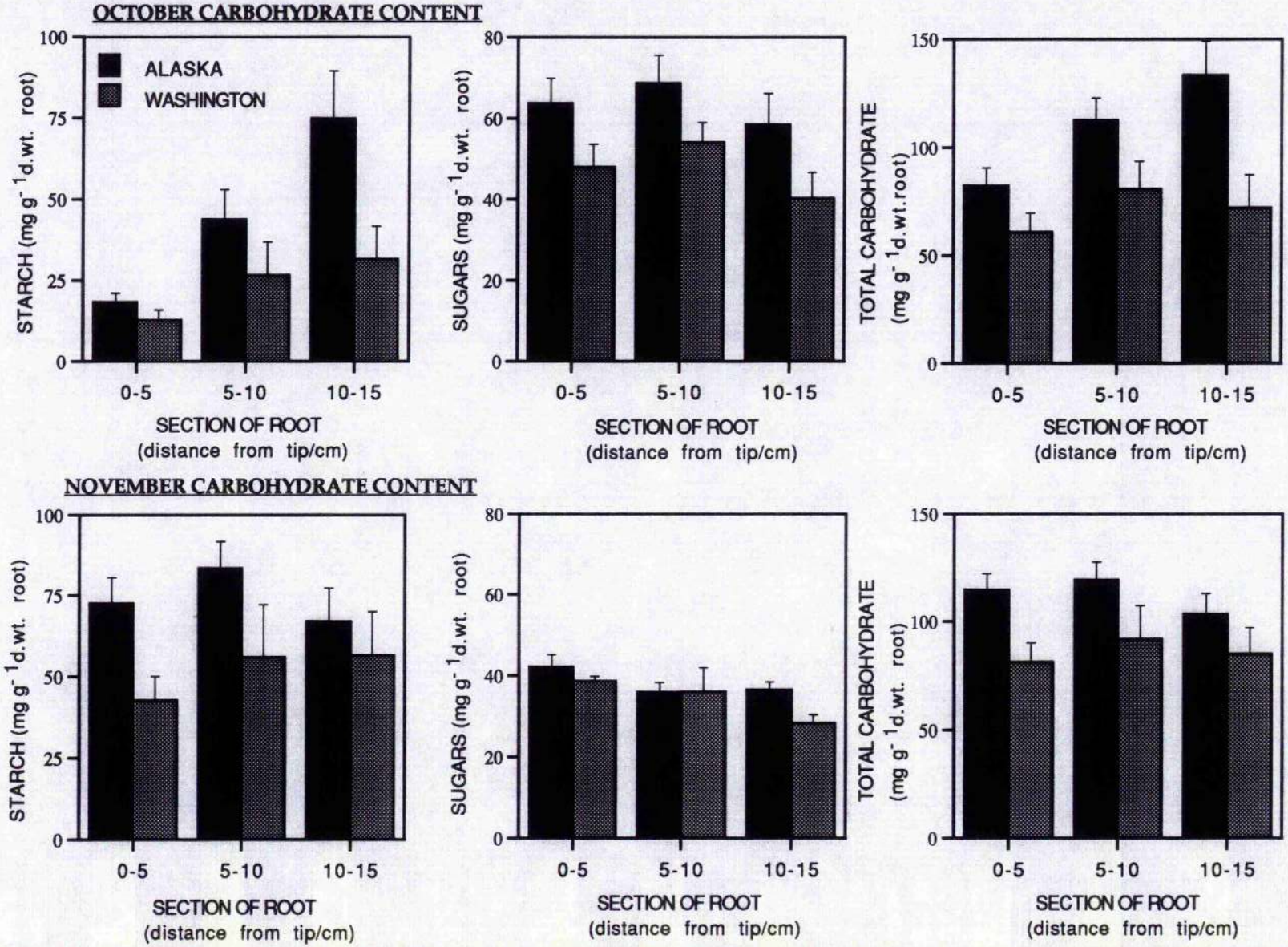
provenances was similar. In November, ethanol leakage and accumulation in the tissues were similar. Ethanol production under 24 hours anoxia may be estimated from the anaerobic respiration rate measured immediately after excision, assuming the rate of glycolysis remains constant over this time (see Chapter 3). Estimated values are much higher than the actual levels of ethanol production measured. This is most likely due to a decrease in respiration rate over time, as explained in Chapter 3 when a similar pattern was observed in Lodgepole pine and Washington Sitka spruce roots.

c) Root Carbohydrate Content

Carbohydrate content of each 5cm root section was determined in units of mg g^{-1} d.wt. root tissue. In October and November each root section of the Alaskan provenance (0-5, 5-10 and 10-15cm from the tip), contained more carbohydrate in terms of both starch and sugars than comparable root sections of the Washington provenance[32] (see Figure 4.5). Mean carbohydrate content and probability values are shown in Table 4.5. Not all means were statistically different at the 5% level, however the pattern was repeated in almost every section and the low probability values are likely to stem from the lack of replicates. In each provenance, starch content of the distal 15cm of root approximately doubled between October and November (Table 4.9), while the sugar content fell considerably. The mean total non-structural carbohydrate content of the distal 15cm of root was compared both between provenances and in October and November using two-way analysis of variance. The increase in reserve noted in each Provenance between October and November was non-significant, however the TNC content

[32] One excpetion, the 5-10cm section of the Washington provenance contains slightly, but not significantly higher glucose at $p \leq 0.05$.

Figure 4.5 Comparison of root carbohydrate content in the two provenances at the initiation of flooding in October and November



of the Alaskan provenance was significantly higher than that of Washington origin (F=8.54, df=1, p=0.015).

Table 4.5. Comparison of root carbohydrate content in the 2 provenances in October and November.(mg g^{-1} d.wt. root)

OCTOBER		ALASKA	WASHINGTON	Probability value/ significance at 5%level
STARCH	0-5cm	18.43 ±2.57	12.84 ±3.12	NS
	5-10cm	43.81 ±9.24	26.7 ±10.2	NS
	10-15cm	75.1 ±14.6	31.6 ±10.1	0.05
SUGARS	0-5cm	63.70 ±6.22	47.88 ±5.78	NS
	5-10cm	68.64 ±6.95	54.03 ±4.89	NS
	10-15cm	58.33 ±7.82	40.26 ±6.40	NS
TOTAL CARBOHYDRATE	0-5cm	82.13 ±8.42	60.72 ±8.84	NS
	5-10cm	112.4 ±10.5	80.7 ±12.9	NS
	10-15cm	133.5 ±15.5	71.9 ±15.5	0.031

n=4 each provenance except Alaskan provenance 5-10cm when n=3

NOVEMBER		ALASKA	WASHINGTON	Probability value/ significance at 5%level
STARCH	0-5cm	72.56 ±8.08	42.77 ±7.48	0.035
	5-10cm	83.38 ±8.34	55.9 ±16.4	NS
	10-15cm	66.9 ±10.5	56.6 ±13.5	NS
SUGARS	0-5cm	42.16 ±3.03	38.67 ±1.25	NS
	5-10cm	35.93 ±2.48	36.00 ±6.02	NS
	10-15cm	36.55 ±2.02	28.40 ±2.01	0.029
TOTAL CARBOHYDRATE	0-5cm	114.73 ±7.65	81.43 ±8.71	0.028
	5-10cm	119.31 ±8.25	91.9 ±15.5	NS
	10-15cm	103.44 ±9.45	85.0 ±12.2	NS

n=4, except Washington provenance 5-10cm when n=3. Table shows mean ±se.
The probability value in column 4 is generated using MINITAB™ to compare the mean carbohydrate content between like sections of root of the two Provenances using a two sample t-test, allowing for equal or unequal variance as appropriate.

4.3.2 Effect of 3 months Winter flooding on root survival and metabolism

a) Respiration rate

When roots were excised from free-drained seedlings and those flooded for three months, the respiration rate was measured under fixed laboratory conditions in either air or nitrogen (Table A 4.1c and d). The

rate of CO_2 production under these conditions may indicate the degree of cellular dysfunction due to flooding (see discussion). Aerobic and anaerobic respiration rate of each sample of previously flooded roots was expressed as a percentage of the mean respiration rate of free-drained roots (Table 4.6).

Table 4.6. Effect of flooding on aerobic and anaerobic respiration capacity

(Measured in terms of CO_2 output relative to that of free drained seedlings.)

Aerobic

	FLOODING FROM OCTOBER		FLOODING FROM NOVEMBER	
SECTION OF ROOT	ALASKA	WASHINGTON	ALASKA	WASHINGTON
0-5 cm	18.9 ±4.7	18.3 ±10.8	30.4 ±4.0	56.9 ±8.5
5-10 cm	21.7 ±3.9	33.3 ±19.3	54.0 ±3.6	76.9 ±8.3
10-15 cm	36.2 ±4.1	28.0 ±12.0	45.1 ±3.6	69.8 ±5.4

Anaerobic

	FLOODING FROM OCTOBER		FLOODING FROM NOVEMBER	
SECTION OF ROOT	ALASKA	WASHINGTON	ALASKA	WASHINGTON
0-5 cm	53.6 ±20.0	45.8 ±27.5	49.9 ±2.8	85.4 ±14.0
5-10 cm	69.7 ±31.9	61.7 ±46.3	82.4 ±4.0	87.9 ±3.9
10-15 cm	56.2 ±27.2	82.5 ±50.7	66.6 ±4.6	106.2 ±6.6

Flooded seedlings n=4 , control in January n=1 , control in February n=2.
Table shows mean ±se.

In almost all cases, three months of flooding caused reduction in post-treatment respiration rate both in air and nitrogen relative to free-drained controls and irrespective of date of flood initiation, origin or root section. Comparing the data, a number of patterns appear:-

i) mean aerobic and anaerobic respiration rates were most reduced relative to the controls in the most distal section. This pattern was seen in each provenance whether flooded from October or November.

ii) the deficit in aerobic respiration rate was always greater than that measured in anoxia, independent of flood date, provenance and root section.

iii) the deficit in aerobic respiration rate was similar in the Alaskan and Washington Provenances when flooded from October, but appeared to be greater in the Alaskan provenance following flooding from November.

iv) in each provenance the reduction in relative aerobic respiration rate was greater when flooded from October than when flooded from November.

In freely drained seedlings measured through the Winter, aerobic respiration rate was always highest in the distal section, reflecting a higher demand for oxygen even when growth had ceased and roots were dormant.

b) Ethanol production

Ethanol production under anoxia was compared in excised roots of seedlings from flooded and non-flooded soil conditions (Figure 4.4). This is considered useful as it also shows the effect of flooding on glycolysis and may indicate cell integrity. The rate of ethanol production in each root section was expressed as a percentage of the mean rate of ethanol production in non-flooded seedlings and the mean 'percentage ethanol production' calculated (table 4.7).

Table 4.7. Total ethanol production after three months flooding from October or November as a percentage of that of free-drained controls

		SECTION OF ROOT/cm		
PROVENANCE	DATE OF FLOOD INITIATION	0-5	5-10	10-15
ALASKA	OCTOBER	9.7 ±0.8 (n=3)	21.5 ±3.6 (n=4)	34.1 ±4.9 (n=4)
	NOVEMBER	54.1 ±5.2 (n=4)	102.1 ±8.0 (n=4)	138.0 ±11.0 (n=4)
WASHINGTON	OCTOBER	19.6 ±13.2 (n=2)	76.0 ±22.5 (n=2)	78.4 ±31.6 (n=3)
	NOVEMBER	61.1 ±11.7 (n=4)	217.2 ±12.4 (n=4)	201.7 ±19.8 (n=4)

REPLICATES:
Number of flooded replicate seedlings shown in brackets, with mean and standard error of mean.
Controls; 2 seedlings per provenance in February, 1 in January.

The greatest deficit in ethanol production in flooded as compared to non-flooded was in the tip, production being reduced to <10% in the Alaskan provenance flooded in October.

In each provenance flooded from October to January, ethanol production capacity of each root section was greatly reduced. In contrast, following flooding from November, ethanol production of the root tip sections decreased to approximately half that of the controls whereas in the more proximal sections 5-15cm from the tip, ethanol production was greater than that in the freely drained seedlings.

Measuring ethanol production and accumulation in roots sealed under anoxia at 20°C only allows comparison of metabolic activity between different treatments and root sections. It does not indicate the activity of roots in the soil or the degree of ethanol accumulation which may occur. Root ethanol accumulation in flooded and drained Sitka spruce seedlings *in vivo* was analysed between late January and early February (Chapter 5). Ethanol accumulation at 20°C in experimental

anoxia was of similar concentration to that in waterlogged soil outside (Table 4.8).

Table 4.8. Comparison of accumulation of ethanol in roots in flooded soil compared to that in sealed roots under experimental anoxia, (μmoles ethanol g^{-1} f. wt. root)

TREATMENT	DATE	MAX. AND MIN. MEAN ETHANOL CONTENT
Washington provenance. *in vivo* analysis	Jan./Feb.	5.2-6.8
Alaskan and Washington provenances, *in vitro* analysis	January	5.0-9.0
Alaskan and Washington provenances, *in vitro* analysis	February	3.9-8.9

* Range of ethanol content in the sections and provenances. In each case ethanol accumulation was determined in the distal 15cm of root.
Table shows mean ±se, where 4 replicate seedlings for each mean.

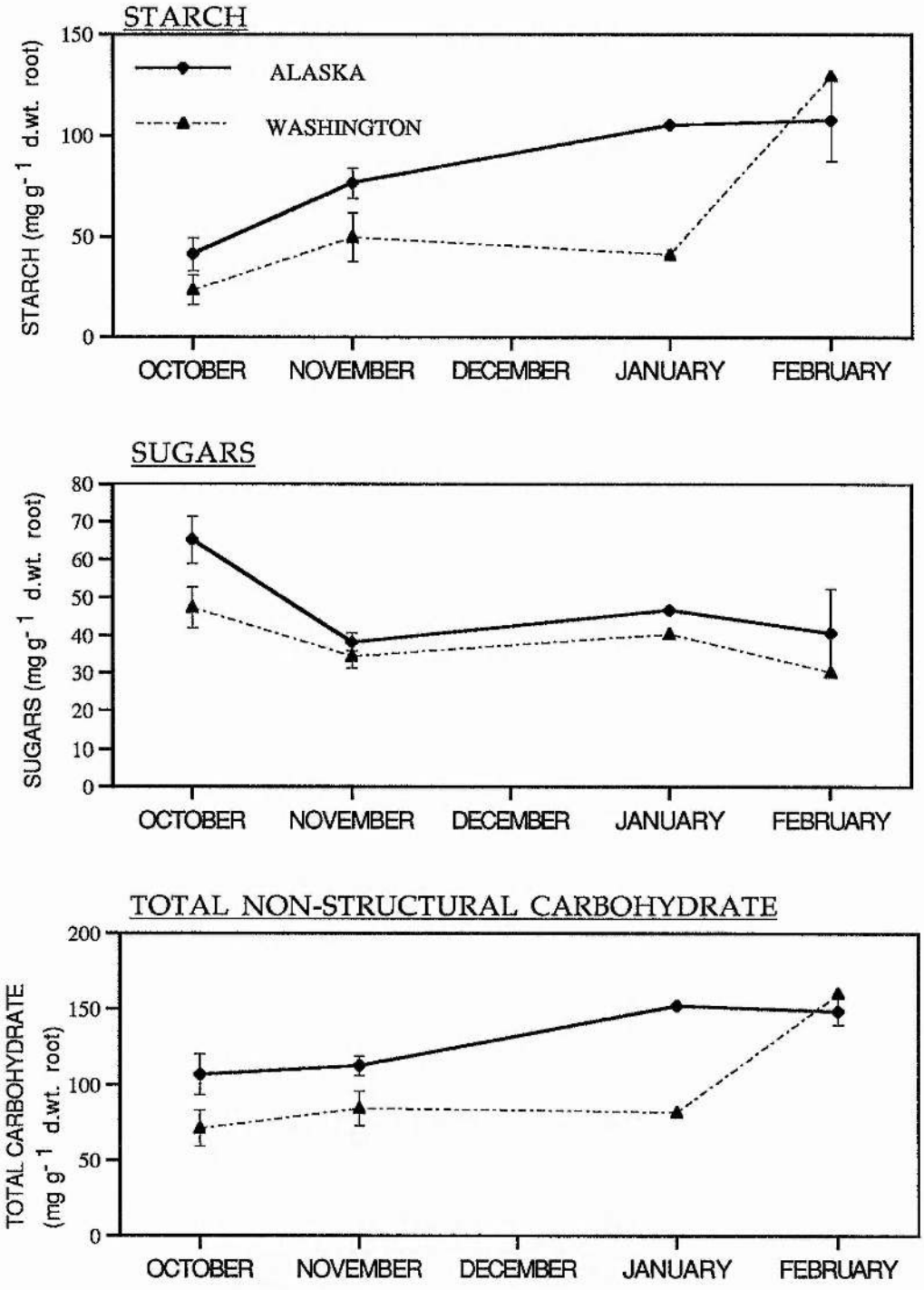
c) Root carbohydrate content

i) Root carbohydrate content of free-drained seedlings during Winter

To compare the changes in root carbohydrate during Winter, the mean carbohydrate content of the three sections of each seedling was calculated. These means were then cumulated for the replicate seedlings and the average calculated. This figure may be different to that obtained if the distal 15cm of root had been analysed as one sample, due to the tapering of the root towards the tip. The proximal 5cm of root generally has the greatest mass. Bearing this in mind however, and assuming that the relative weights of each section do not change significantly through the Winter and do not differ between provenances, the figure calculated provides a useful indication of relative changes in carbohydrate during Winter and any provenance variation (Table 4.9, figure 4.6).

Total carbohydrate content of Alaskan roots was generally higher than that of the Washington provenance through the Winter, reflecting

Figure 4.6 Mean carbohydrate content of three root sections during winter in free-drained Alaskan and Washington Provenances.



REPLICATES: October: 4 seedlings Alaskan prov.; 3 seedlings Washington.
November: 3 seedlings Alaskan prov.; 4 seedlings Washington.
January: 1 seedling each provenance.
February: 2 seedlings Alaskan prov.; 1 seedling Washington

both higher starch and sugar levels, although carbohydrate content of each was similar in February. Starch content increased steadily from mid-October to late February in each provenance, apart from a slight decrease between November and February in the Washington provenance. Sugar levels fell between October and November, rose slightly between November and January, then continued to decrease slightly from January to late February. This pattern is shown by both provenances. Total carbohydrate content showed a general pattern of gradual increase through the Winter.

Table 4.9. Mean over-Winter carbohydrate content of three distal sections of root from free-drained seedlings and those flooded for 94 days from October or November. Drained seedlings shown in plain text, flooded seedlings in *italics*

STARCH (mg g^{-1} d.wt. root)

ANALYSIS TIME	ALASKA	WASHINGTON
OCTOBER	41.4 ±8.17	23.73 ±7.43
NOVEMBER	76.27 ±7.37	49.83 ±12.0
JANUARY	105.24 *0.55 ±0.21*	41.38 *0.42 ±0.14*
FEBRUARY	107.6 ±20.27 *5.46 ±2.32*	129.82 *4.89 ±1.67*

SUGARS (mg g^{-1} d.wt. root)

ANALYSIS TIME	ALASKA	WASHINGTON
OCTOBER	65.3 ±6.3	47.4 ±5.37
NOVEMBER	38.21 ±2.41	34.50 ±3.19
JANUARY	46.78 *8.37 ±1.85*	40.43 *25.6 12.27*
FEBRUARY	40.7 ±11.73 *39.2 ±4.73*	30.27 *33.4 ±3.8*

TOTAL NON-STRUCTURAL CARBOHYDRATE (mg g^{-1} d.wt. root)

ANALYSIS TIME	ALASKA	WASHINGTON
OCTOBER	106.7±13.47	71.1 ±11.97
NOVEMBER	112.5 ±6.3	84.33 ±11.5
JANUARY	152.01 *8.91±1.76*	81.81 *26.03 112.2*
FEBRUARY	148.3 ±8.57 *44.67±4.33*	160.09 *38.3 ±5.3*

Means ±se of mean shown.
Replicates: 4 seedlings except Alaska free-drained October and flooded until January, n=3: Washington free-drained in November, and flooded until February, n=3. Free-drained controls in January and February, n=1 except Alaskan control in February, n=2.

ii) Effect of flooding on root carbohydrate content

94 days flooding, whether initiated in October or November reduced the carbohydrate content of each root section, probability values shown in Table 4.9. In all but one section, the carbohydrate content after flooding was significantly lower than the initial pre-flood levels (when tested at the 5% level).

Table 4.10. Mean total non-structural carbohydrate content after 94 days waterlogging compared to that at flood initiation - probability values

		SECTION OF ROOT/cm		
		0-5	5-10	10-15
ALASKA	*OCTOBER*	0.0027	0.0001	0.005
	NOVEMBER	0.0001	0.0004	0.0034
WASHINGTON	*OCTOBER*	0.024	0.014	NS
	NOVEMBER	0.0009	0.047	0.027

Probability values generated using MINITAB^{TM} to compare the means by a two sample t-test, with equal and unequal sample variances as appropriate.
Means are considered significantly different if $p \leq 0.05$. Number of replicates in table 4.9.

In order to compare the degree of carbohydrate depletion, the carbohydrate contents of the 0-5, 5-10 and 10-15cm root sections were totalled for each seedling, and the mean calculated for each treatment. This value is simply used to compare changes in carbohydrate content in the distal 15cm. The mean carbohydrate contents (starch and sugar) of seedlings were compared before and after flooding. i.e. seedlings flooded from October to January were compared to free-drained seedlings in October, and those flooded from November to February were compared to free-drained seedlings in November (Table A4.6i). The carbohydrate remaining in the root after flooding was also compared to that in control seedlings harvested at the same time (Table A4.6ii).

Carbohydrate remaining in the root, both as a percentage of initial pre-flood levels and those of free-drained controls in January and February, are shown in figure 4.7.

The starch content of seedlings flooded in October was less than 2% of the initial levels, whereas between 7 and 10% remained when flooding was initiated in November. Root sugar depletion showed a similar pattern, when seedlings were flooded from October, 13 and 54% remained in the Alaskan and Washington provenances respectively, compared to 103 and 97% if flooding was initiated in November. Thus if flooded slightly later in the season, although most of the starch reserve was depleted, sugar content was maintained in concentrations similar to those in non-flooded seedlings.

The carbohydrate remaining in each seedling after flooding (either as a percentage of the initial content in October or November or of that of the free-drained controls in January or February), was transformed into the arcsin$\sqrt{}$percentage and the means compared using a 2-sample t-test. This was considered the most appropriate test because arcsin transformation had failed to stabilise the unequal sample variances for both sugar and TNC measurement. The t-test allows for equal or unequal variance as appropriate.

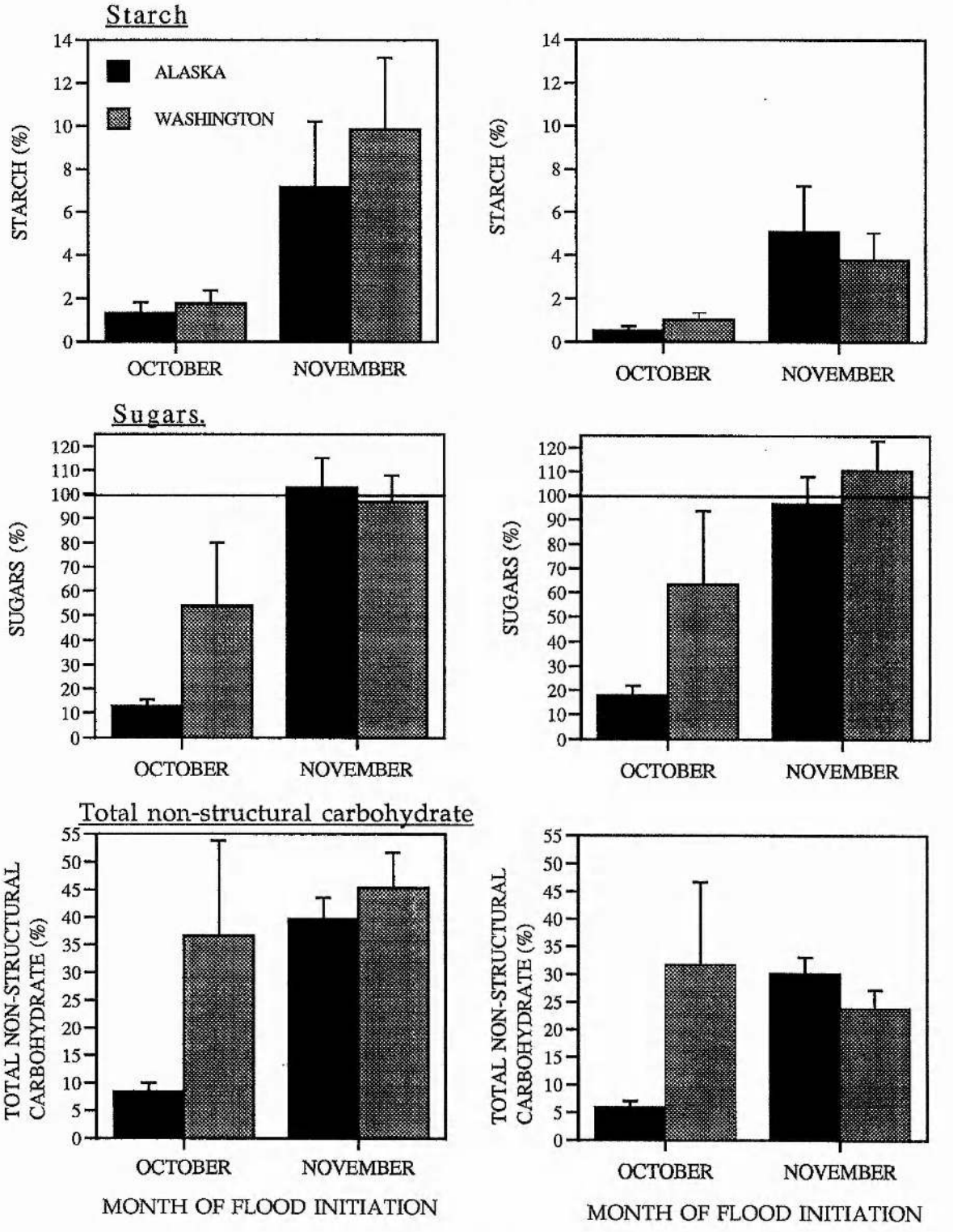
Thus the severity of carbohydrate depletion could be compared both between provenances and dates of flooding. Carbohydrate depletion was significantly higher in the Alaskan provenance when flooding was initiated in October rather than one month later in November. In contrast the depletion of carbohydrates in the Washington provenance did not differ significantly with flood date (probability values shown in Table A4.6i and ii).

Starch was depleted to a greater degree from October in both provenances, but minimal levels were retained and the degree of

Figure 4.7. Root carbohydrate remaining after flooding as a percentage of the initial levels at flood initiation or those of free-drained seedlings in January or February.

a) As percentage of *initial* carbohydrate content

b) As percentage of *control* carbohydrate content



Replicates: Alaskan provenance flooded until January or February,4 seedlings:Washington provenance flooded until January or February, 3 seedlings. Means expressed as a percentage of mean content of free-drained seedlings. (See table 4.9 for replicates of control seedlings).

depletion was not significantly different comparing the two provenances. The depletion of root sugar was significantly greater in the Alaskan provenance when flooded in October rather than November, whereas that in the Washington provenance was not statistically significant at $p \leq 0.05$.

Following October flooding, the starch and sugar depletion was greater in the roots of the Alaskan provenance than those of Washington origin (Figure 4.7). However the degree of starch and sugar depletion did not differ significantly between the two provenances when measured in October or November.

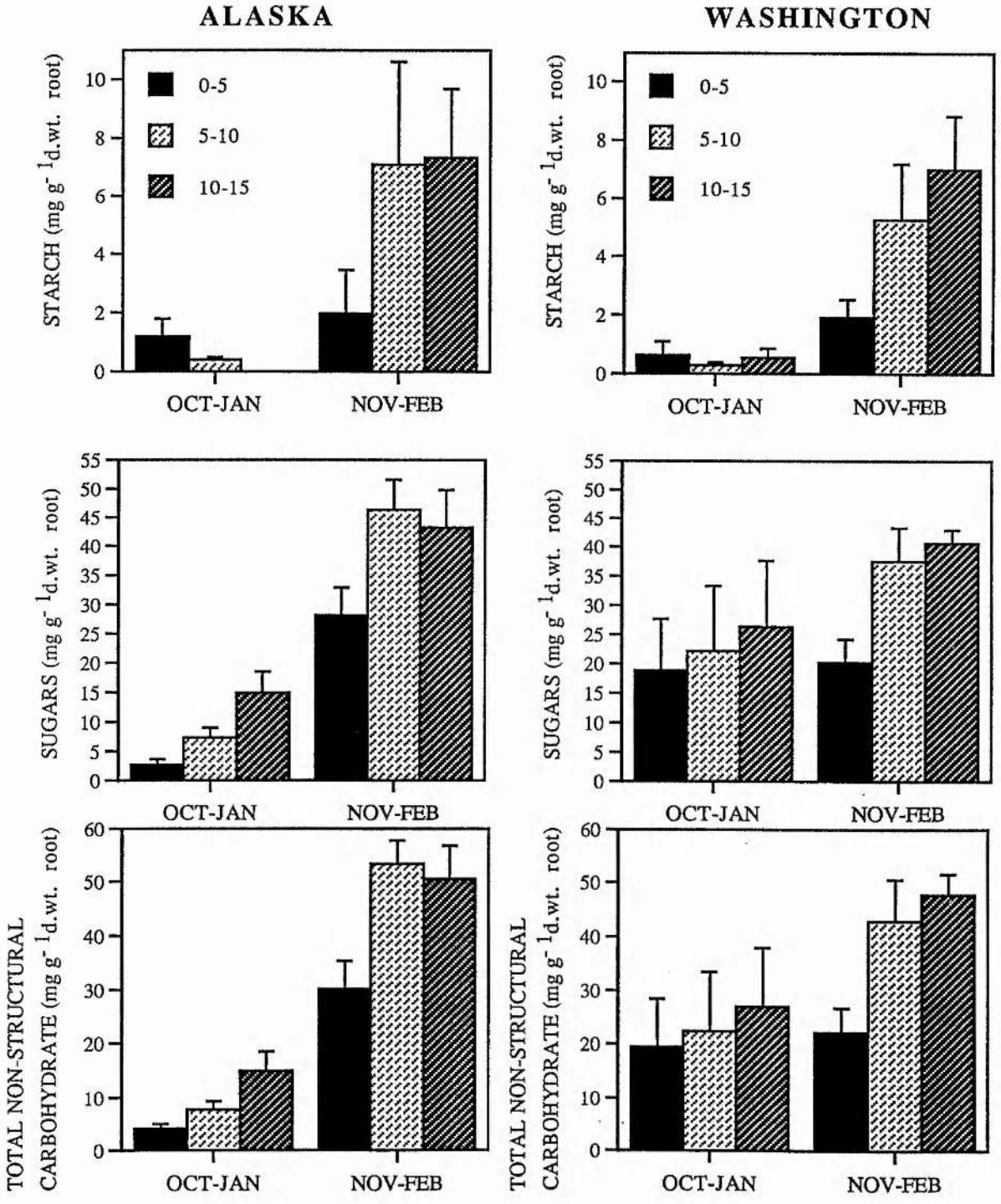
iii) Carbohydrates remaining in the root after flooding

The actual levels of starch and sugars remaining in the roots after flooding are shown in figure 4.8. The graphs compare the level of each carbohydrate component in the three root sections when flooded from October or November. Carbohydrate remaining at this time is essential for cell maintenance and may also be important for the growth of roots in Spring after subsidence of the water table (Philipson 1988).

Comparing the two provenances, roots of the Washington provenance flooded in October generally have more carbohydrate remaining after 94 days than those of the Alaskan provenance. Carbohydrate content remaining in the two provenances after flooding from November is however similar. None of the provenance differences are however significant at the 5% level.

Comparing the levels remaining after flooding either from October or November within one provenance, a distinct pattern can be seen (figure 4.8). In each provenance there is a general pattern of roots retaining more carbohydrate when flooded from November to those flooded from October. The difference is much greater in the Alaskan provenance, much more starch and significantly more sugar and total

Figure 4.8. Root carbohydrate remaining in Alaskan and Washington provenances of Sitka spruce after three months waterlogging from either October to January or November to February.



Each value represents mean ±se of 4 seedlings; except root tips ofWashington flooded October to January and 5-10cm section of Washington provenance flooded November to February when n=3.

carbohydrate remaining in the roots flooded from November. In the Washington provenance much more starch remains following later flooding, but total carbohydrate levels do not differ significantly. Results of the statistical analysis are shown in table 4.11.

Table 4.11. Probability values generated by comparing the mean carbohydrate content of roots after flooding from October with those following flooding from November

	STARCH		SUGARS		TOTAL NON-STRUCTURAL CARBOHYDRATE	
SECTION OF ROOT	ALASKA	WASH.	ALASKA	WASH.	ALASKA	WASH.
0-5cm	NS	NS	0.014	NS	0.017	NS
5-10cm	NS	NS	0.006	NS	0.0001	NS
10-15cm	*	0.040	0.0093	NS	0.0025	NS

Mean carbohydrate content of roots in January and February compared using t-tests with equal or unequal sample variances as appropriate.

* zero starch remaining following flooding from October, therefore impossible to conduct t-test.

In terms of carbohydrate starvation and the necessity of reserves for Spring root establishment, the date of initiation of flooding appears to be important in both provenances, but especially critical in that of Alaskan origin which retains much more reserve if flooded 1 month later in the season.

d) Tissue Viability

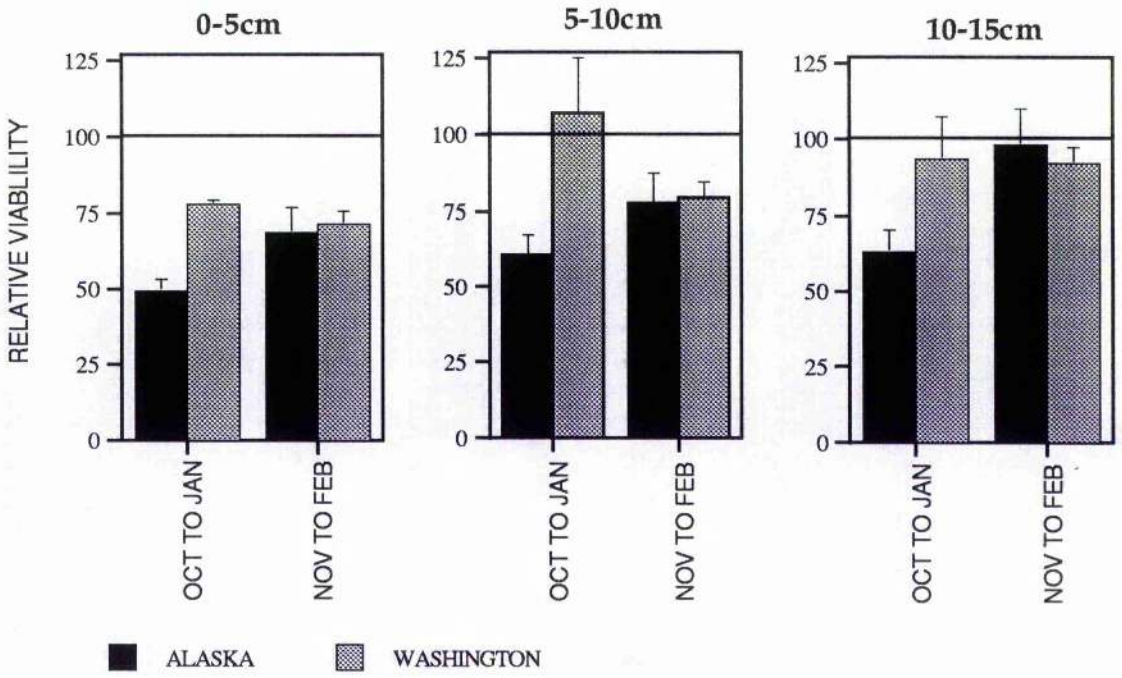
The viability index of each flooded seedling was expressed as a percentage of the mean viability index of free-drained seedlings of the same provenance determined at the time of harvest.

The number of seedlings was limited such that only one control was available for each provenance, except the Alaskan provenance in February where 2 controls were analysed. (Although there were two control seedlings for each provenance in February, only one of the

Washington seedlings had enough roots to assess each parameter - respiration and ethanol production capacity, carbohydrate content and tissue viability.) The low number of replicates puts limitations on the interpretation of the data, as the viability index of the control is variable. Where two control seedlings were available for the Alaskan provenance in February, the standard error was between 0.4 and 5% of the mean. Standard errors varied more in the flooded seedlings where the number of replicates was between 2 and 4. Within these limitations, the relative viabilities were calculated for each treatment. Being expressed as a percentage, the relative viability was arcsin transformed before the relative damage caused by the different treatments could be compared using three-way analysis of variance. The arcsin transformed data showed heterogeneity of variance. However the use of the $F_{MAX.}$ test to compare variances showed that the departure from homogeneity of variance was small and the use of ANOVA justified. The results of ANOVA of this type of data should be viable (see Abbott 1986).

Figure 4.9 shows viability of flooded seedlings relative to free drained controls (data shown in table A4.7). Each section is compared after three months flooding from October or November, and the 2 provenances graphed together to compare survival. The effect of waterlogging for three Winter months varied with the provenance, the date of flood initiation and the root section. Injury to the root by flooding was greatest in the root tip and decreased moving up the root system in each provenance irrespective of the date of flood initiation, e.g. when Alaskan seedlings were flooded in October, the relative viability decreased to ~50% of that of controls, whereas that of the most proximal section was only reduced to ~64%. The difference in flooding injury between the root sections was significant ($F=6.16$, $df=2$, $p=0.006$).

Figure 4.9. Relative viability after 3 months flooding from October or November in Alaskan and Washington provenances.



Viability of each section expressed as percentage of mean viability of freely drained seedlings of same provenance at same time. Details of replicate samples given in Table A4.7 .

Importance of date of flood initiation.

Root viability was reduced in almost every section relative to free-drained controls during three months of Winter flooding. Overall, roots of the Washington provenance retained greater viability than those of Alaskan origin ($F=6.48$, $df=1$, $p=0.017$). However, the two provenances showed a significantly differing response to the date of flood initiation ($F=10.63$, $df=1$, $p=0.003$). The Alaskan seedlings suffered a large reduction in viability following October-initiated flooding, but much greater survival when flooding was postponed for one month. In contrast, the Washington provenance suffered slightly greater loss of viability when flooded from November, although the difference between the two treatments was not as large as that of Alaskan seedlings.

When flooding was initiated in October, after three months the roots of the Washington provenance were least damaged. If flooding was initiated one month later, in November, tissue injury was very similar in the two provenances.

The results only give an estimate of tissue damage and it is important to bear in mind the limitation of results drawn from such a small data sample. It would be very useful to repeat this work with more replicates alongside root growth potential tests, as it is of great practical relevance.

e) Correlation between root viability, respiratory activity and carbohydrate reserve

The mean viability index of each provenance flooded from October or November was plotted against the mean

a) aerobic respiration rate,

b) total ethanol production under 24 hours anoxia.

c) total non-structural carbohydrate content of the root.

Graphs were plotted with and without the data for free drained controls (see figures 4.10a-c and 4.11a-c respectively). Each root section was plotted on the same graph.

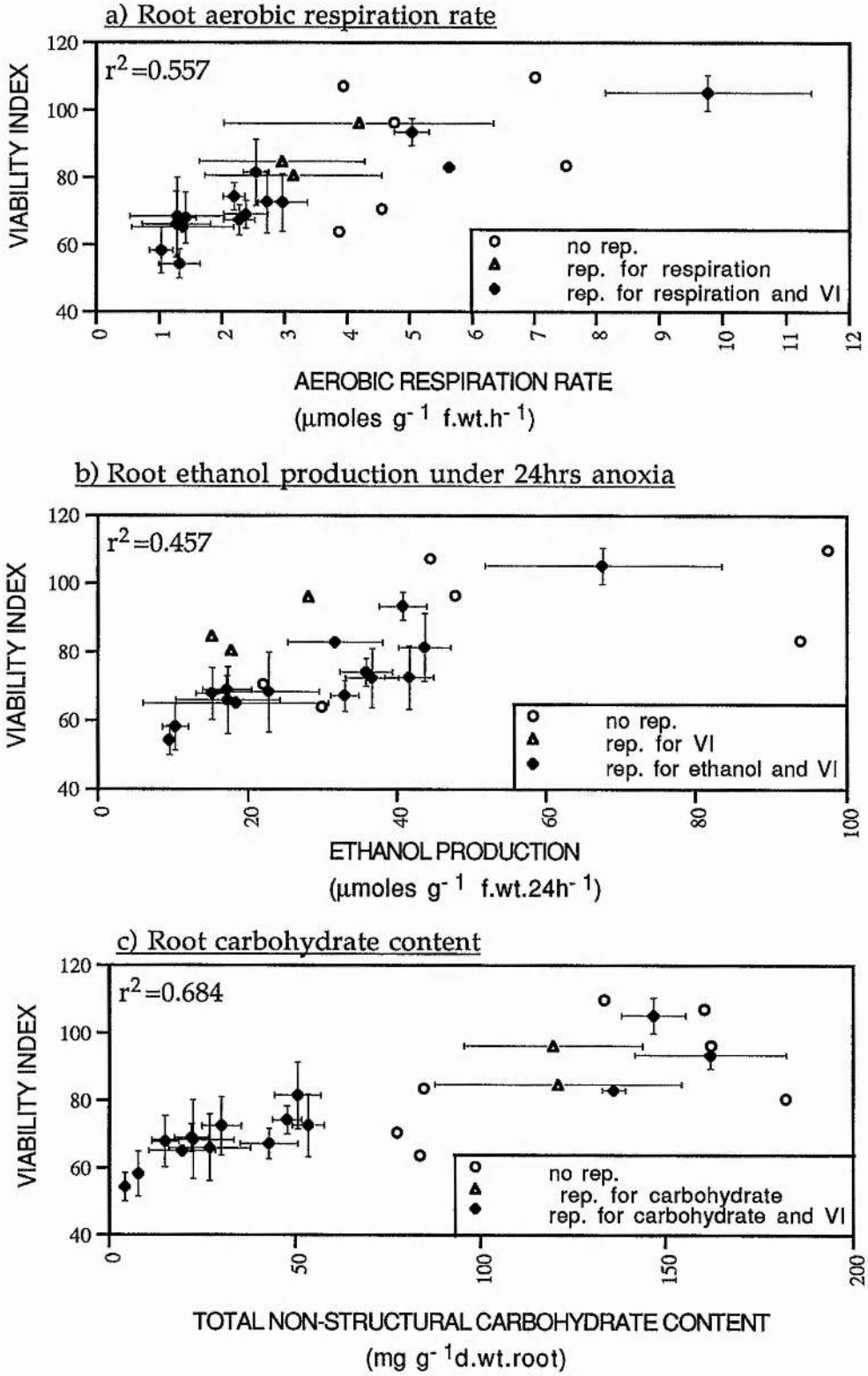
This allowed investigation of any relationship between cell metabolism and tissue viability.

Tissue viability increased with increasing carbohydrate content, aerobic respiration rate and ethanol production. It is likely that the depletion of carbohydrate reserve during flooding reduces the glycolytic rate due to lack of substrate supply, leading to a reduction in cell viability due to a fall in energy charge and inability to sustain normal cell function. The cells may also be damaged by lack of oxygen *per se*, leading to injury of the biochemical pathways, resulting in the correlation of respiration rate with viability. Reduction in viability may also result due to injury from soil toxins and free radicals during post-anoxia, as well as accumulation of anaerobic end-products.

f) Root observations

In general the shoots of flooded seedlings retained a healthy appearance during Winter waterlogging. On close inspection the distal roots of seedlings flooded for three months showed signs of injury. Some appeared to lose their turgidity slightly in comparison to free-drained seedlings. Most noticeable was their lack of cortical browning towards the root tip reported in dormant roots (Philipson and Coutts 1979), and clearly evident in those from free-drained soil conditions (Plates 4.1 and 4.2). Roots of seedlings flooded from either October or November appeared similar.

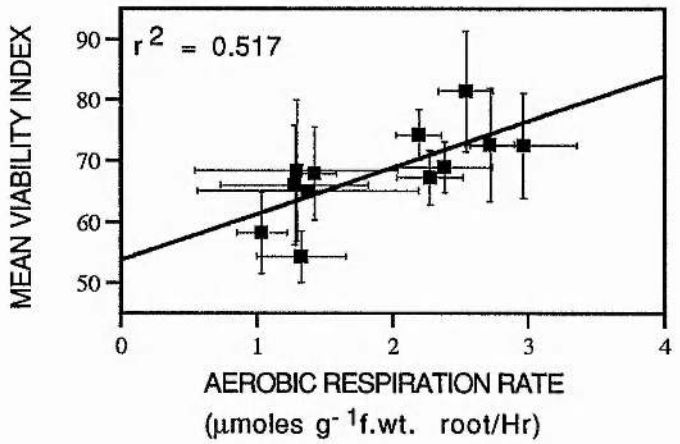
Figure 4.10 Root viability in relation to a) aerobic respiration rate; b) ethanol production under 24 hours anoxia; and c) root total non-structural carbohydrate content. Values are means for seedlings after 3 months flooding and those from free-drained soil conditions. Plot symbols are used to highlight the numberof replicates for that mean. (Correlation coefficient r^2 shown).



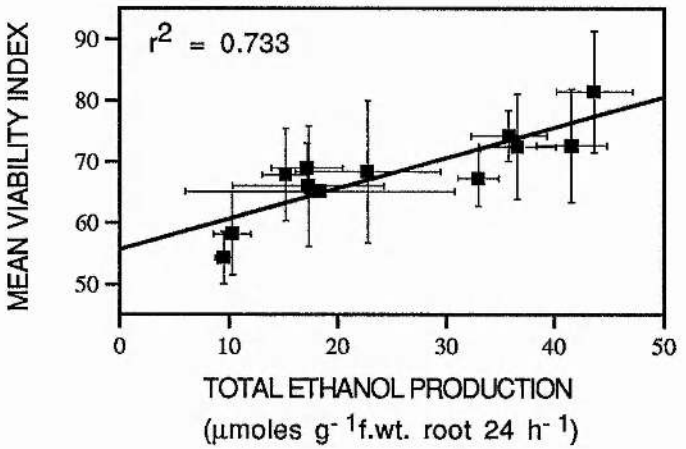
Replicates: 1-4 seedlings.

Figure 4.11. Viability index of roots of Alaskan and Washington Sitka spruce seedlings after 3 months winter flooding, in relation to a) aerobic respiration rate, b) total ethanol production under 24 hours anoxia at 20°C; and c) root carbohydrate reserve. (VI n=2-4; respiration rate, n=4; ethanol production n=2-4; carbohydrate content, n=4). Correlation coefficient r^2 shown for each graph.

a) Aerobic respiration rate.



b) Ethanol production under 24 hours anoxia.



c)Total non-structural carbohydrate content.

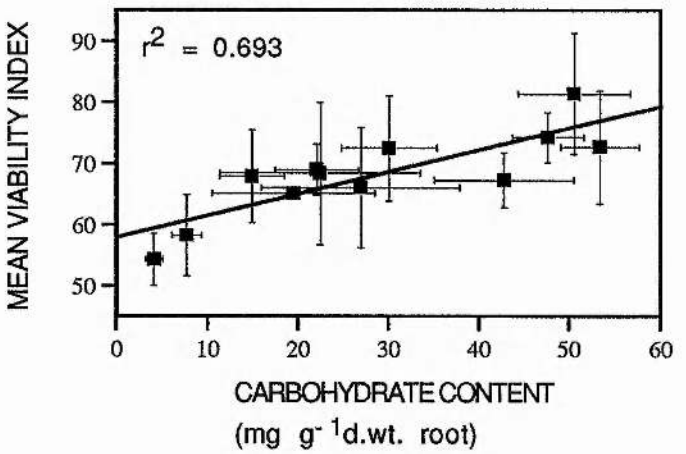
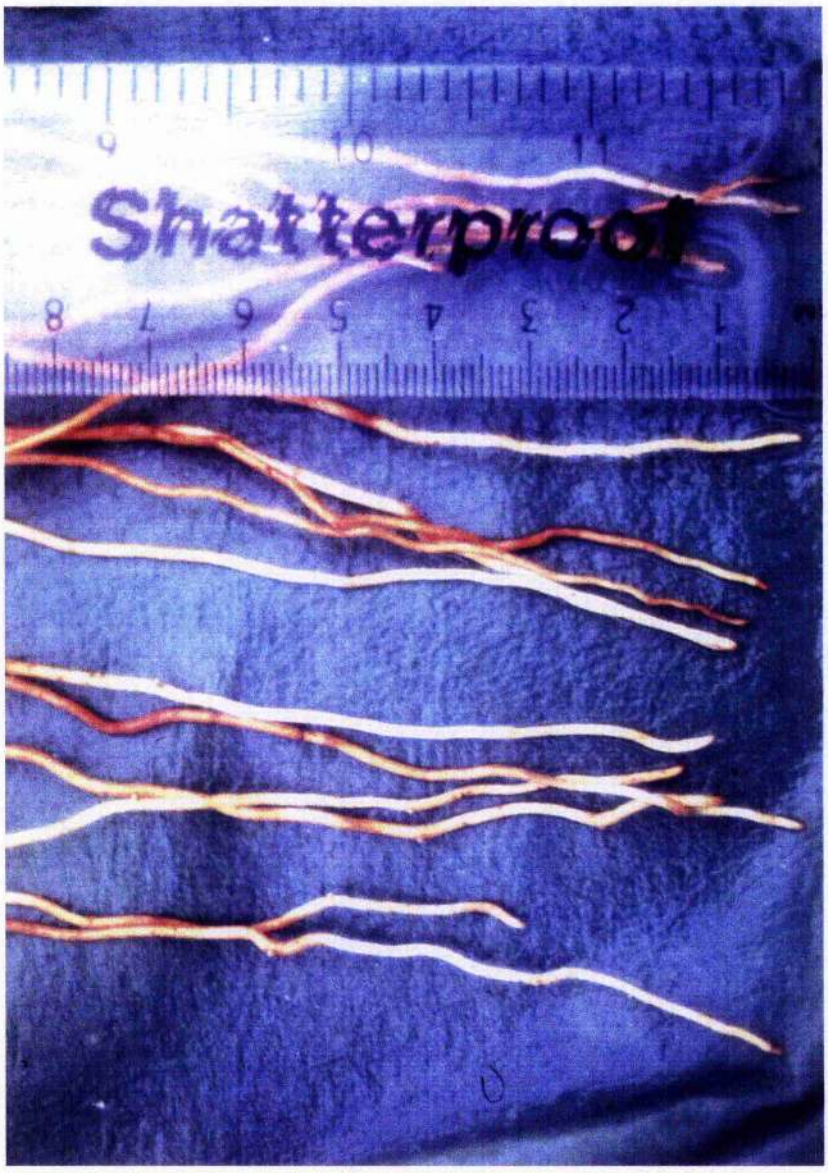
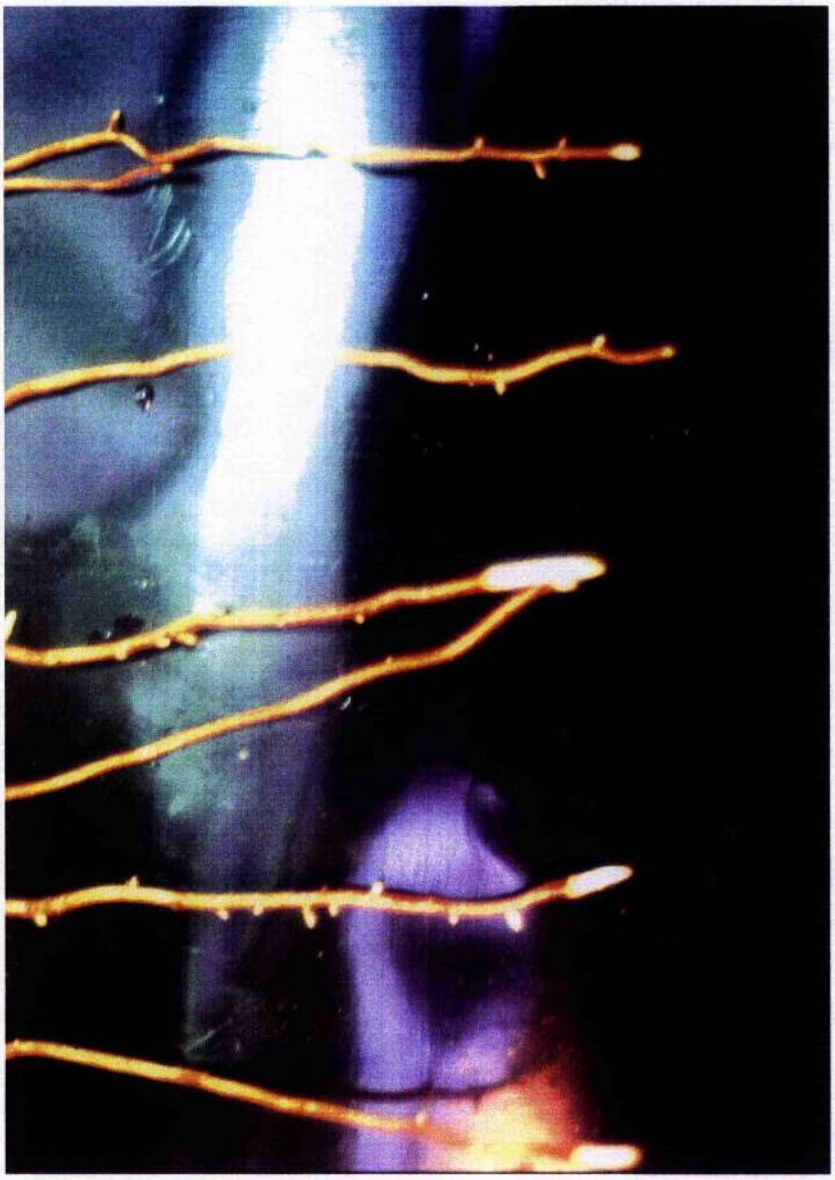


PLATE 4.1



Appearance of the distal roots of Sitka spruce seedlings after three months of winter flooding, initiated in October. Roots lack browning of the cortex clearly visible in roots of free-drained seedlings.

PLATE 4.2



Distal roots of free-drained Sitka spruce seedlings, showing the cortical browning towards the root tip usual in dormant conifers. (Scale as Plate 4.1)

4.4 DISCUSSION

Three months of Winter flooding reduces the viability of spruce roots, the distal tissues being most damaged and injury decreasing moving up the root system. The date of flood initiation appears most important in the Alaskan provenance, with greater loss of viability in each section following flooding from October rather than one month later in November. In contrast injury to the roots of Washington seedlings is slightly greater following November-initiated flooding, although the difference between the two flooding treatments much smaller. Comparing the two provenances, roots of the Washington seedlings retain greater tissue viability than the Alaskan seedlings when flooded in October, whereas damage is very similar following November flooding. Overall the seedlings of Washington origin appeared to withstand flooding better than those from Alaska, when assessed in terms of TTC reduction.

The effect of such a loss of viability measured by TTC reduction should ideally be related to survival and growth of the root and shoot during Spring in free-drained soil. The number of seedlings was limited such that growth potential tests could not be made. However a reduction in relative viability to 50% has been considered the lethal limit in a number of species and tissues where root and shoot survival has been assessed alongside TTC reduction (Steponkus and Lanphear 1967, Lindstrom and Nystrom 1987, Lindstrom and Mattsson 1989).

The response of the Alaskan provenance reflects that observed in cuttings of Queen Charlotte Island origin flooded at similar dates in 1987 (Coutts and Nicoll 1990b). Roots of these cutting survived to greater depth and suffered reduced die-back when flooded from November rather than October. Average dieback of the roots was 12.5cm following October flooding compared to only 2.3cm after November flooding,

(values estimated from diagrammatic representation, Coutts and Nicoll 1990b). These seedlings were flooded for slightly longer than the Alaskan and Washington provenances in this experiment, but provide a useful comparison of the response to flood initiation date.

The viability of seedlings may also be assessed by their respiratory capacity following treatment. Under anoxia, yeast cells suffer deterioration of mitochondrial cristae, cytochrome b and Cytochrome c oxidase, and consequently O_2 consumption (Luzikov *et al.* 1971). These cells cannot synthesise cytochrome c oxidase without oxygen (Ross *et al.* 1976). Similar injury during flooding was suggested to lead to the inability of flood-intolerant sugar maple to utilise oxygen, more tolerant species maintaining their capacity for uptake (Carpenter and Mitchell 1980). Oxygen uptake capacity in sugar maple roots decreased with increasing duration of flooding and could not be restored by feeding with glucose. Thus mitochondrial injury rather than substrate depletion was suggested to be the cause. Reduced oxygen uptake in sugar maple was correlated with shoot death and its measurement gives a useful indication of flood tolerance, root viability and the integrity of the mitochondrial apparatus (Tripepi and Mitchell 1984b). Aerobic and anaerobic respiration rates after flooding were compared to those of freely drained seedlings analysed concurrently. Flooding reduced respiration rates relative to those of controls in almost all cases, aerobic respiration being more severely reduced than that under nitrogen. If substrate shortage were the only cause of impaired respiratory capacity the reduction of aerobic and anaerobic respiration rate would be similar. Therefore injury to the aerobic pathway seems the likely cause of this pattern. A fall in respiratory capacity after flooding, (measured by CO_2 output), is thought to be characteristic of flood intolerant plants (Carpenter and Mitchell 1980).

Relative respiration rates in air and nitrogen were always more severely reduced in the tip section, irrespective of provenance and date of flooding, indicating greater cellular injury in this section in agreement with viability test results. In each provenance, reduction in relative aerobic respiration rate was greatest following flooding from October rather than November.

Ethanol production relative to that of controls showed a similar response, the root tip always showing a deficit in ethanol production following flooding. In each provenance, ethanol production capacity decreased in each root section following flooding from October, but increased in the root 5-15 cm from the tip following November flooding. Again this difference in response may reflect a difference in cellular injury from the two dates of flooding, but also differences in the amount of substrate remaining in the root. Comparing the two provenances, each showing the same pattern of response, although the Alaskan provenance shows the greater deficit in ethanol production relative to the controls when flooded from October.

Aerobic and anaerobic respiration rate and ethanol production of flooded seedlings may reflect cellular injury due to anoxic and/or post anoxic injury, but may also reflect the substrate levels remaining in the tissues. Flooding severely depleted carbohydrate reserves, especially following October flood initiation. Loss of viability is well correlated with loss of substrate for glycolysis, reduced aerobic respiration rate and reduced ethanol production. While such severe depletion of carbohydrate seems likely to cause the loss of tissue viability, injury to the cells may also be caused by factors besides those of carbohydrate metabolism. Uptake of reduced ions in the soil and accumulation of ethanol may lead to cell toxicity. Membrane peroxidation or acetaldehyde production on re-exposure to air may also result in tissue injury. The

greater reduction in aerobic relative to anaerobic respiration rate in flooded seedlings suggests that injury to the aerobic pathway is likely to be an important factor in cell deterioration. This may come about due to the reduced carbohydrate content of the cells because soluble sugars are needed for maintenance of cell organelles as well as oxygen itself (Vartapetian et al. 1976, 1978, Webb and Armstrong 1983 - see General discussion Chapter 7).

During Winter the roots are either extending slowly or dormant and energy is mainly required for maintenance respiration. Flooding injury is temperature dependant, the low Winter temperatures ameliorating stress. However carbohydrate depletion during flooding proved to be severe. The starch reserves in the roots are greatly reduced when flooded from October or November. However, the higher level of root starch at flood initiation in November as compared to October and the slightly lower rate of depletion in November leaves roots flooded from this time with higher levels of starch. Root sugars are also depleted at a higher rate following October flooding. Although root sugar content is lower in November than October in free drained seedlings, levels of sugar similar to those in non-flooded trees remain in roots after flooding from November, whereas they are severely reduced if flooding is initiated in October. Such depletion of reserves is most likely to explain the measured rates of ethanol production and respiration after three months of flooding. If reserves are too low to support respiration rate, adenylate energy charge will fall and the ATP generated may not maintain normal cell function, loss of viability will ensue. Flooding reduced respiration capacity whether initiated in October or November. The degree of reduction however was greater following flooding from October after which little carbohydrate remained. Assessing root viability from results of remaining carbohydrate reserve and respiratory capacity

(in terms of ethanol and CO_2 production), both provenances show superior survival of Winter flooding if initiated in November rather than one month earlier in October. The viability test correlates with this data, except for the Washington provenance apparently suffering less injury following October-initiated flooding. Variation may be due to the limited number of replicates, and the TTC test may not be as sensitive to tissue injury as the measurement of ethanol or CO_2 production. Ideally, these tests should be repeated with a larger number of replicates.

Following flooding from October, the Alaskan provenance suffered the greatest depletion of carbohydrate reserve, viability and ethanol production. Although this provenance contained more carbohydrate reserve than the Washington seedlings, the respiration rate at initiation of flooding was higher.

The Alaskan provenance also showed the greatest response to postponement of the date of flooding. This reflects the greater reduction of respiration rate from October to November when flooding was initiated. The Washington provenance, from a more southerly latitude, showed less of a reduction in respiration rate between the two months. The greatest difference in depletion of carbohydrate, deficit in ethanol production under anoxia and loss of viability are seen in the Alaskan provenance when comparing flooding from October with that initiated in November. It therefore appears that root activity at flood initiation is the most important factor determining root survival. This supports Coutts and Nicoll's (1990b) hypothesis from their observations on root survival during flooding from the same months in Winter 1987-1988. Species with large carbohydrate reserves are not inherently flood-tolerant, this property must be coupled with control of glycolysis and carbohydrate conservation on flooding (Barclay and Crawford 1983). The same response is seen in seeds. Species which survive hypoxia during

germination tend to have lower metabolic rates when placed under anoxia (Crawford 1977).

It appears from this work that the screening of different provenances for high carbohydrate content, but, most importantly, differences in the phenology of root growth, would be advantageous. In areas where the water table rises early in the Winter, the planting of provenances which "shut down" their root activity early in the Autumn may reduce the dieback of the root systems and cut down losses from wind throw. Little data is available on the variation between provenances in phenology of root growth (Coutts and Nicoll 1990a). More research has been conducted on the provenance variation in shoot growth periodicity because of the importance of frost damage to trees in which bud-dormancy is late. In a study of various provenances of Sitka spruce, Lines and Mitchell showed that shoot growth is daylength-controlled and ceases much earlier in Northern provenances compared to those from more Southern latitudes when seedlings are grown together in Britain. The Washington provenance, Hoquiam (47°N), showed a large increase in shoot height growth after mid-September when that of Juneau (58.25°N), Alaska had ceased. The two provenances come from areas of similar rainfall (~60 inches per annum), however the mean annual temperature of the Alaskan provenance is only 5°C compared to that of 10°C in Washington (Lines and Mitchell 1966).

It is possible that the difference in root respiration seen in the two provenances is related to the 'normal temperatures' experienced by seedlings from the two origins. Although influenced by daylength root growth is largely temperature regulated (Coutts and Nicoll 1990a). It is soil-temperature dependant, increasing between 5 and 25°C (Coutts and Philipson 1987). It is likely that the seedlings are adapted to their 'native'

temperature regimes such that roots of the Alaskan provenance may grow and respire faster at lower soil temperature. If flooding occurs whilst root respiration rate is still high, the results suggest that damage will be greater. Seedlings of Washington origin with slightly lower root respiration rates were seen to survive flooding in October better than the Alaskan seedlings.

4.5 APPENDIX

Table A4.1. Aerobic and anaerobic respiration rate of root sections harvested through Winter from freely drained soil or after 3 months waterlogging

(Each table shows mean ±se).

a) OCTOBER MEASUREMENT (μmoles CO_2 g^{-1}f.wt. h^{-1})

	ALASKA		WASHINGTON	
SECTION OF ROOT	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC
0-5cm	17.42 ±0.66	11.18 ±0.39	15.37 ±1.34	8.07 ±0.75
5-10cm	8.92 ±0.60	7.74 ±0.53	7.56 ±1.29	5.47±0.78
10-15cm	8.16 ±0.99	7.14 ±0.39	6.13 ±1.00	4.53±0.63

Number of replicate seedlings; n=4 Alaska
n=3 Washington

b) NOVEMBER MEASUREMENT (μmoles CO_2 g^{-1}f.wt. h^{-1})

	ALASKA		WASHINGTON	
SECTION OF ROOT	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC
0-5cm	8.92 ±0.29	7.76 ±0.97	11.94 ±3.63	7.61 ±1.48
5-10cm	4.85 ±0.43	5.54 ±0.33	6.01 ±1.25	5.69±1.29
10-15cm	4.01 ±0.48	4.46 ±0.46	6.34 ±1.06	6.05±1.29

Number of replicate seedlings; n=4 Alaska and Washington

c) JANUARY MEASUREMENT (μmoles CO_2 g^{-1}f.wt. h^{-1})
Free drained seedlings shown in plain text, those flooded from October for 3 months in *italics*

	ALASKA		WASHINGTON	
SECTION OF ROOT	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC
0-5cm	7.00 *1.32 ±0.33*	5.58 *2.99 ±1.11*	7.51 *1.37 ±0.81*	7.35 *3.37 ±2.02*
5-10cm	4.74 *1.03 ±0.19*	3.35 *2.33 ±1.07*	3.87 *1.29 ±0.75*	6.31 *3.89 ±2.92*
10-15cm	3.93 *1.42 ±0.16*	4.33 *2.44 ±1.18*	4.55 *1.27 ±0.54*	5.42 *4.47 ±2.75*

Number of replicate seedlings; n=4 Alaska and Washington flooded seedlings, n=1 free drained seedlings

d) FEBRUARY MEASUREMENT (μmoles CO_2 g^{-1}f.wt. h^{-1})
Free drained seedlings shown in plain text, those flooded from November for 3 months in *italics*

	ALASKA		WASHINGTON	
SECTION OF ROOT	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC
0-5cm	9.76 ±1.63 *2.96 ±0.40*	8.77 ±1.00 *4.38 ±0.24*	4.18 ±2.16 *2.38 ±0.36*	4.46 ±0.64 *3.81 ±0.62*
5-10cm	5.03 ±0.28 *2.72 ±0.18*	5.10 ±0.52 *4.20 ±0.20*	2.96 ±1.32 *2.27 ±0.25*	4.26 ±1.10 *3.74 ±0.17*
10-15cm	5.63 ±0.03 *2.54 ±0.20*	5.73 ±0.07 *3.81 ±0.27*	3.14 ±1.41 *2.19 ±0.17*	3.37 ±0.51 *3.58 ±0.22*

Number of replicate seedlings; n=4 Alaska and Washington flooded seedlings, n=2 free drained seedlings

Table A4.2. Ethanol production and accumulation in roots analysed under 24 hours experimental anoxia - free drained seedlings in October
* all results shown are mean and standard error of the mean.

a) Total ethanol production (μmoles ethanol g^{-1}f.wt. root 24 h^{-1}).

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA	**86.8** ±9.4	**74.8** ±6.1	**79.0** ±4.3
WASHINGTON	**71.0** ±15.2	**48.7** ±12.5	**52.1** ±7.7

b) Ethanol accumulation in tissue (μmoles ethanol g^{-1}f.wt. root 24 h^{-1}).

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA	**17.2** ±2.3	**16.2** ±2.9	**15.1** ±1.8
WASHINGTON	**20.4** ±2.2	**14.8** ±1.8	**14.6** ±1.6

c) Ethanol accumulation in water around roots as a percentage of total production.

	SECTION OF ROOT/cm		
TREATMENT			
ALASKA	**80.0** ±2.1	**78.7** ±2.3	**81.0** ±1.7
WASHINGTON	**68.5** ±5.1	**66.7** ±4.9	**71.5** ±2.0

NUMBER OF REPLICATES.

ALASKAN PROVENANCE:- 4 trees per section.
WASHINGTON PROVENANCE:- 4 trees per section.

Table A4.3. Ethanol production and accumulation in roots analysed under 24 hours experimental anoxia - free drained seedlings in November

* all results shown are mean and standard error of the mean.

a) Total ethanol production (µmoles ethanol g^{-1}f.wt. root 24 h^{-1}).

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA	**75.6** ±10.4	**55.7** ±6.3	**44.9** ±12.9
WASHINGTON	**81.4** ±19.1	**63.1** ±15.5	**52.7** ±12.7

b)Ethanol accumulation in tissue, (µmoles ethanol g^{-1}f.wt. root 24 h^{-1}).

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA	**8.3** ±2.0	**9.0** ±2.1	**8.2** ±2.0
WASHINGTON	**9.8** ±3.3	**9.4** ±3.1	**8.0** ±2.2

c)Ethanol accumulation in water around roots as a percentage of total production.

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA	**88.4** ±3.5	**83.9** ±3.2	**82.8** ±2.2
WASHINGTON	**87.2** ±4.0	**84.9** ±3.2	**84.4** ±1.9

NUMBER OF REPLICATES.
ALASKAN PROVENANCE:- 4 trees per section*
WASHINGTON PROVENANCE:- 4 trees per section.
*Except10-15cm section when mean calculated from results of 3 trees analysed for ethanol content of water around root.

Table A4.4. Comparison of root ethanol metabolism during 24 hours anoxia when analysed in January after 3 months of flooding or free-drainage

a)Total ethanol production (µmoles ethanol g^{-1}f.wt. root 24 h^{-1}).

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA FLOODED OCT.-JAN.	**9.5** ±0.8 *n*=3	**10.3** ±1.7 *n*=4	**15.2** ±2.2 *n*=4
ALASKA CONTROL	**97.5**	**47.8**	**44.5**
WASHINGTON FLOODED OCT.-JAN.	**18.4** ±12.4 *n*=2	**22.7** ±6.7 *n*=2	**17.3** ±7.0 *n*=3
WASHINGTON CONTROL	**93.9**	**29.9**	**22.1**

Controls: 1 tree per provenance.
Flooded trees: No of trees analysed shown in table (n).

b) Ethanol accumulation in root tissue, (µmoles ethanol g^{-1}f.wt. root 24 h^{-1}).

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA FLOODED OCT.-JAN.	**9.0** ±0.7	**8.1** ±0.9	**8.2** ±0.2
ALASKA CONTROL	**15.6**	**12.2**	**15.7**
WASHINGTON FLOODED OCT.-JAN.	**5.7** ±0.9	**5.1** ±1.1	**5.0** ±1.3
WASHINGTON CONTROL	**7.3**	**4.3**	**4.5**

Controls: 1 tree per provenance.
Flooded trees: 4 trees per provenance.

c)Ethanol accumulation in water around roots as a percentage of total production.

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA FLOODED OCT.-JAN.	**0.4** ±0.4 n=3	**18.5** ±6.7 n=4	**42.4** ±9.2 n=4
ALASKA CONTROL	**84.0**	**74.4**	**64.7**
WASHINGTON FLOODED OCT.-JAN.	**60.3** ±15.3 n=2	**70.2** ±2.2 n=2	**56.8** ±15.1 n=3
WASHINGTON CONTROL	**92.3**	**85.7**	**79.4**

Controls: 1 tree per provenance.
Flooded trees: No of trees analysed shown in table (*n*).

Table A4.5. Comparison of root ethanol metabolism during 24 hours anoxia analysed in February after 3 months of flooding or free-drainage

a) Total ethanol production (μmoles ethanol g^{-1}f.wt. root 24 h^{-1}).

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA FLOODED NOV.-FEB.	**36.6** ±3.5 *n*=4	**41.6** ±3.3 *n*=4	**43.6** ±3.5 *n*=4
ALASKA CONTROL	**67.6** ±15.9 *n*=2	**40.7** ±3.2 *n*=2	**31.6** ±6.3 *n*=2
WASHINGTON FLOODED NOV.-FEB.	**17.2** ±3.3 *n*=4	**32.9** ±1.9 *n*=4	**35.8** ±3.5 *n*=4
WASHINGTON CONTROL	**28.1** ±6.5 *n*=2	**15.2** ±4.0 *n*=2	**17.7** ±1.6 *n*=2

Control + Flooded treatments: No. of trees analysed shown in table (*n*).

b)Ethanol accumulation in root tissue, (μmoles ethanol g^{-1}f.wt. root 24 h^{-1}).

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA FLOODED NOV.-FEB.	**7.6** ±3.2 *n*=4	**8.0** ±2.0 *n*=4	**8.9** ±2.0 *n*=4
ALASKA CONTROL	**6.8** ±1.5 *n*=2	**6.3** ±0.4 *n*=2	**6.3** ±0.6 *n*=2
WASHINGTON FLOODED NOV.-FEB.	**3.9** ±1.0 *n*=4	**5.0** ±0.5 *n*=4	**5.8** ±0.2 *n*=4
WASHINGTON CONTROL	**6.1** ±2.6 *n*=2	**4.0** ±0.8 *n*=2	**4.1** ±0.5 *n*=2

Control + Flooded treatments: No. of trees analysed shown in table (*n*).

c) Ethanol accumulation in water around roots as a percentage of total production.

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA FLOODED NOV.-FEB..	**78.0** ±10.1 *n*=4	**81.2** ±3.5 *n*=4	**80.0** ±3.2 *n*=4
ALASKA CONTROL	**89.9** ±0.2 *n*=2	**84.6** ±0.1 *n*=2	**79.5** ±2.1 *n*=2
WASHINGTON FLOODED NOV.-FEB..	**76.4** ±4.6 *n*=4	85.0 ±0.7	**83.6** ±1.1 *n*=4
WASHINGTON CONTROL	**74.7** ±15.2 *n*=2	**73.3** ±1.7 *n*=2	**77.1** ±0.7 *n*=2

Control + Flooded treatments: No. of trees analysed shown in table (*n*).

Table A4.6. Depletion of root carbohydrate in Alaskan and Washington provenances of Sitka spruce during flooding, comparison with levels on initiation of flooding and those in free-drained controls at end of flood period

i) As percentage of initial levels.

	FLOODED FROM OCTOBER		FLOODED FROM NOVEMBER		P VALUE	
	ALASKA	WASHINGTON	ALASKA	WASHINGTON	ALASKA	WASH.
STARCH	1.32 ±0.50	1.77 ±0.59	7.16 ±3.04	9.82 ±3.35	NS	NS
SUGARS	12.81 ±2.83	54.0 ±25.9	102.6 ±12.4	96.8 ±11.0	0.019	NS
TOTAL CARBOHYDRATE	8.35 ±1.65	36.6 ±17.2	39.69 ±3.86	45.4 ±6.29	0.0002	NS

ii) As percentage of content of free drained seedlings harvested at same time.

	FLOODED FROM OCTOBER		FLOODED FROM NOVEMBER		P VALUE	
	ALASKA	WASHINGTON	ALASKA	WASHINGTON	ALASKA	WASH.
STARCH	0.52 ±0.196	1.02 ±0.34	5.07 ±2.15	3.77 ±1.28	NS	NS
SUGARS	17.88 ±3.94	63.3 ±30.3	96.3 ±11.6	110.3 ±12.6	0.029	NS
TOTAL CARBOHYDRATE	5.86 ±1.16	31.8 ±14.9	30.11 ±2.93	23.91 ±3.31	0.0002	NS

The probability value in column 4 is generated using MINITAB™ to compare the mean depletion of carbohydrate in October and November by a two sample t-test allowing for equal or unequal variance as appropriate. Means are significantly different if $p \leq 0.05$. Percentage of carbohydrate remaining in the root was transformed into the arcsin$\sqrt{}$percentage for statistical analysis. Replicates detailed in table 4.9.

Table A4.7. Relative viability of Sitka spruce roots after 3 months of flooding initiated in October and November

	SECTION OF ROOT/cm		
TREATMENT	0-5	5-10	10-15
ALASKA FLOOD OCT.-JAN.	49.48 ± 3.88 (4)	60.43 ±6.97 (4)	63.40 ±7.05 (4)
ALASKA FLOOD NOV.-FEB.	68.92 ±8.17 (3)	77.69 ±9.89 (3)	98.16 ±11.93(3)
WASH. FLOOD OCT.-JAN.	78.01 ±1.42 (2)	107.10 ±18.18 (2)	93.61 ±13.86 (2)
WASH. FLOOD NOV.-FEB.	71.69 ±4.29 (3)	79.43 ±5.28 (4)	92.22 ±5.11 (4)

RELATIVE VIABILITY = $\frac{\text{MEAN VIABILITY INDEX FLOODED TREE}}{\text{MEAN VIABILITY INDEX CONTROL}} \times 100$

Standard error of mean (SD/$\sqrt{n}$) and number of flooded replicates given below mean R.V. values.

Mean viability of flooded trees compared to mean viability of;

1 Alaskan and 1 Washington non-flooded control in January,

1 Washington and 2 Alaskan controls in February.

CHAPTER 5

Effect of Elevated Soil Temperature on Survival of Winter Flooding in Sitka Spruce

5.1 INTRODUCTION

5.1.1 Predictions of possible climate change

Exactly how increases in atmospheric CO_2 may affect global climate are unknown. Predictions of the many models vary widely, but certain patterns of change seem likely. A doubling of present CO_2 levels (now ~350cm^3m^{-3}) is expected during the next century and is estimated to cause an increase in mean global precipitation of between 3 and 11% (Wigley *et al.* 1990), and an increase in the earth's annual mean surface temperature of a few degrees Celcius (Woodwell 1989). The patterns of rainfall are also likely to change, many dry areas suffering more drought, and wet regions becoming wetter. Rainbelts are likely to move North, causing up to 20% increases in the annual precipitation in wetter areas of Northern Europe, and increasing the rainfall of Northern and Western Britain (Wigley *et al.* 1990). This is an area of concentrated forestry.

Such increases would reduce the level of soil aeration unless accompanied by an equal increase in evapo-transpiration. Whether or not this would happen is very unclear at present, although it is possible that evapo-transpiration may decrease in these circumstances. The increased CO_2 will reduce stomatal aperture and transpiration (Bolin *et al.* 1990), thus the uptake of soil water. Shallower horizons of aerated soil will reduce root depth and cause greater instability in forests. The consequent increase in windthrow and reduced tree cover will again result in reduced transpiration (Crawford 1992). Thus there may well be a positive feedback mechanism accentuating the waterlogging of the soil.

5.1.1 Possible effects on forestry

Increases in temperature and windspeed predicted by many models would also aggravate the injury to trees under increased soil waterlogging. Roots are more susceptible to injury when flooded at

higher temperature (Coutts and Philipson 1978a), and the response of root growth to temperature (an area needing more research) may result in actively growing roots suffering Winter waterlogging. This would result in greater dieback, because before dormancy sets in, tolerance to soil anoxia is not well developed (Coutts and Philipson 1978a).

The increase in CO_2 itself may predispose forest trees to windthrow. Root:shoot ratios of CO_2 enriched trees tends to remain the same (e.g. Engelmann spruce - Chomba *et al.* 1993) or decrease (Sionit *et al.* 1985) and increased temperature reduces C accumulation in the source and sink (Farrar and Williams 1991). If the root to shoot ratio did decrease, the trees would be more 'top-heavy' and more susceptible to wind-throw.

5.1.3 Aims of the experiment

This experiment is designed to investigate the effect of increased soil temperature during Winter waterlogging on survival of Sitka spruce roots. This is very crude in terms of modelling a 'Greenhouse world' situation where root and shoot temperature, rainfall and CO_2 concentration may change. Keeping the seedlings in an environmentally controlled growth room was not possible as their roots were growing in 1 and 2m perspex tubing. It was also desirable to have the seedlings outdoors such that they could respond to the environmental cues (photoperiod and light intensity). The experimental design was therefore very simple in that root temperature alone was increased above ambient.

Elevated soil temperature causes a fall in oxygen content, firstly as oxygen solubility decreases with increasing temperature and secondly due to increased uptake by soil micro-organisms and roots. Maintenance respiration increases with increasing temperature (Lawrence and Oechel 1983, Amthor 1984), thus increased root zone temperature will increase the cost of maintaining even the dormant root system. Roots in warmer

soil may have greater O_2 demand, and may be pre-disposed to greater injury under Winter waterlogging. The temperature of the root also affects the partitioning of reserves (Marshall and Waring 1985). More information is needed on the growth periodicity of the root system in Sitka spruce. It is thought to be primarily temperature dependant although internal cues (e.g. low light intensity, daylength) appear to play an important role in regulating growth. Thus mild mid-Winter temperatures do not initiate root growth which may begin at even lower temperature in early Spring (Coutts and Nicoll 1990a).

This experiment was designed to quantify injury caused by elevated root temperature through its effect on respiration rate and carbohydrate depletion and the accumulation of anaerobic end-products.

5.2 METHODS

5.2.1 Seedling growth and maintenance

Sitka spruce seedlings[33] were planted during late February 1992 in 1 or 2m perspex tubes and top dressed with 2g *osmocote plus*™ slow release fertiliser (supplemented with a further 3g in late May). Tubes were equally distributed between two insulated root boxes (see chapter 2), and watered regularly with tap water.

5.2.2 Experimental design

In mid-November, a thermostated electric heater was placed inside one of the root boxes between the perspex tubes. The minimum temperature was thus maintained at 13°C. The temperature in the second root box was allowed to fluctuate with outside air temperature. Outside air temperature and that inside the root boxes were monitored through the Winter with max-min thermometers.

a) Measurement of root metabolic activity at initiation of flooding treatments

Between mid and late November, 4 seedlings were randomly selected and the root core carefully removed by rinsing with water to prevent root damage. 15cm distal lengths of root were removed, carefully washed in tap water and further sectioned into 3x5cm lengths. Root metabolic activity was then assessed as described in chapter 3 for Lodgepole pine roots. Data was collected for aerobic and anaerobic respiration rate at 10°C, root carbohydrate content (starch and sugars) and ethanol production under 24hrs anoxia at 10°C.

---

33 Hoquiam,Washington provenance, no 71(7972)5.
Seedlings (age 1+1, mean size 29.3cm) lifted 14/01/92 by Forestry Commission Northern Research Station, Roslin, Midlothian. Cold stored until transplanted mid-February at St. Andrews.

(Experimental details as for Chapter 3.)

b) Initiation of waterlogging treatments

Concurrent with the analysis of free-drained seedlings, a sample of those from each root box was flooded from the tube base. Waterlogging was maintained just above the soil surface until April. A sample of seedlings was removed after an average of 65 (±1) days flooding along with freely drained controls from each temperature. The remaining seedlings were then analysed 137 days after flood initiation. The number of replicate seedlings in each treatment is shown in Table 5.1. During February storm damage to the root box maintained at ambient temperature led to the loss of a number of freely drained samples. Thus only 5 seedlings remained as controls for the 2 harvest dates.

Table 5.1. Number of seedlings analysed in each treatment

TREATMENT	65 DAYS FLOODING (analysed mid-Jan/early Feb)	137 DAYS FLOODING (analysed April)
Free drained, ambient temperature	3	2
Free drained, elevated temp.	3	5
Flooded, ambient temperature	4	4
Flooded, elevated temp.	4	4

c) Post-Treatment analysis of seedlings

After 65 days:

After each treatment seedlings were harvested from the perspex tubes without flushing with water. Roots were sectioned and placed into 3 groups as before. From each seedling, one sample of roots was immediately wiped clean of peat (without washing), and plunged into liquid nitrogen. A perchloric acid extract of the frozen root sample was

prepared to determine the actual accumulation of ethanol and malic acid in the tissues *in vivo*. The other roots were washed clean as before and analysed for tissue viability (TTC assay) and carbohydrate content. (Each procedure detailed in chapter 2.)

After 137 days:

All remaining seedlings were washed with tap water to aid removal from the tube. Roots were sectioned as above and assayed for tissue viability and carbohydrate content.

Experimental details given in chapter 2 and detailed in Fig 5.1.

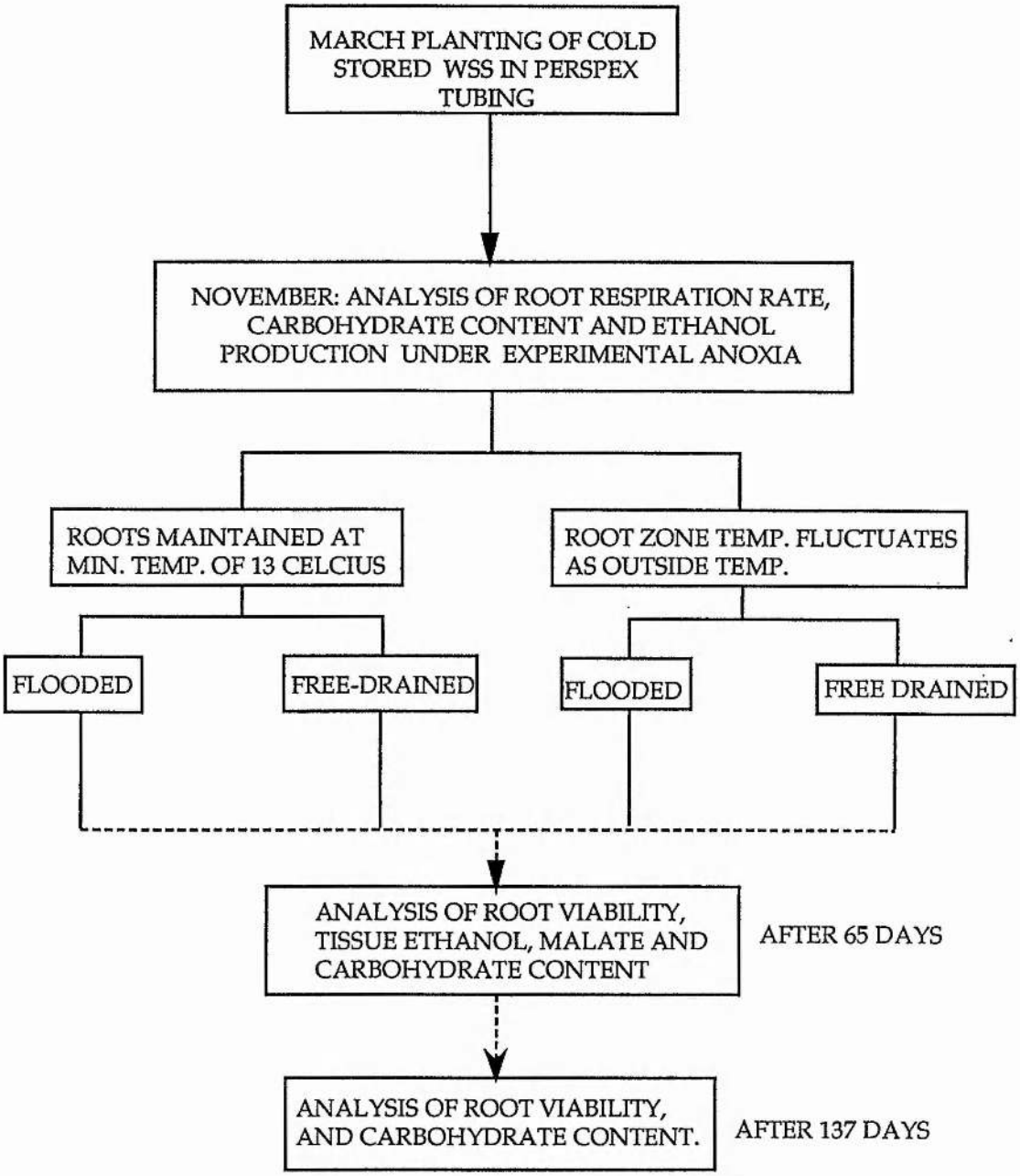


Figure 5.1. Flowchart summary of experimental design.

5.3 RESULTS

5.3.1 Root physiological status concurrent with initiation of flood/temperature treatments

Results of root carbohydrate analysis, ethanol production and respiration rates were presented in chapter 3 to allow a comparison with Lodgepole pine.

5.3.2Analysis of roots after combination of temperature/flood regimes

a) Root temperature maintenance

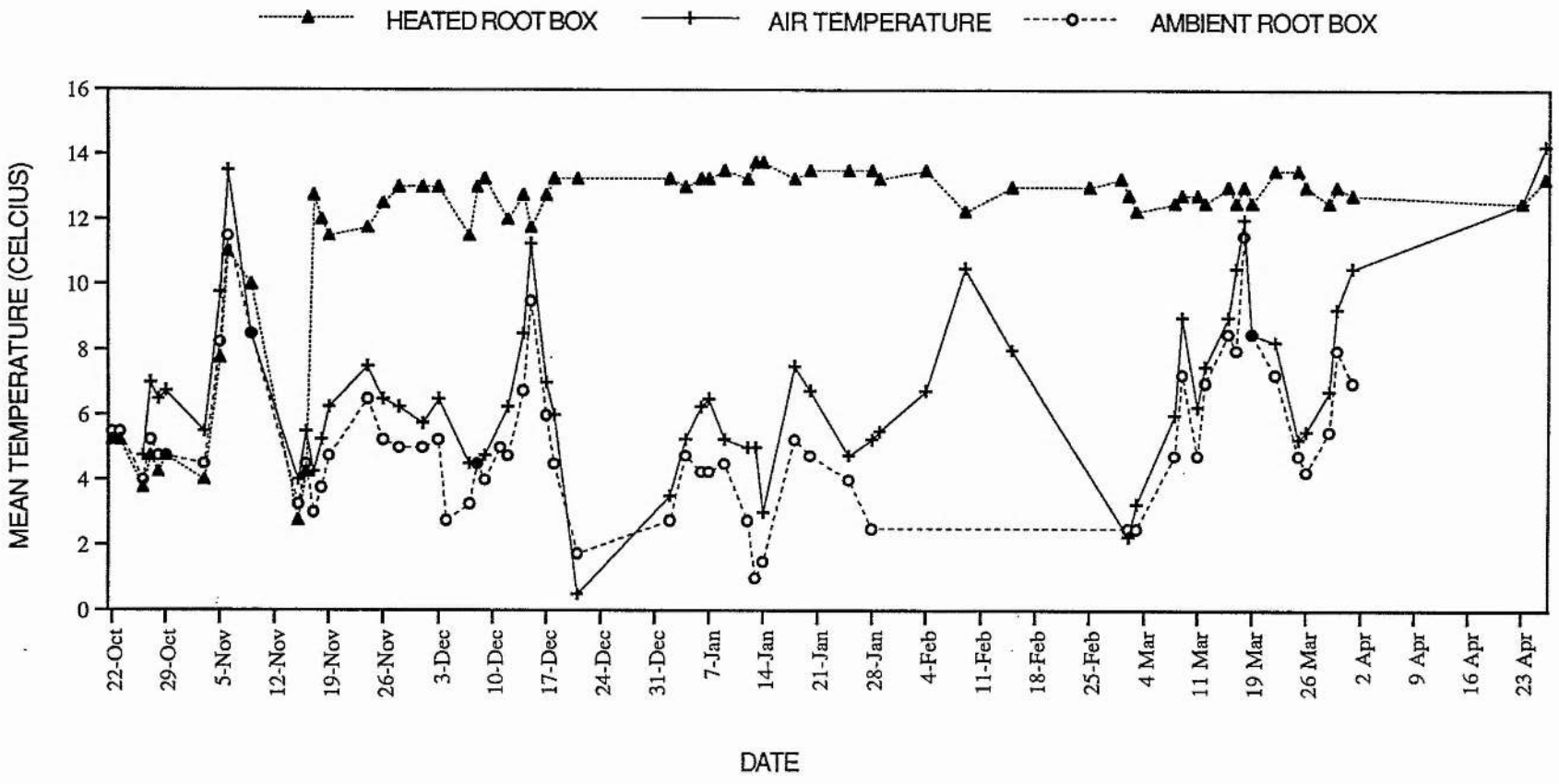
Minimum temperature in the unheated root box was similar to the minimum air temperature, but daily increase in root zone temperature with that of outside air was prevented by insulation with polystyrene sheets and maintenance of roots in the dark. The root box therefore prevented large fluctuations in root temperature through the day and kept the mean root temperature slightly lower than the mean outside air temperature. Thermostatically controlling the root zone temperature proved successful. Comparing the daily max-min temperature, the mean daily flux was maintained at only 2.3 ±0.11°C. The mean daily average over the experiment was 12.9 ±0.08°C. Mean temperatures are shown in Figure 5.2.

b) Observations on seedling performance

The number of seedlings available did not allow a quantitative measurement of flood/temperature treatments on shoot or root growth. However general observations are made.

After 65 days shoots appeared healthy in each treatment, although roots from seedlings flooded at elevated minimum temperature were slightly greying. Total blackening of roots is often reported during

Figure 5.2 Temperature of each root zone during flooding or free-drainage treatment, Winter 1991-1992.



flooding, but has never been noted in the distal 15cm of root analysed in this thesis.

Shoot and root observations in April after 137 days treatment are noted in Table 5.2. New root growth was observed in free-drained seedlings at ambient temperature by 19th March, but was not evident at elevated root temperature.

Table 5.2. General observations of effect of 137 days overwinter treatment on shoot/root appearance

TREATMENT	SHOOT	ROOT
Free drain, ambient temp	Healthy shoot, new buds	New growth visible
Flooded ambient temp	Low degree of needle loss, slight yellowing, some seedlings bear new buds.	No growth, fragile roots
Free drain, elevated temp.	slight to extensive needle loss, some slightly browning, all bear new buds.	No growth, unhealthy appearance, lack cortical browning evident in healthy roots, as shown in Plate 4.1.
Flooded elevated temp.	severe needle loss, some new buds.	No growth, greying roots

c) Accumulation of end products of anaerobic respiration

Ethanol and malate accumulated in flooded and drained roots at ambient or elevated temperature, indicating a degree of anaerobiosis in all treatments. Both end-products were detected *in vivo* after 65 days treatment. See Table A5.1a-c.

i) Ethanol

Ethanol content of harvested roots was similar to that of Lodgepole pine (Chapter 3). Only a fraction of the ethanol produced by the roots accumulates within the tissues as a large percentage leaks through the cell membranes into the soil environment (see *in vitro* analysis at initiation of flooding, results presented in Chapter 3).

Mean ethanol contents of each root section in each treatment were compared using three-way analysis of variance, having confirmed homogeneity of sample variances. In each treatment ethanol showed a general pattern of increasing accumulation moving away from the tip (Figure 5.3), although not necessarily indicating increased production. The increase moving away from the root tip was significant at the 5% level ($F=7.19$, $df=2$, $p=0.003$). It is likely that the rate of leakage from the tissue decreases in this direction due to suberisation and lignification of the root and its increased diameter.

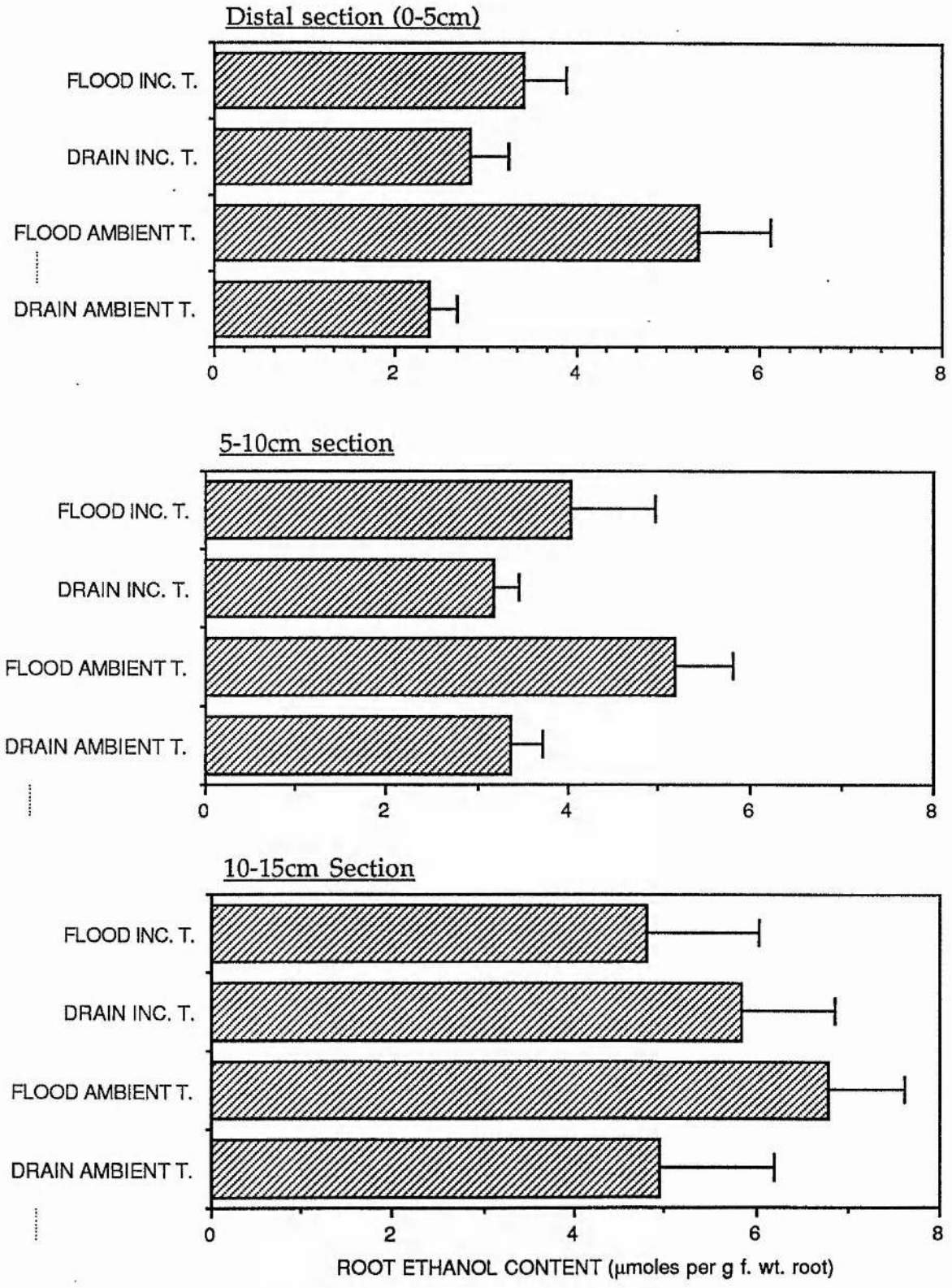
When comparing the 4 treatments obvious differences in accumulation can be seen. Flooding caused a significant increase in root ethanol content ($F=6.04$, $df=1$, $p=0.02$). In all root sections in each temperature treatment, ethanol content of flooded roots was higher than those from free drained soil (except the most proximal at elevated temperature). Flooding at ambient temperature caused a significantly greater increase in ethanol accumulation than flooding at elevated root zone temperature ($F=4.73$, $df=1$, $p=0.038$). Table 5.3 shows the ratio of ethanol production in waterlogged soil to that in freely drained soil at the same temperature. A ratio of >1 indicates increased accumulation under anoxia.

Table 5.3. Ratio of ethanol accumulation under waterlogged compared to that in freely drained soil conditions

	SECTION OF ROOT		
	0-5cm	5-10cm	10-15cm
Ambient temperature	2.3	1.5	1.4
Elevated temperature	1.2	1.3	0.82

Replicates: 4 flooded seedlings and 3 free-drained seedlings at each temperature.

Figure 5.3 Accumulation of ethanol in root tissue after 65 days flooding or free-drainage under ambient or elevated root zone temperature.



INC. T. = Treatment at root temperature elevated above ambient

AMBIENT T. = Treatment at ambient root temperature

n=4 seedlings flooded treatments,
n=3 free-drained seedlings

In general, ethanol accumulated to a similar degree in free drained seedlings irrespective of temperature. Flooding at ambient temperature lead to the highest accumulation of ethanol in the root.

Malate also accumulated in both flooded and free-drained roots, but showed a different pattern to that of ethanol (Figure 5.4). Samples showed heterogeneity of variance for root malate accumulation, reflecting large differences in sample means. Thus means were compared using t-tests allowing for equal or unequal sample variance as appropriate. Most malate accumulated in free-drained roots at ambient temperature, significantly less in freely drained roots at elevated temperature[34]. Flooding significantly reduced malate accumulation in each section, again irrespective of temperature[35]. Table 5.4 shows the ratio of malate production in waterlogged soil to that in freely drained soil at the same temperature.

Table 5.4. Ratio of malate accumulation under waterlogged compared to that in freely drained soil conditions

	SECTION OF ROOT		
	0-5cm	5-10cm	10-15cm
Ambient temperature	0.36	0.26	0.32
Elevated temperature	0.01*	0.03	0.06

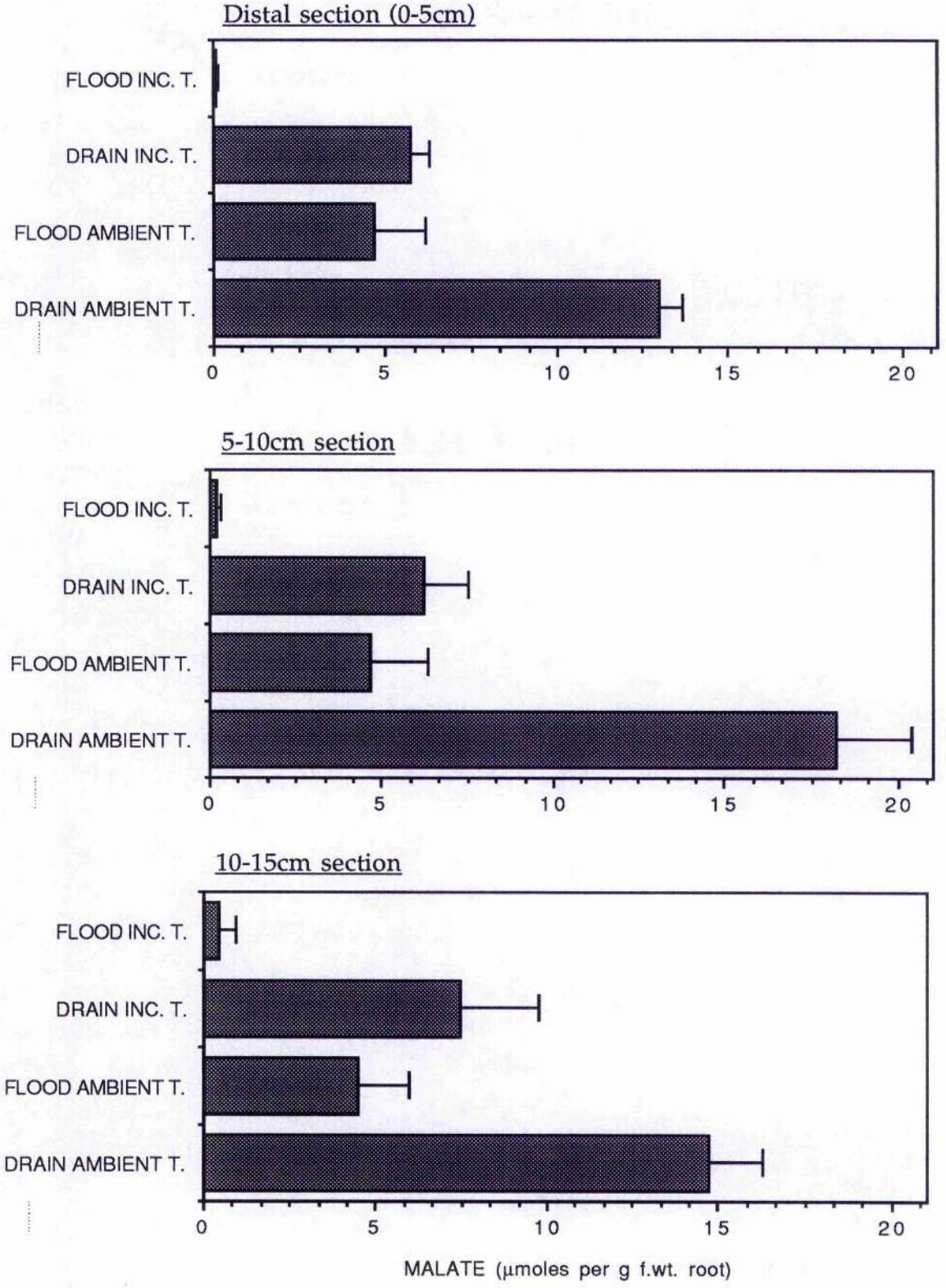
Replicates: 4 flooded seedlings and 3 free-drained seedlings at each temperature, except * flooded at elevated temperature n=3.

The degree of reduction in malate accumulation associated with waterlogging is greater at elevated temperature. The ratio of malate accumulation to that of ethanol is shown in Table 5.5. In free-drained soils, malate accumulated to a higher degree than ethanol, although the

[34] (0-5cm, p=0.0038, 5-10cm p=0.019, 10-15cm NS).

[35] Malate content is significantly lower in flooded roots
At ambient temperature, 0-5cm,p=0.0064, 5-10cm p=0.0081,10-15cm p=0.0093
At elevated temperature, 0-5cm, p=0.010, 5-10cm p=0.046, 10-15cm NS

Figure 5.4 Accumulation of malate in root tissue after 65 days flooding or free-drainage under ambient or elevated root zone temperature



INC. T.= Treatment at elevated temperature
AMBIENT T.=Treatment at ambient temperature

Seedlings flooded at ambient temperature, n=
Seedlings flooded at elevated temperature and all free drained seedlings, n=3

presence of ethanol indicated a degree of anaerobiosis. Flooding lead to a reduction in root malate and increase in ethanol content.

Graph 5.5 shows relative viability of the three root sections from each treatment for comparison with malate and ethanol production. Viability is discussed below.

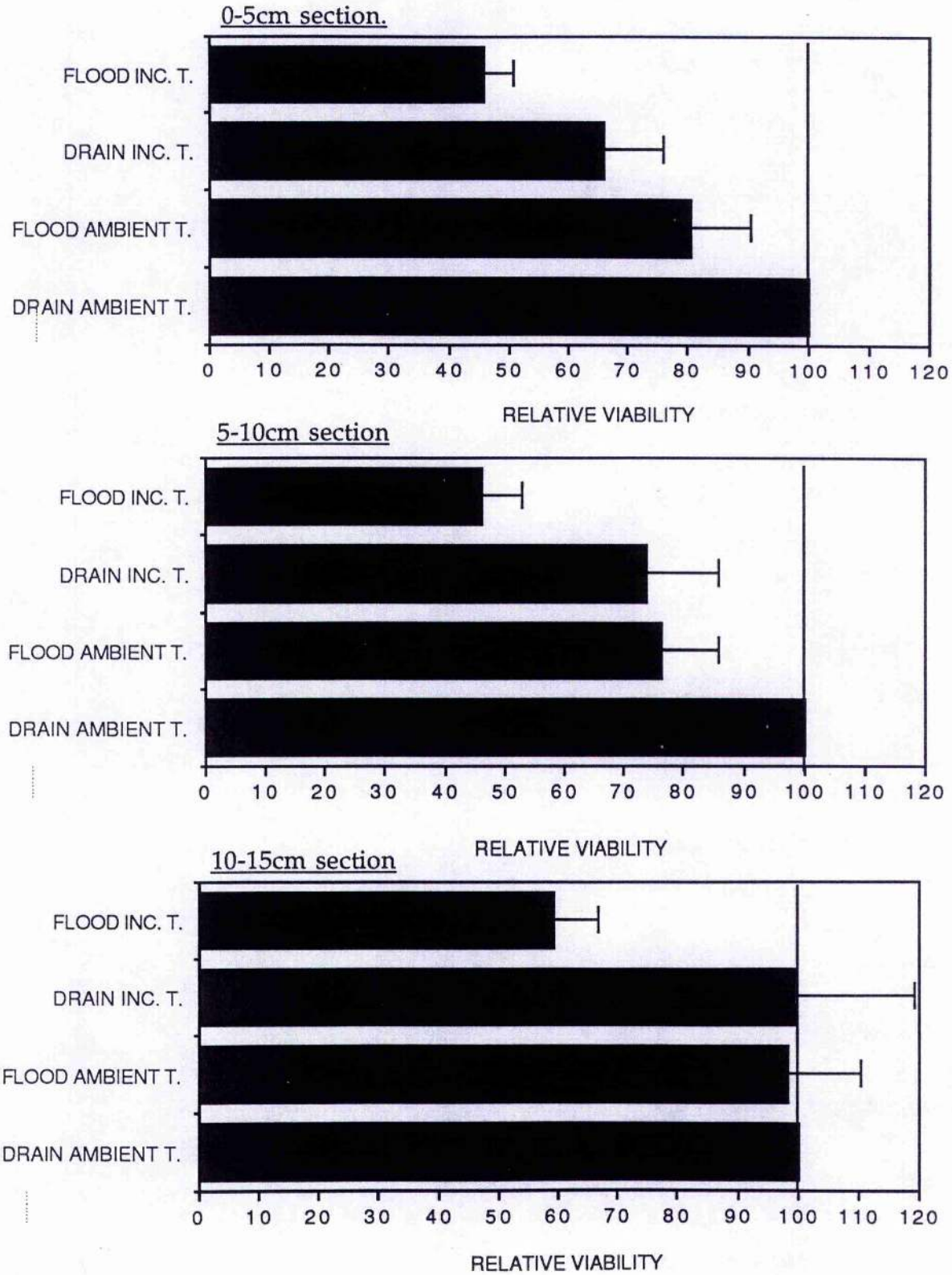
Table 5.5. Ratio of malate/ethanol accumulation in each treatment

	SECTION OF ROOT		
TREATMENT	0-5cm	5-10cm	10-15cm
Free drain, ambient temp.	5.50	5.42	2.98
Waterlogged, ambient temp.	0.88	0.91	0.66
Free drain, elevated temp.	2.04	1.96	1.28
Waterlogged, elevated temp.	0.02	0.04	0.10

d) Carbohydrate reserves over Winter in each treatment

(Table 5.6a-c). Total root carbohydrate was depleted in free drained seedlings between late November and early February (Figure 5.6). Glucose levels remained constant during this time, overall depletion reflecting a fall in starch reserves in each section (Table 5.7). Results were thus contrary to the accumulation seen during the previous Winter in 2 provenances of Sitka spruce (see Chapter 4).

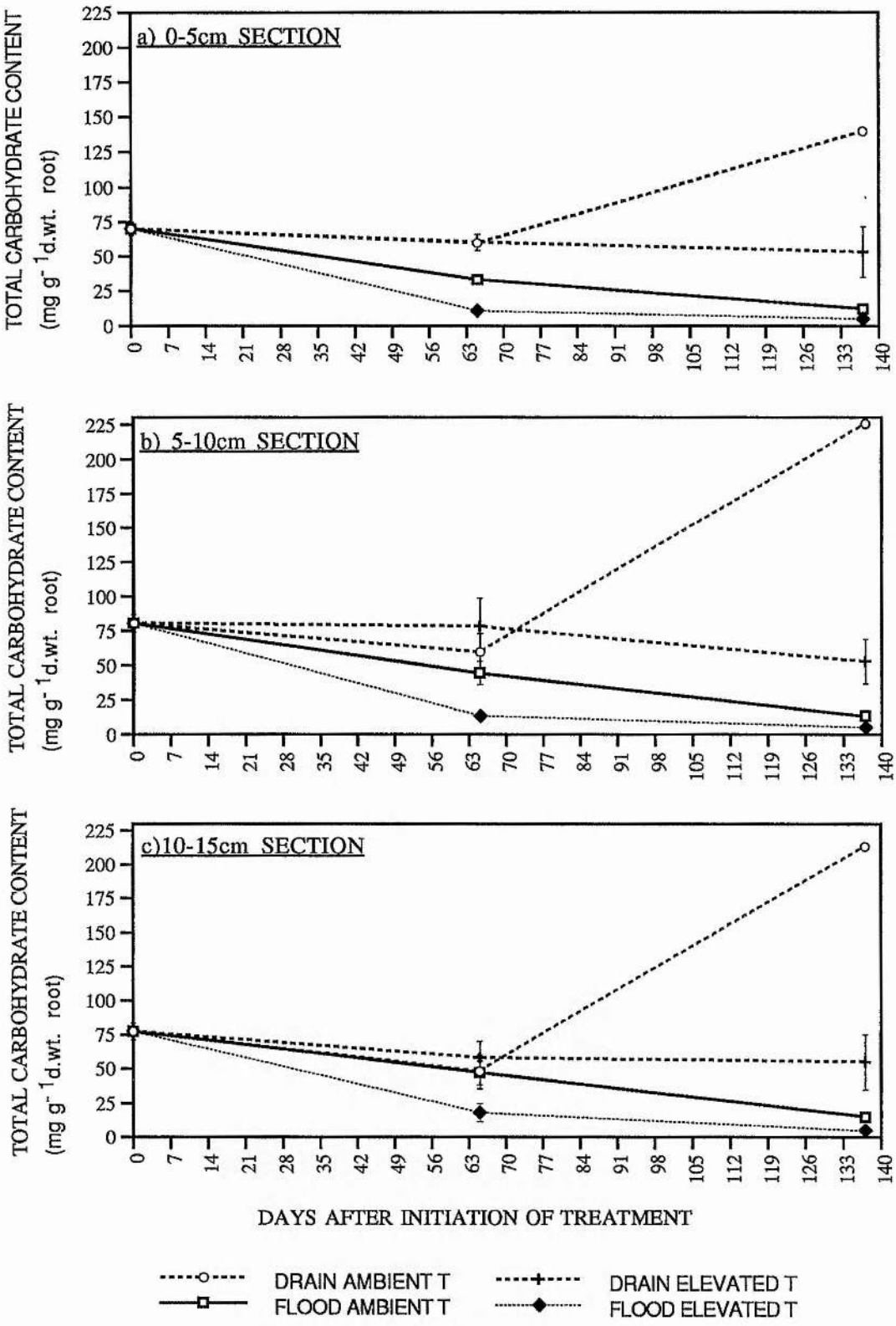
Figure 5.5 Relative viability after 65 days flooding or free-drainage under ambient or elevated root zone temperature



INC. T.=Treatment at elevated root zone temperature
AMBIENT T.=Treatment at ambient root zone temperature

n= 3 all drained seedlings,
n=4 all flooded seedlings

Figure 5.6 Change in total carbohydrate content during flooding or free-drainage in Sitka spruce roots under two temperature regimes.



Number of replicate seedlings = 1-4, (See Table 5.6 a-c)

Table 5.6 a-c. Carbohydrate content in root tissue 65 and 137 days after initiation of treatment

a) Total carbohydrate (mg g^{-1} d.wt. root).

	65 DAYS			137 DAYS		
TREATMENT	0-5cm	5-10cm	10-15cm	0-5cm	5-10cm	10-15cm
Free drain, ambient temp.	59.43 ±3.20 (3)	59.8 ±13.4 (2)	48.1 ±12.3 (3)	139.79 (1)	225.84 (1)	213.06 (1)
waterlogged, ambient temp.	33.02 ±4.28 (4)	44.57 ±8.50 (4)	47.05 ±8.53 (4)	12.10 ±1.43 (3)	13.62 ±2.36 (3)	15.13 ±2.33 (3)
Free drain, elevated temp.	60.18 ±5.96 (3)	78.3 ±20.5 (3)	58.0 ±11.9 (3)	53.2 ±18.2 (4)	52.9 ±16.0 (4)	55.1 ±20.1 (4)
waterlogged, elevated temp.	11.00 ±2.60 (3)	13.87 ±3.22 (4)	18.37 ±6.64 (4)	5.205 ±0.62 (4)	5.514 ±0.19 (4)	5.068 ±0.30 (4)

b) Starch (mg g^{-1} d.wt. root).

	65 DAYS			137 DAYS		
TREATMENT	0-5cm	5-10cm	10-15cm	0-5cm	5-10cm	10-15cm
Free drain, ambient temp.	22.16 ±4.73 (3)	24.73 ±8.85 (2)	17.83 ±9.66 (3)	108.84 (1)	197.99 (1)	185.88 (1)
waterlogged, ambient temp.	5.81 ±2.92 (4)	11.54 ±5.32 (4)	12.23 ±6.49 (4)	1.66 ±0.84 (3)	1.28 ±0.64 (3)	0.55 ±0.13 (3)
Free drain, elevated temp.	29.36 ±7.42 (3)	47.0 ±21.3 (3)	29.7 ±11.6 (3)	53.2 ±18.2 (4)	31.0 ±15.4 (4)	33.4 ±18.7 (4)
waterlogged, elevated temp.	0.82 ±0.54 (3)	2.25 ±0.22 (4)	3.11 ±1.42 (4)	5.21 ±0.62 (4)	1.82 ±0.23 (4)	1.29 ±0.19 (4)

c) Sugars (mg g^{-1} d.wt. root)

	65 DAYS			137 DAYS		
TREATMENT	0-5cm	5-10cm	10-15cm	0-5cm	5-10cm	10-15cm
Free drain, ambient temp.	37.26 ±3.01 (3)	35.06 ±4.54 (2)	30.26 ±7.39 (3)	30.944 (1)	27.86 (1)	27.18 (1)
waterlogged, ambient temp.	27.21 ±3.25 (4)	33.04 ±4.87 (4)	34.82 ±4.91 (4)	10.44 ±2.26 (3)	12.35 ±3.00 (3)	14.57 ±2.45 (3)
Free drain, elevated temp.	30.82 ±3.71 (3)	31.25 ±1.52 (3)	28.32 ±2.84 (3)	22.40 ±2.49 (4)	21.92 ±1.40 (4)	21.76 ±3.11(4)
waterlogged, elevated temp.	10.18 ±2.73 (3)	11.62 ±3.10 (4)	15.26 ±5.29 (4)	3.01 ±0.67 (4)	3.69 ±0.21 (4)	3.782 ±0.24 (4)

Values = mean ±se
Number of replicate samples, each from separate seedlings, shown in brackets.

Table 5.7. Changes in root carbohydrate between late November and early February. (mg g^{-1} d.wt.root)

		SECTION OF ROOT		
	ANALYSIS DATE	0.5cm	5-10cm	10-15cm
Total carbohydrate	*November*	70.02 ±4.8	80.6 ±6.2	77.2 ±6.2
	February	59.43 ±3.2	59.8 ±13.0	48.1 ±12.0
Starch	*November*	32.5 ±6.0	45.8 ±6.0	45.9 ±5.1
	February	22.16 ±4.7	24.7 ±8.9	17.8 ±9.7
Sugars	*November*	37.50 ±1.73	34.82 ±1.08	31.32 ±2.62
	February	37.26 ±3.01	35.06 ±4.54	30.26 ±7.39

Values shown are means ±se for 4 replicate seedlings in November and 2-3 in February. Comparing of each section between November and February, mean contents are not statistically significant at $p \leq 0.05$.

During February and March, starch reserves increased approximately 5-10 fold, sugar content remaining at the steady level of approx. 30mg g^{-1} d.wt. root. This is in agreement with the accumulation of starch during February noted in the same provenance during the previous Winter, as is the level of root sugars.

Maintaining the minimum root temperature at 12°C did not lead to increased carbohydrate depletion in the freely drained roots. After 65 days elevated temperature, the mean carbohydrate content of each root section was not significantly different to those from ambient temperature. However, when roots were maintained at elevated temperature until April, the root reserves were not augmented as they were at ambient temperature, and were consequently much lower.

Flooding caused a reduction in carbohydrate content irrespective of root zone temperature. The importance of soil temperature during flooding was investigated by calculating the degree of carbohydrate depletion in flooded roots relative to those of freely drained seedlings at the same temperature. Total carbohydrate remaining in each root was expressed as a percentage of the mean carbohydrate content of freely drained seedlings at the same temperature. Each percentage was

transformed into the arcsin √percentage such that the degree of depletion could be compared statistically. Arcsin transformed samples showed heterogeneity of variance, such that comparison of results using analysis of variance was invalid. Means were compared using t-tests with equal or unequal variances as appropriate. Actual percentages are shown in table 5.8.

Table 5.8. Total carbohydrate remaining in flooded roots as percentage of that in freely drained controls at the same temperature and time

	65 DAYS			137 DAYS		
	0-5cm	5-10cm	10-15cm	0-5cm	5-10cm	10-15cm
FLOODING AT AMBIENT TEMPERATURE	55.57% ±7.20 (4)	74.84% ±14.21(4)	97.81% ±17.73(4)	8.65% ±1.02 (3)	8.08% ±0.06(3)	7.10% ±1.09 (3)
FLOODING AT ELEVATED TEMPERATURE	18.28% ±4.32 (3)	22.58% ±5.80 (4)	31.68% ±11.45(4)	8.65% ±1.03 (4)	7.04% ±0.24 (4)	8.74% ±0.51 (4)

Values = mean ±se
Number of replicate samples, each from separate seedlings, shown in brackets.
After 65 days flooding, depletion of carbohydrate was greater at higher temperature:- tip p=0.0096; 5-10cm p=0.024), 10-15cm p=0.025).
After 137 days waterlogging, means not statistically significant except 5-10cm section, p=0.018.

The importance of temperature on rate of carbohydrate depletion is clearly seen by comparing its depletion under the 2 temperature regimes during 2 months waterlogging. Flooding alone reduced root reserves (except in the proximal section at ambient temperature), but reserves were depleted to a much greater extent in conjunction with elevation of minimum root temperature. In each case the degree of depletion was more severe moving towards the tip. Two months of flooding at ambient temperature reduced root carbohydrate content to half that in free drained seedlings whereas only one fifth of the reserves remained at elevated temperature. A further 72 days of flooding, however, resulted in equally low levels of carbohydrate at both temperatures, less than 10% remaining in each section.

Actual levels of carbohydrate remaining in the root were compared to see which reserves were depleted in each treatment. Again t-tests were used to compare means because samples showed heterogeneity of variance and did not satisfy the requirements of ANOVA.

After 65 days flooding.

At ambient temperature

When seedlings were flooded at ambient temperature only the most distal section showed significantly reduced total carbohydrate content ($p=0.0056$). This reflected a significant fall in starch reserve ($p=0.027$), sugar levels being maintained in each section.

Elevated minimum temperature.

Flooding at higher temperature resulted in the depletion of both starch and sugars. Total carbohydrate reserves were greater in each section of free drained roots in comparison to flooded seedlings at the same temperature, flooding resulting in large scale depletion, though only statistically significant in the tip, $p=0.017$. Free drained seedlings contained higher sugar in each section[36] and more starch[37].

After 65 days flooding, each section from higher root zone temperature had significantly lower total carbohydrate content than those flooded at ambient temperature, reflecting lower starch reserve and significantly lower sugar content. Probability values are shown in table 5.9.

[36] (0-5cm $p=0.011$, 5-10cm $p=0.0039$, 10-15cm NS)

[37] Drained seedlings contain much more starch,but means not significantly different at $p\leq0.05$ due to low number of replicates.

Table 5.9. Statistical comparison of reserves remaining in flooded seedlings at 2 temperatures. Each carbohydrate was higher in roots of seedlings flooded at ambient temperature

	SECTION OF ROOT		
	0.5cm	5-10cm	10-15cm
TOTAL CARBOHYDRATE	0.010	0.015	0.038
STARCH	NS	NS	NS
SUGAR	0.013	0.010	0.035

After 137 days flooding.

Flooding at ambient temperature greatly reduced carbohydrate reserves relative to free-drained samples analysed concurrently, mainly reflecting the fall in root starch but also lower sugar levels (see table 5.6a-c). High temperature flooding had the same effect, but in addition caused significant reduction in sugar content[38] in comparison to free drained seedlings at the same temperature. After 137 days flooding, roots from the 2 temperature regimes had a similar minimal starch content, though roots waterlogged at ambient temperature did retain more sugar in each section[39], as well as slightly higher TNC.

e) Root relative viability

Viability is measured quantitatively using the TTC test, as described in Chapter 2. Tissue health is determined as the extent of TTC reduction to the red formazan derivative per unit weight tissue. This gives a 'viability index'. This figure is then compared to that of the healthy control plant to give percentage or 'relative viability'. In this case, all viability indices are calculated as a percentage of the mean viability index

[38] Total carbohydrate and starch content: not significantly lower in flooded roots
Sugar content, 0-5cm, p=0.0003, 5-10cm p=<0.0001, 10-15cm p=0.010

[39] Sugar content, 0-5cm , p=0.015; 5-10cm NS, 10-15cm p=0.048
Total carbohydrate content, 0-5cm, p=0.0044, 5-10cm NS, 10-15cm p=0.050.
Starch non-significant except 10-15cm at elevated temperature.

of free-drained seedlings at ambient temperature. This allows the effect of the 3 stress treatments to be compared:

Flooding at ambient root zone temperature (RZT)

Free drainage at elevated minimum RZT

Flooding at elevated RZT.

65 days of Winter stress.

Figure 5.7 and 5.8a.

The mean viability indices of each type of root in each treatment were compared after 65 days using three-way analysis of variance, having confirmed homogeneity of variance between samples. Flooding caused significant reduction in viability ($F=15.89$, $df=1$, $p=0.001$), as did increased soil temperature ($F=24.27$, $df=1$, $p<0.001$). For each root section flooding at increased temperature was most damaging to the root tissue, causing >50% reduction in viability in the distal section. Elevating the minimum temperature of freely drained roots over Winter caused a similar degree of damage to flooding at ambient temperature.

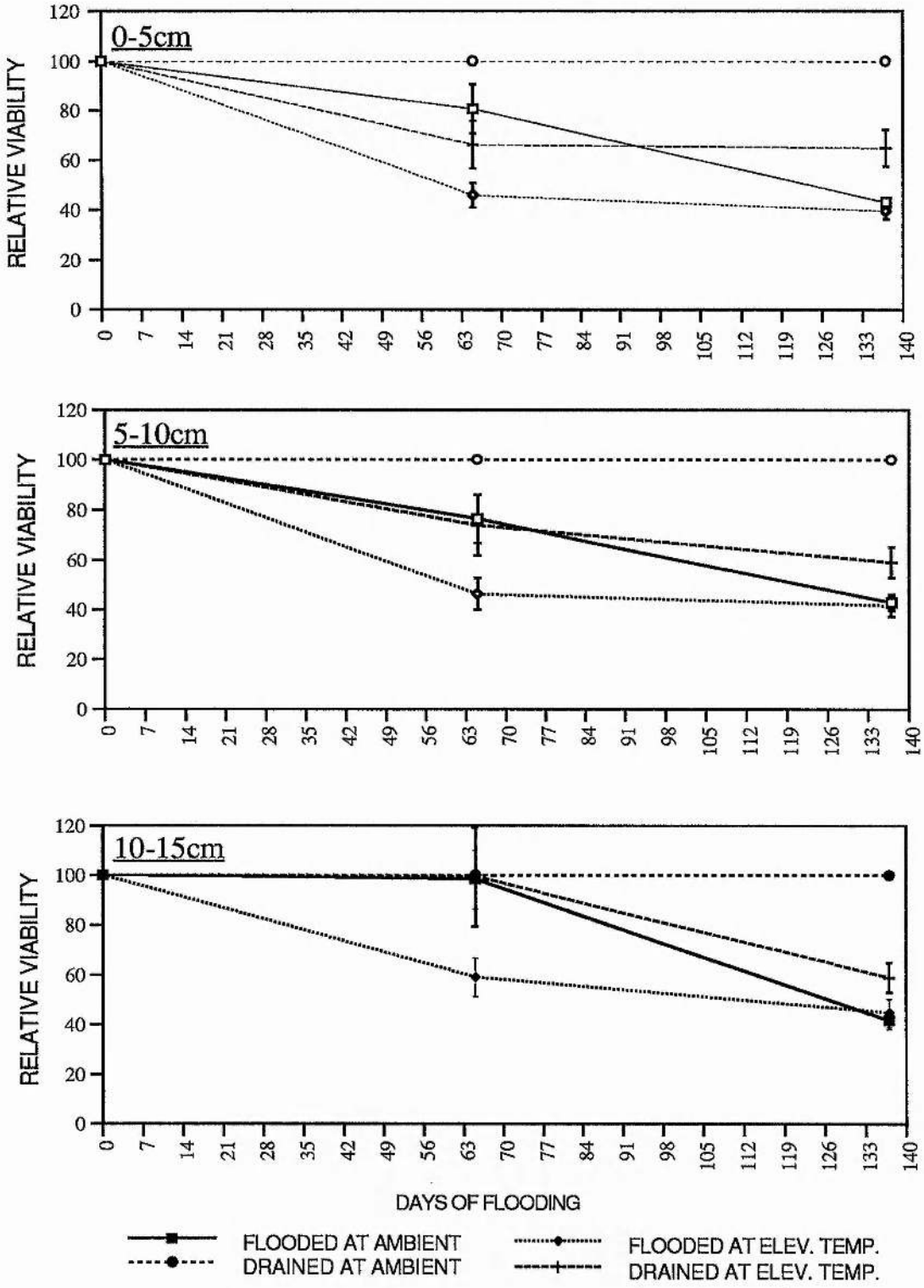
The reduction in viability caused by each overwinter stress was in general greatest in the root tip and decreased moving up the root system. The most proximal section was most hardy to each Winter stress, only roots flooded at high temperature being damaged. Differences between the sections were not however significant at the 5% level.

137 days of Winter stress.

In all sections and all treatments, relative viability is further reduced under continued stress (Figure 5.7 and 5.8b).

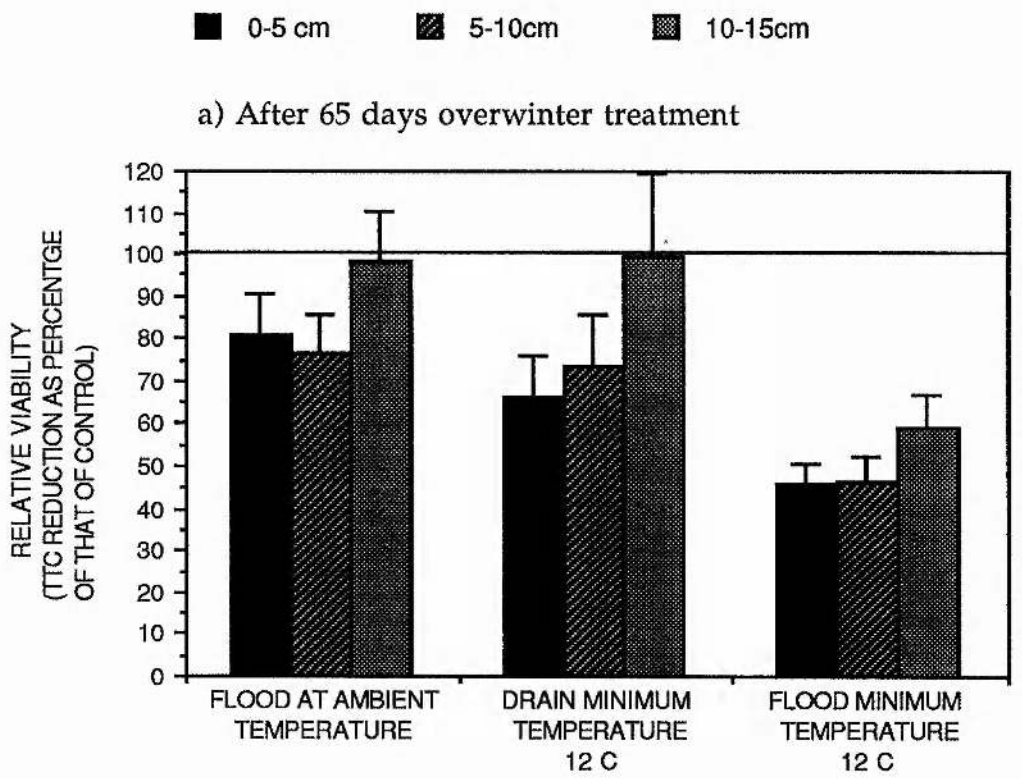
Comparing the mean relative viability values in table A5.2a-c, it appears that values of approximately 45-50% relative viability indicate major tissue damage as they do not fall much further when the extreme stress

Figure 5.7 Root relative viability during overwintering in flooded or drained conditions at ambient or elevated root zone temperature



Number of replicate seedlings:-65 days flooding, n=4 (flooded treatments)
n=3 (drained treatments)
137 days, n=4 (flooded treatments)
n=2 (drained at ambient; n=5 drained at elevated temperature).

Figure 5.8 Effect of elevated temperature and flooding, alone or in combination, on the viability of Sitka spruce roots.



(in this case flooding at high temperature) is maintained. Thus 65 days flooding at high temperature seems to kill each root section.

Continued flooding at ambient temperature reduced relative viability of the root to the same 'minimal' level as those flooded at elevated RZT. The freely drained seedlings at elevated root zone temperature remain least damaged after 137 days, although the viability indices are low and may constitute irreparable damage.

To examine the importance of flood temperature, viability index for the flooded seedlings was calculated as a percentage of the mean viability indices of free drained plants at the same root temperature. Results are shown in Table 5.10.

Table 5.10. Viability index as percentage of mean viability index of free drained seedlings at same root zone temperature (after 65 days of treatment)

	SECTION OF ROOT		
	0-5cm	5-10cm	10-15cm
FLOODING AT AMBIENT TEMPERATURE	80.6% ±9.9	76.4% ±9.5	98.3% ±12.0
FLOODING AT ELEVATED TEMPERATURE	69.4% ±7.3	62.7% ±8.7	59.5% ±7.8

mean ±se for n=3 seedlings drained treatments
n=4 flooded treatments.

Results clearly show that flooding is most damaging to the root when in conjunction with higher root zone temperature.

5.4 DISCUSSION

This experiment aimed to assess the effect of increased temperature or waterlogging, or a combination of each on the physiology of over-wintering Sitka spruce roots. Soil temperature and root metabolic activity are important factors in determining the survival of water-logging. Research by Coutts and Philipson (1978a) has demonstrated that Lodgepole pine and Sitka spruce cuttings suffer most damage when partially flooded during active growth, all root tips and part of the primary root dying over 28 days. Survival was increased by reducing soil temperature, and all tips survived if flooded at low temperature when dormant. Seedlings show the same response, actively growing Sitka spruce are eventually killed by 22 days of flooding at 15°C whereas plants dormant at flood initiation show greater tolerance (Coutts 1981). The control and effects of root dormancy on flood tolerance were detailed in the general introduction (Chapter 1), being of common importance to each chapter in the thesis.

Root zone temperature was recorded using max-min thermometers placed between the perspex tubing in the root boxes. In similar root boxes, Coutts and Nicoll (1990a) found the temperature within the perspex tubes and that around them to be within 0.5°C. Thus the values obtained were taken to indicate root temperature. As root temperature was checked only intermittently to make sure that the thermostated heater was functioning sufficiently, not enough data was obtained to calculate representative means in the 3 treatments during the Winter. However, growth of Sitka spruce has already been monitored throughout a whole Winter and the growth-temperature relationship investigated (see Coutts and Nicoll 1990a). The main objective of this experiment was to investigate the effect of higher root zone temperature on the carbohydrate depletion and metabolite accumulation of the root.

The minimum temperature chosen (13°C) represents a large warming of the soil, and prevents daily fluctuations normally experienced by the roots. It does not represent the likely warming of the soil in a 'Greenhouse world' but is intended to test the root system under an extreme condition which may give an indication of the type of response under more realistic temperature changes. This was a pilot experiment and results on the rate of carbohydrate depletion and decline in root viability proved useful in designing future tests where more realistic soil temperature are maintained.

New root growth was observed by March 19th in freely drained seedlings at normal seasonal temperature. This corresponds to root growth initiation dates reported previously for Sitka spruce, e.g. 10th March (Coutts and Nicoll 1990a) and early March (Cannell *et al.* 1990). Flooded seedlings and those free-drained at elevated minimum temperature showed no signs of growth.

In discussing the results it is important to remember that only the distal 15cm of root was measured. This was chosen as it represents the most sensitive part of the root system which dies back when seedlings are flooded during the Winter (e.g. Coutts and Philipson 1978a, Coutts and Nicoll 1990b). Although changes in the total carbohydrate content of free-drained roots were not statistically significant they decreased slightly between November and February due to a fall in starch reserves. This is not typical of Sitka spruce which maintains a positive carbon balance even at low temperatures (Neilson *et al* 1972). Bradbury and Malcolm (1978) found a dry matter gain between late September and mid-April in Sitka spruce seedlings in Southern Scotland, increases being similar in root and shoot until mid-January. Cannell *et al* (1990) however reported fairly constant whole seedling TNC levels for Sitka spruce lifted over the same period. The large increase in root starch content seen between

February and April may be explained by starch deposition in the growing root, as reported in Douglas fir seedlings at 10°C (Marshall and Waring 1985). Total carbohydrate of the whole seedling is reported to show similar increase, again concurrent with new root and shoot growth (Cannell *et al* 1990). Although maintaining the roots at higher minimum temperature would lead to increased costs of maintenance respiration (Lawrence and Oechel 1983, Amthor 1984), after 65 days the carbohydrate levels of freely drained seedlings was not significantly different from those at normal Winter temperatures. However roots at higher temperature had not incurred starch deposition by April, perhaps as they were not growing and were injured by the treatment (see root viability measurements).

Flooding depletes carbohydrate reserves relative to free drained seedlings because they are respired to meet maintenance respiration needs of the roots and are not augmented by phloem transport from the shoot. The effect of flooding on glycolytic rate cannot be estimated by comparing the carbohydrate content of flooded and free drained roots because of this lack of substrate supply. However, the response of excised roots to anoxia in terms of carbon dioxide production under air and nitrogen suggest that an acceleration of glycolysis under flooding may occur, at least initially, and cause rapid depletion of reserves (see chapter 3). After 65 days flooding, depletion of total carbohydrate is most severe in the root tip when seedlings are flooded at either temperature, and less so moving up the root system. This may indicate higher metabolic activity of the root tip, even under dormant conditions. Greater carbohydrate depletion and increased dieback may also be explained by the limited oxygen transport capacity of the primary root (Coutts and Nicoll 1990b). Sitka spruce roots with secondary growth transport oxygen in their outer tissues (Philipson and Coutts 1980b) and are more tolerant

to waterlogging (Coutts 1982). It may be that more proximal roots have some secondary growth and are benefiting from a certain amount of oxygen supply from the shoot. This could be investigated in future work by microscopic analysis of the distal 15cm section.

Seedlings flooded at ambient temperature for 65 days contained lower starch reserve in comparison to free drained seedlings, but maintained the same glucose levels. Continued flooding caused a drop in glucose content which could not be augmented by the starch reserves, now practically zero. Higher temperature during flooding lead to greater reduction in carbohydrate reserve, such that after only 65 days the total reserve was significantly lower and glucose reserves were dramatically reduced. This greater depletion is likely to be due to the effect of temperature on maintenance respiration rate. In free drained seedlings at elevated temperature root reserves were not depleted in comparison to those at ambient temperature over the 65 day period. These reserves may however be depleted but then augmented by those from the shoot or other parts of the root system. This could only be investigated by analysing whole seedling carbohydrate content.

After 137 days of flooding, seedlings from both temperature regimes retained minimal reserves, though those flooded at lower temperature contain slightly more glucose. Depletion of reserves in the root may be especially damaging for Sitka spruce in terms of establishment of the root system in Spring. Unlike Douglas fir, Sitka spruce root growth may initially be supplied by starch reserves in the roots themselves, rather than by current photosynthate (Philipson 1988).

Data for accumulation of anaerobic end products in the root tissues can be misleading and conclusions must be drawn carefully. In this case the ethanol and malate levels in the root tissue are not necessarily representative of the relative amounts produced by metabolism, due to

leakage from the tissue or movement within the plant. Both temperature and flooding may affect these two processes. Under higher temperature, ethanol leakage may increase, and the removal of malate and ethanol in the transpiration stream may be affected by stomatal closure and reduced membrane permeability of the roots during waterlogging. Ethanol is likely to leak out of the root tissue to a much greater extent than malate. It is lipid soluble and diffuses easily through the cell membrane. When root sections were incubated in anoxia for 24hrs, between 56 and 70% of the total ethanol produced was found in the water bathing the roots (Table A3.2). Similar leakage has been reported for excised roots of swamp tupelo and Loblolly pine incubated in nitrogen for 4 hours. Hook *et al* 1983 found 55% and 85% (of the total ethanol produced in each species respectively) escaped from the roots into the surrounding medium. Malate accumulated in the roots but the authors could not detect any leakage. Loss of ethanol is advantageous as its accumulation within the root tissue may cause cellular injury. However it also causes the loss of a large source of fixed carbon. Debate goes on as to whether ethanol accumulating in root tissues actually reaches directly toxic levels. Jackson, Herman and Goodenough (1982) concluded that artificial application of ethanol, even when greatly in excess of that accumulating naturally does not simulate flooding injury. In contrast, Andrews and Pomeroy (1979) have shown that application of exogenous ethanol and CO_2 to wheat at concentrations equivalent to or lower than those accumulating naturally in the seedlings increases cell permeability and reduces survival. Crawford and Zochowski (1984) report increased survival of a number of crop species by circulating the anaerobic environment, and in effect reducing their ethanol content 13 fold. It is thought that the removal of ethanol alone enhances survival, CO_2 accumulation in flood-intolerant chickpea seedlings being a

compounding factor as it increases ethanol accumulation. The accumulation of ethanol may be more dangerous to plants flooded in the long term due to its intracellular build up (Barclay and Crawford 1981, 1982). and may be injurious due to its oxidation to more toxic acetaldehyde when the water table subsides and tissues are re-oxygenated (Studer and Braendle 1988, Crawford 1989).

Production and accumulation of malate would reduce this loss of fixed carbon to the environment. Malate may be moved up to the shoot in the transpiration stream and used as a carbon skeleton for the synthesis of new compounds. Indeed, high levels of malate are found in the rising sap of birch trees in Spring after the high Winter water tables have subsided (Crawford 1972). The role of malate in anaerobic survival is also controversial. It may act as an alternative end product, thus reducing ethanol accumulation. Its accumulation during waterlogging is associated with flood tolerance in a number of herbaceous species (Crawford and Tyler 1969).

Even during Winter, free drained dormant roots suffer a degree of anoxia apparent by the presence of ethanol. At both ambient and elevated temperature, more ethanol accumulates in flooded roots than free-drained roots, as would be expected due to lack of oxygen. The levels of ethanol found in flooded roots *in vivo* (5.3-6.8μmoles/gFWroot) are in close agreement with those observed by Crawford and Baines (1977) for the same species in unaerated water culture at 24°C. The authors however reported a 12 fold increase in ethanol accumulation during the first 23hrs of flooding, whereas this study observed only 1.4 to 2.3 fold increases, and even less at higher temperature. This may be explained by the different culture media and the time scale of the experiment. Roots growing in freely drained peat are likely to suffer from a greater degree of hypoxia than those in solution with forced aeration. Also the sudden

increase in the rate of ethanol production seen over the first 24 hrs. of anoxia is not likely to be sustained in the long term. In this work ethanol was determined after 65 days waterlogging.

Malate accumulates in much higher concentrations in free drained compared to flooded roots. Its concentration within the tissue is greater than that of ethanol. If malate accumulation is proportional to its production, the metabolism of the roots agrees with Crawford's theory of flood tolerance. Crawford suggested that increased flood tolerance is often associated with minimisation of ethanol accumulation by low ADH content and formation of alternative end-products. The very low ratio of malate to ethanol seen in flooded roots may be expected from a flood-intolerant species such as *Picea sitchensis*. A similar reduction in malate content is reported for flood-tolerant river birch and intolerant European birch when moved from aerated to N_2 gassed media (Tripepi and Mitchell 1984a). After 6 days European birch roots contained 0.7µmole/gFW malate compared to 23.2 in aerated roots. Thus the concentration of malate in aerated roots is similar to that observed in free drained Sitka spruce roots at ambient Winter temperatures (13-18.2µmole/gFW). In both species root malate decreases under anoxia, but the fall is much greater in the birch.

The TTC test provides a very useful measure of tissue viability. By calculating the degree of TTC reduction relative to that of untreated control seedlings a measure of tissue health is obtained. The 'relative viability' of seedlings from different overwinter treatments can then be compared to assess the severity of cellular damage incurred. In this case injury caused by waterlogging or elevated Winter root zone temperature is compared when either alone or in combination. Relative viability values are also useful in determining post-treatment survival. Ideally values of root relative viability should be compared with root growth

potential analysis or at least observations on post-treatment root growth or die-back. Steponkus and Lanphear (1967) were first to determine the correlation between TTC reduction immediately following injury and survival in the long term. After subjection to various sub-zero temperatures, stems and leaves of *Hedera helix* showed a range of relative viabilities, those of 50% and less being associated with future death of the tissue. This figure has since been quoted as the lethal level in the roots of a number of conifers. In Lodgepole pine, Scots pine and Norway spruce roots, Lindstrom and Nystrom (1987) reported that after freezing treatments, a 50% fall in TTC reduction correlated with low root growth capacity and general seedling death*. The 50% level is reported to indicate 'serious injuries to the roots which may be detrimental to water uptake and net photosynthetic capacity and growth' (see Lindstrom and Mattsson 1989). Thus although actual correlations between TTC reduction and future survival are not available for the data presented here, published data on roots of similar species give an indication of the extent of injury.

Flooding at high temperature is most damaging to the root. When compared to the 'lethal levels' recorded for roots of similar species. values of relative viability after only 65 days of flooding suggest tissue death in at least the distal 10cm. High temperature flooding also leads to greatest depletion of root carbohydrate reserves. Such large scale loss of carbohydrate will have great effect on cell function, energy levels are likely to be very low, and processes needed for cell maintenance will fail. The lack of turgor observed in flooded roots may be due to membrane damage. Obviously the injuries associated with anoxia are many and complex (see general introduction). During anoxia soil toxins, metabolite accumulation and mineral nutrient deficiency may all damage the

* Determined by total lack of root growth during 3 week root growth tests under ideal growth room ideal conditions

tissues, as may post-anoxic oxidative injury. However, the large scale depletion of carbohydrates recorded in the roots must be an important factor. The accumulation of ethanol in the roots does not seem to be associated with a large reduction in viability. Roots flooded at ambient temperature for 65 days contained more ethanol than seedlings flooded at higher temperature, but only showed slight reduction in relative viability.

Although carbohydrates were not depleted in drained roots at elevated temperature over the first 65 days, root viability was reduced in the distal 10cm indicating other causes of root damage. After 137 days however these seedlings were more viable than those from each flood treatment, correlating with higher remaining carbohydrate reserves.

The results show that a combination of higher watertables with warmer Winter soil temperatures could strongly aggravate the dieback of Sitka spruce roots. The experimental set up was obviously very basic, but a useful starting point for future investigations and a physiological 'back-up' to the work of Coutts *et al.* described alongside. Future research combining the physiological measurements with observations on root growth after each treatment would be most useful, to see if such depleted carbohydrates are replaced and how the quantification of tissue viability relates to dieback.

5.5 APPENDIX

Table A5.1. Root tissue malate and ethanol accumulation and root viability after 65 days flooding or free-drainage under ambient and elevated root zone temperature

0-5cm SECTION	FREELY-DRAINED SOIL		FLOODED SOIL	
	AMBIENT TEMPERATURE	ELEVATED TEMPERATURE	AMBIENT TEMPERATURE	ELEVATED TEMPERATURE
ROOT MALATE CONTENT (μmoles g^{-1}f.wt.)	12.993 *±0.681*	5.715 *±0.575*	4.686 *±1.437*	0.069 *±0.038*
ROOT ETHANOL CONTENT (μmoles g^{-1}f.wt.)	2.362 *±0.302*	2.806 *±0.447*	5.353 *±0.783*	3.405 *±0.484*
% RELATIVE VIABILITY	100	66.179 *±9.677*	80.624 *±9.856*	45.950 *±0.4.817*

5-10cm SECTION	FREELY-DRAINED SOIL		FLOODED SOIL	
	AMBIENT TEMPERATURE	ELEVATED TEMPERATURE	AMBIENT TEMPERATURE	ELEVATED TEMPERATURE
ROOT MALATE CONTENT (μmoles g^{-1}f.wt.)	18.186 *±2.193*	6.204 *±1.343*	4.688 *±1.671*	0.165 *±0.128*
ROOT ETHANOL CONTENT (μmoles g^{-1}f.wt.)	3.357 *±0.365*	3.174 *±0.278*	5.175 *±0.647*	4.030 *±0.934*
% RELATIVE VIABILITY	100	73.863 *±12.015*	76.417 *±9.497*	46.284 *±6.449*

10-15cm SECTION	FREELY-DRAINED SOIL		FLOODED SOIL	
	AMBIENT TEMPERATURE	ELEVATED TEMPERATURE	AMBIENT TEMPERATURE	ELEVATED TEMPERATURE
ROOT MALATE CONTENT (μmoles g^{-1}f.wt.)	14.710 *±1.573*	7.480 *±2.305*	4.485 *±1.500*	0.465 *±0.465*
ROOT ETHANOL CONTENT (μmoles g^{-1}f.wt.)	4.938 *±1.272*	5.851 *±1.003*	6.796 *±0.834*	4.804 *±1.237*
% RELATIVE VIABILITY	100	99.401 *±19.985*	98.310 *±11.966*	59.124 *±7.705*

Tabulated values are mean ±se of the mean for a number of replicate seedlings:-
Ethanol; flooded seedlings n=4, drained seedlings n=3.
Malate; seedlings flooded at ambient temperature n=4,
free drained seedlings and those flooded at elevated temperature n=3,
Viability; n=4 flooded seedlings, n=3 free drained seedlings.

Table A5.2. Relative viability after over-wintering under flooded / free drained conditions at ambient or elevated root zone temperature

0-5cm SECTION

ANALYSIS TIME	TREATMENT		
	FLOOD AT AMBIENT TEMPERATURE	FLOOD AT HIGH TEMPERATURE	DRAIN AT HIGH TEMPERATURE
65 DAYS	80.624 ±9.856	45.950 ±4.817	66.179 ±9.677
137 DAYS	43.227 ±1.224	39.704 ±3.252	64.889 ±7.522

5-10 cm SECTION

ANALYSIS TIME	TREATMENT		
	FLOOD AT AMBIENT TEMPERATURE	FLOOD AT HIGH TEMPERATURE	DRAIN AT HIGH TEMPERATURE
65 DAYS	76.417 ±9.497	46.284 ±6.449	73.863 ±12.015
137 DAYS	42.920 ±3.297	41.559 ±4.217	59.160 ±6.176

10-15cm SECTION

ANALYSIS TIME	TREATMENT		
	FLOOD AT AMBIENT TEMPERATURE	FLOOD AT HIGH TEMPERATURE	DRAIN AT HIGH TEMPERATURE
65 DAYS	98.310 ±11.966	59.124 ±7.705	99.401 ±19.985
137 DAYS	41.840 ±3.375	44.957 ±5.670	58.951 ±5.891

In each case mean ± se of the mean is shown.

REPLICATES

	65 DAYS	137 DAYS
FLOOD AT AMBIENT TEMP.	4	4
FLOOD AT ELEVATED TEMP.	4	4
DRAIN AT ELEVATED TEMP.	3	5*
DRAIN AT AMBIENT TEMP.	3	2

* Except 10-15cm section, 4 replicates.

CHAPTER 6

Effect of Over-Winter Cold Storage on Survival of Sitka Spruce During Spring Waterlogging

6.1 INTRODUCTION

Refrigerated storage of Spring-lifted conifer seedlings, as a method for extending the planting season (Cram and Lindquist 1981) began early in the 1960's. Cold storage is now a very important process in practical forestry where the lifting and planting seasons are short. In Scotland the 'normal' planting season, during late Winter and Spring, is ~10-15 weeks (Low 1973), and even more restricted in other areas such as the heavily forested Pacific Northwest. Transplants are very susceptible to handling, foresters must ensure that seedlings are handled carefully and do not overheat or dry out between the nursery and plantation. Rough handling and damage to root apices leads to reduced survival and early growth (see Low 1987). Desiccation and rough handling of Sitka spruce seedlings has been shown to reduce the root growth potential (RGP) to 59 and 85% respectively, and in combination RGP is reduced 98% (Deans *et al* 1990).

Cold storage has a number of advantages. If lifted when fully dormant, cold storage at -2 to +2°C prolongs dormancy (Brown 1973). This improves the condition of the seedling for transplanting, as dormant seedlings are less susceptible to rough handling. It also allows extension of the planting season. This is essential in a number of areas, e.g. high elevation sites in the Pacific Northwest (Ritchie 1982). In New Zealand snow coverage until early Summer necessitates the storage of seedlings lifted in the Winter before Spring flushing, until the areas are accessible. If seedlings were lifted from the lower altitude nurseries at the time of planting, growth would have already begun and loss of new tissue and seedling mortalities would be inevitable. Cold storage allows maintenance of dormancy until planting (McCracken 1979).

In Britain, seedlings are typically lifted from mid-November through to December and January, stored in plastic in the dark at -1 or +2°C for periods of around 3 months, until planting out at the end of

March. Recent research has examined the effects of cold storage on a range of physiological parameters in an attempt to relate these to root growth potential (RGP). Results suggest that the RGP depends as much on the morphology of the root (e.g. number of undamaged root apices) as on the physiology of the shoots. Levels of reserve carbohydrate are severely depleted in cold storage, Sitka spruce *whole seedling* TNC being only ~60mg/gDW after 2 months cold storage compared to ~110 in nursery overwintered seedlings (Cannell *et al.* 1990). TNC in the roots showed a slightly different pattern of depletion, cold stored seedlings contained less than half that of the nursery over-wintered seedlings when compared in April after only 2-3 months of cold storage (B. Mason unpubl. data).

The difference in April carbohydrate content of cold stored as compared to nursery overwintered seedlings shown in figure 6.0a is mainly due to the increase in root reserves seen in the latter rather than the depletion of reserves during storage. However depletion of carbohydrate reserve during cold-storage has been shown in other species. In cold-stored Douglas fir (*Pseudotsuga menziesii*), root starch reserves in seedlings lifted in January and cold stored for 3 months at -1°C were approximately half those of seedlings overwintered outdoors (Ritchie 1982). The root TNC of cold stored seedlings showed a slower decline, falling from approximately 150 to 125mg g^{-1} d.wt during 4 months cold storage at -1°C.

Root growth potential is monitored in ideal growth conditions[40] (see Cannell *et al* 1990: Tabbush 1986), which may be very different from those found in the field. Cold storage may have no effect on root viability

[40]E.g.Temperature of 20 ±1°C, 75% relative humidity, 14 hr photoperiod of 300μ$Em^{-2}s^{-1}$ at plant level (Cannell *et al* 1990).

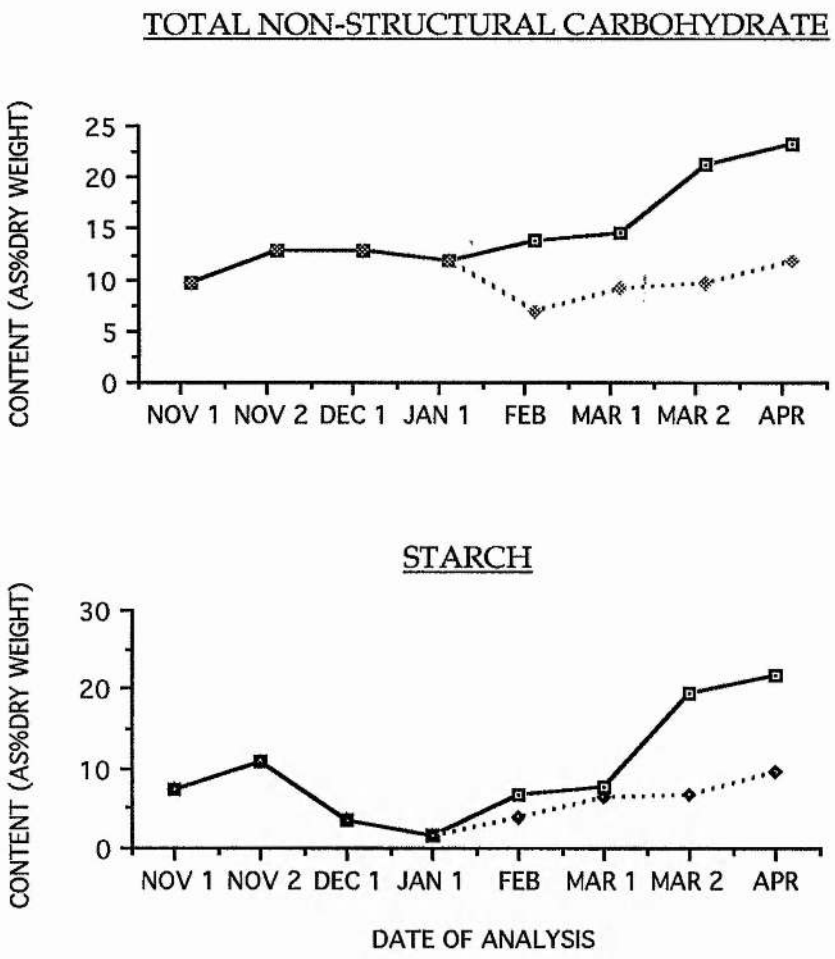


Figure 6.0. Drawn from data supplied by Mason from a study of root carbohydrate reserves in 2yr old Sitka spruce seedlings. Seedlings were lifted in January and either cold stored at +2°C (.....) or overwintered in the nursery (__). Analysis during Winter 1987-88.

under these favourable conditions, while under stress the change in root physiology may have severe consequences for their survival.

Under soil waterlogging roots may become anoxic and phloem transport, an energy requiring process, ceases. Thus the root carbohydrate reserve at the time of flooding must sustain maintenance respiration until the water table subsides.

The initiation of root growth in tree seedlings depends in part on a stimulus originating in the shoot and translocated in the phloem and, in most species studied, this growth utilises current photo-assimilate rather than starch reserves (Ritchie and Dunlap 1980). The importance of root carbohydrate reserves for new root growth in the Spring is controversial. Experiments have been conducted whereby Sitka spruce was exposed to ^{14}C (in the form of CO_2) either in Autumn or in Spring after initiation of root growth, and the amount of label in the new roots monitored (van den Driessche 1987). Results showed that new root growth primarily utilised current photosynthate and only a very small amount of the carbohydrate stored in the older roots. However, bark ringing Sitka spruce and Douglas fir seedlings has provided contradictory evidence (Philipson 1988). Douglas fir appeared to use current photosynthate for new root growth whereas Sitka spruce seedlings used carbohydrate stored in the roots, which continued to emerge and elongate even when the shoot was bark-ringed. Therefore the reserves may not only be essential for maintenance of the roots during Winter but also the establishment of new roots in the Spring after outplanting. Differing results may reflect damage to seedling physiology by the crude process of bark-ringing, but clarification of carbohydrate utilisation is of clear importance due to the consequences of their depletion during Winter on Spring root establishment.

Three months of Winter flooding severely reduced root viability and carbohydrate content in two provenances of *Picea sitchensis*. (Chapter 4). The greatest deficit in root starch and sugar was found in seedlings flooded from October when root activity and therefore oxygen demand was higher. If flooding was initiated in November, roots were in a more dormant state and had slightly more stored carbohydrate, such that more remained after waterlogging. The tip, being the most metabolically active section of root, was most damaged by flooding. Thus carbohydrate status and especially root respiration seemed important factors in determining the survival of roots in anoxia.

It therefore seems possible that the practice of cold storage and consequent impoverishment of root carbohydrate reserves may severely reduce the ability of seedlings to survive soil waterlogging at the time of planting. Although cold storage of Sitka spruce reduces root and total seedling carbohydrate reserves, a concurrent reduction in RGP has not been shown. However, if root survival was assessed under waterlogged conditions, as may be found on forest plantations, then the diminished levels of carbohydrate may not support root maintenance respiration until the water table subsides, and result in greater losses of cold-stored compared to nursery overwintered seedlings. The experiment aims to test this hypothesis.

6.2 EXPERIMENTAL DETAILS

6.2.1 Seedling origin and growth

Sitka spruce seedlings [Juneau, Alaska. Prov. No. 81(7987)1] were collected from the Forestry Commission Northern Research Station, Roslin, Midlothian on 5th April 1993. These seedlings had been grown from seed, maintained for 1.5 seasons in a seedbed followed by 1.5 seasons as transplants before being overwintered under one of two conditions typical of forest practice:

1) Left in peat/sand mixture in the nursery followed by lifting in late March;

2) Lifted in late January and stored in the dark in polythene bags at 0.5°C (±0.5°C) for two months until collection.

Immediately on arrival in St. Andrews (Day 1), 6 cold stored seedlings were re-sealed in black polythene bags and stored at 2-4°C until analysis 2 days later. A sample of 5 nursery overwintered seedlings were also set aside for later analysis, these being placed outdoors out of direct sunlight with only the roots wrapped in black polythene to maintain moisture. Within hours of arrival in St. Andrews, all remaining seedlings (150 cold stored and 164 nursery overwintered) were planted in peat with 1g 'Osmocote plus' slow release fertiliser[41] in 6" pots. Seedlings were well watered and allowed 2 days recovery from handling before treatments began.

On day 3, 29 previously cold stored and 31 nursery overwintered seedlings were placed in water-tight tubs outdoors and waterlogged to the soil surface. An equal number of seedlings were maintained in free-drained peat and watered as appropriate.

[41] 16:8:12 NPK +MgO+traces, 8/9month strength

6.2.2 Metabolic activity at initiation of flooding

The physiological state at the time of flooding was then assessed. Roots of seedlings set aside on day 1 were carefully washed in tap water and separated into three types:

A) main tap root + major laterals >1.5mm diameter,

B) root tips , 5cm sections from tip,

C) fibrous roots <1.5mm diameter.

The main tap root/major laterals were plunged into liquid nitrogen and subsequently freeze-dried for future carbohydrate analysis. Samples of root tips and fibrous roots were then analysed to determine root tissue viability (tri-phenyl tetrazolium chloride test), aerobic respiration rate at 10°C and root starch and D-glucose content.

6 cold stored and 5 nursery overwintered seedlings were analysed.

6.2.3 Carbohydrate content of seedlings after 2 weeks in free drained soil

Two weeks after delivery, seedlings were monitored for signs of root growth and carbohydrate content. 3 cold stored and 3 nursery-overwintered seedlings were harvested and samples prepared for carbohydrate analysis.

6.2.4 Effect of flooding on root survival

After 55 days waterlogging or free-drainage, 10 seedlings from each treatment were transplanted into peat and placed in a controlled environment room and allowed free-drainage. On transplanting, roots were washed with tap water and the lengths of all new roots recorded. Root growth and survival in uniform conditions was then monitored.

A sample of seedlings was removed from flooding and left outdoors in freely draining conditions to monitor survival after a period of post-anoxia at ambient temperature. Seedling survival was also noted under

continued flooding. All treatments were compared to free-drained controls.

Root viability and carbohydrate content were assessed concurrent with the root growth potential tests. A large number of seedlings were analysed, those waterlogged having had flooding initiated on the same date immediately after each overwintering treatment. Seedlings were harvested at the time of measurement, which lead to variation in the exact length of flooding. This procedure was undertaken as it was considered that initiating flooding on the same day and analysing seedlings immediately after flooding was important. (Flooding had to be initiated immediately after over-wintering because of possible physiological changes on outplanting which may affect flood-survival. The effects of varying lengths of post -anoxia were avoided by analysing seedlings immediately after flooding.) However, the variation in the duration of flooding was small in comparison to the total time. After approximately 62 days waterlogging, seedlings were harvested and the viability and carbohydrate content of the tap, fibrous and root tip sections assessed.

The experimental design is summarised in Fig. 6.1.

6.2.5 Physiological measurements

See experimental techniques chapter for details.

a) Viability test

For each seedling one sample of tip sections (containing several roots) was analysed. Fibrous roots were most plentiful, 5cm sections were therefore cut randomly from the root system and each sectioned into 1cm lengths. From these sections 2 to 4 replicate samples were produced to provide an accurate mean viability.

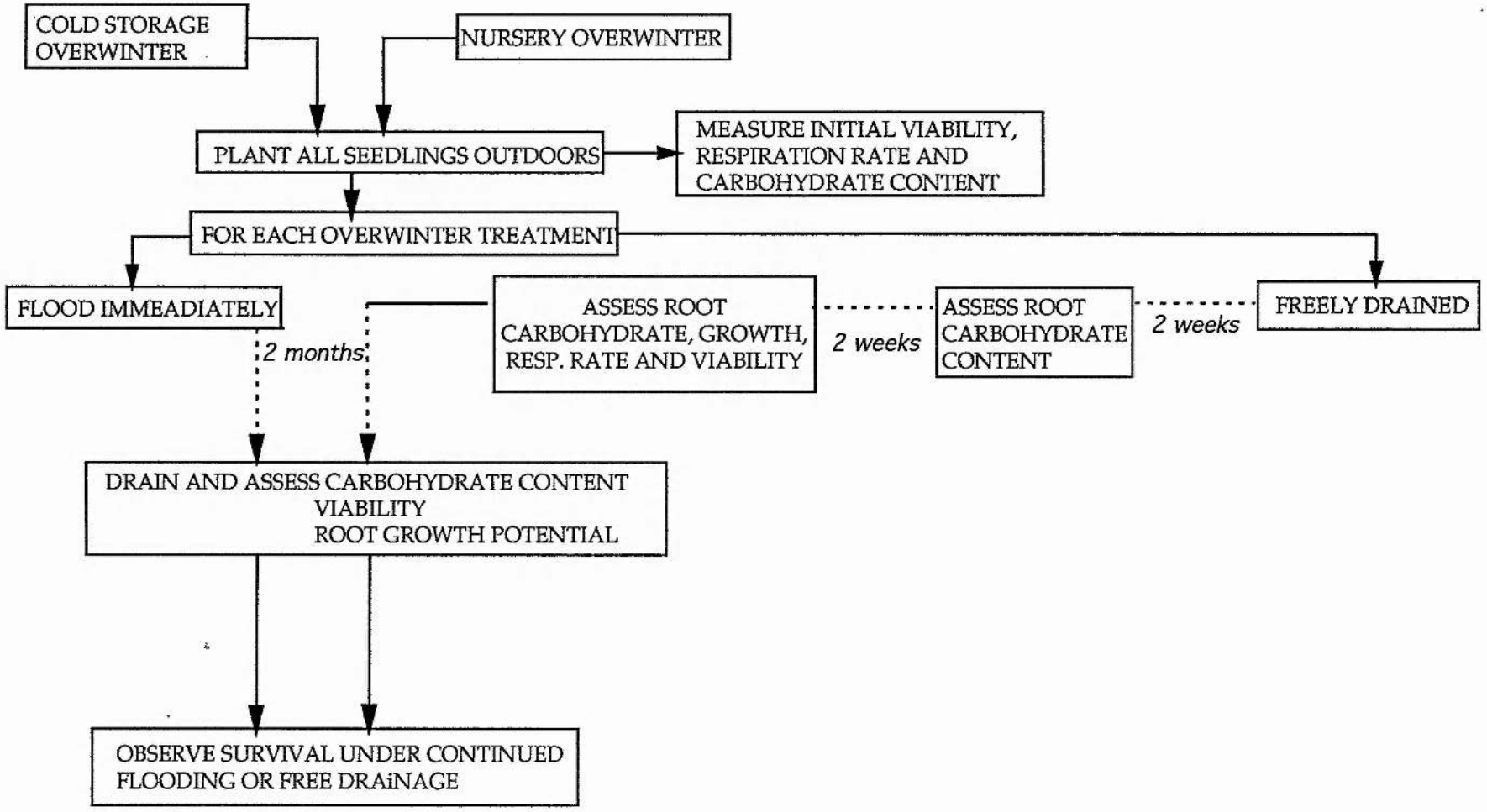


Figure 6.1. Summary of experimental design

b) Respiration rate

One sample of several (> 3) roots each 5cm in length.

c) Carbohydrate analysis

Sampling of roots.

Root tip section:- one sample of several (> 3) roots was analysed.

Fibrous roots:- all roots except those sampled for measurement of respiration and viability were analysed.

Main tap root:- tap root and major laterals (except small sections removed for viability assay) were sampled.

Roots were plunged into liquid nitrogen, freeze-dried and frozen at -20°C until analysis. Roots were then ground to a fine powder in a Glen Creston™ mill (tap and fibrous roots) or a pestle and mortar (root tip sections). Powdered roots were then assayed for carbohydrate content as detailed in the experimental techniques chapter. In some cases, root samples from replicate seedlings were analysed separately but when the number of replicates was large, roots were bulked into a number of sub-samples. Rather than bulking the whole sample (see Cannell *et al.* 1990), in this study an equal weight of ground root from each seedling was bulked . This method removes the error caused by varying root system size and gives a truer mean carbohydrate content.

d) Root growth potential

Containers were designed in which roots could grow against a perspex face, and therefore be marked at intervals and the growth monitored, as in Cannell *et al.* (1990) The containers were constructed from 0.86m lengths of plastic guttering, halved and sealed across the diameter with transparent perspex. The bottom of the tube was filled with gravel to a 5cm depth to cover 3 drainage holes.

After washing away peat from the roots, seedlings were placed against the perspex face and the tube filled with moist peat. Each tube, containing 2 seedlings, was covered in black polythene to keep the roots dark and tubes were maintained in an environmentally controlled room:-

Light Intensity: 245$\mu Em^{-2}s^{-1}$ at mid-stem height,

(monitored using a Macam™ photometer at 400-700nm).

Relative humidity: 70-75%

Daylength: 16 hours.

Temperature: 19.5 ±0.08°C, (daily root temperature recorded using max./min. thermometer.

The tubes were placed in a Dexian™ frame at 30° to the vertical in order to maintain growth against the perspex and allowing equal spacing of the seedlings under the light. Seedlings were watered daily as appropriate.

6.3 RESULTS

6.3.1 Metabolic activity at initiation of flooding

Seedlings were measured immediately to assess the effect of cold storage or overwintering in the nursery on seedling viability, root respiration rate and carbohydrate content. The results are shown in Table 6.1.

Table 6.1. Metabolic activity after over-wintering in the nursery or cold store

	NURSERY SEEDLINGS			COLD STORED SEEDLINGS		
	TIP	FIBROUS	TAP	TIP	FIBROUS	TAP
STARCH (mg g^{-1} d.wt. root)	78.76 ±9.8	82.88 ±8.81	80.06 ±8.04	65.97 ±5.25	73.49 ±3.52	56.38 ±5.18
SUGARS (mg g^{-1} d.wt. root)	31.93 ±1.37	29.38 ±1.65	18.95 ±0.89	28.39 ±2.28	28.94 ±0.44	18.97 ±0.65
STARCH + D-GLUCOSE (mg g^{-1} d.wt. root)	110.70 ±9.15	112.30 ±10.20	99.01 ±7.60	94.35 ±6.07	102.42 ±3.48	75.35 ±5.54
RESPIRATION RATE (μmoles CO_2 g^{-1}f.wt.root h^{-1})	4.56 ±0.56	4.61 ±0.25	_	4.92 ±0.53	4.55 ±0.29	_
VIABILITY INDEX	173.79 ±16.25	172.82 ±11.73	_	141.07 ±5.56 *	158.47 ±10.25	_

REPLICATION:
Samples from 5 nursery seedlings and 6 cold stored seedlings for each parameter analysed, (except *=5).

a) Respiration rate

Overwintering treatment had no effect on root respiration rate, seedlings from cold storage and the nursery had approximately equal aerobic respiration rates at 10°C. Respiration rate did not differ between the root tips and fibrous roots.

b) Viability

Tissue viability was determined quantitatively using the TTC test. Mean viability index of cold stored and nursery overwintered roots was calculated. The sample variances were equal and the mean viability index of each root type from the two overwintering treatments compared using Student's t test. Tissue viability is reduced in the root tip sections and fibrous roots during cold storage, though reduction is only significant in the tip sections at the 10% level ($p=0.093$). Cold storage reduces viability of the root tips and fibrous roots to 81 and 92% respectively of that of nursery overwintered seedlings.

c) Root Carbohydrate content

Cold storage reduced the total carbohydrate content (TNC) of each root type (Figure 6.2). Mean TNC was determined, and having confirmed equal variances for each sample, significant differences between root types and overwinter treatments tested by ANOVA. Irrespective of storage treatment, there was a significant difference in the carbohydrate content of the 3 root types ($F=4.48$, $df=2$, $p=0.021$). Tap roots store less carbohydrate than the rest of the root system per g dry weight of root (Table 6.2).

Table 6.2. Mean TNC content of different root types with 95% confidence intervals

ROOT	TOTAL CARBOHYDRATE CONTENT (mg glucose g^{-1} d.wt. root)	95% CONFIDENCE INTERVAL
TAP	86.11	11.22
FIBROUS	106.89	9.70
TIPS	101.78	11.07

n=11, each root type, cold stored and nursery overwintered seedlings grouped.

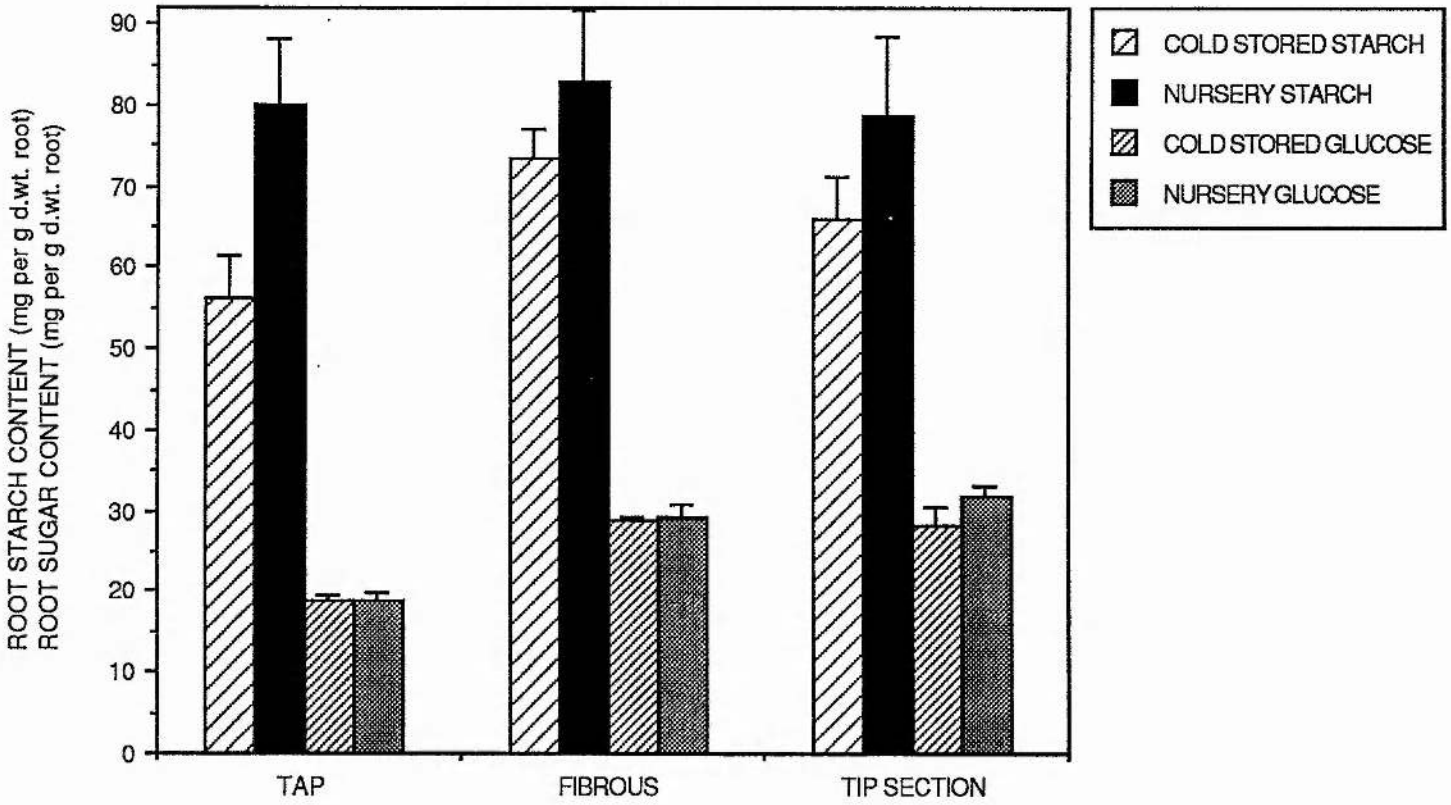


Figure 6.2. Root carbohydrate content immediately following cold storage or over wintering in the nursery. Mean starch and sugar content was calculated from analysis of 6 cold stored seedlings and 5 nursery over wintered seedlings. (se bars shown).

Cold storage caused a significant reduction in root total carbohydrate content ($F=8.37$, $df=1$, $p=0.007$).

For each type of root, levels of starch, sugars and total non-structural carbohydrate were compared between the two treatments using Students t-test with equal or unequal variances as appropriate. Sugar levels were very similar in nursery overwintered and cold-stored roots, whereas cold storage lead to lower starch reserves in each root type, though only significantly in the tap root. Cold storage reduced tap root starch content to 70.4% (± 6.5) that of the nursery overwintered seedlings ($p=0.031$). Consequently, TNC was lower in the tap roots of cold stored seedlings in comparison to those overwintered in the nursery ($p=0.030$).

6.3.2 Growth and carbohydrate content two weeks after transplanting

Two weeks after transplanting outdoors, there was no sign of root growth in free drained seedlings from the nursery or cold store.

Root samples of 3 seedlings were combined to give one bulked sample for each type of root from each overwinter treatment (Table 6.3).

Table 6.3. Root carbohydrate content 2 weeks after transplanting cold stored and nursery overwintered seedlings outdoors in comparison to initial post-storage levels

		POST-STORAGE CARBOHYDRATE CONTENT		CARBOHYDRATE CONTENT 2 WEEKS AFTER OUTPLANTING	
CARBOHYDRATE	ROOT TYPE	COLD STORED	NURSERY	COLD STORE	NURSERY
STARCH	TIP	65.97 ±5.25	78.76 ±9.80	86.66	113.30
(mg g^{-1}d.wt. root)	FIBROUS	73.49 ±3.52	82.88 ±8.81	97.85	115.03
	TAP	56.38 ±5.18	80.06 ±8.04	-	-
SUGARS	TIP	28.39 ±2.28	31.93 ±1.37	28.46	30.61
(mg g^{-1}d.wt. root)	FIBROUS	28.94 ±0.44	29.38 ±1.65	26.00	27.21
	TAP	18.97 ±0.65	18.95 ±0.89	-	-
TNC	TIP	94.35 ±6.07	110.70 ±9.15	115.12	143.91
(mg g^{-1}d.wt. root)	FIBROUS	102.42 ±3.48	112.30±10.2	123.84	142.23
	TAP	75.35 ±5.54	0	-	-
			99.01 ±7.60		
No. replicates		6*	5*	3†	3†

TNC = total non-structural carbohydrate. (Sum of starch and sugar content).
*Roots analysed individually, mean and se shown.
† roots bulked to give one sample.

Due to bulking of the 3 seedlings to measure carbohydrate content, no statistical comparisons between treatments or dates of analysis can be made, although patterns can be seen. Sugar content of the fibrous and root tip sections are approximately equal in the seedlings from the two treatments, and do not change during the 2 weeks after planting. In contrast, starch content increases in each root type by approximately one third of its initial value during the first two weeks after outplanting:

	COLD STORE	NURSERY
TIP SECTIONS	31%	44%
FIBROUS ROOTS	33%	39%

Increases in starch were slightly larger in the nursery seedlings, consequently TNC content in root tip sections and fibrous roots of nursery seedlings remained greater than that in cold stored seedlings,

even after two weeks of outplanting. Thus the deficits resulting from cold storage had not been made-up.

6.3.3. Growth and carbohydrate metabolism one month after transplanting

a) Root growth

The number and length of new roots of free-drained seedlings was determined one month after transplanting. 4 previously cold stored and 4 nursery overwintered seedlings were sampled and the number, mean and total length of new roots recorded for each seedling:

Mean growth of seedlings from the two treatments was then compared. Samples were found to fit a normal distribution and had equal variances therefore were compared using Student's t-test at 5% significance levels (Table 6.4). Cold stored seedlings had a greater number of new roots (p=0.015), of greater mean length. The total length of root growth of cold stored seedlings was therefore much greater (p=0.021) than that of nursery overwintered seedlings (Figure 6.3).

Table 6.4. Root growth one month after transplanting cold stored and nursery overwintered seedlings outdoors

	COLD STORED	NURSERY OVER WINTERED	p value at 95% confidence level
Mean length new roots/mm	17.2 ±4.3	7.4 ±3.0	NS
Total length new roots/seedling	59.1 ±16.1	6.92 ±4.7	0.021
Number new root per seedling	33 ±7	7 ±4	0.015

Each value mean ±se of 4 seedlings.

Root metabolic activity

Four cold stored and four nursery overwintered seedlings were randomly selected for physiological measurements of the roots.

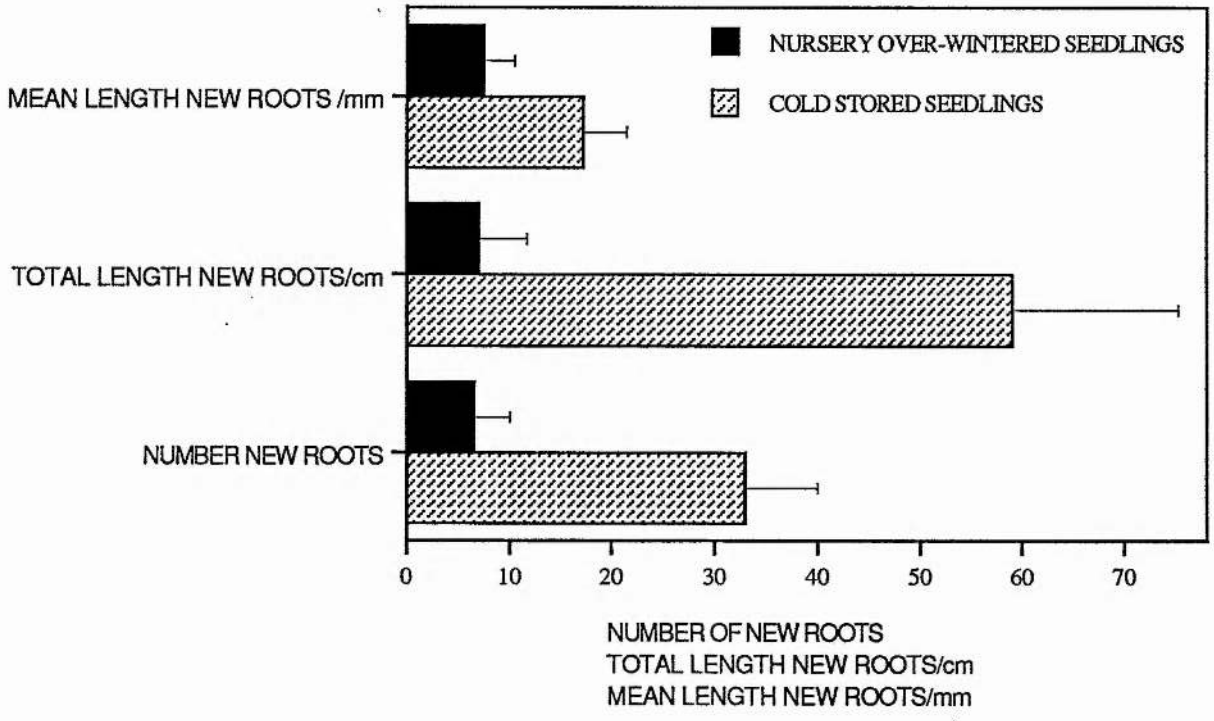


Figure 6.3 Effect of previous overwintering treatment on root growth during one month under ambient Spring conditions. Means and standard errors shown for 4 replicate seedlings.

i) Root viability

The mean viability index of roots from each treatment was calculated. The effect of cold storage on post-transplant root viability was assessed by expressing the viability index of each cold stored plant as a percentage of the mean viability index of nursery overwintered seedlings (=relative viability). Relative viability immediately after storage and after transplanting are compared in Table 6.5.

Table 6.5. Root viability of cold stored seedlings relative to that of controls (nursery overwintered seedlings) immediately after storage and one month after transplanting

	AFTER STORAGE	1 MONTH AFTER OUTPLANTING
ROOT TIPS	81.2 ±3.2*	77.7 ±5.8
FIBROUS ROOTS	91.7 ±5.9	70.0 ±5.7

Immediately following overwintering, n=6 cold stored seedlings, n=5 nursery seedlings and *.
One month after outplanting, n=4 , all treatments.

For each seedling, relative viability was arcsin transformed and the means compared using ANOVA. Viability of cold stored roots (relative to nursery overwintered seedlings) decreased significantly during the first month after transplanting (significant only at 10% level, p=0.077), though cold storage did not lead to differences in degree of damage when comparing the two types of root.

ii) Root respiration rate

Aerobic respiration rate at 10°C was compared in roots from the two treatments. Respiration rate was similar in both nursery and cold stored seedlings and had not changed significantly since storage (Table 6.6). Differences between treatments or dates of analysis were not significant.

iii) Root carbohydrate content

Equal weights of roots of the 4 seedlings were bulked to produce one root sample. Consequently, as above, no statistical analyses were possible. Changes in carbohydrate content during the first month after overwintering are compared in Figure 6.4. Total non-structural carbohydrate showed a gradual increase in nursery overwintered root tip sections and in the fibrous and tap roots of each treatment. Carbohydrate content of the root tips of cold stored seedlings decreased slightly in the latter 14 days, reflecting reduced starch reserve (Table 6.6). This may be explained by the faster growth rate of these seedlings (see discussion).

Table 6.6. Root aerobic respiration rate and carbohydrate content one month after transplanting

		ROOT		
	OVERWINTER CONDITIONS	TIP SECTION	FIBROUS ROOT	TAP ROOT
RESPIRATION RATE ($\mu molesCO_2\ g^{-1}$ f.wt. root h^{-1})	COLD STORE	5.31 ±0.98	3.70 ±0.23	-
	NURSERY	4.08 ±0.79	3.72 ±0.24	-
STARCH ($mg\ g^{-1}$ d.wt. root)	COLD STORE	65.07	123.92	112.58
	NURSERY	122.00	135.81	100.7
SUGARS ($mg\ g^{-1}$ d.wt. root)	COLD STORE	30.41	19.19	17.91
	NURSERY	25.18	23.01	17.50
TNC ($mg\ g^{-1}$ d.wt. root)	COLD STORE	95.47	143.11	130.49
	NURSERY	147.18	158.82	118.2

Respiration rate mean of 4 seedlings ±se
Carbohydrate content, 4 replicate seedlings, roots bulked into one sample for each root type.

6.3.4 Effect of flooding on root survival

a) General observations of seedling health

Flooded seedlings suffered some needle yellowing and loss to varying degrees. Individuals varied widely in their appearance after flooding such that visual comparisons of shoot survival could be misleading. Comparing seedlings after 70 days waterlogging, shoots of nursery overwintered seedlings looked slightly less viable in comparison

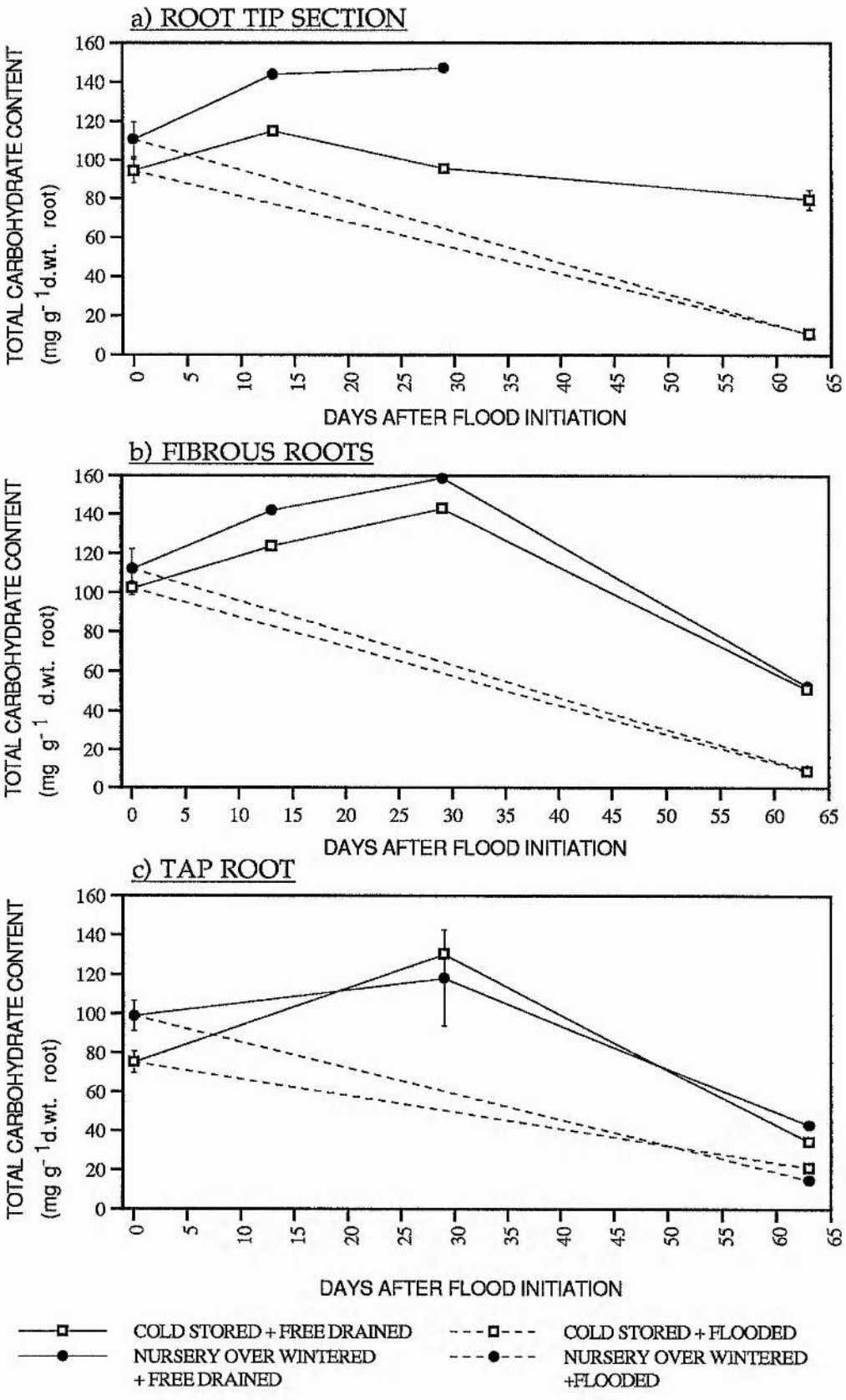


Figure 6.4 . Change in total carbohydrate reserve during Spring after cold storage or nursery over-wintering. Levels are compared in free drained seedlings and those flooded immediately on planting. Data is presented for tap roots, fibrous roots and root tips. Standard error bars shown for initial reserve and that in flooded and drained seedlings after 63 days.
Plain lines represent free drained seedlings, dashed represent flooded.

to previously cold stored seedlings. However some seedlings, especially those from cold storage, retain a generally healthy shoot appearance when compared to free drained controls (see Plate 6.1 a, b c). In early June, after 63 days flooding, seedlings were allowed free drainage and maintained outdoors in ambient conditions. The effect of 7 days post-anoxia was then monitored. In general, cold stored seedlings appeared much healthier than nursery overwintered seedlings, the latter showing greater wilting and needle loss (Plate 6.2 a). This effect was much more noticeable after 53 days post anoxia. After this time, shoots of the nursery overwintered seedlings were completely dead, whilst cold stored seedlings retained a large proportion of green needles (Plate 6.2b). Cross sections of tap roots revealed blackening of the central core of vascular tissue in the flooded seedlings, whilst tissues remained white/yellow in drained trees. It is possible that the blackening of xylem tissue in the flooded seedlings results from the uptake of Fe^{2+} formed in the reducing conditions in the soil.

b) Root viability

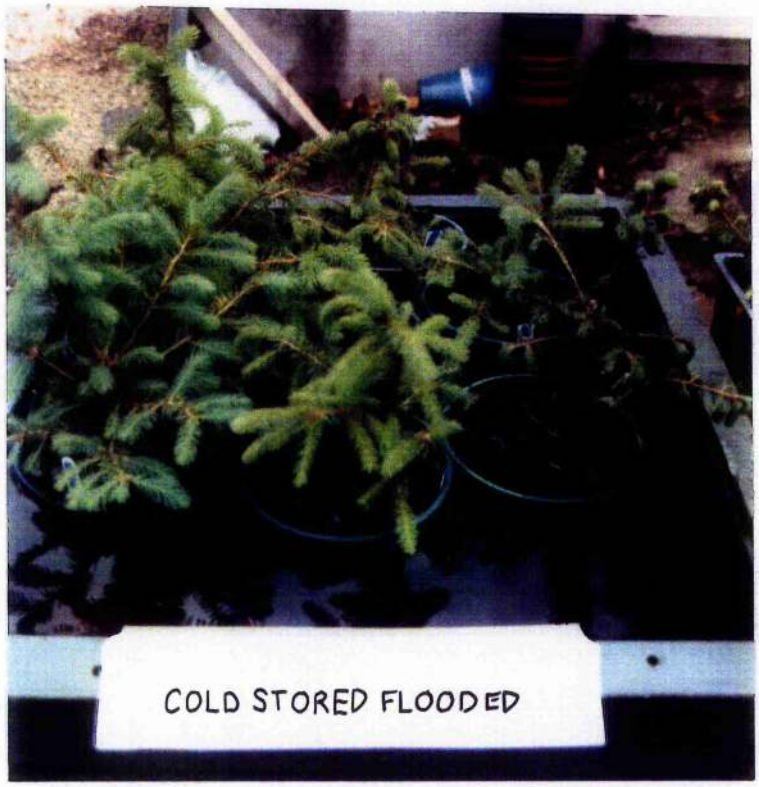
The relative viability of each flooded root sample was calculated by expressing viability index as a percentage of the mean viability index of roots of non-flooded seedlings overwintered under the same conditions. Thus the effect of overwintering treatment on survival of two months flooding could be compared (Table 6.7).

PLATE 6.1 Effect of flooding on shoot appearance.

a) Free drained controls from nursery over wintering and cold storage.

OPPOSITE: Seedlings from each overwinter treatment after 70 days waterlogging.
b) cold storage
c) nursery over wintering

b) cold storage.



c) nursery over wintering.

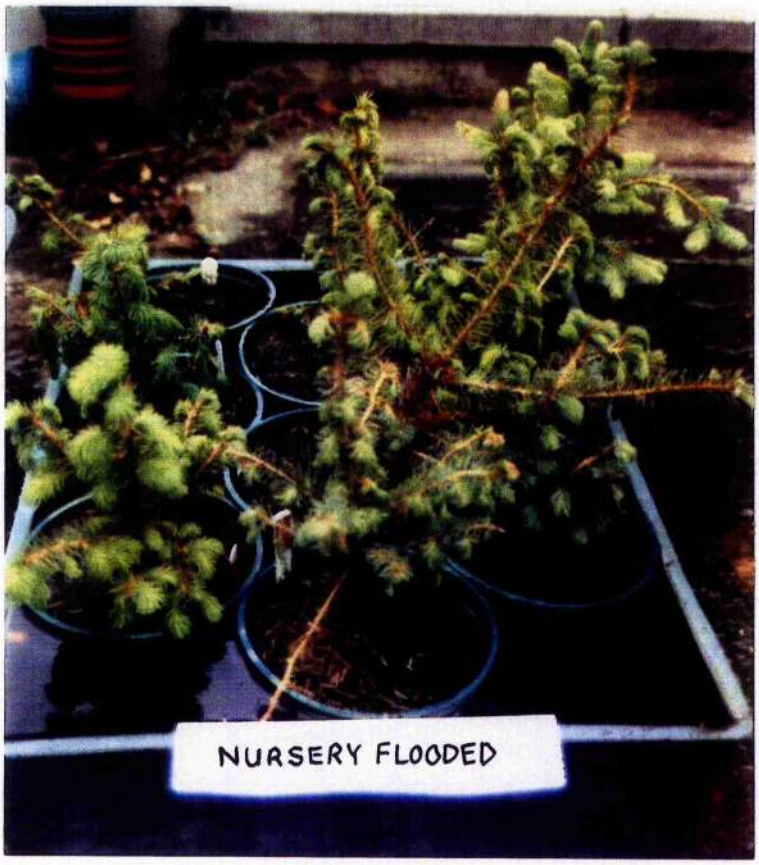
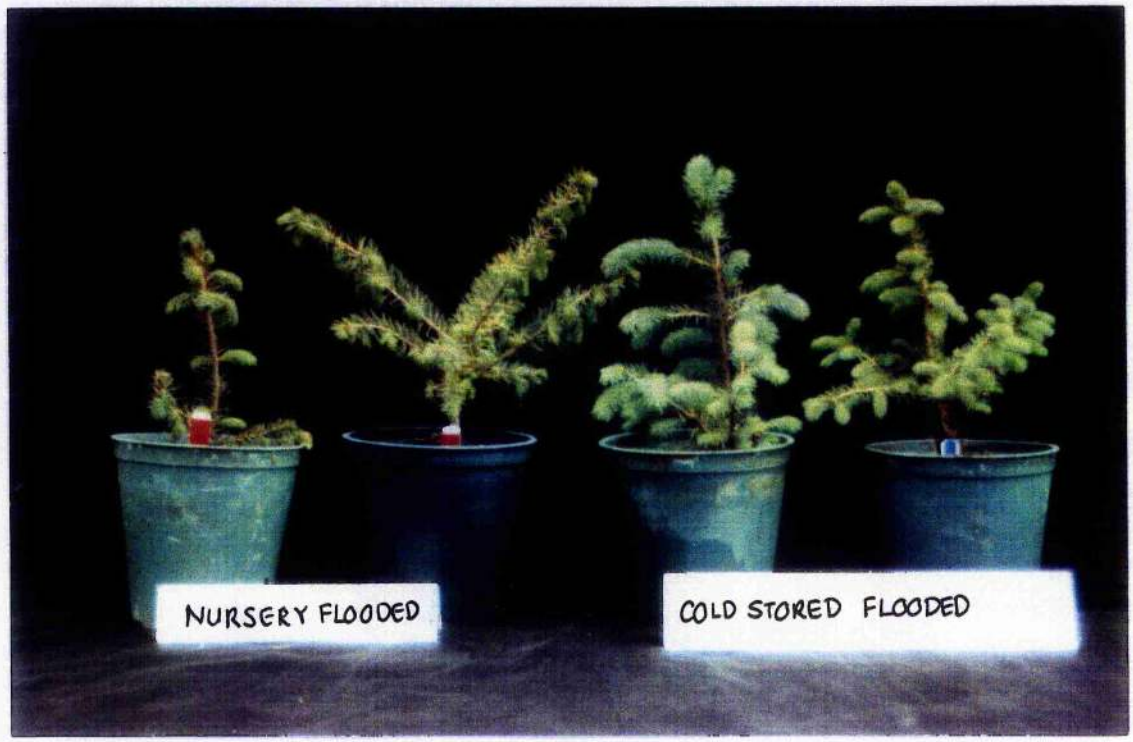


PLATE 6.2 Effect of post-anoxia on shoot appearance.

a) After 7 days post-anoxia.



b) After 53 days post-anoxia

Table 6.7. Comparison of effect of flooding on viability of cold stored and nursery overwintered seedlings

	ROOT		
OVERWINTER CONDITIONS	TIP SECTION	FIBROUS ROOT	TAP ROOT
COLD STORE	71.5 ±9.9 (7)	71.2 ±7.3 (7)	103.1 ±15.0 (5)
NURSERY	72.6 ±4.6 (7)	62.0 ±6.7 (7)	77.6 ±13.5 (4)

Mean relative viability calculated from (n) replicates under flooded conditions. Viability Index of each flooded seedling expressed as a percentage of mean VI of free drained seedlings where n=6.

In all except the previously cold stored tap roots, flooding reduced root viability to less than 80% of free drained seedlings (Figure 6.5). Loss of viability was similar in seedlings from cold storage and nursery overwintering. Relative viabilities were arcsin transformed and the samples tested for equal variance. Samples showed heterogeneity of variance, but bearing this in mind, ANOVA was conducted on the transformed data. Mean relative viability of the tip and fibrous roots was lower than that of the tap roots, the effect of flooding differing significantly between the three root types ($F=5.07$, $df=2$, $p=0.012$). An assessment of the damage to the root system showed that roots of the nursery overwintered seedlings were injured to a significantly greater degree than the cold stored seedlings, though differences were only significant at the 10% level ($p=0.097$).

c) Growth

At initiation of RGP tests, root growth of free-drained seedlings during the two months since transplanting was assessed as above. Flooded seedlings showed zero growth in comparison to substantial growth of free drained seedlings from each overwinter treatment.

Effect of flooding on relative viability of roots of previously cold stored and nursery over wintered seedlings.

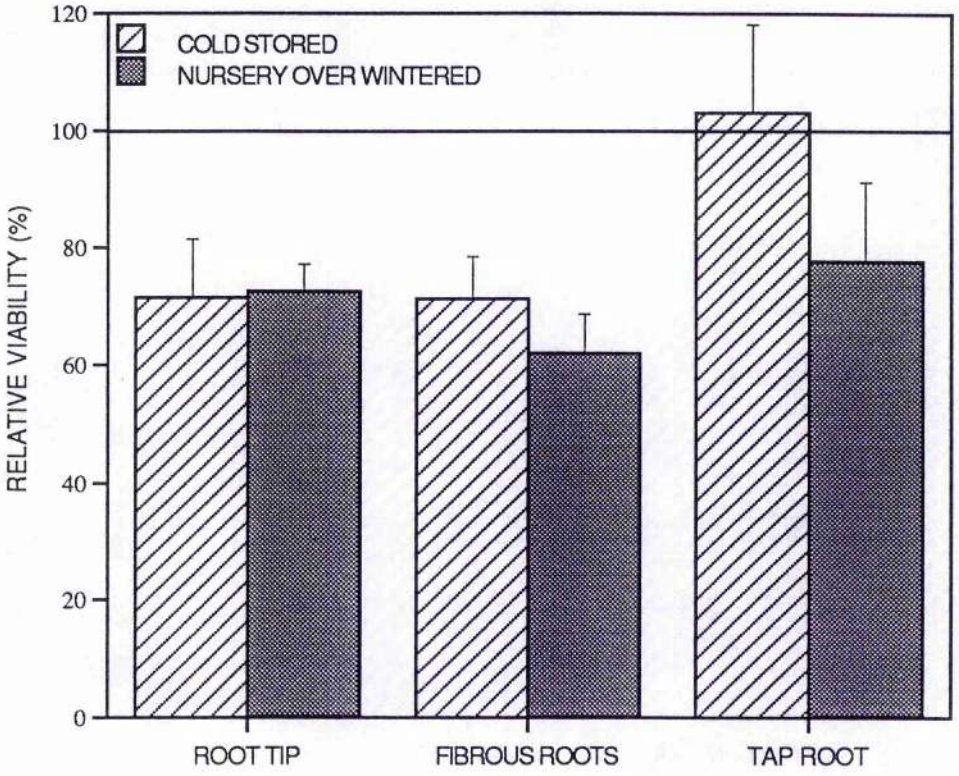


Figure 6.5. Root viability after 2 months flooding following either cold storage or overwintering in the nursery. Viability of flooded seedlings is expressed as a percentage of that of free drained controls from the same over wintering treatment. Means and se bars shown. n=7, root tips and fibrous roots; n=5 tap roots of cold stored seedlings; n=4 tap roots of nursery seedlings.

Number, mean and total length of new roots was recorded for the ten free-drained seedlings from each overwinter treatment. Means were calculated and the fit of the samples to a normal distribution confirmed.

Growth of nursery and cold stored seedlings was compared using Student's t-test with equal or unequal variances as appropriate.

Table 6.8. Root growth of free drained seedlings during two months after outplanting

	COLD STORED	NURSERY OVER WINTERED	p value at 95% confidence level
Mean length new roots/mm	44.6 ±3.4	31.2 ±1.5	0.004
Total length new roots/seedling (cm)	208.3 ±46.0	74.7 ±15.5	0.019
Number new root per seedling	45 ±8	24 ±5	0.04

n=10 replicate seedlings, each treatment.

Roots continued to show substantial growth during May. Growth of previously cold stored seedlings was significantly greater than that of nursery overwintered seedlings in terms of the number, mean and total length of new roots (Table 6.8). Thus the pattern seen after one month of growth continued for the subsequent month.

Neither cold stored nor nursery overwintered seedlings showed new root growth when transferred to free drained peat in ideal growth conditions after 55 days waterlogging. After one week in the controlled environment room, the majority of previously flooded seedlings showed varying degrees of fungal growth on the root system. In contrast, previously free-drained seedlings showed no sign of fungal infection and many showed growth of new roots. Growth of fungus on the roots seems to indicate cellular injury and leakage of substrate to the soil. Injury is unlikely to result from damage during handling as the flooded roots were quickly transplanted with minimal disturbance. Free-drained

controls in contrast suffered much greater handling whilst new root growth was measured before transplanting (6.3.4 c), yet showed no signs of fungal growth. Thus tissue damage during waterlogging seems to be the likely cause.

After 25 days in ideal growth conditions, each seedling was scored for shoot health and number of new roots >1cm length. Growth of all new roots against the perspex face was measured and the mean and total length calculated (Table 6.9).

Previously flooded seedlings showed no signs of root growth after 25 days whereas previously free-drained seedlings showed varying degrees of root growth. Flooding lead to shoot damage in terms of needle browning and loss during RGP tests, but the extent of damage varied from slight to total. The degree of damage did not however differ between seedlings from the two overwintering treatments.

Table 6.9. Root growth of cold stored and nursery overwintered seedlings after 25 days in the controlled environment room

	COLD STORED	NURSERY OVER WINTERED	p value at 5% significance level
Mean length new roots (mm)	24.1 ±2.3	14.06 ±2.04	0.0016
Total length new roots/seedling (cm)	42.78 ±7.16	28.1 ±14.9	0.022
Number new roots per seedling	17 ±2	15 ±5	NS

n=10 replicates per treatment.
New roots are those visible and growing against perspex only.

After approximately 2 months, roots were again monitored for signs of growth. Previously flooded seedlings showed zero growth. Growth of previously drained cold stored and nursery overwintered seedlings were compared by measuring root collar diameter using vernier callipers. This statistic is a good indicator of survival and growth and is superior to measurement of shoot height (Thompson 1985). Each sample was

confirmed to fit a normal distribution and the treatments showed homogeneity of variance. Mean collar diameters (Table 6.10) were therefore compared using ANOVA.

Table 6.10. Root collar diameter of previously flooded and free drained seedlings after 2 months in RGP test

TREATMENT PRIOR TO RGP TEST	ROOT COLLAR DIAMETER (cm)	95% CONFIDENCE INTERVAL
COLD STORAGE +FREE DRAINAGE	0.96 ±0.06	0.11
COLD STORAGE + FLOODING	0.68 ±0.06	0.12
NURSERY +FREE DRAINAGE	0.91 ±0.05	0.10
NURSERY +FLOODING	0.79 ±0.05	0.09

n = 10 all treatments.

In both cold stored and nursery overwintered treatments, free drained seedlings have greater mean root collar diameters than flooded seedlings reflecting growth of the root system of the former ($F=14.15$, $df=1$, $p=0.001$). Seedlings from each overwintering regime reacted similarly to flooding in terms of root collar diameter changes. In cold stored seedlings, root collar diameter of previously drained seedlings was significantly greater than those following waterlogging (Student's t-test, $p=0.0023$). The difference in collar diameter in nursery overwintered seedlings showed the same pattern though the difference was non-significant.

d) Carbohydrate content

Root carbohydrate content of free drained seedlings decreased considerably between overwintering and early June when roots were analysed for comparison with those of flooded seedlings (Figure 6.4). This fall in reserve, especially in the fibrous and tap roots, presumably reflects the demand on stored carbohydrate for root growth. It reflects a decrease

in starch reserves, sugar levels remaining approximately constant. Ford and Deans (1977) also found a correlation of root starch depletion with new root growth. A similar pattern is reported in Douglas fir seedlings, starch accumulating in Autumn and Spring until it is rapidly depleted during root elongation. In Douglas fir however, reducing sugars were also depleted, the reducing sugar content being inversely related to the number of new roots per seedling (Krueger and Trappe 1967).

Effect of flooding:

Comparing roots of flooded seedlings to those from free drained soil analysed concurrently, levels of both sugars and starch are greatly depleted by waterlogging. Flooded seedlings showed no root growth, depletion is therefore presumably due to high metabolic costs of maintenance respiration under anaerobic conditions (Table 6.11).

Table 6.11. Carbohydrate remaining in flooded roots of cold stored and nursery overwintered seedlings in comparison to those from free drained soil

		COLD STORED SEEDLINGS		NURSERY OVERWINTERED SEEDLINGS	
CARBOHYDRATE	ROOT TYPE	FREE DRAINED	FLOODED	FREE DRAINED	FLOODED
STARCH	TIP	17.84 ±3.66	3.97 ±0.29	-	5.23 ±0.83
(mg g^{-1} d.wt. root)	FIBROUS	22.24 ±2.75	1.83 ±0.56	22.96 ±1.25	3.93 ±0.44
	TAP	14.93 ±2.20	11.49 ±0.98	23.59 ±1.93	7.50 ±1.07
SUGARS	TIP	61.68 ±4.00	6.951 ±0.44	-	5.15 ±0.52
(mg g^{-1} d.wt. root)	FIBROUS	29.05 ±2.37	6.92 ±1.28	29.68 ±1.01	5.40 ±1.24
	TAP	19.55 ±1.45	9.74 ±1.94	19.42 ±1.49	7.60 ±1.29
TNC	TIP	79.53 ±5.12	10.92 ±0.73	-	10.37 ±0.32
(mg g^{-1} d.wt. root)	FIBROUS	51.29 ±1.76	8.75 ±1.08	52.64 ±2.23	9.33 ±1.65
	TAP	34.47 ±3.26	21.23 ±2.42	43.01 ±1.32	15.11 ±2.36

Mean ±se of n samples.
Free drained samples = 9 seedlings bulked into 3 samples for carbohydrate analysis, flooded samples =12 seedlings bulked into 3 samples.

Carbohydrates remaining after flooding:

Root starch, sugar and TNC content after flooding do not differ significantly between seedlings from cold storage or nursery overwintering[42] when compared at the 5% level.

Degree of carbohydrate depletion:

The degree of carbohydrate depletion in cold stored and nursery overwintered seedlings during flooding was calculated by expressing mean carbohydrate content remaining in each root as a percentage of that in free drained seedlings (Table 6.12). Thus the effect of overwinter storage treatment on depletion of carbohydrate reserve during flooding could be compared (Figure 6.6).

Table 6.12. Carbohydrate remaining in seedlings after flooding as a percentage of those in free drained controls

	COLD STORED SEEDLINGS		NURSERY OVERWINTERED SEEDLINGS	
CARBOHYDRATE	TAP ROOT	FIBROUS ROOTS	TAP ROOT	FIBROUS ROOTS
STARCH	76.9%	8.2%	31.8%	17.13%
(mg g^{-1} d.wt. root)	±6.57	±2.53	±4.5	±1.9
SUGARS	49.8%	23.8%	39.5%	17.8%
(mg g^{-1} d.wt. root)	±9.94	±4.4	±6.4	±4.5
TNC	61.6%	17.1%	35.1%	17.7%
(mg g^{-1} d.wt. root)	±7.01	±2.1	±5.5	±3.1

Percentages were arcsin transformed and the means compared by analysis of variance.

In all seedlings, carbohydrates were depleted to a greater degree in fibrous roots than in tap roots. Fibrous root starch and sugar content was reduced to less than 25% that of free drained seedlings. In contrast, the

[42] except the fibrous roots of the cold stored seedlings contain significantly less starch than those of the nursery over wintered seedlings, (p=0.042)

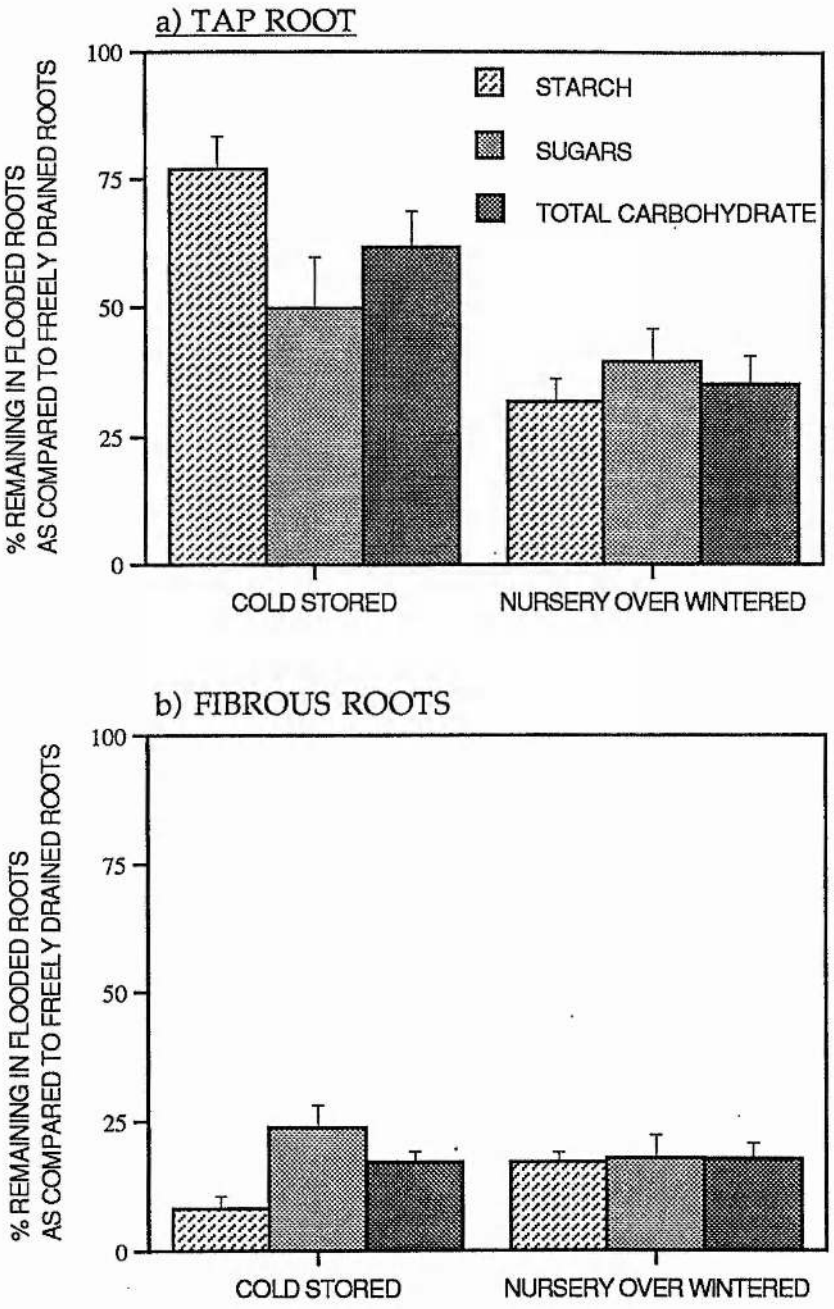


Figure 6.6 Depletion of root carbohydrate during 2 months flooding following over wintering either in cold storage or the nursery. Means ±se for replicate seedlings, (see Table 6.11)

tap roots retain a greater proportion in each root section. Using ANOVA, the degree of TNC depletion between tap and fibrous roots for both cold stored and nursery overwintered seedlings were compared. Tap roots retain significantly more total carbohydrate than fibrous roots ($F=41.92$, $df=1$, $p<0.001$), reflecting significantly greater conservation of both starch and sugars (starch: $F=76.28$, $df=1$,$p<0.001$, sugars: $F=13.21$, $df=1$,$p<0.007$).

During flooding, previously cold stored seedlings suffer less carbohydrate depletion in the tap root in terms of starch, sugar and total non-structural carbohydrate than seedlings overwintered in the nursery. ANOVA revealed that tap roots differ in the degree of starch and TNC depletion depending on overwinter treatment (Starch; $F=31.74$ $df=1$, $p<0.001$, TNC $F=7.07$, $df=1$, $p=0.029$), depletion of carbohydrate being similar in the fibrous roots irrespective of previous overwintering treatment.

The fibrous and tap roots were analysed together (these making up the majority of the root system in terms of mass) it was shown that previously cold stored seedlings suffer significantly less carbohydrate depletion in terms of both starch and total non-structural carbohydrate than the nursery overwintered seedlings (Starch $F=76.28$, $df=1$, $p<0.001$; TNC $F=41.92$, $df=1$, $p<0.001$). Sugar depletion is greater in nursery overwintered seedlings, but the difference between means non-significant at the 5% level.

6.4 DISCUSSION

Two months cold storage did not reduce the capacity to withstand Spring flooding in comparison to nursery over-wintered seedlings, contradicting the initial hypothesis. In fact, cold storage appeared to slightly enhance survival. Photographic evidence of seedling survival certainly showed that shoots of cold stored seedlings appeared much healthier after flooding and especially after a period of post-anoxia. In drawing conclusions, the restrictions in the scale of the experiment must be considered. Physiological measurements of root viability mirrored these observations although differences were only slight. Considering the large visual difference in survival and the small number of replicate samples, this line of the investigation may be fruitful if repeated with a large number of seedlings.

Cold storage in itself slightly reduced the viability of root tissues (as measured by reduction of tri-phenyl tetrazolium chloride) relative to those from nursery over-wintering. A further decrease in viability was apparent two weeks after transplanting outdoors although there was no visible difference in root or shoot health.

It was not possible to measure initial levels of carbohydrate reserve before cold storage, therefore comparisons are made only between levels remaining after nursery overwintering or cold storage. Cold storage only lead to significantly lower carbohydrate reserve in the tap root (to ~70% that of nursery overwintered seedlings), starch levels being lower in fibrous roots and root tips of cold stored seedlings though not significantly. Differences in root carbohydrates between cold stored and nursery overwintered seedlings appear slightly lower than those measured by Mason (pers. comm., See Introduction). Carbohydrate reserve in the cold stored roots was similar in the present and Mason's study, while nursery seedlings measured in the latter had much higher

carbohydrate content. This may be explained by poorer conditions for photosynthesis in Spring, either by position in the nursery or due to different weather conditions during the two experiments. It cannot be concluded from this work whether depletion of reserves during storage or their accumulation in nursery over-wintered seedlings results in the observed differences in reserve when seedlings are compared in April. For practical purposes however, the fact that a difference exists and may be related to survival is important. In many species cold storage does severely deplete reserve. Storage of Engelmann spruce at -5°C for 2 months reduces root reserve to a greater degree than in the rest of the seedling. Storage reduces starch by >50% in all plant parts and root soluble sugars by 20% (Chomba *et al.*1993). During 12 weeks copl storage at 1°C, TNC reserves of *Pinus mugo* were reduced to 66% of initial levels whereas those of *Pinus radiata* were depleted to only 38% (McCracken 1979).

Flooding of Sitka spruce initiated in successive months in late Autumn/Winter lead to differences in survival of seedlings of Alaskan origin (Chapter 4). Higher carbohydrate reserve and lower root respiration rates at the time of flood initiation correlated with increased survival. In this experiment, although cold stored seedlings were flooded at a time when root carbohydrate reserves were lower than those of nursery over-wintered seedlings, root respiration rate at a fixed temperature (10°C) was unchanged. The stress of imposing anoxia would therefore be expected to be similar in terms of the oxygen demand of the two types of seedlings. It is possible that the relationship between respiration rate and temperature varies between cold stored and nursery over-wintered seedlings. Retrospectively, it would be interesting to measure the respiration rate in excised roots from the two treatments during flooding. This could be done at ambient outdoor temperature. It

may be that more dormant cold stored seedlings take longer to increase their metabolic rate with increasing temperature. It has been suggested that the lower photosynthetic capacity of previously cold stored *Pinus mugo* and *Pinus radiata* seedlings was mainly due to disorganisation of the CO_2 fixing process (McCracken 1979).

Cold storage lead to much more extensive root growth in comparison to nursery over-wintered seedlings when assessed in early May, one month after transplanting outdoors. This phenomenon has previously been noted for Sitka spruce (Cannell *et al.* 1990, Deans *et al.* 1990). RGP varies with lifting date, storage and handling (see Deans *et al.* 1990, Ritchie and Dunlap 1980). RGP after storage depends on the physiological condition of the seedlings at lifting (Stone 1970). RGP may be reduced if seedlings are lifted in an early phase of dormancy when not responsive to chilling whereas later in the dormancy cycle plants are chill-responsive and their RGP increases in storage (Ritchie and Dunlap 1980). In Sitka spruce, RGP increases after the buds have ceased growth, become dormant and been chilled. Changes in RGP during cold storage then depend on the lifting date, increasing after lifting in November and remaining constant if stored from December or January. RGP of nursery over-wintered seedlings falls to a low level from mid-April, such that after this time, root growth of cold stored seedlings is superior (Cannell *et al.*1990). Cold stored Sitka spruce seedlings are also more tolerant of desiccation and rough handling, possibly reflecting better physiological quality of cold stored stock (Deans *et al.* 1990).

Root growth potential is not enhanced by cold storage in all species and in all conditions. Douglas fir RGP decreases following storage from November and December and is only maintained for one month following storage in January (Cannell *et al.* 1990). However, RGP may depend on storage temperature and pre-storage root development, as

conflicting results have been presented by Ritchie (1982) who noted increased RGP in Douglas fir in the first 6 months of cold storage from January. Storage temperature in this experiment was -1°C whereas that of Cannell *et al.* was +0.5°C, seedlings being of equal age. Cannell *et al.* suggest that the poor RGP of Douglas fir seedlings may have been partly due to poor root development in saturated soil before storage and that Douglas fir normally maintains high RGP in storage (Mason - See Cannell *et al* 1990).

Root carbohydrate content increased during April and early May in both cold stored and nursery over-wintered seedlings. The increase in root starch was slightly greater in the nursery over-wintered seedlings than those from cold storage. This may be explained by slower initiation of photosynthesis after the two months cold-storage and/or the faster depletion of root reserve with more extensive growth. Photosynthetic rate was not monitored after the two treatments although in *P. mugo* and *P. radiata* lower photosynthetic capacity after cold storage is reported (McCracken 1979). Reduced photosynthesis during cold storage of Pinus sylvestris during the first week after outplanting has been attributed to light acclimation and water stress (leading to reduced stomatal resistance), especially in freezer stored seedlings at -4°C (Mattsson and Troeng 1986).

The decline in carbohydrates between May and June coincides with extensive root growth. Sitka spruce roots may grow using carbohydrate stored in the root (Philipson 1988) and depletion of root carbohydrate with growth has previously been reported by McCracken (1979).

Flooding caused large depletion of root carbohydrate in both cold-stored and nursery over-wintered seedlings. The degree of depletion was significantly lower in cold stored seedlings, although this figure reflects both remaining reserve and that in free drained controls with which they

were compared. The levels of reserve remaining after flooding are very similar, these being more important than the degree of depletion as they must support the root until reserves are supplemented from the shoot.

The loss in viability inferred by the TTC test results suggests that the seedlings may survive 65 days flooding after storage. Other researchers have suggested that such decreases (approximately 70% in the root tips and fibrous roots) would not constitute tissue death. However flooded roots never recovered growth during ideal conditions in the RGP test and showed signs of fungal infection, suggesting leakage of cell contents into the soil. *Phythophora* fungi can tolerate the low oxygen in poorly aerated soils and attack dead roots. The fungal zoospores are attracted to root exudates such as ethanol, sugars and amino-acids. These leak out as membrane permeability changes as energy charge falls (Stolzy and Sojka 1984). Seedlings declined rapidly during post-anoxia, especially those over-wintered in the nursery.

In conclusion, cold stored seedlings survive 2 months flooding immediately on transplanting slightly better than those from the nursery. The lower level of carbohydrate reserve in cold stored seedlings compared to those over-wintered in the nursery therefore does not disadvantage Sitka spruce in these conditions as hypothesised. Longer term storage or flooding may yield slightly different results if carbohydrate reserves fell to a threshold value. Superior survival of cold stored seedlings may be due to their more dormant condition at flood initiation which makes them more resilient to damage (Coutts 1981). Cold storage thus seems beneficial for Sitka spruce as it causes rapid root growth and establishment early in the season and reduces slightly the effects of soil waterlogging as the seedlings are transplanted.

Chapter 7

General Discussion

7.1 PRACTICAL RELEVANCE OF AREA INVESTIGATED

The physiology of the over-wintering tree root is an important area for research. Trees and especially their root systems are mainly studied during the growing season and studies of Winter survival concentrate mainly on the extremes, e.g. at the altitudinal tree line or in very low temperatures in the Taiga regions. In temperate areas, where Winter mortality is substantial but not catastrophic, root physiology is relatively neglected. However, when plants are surviving under stress, a very small increase in stress may tip the balance and lead to large scale die-back. This increase may result from increasing susceptibility to stress or from environmental fluctuations.

At present, Sitka spruce in British forestry plantations suffer large-scale windthrow. Soil waterlogging during Winter and the flood-intolerance of submerged roots results in shallow rooting depth and instability. The advantages of the species in terms of productivity and tolerance of soils and climate make it the most planted in Scotland, although others have greater waterlogging tolerance and would suffer less windthrow. Thus Scottish forestry may be fragile to change. Climate change or forest practice may make seedlings more susceptible to injury. As emphasised in Chapter 1, there are many aspects of anoxic injury and mechanisms of anoxia tolerance. This thesis has examined the effect of Winter flooding on the carbohydrate metabolism of the root and aims to test how further stresses of increased temperature or cold store pretreatment may affect survival.

Whether or not a cell survives anoxia may depend on generation of sufficient energy, the compatibility of the end-products of anaerobiosis with cell function, the availability and supply of assimilates and the maintenance of supply of intermediates needed in the TCA cycle (Jackson and Drew 1984).

7.2 EFFECT OF WINTER WATERLOGGING ON CARBOHYDRATE METABOLISM

7.2.1 Carbohydrate content

The starch content of both Lodgepole pine and Sitka spruce roots was similar to that reported for various conifers. In comparing storage carbohydrates, it must be noted that the content depends on the date of measurement, section of root and method of analysis. In this thesis, the sugar content represents glucose present and that released by the acid hydrolysis of sucrose, maltose and raffinose in the root tissue during starch solubilisation (see chapter 2). It does not include fructose and galactose content and is expressed in terms of glycosyl content (molecular weight 162.1g). The sugar content therefore cannot strictly be compared to sugar contents published where each component is determined separately and in this thesis is merely measured to compare changes during flooding. In Lodgepole pine, starch content of the distal 15cm of root was approximately 59mg g^{-1} d.wt and 74mg g^{-1} d.wt in Sitka spruce. Levels of starch reserve were similar to those reported for conifers although most reported are that of the whole root system:

Pinus mugo	43 mg g^{-1} d.wt root	McCracken (1979)
Pinus radiata	62	McCracken (1979)
Picea sitchensis	92	Philipson (1988)
Pseudotsuga menziesii	95	Marshall and Waring (1985)

In both Lodgepole pine (flood-tolerant) and Sitka spruce (flood-sensitive), 3 months of Winter flooding, initiated after cessation of root growth, severely depleted the root carbohydrate reserve. In each species TNC was reduced to less than half that of free-drained controls, reflecting almost total depletion of starch. Sugar content of flooded

seedlings was similar to those from free-drained soil. In some species, waterlogging of the roots can lead to an accumulation of carbohydrates. This occurs when the depletion through accelerated glycolysis and reduced photosynthesis is outweighed by the amount of carbohydrate saved due to cessation of growth (Setter *et al.* 1987). Starch often accumulates in the shoot due to lack of translocation to the roots (e.g. Castonguay 1993). However in dormant Winter seedlings reserve is expended only for maintenance respiration. The depletion of reserves presumably reflects the high costs of anaerobic respiration and impaired phloem transport. Under anaerobic conditions phloem transport in roots is slowed or inhibited (Wilkins and Martin 1967, Vartapetian *et al.* 1978, Drew and Sisworo 1979, Saglio 1985), such that lack of substrates for respiration may become critical for cell survival (Jackson and Drew 1984). In dormant roots, a supply of substrate is essential for maintenance respiration. Amthor (1984) has defined the function of maintenance respiration. It supplies energy for resynthesis of substrates constantly processed in metabolism, e.g. enzymatic proteins, ribonucleic acids and membrane lipids and maintains ion and metabolite gradients required for normal cell function. The energy generated is also needed for physiological adaptation to changing environments. Soil waterlogging is obviously demanding in terms of energy, as anaerobic respiration is inefficient and more substrate is needed to produce each mole of ATP. Starch reserves have here been shown to be quickly depleted under waterlogging.

The availability of stored starch for root respiration has been questioned. Various authors have evidence that root starch is not readily mobilised for respiration such that glycolysis is dependant on assimilate supply by phloem transport (see Jackson and Drew 1984). It is unclear what substance(s) controls the mobilisation of starch to sugar in freely

drained Sitka spruce roots, though indole-acetic acid may be involved (Philipson 1988). In *Phaseolus vulgaris* cuttings, Altman and Waring (1975) demonstrated that exogenous IAA application increased rooting and the availability of sugars where roots were forming, presumably through its enhancement of the breakdown of starch. The almost complete depletion of starch reserves after three months flooding of both Lodgepole pine and Sitka spruce seedlings in chapters 3 and 4 suggests that these reserves *can* be utilised in flooded roots. Large scale depletion of total non-structural carbohydrate reserves during anaerobic incubation has also been noted in anoxia-intolerant *Glyceria maxima* storage tissue. TNC and sugar content of rhizome and swollen stem base tissue were determined in species of varying flood tolerance. *G. maxima* showed depletion of total non-structural carbohydrates to approximately 50% its initial levels during only 4 days incubation, whereas TNC content of *Phalaris arundinacea* and *Scirpus maritimus* was unchanged. The reduction was mainly at the expense of storage carbohydrates, starch or fructosan (Barclay and Crawford 1983). In rice seedlings Atwell (1982) has also shown metabolic starch consumption during anaerobic incubation (see Setter *et al.* 1987). Thus research is needed to clarify the 'usability' of starch under anaerobiosis.

Depletion of reserves may be directly damaging to the tissue. The rate of depletion of carbohydrates after flooding was not investigated. This may change due to metabolic changes in the cell, such as pH fluctuations affecting enzyme activity, changes in energy charge or substrate supply. Tissue sucrose or glucose content can exert coarse control over respiratory rate, thus regulate the metabolism to match substrate availability (Farrar and Williams 1990). Sucrose may set the capacity of mitochondrial respiration, and adenylates the degree of expression of the respiratory capacity by their fine control. The observed

depletion of root carbohydrate reserve during Winter flooding (and the probable fall in energy charge) may cause changes in the rate of carbohydrate consumption. Root carbohydrate after 65 and 137 days flooding was measured in Sitka spruce (Chapter 5). The daily rate of carbohydrate consumption was calculated from the initial (mid/late November), intermediate (late January) and final (mid April) TNC content of roots, assuming total absence of phloem translocation to the roots. During the first 65 days, carbohydrate consumption averaged 1.0mg glucose g^{-1}d.wt. day^{-1} in comparison to only 0.4 during the latter 72 days of waterlogging. This may reflect a slowing of glycolysis as substrate level declines and/or the observed loss of tissue viability recorded over this period. Control of glycolysis may not be well developed in Sitka spruce. Crawford suggested that flood-tolerant plants tend to survive by control of glycolytic rate and carbohydrate conservation whereas flood-sensitive species accelerate glycolysis and quickly deplete their carbohydrate reserve (Barclay and Crawford 1983, Crawford 1989). Sitka spruce may therefore fit this pattern.

Lodgepole pine and Sitka spruce of Washington origin were compared in Chapter 3 in order to investigate whether carbohydrate metabolism under anoxia differed in the two species and aided superior survival of Lodgepole pine. The distal 15cm of root of Lodgepole pine contains more starch and sugar than Sitka spruce roots. Storage carbohydrates are important for flood survival (Setter *et al.* 1987). Reducing the growth of rice seedlings through low nitrogen supply increases the carbohydrate content and their survival after submergence (Palada and Vergara 1972).

When placed under anoxia, excised roots of each species showed a similar Pasteur effect, glycolysis increasing approximately 2 fold. However the roots were detached from the shoot preventing any oxygen

supply which may normally occur under soil waterlogging. This has been shown to be superior in Lodgepole pine to Sitka spruce roots (Philipson and Coutts 1978). Also, measurement of roots excised from freely drained seedlings and those placed immediately under anoxia does not allow any metabolic adaptation which may occur naturally under longer term waterlogging. The degree of carbohydrate depletion under total root waterlogging can be compared from Chapters 3 and 4. These experiments were performed in consecutive years, but flooding treatments were initiated at similar dates and for the same duration. A comparison of results is considered useful, bearing in mind that variations in weather may affect results and experiments should ideally be conducted together.

Root carbohydrate content after 3 months flooding expressed as a percentage of initial pre-flood content

Lodgepole pine	*-uncovered stem*	34.3%
	-blocked lenticels on stem	22.3%
Sitka spruce (Alaskan origin)		39.7%
Sitka spruce (Washington origin)		45.4%

There does not seem to be any metabolic adaptation to waterlogging in terms of control of glycolytic rate. Although Lodgepole pine roots contain more carbohydrate reserve, its depletion under waterlogging is greater than in Sitka spruce and was exacerbated by prevention of aeration from the stem. Thus Lodgepole pine's superior flood tolerance does not seem to result from carbohydrate metabolism, although larger carbohydrate supply may prove advantageous. Neither does aeration of the root system lead to reduced carbohydrate depletion. It seems to convey flood tolerance through another mechanism. Since oxygen appears to reach the distal sections of primary root (Chapter 3), more energy will be supplied per unit of hexose respired, even though

anaerobic respiration occurs (ethanol accumulation increased 2-3 fold in flooded Lodgepole pine roots compared to those under free drained conditions). Thus Lodgepole pine roots may maintain higher energy levels than Sitka spruce roots through their superior internal gas transport. It is possible that oxygen may allow a degree of phloem transport and greater aeration of the rhizosphere, therefore protecting the root from soil toxins. A comparison of root adenylate energy charge under waterlogging would be an interesting follow-up to this work.

Maintenance of sugar content is important both in terms of energy supply and for conservation of cell ultrastructure. Soluble carbohydrates are needed for preservation of mitochondria (Vartapetian *et al.* 1976, 1978; Webb and Armstrong 1983) and can prolong tissue survival under anoxia. It can be essential for survival under low oxygen (Setter *et al.* 1987) and for root establishment after the water table has receded and soil is re-oxygenated (Philipson 1988).

If flooding does not reduce the carbohydrate content of roots enough directly to injure the tissues, the depletion during Winter waterlogging may severely disadvantage the seedlings in Spring. Philipson (1988) has shown that Sitka spruce is unusual in that new root growth utilises starch stored in the roots. In most seedlings studied, Ritchie and Dunlap (1980) concluded that new root growth utilises current photosynthate rather than starch reserves. In Philipson's study, bark ringed Sitka spruce roots grew whilst depleting starch reserves from the pericycle cells of the roots of the previous season's growth. Seedlings growing in this way may have an advantage in terms of establishment success in forestry plantations. During transit, rough handling can cause stomatal closure and reduction in post-planting photosynthesis. Since Sitka spruce root growth depends only on root starch reserves, roots may grow until the shoot has recovered and photosynthesis can supply

assimilates on which root growth ultimately depends. In other species such as Douglas fir, root initiation may be postponed until photosynthesis recovers from handling and supplies the necessary assimilates. Early growth of new roots is important in Spring in terms of water absorption and reduction in water stress which may develop during transit (Philipson 1988). Therefore any depletion of carbohydrate reserves during Winter may disadvantage the seedlings' growth and establishment in Spring.

7.2.2 Ethanol accumulation

Ethanol accumulation occurred in both flooded and freely drained roots *in vivo*, indicating a degree of anaerobiosis even in aerated soil in Winter when metabolic activity is lower. Flooding leads to greater accumulation of ethanol, approximately 6µmoles g^{-1} f.wt. in Lodgepole pine roots after 90 days flooding and 5.8µmoles g^{-1} f.wt. in Sitka spruce after 60 days. These results are in contrast to those recorded by Crawford and Baines (1977) for pot-grown seedlings in the greenhouse. They analysed ethanol content of the whole root system after a period of flooding and observed a much lower accumulation of ethanol in Lodgepole pine roots, only 0.7µmoles g^{-1} f.wt. However ethanol accumulation in Sitka spruce was very similar, 5µmoles g^{-1} f.wt. Crawford and Baines suggested that the lower accumulation in Lodgepole pine indicated its ability to limit ethanol production by control of glycolysis under anoxia. It is possible that the seedlings in Crawford's study responded differently to anoxia under the higher temperature (20°C) and longer daylength (16hrs) used in their experiment. Aeration of the root from the shoot may be superior in these seedlings, therefore reducing the oxygen stress of the roots of Lodgepole pine. Alternatively the warmer soil may enhance the leakage of ethanol to the roots. It is therefore important to consider the climate conditions when comparing

ethanol accumulation. Dormant roots in Winter may be affected differently from active plants under greenhouse conditions.

Similar tissue accumulation of ethanol has been reported in a number of herbaceous species, e.g. 7.3μmoles g^{-1} f.wt. in *Ranunculus scleratus*, 8.0 in *Senecio aquaticus* (Smith and ap Rees 1979). Most of the ethanol produced by flooded roots is likely to leak into the surrounding medium. This may be advantageous as ethanol, or its oxidation product acetaldehyde, may be toxic, but it does represent the loss of fixed carbon. When ethanol production was assessed *in vitro* (Chapters 3 and 4), between 56 and 92% of the ethanol measured was present in the water around the roots. This is comparable with leakage from alfalfa roots (77%) and bird's foot trefoil (84%) (Barta 1984).

Whether the concentration of ethanol accumulating in flooded roots is toxic is a controversial issue. Much evidence suggests that its accumulation in plant tissues reduces viability. A pattern of ADH induction and ethanol accumulation has been noted in many flood intolerant herbaceous species contrasting with no induction or accumulation in those withstanding anoxia (Crawford 1978). Circulation of air during anaerobic incubation and prevention of its accumulation has been shown to prolong life in a number of species of germinating seeds (Crawford *et al.* 1987). Ethanol and lactic acid accumulation may be detrimental to the selectivity of root membranes (Jackson and Drew 1984), causing damage by lipid solubilisation and inactivation of mitochondrial enzyme activity. This feeds-back by further increasing glycolysis (Crawford 1978). However this pattern has been contradicted by a number of authors. Jackson, Herman and Goodenough (1982) applied up to 100x the concentration of ethanol normally found in flooded soil to roots of peas and observed no injury, concluding that typical levels of ethanol could not explain observed flooding injury. It is possible

however that 'artificial' application of ethanol may be less harmful than an equal concentration produced internally (see Crawford 1982). The return of ethanol-rich tissues to oxygen results in a surge of acetaldehyde which is considered more toxic (Studer and Braendle 1988).

Flooding of Sitka spruce seedlings caused greater injury to the root tip sections in terms of reduction of triphenyl tetrazolium chloride solution than that incurred by equivalent roots of Lodgepole pine seedlings. In contrast, the distal 15cm of Lodgepole pine root appeared to be only slightly injured after 3 months flooding and there was no difference in damage between the sections. After 3 months, flooding caused more injury to Sitka spruce root tips than the proximal section, whether at ambient or elevated temperature (Chapters 4 and 5), although continued flooding for over 4 months reduced the viability of all sections to a presumably minimal level (Chapter 5). After only two months flooding in Spring, the 5cm distal section of Sitka spruce root was similarly damaged to the fibrous roots, although each was significantly more damaged than the tap root (Chapter 6). Coutts and Philipson (1978a) suggested that the greater susceptibility of the root tip may be due to greater demand for oxygen. Even in Winter, when the roots are dormant, the physiological measurements support this theory. In general, the results of this thesis indicate that the respiratory demand of the tissues as flooding is initiated is the most important factor determining survival under Winter flooding rather than the carbohydrate content. This is seen in a number of cases, each of which will be discussed briefly below to support this statement.

Comparing the different root sections, those which respire more slowly and conserve their carbohydrate supply generally retain tissue viability. Possession of a larger carbohydrate reserve does not alone enhance survival. The depletion of reserve in the tap roots during cold

storage did not reduce survival of the roots when they were waterlogged in the Spring. In fact cold stored seedlings fared better than those over-wintered in the nursery. Neither did their superior survival reflect lower respiration rates at flood initiation, but may reflect greater dormancy. However carbohydrate depletion in the tap roots of nursery overwintered seedlings was significantly greater than that of cold stored seedlings, suggesting that the root respiration rate was higher in nursery seedlings during flooding. This correlates with increased injury during flooding after nursery over-wintering. The tap roots had lower carbohydrate content initially in comparison to the fibrous roots and root tip sections. However the fibrous roots suffered greater carbohydrate depletion during flooding and had less carbohydrate remaining when compared to the tap roots. In this experiment, root tips and fibrous roots showed a similar degree of injury, correlating with similar carbohydrate content and initial respiration rate.

More evidence supporting the conclusion that respiratory rate and depletion of carbohydrates are an important determining factor in survival of Winter waterlogging come from Chapters 3 and 4. Comparing the injury to the 3 root sections in the two species, Sitka spruce, with much higher respiration rate in the root tip section at flood initiation, showed greater injury in this section than those proximal. In contrast, Lodgepole pine in which the root sections showed fairly uniform respiration rate showed uniform viability up the root length. These results were also reflected in greater carbohydrate depletion in the tip sections of Sitka spruce, but similar in each section of Lodgepole pine.

When the importance of flooding date on root survival was investigated, the respiration rate decreased from October to November in each provenance. Injury due to flooding also decreased if flooding was initiated in November rather than October. The greatest difference in

respiratory activity between October and November was seen in the Alaskan provenance which also showed the greatest susceptibility to the date of flood initiation, being most severely damaged following flooding from October. Even though the Alaskan provenance contained more carbohydrate reserve than that of Washington origin when flooded in October, it showed a higher rate of depletion and greater injury.

The injury to the tissues may be due to a number of factors. High respiratory activity leads to faster depletion of the carbohydrate reserve needed for cell maintenance. It also results in accumulation of potentially toxic metabolites. The actual cause of injury cannot be concluded from this work. After flooding, injured roots had lower carbohydrate content and reduced respiratory activity, both in terms of ethanol and CO_2 production (Chapter 4). The relationship between injury and carbohydrate content and its depletion during respiration is important in considering possible effects of global warming.

Waterlogging is prevalent in Winter when roots are more tolerant to flooding, seemingly due to lower oxygen demand in the non-growing roots at lower temperature. Roots are known to survive better when flooding is postponed until later in the Winter when roots have entered dormancy. Environmental changes which may affect root dormancy or demand for oxygen may therefore tip the balance and result in greater die-back of flooded roots. Predictions of CO_2 increase and global warming are many and varied and the impact of each on plant biochemistry complex. One scenario of global warming may be increased respiration rates and later root dormancy. Roots do not have a period of dormancy in the same sense as shoots as they have no chilling requirement and will grow providing their minimum temperature requirement is met. Growing roots can therefore be found most of the year, provided that the soil is warm enough (Coutts and Philipson 1987). Coutts and Nicoll

(1990a) have observed the periodicity of root growth in Sitka spruce. Unlike shoot growth, cessation and initiation of root extension is largely controlled by temperature. However control of root growth is not fully understood and there may be a degree of control by the shoot. The authors found that roots remained dormant during Winter in warmer soils than those in which growth was initiated in March. They suggested that internal regulation of growth may occur in roots which have been actively growing for a long period. In Winter the shoot may sense shortening daylength and low light intensity and reduce the tendency for root growth. An inhibitor in the root system may prevent root growth until it is removed to the shoot in the transpiration stream (Philipson 1988). Coutts (1980) has suggested abscissic acid to be a possible inhibitor. Thus the effects of global warming on root growth are uncertain. It seems likely from the evidence that roots will stop growing during Winter irrespective of temperature increases. However growth during Autumn may proceed at a faster rate.

Root respiration increases with increasing temperature (e.g. Lawrence and Oechel 1983). Root respiration rate of Sitka spruce was analysed at similar dates in 2 consecutive years (see chapters 3 and 4). Non-growing roots were measured either at 10 or 20°C, respiration rate approximately doubling with temperature. Thus increases in temperature may proportionately increase the maintenance respiration rate of dormant roots. This response may tip the balance, carbohydrate being depleted at a faster rate and resulting in greater root injury, as demonstrated in Chapter 5. Species which are just surviving under current environmental stresses may be pushed beyond their limit by environmental change. Excessive nitrogen supply in eutrophic lakes leads to reduced carbon allocation to the structural tissues of reeds. Root anaerobiosis causes rapid depletion of carbohydrate reserves such that

episodic stresses such as shoot flooding tip the survival balance and lead to reed decline (Cizkova-Koncalova *et al.* 1992). It is easy to see how small environmental changes could have large consequences when examining the physiological responses of forest seedlings to Winter flooding.

Understanding the underlying physiology of forest seedlings is essential for predicting responses to changing environments and planning future forestry.

REFERENCES

Abbott, R. J. (1986). Life history variation associated with the polymorphism for capitulum type and outcrossing rate in *Senecio vulgaris* L. Heredity. **56**: 381-391.

Adams, S. N., Dickson, D. A. and Cornforth, I. S. (1972). Some effects of soil water tables on the growth of Sitka spruce in Northern Ireland. Forestry. **45**: 129-133.

Altman, A. and Waring, P. F. (1975). The effect of IAA on sugar accumulation and basipetal transport of ^{14}C-labelled assimilates in relation to root formation in *Phaseolus vulgaris* cuttings. Physiol. Plant. **33**: 32-38.

Amthor, J. S. (1984). The role of maintenance respiration in plant growth. Plant Cell and Environ. **7**: 561-569.

Andrews, C. J. and Pomeroy, M. K. (1979). Toxicity of anaerobic metabolites accumulating in Winter wheat seedlings during ice encasement. Plant Physiol. **64**: 120-125.

Armstrong, W. and Read, D. J. (1972). Some observations on oxygen transport in conifer seedlings. New Phytol. **71**: 55-62.

Armstrong, W. and Gaynard, T. J. (1976). The critical oxygen pressures for respiration in intact plants. Physiol. Plant. **37**: 200-206.

Armstrong, W., Booth, T. C., Priestley, P. and Read, D. J. (1976). The relationship between soil aeration, stability and growth of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) on upland peaty gleys. J. Appl. Ecol. **13**: 585-591.

Armstrong, W. and Armstrong, J. (1990). Light enhanced convective throughflow increases oxygenation in rhizomes and rhizosphere of *Phragmites australis* (Cav.) Trin. ex Steud. New Phytol. **114**: 121-128.

Atwell, B. J. (1982). Growth and metabolism of rice seedlings at low oxygen concentrations. PhD Thesis, University of Western Australia, Nedlands, 6009.

Barclay, A. M. and Crawford, R. M. M. (1981). Temperature and anoxic injury in pea seedlings. J. Exp. Bot. **32**: 943-949

Barclay, A. M. and Crawford, R. M. M. (1982). Plant growth and survival under strict anaerobiosis. J. Exp. Bot. **33**: 541-549.

Barclay, A. M. and Crawford, R. M. M. (1983). The effect of anaerobiosis an carbohydrate levels in storage tissues of wetland plants. Ann. Bot. **51**: 255-259.

Barta, A. L. (1984). Ethanol synthesis and loss from flooded roots of *Medicago sativa* L. and *Lotus corniculatus* L. Plant Cell and Environ. **7**: 187-191.

Bennett, A. B. and Spanswick, R. M. (1984). H^+-ATPase activity from storage tissue of Beta vulgaris. Plant Physiol. **74**: 545-548.

Bergmeyer, H.U. (1963). Methods of enzymatic analysis. Verlag Chemie, Academic Press Inc.

Black, R. A. (1984). Water relations of *Quercus palustris*: field measurements on an experimentally flooded stand. Oecologia. **64**: 14-20.

Boehringer (1989). L-malic acid - Test kit instructions for the determination of L-malic acids in food stuffs and other materials. Boehringer Mannheim.

Boehringer (1989). Starch - Test kit Instructions for the determination of native starch in foodstuffs and other materials . Boehringer Mannheim.

Boggie, R. (1972). Effect of water-table height on root development of *Pinus contorta* on deep peat in Scotland. Oikos. **23**: 304-312.

Bolin, W., Jaer, J. and Doos, B. R. (1990). The Greenhouse Effect Climatic Change and Ecosystems. In: (eds. W. Bolin, B. R. Doos, J. Jager and R. A. Warwick), Scope 29: The Greenhouse Effect Climatic Change and Ecosystems. Chichester, John Wiley. pp1-32.

Boyer, W. D., Romancier, R. M. and Ralston, C. W. (1971). Root respiration rates of 4 tree species grown in the field. Forest Sci. **17**: 492.

Bradbury, I. K. and Malcolm, D. C. (1978). Dry matter accumulation by *Picea sitchensis* seedlings during Winter. Can. J. For. Res. **8**: 207-213.

Buchel, H. B. and Grosse, W. (1990). Localization of the porous partition responsible for pressurised gas transport in *Alnus glutinosa* (L.) Gaertn. Tree Physiol. **6**: 247-256.

Burdett, A. N. (1987). Understanding root growth capacity: theoretical considerations in assessing planting stock quality by means of root growth tests. Can. J. For. Res. **17**: 768-775.

Buwalda, F., Thomson, C. J., Steigner, W., Barrett-Lennard, E. G., Gibbs, J. and Greenway, H. (1988). Hypoxia induces membrane depolarisation and potassium loss from wheat roots but does not increase their permeability to sorbitol. J. Exp. Bot. **39**: 1169-1183.

Cannell, M. G. R., Tabbush, P. M., Deans, J. D., Hollingsworth, M. K., Sheppard, L. J., Philipson, J. J. and Murray, M. B. (1990). Sitka spruce and Douglas fir seedlings in the nursery and in cold storage: root growth potential, carbohydrate content, dormancy, frost hardiness and mitotic index. Forestry. **63**: 9-27.

Carpenter, J. R. and Mitchell, C. A. (1980). Root respiration characteristics of flood-tolerant and intolerant tree species. J. Amer. Soc. Hort. Sci. **105**: 684-687.

Castonguay, Y., Nadeau, P. and Simard, R. R. (1993). Effects of flooding on carbohydrate and ABA levels in roots and shoots of alfalfa. Plant Cell and Environ. **16**: 695-702.

Chomba, B. M., Guy, R. D. and Weger, H. G. (1993). Carbohydrate reserve accumulation and depletion in Engelmann spruce (*Picea engelmannii* Parry): effects of cold storage and pre-storage CO_2 enrichment. Tree Physiol. **13**: 351-364.

Cizkova-Koncalova, H., Kvet, J. and Thompson, K. (1992). Carbon starvation: a key to reed decline in eutrophic lakes. Aquat. Bot. **43**: 105-113.

Coutts, M. P. and Armstrong, W. (1976). Role of oxygen transport in the tolerance of trees to waterlogging. In: (eds. M. G. R. Cannell and F.T. Last), Tree Physiology and Yield Improvement. London, Academic Press. pp361-385.

Coutts, M. P. and Philipson, J. J. (1978a). Tolerance of tree roots to waterlogging I. Survival of Sitka spruce and Lodgepole pine. New Phytol. **80**: 63-69.

Coutts, M. P. and Philipson, J. J. (1978b). Tolerance of tree roots to waterlogging II. Adaptation of Sitka spruce and Lodgepole pine to waterlogged soil. New Phytol. **80**: 71-77.

Coutts, M. P. (1980). Control of water loss by actively growing Sitka spruce seedlings after transplanting. J. Exp. Bot. **31**: 1587-1597.

Coutts, M. P. (1981). Effects of waterlogging on water relations of actively-growing and dormant Sitka spruce seedlings. Ann. Bot. **47**: 747-753.

Coutts, M. P. (1982). The tolerance of tree roots to waterlogging V. Growth of woody roots of Sitka spruce and Lodgepole pine in waterlogged soil. New Phytol. **90**: 467-476.

Coutts, M. P. (1983). Root architecture and tree stability. Plant Soil. **71**: 171-188.

Coutts, M. P. and Philipson, J. J. (1987). Structure and physiology of Sitka spruce roots. Proc. Roy. Soc. Edinb. **93B**: 131-144.

Coutts, M. P. and Nicoll, B. C. (1990a). Growth and survival of shoots, roots, and mycorrhizal mycelium in clonal Sitka spruce during the first growing season after planting. Can. J. For. Res. **20**: 861-868.

Coutts, M. P. and Nicoll, B. C. (1990b). Waterlogging tolerance of roots of Sitka spruce clones and of strands from *Thelephora terrestris* mycorrhizas. Can. J. For. Res. **20**: 1894-1899.

Cram, W. H. and Lindquist, C. H. (1981). Overwinter and Spring storage of pine and spruce seedlings. For. Chron. 162-164

Crawford, R. M. M. (1967). Alcohol dehydrogenase activity in relation to flooding tolerance in roots. J. Exp. Bot. **18 No.56**: 458-464.

Crawford, R. M. M. and Tyler, P. D. (1969). Organic acid metabolism in relation to flooding tolerance in roots. J. Ecol. **57**: 235-244.

Crawford, R. M. M. (1972). Some metabolic aspects of ecology. Trans. Bot. Soc. Edinb. **41**: 309-322.

Crawford, R. M. M. (1976). Tolerance of anoxia and the regulation of glycolysis in tree roots. In: (eds. M. G. R. Cannell and F.T. Last), Tree physiology and yield improvement. London. Academic Press. pp387-401.

Crawford, R. M. M. and Baines, M. A. (1977). Tolerance of anoxia and the metabolism of ethanol in tree roots. New Phytol. **79**: 519-526.

Crawford, R. M. M. (1977). Tolerance of anoxia and ethanol metabolism in germinating seeds. New Phytol. **79**: 511-517.

Crawford, R.M.M. (1978). Metabolic adaptation to anoxia. In (eds. D.D. Hook and R.M.M. Crawford) Plant Life in Anaerobic Environments. Ann Arbor Science, Michigan. pp119-136.

Crawford, R. M. M. (1982). Physiological responses to flooding. In: (eds. O. L. Lange, P. S. Nobel, C. B. Osmond and H. Zeigler), Encyclopaedia of plant physiology .Physiological Plant Ecology II. Water relations and carbon assimilation. Berlin, Springer Verlag. pp453-477.

Crawford, R. M. M. (1983). Root survival in flooded soils. In: (eds. A. J. P. Gore), Mires; swamp, bog, fen, and moor, A. General studies. Amsterdam, Elsevier. Amsterdam. pp257-283

Crawford, R. M. M. and Zochowski, Z. M. (1984). Tolerance of anoxia and ethanol toxicity in chickpea seedlings (*Cicer arietinum* L.). J. Exp. Bot. **35**: 1472-1480.

Crawford, R. M. M., Monk, L. S. and Zochowski, Z. M. (1987). Enhancement of anoxia tolerance by the removal of the volatile products of anaerobiosis. In: (ed. R.M.M. Crawford). Plant life in aquatic and amphibious habitats. Special publications series of the British Ecological Society. No.5. Oxford, Blackwell. pp375-384.

Crawford, R.M.M. (1989) Studies in plant survival. Blackwell Scientific Publications, Oxford.

Crawford, R. M. M. and Finegan, D. M. (1989). Removal of ethanol from Lodgepole pine roots. Tree Physiol. **5**: 53-61.

Crawford, R. M. M. (1992). Oxygen availability as an ecological limit to plant distribution. Adv. Ecol. Res. **23**: 93-185.

Dacey, J. W. H. (1980). Internal winds in water lilies: an adaptation for life in anaerobic sediments. Science. **210**: 1017-1019.

Deans, J. D., Lundberg, C., Tabbush, P. M., Cannell, M. G. R., Sheppard, L. J. and Murray, M. B. (1990). The influence of desiccation, rough handling and cold-storage on the quality and establishment of Sitka spruce planting stock. Forestry. **63 (2)**: 129-141.

Drew, M. C. and Sisworo, E. J. (1979). The development of waterlogging damage in young barley plants in relation to plant nutrient status and changes in soil properties. New Phytol. **82**: 301-314.

Everard, J. E., Neustein, S. A. and Taylor, G. G. M. (1970). Crop stability. Forestry Commission Report on Forest Research 1970, London: HMSO. 96-97.

Farrar, J. F. and Williams, J. H. H. (1990). Control of the rate of respiration in roots: compartmentation, demand and the supply of substrate. In: (ed. M. J. Emes), Compartmentation of plant metabolism in non-photosynthetic tissues. Society for experimental biology seminar series. CUP. pp167-188.

Farrar, J. F. and Williams, M. L. (1991). The effects of increased atmospheric carbon dioxide and temperature on carbon partitioning, source-sink relations and respiration. Plant Cell and Environ. **14**: 819-830.

Ford, E. D. and Deans, J. D. (1977). Growth of a Sitka spruce plantation:spatial distribution and seasonal fluctuations of lengths, weights and carbohydrate concentrations of fine roots. Plant Soil. **47**: 463-485.

Fraser, A. I. and Gardiner, J.B.H. (1967). Rooting and stability in Sitka spruce. Bull. For. Commn, Lond. **40**:

Geiger, D. A. and Savonick, S. A. (1975). Effect of temperature, anoxia and other metabolic inhibitors on translocation. Enc. Plant Physiol. **New series 1**: 256-286.

Gill, C. J. (1970). The flooding tolerance of woody species-a review. For. Abstr. **31**: 671.

Green, M. S. and Etherington, J. R. (1977). Oxidation of ferrous iron by rice (Oryza sativa L.) roots: a mechanism for water-logging tolerance. J. Exp. Bot. **28**: 678-690.

Grosse, W., Frye, J. and Lattermann, S. (1992). Root aeration in wetland trees by thermo-osmotic gas transport. Tree Physiol. **10**: 285-295.

Hendry, G. A. F. and Brocklebank, K. J. (1985). Iron induced oxygen radical metabolism in waterlogged plants. New Phytol. **101**: 199-206.

Hook, D. D., Debell, D. S., McKee Jr, W. H. and Askew, J. L. (1983). Responses of loblolly pine (mesophyte) and swamp tupelo (hydrophyte) seedlings to soil flooding and phosphorus. Plant Soil. **71**: 387-394.

Hook, D. D. and Denslow, S. (1987). Metabolic responses of four families of loblolly pine to two flood regimes. In: (ed. R.M.M. Crawford) Plant life in aquatic and amphibious habitats. Special publications series of the British Ecological Society, No. 5. Oxford, Blackwell. pp281-292.

Jackson, M. B., Herman, B. and Goodenough, A. (1982). An examination of the importance of ethanol in causing injury to flooded plants. Plant Cell and Environ. **5**: 163-172.

Jackson, M. B. and Drew, M. C. (1984). Effects of flooding on growth and metabolism of herbaceous plants. In: (ed. T. T. Kozlowski), Flooding and plant growth. London, Academic Press. pp47-128.

Jarvis, P. G. and Jarvis, M. S. (1964). Growth rates of woody plants. Physiol. Plant. **17**: 654-666.

King, J. A., Smith, K. A. and Pyatt, D. G. (1986). Water and oxygen regimes under conifer plantations and native vegetation an upland peaty gley soil and deep peat soils. J. Soil Sci. **37**: 485-497.

Kozlowski, T. T. and Pallardy, S. G. (1984). Effects of flooding on water, carbohydrate and mineral relations. In: (ed. T. T. Kozlowski), Flooding and plant growth. London, Academic Press. pp165-193.

Kozlowski, T. T. (1986). Soil aeration and growth of forest trees. Scand. J. For. Res. **1**: 113-123.

Krueger, K. W. and Trappe, J. M. (1967). Food reserves and seasonal growth of Douglas fir seedlings. Forest Sci. **13**: 192-202.

Lahde, E. (1966). Studies on the respiration rate in the different parts of the root system of pine and spruce seedlings and its variation during the growing season. Acta For. Fenn. **81**: 1.

Lawrence, W. T. and Oechel, W. C. (1983). Effects of soil temperature on the carbon exchange of taiga seedlings. I. Root respiration. Can. J. For. Res. **13**: 840-849.

Lees, J. C. (1972). Soil aeration and Sitka spruce seedling growth in peat. J. Ecol. **60**: 343-349.

Lindstrom, A. and Nystrom, C. (1987). Seasonal variation in root hardiness of container grown Scots pine, Norway spruce, and Lodgepole pine seedlings. Can. J. For. Res. **17**: 787-793.

Lindstrom, A. and Mattsson, A. (1989). Equipment for freezing roots and its use to test cold resistance of young and mature roots of *Picea abies* seedlings. Scand. J. For. Res. 59-66.

Lines, R. and Mitchell, A.F. (1966). Differences in Phenology of Sitka Spruce Provenances. Forestry Commission Report on Forest Research 1965. (HMSO Lond.). 173-184.

Lines, R. (1987). Seed origin variation in Sitka spruce. Proc. Roy. Soc. Edinb. **93B**: 25-39.

Low, A. J. (1973). The effective planting season in Scotland. Scottish For. **27**: 4-8.

Low, A. J. (1987). Sitka spruce silviculture in Scottish forests. Proc. Roy. Soc. Edinb. **93B**: 93-106.

Luzikov, V. N., Zubatov, A. S., Rainina, E. and Bakeyeva, L. E. (1971). Degradation and restoration of mitochondria upon deaeration and subsequent reaeration of aerobically grown *Saccharomyces cerevisiae* cells. Biochim. Biophys. Acta. **245**: 321-334.

Malcolm, D. C. (1987). Some ecological aspects of Sitka spruce. Proc. Roy. Soc. Edinb. **93B**: 85-92.

Marshall, J. D. and Waring, R. H. (1985). Predicting fine root production and turnover by monitoring root starch and soil temperature. Can. J. For. Res. **15**: 791-800.

Mattsson, A. and Troeng, E. (1986). Effects of different overwinter storage regimes on shoot growth and net photosynthetic capacity in *Pinus sylvestris* seedlings. Scand. J. For. Res. **1**: 75-84

McCracken, I. J. (1979). Changes in the carbohydrate concentration of pine seedlings after cool storage. N.Z. J. For. Sci. **9**: 34-43.

McKay, H. and Coutts, M. P. (1989). Limitations placed on forestry production by the root system. Asp. Appl. Biol. **22**: 245-253.

Miller, K. F. (1987). Forestry practice in relation to windthrow. Comm. Bull.

Minore, D. (1968). Effects of artificial flooding on seedling survival and growth of six Northwestern tree species. U.S. Dept. Agric. Forest Service Research note PNW-92.

Monk, L. S., Fagerstedt, K. V. and Crawford, R. M. M. (1989). Oxygen toxicity and superoxide dismutase as an anti-oxidant in physiological stress. Physiol. Plant. **76**: 456-459.

Nature Conservancy Council, (1986). Nature conservation and afforestation in Britain. 22-23.

Neilson, R. E., Ludlow, M. M. and Jarvis, P. G. (1972). Photosynthesis in Sitka spruce (*Picea sitchensis* (Bong.) Carr.). II Response to temperature. J. Appl. Ecol. **9**: 721-745.

Palada, M. C. and Vergara, B. S. (1972). Environmental effects on the resistance of rice seedlings to complete submergence. Crop Sci. **12**: 209-212.

Philipson, J. J. and Coutts, M. P. (1978). The Tolerance of tree roots to waterlogging. III. Oxygen transport in Lodgepole Pine and Sitka spruce roots of primary structure. New Phytol. **80**: 341-349.

Philipson, J. J. and Coutts, M. P. (1979). The induction of root dormancy in *Picea sitchensis* (Bong.) Carr. by Abscissic Acid. J. Exp. Bot. **30**: 371-380.

Philipson, J. J. and Coutts, M. P. (1980). The tolerance of tree roots to waterlogging. IV. Oxygen transport in woody roots of Sitka Spruce and Lodgepole pine. New Phytol. **85**: 489-494.

Philipson, J. J. (1988). Root growth in Sitka spruce and Douglas fir transplants - dependence on the shoot and stored carbohydrates. Tree Physiol. **4 (2)**: 101-108.

Ponnamperuma, F.N. (1984). Effects of flooding on soils. In (ed. T.T. Kozlowski), Flooding and plant growth. London, Academic Press. pp9-45.

Pyatt, D. G. and Smith, K. A. (1983). Water and oxygen regimes of four soil types at Newcastleton forest, south Scotland. J. Soil Sci. **34**: 465-482.

Qureshi, F. A. and Spanner, D. C. (1973). The effect of nitrogen on the movement of tracers down the stolon of *Saxifraga sarmentosa*, with some observation on the influence of light. Planta. **110**: 131-144.

Ritchie, G. A. and Dunlap, J. R. (1980). Root growth potential: its development and expression in forest tree seedlings. N.Z. J. For. Sci. **10**: 218-248.

Ritchie, G. A. (1982). Carbohydrate reserves and root growth potential in Douglas fir seedlings before and after cold storage. Can. J. For. Res. **12**: 905-912.

Roberts, J. K. M., Callis, J., Jardetzky, O., Walbot, V. and Freeling, M. (1984). Cytoplasmic acidosis as a determinant of flooding intolerance in plants. Proc. nat. Acad. Sci. **81**: 6029-6033.

Ross, E. M., Docktor, M. E. and Schatz, G. (1976). Synthesis and degradation of mitochondrial cytochromes. In: (ed. J. S. Cook), Biogenesis and turnover of membrane macromolecules. N.Y., Raven Press. pp37-48.

Saglio, P. H. and Pradet, A. (1980). Soluble sugars, respiration and energy charge during ageing of excised maize root tips. Plant Physiol. **66**: 516-519.

Saglio, P. H. (1985). Effects of path or sink anoxia on sugar translocation in roots of maize seedlings. Plant Physiol. **77**: 285-290.

Sanderson, P. L. and Armstrong, W. (1978). Soil waterlogging, root rot and conifer windthrow: oxygen deficiency or phytotoxicity? Plant Soil. **49**: 185-190.

Sanderson, P. L. and Armstrong, W. (1980). The responses of conifers to some of the adverse factors associated with waterlogged soils. New Phytol. **85**: 351-362.

Sanderson, P. L. and Armstrong, W. (1980). Phytotoxins in periodically waterlogged soils. J. Soil Sci. **31**: 643-653.

Savill, P. S. (1976). The effect of drainage and ploughing of surface water gleys on rooting and windthrow of Sitka spruce in Northern Ireland. Forestry. **49**: 133-141.

Scholander, P. F., van Dam, L. and Scholander, S. I. (1955). Gas exchange in the roots of mangroves. Amer. J. Bot. **42**: 92-98.

Schroder, P., Grosse, W. and Woermann, D. (1986). Localisation of thermo-osmotically active partitions in young leaves of Nuphar lutea. J. Exp. Bot. **37**: 1450-1461.

Setter, T. L., Waters, J., Atwell, B. J., Kapanchanakul, T. and Greenway, H. (1987). Carbohydrate status of terrestrial plants during flooding. In: (ed. R. M. M. Crawford), Plant life in aquatic and amphibious habitats. British Ecol. Soc. Special Publication No. 5. Oxford, Blackwell. pp411-433.

Sieber, M. and Braendle, R. (1991). Energy metabolism in rhizomes of *Acorus calamus* (L.) and in tubers of *Solanum tuberosum* (L.) with regard to their anoxia tolerance. Bot. Acta. **104**: 279-282.

Sionit, N., Strain, B. R., Hellmers, H., Riechers, G. H. and Jagger, C. H. (1985). Long term atmospheric CO_2 enrichment affects the growth and development of *Liquidambar styraciflua* and *Pinus taeda* seedlings. Can. J. For. Res. **15**: 468-471.

Slatyer, R. O. (1967). *Plant-water relationships.* N.Y., Academic Press.

Smith, A. M. and ap Rees, T. (1979). Pathways of carbohydrate fermentation in the roots of marsh plants. Planta. **146**: 327-334.

Steponkus, P. L. and Lanphear, F. O. (1967). Refinement of the triphenyl tetrazolium chloride method of determining cold injury. Plant Physiol. **42**: 1423-1426.

Stolzy, L. H. and Sojka, R. E. (1984). Effects of flooding on plant disease. In: (eds. T. T. Kozlowski), Flooding and Plant growth. N.Y., Academic Press. pp221-264.

Stone, E. C. (1970). Variation in the root growth capacity of ponderosa pine transplants. In: (eds. R. K. Hermann), Regeneration of ponderosa pine. Oregon State University, Corvallis, pp40-46.

Studer, C. and Braendle, R. (1987). Ethanol, acetaldehyde, ethylene release and ACC concentration of rhizomes from marsh plants under normoxia, hypoxia and anoxia. In: (ed. R. M. M. Crawford), Plant life in aquatic and amphibious habitats. Oxford, Blackwell. pp293-301.

Studer, C. and Braendle, R. (1988). Postanoxische effeckte von aethanol in rhizomen von *Glyceria maxima* (Hartm.) Holmberg, *Iris germanica* (Cav.) Trin. Bot. Helvet. **98**: 111-112.

Tabbush, P. M. (1986). Rough handling, soil temperature, and root development in outplanted Sitka spruce and Douglas fir. Can. J. For. Res. **16**: 1385-1388.

Taylor, G. G. M. (1970). Ploughing practice in the forestry commission. For. Commn. forest record. **70**:

Toleman, R.D.L. and Pyatt, D.G. (1974). Site classification as an aid to silviculture in the Forestry Commission of Great Britain. Proc. Tenth Commw. For. Conf. U.K. H.M.S.O. London.

Topa, M. A. and McLeod, K. W. (1986). Aerenchyma and lenticel formation in pine seedlings: A possible avoidance mechanism to anaerobic growth conditions. Physiol. Plant. **68**: 540-550.

Tripepi, R. R. and Mitchell, C. A. (1984a). Metabolic responses of River birch and European birch roots to hypoxia. Plant Physiol. **76**: 31-35.

Tripepi, R. R. and Mitchell, C. A. (1984b). Stem hypoxia and root respiration of flooded maple and birch seedlings. Physiol. Plant. **60**: 567-571.

van den Driessche, R. (1976). Survival of coastal and interior Douglas fir seedlings after storage at different temperatures, and effectiveness of cold storage in satisfying chilling requirements. Can. J. For. Res. **7**: 125-131.

van den Driessche, R. (1987). Importance of current photosynthate to new root growth in planted conifer seedlings. Can. J. For. Res. **17**: 776-782.

Vartapetian, B. B., Andreeva, I. N. and Kozlova, G. I. (1976). The resistance of anoxia and the mitochondrial fine structure of rice seedlings. Protoplasma. **88**: 215-224.

Vartapetian, B. B., Andreeva, I. N. and Nurutdinov, N. (1978). Plant cells under oxygen stress. In: (eds. D. D. Hook and R. M. M. Crawford), Plant life in anaerobic environments. Michigan, Ann Arbor Science. pp12-88.

Webb, T. and Armstrong, W. (1983). The effects of anoxia and carbohydrates on the growth and viability of rice, pea and pumpkin roots. J.Exp. Bot. **34**: 579-603.

Wigley, T. M. W., Jones, P. D. and Kelly, P. M. (1990). Empirical climate studies. In: (eds. B. R. Bolin, J. Doos, J. Jaer and R. A. Warwick), Scope 29: The Greenhouse Effect Climate Change and Ecosystems. Chichester, John Wiley. pp1-32.

Wilcox, H. (1954). Primary organization of active and dormant roots of noble fir, *Abies procera*. Amer. J. Bot. **41**: 812-821.

Wilkins, M. B. and Martin, M. (1967). Dependence of basipetal polar transport of auxin upon aerobic metabolism. New Phytol. **42**: 831-839.

Woodwell, G. M. (1989). The warming of the industrial middle latitudes 1895-2050. Causes and consequences. Climatic change. **15**: 31-50.