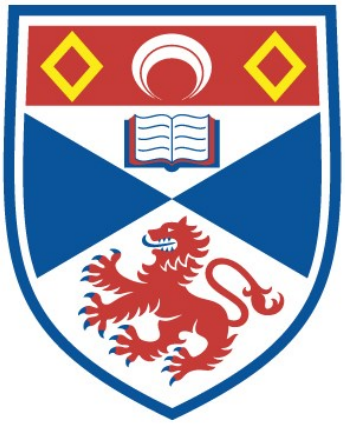
THE PRODUCTION AND REMOVAL OF ANAEROBIC METABOLITES FROM FLOODED TREE ROOTS WITH SPECIAL REFERENCE TO 'PINUS CONTORTA'

Donal Michael Finegan

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



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by

Donal Michael Finegan

Declaration

I Donal Michael Finegan 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 of professional qualification.

Donal M. Finegan

March 1989

Statement

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 in October 1985 and as a candidate for the degree of Ph.D. in October 1986.

Donal M. Finegan

March 1989

Certificate

I hereby certify that the Candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

Robert M. M. Crawford

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ABSTRACT

The production and removal of anaerobiotic compounds, principally ethanol from flooded tree roots was examined in lodgepole pine (Pinus contorta) from known provenances.

Contrary to earlier suggestions, the gaseous pathway, with ethanol exiting from the tree through the lenticels, removes only a very small proportion (less than 0.2%) of the ethanol generated in the flooded roots. The transpiration stream provides the major route for ethanol transport from the roots. Although the lenticels do not contribute significantly to the removal of ethanol from tree roots, analysis of the gas exiting from them provides a sensitive indicator of the existence of oxygen within roots.

Within the xylem sap and root tissue, changes in ethanol, amino acid (alanine, ∝amino butyrate and glutamine) and organic acid (malate and shikimate) composition under flooded conditions were analysed and discussed. For example during flooding glutamine (the major constituent amino acid) was shown to decrease, and alanine and ∝ amino butyrate to increase. From the data obtained it was concluded that the health and the metabolism of the root tissue during flooding can be diagnosed by examination of the xylem sap.

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

World climatic conditions are changing. For example global temperatures are increasing. By comparing records from land and sea surface temperatures it was estimated that the 3 warmest years have all occurred in the 1980s (Jones et al. 1986). The greenhouse effect, resulting from increasing levels of CO_2, CH_4, N_2O_2, and chlorofluoromethanes in the atmosphere as described by Ramanathan (1988) will cause a vigorous climatic system with a more active hydrological cycle probably resulting in windier conditions in northern Europe. Poorer anchorage afforded by waterlogged soils may consequently cause trees to be potentially unstable.

The stability of a tree to wind exposure is dependent upon root depth and this is severely affected in poorly drained soils and peat (Pyatt 1966). In north America sitka spruce grows in comparatively more fertile soils than lodgepole pine (Lines 1980) and can have a tap-root of 6-7 feet but on shallow peat this can be restricted to 9 inches (Day 1963). Stability of lodgepole pine is enhanced by the capacity to grow roots into waterlogged soils. Although root development was mostly confined to above the watertable lodgepole pine root penetration into waterlogged horizons was evident (Boggie 1972). As a result of sitka spruce intolerance to waterlogging (Coutts and Philipson 1978) lodgepole pine is comparatively more stable in wet conditions to windthrow. In 1928 an attempt to nurse sitka spruce with lodgepole pine by planting one pine

with one spruce in peat failed and the experimental block was, in 1968, almost completely pure pine (Binns 1968).

Roots cannot become established during waterlogged conditions. In late summer and autumn and in wet spring periods tree roots are at most risk of being exposed to anoxic conditions. The sulphide stain upon sulphide staining rods appeared 19.8 cm and 29.0 cm below the surface in May and in September respectively indicating that anaerobic conditions as a result of waterlogging were nearer the surface in spring (Boggie 1972).

Many ecotypes occur within the species Pinus contorta (Lines 1980) with presumably different degrees of suitability for Scottish conditions. Lines (1980) emphasised that although Pinus contorta is susceptible to various insects such as pine beauty moth, which can be controlled (anon. 1979), it is probably better to increase numbers of this species in poorer soils than to attempt to grow the more nutritionally demanding sitka spruce. Phillips (1984) gave an example where 5 hectares of lodgepole pine known as 'la Pine' were planted in 1956 on an elevation of 425 m; the trees appeared healthy and straight and had acheived almost 100 % survival but, in contrast, neighbouring crops originating from Long Beach Washington did not have 100 % survival and suffered severely from wind damage. Morphologically distinct populations of lodgepole pine exist and, therefore, there is a strong likelihood that metabolically distinct populations also exist.

Plants have two main mechanisms to endure oxygen stress during flooding. The first is to avoid stress by becoming dormant or to produce aerenchyma to supply roots with sufficient O_2. The second is to tolerate O_2 stress. This can be achieved through adaptive metabolism which may occur in Pinus contorta. Neither category is mutually exclusive, for example Pinus contorta has been shown to have the ability to aerate roots (Philipson and Coutts 1980) and also have the ability to produce less ethanol compared with sitka spruce of comparative age.

Differences in physiological response of loblolly pine seedlings have been obtained as a result of different flooding regimes, for example, malate levels were significantly higher in continuously flooded than in cyclic conditions and one family of trees (F_2) produced 144 nmol malate mg^{-1} fresh weight $hour^{-1}$ while trees from other sources produced less than 40 nmol malate mg^{-1} fresh weight $hour^{-1}$ (Hook and Denslow 1987). Although an analysis of variance between families showed no significant differences perhaps future research may reveal one. Different physiological responses to flooding using lodgepole pine, a closely related species, may also be found.

Perhaps adaptive metabolism such as the production of malate, or another compound, which is similarly a carrier of hydrogen and a regenerator of oxidised nicotinamide nucleotides, coupled to the removal of these compounds may facilitate root survival under O_2 stress. Debell et al. (1984) showed that loblolly pine root tips from

continously flooded conditions increased CO_2, malate and ethanol production and also increased the relative content of glutamic acid, ∝amino butyrate, asparagine and alanine in the xylem sap. Removal of hydrogen by compounds produced within the root system during flooding may serve the same purpose as O_2, a hydrogen accepting molecule in aerobic respiration, diffusing downwards and thus may facilitate root survival.

CHAPTER 2

REMOVAL OF ETHANOL FROM LODGEPOLE PINE ROOTS

The tree structure by its size alone inevitably causes problems for gas exchange between the atmosphere and living tissues. The protection of tissues such as the radial cambium and phloem by the differentiation of successive peridermal layers from the phellogen adds further barriers to the free exchange of gases. The consequent existence of anaerobic conditions inside woody tissues has long been known. Devaux (1899) was able to detect appreciable quantities of ethanol in the interior regions of wood. Ever since the evolution of the earliest trees their anatomy has contained provision for aeration channels. In the vascular plants of the Middle and Upper Devonian the interior of woody stems had aeration channels termed 'parichnos' connecting the outer and middle cortex with the mesophyll tissues of the leaf (Hook and Scholtens 1978). In the angiosperms the lenticels on the bark of tree trunks provide a means of gas-exchange through otherwise impermeable cork and if present are usually visible without magnification as small crater like structures or irregular protuberances.

The importance of lenticels as aerating structures in a wide range of species is clearly evident. The extent to which they are effective in providing ventilation in woody tissues has been much discussed (Topa and McLeod 1986; Philipson and Coutts 1980; Hook, Brown and Kormanik 1971). Many tree species when flooded show a marked proliferation of lenticel tissue (Hook and Scholtens 1978; Grosse and Schroder 1985).

Although this is frequently a consequence of flooding it is not certain whether or not lenticel production aids survival when trees are waterlogged. There is some doubt as to how far into the tree the ventilating capacity of the lenticels is effective. It may be that the lenticel functions mainly to provide aeration to the radial living tissues of the trunk. Back (1969) in an examination of the pathways for air diffusion into the tree from the lenticels points out that despite their presence the oxygen concentration in woody tissues is low and that of carbon dioxide high (up to 10 per cent). Consequently the lenticels at the base of the stem may function only as gas exchange sites for the stem and not for the roots. Tripepi and Mitchell (1984) have suggested that waterlogging tolerance of river birch (Betula nigra) and red maple (Acer rubrum) may not depend on lenticel development and aeration around the base of the stem because excluding O_2 from the the base of the stem did not inhibit root respiratory capacity. In a study of the effects of flooding on alder Gill (1975) pointed out that although flooding may induce morphological changes such as adventitious root production these are not necessary for survival. A similar situation may exist in relation to lenticels.

Gas exchange, or ventilation, as with all respiratory activity includes the supply of oxygen and the removal of carbon dioxide. When soils are compacted or flooded the restricted supply of oxygen to roots increases the likelihood of anaerobic conditions and therefore there will be additional volatile compounds from anaerobic metabolism including ethanol and acetaldehyde which may leave the tree via the lenticels.

Chirkova and Gutman (1972) have qualitatively demonstrated the release of these compounds from branch cuttings of lenticels of willow (Salix alba) and poplar (Populus petrowskiana). However the quantitative efficiency of this route for ethanol removal from roots has not hitherto been examined and therefore it is not yet clear whether the lenticels serve as a means of removing toxic volatile compounds from anaerobic roots as suggested by Chirkova and Gutman (1972). In addition the distance over which lenticels serve to aerate stem tissues has not been clearly established.

Whether or not ethanol accumulation is a hazard to plant survival has also been the subject of much discussion. Although it is difficult to prove that the addition of small physiologically relevant concentrations of ethanol to aerobic tissues is harmful, it is possible to show that the removal of ethanol can enhance anaerobic survival. In chickpea seedlings (Cicer arietinum) (Crawford and Zochowski 1984, Crawford et al. 1987) it has been shown that when anaerobic atmospheres are circulated around seedlings then their anaerobic survival rate is greatly increased. Some of the confusion concerning ethanol toxicity has been due to insufficient evidence as to when it is injurious to plants. It appears that the dangers of ethanol accumulation are greatest on the restoration of air after a period of oxygen deprivation. The re-availability of oxygen, generates oxygen radicals (Monk et al. 1987b) and causes a rapid increase in acetaldehyde concentrations by oxidation of the ethanol accumulated during anoxia. The consequent post-anoxic injury is reduced if tissues, when they re-enter the

aerobic environment, do so with minimal concentrations of ethanol (Monk et al. 1987a). It is therefore of interest to note the adaptations that are found in plants to minimise the accumulation of ethanol during periods of restricted aeration. These adaptations fall into three classes (1) restriction in the rate of ethanol production, (2) dispersion of ethanol, either through porous tissues or tissues with relatively large surface area to volume ratios, as in adventitious roots or (3) forced ventilation. Water lilies provide an example of plants which have evolved a system to force-ventilate their submerged rhizomes using solar energy to drive an air flow down young petioles, into the rhizome and up older petioles where the resistance to gas flow is less (Dacey and Klug 1982). The effectiveness of this ethanol removal system is evident in the popular name of 'brandy bottle' given to the yellow water lily (Nuphar lutea). The development of large air spaces in roots and underground rhizomes in many wetland species is commonly attributed to the need for the downward diffusion of oxygen from shoot to root (Armstrong 1979). However as pointed out by Williams and Barber (1961) it is possible to question the need for such large air spaces in terms of providing a path for oxygen diffusion. An alternative explanation might be the need to disperse the volatile products of anaerobic respiration or aid their upward dispersion in order to avoid accumulating products that would become rapidly toxic when exposed to oxygen.

Trees are particularly relevant plants for the study of ethanol removal as they have the double hazard of not only having roots that are liable to encounter anaerobic soil conditions, but also the mass of the

tree itself is likely to be the cause of hypoxic or anoxic conditions within its tissues (Devaux 1899; Back 1969). Lodgepole pine (Pinus contorta) is an economically important tree in the U.K., that suffers frequently from waterlogging in late autumn when dormancy has not yet taken place (Miller 1984). In comparison to variability studies on above ground growth, root-metabolism and its relation to the role of lenticels at the base of the stem has received relatively little attention in trees.

The functional role of any organ cannot be substantiated by qualitative measurements alone. A quantified study, examining the rate of production of ethanol in the root, coupled with an examination of the various modes of exit, either gaseously via the lenticels or in solution in upward moving xylem sap is essential before any conclusion can be reached on the functional significance of lenticels in adapting trees to periods of flooding. This chapter therefore examines the effectiveness of ethanol removal from tree roots by measuring the rate at which ethanol is dispersed within the tree, either gaseously or in solution, and compares this with the capacity of flooded roots to generate ethanol.

MATERIALS AND METHODS

Plant Material

Young saplings of known provenance (2-3 years old) of inland, central and northern lodgepole pine (Pinus contorta) were supplied by the Forestry Commission. Trees were grown in 15 cm diameter pots in a 1:1 mixture of peat and sand under glass with daylight supplemented to 16 hours between 18 and 25 ^{o}C. During periods when sap was being extracted transpiration rates were maintained at as constant a rate as possible by keeping the trees in a constant environment growth room at 20 ^{o}C under 16 hours light with a quantum flux density of 105 μM m^{-2} s^{-1} at 22 cms above the bench and 135 μM m^{-2} s^{-1} above the growing tip. Trees were watered monthly with 200-300 ml Hoagland's solution. All other waterings were with tap water and were given depending on conditions, either each or every second day. In all cases careful attention was paid to ensure that the pots were freely drained. For flooding the 15 cm diameter pots were immersed in 17.5 cm diameter vessels with tap water which ensured that the trees were flooded to soil level. Five trees were used in each flooding treatment and 3 trees were kept as non-flooded controls. In order to assess provenance as well as individual variation two provenances were used in every experiment.

Gas Collecting Structures and Ethanol Quantification

The effectiveness of lenticels as releasing areas for ethanol was assessed by calculating the release rate into collecting structures (collars) constructed around the base of the stem (see Fig. 2.1). The collars had an internal volume of 124 ml. On average the volume of the stem was 2.2% ± 0.08% of the collar volume. In the calculations the internal gas volume of the collar was therefore taken as 120 ml after allowing for stem volume. Perspex glue was used to ensure a gas-tight seal. The collars were left for 2 weeks to allow the glue to cure thus removing any volatiles compounds which might interfere with the estimation of gaseous substances coming from the lenticels. Paraffin wax and then vaseline were used to seal all joined margins. During the measurement period the ethanol concentrations were determined at 24, 48, 72 and 120 hours after flooding. Gaseous one ml samples were taken from the sealed ports on the collars with preheated gas syringes which were immediately injected into a gas liquid Pye series 104 chromatograph for ethanol and acetaldehyde determinations using a column 1.4 m long and 7 mm in diameter filled with 100 mesh Porapak Q. The estimation of the ethanol was carried out isothermally at 150 ^{o}C with a gas flow of 40 ml. per min. Analytical ethanol preparations (Sigma) diluted by 10 were used to obtain a standard curve. Ethanol had a retention time (R.T.) of 2.45 ±0.03 min (Fig. 2.2). An unknown gas due to glue had a R.T. of 6.88 ±0.10 (+ S.E.) which thus did not affect ethanol quantification.

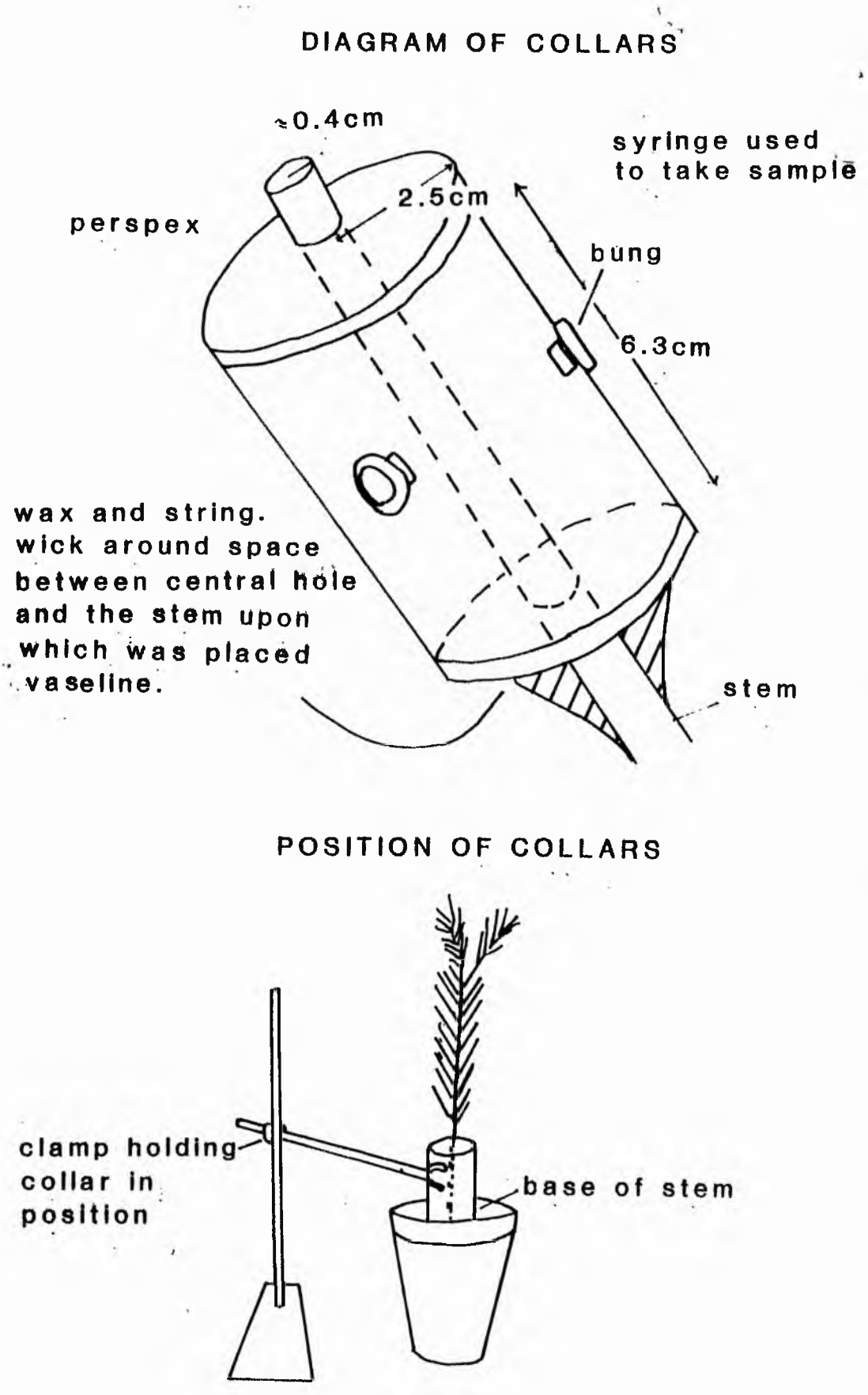


Figure 2.1. Construction of collar for collecting gas exiting from lenticels.

The rate of ethanol removal from the root system in the xylem sap was also quantified for comparison with that removed gaseously via the lenticels. Trees were cut as near to soil level as possible and placed immediately in a pressure bomb with the cut end protruding. Sap was then extracted with a pressure of 14-18 x 10^5 Pa. The extracted sap was immediately put on ice and analysed within 5 minutes by injecting 1.0 µl samples into the gas chromatograph using the same procedure as described above for the gaseous samples. Denaturation of any enzymes present in the xylem sap with perchloric acid proved unnecessary due to the short time before analysis. In order to calculate the rate of ethanol removal from the roots the transpiration rate of the trees was obtained by weighing a sample of at least 7 trees in which the pot system had been sealed with 2 polythene bags tightly closed with elastic bands and vaseline sealing. Previously non-flooded trees were flooded for 48 hours and weight loss was averaged over the later 24 h period.

Ethanol Quantification within Root Tissue

Non-lignified root tissue was used. After 24 hours of flooding root tips were harvested without washing but wiped clean of the peat and gravel mixture and within two minutes immersed in liquid N_2. While in the liquid N_2 the roots were ground up with a mortar and pestle. The root material was then dropped into ice-cold 6% (w/v) perchloric acid. This was sealed and kept under refridgeration until analysed. The perchloric acid removal followed (Bergmeyer 1963). Ethanol content of

root tips was then analysed by gas-liquid chromatography as described earlier.

RESULTS

Fig. 2.2 shows typical chromatographs of the volatile compounds detected in the gas released from the lenticels of flooded and non-flooded trees of inland lodgepole pine. Methane, probably of soil origin, can always be detected. Very noticeable however is the appearance of both acetaldehyde and ethanol after only 24 hours of flooding. The quantities of ethanol released into the collar per gram fresh weight of root over a 120 hour flooding period are shown in Fig 2.3 . In the unflooded controls only trace amounts of ethanol can be detected whereas on flooding there is a significant rise in ethanol in both the provenances tested. Although over the first 72 hours of flooding the mean values for ethanol venting from the lenticels is regularly higher in the inland provenance the extent of the variation is too great to establish any significant differences between the two provenances. Fig 2.4 shows the concentrations of ethanol in the xylem sap of inland compared with northern lodgepole pine over a 120 hour flooding experiment. No ethanol could be detected in the unflooded controls. However on flooding there is again the same rapid increase in ethanol concentration during the first 72 hours of flooding with again the inland provenance showing a tendency to higher concentrations.

In order to be able to compare the ethanol lost from the

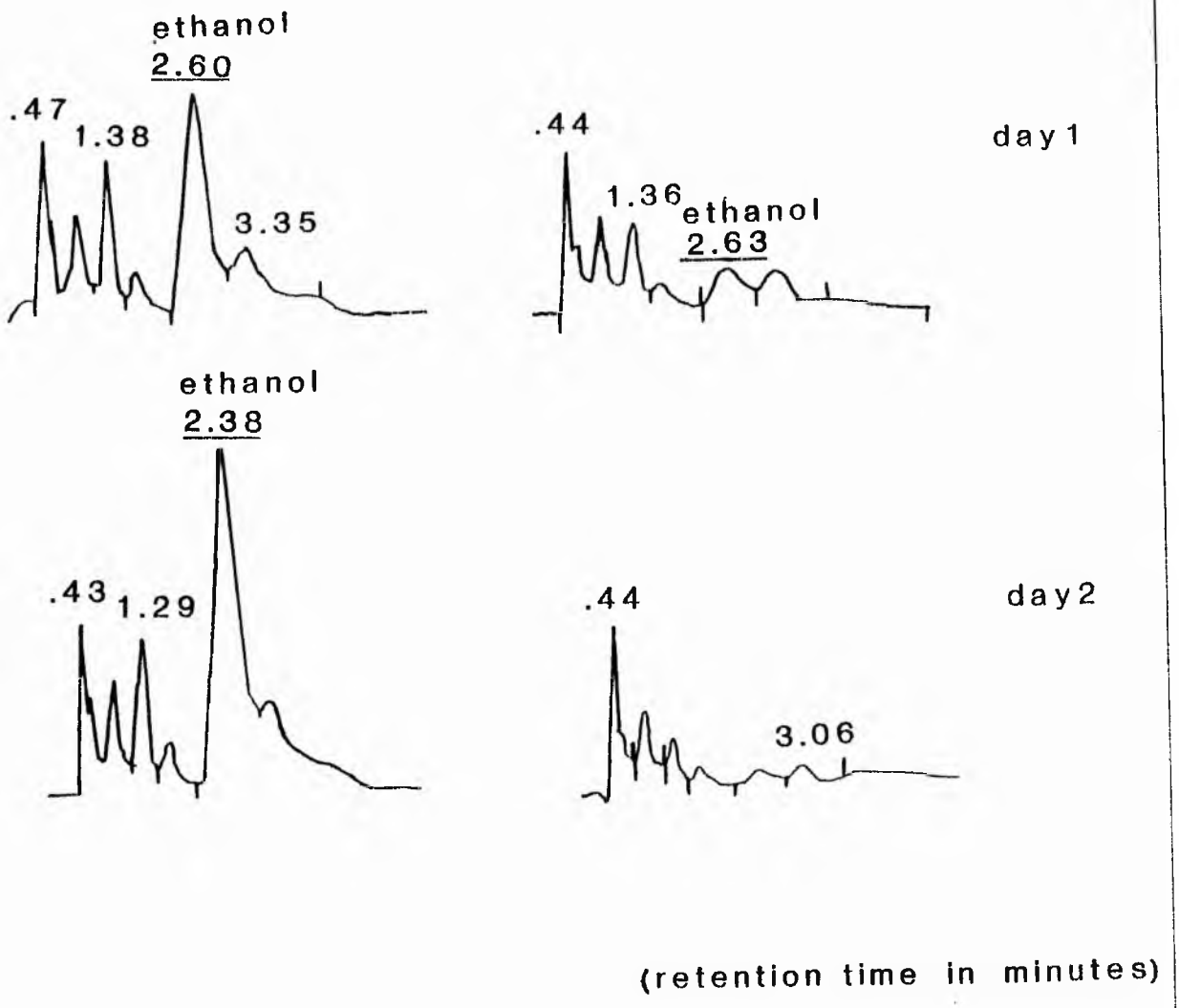


Figure 2.2. Chromatographs obtained from the Gas Liquid Chromatoraph after 24 and 48 hours of flooding of Inland Lodgepole pine (left) and unflooded controls (right). First retention time on each chromatograph reading from left to right equals methane, second equals acetaldehyde and third equals ethanol.

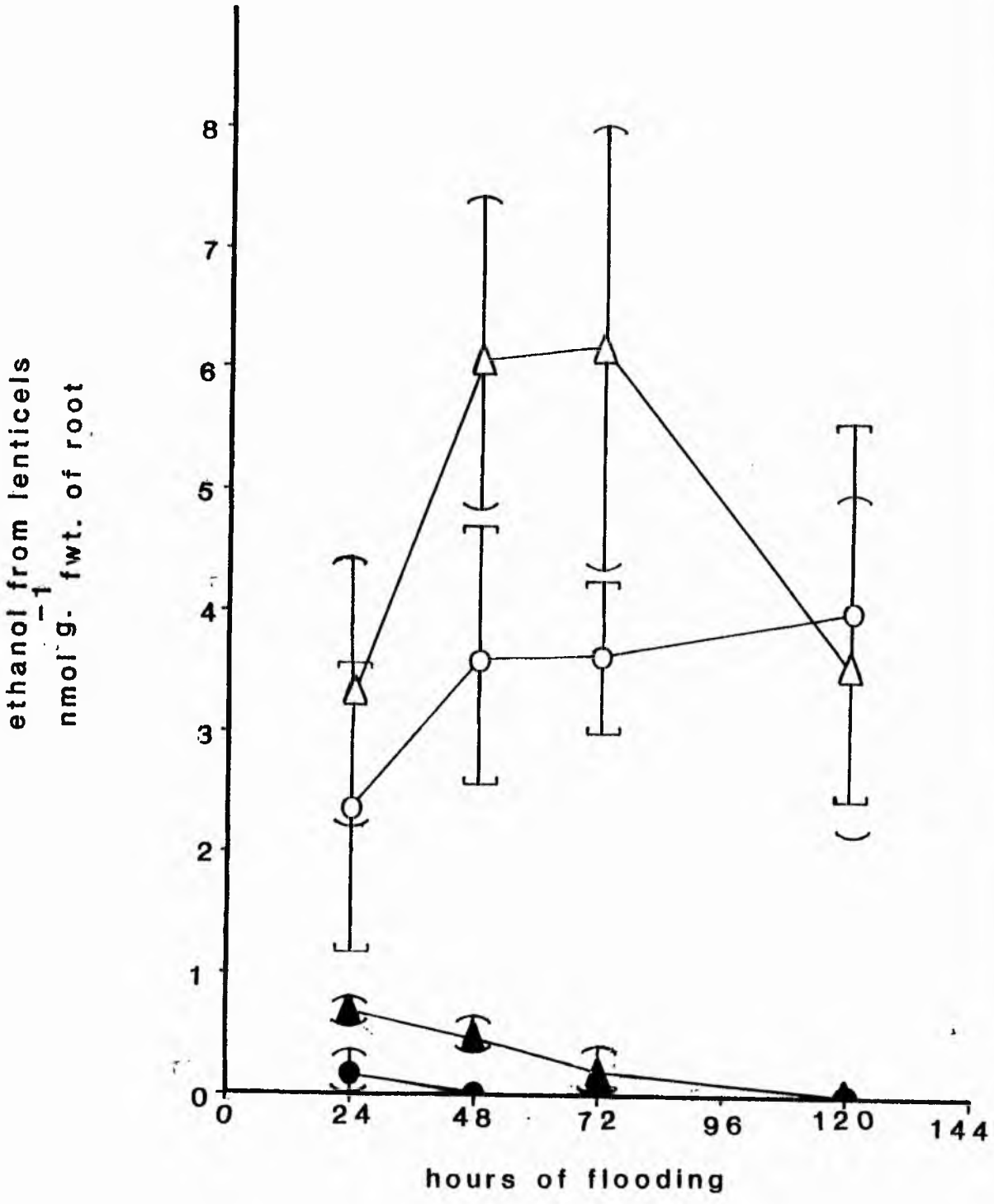


Figure 2.3. Release of ethanol into a 120 ml collar attached around the base of the stem (see Figure 2.1). Non-flooded inland lodgepole pine = ▲; flooded = △. Non-flooded Central Lodgepole pine = ● ; flooded = ○. Under flooded conditions there were 5 replicates samples and under control conditions there were 3 replicate samples.

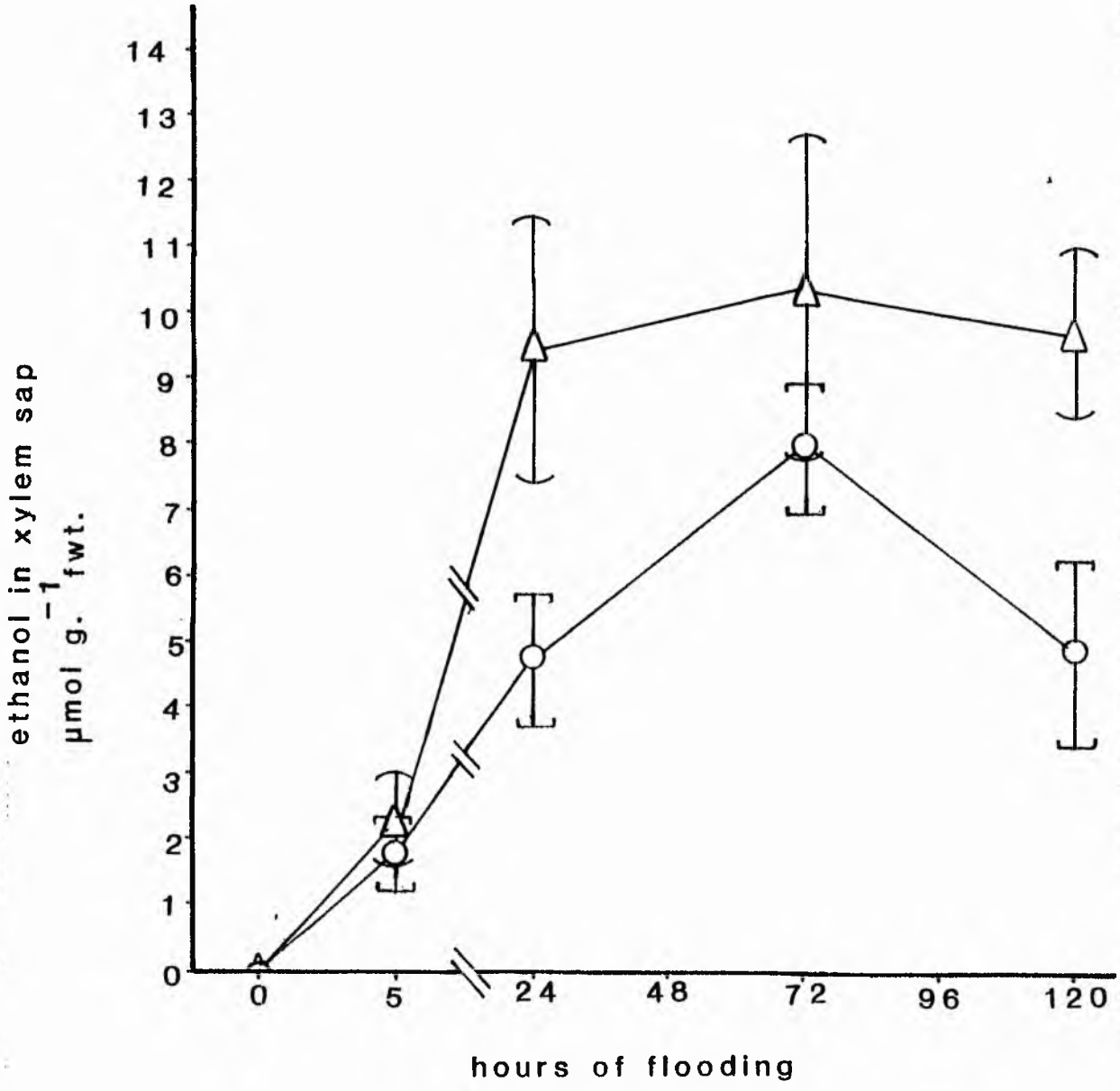


Figure 2.4. Accumulation of ethanol umol g.-1fwt. of xylem sap during flooding. O = Northern Lodgepole Pine; Δ = Inland Lodgepole Pine. There were 10 replicate samples used apart from after 0 hours of flooding when 6 were used.

trees through the lenticels with that passing upwards in the xylem it is necessary to estimate the transpiration rate of the trees. Measurements of water loss in the standardised conditions in the growth cabinet gave mean values of 9.9 g per day per tree for flooded trees of the inland provenance and 13.3 g per day per tree for the northern provenance. There was no evidence of any change in the relative water content of the upper needles of the trees as a result of flooding and transpiration appeared to continue at rates which were comparable with the unflooded trees. When these rates are applied to the concentrations of ethanol found in the rising xylem sap it is possible to estimate the ethanol removal from roots via the transpiration stream. Table 2.1 shows that the rate of removal of ethanol from the roots in the transpiration stream is three orders of magnitude greater than that passed out gaseously from the lenticels. Thus although lenticel emissions are sensitive indicators of the onset of anaerobic activity in flooded roots they do not represent a major exit pathway for the volatile end-products of glycolysis.

To assess properly the effectiveness of lenticels and transpiration stream in removing ethanol from anaerobic roots it is necessary to obtain some estimation of the probable amounts of ethanol generated by the roots. To obtain a figure for the extent of glycolysis in flooded roots the ethanol accumulation in root tips after 24 hours flooding was determined as described above. The root tip being the more metabolically active region of the root is also likely to over-estimate the ethanol production of the root system as a whole. Using this figure however it is possible to obtain an estimate of the maximum ethanol

Table 2.1. Estimated ethanol loss in the transpiration stream (μmol $g.^{-1}$ f. wt of root ± standard error) assuming a mean rate of 13.34g per tree per 24 hours for the northern provenance and 9.9 g for the inland provenance and root weights of 12.1 and 9.4 g respectively. There were 10 replicate samples used apart from the harvest at 0 hours of flooding when 6 were used.

	Duration of flooding (h)				
Provenance	0	8	24	72	120
Inland	0.01	2.5	10.1	11.0	10.3
Lodgepole Pine	0.01	0.7	2.0	2.5	1.4
Northern	0.02	2.0	5.3	8.8	5.5
Lodgepole Pine	0.01	0.7	1.0	1.0	1.7

Table 2.2. Comparison of rates of ethanol evacuation from flooded roots gaseously via the lenticels and in solution via the xylem sap during the first 24 h of flooding. The amounts of ethanol evacuated are also compared with the ethanol accumulated in the root tips for 24 h.

	Ethanol μmol $g.^{-1}$ f.wt (± standard error)		
	leaving lenticels (n = 5)	removed in xylem sap (n = 10)	accumulated in root tip (n = 5–8)
Inland Lodgepole Pine	0.003 ±0.001	10.1 ±2.0	2.1 ±0.3
Northern Lodgepole Pine	0.001 ±0.001	5.3 ±1.0	2.7 ±0.2
Central Lodgepole Pine	0.002 ±0.001	*	1.9 ±0.3

* missing data

concentrations with which a root system may have to contend. In Table 2.2 it can be seen therefore that the rate of ethanol removal by xylem sap is of an order of magnitude that is likely to have an effect on the level that is known to accumulate in the flooded root tips.

DISCUSSION

The importance of the transpiration stream for removing compounds from roots is already well documented. Fulton and Erickson (1964) demonstrated that tomato plants flooded in subdued light accumulated less ethanol in the xylem sap than plants flooded in full sunlight. Unlike herbaceous species, woody plants do not reduce transpiration as an immediate response to flooding. Kramer (1951) showed that in seedlings of loblolly pine (Pinus taeda) transpiration was still above the control rate after nearly one month of flooding. Stomatal closure was not observed during the flooding of red alder (Alnus rubra) and black cottonwood (Populus trichocarpa) (Harrington 1987). Similarly in this study flooding caused no reduction in the relative water content of the upper needles of lodgepole pine. It therefore appears that the transpiration stream is likely to serve as a possible exit route for volatile toxic substances such as ethanol and acetaldehyde which will be generated during flooding in the roots of woody species. Until now the evidence has been limited to detecting the presence of the volatile compounds and no quantitative assessment has hitherto been made of the physiological efficiency of this pathway for the removal of glycolytic products such as ethanol or acetaldehyde. This is also the case in relation to the physiological role of lenticels as possible ventilating ports for volatile anaerobic products. Chirkova and Gutman (1972) detected ethanol and acetaldeyhde in the gas coming from lenticels after flooding but no quantitative assessment was made of the efficiency of this pathway

for venting anaerobic products.

The data presented in this chapter compares for Pinus contorta the relative amounts of ethanol that escape from the underground tissues either gaseously via the lenticels or else in the transpiration stream. The amounts exiting from the roots are compared with the quantities that can be generated during flooding. From Table 2.2 it can be seen that the amount of ethanol exiting from the lenticels was only one thousandth of that passing upwards in the xylem sap. Thus the concentrations of ethanol, together with the transpiration rate of the flooded trees indicate that the xylem sap is a relatively more important pathway for the transport of ethanol away from the roots. Transpiration of woody species does not stop rapidly during flooding and the high transpirational velocities observed in trees which can exceed 1 m h^{-1} may be an additional adaptive factor (Huber and Schmidt 1936; and review by Zimmerman 1983). Ethanol released gaseously from the lenticels may be derived from the solution in the transpirational stream as well as from the gas phase in air spaces in the permeable tissue outwith the vascular cambium such as the cortex. However, the partition coefficient for ethanol between the liquid and gas phases will not favour the transfer of ethanol from solution into the gas phase unless there is some forced form of aeration. So far it is only in aquatic plants such as water lily (Nuphar lutea) (Dacey and Klug 1982) that a possible solar energy-driven venting system has been demonstrated, where air moves down young petioles to the rhizome and then up the old ones due to the reduced pressure gradient in the older tissues. In alder, gas

transport can be driven downwards by a thermo-osmotic pressurisation within the air space system of the stems (Grosse and Schroder 1984). However in trees this movement is only downwards and therefore does not serve to bring volatile substances to the surface. It would appear therefore that the hypertrophy of lenticels, although it can facilitate the downward diffusion of oxygen (Grosse and Schroder 1985), is not effective in the reverse direction presumably due to the adverse partition coefficient for the dispersal of ethanol into the gas phase from solution in water.

Knowing the transpiration rates of the intact trees the amounts of ethanol removed in the sap can be compared with the amount accumulating in the metabolically active root tips. The amount leaving in the transpiration stream is 2-5 times greater than that acumulating in the root tips and is therfore likely to serve as an important means for preventing excessive accumulations of ethanol in flooded roots. Comparison of release rate of ethanol into the collars against the ethanol accumulation rate in the root tips, the main site of production, however confirms the inefficiency of this pathway for the release of ethanol. The amounts accumulating in collars were always low and reached only 0.14% of the maximum production rate for I.L.P. and 0.1% for C.L.P. These low percentages reaffirm the conclusion that the rate of ethanol release from the lenticels is of minor physiological importance to the root system.

The gaseous release of ethanol from the lenticels can however be used as a reliable and sensitive indicator of the development of hypoxia

in tree roots. From Figs 2.3 and 2.4 it can be seen that the time course for ethanol production as detected in either the rising sap or escaping lenticel gas follows the same pattern. The release of ethanol into the collars tended to decrease by 120 hours. This was also true of the sap ethanol content. This probably indicates the death of root tips. Kimmerer (1987) using Populus deltoides roots noticed that ADH activity increased within 24 hours of anaerobic conditions being imposed but then decreased dramatically. This decrease was accompanied by the appearance of gross necrotic tissues. In Nyssa sylvatica during flooding there was a decrease in ethanol production also accompanied by a deterioration of the root systems which was marked in the intolerant upland populations (Keeley and Franz 1979). Thus although gaseous release is reliable and convient guide to the state of root aeration, as it can be sampled continuously and non-destructively it does not represent a significant pathway for the removal of ethanol from flooded roots. The xylem sap however does make a significant contribution to the removal of glycolytic end-products such as ethanol from hypoxic roots.

Summary

Contrary to earlier suggestions, the gaseous pathway, with ethanol vapour exiting from the tree through lenticels, removes only a very small proportion (less than 0.2%) of the ethanol generated in flooded roots. The transpiration stream provides the major route for ethanol transport from the roots. Although the lenticels do not contribute significantly to the removal of ethanol from tree roots, analysis of gas exiting from them

provides a sensitive indicator of the existence of oxygen deficits in roots.

CHAPTER 3

ANAEROBIC PRODUCTS IN THE XYLEM EXUDATE OF LODGEPOLE PINE

Downward diffusion of oxygen due to aerenchyma is of vital importance to the long-term tree survival under waterlogged conditions (Crawford 1972). All species (Salix nigra, Ulmus americana, Populus deltoides and Eucalyptus camaldulensis, apart from Eucalyptus globulus, examined by Pereira and Kozlowski (1977) developed aerenchyma to allow diffusion of O_2 after several days of flooding. However, short term survival may be enhanced by adaptive root metabolism and removal of reduced compounds upwards. Potential respiratory end-products which are derived from hydrogen accepting compounds would be beneficial to survival under waterlogging. Differences between provenances of the flood-tolerant lodgepole pine may be due to such a complementary enhancement of compounds being removed upwards as well as O_2 diffusion downwards. It must be remembered that velocities of $>$ 1 m per hour have been recorded within the xylem (Huber and Schmidt 1936). This is therefore an efficient removal system for various types of products and thus should be examined.

Some trees can survive long term flooding. Broadfoot and Williston (1973) have noted that trees such as green ash and sweet gum can survive up to 3 years of waterlogging. Continual flooding eventually proves fatal even for the most flood-tolerant tree species because new growth of absorbing roots cannot proceed in the absence of oxygen. Lodgepole pine roots can grow into waterlogged soil and survive for at least 4

weeks under waterlogged conditions (Coutts anf Philipson 1978). These trees mentioned above have various methods to aerate roots under waterlogging. Green ash have prominant intercellular spaces within stem tissues including the cambial ray initials (Hook and Brown 1972). Pinus contorta as well as other pines develop late wood tracheids in each growth ring which become gas filled and under flooded conditions this could be a pathway for longitudinal transport of gases such as O_2(Coutts and Armstrong 1976).

Short term survival, may however be due to adaptive metabolism in the root system. For example, before aerenchyma development, malate levels increased in root tissues of Nyssa sylvatica var. biflora under flooding (Keeley, 1978). Tripepi and Mitchell (1984) have shown that aeration via aerenchymatous roots is not necessary for survival because the exclusion of oxygen from the lower stem of flooded red maple and river birch did not affect root respiration capacity indicating a root metabolic adaptation in the absence of oxygen. Gill (1975) has shown that excision of adventitious roots during flooding affected only leaf number and then only marginally in Alnus glutinosa seedlings showing again the lack of necessity of such aerating tissue.

In the literature it is frequently asserted that the main terminal hydrogen acceptor during anaerobic respiration is acetaldehyde which results in the production of ethanol. This has been shown to be produced anaerobically by roots of various plant species: rice (John and Greenway 1976); Glyceria maxima, Ranunculus sceleratus and

Senecio aquaticus (Smith and ap Rees 1979) and swamp tupelo (Hook et al. 1971). Ethanol production yields 2 mol ATP (adenosine triphosphate) per mol hexose respired. Bertani, Brambilla and Menagus (1980), have shown that 98% of the ethanol produced by rice seedlings could be lost to the growth medium while only 2% accumulated in the tissues indicating the diffusability of this compound. Membranes serve as no great barriers to compounds such as ethanol due to their small size and lipid solubility (Collander 1949).

A possible metabolic adaptation to short periods of flooding and an alternative to the large carbon loss of ethanol could be the production and transportation of another hydrogen carrier, for example malate or shikimate, produced within the tree root system. This would relieve the necessity of O_2 transport downwards. Hook et al. (1983) have indicated that 85% of the total ethanol produced by loblolly pine roots was leaked into solution while essentially no malate was leaked. Malate may be produced in the root system and could be transferred with its constituent hydrogen and carbon upwards. In the literature there are numerous examples of malate accumulation during low O_2 availability. Joly and Crawford (1982) have shown that the flood tolerant tree species Hymenaea coubaril, which experiences short periods of flooding in its natural habitat, showed no change in ethanol accumulation but a significant increase in malate, lactate and succinate accumulation within the roots under flooding after 2 days. In roots of helophytes (Erica tetralix, Filipendula ulmaria and Glyceria maxima) under naturally flooded conditions during spring malate was

reported to accumulate (Crawford and Tyler 1982). Crawford (1972) has also shown that birch grown in wet soils had more malate within the spring sap than those grown in drier soils.

Accumulation and transport of hydrogen carriers in the transpiration stream such as malate, may be advantageous in several ways to tree survival under low O_2 root environments. First, there would be less of a necessity for O_2 diffusion downwards. Second, there would be a reduction of carbon loss to the environment unlike ethanol production. Third, the supply of carbon to the needles would provide a valuable carbon source especially in spring when growth rate of needles is most pronounced. Fourth, transport of malate and other organic acids in the xylem may alleviate acidosis in the roots. It has been suggested that ethanol production may not be a result of anoxia *per se* but may be due to acidosis at the onset of anaerobiosis in roots (Roberts *et al.* 1984). It is of note that if malate was such an acid then its production and transport would regenerate NAD (nicotinamide adenine triphosphate) allowing glycolysis to continue as well as alleviating acidosis in roots.

In many species there is an upward surge of organic compounds within the xylem at certain periods of the year. Mobilisation of carbohydrate reserves in woody tissues during spring is one such example. It is interesting to note that wetland trees such as *Betula* species and *Acer saccharinum*, the silver maple, transport large quantities of organic compounds which may be connected to tolerance of wet conditions.

Anaerobic conditions in the soil occur at this period due to increased metabolic activity and due to the wet conditions in the soil restricting O_2 downwards. Upward transport of organic compounds which may alleviate O_2 stress of the tree root systems has seldom been discussed or quantified.

In this study ethanol, malate, lactate and shikimate are examined in xylem exudate and non-lignified root material of lodgepole pine. Analysis of organic acids and ethanol in the root tissue and xylem exudate of pine trees during short periods of flooding has not been carried out previously.

Amino acids that are also found in the xylem sap that are produced by roots under waterlogged conditions are dealt with in the next chapter.

MATERIALS AND METHODS

Plant Material

Inland lodgepole pine (I.L.P.) and northern lodgepole pine (N.L.P.) trees were grown under control conditions as described in chapter 2.

Harvest of Root Material

Non-lignified root material after having been rapidly wiped clean of peat and gravel was immediately placed into liquid N_2 and ground to a powder. Perchloric acid was then added to denature enzymes as

in chapter 2. The sample was then stored under refridgerated conditions until analysis by G.L.C. (Gas Liquid Chromatography) and H.P.L.C. (High Precision Liquid Chromatography).

Harvest of Xylem Exudate

The trees were cut and the shoots placed in a pressure bomb (Crump Scientific Instruments) with the cut end protruding. Sap was then extracted using 14-18 x 10^5 Pa. The sap was immediately put into an ice bath and analysed within 5 mins using 104 Pye series chromatograph for ethanol quantification as described and within an hour the sap was also analysed for organic acids after having been spun at 10 000 rpm for 5 mins to remove solid particles.

Ethanol Quantification

The root and xylem exudate samples were examined for ethanol using a Gas Liquid Chromatograph as described in chapter 2.

Organic Acid Quantification by High Precision Liquid Chromatography

An Aminex HPX-87H column specifically for organic acids was used to quantify organic acids in root and xylem exudate samples. The stationary phase is a strong cation exchange resin and the use of a disposable guard column removes positively charged molecules, such as amino acids also present in xylem exudate, reducing the necessity for sample preparation. The quantification was carried out isocratically using 0.01 N Sulphuric acid at a flow rate of 0.6 ml per min. All acids

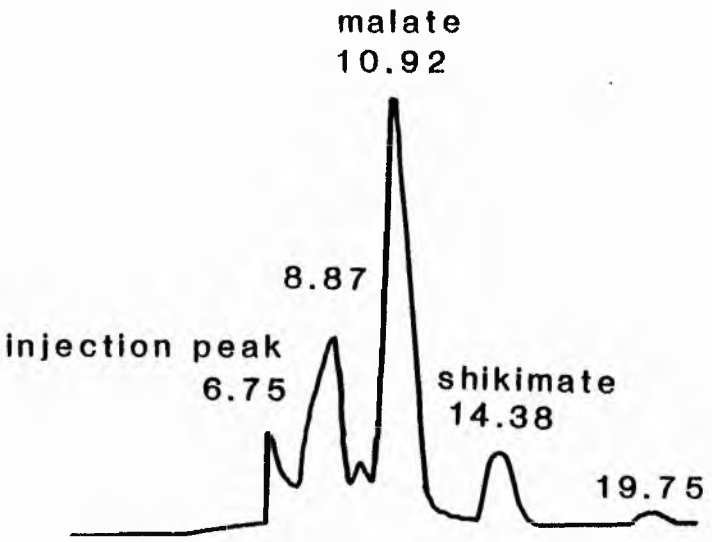
were detected by U.V. at 254 nm. Using a modified method, Jessop and Scaife (1985) resolved 10 organic acids indicating the effectiveness of the HPX-87H column. As a cross check on the quantification reliability malic acid was also analysed enzymatically (Bergmeyer 1963).

RESULTS

Fig.3.1 shows a typical chromatograph and retention times (R.T.s) of acid standards for comparison. Lactate, a potential product of anaerobic respiration, is absent from the xylem exudate of trees of both provenances under flooding although its presence can be detected in the root tip extracts. Lactate in the root extracts produced a broad peak which partly obscured the shikimate peak preventing quantification of both these acids in the root tissue.

Table 3.1 compares ethanol, malate and shikimate within the xylem exudate after 8, 24, 72 and 120 hours of flooding with control conditions for northern and inland lodgepole pine. N.L.P. after 120 hours under flooding contained slightly less malate than under control conditions. Except for this I.L.P. and N.L.P. trees under flooding showed no indication of a decrease of shikimate or malate compared with control conditions. N.L.P. trees under flooding tended to show a peak in ethanol accumulation after 72 hours of flooding unlike I.L.P. trees.

Table 3.2 illustrates the concentration of ethanol and malate within the xylem exudate and root tip tissue under 72 hours of flooding and under control conditions. After 72 hours under flooding the ethanol



ORGANIC ACID	AVERAGE R.T. (mins)
Lactate	13.75 ±0.04
Shikimate	14.36 ±0.02
Oxaloacetate	9.27 ±0.03
Oxalate	9.81 ±0.03
Citrate	9.20 ±0.08

Figure 3.1. A typical chromatograph of lodgepole pine xylem exudate using a H.P.L.C. aminex HPX-87H column for organic acids. Examples of organic acid retention times (R.T.) ± standard error are shown below.

Table 3.1 Comparison of ethanol, malate and shikimate in the xylem exudate (μmol $g.^{-1}$fwt.) ± S.E. after 0, 8, 24, 72 and 120 hours of flooding using northern (N.L.P.) and inland lodgepole pine (I.L.P.). There were 5 replicate samples for each harvest apart from after 0 hours of flooding when there were 3 replicates for each harvest.

	Duration of flooding (h) μmol $g.^{-1}$fwt.				
	0	8	24	72	120
Ethanol using	0.2	0.2	2.5	7.1	3.1
N.L.P.	0.0	0.1	0.7	1.0	1.6
Ethanol using	0.0	1.7	6.1	8.7	9.1
I.L.P.	0.0	0.4	0.8	2.8	2.1
Malate using	10.1	14.4	11.8	16.7	8.6
N.L.P.	2.1	7.1	2.5	2.0	1.9
Malate using	5.5	12.9	12.7	18.2	8.3
I.L.P.	0.3	2.1	3.6	4.1	1.2
Shikimate using	1.2	1.3	1.8	1.5	1.2
N.L.P.	0.3	0.3	0.4	0.1	0.2
Shikimate using	1.2	0.8	1.5	2.3	1.5
I.L.P.	0.3	0.1	0.4	0.7	0.2

Table 3.2. Comparison of the concentrations of malate and ethanol within the xylem exudate and root tip tissue after 72 hours of flooding and under control conditions.

ROOT TIP TISSUE μmol $g.^{-1}$fwt.(average ± S.E.)

	N.L.P.		I.L.P.	
	ethanol	malate	ethanol	malate
control	0.0	25.9	0.2	29.6
(n=3-4)	0.0	10.8	0.2	7.4
72 hours	4.3	50.3	5.9	27.6
flooding	2.2	5.5	2.7	4.5
(n=4-6)				

XYLEM EXUDATE μmol $g.^{-1}$wt.(average ± S.E.)

	N.L.P.		I.L.P.	
	ethanol	malate	ethanol	malate
control	0.2	7.6	0.1	5.6
(n=3)	0.1	3.0	0.0	0.8
72 hours	22.1	7.9	19.8	5.8
flooding	3.5	2.1	4.2	0.9
(n=4-6)				

within the xylem exudate increased from 0.2 to 22.1 and from 0.1 to 19.6 μmol $g.^{-1}$fwt. for N.L.P. and I.L.P. trees respectively. After 72 hours under flooding the malate within the xylem exudate changed fractionally for trees of both provenances. After 72 hours of flooding ethanol accumulation in the site of production, the root increased from 0 to 4.3 and from 0.2 to 5.9 μmol $g.^{-1}$fwt. for N.L.P. and I.L.P. trees respectively. Malate increased from 25.9 to 50.3 μmol $g.^{-1}$fwt. of root tip using N.L.P. trees and using I.L.P. trees there was little change from 29.6 to 27.6 μmol $g.^{-1}$fwt. of root tip.

Table 3.3 illustrates malate quantification within the xylem exudate after 72 hours of flooding and under control conditions at different periods in the growing season of 2 batches of I.L.P. trees obtained from the Forestry Commission. An analysis of variance was carried out between all control harvests and no significant difference was found ($F=1.7$, $P>0.01$ with 3 and 9 degrees of freedom). From this it should be noted that there was no significant difference between the 2 batches using an enzymatic (MDH) and a high precision liquid chromatographic technique to quantify malate. The % recovery of malate was 110.6% and 102.1% using the enzymatic (MDH) and chromatographic techniques respectively. After 72 hours of flooding malate quantities within the xylem exudate were shown to be variable compared with quantities obtained under control conditions. This appeared to be due to the time of harvest within the growing season. Student's t-tests were carried out between flooded and control trees of each harvest. There was a significant decrease between the control trees, 9.1 μmol

Table 3.3. Malate quantification in the xylem exudate of inland lodgepole pine after 72 hours of flooding and under control conditions. The malate quantities are examined during different periods within the growing season of 2 batches of three year old trees obtained from the Forestry Commission. Enzymatic and chromatographic methods used are indicated.

Malate $\mu mol\ g.fwt^{-1}$ ($\pm$ S.E.)

Harvest	control	after 72 hours of flooding	method of analysis
2months after	4.6	1.7	enzymatic
potting (n=4)	1.3	0.2	
4 months after	9.1*	2.8*	enzymatic
potting (n=3-4)	2.9	0.4	
3 months after	5.5*	18.2*	H.P.L.C.
potting (n=3-5)	0.4	4.1	
4 months after	5.6	5.8	H.P.L.C.
potting (n=3-6)	0.9	0.8	

* significantly different $P<0.1$

$g.^{-1}$fwt. and flooded trees, 2.8 μmol $g.^{-1}$fwt. 4 months after potting ($t=2.5$, $P<0.1$ with 5 degrees of freedom). All other harvests showed no such significant decrease between control and flooded trees. There was a significant increase between the control trees, 5.5 μmol $g.^{-1}$fwt. and flooded trees, 18.2 μmol $g.^{-1}$fwt. 3 months after potting ($t=2.3$, $P<0.1$ with 6 degrees of freedom).

DISCUSSION

Lactate was not detected within the xylem exudate of lodgepole pine although its presence was detected in the root tissue. Lactate production within the root tissue was perhaps only present at the onset of oxygen stress. Wager (1961) has reported that pea seeds under anaerobic conditions accumulated initially lactate and succinate before ethanol. This was probably due to pyruvate decarboxylase (pH optimum 5.8) becoming more active due to a pH decrease as explained by Davies (1985 and 1973). Bertani, Brambilla and Menegus (1980) using rice under anoxia have shown that lactate doubles in 6 hours then ethanol production proceeds.

Shikimate was detected in xylem exudate of both provenances (table 3.1) All phenols, except flavanoids, are derived from shikimate and give rise to such substances as pinene producing the characteristic aromatic odour of pine trees. There are examples of shikimate accumulating within tissues under low oxygen availability but this did not occur in the xylem exudate of Pinus contorta. Shikimate was reported to have increased in Nuphar lutea rhizomes during April under low oxygen availability then decreased when more oxygen was available in May (Crawford and Tyler 1982). Boulter et al. (1963) using Iris pseudacorus rhizomes under low oxygen levels have shown an increase in shikimate accumulation but there was no increase in malate accumulation. Both provenances of lodgepole pine under flooding showed no reduction of shikimate quantities within the xylem exudate

(table 3.1).

Under waterlogging the malate quantity within the xylem exudate of both provenances did not decrease significantly (tables 3.1 and 3.2). The ethanol concentration tended to decrease after 120 hours of flooding for N.L.P. but not for I.L.P. In table 3.1 malate ranged from 8.6 to 16.2 μmol $g.^{-1}$ fwt. of xylem exudate and from 5.5 to 18.2 μmol $g.^{-1}$ fwt. of xylem exudate for N.L.P. and I.L.P. trees respectively. The presence of large quantities of ethanol and malate within the xylem implies that a large carbon source can reach the growing shoots. Mc Gregor et al. (1963) noticed that white pine seedlings had completed 88% of stem elongation by the 15th of May although the rate of net photosynthesis more than doubled after that date and also using Loblolly pine seedlings it was noted that 42% of the stem elongation was completed before maximum photosynthesis indicating that early growth was not due solely to carbon derived from current photosythesis but possibly from another source such as soluble carbohydrates and organic acids transported in the xylem from storage tissue. The occurrence of relatively large quantities of malate within the xylem during spring growth period is obviously dependent upon the amount of starch stored within woody root and stem tissue from the previous autumn and may hence be affected by the trees' past development in a nursery. For example, Taylor and May (1967) have cited that organic N transported from woody tissue of peach trees to the shoots was dependent on the amount of stored N from last autumn. This example is perhaps analogous to the supply of organic C such as malate cited here.

The production of malate indicated by the accumulation of malate within the root tissue (table 3.2) did not decrease significantly during these short periods of flooding. After 72 hours of flooding I.L.P. and N.L.P. root tip tissue contained 92.3% and 194.2% of the quantity under control conditions. Malate production would allow the regeneration of NAD from NADH. Ethanol production, indicated by accumulation of ethanol within the root tip tissue would also allow regeneration of NAD, increased from 0.2 to 5.9 μmol $g.^{-1}$fwt. and from 0 to 4.3 μmol $g.^{-1}$fwt. for I.L.P. and N.L.P. trees respectively. The above figures indicate that the ethanol production was relatively more responsive to flooding. Ethanol in the xylem exudate increased from 0.1 to 19.8 and from 0.2 to 22.1 μmol $g.^{-1}$ fwt. of xylem exudate for I.L.P. and N.L.P. trees respectively. Malate within the xylem exudate showed little change for trees from both provenances. Basically quantities of ethanol accumulating in both xylem exudate and root tip tissue was comparatively more responsive to flooding than malate quantities measured.

Table 3.3 illustrates differences in malate content within the xylem exudate after 72 hours of flooding and under control conditions using 2 different batches of 3 year old I.L.P. trees harvested at different periods during the growing season. Using one batch of trees there was a significant increase of malate from 5.5 to 18.2 μmol $g.^{-1}$fwt. under flooding ($t=2.3$, $P<0.1$ with 6 degrees of freedom) however there was little change under flooding 4 months after potting. The figure of 18.2 μmol $g.^{-1}$fwt. of xylem exudate illustrates that

malate can reach equimolar levels to ethanol during flooding (compare tables 3.2 and 3.3) but it is noteworthy that for every hydrogen transfered from NADH there is 3 times more carbon for malate than for ethanol. During a second year using a different batch of 3 year old trees obtained from the Forestry Commission (table 3.3) the quantity of malate on average after 72 hours under flooding decreased from 4.6 to 1.7 μmol $g.^{-1}$fwt. and from 9.1 to 2.8 μmol $g.^{-1}$fwt. 2 and 4 months after potting respectively. Only the latter decrease proved to be significant ($t=2.5$, $P<0.1$ with 5 degrees of freedom). Table 3.3 demonstrates that the malate content under flooding was variable and may be affected by the harvest time during the growing period. Malate quantities, as previously mentioned may also be affected by growth conditions during the previous year.

Summary

There was no significant difference between F.L.P. and I.L.P. trees with regard to malate, shikimate or ethanol within the xylem exudate. Seasonally the quantity of malate within the xylem exudate can be approximately 2 to 3 times greater than the quantity of ethanol during flooding. Ethanol must be a better indicator of waterlogging stress because malate may be produced mainly at springtime, and malate is not produced necessarily by the most stress sensitive non-lignified roots unlike ethanol.

CHAPTER 4

AMINO ACIDS WITHIN THE XYLEM EXUDATE DURING FLOODING OF LODGEPOLE PINE

The main objectives of this chapter were to quantify amino acids within the xylem exudate and examine the compositional changes that occur under flooding.

In forests nitrate is not as available as ammonium. Ammonium has even been shown to be accumulating on forest soils (Roelofs *et al.* 1985). As a result trees utilise to a greater extent the more toxic ammonium. It has been shown that lodgepole pine and Engelmann spruce grew more rapidly using ammonium or ammonium plus nitrate than nitrate alone (Bigg and Daniel 1978) indicating a possible adaptation. Nitrate compared with nitrate and ammonium or ammonium alone proved to be an inferior form of nitrogen source for growth of sitka spruce and Scots pine (Nelson and Selby 1974). Indirect evidence for root assimilation comes from the calculation that for every mol of ammonium assimilated within the plant root system there is a corresponding 1.22 mol H^+ produced which is unlikely to be disposed of biochemically but rather extruded into the rooting medium (Raven 1988). This is supported further by Allen and Raven (1987) and Allen *et al.* (1988). Bigg and Daniel (1978) have shown that *Pinus contorta* supplied with ammonium decreased the pH of the rooting medium. Further evidence for root assimilation of N in pines comes from $^{15}NO_3$ tracing. Martin *et al.* (1981) using Austrian pine (*Pinus nigra*) have shown that there was a predominance of ^{15}N in a carboxylated form being transported upwards

as opposed to $^{15}NO_3$.

Different amino acids predominate within xylem sap of different families. For example, citrulline is the major amino acid in the xylem exudate of the Betulaceae (Barnes 1963 and Reuter 1954). In apple trees, the Rosaceae, the major amino acid within the sap is arginine (Tromp 1970). Barnes (1963) has shown glutamine to be the major constituent in the xylem sap of pines.

During a wet spring when roots may be under O_2 stress additional amino acids may be transported within the xylem from tree roots. Amino acids may act as alternative hydrogen acceptors and then be transported upward relieving the necessity of O_2 diffusion downwards. Amino acids may accumulate within the xylem sap due to protein degradation within the root system. Amino acid accumulation within the xylem of Pinus contorta during periods of waterlogging has not been examined previously. Alanine and ∝ amino butyrate are cited in the literature as amino acids produced during oxygen stress and may be transported in the xylem. Streeter and Thompson (1972) using radish leaves under anoxia measured increases in both these amino acids. Reggiani et al. (in press 1988) have shown that when rice roots were under anoxia there was an increase in ∝ amino butyrate which was likely to be produced by glutamate decarboxylase,with a pH optimum of 5.9, becoming more active. Alanine was also produced from protein degradation as well as from transamination of acidic amino acids. Smith and ap Rees (1979) have also shown that in pea roots under anoxia there was an

increase in alanine in pea root tissue under anoxia.

To investigate transportation of potential amino acids produced under waterlogging the composition of the xylem exudate for amino acids was analysed from 2 provenances of logdepole pine.

MATERIALS AND METHODS

Skeena river lodgepole pine (S.L.P.) and northern coastal lodgepole pine (N.C.L.P.) were grown under control conditions as described in chapter 2. Trees were flooded with Hoagland's solution.

Quantification of Specific Amino Acids by High Precision Liquid Chromatography

Amino acids in the xylem exudate (obtained as in chapter 2) and the extracts of thoroughly washed non-lignified root tissue (prepared as in chapter 2) were analysed using the OPT (o-Phthaldialdehyde thiol) derivatisation method (Joseph and Marsden 1986). An Ultrapac column TSK ODS-120T with 5 µm particles was used. All amino acids were detected at 280 nm. A gradient using 2 buffers was used. Buffer A and buffer B contained 20% and 80% methanol respectively in 0.05 Molar sodium dihydrogen phosphate (pH 5.5). The gradient used ran from 0% of buffer B to 10% buffer B in 10 minutes, 10% to 85% in 30 mins, 85% to 0% in 5 mins and then 10 minutes were allowed for re-equilibrium. The flow rate was

0.6ml per minute.

Confirmatory qualitative analysis of amino acids present was carried out using either 1 or 2 dimensional paper chromatrography. The solvent used was ethanol:water:ammonia solution 0.88 in the volume ratio of 80:10:10. The amino acids were located by a ninhydrin solution containing 200 mg in 100 ml of acetone (Feinberg and Smith 1963). Relative fronts (R.F.) values were calculated. A R.F. value represents the ratio between the relative movement of a standard amino acid or sample and the movement of the solvent used for the chromatograph.

Quantification of total amino acids

Total amino acids were quantified by a ninhydrin method (Cramer 1958). Detection of amino acids was carried out at 578 nm. Moore and Stein (1948) have shown that ninhydrin reacts with a variety of compounds containing NH_2 groups, including amino acids, urea, peptides and ammonia and is therefore non-specific.

RESULTS

Qualitative analysis of amino acids within the xylem exudate

Paper chromatography helped to confirm qualitatively with the H.P.L.C. what amino acids were present within the xylem exudate. Using paper chromatography the relative movement of standards was compared with unknown amino acids within the xylem exudate.

Asparagine which stains brown with ninhydrin, as explained by Conn and Stumpf (1972), was not detected within the xylem exudate of trees from both provenances. Arginine was also not detected by examining R.F. values of the standards and xylem exudate samples. The R.F. value of glutamine standards coincided almost exactly with an unknown acid which stained the most intensively and therefore it appeared that glutamine was the major amino acid within the xylem exudate of both provenances. On one occasion the R.F. value was identical to that of the glutamine standard. Using the H.P.L.C. the glutamine standard had a retention time (R.T.) value which coincided with that of the largest peak, or optical density. This gave confirmatory evidence that glutamine was the major amino acid present. αAmino butyrate was shown to be present taking evidence from R.F. values of standards and xylem exudate samples and also by using R.T. comparison from the H.P.L.C.. Using R.T. comparison alanine, glutamate, aspartate and glycine were also shown to be present within the xylem exudate.

Glutamine, α amino butyrate and alanine were quantified within the

xylem exudate and the root tip extracts after 24 and 72 hours of flooding due to their peak clarity using the H.P.L.C. which was not always the case for other amino acids mentioned above.

Examination of amino acids within the xylem exudate and root tip samples after 24 hours of flooding and under control conditions

Table 4.1 shows that for trees of both provenances after 24 hours of flooding amino acids in the xylem exudate altered fractionally compared with controls. The average percentage (± standard error) of glutamine against the total amino acids within the xylem exudate samples was 20.6% ±1.8 and 31.1% ±9.1 under control and flooded conditions respectively for S.L.P. trees. The percentage (± standard error) of glutamine against the total amino acids within the xylem exudate samples was 30.9% ±6.0 and 22.5% ±1.9 under control and under flooded conditions respectively for N.C.L.P. trees.

Unlike the xylem exudate, root tip extracts after 24 hours of flooding tended to show an increase of ∝ amino butyrate and alanine for trees of both provenances (fig.4.1 and 4.2). There was an increase of glutamine after 24 hours of flooding from 5.9 to 9.5 μmol $g.^{-1}$fwt. for S.L.P. trees and there was a decrease from 6.7 to 4.0 μmol $g.^{-1}$ fwt. for N.C.L.P. trees. The average percentage (± standard error) of glutamate against the total amino acids within the root tip samples was 38.5% ± 5.5 and 26.6% ±3.8 under control and under flooded conditions respectively for S.L.P. trees. The average percentage (± standard error) of glutamine against the total amino acids within the root tip samples was 48.1% ±7.4 and 25.1% ±6.2 for control and flooded conditions respectively for N.C.L.P. trees.

Table 4.1. Comparison of amino acids within the xylem exudate, μmol $g.^{-1}$fwt.(± standard error) for northern coastal lodgepole pine (N.C.L.P.) trees and for skeena river lodgepole pine (S.L.P.) trees after 24 hours of flooding and under control conditions. Gln.= Glutamine, Am.B.= ∝ amino butyrate, Glut.= glutamate, Glyc.=glycine.

	μmol $g.^{-1}$fwt. of xylem exudate					
Treatment	Gln.	Am.B.	Alanine	Glut.	Glyc.	Total Amino Acids
Skeena river	1.04	0.13	0.02	0.09	0.14	5.05
lodgepole pine	0.10	0.01	0.02	0.01	0.08	0.07
control (n = 3)						
Skeena river	1.57	0.13	0.05	0.07	0.28	5.96
lodgepole pine	0.19	0.01	0.02	0.02	0.06	0.84
flooded (n = 5)						
Northern coastal	1.68	0.16	0.06	0.09	0.25	5.43
logepole pine	0.12	0.01	0.02	0.01	0.13	0.93
control (n = 4)						
Northern coastal	1.33	0.16	0.06	0.09	0.25	5.43
lodgepole pine	0.15	0.00	0.02	0.02	0.04	0.36
flooded (n = 5)						

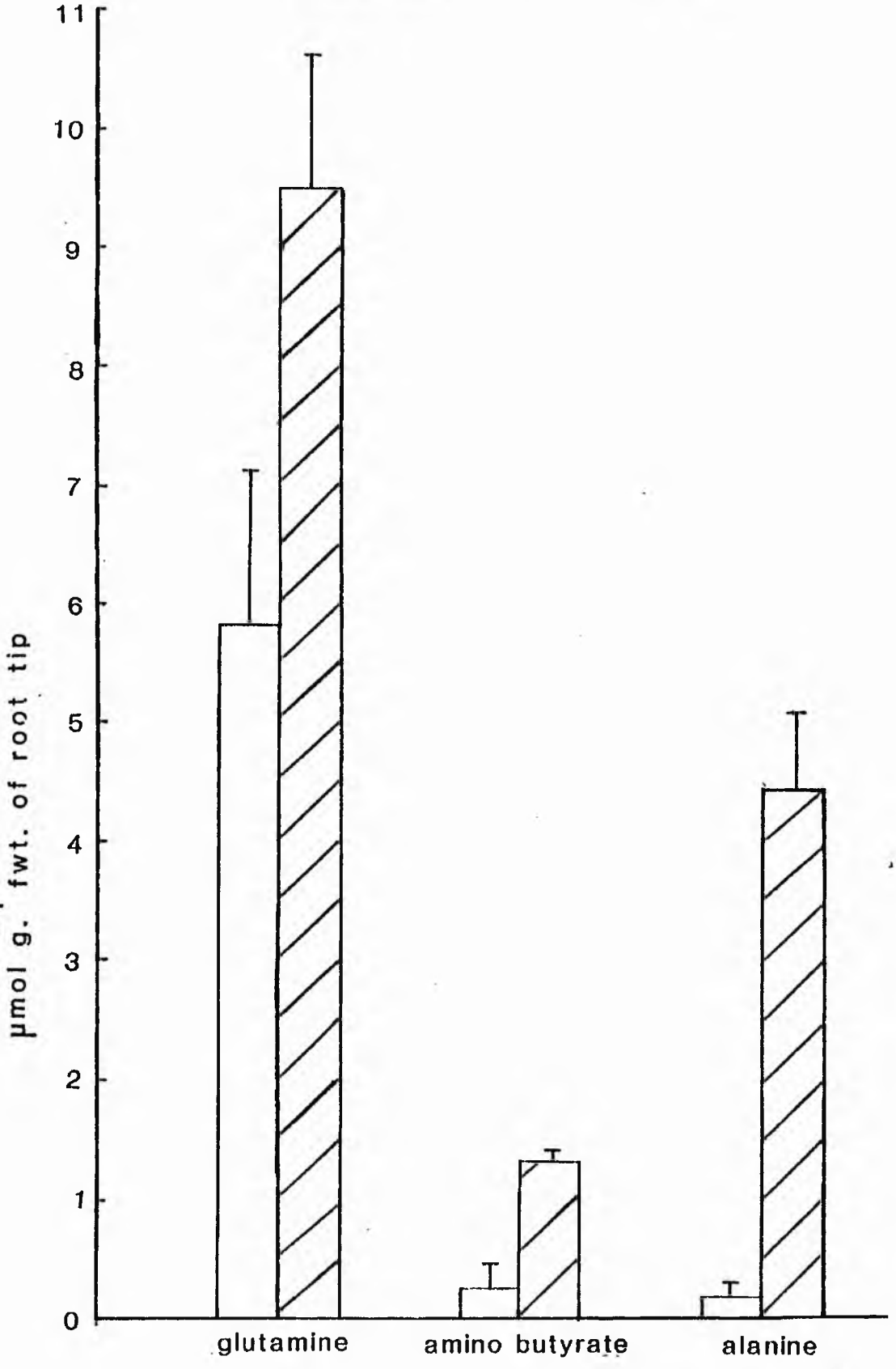


Figure 4.1. Accumulation of amino acids µmol g.$^{-1}$fwt.(standard error = bars) within root tip tissue of trees from skeena river provenance after 24 hours of flooding ☒ (n = 5-6) and under control conditions ☐ (*n* = 3).

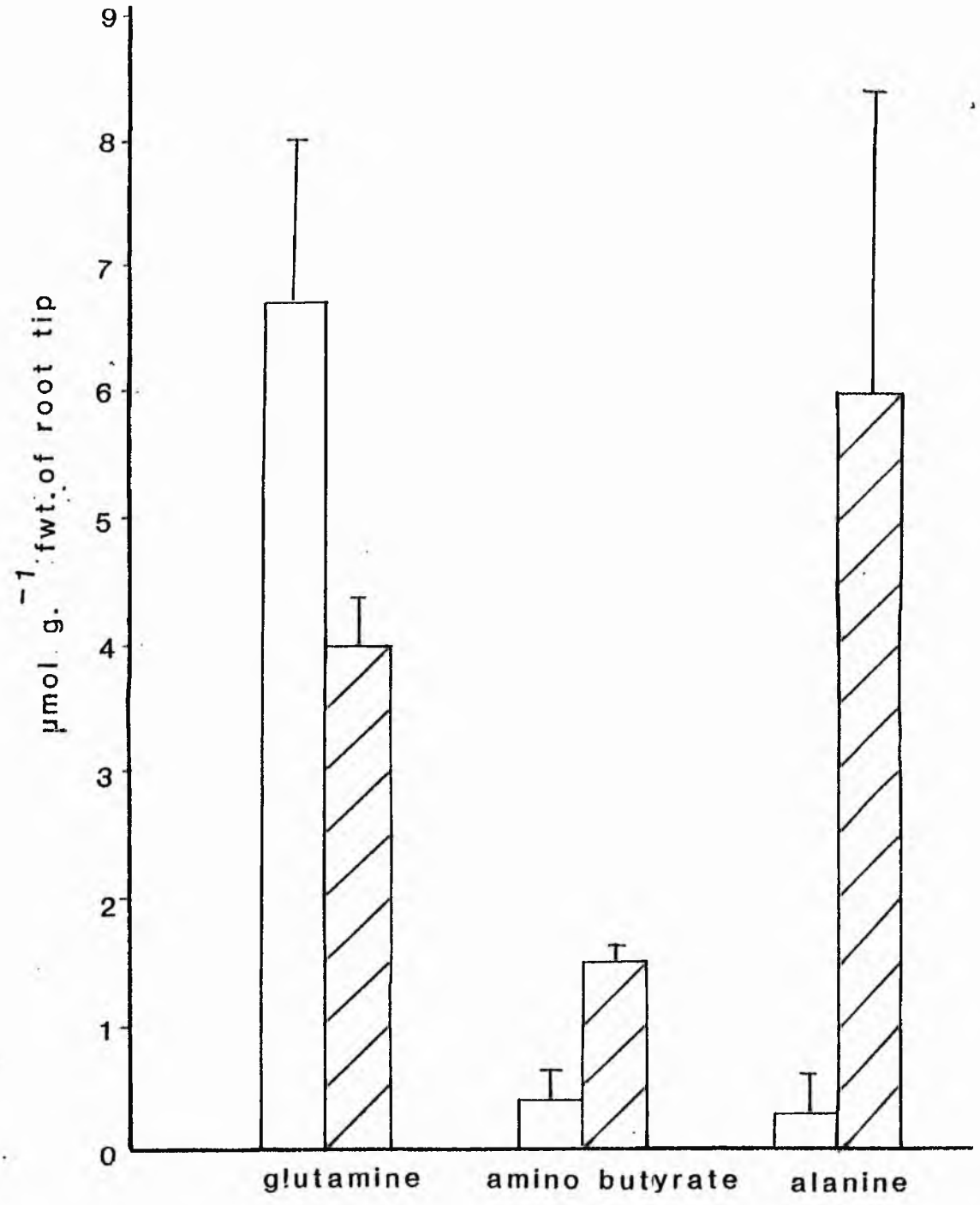


Figure 4.2. Accumulation of amino acids µmol g.$^{-1}$ fwt.(standard error = bars) within root tip tissue of trees from northern coastal provenance after 24 hours of flooding ☒ (n = 6) and under control conditions ☐ (n = 4).

Examination of amino acids within the xylem exudate and root tip samples after 72 hours of flooding and under control conditions

Table 4.2 shows that within the xylem exudate trees of both provenances after 72 hours of flooding showed approximately a doubling of ∝ amino butyrate. For N.C.L.P. trees the quantity of alanine within the xylem exudate remained constant after 72 hours of flooding. For S.L.P. trees the quantity of alanine increased by approximately 5 fold.

For trees of both provenances after 72 hours of flooding glutamine decreased to approximately third within the root tip tissue (fig.s 4.3 and 4.4). ∝ Amino butyrate and alanine increased after flooding for trees of both provenances (fig.s 4.3 and 4.4). The average percentage (± standard error) of glutamine against the total amino acids within the root tip extracts was 25.9% ± 5.9 and 14.6% ± 3.3 under control and flooded conditions respectively for S.L.P. trees. For N.C.L.P. trees the average percentage (± standard error) of glutamine against the total amino acids within the root tip extracts was 49.5% ± 18.7 and 8.5 % ± 4.4 under control and under flooded conditions respectively. The proportion of glutamine was clearly decreasing within the root tip tissue after 72 hours.

Table 4.2. Comparison of amino acids within the xylem exudate, μmol $g.^{-1}$fwt.(± standard error) for northern coastal lodgepole pine (N.C.L.P.) trees and skeena river lodgepole pine (S.L.P.) trees after 72 hours of flooding and under control conditions (n =3).

	μmol $g.^{-1}$fwt.of xylem exudate		
Treatment	Glutamine	∝Amino Butyrate	Alanine
Skeena river	1.79	0.07	0.04
lodepole pine	0.54	0.03	0.01
control			
Skeena river	1.31	0.14	0.19
lodgepole pine	0.46	0.12	0.13
flooded			
Northern coastal	2.55	0.08	0.04
lodgepole pine	1.59	0.02	0.04
control			
Northern coastal	1.11	0.15	0.04
lodgepole pine	0.55	0.08	0.02
flooded			

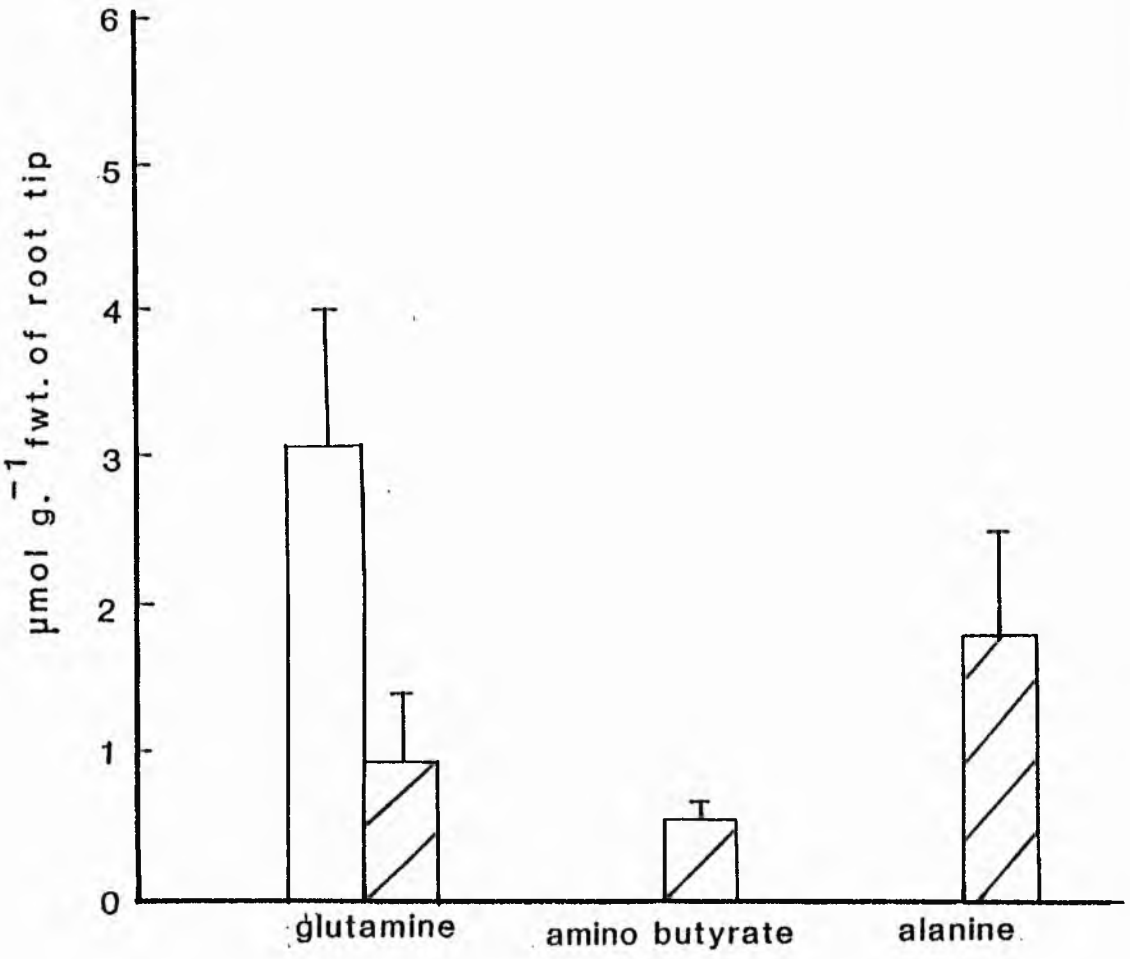


Figure 4.3. Accumulation of amino acids µmol g.$^{-1}$ fwt. (standard error = bars) within root tip tissue of trees from northern coastal provenance after 72 hours of flooding ▨ and under control conditions □ (n = 3).

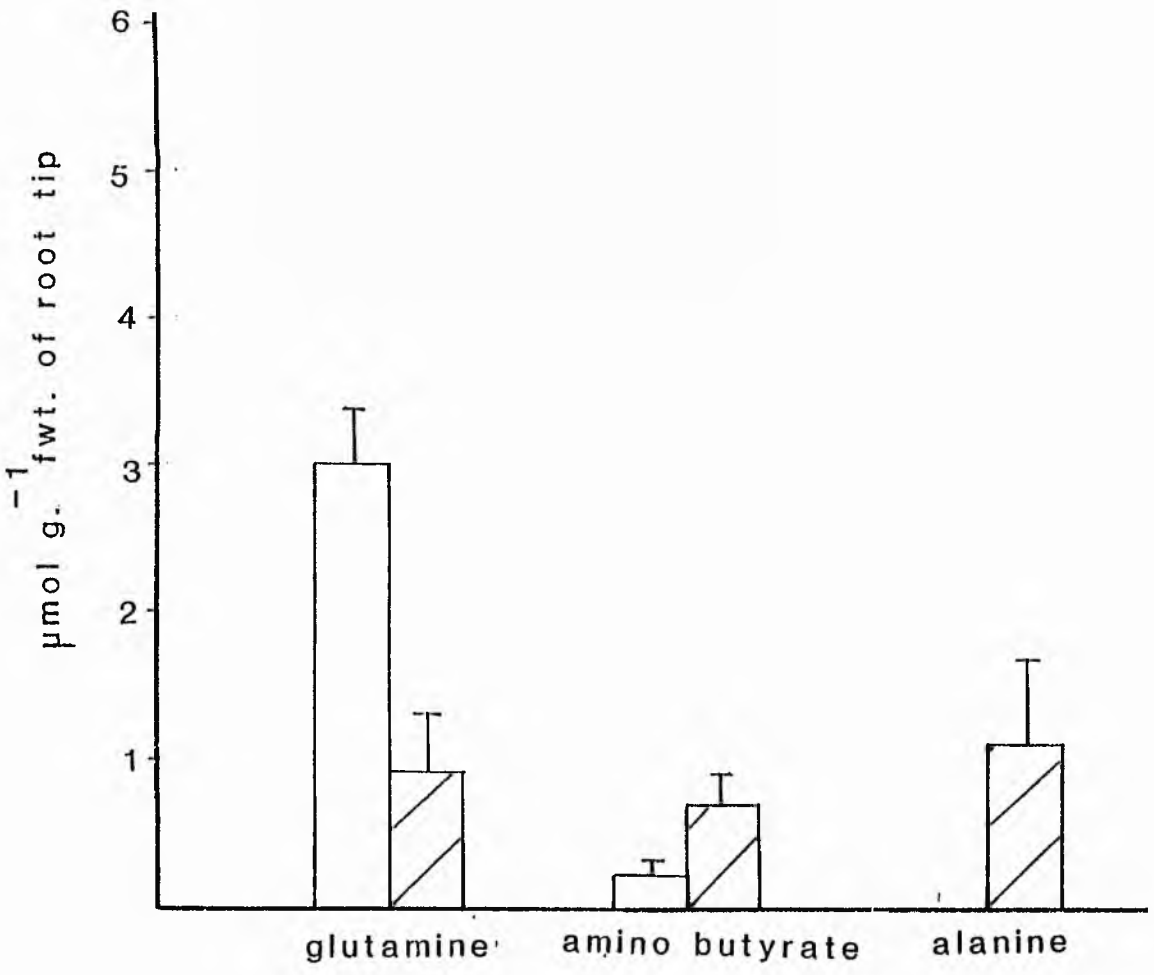


Figure 4.4. Accumulation of amino acids µmol $g.^{-1}$ fwt. (standard error = bars) within root tip tissue of trees from skeena river provenance after 72 hours of flooding ☒ and under control conditions ☐ (n = 3).

DISCUSSION

Evidence from the H.P.L.C. method and paper chromatography indicated that glutamine was the major amino acid within the xylem exudate. Using different pine species Barnes (1962 and 1963) found also that the major amino acid within the xylem exudate was glutamine. It is only after 72 hours of flooding that there was a change in composition of amino acids within the xylem exudate (tables 4.1and 4.2). For example, there was an increase of alanine from 0.04 to 0.19 μmol $g.^{-1}$fwt. and an increase of ∝ amino butyrate from 0.7 to 0.14 μmol $g.^{-1}$fwt. for S.L.P. trees after 72 hours of flooding. Glutamine within the xylem exudate decreased after 72 hours of flooding from 1.7 to 1.3 μmol $g.^{-1}$fwt. for S.L.P. trees. In the xylem exudate of N.C.L.P. trees after 72 hours after flooding there was also a decrease of glutamine from 2.5 to 1.1 μmol $g.^{-1}$ fwt. and an increase of ∝ amino butyrate but alanine remained constant.

∝ Amino butyrate and alanine are known to be produced by plant tissue under O_2 stress (Streeter and Thompson 1972 and Effer and Ranson 1967). For trees of both provenances within the xylem exudate these amino acids did not accumulate rapidly. By comparison a better indicator of stress of roots would be ethanol accumulation within the xylem exudate. Fulton and Erikson, (1964) have shown ethanol accumulation within the xylem exudate of tomato plants was rapid (hours) and varied according to the level of flooding imposed indicating its responsiveness to O_2 stress within the roots.

In contrast to the slow responsiveness of 'stress' amino acid accumulation within the xylem exudate as mentioned above, after 24 hours of flooding the root tip tissue showed a more immediate response (fig.s 4.1 and 4.2). The root tip tissue, the most metabolically active part of the root, as shown by having the highest respiration (Lähde 1967 and Johnson-Flanagan and Owens 1986) accumulated within 24 hours ∝ amino butyrate and alanine in trees of both provenances. Fagerstedt and Crawford (1987) using barley have indicated that the root tips are the most sensitive to the lack of O_2 and the accumulation of alanine and ∝ amino butyrate tended to indicate this here.

As stated in the result section there was a large difference within the xylem exudate and within the root tip tissues between the percentage of glutamine against the total amino acids quantified using the ninhydrin method. Possibly, but unlikely, an undetected amino acid may account for this difference. Barnes (1962) has shown that 80% of the amino acids present within the xylem exudate of loblolly pine was in the form of glutamine. This difference cannot be removed if other amino acids quantified by the H.P.L.C. together with glutamine are then compared with the total amino acids quantified by the ninhydrin method. Under control conditions the percentage of glutamine, glutamate, ∝ amino butyrate, glycine and alanine against the total amino acids quantified within the xylem exudate samples ranged from 25.4% to 29.6% and from 35.2% to 70.0% for S.L.P. and N.C.L.P. trees respectively.

Trees were watered monthly with Hoagland's which contains ammonium ions. Ammonium ions if they were within the xylem sap or root tip tissue would react with the ninhydrin making therefore the total amino acids quantified an over estimation thus the exact quantities within the root tip tissue were not mentioned here. Within the root tip tissue samples after 72 hours of flooding there was a decrease of glutamine compared with the total amino acids quantified which ranged from 25.9% to 14.6% and from 49.5% to 8.5% for S.L.P. and N.C.L.P. trees respectively. This decrease may have indicated flood damage to the root tips. There may have been a inward leakage of ammonium ions from Hoagland's solution in which the trees were flooded coupled with an outward leakage of glutamine to the Hoagland's solution. Hiatt and Lowe (1967) have demonstrated that barley roots when exposed to anoxia rapidly leak amino acids, organic acids and potassium ions to the surrounding medium. This may have occured during flooding in addition to a decline in glutamine production.

Summary

Glutamine was the major amino acid present within the xylem exudate from both provenances. There appeared to be no significant difference between the trees from N.C.L.P. and S.L.P. provenances with regard to amino acid composition within root tip tissue and within xylem exudate under control and under flooded conditions. Root tip tissue after 24 hours of flooding showed compositional changes but the xylem exudate did not. In the xylem exudate and root tip tissue there was a marked tendency,

after 72 hours of flooding, for glutamine to decrease and for alanine and α amino butyrate to increase.

CHAPTER 5

ETHANOL ACCUMULATION WITHIN ROOT TISSUE OF GREEN ASH AND LODGEPOLE PINE

Concentration within tissue of ethanol and other possible carriers of hydrogen is not only controlled by the rate of removal as described in previous chapters but by the rate of production and further metabolism. Lack of dissipation and further metabolism may cause an increase in the rate of accumulation within root tissue of potential toxic compounds, such as ethanol which may damage tissues for example during the post anoxic re-exposure to air (Monk et al. 1987a and Crawford et al. 1987). Removal of toxic compounds and accumulation then act together not separately with each having a potential effect on survival.

Lodgepole pine, although tolerant of low O_2 levels within the rooting medium compared with sitka spruce (Boggie 1974 and Coutts and Philipson 1978), is far from tolerant if compared with wetland species such as green ash which can withstand 3 years of flooding (Broadfoot and Williston 1973). Wetland species have various adaptive features to facilitate survival during long periods of flooding. Cypress, Taxodium distichum, for example has adaptive pneumatophores which have been suggested as ventilating organs for waterlogged roots but this function has come under doubt (Kramer and Riley 1952). Hook (1972) has shown that green ash can ventilate flooded roots by having gas-permeable stem tissues allowing diffusion of O_2 to pass freely from the lenticels to gas filled xylem and thence to the roots. Due to gas permeable tissues aerating roots ethanol may not be detectable within the xylem exudate or

root tissues during flooding.

Although considered less tolerant than the above mentioned species lodgepole pine can tolerate extensive periods of flooding. After 100 days of flooding lodgepole pine shoot growth continued (Crawford and Baines, 1977). During flooding roots can penetrate the watertable and actively grow deeper due to the trees' ability to aerate its root system (Coutts and Armstrong 1976). Variation in ethanol production between different trees from 2 provenances may indicate different levels of aeration or root metabolic adaptation. Crawford and Baines (1977) have shown that ethanol quantities within tissues reflected the relative tolerance of lodgepole pine and sitka spruce and they have also shown different ethanol quantities within wood tissue of pines growing in comparatively wet and dry conditions reflecting the incapacity to sustain aerobic metabolism when flooded. Investigation of intraspecific differences in accumulation of ethanol and other metabolic indicators of O_2 deficiency within the root system during flooding may enable an assessment of lodgepole pine's suitability in Scottish conditions.

During flooding anoxia within tree roots is most likely to occur in the root tip tissue which is farthest from the shoot. Webb and Armstrong (1983) have shown that root tip tissue of various herbaceous species such as pea, rice and pumpkin can only survive a few hours of anoxia without added glucose illustrating the high demand for carbohydrate when oxygen is limiting. The root tip is the most metabolically active region of the root system in plants as has been demonstrated in Picea with its high

respiratory capacity (Johnson-Flanagan and Owens 1986). There was a positive correlation between anoxia and flooding stress using root tip tissue of different barley cultivars (Fagerstedt and Crawford 1987). To compare the possibilty of limited oxygen during flooding, ethanol within root tips of lodgepole pine under flooded conditions was compared with root tips under total anoxia.

In the following experiments ethanol accumulation is quantified using root tip tissue under flooded conditions from trees of two lodgepole provenances and, for the reason stated above, also under anaerobic conditions. In comparison with lodgepole pine, ethanol within root tissue and xylem exudate of green ash, a highly flood-tolerant American species, was also examined under flooded conditions. CO_2 production under N_2, as an indicator of the maximum metabolic activity of the roots to produce ethanol was examined using lodgepole pine and green ash.

Materials and Methods for northern lodgepole pine (N.L.P.) and inland lodgepole pine (I.L.P.) comparison

Trees were grown in controlled conditions as in chapter 2. Trees were flooded with Hoagland's solution. Hoagland's solution was applied to trees for 2 days before anaerobic treatment described below.

Anaerobic treatment

Roots within soil were wrapped in muslin and sealed within an anoxic root box (plate 1). Roots were sealed within this box (plate 1) in an anaerobic work-bench (Forma Scientific, U.S.A.) within 30 minutes to minimise stress to the tree. After having sealed the roots within the box, the whole tree and sealed root system was removed from the anaerobic work-bench and immediately a flow through of N_2 at approximately 300 ml per minute was then applied to the box in order to provide a positive pressure thus maintaining total anoxia. O_2 levels within the root box were shown to be less than or equal to 0.22% at the beginning of the anaerobic treatment (compare plate 2 with 1). Low levels of O_2 indicated by the methylene blue strips were probably due to O_2 coming from the soil and would be removed after a short period because of the N_2 flow.

Good (1985) described a more elaborate method to surround tree roots with gases but this proved unreproducible. The above method was simple but satisfactory because of its reproducibility.

Ethanol quantification

Ethanol was analysed using root tissue under anaerobic and under flooded conditions as described in chapter 2 using a gas liquid chromatograph (G.L.C.). The ethanol vapour flowing from the anaerobic root box was captured with an ice cold 20 ml water trap.

Plate 1. This is a picture of an anaerobic root box. The box was made gas tight by securing the top surface down (with wing nuts) upon blue silicone sealant smeared upon the base. The silicone sealant was also smeared around a rubber bung on the top surface through which the stem protruded. Bubbles indicate N_2 flow and the methylene blue indicator strip indicated O_2 presence.

Plate 2. Different O_2 concentrations represented by methylene blue indicators.

Materials and Methods for green ash and central lodgepole pine (C.L.P.) comparison

Green ash and C.L.P. were watered weekly with Hoagland's. All other conditions were as described in chapter 2 for C.L.P. except the quantum flux density for green ash ranged from 90 to 200 $\mu M\ m^{-2}\ s^{-1}$ from 100 to 130 cm respectively above the bench. Most of the foliage was between this height range.

Ethanol Quantification

Ethanol within the root tip tissue and within < 2 mm diameter root tissue of green ash was analysed using the G.L.C. as described. Control and flooded roots were harvested 24, 48, 96 and 144 hours after flooding.

Green ash xylem sap was extracted from lignified root material using a pressure bomb. Pressures were often higher than those stated for those necessary to extract xylem sap from lodgepole pine stem tissue. Flooded and non-flooded root material was excised from pots and then used. No xylem sap could be obtained from green ash stem tissue due to its porosity.

Carbon Dioxide Quantification

The tissue, root tips of lodgepole pine and < 2 mm diameter roots of green ash, was washed thoroughly with tap water and also with 10 mMolar chloramphenicol. Root tissue was then sealed within a vessel of known volume (25 ml). The vessel was then attached to an Infra Red Gas Analyser (I.R.G.A.) and after 30 mins within a 20 ^{o}C waterbath CO_2 production within a closed loop system was measured. After this period the closed loop was flushed with N_2 and after an equilibration time of 5-10 mins CO_2 production was measured for 30 mins.

RESULTS

Comparison of ethanol accumulation using northern lodgepole pine (N.L.P.) and inland lodgepole pine (I.L.P.)

Table 5.1 shows no differences were obtained for ethanol accumulation within root tip tissue after 24 hours of flooding compared with after 24 hours of the anaerobic treatment for trees of both provenances. It can be concluded therefore that root tips from both provenances were under similar O_2 stress under flooding as under anaerobic conditions. The ice cold water trap contained 23.0% and 20.7% of ethanol generated from the anaerobically treated root tissue for N.L.P. and I.L.P. trees respectively.

Table 5.1. Comparison of ethanol accumulation within root tips from trees from two provenances (C.L.P. and I.L.P.) μmol $g.^{-1}$fwt. ± standard error (S.E.) after 24 hours of flooding and anaerobic treatment (n = 4-5). Also the ethanol μmol per ml of water obtained from N_2 flow from box captured within water trap (n = 4-5).

treatment	I.L.P.	N.L.P.
anaerobic	1.49	1.71
	0.23	0.29
flooded	1.66	1.90
	0.19	0.50
non -flooded	0.10	0.00
	0.09	0.00
trap water	0.39	0.51
	0.16	0.23

Comparison of ethanol accumulation using green ash and central lodgepole pine (C.L.P.)

Table 5.2 shows CO_2 production decreased under N_2 compared with under air for root tissue from trees of C.L.P.and increased for green ash root tissue. For C.L.P. root tips the rate of ethanol accumulation to 96 hours after flooding obtained from figure 5.1 against the CO_2 production under N_2, which represents the maximum potential ethanol production, equals 5.3%. This implies potentially 5.3% of the ethanol production can accumulate within the root tip tissue.

Figure 5.1 illustrates the difference in ethanol accumulation within root tissue of the two tree species. Using C.L.P. trees ethanol accumulation peaked 96 hours after flooding. There was no similar change in ethanol accumulation within green ash root tissue during flooding. Green ash root material under flooded conditions accumulated more ethanol than under control conditions.

Table 5.3 shows that on average there were similar concentrations of ethanol within the green ash xylem exudate under control and flooded conditions. Only one out of 5 replicate control samples contained detectable ethanol but 5 out of 5 replicate samples from flooded plants contained detectable ethanol within the xylem exudate. Table 5.3 shows that ethanol within the root tissue under flooded conditions was higher than under control conditions as is shown in figure 5.1.

Table 5.2. The CO_2 production nmol $g.fwt^{-1}$ min^{-1} ± standard error (S.E.) from root tips of central lodgepole pine (C.L.P.) and fine roots of green ash exposed to air then exposed to N_2 for 30 mins in each case.

nmol CO_2 $g.^{-1}fwt.$ min^{-1}

species	air	N_2	N_2/air
green ash	13.0	23.4	1.75
S.E.(n=8)	2.0	4.2	0.21
C.L.P.	124.0	73.2	0.61
S.E.(n=5)	12.3	7.4	0.07

Figure 5.1. Comparison of ethanol accumulation μmol $g.^{-1}$fwt. + standard error within root tip of central lodgepole pine (○ = control, n = 3; ● = flooded, n = 4-6) and with < 2mm diameter green ash roots (△ = control, n = 3; ▲ = flooded, n = 4-6).

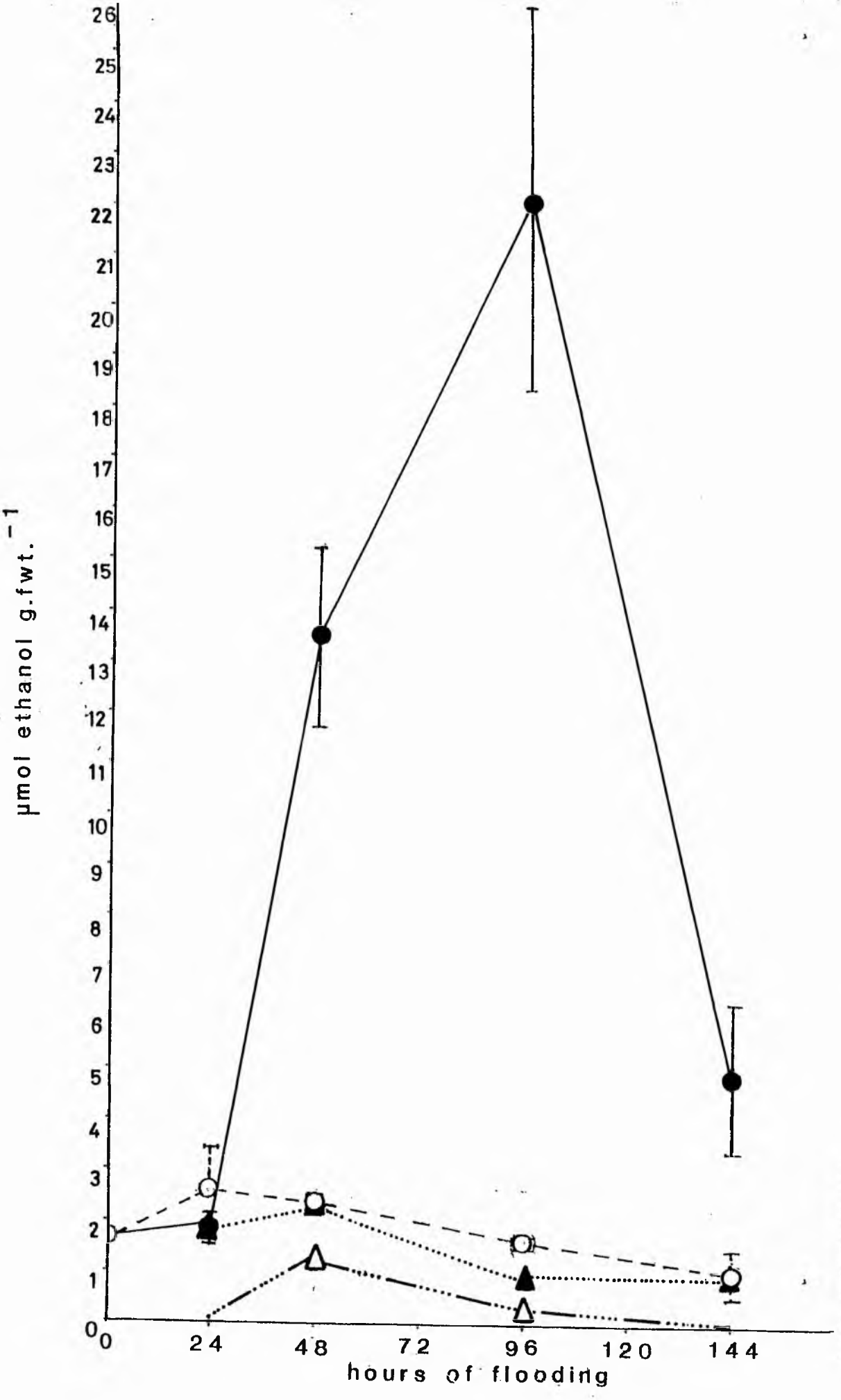

µmol ethanol g.fwt.$^{-1}$
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
0
24
48
72
96
120
144
hours of flooding

Table 5.3. Ethanol μmol $g.^{-1}$fwt. ± standard error of xylem exudate and within root tissue from green ash after 72 hours of flooding and under non-flooded conditions.

	non-flooded	flooded
Xylem	0.35	0.38
exudate(n=5)	0.13	0.36
Root	0.0	0.13
tissue	0.0	0.08
	(n=7)	(n=4)

DISCUSSION

Table 5.1 illustrates that root tips under flooded conditions were under similar O_2 stress to those under anaerobic conditions. Only slight differences were obtained for ethanol accumulation between trees from the two provenances. Root tips, as already mentioned are the most metabolically active regions of the root. A large proportion of ethanol generated in the anaerobic root tips, 23.0% and 20.7% for N.L.P. and I.L.P. respectively, accumulated within the trap containing ice cold water. This might suggest that at least or a greater proportion of ethanol, because of its solubility, would presumably leak from flooded root tip tissue into the surrounding solution.

If the rate of carbohydrate consumption under nitrogen remained unchanged as compared with that in air then CO_2 production should decrease to a third. However in order to sustain cell activities during O_2 stress glycolysis can be accelerated giving what is termed the Pasteur effect. An increase above this value of one third as shown by green ash roots and lodgepole pine root tip tissue under N_2 (table 5.2) indicates the occurrence of the Pasteur effect. A completely anoxic green ash root environment would not occur naturally because O_2 would be supplied via permeable stem tissue as described by Hook and Brown (1972). A wide range of species and tissues have been cited in the literature as showing evidence for the Pasteur effect. For example, Joly and Crawford (1982) using Brazilian tree roots and also Crawford (1977)

using germinating seeds of various species have given evidence for the occurence of the Pasteur effect. The Pasteur effect appeared not to be present using Buckwheat roots (Effer and Ranson, 1967). The Pasteur effect also increases ethanol production coupled to CO_2 production which may then be accumulated or removed by dissolution into the soil medium or within the transpiration stream. Hook et al. (1963) have shown higher quantities of ions such as Na, Mn and Fe arise in the shoots of loblolly pine under flooding, illustrating the incapacity of the plant to control upward movement of potential toxins.

Figure 5.1 clearly shows that during flooding green ash root tissue did not accumulate as much ethanol as root tip tissue of C.L.P. trees. There are a number of probable reasons for this. First, little ethanol may have been produced. The metabolic rate of green ash root tissue under N_2 was lower than lodgepole pine root tip tissue under N_2 (table 5.2) indicating that ethanol production under O_2 stress would also be relatively low. Second, Hook and Brown (1972) have demonstrated the ability of of green ash to aerate flooded roots due to gas-permeable stem tissue. Due to high porsity of stems, xylem sap was extracted in this study from excised root tissue as mentioned earlier. Nevertheless the incapacity to sustain aerobic respiration was apparent because ethanol content within the root tissue did increase during flooding. Third, root tissue of green ash used was finer and more branched and therefore had a larger surface to volume ratio than lodgepole pine root tips thus allowing ethanol to dissolve more easily from root tissue. Lodgepole pine roots had been taken from a root system which was virtually pot-bound. This may

have also hampered ethanol removal. Harms (1973) has shown that Nyssa sylvatica, a flood tolerant species grew better while flooded in flowing water than in static water. This may perhaps be an analogous to the removal of volatile compounds from germinating chickpea seedlings allowing more growth and better survival on reexposure to aerobic conditions as descibed by Crawford and Zochowski (1984).

Ethanol accumulation within lodgepole pine root tip tissue increased and reached a peak approximately after 96 hours of under flooding. This peak probably coincided with root tip death. Keeley and Franz (1979) using Nyssa sylvatica showed that a decrease in ethanol production was accompanied by a marked deterioration of the root system especially in intolerant upland populations.

Table 5.3 shows that ethanol content within the green ash xylem exudate did not increase after flooding. This suggests that potential hydrogen carriers such as ethanol and potential toxic compounds are not transferred upwards. Kimmerer and Stringer (1988) stated that the ethanol and acetaldehyde within the xylem exudate of Populus deltoides also showed no significant change after 3 days of flooding.

Summary

Green ash and lodgepole pine have been shown to be completely different in regard to ethanol accumulation within root tissue. First, ethanol accumulation within the root tip tissue of central lodgepole

pine trees reached a peak approximately after 96 hours of flooding. Green ash tissue displayed no similar peak although ethanol within flooded root tissue was greater than in control root tissue. Second, xylem exudate of green ash showed little change in ethanol content after 72 hours of flooding unlike the xylem exudate of lodgepole pine in chapters 2 and 3. This was partly due to the low metabolic rate of fine roots of green ash. Under N_2 CO_2 production was a third of production from root tip tissue of lodgepole pine (table 5.2) indicating the potential maximum quantity of ethanol that could enter the transpirational stream of green ash to be small compared to that for lodgepole pine. Third, although green ash root tissue increased CO_2 production under N_2 and increased ethanol production under flooded conditions showing O_2 stress, green ash would have been able to aerate at least some of its root system due to the stem gas-permeablity. Thus perhaps hypoxic conditions rather than anoxic conditions, as in lodgepole pine root tips, would result. Lodgepole pine root tips after 24 hours of anaerobic treatment had similar ethanol quantities to those subjected to flooding after 24 hours indicating that the O_2 stress was the same.

CHAPTER 6

CONFIRMATION OF ETHANOL WITHIN THE TRANSPIRATIONAL STREAM

Ethanol accumulation within flooded root tip tissue, the main site of production, peaked after approximately 96 hours (chapter 5). The gaseous ethanol released from lenticels and ethanol content within xylem exudate both peaked after approximately 72 hours of flooding (chapter 2) suggesting this may be due to transportation from the source of production mentioned above. Ethanol within the needle tissue should, if detected, therefore accumulate after 72 to 96 hours of flooding thus demonstrating again an upward transport from the source of production.

During flooding transpiration in woody species does not stop immediately as with herbaceous species. Harrington (1987) using red alder and black cottonwood noted that stomatal closure following flooding was not observed in either species. Dobbs and Scott (1971) using recordings of diurnal changes of girth from 35 year old Douglas fir have shown that greater suction pressure indicated by constriction of girth starts earlier in higher than lower canopy levels. This suggests that if suction pressures caused by transpiration were large, root conditions, such as flooding may have a small effect upon reducing transpiration and also upward transport of compounds within the xylem. Coutts (1981) has shown that when sitka spruce seedlings were flooded there followed a sudden decrease and then an increase in transpiration accompanied by an increase in needle water potential. In fact

transpiration increased for 7 days before finally decreasing again.

Direct measurements of weight loss from trees sealed within polythene and covered with aluminium foil can give estimates for transpiration as carried out, for example, by Coutts (1980). Weyers and Johansen (1985) have demonstrated using Commelina communis that accurate estimation of stomatal aperture from silicone rubber impressions can be made thus giving an estimation of transpiration, however due to sunken stomata and needle shape this technique cannot be used for pines. Diffusion porometers, to measure leaf resistance giving an indication of stomatal aperture, have not been developed for needles. The leaf water potential measured by the use of pressure bombs, and relative water content give an indication of the water supply to leaves and this can be carried out for pine needles.

In a review article Hsiao (1973) stated that a decrease in the relative water content (R.W.C.) of leaves by 15-20% causes a sharp increase in stomatal closure which can be shown by higher resistance measured by diffusion porometers and thus indirectly the R.W.C. can be considered an indication of transpiration.

In the following the R.W.C. of young and old needles of trees from skeena river lodgepole pine and northern coastal lodgepole pine provenances was examined under control and under flooded conditions. Ethanol content of needles taken from different positions upon trees from central lodgepole pine and inland lodgepole pine provenances was

quantified under control and under flooded conditions.

MATERIALS AND METHODS

Examination of the relative water content (R.W.C.) of needles

Three year old trees from Skeena river lodgepole pine (S.L.P.) and northern coastal lodgepole pine (N.C.L.P.) provenances were kept under control conditions as described in chapter 2. In addition the relative humidity was kept artificially low between 60 and 70%. Trees were flooded with Hoagland's solution.

Needles were taken from the top of the trees and from the base where the needles were older. Needles were dried at 68 ^{o}C for 1-2 weeks. The difference between the fresh weight and dry weight represents the water content. The relative water content (R.W.C.), as described here in this chapter, refers to percentages which were calculated by comparing the water content of needles from non-flooded trees with needles from more non-flooded trees and with needles from trees flooded for 48, 96, 144 and 192 hours.

Examination of ethanol content of needles

Three year old trees from central lodgepole pine (C.L.P.) and inland lodgepole pine (I.L.P.) provenances were watered weekly with Hoagland's and flooded with tap-water. The trees were grown in controlled conditions as described in chapter 2.

Needles were taken from the top of the growing stem and form the base. In addition needles from a branch tip between the top and the base positions were taken for comparison.

Using unwashed needles the method as described in chapter 2 for root tip tissue ethanol quantification, which entailed using liquid N_2 to grind the tissue, was followed here. Ethanol was quantified using the G.L.C..

RESULTS

Relative water content

Table 6.1 shows the relative water content (R.W.C.) of needles from trees from two provenances under control and under flooded conditions. The R.W.C. data shown for S.L.P. and N.C.L.P. trees although variable demonstrated during flooding no tendency to decrease water content for young top needles.

Ethanol content of needles

Figure 6.1 and 6.2 show ethanol content within needles from 3 different positions from C.L.P. and I.L.P. trees respectively, which were exposed to flooded conditions. Lower older needles from trees of C.L.P. and I.L.P. accumulated most ethanol, 0.81 and 0.59 μmol $g.^{-1}$fwt. of needle tissue respectively after 72 hours of flooding.

The ratio of the number of needle samples containing ethanol against the total number of needle samples given as a percentage for different positions in trees from C.L.P. and I.L.P. provenances are shown in figures 6.3 and 6.4 respectively. For trees of C.L.P. there was a

Table 6.1. The Relative Water Content (R.W.C.) percentages (+ standard error) for needles taken from top and lower positions from trees from skeena river and northern coastal lodgepole pine provenances (at least 9 replicates samples used).

		hours of flooding				
	position of needle	0	48	96	144	192
Skeena river	top	81.1	77.9	81.0	70.7	84.7
		9.1	7.3	15.1	7.9	9.6
	lower	121.3	117.7	93.9	96.3	99.4
		19.8	21.3	12.6	13.5	13.4
Northern coastal	top	103.0	101.6	102.5	100.3	99.4
		11.9	8.1	5.5	10.6	15.5
	lower	111.5	98.6	104.8	100.0	97.5
		10.1	9.2	10.4	13.9	10.1

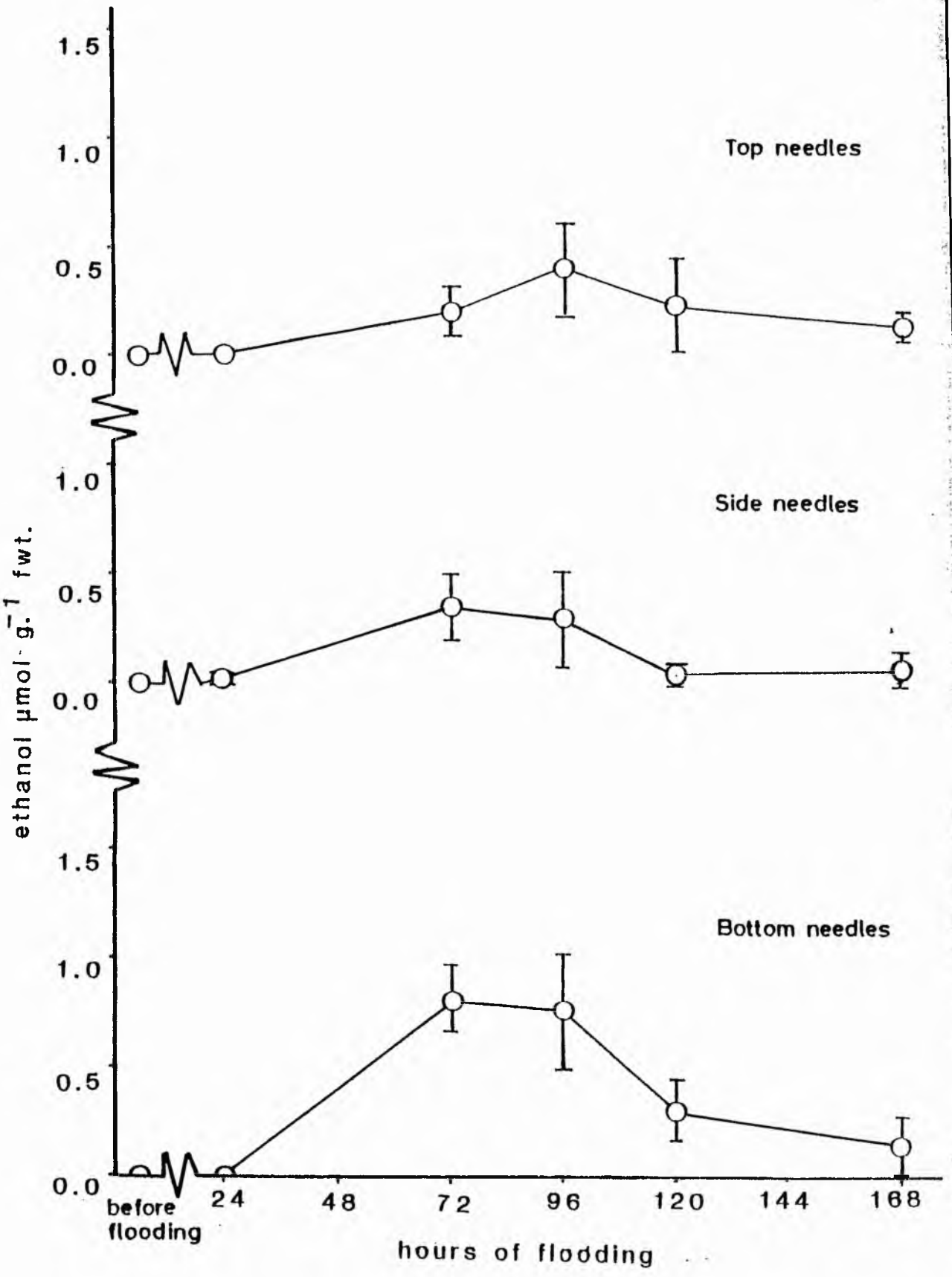


Figure 6.1. Quantitative analysis: needles from different positions were taken and examined for ethanol during flooding of trees from central lodgepole pine provenance (at least 11 replicate samples used; bars = standard error).

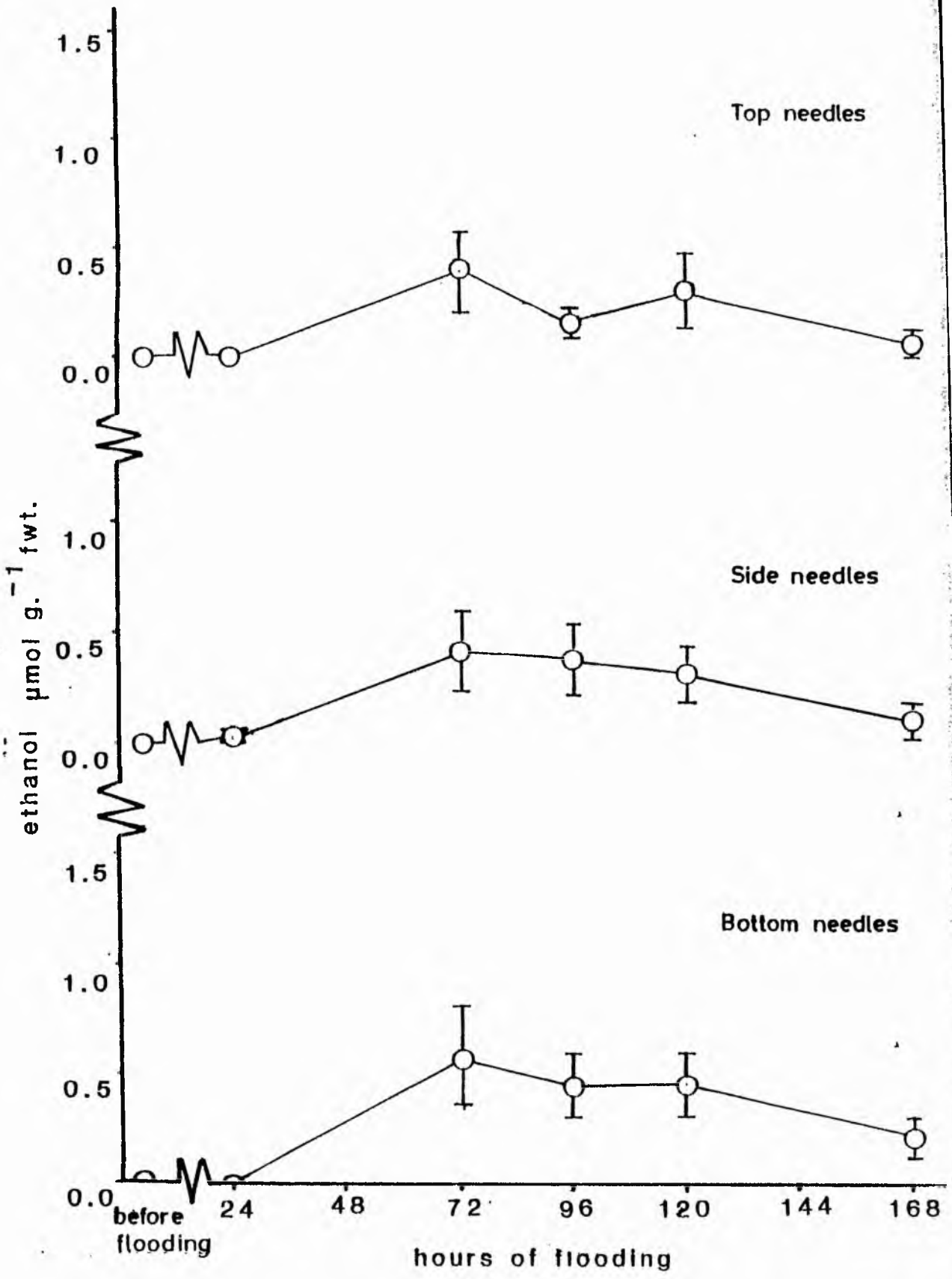


Figure 6.2. Quantitative analysis: needles from different positions were taken and examined for ethanol during flooding of trees from inland lodgepole pine provenance (at least 11 replicate samples used; bars = standard error).

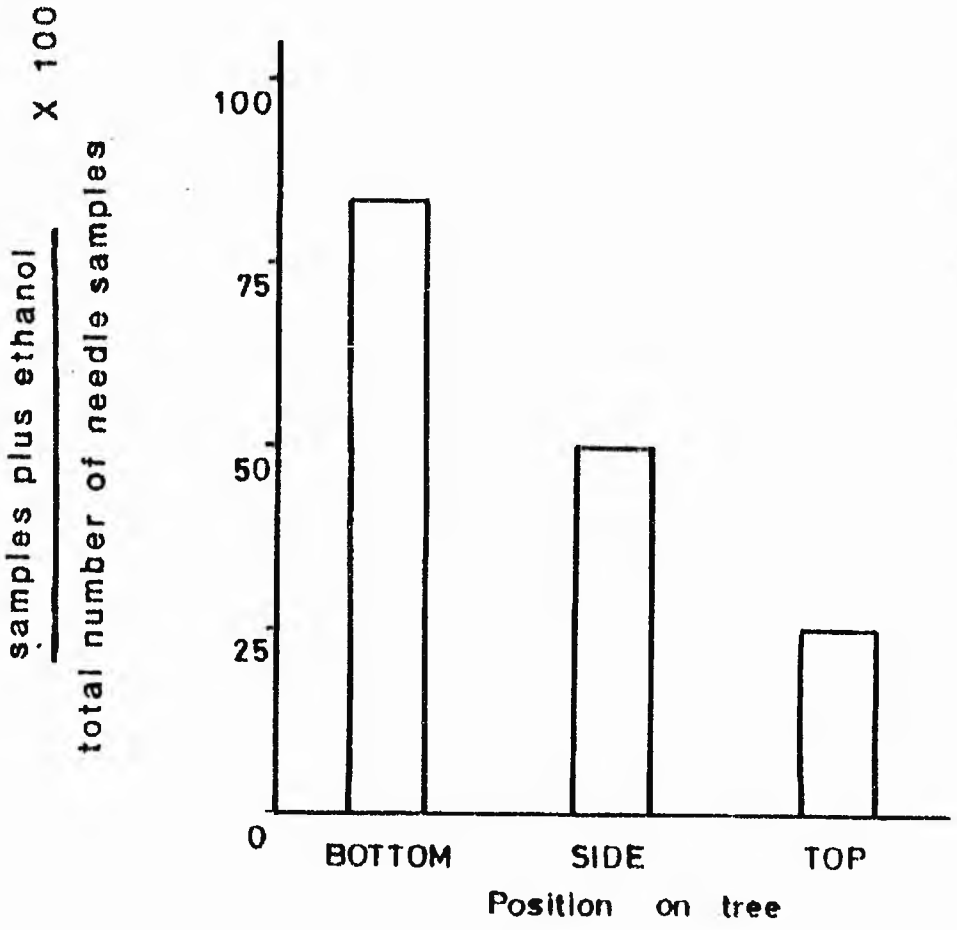


Figure 6.3. Qualitative analysis: needles from different positions were taken and examined for ethanol after 72 hours of flooding using trees from central lodgepole pine provenance.

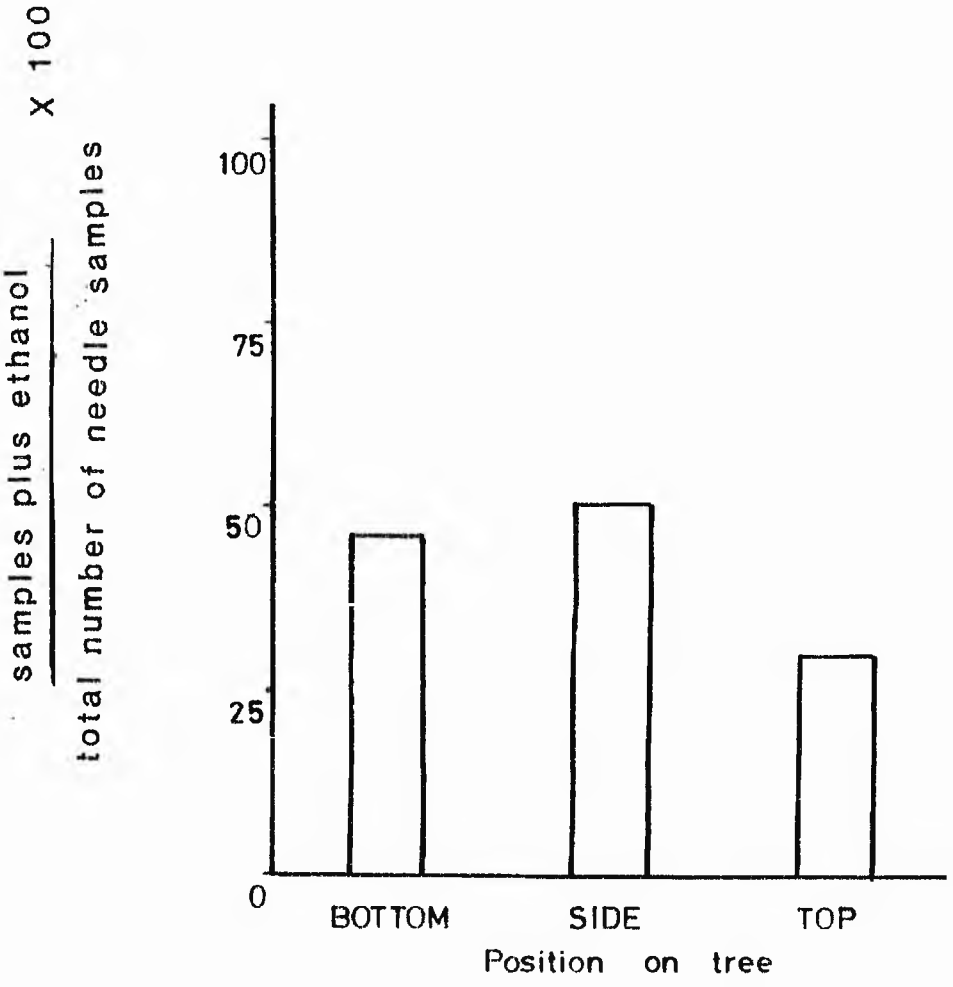


Figure 6.4. Qualitative analysis: needles from different positions were taken and examined for ethanol after 72 hours of flooding using trees from inland lodgepole pine provenance.

marked tendency for the percentage of samples with ethanol to decrease from old to young needles. For trees of I.L.P. young top needles tended to have less needle samples containing ethanol.

A statistical test of proportions, as described by Freund (1972), was carried out on samples from different positions of trees from C.L.P. and I.L.P. provenances. For C.L.P. trees significantly top needle samples contained a smaller proportion of samples containing ethanol than bottom needle samples; $X^2=8.2$, $P<0.05$ with 1 degree of freedom.

DISCUSSION

Table 6.1 shows that the relative water content (R.W.C.) of young top needles from both provenances did not decrease during flooding which clearly demonstrates that the water supply to needles was not impeded by flooding although the transpiration rate may have been decreasing. Coutts (1980) for example, has noted that the water potential, a measure of the water content, remained unchanged or increased using root damaged unflooded sitka spruce. Raschke (1970) has shown using Zea mays that a reduction in the relative humidity decreases transpiration which would presumably eventually reduce growth. Lange et al. (1971) have shown that a reduction in the relative humidity decreases the stomatal aperture of Polypodium vulgare and Valerianella locusta. With the added potential stress of artificially lowered humidity the R.W.C. of young top needles from trees of skeena river and northern coastal lodgepole pine provenances under flooding did not decrease sharply. This would enable

lodgepole pine shoot growth under waterlogged conditions (Crawford and Baines, 1977). Harrington (1977) has shown leaf expansion of black cottonwood and red alder was detected from flooded trees. By contrast to the younger needles there was a tendency for older lower needles to reduce the R.W.C. during flooding giving an indication of more water stress.

By examining ethanol content within needles it was discovered that lower older needles accumulated most ethanol from trees from central lodgepole pine (C.L.P.) and inland lodgepole pine (I.L.P.) provenances as shown in figures 6.1 and 6.2 respectively. The ethanol rose to a peak after 72 hours of flooding within needle tissue as shown in figures 6.1 and 6.2 for C.L.P. and I.L.P. trees respectively. The accumulation within the root tip tissue, the source of ethanol production peaked after approximately 96 hours (figure 5.1 chapter 5) suggesting a transport upwards to the needles from this source. Needles from trees of C.L.P. tended to accumulate more ethanol within lower needles than corresponding needles from trees of I.L.P..

Some needle samples contained no detectable ethanol. The ratio of needle samples with ethanol to the total number of needle samples is given as a percentage in figures 6.3 and 6.4 for trees from C.L.P. and I.L.P.. There was a percentage decrease towards young top needles from old lower needles from trees of C.L.P.. Needles from trees of I.L.P. provenances showed a less pronounced tendency to have fewer samples with detectable ethanol within young top needles than within old lower needles. Old needles may not metabolise or release ethanol so readily. The R.W.C.

decreased in older needles with trees from N.C.L.P. and S.L.P. provenances indicating a possible reduction in transpiration or release of ethanol.

Ethanol here was examined using a chromatographic technique. Examining ethanol quantities enzymatically would have proved probably less sensitive for small quantities of ethanol within needles. Pitel and Cheliak (1985) have shown that to optimise enzymatic analysis of malate from spruce needles necessitates the use of specific concentrations of various polymers and detergents because of the presence of phenols and tanins.

In summary the above clearly verifies the capacity of 3 year old lodgepole pine to transport ethanol and potentially other compounds to needles within the transpirational stream.

CHAPTER 7

Xylem exudate comparison with gaseous release from lenticels

The relatively small importance of lenticels compared with xylem exudate in removing ethanol from a root system under flooding has been demonstrated in chapter 2. Less than 0.2% of ethanol generated in flooded root systems is released by lenticels. Transport from the root and subsequent release of gaseous compounds ** such as ethanol from lenticels can be diagnostic of root health under waterlogging although measurements of gaseous released compounds in field conditions would prove impossible. Xylem exudate may be likened to a blood sample and it can, by contrast, be adapted to diagnose the root health under waterlogged field conditions using the presence of certain metabolites.

** See appendix A. (Additional figure showing the relative amounts of ethene released from lenticels. Flooded roots had less hypoxia therefore less ethene).

Xylem exudate as an useful indicator of Root health

The ethanol content within the xylem exudate (chapter 2) and ethanol accumulation within the root tip tissue of lodgepole pine (chapter 5) immediately increased in response to flooding. After approximately 96 hours of flooding root accumulation decreased as did the ethanol content within the xylem exudate. Within the flooded root system O_2 stressed cells were presumably unable to sustain ethanol

production through glycolysis and death would have then have followed. Keeley and Franz (1979) noted that with intolerant upland populations of Nyssa sylvatica the decrease in ethanol production was also accompanied by a deterioration of the root systems. Using the xylem exudate from lodgepole pine the compositonal changes in amino acids occured, not after 24 hours, but distinctly after 72 hours of flooding indicating again further degeneration such as protein breakdown within root tissue.

Xylem exudate from the flood tolerant tree, green ash showed no increase in ethanol content after 72 hours of flooding in contrast to the xylem exudate of lodgepole pine. Kimmerer and Stringer (1988) using another flood tolerant species, Populus deltoides found that ethanol and acetaldehyde also did not increase within the xylem exudate after 72 hours of flooding. The above illustrates that the xylem exudate can indicate the flood tolerance of different trees.

Removal of hydrogen and its importance within xylem sap

Figure 7.1 is a résumé of previous chapters and emphasis is placed upon the removal of hydrogen. The percentages in this figure are relative to each other and therefore give an indication of the importance of certain compounds within the xylem to remove hydrogen from the root system under flooding. Knowing the waterloss in transpiration and the content of ethanol, for example, within the xylem exudate it is thus possible to estimate the amount removed via the transpiration stream and

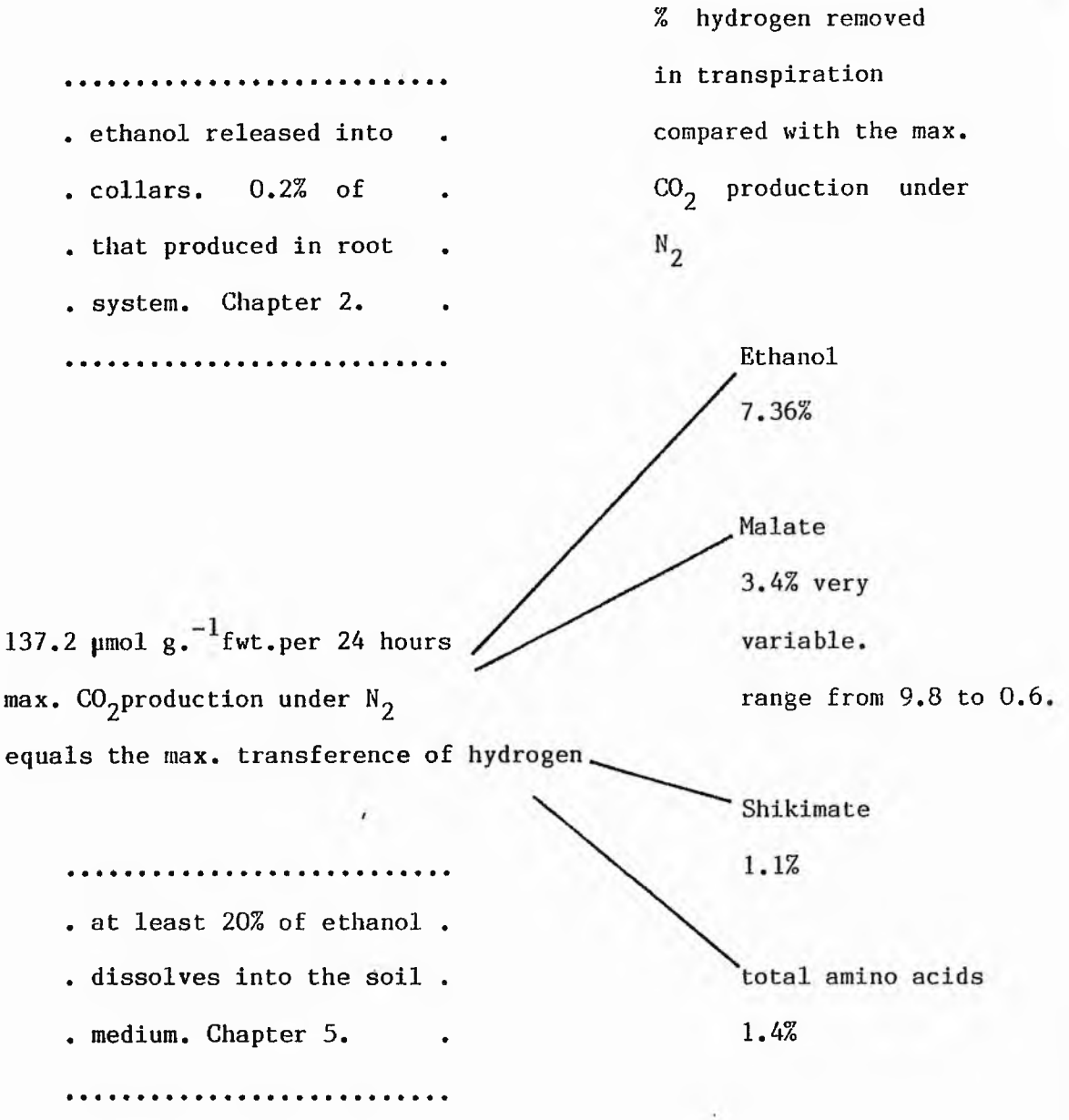


Figure 7.1 illustrating the importance of xylem exudate for the removal of hydrogen. 137 µmol $g.fwt.^{-1}$ equals the maximum transference of hydrogen from NADH to regenerate NAD within the root system from a flooded inland lodgepole pine tree. Other figures enclosed were obtained from other chapters of this study.

compare this with the maximum potential production. The maximum potential ethanol production and thus the maximum transference of hydrogen from NADH was calculated by obtaining the CO_2 production under N_2 from whole roots that had been previously flooded for 120 hours. This was carried out using an infra red gas analyser (I.R.G.A.).

Malate removed via the transpiration was also compared with this maximum potential rate of hydrogen removal from NADH but it must be noted that for every hydrogen moved to malate there is three times as much carbon involved making malate three times more costly in carbon. Other compounds such as shikimate and total amino acids quantified by the ninhydrin method are also included in this comparison.

Figure 7.1 shows that more than 20% of hydrogen can be removed as a constituent part of ethanol into the soil medium (derived from Chapter 5) however the transference of hydrogen to form malate and ethanol, and subsequent transport within the transpiration stream can represent $\geq$ 10% of the maximum transference rate of hydrogen from NADH. During spring growth NAD regeneration from NADH may in fact contribute to tree root survival although this may only be one of many factors involved.

Metabolic differences within Pinus contorta

The geographical range of Pinus sylvestris extends over an area from northern Europe to Spain and from the British isles eastwards across Europe and Asia to Kamchatka. Within this large range different ecotypes

occur. Similarly Pinus contorta has a large range which covers large tracts of western Canada and north western U.S.A. including Alaska. Morphologically distinct populations exist and there is a strong likelihood of metabolic differences which may have a bearing on flooding-tolerance.

By quantifying the production and dissipation of products such as ethanol during flooding, it was thought differences in metabolism as a result of flooding between trees from different provenances would be discovered. The results, as summarised in table 7.1, were inconsistent. Table 7.1 shows that 4 out of 6 examples of trees from inland lodgepole pine provenance had a tendency to produce more ethanol when compared with trees from other provenances.

Malate content within the xylem exudate after 72 hours of flooding shown in table 3.3 chapter 3 also showed inconsistency. The reason for this, as well as the inconsistency with regard to ethanol, may be due to previous differing nursery conditions and may also be due to the time of flooding within the growing season. Harms (1973) has shown using Nyssa that the growth effect of flooding trees in stagnant and moving water did not become apparent until later in the growing season after the initial growth period in April. It was suggested that this was due to the high CO_2 concentrations and O_2 becoming less available during the latter part of the growing season. Possibly growth may also be affected by decreasing transport of hydrogen carrying compounds within the transpiration stream also later in the growing season making it more of a necessity for O_2 to diffuse downwards.

Table 7.1. This summarises ethanol results within this study. Trees from a provenance are categorised as either having more or less ethanol compared with trees from the other provenance.

Reference in study	difference between trees from different provenances.	tissue used
chapter 2 Fig. 2.3	I.L.P.> C.L.P.	lenticels gaseous released ethanol
chapter 2 Fig. 2.4	I.L.P.> N.L.P.	xylem exudate
chapter 3 table 3.1	I.L.P.> N.L.P.	xylem exudate
chapter 3 table 3.2	I.L.P.> N.L.P.	root tissue
chapter 3 table 3.2	I.L.P.< N.L.P.	xylem exudate
chapter 5 table 5.1	I.L.P.< N.L.P.	root tissue

Due to insignificant and inconsistent differences from trees of different provenances the general conclusions from this study are therefore primarily based on lodgepole pine considering its response as a species and not as provenances.

Thoughts on future research

Xylem exudate is readily obtainable and provides a source of information from which to diagnose root health. It would be necessary to examine seedlings from different provenances within a variety of field conditions coupled to the examination of seedlings in the laboratory to forecast the potential expansion of this technique to field conditions.

To thoroughly examine potential differences between trees from different provenances with respect to metabolites accumulated within the root system and xylem exudate, or released in gaseous form from lenticels it would be necessary to grow trees under controlled conditions from seed to the age of approximately 3 years. This then would provide more uniform plant material. This was not carried out because the time involved would have exceeded the time provided for the project but any future experiments should use trees kept under controlled conditions for at least the previous growing season. This would provide more consistent differences.

Analysis of xylem exudate from different tree species with a range

of tolerance to waterlogging would indicate clearly differences between tolerance and intolerance.

There is a widescope for further investigation within this field of research. For instance, analysis of the reduced iron and manganese levels within the xylem could be conducted from trees under various flooding regimes, for example either under stagnant or cyclic flooding regimes. Tree root health and response to flooding after having been starved of nutrients in the previous growing season or kept under normal conditions in various plantations could be studied through the xylem exudate. Investigation of tree crops from not only the United Kingdom but other regions of the world could be conducted.

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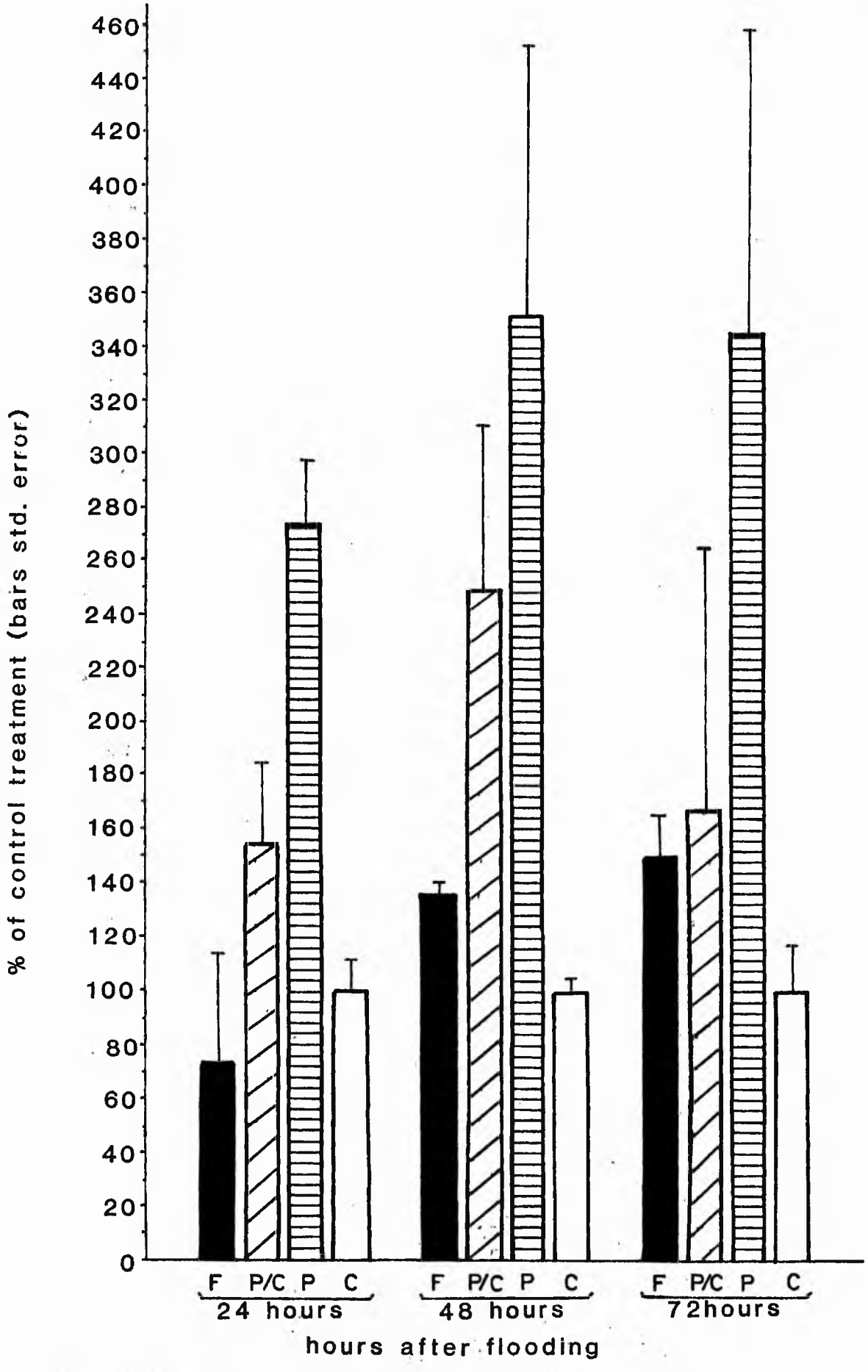
APPENDICES

APPENDIX A

Additional figure, overleaf, represents ethene released into collars at the base of the stem of inland lodgepole pine under various conditions (F = Flooded with tap water to soil level; P/C = Partially flooded (3.5 cm of the pot base immersed) for 20 days and then completely flooded for the duration of the experiment; P = Partially flooded (3.5 cm of pot base was immersed) and C = under control conditions).


% of control treatment (bars std. error)
460
440
420
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
F P/C P C
24 hours
F P/C P C
48 hours
F P/C P C
72hours
hours after flooding

APPENDIX B

Nutrients

Hoagland's Solution

6ml of 1 Molar KNO_3 per litre

2ml of 1 Molar $NH_4H_2PO_4$ per litre

1ml of 1 Molar $MGSO_4$ per litre

1ml of 20 mMolar Fe -EDTA per litre

4ml of 1 Molar $Ca(NO_3)_2$ per litre

1ml of micronutrients per litre

Micronutrients contained 50 mMolar KCL,
25 mMolar H_3BO_3,
20 mMolar $MnSO_4(x\ 4H_2O)$,
2 mMolar $ZnSO_4(x\ 7H_2O)$,
0.5 mMolar $CuSO_4(x\ 5H_2O)$,
and 0.1 mMolar H_2MoO_4.