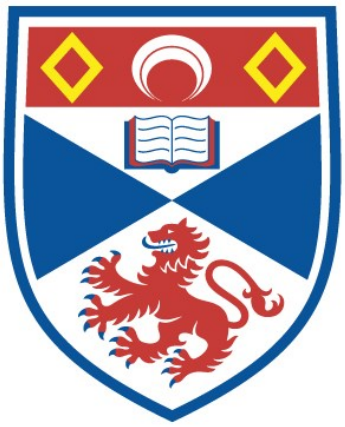
IRON TOLERANCE AND THE ROLE OF AERENCHYMA IN WETLAND PLANTS

Nicholas Smirnoff

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



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by
NICHOLAS SMIRNOFF

ABSTRACT

The relative iron (II) tolerance of a range of wetland plants was determined and compared with some species characteristic of well drained soils. A wide range of tolerance occured amongst the wetland species but they were generally more tolerant than those from well drained soils. No correlation was found between iron (II) tolerance and the amount of air space (% v/v)(aerenchyma) in the roots of these species. There was a significant negative correlation between air space and iron uptake by roots. This may have been caused by iron (II) oxidation in the rhizosphere resulting in decreased availability. There was evidence that differential iron (II) tolerance of excised root tips was maintained under aerobic and anaerobic conditions. It was thus suggested that iron (II) tolerance may not be dependent on iron exclusion or oxidation of iron (II) by oxygen diffusing through the aerenchyma.

Levels of malic and citric acids in roots were altered by iron (II) sulphate, but the absolute levels and changes in levels had no correlation with the iron (II) tolerance of the species.

Peroxidase and catalase activities in root tips of plants gown in drained and flooded sand culture were measured and considered in relation to the oxidising power of roots. Activity was detected in all species examined but was generally unaffected by flooding. Evidence from the literature suggested that these enzymes of peroxide metabolism are unlikely to be active in flooded roots and so could not mediate their oxidising power.

The structure of root aerenchyma had great variability between species. The Cyperaceae had the most complex and well organised structure. Growth under flooded conditions increased air space in most species, but there were exceptions. In *Eriophrum angustifolium* and *E. vaginatum* air space was high under drained conditions and was not increased by flooding. In *Filipendula ulmaria* the small amount of air space was not increased by flooding. Low nutrient levels increased air space production in *Nardus stricta*. The function of aerenchyma and the influence of environmental factors on its production are discussed.

Iron Tolerance and the Role of Aerenchyma in Wetland Plants.

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Declaration

I hereby declare that this thesis has been composed by myself, and that it is a record of work done by myself. None of the work has been accepted in any previous application for a degree. Any other sources of information have been acknowledged.

Signed:

Nicholas Smirnoff

I, Nicholas Smirnoff, was admitted as a research student of the University of St. Andrews in October 1977 in accordance with Ordinance General No. 12 and the resolution of the University Court, 1967, No. 1. The thesis was completed in April 1981.

Certificate

I hereby declare that Nicholas Smirnoff has been engaged upon research from October 1977 onwards to prepare the accompanying thesis for the degree of Doctor of Philosophy.

Signed:

R.M.M. Crawford

St. Andrews

April 1981.

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Above all, I thank Gill Swales who was a support and inspiration to me for two years.

This thesis is dedicated to my Mother

Contents

CHAPTER 1

Introduction

Waterlogged soils are widespread in almost all parts of the world and they support a wide variety of communities. Many wetland communities have a high productivity or high diversity of plant and animal species. With increasing human pressure on land for living and agriculture they represent, particularly in temperate climates, much of the remaining "natural" vegetation. The only major crop plant grown in waterlogged soils is rice. Waterlogging is unfavourable for the growth of all the other major crops, so wetlands are useless for agriculture unless drained. Many crop plants have been selected or bred to grow well in drained and fertile soils and their yield is severeley reduced under "unfavourable" conditions. It is clear from examining the distinctive flora of wetlands that many species can grow under these "unfavourable" conditions and also have a high productivity. Phragmites communis in freshwater marshes and Spartina alterniflora in salt marshes can produce strands of extremely high productivity (Linacre, 1976).

What are the potentially unfavourable conditions for plant growth in waterlogged soils? As Arber (1920) has pointed out, plants growing in wetland habitats have their roots submerged in the "asphyxiating mud". This phrase aptly describes conditions in waterlogged soils. The pore space of waterlogged soils is completely filled with water. Because the rate of diffusion of oxygen in water is ten thousand times less than in air, any oxygen dissolved in the soil water is more rapidly used up by soil microorganisms and plant roots than it can diffuse from the air. Waterlogged soils are essentially anaerobic as a result of this. Lack of oxygen will immediately cause a problem for higher plants because they are thought to be obligately aerobic organisms. Much attention has been paid recently to metabolic pathways in wetland plants under anoxia

and hypoxia (Crawford, 1978; Hook and Crawford, 1978; Smith and ap Rees, 1979). The evidence suggests that wetland plants may show various metabolic adaptations to hypoxia and anoxia (Crawford, 1978; Hook and Crawford, 1978). Characteristic of wetland plants is the production of aerenchyma (tissue containing large intercellular spaces or lacunae). In roots this tissue could possibly function in aeration (Armstrong, 1979).

The other very important characteristic of the "asphyxiating mud" is the presence of elevated levels of potentially phytotoxic substances produced under anaerobic conditions. In the absence of oxygen, certain soil microorganisms turn to other electron acceptors for their anaerobic respiration. Many substances normally present in aerated soils disappear and their reduced forms can be phytotoxic. A major change is the reduction of relatively insoluble Fe (III) to the more soluble Fe(II). High levels of Fe(II) are potentially phytotoxic. Some aspects of the chemistry of waterlogged soils and the evidence for this will be reviewed in the following sections.

The Chemistry of Waterlogged Soils

This subject has been well reviewed (e.g. Armstrong, 1975; Gambrell and Patrick, 1978; Ponnamperuma, 1972), so only a short outline will be given here. A convenient measure of the degree of soil reduction is the redox potential. Its use was first suggested by Pearsall (1938) and since then it has become a widely-used technique. Measurement is made with platinum electrodes and the values expressed as electrical potentials. The redox potential is dependent on pH and can be corrected to a standard pH. The redox potential of a soil measured in this way represents a weighted average for all the redox couples present, but it has been found useful for predicting which chemical species will be present in a particular soil (Gambrell and Patrick, 1978). As the redox potential becomes more negative the soil is more reduced.

The potentials at which various oxidised forms of some important soil redox couples become unstable and undergo reduction are shown in figure 1.1.

Most waterlogged soils do not contain nitrate, Mn(IV) or Fe(III). Nitrate is denitrified to produce N_2 and the NH_4^+ present in reduced soils is derived from the breakdown of organic matter. The insoluble oxidised species Mn(IV) and Fe(III) are present as more soluble Mn(II) and Fe(II). In soils of lower redox potential sulphate is reduced to sulphide. In soils high in iron, Fe(II) sulphide may be precipitated, but it is possible for appreciable amounts of hydrogen sulphide to coexist with Fe(II) (Joshi et al, 1975). Methane (marsh gas) is produced from carbon dioxide only under extremely reducing conditions.

The redox potential of a newly-flooded soil generally decreases with time and reduced species are formed in the sequence shown in figure 1.1. In permamently waterlogged soils redox potential decreases with increasing depth and the sequence shown in figure 1.1 will be followed with increasing depth. The soil surface may have a thin oxidised zone. The reductions referred to above are mainly mediated by microorganisms. In addition to these reductions various organic compounds are produced by the anaerobic metabolism of microorganisms, e.g. fatty acids (acetic acid, butyric acid), ethylene, aldehydes, ketones and alcohols. Many of these are potentially phytotoxic.

The Potential Phytotoxicity of Some of the Reduced Substances in Waterlogged Soils.

It has been realised for some time that waterlogged soils contain substances toxic to some plants. For example, Dachnowski (1908, 1909) found that bog water could be toxic, but he did not discover what the active principles were. Further investigations have shown that the major toxins in reduced soils are sulphide (Allam and Hollis, 1972; Goodman and Williams, 1961; Sheikh, 1970; Vamos and Koves, 1972), manganese (II) (Ernst and Lugtenborg, 1980; Jones 1972b), iron (II) (see next section) and lower fatty acids, e.g. acetic and butyric acids (Lynch, 1977; Sanderson and Armstrong, 1980). Iron and manganese

can reach toxic levels because their divalent reduced forms are much more soluble than their oxidised forms (Ponnamperuma, 1967, 1969). The greater solubility makes these elements more available for uptake by roots.

Iron in Waterlogged Soils and Plants

a) Iron Availability and Uptake. In well-aerated soils iron exists predominantly in the Fe(III) condition and is sparingly soluble. It does not become appreciably soluble until the pH drops to three or below. The solubility product of Fe(III) hydroxide is 6×10^{-38} g ion per litre whereas for Fe(II) hydroxide it is 1.8×10^{-5} - 8×10^{-6} g ion per litre (Williams, 1971). Fe (II) is more soluble than Fe(III) and can remain soluble at higher pH values. Because of these properties high levels of soluble Fe (II) can occur in reduced soils over a wide range of pH. These relationships are summarised in table 1.1.

Measurements of the levels of Fe(II) or soluble Fe in waterlogged soils have confirmed these theoretical relationships. The occurence of high levels in rice paddy fields is well known (Mandal, 1961; Ponnamperuma, 1972). In sand dune slacks, where the water table fluctuates seasonally, the levels of exchangeable iron are greatest in early summer when the water table is above the sand surface. In late summer the water table drops below the surface and exchangeable iron levels are very low (Jones, 1972c). In this study the exchangeable iron was mainly Fe(II) because it remained soluble in the neutral ammonium acetate extractant. Experimental flooding of dune and dune slack soils increases soluble and exchangeable iron levels (Jones, 1972a). Flooded peat also has an increase in soluble and exchangeable iron levels (Jones and Etherington, 1970). Higher Fe(II) levels are found in waterlogged woodland soils than in well-drained areas nearby (Martin,1968)

The increased soluble and exchangeable iron levels leads to greater uptake by plants. Mayer and Gorham (1951) found that, among various species in the English Lake District, those from waterlogged habitats

had more iron in their shoots. The results of a number of investigations of the effect of flooding on iron uptake by various species are shown in table 1.1. With only one exception (the swamp population of Nyssa sylvatica) flooding has increased iron concentration in roots. Iron concentration in shoots has also been increased (with the exception of Stellaria media). Diverse types of soil have been used in these investigations and this can have a great effect on iron uptake. For example, flooding Erica cinerea in peat and mineral soil results in different levels in the plants. The iron concentration in roots is usually an order of magnitude greater than in shoots.

b) The Occurence of Iron Toxicity. Some of the species shown in table 1.1 have a reduction in growth when flooded (e.g. Stellaria media, Festuca rubra and Erica cinerea), but this does not correspond with any particular pattern of iron uptake and it cannot be concluded for certain that growth reductions are a result of iron toxicity. Anaerobic conditions and other toxins could contribute to growth inhibition. There is evidence that iron can reach toxic concentrati Iron toxicity can occur even in the wetland plant rice. Ponnamperuma et al (1955) found that various physiological diseases of rice were caused by iron toxicity. Physiological diseases of rice have many local names, for example Mentek, Penyakit merah, bronzing, akiochi and alkagare (Foy et al, 1978; Ponnamperuma et al , 1955; Tanaka et al, 1966) and many of them are the result of iron toxicity. There have been few detailed field studies, so there is little information on iron toxicity or tolerance in plants from natural or semi-natural vegetation. Three investigations, described below, have shown that iron toxicity is a factor influencing survival of species in waterlogged soils.

(i) Martin (1968) showed that Fe(II) level was one factor influencing the composition of the ground flora in a Quercus robur woodland on boulder clay. The ground flora consisted of various communities. Those on well-drained soils contained Mercurialis pernnis, while those on

soils liable to flooding contained Primula elatior and Deschampsia caespitosa. Soluble Fe(II) levels were greater in the waterlogged communities. Water culture experiments showed that Fe(II) was toxic to M. perennis. P. elatior and D. caespitosa were more tolerant to Fe(II). These observations suggested that Fe(II) toxicity could control the distribution of M. perennis in the field. M. perennis was able to survive flooding with distilled water in sand culture, but rapidly showed toxicity symptoms when flooded with Fe(II) sulphate solution.

(ii) Etherington and Jones have shown that the distribution of two closely related species, Erica cinerea and E. tetralix is partly controlled by differential iron tolerance (Jones and Etherington, 1970; Jones, 1971a and b). Both species grow in peat soils, but E. cinerea is generally confined to well-drained sites. E. tetralix can grow under waterlogged conditions. Flooding on a mineral soils increases iron uptake by E. cinerea to a greater extent than E. tetralix (table 1.1) and also eventually results in the death of E. cinerea but not E. tetralix. However, when E. cinerea is flooded in an ombrogenous peat of extremely low soluble iron content it survives. The results suggest that Fe toxicity contributes to the death of E. cinerea in the flooded mineral soil. E. tetralix is thus more tolerant to Fe than E. cinerea and can grow in waterlogged sites from which E. cinerea is excluded.

(iii) Sanderson and Armstrong (1980) have found that rooted cuttings of Pinus contorta are slightly more tolerant to Fe(II) than Picea sitchensis. Both species are widely planted for timber and P. contorta is used on sites prone to waterlogging because it is more tolerant to hypoxia (Crawford and Baines, 1977; Coutts and Philipson, 1978 a and b). In aerated water culture P. contorta was more tolerant to low levels of Fe(II) (0.18 - 0.36mM) than P sitchensis. P. contorta was also better able to regenerate its root system by the growth of laterals after the tips of the primary roots had been killed by a lethal dose of Fe(II) (1.8 mM).

The Mechanism of Iron Toxicity.

Iron is an essential element for all living things. Its biological usefulness arises from its properties as a transition metal. It can easily undergo changes in oxidation state and form complexes with various organic ligands. Because of this, iron has a prominent function in electron transfer. For example, it occurs in the cytochromes and ferredoxin. Apart from being complexed in electron transfer proteins it also functions in other ways. Fe(II) is a cofactor for aconitase (Clarkson and Hanson, 1980) and it is involved in the control of Chlorophyll synthesis (Ryberg and Sundqvist, 1976). The typical symptom of iron deficiency is the chlorosis of young leaves.

The toxicity of iron, if it is available to plants in large amounts, is a feature it shares with other micronutrients. Most micronutrients, for example copper, manganese, zinc (Antonovics et al, 1971; Foy et al, 1978), boron (Bennett, 1974) and magnesium (Proctor, 1970), can be toxic under certain conditions. Other with no, or doubtful, physiological function can also be toxic. These include aluminium (Foy et al, 1978) and many of the heavy metals (those with a density greater than 5), for example lead, cadmium, and mercury (Antonovics et al, 1971).

The mechanism of metal toxicity has been studied little. As with all studies of the effect of toxic substances, it is difficult to separate primary and secondary effects. There is no reason to suppose that all toxic metals have the same effect, or even that there is only one primary effect. Consideration of the chemistry of the toxic metals can give some clue to their primary effects on plants. It appears that the toxicity of various metals follows their order in the Irving-Williams series (Williams, 1971). For divalent cations, the order is:

$$Ca < Mg < Mn < Fe < Co \leqslant Zn \leqslant Ni < Cu$$

In this series the stability of the complexes formed with organic ligands (e.g. O^- S^- NH_2 N) increases from calcium to copper. If excess

metal enters a cell, such complexes are made with exposed functional groups on enzymes and inhibition or inactivation will result. Additionally, physiologically active metal cofactors could be displaced by excess of a toxic metal with a similar size or charge, again causing inhibition or inactivation. The degree of toxicity will depend on the stability of the complex formed by a particular metal. Investigations so far have found that the toxicity of metals to plants approximates the Irving-Williams series. Copper is much more toxic to Agrostis tenuis than zinc (Wainwright and Woolhouse, 1977). Various enzymes extracted from Silene cucubalus have been tested for their sensitivity to inhibition by various metals. The range of metals tested ranged over several orders of magnitude in the concentration needed to inhibit activity (Ernst, 1976; Mathys, 1975). The degree of toxicity of the metals also roughly follows the Irving-Williams series. The sensitivity of the various enzymes also varies. Peroxidase and malate dehydrogenase are more resistant to inhibition than nitrate reductase. The latter enzyme is very rich in sulphydryl groups to which metals can bind (Ernst, 1976).

The basis for iron toxicity is probably similar to that described above. Iron will be available in waterlogged soils as Fe(II). However, there is evidence that excess iron supplied either as Fe(II) or Fe(III) can be toxic (Brown and Jones, 1977; Jones and Etherington, 1970; Olsen, 1958 and Skeen, 1929). These points will be discussed in further chapters.

Plan of the Thesis.

The objectives of the experiments described in this thesis were to examine Fe(II) tolerance and toxicity in a range of wetland species and to compare these with some species characteristic of well-drained soils and to investigate the possible basis of Fe(II) tolerance, with special reference to root aerenchyma. A more general study was also made of the ability of roots to oxidise their environment and of the role of root aerenchyma.

Work on Fe(II) tolerance and toxicity is described in Part I (Chapters 2 - 5). Aerenchyma and root oxidising activity are discussed in Part II (Chapters 6 and 7). The most extensive study of Fe(II) tolerance published is that of Martin (1968) described previously, and in his study most of the species tested were not typical wetland plants. Other studies have been limited to pairs of species (*Erica cinerea* and *E. tetralix* (Jones and Etherington, 1970; Jones, 1971a and b) and *Pinus contorta* and *Picea sitchensis* (Sanderson and Armstrong, 1980)). In this investigation Fe(II) tolerance has been compared in a wide range of plants characteristic of different types of wetland habitat. No extensive field work has been carried out, so the results must be used with caution in making conclusions about the ecology of the plants.

Table 1.1 - The effect of flooding on iron uptake by various species.

Species	Organ	Iron concentration umol. g. dry wt.$^{-1}$ Drained	Iron concentration umol. g. dry wt.$^{-1}$ Flooded	Ratio Flooded:drained	Soil type	Duration of experiment, weeks	Reference
Agrostis stolonifera	Shoots	3.24	3.60	1.11	Desalinated salt marsh sand	6	Rozema and Blom, 1977
	Roots	18.90	156.60	8.29			
Agrostis stolonifera*	Shoots	1.98	6.66	3.36	Dune and dune slack sand		Jones, 1972 a
	Roots	144.00	432.00	3.00			
Carex flacca*	Shoots	1.62	2.70	1.67	"	"	"
	Roots	135.00	414.00	3.07			
Carex nigra*	Shoots	2.16	3.78	1.75	"	"	"
	Roots	99.00	108.00	1.09			
Erica cinerea (FI)*	Shoots	3.60	19.80	5.5	Mineral soil	12	Jones and Etherington, 1970
	Roots	10.80	97.20	9.0			
Erica cinerea (FI)*	Shoots	5.40	13.50	2.50	Ombrogenous peat	12.5	Jones, 1971 b
	Roots	19.80	21.60	1.09			
Erica tetralix*	Shoots	14.40	21.60	1.50	Mineral soil	12	Jones and Etherington, 1970
	Roots	21.60	39.60	1.83			
Eucalyptus ovalis (FI)	Leaves	3.78	10.80	2.86	Mineral soil	40	Ladiges and Kelso, 1977
Eucalyptus viminalis (FI)							
Population 1	Leaves	4.32	19.80	4.58	"	"	"
Population 2	Leaves	9.00	12.60	1.40	"	"	"
Festuca rubra (FI)*	Shoots	0.90	4.50	5.00	Dune and dune slack sand		Jones, 1972 a
	Roots	90.00	459.00	5.10			

continued

Holcus lanatus	Shoots	1.97	2.86	1.45	Mineral soil	13	Ernst, 1978
	Roots	18.44	27.57	1.50			
Juncus articulatus	Shoots	2.54	2.65	1.04	"	"	"
	Roots	9.67	17.01	1.76			
Juncus geradii	Shoots	5.04	7.56	1.50	Desalinated salt	6	Rozema and
	Roots	11.16	26.28	2.35	marsh sand		Blom, 1977
Nyssa sylvatica							
Upland population(FI)	Roots	25.65	613.44	23.92	Mineral soil	64	Keeley, 1979
Flood plain population(FI)	Roots	11.07	52.02	4.70	watered with nutrient soln.		
Swamp population	Roots	8.95	7.00	0.78			
Stellaria media (FI)	Shoots	1.46	1.43	0.98	Mineral soil	13	Ernst, 1978
	Roots	12.39	19.46	1.57			

* Iron concentration estimated from histograms in the original publications.

FI Flood-intolerant species, i.e. those showing growth inhibition under flooded conditions. All other species are flood-tolerant.

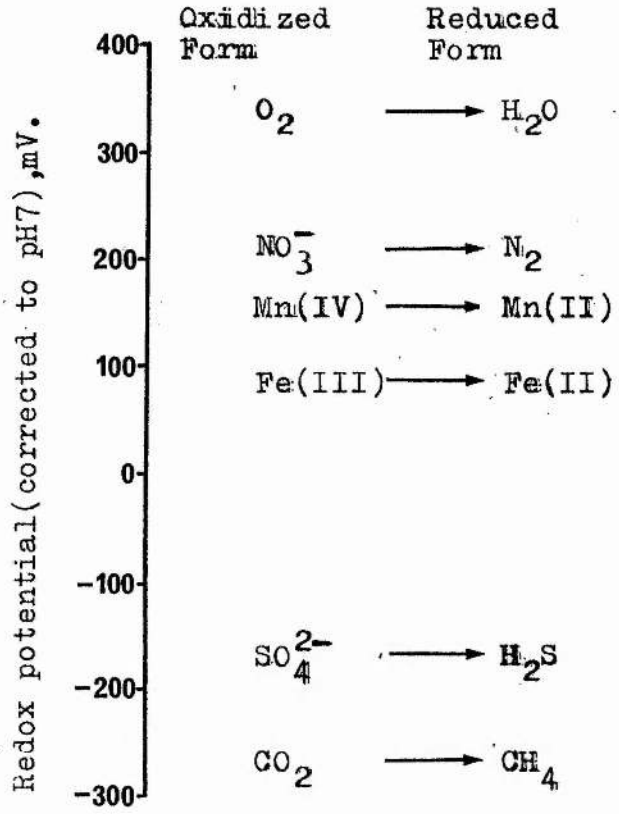


Fig.1.1. Approximate potentials at which the oxidised forms of some redox couples in soils become unstable.(Modified from Gambrell and Patrick, 1978.).

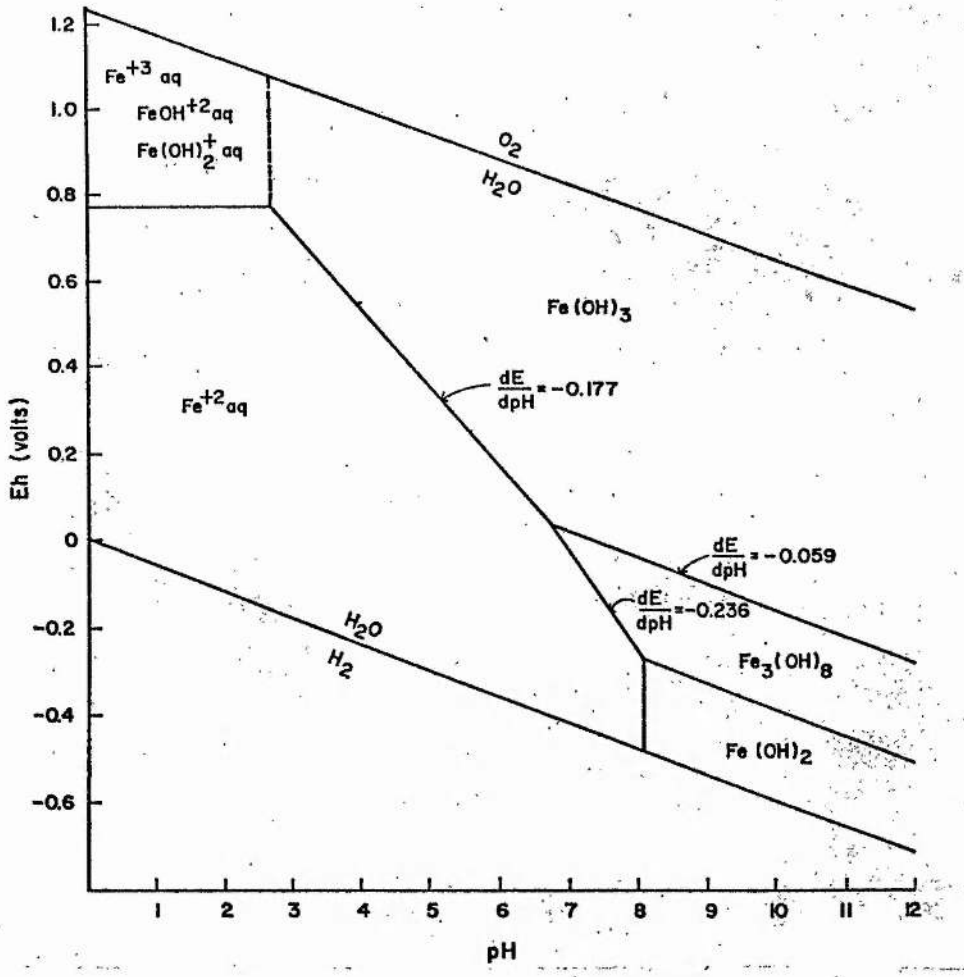


Fig.1.2. The stability areas of ferric, ferrosoferric and ferrous hydroxides relative to Eh (redox potential), pH, and an aqueous Fe(II) activity of 1mM at 25°C. From Ponnamperuma *et. al.*, 1967.

Part I

CHAPTER 2

Iron(II) Toxicity and Tolerance in Various Species

Introduction

The experiments described in this Chapter were designed to establish the relative tolerance of a range of species to iron(II), and to see if this tolerance has any relationship to the amount of air space tissue (aerenchyma) in their roots. "Tolerance" is used here as a general term and does not imply any particular mechanism of resistance to iron toxicity. The species chosen for study were from a range of different habitats. In addition, a cultivar of rice (*Oryza sativa* L.) was included.

Two hypotheses have been put forward to explain the mechanism of iron(II) tolerance. The first suggests that the possession of a large amount of air space tissue confers iron tolerance. This is thought to be because plants with well-developed air space allow oxygen to diffuse from their shoots to their roots. The oxygen can leak out of the roots and may oxidise, and thus immobilise iron in the rhizosphere or on the root surface. In this way, uptake into cells, and toxicity, may be prevented (Armstrong, 1975, 1979; Martin, 1968; Green and Etherington, 1977). The second hypothesis has been applied to only one pair of species: *Erica cinerea* and *E. tetralix*. The latter is more iron tolerant and is found to have a lower transpiration rate. It was suggested that the latter species was more tolerant because the lower transpiration rate decreased the amount of iron taken into the plant. In addition, spraying *E. cinerea* with silicone oil reduced the transpiration rate and also increased the time that this species could survive waterlogging (Jones, 1971a). Waterlogging damage was probably mainly caused by iron because *E. cinerea* could survive waterlogging in peat of very low iron content (Jones, 1971b). In peas iron translocation to the shoots is decreased if transpiration rate is lower (Branton and Jacobson, 1962a). There is no evidence that having a low

transpiration rate is likely to explain iron tolerance in most species. Many species from peat bogs have apparently xeromorphic leaf characteristics (Yapp, 1912; Caughey, 1945) and early investigators suggested that bogs were "physiologically dry" because low temperature, lack of oxygen, or the production of toxins inhibited water absorption (Walter, 1973). Small (1972a) has shown that a group of woody xeromorphic bog species did not differ in leaf resistance or water potential (indication of water stress) from mesophytes growing on a site nearby. So transpiration rate is just as high in these species and may have no significance in reducing iron uptake. Also, many marsh plants have no obvious xeromorphic features and they are more likely to be exposed to high iron concentrations in the mineral soil. It seems more likely that xeromorphy is an adaptation in plants from oligotrophic habitats (Beadle, 1966; Small, 1972b). The two hypotheses appear to be incompatible when it is considered that aerenchyma, which is produced in greater amounts in flood tolerant or flooded plants (Chapter 7) can increase transpiration rate by lowering root resistance to water flow at least in rice (Tomar and Ghildyal, 1975). Since there is little evidence to support the second hypothesis, no measurements of transpiration rate were made in the present series of experiments. The important parameter would be the rate of water uptake per unit area of root surface.

Materials and Methods

All the species used in these investigations were collected from their natural habitats, except Oryza sativa. The plants and their origins are shown in Table 2.1. Plants from the same populations were used throughout the experiments. Plants and germinated seeds were maintained in the glasshouse in sand culture and watered weekly with one fifth strength Hoagland's solution (Johnson et al, 1957). Supplemental lighting and heating gave a temperature of about 20 °C and a daylength of 18 hours.

Fe(II) Tolerance Experiment: Uniform individuals were selected from the sand culture material and transferred to a water culture with one fifth strength Hoagland's solution. Iron was supplied as 20 μmol l^{-1} Fe(III) sodium EDTA and the solution was changed once a week. The plants were kept in this solution for at least three weeks, during which time sufficiently large root systems were produced. After this time the plants were subjected to the Fe treatments. They were transferred to 250 ml Erlenmeyer flasks (2 plants per flask) where they were held in place by non-absorbent cotton wool. The flasks were filled with a deoxygenated solution of 4 mmol l^{-1} $Ca(NO_3)_2$ to which Fe(II) had been added and the pH adjusted to 5.5 with KOH or H_2SO_4. Fe(II) was added as $FeSO_4$ in concentrations from 0 - 6.0 mmol l^{-1}. The solutions were changed daily to prevent excessive Fe(II) depletion by uptake or oxidation. In flasks without plants at least 70% of the Fe remained as Fe(II) for one day. A full nutrient solution was not used because iron phosphates are not very soluble and the Fe would have been largely precipitated. $Ca(NO_3)_2$ was chosen because, apart from being used widely in studies of metal tolerance (Wainwright and Woolhouse, 1977; Coughtrey and Martin, 1978), Ca can protect roots against injury by H^+ ions (Marschner et al, 1966). This was important in these experiments because oxidation of Fe(II) results in increased acidity. Nitrate is known to alleviate any adverse effects of hypoxia in culture solutions (Gilbert and

Shive, 1942).

The plants were kept in these solutions for 4 days in a growth cabinet. The cabinet was kept at 20 °C with a light intensity of 14,000 lux during the 12 hour light period. At the end of this period the plants were examined for root growth and Fe(II) toxicity symptoms. In addition root tips were cut off and tested for their ability to reduce triphenyl tetrazolium chloride (T.T.C.).

Reduction of T.T.C. by Root Tips. The root tips, after excision from the plants, were incubated in a solution of T.T.C. The method was modified from Steponkus and Lamphear (1967). 1cm root tips were placed in 0.6% T.T.C. in 50 mmol l^{-1} potassium phosphate buffer at pH 7.4 containing 2% sucrose. They were then incubated in the dark at 30 °C for 2 hours. After this they were examined for the presence or absence of red staining. Reduction of T.T.C. forms an insoluble red formazan derivative (Pearse, 1972) which is deposited within the tissues.

Estimation of air space in the Root Systems. Plants were taken from the water cultures at the s ame time as others were taken for tolerance testing and used for air space estimation. The root systems were cut off and their air space (% v/v) estimated using 40 ml density bottles after the method of Jensen et al (1967). The procedure was as follows:

a) Roots placed in the bottle with water, the stopper put on, and then weighed.

b) Roots removed from bottle, blotted dry and fresh weight determined.

c) Roots ground with a pestle and mortar until all the structure destroyed and then carefully washed back into the bottle. The bottle was then refilled with water, the stopper put on, and weighed.

d) Bottle emptied and weighed filled with water only.

From these four weights the air space (%v/v) in the roots was

calculated from the formula:

$$\% \text{ air space (v/v)} = \frac{c - a}{d - a + b} \times 100\ \%$$

The Effect of Fe(II) on Root Growth of Nardus stricta. Tillers of N. stricta were established in unaerated one fifth strength Hoagland's solution for 1 week. After this period Fe(II) was added to the culture solution as $FeSO_4$. The pH was adjusted to 6.0 using KOH or H_2SO_4. The solution was changed every two days. The increase in length of the longest root on each plant was measured over 1 week.

Results

General. The experiments were carried out using culture solution containing 4mM $Ca(NO_3)_2$ which was deoxygenated before use. In this respect they differ from the previous experiments on iron(II) tolerance which were carried out in aerated, or at least not deoxygenated, full nutrient solution (Martin, 1968; Sanderson and Armstrong, 1980). The relative tolerance of the species to iron(II) was decided by using three criteria chosen from preliminary experiments. These were:

1. The occurence of blackened root tips.
2. A qualitative assessment of root growth inhibition.
3. Loss of the ability of excised root tips to reduce triphenyl tetrazolium chloride (T.T.C.).

These characteristics appeared to give a repeatable indication of damage by toxic iron concentrations. Evidence of toxicity rarely appeared in the shoots during the short period of the experiments. The primary effect of iron toxicity was on the root tips.

Fe(II) Toxicity Symptoms in Roots.

The most obvious visual effect of Fe(II) toxicity was blackening of the root tips, sometimes accompanied by flaccidity. Blackening occured in all the species studied except Zerna ramosa, Oryza sativa and Glyceria maxima (Table 2.2). In toxic concentrations of iron the blackening appeared after 24 hours, and the length of root tip affected increased with time and iron concentration. The length of blackened tip was from 2 to 10 mm.

The effect of various concentrations of Fe(II) on the root tips of Senecio aquaticus and Glyceria maxima are shown in Plates 2.1 and 2.2. In both species the control root tips grown in the absence of Fe(II) were white and apparently healthy. In S. aquaticus blackening at this stage was evident in the 1.0 and 6.0 mM Fe(II) treatments, however iron(II) concentrations of up to 6.0mM did not cause blackening in

G. maxima.

The roots of most species became progressively more coloured by orange-brown deposits as the Fe(II) concentration in the culture solution increased. These deposits stained blue with acidified potassium ferrocyanide suggesting that they contained Fe(III). Not all species stained with the same intensity and it was particularly noticeable that Eriophorum angustifolium and E. vaginatum were poorly stained.

Root growth was inhibited by high Fe(II) concentrations and was eventually stopped altogether. The concentration at which this occured corresponded with the blackening of the tips (Table 2.2). The effect of Fe(II) on root growth was quantified only for Nardus stricta (fig 2.1). High concentrations of iron inhibited root growth of N. stricta. It is also clear from the graph that there is an optimum iron concentration for root growth. The Fe(II) concentration causing almost complete inhibition of root growth did not correspond with that in the iron tolerance experiment (Table 2.2). This could be because the Nardus experiment differed in four ways: the culture solution was a complete nutrient medium; pH was adjusted to 6 instead of 5.5; it was not deoxygenated and the solution was changed every two days instead of daily. All these factors would tend to reduce Fe(II) availability by oxidation and precipitation.

It was normally apparent when root growth had been almost completely inhibited in the iron tolerance experiment. When the toxic concentration was reached production of adventitious roots from the stem bases stopped. Also, as root growth became more severly inhibited, the Fe(III) deposits on the roots extended nearer to the root tips. It seemed that growing roots were able to keep their tips free from the deposits. In roots that were not grwoing the deposit often covered the whole tip.

In some of the species different optimum Fe(II) concentrations could be observed for root growth. For example, Glyceria maxima produced the most prolific new root growth in between 60 and 80μM

whereas the optimum for Schoenus nigricans was 10 μM with complete inhibition at 60 μM.

The Assessment of Root Tip viability using T.T.C.

The control root tips of all the species in the iron tolerance experiment, except Senecio jacobaea and Myosotis scorpioides, were able to reduce T.T.C. to its red formazan derivative. They lost this ability near the Fe(II) concentrations which caused root tip blackening and inhibition of new root growth, suggesting that viability as determined by T.T.C. was a good indication of iron toxicity (Table 2.1). However, the method was not used on its own because in some species staining was erratic and not all root tips stained, even from Fe(II) treatments that were clearly not toxic.

Fe(II) Tolerance in Roots.

The three criteria previously discussed have been taken together to give a mean toxic iron(II) concentration for each species (Table 2.2). The species have been arranged in order of increasing tolerance as judged by the mean toxic concentration. A very wide range of tolerance has been found between species, ranging from Chamaenerion angustifolium, at a toxic concentration of 20 μM, to Glyceria maxima which was unaffected up to 6000 μM. This was the highest concentration used in the experiment.

Fe(II) Toxicity Symptoms in Shoots.

Symptoms in the shoots were rarely seen during these experiments, and, if they did occur, they were at very much higher concentrations than those which affected the roots. The occurence of symptoms is summarised in Table 2.3. It is possible that some of the less tolerant species would have shown symptoms if the experiment had lasted longer or if higher concentrations had been used.

In another experiment plants were kept in nutrient agar with added

Fe(II) sulphate at much higher concentrations.

In this case symptoms were seen in the shoots. Treatments with iron up to 20mM were used. The symptoms took the form of wilting and desiccation of the leaves. The species, and concentration at which wilting occured, were Senecio jacobaea (4.0 mM); Lychnis flos-cuculi (4.2 mM); Nardus stricta (8.0 mM) and Ranunculus flammula (16 mM).

In the iron tolerance experiment (Table 2.3) wilting and some desiccation occured in the 6.0 mM treatments in Schoenus nigricans, Eriophorum vaginatum and Oryza sativa. In E. angustifolium the leaves in the 6.0 mM treatment had brown patches and spots, particularly on the abaxial surfaces. The deaths of isolated plants of Senecio aquaticus and Ranunculus flammula may not have been connected with iron toxicity since the other replicate remained healthy-looking and, in the case of S. aquaticus, plants in the higher concentrations were unaffected.

The Effect of Fe(III) on Cut Leaves and Shoots.

A brief experiment was carried out in Orkney (June 1980) on the effect of Fe(III) citrate on various species. Leaves and shoots were collected and placed in Fe(III) citrate solutions in an unheated greenhouse. The solutions contained 0.5, 5 and 50 mM Fe(III). Controls were also set up in water and 50 mM potassium citrate. The shoots were left for up to 64 hours with occasional examination for symptoms.

The species are listed in Table 2.4. Over this period plants in the potassium citrate had no symptoms. In all the species except Eriophorum angustifolium bronzing of the leaves occured. The bronzed leaves had red-brown spots and blotches on them. (Bronzing is a term used for similar symptoms in rice, see Discussion.) In addition to the bronzing the leaves wilted and sometimes became desiccated round the margins. The symptoms took different times to appear in the various species. Eriophorum angustifolium was unaffected under these conditions, whereas Anthriscus sylvaticus was very sensitive.

Air Space in the Roots of Various Species and its Relationship with Fe(II) Tolerance.

The amount of air space in the root systems of various species is shown in Table 2.5. The results represent an average value for the whole root system because air space is not evenly distributed along the length of the roots (Armstrong, 1971), and primary roots may have more than laterals (see Chapter 7). Values are given for all the species in the iron tolerance experiment except Nardus stricta and Glyceria maxima. Insufficient root was produced in the culture solution by these species for air space measurements.

The species in this experiment had a wide range of air space in their roots when grown in unaerated Hoagland's solution. The values for air space obtained for some species differed when they were grown in either water or sand culture (see Chapter 7). For example, Deschampsia caespitosa (Chapter 7) and Oryza sativa (A. Barclay, personal communicatin) had less air space in unaerated solution culture than in sand culture. Because of the unexpectedly low amount of air space in these two species, further plants were grown under the same conditions. They produced the same amount of air space.

The relationship between air space in the roots and tolerance (as defined by the mean toxic iron concentration) was examined using several transformations of the data. The results are shown in Table 2.4 and Fig. 2.2. Tolerance was transformed logarithmically and an arcsin transformation was used for air space. In no case was the correlation coefficient significant at the 5% level of probability (Table 2.6). The best fit was found between the arcsin transformation of air space and the natural logarithm of tolerance, however, the relationship between untransformed air space and $\log_{10}$ tolerance is shown in fig 2.2. (For statistical purposes the arcsin transformation of data expressed as percentages should normally be used (Bishop, 1971).) The statistical analysis does not support the hypothesis that there is a correlation between the amount of air space in the roots of a species and its

tolerance to Fe(II).

Although there is no significant relationship, some points can be made from fig 2.2. (a) Species with a low amount of air space can have an extremely wide range of iron tolerance (Chamaenerion angustifolium, Senecio jacobaea, Senecio aquaticus and Deschampsia caespitosa). (b) Species with relatively high air space always had a relatively high tolerance (easily seen if a line is drawn between Chamaenerion angustifolium and Eriophorum angustifolium on fig 2.2). However, this relationship could have broken down if more species had been examined.

Because the data for mean toxic concentration and air space may not be normally distributed, Spearman's rank correlation coefficient was also calculated. The rank correlation coefficient between air space and mean toxic iron (II) concentration for the various species was 0.243 and is not significant at the 5% probability level.

Table 2.1 - Species used in the iron (II) tolerance experiment

Species*	Habitat	Location	Form Collected
Chamaenerion angustifolium	Sand dunes	Tentsmuir, Fife NO500242	Seeds
Deschampsia caespitosa	Lagg vegetation by raised bog	Peat Inn, Fife NO448103	Seeds
Eriophorum angustifolium	Raised bog	Peat Inn, Fife NO448103	Rhizomes
Eriophorum vaginatum	Raised bog	Peat Inn, Fife NO448103	Plants
Glyceria maxima	Reedswamp	Lindores Loch, Fife NO268165	Rhizomes
Lychnis flos-cuculi	Marsh	Ceres, Fife NO400085	Seeds
Myosotis scorpioides	Reedswamp	Lindores Loch, NO268165	Plants
Nardus stricta	Acidic grassland	Drumcarrow Craig, NO460133	Tillers
Oryza sativa cv. Oeiras	Estacao Agronomica Nacional, Portugal		Seeds
Ranunculus flammula	Marsh	Drumcarrow Craig, Fife NO460133	Seeds
Schoenus nigricans	Mesotrophic mire	Orkney mainland HY252242	Plants
Senecio aquaticus	Marsh	Tweeddale, NT122320	Seeds
Senecio jacobaea	Sand dunes	Tentsmuir, Fife NO500242	Seeds
Zerna ramosa	Woodland	Dura Den, Fife NO417140	Seeds

* Nomenclature follows Clapham, Tutin and Warburg (1962) throughout the thesis - the only exception being the spelling of Deschampsia caespitosa.

Table 2.2 - Iron (II) concentration (uM) causing toxicity symptoms in roots. The toxic concentration was assessed using three criteria: blackening of root tips, loss of ability to reduce triphenyl tetrazolium chloride (TTC) and lack of new root growth.

	Iron (II) Concentration, uM			
	Root Tip Blackening	TTC Test	Root Growth Inhibition	Mean Toxic Concentration*
Chamaenerion angustifolium	10	40	-	20
Senecio jacobaea	40	-	80	60
Schoenus nigricans	60	60	60	60
Myosotis scorpioides	-	-	80	80
Zerna ramosa	n/a	100	100	100
Senecio aquaticus	80	200	-	140
Ranunculus flammula	200	600	-	400
Eriophorum angustifolium	800	800	-	800
Eriophorum vaginatum	800	1000	800	800
Nardus stricta	-	800	600	800
Oryza sativa cv. Oeiras	n/a	600	1400	1000
Deschampsia caespitosa	1200	2000	1200	1400
Glyceria maxima	n/a	n/a	n/a	n/a

* Mean value from the criteria used to assess the toxic concentration rounded to the nearest test solution concentration.

\- Not assessed

n/a Not affected

Table 2.3 - Iron (II) toxicity symptoms in shoots

	Range of iron (II) Concentration tested (mM)	Symptoms
Chamaenerion angustifolium	0-2.0	-
Senecio jacobaea	0-2.0	-
Schoenus nigricans	0-6.0	Leaves brown at 6.0 mM
Myosotis scorpioides	0-4.0	-
Zerna ramosa	0-2.0	-
Senecio aquaticus	0-6.0	1 plant dead at 4.0 mM
Ranunculus flammula	0-2.0	1 plant dead at 2.0 mM
Eriophorum angustifolium	0-6.0	Leaves with blackish patches and spots, particularly on abaxial surfaces at 5.0 and 6.0 mM
Eriophorum vaginatum	0-6.0	Leaves dessicated at 6.0 mM
Nardus stricta	0-6.0	-
Oryza sativa cv. Oeiras	0-6.0	Slight wilting at 6.0 mM
Deschampsia caespitosa	0-6.0	-
Glyceria maxima	0-6.0	-

Table 2.4 - Time (hours) taken for bronzing symptoms to appear in cut shoots exposed to 5 mM iron (II) citrate.

	Hours
Eriophorum angustifolium	No symptoms after 64 hours
Mentha aquatica	39
Caltha palustris	39
Ranunculus repens	36
Potentilla anserina	36
Filipendula ulmaria	18
Angelica sylvestris	18
Myosotis caespitosa	16
Anthriscus sylvestris	10*

* In 0.5 mM iron (II) citrate

Table 2.5 - Air space (% v/v) in the roots of plants grown for at least three weeks in unaerated one-fifth strength Hoagland's solution.

	Air space in roots, % v/v (n = 3) with range
Senecio aquaticus	6.9 (5.9-7.9)
Senecio jacobaea	7.8 (6.5-8.9)
Deschampsia caespitosa	8.3 (7.6-9.4)
Chamaenerion angustifolium	9.7 (8.2-11.1)
Oryza sativa cv. Oeiras	14.1 (12.7-15.9)
Schoenus nigricans	14.6 (12.6-16.7)
Myosotis scorpioides	17.6 (16.9-19.6)
Ranunculus flammula	18.3 (16.4-21.3)
Zerna ramosa	21.6**
Eriophorum vaginatum	31.2**
Eriophorum angustifolium	44.4 (41.6-48.5)

* n = 2

** n = 1

Table 2.6 - Correlation coefficients (r) between air space in roots and iron (II) tolerance (mean toxic iron (II) concentration) for various species.

Comparison	r
Air space (%) v toxic concentration	0.250
Air space (%) v $\log_{10}$ concentration	0.441
Air space (%) v In. toxic concentation	0.414
Air space (arcsin transformation) v toxic concentration	0.279
Air space (arcsin transformation) v $\log_{10}$ concentration	0.443
Air space (arcsin transformation) v In. toxic concentration	0.447

Table 2.7 - Tolerance of some woodland species to iron (II) in water culture. From Martin, 1968.

	Concentration causing root death, uM
Mercurialis perennis	72
Endymion non-scriptus	179
Brachypodium sylvaticum	269*
Geum urbanum	179-358
Circaea lutetiana	269
Primula vulgaris	358
Primula elatior	538
Carex sylvatica	538-717
Deschampsia caespitosa	1434-1792

* Not tested below 269 uM

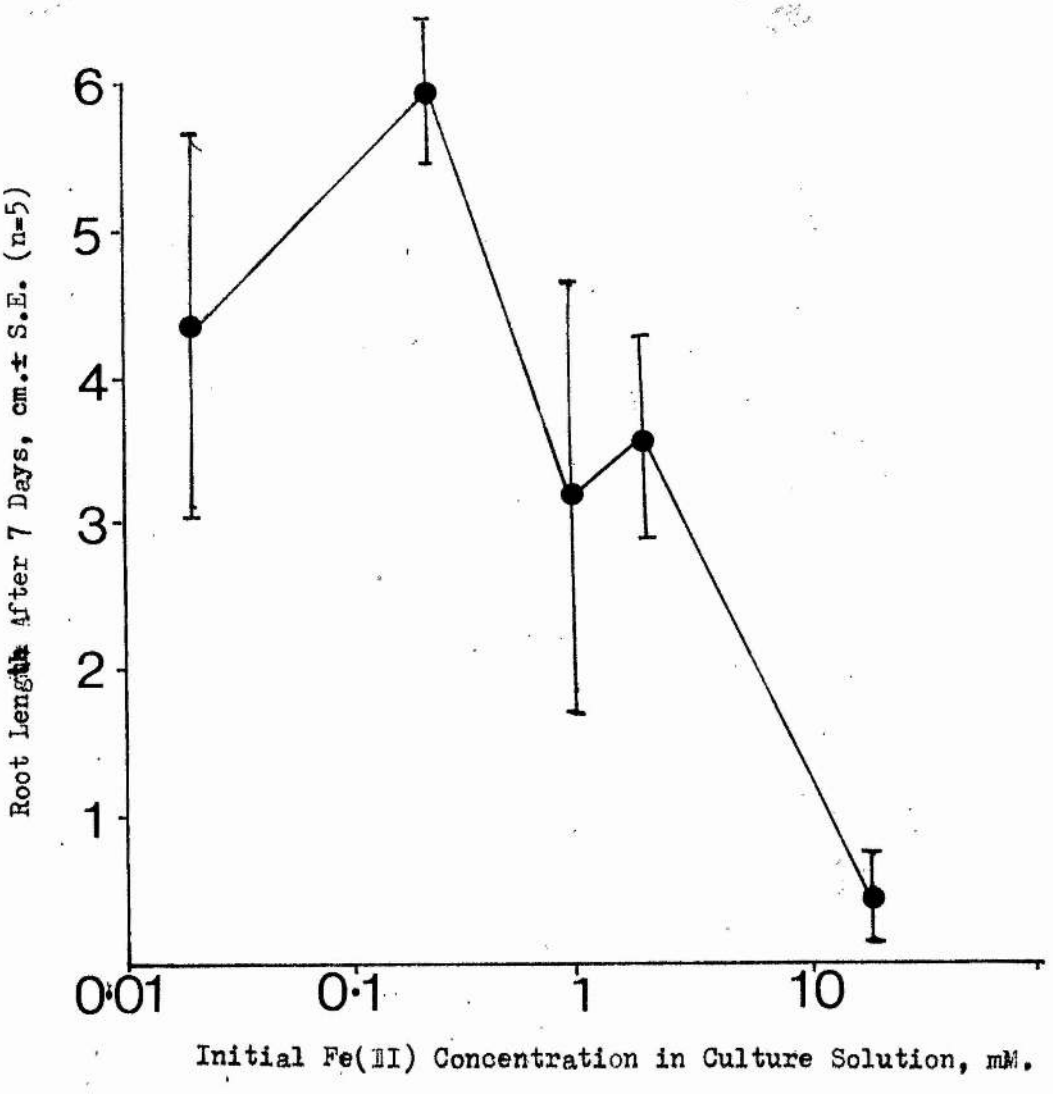


Fig.2.1. The Effect of Fe(II) sulphate on root Extension in Nardus stricta

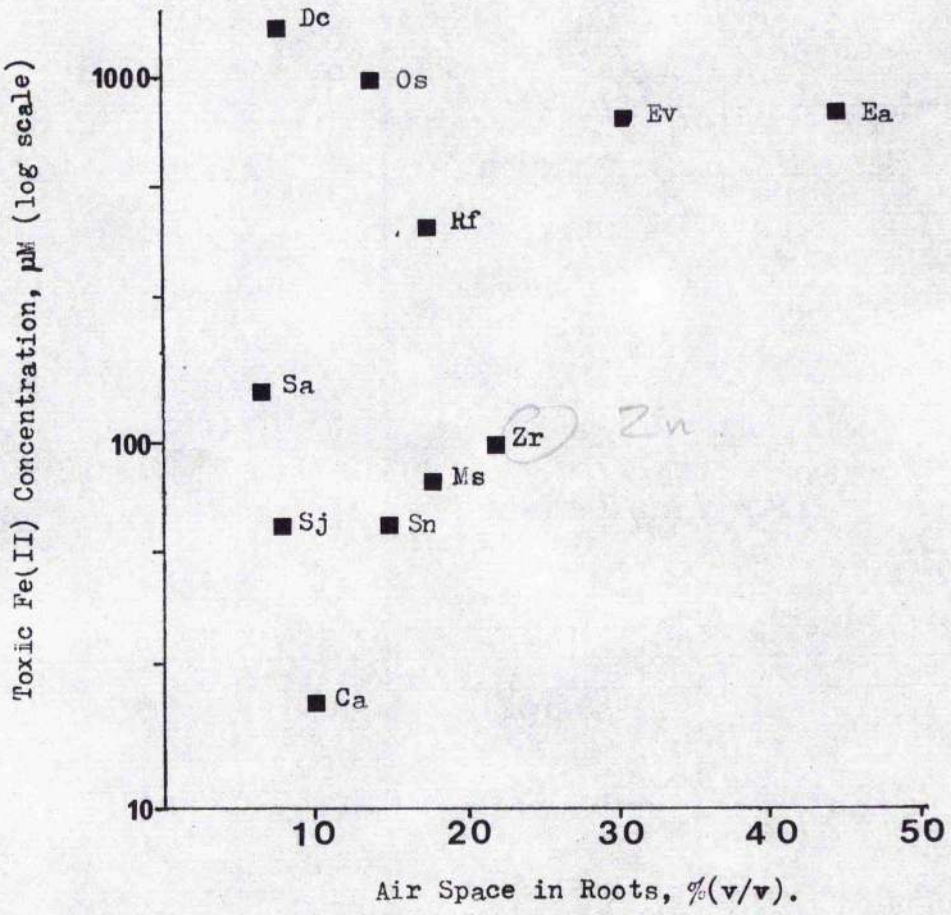
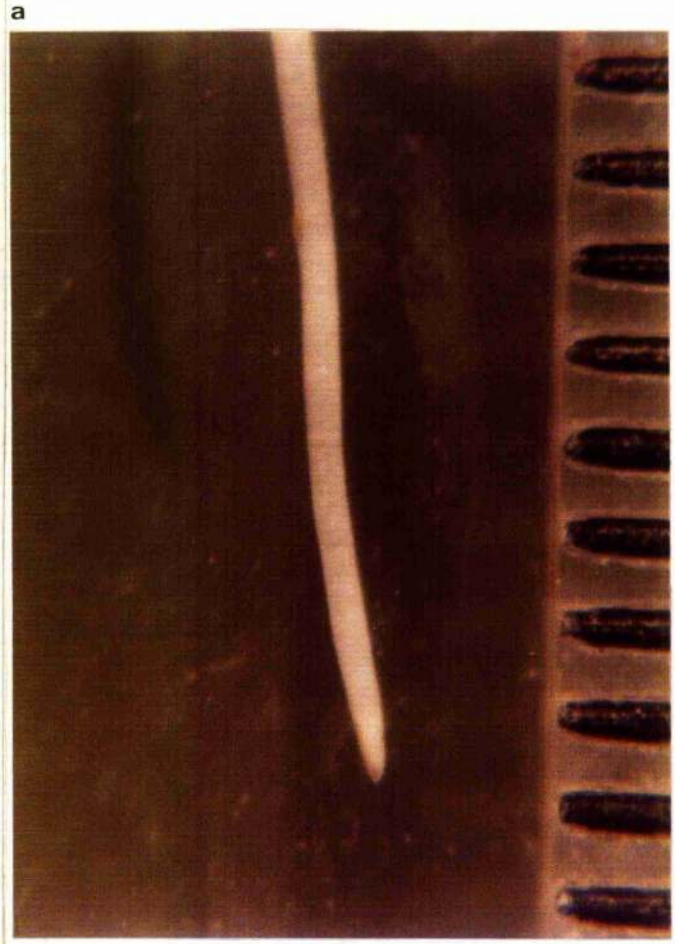


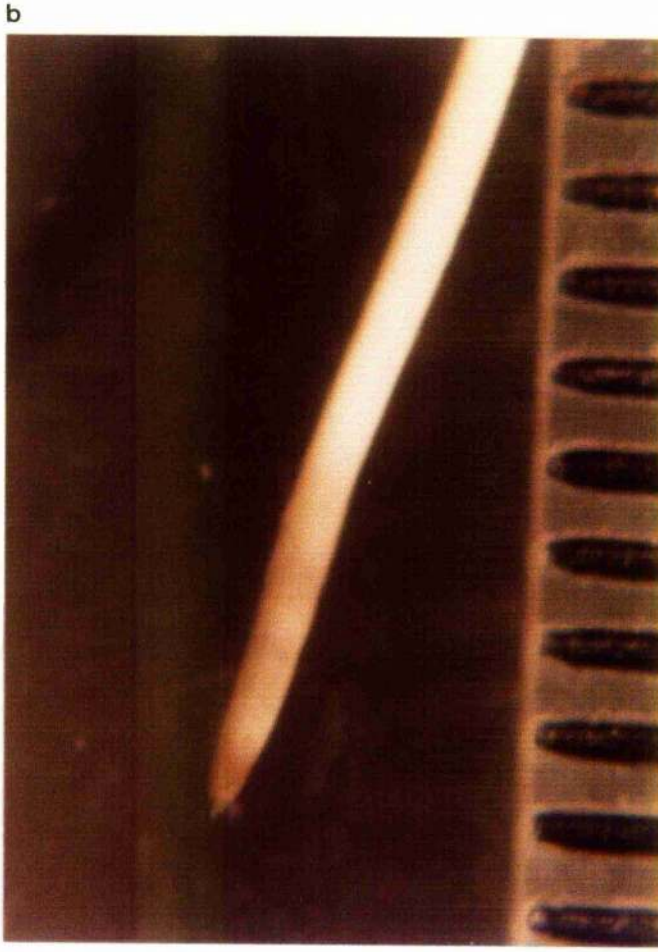
Fig. 2.2. The Relationship Between Air Space in the Roots and Fe(II) Tolerance of Various Species.
r=0.411 Not Significant (p=0.05)

Key Ca. Chamaenerion angustifolium
Dc. Deschampsia caespitosa
Ea. Eriophorum angustifolium
Ev. Eriophorum vaginatum
Ms. Myosotis scorpioides
Os. Oryza sativa
Rf. Ranunculus flammula
Sa. Senecio aquaticus
Sj. Senecio jacobaea
Sn. Schoenus nigricans
Zn. Zerna ramosa

Plate 2.1 Iron toxicity symptoms in roots of *Senecio aquaticus*. Scale divisions = 1mm. Plants were exposed to various iron (II) sulphate concentrations in deoxygenated 4mM calcium nitrate for 3 days.

a) 0mM Root tip white and healthy
b) 0.01mM Root tip healthy. Some orange-brown iron (III) deposits evident.
c) 0.1mM Root darkened, but tip apparently healthy.
d) 1.0mM Root darkened. Root tip blackened.


a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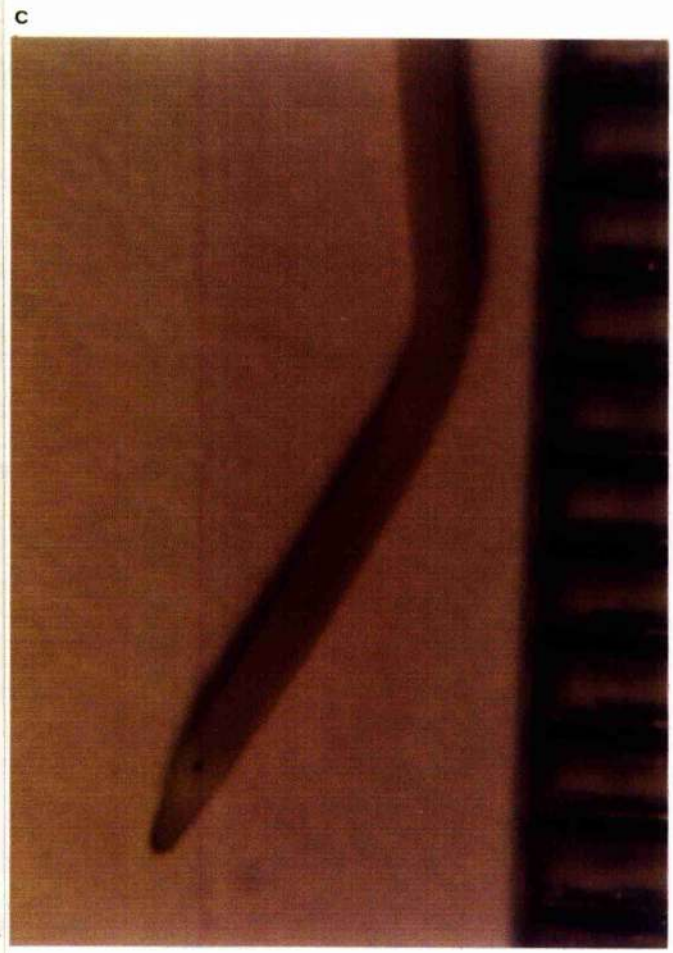
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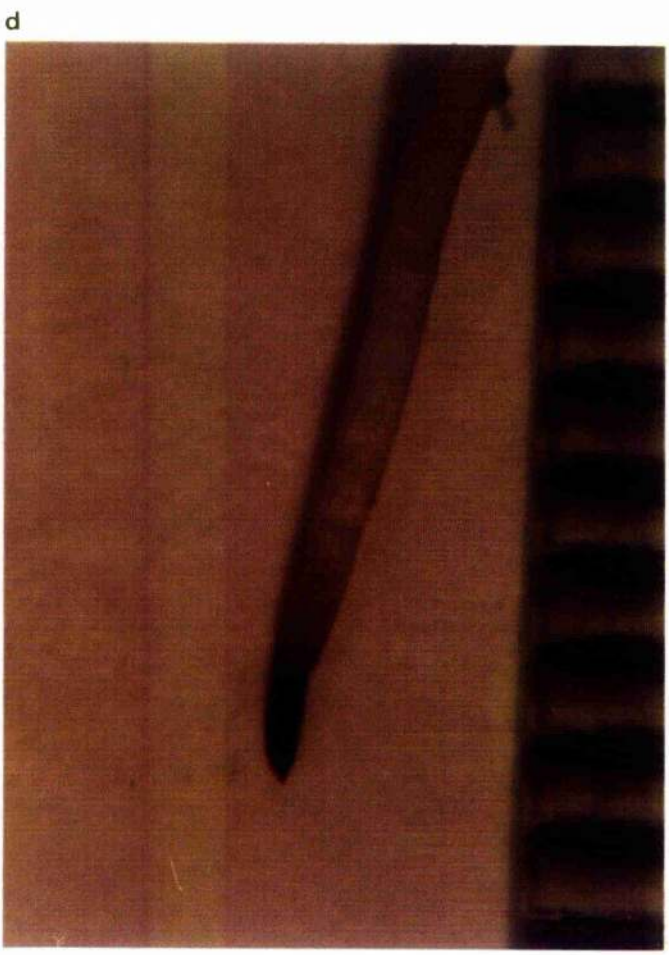

d

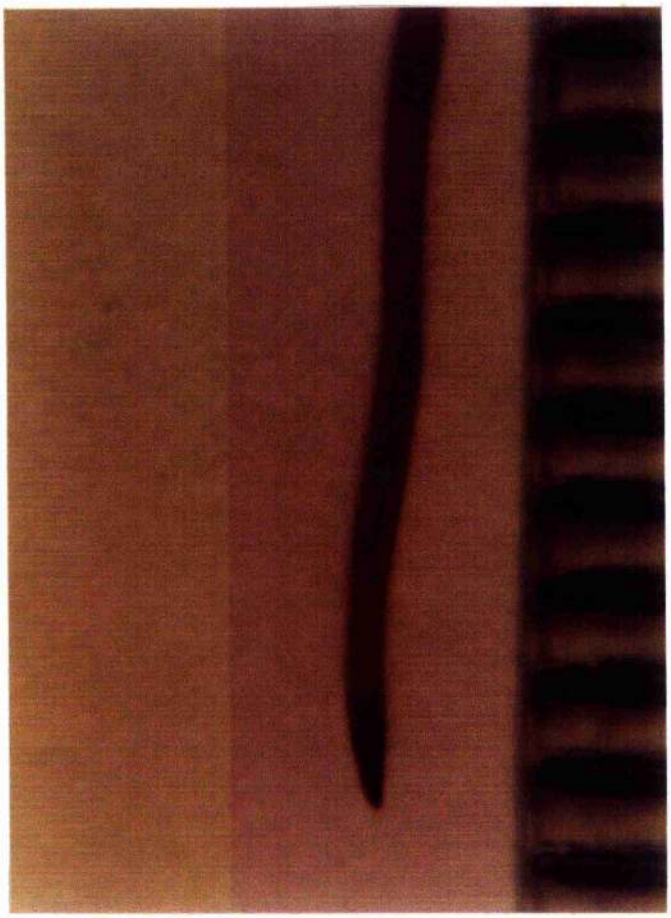
Plate 2.2 Iron (II) toxicity symptoms in roots of *Senecio aquaticus* and *Glyceria maxima*. Scale divisions = 1mm. Plants were exposed to various iron (II) sulphate concentrations in deoxygenated 4mM calcium nitrate for 3 days.

a) *Senecio aquaticus*, 6.0mM. Root darkened and root tip blackened.

b) *Glyceria maxima*, 1.0mM. Root tip white and healthy (c.f. *S. aquaticus* in 1.0mM, Plate 2.1d). Orange-brown iron (III) deposits are evident behind the root tip.

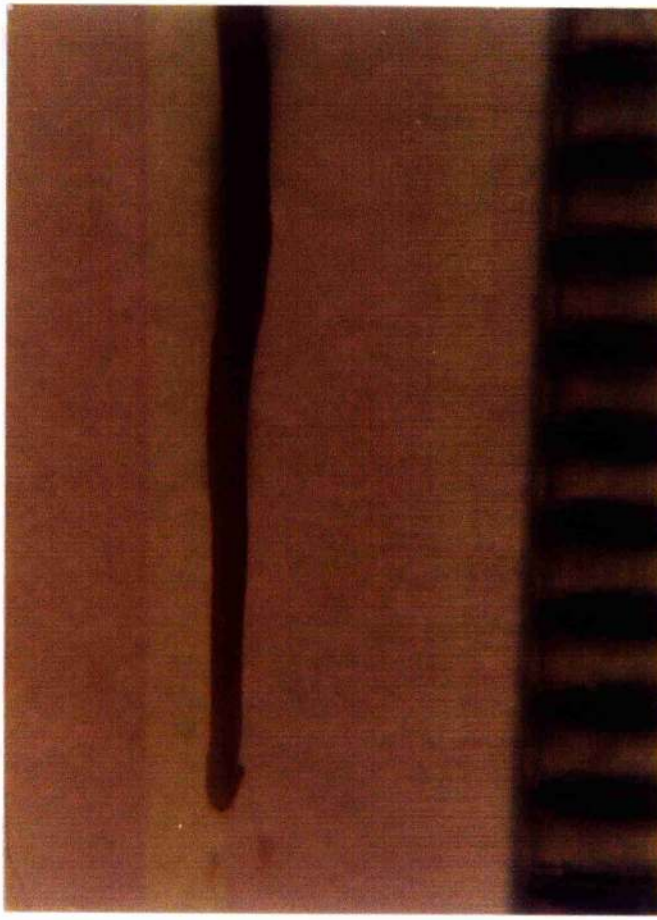
c) *G. maxima*, 6.0mM. Root tip healthy. Root behind tip darkened with iron(III) deposits.

a



b

c



Discussion

Fe(II) Toxicity Symptoms in Roots

In the short duration of these experiments symptoms were mainly confined to the root tips. Above the optimum concentration for root growth, which was observed for some species, there was a progressive reduction in root growth, until at the toxic concentration root growth stopped and the tips became blackened. The tip is the most physiologically active part of the root so it is not surprising that this is where symptoms are first seen. Copper and zinc toxicity also cause root tip blackening (Bennett, 1974). Inhibition of root growth is a general effect of phytotoxic metals (Wainwright and Woolhouse, 1977) and Fe(II) has the same effect.

The primary cause of metal toxicity is not known for certain, but as discussed in Chapter 1, the mechanism may involve binding to ligands on enzymes and proteins resulting in inactivation. Skeen (1929) found that root tips of *Phaseolus vulgaris* and *Lupinus albus* seedlings became flaccid under conditions of Fe(II) toxicity. Fe(III) toxicity in *Sinapsis alba* and *Cannabis sativa* stopped root growth and root hair development (Olsen, 1958). Blackening of root tips was not mentioned by Skeen or Olsen for Fe(III) toxicity. It has been suggested that Fe(III) must be reduced at the root surface before it can be absorbed (Brown, 1978), so it is possible that toxicity resulting from both oxidation states is the same. But the evidence is not conclusive because it is not known if Fe(III) can be directly absorbed without reduction from high external concentrations. There is evidence that Fe(II) and Fe(III) fed to cut shoots result in similar toxicity symptoms (see later).

The use of T.T.C. to determine root tip viability after exposure to phytotoxic metals has not been reported before. The results of these experiments suggest that the loss of T.T.C. reducing activity correlated well with the appearance of other symptoms. The reduction of T.T.C.

to the red formazan derivative is probably by the activity of dehydrogenase enzymes (Roberts, 1951; Pearse, 1972). Lack of T.T.C. reduction by root tips suggests that they have lost their dehydrogenase activity and are probably dead. T.T.C. reduction has previously been used for determining cold injury in leaves and stems (Steponkus and Lamphear, 1967) and as a viability test for seeds (Porter et al, 1947).

There is evidence, discussed below, that iron toxicity in the field may be indirect through the action of low concentrations of iron for a longer time resulting in inhibition of nutrient uptake. However, because of the rapid development of symptoms in this experiment, toxicity was likely to be a direct effect of Fe. The evidence for indirect effects is from the following studies. Howeler (1973) found that Oranging disease of rice in Columbia was a result of P, K, Ca and Mg deficiency. This came about because, although Fe concentrations in the soil were not high enough to cause direct toxicity, root growth was stunted and they became coated with thick Fe(III) deposits. This resulted in decreased nutrient uptake. Similarly, Jones (1975) suggested that growth reduction in flooded Festuca rubra could have been caused by inhibition of phosphate uptake at high Fe levels, perhaps by precipitation of Fe(III) phosphate. He found a negative correlation between Fe concentration in the roots and P concentration in the shoots. Finally, Tanaka et al (1966) found that high Fe(II) concentrations in solution culture inhibited P, K and Mg uptake by rice.

In contrast to other experiments on Fe tolerance (Martin, 1968; Sanderson and Armstrong, 1980) these experiments were done using deoxygenated $Ca(NO_3)_2$ solution. The other authors used full nutrient solutions that were not deoxygenated. The solutions used here would not have been fully anoxic because of oxygen diffusion from the atmosphere or roots. The control plants of all the species could survive this hypoxic treatment and produce new root growth. It seems that many species can survive hypoxia in water culture for at least a short period.

If a further stress is added then toxicity symptoms may develop. For example peas could survive in anaerobic water culture, but quickly developed symptoms of waterlogging damage when flooded in soil. Anaerobically produced toxins from the soil seem to produce waterlogging symptoms (Drew, 1979). There are two examples of this where Fe(II) acts as the additional stress to hypoxia. Mercurialis perennis survived flooding with distilled water in sand culture, but died within 3 weeks when flooded with 90 μM $FeSO_4$ (Martin, 1968). Erica cinerea is normally flood-intolerant but can survive flooding in peats of very low Fe content. Flooding in peat with higher iron content results in death (Jones, 1971b).

Fe(II) Toxicity Symptoms in Shoots.

The occurence of symptoms was rare, but they were usually wilting and desiccation of leaves. Olsen (1958) found Fe(III) toxicity caused wilting in Sinapsis alba and Cannabis sativa and suggested that water uptake by roots was impaired. However, the experiment using cut shoots showed that wilting and desiccation occured without roots. Fe toxicity still upset the water balance of the leaves. Jones and Etherington (1970) also found that Fe(II) or Fe(III) solutions upset the water balance of cut shoots of Erica cinerea and Erica tetralix.

The production of spots on the leaves of intact Eriophorum angustifolium plants resembled the symptoms of bronzing disease of rice caused by Fe(II) (Ponnamperuma et al, 1955; Tanaka et al, 1966). Bronzing also occured on most of the species whose cut shoots were treated with Fe(III) citrate. Tanaka et al (1966) could also induce bronzing on excised rice leaves fed with Fe(II) sulphate. Using ^{59}Fe they showed that Fe was localised in the brown spots.

The different times for various species in the cut shoot experiment to show bronzing could be explained in two ways. Either the shoots are differentially tolerant to Fe or different transpiration rates could affect the amount of Fe absorbed. Further experiments are needed

before any conclusions can be drawn. Comparison of these results with cut shoots with those of Jones and Etherington (1970) and Tanaka et al (1966) suggests that Fe(II) and Fe(III) produce similar toxicity symptoms in leaves. In the present experiments Fe(III) citrate was used because this is the form of Fe translocated in the xylem (Clark et al, 1973).

Fe(II) Tolerance.

There was a wide range of tolerance among the species. The only other comparable study of Fe tolerance is that of Martin (1968). For comparison his results are shown in Table 2.7. None of the species he studied, except Deschampsia caespitosa are typical wetland plants. D. caespitosa is the only species common to both investigations. Its tolerance in this investigation was similar to that found by Martin. Some of the other species studied by Martin are not commonly considered as flood tolerant but they have relatively high tolerance when compared with more typically wetland (and flood tolerant) species in the present experiments. This could be because Martin's experiments were not carried out in deoxygenated medium, so his plants were only subjected to high Fe stress, and not to the extra stress of hypoxia. The plants could have been more sensitive to Fe(II) under hypoxic conditions.

Fe(II) Tolerance and Air Space in Roots.

There was no correlation between air space and tolerance, but none of the species with high air space had low tolerance. Senecio jacobaea and S. aquaticus make an interesting comparison. They are morphologically very similar and under these conditions they have the same amount of air space in their roots, but S. aquaticus can withstand twice the Fe concentration. The results do not support the hypothesis that tolerance is correlated with air space in the roots. However, these results do not disprove that radial oxygen loss from the roots (ROL = diffusion of oxygen out of the roots) is a factor in Fe(II)

tolerance. No measurements were made of ROL or of the relationship between ROL and air space in the roots. There is much evidence that they are correlated, but further discussion of this point will be left until Chapter 3. To test the relationship between tolerance and air space further, tolerance of plants whose air space has been experimentally altered by changing oxygen or nutrient levels (Chapter 7) could be tested.

CHAPTER 3

Iron Uptake by Various Species and its Relationship to Fe(II) Tolerance and Root Aerenchyma

Introduction

In the previous chapter it was shown that Fe(II) tolerance did not have a significant correlation with the amount of air space in roots of various species. This does not support the hypothesis that Fe(II) tolerance is the result of oxygen diffusion through the root aerenchyma from the shoot (Martin, 1968; Armstrong, 1979; Green and Etherington, 1977). These authors have suggested that aerenchyma allows oxygen diffusion through roots and thus radial oxygen loss (ROL) from the roots. This causes oxidation and precipitation of Fe(III) in the rhizosphere, on the root surface or in cell walls adjacent to the lacunae in the aerenchymatous cortex. The precipitated Fe is thought to be harmless. These conclusions have been based only on the comparison of very few species by the above authors. For example, Martin (1968) found that Deschampsia caespitosa was more Fe(II) tolerant than Mercurialis perennis. When he compared the rate of oxygen diffusion from the root tips of these two species, he found that it was greater in D. caespitosa. Sanderson and Armstrong (1980) found that Pinus contorta was slightly more tolerant to Fe(II) than Picea sitchensis and it is known that the oxidising activity of the primary roots of P. contorta is greater than P. sitchensis (Philipson and Coutts, 1978); the former species can produce continuous air spaces in its pericycle (Coutts and Philipson, 1978). Fe(II) oxidation can occur in the root or the rhizosphere. Fe precipitation can easily be seen on the roots of many species growing in waterlogged soils, giving them an orange-brown colour. Occasionally these deposits build up to such an extent that concretions up to 5mm in diameter, rich in Fe(III) compounds (probably hydrated oxides), encase the roots. These have been seen on roots of

Molinia caerulea (Armstrong and Boatman, 1967) and Ranunculus flammula (personal observation). The deposits can extend into the root: Armstrong and Boatman (1967) found Fe deposits in the intercellular spaces of the outer cortex and in the cell walls of the epidermis in Menyanthes trifoliata. Fe deposits have been detected using electron probe analysis in the cell walls and middle lamellae of the diaphragms of tissue traversing the aerenchymatous cortex of rice roots. The deposits did not extend as far as the endodermis (Green and Etherington, 1977). These authors concluded that Fe(II), drawn in passively with water and travelling in the apoplast, was oxidised and precipitated in the cell walls adjacent to the air spaces, preventing uptake into the stele.

Precipitation of Fe(III) in the rhizosphere can be easily seen in any Fe-rich waterlogged soil. Orange-brown stains of Fe(III) can be seen surrounding the root channels. Eriophorum angustifolium roots produce a zone of Fe(III) deposits at some distance from their tips (Armstrong, 1967a). Bartlett (1961) found that plants growing in Fe(II) solutions or waterlogged soils varied in their ability to oxidise Fe(II). Rice and reed canary grass (Phalaris?), species tolerant of waterlogging, were more effective at this than the flood-intolerant species alfalfa. However, he did not compare this ability to oxidise Fe(II) with air space or oxygen diffusion from the roots.

The diffusion of oxygen from roots and their resulting oxidising activity has been known for many years and has been thoroughly reviewed by Armstrong (1979). Oxygen diffusion from roots has been measured by several techniques: the oxidation of redox dyes (Goto and Tai, 1957; Armstrong, 1967; Philipson and Coutts, 1978); measurement of $^{15}O_2$ transport from shoots (Evans and Ebert, 1960; Barber et al, 1962) and the detection of O_2 diffusion from root tips submerged in anaerobic media using a platinum electrode (Armstrong, 1979). Oxygen diffusion from roots is not limited to wetland species, for example oxygen diffusion can be detected from pea roots (Healy and Armstrong, 1972). The rate of oxygen diffusion is far greater from wetland plants and this corresponds

to the greater development of aerenchyma (Armstrong, 1970, 1971, 1972).

The relationship between air space in roots and Fe(II) uptake needs further investigation in a wide range of species. In this chapter experiments showing the pattern of Fe uptake by various species are described and this pattern is compared with air space in the roots and the Fe(II) tolerance of the plants.

Materials and Methods

The Effect of Flooding in Sand Culture on Fe Uptake by *Deschampsia caespitosa* and *Glyceria maxima*.

Small plants of *D. caespitosa* and *G. maxima* were established in sand culture in 15 litre rubber buckets in the glasshouse. After 6 weeks they were subjected to 4 treatments:

1. Flooded to surface of sand with full-strength Hoagland's solution
2. Watered, but kept freely-drained, with full-strength Hoagland's solution.

3 and 4. As above, but with 1/50th strength Hoagland's solution.

Fe was added as $FeSO_4$ giving final concentrations of 4.46 and 0.09mM in the high (full-strength) and low (1/50th strength) nutrient solutions respectively. Nutrient solution was given once a week. The plants were harvested after eleven weeks and the roots carefully washed free of sand.

Roots and shoots were separated and washed for 20s in 0.3% 'Teepol' followed by distilled water to remove surface contamination (Nicholas *et al*, 1956). They were dried at 100 °C for 24 h and then digested in concentrated nitric acid on a hot-plate in 50ml flasks. The extract was made up to 50ml with distilled water and 10ml aliquots were taken for Fe determination. Fe was measured using the red complex formed between Fe(II) and 2,2'-bipyridyl between pH 4.0 and 7.0. The method was modified from Mason (1950). The 10ml aliquot was placed in a 50ml volumetric flask with 5ml 4M sodium acetate-acetic acid buffer, pH 4.8; 2ml 0.1% 2,2'-bipyridyl in 0.1M HCl; 5ml 10% hydroxylamine hydrochloride (a reducing agent to convert Fe(III) to Fe(II)). The mixture was made up to 50ml and the absorbance read at 520nm. The Fe concentration was calculated using a calibration curve prepared from standard Fe solutions.

Fe Uptake from a range of Concentrations in Hypoxic Culture Solution.

Fe uptake by the plants from the Fe(II) tolerance experiment described in Chapter 2 was measured. The plants were washed and dried as in the previous experiment, but the procedures for acid digestion and Fe determination were different.

The dried tissue was ground with a pestle and mortar or cut into small pieces and samples (c. 0.4g) weighed into 50 ml boiling tubes. 4 ml 2M nitric acid was added to each tube. The tubes were then covered with glass bubbles and kept in a water bath at 96°C for 6 h. The use of this mild digestion technique prevents the loss of Fe as volatile organic complexes and decreases replicate variablity (Etherington and Davies, 1978). The digest was centrifuged at full speed with an MSE Bench Centrifuge for 25 minutes. The supernatant was decanted and made up to 25 ml with distilled water. For some species (Eriophorum vaginatum, E. angustifolium, Chamaenerion angustifolium, Myosotis scorpioides, Zerna ramosa and shoots of Nardus stricta) the digest was filtered through Whatman no. 44 filter paper and diluted to 25 ml.

The resulting solutions were analysed for Fe by atomic absorption spectrophotometry using a Shandon Southern A3400 atomic absorption spectrophotometer. Measurements were made with the 248.3 nm absorption line in an air-acetylene flame. The standard Fe solutions (40 - 400 μM) were made up from a 2.0mM stock solution of $FeCl_3$. Nitric acid was added to the standards to give an acid concentration equivalent to that in the extracts. Blank digests were run with no tissue. Interference in Fe determination by sulphate has been reported (Curtis, 1969). Trials showed that under the conditions used here there was none. The results for Fe uptake are the mean of determinations on two plants (μmol Fe g^{-1} dry weight).

Short-term Experiments on Fe(II) uptake and Oxidation in Solution Culture.

Two species from unflooded sand culture, Eriophorum angustifolium and Ranunculus flammula were used in these experiments. Plants were carefully removed from the sand, their root systems washed and then surface sterilized for 2 minutes in 0.02% mercuric chloride followed by a thorough rinsing in distilled water. The plants were placed in 250ml Erhlenmeyer flasks containing 0.1 mmol $Fe(II)SO_4$ per litre with or without 4.0mM $Ca(NO_3)_2$. The plants were held in place by non-absorbant cotton wool. Control flasks were plugged with cotton wool but contained no plants. The flasks were covered with black polythene to exclude light and weighed. 2ml aliquots of the solution (4 replicates per flask) were taken at intervals over the two hours and the concentration of Fe(II) and Fe(III) measured using the 2,2'-bipyridyl method. At the end of the experiment the flasks were reweighed to determine water loss by transpiration. This was used to correct the Fe concentrations. Using the 2,2'-bipyridyl assay the concentration of Fe(II) and Fe(III) in the solutions can be measured separately. Fe(II) was determined directly, and then total Fe was determined by reducing any Fe(III) with hydroxylamine hydrochloride. The 2ml aliquots were placed in 10ml volumetric flasks to which was added 2ml 4M sodium acetate-acetic acid buffer, pH 4.8, and 1ml 0.1% 2,2'-bipyridyl in 0.1M HCl. 1ml 10% hydroxylamine hydrochloride was added to some samples to determine total Fe. The volume was made up to 10ml with water and the absorbance measured at 520nm. The concentration of Fe was determined using a calibration curve prepared from standard Fe solutions. At the end of the experiment the root systems were cut off and their fresh weight and the amount of air space in the roots determined (method as in Chapter 2). The results are expressed as μmol Fe g^{-1} fresh weight of roots. No Fe(III) was produced in the control flasks during the experiments.

Results

The Effect of Flooding in Sand Culture at Low or High Nutrient Level on Growth and Fe Uptake by Deschampsia caespitosa and Glyceria maxima.

The effects of flooding at two nutrient levels on growth, judged from dry weight of the plants after 11 weeks treatment, are shown in table 3.1. Flooding increased growth of D. caespitosa at both nutrient levels. The effects were significant for all treatments except shoot growth under low nutrient levels. In G. maxima flooding had no significant effect on growth. Flooding had no effect on root:shoot ratios in either species under low or high nutrient levels, but both species had a lower root:shoot ratio under high nutrient conditions. The difference was significant ($p = 0.02$) in D. caespitosa.

The effect of flooding on Fe concentration in the tissues is shown in table 3.2. Flooding generally increased Fe concentration in roots and shoots, but the only significant effect ($p = 0.05$) was the increase in shoot Fe concentration with flooding under low nutrient levels. Reference to the flooded:drained ratios in table 3.2 shows that the effect of flooding on root Fe concentration was greater in the low nutrient treatments.

No Fe(II) toxicity symptoms were shown by either species, although the Fe(II) concentration in the high nutrient treatment (4.46mM) exceeded that found to be toxic in D. caespitosa in the Fe(II) tolerance experiment (Chapter 2). The use of full strength Hoagland's solution in the high nutrient treatment could have decreased the toxic effect of Fe(II).

Fe Uptake from Hypoxic Culture Solution.

a) Patterns of Fe uptake by roots.

Uptake of Fe by roots followed a similar pattern in all the species. The results are presented in fig 3.1. Fe uptake by roots has been plotted against the logarithm of Fe concentration in the culture solution so as to include the whole range of concentrations tested. In Fe(II) concentrations up to 1 or 2mM the uptake was linear with increasing concentration. Above 1 or 2mM the gradient of the line appeared to increase. This was particularly marked in Glyceria maxima Eriophorum vaginatum, Senecio aquaticus and Zerna ramosa. However, because of the logarithmic scale, this does not indicate an increase in Fe uptake with a similar increase in Fe concentration. This is made clear in fig 3.2 where uptake by the roots of some species has been plotted with Fe(II) concentration in the solution on a linear scale. Uptake follows a roughly hyperbolic curve and shows saturation. The saturation effect is particularly marked in Schoenus nigricans, Nardus stricta and Deschampsia caespitosa above 2.0mM Fe(II) in the culture solution (figs 3.1 & 3.2).

The hyperbolic uptake pattern shown in fig 3.2 suggested that the uptake of Fe by roots followed Michaelis-Menten kinetics, as observed for many other nutrients (Epstein, 1976). But, using Lineweaver Burk or Hofstee plots (Epstein, 1976), a good fit to the data was not obtained. The use of Michaelis-Menten kinetics is not justified in this case because its application assumes that there are a finite number of binding sites for Fe and that there will be a turnover of Fe in these sites. Much of the Fe is precipitated onto the root surface and so does not fit these requirements (Green and Etherington, 1977; Clarkson and Sanderson, 1978). Although the uptake by roots does not follow conventional kinetics in whole plants, the semi-log plots (fig 3.2) do suggest that there are two uptake phases, the second of which starts between 1.0 and 2.0mM Fe(II). The position of the break

seems to be similar for all the species. The second phase may reach saturation (e.g. S. nigricans, N. stricta and D. caespitosa). It is possible that in the lower Fe(II) concentrations (~ 1.0mM) absorption is limited by boundary layer effects because the solutions were unstirred. This effect becomes marked at lower concentrations if the rate of uptake exceeds the rate of diffusion across an unstirred boundary layer next to the root surface (Clarkson, 1974). This could have affected Fe uptake by the roots from the lower concentrations. An additional complication is provided by the presence of 4.0mM $Ca(NO_3)_2$ in the culture solution. This results in different Fe(II):Ca ratios in the different Fe(II) concentrations. This could interfere with Fe(II) uptake, but at the higher Fe(II) concentrations when Fe(II) Ca no marked increase in Fe(II) uptake occurred. The effect of Ca cannot be ruled out.

Although the Fe uptake patterns by roots of all the species were similar, there was variation in the amount of Fe taken up. Considering uptake in the "first phase" (0 - 2.0mM), regression lines were fitted to the semi-log plots (fig 3.2) and the Fe uptake from a concentration of 2.0mM in the culture solution was calculated (Table 3.3). The regression lines gave good fits and predicted values for uptake similar to those measured. Values calculated from the regression equations were used because the variability of the results did not allow an accurate estimate of uptake to be made for any particular concentration and it avoids any subjective bias.

b) The relationship between Fe uptake, Fe(II) tolerance and air space in roots of various species.

Fig 3.3 shows the relationship between Fe(II) tolerance and Fe uptake by the roots of various species. The Fe(II) tolerance data is taken from Chapter 2. There was no significant correlation between $\log_{10}$ tolerance and uptake ($r = 0.366$, p 0.1). Species of high tolerance had a wide range of uptake values. The Eriophorum species took up little

Fe while species of equal or greater tolerance (Nardus stricta, Oryza sativa and Deschampsia caespitosa) took up more Fe.

In contrast to the relationship with tolerance, a significant negative correlation was found between Fe uptake or $\log_{10}$ Fe uptake and the amount of air space in the roots (fig 3.4a & b). The relationship between $\log_{10}$ uptake and air space gave a better fit. Glyceria maxima and Nardus stricta were not included in the statistical analysis because there was no data for air space in the roots of the plants used in this experiment. The values for these species plotted in fig 3.4 were obtained from Chapter 7. These results suggest that species with a higher amount of air space tend to take up less Fe.

c) Patterns of Fe uptake by shoots.

The Fe concentrations in the shoots of plants grown in various Fe(II) concentrations are shown in fig 3.1 as log-log plots. In general the concentration of Fe in shoots was about one tenth of that found in the roots. The uptake pattern is similar in all the species and can be divided into two phases. In the first, from 0.01 to approximately 1.0mM Fe(II), uptake increases little with increasing Fe(II) concentration. In the second phase, from 1.0 - 6.0mM Fe(II), there is a greater increase in Fe concentration in the shoots. This has been quantified by comparing the gradient of the regression lines in the two concentration ranges (Table 3.5). The division between the concentration ranges at 1.0mM was arbitrarily chosen by looking at the graphs. In all species, except Eriophorum vaginatum, the gradient was greater in the 1.0 - 6.0mM range.

In the Fe(II) tolerance and uptake experiment shoot systems of Ranunculus flammula and Senecio aquaticus in the 2.0 - 4.0mM Fe(II) concentrations respectively appeared very unhealthy, whereas the other replicates were apparently healthy. The Fe concentration in the shoots of the unhealthy replicates was very much greater than in the healthy ones (Table 3.6).

d) The relationship between Fe uptake by shoots, Fe(II) tolerance and air space in the roots of various species.

Fe concentration in the shoots varied between species (Table 3.4). The uptake from 1.0mM Fe(II) was calculated from regression lines of $\log_{10}$ concentration in shoots on $\log_{10}$ Fe(II) concentration in the culture solution from 0.01 - 1.0mM Fe(II). Because of the variation in shoot Fe concentration, the regression lines were not as good a fit as for roots (c.f. Table 3.3). There was no significant correlation between Fe concentration in the shoots in 1.0mM Fe(II) and Fe(II) tolerance ($r = 0.03$) or air space in roots ($r = -0.087$).

Fe Uptake and Oxidation over Short Periods in Solution Culture.

Fe uptake by roots of Ranunculus flammula from 0.1mM $Fe(II)SO_4$ was linear over a 2.5 hour time course (fig 3.5). Over this period the concentration of Fe(III) in the culture solutions increased. Except for one possibly abberant measurement at 1 hour the concentration increased linearly over the time course (fig 3.5). The Fe(III) was produced by oxidation of Fe(II). The culture solution also contained 4.0mM $Ca(NO_3)_2$ as in the Fe(II) tolerance and uptake experiments described previously. In a further experiment the effect of calcium on Fe uptake was examined (Table 3.7). Fe uptake by R. flammula was decreased by 4.0mM calcium nitrate, but the effect was not significant. Oxidation of Fe(II) in the culture solution was significantly ($p = 0.001$) less in the presence of calcium.

The ability of R. flammula and Eriophorum angustifolium to oxidise Fe(II) in the culture solution in the absence of calcium was compared (Table 3.8). After one hour E. angustifolium oxidised significantly ($p = 0.05$) more Fe(II) than R. flammula. The roots of E. angustifolium grown under these conditions (unflooded sand culture) contained a greater amount of air space.

Table 3.1 - The effect of flooding and nutrient level on dry weight and root:shoot ratio of Deschampsia caespitosa and Glyceria maxima. Plants were harvested eleven weeks after beginning the flooding treatment.

Deschampsia caespitosa (n = 8)

Treatment	Dry weight mg. plant^{-1} ± S.E.		Root:shoot ratio ± S.E.
	Shoots	Roots	
Low nutrient - drained	597 ± 59	107 ± 28	0.128 ± 0.011
Low nutrient - flooded	801 ± 110	141 ± 23	0.172 ± 0.017
High nutrient- drained	448 ± 41	47 ± 9	0.100 ± 0.013
High nutrient- flooded	798 ± 91	105 ± 10	0.125 ± 0.013

Glyceria maxima (n = 3)

Low nutrient - drained	656 (622-1106)	356 (121-722)	0.461 ± 0.119
Low nutrient - flooded	716 (113-1021)	301 (46-622)	0.417 ± 0.008
High nutrient- drained	1531 (705-2230)	452 (232-694)	0.301 ± 0.016
High nutrient- flooded	817 (524-1209)	246 (213-384)	0.323 ± 0.105

Table 3.2 - The effect of flooding and nutrient level on iron concentration in Deschampsia caespitosa and Glyceria maxima. Plants were harvested eleven weeks after beginning the flooding treatment.

Deschampsia caespitosa (n = 4)

Treatment	Iron concentration, μmol. g dry wt.$^{-1}$ ± S.E.		Ratio Flooded:drained	
	Shoots	Roots	Shoots	Roots
Low nutrient - drained	2.29 ± 0.11	18.00 ± 2.72		
Low nutrient - flooded	2.39 ± 0.27	46.28 ± 14.96	1.04	2.57
High nutrient- drained	7.78 ± 0.79	71.73 ± 5.90		
High nutrient- flooded	8.96 ± 0.59	88.04 ± 11.48	1.16	1.23

Glyceria maxima (n = 3)

Low nutrient - drained	0.61	6.89		
Low nutrient - flooded	2.72	30.91	4.46	4.49
High nutrient- drained	3.83	25.92		
High nutrient- flooded	1.13	28.17	0.30	1.09

Table 3.3 - Iron uptake by roots of various species from 2mM iron (II) sulphate in solution culture. Values calculated from the regression equation of iron uptake on $\log_{10}$ external iron (II) concentration from 0.01 - 2.0mM. Species are arranged in order of increasing iron (II) tolerance.

	Iron uptake μmol. g dry wt.$^{-1}$ 4 days^{-1} ± S.E.	Correlation coefficient for uptake v. external iron (II) concentration		
		r	n	p
Chamaenerion angustifolium	507.1 ± 46.6	0.966	9	0.001
Senecio jacobaea	758.4 ± 143.5	0.889	11	0.001
Schoenus nigricans	579.9 ± 69.8	0.966	10	0.001
Myosotis scorpioides	924.0 ± 82.6	0.938	13	0.001
Zerna ramosa	787.3 ± 153.9	0.203	11	0.01
Senecio aquaticus	1195.4 ± 283.6	0.854	9	0.01
Ranunculus flammula	806.6 ± 145.8	0.854	12	0.001
Eriophorum angustifolium	80.6 ± 13.9	0.910	7	0.01
Eriophorum vaginatum	216.8 ± 32.3	0.915	9	0.001
Nardus stricta	541.4 ± 96.7	0.922	6	0.01
Oryza sativa	607.1 ± 70.0	0.958	9	0.001
Deschampsia caespitosa	665.8 ± 63.6	0.966	9	0.001
Glyceria maxima	335.4 ± 97.3	0.744	10	0.01

Table 3.4 - Iron concentration in shoots of various species after expsoure to 1mM iron (II) sulphate in solution culture for four days. Values calculated from the regression equation of $\log_{10}$ concentration in shoots on $\log_{10}$ external iron concentration from 0.01-1.0mM. Species are arranged in order of increasing iron (II) tolerance.

	Iron concentration in shoots, μmol. g. dry wt.$^{-1}$	Correlation coefficient for $\log_{10}$ shoot iron concentration v $\log_{10}$ external iron (II) concentration		
		r	n	p
Chamaenerion angustifolium	5.1	0.276	8	0.05
Senecio jacobaea	15.1	0.722	8	0.05
Schoenus nigricans	3.8	0.356	8	0.05
Myosotis scorpioides	5.2	0.408	11	0.05
Zerna ramosa	25.00	0.864	9	0.01
Senecio aquaticus	6.2	0.253	6	0.05
Ranunculus flammula	7.1	0.371	10	0.05
Eriophorum angustifolium	4.2	0.646	66	0.05
Eriophorum vaginatum	12.3	0.879	8	0.01
Nardus stricta	4.3	0.842	5	0.05
Oryza sativa	8.6	0.998	5	0.001
Deschampsia caespitosa	15.6	0.512	6	0.05
Glyceria maxima	3.7	0.143	4	0.05

Table 3.5 - Gradients of the regression lines of $\log_{10}$ iron concentration in shoots on $\log_{10}$ external iron (II) concentration in the ranges 0.01 - 1.0 and 1.0 - 6.0 mM. The gradient gives an indication of the responsiveness of shoot iron concentration to increases in the external concentration in the two ranges.

	Gradient		Ratio of gradients
	0.01-1.0mM	1.0-6.0mM	1.0-6.0:0.01-1.0
Chamaenerion angustifolium	0.08	-	-
Senecio jacobaea	0.45	-	-
Schoenus nigricans	0.08	0.43	5.38
Myosotis scorpioides	0.10	-	-
Zerna ramosa	0.36	-	-
Senecio aquaticus	0.06	1.79	29.83
Ranunculus flammula	0.17	-	-
Eriophorum angustifolium	0.15	0.81	5.40
Eriophorum vaginatum	0.76	0.74	0.97
Nardus stricta	0.12	1.09	9.08
Oryza sativa	0.45	1.00	2.22
Deschampsia caespitosa	0.05	0.62	12.40
Glycera maxima	0.06	0.73	12.17

Table 3.7 - The effect of 4mM calcium nitrate on iron uptake and iron (III) production (iron (II) oxidation) in deoxygenated 0.1mM iron (II) sulphate by Ranunculus flammula.

	μmol.g. fresh wt.$^{-1}$ $2.5h^{-1}$. (n = 3)	
	no Ca	4mM Ca
Uptake	3.853	2.672
Iron (III) production	0.573*	0.423*

* significantly different (p = 0.001 t = 16.476)

Table 3.6 - Iron concentration in healthy and unhealthy shoots of *Ranunculus flammula* and *Senecio aquaticus*.

	Iron (II) concentration in culture soln., mM	Iron concentration in shoots umol. g. dry wt.$^{-1}$	
		Healthy replicate	Unhealthy replicate
Ranunculus flammula	2.0	28.9	112.5
Senecio aquaticus	4.0	29.7	1200.0

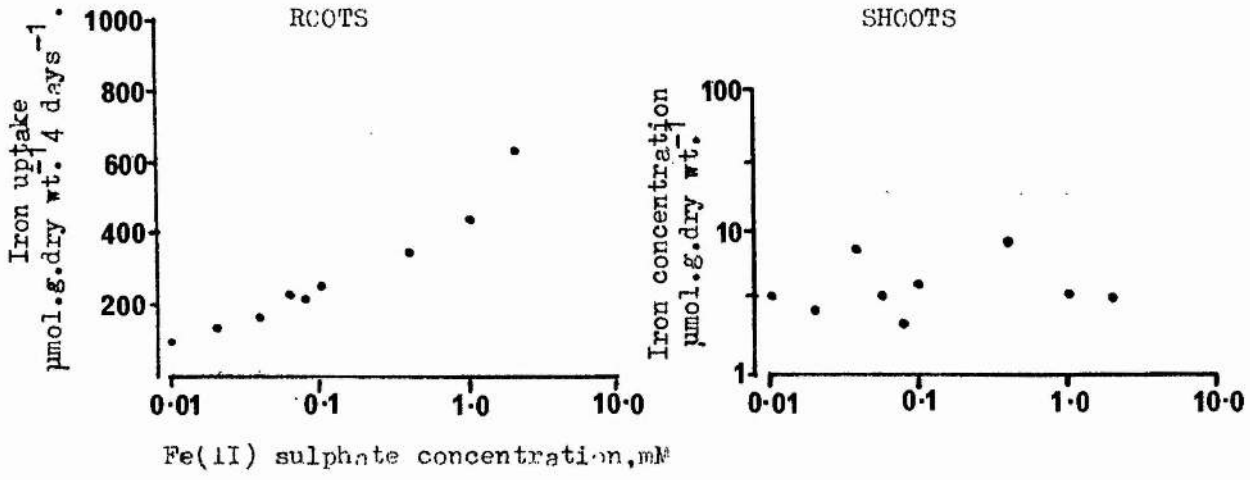
Table 3.8 - Iron (III) production (iron (II) oxidation) in deoxygenated 0.1mM iron (II) sulphate containing 4mM calcium nitrate by Ranunculus flammula and Eriophorum angustifolium.

	Iron (III) production µmol. g. fresh wt. roots^{-1} h^{-1} (n = 3)		Air space in roots, % v/v **
Ranunculus flammula	0.169	*	9.1 (9.0-9.4)
Eriophorum angustifolium	0.328	*	48.2 (47.5-49.7)

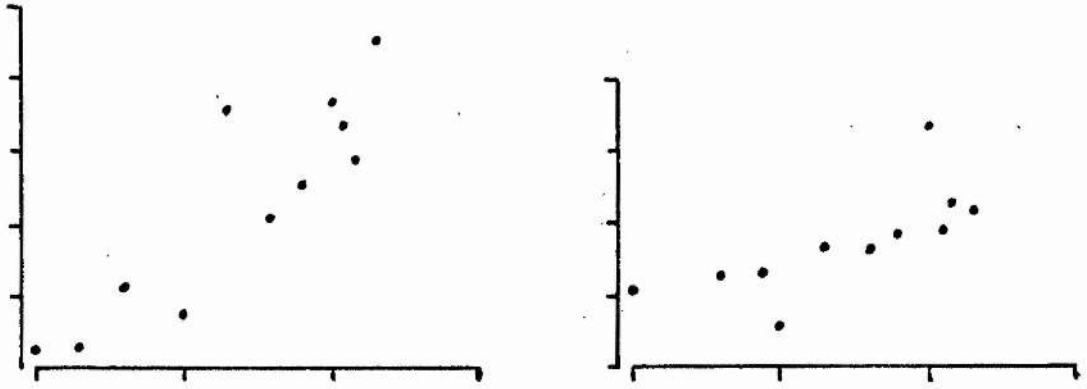
* significantly different (p = 0.001 t = 128.2)

** data from Chapter 7

CHAMAENERION ANGUSTIFOLIUM



SENECIO JACOBAEA



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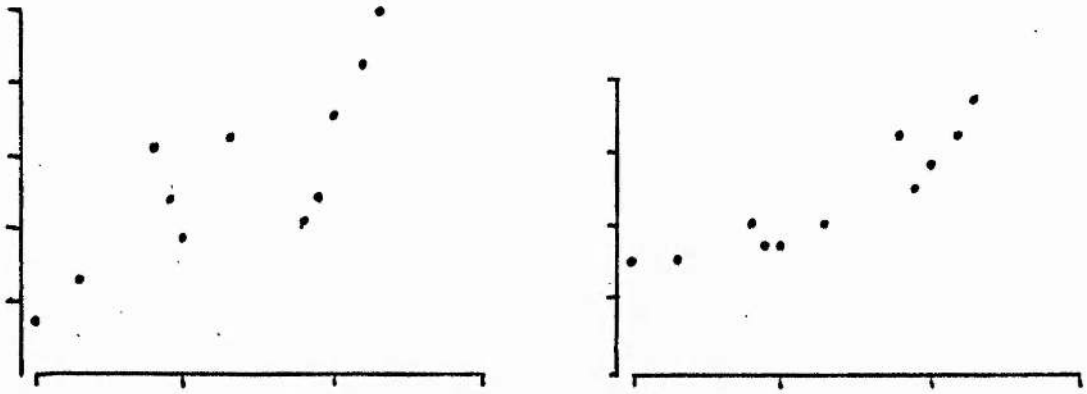


Fig.3.1. Iron uptake by roots and shoots of various species. The plants were kept for 4 days in deoxygenated calcium nitrate solution (4mM) containing Fe(II) sulphate (0.01-6.0mM).
Uptake by roots. Semi-log plot. Uptake calculated by sutracting iron concentration in control roots (no Fe(II)) from iron concentration in roots from the Fe(II) treatments.
Uptake by shoots. Log-log plot. Uptake taken as the iron concentration in the shoots after 4 days.

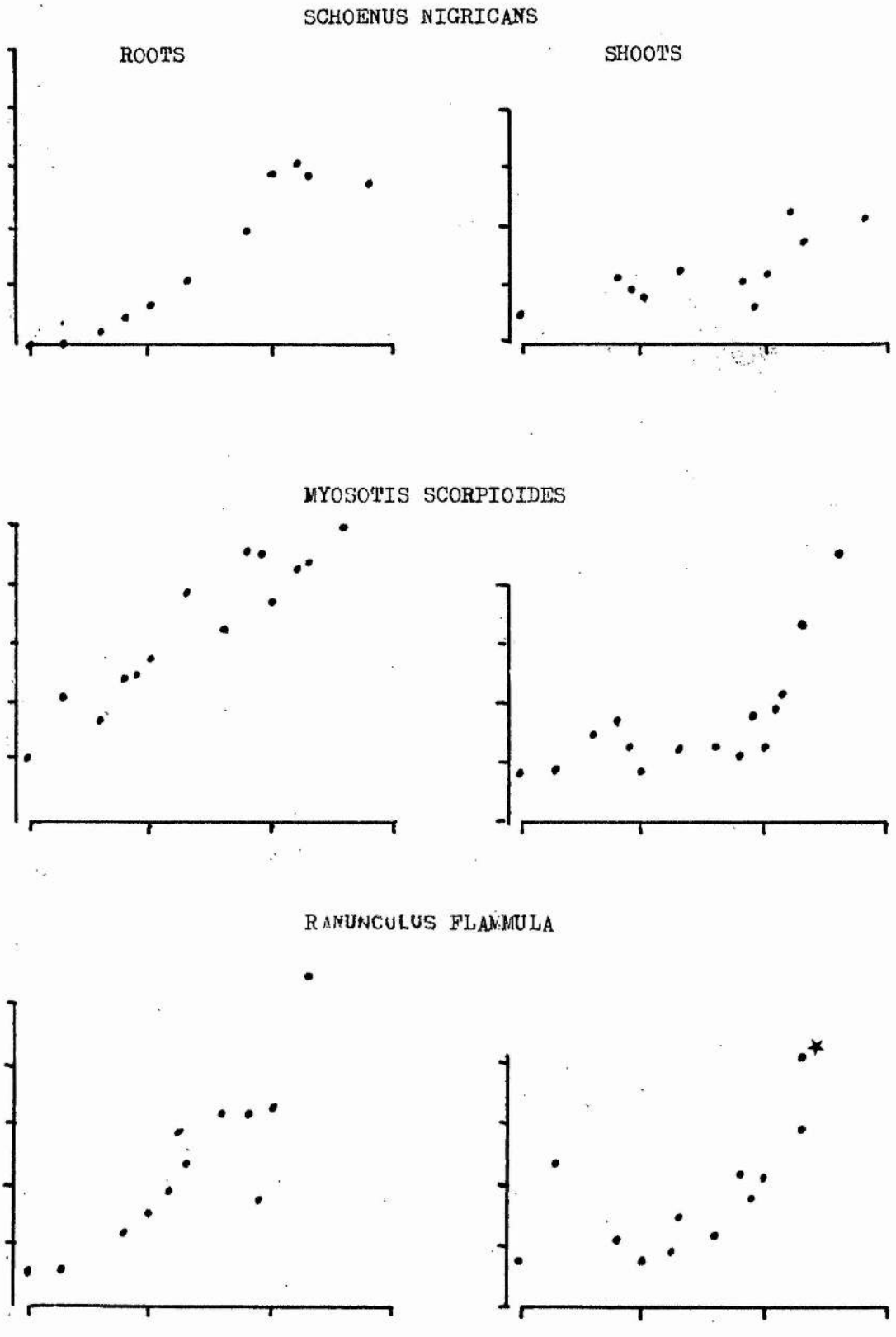
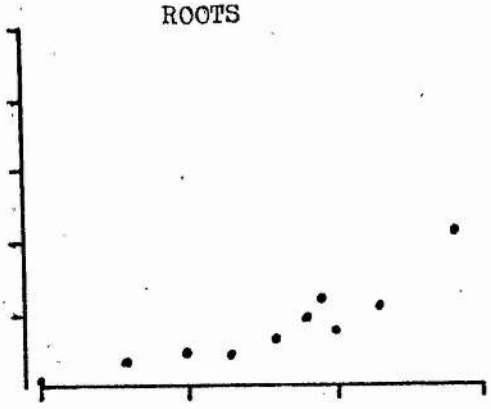


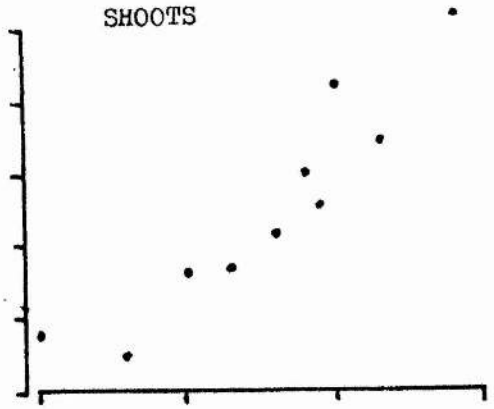
Fig.3.1. Continued.

ERIOPHORUM VAGINATUM

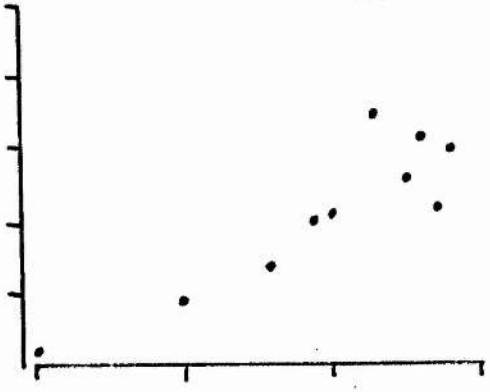
ROOTS

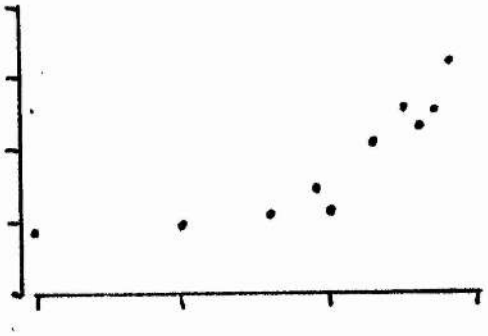


SHOOTS

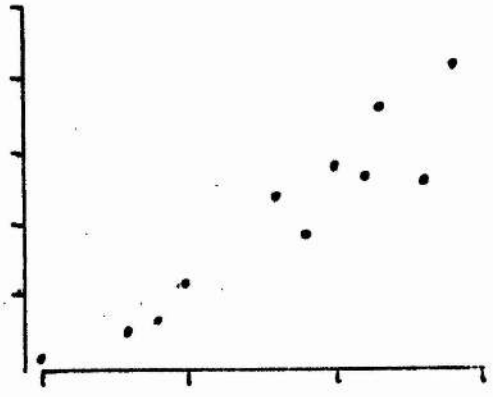


NARDUS STRICTA





ORYZA SATIVA



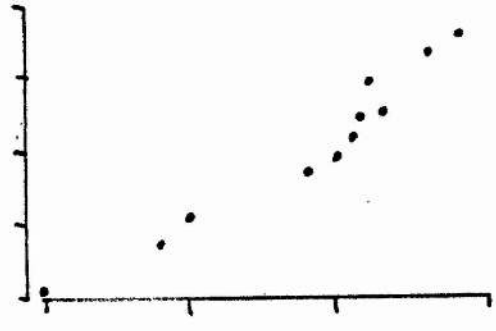
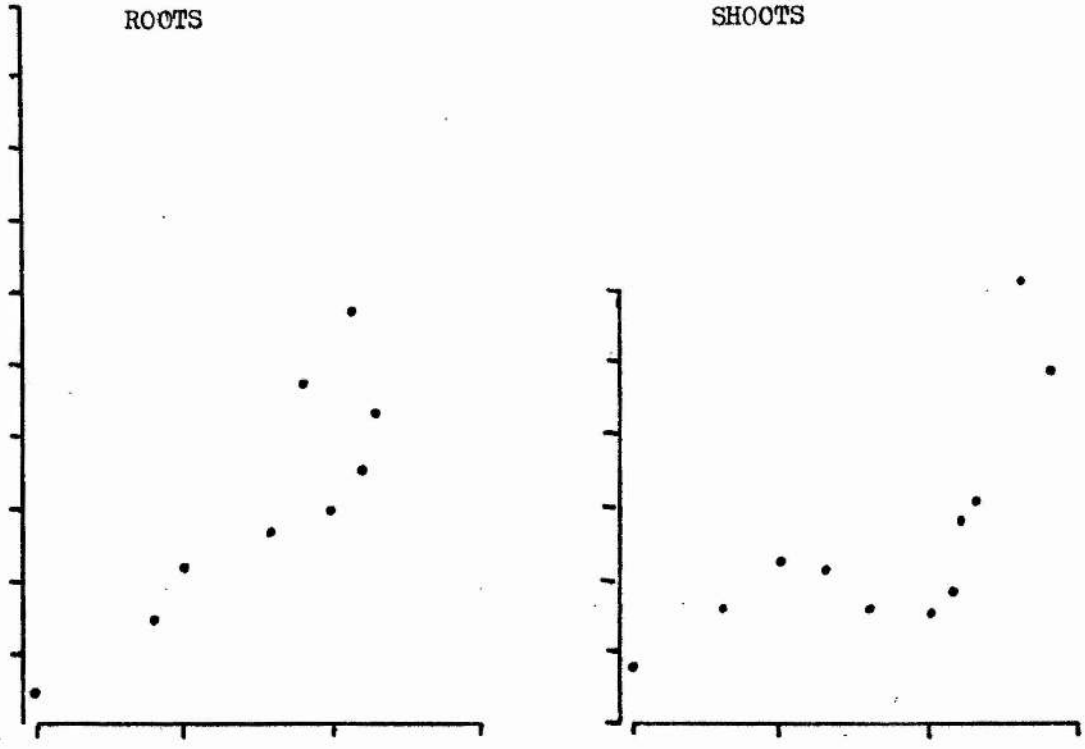


Fig.3.1. Continued.

SENECIO AQUATICUS



ERIOPHORUM ANGUSTIFOLIUM

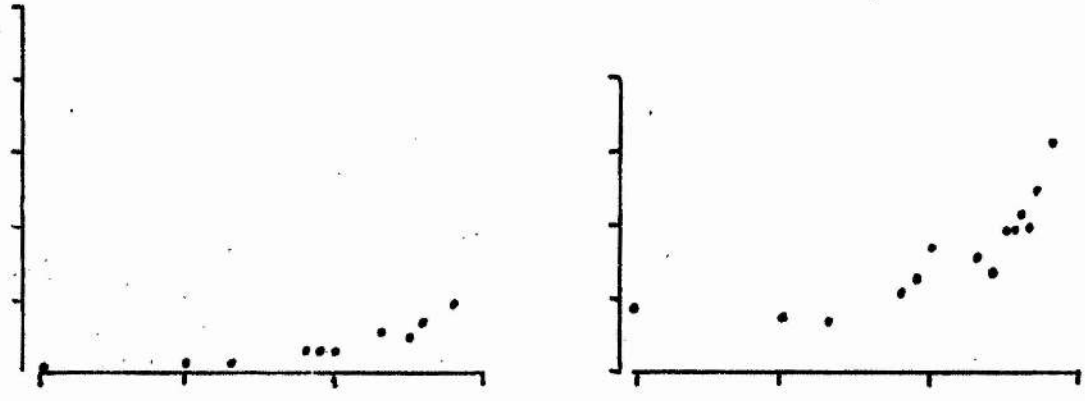
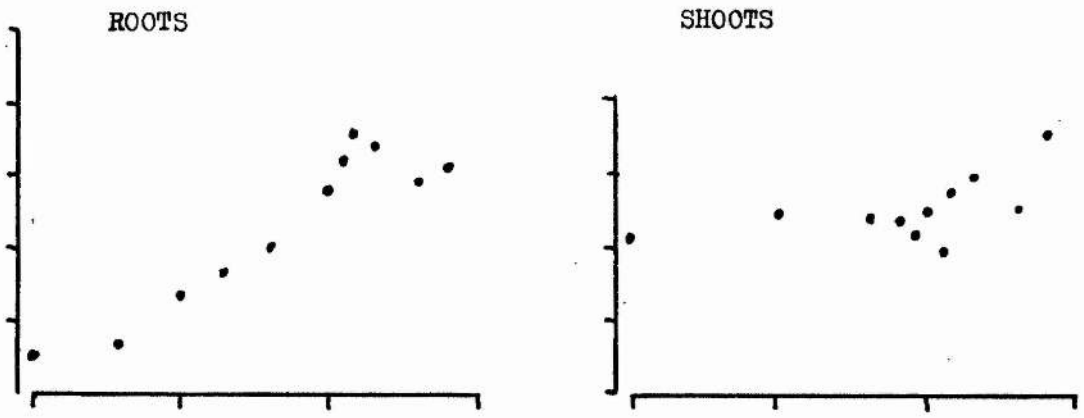
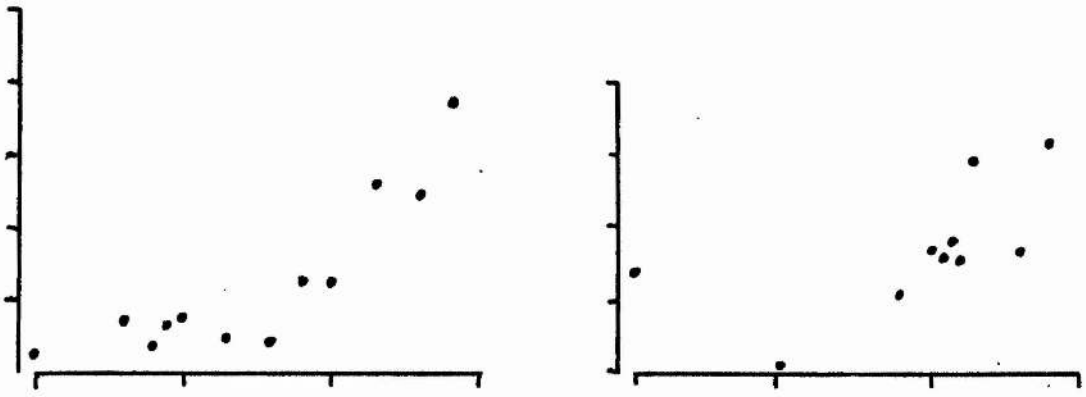


Fig.3.1. Continued.

DESCHAMPSIA CAESPITOSA



GLYCERIA MAXIMA



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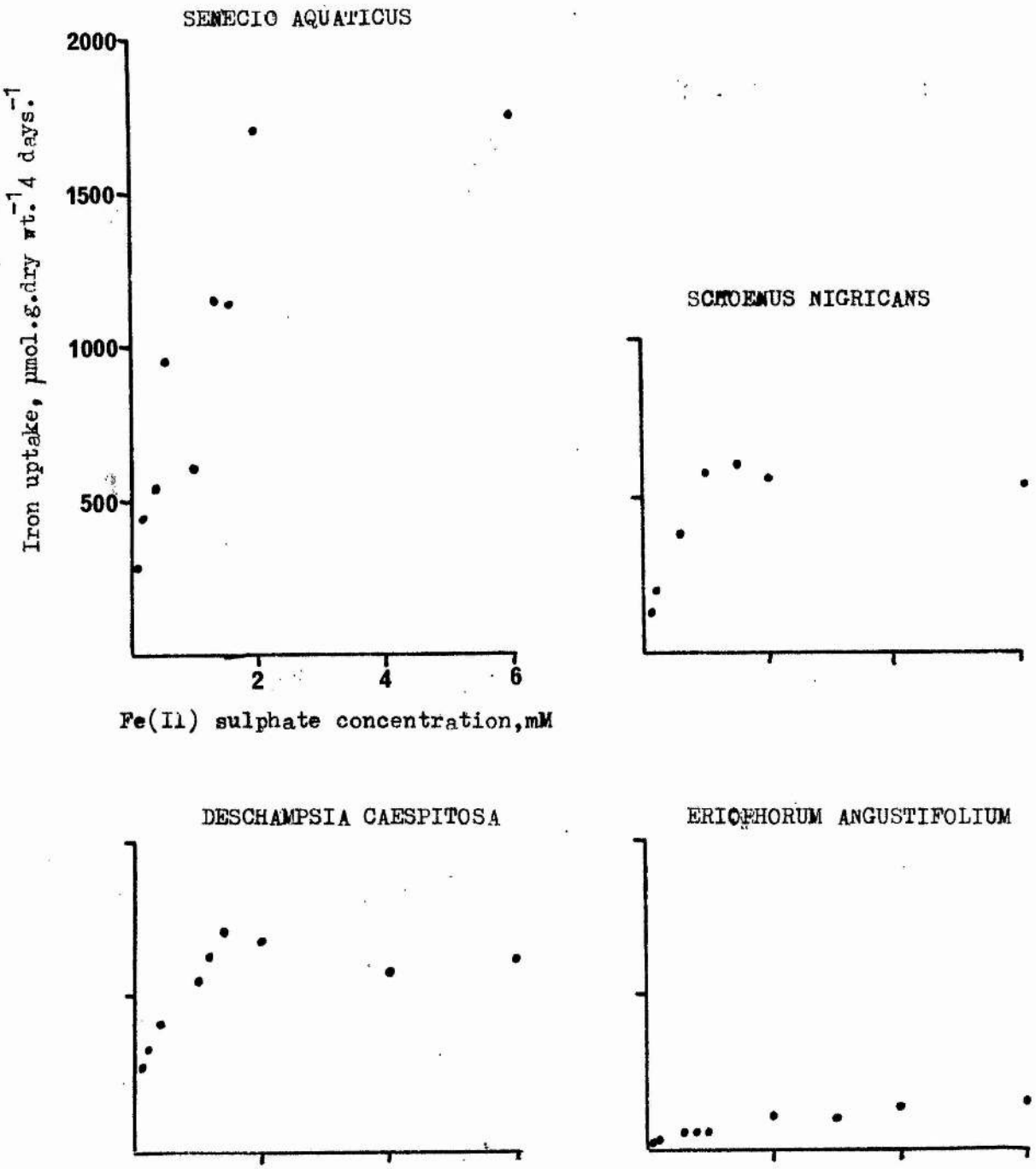


Fig.3.2. Iron uptake by roots of various species. Data as in fig.3.1, but both axes with a linear scale.

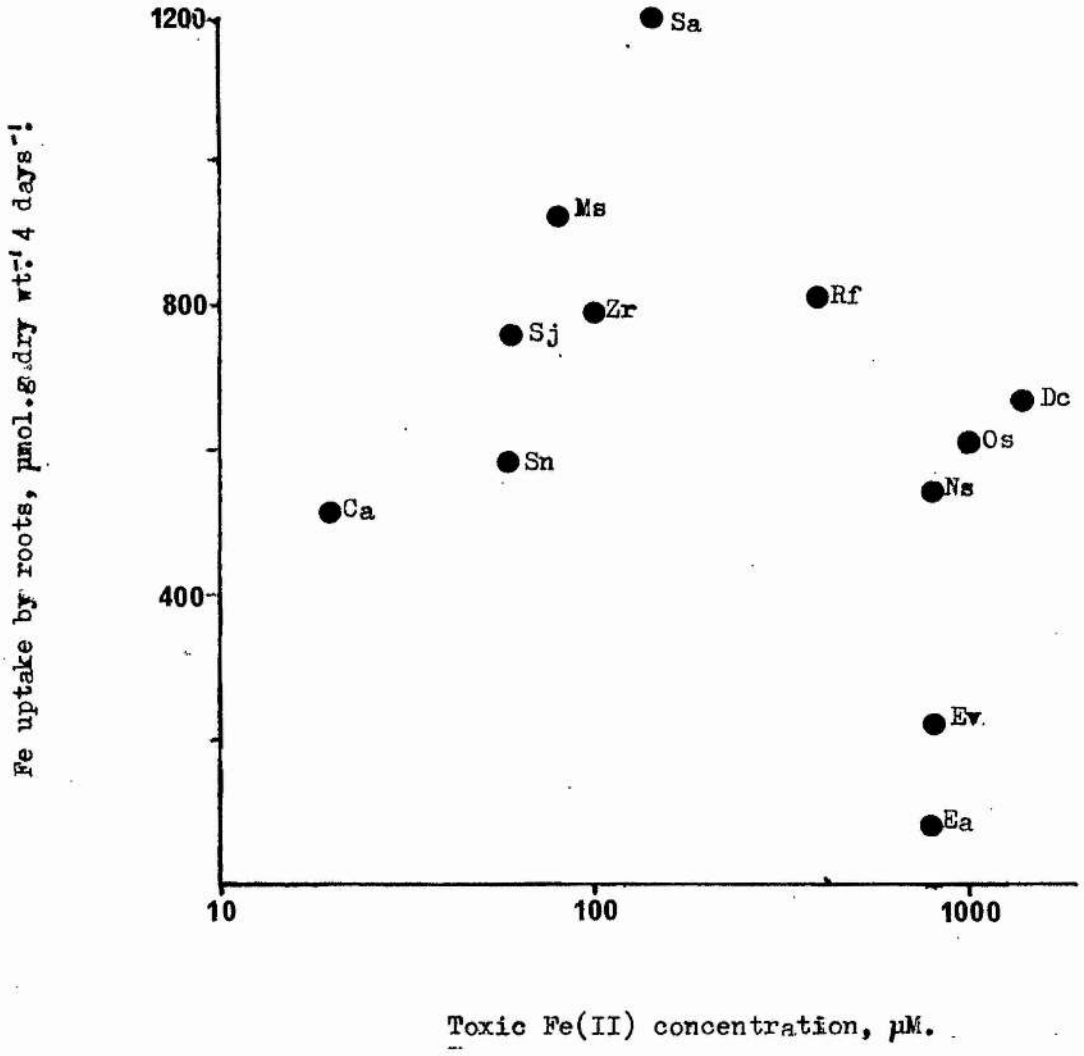


Fig.3.3. The relationship between Fe(II) tolerance and Fe uptake by roots from 2 mM Fe(II) sulphate in various species.

Key. Ca. Chamaenerion angustifolium
Dc. Deschampsia caespitosa
Ea. Eriophorum angustifolium
Ev. Eriophorum vaginatum
Gm. Glyceria maxima
Ms. Myosotis scorpioides
Ns. Nardus stricta
Os. Oryza sativa
Rf. Ranunculus flammula
Sa. Senecio aquaticus
Sj. Senecio jacobaea
Sn. Schoenus nigricans
Zn. Zerna ramosa

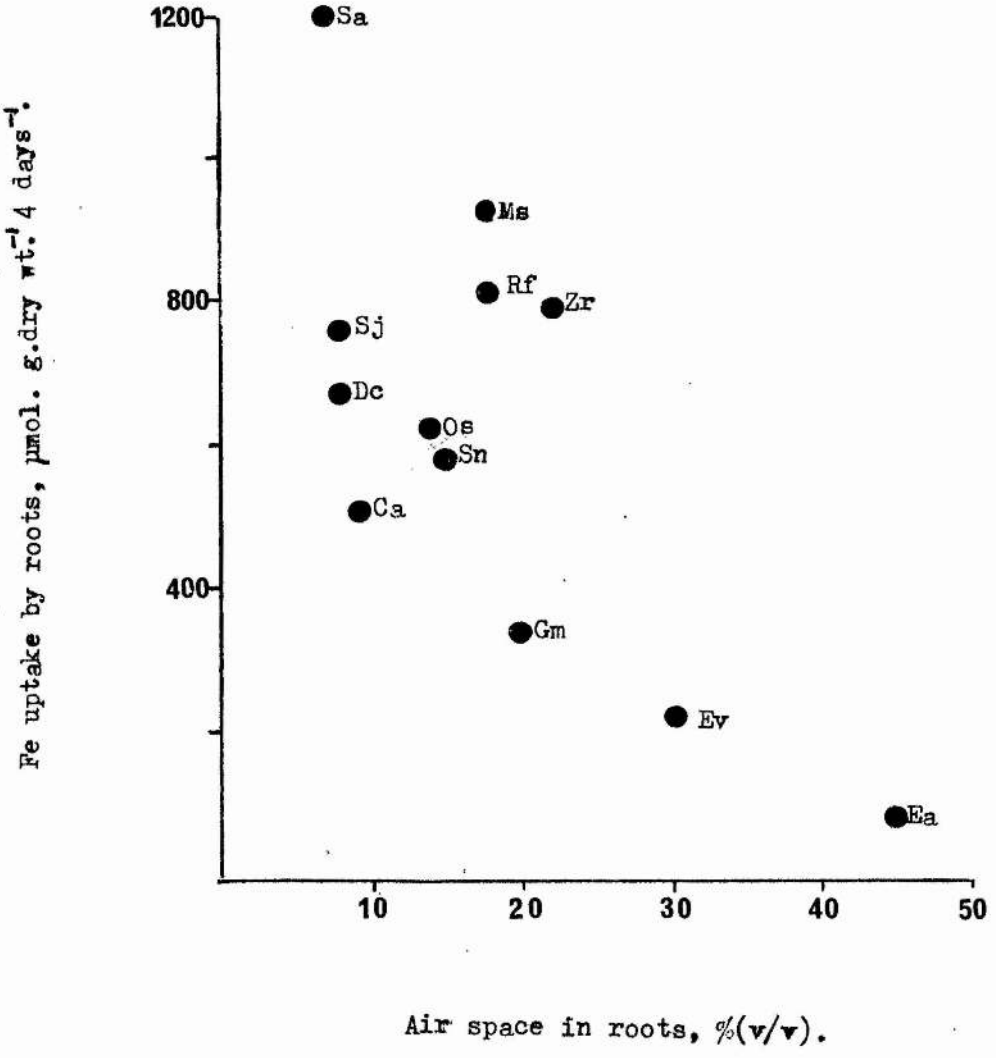


Fig.3.4a. The relationship between air space and Fe(II) uptake by the roots of various species from 2mM Fe(II) sulphate. r=0.735, p=0.01

N.B. No air space data were obtained for Glyceria maxima in this experiment. The value on the graph is taken from Chapter 7, but was not used to calculate the correlation coefficients.

For key to species see Fig.2.3.

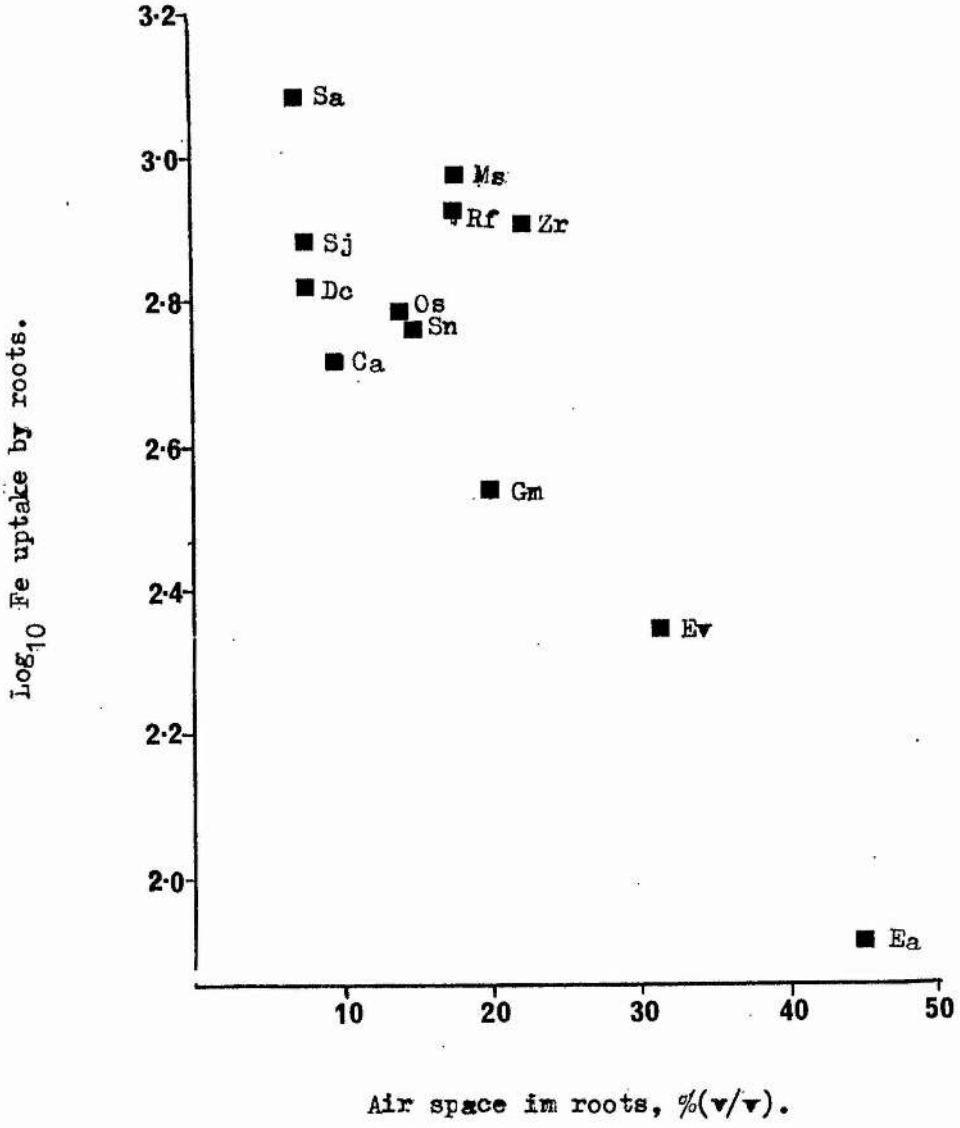


Fig.3.4b. The relationship between air space in roots and $\log_{10}$ Fe uptake by the roots of various species from 2mM Fe(II) sulphate.
r=0.863, p=0.001

For notes see Fig.3.4a.

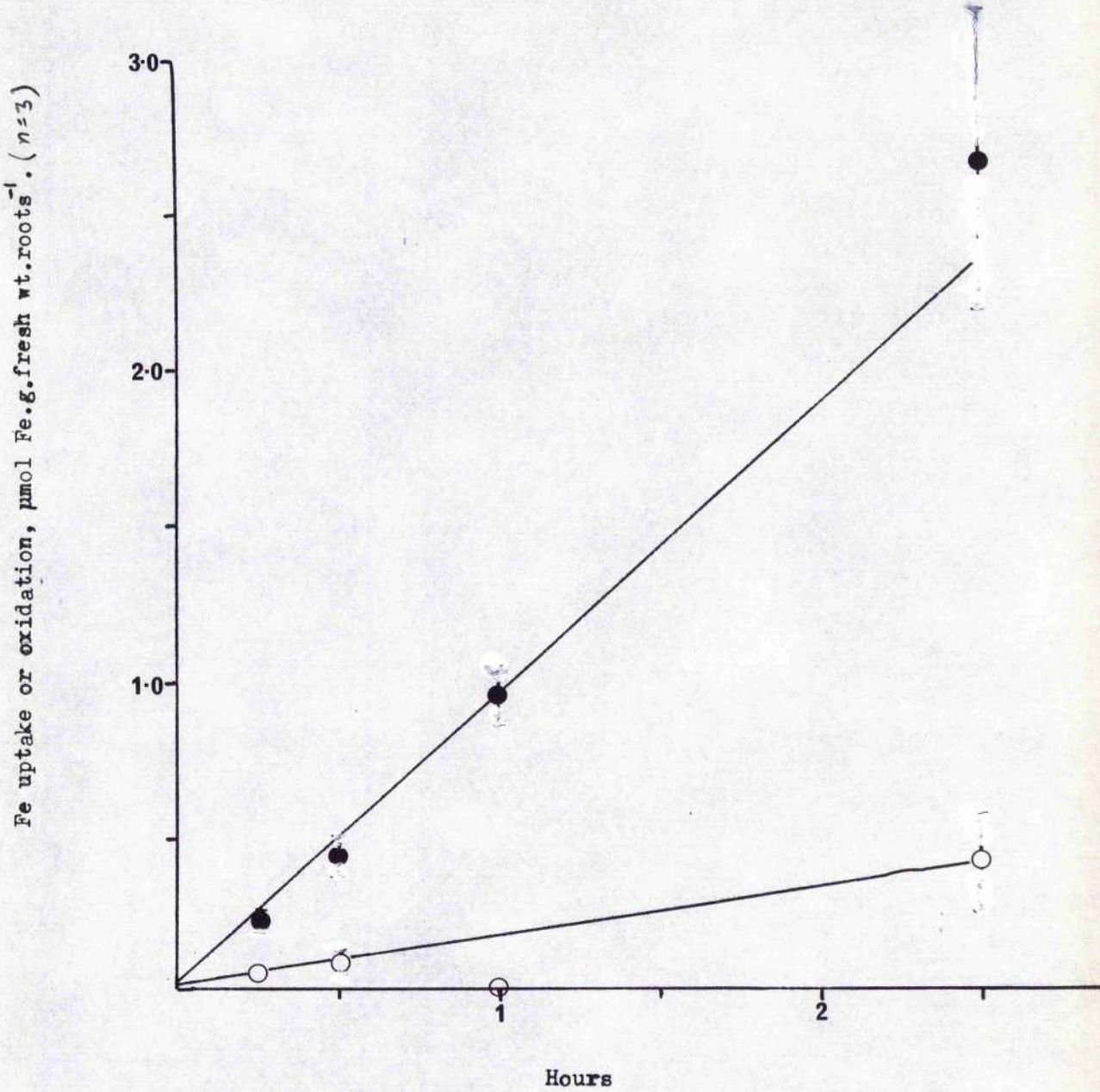


Fig.3.5. Iron uptake and oxidation by Ranunculus flammula from a deoxygenated solution containing 0.1mM Fe(II) sulphate and 4mM calcium nitrate

● Iron uptake

○ Iron oxidation (increase in Fe(III) in the solution)

Lines fitted by eye.

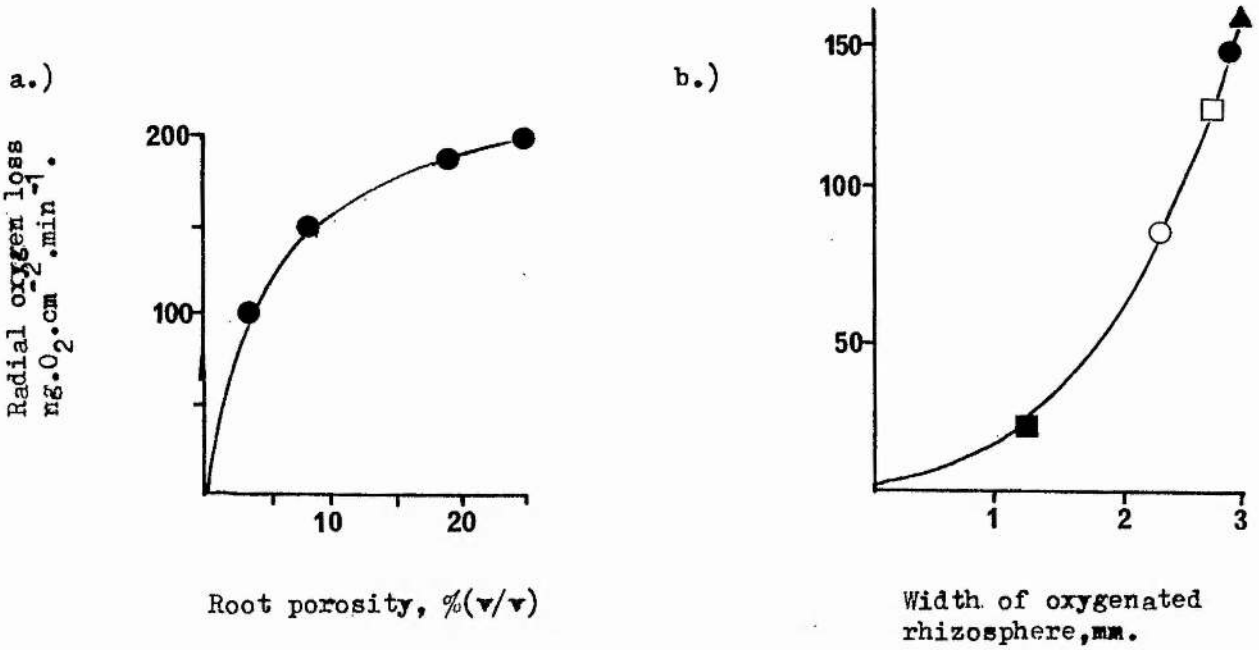


Fig.3.6. a) Radial oxygen loss from the apex of an artificial silicone root of various porosities. (From Armstrong, 1972.)

b) Width of the oxygenated rhizosphere around the root tips of various species (predicted from a mathematical model) plotted against measured radial oxygen loss. (From Armstrong, 1970.)

- ■ Molinia caerulea
- ○ Oryza sativa cv. Norin
- □ Eriophorum angustifolium
- ● O. sativa cv. Yubae
- ▲ Menyanthes trifoliata

Discussion

The Effect of Flooding on Growth and Fe Uptake by Deschampsia caespitosa and Glyceria maxima.

The results are comparable to previous studies in that flooding generally increased Fe concentration in the tissues (see Table 1.1). Flooding increases uptake by increasing the availability of Fe by reducing it to the more soluble Fe(II) (see Chapter 1) which is the form absorbed into plant cells (Brown, 1978). In this experiment Fe was supplied as $Fe(II)SO_4$ and uptake was still increased by flooding. It is likely that in the unflooded treatments the Fe(II) was rapidly oxidised and precipitated. The greater effect of flooding in the low nutrient treatments was probably because at the high level, there may have been enough available Fe to saturate the root uptake capacity before flooding.

In their growth responses both species behaved as flood-tolerant plants. G. maxima was able to maintain its growth under flooded conditions and D. caespitosa had increased growth when flooded. The reason for increased growth under flooded conditions is unknown, but in this case increased availability of nutrients can probably be ruled out because the same response occured at low and high nutrient levels. Because the growth of D. caespitosa was greater in the low nutrient treatments, it is unlikely that an increase in nutrient availability on flooding under low nutrient conditions would then increase its growth. D. caespitosa is known to occur in nutrient poor soils. It is able to continue growth under low nutrient conditions by efficiently recycling nutrients within the plant (Davy and Taylor, 1975). The lower growth under high nutrient conditions may be because these levels are toxic to D. caespitosa. Many oligotrophic species show growth inhibition at high nutrient levels (Bradshaw et al, 1960, 1964). Although there were no visible symptoms, decreased growth in the high nutrient treatment could have resulted from Fe(II) toxicity under both drained and flooded conditions.

Root:shoot ratios were greater under low nutrient conditions, and the effect was significant in D. caespitosa. This effect on root:shoot ratios has been observed frequently (Scott Russell, 1977) and may be a compensatory mechanism to allow roots to exploit a greater volume of soil.

Fe uptake from Hypoxic Culture Solution.

The uptake patterns of Fe uptake by roots of all the species over a 4 day period were similar and followed a hyperbolic curve. Semi-log plots suggested that there were two uptake phases (Epstein, 1976). The break occured between 1.0 and 2.0mM Fe(II). In the first phase (0.01 - 1.0 or 2.0mM) uptake increased greatly with increasing concentration. The second phase (1.0 or 2.0 - 6.0mM) was less responsive, and often reached saturation. A biphasic uptake pattern has been found in excised rice roots for Fe(II) (Kannan, 1971) and Fe(III) uptake (Shim and Vose, 1965). Kannan found that the two phases were separated between 0.5 and 1.0mM. This was in a similar concentration range to the present experiments. Uptake of many nutrients follows a biphasic pattern with each phase conforming to Michaelis-Menton Kinetics (Epstein, 1976), however, as suggested (p 33) in the results, there is no justification for fitting these kinetics to the data. Without further investigation the significance of the two phases is not clear. Fe uptake from 0.1mM Fe(II) by Ranunculus flammula was nearly linear over 2.5 hours. Uptake by excised rice roots followed a similar time course and was of the same magnitude (Kannan, 1971). Calcium (4mM) inhibited Fe uptake from 0.1mM Fe(II) by R. flammula, but the effect was not significant. Calcium (0.5mM) stimulated Fe(II) uptake by excised rice roots from 0.1mM Fe(II) (Kannan, 1971), so the effect of calcium was not identical in these species. Time courses of uptake over 4 days were not measured so it is not known if the uptake pattern will change after this period. It is still possible to compare uptake between species.

The measurements of uptake represented total Fe in the roots: the location of the Fe in the roots was not determined. There are three possible sites for Fe in roots. a) Precipitated on the root surface and in cell walls or middle lamellae; b) Ionically held in the cation exchange complex of the cell walls (Haynes, 1980); c) Intracellularly located, for example in cytoplasm, vacuoles or in an Fe storage protein, phytoferretin (Hyde et al., 1963). Fe in the first two locations is entirely apoplastic. Kannan (1971) showed that Fe uptake by excised rice roots was dependant on respiration. Uptake under the conditions used by Kannan must have been into cells because uptake into the apoplast is not likely to be directly dependant on metabolic energy. The evidence from various studies suggests that much of the Fe in roots is precipitated on the root surface and in cell walls. Precipitation of Fe in these locations in Molinia caerulea (Armstrong and Boatman, 1967) and rice (Green and Etherington, 1977) was discussed in the introduction. In peas Fe is precipitated on the root surface (Branton and Jacobson, 1962b). In barley Fe accumulates on the root surface, and is most abundant from 4cm behind the tip. Surface deposits nearer the tip were less, and were associated with bacterial colonies in the mucigel (Clarkson and Sanderson, 1978). These authors provided evidence that the deposits were precipitates rather than ionically bound on the cell wall cation exchange sites. Only 20 - 25% of the labeled Fe could be removed from roots, previously exposed to ^{59}Fe, by desorbing with unlabeled $Fe(NO_3)_2$, but treatment with the reducing agent, ascorbic acid, removed 87% of the Fe. Woolhouse (1966) found that only 1% of the Fe absorbed by roots of Deschampsia flexuosa, Holcus mollis, Koeleria cristata and Arrhenatherum elatius was translocated to the shoots, suggesting that most was in an unavailable form, perhaps precipitated on the root surfac Rice roots, grown for 3 days in 0.04mM $Fe(II)SO_4$, accumulated Fe on the root surface of mature root segments, but at the tip little Fe was deposited on the surface and was mostly inside the roots (Tanaka et al., 1966 This resembles the pattern found with barley (Clarkson and Sanderson, 1978). The results of the present experiments support

these observations. The occurence of Fe(III) deposits on roots, particularly of plants grown in high Fe(II) concentrations, was noted in Chapter 2. Fe deposits did not extend over the root tips unless root growth had been inhibited by high Fe(II) concentrations. This suggests that root tips remain free from deposits because they are growing fast enough to outstrip the rate of Fe precipitation.

Fe seems to accumulate in the cytoplasm of cells adjacent to the endodermis, at least in regions of the root from which Fe is being translocated (Branton and Jacobson, 1962b; Clarkson and Sanderson, 1978). There is some circumstantial evidence that Fe could be held in exchange sites in cell walls of rice roots. Nagai and Matano (1959) found a positive correlation between root cation exchange capacity and the amount of Fe taken up by the roots of a variety of rice cultivars. Further experiments are required to confirm this, and to give a quantitative estimate of Fe uptake into the various locations in roots over a range of Fe concentrations. Most of the studies cited above have used low concentrations of chelated Fe(III) as the Fe source.

If a large proportion of the Fe content of roots is present as extracellular precipitates, it is difficult to explain why uptake shows saturation at higher concentrations in many of the species. The roots may not have an unlimited capacity to absorb Fe precipitates. It is possible that washing the roots in detergent before Fe analysis could have removed loosely-held deposits. Another factor was the presence of $Ca(NO_3)_2$ in the culture solution. This could have had an influence on the uptake of Fe. Assimilation of nitrate, assuming this is not inhibited by Fe(II), results in a net efflux of OH^- from roots (Raven and Smith, 1976; Smith and Raven, 1979). In an unstirred solution this would result in a localised increase in pH around the roots and this would favour Fe precipitation as hydroxide, or in combination with CO_2 produced by root respiration, as carbonate. However, Fe(II) must first be oxidised before it is precipitated. Oxidation of Fe(II) with oxygen and water results in the net production of H^+ by hydrolysis of the resulting Fe(III)

(Spiro and Saltman, 1974). Interactions in the rhizosphere resulting from nitrate assimilation and Fe(II) oxidation are likely to be complex.

The Fe concentrations in shoots were usually one tenth of the roots and in comparison with the roots increased very little with increasing Fe(II) concentration. Fe translocation seems to be under close control by the roots. With low external levels of Fe (c. 10μM) translocation is decreased by metabolic inhibitors in peas (Branton and Jacobson, 1962a) and barley (Clarkson and Sanderson, 1978). Clark and Brown (1974) suggested that the efficiency of Fe use in maize cultivars was under the control of the roots. In decorticated pea roots Fe is translocated passively in the transpiration stream (Branton and Jacobson, 1962a). The results suggest that translocation is under metabolic control in the cortex or endodermis. In connection with this, accumulation of Fe in cells adjacent to the endodermis has been noted (Branton and Jacobson, 1962b), particularly in regions active in translocating Fe (Clarkson and Sanderson, 1978).

In contrast to the above results, experiments with higher levels of Fe(II) have shown that inhibition of metabolism can increase translocation of Fe to shoots. The excessive uptake of Fe by the unhealthy plants of Ranunculus flammula and Senecio aquaticus supports this. Additional evidence was provided by Tanaka et al (1968). They found that treatment of rice roots in solution culture with H_2S, a powerful inhibitor of respiration by inactivation of enzymes with metallic cofactors, greatly increased Fe uptake into the shoots from 5.4mM $Fe(II)SO_4$. At high, potentially toxic levels of Fe(II) metabolic control may be needed to prevent too much Fe translocation into shoots. If the experiments had been continued for longer than 4 days, it is possible that Fe translocation into shoots at toxic Fe(II) concentrations would have increased. The biphasic pattern of Fe uptake by the shoots could be the initial signs of the breakdown of such a control mechanism. A similar biphasic uptake pattern for copper and cobalt has been demonstrated in some African metallophytes (Morrison et al, 1979). These metallophytes, Haumeniastrum

katagense, H. robertii and Aeolanthus biformifolius, are able to tolerate high metal levels and to accumulate high concentrations in their tissues. Over a wide range of copper and cobalt concentrations in the soil there was little increase in concentration in leaves, but once a certain soil concentration was exceeded (different for copper and cobalt), shoot concentration increased greatly. The resulting biphasic uptake pattern is the same as observed for Fe uptake. A further similarity is that the change in the gradient occured at similar concentrations in all species irrespective of their tolerance. The pattern for Fe in these experiments was evident after 4 days treatment, but for the metallophytes exposure was for 2 months. This suggests that an increased length of exposure to Fe would not affect the results. Further studies on the control of uptake and translocation of Fe and other heavy metals in relation to tolerance would be valuable.

Fe Uptake and Oxidation in Relation to Tolerance and Air Space in the Roots.

There were large differences in the amount of Fe taken up by the various species. The Eriophorum species took up very little in comparison with the others. This is probably not a characteristic of the Cyperaceae, because Schoenus nigricans took up more Fe.

The negative correlation between Fe uptake and air space in the roots supports the suggestion of Green and Etherington (1977) and Armstrong (1979) that possession of air space allows Fe oxidation by oxygen diffusing from the roots. Oxidised Fe is less available to plants because it may be precipitated in the soil or culture solution. Oxygen diffusion from the roots was not measured in these experiments, but there is evidence to suggest that a greater development of aerenchyma leads to increased ROL from roots. In two theoretical studies, using artificial silicone roots and a mathematical model, Armstrong (1971, 1972) has predicted that increasing root porosity (air space) can lead to greater ROL from roots and that this in turn produces an oxygenated

rhizosphere of greater radius. The relationships between air space, ROL and oxygenated rhizosphere dimensions are shown in fig 3.6. Waterlogging increases the amount of air space in rice roots and also increases ROL from the root tips (Armstrong, 1971).

Over a short period Eriophorum angustifolium oxidised twice as much Fe(II) in deoxygenated solution culture as Ranunculus flammula. No precipitation was observed over this period, but it would eventually occur as Fe(III) builds up in solution and the low solubility product of $Fe(OH)_3$ is exceeded. Bartlett (1961) found that seedlings of various species varied in their ability to oxidise Fe(II) in culture solution. In the light those species with greater oxidising power absorbed less Fe into their shoots. Unfortunately he made no measurements of air space or ROL from the roots. E. angustifolium had four times more air space in its roots than R. flammula but, because only 2 species were compared, it is not possible to determine the relationship between air space and Fe(II) oxidation. The results do support the hypothesis that Fe exclusion by increased air space is the result of Fe(II) oxidation by oxygen diffusing from the roots. Armstrong's predictions (fig 3.6) suggest that the relationship is not linear and could explain why there is a stronger relationship between $\log_{10}$ Fe uptake and air space.

Evidence in support of Fe exclusion from roots by oxidation has been provided by Keeley (Keeley, 1979; and see table 1.1) in two populations of the tree Nyssa sylvatica. He compared oxygen diffusion and Fe uptake by roots of the two populations after growth in flooded or drained soil for one year. After a year the swamp (flood-tolerant) population had a lower Fe concentration in the roots of the flooded treatment. In contrast, a floodplain (less flood-tolerant) population had increased Fe concentration in roots of flooded plants. Comparison of oxygen diffusion rates from the roots of flooded plants showed that the oxygen diffusion rate from roots of swamp plants was much greater than from floodplain plants. It can be concluded that the greater ROL from

the swamp population oxidised and precipitated Fe(III) in the rhizosphere and resulted in Fe exclusion. The results of Nagai and Matano (1959) give some support to this idea. They found that various rice cultivars grown in solution culture had more Fe in their roots when the shoots had been cut off. Cutting off shoots would decrease or stop oxygen diffusion to the roots, and this could have prevented Fe(II) oxidation in the rhizosphere.

ROL from wetland plants may be confined to the terminal 2 cm of the root (Armstrong, 1964; 1967; 1979), although oxygen loss can be detected where lateral roots emerge (Armstrong 1979; Healy and Armstrong, 1972). Armstrong suggests that this is because the surface layers of the roots are suberized approximately 2cm behind the tip and this makes the root wall impermeable to oxygen. This would suggest that in soil the production of an oxygenated rhizosphere would only occur around root tips or where laterals emerge. In solution culture the oxygen may more rapidly diffuse throughout the culture vessel. Increased air space and thus increased ROL leads to an oxygenated rhizosphere of greater radius round root tips (fig 3.6). This would allow a larger residence time for Fe(II) in the oxygenated rhizosphere, so it is more likely to be oxidised and precipitated before it reaches the root surface. But these predictions do not take into account the possible effect of soil microorganisms. In further experiments, using a silicone root in waterlogged soil impregnated with the redox dye methylene blue, Armstrong (1979) found that an oxidised halo forms round the tip of the root, but after a while it contracts, and reducing conditions reach the root surface. An increase in aerobic microorganisms in the oxygenated rhizosphere could rapidly deplete oxygen diffusing from the root. However a further complication arises because silicone roots do not grow. An actively growing root tip may be able to maintain the oxidised zone round its tip ahead of the growth of aerobic microorganisms. However, Keeley's (1979) results showing Fe exclusion over a long period in soil suggest that Fe exclusion can still occur. Rhizosphere oxidation

in soils and its relationship with microorganisms needs further study.

If there is no rhizosphere oxidation behind the root tip, reduced phytotoxins such as Fe(II) will be able to come into contact with the root. Armstrong (1979) has suggested, using Eriophorum angustifolium as an example, that suberization prevents the absorption of Fe(II). No evidence has been produced for this. In contrast, Chapin (1978) showed that the roots of E. vaginatum are not suberized under flooded conditions, and that they retain their ability to absorb phosphate along their whole length. The root surface of this species only became suberized under well-drained conditions. Ability to absorb phosphate suggests that the roots might also be able to absorb Fe. This would apply to E. angustifolium which has identical root anatomy (see Chapter 7). Observations on Fe uptake and translocation from root sections at various distances along roots have been made on two dryland species, maize and barley (Clarkson and Sanderson, 1978 and Kashirad et al, 1972, cited in Clarkson and Sanderson, 1978). However, there was no parallel information on suberization, if any, in the root surface layers. Maize was able to absorb and translocate Fe along the entire length of its roots. Barley could also absorb Fe along its whole length. Absorption was less near the tip because there was less surface precipitation. Most Fe was translocated from a zone 1.5cm from the tip, but microautoradiography showed that Fe still penetrated into the cortex in older, non translocating, zones. Evidence that Fe can penetrate into the cortex of older root segments of wetland plants comes from the study of Green and Etherington (1977) with rice. Their micrographs, from roots grown in deoxygenated agar, showed Fe deposits in the cortex of roots with well-developed aerenchyma and a prominent sclerenchymatous sheath under the epidermis. Aerenchyma is not well-developed and mature in rice roots until at least 2cm beyond the tip (Armstrong, 1971). The evidence does not support Armstrong's hypothesis that Fe cannot penetrate into the root behind the tip. Further investigation is needed of the relationship between Fe uptake along the length of roots of wetland plants and its relationship to the possible suberization of

the root surface.

Although species with more air space take up less Fe, possibly as a result of increased ROL from their roots, it was shown in Chapter 2 that there was no correlation between air space and tolerance to Fe(II). This is further confirmed by the lack of correlation between Fe uptake and tolerance (fig 3.4). It would seem that Fe exclusion from roots cannot entirely explain tolerance. In species with a very large amount of air space, for example Eriophorum spp., the very small amount of Fe taken up could contribute to tolerance. Even if Fe(II) oxidation does reduce the availability of Fe, the observations in Chapter 2 suggest that the root tip is the primary site of Fe(II) toxicity. Any exclusion or precipitation of Fe(II) must operate in the root tip if it is to contribute to tolerance. The results do not support the idea of Green and Etherington (1977) that oxidation and precipitation of Fe in cell walls adjacent to the air spaces in the cortex result in Fe(II) tolerance. The air space tissue may make some contribution to tolerance by oxidising and sequestering Fe. In this case the structure, rather than the amount, of air space would be the important factor. The structure of air space in the tolerant species may more efficient for this function. The structure of air space tissue will be considered further in Chapter 7. Root tips do not contain extensive intercellular air spaces (Armstrong, 1971) and it is possible that any oxygen penetrating the root to the meristematic zone will be used up by cytochrome oxidase, which has a very high affinity for oxygen (Crawford, 1978) rather than for Fe oxidation. So any Fe(II) penetrating the oxygenated rhizosphere around the root tip may not be oxidised and precipitated within the tip. Fe has been found to accumulate in the root tips of rice (Tanaka et al, 1966), so rhizosphere oxidation is not sufficient to exclude it.

CHAPTER 4

The Effect of Iron(II) Sulphate on Malate and Citrate Levels in Roots

Introduction

The levels of organic acids in roots can be greatly affected by various factors. These include the ionic composition of the external solution, the availability of oxygen and Fe nutrition. Both malic acid and citric acid are strong acids occuring as malate and citrate anions in the physiological pH range. They are intermediates in the TCA cycle and, in addition, malate takes part in reactions outside the cycle, for example in Crassulacean Acid metabolism (Osmond, 1978) and as a key intermediate in the biochemical pH stat (Raven & Smith, 1976).

The ionic composition of the external solution affects organic acid levels, particularly malate, in roots and xylem exudates (Hiatt and Leggett, 1974; Osmond, 1976; Triplett et al, 1980). Under conditions where excess cations are absorbed the organic acid content of the roots increases. If excess chloride is absorbed levels may decrease. Hiatt & Leggett (1974) suggest that these changes have a causal relationship with ion accumulation, but whatever the reason, organic acid levels are certainly affected. Plants grown with nitrate as the nitrogen source as opposed to ammonium have higher concentrations of organic acids, including citrate and malate (Kirkby & Mengel, 1967; Raven & Smith, 1976; Kirkby & Knight, 1977). These changes can be explained in terms of pH regulation during NO_3^- assimilation using the biochemical pH stat (Raven & Smith, 1976; Smith & Raven, 1979). Flood-tolerant marsh plants can accumulate malate under hypoxia and it may serve as an end product of anaerobic metabolism (Crawford and Tyler, 1969; McManmon and Crawford, 1971; Linhart and Baker, 1973). The physiological significance of malate accumulation (Keeley, 1978) and even its occurence

(Smith and ap Rees, 1979) in flooded plants has been questioned. Smith and ap Rees could not demonstrate malate accumulation in several flood-tolerant species. However, they used excised root tips under complete anoxia. These conditions are not ecologically realistic, and Tyler (1969) found that malate levels in excised roots under anoxia usually decreased. This could have been because of decreased synthesis or leakage from the roots (Hiatt & Lowe, 1967).

The Fe nutrition of plants has a large effect on levels of organic acids in roots and xylem exudates. Fe-deficient plants generally contain higher levels of organic acids, notably citrate and malate (Iljin, 1951; De Kock and Morrison, 1958; Van Egmond & Atkas, 1977). Because Fe is insoluble in the physiological pH range it must be held in an organic complex to keep it soluble. Fe forms a stable complex with citrate and is translocated in the xylem in this form (Brown & Tiffin, 1965; Tiffin 1966a & b; Brown & Choney, 1971; Clark et al, 1973). In the previous investigations much attention has been given to acid levels under conditions of Fe-deficiency or in response to low levels of Fe(III). The experiments described in this Chapter concern the effects of higher levels of Fe(II) added as Fe(II) sulphate on malate and citrate levels of species varying in Fe(II) tolerance. The experiments were designed to test three possibilities. These are that the more Fe(II) tolerant species i) contain higher malate and citrate levels in their roots or (ii) can increase their acid levels when subjected to high Fe(II) levels or (iii) can maintain their acid levels when subjected to high Fe(II) levels. If acid levels are maintained or increased they could possibly chelate excess intracellular Fe and prevent toxicity.

Materials and Methods

The Effect of $Fe(II)SO_4$ on Malate and Citrate Levels in roots of Plants Grown in Solution Culture.

To be comparable to the Fe tolerance experiment the plants were grown, pretreated and exposed to $Fe(II)SO_4$ in exactly the same way as described in Chapter 2. The plants were kept in the Fe solutions (0, 0.01, 0.1, 1.0 and 10.0 mmol l^{-1}) for three days before harvesting. All the plants were harvested at midday to allow for any diurnal fluctuations in acid content (Triplett et al, 1980). The root systems were excised, washed, blotted and weighed and then frozen with liquid nitrogen. Cold 6% perchloric acid was added to cover the roots which were then deep-frozen in small plastic bottles. Later the samples were ground in a pestle and mortar with a little sand. The resulting slurry was centrifuged at 30,000g for 30mins at 4 °C. The supernatant was decanted and neutralized with 5M potassium carbonate using methyl orange as the indicator (pink → yellow). This treatment precipitated potassium perchlorate. The volume of the extracts was measured and then the potassium perchlorate crystals were allowed to settle out at 4 °C for 2 hours. The resulting clear extracts were decanted and deep frozen until they were assayed for malate and citrate. Fe in the extracts could not be accurately measured because neutralization resulted in some Fe precipitation.

Malate and citrate were assayed enzymatically using ready-prepared Boehringer food analysis kits (Boehringer Corporation Ltd.). Both reactions involved the oxidation or reduction of NAD/H which was followed at 340nm using a Unicam SP1800 UV Spectrophotometer.

Malate. The assay used malate dehydrogenase (MDH) and glutamate-oxaloacetate transaminase (GOT) in the following reactions:

1. L-malate + $NAD^+ \xrightleftharpoons{MDH}$ oxaloacetate + NADH + H^+

2. Oxaloacetate + L-glutamate $\xrightleftharpoons{GOT}$ L-aspartate + α-ketoglutarate

The equlibrium of reaction 1 lies towards malate. Reaction 2 consumes oxaloacetate and shifts the equilibrium of reaction 1 towards malate oxidation. The reactions were carried out in 1cm glass cuvettes which contained the following in a total volume of 2.22ml: 1ml glycylglycine buffer, pH 10.0, 0.2-1.0 ml of sample, 14.7mg glutamic acid, 7mg NAD^+, 4 I.U. (International Units) GOT and 60 I.U. MDH. The volume was made up with distilled water and the reaction was started by adding MDH. Blanks without samples were run as controls. The increase in absorption at 340nm, from NAD^+ reduction after MDH addition was read when the reaction was complete (10 - 15 minutes). The amount of NADH produced, which is stoichiometric with malate in the sample, was calculated using the extinction coefficient of NADH at 340nm (6.3 $mmol^{-1}$ cm^{-1})

Citrate. The assay used citrate lyase (CL) in combination with malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) in the following reactions:

1. Citrate $\xrightarrow{CL}$ oxaloacetate + acetate
2. Oxaloacetate + NADH + H^+ $\xrightarrow{MDH}$ L-malate + NAD^+
3. Pyruvate + NADH + H^+ $\xrightarrow{LDH}$ L-lactate + NAD^+

The amount of NADH oxidised by oxaloacetate and its decarboxylation product pyruvate is stoichiometric with the amount of citrate. The cuvette contained the following in a total volume of 3.02ml: 1ml glycylglycine buffer, pH 7.8, 0.5mg NADH, 0.8 I.U. CL, 11.3 I.U. MDH, 23.7 I.U. LDH and 0.1-2.0ml sample. The volume was made up with distilled water and the reaction started by adding CL. Control cuvettes contained no sample. The decrease in absorption after CL addition was read after 30 minutes. The amount of NADH oxidised, and hence the amount of citrate in the sample was calculated as for malate.

The methods were checked using standard solutions of malic and citric acids and found to give at least 96% recovery. The possibility

that Fe could interfere with the citrate analysis was checked by adding excess $FeCL_3$ to the cuvettes. There was no interference. Citrate levels and citrate malate ratios were higher than has previously been found in other experiments (Tyler, 1969). This may have been because the citrate assay using the extracts did not come to an obvious end point. So the reading taken after 30 minutes could result in an overestimation of citrate. However, the citrate levels measured were proportional to the amount of extract added and the results are comparable within the experiment. The results are the mean of determinations with three separate plants.

The Effect of Flooding Natural Vegetation Turves with $Fe(II)SO_4$.

This experiment was carried out in Orkney, in the open, during June 1980. The vegetation turves were cut from the Loons, an extensive mire developed in a loch basin on Orkney Mainland (grid reference HY 252 242). Much of the surface supports oligotrophic peat bog vegetation (pH 5.0 - 5.5, conductivity 1 x 10^2 µmhos. Calluna vulgaris, Empetrum nigrum, Eriophorum angustifolium, Narthecium ossifragum). The mire has extensive peat cuttings and in which a more eutrophic vegetation has developed because of contact with the groundwater (pH 6.3 - 6.8, conductivity 40 x 10^2 µmhos. Potentilla palustris, Ranunculus flammula, Caltha palustris and Senecio aquaticus.). The vegetation turves were from the more eutrophic part. 2 similar turves were cut, each 25 x 25cm square. They contained Ranunculus flammula, Carex nigra and Senecio aquaticus. The turves were placed in 2 litre polythene cartons and flooded to the surface of the peat with water or water plus 100 mmoles $Fe(II)SO_4$. This gave a concentration of about 100 mmol l^{-1}. After 3 days the plants were examined for toxicity symptoms. The roots of Ranunculus flammula were carefully washed free of peat, weighed and stored in plastic bottles containing 6% perchloric acid. They were later analysed for malate and citrate, as described previously.

The Effect of $Fe(II)SO_4$ on the Aerobic Respiration Rates (QO_2) of Roots.

This was a preliminary experiment to determine the effect of Fe(II) in solution cultures on subsequent aerobic respiration of the roots of Ranunculus flammula, Deschampsia caespitosa, Oryza sativa and Glyceria maxima. The plants were taken from sand culture and pretreated for 1 week in 250 ml Erhlenmeyer flasks containing 1/5 strength Hoagland's solution (one plant per flask). After this period the nutrient solution was replaced with various concentrations of deoxygenated $Fe(II)SO_4$ adjusted to pH 5.5 with KOH or H_2SO_4. The solution was changed every two days. No $Ca(NO_3)_2$ was added in this experiment. After 4 days the roots were excised, washed in distilled water, and their oxygen uptake measured using a Gilson Differential Respirometer (Williams and Wilson., 1975). Roots were placed in respiration flasks and were moistened with 2 ml distilled water. The central well of the flask contained 0.3 ml of 10% KOH and a filter paper wick. QO_2 was measured at 25 °C. There was not enough root material to replicate the measurements for each $Fe(II)SO_4$ concentration, but with the wide concentration range used the trends can be seen.

Results

The Effect of $Fe(II)SO_4$ on Malate and Citrate Levels in Roots of Plants Grown in Solution Culture.

Malate. Variation in $Fe(II)SO_4$ concentration had a significant depressing effect on malate levels in the roots of all the species except Chamaenerion angustifolium and Ranunculus flammula (fig 4.1 - 6). The F ratios and their significance from the analysis of variance are shown in Table 4.1. The general trend was similar in all the species except R. flammula. Malate levels fell with increasing $Fe(II)SO_4$ concentrations. C. angustifolium and Glyceria maxima had more malate in the 0.01mM treatment than the controls, but the differences were not significant. There was no relationship between absolute levels of malate in the control treatments and Fe(II) tolerance. There also appeared to be no relationship between the response of malate levels to increasing levels of $Fe(II)SO_4$ and the Fe(II) tolerance of the plants (fig 4.7).

Citrate. Variation in $Fe(II)SO_4$ concentration had a significant depressing effect on citrate concentration in the roots of Chamaenerion angustifolium, Ranunculus flammula and Glyceria maxima (fig 4.1 - 4.6, Table 4.1). Citrate levels in Eriophorum angustifolium were too low for accurate measurement and the data have not been presented. The general pattern of response of citrate was similar to malate. Above 0.01 mM citrate levels fell with an increase in $Fe(II)SO_4$ concentration. There was an increase in citrate levels in C. angustifolium and G. maxima from the controls to the 0.01 mM treatment. This parallels the increase in malate noted above. Ranunculus flammula was a notable exception to this pattern (fig 4.3). Citrate levels were higher in the 1.0 & 10.0 mM treatments. The responses of all the species are compared with their Fe(II) tolerance in fig 4.8. Except for R. flammula, changes in all the species were similar and there was no obvious relationship between tolerance and the response of citrate to $Fe(II)SO_4$ concentration in

the external solution. As with malate, there was no relationship between the absolute levels of citrate in the control treatments and Fe(II) tolerance.

Citrate:malate ratios in roots. The ratio of citrate:malate varied between species in the controls by nearly six-fold and $Fe(II)SO_4$ had an effect on the ratios (Table 4.2). The pattern of changes in the ratios can be seen in fig 4.9 where they are plotted on a percentage increase or decrease relative to the control (no $Fe(II)SO_4$). The pattern is variable, but Chamaenerion angustifolium and Senecio aquaticus always had a decrease in the ratio in $Fe(II)SO_4$. The large increase in citrate levels in R. flammula is reflected in the increase of its ratio in all except the 0.01 mM treatment. Deschampsia caespitosa had an increase in the ratio in all the treatments except 1.0 mM. Glyceria maxima had a small decrease in all treatments except 0.01 mM. The mean ratio for all the treatments, in comparison with the control, is shown in fig 4.9e. C. angustifolium and S. aquaticus had a decrease in the ratio. D. caespitosa and R. flammula had an increase and G. maxima had a small decrease. Comparing these results with Fe(II) tolerance (Chapter 2) it seems that low tolerance (C. angustifolium & S. aquaticus) is associated with a decrease in the citrate:malate ratio. The more tolerant species (Ranunculus flammula, Deschampsia caespitosa) have increased ratios. The most tolerant species, G. maxima, has a small decrease and does not fit into this pattern.

Malate and Citrate Levels in the Roots in Relation to Fe uptake.

Because the Fe uptake by the roots in this experiment could not be determined, the results are compared with Fe uptake in a previous experiment (Chapter 3). The Fe concentration in the roots of the various species in the 0 - 1.0 mM treatments was calculated using the equations for the linear regressions of Fe uptake and Fe(II) concentration in the culture solution between 0 and 2.0 mM. The values obtained have been plotted against malate or citrate levels in the roots (as percentages

of the control values) and are shown in fig 4.10 and 4.11. Three points can be made from these graphs. a) There is no correlation between Fe(II) tolerance and the relationship of acid and Fe concentrations in the roots. b) A small increase in Fe concentration in the roots between the 0 and 0.01 mM treatments (the two points with the lowest root Fe concentration) is often associated with a relatively large change in malate or citrate levels. c) With higher Fe concentrations in the roots, Fe concentrations and acid levels are usually inversely and linearly related. The exception again was R. flammula where malate levels decreased little and citrate levels increased.

The Effect of Flooding Natural Vegetation Sods with $Fe(II)SO_4$.

After the turves had been flooded for three days with water or $Fe(II)SO_4$ the flood water was tested qualitatively for the presence or absence of Fe(II) and Fe(III). Samples were drawn off, spotted onto filter paper and acidified with a few drops of 4% HCl. Fe(II) was detected by adding 4% potassium ferricyanide. A blue complex (Turnbull's blue) indicates Fe(II). Fe(III) gives a red complex when 4% potassium thiocyanate is added. In the control turf Fe(III) was not detectable and only a trace of Fe(II) was present. In the $Fe(II)SO_4$ treatment a trace of Fe(III) was present and Fe(II) was abundant. So the control contained very little Fe and the treated turf contained high levels of Fe(II).

After three days the shoots of Ranunculus flammula appeared healthy in both treatments, but the other species appeared unhealthy in the Fe treatment. The leaves of Carex nigra had become blackened and were beginning to wither. Senecio aquaticus had dark brown spots on its leaves. Both species were healthy in the control treatment. These symptoms resembled the bronzing caused by Fe toxicity described in Chapter 2. The roots of Ranunculus flammula appeared healthy in both treatments. These results agree with the conclusions of Chapter 2, where R. flammula was found to be more tolerant than S. aquaticus in

solution culture. But if the Fe concentrations are compared it appears that the root tips of R. flammula showed toxicity symptoms at 0.4 mmol Fe per litre in solution culture, but withstood approximately 100 mmol l^{-1} in peat over the same period. No definate explanation can be put forward for this and, although relative tolerance between the species is maintained in peat, the actual levels of Fe causing toxicity cannot be compared.

The malate and citrate levels in the roots of R. flammula after three days of flooding are shown in Table 4.3. Malate was slightly decreased in the Fe treatment, but citrate levels were increased 1.7 times. The levels of both malate and citrate were higher in these plants than those used in the solution culture experiment (fig 4.3). The response to Fe was similar in this experiment and the water culture experiment.

The Effect of Fe(II)SO_4 on the Aerobic Respiration Rates (QO_2) of Roots.

The effect of Fe(II)SO_4 on QO_2 was variable (fig 4.12). In general respiration was inhibited by Fe addition, although in Deschampsia caespitosa it was stimulated above 0.1 mM. Microbial contamination cannot be ruled out because the root systems were not surface-sterilised before the respiration measurements. D. caespitosa may have been contaminated since, unlike the other species, the rate increased towards the end of the measurement period. QO_2 of Oryza sativa was completely inhibited at 10 & 20 mmol l^{-1} . As with the acid levels there was no apparent relationship between tolerance and the effect of Fe on QO_2.

Table 4.1 - Variance ratios (F) and their significance (p) for the effect of iron (II) sulphate on malate and citrate levels in roots of various species. The analysis of variance refers to the data shown in figs. 4.1 - 4.6.

	Malate		Citrate	
	F	p	F	p
Chamaenerion angustifolium	2.98	0.05	7.76	0.01
Senecio aquaticus	9.03	0.01	2.26	0.05
Ranunculus flammula	1.62	0.05	4.33	0.05
Eriophorum angustifolium	15.72	0.001	-	-
Deschampsia caespitosa	6.32	0.05	2.54	0.05
Glyceria maxima	5.42	0.05	7.38	0.01

Table 4.2 - Citrate:malate ratios in the roots of various species after exposure to various concentrations of iron (II) sulphate in deoxygenated 4mM calcium nitrate.

	Iron (II) concentration, mM				
	0	0.01	0.1	1.0	10.0
Chamaenerion angustifolium	1.47	1.15	1.05	0.22	0.06
Senecio aquaticus	2.95	1.72	1.32	2.15	0.86
Ranunculus flammula	0.56	0.41	0.90	1.75	2.36
Deschampsia caespitosa	0.47	0.81	0.51	0.21	0.88
Glyceria maxima	1.00	1.14	0.52	0.53	0.82

Table 4.3 - The effect of flooding for three days with water and iron(II) sulphate (approximately 100mM) on malate and citrate levels in the roots of Ranunculus flammula growing in turves of natural vegetation.

	μmol. g. fresh wt.$^{-1}$ (with range) n = 2		citrate / malate ratio
	Malate	Citrate	
Flooded with water	1.83(1.62-2.05)	4.43(4.23-4.64)	2.42
Flooded with iron (II) sulphate	1.32(1.08-1.57)	7.52(7.07-7.98)	5.70

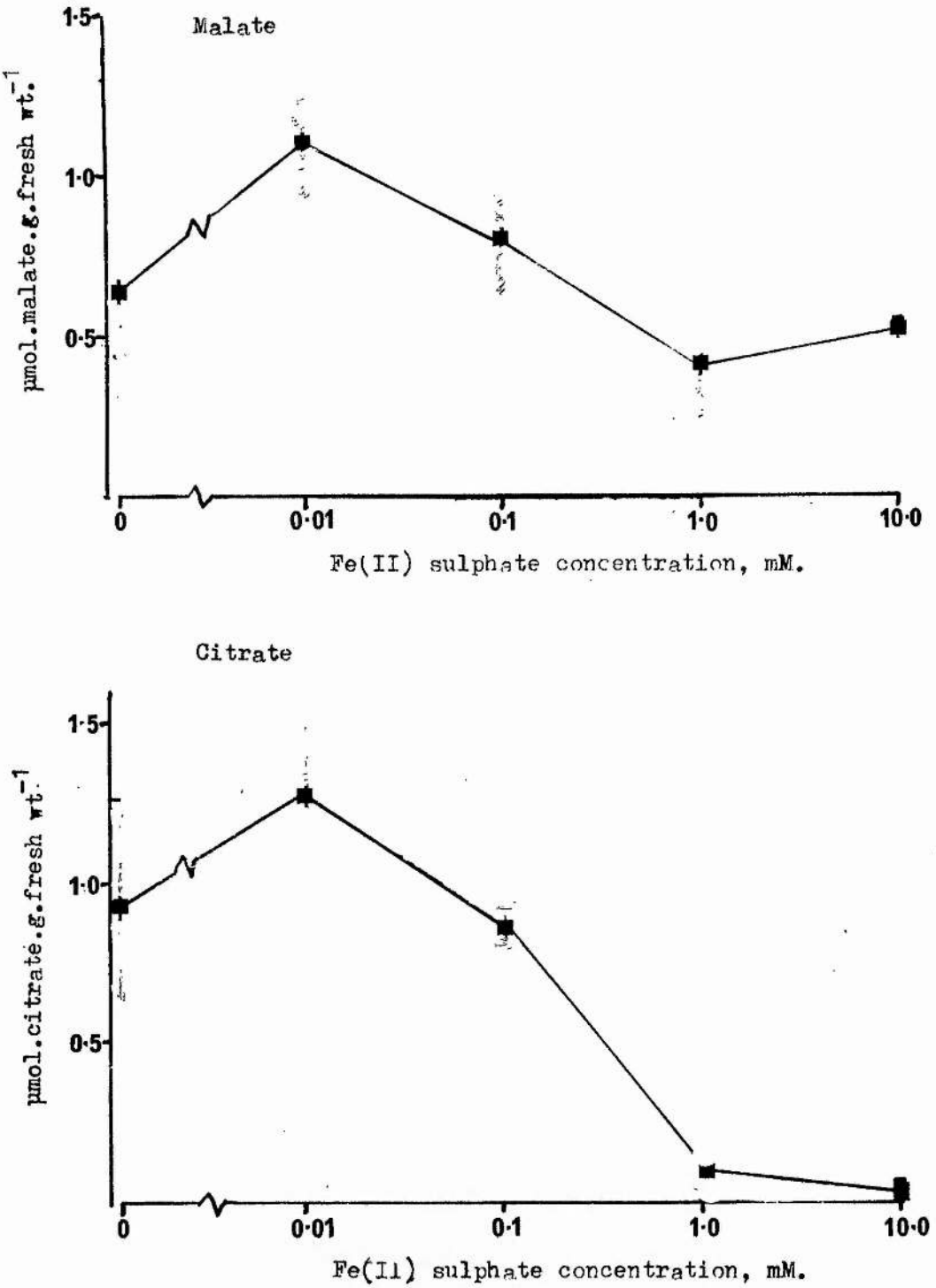
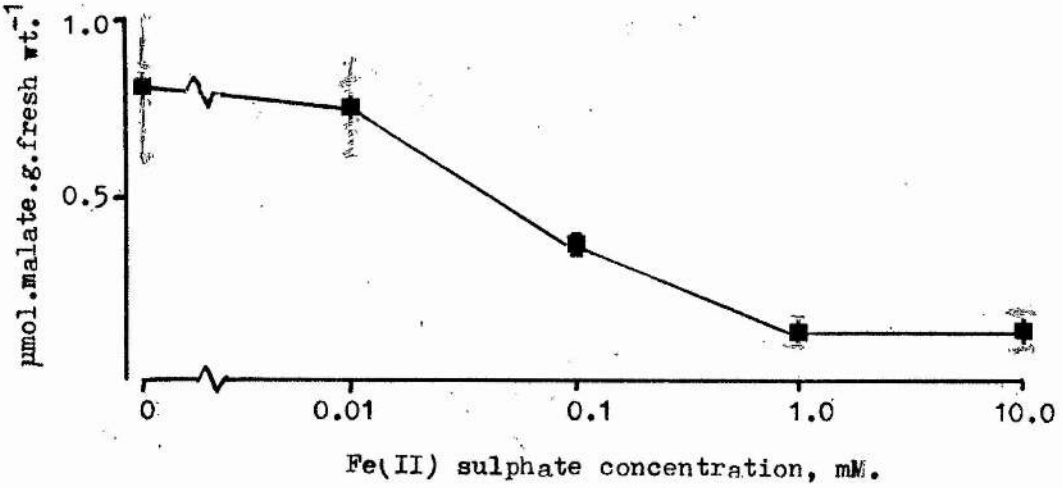


Fig.4.1. The effect of treatment for 3 days in deoxygenated 4mM calcium nitrate solution containing Fe(II) sulphate on malate and citrate levels in the roots of Chamaenerion angustifolium.



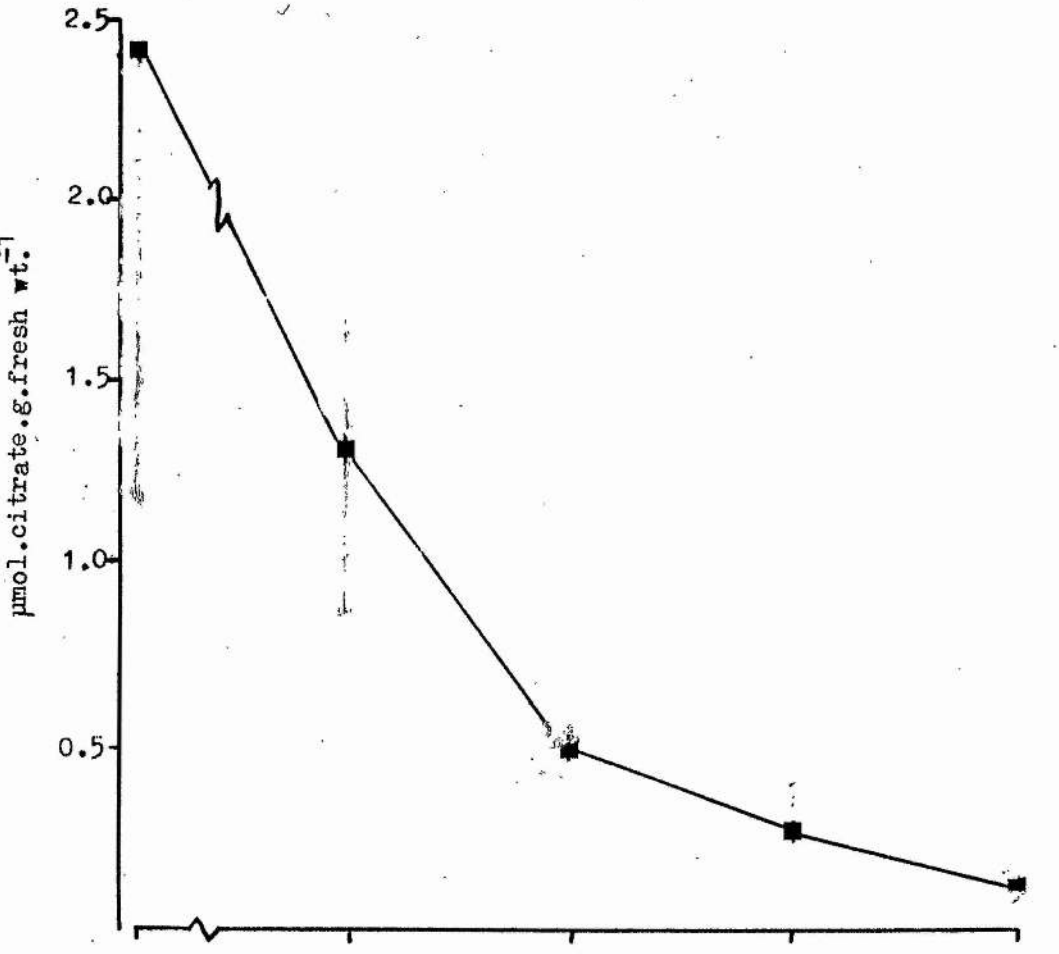


Fig.4.2. The effect of treatment for 3 days in deoxygenated 4mM calcium nitrate solution containing Fe(II) sulphate on malate and citrate levels in the roots of Senecio aquaticus.

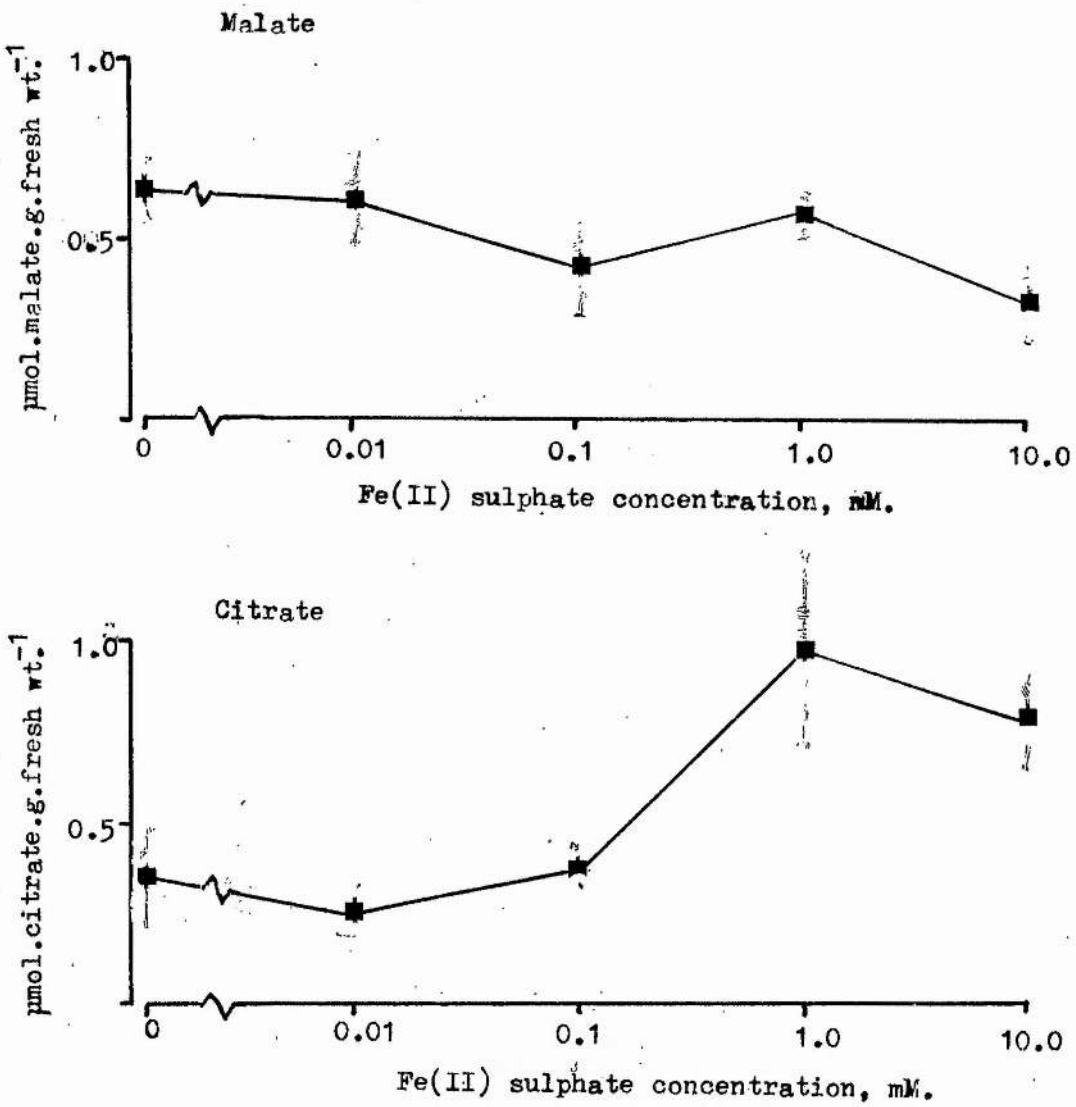


Fig.4.3. The effect of treatment for 3 days in deoxygenated 4mM calcium nitrate solution containing Fe(II) sulphate on malate and citrate levels in the roots of Ranunculus flammula.

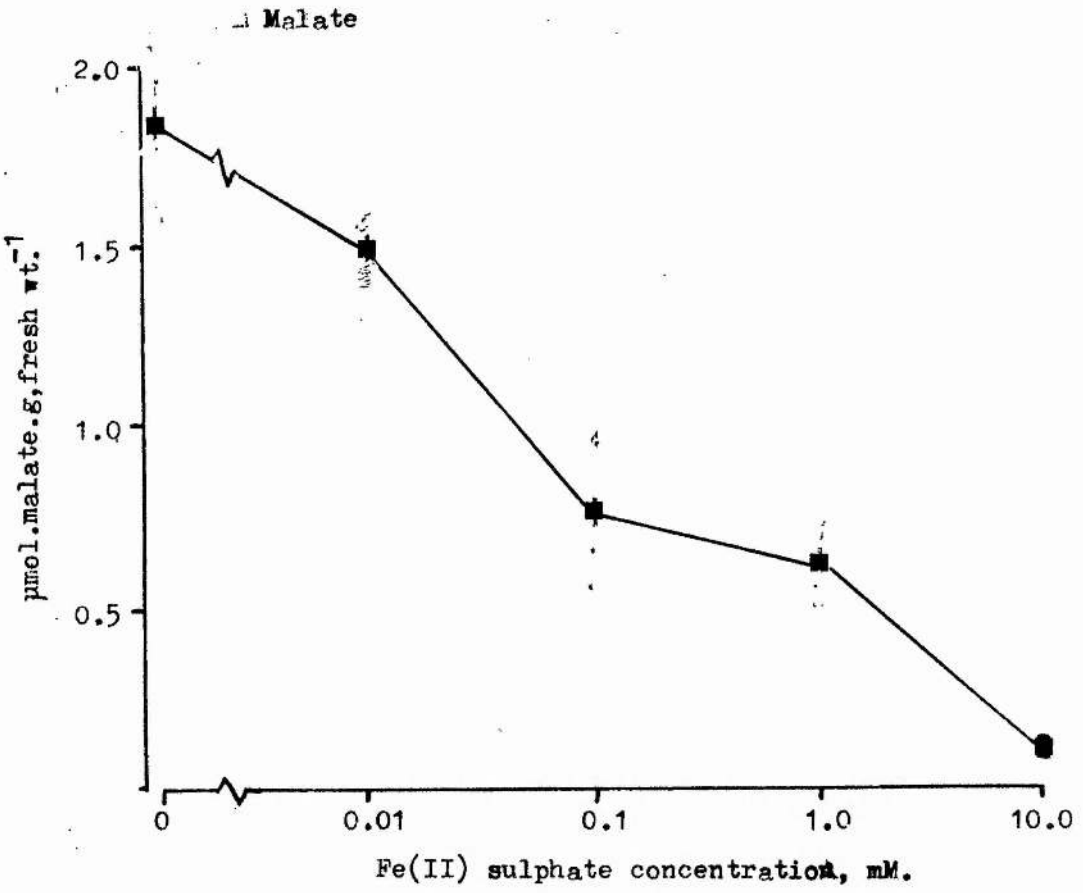


Fig.4.4. The effect of treatment for 3 days in deoxygenated 4mM calcium nitrate solution containing Fe(II) sulphate on malate levels in the roots of Eriophorum angustifolium.

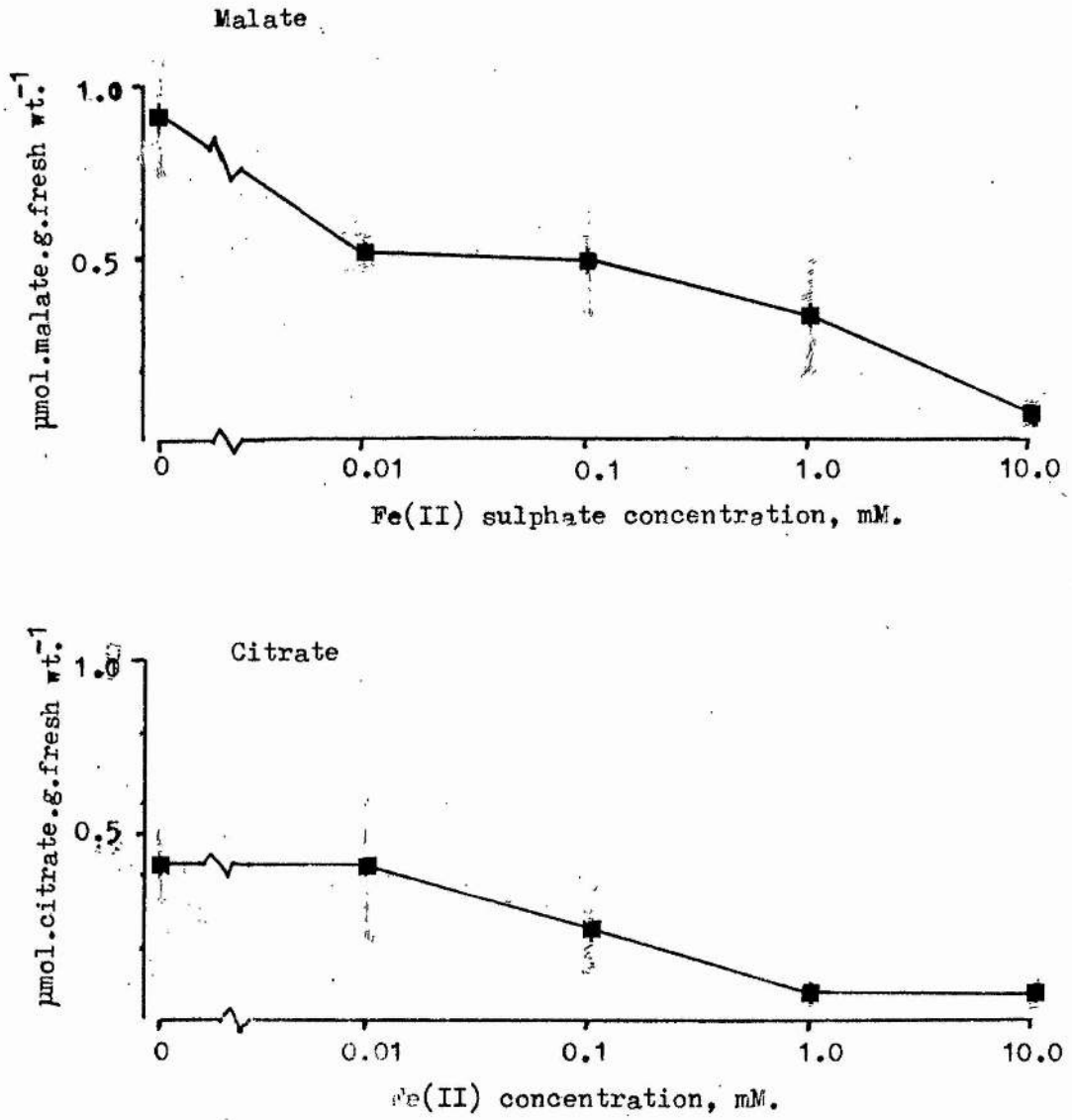


Fig.4.5. The effect of treatment for 3 days in deoxygenated 4mM calcium nitrate solution containing Fe(II) sulphate on malate and citrate levels in the roots of Deschampsia caespitosa.

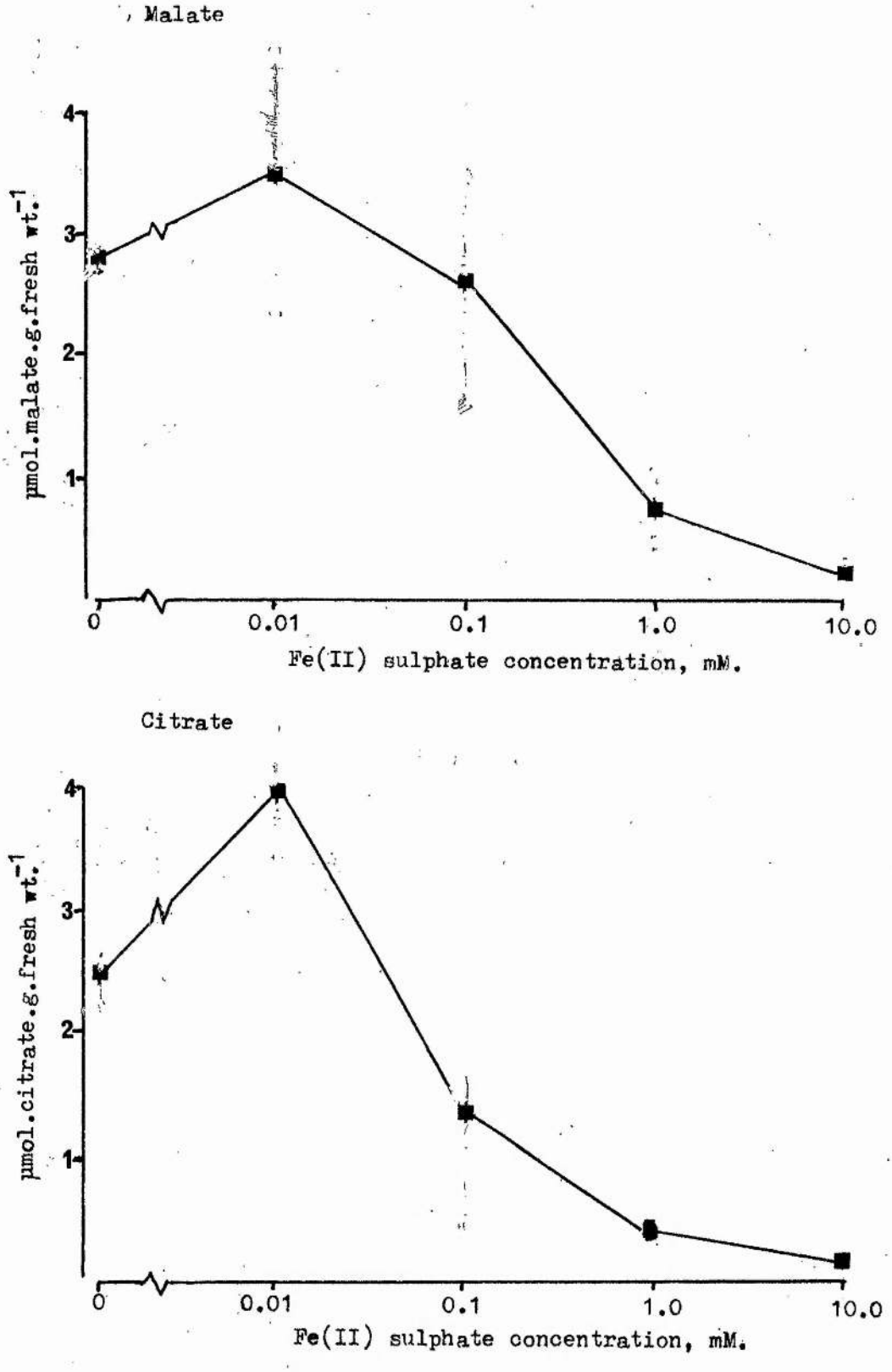


Fig.4.6. The effect of treatment for 3 days in deoxygenated 4mM calcium nitrate solution containing Fe(II) sulphate on malate and citrate levels in the roots of Glyceria maxima.

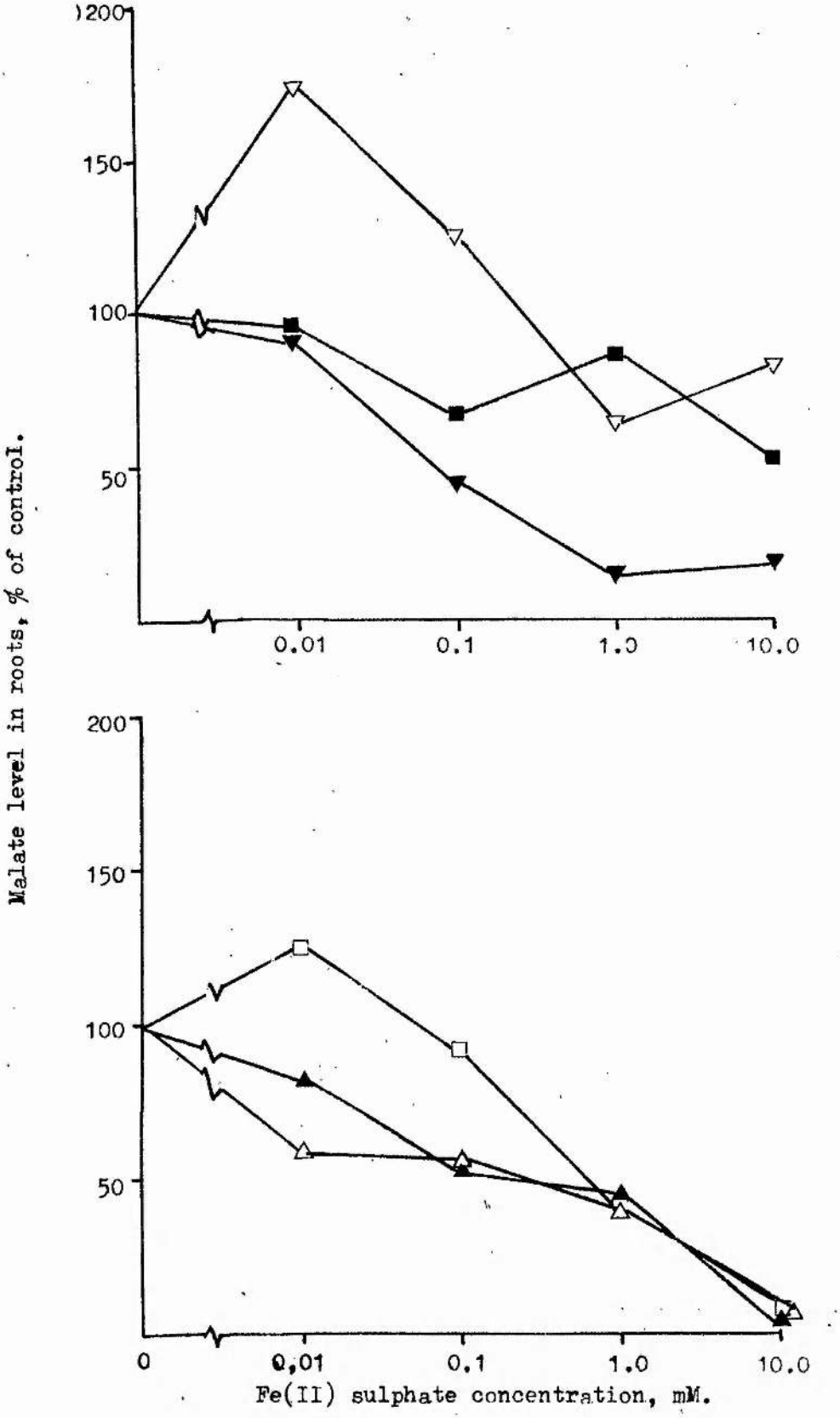


Fig.4.7. The effect of treatment for 3 days in deoxygenated 4mM calcium nitrate containing Fe(II) sulphate on malate levels in the roots of various species

▽ Chamaemerion angustifolium
△ Deschampsia caespitos
▼ Senecio aquaticus
□ Glyceria maxima
■ Ranunculus flammula
▲ Eriophorum angustifolium

Each point is the mean of 3 observations

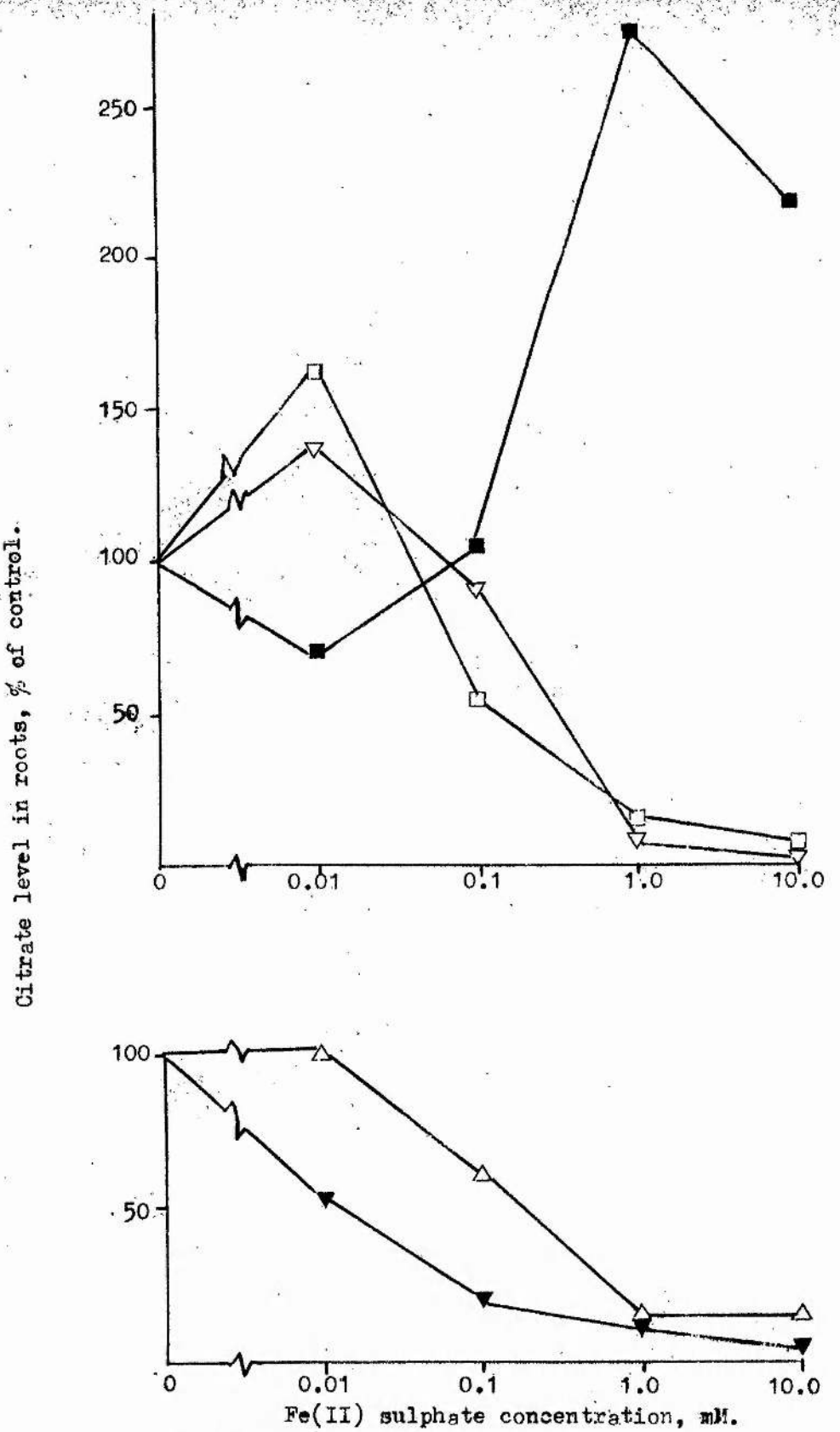


Fig.4.8. The effect of treatment for 3 days in deoxygenated 4mM calcium nitrate containing Fe(II) sulphate on citrate levels in the roots of various species. For symbols see fig.4.7.

Each point is the mean of 3 observations

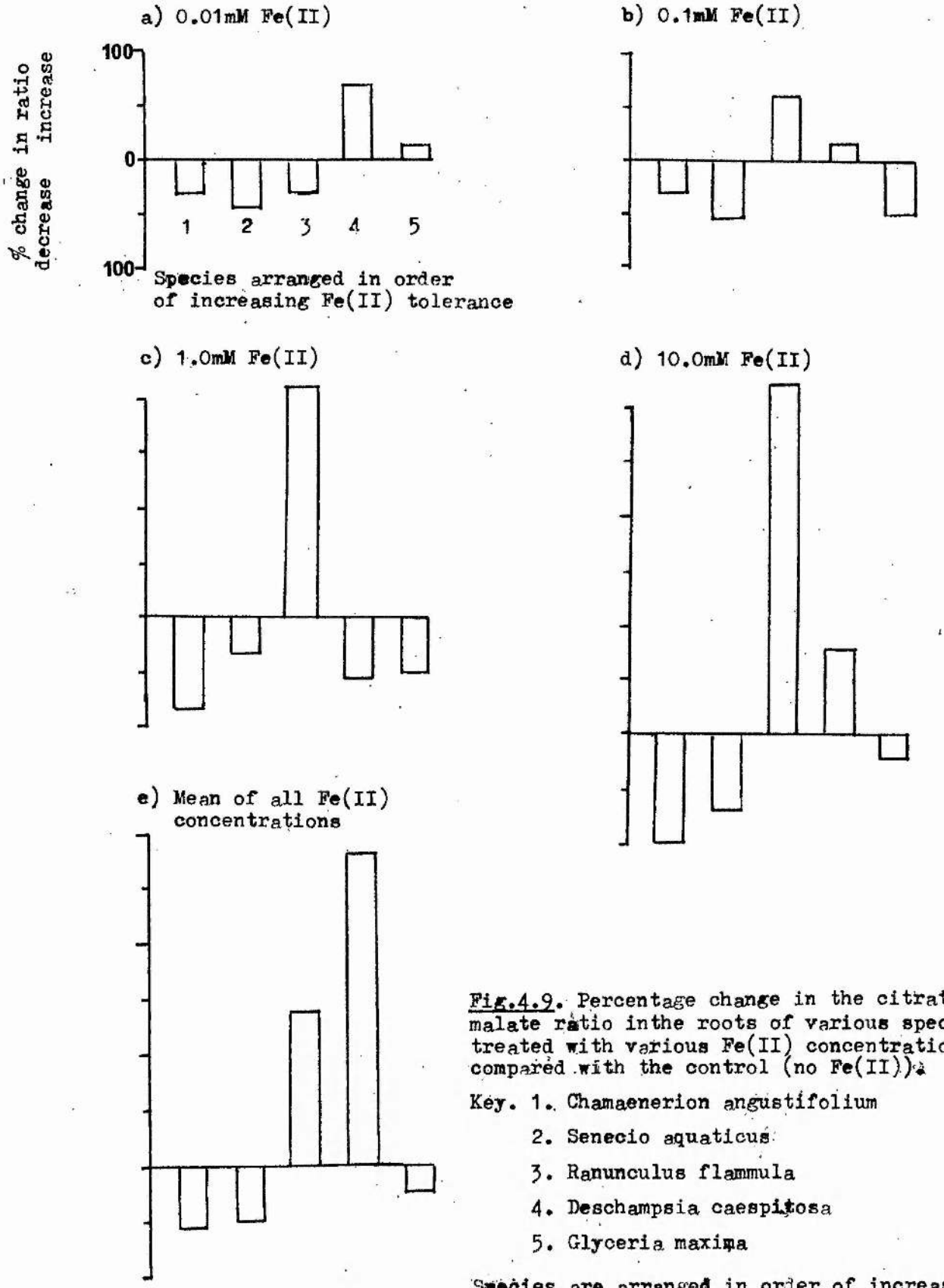


Fig.4.9. Percentage change in the citrate: malate ratio in the roots of various species treated with various Fe(II) concentrations compared with the control (no Fe(II)).

Key. 1. Chamaenerion angustifolium
2. Senecio aquaticus
3. Ranunculus flammula
4. Deschampsia caespitosa
5. Glyceria maxima

Species are arranged in order of increasing Fe(II) tolerance.

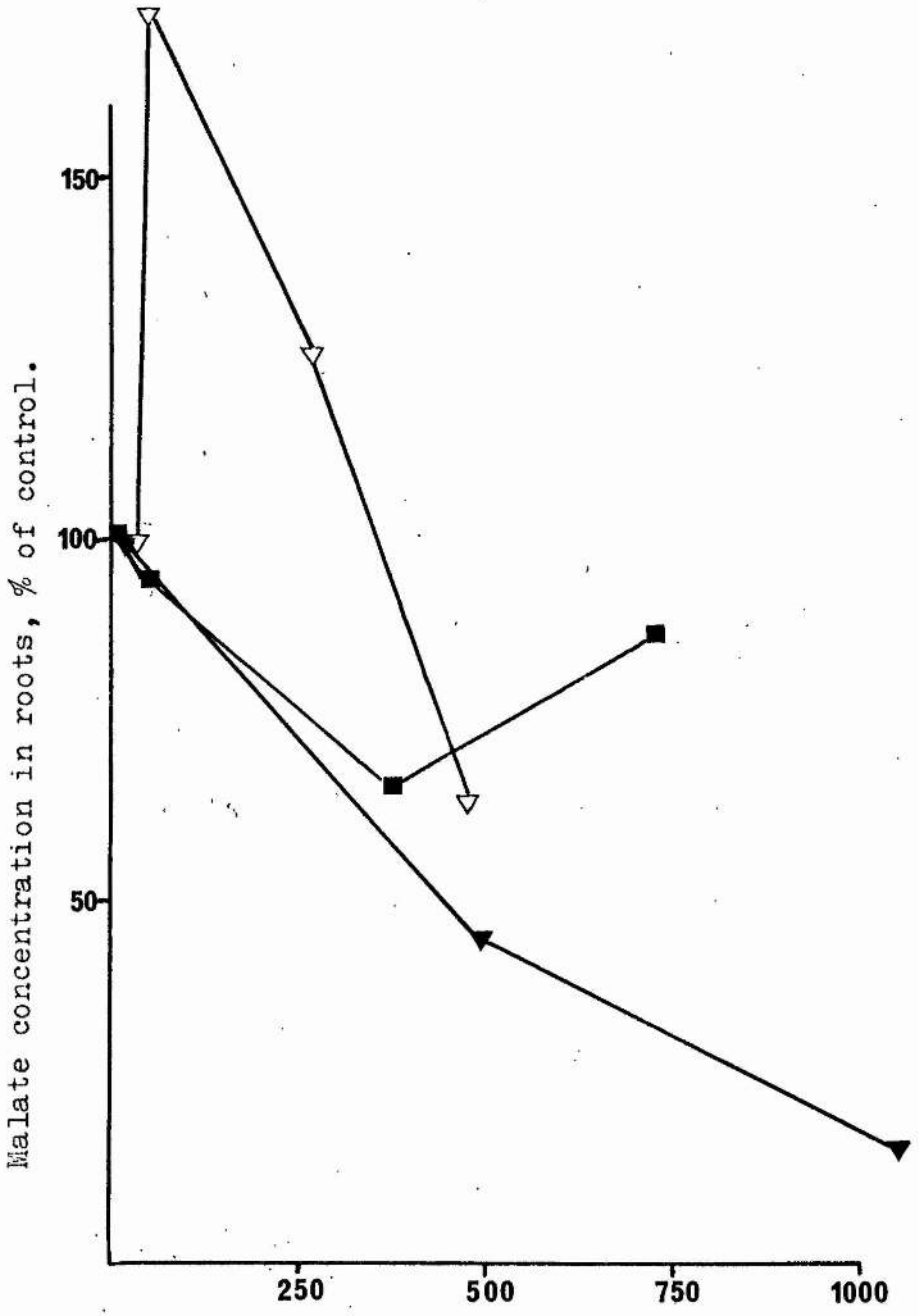


Fig.4.10. The relationship between iron concentration and malate concentration (% of the no Fe(II) treatment) in the roots of various species. The plants were kept for 3 days in deoxygenated 4mM calcium nitrate containing 0, 0.01, 0.1, and 1.0 mM Fe(II) sulphate. Data for iron concentration are from Chapter 3.

▽ Chamaenerion angustifolium

▼ Senecio aquaticus

■ Ranunculus flammula

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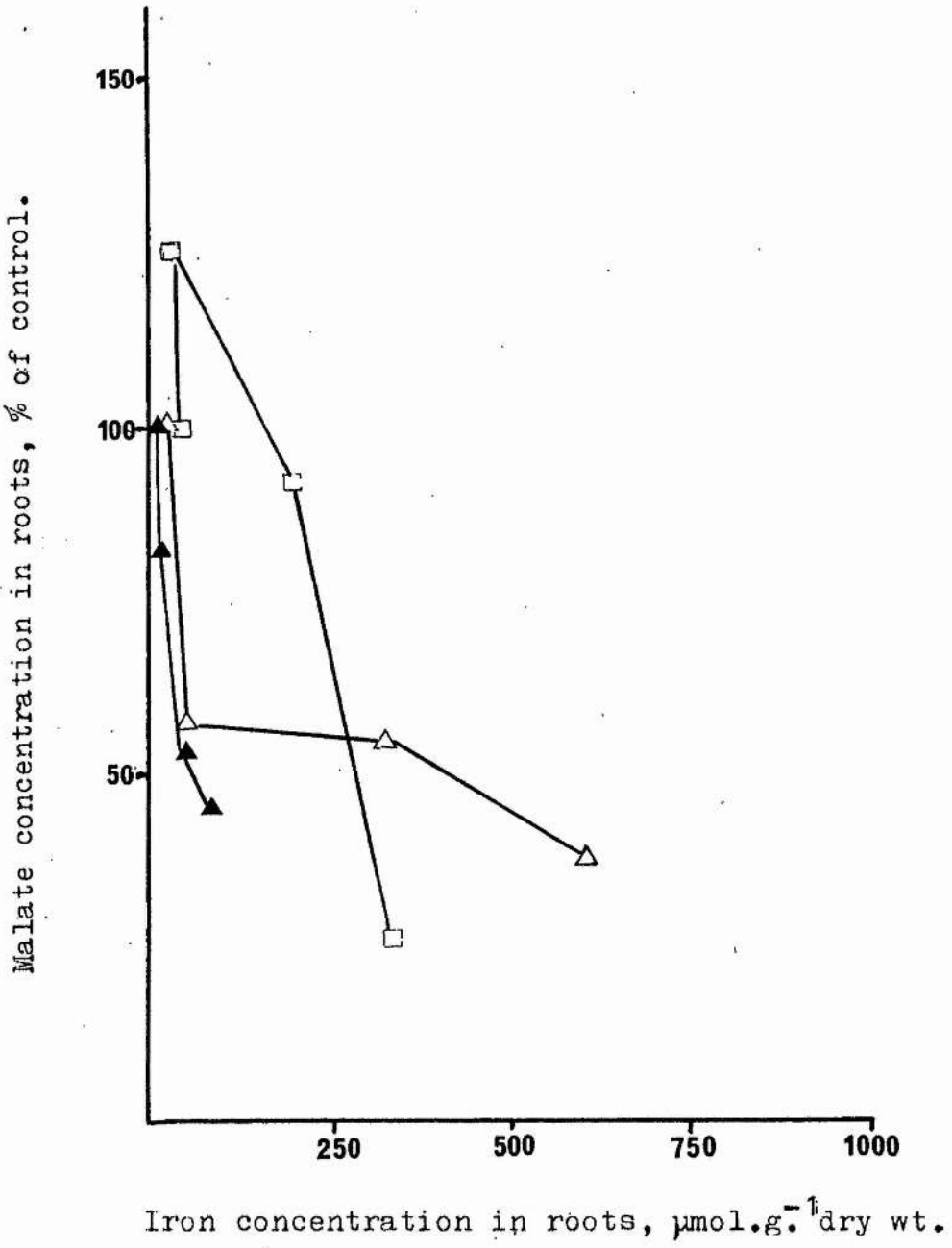

Fig.4.10. Continued.

▲ Eriophorum angustifolium
△ Deschampsia caespitosa
□ Glyceria maxima

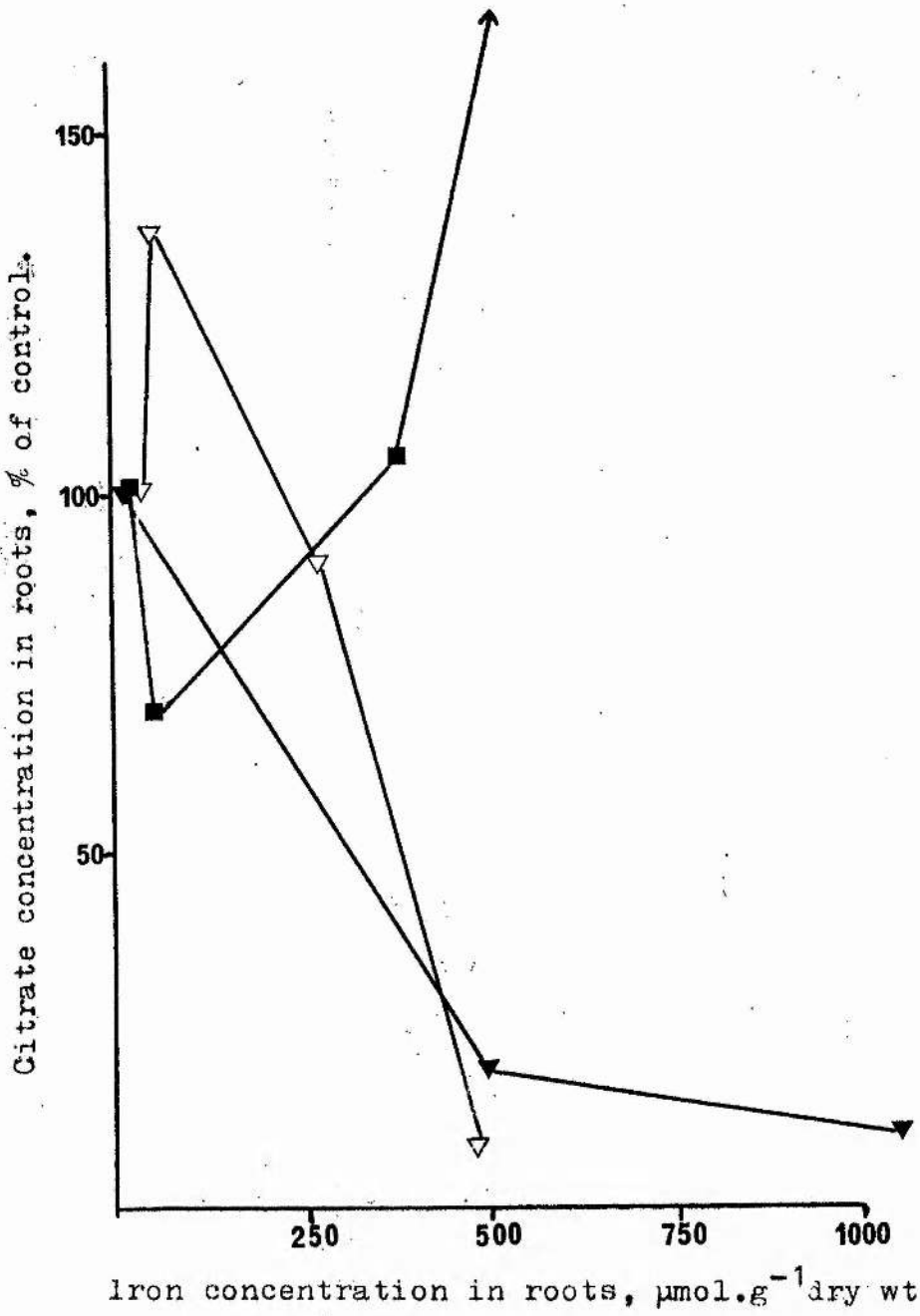

Fig.4.11. The relationship between iron concentration and citrate concentration (% of the no Fe(II) treatment) in the roots of various species. The plants were kept for 3 days in deoxygenated 4mM calcium nitrate containing 0, 0.01, 0.1, and 1.0mM Fe(II) sulphate. data for iron concentration are from Chapter 3.

▽ Chamaenerion angustifolium
▼ Senecio aquaticus
■ Ranunculus flammula

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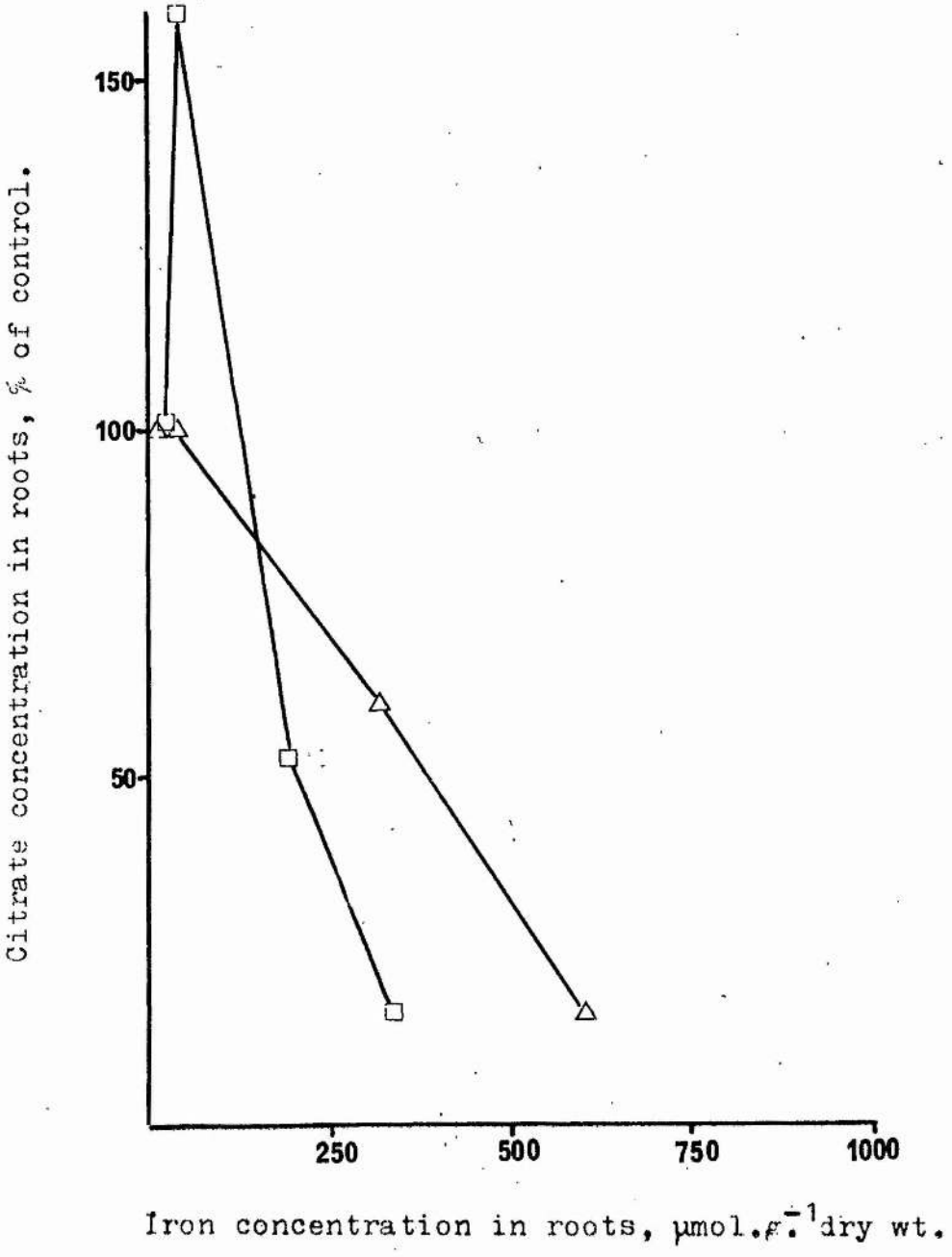


Fig. 4.11. Continued.

△ Deschampsia caespitosa

□ Glyceria maxima

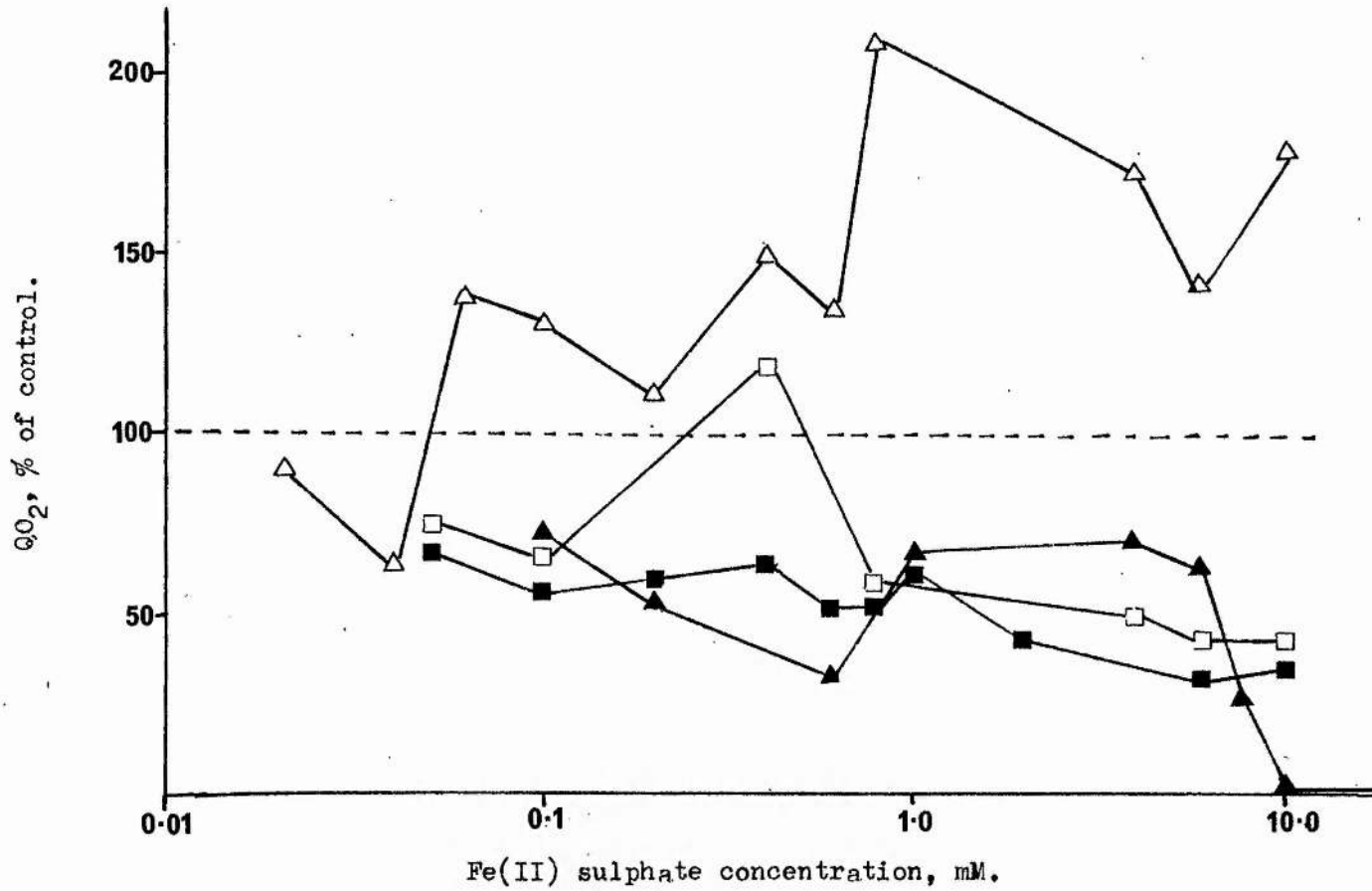


Fig.4.12. The effect of treatment for 4 days in deoxygenated 4mM calcium nitrate, pH 5.5, containing Fe(II) sulphate on the subsequent aerobic respiration rate (QO_2) of roots.

▲ Oryza sativa

△ Deschampsia caespitosa

■ Ranunculus flammula

□ Glyceria maxima

Discussion

Before the results are discussed in detail, three points must be raised concerning their interpretation. Firstly, Fe(II) was not the only variable in the culture. Sulphate levels also increased in exactly the same way. Secondly K^+ levels increased as the Fe(II) concentration increased because KOH was used to adjust the pH. The concentration of K^+ in the 10mM Fe(II) treatment did not exceed 0.5 mM and was considerably less in the other treatments. Thirdly, in the higher Fe(II) concentrations the root tips of some of the species had toxicity symptoms, so it can be suggested that the results could be caused by the loss of metabolites from dead roots. The importance of these factors is discussed below.

Excess uptake of anions by roots can lead to a decrease in organic acid levels. This has been demonstrated in the case of excess chloride uptake (Hiatt and Leggett, 1974). The decrease in malate observed in these experiments could be explained by assuming that sulphate is absorbed in excess of the other cations in the solution (Fe, Ca, K). Although sulphate is only absorbed slowly by plants, if its uptake exceeds its rate of reduction it will remain as sulphate within the plant (Kylin, 1960; Dijkshoorn, 1969). In an investigation of the effect of nitrate on organic acid levels in tomatoes Kirkby and Knight (1977) used a culture solution in which sulphate concentration varied greatly (3 - 26.5 mM). They concluded that this had little effect on their results. K^+ is rapidly absorbed in comparison with Ca or sulphate and excess cation uptake can lead to an increase in organic acid levels (Hiatt & Leggett, 1974). But since there was a general trend to decreasing malate and citrate levels (except for Ranunculus flammula) in this experiment, it would seem that this effect did not occur.

The root tips of Glyceria maxima had no toxicity symptoms in the 10 mmol l^{-1} treatment and the response of its malate and citrate levels to $Fe(II)SO_4$ was similar to other species which developed toxicity symptoms. Respiration was not completely inhibited in any of the species used for the malate and citrate experiment, so it would

seem that, even if toxicity symptoms do appear in the root tips over the short experimental period, the remainder of the root system can continue to metabolise, although at a reduced rate. In all the species except Chamaenerion angustifolium the root tips were healthy up to 0.1 mM. Deschampsia caespitosa and Eriophorum angustifolium were healthy up to 1.4 & 0.8 mM respectively (see Chapter 2).

If the results from the highest Fe(II) treatments are ignored, then the possible artefacts from sulphate, potassium and dead root tips can be minimised. The following discussion assumes that the effects can be mainly attributed to Fe(II).

The Effect of Fe(II) on Malate and Citrate Levels in Roots.

In most cases malate and citrate levels declined with increasing Fe(II) concentration. The exceptions were the increase in citrate in Ranunculus flammula and the initial increases in malate and citrate in Chamaenerion angustifolium and Glyceria maxima in the lowest Fe(II) concentration. G. maxima and R. flammula also had a decline in QO_2. The increase in QO_2 of Deschampsia caespitosa could have been caused by microbial contamination. If respiration is inhibited the decline of malate and citrate, as intermediates in aerobic respiration, could be the result. Copper inhibits respiration in Agrostis stolonifera roots and there is a decrease in malate dehydrongenase activity probably as a result of a general decline in protein content (Wu et al, 1975). Similar effects of Fe(II) could result in a general decrease in metabolic rate and lower levels of respiratory intermediates. However, this does not explain the increase in citrate levels in R. flammula.

Inhibition of nitrate reduction and assimilation could result in a decrease in malate and perhaps citrate levels. Nitrate assimilation involves the production of excess OH^- and the consequent synthesis of malate (which can be transformed to citrate). Roots assimilating nitrate have a higher organic acid content than those assimilating ammonium (Kirkby and Mengel, 1967; Raven and Smith, 1976) and increasing

NO_3^- results in increased organic acid (Kirkby and Knight, 1977). So if nitrate assimilation is inhibited organic acid levels could drop. Nitrate reductase is very sensitive to inhibition by metals *in vivo* or *in vitro* because it has a high sulphydryl content (Mathys, 1975; Ernst, 1976). Fe may inhibit nitrate reductase and this could lead to lower malate and citrate levels. Further experiments would be needed to check this specific effect. Fe could have a less specific effect by causing leakage of metabolites from the roots. Hiatt and Lowe (1967) found that metabolic inhibitors (sodium cyanide and dintrophenol) caused leakage of organic acids from excised barley roots.

Previous investigations have concentrated on the effects of Fe-deficiency and the addition of low levels of Fe, usually as Fe(III) chelate, on organic acid levels. Fe-deficient plants usually have higher organic acid levels, particularly citrate and malate (Iljin, 1951; De Kock and Morrison, 1958; Van Egmond and Atkas, 1977). Van Egmond and Atkas found that Fe-efficient species and varieties could accumulate malate, citrate, malonate and oxalate under Fe-deficiency. In sugar beet the effect was specific to each acid and not because of a general increase in levels. Addition of low concentrations of Fe(III) to plants has various effects on acid levels. Clark *et al* (1973) found that FeHEDTA from 1.8 - 53 μM in solution culture increased malate (x 2) and citrate (x 1.4). Succinate and fumanate were unaffected (De Kock and Morrison, 1958). In tomato the effect of added Fe(III) depended on the Fe-efficiency of the variety. Adding Fe(EDDHA or HEDTA) to an Fe deficient Fe-efficient variety caused a decrease in citrate levels at 1 and 10 μM, but a return to the levels present without Fe at 60 μM. Levels in an Fe-inefficient variety were unaffected (Brown and Chaney, 1971). The results of these investigations do not present a clear picture and suggest that the response to added Fe depends on the Fe status of the plant. Deficient plants may not increase their acid levels because they start with higher levels. The increases in malate and citrate observed in the above experiments do not agree with the effects of Fe(II) under hypoxia in the present experiment, except

in the cases of the initial increases in Glyceria maxima and Chamaenerion angustifolium and the increase in citrate in Ranunculus flammula.

Malate and Citrate Levels in Roots and Fe(II) Tolerance.

Malate was originally suggested as being the natural chelating agent for Fe in soybeans (Tiffin and Brown, 1962) however investigations since then have shown that the Fe citrate complex has a higher stability and is likely to form an Fe chelate in plants (Brown and Tiffin, 1965; Tiffin, 1966a and b; Clark et al, 1973). It is possible that high citrate or malate levels in the roots of Fe(II) tolerant plants could chelate excess Fe absorbed and render it less toxic. The results do not support this hypothesis since there was no relationship between tolerance and the acid levels. Ernst (1976) and Mathys (1977) have suggested a protective role for malate in zinc tolerant clones of Agrostis tenuis, Silene cucubalus and Thlaspi alpestre. These clones have higher malate levels than non-tolerant clones. The malate could chelate excess zinc and store it in the vacuole.

The results do not support the hypothesis that the more tolerant species could maintain their citrate and malate levels at higher Fe(II) concentrations than the less tolerant species. The decline in levels was general in all the species except R. flammula and apparently bore no relationship to tolerance. With the present evidence these results cannot be explained. The increase in citrate levels in R. flammula occurred in the solution culture experiment and the field experiment. Again there is insufficient data to give an explanation. Absolute levels of citrate and malate differed in R. flammula between the field and solution culture experiments. Levels were higher in the latter. There are two possible explanations for this. The plants may have inherently different levels because they come from different populations. Alternatively it could have been because of differing nitrogen nutrition: the solution culture plants were in a high nitrate medium, whereas it is possible that the plants in waterlogged peat were subjected

to lower levels of ammonium nitrogen. Plants grown on nitrate have higher organic acid levels than those on ammonium (Kirkby and Mengel, 1967; Raven and Smith, 1976).

Citrate:Malate Ratios in the Roots.

The citrate:malate ratios varied in magnitude between species and the change in the ratio when Fe(II) was added differed between species. The two least Fe(II) tolerant species, Chamaenerion angustifolium and Senecio aquaticus always had decreased citrate:malate ratios in Fe(II). The response of the other species was more variable, but with the exception of G. máxima, the more tolerant species had an increased ratio. It could be suggested that the less tolerant species tend to have their citrate levels decreased more than their malate levels when Fe is added. In contrast to the large changes in the ratio observed here in the 0.01mM treatment, Clark et al (1973) found that increasing Fe(III)HEDTA in the culture solution did not alter the citrate:malate ratio in maize roots, but De Kock and Morrison (1958) found a small decrease in the ratio in mustard leaves from plants grown in 1.8 as opposed to 222.5 μM Fe(III). Further investigation is needed to determine the cause of variations in the citrate:malate ratio between species.

Malate and Citrate Levels in the Roots in Relation to Fe Uptake.

If Fe(II) is to have an effect on malate and citrate levels some of it must be absorbed into the root cells. Fe precipitated onto cell walls will not be metabolically active. The measurements of total Fe in the roots does not give a direct measurement of intracellular Fe. Much of the Fe is probably precipitated onto the cell walls, and a small amount may be ionically bound (Clarkson & Sanderson, 1978). This also occurs with other metals. 24 - 90% of zinc in roots of Agrostis tenuis is bound to cell walls, the proportion depending on the external concentration (Ernst, 1976), and about 50% of the copper content of the roots of the same species is bound to the cell wall (Turner, 1970).

In an Fe-efficient tomato cultivar intracellular Fe concentration increased with a range from 0 - 60 μM FeHEDTA in the culture solution, but there was no increase in an Fe-inefficient cultivar (Brown and Chaney, 1971). So it cannot be assumed that there is a simple relationship between external concentration and intracellular Fe content of the roots in this investigation. Although Eriophorum angustifolium can exclude Fe from its roots and absorb very little (see Chapter 3) the effect on malate levels is just as great as in Senecio aquaticus which absorbs a large amount of Fe. An increase in the external Fe(II) concentration probably increases intracellular Fe concentration in all the species resulting in the direct relationship between Fe concentration in the roots and changes in the levels of malate and citrate.

Fe Uptake and Malate Accumulation in Flooded Plants.

Keeley (1978) suggested that malate accumulation in roots under flooded conditions could be the result of increased cation uptake because of the increased availability of Mn^{2+} in waterlogged soils. But if this conclusion is extended to Fe(II), the results here do not support this idea. Except in Ranunculus flammula, malate was decreased by high levels of Fe(II). The results do suggest that it is important to control the ionic environment of the roots when investigating organic acid metabolism under flooded conditions or in solution culture.

Further studies are necessary to establish why the response of malate and citrate levels to Fe differs little between most of the species, and why R. flammula differs. More detailed studies of metabolism under different Fe levels such as respiration rates, enzyme activities and protein content, could clarify some of the results. The same responses may not have occured if a different nitrogen source had been used (ammonium or ammonium plus nitrate). This could also be investigated.

CHAPTER 5

Iron (II) Tolerance in Excised Root Tips of Various Species

Introduction

Evidence has been presented in Chapter 2 that there is no relationship bweteen Fe(II) tolerance and the amount of air space in the roots of a range of species. Assuming that there is a relationship between air space and ROL (Armstrong, 1972), this result does not support the hypothesis that Fe(II) tolerance is a result of Fe(II) oxidation and immobilization as suggested by some authors (Armstrong, 1979; Green and Etherington, 1977; Martin, 1968). Although oxygen diffusion from roots has not been measured in the present experiments, it was shown in Chapter 3 that there was an inverse relationship between air space and Fe uptake by the roots of various species. It was suggested that this exclusion of Fe was the result of increased ROL from the roots of species with more air space. However, this exclusion cannot explain tolerance because there was no relationship between tolerance and either air space or Fe uptake.

An alternative approach to assessing the role of aerenchyma and oxygen diffusion in Fe(II) tolerance is to eliminate any oxygen supply from the shoot by using excised roots or root tips. In this way the roots of various species can be treated with controlled amounts of oxygen or with anoxia. If a suitable indicator of Fe(II) toxicity can be found, the hypothesis that roots from species of differing Fe(II) tolerance do not retain their differential tolerance when excised can be tested. The effect of Fe(II) on various processes in excised root tips under aerobic or anoxic conditions have been tested to find a suitable indicator for Fe(II) toxicity. Results are presented in this Chapter for potassium leakage under anoxia, viability tested with triphenyltetrazolium chloride under anoxia or aerobic conditions and the elongation of root tips under aerobic conditions.

Materials and Methods.

In all the experiments healthy white tips from primary roots were used. The plants were grown in sand culture and watered once a week with 1/5 strength Hoagland's solution.

Potassium leakage from excised root tips. The method was based on Wainwright and Woolhouse (1977). Root tips, 1.5 cm long, were excised and preincubated for 4 h in an aerated solution of 30 mM KCl and 0.5 mM $CaSO_4$. This ensured that the roots were equally loaded with K^+. After this treatment the root tips were given 3 five-minute washes in distilled water to remove K^+ from the free space. Two root tips were placed in a small vial containing 2 ml of deoxygenated $Fe(II)SO_4$ solution. The composition of the Fe solutions and times of incubation are given in the results. After incubation in the Fe(II) solutions in a shaking water bath at 25 °C, the root tips were removed from the vials and their diameters measured under a microscope. The surface area of the tips was calculated assuming that they were cylindrical. The K^+ released into the incubation medium was measured by atomic absorption spectrophotometry using the 766.5 nm absorption line with a Shandon Southern A3400 Atomic Absorption Spectrophotometer. The results are expressed as K^+ loss per mm^2 of root surface per hour and are the means of two replicates.

Elongation of excised root tips. The method was based on Brown and Sutcliffe (1950) and Wainwright and Woolhouse (1977). Root tips were excised using a mounted razor blade. The mean length of the tips was 2.26 mm (standard deviation of 0.15mm). Tips of this length were used so as to include both the zone of cell division and the zone of cell elongation. Wainwright and Woolhouse found that both those zones were included in the terminal 1.8mm of Agrostis tenuis roots. After excision the root tips were preincubated in 0.5 mM $CaSO_4$ for 1 h. Since the Fe (II) solutions did not contain calcium, this pretreatment was found to enhance root elongation. After the pretreatment the length of each

tip was measured using a travelling microscope. The measured tips were placed in 250ml beakers each containing 4 ml of incubation medium (3 tips per beaker) which contained various concentrations of Fe(II). The incubation medium contained 2mM potassium acetate buffer, pH 5.0, 2.5 mM KCl (total K concentration was 4mM) and 2% sucrose. Fe(II) was added as $Fe(II)SO_4$. The incubation medium was not deoxygenated. Ascorbic acid was added in preliminary experiments to prevent Fe(II) oxidation, but since it inhibited root elongation, it was not included in the final incubation medium. The addition of KCl enhanced root elongation. It was necessary to use a buffer because the pH of the medium would be decreased by increasing Fe(II) concentration, and Fe(II) oxidation also results in acidification. Acetate buffer was chosen because it does not precipitate Fe(II) and was not likely to chelate it to any extent (Wainwright and Woolhouse, 1975). Acetic acid can be toxic and may inhibit root growth (Lynch, 1977; Sanderson and Armstrong, 1980). However at a concentration of 2 mM no inhibition occurs with pea (C. Mawer, personal communication) or barley roots (Lynch, 1977). The beakers, containing 3 root tips in 4 ml of incubation medium, were covered with "cling film", through which holes were punctured to allow free gas exchange, and incubated in the dark for 24 h at 25 °C in a shaking water bath. After this period the tips were removed from the beakers and remeasured. Each treatment was replicated 3 times, with 3 root tips in each replicate. Elongation is expressed as $mm.24h^{-1}$.

Viability of root tips. The viability of the root tips was tested using triphenyl tetrazolium chloride (TTC). Its use was discussed in Chapter 2. Tips from the elongation experiment were rinsed with distilled water and placed in vials containing 3 ml of 0.6% TTC in 50 mM potassium phosphate buffer, pH 7.4 with 2% sucrose. They were vacuum infiltrated in a desiccator for 10 minutes and then transferred to a dark incubator at 25 °C for 1 hour. After this they were examined for the presence or absence of staining. Results are expressed as % of root tips from each treatment stained. Root tips from the potassium

leakage experiment were not vacuum infiltrated and were incubated at 30 °C.

Results

Potassium Leakage from Excised Root Tips.

Two experiments were carried out to investigate the effect of Fe(II) on potassium leakage from excised root tips and its usefulness as an indicator for Fe(II) toxicity. In the first experiment root tips of Senecio jacobaea and Glyceria maxima were incubated for 1 hour in deoxygenated water containing various concentrations of $Fe(II)SO_4$. The results are shown in fig 5.1. Under these conditions potassium loss from G. maxima was unaffected by Fe(II) up to 100 mM, whereas S. jacobaea had greatly increased leakage in all the Fe(II) treatments. The experiment was not entirely satisfactory because the pH varied between 6.62 and 3.76 (fig 5.1). This could itself contribute to potassium leakage. There was still a big difference in the response of the two species in the 0.1 mM treatment where the pH was little different from the control.

The second experiment attempted to overcome the variation in pH by using a buffered incubation medium. It proved difficult to find a non-toxic buffer which did not contain potassium or precipitate Fe, so a deoxygenated 50mM glycylglycine solution was used. This stabilised the pH of the Fe(II) solutions between 5.11 and 6.47 (fig 5.2). Nevertheless it was still not satisfactory because glycine chelates Fe(II) and (III) and metals have reduced toxicity when chelated (Ernst, 1978). The results of the experiments with root tips of various species incubated in deoxygenated 50mM glycylglycine for 6.5 hours are shown in fig 5.2. Increasing Fe(II) concentration resulted in an increase in potassium leakage in all the species. In the 0.1 and 1.0mM treatments S. jacobaea had a greater increase in leakage than the other species. At 1.0mM the leakage in G. maxima was similar to S. jacobaea but the other species remained lower. There appeared to be a differential response between S. jacobaea and the other species, but the responses of R. flammula, N. stricta and G. maxima could not be distinguished from each other. The maximum

leakage in all species was never more than 20% of the leakage induced by killing the roots in 2M HCl, so in no case did Fe(II) cause a serious disruption of membrane semipermeability.

Elongation of Excised Root tips.

The excised root tips of all the species tested were able to elongate in the incubation medium. Addition of Fe(II) caused significant inhibition of elongation in all the species except Glyceria maxima (fig 5.3). The variance ratios, showing the effect of Fe(II) on root tip elongation, are shown in Table 5.1. The response of the species is compared in fig 5.4 where elongation is expressed as a percentage of the no Fe control. The response of the species to Fe(II) is clearly different. Glyceria maxima was unaffected whereas Senecio aquaticus was severly inhibited in all the Fe(II) treatments. Ranunculus flammula and Eriophorum angustifolium were intermediate in their response. Considering the concentration of Fe(II) resulting in 50% inhibition of root elongation (read from fig 5.4), the order of sensitivity to Fe(II) was (with Fe(II) concentrations in μM):

S. aquaticus (10) < E. angustifolium (18) < R. flammula (32) < G. maxima (> 2000)

The results do not support the hypothesis that excised roots will lose their differential tolerance to Fe(II).

Viability of Excised Root Tips.

The viability of root tips from the elongation experiment which were exposed to an aerobic solution of Fe(II) was determined using TTC reduction. The percentage of root tips staining after each treatment is shown in fig 5.5. Except in G. maxima, Fe(II) decreased the viability of the root tips and the degree of sensitivity followed the same order as determined from root tip elongation. The greater elongation of E. angustifolium tips in the 2.0mM treatment as opposed to the 0.2mM treatment (fig 5.3) is reflected in the greater proportion of tips staining in TTC (fig 5.5).

Some of the tips had become blackened in the terminal 1mm, and this symptom was similar to the blackening of root tips from intact plants caused by Fe(II) toxicity (Chapter 2). The occurence of blackened tips is shown in Table 5.2. No blackening occured in G. maxima. In S. aquaticus all the treatments except the control were blackened. R. flammula was intermediate. Tips were blackened in all treatments including the control of E. angustifolium, so it may have had another cause. These results provide further evidence that excised root tips from different species respond differentially to Fe(II).

The viability of root tips after incubation in deoxygenated 50mM glycylglycine is shown in Table 5.3. Again there was some evidence for differential tolerance between species. Senecio jacobaea was the most sensitive, R. flammula intermediate while Nardus stricta and G. maxima remained viable in all the Fe(II) treatments.

Respiration Rate of Excised Roots.

Several preliminary experiments were carried out on the effect of Fe(II) on respiration rate (QO_2 & QCO_2). Respiration was not inhibited in Senecio jacobaea or Ranunculus flammula after exposure for 24 hours to deoxygenated solutions of Fe(II). Respiration was not a useful indicator of toxicity in excised roots. Similar results were obtained by Wu et al., (1975) for the effect of short term exposure to copper on excised roots of Agrostis stolonifera. Respiration was not affected, although it was inhibited after longer exposures of the whole plant to copper.

Table 5.1 - Variance ratios (F) and their significance (p) for the effect of iron (II) sulphate on the elongation growth of excised root tips. The analysis of variance refers to the data shown in fig. 5.3.

	F	p
Senecio aquaticus	8.97	0.01
Ranunculus flammula	8.66	0.01
Eriophorum angustifolium	4.24	0.05
Glyceria maxima	0.70	0.05

Table 5.2 - The occurrence of root tip blackening after exposure for 24 hours to aerobic iron (II) sulphate in the excised root tip elongation experiment (see figs. 5.3 and 5.4).

- = no symptoms + = tips blackened

	Iron (II) sulphate concentration, mM			
	0	0.02	0.2	2.0
Senecio aquaticus	-	+	+	+
Ranunculus flammula	-	-	+	+
Eriophorum angustifolium	+	+	+	+
Glyceria maxima	-	-	-	-

Table 5.3 - The viability of excised root tips after exposure to anaerobic iron (II) sulphate buffered with 50mM glycylglycine for 6.5 hours. Viability was determined using the T.T.C. assay.

+ = root tips stained (viable) - = no root tips stained (non viabl

	Iron (II) sulphate concentration, mM			
	0	0.1	1.0	10.0
Senecio jacobaea	+	+	-	-
Ranunculus flammula	+	+	+	-
Nardus stricta	+	+	+	+
Glyceria maxima	+	+	+	+

Table 5.4 - A comparison of the relative iron (II) tolerance of intact plants with the relative tolerance of excised roots of various species. Tolerance of excised tips was determined by elongation growth, viability and potassium leakage. The species are ranked in order of increasing tolerance.

	Intact plants*	Excised root tips			
		Root elongation[a]	viability[a]	K leakage[b]	viability[b]
Senecio jacobaea	1	-	-	1	1
Senecio aquaticus	2	1	1	-	-
Ranunculus flammula	3	3	3	2=	2
Eriophorum angustifolium	4	2	2	-	-
Nardus stricta	5	-	-	2=	3=
Glyceria maxima	6	4	4	2=	3=

- = not tested

* data from Chapter 2

a = under aerobic conditions

b = under anaerobic conditions

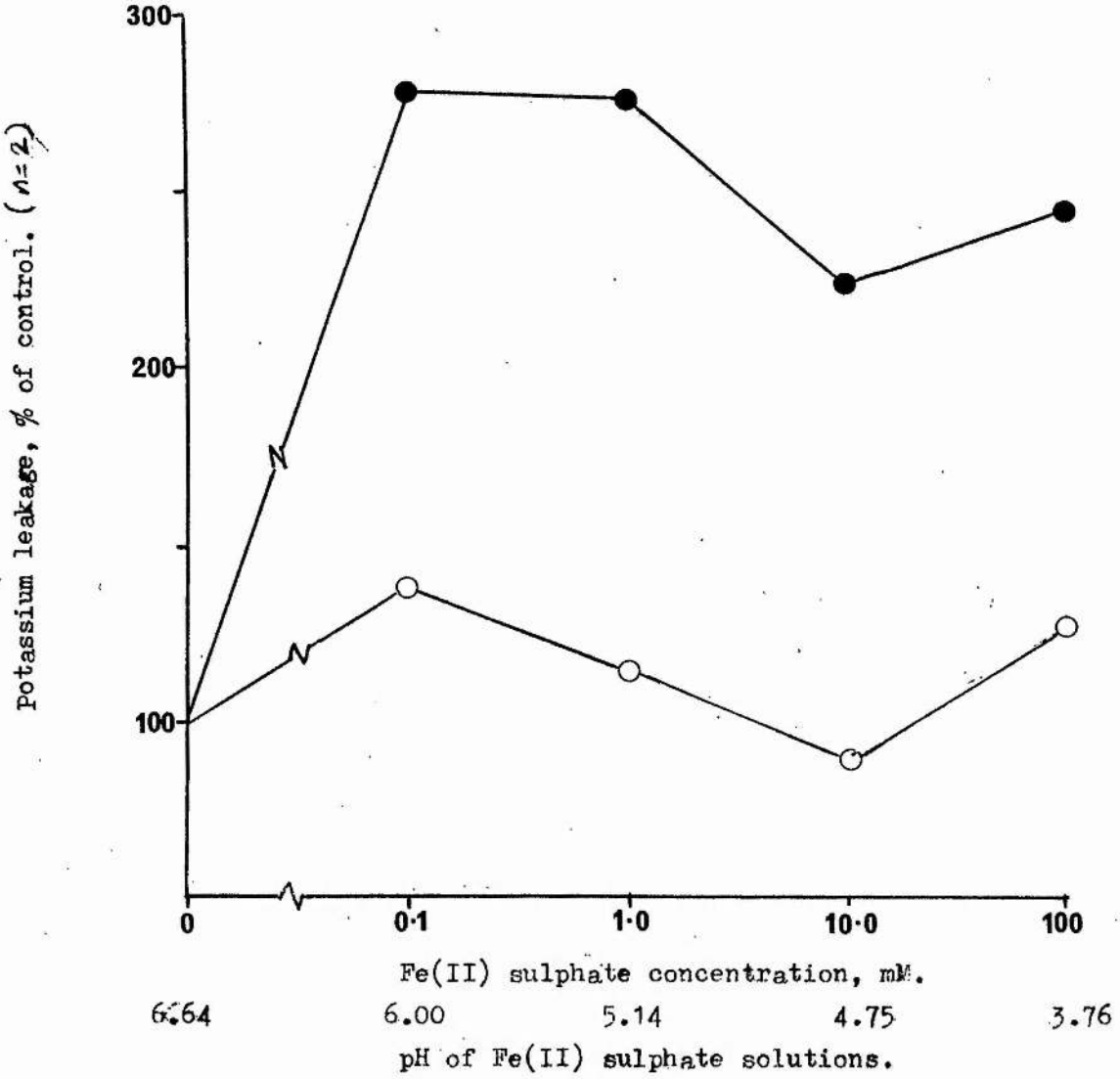


Fig.5.1. Potassium leakage from excised root tips of Senecio jacobaea and Glyceria maxima treated for 1 hour in anaerobic Fe(II) sulphate solution.

Control leakage rates: Senecio jacobaea 65 nmol K^+ (2 root tips)$^{-1}$.hr^{-1}
Glyceria maxima 97 "

● Senecio jacobaea
○ Glyceria maxima

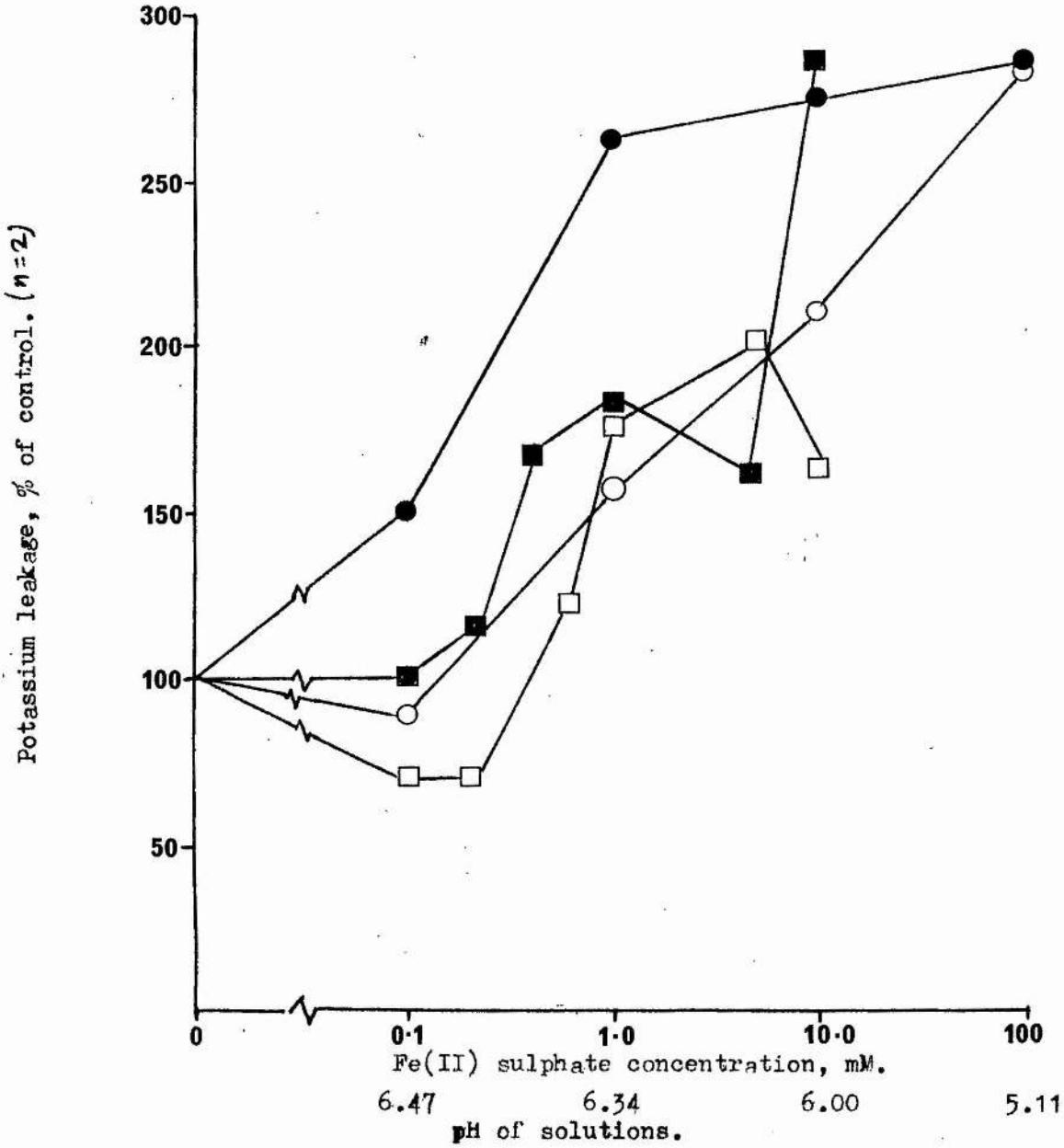


Fig.5.2. Potassium leakage from excised root tips of various species treated for 6.5 hours in anaerobic 50mM glycylglycine containing various concentrations of Fe(II) sulphate.

● Senecio jacobaea Control leakage rate:0.35 nmol.mm^{-2}.h^{-1}

○ Ranunculus flammula " " " 0.33 "

□ Nardus stricta " " " 0.34 "

■ Glyceria maxima " " " 0.28 "

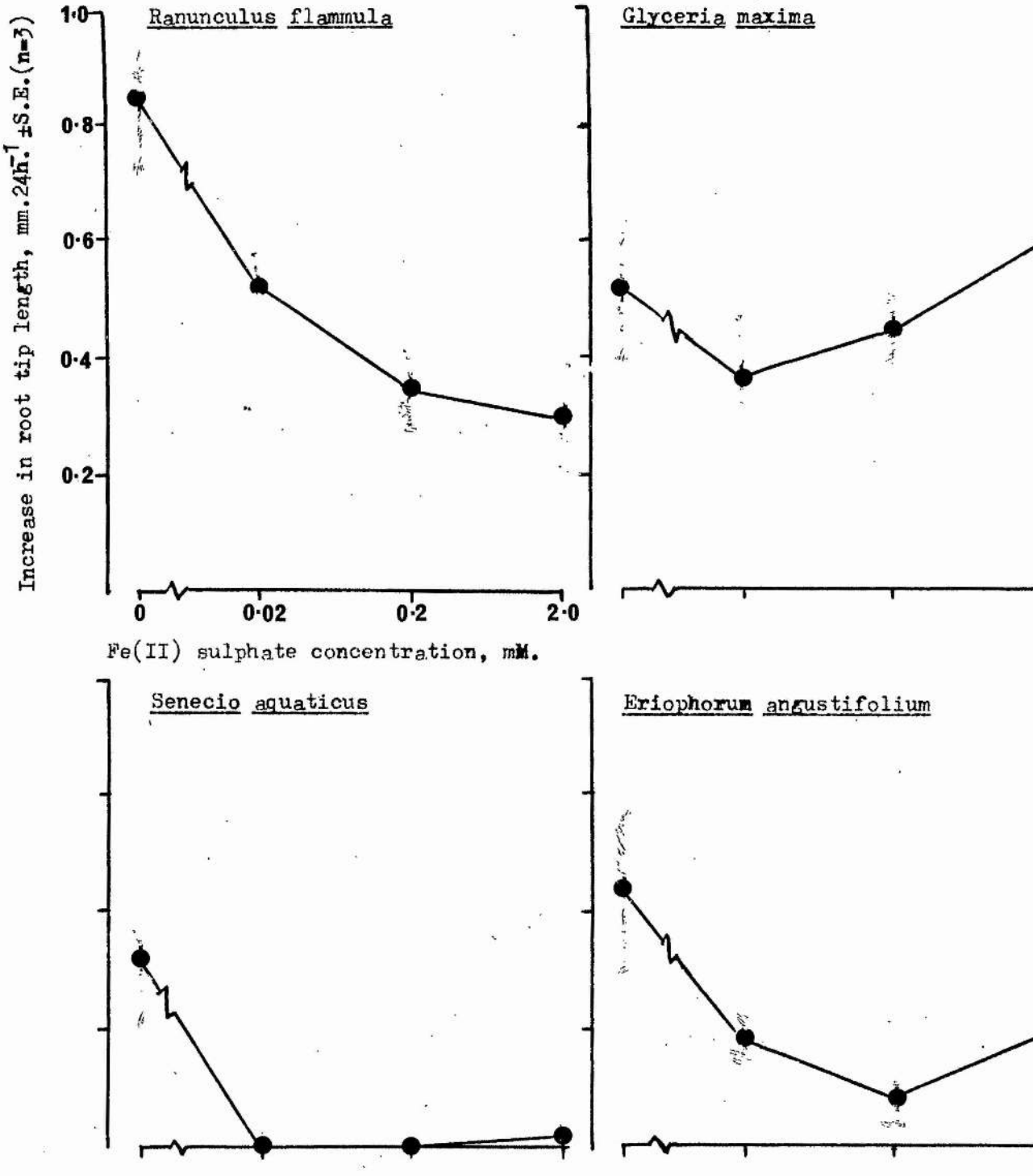


Fig.5.3. Elongation of excised root tips incubated aerobically for 24 hours in a buffered medium containing various concentrations of Fe(II) sulphate.
See table 5.1 for the analysis of variance.

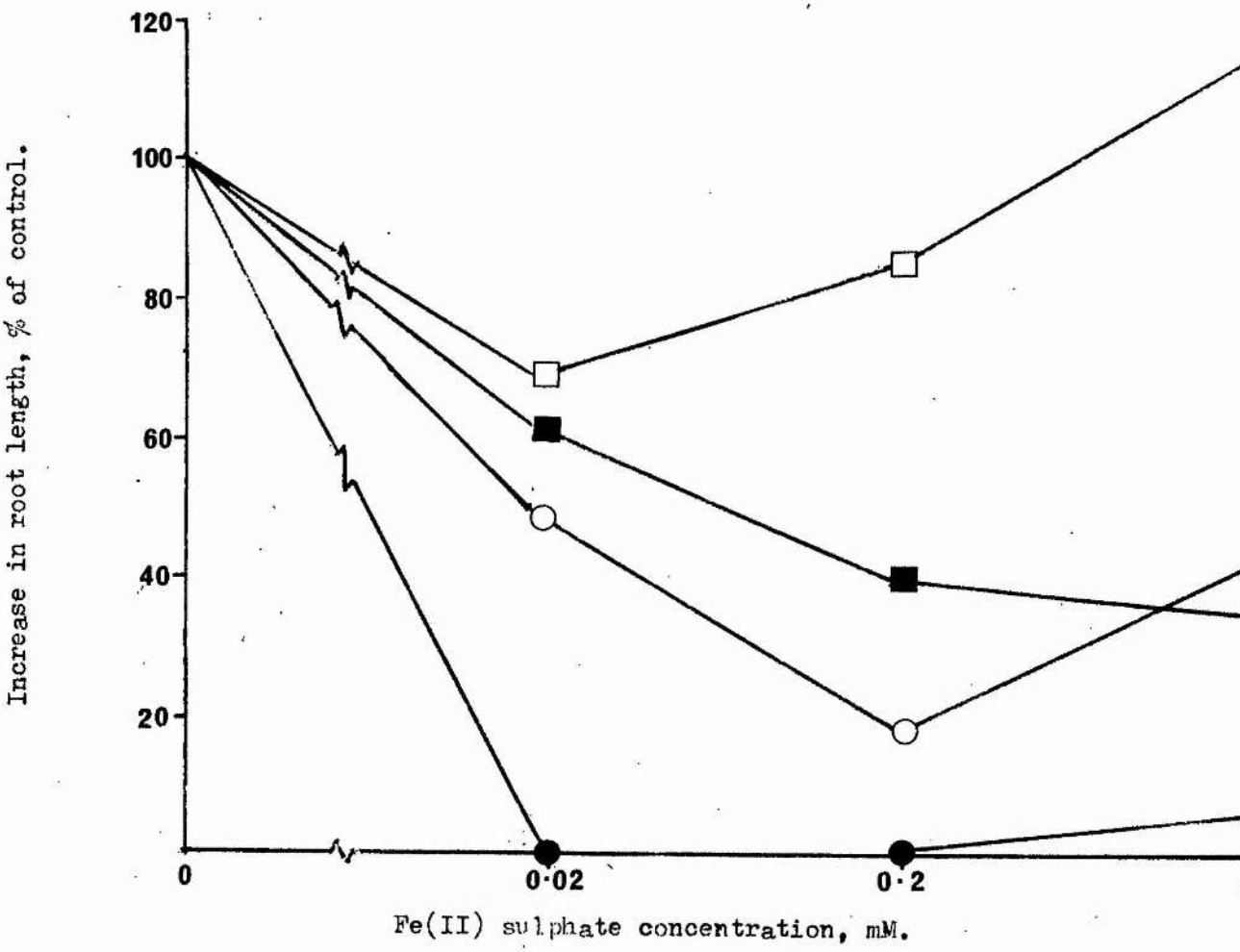


Fig.5.4. Elongation of excised root tips incubated aerobically for 24 hours in a buffered medium containing various concentrations of Fe(II) sulphate. Elongation expressed as % of control (no Fe(II)) to allow comparison of species. ($n=3$)

● Senecio aquaticus
○ Eriophorum angustifolium
■ Ranunculus flammula
□ Glyceria maxima

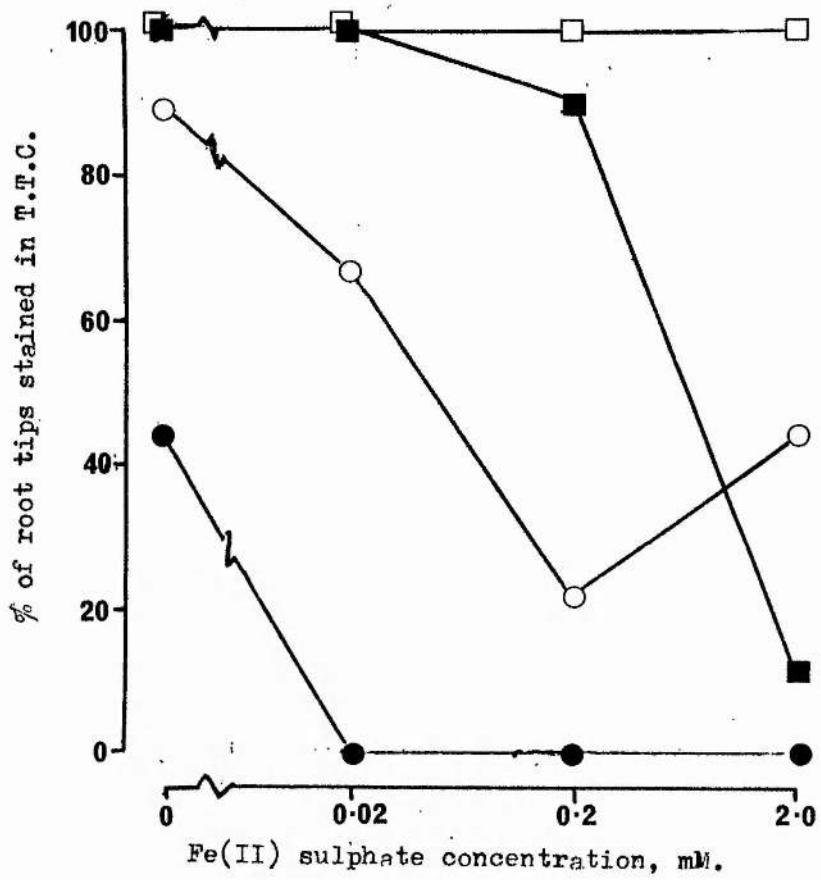


Fig.5.5. The percentage of root tips staining in triphenyltetrazolium chloride after incubation for 24 hours in an aerobic buffered medium containing various concentrations of Fe(II) sulphate.

● Senecio aquaticus
○ Eriophorum angustifolium
■ Ranunculus flammula
□ Glyceria maxima

Discussion

Increasing concentrations of Fe(II) caused K leakage from the root tips, although it was not a marked effect because only 20% or less of the total potassium content of the tips leaked out. Potassium leakage has been used as an indicator of metal toxicity in higher plants (Wainwright and Woolhouse, 1977) and lower plants (Brown & House, 1978). In these studies metal tolerant ecotypes had less potassium leakage induced by the toxic metal than the intolerant ecotypes. There was also a differential effect in these experiments between Senecio jacobaea and the other species. S. jacobaea was more sensitive to Fe(II): potassium leakage was greater and induced at lower Fe(II) concentrations, suggesting that the excised tips of this species are less tolerant to Fe(II). The tips were incubated in a deoxygenated medium, so it could be suggested that S. jacobaea is more sensitive to Fe(II) because it is also flood-intolerant (i.e. it cannot withstand hypoxia) whereas the other species are all flood-tolerant (Crawford, 1967). The effects of low oxygen tension and Fe(II) may interact to make S. jacobaea more sensitive. Even if this is true for S. jacobaea, differences could be seen between species in the root elongation experiment under aerobic conditions.

The cause of K leakage is not known for certain, but probably involves damage to the plasma membrane, either directly or as a secondary effect after disruption of metabolism (Wainwright and Woolhouse, 1977). These authors also showed that copper and zinc were not equally effective at causing K leakage from root tips of Agrostis tenuis. 1 mmol l^{-1} Cu caused leakages 1140 and 2755% of the control for tolerant and non-tolerant clones respectively, whereas the equivalent percentages for zinc were 60 and 175%. The effect of Fe(II) was similar in magnitude to zinc (150 - 250%).

It was shown in Chapter 2 that Fe(II) inhibited root growth and the results here show that this effect also occurs in excised root

tips. Because the tips contained the meristem and the region of cell elongation, it cannot be determined if Fe(II) inhibits cell division, cell elongation or both. Zinc and copper inhibit cell elongation in Agrostis tenuis (Wainwright and Woolhouse, 1977) and aluminium inhibits cell division in various species (Clarkson, 1969). Whatever the cause of inhibition, the root tips of the various species had different sensitivities to Fe(II) and the degree of sensitivity corresponded well with that of the whole plants (Chapter 2), except that the sensitivity of Ranunculus flammula and Eriophorum angustifolium was reversed (Table 5.4). This method was able to distinguish copper and zinc tolerant clones from intolerant clones in Agrostis tenuis (Wainwright and Woolhouse, 1977) and has distinguished variations in Fe(II) tolerance between species in the present experiments.

The use of TTC to test root tip viability was discussed in Chapter 2. These results, and the occurence of toxicity symptoms paralleled the results of the elongation experiment. Tolerance of excised root tips varied between species incubated under aerobic conditions or in a deoxygenated medium. The order of tolerance again followed that of intact plants. The results are summarised in Table 5.4. The species have been ranked in order of increasing tolerance of whole plants or excised root tips. In all cases there was good agreement between whole plants and excised root tips. Not enough species were tested to apply Spearman's Rank Correlation test. In the elongation experiment the tolerance of root tips from E. angustifolium and R. flammula was reversed when compared with the whole plants. E. angustifolium has highly developed aerenchyma, and air space occupies almost 50% of the root volume (see Chapters 2 & 7). It was shown in Chapter 3 that this large amount of air space was correlated with exclusion of Fe from the roots, perhaps by oxidation of the rhizosphere, so under natural conditions E. angustifolium may not be exposed to very high Fe(II) levels, and, as a consequence, its roots may not have developed a very high tolerance.

The results show that differential tolerance is maintained under aerobic conditions (Table 5.2, figs 5.3 - 5.5), or under reduced oxygen tension (Table 5.3, figs 5.1 - 5.2). It could be argued that excised root tips under aerobic conditions can maintain a range of tolerance because they vary in the efficiency with which they can use oxygen to oxidise Fe(II) and prevent its uptake. Maintenance of a differential tolerance in the deoxygenated Fe(II) solutions does not support this idea (but the possibility still exists that oxygen diffused into the solutions during incubation although efforts were made to prevent this by flushing the vials with nitrogen before putting the lids on). It has been suggested that the oxidising power of some roots is greater than can be explained by ROL (Armstrong, 1967) and rice root extracts can oxidise Fe(II) enzymatically (Yamada & Ota, 1958). This will be discussed further in Chapter 6.

The results do not support the hypothesis that root tips from species of varying Fe(II) tolerance do not maintain their differential tolerance after excision. This, taken with the evidence from Chapter 2 where no relationship between tolerance of whole plants and the amount of air space in their roots was found, suggests that oxygen diffusion from the shoot and through the air spaces of the root is not necessary for tolerance. This does not rule out completely a function for aerenchyma, because species with particularly large amounts of air space (e.g. Eriophorum angustifolium) may, in addition, be able to exclude sufficient Fe for it to contribute to tolerance. It is perhaps not surprising that the species investigated have developed varying tolerance to Fe(II). Interspecific differences in tolerance to metals are common in plants. Species which occur in soils containing elevated metal levels are more tolerant than those which do not. This has been demonstrated for many metals, for example: manganese (Mohmond & Grime, 1977; Ernst and Lugtenborg, 1980); aluminium (Clarkson, 1966); zinc (Ernst, 1976; Mathys, 1977); nickel and cobalt (Morrison et al., 1979). In no case does tolerance to these metals involve oxidation and exclusion.

The mechanism of Fe(II) tolerance cannot be inferred from these results, but as was pointed out by Wainwright & Woolhouse (1977) the amount of metal in the incubation medium of the elongation experiment is far in excess of that which could be bound on cell wall sites. This could also be the case for Fe, although precipitation probably occurs in addition. The ratio of root tip volume to solution volume, assuming a root tip diameter of 0.5mm, was 1:3000 which results in a large excess of Fe(II). Binding to exchange sites on cell walls can possibly be ruled out as a tolerance mechanism.

PART II

The Oxidising Activity of Roots and Root Aerenchyma

In Part I the tolerance of various species to Fe(II) was reported, and evidence that tolerance was not dependent on air space tissue in the roots was presented. It is possible that aerenchyma could still play a part in oxidising and excluding Fe from the roots of some species. In Part II various aspects of the oxidising activity of roots (Chapter 6) and the structure and function of root aerenchyma will be discussed (Chapter 7).

Factors Affecting the Oxidising Power of Roots.

The oxidising power of roots must ultimately depend on the supply of oxygen. Under flooded conditions oxygen is supplied from the shoot by diffusion through the air spaces in the root cortex. Diffusion of oxygen from the roots (ROL) can be detected in many species. Species with a large amount of air space in their roots have a greater ROL from their roots than those which do not. The evidence for this was reviewed in Chapter 3. Various environmental factors can affect the oxidising power of roots towards redox dyes or Fe(II). The studies on the effects of light, nutrient deficiency and metabolic inhibitors have been mainly carried out with rice. They will be reviewed here as a preface to Chapter 6.

Excised rice roots have the ability to oxidise Fe(II) (Yamada and Ota, 1958; Tadano, 1975) and the redox dye α-naphylamine (IRRI, 1966). This could be caused by oxygen stored in the root air spaces because freshly excised roots were used in all the experiments. But Tadano (1975) found that the oxidation was inhibited in the presence of 1000 ppm Na-NaCl. Yamada and Ota (1958) found that the oxidising activity increased up to 90 minutes after excision. By this time the internal oxygen supply of the roots is likely to be depleted (see

Chapter 7). It is possible that the presence of the rice roots could catalyse Fe(II) oxidation using dissolved oxygen from the incubation medium.

Light intensity affects dye and Fe(II) oxidation by roots of intact plants. Bartlett (1961) showed that Fe(II) oxidation by seedlings of various species, including rice, alfalfa and a Phalaris species, was greater over 72 hours in the light than in the dark. Rice plants grown for 2 days in darkness oxidised less α-naphylamine than light-grown plants. Also, rice seedlings grown for 1 week under various light intensities had different oxidising powers. Oxidation of α-naphylamine was greater at higher light intensities (Irri, 1966). It is not clear if measurements were made under the pretreatment light intensity or at a uniform intensity. It is possible that oxygen generation by photosynthesis could increase oxygen transport to roots. This is not the case for Eriophorum angustifolium. Varying light intensity or placing the plants in the dark did not alter ROL from the roots (Armstrong, 1978; 1979). Photosynthesis can only increase the oxygen supply of roots if the shoots are submerged in water, preventing oxygen loss through the stomata (Armstrong, 1979). Without further data the effect of light cannot be interpreted.

Treatment of rice roots with various metabolic inhibitors (azide, cyanide, 2,4-dinitrophenyl and hydrogen sulphide) increases uptake of Fe into the plants (fig 1; Tanaka et al., 1968; Tadano, 1975). The effect of inhibitors is difficult to interpret: the ability of the roots to oxidise Fe(II) may have been affected but the inhibitors could affect other aspects of uptake. Joshi et al., (1975) found that pretreating seedlings of various rice varieties with hydrogen sulphide caused a decrease in the diffusion of oxygen from the roots as measured with an oxygen electrode. If oxygen diffusion from the roots is a purely physical process it is difficult to see why this metabolic inhibitor should affect it. They gave no explanation for their results, but it could be that the accumulation of hydrogen sulphide in the tissues

acted as an "oxygen debt" and absorbed extra oxygen as it diffused through the roots. Further investigation is needed.

The growth of rice under nutrient-deficiency decreases their ability to oxidise Fe(II) (Trolldenier, 1973, 1977). In this case the effect has been ascribed to rhizosphere microorganisms. Nutrient deficiency, particularly potassium, increases leakage of organic solutes from roots and increases the abundance of rhizosphere microorganisms. These reduce the oxidising power of the roots by consuming oxygen diffusing from them and by directly reducing any Fe(III) produced by the oxygen diffusing from the roots.

Some of these results suggest that the oxidising power of roots could depend on metabolic activity and not simply on oxygen diffusion from the roots.

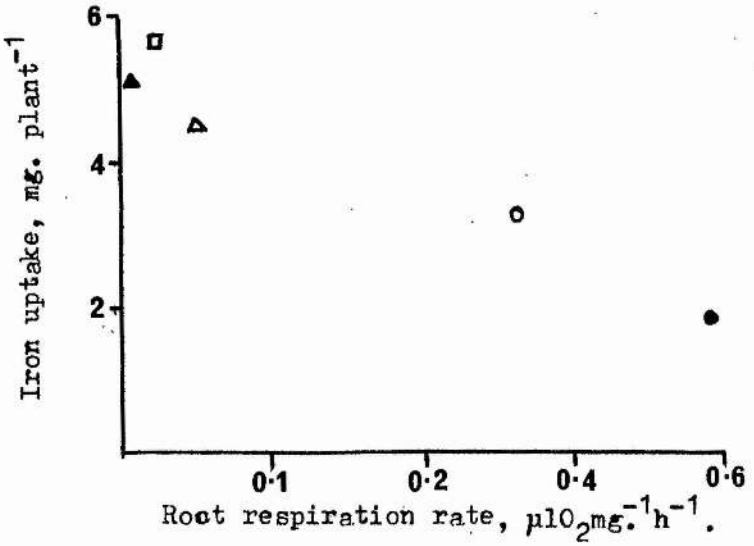


Fig.1. The relationship between respiration rate and iron uptake by rice plants treated with the metabolic inhibitors potassium cyanide and sodium azide. Plants were kept in a culture solution containing 5.4mM Fe(II) (labelled with ^{59}Fe) and various concentrations of the inhibitors. Drawn from the data of Tadano, 1975.

- ● Control
- ○ 10^{-4} M KCN
- □ 10^{-3} M KCN
- △ 10^{-4} M NaN_3
- ▲ 10^{-3} M NaN_3

CHAPTER 6

The Role of Metabolism in the Oxidising Power of Roots

Introduction

It has been realised for some time that roots can have an oxidising effect on their environment. The literature has been reviewed by Armstrong (1979). There is some evidence to suggest that the oxidising power of roots is greater than would be expected if only oxygen diffusion through the roots was involved so metabolic mediation of oxidation has been postulated. The ability of roots to oxidise redox dyes has been studied and plants show inter- and intraspecific variation in their oxidising power (Schreiner and Reed, 1909; Fukui, 1953; Goto and Tai, 1957; Armstrong, 1967). Armstrong (1967) showed that the ability of roots of *Menyanthes trifoliata* and *Molinia caerulea* to oxidise methylene blue was nine times greater than would be expected from measurements of ROL from the roots. Schreiner and Reed (1909) suggested that oxidising activity towards dyes was mediated by peroxidase enzymes on the root surface or secreted into the culture solution. The roots in their experiments were not sterile and the activity may have been microbial. Recent studies have shown that peroxidase can occur in root cell walls, particularly those of the epidermis (Fielding and Hall, 1978b). Secretion of peroxidase from cells has been demonstrated, for example into the intercellular spaces of leaves (Rathmell and Sequiera, 1974) and from sterile roots (Collet, 1975). Sterile rice roots also secrete catalase (Pitts *et al.*, 1972).

The function of peroxidases in plants is still unclear although they have been widely studied. Functions that have been suggested include: IAA oxidation, ethylene biosynthesis; hydroxylation of proline; lignification; wound-healing and disease resistance (Fielding and Hall, 1978a). Peroxidase has many isoenzymes and these could have different functions. Catalase is thought to decompose hydrogen peroxide to

prevent it building up to toxic levels, but it may also act as a peroxidase in the oxidation of methanol and formate (Halliwell, 1974).

There are two ways in which peroxidase and catalase could mediate the oxidising power of roots: i) catalase could decompose hydrogen peroxide to produce oxygen; (ii) peroxidase could use hydrogen peroxide to oxidise various substrates. Peroxidases have an extremely wide range of substrates (Saunders et al., 1964). Both these processes depend on the availability or generation of hydrogen peroxide. Hydrogen peroxide accumulation has been demonstrated in some tissues (Sagisaka, 1976; Brennan and Frenkel, 1977), but generation of only small amounts would be sufficient to supply it as a substrate. The main hydrogen peroxide-producing reactions are oxidations catalysed by oxidases with a flavin micronucleotide (FMN) cofactor such as glycolate oxidase, xanthine oxidase and amino acid oxidase (Dixon, 1971; Brennan and Frenkel, 1977). It had been suggested that the alternative oxidase generated hydrogen peroxide (Rich et al., 1976) but recent evidence suggests that an FMN-linked oxidase is involved (Huq and Palmer, 1978). Glycolate oxidase and catalase are both located in microbodies (Breidenbach, 1976) but Halliwell (1974) suggested that the Km for hydrogen peroxide of catalase was high enough to allow some of it to excape from the microbody before being decomposed.

In this chapter the possibility that the oxidising power of roots is mediated by some enzymes of peroxide metabolism, particularly peroxidase, catalase and glycolate oxidase, is examined. This follows the suggestion of Vamos and Koves (1972) that rice could generate oxygen in its roots metabolically by using glycolate oxidase and catalase or by producing peroxides from $^{\cdot}OH$ radicals produced by photosynthesis. It was suggested that the peroxides could be translocated to the roots where they would release their oxygen. A preliminary investigation of the ability of the root extracts of several species to consume Fe(II) is also described. This follows from the discovery of Yamada and Ota (1958) that rice root extracts could catalyse the oxidation of Fe(II)

using a peroxidase-like enzyme.

Materials and Methods

Peroxidase and Catalase Activity in Root Tips of Various Species.

The origin of the additional species not used in the previous experiments is shown in Table 6.1. Small similarly-sized plants were established in sand culture in 15 litre rubber buckets. Two buckets were set up for each species. They were kept in the glasshouse at 20 °C with supplementary lighting to give a daylength of 18 hours. Hoagland's solution (1/5 th strength) was given weekly, and after three weeks one bucket of each species was subjected to a flooding treatment. The bucket was flooded with 1/5th strength Hoagland's solution to the sand surface. After this both treatments were given the nutrient solution once a week. At other times the water level was maintained by adding tap water.

The plants were harvested after 9 weeks (21 weeks for the Eriophorum species). The root systems were carefully washed free of sand. White, apparently healthy, 3cm root tips were excised and used for enzyme extraction. The root tips were washed thoroughly with distilled water. Between one and seven tips were ground with a chilled pestle and mortar with a little sand and chilled extraction buffer (see below). The tips were ground in 2ml extraction buffer, strained through muslim and the residue re-extracted with another 2ml buffer. The two extracts were combined and centrifuged for 2 minutes in 1.5ml tubes using a Beckman Microfuge B. This short spin in small tubes resulted in supernatants with no turbidity. The supernatant was decanted and stored on ice until it was used for the enzyme assays. Assays were made within 40 minutes of extraction without further purification of the extracts.

Peroxidase. The extraction buffer was 25mM tris-HCl, pH 7.2 + 7% polyclar AT (insoluble polyvinylpyrrolidone). The assay was based on the method of Fielding and Hall (1978a). Peroxidase catalyses the oxidation of guaiacol by hydrogen peroxide to give a red-brown quinone (Saunders et al., 1964). The reaction was followed by measurement of

the production of the quinone at 470nm with a Unicam SP1800 UV Spectrophotometer with a water-jacketted cuvette holder at 25°C. The reaction mixture, in 1cm path length glass cuvettes, contained the following in a final volume of 3.00ml: 83.3mM potassium phosphate buffer, pH 7.0; 20mM guaiacol; 5mM hydrogen peroxide and 0.1 - 0.2ml root extract. The reaction was started by adding extract.

Catalase. The extraction buffer was 10mM potassium phosphate buffer, pH 7.0 plus 7% polyclar AT. The assay was based on the method of Luck (1963). Catalase catalyses the decomposition of hydrogen peroxide to form water and oxygen. Hydrogen peroxide absorbs strongly in the ultra-violet and the assay measured the decrease in absorbance at 240nm when it decomposes. Hydrogen peroxide solutions were prepared freshly before each assay and standardized using an extinction coefficient of 0.067 $cm^{-1}mM^{-1}$ at 230nm (Maehley and Chance, 1954). Reactions were carried out under the same conditions as peroxidase except that 1cm path length silica cuvettes were used. The reaction mixture contained in a final volume of 3ml: 83.3 mM potassium phosphate buffer, ph 7.0; 30mM hydrogen peroxide (8mM for the Eriophorum species) and 0.1ml extract. The reference cuvette contained buffer and extract.

Protein concentration in the extracts was measured by the method of Lowry et al.,(1951). All reaction rates were proportional to the amount of extract added. The activity of both enzymes is expressed as ΔOD (change in absorbance) min^{-1} $(mg\ protein)^{-1}$ and are the mean of three plants (two plants in the case of Eriophorum species). The extract from each plant was assayed two or three times.

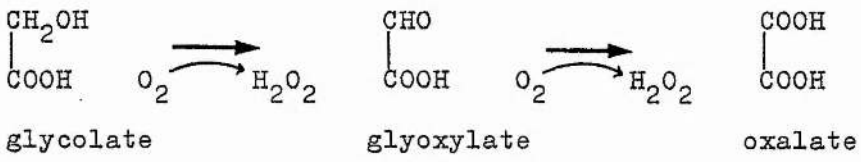
Preliminary experiments showed that the assay conditions (pH and substrate concentrations) were optimum for Glyceria maxima (figs 6.1 and 6.2). It would not have been practical to optimize conditions for all the species. Catalase activity (fig 6.2) increased in an almost linear fashion over the range 0 - 40mM hydrogen peroxide, but these higher concentrations rapidly inactivated the enzyme.

Glycolate Oxidase Activity in Oryza sativa.

Rice seedlings, one week old, were transplanted to 10cm plastic pots (6 per pot) and allowed to establish for one week. After this period some of the pots were flooded by submerging them in basins containing 1/5th strength Hoagland's solution. The controls were freely drained and watered once a week with the nutrient solution.

After one month the plants were harvested and the root systems washed free of sand. The root systems and shoots were used for estimating glycolate oxidase activity. A crude extract was prepared using the method of Osmond (1969). The tissue was ground in a chilled mortar and pestle with a little sand and the following extraction medium: 50mM tris-HCl buffer pH 8.0 containing 10mM mercaptoethanol; 8.0mM magnesium chloride and 8% polyclar AT. The resulting slurry was strained through muslim and centrifuged for 2 minutes in 1.5ml tubes using a Beckman Microfuge B. The clear supernatant was used for the assay directly.

Glycolate oxidase catalyses the oxidation of glycolate by oxygen to form glyoxylate and hydrogen peroxide. Glycoxylate can be further oxidised by the same enzyme to give oxalate and hydrogen peroxide.



glycolate glyoxylate oxalate

The activity of the enzyme in the extracts was measured by following oxygen consumption in a Rank oxygen electrode. The assay was modified from Zelitch (1955). The assay medium contained the following in a final volume of 2.8ml: 560mM potassium phosphate buffer, pH 8.0; 14.3mM potassium cyanide; 35.7mM potassium glycolate and 0.5ml extract. The medium was placed in the chamber of the electrode without glycolate. After a steady trace was produced a small volume of glycolate solution was added through the capillary in the lid to start the reaction. Water at 25 °C was circulated round the jacket of the electrode. The assay

buffer was saturated with air at 25 °C before use. The stock potassium cyanide solution (0.2M) was made up in 2mM ammonium hydroxide to prevent loss of hydrogen cyanide. Cyanide was necessary to inhibit catalase activity in the assay. Catalase would break down any hydrogen peroxide formed in the reaction and release oxygen.

Fe(II) Consumption by Root Extracts from Various Species.

Roots were taken from plants in sand culture and washed well in distilled water. They were homogenized using an Ultra-Turrax homogenizer in cold 0.2M sodium acetate-acetic acid buffer, pH 5.5. The homogenate was strained through muslim and centrifuged at 2000g for 10 minutes at 4 °C. The supernatant was used for the assay.

The assay measured the disappearance of Fe(II) from the solution as determined by the 2,2'-bipyridyl assay. Total Fe or Fe(III) in solution was not measured, so disappearance of Fe(II) cannot for certain be attributed to oxidation. Activity of the extracts was compared with extracts boiled for 10 minutes to act as controls. Over the time of the measurements (30 minutes) the Fe(II) concentration in the buffer without added extract remained constant.

The assay was conducted as follows: 1ml of stock Fe(II) solution was added to 5ml of extract in test tubes in a water bath at 25 °C. The final Fe (II) concentration was 0.5 or 1.0mM. 1ml aliquots were withdrawn from the test tubes at 0, 15 and 30 minutes after Fe(II) addition. These were analysed for Fe(II) by the 2,2'-bipyridyl assay (see Chapter 3). The results are the mean of two determinations and are expressed as μmol Fe(II) consumed g^{-1} fresh weight of roots.

Results

Peroxidase and Catalase Activity in the Root Tips of Various Species.

A small scale method of extraction was developed to allow a well-defined part of the root system to be measured: the root tips. This is of particular advantage for peroxidase, where activity may vary along the length of a root (Fielding and Hall, 1978b). If flooding altered the proportion of old to young roots, interpretation of any changes in activity could be complicated. The method was found to give repeatable results. The use of a microcentrifuge allowed small volumes of extract to be processed rapidly.

Peroxidase activity in root tips of various species and the effect of flooding is shown in Table 6.2. Peroxidase activity varied greatly between species. Flood-tolerant and flood-intolerant species had a similar range of activity. Flooding had no effect on activity except in Brachypodium sylvaticum. In this species flooding caused a large significant increase ($p = 0.01$) in activity. Interspecific variation in activity could be related to taxonomic groups. The Gramineae had activities an order of magnitude greater than the dicotyledonous species (except Nardus stricta). In the drained treatment the mean activity in the Gramineae was significantly greater ($p = 0.02$) than in the dicotyledons. The Eriophorum species (Cyperaceae) occupied an intermediate position. There was no relationship between Fe(II) tolerance and peroxidase activity (for those species described in Chapter 2) or air space in the roots (see Chapter 7) and peroxidase activity.

Catalase activity in root tips of various species and the effect of flooding is shown in Table 6.3. There was no difference between activity in the Gramineae and the dicotyledons. Flooding had a significant effect ($p = 0.05$) on only three species. In Phalaris arundinacea and Nardus stricta flooding inhibited catalase. In Brachypodium sylvaticum there was no activity in the flooded root tips. This contrasted greatly with the large increase in peroxidase activity. A lower hydrogen peroxide

concentration was used in the Eriophorum species so they cannot be compared with the others. In these species flooding doubled the activity of catalase, but the difference was only significant with a probability of 0.1. There was no relationship between Fe(II) tolerance and catalase activity or air space in roots and catalase activity.

The results suggest that the activity of both enzymes has no relationship to Fe(II) tolerance or flooding tolerance. Except for Brachypodium sylvaticum, activities were little affected by flooding. The root system of B. sylvaticum was severly affected by flooding. Few roots were produced and many of these had blackened tips and red-brown Fe deposits. The symptoms suggested possible Fe(II) toxicity.

Glycolate Oxidase Activity in Oryza sativa.

The effect of flooding for one month on glycolate oxidase activity in roots and shoots is shown in Table 6.4. Flooding had no effect on activity in the shoots. In roots the activity decreased, but the effect was not significant. Activity in the shoots was in the range reported for other species (Tolbert et al., 1971). Activity in roots was an order of magnitude less and very near the detection limit of the assay, so the rates are doubtful. The possibility that there were inhibiting substances in roots decreasing activity was tested by mixing root and shoot extracts and assaying after 30 minutes. Activity of the shoot enzyme was not inhibited. Roots of Oryza sativa have very low activity of glycolate oxidase.

Fe(II) Consumption by Root Extracts From Various Species.

The results are shown in Table 6.5. Untreated and boiled extracts caused Fe(II) to disappear from the solution as measured by the 2,2'-bipyridyl assay. In Ranunculus flammula there was no difference between the untreated and boiled extracts. In Oryza sativa Fe(II) consumption was significantly greater ($p = 0.05$) in the untreated extracts. Untreated extracts of Glyceria maxima had significantly greater ($p = 0.05$)

Fe(II) consumption after 30 minutes, but the effect was not as great as in O. sativa. Aeration of boiled O. sativa extracts did not change their Fe(II) consumption.

The results show that boiled or untreated extracts can cause Fe(II) to disappear from solution. There are three possible reasons for this a) Extracts stimulate Fe(II) oxidation b) The extracts form a complex with a substance(s) in the extract with a higher stability constant than the Fe(II)-bipyridyl complex c) The extracts cause a combination of (a) and (b). In O. sativa and G. maxima this activity decreased when the extracts had been boiled. Aeration of the extract did not restore activity, so a heat-labile factor in these extracts contributed to the activity. The activity of the heat-labile factor was greater in O. sativa than in G. maxima.

Table 6.1 - Species, in addition to those listed in Table 2.1, used for the measurement of peroxidase and catalase activity.

	Habitat	Location	Form Collected
Brachypodium sylvaticum	Well-drained woodland	Dura Den, Fife	Tillers
Ammophila arenaria	Sand dunes	Tentsmuir, Fife	Plants
Hieracium pilosella	Sand dunes	Tentsmuir, Fife	Plants
Phalaris arundinacea	Fen	Lindores Loch, Fife	Rhizomes

Table 6.2 - The activity of peroxidase in the root tips of various species after nine weeks growth in drained and flooded sand culture.

	Peroxidase activity $\Delta OD\ min^{-1}\ mg.\ protein^{-1}$ with range (n = 3)	
Flood-tolerant species[a]	Drained	Flooded
Glyceria maxima	27.42 (15.35-39.01)	11.97 (9.97-13.31)
Phalaris arundinacea	31.87 (16.71-29.67)	32.63 (15.28-55.76)
Eriophorum angustifolium[b]	5.32 (2.50-8.34)	5.28 (4.29-6.21)
Eriophorum vaginatum[b]	3.55 (1.06-4.86)	3.59 (3.00-4.22)
Nardus stricta	3.65 (3.16-4.27)	5.65 (3.37-7.74)
Ranunculus flammula	2.37 (0.73-3.22)	1.09 (0.58-1.74)
Myosotis scorpioides	1.18 (0.45-1.52)	1.85 (1.00-2.69)
Flood-intolerant species[a]		
Ammophila arenaria	13.23 (5.97-20.70)	15.14 (11.26-19.0)
Brachypodium sylvaticum	19.62 (18.4-22.11)	132.31 (42.44-160.02)
Senecio jacobaea	2.20 (1.52-3.01)	2.53 (1.76-4.61)
Hieracium pilosella	1.31 (0.74-1.97)	1.17 (0.45-1.38)

[a] Classification from Crawford (1967) and Chapter 7

[b] Flooded for 21 weeks

** Drained and flooded treatments significantly different, p = 0.01 (student's t-test). Effect of flooding was insignificant ($p > 0.05$) for all other species.

Table 6.3 - The activity of catalase in the root tips of various species after nine weeks growth in drained and flooded sand culture.

	Catalase activity ΔOD min^{-1} mg. $protein^{-1}$ with range (n = 3)	
Flood-tolerant species[a]	Drained	Flooded
Glyceria maxima	1.420 (1.127-1.781)	1.137 (0.938-1.393)
Phalaris arundinacea	0.474 (0.452-0.494)***	0.157 (0.083-0.144)***
Eriophorum angustifolium[b]	0.097 (0.056-0.182)**	0.236 (0.128-0.412)**
Eriophorum vaginatum[b]	0.061 (0.040-0.086)	0.140 (0.087-0.185)
Nardus stricta	1.013 (0.945-1.074)	0.731 (0.637-0.812)
Ranunculus flammula	0.290 (0.236-0.360)	0.269 (0.206-0.355)
Myosotis scorpioides	0.289 (0.165-0.386)	0.241 (0.207-0.292)
Flood-intolerant species[a]		
Ammophila arenaria	0.429 (0.391-0.492)	0.334 (0.233-0.432)
Brachypodium sylvaticum	0.278 (0.268-0.292)***	0.000 (0-0)***
Senecio jacobaea	0.325 (0.248-0.385)	1.041 (0.594-0.672)
Hieracium pilosella	0.250 (0.209-0.312)	0.379 (0.296-0.537)

[a]Classification from Crawford (1967) and Chapter 7

[b]Flooded for 21 weeks. H_2O_2 concentration in catalase assay mixture was 8mM

Drained and flooded treatments significantly different, **p = 0.01, ***p = 0.001 (student's t-test). Effect of flooding was insignificant ($p > 0.05$) for all other species.

Table 6.4 - Glycolate oxidase activity in Oryza sativa cv. Oerias after four weeks growth in drained and flooded sand culture.

	Glycolate oxidase activity nmol. O_2 min^{-1} mg. $protein^{-1}$ with range	
	Drained	Flooded
Shoots	33.14 (21.43-31.85) a	39.16 (34.13-44.18) a
Roots	5.09 (3.61-6.57) b	1.86 (0-3.72) b

Figures followed by different letters are significantly different (p = 0.02)

Table 6.5 - Iron (II) "consumption" by root extracts of various species. The initial iron (II) concentration in the assay mixture was 1mM (0.5mM for Ranunculus flammula).

μmol. iron (II) g. fresh wt.$^{-1}$ . . (n = 2)

	Incubation time, min	Untreated extract	Boiled Extract	Difference between untreated & boiled
Ranunculus flammula	15	2.171	2.400	0
	30	2.914	2.857	0.057
Oryza sativa	15	3.933	0.693	3.240*
Glyceria maxima	15	3.612	2.408	1.204
	30	5.056	3.611	1.455*

* Difference significant, p = 0.05. Student's t-test.

Table 6.6 - The affinity (Km) of various oxidase enzymes for oxygen.

Enzyme	Cofactor at active centre	Km (M)	Reference
Cytochrome oxidase	Fe	c. 10^{-8}	Longmuir, 1954
Terminal oxidase of alternative pathway	Fe	c. 10^{-7}	Solomos, 1977
Phenol oxidase	Cu	1.3×10^{-5}	Beevers, 1961
Ascorbic acid oxidase	Cu	1.5×10^{-4}	Beevers, 1961
Glycolate oxidase	FMN*	1.33×10^{-4}	Kerr & Groves, 1975; Beevers, 1961.
Amino acid oxidase	FMN	?	Dixon, 1971.
Xanthine oxidase	FMN	?	Dixon, 1971; Kumer & Taneja, 1977

* = flavin mononucleotide

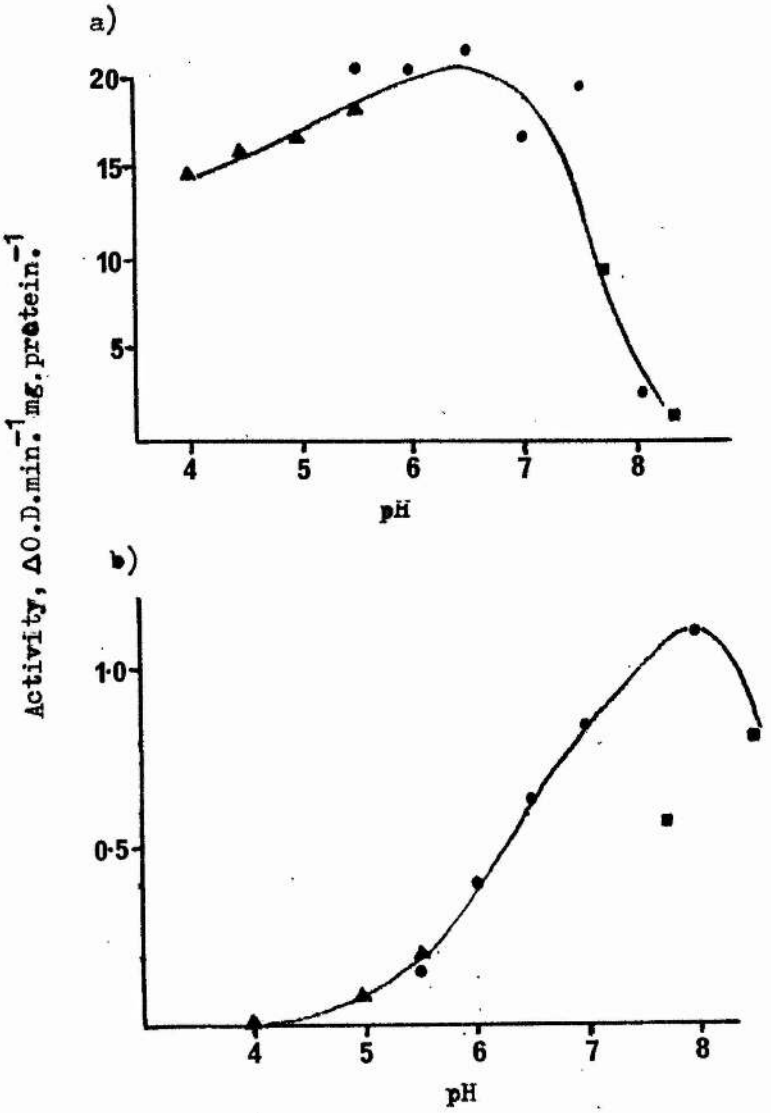


Fig.6.1. pH - activity curves for (a) peroxidase and (b) catalase from Glyceria maxima.
Peroxidase assayed with 5mM guaiacol and 2mM H_2O_2.
Catalase assayed with 20mM H_2O_2.

Buffers: ▲ 100mM citrate - phosphate.
● 100mM potassium phosphate.
■ 50mM tris - HCl.

Each point represents 1 measurement
Lines fitted by eye

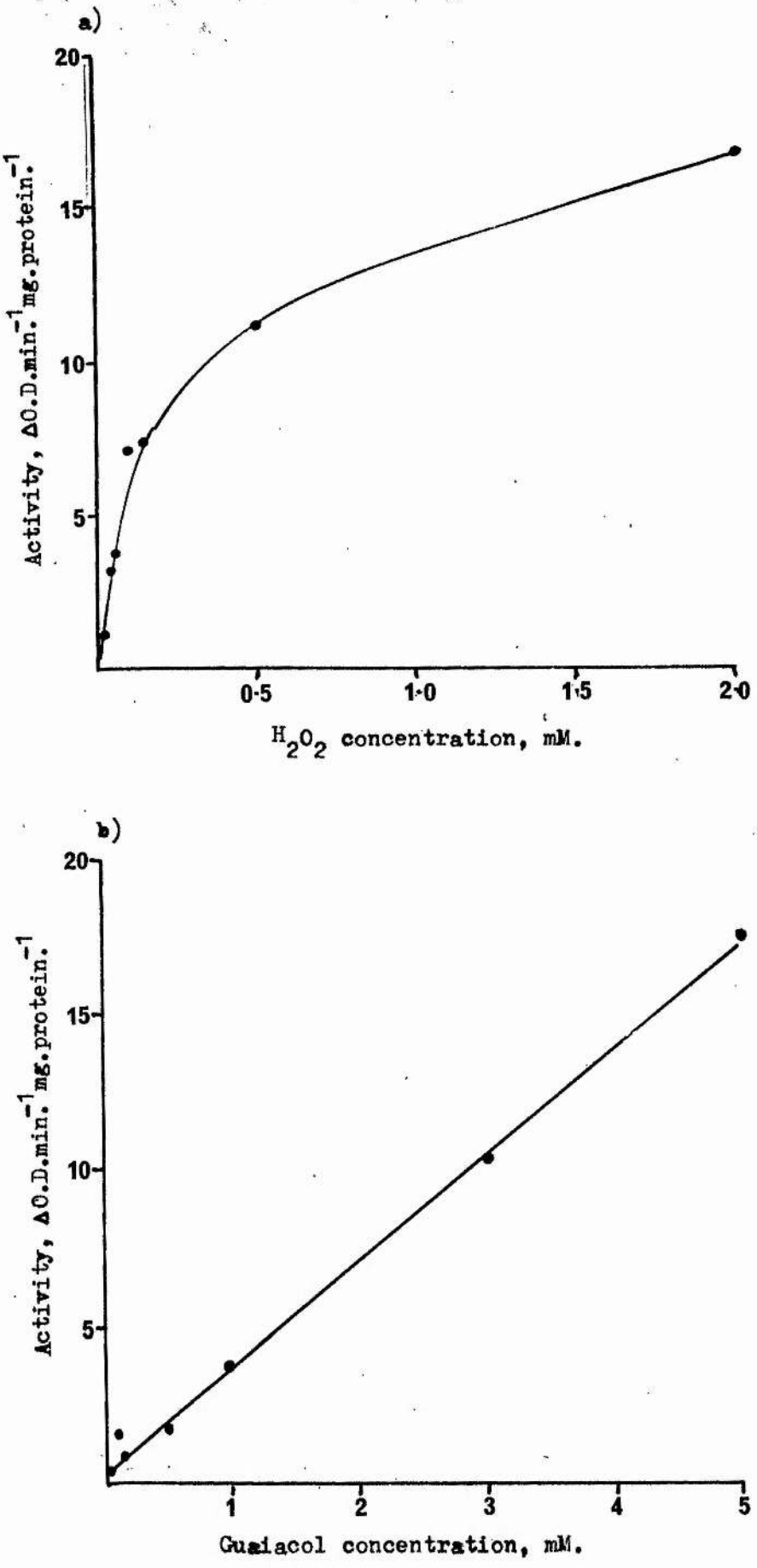


Fig.6.2. The effect of substrate concentration on the activity of peroxidase and catalase from Glyceria maxima roots.

a) Effect of H_2O_2 concentration on peroxidase activity. Guaiacol concentration 5mM.

b) Effect of guaiacol concentration on peroxidase activity. H_2O_2 concentration 2mM.

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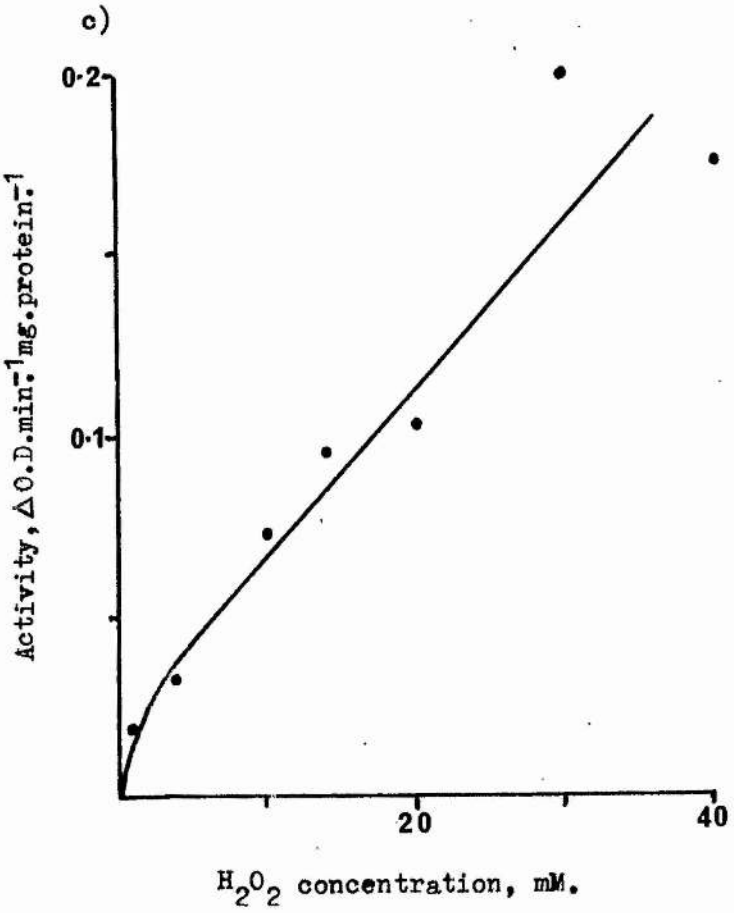


Fig.6.2. Continued from previous page.

c) Effect of H_2O_2 concentration on catalase activity.

Each point represents 1 measurement
Lines fitted by eye.

Discussion

Peroxidase. Flooding had no effect on peroxidase activity in the root tips of any of the species except Brachypodium sylvaticum. In this species two explanations for the increase can be put forward. First, many of the root tips were visibly damaged by flooding and the symptoms resembled Fe(II) toxicity. Toxicity could result in an increase in peroxidase activity. Toxic concentrations of zinc caused an increase in peroxidase activity in roots of a non-tolerant clone of Silene cucubalus (Mathys, 1977). The second possibility is that the damaged roots had been heavily contaminated with bacteria and these contributed to the activity. This explanation may be less likely because catalase activity was completely lost in flooded roots, and it is unlikely that bacteria with peroxidase activity will lack catalase.

Chirkova et al., (1973) investigated the effect of hypoxia and anoxia on peroxidase activity in the roots of various species in solution culture. They compared two flood-tolerant plants (Populus petrowskiana and Phaseolus vulgaris)with two flood-tolerant plants (Salix alba and Glyceria aquatica). The results of Chirkova et al. agree with these experiments. Over a short period (3 - 7 days) they found that subjecting the roots to nitrogen bubbling (a hypoxic treatment, comparable to flooding) had little effect on peroxidase activity in any of the species except P. vulgaris. In this species activity doubled after 20 hours. Peroxidase activity did increase when whole plants were subjected to anoxia. This is a completely unnatural situation, and suggests that increase in peroxidase activity is an indication of cell damage.

The activity of peroxidase assayed with guaiacol as substrate had no correlation with Fe(II) tolerance, flood-tolerance or the amount of air space in the roots of various species. There was a clear difference in activity between taxonomic groups. The Gramineae had greater specific activity than the Dicots. The Eriophorum species (Cyperaceae) were intermediate. Differences in specific activity of peroxidase have been

found in a survey of activity throughout the plant kingdom (Georgiev et al., 1977). They found that activity increased from the lower plants to higher plants. Saunders et al. (1964) suggested that plants could be classified into two groups on the basis of peroxidase and phenol oxidase activity: i) species with high peroxidase activity; ii) species with low peroxidase activity and high phenol oxidase activity.

Catalase. Flooding had no effect on catalase activity except in Phalaris arundinacea, Nardus stricta and Brachypodium sylvaticum. In these species activity was inhibited. Inhibition had no relationship with flooding tolerance of the species or air space in their roots. The activity of catalase had no relationship with Fe(II) tolerance.

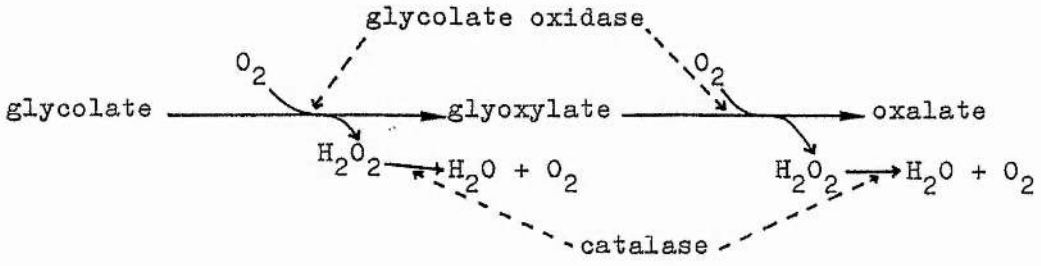
Peroxidase and catalase activity was detected in all the species, but their role, if any, in Fe(II) tolerance cannot be deduced from these experiments.

Glycolate oxidase in Oryza sativa.

Glycolate oxidase activity was easily detected in shoots. Activity in the roots was very low and near the detection limit of the assay, so the results must be treated with caution. Previous investigations have shown that glycolate oxidase activity in roots may be low or absent. Glycolate oxidase and catalase are located in microbodies (Breidenbach, 1976). Microbodies isolated from Daucus carota and Ricinus communis roots had glycolate oxidase activity (Huang and Beevers, 1971), but no activity was detected in microbodies from Zea mays roots (Parish, 1972).

Peroxidase Metabolism and Oxidase Activity in roots under Hypoxic Conditions.

Vamos and Koves (1972) suggested that oxygen could be generated metabolically by rice. This oxygen could then be used to oxidise and thus detoxify hydrogen sulphide. They suggested that oxygen was produced by the combined action of glycolate oxidase and catalase as follows:



Oxidation of glycolate to oxalate by glycolate oxidase produces hydrogen peroxide. This is then decomposed to oxygen and water by catalase. They produced no experimental evidence that this pathway occured in rice roots. The low levels of glycolate oxidase activity found in these experiments suggest that the pathway may not be important. There is other evidence to suggest that this pathway has no importance in rice roots (or in other species) under flooded (hypoxic) conditions. The affinity of glycolate oxidase for oxygen is very low compared with cytochrome oxidase (table 6.6). The affinity of cytochrome oxidase is about 1,000 times greater than glycolate oxidase. Under flooded conditions oxygen availability in roots will be low, it is likely that any oxygen diffusing into cells will be more efficiently used by cytochrome oxidase. There is evidence that this could be so. Dubinina (1961) found that subjecting tomato roots to anoxia in solution culture caused an increase in glycolate levels. This suggests that the activity of glycolate oxidase was inhibited. But tomato is a flood-intolerant species and may have little ability to allow oxygen transport to its roots. Senecio aquaticus is a flood-tolerant species and transports enough oxygen to its roots to allow some to diffuse out into the external medium (lambers, 1976). When this plant is grown in anaerobic solution culture its aerobic respiration rate is reduced by 50%. Evidence has been produced to show that this decrease can be attributed to the loss of alternative oxidase activity under hypoxia (Lambers and Smakman, 1978; Lambers et al., 1978). The affinity of the alternative terminal oxidase is only 10 times less than cytochrome oxidase (table 6.6) but it is inhibited under hypoxia. This evidence suggests that glycolate oxidase, localized in microbodies

and with a very low affinity for oxygen, will have little chance of being active under hypoxia. Rice, which has a large capacity for oxygen diffusion and ROL from its roots (Armstrong, 1969), may suffer from oxygen shortage when its roots are subjected to anoxia. Oxygen diffusion from the shoots was not sufficient to maintain the energy charge at the same level as when the rooting medium is aerated (Raymond et al., 1978). This suggests that even cytochrome oxidase activity is partly inhibited. Glycolate oxidase is likely to be almost inactive. The same argument can be applied to the other flavin mononucleotide-linked enzymes which produce hydrogen peroxide (Xanthine oxidase and amino acid oxidase). The other suggestion of Vamos and Koves (1972) was that peracids are produced by the action of free $^{\cdot}OH$ radicals formed during photosynthesis, on organic acids:

$$R-\underset{\underset{O}{\|}}{C}-OH \;+\; 2\,{}^{\cdot}OH \longrightarrow R-\underset{\underset{O}{\|}}{C}-O-OH \;+\; H_2O$$

peracid

If these peroxides were then translocated to the roots they could release their oxygen where it would be available to oxidase hydrogen sulphide. They gave no evidence for this scheme and, using the same arguments as before, it is likely that if any oxygen was released in this way inside the root cells, it would be rapidly used by cytochrome oxidase.

The considerations above call into question the extent of peroxide metabolism in the roots of flooded plants. If very little hydrogen peroxide can be generated, then peroxidase and catalase will be completely inactive. Both these enzymes were detected in flooded roots and this underlines the difficulty in deciding the relationship of *in vitro* enzyme assays to their activity in the whole plant.

Fe(II) Consumption by Root Extracts from Various Species.

All the species (*Oryza sativa*, *Ranunculus flammula* and *Glyceria*

maxima) produced root extracts which caused the disappearance of Fe(II) from solution. When the extracts were boiled the activity decreased only in Oryza sativa and Glyceria maxima. This suggests that in these species a heat-labile factor was active. From these experiments it cannot be said if the extracts caused Fe(II) oxidation, or formed a complex with the Fe(II) stronger than the Fe(II)-bipyridyl complex.

The results with Oryza sativa confirm those of Yamada and Ota (1958) who first demonstrated this phenomenon. They produced evidence that extracts catalysed Fe(II) oxidation: Oxygen was consumed during the reaction, indicating that it was the electron acceptor. The active factor could be partly purified by ammonium sulphate and acetone precipitation and they suggested that it was a peroxidase-like enzyme. Activity was inhibited by cyanide and azide and competitively inhibited by p-phenylenediamine and pyrogallol. However, oxidation occured in the absence of added hydrogen peroxide.

It is possible that Glyceria maxima has a similar activity, but further investigation is needed to show that the reaction involves oxidation. Kenten and Mann (1950, 1952) have found that pea root extracts and purified peroxidase can catalyse the oxidation of Mn(II) to Mn(III) or (IV) and ferrocyanide (a complex containing Fe(II)) to ferricyanide (a complex containing Fe(III)). Oxidation also occured in pea plants grown with high Mn(II) levels (Kenten and Mann, 1957). But the reactions needed exogenous hydrogen peroxide and were mediated by the oxidation and reduction of certain phenols (fig 6.3). The requirement for hydrogen peroxide suggests that this may not be the mechanism causing Fe(II) oxidation in the extracts. Also it is not possible to decide from this evidence if such a system would operate in vivo.

Some bacteria are able to catalyse the oxidation of Fe(II) directly by oxygen. Two of these iron bacteria, Ferrobacillus ferrooxidans and Thiobacillus ferrooxidans, use this oxidation as an energy source. Other microorganisms oxidise Fe(II) including Gallionella, Siderocapsa and Leptothrix but their biochemistry has not been studied (Aristovskaya

and Zavarin, 1971; Doelle, 1975). F. ferrooxidans and T. ferrooxidans are confined to habitats of extremely low pH and their Fe(II) oxidising mechanism may be dependent on this. In F. ferrooxidans Fe(II) is complexed in the cell wall where it is oxidised by molecular oxygen. The reaction is catalysed by an Fe(II) oxidase or oxygenase. The electron from the oxidation is carried by a modified cytochrome chain and reduces oxygen (Doelle, 1975). It could be speculated that a similar mechanism is active in Oryza sativa and Glyceria maxima. If the enzyme was located in cell walls adjacent to the air space cavities of aerenchymatous roots it would be in the ideal position to intercept oxygen before it could diffuse into the cell and be reduced by cytochrome oxidase. Gallionella can oxidise Fe(II) and lives in swamps and the surface layers of gleyed soils where it is associated with spots and streaks of oxidised Fe (Aristovskaya and Zavarin, 1971). How it oxidises Fe is not known, but the mechanism must work in the pH range of waterlogged soils. A similar mechanism could occur in plant roots. There is little evidence for these suggestions and further investigation is needed.

If oxygen diffusion from roots produces and oxidised rhizosphere it is possible that Fe(II) oxidising microorganisms could occur at the interface of the rhizosphere and reduced soil. Here they could use oxygen from the roots to oxidise Fe(II) diffusing in from the reduced areas of soil. Such a symbiosis has been found between rice and Beggiatoa, a hydrogen sulphide oxidising microorganism to prevent sulphide toxicity to the rice (Pitts et al., 1972; Joshi and Hollis, 1977). Beggiatoa occurs in the rice rhizosphere where it uses oxygen from the roots to oxidise hydrogen sulphide to sulphur. The reactions produce hydrogen peroxide and, because Beggiatoa lacks catalase, this is autotoxic. However, the rice roots secrete catalase which breaks down the hydrogen peroxide and allows good growth of Beggiatoa. Beggiatoa does not grow well in drained soil or in flooded soil in the absence of rice. Spartina may also have an association with Beggiatoa.

Evidence from the previous chapters has suggested that Fe(II) oxidation does not contribute greatly to tolerance. The evidence in this chapter suggests that root extracts of some species can catalyse Fe(II) oxidation. Further investigation is needed to confirm this and to determine its role, if any, in Fe(II) tolerance.

CHAPTER 7

Root Aerenchyma

Introduction

The occurence of aerenchyma in the roots and shoots of plants has been a matter of interest for many years. Much has been written about the structure, occurence and function (Arber, 1920; Sifton, 1945, 1957; Williams and Barber, 1961; Sculthorpe, 1967). Aerenchyma in a wide sense denotes tissue in which there are greatly enlarged intercellular spaces or lacunae formed by separation or degeneration of cells. The occurence of aerenchyma is generally associated with plants from aquatic or waterlogged habitats. Aerenchyma was originally defined by Schenck in 1889 as a non-suberized "ventilating tissue" produced from a phellogen (Arber, 1920). Now aerenchyma is defined in a wider sense as described before. Aerenchyma in Schenck's sense is common in certain families, for example the Onagraceae, Lythraceae, Leguminosae, Salicaceae (Salix and Populus). It is also formed in the tropical tree Chorisia speciosa (Bombaceae) (C.A. Joly, personal communication). Aerenchyma forms in these woody species when they are flooded. The base of the stem or trunk becomes swollen with spongy aerenchyma and lenticels become hypertrophies (Arber, 1928). Aerenchyma, in the wide sense, is found in almost all plant organs: roots; rhizomes; stems; petioles and leaves (Arber, 1920; Sculthorpe, 1967) and in specially developed organs such as the pneumatophores of mangroves (Scholander et al., 1955).

In some herbaceous species aerenchyma development may result in a continuous system of air spaces from the stomata in the leaves to the roots, for example in Spartina alterniflora (Teal and Kanwisher, 1966) and Cladium mariscus (Conway, 1937), interrupted by only a few cell diaphragms. The diffusion of oxygen through the air spaces from shoots to roots occurs in this species and in many others with aerenchyma (Armstrong, 1979). Oxygen can also diffuse from the shoots to the

roots in non-aerenchymatous species such as peas (Healy and Armstrong, 1972), but to a lesser extent.

Knowledge of the function of aerenchyma was critically examined in a review by Williams and Barber (1961). They argued that the development of aerenchyma was excessive if its primary function was oxygen transport. Instead of this they suggested that aerenchyma was formed as a mechanical - cum - metabolic adaptation. Their discussion considered aerenchyma in shoots as well as roots. From the point of view of roots this mechanical - cum - metabolic adaptation was supposed to fit the plant to two characteristics of waterlogged or submerged soils: bending stresses and the decrease in frictional resistance of the soil and the lack of oxygen. Aerenchyma formation will decrease the amount of respiring tissue in a given volume of root and at the same time will allow the production of a reasonably thick root to give adequate anchorage. Additionally they suggest that, because bending stresses from movement of aerial parts die away less rapidly in wet soils, a honeycomb structure with regular transverse diaphragms will be able to withstand them. An objection to this part of the theory arises because diaphragms of this sort have never been observed in root aerenchyma (personal observation). Williams and Barber suggested that, although the development of aerenchyma would be determined by the above factors, it would incidentally serve as a gas transport pathway and an oxygen reservoir. Armstrong (1972), using model silicone roots with variable porosity, showed that the assumption of Williams and Barber that the devlopment of aerenchyma was too extensive if it was only required for gas transport was unfounded. ROL (radial oxygen loss from root tips) from the roots increased with increases in porosity up to high porosity levels. This effect would be exaggerated in living roots because of oxygen consumption by the root tissue and the soil. The other evidence that increased air space in roots leads to increased ROL from the root tips was discussed in Chapter 3.

The proposals which have been put forward for the functions of

aerenchyma in roots are as follows:

1. The mechanical - cum - metabolic compromise (Williams and Barber, 1961).
2. To allow increased gas (oxygen) transport to supply oxygen for aerobic respiration when roots are in waterlogged soils.
3. As a consequence of (2) oxygen can leak out of the root (ROL) and oxidise the rhizosphere and thus protect against phytotoxins produced under reducing conditions.
4. Act as an oxygen reservoir when the external supply from the shoots is cut off.

In this chapter the structure and occurence and effect of some environmental factors on root aerenchyma in some herbaceous wetland species will be examined. The possible functions of aerenchyma listed above will be discussed.

Materials and Methods

The origin of the plants used in the experiments is given in Chapters 2 and 6. Plants grown in flooded or drained sand culture were treated in the same way as described in Chapter 3.

Measurement of Air Space in Roots. Plants were either dug up freshly from the field or taken from sand culture and the root systems carefully washed free of soil or sand. Air space content of the roots was measured as described in Chapter 2. In some cases the root systems were carefully separated into primary and lateral roots and the air space in these measured separately. In this case primary roots are defined as those roots attached directly to the stem or root stock. Lateral roots are those arising from the primary roots.

Root Anatomy. Roots, collected from the field or from sand culture, were fixed and preserved in formalin-acetic-alcohol fixative (Purvis et al., 1966). To make transverse sections, root segments were dehydrated in a tertiary butyl alcohol series (50, 70, 85, 95 and 100%), embedded in paraffin wax (m.p. 60 °C) and sections cut using a microtome (15 or 20 μm thick). The sections were fixed to slides with Haupt's adhesive, and stained with safranin and light green (Purvis et al., 1966) and mounted Canada Balsam.

Root Respiration Rates. Roots were washed free of adhering soil particles and thoroughly rinsed in distilled water. The rate of oxygen uptake was measured at 18 °C using a Gilson Differential Respirometer. The respiration flasks contained 2ml 0.05M citric acid - 0.1M dipotassium hydrogen phosphate buffer, pH 5.4 and 0.3ml 10% KOH in the centre well. Rates of oxygen uptake were measured over one hour after equilibration for 30 minutes. The values given (μl O_2 mg^{-1} dry weight h^{-1}) are the mean of three replicates.

Results

The Occurence of Aerenchyma in Various Species Collected from their Natural Habitats.

Plants were carefully dug up from their natural habitats. Root systems were washed free of soil or peat and their air space content measured. The results are shown in table 7.1. There was a continuous variation in air space from 0 - 40% (v/v) in the range of species examined. Roots of species from well-drained soils (e.g. Brachypodium sylvaticum, Mercurialis perennis, Geum urbanum and Senecio jacobaea) contained relatively small amounts of air space (5%). The species from the wetland habitats had a wide range of air space in their roots. These differences could result from innate differences between species in their capacity to produce aerenchyma, or from differences in their habitats. Mentha aquatica and Myosotis scorpioides were growing closely to Ranunculus lingua in the fen at Lindores Loch. The latter species contained twice as much air space as the other two. The root systems of M. aquatica and M. scorpioides were located near the surface of the peat, whereas the roots of R. lingua penetrated deeply into the waterlogged peat. Waterlogging is probably an important factor in inducing aerenchyma formation (see later), so the development of superficial root systems may avoid waterlogging to some extent. This could be the case for Cardamine pratensis which, although it was surrounded by waterlogged vegetation, was elevated on a hummock above the water table.

Among the species from wetland habitats, the monocotyledons generally developed more air space than the dicotyledons (see also fig 7.3). The monocotyledons may have a greater capacity for aerenchyma development, but they do not always produce it. Brachypodium sylvaticum from a well-drained soil had no aerenchyma (table 7.1) and additionally Luzula campestris and some Carex species, all characteristic of well-drained soils, do not produce aerenchyma (Cutler, 1969). Some dicots, for example Senecio aquaticus, can produce a large amount of aerenchyma.

In three species (Glyceria maxima, Narthecium ossifragum and Lycopus

europaeus) air space in the primary and lateral roots was measured separately. In all cases air space was less in the laterals. It is possible that the more delicate lateral roots were squashed during preparation.

The results show that a wide range of air space occurs and this variation is probably the result of interspecific differences and environmental factors. When the same species were collected from separate localities (Eriophorum angustifolium and Ranunculus lingua), their air space content was similar. The only published study of air space in the roots of plants from different habitats is that of Iversen (1949). He examined the specific gravity of roots of species from swamp, meadow and dry-soil plants and found that in general the specific gravity increased in the order swamp < meadow < dry-soil. The present results are essentially in agreement with this.

The Structure of Aerenchyma in Various Species.

In all the herbaceous species examined in this study the aerenchyma was formed in the primary cortex of the roots. The only species where sloughing off of the primary cortex occured was Ammophila arenaria. All the other species retained their cortex along the entire length of their roots. The structure of the aerenchyma was examined in transverse sections of the primary roots taken from the mature zone at least 2cm behind the tip. The roots were from plants collected from the field or grown in sand culture in the glasshouse. The transverse sections of the roots are shown in plates 7.1 - 7.5.

The cortical aerenchyma had a variety of forms and the structure of the dicotyledonous and monocotyledonous roots will be described seperately.

Dicotyledons. Senecio jacobaea and Hieracium pilosella (plates 7.1 - 7.3a) were all collected from a sand dune: a well aerated soil. Their cortex contains no well-developed aerenchyma, although small intercellular spaces can be seen. These species represent the typical anatomy text-book structure. Galium palustre and Filipendula ulmaria have a similar

structure to the previous species (plate 7.1c and d) and the cavities formed in cell breakdown in Filipendula ulmaria may have been the result of damage during preparation. Species with this structure tended to have a small amount of air space (5%) when quantitative estimates were made (table 7.1 & 7.2). G. palustris is a wetland plant but air space has not been developed. The roots of this species are thin and ramify in the surface layers of the soil. Hydrocotyle vulgaris, another wetland plant also has thin roots and Iversen (1949) found that this species had roots of high spefific gravity.

Caltha palustris (plate 7.2a) has greatly enlarged intercellular spaces in the cortex except in the outer cell layers where the cells remain more tightly-packed. This structure allows the roots of this species to contain more air space (15 - 20%, page). The remaining dicotyledonous species, Mentha aquatica, Ranunculus flammula, Lythrum salicaria and Potentilla palustris have structures which can be better described as aerenchyma than simple intercellular spaces (plate 7.3a). In these species lacunae have formed in the cortex. This process is apparently aided by cell breakdown (lysigany) because remnants of broken cells can be seen. The process must be under physiological control to produce a regular pattern, as in Potentilla palustris where the lacunae are seperated by rapidly-spreading diaphragms one cell thick. In R. flammula this structure allows a large proportion of the root to be taken up by air space (up to 30%, table 7.2). Small air space values were found for P. palustris and M. aquatica (table 7.1) possibly because the stele occupies a large portion of the root in these species.

Monocotyledons. The general structure of the monocot roots examined differs from the dicots in the presence of a sheath of cells with secondarily thickened walls in the hypodermis. This sheath is sometimes referred to as the exodermis (Metcalfe, 1971; Cutler, 1969). It is not clear if it functions as an apoplastic barrier in the same way as the endodermis. The cortex of these species can be divided into three zones:

the outer is the exodermis; the middle portion is of unthickened cells and the air cavities form in this part; the inner zone next to the endodermis has more closely-packed cells which may become secondarily thickened in the Juncaceae (Cutler, 1969). The thickening of the inner zone can be seen in Juncus effusus (plate 7.4 a & b).

In Ammophila arenaria (plate 7.3b) the structure of the cortex is similar to the dicots with little air space (Senecio jacobaea and Hieracium pilosella). A small amount of air space (c. 5% table 7.2) is present as small intercellular spaces. All the other monocots examined have an elaborate aerenchyma structure which can be divided into two types: i) that in the grasses, Juncus and Narthecium (Gramineae, Juncacaea & Liliaceae) and ii) that in the Cyperaceae (Eriophorum, Trichophorum & Carex). In type (i) (plates 7.3c - 7.4c) the lacunae are seperated by radically extending plates of cells. Many of these cells are broken, but in places chains of living cells traverse the cortex. The lacunae appear to have formed by cell seperation (schizogeny) and cell breakdown (lysigeny). In type (ii) there is a structure with a very well-organised appearance (plates 7.4d - 7.5c). Regularly-arranged chains of cells traverse the cortex in Eriophorum angustifolium. In longitudinal section these can be seen as long plates (Armstrong, 1979). Between these radiating plates of cells are stretched the cell walls of broken cells forming a structure like a spider's web. In the previous type the dead cell walls stretched from the inner to the outer cortex.

Sections of Phalaris arundinacea and Eriophorum angustifolium roots were also examined (not shown) and had similar structures to Glyceria maxima and Eriophorum vaginatum respectively. Both types of aerenchyma structure in the monocotyledons allow large amounts of air space in the roots. The greatest amount of air space, up to 50%(v/v), was found in species with this structure (Tables 7.1, 7.2, 7.3).

On the basis of the species examined here, the structures of aerenchyma can be grouped into four categories. These are listed below

and shown diagramatically in fig 7.1.

1) Aerenchyma of loosely-packed cells with large intercellular spaces formed schizogenously. e.g. Caltha palustris.
2) Aerenchyma of lacunae formed schizogenously & lysigenously with varying degrees of organisation. In dicotyledons with no exodermis. e.g. Mentha aquatica, Ranunculus flammula (fig 7.1a & b).
3) Aerenchyma of radially-arranged lacunae formed as in (2), but in monocotyledons except for the Cyperaceae. Exodermis present. e.g. Glyceria maxima, Juncus effusus (fig 7.1c).
4) Aerenchyma of radially-arranged lacunae formed as in (2), but the dead cells stretched between the radial cell plates. In the Cyperaceae. e.g. Eriophorum spp., Carex curta and Trichophorum caespitosum (fig 7.1d).

The classification is based on only a small number of species. To confirm it many other species must be examined. Other types of structure may be found.

In plates 7.4a and 7.4b two stages in the formation of aerenchyma in Juncus effusus are shown. Plate 7.4a shows a younger root segment with less extensive formation of lacunae. The lacunae appear to form by cell seperation along the radial walls followed by extensive lysigeny to give the mature structure seen in Plate 7.4b.

In every section examined the cortex of a normally aerenchymatous root remained intact around the area where lateral roots emerged (Plates 7.3c & d; 7.4b; 7.5b). In Carex curta (plate 7.5b) the diaphragms between lacunae are usually one cell thick, but where the laterals emerge many rows remain. This suggests that the formation of lacunae is a well-coordinated process. Konings and Verschuren (1980) note that in Zea mays and some other Gramineae & Cyperaceae the cortical areas opposite the xylem poles often remain intact. Aerenchyma formation in Zea mays may be under hormonal control (Drew et al., 1979). The development of laterals and lacunae may be coordinated hormonally, at least in this species.

The Effect of Some Environmental Factors on the Amount of Air Space in Roots.

a) Flooding in Sand Culture. The air space in the roots of plants grown for eleven weeks in drained and flooded sand culture is shown in table 7.2. Except for Myosotis scorpioides, primary and lateral roots are shown seperately. Generally the lateral roots contained less air space. This could have been an artifact because they are easily squashed during preparation. In the drained treatment only Glyceria maxima, Brachypodium sylvaticum and Ammophila arenaria had the same amount of air space in their primary and lateral roots. The species have been divided into flood-tolerant and flood-intolerant types on the basis of their growth response to flooding in sand culture. Growth of intolerant plants is decreased by flooding and growth of tolerant plants is unaffected or increased by flooding (Crawford, 1967; McManmon and Crawford, 1971; tables 7.4, 7.5 and 3.1). The flood-tolerant species had a wide range of air space before flooding. Flooding increased the amount of air space in the primary roots of all the flood-tolerant species. The effect on Glyceria maxima was not significant because of the variation in the flooded treatment. In the intolerant species, flooding increased air space in Senecio jacobaea and Ammophila arenaria. Air space in Hieracium pilosella and Brachypodium sylvaticum was slightly decreased by flooding. In these two species flooding greatly inhibited root growth and many of the roots were unhealthy. The low amount of air space found in Myosotis scorpioides agrees with values in plants from the field (table 7.1). The root system in this species was too fibrous to divide into primary and lateral roots. The effects of flooding on lateral roots were erratic and, as mentioned above, the results may be unreliable.

In a further experiment, plants were grown in flooded or drained sand culture for a longer period of 21 - 32 weeks. The results are shown in table 7.3. In this case air space in whole root tystems was measured. The Eriophorum species had the most air space, and the amount was not increased by flooding. Air space in G. maxima and Deschampsia

caespitosa was also unaffected by flooding. In the previous experiment (table 7.2) the air space in flooded G. maxima was 38.9%. In this experiment it was 25.8%. No clear explanation can be offered for this, but the inclusion of lateral roots in these measurements may have decreased the average air space value for the whole root system. Air space in Nardus stricta and Ranunculus flammula was increased by flooding in the same way as the previous experiment. Again the absolute values were lower, possibly for the same reason. Air space in the roots of the M. scorpioides drained treatment was not measured because the roots had become too matted to be extracted from the sand without damage. The flooded treatment had an air space content comparable to the previous experiment. The roots of Senecio jacobaea in the flooded treatment were almost completely dead after 32 weeks of flooding so air space measurement was not possible. The roots of all the other species shown in table 7.3 were healthy after this period. Roots of the Eriophorum species showed markedly more growth and penetrated deeper into the sand in the flooded treatments. Shoot growth of these species is shown in Table 7.4. Flooding stimulated shoot growth in both species, measured as the number of new shoots produced by E. angustifolium and as leaf length in E. vaginatum.

Filipendula ulmaria (a flood-tolerant species (Crawford, 1967)) was grown for 16 weeks in flooded and drained sand culture (watered with 1/50th strength Hoagland's solution). After this period the air space in the roots of the drained treatment was 9.6 $\pm$ 1.3% and in the flooded treatment 10.4 $\pm$ 4.2% (difference not significant, $p = 0.05$). These values are comparable to those in plants collected from the field (Table 7.1). It appears that this species, although flood-tolerant, lacks the ability to form extensive air space.

The results show that the volume of root occupied by air space varies between species under flooded or drained conditions. Air space can be increased by flooding, but not in all species. The Eriophorum species retained the same large amount of air space in drained conditions, suggesting

that the production of aerenchyma with a complex structure (plate 7.5c) is not under environmental control.

b) Air space in the roots of Senecio aquaticus and Caltha palustris subjected to various degrees of waterlogging in their natural habitats.

Plants of Senecio aquaticus were collected from the Loons, a complex mire on Orkney Mainland (described in Chapter 4), in June 1980. S. aquaticus is widespread in this mire, but mainly confined to the eutrophic areas. It could be found growing in areas where the watertable was at or above the peat surface to areas where the watertable was well below the rooting zone. To investigate the effect of the degree of waterlogging on root air space in S. aquaticus, plants were carefully dug up along with a sample of soil from the rooting zone. The root systems were carefully washed free of peat and their air space measured. Soil samples were collected into polythene bags to minimize loss of water and their water content determined after drying for 12h at 100 °C. The water content of the soil was intended to give an estimate of the degree of waterlogging. Although water content and watertable are likely to vary during the growing season, measurement at a particular time will be a reflection of the relative wetness of the sites throughout the season.

The results are shown in fig. 7.2. A significant positive correlation ($r = 0.667$, $p = 0.05$) was found between soil water content and air space in the roots. This suggests that the development of air space in this species is influenced by the degree of soil waterlogging. All the plants were collected within 150 metres of each other and are likely to belong to the same population, suggesting that the difference in air space is a phenotypic response to soil wetness. The closely-related species, S. jacobaea, also showed a marked increase in air space when flooded (table 7.2).

Caltha palustris, from the same site, showed a similar response to soil water content, although in this case only two plants were examined. A plant from a dry site (73.1% water content) contained 17.3% air space and a plant from a waterlogged pool nearby (82.4% water content) had 23.8% air space in its roots.

c) Growth and air space in the roots of Nardus stricta grown in flooded and drained sand culture under low and high nutrient levels.

The plants were grown in the same way as described for Deschampsia caespitosa and Glyceria maxima (Chapter 3 p. 29). Growth is shown in Table 7.5. Flooding had no effect on shoot or root growth at either nutrient level. On this basis N. stricta can be classified as flood-tolerant. At both nutrient levels flooding slightly decreased the root:shoot ratio, but nutrient level had a large effect on root:shoot ratio under flooded and drained conditions. The root:shoot ratio was less in the high nutrient treatment. The response was similar to D. caespitosa and G. maxima (table 3.1), but more marked.

Flooding and nutrient level both had an effect on air space in the roots (table 7.6). The flooded roots contained more air space than drained roots. In each of the flooded and drained treatments the roots from the low nutrient treatment contained more air space. Analysis of variance (table 7.6) showed that the effects of nutrient level and flooding were significant ($p = 0.01$). Flooding and nutrient level acted on air space independently because the analysis of variance showed no interaction between these factors.

d) The effect of temperature on the air space in roots of Lolium perenne.

Lolium perenne seedlings grown at 12 °C and 20 °C for 3 weeks in unaerated Hoagland's solution had different amounts of air space in their roots (table 7.7). Plants grown at 20 °C had more air space than plants grown at 12 °C. The weight of roots after this period was 3.9 times greater at 20 °C (Schneider, 1980).

The Oxygen Reservoir Capacity of Root Aerenchyma.

It has been suggested that aerenchyma could act as an oxygen reservoir during periods when the external supply of air is cut off (Williams and Barber, 1961). In this experiment an attempt has been made to provide a quantitative estimate of the capacity of root aerenchyma to act as an oxygen reservoir in a number of species.

The aerobic respiration rate and air space in roots of various species freshly collected from the field was measured as described in the "Materials and Methods." From these measurements the length of time that the oxygen supply in the root air spaces would support aerobic respiration was calculated. The results are shown in table 7.8 and fig 7.3. The oxygen reservoir time was calculated using two assumptions:
1. The initial oxygen concentration in the air spaces is 20% (v/v)
2. The rate of oxygen consumption remains constant until the supply is exhausted. Neither of these assumptions are entirely accurate. The oxygen concentration in air spaces is probably much less (Coult and Vallance, 1958; Teal and Kanwisher, 1966) and, although the rate of aerobic respiration may stay constant down to low oxygen concentrations, it does fall off after a certain concentration has been reached (Armstrong and Gaynard, 1976; McCreath, 1980).

The results show that oxygen in the air spaces will be very quickly used up once the external supply is cut off. The longest supply, in Eriophorum angustifolium, is less than 2 hours. A reservoir time of this magnitude is ecologically unimportant. If allowance is made for the two assumptions above, and also for the ecologically unrealistic temperature of 18 °C at which the respiration rates were measured, this reservoir time will not be altered substantially. Armstrong (1978) measured the oxygen reservoir capacity of E. angustifolium leaves. He found, by direct measurement of oxygen concentration in the leaf aerenchyma, that oxygen was exhausted after 60 minutes at 23 °C. These results are in agreement with the present data for E. angustifolium

roots and suggest that aerenchyma in roots, or in the submerged shoots of aquatic plants at night, will have no importance as an oxygen reservoir.

Table 7.1 - Air space in the root systems of various species growing in their natural habitats.

	Air space in roots*, %(v/v) ± S.E. (n = s)	Habitat	Location
Eriophorum angustifolium	41.7 ± 1.9	Raised bog, pool	Bankhead Moss, Fife
	40.0 ± 3.6	Raised bog, pool	Bankhead Moss, Fife
	31.4	Valley mire	The Loons, Orkney Mainlan
Glyceria maxima	35.2 ± 0.3(P)	Loch margin	Lindores Loch, Fife
	10.9 ± 0.8(L)		
Eriophorum vaginatum	31.0 ± 0.8	Raised bog	Bankhead Moss, Fife
Trichophorum caespitosum	25.8 ± 3.1	Raised bog	Perthshire
Juncus effusus	25.4 ± 3.5	Raised bog, pool	Bankhead Moss, Fife
Senecio aquaticus	27.7	Valley mire	The Loons, Orkney Mainlan
Spartina townsendii	24.7 ± 1.4	Flooded sand culture in glasshouse	
Carex curta	22.2 ± 2.0	Raised bog, pool	Bankhead Moss, Fife
Narthecium ossifragum	19.0 ± 1.5(P)	Raised bog	Perthshire
	3.3 ± 1.4(L)		
Ranunculus lingua	12.3 ± 1.7	Fen, watertable at surface	Lindores Loch, Fife
	19.1 ± 0.4	Marsh on pond margin	St. Leonard's Forest, Sussex
Lythrum salicaria	11.8 ± 1.8	Fen, watertable at surface	Lindores Loch, Fife
Potentilla palustris	9.6[b]	Fen, watertable at surface	Lindores Loch, Fife
Lycopus europaeus	16.7 ± 3.3[a](P)	Fen, watertable at surface	Lindores Loch, Fife
	3.7 ± 0.3(L)		
	8.8 (P + L)		
Phalaris arundinacea	7.7 ± 0.6	Dry fen	Lindores Loch, Fife
Mentha aquatica	6.9 ± 0.6	Fen, watertable at surface	Lindores Loch, Fife
Senecio jacobaea	5.2[b]	Sand dunes	Boy of Skail, Orkney Mainland
Geum urbanum	6.2 ± 5.4[a]	Well-drained woodland soil	St. Leonard's Forest, Sussex
Myosotis scorpioides	4.9 ± 0.7	Fen, watertable at surface	Lindores Loch, Fife

continued

Filipendula ulmaria	6.1 ± 3.5	Dry fen	Lindores Loch, Fife
Mercurialis perennis	0.5 ± 0.1	Well drained woodland soil	Boarhills, Fife
Brachypodium sylvaticum	0.5 ± 0.1	Well drained woodland soil	Boarhills, Fife
Cardamine pratensis	0.0	Valley mire	The Loons, Orkney Mainla

* Mean values for whole root systems unless indicated

P Primary roots only

L Lateral roots only

[a] n = 2

[b] n = 1

Table 7.2 - Air space in the roots of various species grown for eleven weeks in drained and flooded sand culture.

	Air space in roots, % (v/v) (range) (n = 3)		"t" for comparison between flooded & drained treatments
	Drained	Flooded	
(a) Primary roots			
Flood-tolerant species[a]			
Nardus stricta	37.8 (32.7-44.1)	54.8 (52.9-55.2)	8.03**
Glyceria maxima	18.8 (18-19.9)	38.9 (22.7-55.5)	2.57
Ranunculus flammula	14.4 (11.0-17.0)	36.0 (35.1-37.4)	9.53**
Phalaris arundinacea	9.7 (7.5-11.4)	29.9 (27.4-34.6)	8.14**
Myosotis scorpioides[c]	3.8 (2.8-4.3)	6.4 (5.8-7.6)	3.14*
Flood-intolerant species[a]			
Brachypodium sylvaticum	8.9 (4.2-12.7)	2.3 (0.6-4.0)[b]	2.06
Senecio jacobaea	8.3 (7.6-8.9)	20.2 (14.8-26.2)	4.22*
Ammophila arenaria	6.2 (5.1-6.5)[b]	12.3 (9.6-15.0)[b]	2.45
Hieracium pilosella	5.8 (4.6-6.2)	4.8 (1.4-7.0)	0.59
(b) Lateral roots			
Flood-tolerant species[a]			
Nardus stricta	2.8 (0-5.0)	10.9 (8.2-14.9)	3.86*
Glyceria maxima	19.4 (13.4-30.4)	0.0 (0-0)	6.34**
Ranunculus flammula	0.0 (0-0)	5.4 (2.8-7.5)	7.00**
Phalaris arundinacea	0.1 (0-0.3)	0.0 (0-0)	1.00
Myosotis scorpioides	-	-	-
Flood-intolerant species[a]			
Brachypodium sylvaticum	7.3 (5.1-11.4)	8.0 (0-14.1)[b]	0.06
Senecio jacobaea	2.9 (0-5.0)	10.8 (7.2-14.5)	2.42
Ammophila arenaria	8.4 (4.8-11.9)	1.0 (0.4-1.5)	2.71
Hieracium pilosella	0.0 (0-0)	1.2 (0.6-3.0)	3.82*

[a] Data from Crawford (1967), McManmon and Crawford (1971) and Chapter 7

[b] n = 2

[c] Primary and lateral roots together

Air space content significantly different between flooded and drained treatments at the following probability levels:

* p = 0.05 ** p = 0.01 *** p = 0.001

"t" was calculated using an arcsin transformation of the data

Table 7.3 - Air space in the roots of various species grown for thirty-two weeks in drained and flooded sand culture.

	Air space in roots, % (v/v) range. (n = 3)		"t" for comparison between flooded & drained treatments
	Drained	Flooded	
Flood-tolerant species[a]			
Eriophorum angustifolium[d]	48.2 (47.5-49.7)	50.6 (49.4-52.4)	2.15
Eriophorum vaginatum[d]	43.2 (40.5-45.9)[b]	49.1 (47.1-50.1)	0.62
Nardus stricta	29.1 (27.2-30.7)	41.6 (39.5-44.3)	7.75**
Glyceria maxima	26.4 (25.6-27.5)	25.8 (19.5-31.7)	0.21
Deschampsia caespitosa	24.0 (21.0-28.1)	24.5 (23.9-25.1)	0.26
Ranunculus flammula	9.1 (9.0-9.4)	17.1 (16.0-17.8)	14.95***
Myosotis scorpioides	-	8.4 (7.0-10.6)	-
Flood-intolerant species[a]			
Senecio jacobaea	6.9 (6.3-7.8)	-	-

[d] Grown for 21 weeks

For other notes see Table 7.2

Table 7.4 - The growth of Eriophorum angustifolium and Eriophorum vaginatum after 21 weeks in flooded and drained sand culture.

Eriophorum angustifolium

Treatment	Number of shoots per bucket (n = 2). Initially 8 shoots per bucket
Drained	52.5 (43-62)
Flooded	86.5 (76-97)

"t" for comparison between drained and flooded treatments = 2.4
Not significant, $p > 0.05$

Eriophorum vaginatum

Treatment	Length of longest leaf on each clump cm ± S.E.
Drained	35.8 ± 1.3 (n=10)
Flooded	48.1 ± 2.3 (n=8)

"t" for comparison between drained and flooded treatments = 4.96
Significant, $p = 0.001$

Table 7.5 - Growth (final fresh weight) of Nardus stricta grown for eleven weeks in drained and flooded sand culture at low (one-fiftieth strength Hoagland's solution) and high (full-strength Hoagland's solution) nutrient levels.

	Fresh weight g. plant^{-1} ± S.E. (n = 7)		Root:shoot ratio
	Shoots	Roots	± S.E. (n = 7)
Low nutrient - Drained	7.004 ± 1.102	6.842 ± 1.413	0.95 ± 0.06
Low nutrient - Flooded	6.946 ± 0.508	6.098 ± 0.491	0.88 ± 0.04
High nutrient - Drained	7.462 ± 1.734	4.462 ± 1.734	0.53 ± 0.06
High nutrient - Flooded	9.129 ± 2.646	3.993 ± 1.085	0.42 ± 0.04

Table 7.6 - Air space in the primary roots of Nardus stricta grown for 11 weeks in drained and flooded sand culture at low (one-fiftieth strength Hoagland's solution) and high (full-strength Hoagland's solution) nutrient levels.

	Air space in roots % (v/v) with range (n = 3)	
	Low nutrient	High nutrient
Drained	32.5 (31-34)	23.7 (22-28)
Flooded	44.9 (43-47)	34.3 (28-39)

Analysis of variance for the effect of watertable and nutrient level on air space (using arcsin transformation of the data).

Source of variance	Mean squares	Degrees of freedom	F
Nutrient level	106.8	1	16.7**
Watertable	148.4	1	23.2**
Interaction	0.3	1	0.05
Residual	6.4	8	

** significant effect p = 0.01

Table 7.7 - Air space in the roots of Lolium perenne seedlings grown in unaerated solution culture (full-strength Hoagland's solution) at 20°C and 12°C for three weeks (14h daylength. Light intensity 14,000 lux).

Temperature	Air space, % (v/v) . (n = 2)
12	2.0 (1.0 - 2.9)
20	5.6 (5.3 - 5.8)

Table 7.8 - The oxygen reservoir capacity of the air space in roots of various species. The reservoir capacity was calculated as the time taken for oxygen in the air spaces to be completely consumed, assuming a constant respiration rate at 18°C and an initial concentration of 20% O_2 in the air space atmosphere.

Species	Oxygen reservoir capacity, minutes	Oxygen consumption $\mu l O_2$ mg. dry wt.$^{-1}$ h.$^{-1}$	Air space ml. g. dry wt.$^{-1}$
Eriophorum vaginatum	126	0.362	3.81
Eriophorum angustifolium	102	0.781	6.62
Trichophorum caespitosum	67	0.168	0.94
Glyceria maxima	37	0.520	1.61
Ranunculus lingua	32	0.670	1.76
Juncus effusus	34	0.645	1.84
Carex curta	22	0.633	1.15
Lythrum salicaria	22	0.515	0.94
Narthecium ossifragum	14	0.388	0.46
Mentha aquatica	13	0.650	0.71
Lycopus europaeus	12	0.755	0.76
Potentilla palustris	9	0.520	0.39
Filipendula ulmaria	2	0.375	0.07

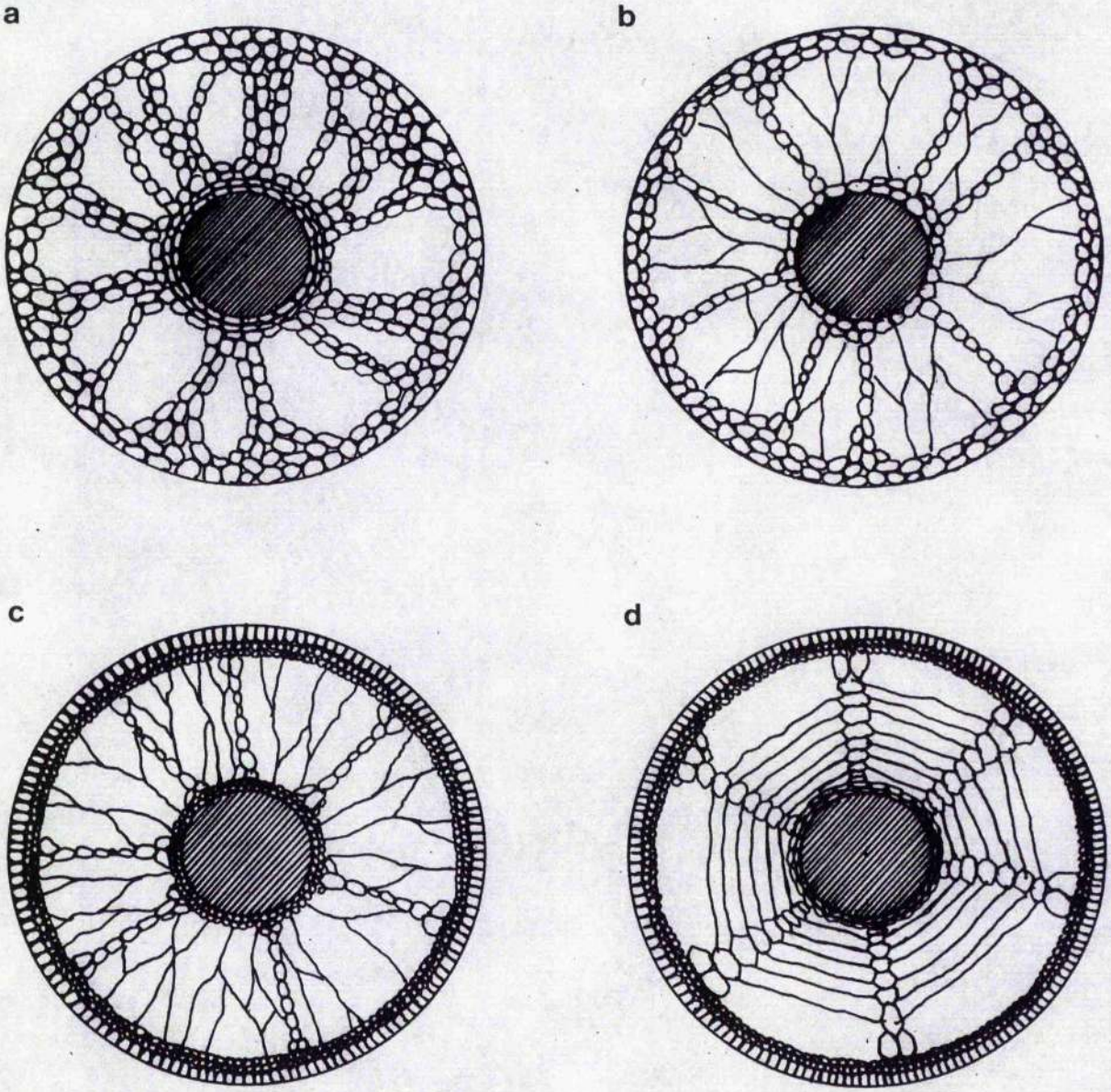


Fig.7.1. Diagrams showing various forms of aerenchyma in roots.

Types a. and b. Aerenchyma in dicotyledonous roots showing differing degrees of lacuna development. In type b.cell walls of broken cells are more evident, mainly crossing the cortex radially. There is no exodermis.

Types c. and d. Aerenchyma in monocotyledonous roots. Lacunae are well-developed in both types. There is an exodermis in both types. In type c. the broken cell walls extend radially (e.g. Gramineae and Juncaceae.), but in type d. they extend tangentially between the radiating cell plates. (e.g. Cyperaceae.)

The cross hatched central area is the stele.
The single lines represent broken cell walls.
The exodermis is the layer of thick walled cells beneath the epidermis.

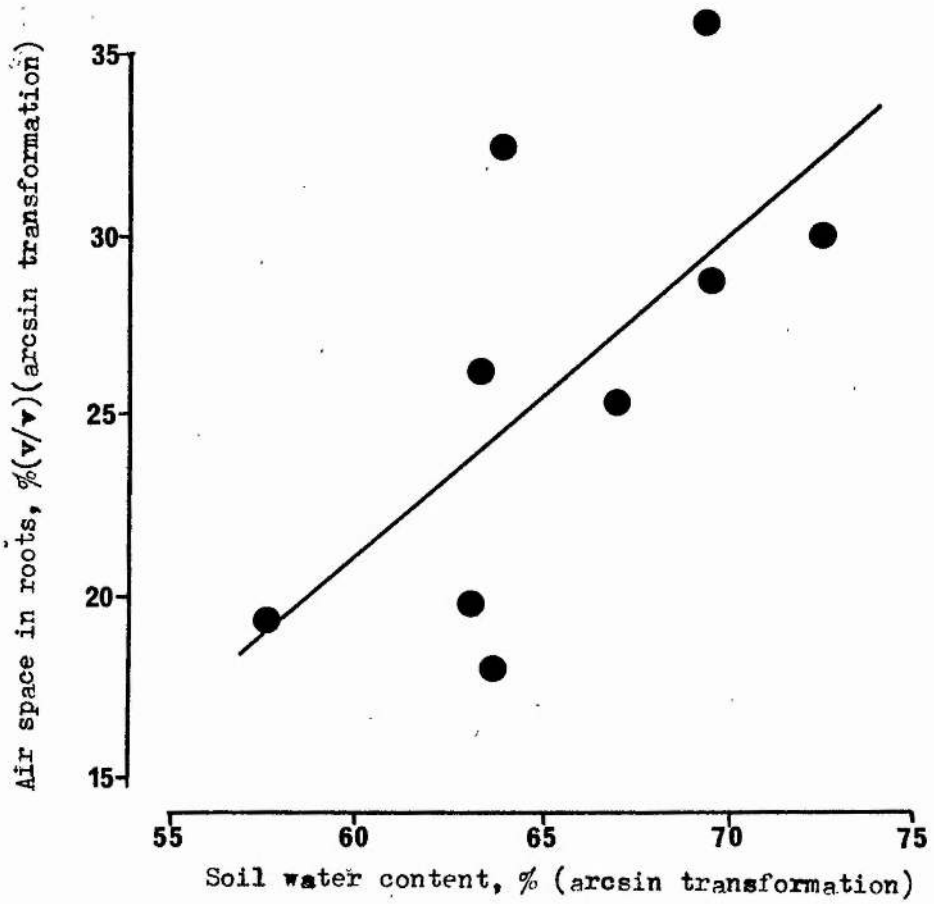


Fig.7.2. The relationship between soil water content and the amount of air space in the roots of Senecio aquaticus.

Regression equation: $y=0.91x-33.48$

$r=0.667$ $p=0.05$

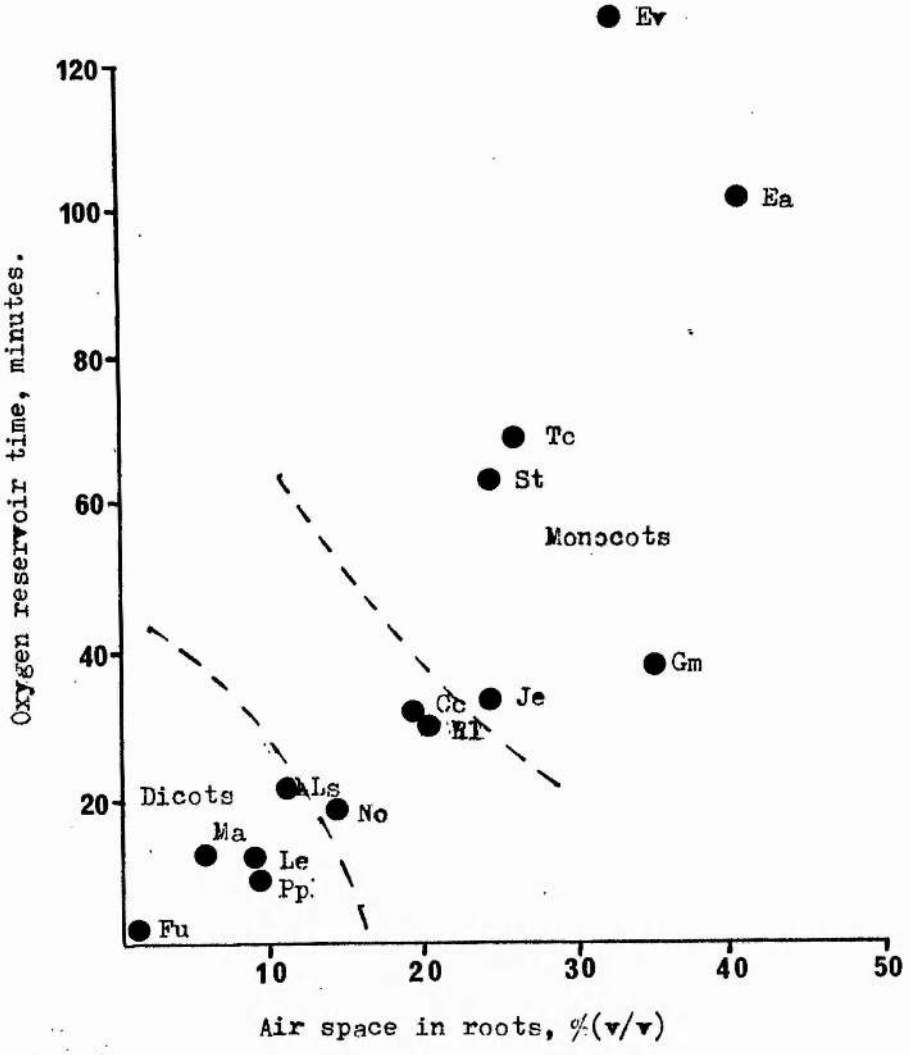


Fig.7.3. The relationship between air space in roots of various species and the oxygen reservoir capacity of the air spaces. The reservoir capacity is the time taken for oxygen in the air spaces (assumed to contain 20% O_2 initially) to be exhausted by aerobic respiration.

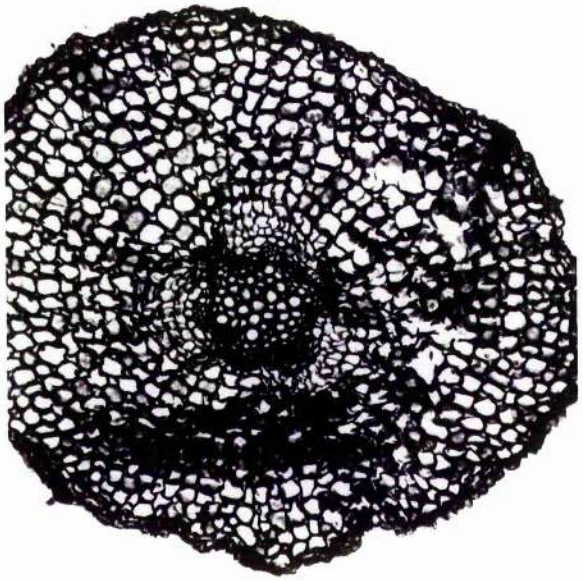
Cc Carex curta	No Narthecium ossifragum
Ea Eriophorum angustifolium	Le Lycopus europaeus
Ev Eriophorum vaginatum	Ls Lythrum salicaria
Fu Filipendula ulmaria	Ma Mentha aquatica
Gm Glyceria maxima	Pp Potentilla palustris
Je Juncus effusus	St Spartina townsendii
Rl Ranunculus lingua	Tc Trichophorum caespitosum

Plate 7.1 Transverse sections of the roots of various species. All sections were cut at least 2cm behind the root tip. Roots were taken from plants growing in their natural habitats.

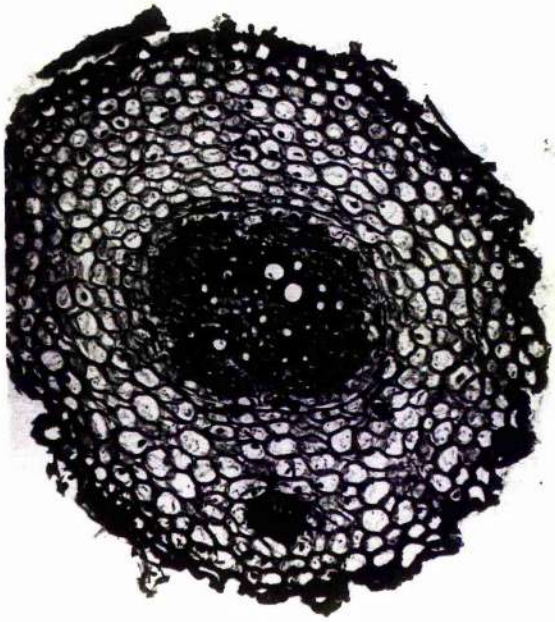
a) Senecio jacobaea
b) Hieracium pilosella
c) Galium palustre
d) Filipendula ulmaria

In all these species cortical aerenchyma is not well-developed. Small intercellular spaces occur in the cortex.

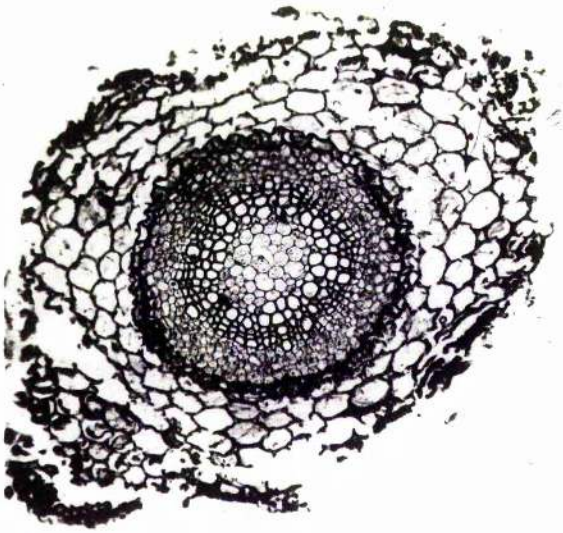
a



b



c



d

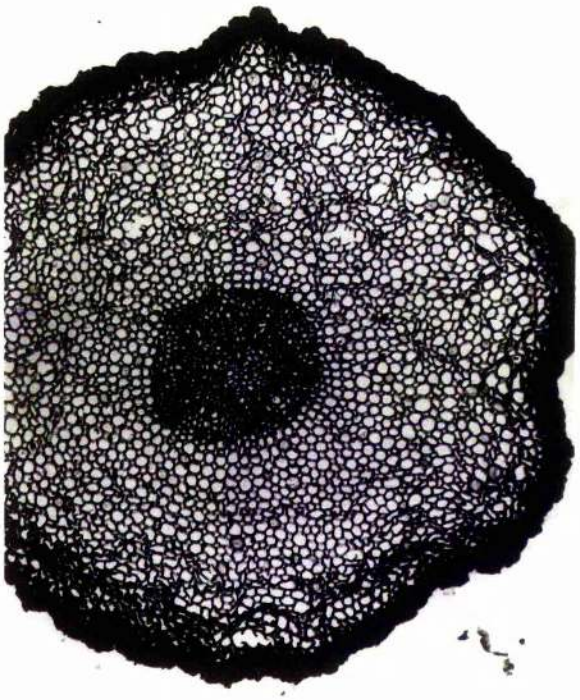
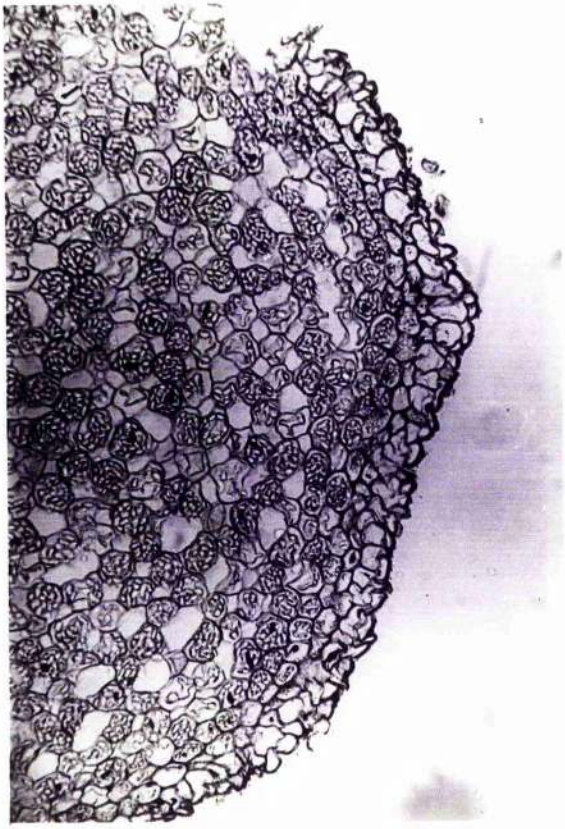


Plate 7.2 Transverse sections of the roots of various species. All sections were cut at least 2cm behind the root tip. Roots were taken from plants growing in their natural habitats.

a) Caltha palustris. Portion of the cortex showing extensive development of schizogenous intercellular spaces.
b) Mentha aquatica. Large, probably lysigenous, lacunae have developed. Many cortical cells remain intact.
c) Ranunculus flammula. More extensive development of lacunae than in (b). Strands of broken cells traverse the cortex.
d) Lythrum salicaria. A thin lateral root showing a similar structure to (c).

a



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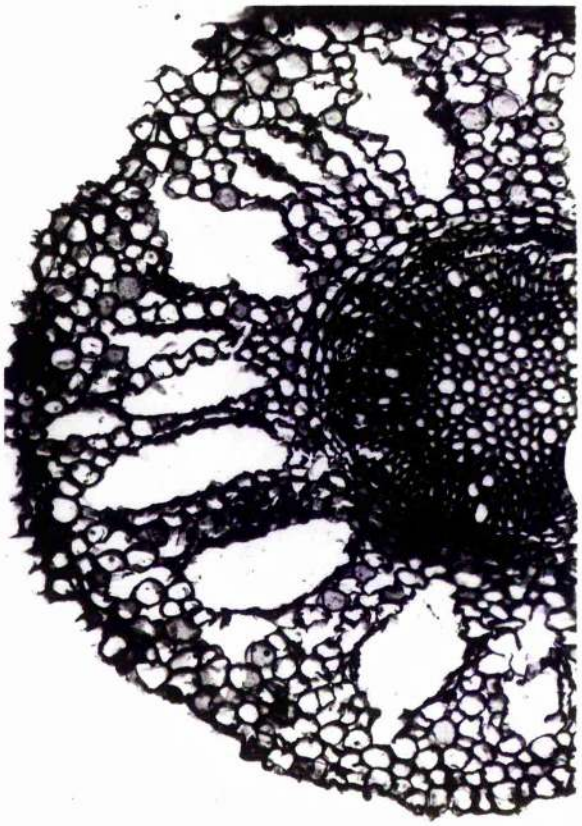

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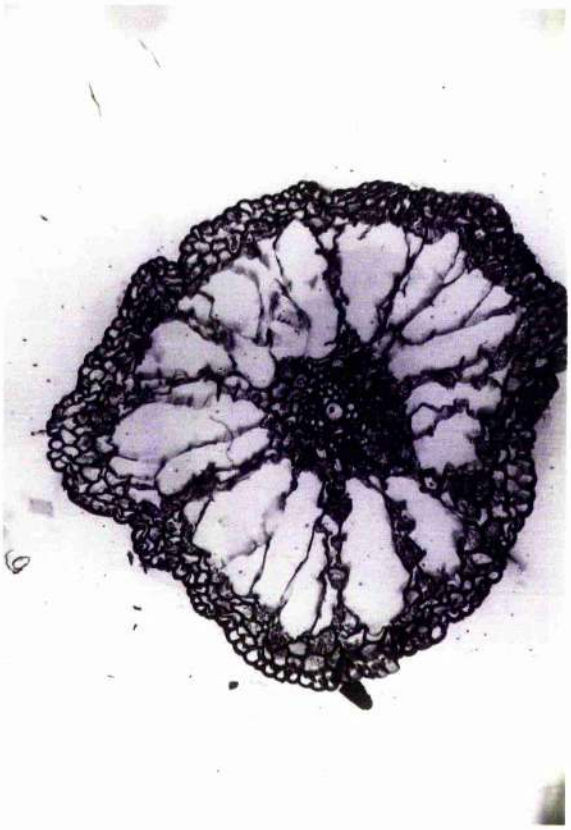

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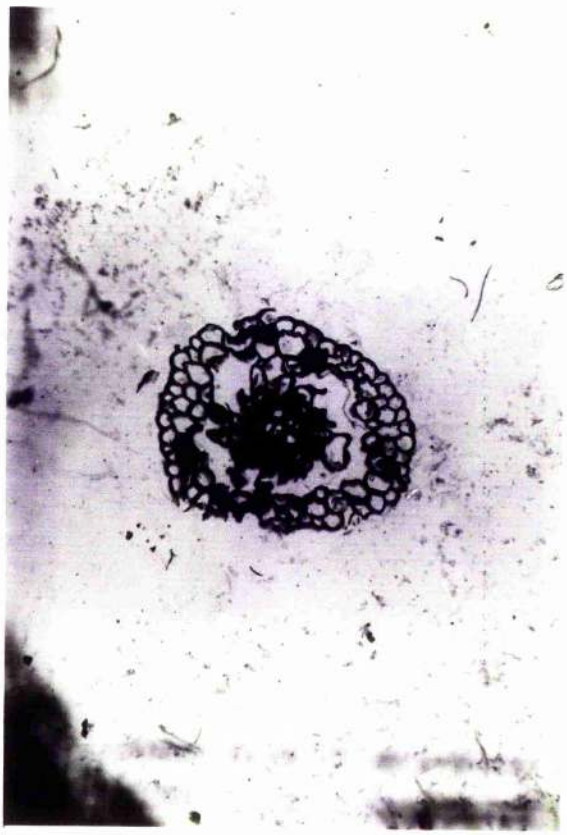


Plate 7.3 Transverse sections of the roots of various species. All sections were cut at least 2cm behind the root tip. Roots were taken from plants growing in their natural habitats.

a) Potentilla palustris. Cortical aerenchyma well-developed, consisting of numerous lacunae seperated by diaphragms one cell wide. The stele occupies a large area of the section, explaining the low amount of air space (%v/v) found in this species (Table 7.1).

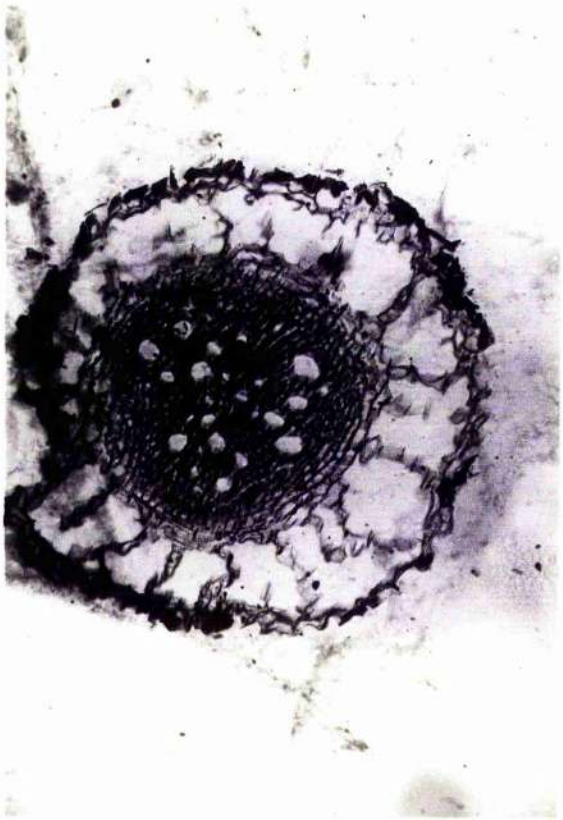
b) Ammophila arenaria. No development of aerenchyma. Air space confined to intercellular spaces. In this species the cortex is sloughing off.

c) Nardus stricta. Aerenchyma well-developed. The lacunae are seperated by radially extending cell diaphragms. Note the thick-walled cells below the epidermis forming an exodermis. This occurs in all the roots of the Gramineae, Juncaceae & Cyperaceae examined.

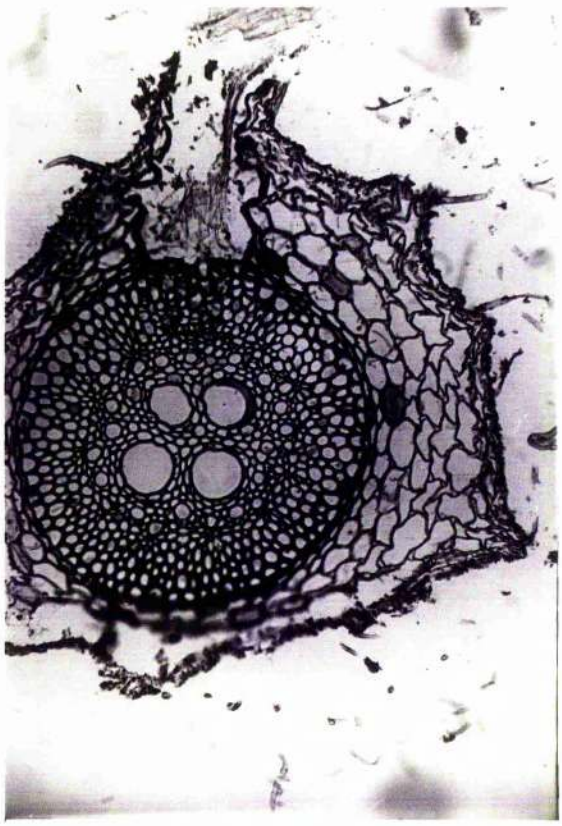
The cortical cells remain intact in the segment where a lateral root is emerging (roots taken from unflooded sand culture).

d) Glyceria maxima. This has a similar structure to (c). In addition, strands of intact cells traverse the cortex.

a



b



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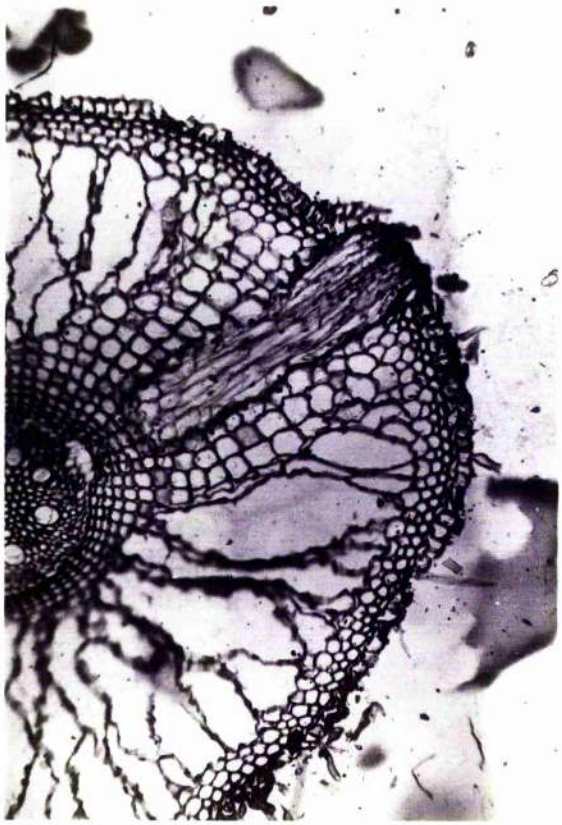

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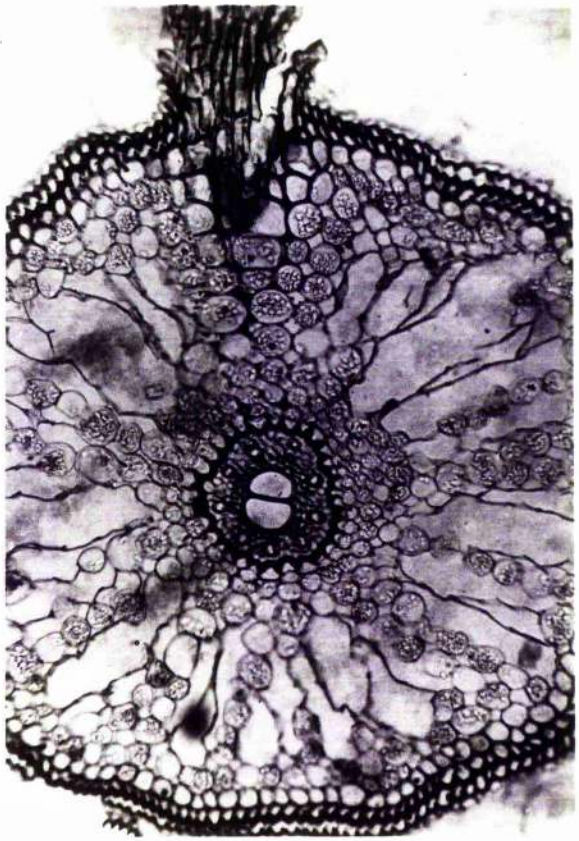


Plate 7.4 Transverse sections of the roots of various species. All sections were cut at least 2cm behind the root tip. Roots were taken from plants growing in their natural habitats.

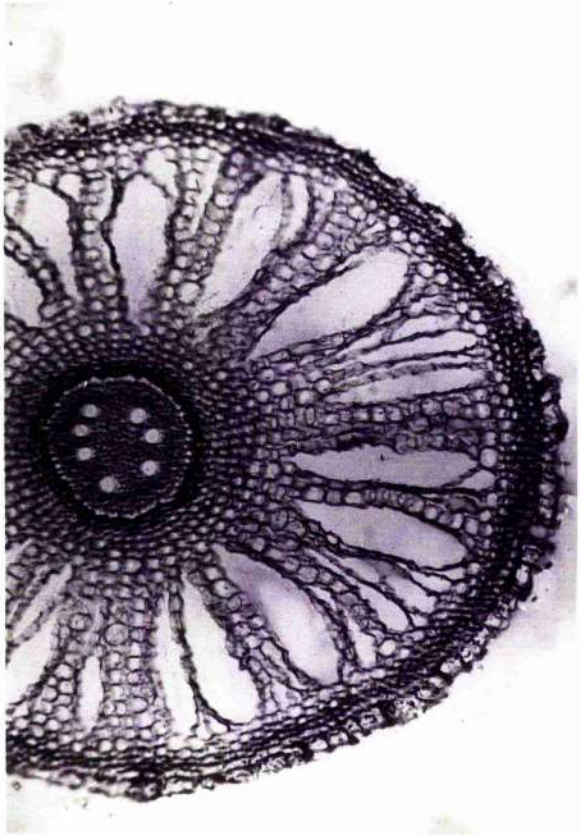
a) Juncus effusus. Young root section, cut nearer the tip.

b) Juncus effusus. Older root section. The lacunae develop by lysigeny. In the younger section more intact cells remain. Note exodermis. In (b) the cortex has remained intact where the lateral root emerges.

c) Narthecium ossifragum. Numerous lacunae are separated by radially arranged cell diaphragms. The exodermis has several layers.

d) Trichophorum caespitosum. The structure of the aerenchyma is delicate and not easily seen in this section. It resembles the structure of Carex curta (Plate 7.5a &b) and Eriophorum vaginatum (Plate 7.5c).

a



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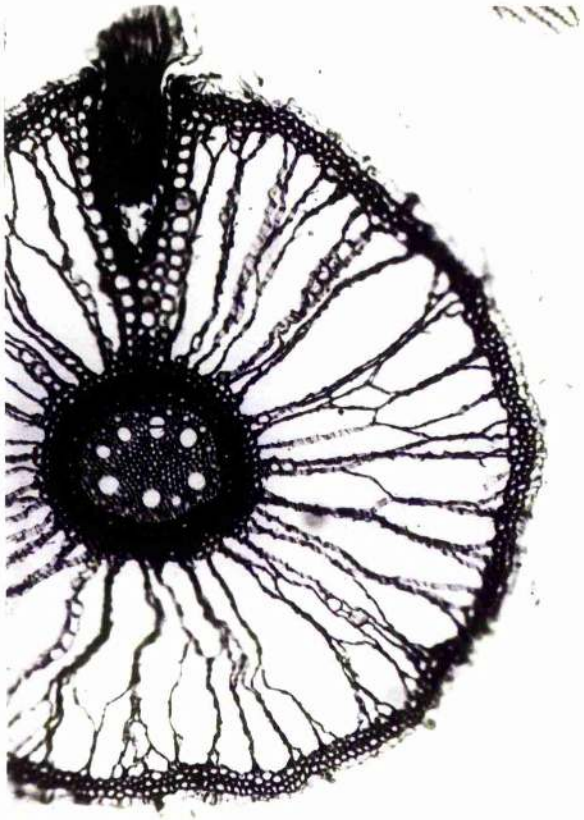

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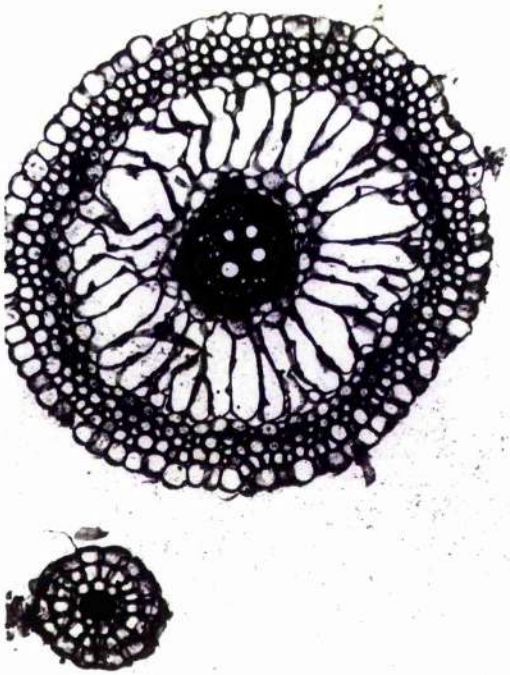

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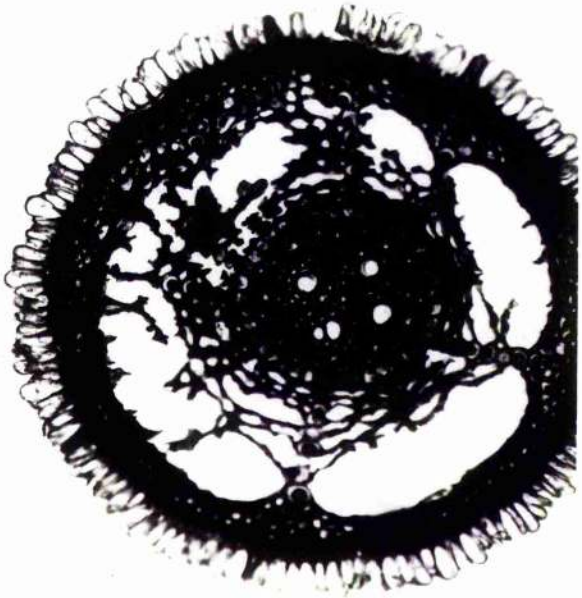
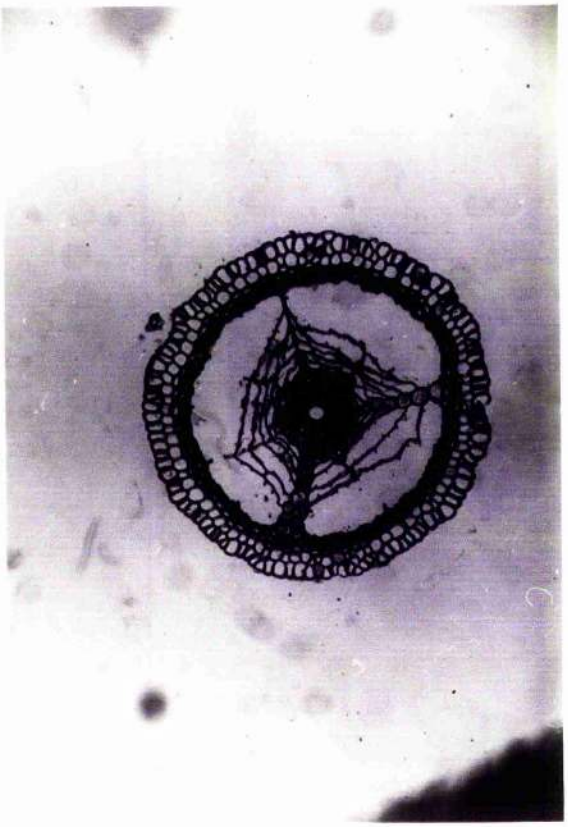


Plate 7.5 Transverse sections of the roots of various species. All sections were cut at least 2cm behind the root tip. Roots were taken from plants growing in their natural habitats.

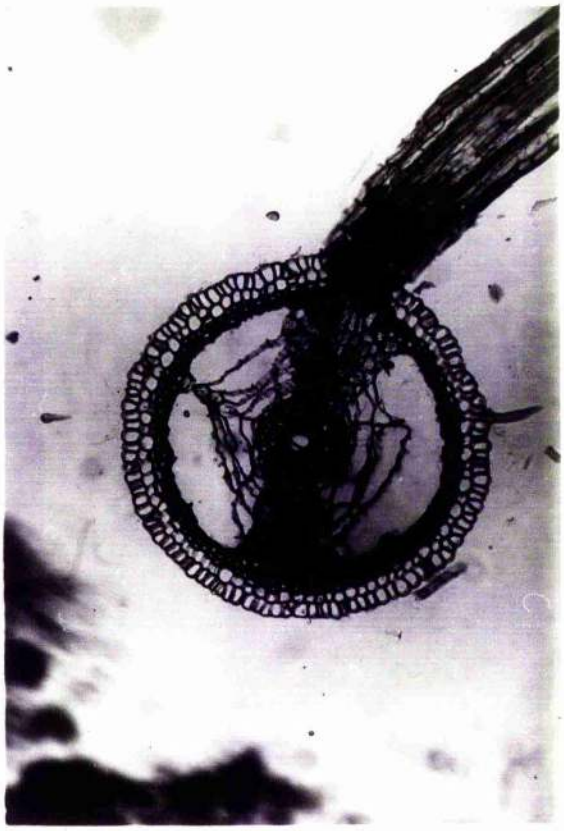
a) Carex curta
b) Carex curta with lateral root
c) Eriophorum vaginatum

The roots of these species, and Trichophorum caespitosum (Plate 7.4d), all have the same type of aerenchyma structure. Radially extending plates of intact cells are joined by diaphragms of broken cells stretching tangentially across the cortex, forming a delicate "spider's web" type structure. There is a well-developed exodermis in all these species. In Carex curta (b) the cortex has remained intact where the lateral root emerges.

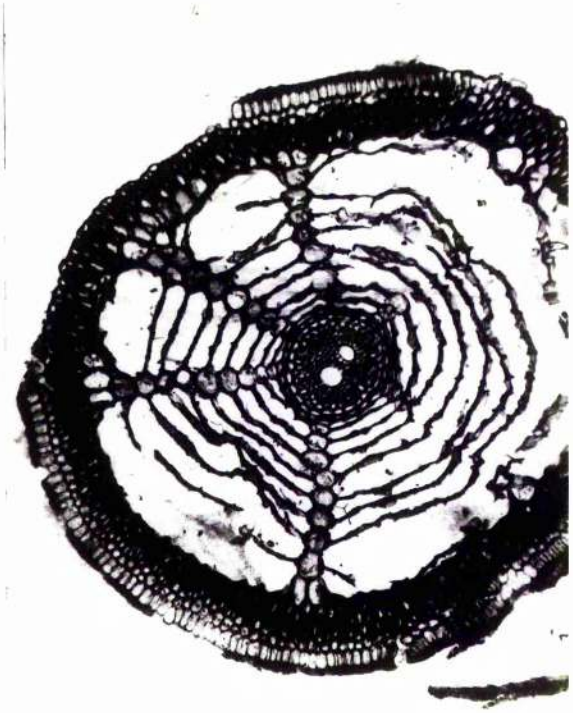
a



b



c



Discussion

Factors Affecting the Development of Aerenchyma.

The results show that there is great interspecific variation in the structure of aerenchyma and consequently in the amount of air space in the roots. The values measured by the density bottle method are mean values for the entire length of the roots, but air space is not evenly spread along this length. The lacunae in *Oryza sativa*, *Zea mays* and *Salix* are not fully-developed until about 4cm behind the apex (Armstrong, 1971; John, 1977; Brouwer, 1977). The results also show that air space in many of the species is greatly affected by various environmental factors. These include waterlogging, nutrient level and temperature. A major environmental factor not tested was light intensity, but Luxmoore *et al.* (1972) have shown that various wheat cultivars developed more air space in high as compared with low light intensities.

In many, but not all of the species tested, flooding increased air space in the root. This effect has been well documented for many other plants, but so far only cultivated plants have been examined. With the exception of *Oryza sativa*, these are not normally found in waterlogged conditions. Amongst cultivated plants waterlogging has been found to increase air space in the roots of *Oryza sativa* (Armstrong, 1971; Brouwer, 1977), *Zea mays* (Brouwer, 1977; Yu *et al.*, 1969), *Helianthus annuus*, tomato and wheat (Yu et al., 1969). Since the primary characteristic of waterlogged soils is a lack of oxygen, it is possible that this induces the formation of aerenchyma. This hypothesis is supported by results from experiments where air space in plants growing in aerated or unaerated culture solution has been compared. In unaerated culture solution plants generally produce roots with more air space. For example this has been demonstrated in *Hordeum vulgare* (Dunn, 1921; Bryant, 1934; Benjamin & Greenway, 1979), *Zea mays*

(McPherson, 1939; Drew *et al.*, 1979) and *Lolium perenne* (Troughton, 1972). The results are not always in agreement: Luxmoore & Stolzy (1969) found no increase in air space in *Zea mays* and *Oryza sativa* after 3 weeks in unaerated culture solutions. Additionally, Luxmoore *et al.* (1972) found that some wheat cultivars only had more air space in the roots under unaerated conditions if the plants were under relatively low light intensity. It is possible that in experiments of this type that various factors can interact. More direct evidence that air space formation is controlled by oxygen concentration has been given by McPherson (1939) and Benjamin and Greenway (1979). McPherson found that air space formation in *Zea mays* could be prevented by bubbling the culture solution with oxygen or a 1:1 mixture of oxygen and nitrogen. Benjamin and Greenway measured air space in *Hordeum vulgare* grown in a range of oxygen concentrations. They found a significant negative correlation between air space and oxygen concentration in the culture solution.

The results suggest that in some cases lateral roots contain less air space than primary roots. It is possible that in the thinner laterals the stele occupies a larger proportion of the root volume. Differences in air space have been observed between the seminal and nodal (adventitious) roots of cereals. The nodal roots contain more air space in *Hordeum vulgare* (Benjamin and Greenway, 1979), *Oryza sativa* and *Zea mays* (Luxmoore and Stolzy, 1969). A common response to flooding is the production of adventitious roots (Jackson, 1955) and this could to some extent explain the increase in air space. Adventitious roots usually have a greater diameter than seminal roots, and, if this is a result of increased radius of the cortex, these roots will show a higher air space, even if the cortical lacunae are developed to the same extent. Aerenchyma formation can take place in mature stem tissue by cell breakdown (Kawase and Whitmoyer, 1980). However, the increase in air space observed in these experiments is probably the result of

the production of new roots. Existing roots were trimmed back before planting and the growth period was long enough for most of the root system to consist of new roots.

In the 32 week flooding experiment air space was not increased in all the species. The Eriophorum species had a large amount of air space even when grown under drained conditions. It would seem that in these species the formation of well-developed aerenchyma is constitutive. These species have a highly organised aerenchyma structure which may have become relatively insensitive to environmental factors. Eriophorum has become highly adapted to growing under waterlogged conditions. Growth of E. angustifolium and E. vaginatum was stimulated by flooding (table 7.4) and Gore and Urquart (1966) found that flooding stimulated growth of E. vaginatum. In the drained treatments both species had greatly reduced root penetration. It is possible that highly aerenchymatous roots do not have the necessary mechanical properties to penetrate drained sand. Waterlogging may decrease frictional resistance to root penetration. Alternatively other factors could contribute to reduced root penetration and further investigation is required. In this experiment air space was also unaffected by flooding in Glyceria maxima and Deschampsia caespitosa. In the case of G. maxima this was in contrast to the previous experiment where plants were treated for 11 weeks (table 7.2). Without further investigation these results cannot be explained. There was also no change in the air space in the roots of the two flood-intolerant species Brachypodium sylvaticum and Hieracium pilosella. In both species the root systems were severeley damaged by flooding, and very few new roots were produced.

The variation in air space in Senecio aquaticus and Caltha palustris collected from the field shows that the response to flooding does not occur only in experimental treatments. Whatever the function of aerenchyma in adapting plants to waterlogged soils, it is apparent that these plants are able to develop it in direct response to the degree of wetness of their habitat.

Of the flood-tolerant species examined, Filipendula ulmaria was an exception. Very little air space was found in the roots of plants collected from the field or those growing in flooded or drained sand culture. The results show that species are not similar in their response to flooding. The responses of root air space to flooding can be summarised as follows.

1. No increase because roots become unhealthy
2. No increase but roots remain healthy
3. Air space increased
4. Air space not increased but large amount present under drained conditions.

Nutrient level had an effect on air space formation in Nardus stricta. Air space formation was greater under low nutrient levels in flooded and drained treatments. The plants produced more roots under the low nutrient conditions, so this response did not decrease the actual amount of root tissue. A similar effect has recently been shown by Konings and Verschuren (1980) with Zea mays in aerated water culture. They found that nutrient deficiency increased air space formation. By varying the composition of the nutrient solution they showed that this was almost entirely the result of nitrogen (nitrate or ammonium) deficiency. McPherson (1939) was able to decrease air space formation in Zea mays by adding extra calcium to the medium. The results with Nardus stricta show that nutrient level can also affect air space in a flood-tolerant plant under flooded as well as drained conditions. Nutrient deficiency, particularly nitrogen, may increase the mortality of cortical cells and lead to the formation of larger lacunae. Konings and Verschuren (1980) interpreted their results with Zea mays in this way, but this explanation may not hold for N. stricta. This species is characteristic of nutrient deficient peats and is an oligotrophic plant showing little response to increasing nitrogen levels (Bradshaw et al., 1964). The results here show that it has a marked ability to increase its root growth under the low nutrient conditions. This suggests

that it may not be suffering from nutrient deficiency. Further experiments with N. stricta are needed to discover the nutrient(s) responsible for air space formation. Extensive air spaces form in the roots of certain grasses from dry and nutrient deficient soils in N. America and S. Africa and India including Bouteloua gracilis (Beckel, 1956; Pillai and Pillai, 1962). In B. gracilis the air spaces form under well aerated conditions and their formation is retarded by oxygen deficiency. In this case air space formation could be adaptive to low nutrient levels, enabling the plant to produce a certain surface area of root for nutrient absorption while using the minimum of tissue. A similar explanation could be given for the constitutive formation of air space in the Eriophorum species. Both these species are from nutrient-deficient habitats.

Lolium perenne had more air space in its roots when grown at a higher temperature. Sojka et al., (1972) found that a short heat shock (42 °C for 36 hours) during the growth of wheat (Triticum aestivum) doubled air space in the roots of control plants and plants growing in soil flushed with nitrogen. If oxygen deficiency can lead to air space formation, these results could be explained by suggesting that increased respiration rate at higher temperatures will deplete oxygen more quickly and result in lower oxygen tensions within the roots.

The Mechanism of Aerenchyma Formation.

It can be seen from the previous discussion that many factors are able to affect air space formation. These are : oxygen concentration; nutrient level; temperature and light intensity. Not all species behave in the same way and some produce air space even under well aerated conditions for example Eriophorum species and others including plants from well drained or arid soils e.g. Bontelona gracilis (Beckel, 1956), Zingibar offinale, Phoenix dactylifera and Cymbopogon flexuous (Pillai and Pillai, 1962). In these air space formation may be genetically-determined and independent of environmental factors.

The results of the flooding experiments support the hypothesis that increased air space formation is a result of low oxygen concentrations, but the effects of the nutrient level is not so easy to explain. It is possible that these factors cause air space formation in different ways. However, one way that diverse factors could affect air space is by hormonal mediation. Recent work has suggested that the formation of aerenchyma in adventitious roots of Zea mays could be mediated by ethylene (Drew et al., 1979). Aerenchyma formed to an equal extent in roots from unaerated culture solution and those in culture solution bubbled aerobically with ethylene. Bubbling with pure nitrogen or air prevented aerenchyma formation. They suggested that ethylene produced endogenously would be trapped in unstirred layers round the roots in the unaerated treatment would stimulate aerenchyma formation. They also showed that under complete anoxia ethylene biosynthesis is inhibited explaining the lack of aerenchyma formation in the nitrogen-flushed treatment. Lack of oxygen may stimulate ethylene production in soils or in shoots which could then induce aerenchyma formation in the roots. How ethylene has its effect is unknown, but Kawase (1979) suggested that, in Helianthus annuus stems, ethylene production in waterlogged plants stimulates cellulase activity which leads to the weakening and collapse of cortical cells. Further work is needed, particularly to see if aerenchyma formation is related to ethylene production in flood-tolerant plants.

The Functions of Aerenchyma.

Four possible functions of aerenchyma were listed in the introduction. They will be discussed here.

Aerenchyma as an oxygen reservoir. The results of the oxygen reservoir experiment show that aerenchyma cannot act as a significant oxygen reservoir if external supplies have been cut off. Wetland plants are able to maintain significant amounts of oxygen in their rhizome and root aerenchyma by diffusion (Barber, 1961; Buttery et al., 1965; Conway, 1937; Coutt and Vallance, 1958; Laing, 1940; Teal and Kanwisher, 1966).

During a normal growing season it is unlikely that the supply from the shoot will be cut off unless it is completely submerged in the dark. Stomatal closure at night has relatively little effect on the oxygen content of the air spaces (Coutt and Vallance, 1958; Laing, 1940). At the end of a growing season when aerial shoots die back the oxygen supply will not be sufficient for more than a few hours. A build up of carbon dioxide in the air spaces depresses the rate of oxygen consumption in Menyanthes trifoliata (Coutt and Vallance, 1958), but this would not be sufficient to significantly prolong the oxygen supply.

The supply of oxygen for aerobic metabolism. Oxygen can be detected leaking from the root tips of all wetland plants so far examined, and this has led to the suggestion that there must be sufficient oxygen in the root to maintain aerobic metabolism (Armstrong, 1979). This appears to be a reasonable assumption, but there is some metabolic evidence to suggest that oxygen transport cannot maintain full aerobic metabolism when aerenchymatous roots are in an anaerobic medium. In Oryza sativa oxygen diffusion from the shoots is insufficient to maintain fully aerobic rates of nutrient uptake (John et al., 1974) and energy charge (Raymond et al., 1978). Additionally, alcohol dehydrogenase can be induced in rice roots when the culture solution is flushed with nitrogen (Wignarajah et al., 1976), suggesting that there is some degree of anoxia in the roots. In a non-aerenchymatous plant, cotton, oxygen transport would only be sufficient to supply 8% of the roots' oxygen requirement (Vartapetian and Nuritidinov, 1976). Stelzer and Lauchli (1980) come to a similar conclusion for the aerenchymatous salt-marsh grass Puccinellia peisonis. If the root system of this species was completely submerged the oxygen supply would be similar to cotton. Only when the root bases were exposed to air was there oxygen diffusion out of the roots. In this species oxygen supply would not be sufficient under fully flooded conditions. Further circumstantial evidence that flooding induces an oxygen shortage in roots of flood-tolerant plants is given by the increase in nitrate reductase activity in roots and shoots on flooding (Garcia-

Novo and Crawford, 1973).

Filipendula ulmaria is a flood-tolerant species (Crawford, 1967), but in this investigation was found to produce very little air space in its roots. This provides some evidence that aerenchyma may not be necessary for growth under waterlogged conditions. Metabolic adaptations to hypoxia may also be necessary (Crawford, 1978). Glyceria maxima and Juncus effusus are both flood-tolerant species (Crawford, 1978) and were found in this investigation to have aerenchyma. Roots of both these species can penetrate well below the watertable of a flooded soil (Brouwer, 1977). However, Brouwer (1977) also found that the flood-intolerant species Zea mays could not penetrate far below the watertable although it was able to produce aerenchyma. Assuming that the aerenchyma in these species is equally effective in oxygen transport, this suggests that metabolic adaptation, as well as the formation of aerenchyma, is necessary for roots to be able to penetrate waterlogged soils. In highly reduced soils the soil could have a higher affinity for oxygen than the root cells. Even if oxygen is present in the lacunae the soil may act as a stronger sink than the root cells. Teal and Kanwisher (1966) found that the oxygen concentration in the air spaces of roots of Spartina alterniflora was less in more reduced than in less reduced soils. Oxygen may diffuse more readily out of the root than into the root cells. Various metabolic adaptations have been proposed by Crawford and co-workers (Crawford, 1978), but they have been questioned (Smith and ap Rees, 1979). It is clear that in some species there must be metabolic adaptation. The perennating organs of aquatic or semi-aquatic plants must grow while buried in anaerobic mud with no external oxygen supply, and recently evidence has been produced that two of these species, the bulrushes Schoenoplectus lacustris and S. tabernaemontani, can not only survive, but grow, under complete anoxia (Crawford and Barclay, personal communication).

In conclusion, it seems that for most marsh and bog herbaceous species that aerenchyma can allow the supply of some, but not all the

oxygen requirement of the roots. However metabolic adaptation may also be necessary for successful growth under waterlogged conditions. Buried perennating organs may be able to grow for some time under complete anoxia.

The reduction of oxygen demand. Williams and Barber (1961) suggested that this was the primary function of aerenchyma. The formation of large lacunae will certainly reduce the amount of respiring tissue in a root, so this function cannot be denied when oxygen is in short supply. Even so, the evidence above suggests that oxygen shortage still occurs, and this may be in the apical meristem where there is no aerenchyma.

Rhizosphere oxygenation and tolerance to reduced phytotoxins. This function of aerenchyma was examined in Part I in relation to Fe(II) tolerance. The evidence suggested that Fe(II) tolerance was not directly correlated with air space in roots, although the possession of air space could decrease Fe uptake, probably by oxidation in the rhizosphere. The conclusion that aerenchyma may not be necessary for Fe(II) tolerance does not mean that this is so for other toxins. Hydrogen sulphide is a powerful metabolic inhibitor and the evolution of cellular tolerance mechanisms may not be as easy as it is for potentially phytotoxic metals. Oxidation in the rhizosphere may be the only way plants can tolerate this toxin. The amount of ROL from roots of various cultivars of Oryza satiwa shows a direct correlation with their resistance to physiological diseases induced by hydrogen sulphide. The greater the ROL the greater the resistance to hydrogen sulphide toxicity (Armstrong, 1969; Joshi et al., 1975). There is a potential for hydrogen sulphide toxicity in all waterlogged soils, even in the presence of high concentrations of Fe(II) (Joshi et al., 1975).

It can be concluded from the above discussion that aerenchyma in roots does not have one particular function. Its degree of development for oxygen transport is not excessive as supposed by Williams and Barber (1961) but this does not invalidate their conclusion that it could act as a mechanical-cum-metabolic compromise and reduce oxygen demand.

Some of the oxygen requirement of the root will be satisfied by oxygen transport and rhizosphere oxidation probably contributes to hydrogen sulphide, but not Fe(II) tolerance. Oxygen shortage is probably the most important environmental factor inducing aerenchyma formation under natural conditions. Cell breakdown in response to anoxia would give the plant the advantages listed above. Aerenchyma formation would thus have a selective advantage and lead to the production of well-organised structures as seen in Eriophorum.

If an aerenchymatous root can give the same surface area of root for a smaller amount of tissue then, why do not all plants have aerenchyma? Two possible explanations can be put forward for this. Aerenchymatous roots may not have suitable mechanical properties to penetrate hard dry soils and, at least in Oryza sativa, an increase in root air space greatly decreases the resistance of the root to water flow (Tomar and Ghildyal, 1975). Aerenchymatous plants could more easily suffer from water deficits in dry soils.

PART III

CHAPTER 8

General Discussion and Summary

Physiological Aspects of Fe(II) Tolerance.

Among the species choosen for investigation there was a wide range of tolerance to Fe(II). Glyceria maxima was remarkable in not showing toxicity symptoms although this species was tested in Fe(II) concentrations as high as 10mM. Concentrations of less than 1mM induced toxicity symptoms in the roots of most other species. Inhibition of root growth occurred before toxicity symptoms were evident. In some cases differing optimum concentrations of Fe(II) for root growth could be distinguished. These features have also been demonstrated in a recently published study by Chinnery and Harding (1980). They found that root and shoot growth of Juncus effusus was inhibited by high levels (0.14 - 2.3mM) of Fe(II) in solution culture. There was an optimum concentration for growth around 0.04mM Fe(II) which is comparable to the optimum found for Glyceria maxima in the present experiments. Fe(II) toxicity appeared within four days in the calcium nitrate solutions, so it is unlikely that it can be attributed to an imbalance in nutrient uptake.

Iron uptake or translocation appeared to have no relationship with Fe(II) tolerance of the various species. There were large differences between species in the amount of iron taken up by the roots. There was a fifteen-fold difference between the greatest (Senecio aquaticus) and the least (Eriophorum angustifolium) concentrations in the roots after exposure to 2mM Fe(II). A significant negative correlation was found between iron uptake by the roots and the amount of air space in the roots of the various species. The evidence suggests that this can be attributed to the greater oxidation of Fe(II) in the culture solution by species with a greater amount of air space in their roots. Oxidation of Fe(II) by oxygen diffusing from the roots into the culture solution

decreases the availability of iron for uptake by roots. It remains to be seen if this mechanism would work in soil. There was no relationship between Fe(II) tolerance and the amount of air space in roots, suggesting that the exclusion mechanism has no role in determining tolerance. The concentration of intracellular iron will be the important factor in determining toxicity and this cannot be deduced from the total root concentration. Although the roots can oxidise Fe(II) and form precipitates on or within themselves, (Green and Etherington, 1977) this may not prevent a buildup of intracellular iron to toxic levels. Using labelled iron, Tanaka et al. (1966) found that, although iron was precipitated on the root surface behind the tips, iron could actually penetrate into the tips where it could be easily available for absorption by cells of the apical meristem. Even in aerenchymatous roots, such as those of rice, the evidence reviewed in Chapter 7 suggests that root tips are likely to suffer from anoxia or hypoxia under flooded conditions although oxygen can be detected diffusing out of the roots. Iron taken up into the root tips under these conditions could easily be reduced and absorbed into the cells in potentially toxic quantities.

The root tip, where aerenchyma is least well-developed, is the primary site for the toxic action of Fe(II) and any explanation of tolerance must take this into account. The experiments with excised root tips showed that species were able to retain differential tolerance under aerobic or anaerobic conditions in the absence of an internal oxygen supply to the air spaces. The relative tolerance of the excised root tips corresponded well with that of the intact plants.

In summary, the following results lead to the conclusion that aerenchyma is not essential to explain Fe(II) tolerance:

a) There is no correlation between aerenchyma and Fe(II) tolerance;

b) There is no correlation between iron exclusion from the roots and Fe(II) tolerance;

c) Excised root tips are able to maintain the same relative tolerance to Fe(II) as intact plants.

It can be concluded that the tolerance of the root tips of a particular species to Fe(II) must depend on physiological or metabolic adaptations of their cells to high Fe(II) levels.

Ecological Aspects of Fe(II) Tolerance.

The occurence of a range of Fe(II) tolerance among wetland species is of ecological interest. The relative tolerance of the species (see table 2.2) will be compared with three other features 1) Their flooding tolerance 2) Iron levels in the soil of their characteristic habitats 3) Iron efficiency.

1. Fe(II) tolerance and flooding tolerance. All the species except Schoenus nigricans have been classified as flood-tolerant or intolerant according to their growth response to flooding in sand culture (Table 8.1). Comparison with Fe(II) tolerance shows that the flood-intolerant species, all collected from well-drained soils, are the least tolerant to Fe(II). There is an overlap between Zerna ramosa and Myosotis scorpioides. This general pattern would be expected from the greater availability of Fe(II) in waterlogged soils.

2. Fe(II) tolerance and Fe levels in the soil. Among the flood-tolerant species there is a wide range of Fe(II) tolerance. The possibility arises that could be correlated with the level of Fe(II) available in their natural habitats. Differences between species in their tolerance to aluminium (Clarkson, 1966; Grime and Hodgson, 1969) and manganese (Mohmoud and Grime, 1977) can be correlated with the levels of these metals in their natural habitats.

No field work was undertaken so further work would be necessary to test this hypothesis properly. The species can be divided on a subjective basis into those likely to come from habitats low or high in Fe(II). To do this the habitats the species were collected from (table 2.1) and their typical distribution (Clapham et al., 1962) was considered. The classification for the flood-tolerant species and Schoenus nigricans is shown in table 8.2. The distinction was made on

the assumption that organic soils are generally likely to be more oligotrophic and low in Fe(II) than organic soils (or very rich fen peats). (Jones, 1971b). There is no apparent correlation between these categories and Fe(II) tolerance. Myosotis scorpioides is relatively intolerant but was collected from the same site as the extremely tolerant species Glyceria maxima. Compared with G. maxima, M. scorpioides is very shallow rooting. The roots of G. maxima penetrate deeply into the soil and are well below the watertable (personal observation). In this way the roots of M. scorpioides could avoid the more highly reduced zone of the soil and not be exposed to such high Fe(II) levels as G. maxima. In the field the Fe(II) tolerance of a species will be affected by many factors, particularly its rooting habit. Wetland species show a great variation in their depth of rooting (Emmerson, 1921; Boggie et al., 1958). Predictions about plant distribution cannot easily be made from the results of laboratory experiments. There is however some evidence that iron rich soils may be frequented by particular species. Pearsall (1950) pointed out that iron-rich flushes in upland areas often have a particular flora (Molinia caerulea, Carex panicea and Juncus acutiflorus). He suggested that these species either had a high iron tolerance or required a high iron supply. The flora of the woodland iron flushes in the Sussex Weald is also distinctive (D.T. Streeter, pers. comm. Species include Ranunculus flammula, Mentha aquatica, Glyceria fluitans, Myosotis secunda, Carex laevigata and Carex remota). Detailed investigations of the ecology of these flushes have not been published.

3. Fe(II) tolerance and iron efficiency. Plants differ in their ability to use iron in aerated soils when it is present as sparingly soluble Fe(III) compounds. Iron deficiency symptoms, chlorosis of the young leaves, can result if insufficient iron is absorbed (Brown, 1972). In calcareous soils plants can suffer from lime-chlorosis, also induced by iron deficiency (Grime and Hodgson, 1969). Some species, ecotypes or cultivars are more resistant to chlorosis than others. These plants have been termed "Fe-efficient" and can use lower levels of iron than

"Fe-inefficient" plants (Brown, 1978). Fe-efficient plants have a complex of characteristics which are induced during low iron stress. They all have the effect of increasing the solubility and availability of Fe(III). These responses are not induced, or only induccd weakly, in Fe-inefficient plants. The characteristics of Fe-efficient plants induced by low iron stress are:

a) The release of protons from the roots (Brown, 1978).

b) The release of Fe(III) reducing substances from the roots and the development of reducing activity at the root surface (Ambler et al., 1971). The reducing substances may be phenols (Kramer et al., 1980).

c) The release of Fe(III) chelating agents by roots. An Fe(III) chelating amino acid (mugineic acid) has been identified in this role (Takagi, 1976; Takemoto et al., 1978). The chelating agent solubilizes Fe(III), but it must probably be reduced at the root surface before it can be absorbed from the complex (Brown, 1978).

d) The Fe-efficient species Helianthus annuus produces epidermal transfer cells under low iron stress. This can be correlated with an increased capacity for the release of protons and reductants and for iron uptake (Kramer et al., 1980).

There is some evidence from the present experiments to suggest that some of the more Fe(II) tolerant species are also Fe-inefficient. Glyceria maxima, when cultivated in sand culture in the glasshouse, was very susceptible to chlorosis in its young leaves. This could be easily reversed by increasing the iron supply (Fe(III)EDTA) in the nutrient solution. It also showed optimum root growth at concentrations of Fe(II) toxic to other species. These observations suggest that G. maxima is an Fe-inefficient species. Some of the other Fe(II) tolerant species have been reported as being Fe-inefficient. These are Oryza sativa (Okajima, 1965; Takagi, 1976), Eriophorum angustifolium and E. vaginatum (Grime and Hodgson, 1969). Grime and Hodgson found that Nardus stricta, also an Fe(II) tolerant species, was not susceptible

to iron chlorosis, but they suggested that this was because of its extremely slow growth rate. Among soybean cultivars, Brown and Jones (1977) found that an Fe-efficient variety was more susceptible to Fe(III) toxicity than two Fe-inefficient varieties.

Iron-efficiency and Fe(II) tolerance may not be compatible strategies for a particular plant, and perhaps some Fe(II) tolerant wetland plants are excluded from dry soils because of their inability to use low levels of Fe(III) efficiently. Fe(II) tolerant plants have not developed the efficient mechanisms to absorb iron because this could work against their own tolerance mechanism. Additionally the ability of plants with aerenchymatous roots to oxidise their rhizosphere will oppose the Fe(III) reducing mechanisms listed above. Much more investigation is needed to determine if there is an inverse relationship between tolerance and efficiency to test these speculations.

The differences between wetland species in the response of their root growth to Fe(II) suggests that Fe(II) levels in the soil could influence competition through seedling establishment and root growth and hence the composition of the community. Fe(II) would, of course, work in conjunction with many other factors.

Table 8.1 - Flooding-tolerance of the species used in this investigation. (Data from Chapters 3 and 7 and Crawford, 1967; McManmon and Crawford, 1971).

Flood-tolerant species	Flood-intolerant species
Deschampsia caespitosa	Chamaenerion angustifolium
Eriophorum angustifolium	Senecio jacobaea
E. vaginatum	Zerna ramosa
Glyceria maxima	
Myosotis scorpioides	
Nardus stricta	
Oryza sativa	
Ranunculus flammula	
Senecio aquaticus	

Table 8.2 - Possible Fe(II) levels in the habitats of the flood-tolerant species used in this investigation.

Low iron (organic soils)	High iron (mineral soils or rich fen peat)
Eriophorum angustifolium	Deschampsia caespitosa
E. vaginatum	Glyceria maxima
Nardus stricta	Myosotis scorpioides
Schoenus nigricans	Ranunculus flammula
	Senecio aquaticus

Summary

Part I

1. A review of the literature suggested that potentially phytotoxic levels of Fe(II) can occur in waterlogged soils.

2. Fe(II) tolerance of a range of species characteristic of waterlogged and well-drained soils was assessed. Tolerance was assessed by exposing the plants for 4 days to Fe(II) sulphate in a deoxygenated solution containing 4mM calcium nitrate. The concentration of Fe(II) required to produce toxicity symptoms in the root tips was determined. A wide range of tolerance was found. Species from well-drained habitats were sensitive to Fe(II). Some wetland species (e.g. Schoenus nigricans and Myosotis scorpioides) were also relatively intolerant. Glyceria maxima, the most tolerant species did not develop toxicity symptoms in concentrations of Fe(II) as high as 10mM. In contrast the most sensitive species, Chamaenerion angustifolium, showed symptoms in 0.02mM Fe(II).

3. Several authors have suggested that Fe(II) tolerance is the result of Fe(II) oxidation by oxygen diffusing through and out of roots. Oxidation renders the iron less available for absorption. The possession of an increasing amount of air space allows increasing oxygen transport through roots. However, no correlation was found between Fe(II) tolerance and root air space. The results do not support the above hypothesis.

4. Iron uptake from deoxygenated 4mM calcium nitrate with a range of Fe(II) sulphate concentrations (0 - 6mM) was measured over a four day period. Iron concentrations in roots were generally an order of magnitude greater than in the shoots. Much of the iron in roots was precipitated on the root surface. In some species iron uptake by roots was saturated at concentrations greater than 2.0mM Fe(II) in the external solution. Semi-log plots showed that uptake by the roots showed a biphasic pattern with the break between

1.0 and 2.0mM Fe(II). Log-log plots of uptake by shoots also revealed a biphasic pattern with the break between 1.0 and 2.0mM Fe(II). In the first phase (0.01 - 1.0mM) uptake into shoots hardly increased with increasing external Fe(II) concentration. Further experiments would be required to establish the physiological significance of these results.

5. There was no relationship between Fe(II) tolerance and the amount of iron absorbed by the roots or shoots of the various species. A significant negative correlation was found between root air space and iron uptake by the roots in the first phase (0.01 - 2.0mM). Those species with a greater amount of air space took up less iron into their roots. This exclusion was particularly marked in the Eriophorum species. Eriophorum angustifolium had a greater capacity to oxidise Fe(II) in deoxygenated culture solution than Ranunculus flammula, furthermore, roots of E. angustifolium contain more air space than R. flammula. Along with evidence from other authors, this suggests that the exclusion mechanism is the result of Fe(II) oxidation and a consequent decrease of availability in the culture solution. Because there was no correlation between Fe(II) tolerance and root air space or Fe(II) tolerance and iron uptake by roots this exclusion mechanism cannot explain tolerance.

6. To examine further the relationship between Fe(II) tolerance and root air space (hence oxygen diffusion) attempts were made to assess the tolerance of excised root tips from various species. In this way oxygen diffusion through the root air space was prevented. Three parameters were choosen to assess tolerance of excised root tips a) potassium leakage, b) vital staining in triphenol-tetrazolium chloride (T.T.C.) and c) elongation. Root tips of various species incubated under anaerobic conditions with Fe(II) sulphate maintained differential tolerance as assessed by T.T.C. staining and potassium leakage. Root tips of various species incubated under aerobic conditions with Fe(II) sulphate also

maintained differential tolerance as assessed by root tip elongation. Under aerobic and anaerobic conditions the relative tolerance of the excised root tips of the species showed close agreement with the relative tolerance of intact plants. The results suggest that Fe(II) tolerance is not directly dependent on aerenchyma and oxygen diffusion through the roots but depends on the physiological or metabolic adaptations of individual cells.

7. Malate and citrate levels in roots were generally depressed by treatment with Fe(II) in solution culture. There was no relationship between the levels of these acids and Fe(II) tolerance or between tolerance and the decrease in acid levels. It was tentatively suggested that the more tolerant species were able to maintain a higher citrate:malate ratio in high Fe(II) concentrations. There was no evidence that malate or citrate accumulated in tolerant species to detoxify iron by chelation.

Part II

8. The literature on the oxidising power of roots was reviewed. Previous work suggested that the oxidising activity of roots could be partly dependent on metabolism. Peroxidase-like enzymes and the metabolic production of oxygen *via* pathways using flavin-linked oxidases and catalase have been implicated.

9. Peroxidase and catalase activity in the root tips of flood-tolerant and flood-intolerant species grown in flooded and drained sand culture was measured. There was no relationship between flood-tolerance and the activity of these enzymes or Fe(II) tolerance and activity. Flooding generally had little effect on the activity of these enzymes. The exception was *Brachypodium sylvaticum*. Root growth was greatly inhibited by flooding; peroxidase activity was stimulated and catalase activity was undetectable. Arguments were put forward suggesting that the metabolic production of oxygen in flooded roots is unlikely because of the low affinity for oxygen

of flavin-linked hydrogen peroxide-generating oxidase enzymes. Additionally if oxygen, produced intracellularly by catalase from hydrogen peroxide, was released it would be more likely to be reduced by cytochrome oxidase than to be excreted from the root cells for use in toxin oxidation.

10. A preliminary study was made of the ability of root extracts to catalyze the removal of Fe(II) from solution. This activity, which was eliminated by boiling the extracts, was found in Oryza sativa, Glyceria maxima and Ranunculus flammula. In Oryza sativa the removal of Fe(II) is an oxidation catalysed by a peroxidase-like enzyme (Yamada and Ota, 1958). The significance of these reactions in Fe(II) tolerance is unknown and further experiments are necessary.

11. The occurence and structure of aerenchyma in a wide variety of species were examined. Anatomical studies showed that aerenchyma can have a well-organized structure, particularly in the Cyperaceae.

12. The effect of various environmental factors on aerenchyma development in the roots of various species was investigated. Growth under flooded conditions generally increased air space in roots. Notable exceptions were Eriophorum angustifolium and E. vaginatum. Air space in these species was unaffected by flooding and was even high (c. 50%) in unflooded conditions. It was suggested that aerenchyma, with a complex structure as is found in these species, is formed constitutively. In Nardus stricta flooding increased air space. This species was also grown at two nutrient levels. The roots contained more air space under low nutrient level than high nutrient level in flooded or drained treatments. There was no interaction between nutrient level and flooding. Roots of Lolium perenne grown at 20 °C had more air space than when grwon at 12 °C. The amount of air space in roots is sensitive to major environmental factors. In the case of flooding, the stimulus for air space formation is low oxygen tension.

13. The possible functions of aerenchyma were discussed. In many cases oxygen diffusion from the shoot through the root aerenchyma might not be sufficient to prevent oxygen shortage in roots even though oxygen can be detected diffusing from the tips of aerenchymatous roots. Rhizosphere oxidation caused by radial oxygen loss may not be necessary for Fe(II) tolerance (see Part I).
It was suggested that rhizosphere oxidation could prevent hydrogen sulphide toxicity.

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