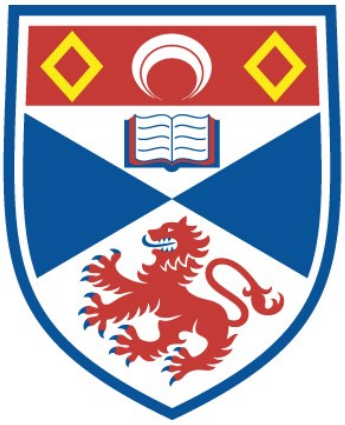
FLOODING AND ANOXIA TOLERANCE OF BARLEY IN COMPARISON WITH RICE, BEAKED SEDGE AND YELLOW FLAG

Kurt Valter Fagerstedt

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



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FLOODING AND ANOXIA TOLERANCE OF BARLEY IN COMPARISON WITH RICE, BEAKED SEDGE AND YELLOW FLAG

by

Kurt Valter Fagerstedt

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

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Kurt V. Fagerstedt

I was admitted to the Faculty of Science of the University of St Andrews under Ordinance General No 12 in October 1984 and as a candidate for the degree of Ph.D. in August 1986.

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ABSTRACT

The flooding and anoxia tolerance of three barley cultivars (Hordeum vulgare L. cv. Kustaa, Hankkija-673 and Pokko), beaked sedge (Carex rostrata Stokes), rice (Oryza sativa L. cv. FR13A) and yellow flag (Iris pseudacorus L.) have been assessed by determining the length of anoxia tolerance and the productivity of the barley cultivars under flooding. Other physiological parameters related to flooding and anoxia tolerance were also determined, and they included alcohol dehydrogenase activity and its enzyme kinetic parameters K_M and V_{max} (for reaction acetaldehyde to ethanol) in the barley cultivars and beaked sedge, production of ethanol, CO_2 and some organic acids in seedlings of the barley cultivars under anoxia, and superoxide dismutase activity in barley, rice and yellow flag under hypoxia and anoxia. A study of the root anatomy of barley and beaked sedge was also conducted.

The anoxia tolerance experiments revealed differences in the three barley cultivars and the flooding tolerance experiment gave very similar results. Thus, Kustaa proved to be the most anoxia tolerant and most productive under flooding, Hankkija-673 being intermediate

and Pokko the least anoxia tolerant and the least productive during flooded conditions.

Simultaneous measurements of ethanol, carbon dioxide and organic acid production during anoxia together with determination of dry weight loss during the period of oxygen deprivation showed that the rate of ethanolic fermentation was significantly faster in the more intolerant cultivars. Also, the more anoxia intolerant cultivars lost more of their dry weight during the anoxic incubation than the most tolerant cultivar. These results were in agreement with the metabolic theory of flooding tolerance.

Superoxide dismutase activity measurements in the barley cultivars as well as in rice under hypoxia did not reveal any changes in the activity with the onset of oxygen deprivation. Neither did incubation under total anoxia increase SOD activity. In yellow flag rhizomes, which are very flood and anoxia tolerant, large increases in SOD activity took place during and after oxygen deprivation indicating greater protection against subsequent oxidative damage. Injuries of this nature have been noticed earlier in flood intolerant species.

The anatomical study of the roots and rhizomes of beaked sedge and roots of barley cultivars revealed the larger percentage of aerenchyma in beaked sedge.

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1. INTRODUCTION

According to currently approved theories on the evolution of life and especially plant life on Earth, it generated in an environment totally free of gaseous oxygen. The primitive plants had to possess the means to operate fully - produce energy rich compounds and grow - under permanent anoxia. Thus anaerobic metabolism was and possibly still is a part of a plant's natural response to oxygen deprivation. It is known that severely hypoxic conditions do exist during normal life cycles in many plants organs. Seeds often germinate under partial or total anoxia due to the seed coat often being impervious to gases. Indeed, it has been noticed that seeds of Erythrina caffra germinate as well under anoxia as in air (Small et al., 1986). Also, hypoxic conditions readily occur in many fruits especially during the respiratory climacteric (Soldatenkov, 1941), and ethanol accumulates (Fidler, 1951). Also, plants growing in marshes or otherwise waterlogged positions experience hypoxic or anoxic stress and are in many instances capable of managing well in these adverse conditions. However, many plant species are flood- or anoxia-intolerant, and cannot continue living under total oxygen deprivation for longer that 24 to 48 hours (Levitt, 1972). From this it can be seen that anoxic or hypoxic conditions are a common hazard in the life of plants.

Most of our crop species belong to the group of not very flood- or anoxia-tolerant plants. Therefore, it would be of interest to develop flood-tolerant varieties of crop species that are at present considered flood-intolerant. This could conceivably involve the introduction of traits leading to the increased production of adventitious roots with well-developed air spaces, aerenchyma. There are two major arguments against such a strategy. First, the production of aerenchyma is a complex developmental process that would be a difficult trait for which to screen and select; the selection procedure would require screening over much of the plants life. Second, one could argue that such adaptation to a waterlogged habitat is unnecessary for crop plants that may experience flooding for only a few days in the entire growing season; more relevant would be less extreme adaptations permitting survival of hypoxic roots for those few days. This view leads to a consideration of the differences between plant species in their short-term tolerance of hypoxia. Such differences in sensitivity to flooding are due to biochemical, not anatomical, differences (Roberts et al., 1984b). Therefore, one of the aims of this study was to examine whether the Finnish barley cultivars, which are very similar to each other anatomically, differ in their metabolic tolerance of hypoxia. In addition, it was of interest to study the responses of barley root tissue to oxygen deprivation in comparison with the very flood-tolerant beaked sedge which develops spacious aerenchyma.

The means to reveal the possible differences were based on previous

research into the flooding tolerance of barley and other crop species. At the beginning, anoxia and flooding tolerance of the three barley cultivars were explored, and then a search for the underlying physiological differences commenced. Since alcohol dehydrogenase (ADH), carbon dioxide, and ethanol production under oxygen deprivation have been considered as being of importance in flooding tolerance, they were once again scrutinised. Also, the production of some organic acids was explored. Since it has been noticed earlier that many plants suffer from oxidative damage after oxygen deprivation, it was of interest to compare barley with some more flood-tolerant species to evaluate the importance of oxygen protecting superoxide dismutase (SOD) in these plants.

2. PLANT MATERIAL

During the latter half of the 1970's, barley has become the leading cereal crop in Finland, surpassing the acreage of oats. In 1980 the total barley area was 600 000 hectares which represents 20% of the arable acreage in Finland. For comparison, spring wheat acreage was only 100 000 hectares at the same time.

Due to the very cold winters only annual spring barley can be grown in Finland. In cultivation six-rowed varieties maintain a predominant role. On the basis of statistics from the State Seed Testing Station

in 1982 only 15% of the total production of barley was attributed to two-rowed cultivars. The good adaptation to the northern climate and soil conditions give the rapidly developing six-rowed barley cultivars an advantage over the two-rowed varieties. Already in Sweden the two-rowed varieties occupy a major part of barley production, the six-rowed cultivars being of importance only in the central and northern parts of the country.

The barley cultivars used in this study were selected to represent different types of barley cultivated in Finland. They have all been bred for the best productivity in Fennoscandian conditions. However, flooding resistance of these cultivars has never before been tested. The cultivars were Hordeum vulgare L. cv. Hankkija-673 (for zones I to V), Pokko (for zones I to III) and Kustaa (for zones I to II). The agricultural zones are shown in figure 2.1. Hankkija-673 is a six-rowed variety cultivated mainly for fodder. Also Pokko is six-rowed and it is being used for fodder as well as for enzyme malting. Kustaa is a new two-rowed cultivar for malting (Fig. 2.2). Hankkija-673 and Pokko have been bred in Finland, whereas Kustaa is of Swedish origin. Since its release in 1980, the cultivation of Kustaa has increased to 14% of the total production of barley grain in Finland. The percentages for Hankkija-673 and Pokko are 12% and 16%, respectively (Fig. 2.3).

Figure 2.1. The agricultural zones in Finland (Lantbrukskalender, 1983).

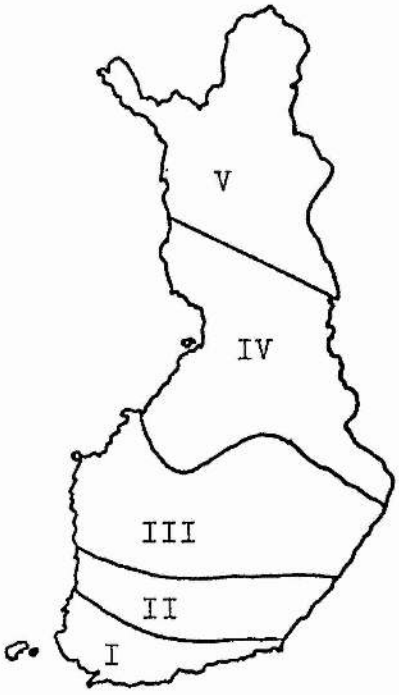


Figure 2.2. A photograph of the heads of the three barley cultivars, Hordeum vulgare L. cv. Kustaa, Hankkija-673 and Pokko, grown in solution cultures in a growth cabin at +20°C and at a 16 h daylight regime.

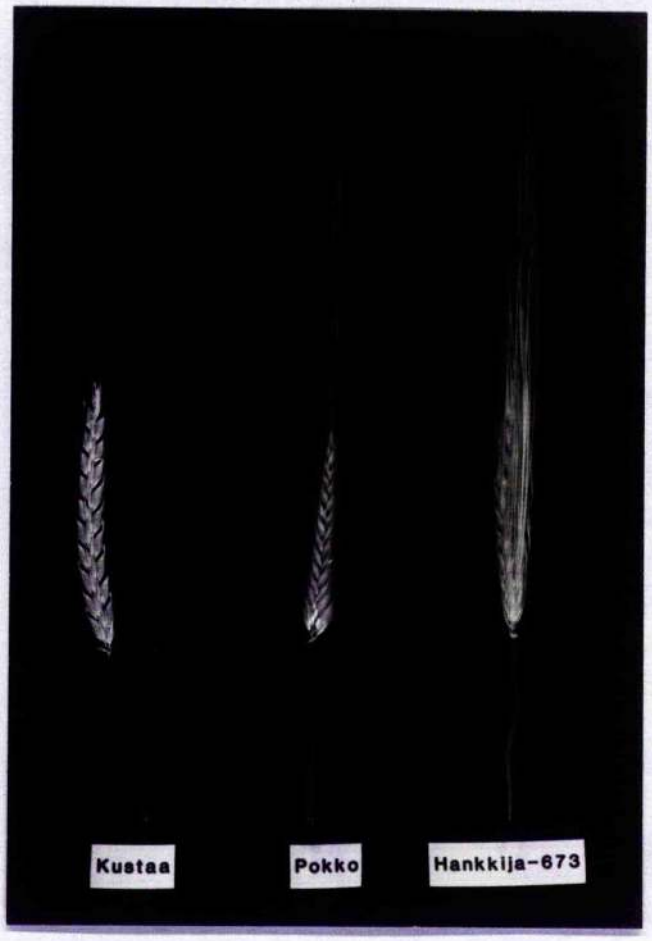
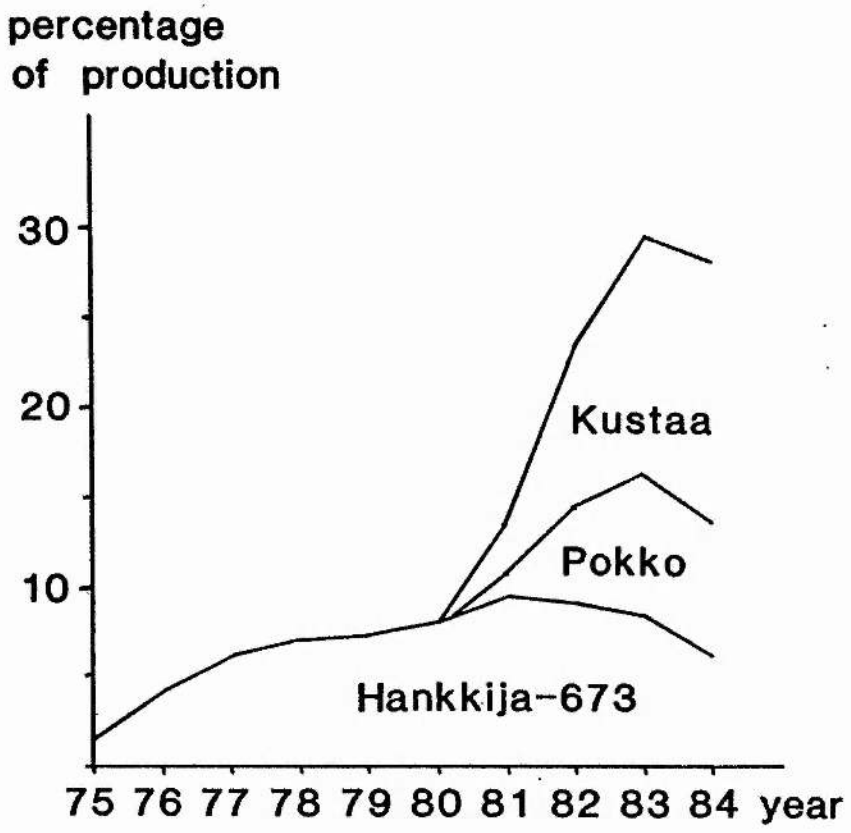


Figure 2.3. The production of barley cultivars Kustaa, Pokko and Hankkija-673 as percentage of total barley production in Finland (Hankkija Breeding Station, Siemenluettelo 1985, reproduced with the permission of the seed company Hankkija.)



The main aims of the barley breeding programmes in Finland are still the further enhancement of straw stiffness and resistance to sprouting in the ear, improvement of the outer quality of the grain in six-rowed barley used for industrial purposes, improvement of the enzyme characteristics of both two-rowed and six-rowed malting barley and raising the protein content and modifying the amino acid composition of fodder barley, and strengthening the resistance to mildew and leaf-spot disease. These characteristics are still of greater importance for the barley breeder than flooding tolerance, hence no effort has been made to improve the flooding tolerance of the barley cultivars in Finland. Only very recently has flooding tolerance emerged on the pages of the programme of Hankkija plant breeding station in Finland.

Carex rostrata Stokes, the beaked sedge, was chosen as a flood tolerant control species to enable comparisons between barley and a true wetland plant. This sedge, which is native to both Finland and Scotland, was collected from Fife at the Black Loch near Loch Lindores (Grid ref. NO269 142). Since the beaked sedge proved to be very difficult to grow from seed, mature plants were collected and grown in the glasshouse and side shoots used in the experiments.

The seeds of a rice cultivar Oryza sativa L. FR13A were obtained from the International Rice Research Institute, Manila, the Philippines. FR13A is considered as being one of the most tolerant cultivars to submergence under water (hypoxia). Rice seedlings are

also very tolerant of anoxia and even growth (elongation) of the hypocotyl can take place during anoxic incubation (Pradet and Bomsel, 1978). Rice was used as a flood tolerant control for comparisons with barley in the determinations of superoxide dismutase activity in plants under oxygen deprivation.

Since induction of superoxide dismutase activity has been observed in Iris pseudacorus L., the rhizomes of this plant were used in a further study to determine whether ther is any de novo synthesis of this enzyme. The rhizomes were obtained from the Botanic Garden here in the University of St Andrews and grown further in the greenhouse until used in the experiments.

3. ANOXIA AND FLOODING TOLERANCE OF THREE BARLEY CULTIVARS

In flooded soils the concentration of oxygen decreases quickly due to microbial activity and can reach a zero point, especially if there is no lateral flush of aerated water (Armstrong and Boatman, 1967). Microbial respiration can also result in the production of reduced chemical species such as ferrous and manganous ions, which have a toxic effect on plant roots. Furthermore, the production of volatile substances and aromatic compounds can cause damage to field crops (Drew, 1979). In plants with aerial stems and leaves, oxygen is conducted to roots via aerenchyma, but flooded seedlings of field crops such as barley do not possess this means of avoiding oxygen stress in the early stages of germination. Conditions such as these frequently arise in Scandinavia in spring, when large amounts of water are released from melting snow. The temperatures can be relatively low but high enough to allow growth to start. Consequently, there has been considerable interest in the emergence capacity of barley cultivars under oxygen stress.

Although there are a number of studies of adaptive interspecific differences in flooding or anoxia tolerance (e.g. Jones and Etherington, 1970, 1971; Jones 1971 a, b; Jones, 1972; Crawford, 1982; Etherington, 1984; Davies, 1984), there have been few investigations

into intraspecific differences in this area. In particular, no investigations have been reported as to whether any Finnish barley cultivars differ in their ability to tolerate anoxia or whether anoxia tolerance is related to flooding tolerance in barley. Interestingly, some Australian barley cultivars have been screened for their flooding tolerance, and differences were recorded (Wignarajah et al., 1976). Some studies with natural populations, however, suggest that selection does take place between neighbouring populations in relation to flooding tolerance. Linhart and Baker (1973) have found variation in the physiological response to flooding in a population of Veronica peregrina L.. Torres and Diedenhofen (1981) have reported some differences within a sunflower population after a series of dry and wet years and Francis, Devitt and Steele (1974) observed a differential influence of flooding on Trifolium subterraneum L. subspecies. Also, populations of Nyssa silvatica vary in their physiological adaptation to flooding (Keeley, 1979) as well as Festuca rubra, Agrostis stolonifera (Davies and Singh, 1983), Geum rivale, G.urbanum (Waldren, 1985) and Dactylis glomerata (Etherington and Thomas, 1986).

Tolerance of flooding during winter has been studied in winter wheat and barley (Pomeroy and Andrews, 1979), but different cultivars were not researched. In barley and maize (Marshall et al., 1973) some mutants which lack certain ADH isozymes have been investigated, but these were not commercial cultivars. Fagerstedt (1984) has reported increases in alcohol dehydrogenase activity in flooded

Finnish barley cultivars, but no differences between cultivars were recorded.

The aim of this present study was to investigate differences in anoxia tolerance in relation to flooding tolerance in plants at an intraspecific level. Three barley cultivars were used in the tests. The environment for the anoxia tolerance experiment was planned to be devoid of the harmful effects of microbial activity and soil toxins, thus concentrating on the effects of strict anoxia only. Also, the complication of air moving through aerenchyma to the roots was avoided by placing the plants in anaerobe jars at +5°C, thus simulating the environment of a flooded field in spring. As the seedlings were only three days old, they were kept in the dark, as if they were buried in soil. The tolerance of anoxia was determined at three levels: survival after the treatment, fresh and dry weights of the plants after a growth period subsequent to anoxic stress, and localization of anoxic or post-anoxic damage. Furthermore, to observe whether anoxia tolerance is correlated with flooding tolerance, a flooding tolerance test with the same barley cultivars was carried out.

3.1. Methods and results

3.1.1. Anoxia tolerance experiment

Seeds of the barley cultivars Hordeum vulgare L. cv. Hankkija-673, Kustaa and Pokko were stored at room temperature in paper bags within plastic bags. Before experimentation they were sterilized with 6% hypochlorite for 10 minutes in a Griffin shaker, washed with tap water for as long as the strong smell of chlorine remained, subsequently rinsed with distilled water, and placed in petri dishes with a Whatman no 3 filter paper moistened with 5 $x10^{-6}$ m^3 distilled water. The seeds were allowed to germinate in a growth cabinet at +20°C in the dark for three days.

Prior to the anaerobic treatment the germinated seeds were placed in new petri dishes with a Whatman no 3 filter paper, 10 seeds per dish and moistened with 5 $x10^{-6}$ m^3 distilled water. The petri dishes were placed in plastic anaerobe jars (GasPack, Becton Dickinson and Co., USA, Figure 3.1), and put into an anaerobic workbench (Forma Scientific, USA) with two evacuations under nitrogen, and then left there for half an hour to allow any remaining oxygen to diffuse out of the plant material and containers. The atmosphere in the workbench was 90% nitrogen and 10% hydrogen. After methylene blue oxygen-indicator strips were placed in the jars, they were sealed and put in a growth cabinet at +5°C in the dark, thus

simulating the dark environment of seedlings buried in soil. Aerobic controls were put into similar jars and placed in the same cabinet. During the incubation period great care was taken to keep the environment completely anoxic, and this was monitored with the methylene blue oxygen-indicator strips. Palladium catalysts inside the anoxic jars were changed regularly.

After the appropriate time in the anaerobic environment (controls in air) the seeds were taken out, rinsed with distilled water and planted in trays in Levington's soil mixture. The seedlings were then allowed to grow for 20 days at +20°C in a 16 hour day light regime in a growth cabinet (Fig. 3.2), after which time the surviving seedlings were counted (Fig. 3.3) and fresh weights of all shoots and roots measured (Fig. 3.4 and 3.5). Dry weights were determined after drying at +60°C for 48 h. Control plants were treated in the same way. The experiment was replicated thrice, 720 seedlings being used on each occasion.

A binomial test for differences among proportions was used to determine the statistical significance of survival values (Freund, 1971).

FIGURE 3.1. A photograph of an anaerobe jar (Gaspack, Becton Dickinson and Co., USA) loaded with petridishes containing barley seedlings of the three cultivars. The jar was filled with gas consisting of 90% N_2 and 10% H_2.

FIGURE 3.2. A photograph of the three barley cultivars growing in a tray of soil after the anoxic incubation. From left to right Hordeum vulgare L. cv. Hankkija-673, Kustaa and Pokko.

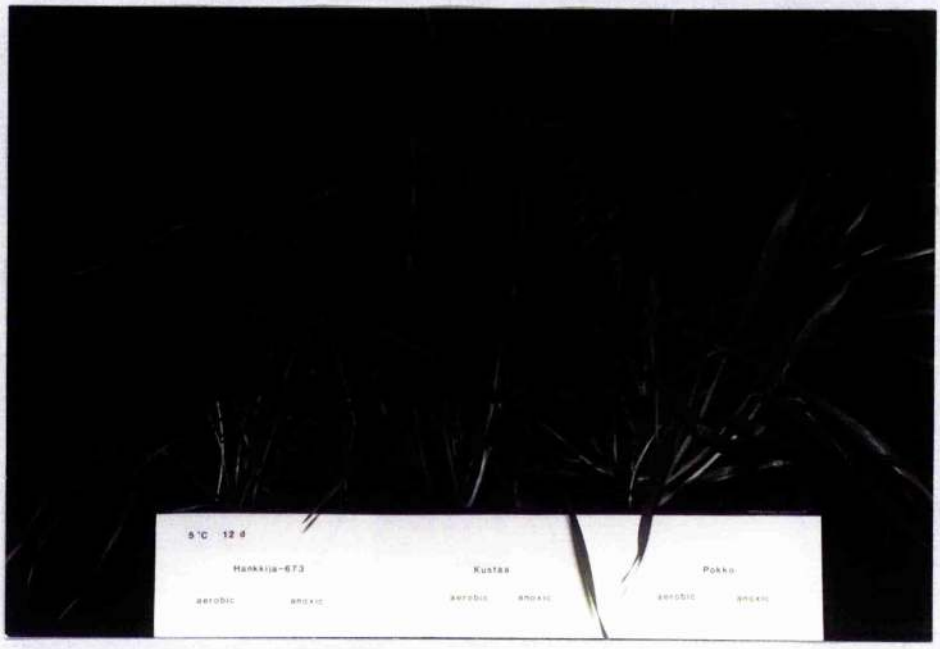


FIGURE 3.3. The survival of seedlings of three barley cultivars Hordeum vulgare cv. Hankkija-673, Kustaa and Pokko under control (air) and anoxic conditions and a 20 day recovery period. The histograms show the recovery out of 20 seedlings. The experiment was replicated thrice. The bars indicate standard error (S.E.) and the asterisks statistically significant differences at 0.1% level.

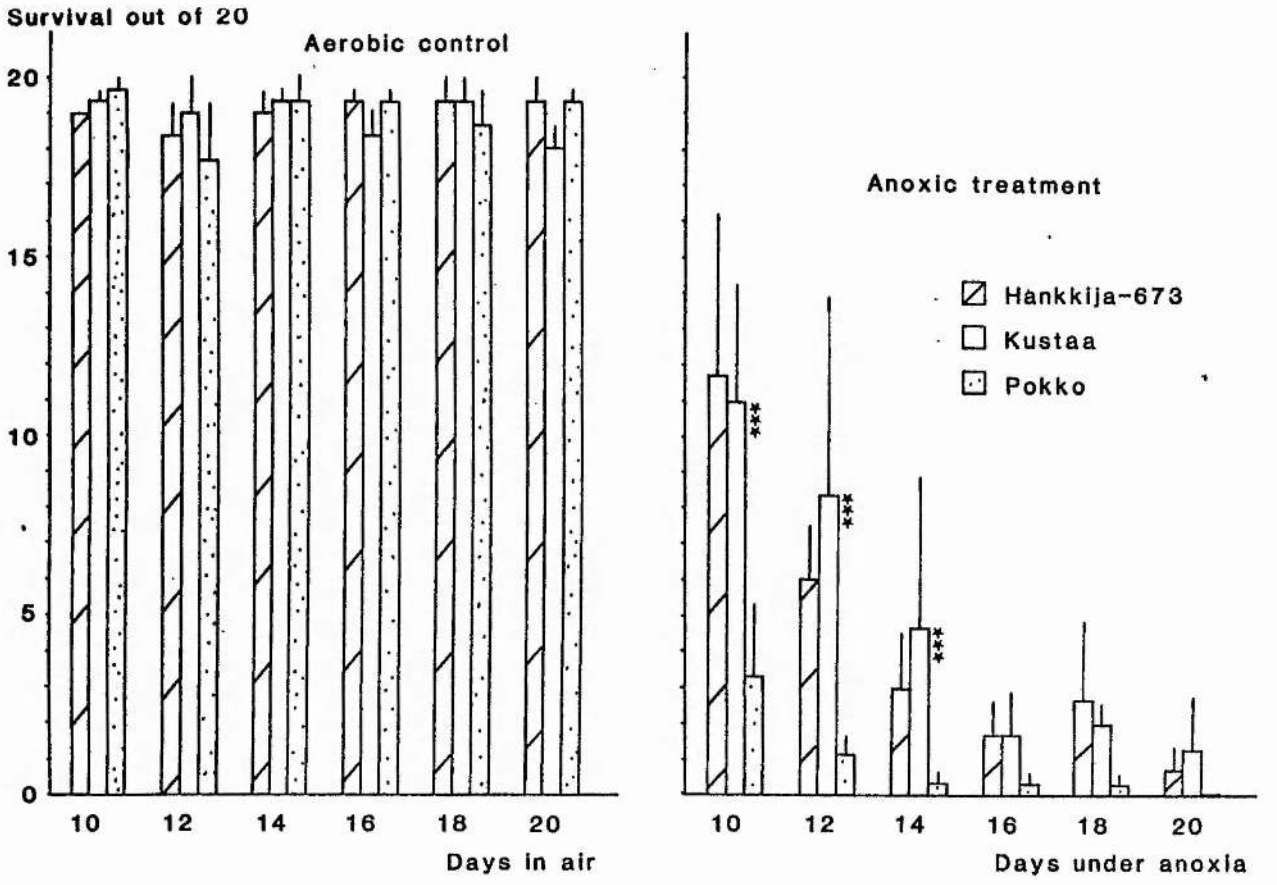


FIGURE 3.4. Fresh and dry weights of the shoots of 20 seedlings of three barley cultivars Hordeum vulgare L. cv. Hankkija-673 (▲), Kustaa (●) and Pokko (■) after control (air) and anoxic treatment and a 20 day recovery period. Twoway analysis of variance showed significant differences between the cultivars at 0.1% level for the anoxic treatment. In the control data significant differences were not found.

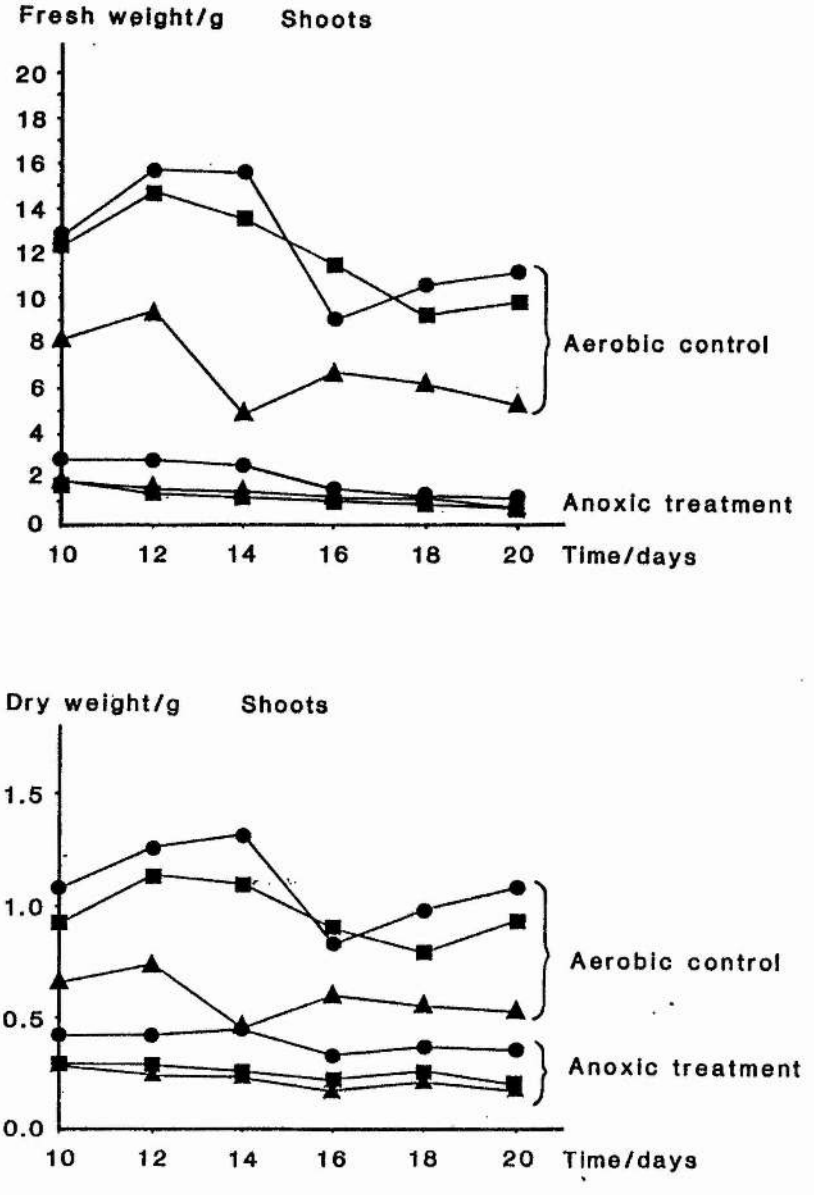
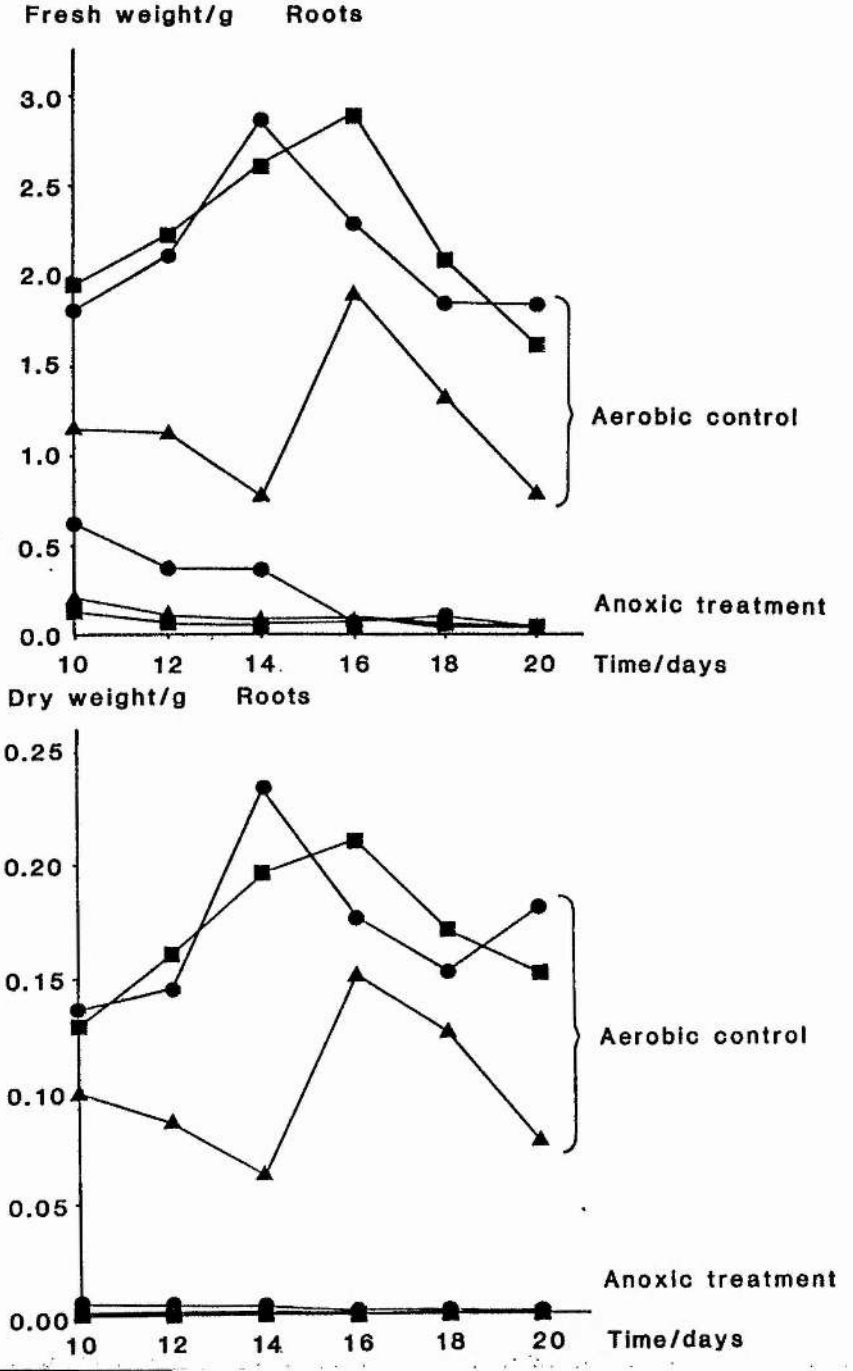


FIGURE 3.5. Fresh and dry weight of the roots of 20 seedlings of the three barley cultivars Hordeum vulgare L. cv. Hankkija-673 (▲), Kustaa (●) and Pokko (■) after a control (air) and an anoxic treatment and a 20 day recovery period. Twoway analysis of variance showed significant differences between the cultivars at 5% level for the anoxic treatment. In the control data significant differences were not found.

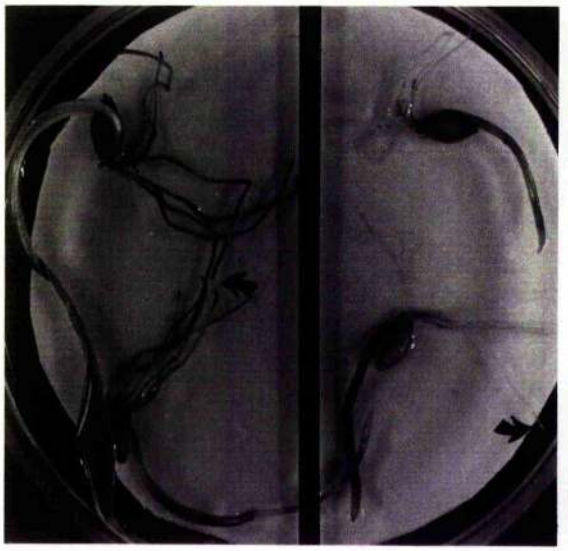


3.1.2. TTC-test

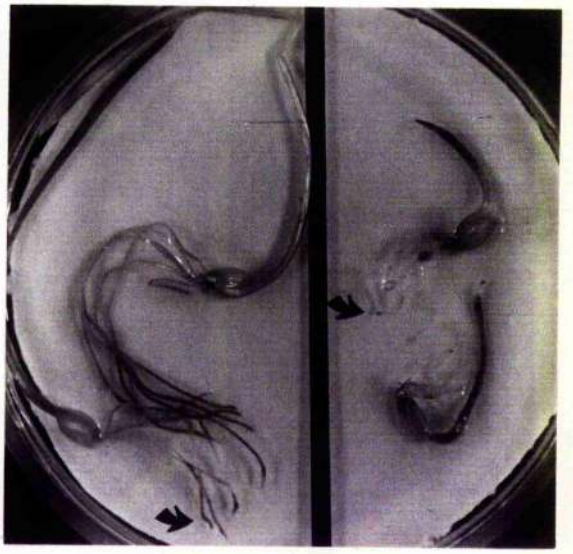
To observe the tolerance of anoxia at the level of tissues, 60 seedlings of each cultivar were germinated as stated above and placed in the anaerobe jars for 12 days at +5°C. After the anoxic treatment the seedlings were placed in new Petri dishes and growth was allowed to start over a period of three days at +20°C in the dark. The seedlings were then rinsed and immersed in 0.5% tetrazolium chloride solution overnight for staining of the living tissues. Dehydrogenase activity reduces tetrazolium chloride to insoluble red formazan. The results were observed with a binocular microscope and subsequently photographed (Fig. 3.6).

Also, some side shoots of Carex rostrata were prepared for anoxia tolerance test by first washing them with tap water and then rinsing the explants with 0.16 mol m-3 chloramphenicol in distilled water to prevent bacterial growth. The explants were further treated in the same way as the barley seedlings (Fig. 3.6).

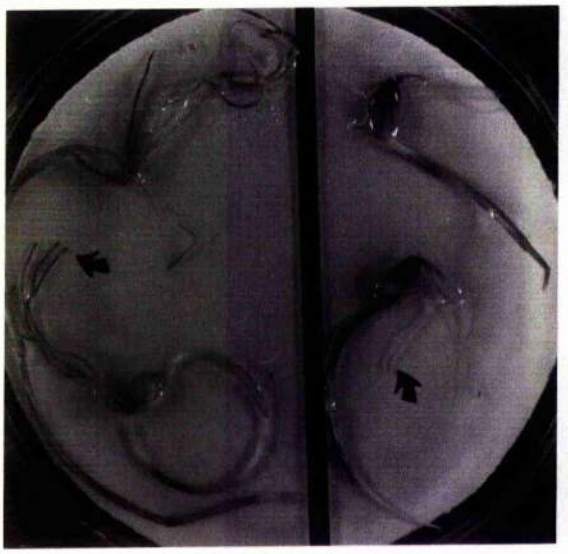
FIGURE 3.6. Photographs of the tetrazolium chloride (0.5%) test for barley seedlings Hordeum vulgare L. cv. Hankkija-673, Kustaa and Pokko after a 12 day anoxic and a three day recovery period. Control material is on the left and anoxic on the right in the petri dishes. Bottom right hand corner shows a Kustaa seedling with seminal roots with dead tips, but live bases (arrow) exhibiting red precipitated stain. Arrows show living control roots and dead anaerobically treated root tips. All shoots have remained alive. Also, in Carex rostrata root tips died during the anoxic treatment.



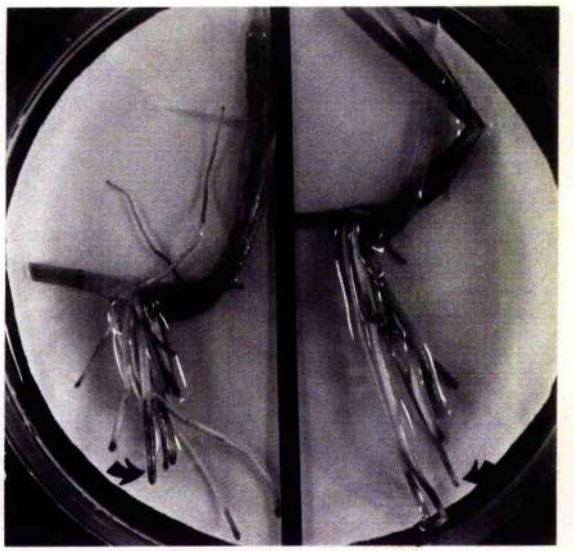
Kustaa



Hankkija-673

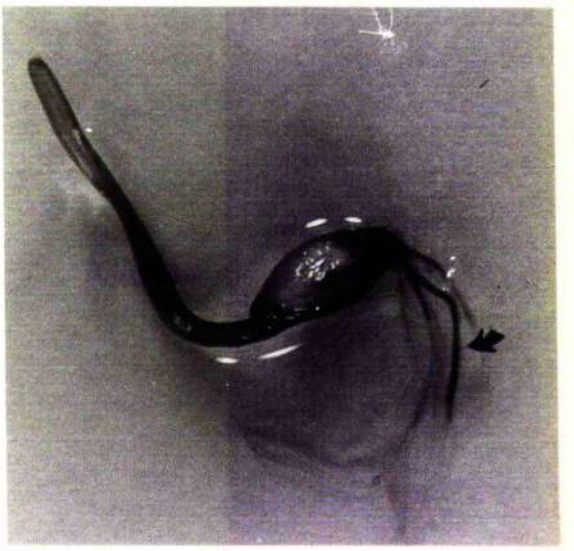


Pokko



Beaked sedge

Kustaa



3.1.3. Flooding tolerance experiment

Flooding tolerance and productivity under flooding was tested as follows: The seeds were surface sterilized with 6% hypochlorite as stated previously and planted in moist coarse sand in 5 $x10^{-3}$ m^3 rectangular plastic trays. The seedlings were allowed to germinate at +15°C until the first leaf was 3 cm tall and the tray was flooded with tap water up to the surface of the sand. The plants were allowed to grow for three months under flooding at +15°C in 16 h artificial light, by which time the seeds had ripened and the shoots started to turn yellow (Fig. 3.7). All three cultivars were grown in the same tray and their places within the tray were randomized in the three replicates. The number of seeds produced were counted and fresh and dry weights of shoots were measured (Table 3.1).

TABLE 3.1. Flooding tolerance experiment with three barley cultivars Hordeum vulgare L. cv. Hankkija-673, Kustaa and Pokko. The seeds were surface-sterilized and germinated in moist sand and flooded with tap water after the first leaf had reached 3 cm length. The plants were harvested after a period of three months under flooded conditions. Each cultivar was represented by 12 seedlings. The statistical significance of the figures of the cultivars Hankkija-673 and Pokko was tested with student's T-test against values of the cultivar Kustaa. N.S. = not significant, * = P 0.05, ** = P 0.01 and ** = P 0.001. The experiment was replicated thrice.

	HORDEUM VULGARE L. cv. Kustaa x±S.E.	Hankkija-673 x±S.E.	Pokko x±S.E.
No of plants	10.7±0.3	7.7±0.3 **	10.0±0.6 N.S.
No of seeds produced	30.3±5.2	7.0±3.8 *	0.0±0.0 **
No of seeds produced/ plant	2.9±0.5	1.0±0.6 N.S.	0.0±0.0 **
D.W. of all heads/g	1.07±0.16	0.31±0.14 *	0.0±0.0 **
F.W.of all shoots/g	4.09±0.56	3.43±0.80 N.S.	3.35±1.13 N.S.
D.W. of all shoots/g	1.64±0.19	0.98±0.22 N.S.	0.95±0.25 N.S.

FIGURE 3.7. A photograph of one of the flooding tolerance experiment replicates. From left to right Hordeum vulgare L. cv. Pokko, Kustaa and Hankkija-673. The seedlings were grown under flood in coarse sand for the period of three months, in which time they seeds had ripened and the shoots turned yellow.

3.2. Discussion

3.2.1. Which plant organ suffers first?

The TTC-test showed clearly that roots, and especially root tips, are the most intolerant organs in the young barley seedlings. In all the seedlings which underwent anoxic stress the tips of the original seminal roots died and only the shoots were able to resume growth. Subsequently, new adventitious roots emerged from the stem bases. In some of the seedlings new roots emerged already during the three day recovery period (Fig. 3.4). Kustaa seemed to start growing new roots more rapidly, which may be one reason for its better recovery after the anoxic period. The TTC-test revealed that in Kustaa the bases of the seminal roots stayed alive after the anoxic period and may subsequently start producing new lateral roots. In fact it may be the case that seedlings of different cultivars emerge after an anoxic period with very similar injuries, and the differences only become apparent during a post-anoxic period in the ability to resume shoot and root growth.

The Carex rostrata explants showed very similar injuries to the more tolerant barley cultivar Kustaa. The root tips were the most intolerant organs and they died during the 12 day anoxic period.

However, immediately above the root tip some red precipitate was to be seen, indicating that the more mature parts of the roots were more tolerant of anoxia. Aerobic control plants exhibited large amounts of precipitated stain in the root tips. These experiments suggest that the higher the metabolic activity of a given tissue is, in this case growing tips of roots vs. mature root tissue, the more susceptible the cells are to anoxic damage.

3.3. Anoxia tolerance vs flooding tolerance

The first sign of differences between the studied cultivars in respect of flooding tolerance was observed during a previous experiment (data not shown) where seeds of the same cultivars were germinated in petri dishes on filter paper moistened with distilled water. It was noticed that one of the cultivars, namely Pokko, died off very easily if the amount of water in the petri dish was enough to keep the seeds inundated. This prompted more precise experiments reported here to determine if the seedlings differed in their tolerance of anoxia, and whether this is related to flooding tolerance. Although rhizomes of different species have been shown to have varying tolerance of anoxia related to flooding tolerance (Barclay and Crawford, 1982), there is no study on dry land crop plants in which anoxia tolerance is assessed as a component of flooding tolerance. We must remember that this anoxia tolerance

experiment was devoid of the harmful effect of soil toxins. We can say that the cultivars differ in their tolerance of anoxia, but it does not necessarily mean that these plants vary in their tolerance of flooding. Anoxia is just one of the many important factors in flooded soil. It is therefore a significant finding of this particular study that anoxia tolerance was positively correlated to flooding tolerance, which suggests that anoxia is one of the determining factors in flooding tolerance.

The survival of the seedlings in the anoxia tolerance test is shown in Figure 3.2. In the control data differences between the cultivars are very small, and the statistical test does not reveal any significant differences between the cultivars. In the anoxic survival data the differences are clear and the test shows statistically significant differences between the cultivars at 10, 12 and 14 days under anoxia. The increase in S.E. values in the anoxic data is possibly due to the fact that when the plants are under a severe stress such as complete anoxia, individiual differences become apparent and affect the survival of the seedlings. In every replicate the cultivar Pokko showed the lowest performance in survival of anoxic stress. Also, fresh and dry weight of Pokko seedlings after a post-anoxic growth period dropped from very high levels in the control environment to the lowest of the three cultivars after anoxia.

Similar experiments with several species including Hordeum

murinum L., where anoxia tolerance varied between species and was greatest at low temperatures, have been reported by Barclay and Crawford (1982). In addition, the tolerance of flooding by winter barley at +2°C (Pomeroy and Andrews, 1979) was very similar in length to our observations. The results of the flooding tolerance experiment (Table 3.1) correlate positively with anoxia tolerance, Kustaa being the most and Pokko the least productive under permanently flooded conditions.

Wignarajah et al. (1976) have pointed out similar differences in flooding tolerance of barley to our results with Finnish barley cultivars. Some of their cultivars suffered more than others of flooding and this was especially noticeable in the reduction of root growth, which was more pronounced than the reduction in shoot growth.

No growth was observed under anoxia. The ultimate cause for the death of the seedlings remains uncertain, the possible factors being changes in the cytoplasmic pH (Roberts et al., 1985), very low energy charge (Al-Ani et al.,1985) and metabolite toxicity. Whether or not metabolites such as ethanol are directly toxic or act via their post-anoxic oxidation products e.g. acetaldehyde is still uncertain (Crawford, 1977; Monk et al., 1987, vs. Jackson et al., 1982). Our determinations of ethanol production under anoxia in the three studied cultivars showed a negative correlation between tolerance and amount of ethanol produced; however, the differences between the cultivars were very small (see chapter 5).

Carbohydrate starvation does not seem either to be applicable in the case of barley seeds, which have large carbohydrate reserves. No correlation in the size of the seeds (Table 3.2) was obvious with the survival data. Barclay and Crawford (1982, 1983) have also noticed the lack of correlation with anoxia tolerance and seed size. Differences in ADH isozyme content may be one reason for the varying anoxia tolerance of the barley cultivars (see chapter 4). Marshall et al. (1973) have noticed that in maize strains alternative forms of Adh1 gene may markedly affect the fitness of their carriers in flooded soils.

The present data show that barley is very susceptible to an anoxic environment. However, differences between cultivars are significant and may prove to be a useful piece of agricultural knowledge for both winter barley culture and early spring sowing, and for the use of wet or irrigated fields especially in spring and autumn temperatures.

Table 3.2. Weight of 100 air dried seeds of the barley cultivars.

Cultivar	Weight g/100 seeds
Hankkija-673	3.109
Kustaa	4.397
Pokko	5.119

4. ALCOHOL DEHYDROGENASE (ADH) ACTIVITY AND ITS KINETIC PROPERTIES IN BARLEY AND BEAKED SEDGE DURING HYPOXIC CONDITIONS

Studies of alcohol dehydrogenase (ADH, alcohol:NAD+ oxidoreductase, E.C. 1.1.1.1.) have been reported in many recent articles in connection with flooding tolerance, and in most plant species an induction of ADH activity has been observed during flooding or hypoxia (Smith and ap Rees, 1979a,b; Harberd and Edwards, 1983; Jenkin and ap Rees, 1983; Lazlo and Lawrence, 1983; Fagerstedt, 1984). However, the significance of these changes in metabolism is still unclear. In maize, anaerobic treatment of root tissue results in the cessation of general (aerobic) protein synthesis together with the initiation of the synthesis of a few polypeptides designated as anaerobic polypeptides (ANPs). Amongst these is ADH (Sachs and Freeling, 1978; Sachs *et al.*, 1980). It seems likely that barley is similar in this respect (Mayne and Lea, 1984). Indeed, Harberd and Edwards (1983) have shown, by electrophoretical and *in vivo* labelling studies, that new ADH isozymes are synthesized during hypoxic conditions in barley tissue.

In maize the new ADH isozymes that emerge during flooding have different biochemical properties, although there are only small alterations in the primary structure (Felder *et al.*, 1973). This, together with cytoplasmic acidosis caused by lactic acid fermentation (Roberts *et al.*, 1984a), may lead to modifications in metabolism.

The oxidation of ethanol by ADH declines drastically in acidic conditions, the pH optimum for this reaction being 9.0. On the other hand, at pH 6.0 the reaction from acetaldehyde to ethanol still occurs readily, the optimum being at pH 8.5. (Felder et al., 1973). Thus, in hypoxic conditions cytoplasmic ADH reacts to produce ethanol, and is consequently competing with lactate dehydrogenase (LDH) for the coenzyme NADH. Indeed, recent studies of barley root tissue have shown that the K_M of the ADH1.ADH1 homodimer for NADH is higher than the K_M of the isozymes induced under anaerobic conditions (Mayne and Lea, 1984).

This part of the thesis investigates the changes in the enzyme kinetic parameters of ADH for acetaldehyde in aerobically grown root tissue of both barley and beaked sedge compared with tissue subjected to hypoxic conditions. Only one barley cultivar (Hordeum vulgare L. cv. Hankkija-673) of the three subjected to study in this thesis was used in the ADH activity determinations, since a comparison of ADH activity in the roots of the same three cultivars had been conducted earlier (Fagerstedt, 1984). In that study no statistically significant differences were found in ADH activity between the cultivars.

As so many previous studies have involved only young seedlings, germinating seeds or aleurone layers, these experiments were carried out with young barley plants possessing seminal roots as well as with mature plants of both barley and beaked sedge with

adventitious root systems.

4.1 Solution cultures

To avoid the well documented harmful effects of changes in the soil due to flooding (Ponnamperuma, 1972; Russell, 1973), the plants were grown in solution culture in Ruakura nutrient solution. In addition, this facilitated easy measurements of oxygen concentration around the roots.

Seeds of the barley cultivar Hordeum vulgare L. Hankkija-673 were supplied by the Finnish seed company, Hankkija. The seeds were not pretreated with a dressing agent.

Seeds were first surface-sterilized with fresh 6 % sodium hypochlorite for 10 min in a Griffin flask shaker. They were then rinsed with cold tap water until the smell of chlorine had reduced, c. 10 min. After rinsing twice with distilled water, the seeds were placed on petri dishes lined with a Whatman no. 3 filter paper to which 1 $x10^{-5}$ m^3 of distilled water was added. The dishes were placed in a growth cabinet in the dark at +20°C for three days to germinate before the solution cultures were started.

Since an optimum nutrient solution for the growth of barley has not yet been described, the barley seedlings were grown in Ruakura nutrient solution, which is the best for maize and ryegrass (Smith et al., 1983). Hoagland solution which is still often used, was considered as insufficient for iron in this study. The plants were grown in 1 $x10^{-2}$ m^3 tanks, 44 specimens in each, with aeration provided (50±5 $x10^{-6}$ m^3 min^{-1}) by means of aquarium pumps (Fig. 4.1). The solution was changed once a fortnight. Meanwhile distilled water was added daily to account for losses via transpiration and evaporation. Light was provided by mercury vapour lamps, 130 umol m^2 s^{-1}, for 16 h daily. The temperature was +20±2°C.

Plants for the experiment with seminal roots were allowed to grow for 30 d, whereas for the experiment with adventitious roots the plants were grown for two months.

Ruakura solution

Macronutrients:

$Mg(NO_3)_2 * 6 H_2O$	0.86 mol m^{-3}
$Ca(NO_3)_2 * 4 H_2O$	3.16 mol m^{-3}
NH_4NO_3	4.71 mol m^{-3}
KNO_3	1.00 mol m^{-3}
KH_2PO_4	0.87 mol m^{-3}
K_2HPO_4	0.42 mol m^{-3}
K_2SO_4	1.69 mol m^{-3}

Na_2SO_4	0.19 mol m^{-3}
NaCl	0.25 mol m^{-3}

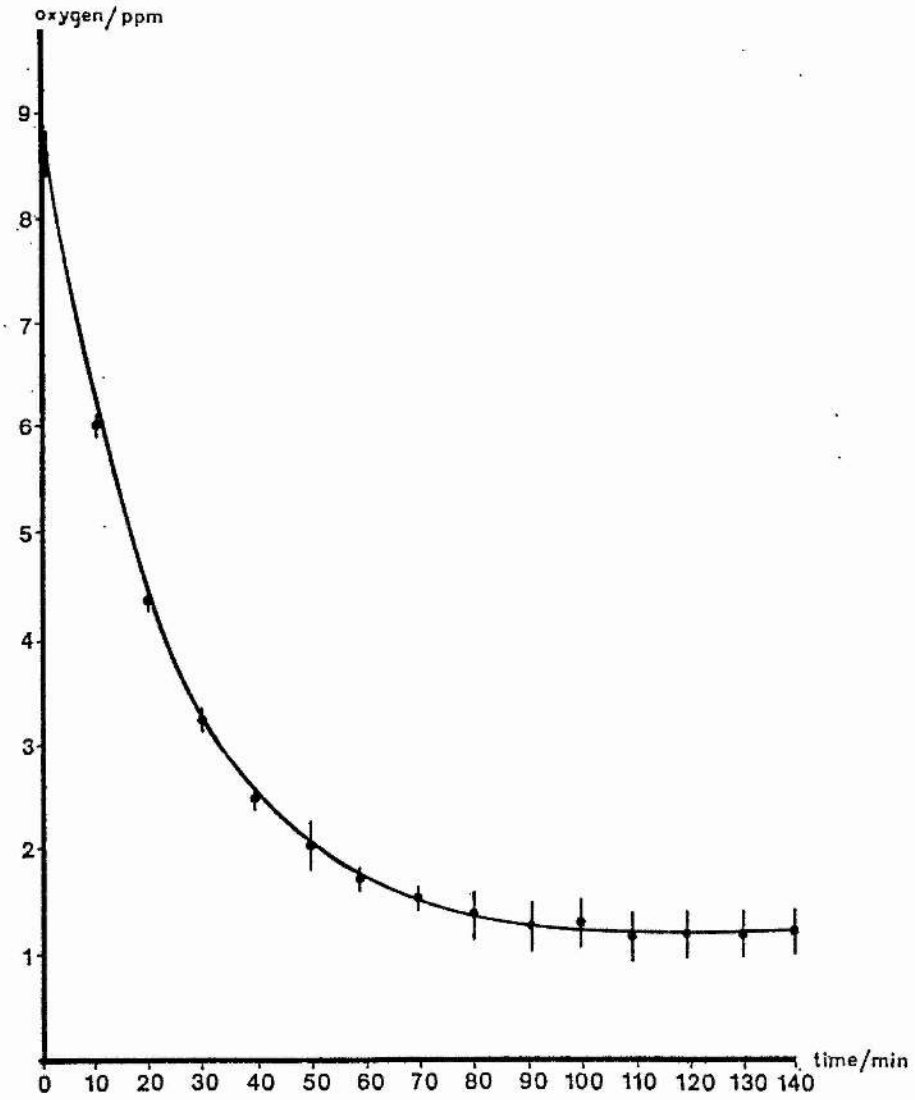
Micronutrients:

H_3BO_3	46.28 $x10^{-3}$	mol m^{-3}
$CuCl_2 * 2 H_2O$	0.63 $x10^{-3}$	mol m^{-3}
$MnCl_2 * 4 H_2O$	9.11 $x10^{-3}$	mol m^{-3}
$(NH_4)_6Mo_7O_{24} * 4 H_2O$	0.015 $x10^{-3}$	mol m^{-3}
$ZnCl_2$	3.82 $x10^{-3}$	mol m^{-3}
$FeC_6H_5O_7 * 5H_2O$	53.71 $x10^{-3}$	mol m^{-3}

The oxygen concentration of the nutrient solution was measured with an oxygen electrode (Beckman Fieldlab Oxygen Analyzer) during both aeration and flooding, which was simulated by passing nitrogen through the solution. Flow of air and nitrogen was in each case 50±5 $x10^{-6}$ m^3 min^{-1}. Results are means and standard errors of four replicates (Fig. 4.2).

FIGURE 4.1. Two photographs of solution cultures of barley cultivars Hordeum vulgare L. cv. Kustaa, Hankkija-673 and Pokko growing in 10 $x10^{-3}$ m^3 vessels in Ruakura nutrient solution with aeration by aquarium pumps. Above a culture of Hankkija-673 and below all three cultivars next to each other.

FIGURE 4.2. Concentration of oxygen in the nutrient solution during hypoxic treatment of barley and beaked sedge solution cultures. The results are means and standard errors of four replicates.



4.2. Optimisation of ADH activity determination

4.2.1. Barley ADH

The extraction procedure for barley root tissue ADH was optimised by testing the effect of polyvinylpyrrolidone (insoluble PVP), 2-mercaptoethanol and cysteine on the activity. The concentrations were 1% and 3% PVP, 5, 10, 20, 30 and 40 mol m^{-3} 2-mercaptoethanol, and 10, 20, 30 and 40 mol m^{-3} cysteine. The enzyme exhibited a rather broad pH range of activity in the direction acetaldehyde to ethanol, the peak lying near pH 8.0, which was used in the activity determinations. A similar broad bell-shaped activity curve has also been observed with maize ADH (Felder et al., 1973). The highest activities were recorded with 10 mol m^{-3} 2-mercaptoethanol and 10 mol m^{-3} cysteine concentrations, hence these concentrations were used in the determinations. Addition of PVP did not seem to affect ADH activity in barley root tissue extracts, which was possibly due to absence of phenolic substances in young barley root tissue. The final reaction mixture was as follows:

83	mol m^{-3}	Tris
8.30	mol m^{-3}	2-mercaptoethanol
8.30	mol m^{-3}	cysteine

0.19 mol m^{-3} NADH

23.0 mol m^{-3} acetaldehyde

For K_M value estimation acetaldehyde concentration was varied between 1.15-46.0 mol m^{-3} (8-10 different concentrations). Total volume of the reaction mixture was 3.00 $x10^{-6}$ m^3. The absorbance was measured at 366 nm wavelengt.

For the determination of ADH activity and K_M values the entire root systems were first removed from the plants, rinsed with distilled water and cut into 50 mm pieces. Root samples (0.50 g) were weighed quickly and then ground at +5°C in 2.5 $x10^{-6}$ m^3 of 0.1 $x10^{-3}$ mol m^{-3} Tris-HCl buffer pH 8.0 (containing 10 mol m^{-3} 2-mercaptoethanol and 10 mol m^{-3} cysteine), first with a pestle and mortar and subsequently with an all-glass homogeniser. The extracts were decanted into 1.5 $x10^{-6}$ m^3 plastic tubes and centrifuged at 11600 rpm = 8730 g for 20 mins at +5°C. The supernatant was used immediately for K_M value estimations. The K_M values were determined by means of a Unicam SP1800 Ultraviolet Spectrophotometer attached to a waterbath at +25±1°C, which kept the temperature of the cuvettes constant. Acetaldehyde concentration was measured enzymatically with yeast ADH (Boehringer), whereas the concentration of NADH was determined with the spectrophotometer. The reaction rate was measured twice in each acetaldehyde concentration.

K_M and V_{Max} values were computed with a Texas Intruments SR-56 programmable calculator. Despite the fact that the measurements were performed with crude extracts, double reciprocal plots of reaction rate against substrate concentration were found to be linear. The correlation coefficients for the lines varied between 0.80 and 0.99. However, all the figures were plotted on paper to ensure correct interpretation of results. Scandalios (1977) has tested purified and partially purified maize extracts and noted that the apparent K_M values did not differ significantly from the K_M values obtained from purified enzymes.

An experiment was carried out to see how much of the ADH activity was lost or inhibited during the extraction of roots. Commercial yeast ADH (Boehringer) was used as a reference added to the extraction medium at the same time as the roots. Root extracts were prepared in the same way as for the K_M value determinations. The reaction mixture consisted of:

83.0 mol m^{-3} Tris pH 8.0
8.30 mol m^{-3} 2-mercaptoethanol
8.30 mol m^{-3} cysteine
0.13 mol m^{-3} NADH
23.0 mol m^{-3} acetaldehyde

The total volume was 3.00 $x10^{-6}$ m^3. Results are presented in Table 4.1. The recovery percentage of the activity of

added known ADH was found to be 82.2±3.3%. Whether or not inhibitors had an effect on the kinetics of ADH cannot be commented on on the basis of these experiments.

Soluble protein content of the extracts were measured by the binding of bromophenol blue to proteins under acidic conditions. The bound form absorbs light at 610 nm wavelength. The reaction mixture consisted of: 2.7 $x10^{-6}$ m^3 of bromophenol reagent (0.0075 % bromophenol blue in 15 $x10^{-6}$ m^3 of 94 % ethanol, 2.5 $x10^{-6}$ m^3 glacial acetic acid and 82.5 $x10^{-6}$ m^3 of distilled water) and 0.3 $x10^{-6}$ m^3 of enzyme extract (Flores, 1978).

TABLE 4.1. Results of the ADH recovery test with barley root ADH. Values represent means and standard errors of three or four independent samples of each kind (i.e. four root extracts and four roots extracts with commercial ADH). Each sample was measured twice.

Extract	Change in absorbance per minute		x ± S.E.
1 roots only	0.0118	0.0130	
2 "	0.0125	0.0120	
3 "	0.0150	0.0120	0.0126 ± 0.0004
4 "	0.0113	0.0128	
1 roots + ADH	0.0364	0.0354	
2 "	0.0364	0.0369	
3 "	0.0364	0.0328	0.0361 ± 0.0005
4 "	0.0372	0.0369	
1 ADH only	0.0300	0.0320	
2 "	0.0343	0.0300	
3 "	0.0300	0.0313	0.0313 ± 0.0007

Predicted activity for roots + ADH = 0.0126±0.0004 + 0.0313±0.0007 = 0.0439±0.0011

Recorded activity of root + ADH = 0.0361±0.0005

Recovery percent (percentage of that predicted)

$$\frac{0.0361 \pm 0.0005}{0.0439 \pm 0.0011} \times 100\% = 82.2 \pm 3.3\%$$

4.2.2. Beaked sedge ADH

The reaction mixture for ADH activity measurement and K_M value estimations was the same as described earlier for barley. To optimise the extraction of sedge root tissue the effect of PVP soluble and insoluble, 2-mercaptoethanol, cysteine and dithiotreitol (DTT) in several combinations were tested. The concentrations used were 4, 8, 12% insoluble PVP and 4% soluble PVP, 10, 20, 30 mol m^{-3} 2-mercaptoethanol, 2, 5, 10, 20 mol m^{-3} cysteine and 5, 10 and 20 mol m^{-3} DTT.

Insoluble PVP (polyclar AT) had a more beneficial effect on the ADH activity than soluble PVP. The final concentrations used in the measurements were as follows: 5 mol m^{-3} DTT, 5 mol m^{-3} cysteine and 8% insoluble PVP. DTT and cysteine protected ADH better when together than when applied separately, even though they are both supposed to safeguard SH-groups. Also, the effect of all the additives on pH was tested, and it was noticed that PVP lowered the pH slightly, about 0.09 pH units in the concentration used for ADH activity determinations.

The roots were extracted the same way as is described earlier for barley root tissue, except that after grinding with a pestle and mortar and some quartz sand the extracts were centrifuged at once

without further grinding with an all-glass homogenizer. Carex root tissue was too hard to be effectively ground with a glass homogenizer and quartz sand was used instead. Root tissue (0.50 g) was gound in 2.5×10^{-6} m^3 of buffer with the above mentioned additives.

The reaction mixture for the activity determinations and K_M estimations was the same as with barley root extracts, except that the additives and their concentrations were different as mentioned above.

A recovery experiment was done to see how much of the ADH activity is lost during the extraction of roots (Table 4.2). Commercial yeast ADH (Boehringer) was used as a reference that was added to the extraction medium at the same time as the roots. The roots were prepared as described above. The relatively low recovery of ADH activity was possibly due to the high phenol content of the Carex rostrata roots.

TABLE 4.2. The results of the ADH recovery test with beaked sedge root ADH. The values represent means and standard errors of four independent samples of each kind (i.e. four root extracts and four roots extracts with commercial ADH).

Extract	Change in absorbance per minute		x ± S.E.
1 roots only	0.0567	0.0600	
2 "	0.0538	0.0550	
3 "	0.0583	0.0583	0.0563±0.0009
4 "	0.0550	0.0533	
1 roots + ADH	0.0617	0.0651	
2 "	0.0701	0.0691	
3 "	0.0617	0.0751	0.0672±0.0016
4 "	0.0657	0.0691	
1 ADH only	0.0540		
2 "	0.0540		
3 "	0.0570		0.0505±0.0046
	0.0369		

Predicted activity for roots + ADH = 0.0563±0.0009 + 0.0505± 0.0046 = 0.1068±0.0055

Recorded activity of root + ADH = 0.0672±0.0016

Recovery percent (percentage of that predicted)

$$\frac{0.0672 \pm 0.0016}{0.1068 \pm 0.0055} \times 100\% = 62.92 \pm 4.75\%$$

4.3. ADH activity in roots of barley and beaked sedge

4.3.1. Results

The concentration of oxygen in the nutrient solution was measured during the simulated flooding. The solubility of oxygen in water at normal atmospheric pressure at +20°C is 9.07 ppm. When the cultures were aerated, the nutrient solution was nearly saturated with oxygen, the concentration being 8.6±0.2 ppm. Passing nitrogen through the solution (50±5 $x10^{-6}$ m^3 min^{-1}) quickly reduced the oxygen concentration to a stable value of 1.2±0.2 ppm within two hours (Fig. 4.3).

The activity of ADH and K_M values were at first recorded for four days with aeration. Hypoxic conditions were then induced by bubbling with N_2 gas, and ADH measured after 24, 48 and 72 h of N_2 treatment. In addition, to observe recovery from hypoxic stress, ADH activity was also determined during the course of four days of aeration after the N_2 treatment had ceased. The results for barley are shown in figure 4.3 and for beaked sedge in figure 4.4. The K_M and V_{Max} values are presented in table 4.3. Some of the plants were allowed to grow on, and the effect on the growth of the roots observed visually. After nitrogen treatment the seminal roots had an unhealthy appearance and soon died. They were replaced by adventitious roots which grew quickly from the lower nodes of the

plants.

The pH of the nutrient solution was measured every day during the experiment. It decreased during the period of aeration from 6.0 to c. 4.5, increased slightly (0.1-0.5 pH units) during the hypoxic treatment, and decreased again during the four day recovery period.

The soluble protein content of the root extracts was very stable throughout all the experiments, decreasing slightly during the hypoxic period. The mean soluble protein content in extracts of barley seminal roots was 0.563±0.040 x103 g m^{-3}, and adventitious roots 0.445±0.025 $x10^3$ g m^{-3}. Total soluble protein concentration of the beaked sedge root extracts was 0.483±0.011 g $x10^3$ m^{-3}.

TABLE 4.3. K_M and V_{max} values for barley (Hordeum vulgare cv. Hankkija-673) and Beaked sedge (Carex rostrata) root ADH during aeration, N_2-bubbling and recovery periods. Values represent means and standard errors of six root extracts. The statistical significance of the values was tested with student's T-test against values from aerated cultures. N.S. = not significant, * = P 0.05, ** = P 0.01 and ** = P 0.001.

Barley seminal roots	K_M mol m-3	S.E.	V_{max} mol m-3 min-1	S.E.
Aerated culture	2.89	0.71	3.93	0.22
N_2-bubbled culture (24-72 h)	1.57 N.S.	0.26	9.76***	0.62
Recovery period (1-4 days)	2.38 N.S.	0.35	9.95*	2.01
Barley adventitious roots				
Aerated culture	4.29	0.54	4.21	0.39
N_2-bubbled culture (24-72 h)	0.76***	0.09	12.58***	0.66
Recovery period (1-4 days)	0.78***	0.03	11.22***	2.36
Beaked sedge adventitious roots				
Aerated culture	2.94	0.80	9.07	0.71
N_2-bubbled culture (24-72 h)	2.02 N.S.	1.01	21.80**	2.70
Recovery period (1 to 4 days)	1.28 N.S.	0.10	13.50*	1.31

FIGURE 4.3. Enzyme units (U) and specific activity (U/mg protein) of barley root ADH during aerobic and hypoxic conditions. (●) seminal roots: enzyme units. (○) seminal roots: specific activity. (■) adventitious roots: enzyme units. (□) adventitious roots: specific activity. Values represent means and standard errors of two root samples.

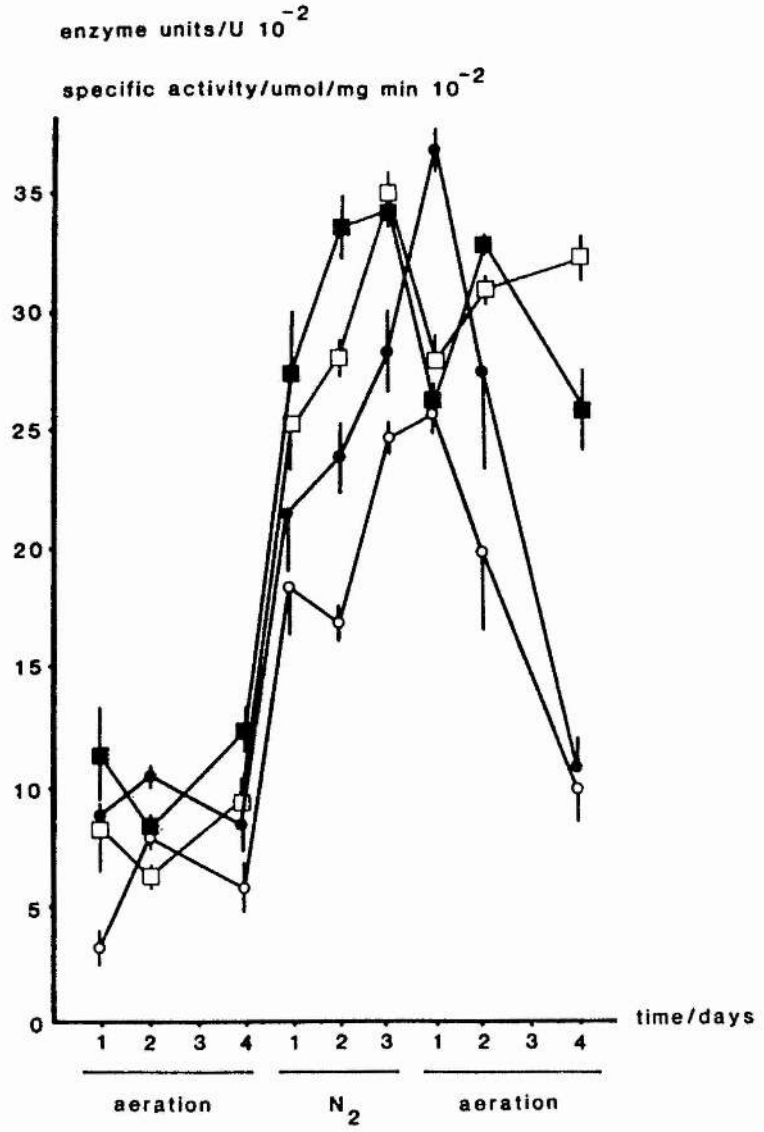
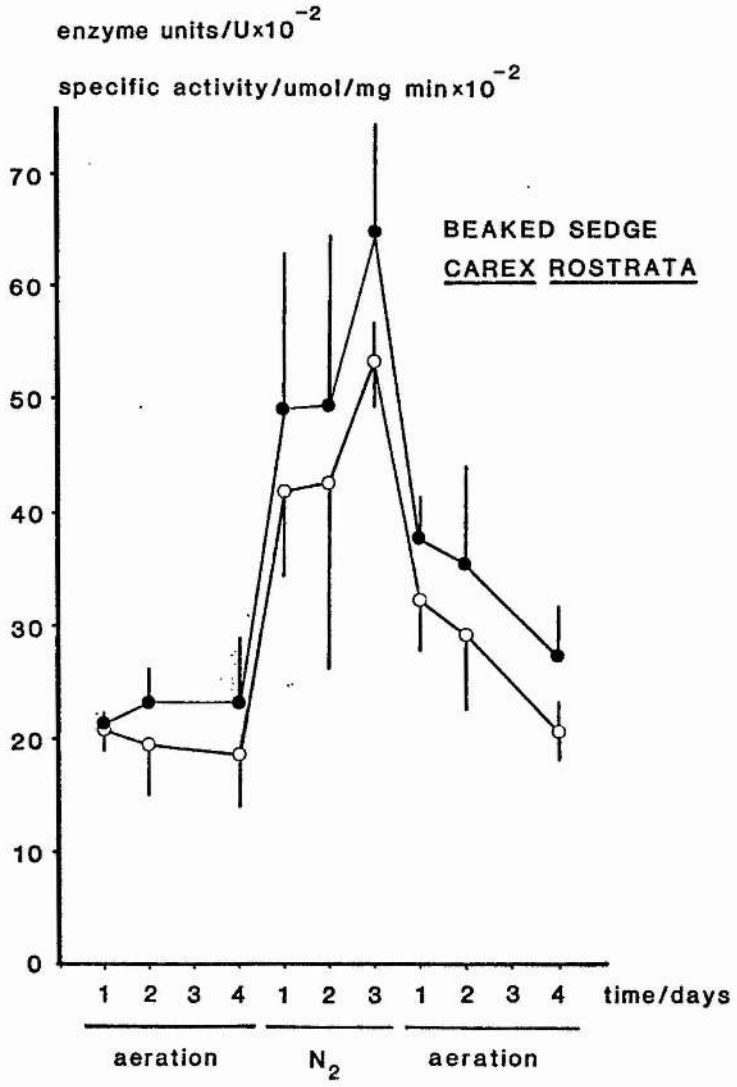


FIGURE 4.4. Enzyme units and specific activity of beaked sedge (Carex rostrata) root ADH under aerobic and hypoxic conditions. (●) Enzyme units. (O) Specific activity. Values represent means and standard errors of two root samples.



4.3.2. Discussion

Previous studies of ADH activity in barley root tissue have involved detailed investigations into the genetic background of barley ADH (Brown, 1980; Harberd and Edwards, 1982a,b, 1983; Hanson and Brown, 1984). However, little attention has been paid to the developmental stage of the plants. Most of the work has involved seedlings or germinating seeds rather than mature plants. This study shows the differences in ADH activity between seminal and adventitious roots as well as the changes in the kinetic properties of ADH in roots subjected to hypoxia. As a flood-tolerant comparison, the same experiment was carried out with beaked sedge, Carex rostrata.

In some preliminary experiments it was noticed that barley plants of different age showed differences in ADH activities. This prompted more precise experiments, the results of which are presented here. Young barley plants, which had mainly seminal roots, did not develop quite as high ADH activities as older plants with well-developed adventitious root systems. During the four day period of recovery, ADH activity in the adventitious roots did not fall back to control levels, but did so in the seminal roots. In addition, the K_M values of ADH extracted from these roots showed a different pattern. In adventitious roots the apparent K_M dropped at the

same time as the ADH activity increased four-fold, whereas in seminal roots the K_M for acetaldehyde did not change significantly during the hypoxic treatment even though the ADH activity increased considerably. Interestingly, very similar changes in K_M have been measured in maize ADH isozymes (Scandalios, 1977).

Carex rostrata root ADH activity developed similarly to barley seminal root ADH, except that the level of ADH activity was constantly c. twice as high in the beaked sedge as in barley root tissue. However, the roots of beaked sedge, which contain large amounts of aerenchyma, did not seem to suffer from the hypoxic period, and continued to grow vigorously during and after the imposed oxygen stress. However, even in the roots of this highly flood tolerant species, ADH activity was induced indicating some degree of oxygen stress being experienced by the root tissue. Similar results with flood tolerant plant species have been recorded by Smith et al. (1986). They measured higher ADH activitities in roots of Filipendula ulmaria the deeper in hypoxic soil they were growing. These results do not support the old theory of flooding tolerance by McManmon and Crawford (1971) and since then many articles have been written against that theory (Keeley, 1978; Smith and ap Rees, 1979a, b; Smith et al., 1984, 1986; Jenkin and ap Rees, 1983, 1986).

Changes in the ADH isozyme content of barley roots in hypoxic treatments have been observed in some earlier genetic studies. It seems that in aerobic conditions ADH activity is associated with Adh1

and Adh2 genes. In anoxia this activity is strengthened, but other isozymes also appear (Hanson and Brown, 1984), and the isozyme content changes (Mayne and Lea, 1984). Recent work suggests that differences in the isozyme complement are metabolically significant. Mayne and Lea (1984) have reported large changes in the K_M values for NADH of ADH isozymes isolated from barley leaf tissue, which means that the induced isozymes have a higher affinity for NADH than the constitutive isozymes. Working with only 8-day old seedlings they failed to show any differences in the K_M values of ADH for acetaldehyde, a result which is similar to our data with seminal roots. Interestingly, adventitious roots in our series of experiments showed a totally different pattern. In these mature roots the K_M of ADH for acetaldehyde decreased considerably, indicating some new changes in the isozyme content of the root tissue. Similar changes in K_M in several flood intolerant species have been noticed earlier by McManmon and Crawford (1971).

A model for the possible reactions taking place during short term oxygen deficiency in root tissue is shown in figure 4.4. It is based on the suggestion by Hanson et al. (1984), which states that as lactate dehydrogenase (LDH) activity rises during oxygen deficit, lactate glycolysis begins to compete with ethanol glycolysis for pyruvate (Davies, 1980) and NADH. Thus, the higher affinities for NADH and acetaldehyde in the induced ADH isozymes could increase the ability of ethanol glycolysis to compete with lactate glycolysis, which would otherwise lead to the acidification of the cytoplasm

and, in anoxia intolerant plants, to rapid cell death (Roberts et al., 1984a,b, 1985). In maize mutants that lack ADH, cytoplasmic acidification continues and finally leads to cell death (Roberts et al., 1985). This phenomenon is also of interest, since ADH works much more effectively in the direction acetaldehyde to ethanol in acidic conditions than in the reverse reaction (Felder et al., 1973). Also, pyruvate decarboxylase is activated by low pH (Davies et al., 1974a), which would increase the total production of ethanol, provided that ADH is able to compete with LDH of NADH. However, if we consider the overall rate of glycolysis and production of ethanol, we must remember that the major control points are at the reactions catalysed by phosphofructokinase (PFK) and pyruvate decarboxylase (PDC) (John and Greenway, 1976) (see chapter 5 for more discussion on glycolysis and production of ethanol).

In this work, during the recovery period, the ADH activity in the root tissues did not return to the control level in adventitious roots. This was probably due to substances passing from the roots into the medium. Acetaldehyde has, for instance, been shown to induce ADH activity in root tissues (Crawford and McManmon, 1968). Also, if ADH activity disappeared only due to a daily turnover of 10% (Hanson et al., 1984) there should still be about 66% left after a four day recovery period.

After the hypoxic period the seminal roots developed an unhealthy

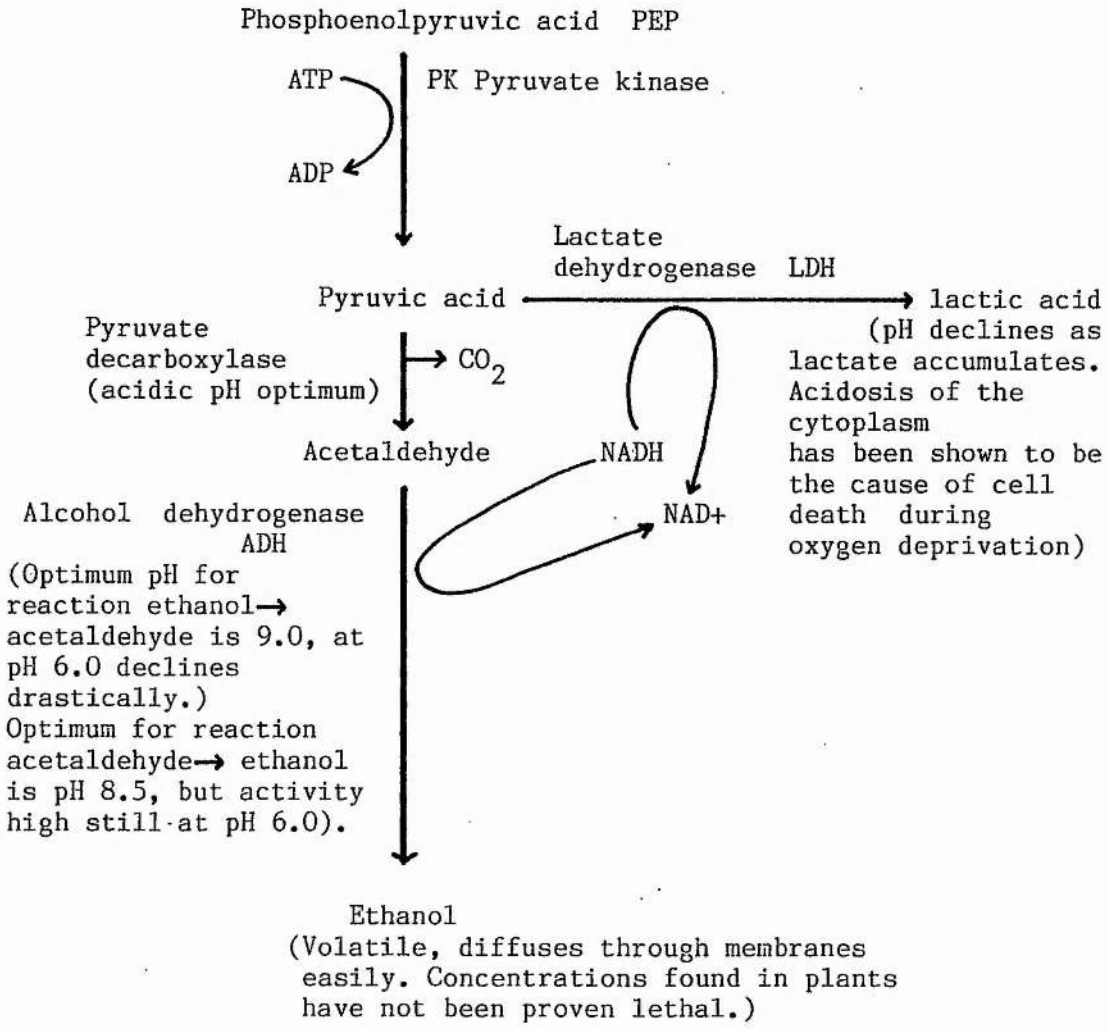
appearance and died, which prompted the emergence of new adventitious roots from the stem bases. Similar symptoms have previously been observed in wheat seedlings suffering from hypoxic conditions by Trought and Drew (1980a). They stated that further growth of seminal root tissue was prevented by waterlogging for two days and this was followed by a breakdown of tissue. However, adventitious roots grew into the anaerobic soil to a maximum depth of 20 cm by the 15th day of waterlogging. The symptoms and damage to shoots and roots were attributed to the fall in soil oxygen concentration, rather than to any decrease in concentration of inorganic nutrients in the soil water, or to the accumulation of CO_2, ethylene or nitrous oxide to toxic concentrations. Several other researchers have noticed the accelerated growth of adventitious roots in cereals under waterlogging. Arikado (1955a,b) has noticed similar production of adventitious roots in barley and clover, Karishnev (1958) in wheat, Kar et al. (1974) in rice and Jackson et al. (1981) and Jat et al. (1975) in maize. In very flood tolerant species, such as Ranunculus scleranthus, R.abortivus and Cyperus alternifolius the original roots survive waterlogging and no adventitious roots emerge (Bergman, 1920).

It is suggested that the changes in the activity and kinetics of barley root ADH are an adaptation to short-term oxygen deficiency. The increase in ADH activity during oxygen deprivation reflects the overall increase in the rate of glycolysis to produce energy for the demands of metabolism while citric acid cycle is blocked. Why the

induction of ADH activity is so great while ADH is not even any of the "bottlenecks" of glycolysis, is not yet known (see chapter 4.5 for more discussion). However, the activation of pyruvate decarboxylase by low pH (caused by lactate) and the increase in ADH activity as well as the greater affinity to its substrates (lower K_M) may retard cytoplasmic acidification by producing ethanol, which is volatile, passes through membranes easily and has previously been shown not to be lethal in short-term hypoxia (Jackson et al., 1982; Barta, 1984; Alpi et al., 1985). Ethanol may, however, have an effect in bulky rhizomes, as opposed to the thin roots of barley, which are deprived of oxygen for longer periods (Monk et al., 1984). There still exists considerable controversy about the toxicity of ethanol in plant cells under hypoxic or anoxic conditions. Recently, Perata et al. (1986) published an article on ethanol toxicity, which explored the effect of ethanol on Helianthus tuberosus discs, protoplasts of Daucus carota and barley aleurone layers. The growth and differentiation of the cell cultures were significantly affected by the added ethanol and the production of α-amylase by the aleurone layers was strongly inhibited. The ethanol concentrations used were of the same order of magnitude as found in plant tissues under oxygen stress (e.g. Avadhani et al., 1978; Crawford, 1978; Alpi and Beevers, 1983). These results indicate that ethanol is harmful for the cells in physiological concentrations, but still it is to be doubted whether ethanol toxicity is the foremost factor in tissue death under hypoxic or anoxic conditions (see also chapter 5 for discussion of the role of ethanol in flooding

tolerance).

FIGURE 4.5. A composite model for the significance of ADH and LDH in the metabolism of barley root tissue during short-term oxygen deficiency. Based on Felder et al. (1973), Jackson et al. (1982), Hanson et al. (1984) and Roberts et al. (1984a,b and 1985).



4.4. Isozyme composition of barley and beaked sedge ADH

As already mentioned in chapter 4.3 on ADH activity and its significance in flooding tolerance and survival from oxygen stress, the isozyme content responds to flooding and may therefore have a significant role in the survival of plants in a waterlogged environment. In maize this has been convincingly shown with studies on mutant individuals lacking one of the alleles to produce an active ADH isozyme (Schwartz, 1969). This present study was conducted to observe whether there are any differences in the isozymes between the three barley cultivars, and also to examine the beaked sedge ADH isozyme system which has not been studied before.

4.4.1. Electrophoresis

The barley seedlings were grown in Ruakura nutrient solution (see chapter 4.1) in conical flasks under sterile conditions. The seeds were sterilized with 6% hypochlorite (see chapter 4.1) and planted aseptically in a laminar flow cabin in 0.1 $x10^{-3}$ m^3 flasks in 25 $x10^{-6}$ m^3 of Ruakura solution autoclaved earlier. Sterility was essential, since frequent infections by fungi

of unsterile growth media took place in earlier experiments and brought about fungal ADH bands in electrophoresis gels. The flasks were aerated by inserting autoclaved Pasteur pipettes filled with cotton wool through the cotton wool and aluminium foil cap of each flask. The pipettes were connected to an aquarium pump (Fig. 4.6). The seedlings were allowed to grow for 10 days at +20°C in 16 H daylight regime in a growth cabin before experimentation. Hypoxic conditions were brought about by bubbling nitrogen gas through the medium for three days to induce ADH activity before electrophoresis.

Beaked sedge was grown in the glasshouse in coarse sand under flooding for a year before root tips were used for electrophoresis. Due to the sand and flooding no fungal contamination interfered with the ADH electrophoresis unlike in the experiment with barley seedlings, which had to be grown under sterile conditions.

The native polyacrylamide gel electrophoresis was done with a GE-2/4 LS apparatus (Pharmacia Fine Chemicals). The gel consisted of 12% acrylamide, 0.16% bisacrylamide, 0.44 $x10^{3}$ mol m^{-3} Tris and 12% glycerol. pH of the gel was 8.8. The stacking gel contained 4% acrylamide, 0.053% bisacrylamide, 0.06 $x10^{3}$ mol m^{-3} and 12% glycerol. Polymerisation was started by adding 0.001% TEMED and 0.01% of 10% fresh ammonium persulphate solution. The electrophoresis buffer contained 25 mol m^{-3} Tris, 192 mol m^{-3} glycine, pH 8.3. The gels were run overnight (c. 20 h) at +5°C in the dark at 14

mA constant current.

The root extracts (c. 0.400 g root tissue in 1.00 $x10^{-9}$ m^3 buffer) were prepared with a pestle, mortar and a little quartz sand in a bed of ice. For sedge roots 20% insoluble PVP was used to counteract the effect of phenols. The extracts were centrifuged at 4000 g at +5°C before injecting into the wells. The buffer contained 0.1 mol $x10^3$ m^{-3} Tris, 5 mol m^{-3} dithiotreitol and 12% glycerol. 20 $x10^{-9}$ m^3 of each extract were injected into the wells in the stacking gel.

The gels were stained with an ADH specific stain which consisted of 0.1 $x10^{-3}$ m^3 0.1 mol $x10^3$ m^{-3} Tris, 8 $x10^{-6}$ m^3 ethanol, 30 $x10^{-3}$ g NAD, 20 $x10^{-3}$ g nitroblue tetrazolium and 5 $x10^{-3}$ g phenazine methosulphate (PMS) (Brown et al., 1978). The reaction which takes place in the presence of active ADH is described in figure 4.7 (Geyer, 1973). To ascertain that the bands in the gels were truly ADH, some gels were stained for peroxidase and superoxide dismutase, since these enzymes may affect nitroblue tetrazolium staining. However, no interference was observed. A typical gel of barley and beaked sedge ADH induced by hypoxia is shown in figure 4.8.

FIGURE 4.6. A photograph of the set-up for sterile aerated culture of barley seedlings. The 0.1 $x10^{-3}$ m^3 conical flasks contained 20 $x10^{-6}$ m^3 Ruakura nutrient solution and 10 seedlings each. The rubber tubing and pasteur pipettes provided aeration as well as an entry for N_2 gas to create anaerobiosis for induction of ADH activity.

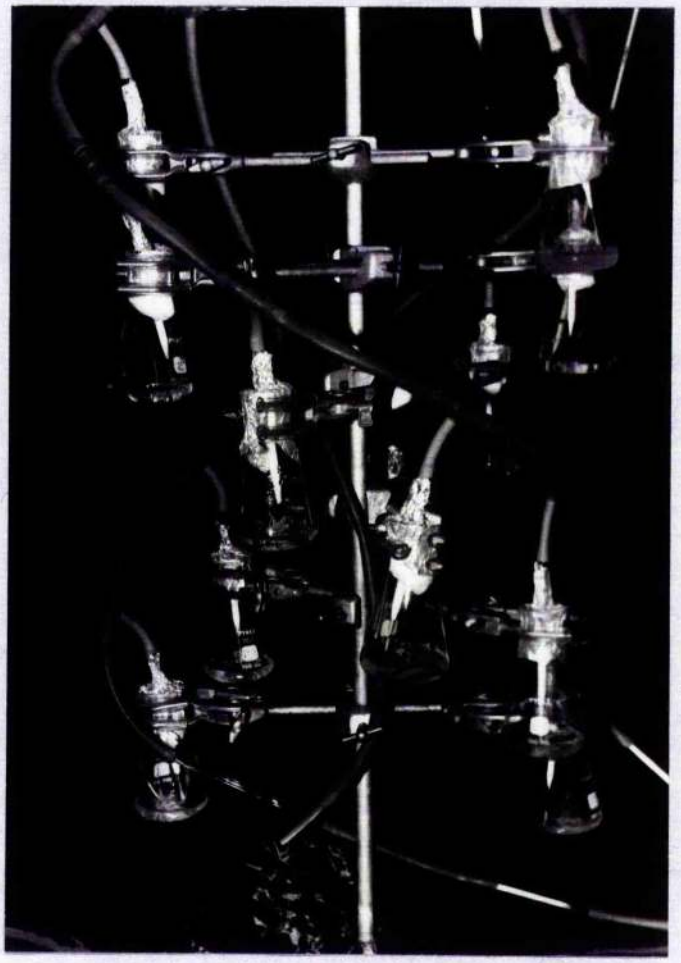


FIGURE 4.7. The reaction catalysed by ADH which was used to stain the PAGE gels (Geyer, 1973).

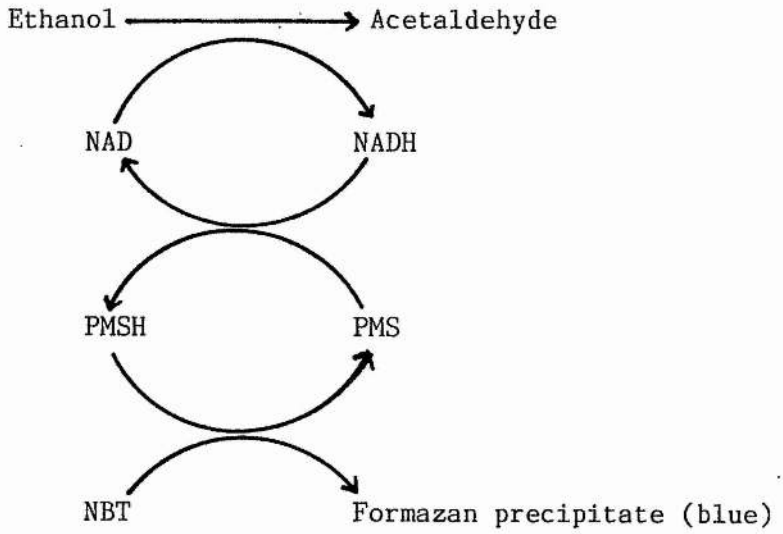
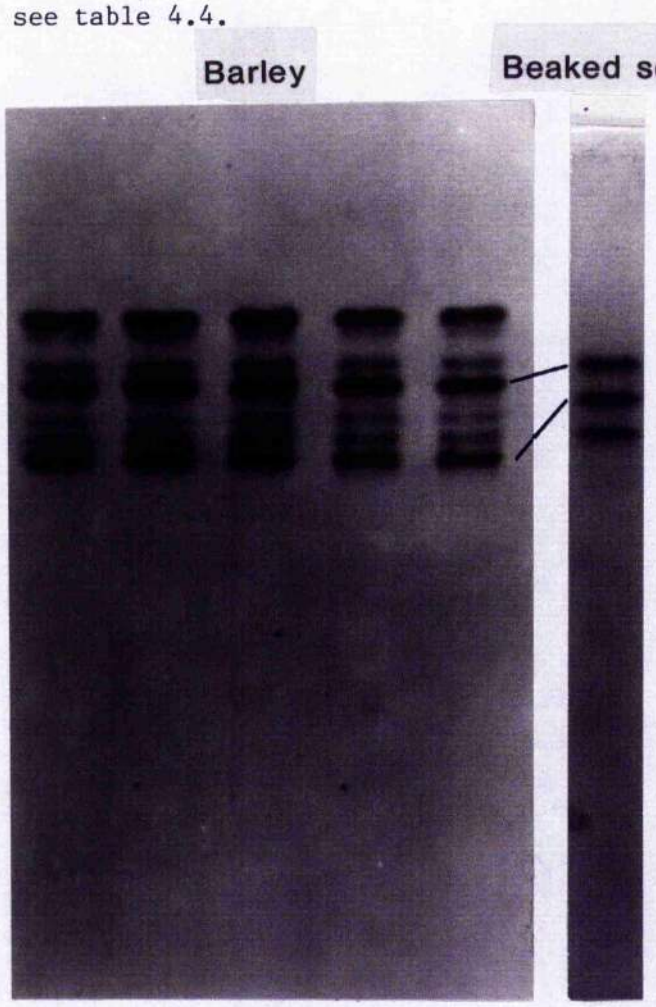


FIGURE 4.8. A typical PAGE gel of barley and beaked sedge ADH. For explanation see table 4.4.



4.4.2. The importance of ADH isozymes in flooding tolerance

The physiological adaptation of plants to flooding has long been known to be connected to their genetic information, and especially to the polymorphism of alcohol dehydrogenase. First Schwartz (1969) noticed this in maize seeds and in their ability to germinate if immersed in water. Maize ADH is a dimeric enzyme consisting of Adh_1^F (fast) and Adh_1^S (slow) alleles. Mutants lacking ADH tolerated much less flooding than normal plants with active ADH. Later, differences between Adh_1^S and Adh_1^F homozygotes have been observed in relation to flooding tolerance (Marshall *et al.*, 1973). The enzyme coded by Adh_1^F has a higher specific activity and is more heat stable than Adh_1^S, and hence plants carrying this fast dimer are more tolerant of inundation than plants with the slower dimer (Marshall *et al.*, 1973). A very similar phenomenon is known to take place in *Bromus mollis* (Brown *et al.*, 1974) and in *Trifolium subterraneum* (Francis *et al.*, 1974). However, in all the studied cases ADH has been found to be induced by hypoxic or anaerobic conditions and is definitely an anaerobic polypeptide (ANP) (Sachs *et al.*, 1980; Sachs and Ho, 1986); one of the 20 proteins synthesized in maize during anaerobic conditions.

Barley ADH is a polymorphic enzyme coded by three loci, which have been named Adh1, Adh2 and Adh3 (Table 4.4.) (Hanson et al., 1984). Barley ADH as well as the ADH of many other plants, is a dimeric enzyme (Fisher and Schwartz, 1973; Hart, 1971, Banuett-Bourrillon and Hague, 1979; Harberd and Edwards, 1982a). It has been noticed that mutants lacking ADH1 have only 55% of the total root ADH activity of a normal Adh1+/Adh1+ specimen. Also, the mutants tolerate much less flooding than the normal plants. The barley cultivars Kustaa, Hankkija-673 and Pokko all showed similar isozyme composition of ADH. No mutations were apparent (Fig. 4.8).

In contrast, beaked sedge ADH showed a different pattern rather similar to the one described for maize ADH. The gels produced three bands (flooded plants) but since the plants did not produce viable seed, further study of the isozymes by cross-breeding was impossible. (Stratification and gibberellic acid treatment were tried, but still the seeds did not germinate).

Recently, some light has been thrown on the background and reasons for induction of ADH activity during oxygen stress. The new methods in present day genetics have revealed the location of ADH gene in maize and even the promoter sequence is now known (Ellis et al., 1987, Ferl et al., 1987). ADH gene expression is controlled primarily at the level of transcription, and, furthermore, the promoter of maize ADH works (under anaerobiosis) even if transferred to tobacco tissue, provided that some upstream elements

from constitutive genes are added at the same time (Ellis et al., 1987). However, we still do not know what is the final stimulus that opens the promoter site and begins the production of new ADH m-RNA.

TABLE 4.4. ADH isozyme composition in barley (Hordeum vulgare L. cv. Hankkija-673, Pokko and Kustaa) and Carex rostrata as seen in a polyacrylamide gel after electrophoresis (arrow) and staining for ADH activity.

Bands in gel of barley root extract	Corresponding barley ADH isozyme	Bands in gel of beaked sedge root extract
-		-
-------	ADH1.ADH1	
-------	ADH1.ADH3	
-------	ADH1.ADH2	-------
-------	ADH3.ADH3	
-------	ADH2.ADH3	
-------	ADH2.ADH2	-------

+		+

4.5. Induction of ADH activity in plant tissues under oxygen deprivation: why?

ADH activity and flooding tolerance have been vigorously investigated during the past 20 years. As early as in 1960 Hageman and Flesher and later Crawford (1967) observed induction of ADH activity in flooded roots of some higher plants and since then several research papers on this matter have been published. At first induction of ADH activity was noticed in flood intolerant plants (Crawford, 1967, 1969), but as biochemical methods improved, anaerobic induction of ADH activity was observed in nearly all plant species studied (Smith and ap Rees, 1979a,b). Not only flooding, but also certain substances like acetaldehyde have been noticed to induce ADH activity in plant root tissue (Crawford and McManmon, 1968). Recently, it has been noted, however, that the amount of ADH produced under flooding stress exceeds the amount needed for alcoholic fermentation and no correlation was found between activity of ADH and rate of production of ethanol under anoxia in Urtica roots, even though they differed from the control treatment by up to 26-fold in their ADH activity (Smith et al., 1986).

ADH is not the only protein synthesized under oxygen deprivation, there are others, but the total number of these ANP's is only 20

(in maize, Sachs et al., 1980). Until now only 5 of these have been identified in maize and they include ADH (Sachs and Freeling, 1978; Laszlo and St Lawrence, 1983; Hake et al., 1985), pyruvate decarboxylase (PDC) (Wignarajah and Greenway, 1976; Laszlo and St Lawrence, 1983), glucose phosphate isomerase (Kelley and Freeling, 1984), sucrose synthase and aldolase (Kelley and Tolan, 1986). All these belong to glucose metabolism and their induction under anaerobiosis is most probably an adaptive feature of the metabolism evolved to ensure sufficient production of energy under oxygen deficiency.

The amount of induced ADH seems to be even more perplexing if one considers the control of glycolysis. The control points are at the reactions catalysed by hexokinase, phosphofructokinase, pyruvate kinase and pyruvate decarboxylase, of which phosphofructokinase is the most important. It is inhibited by ATP and this inhibition is reversed by AMP; in other words, the activity of the enzyme is stimulated when the energy charge of the cell is low. Also pyruvate kinase is inhibited by ATP. In general, the control of glycolysis is a property of the whole system. However, during oxygen deprivation the energy charge of the cell is lowered and hence the rate of glycolysis increases (Pasteur effect). In situations such as this more enzyme activity is needed, especially at the control points in glycolysis. Therefore, it is not easy to understand why the activity of ADH increases several fold during oxygen deprivation. Would it not be selected against during evolution, if production of ADH were not of

any adaptive value to the plant under flooded conditions? Surely these plants, whether or not they are adapted to flooding, would be better off if they did not produce useless amounts of ADH under a severe stress. The fact that a balanced polymorphism of ADH has been described in some plant species (Brown et al.,1974, 1976; Dolferus and Jacobs, 1984; Torres and Diedenhofen, 1979, 1981) supports a theory that induction of ADH is of adaptive value to the plants in suboptimal conditions. In addition, studies on the kinetic properties of ADH isozymes in plants under flood (Mayne and Lea, 1984; Scandalios, 1977) give some evidence of the importance of ADH in flooded conditions (see chapter 4.3.2 for further discussion of the importance of ADH in the production of ethanol vs. lactate).

Much more accurate work is still needed to reveal the background for the induction of ADH isozymes. Plant molecular biology will eventually provide us with the information after all the technical difficulties have been overcome.

5. PRODUCTION OF ETHANOL, CO_2 AND ORGANIC ACIDS IN BARLEY UNDER ANOXIA

Under aerobic conditions the oxidation of 1 mol of glucose to CO_2 and water produces 38 mol of ATP. In waterlogged conditions under total lack of oxygen, when oxidative phosphorylation has been blocked, only two mol of ATP is produced from each mol of glucose by alcoholic fermentation. Furthermore, coenzymes such as NAD and FAD are no more regenerated by the electron transport system. However, glycolysis can continue provided that there is a way to oxidize NADH, which has been generated in the dehydrogenation of phosphoglyceraldehyde to 1,3-diphosphoglycerate. This can be provided by ethanolic or lactate fermentation (Fig. 5.1).

Since fermentation seems to play the major role in the production of energy in anoxia-intolerant species such as barley seedlings under oxygen stress, some experiments were carried out to clarify whether there are any differences in the three barley cultivars in their production of ethanol, which may have affected their tolerance of anoxia and flooding (see chapter 3). ADH activity has earlier been connected with ethanol production and ethanol toxicity. However, it has been noted that ADH activity in barley roots is so variable that any differences between the cultivars would disappear within the variation (Fagerstedt, 1984). Other researchers have pointed out

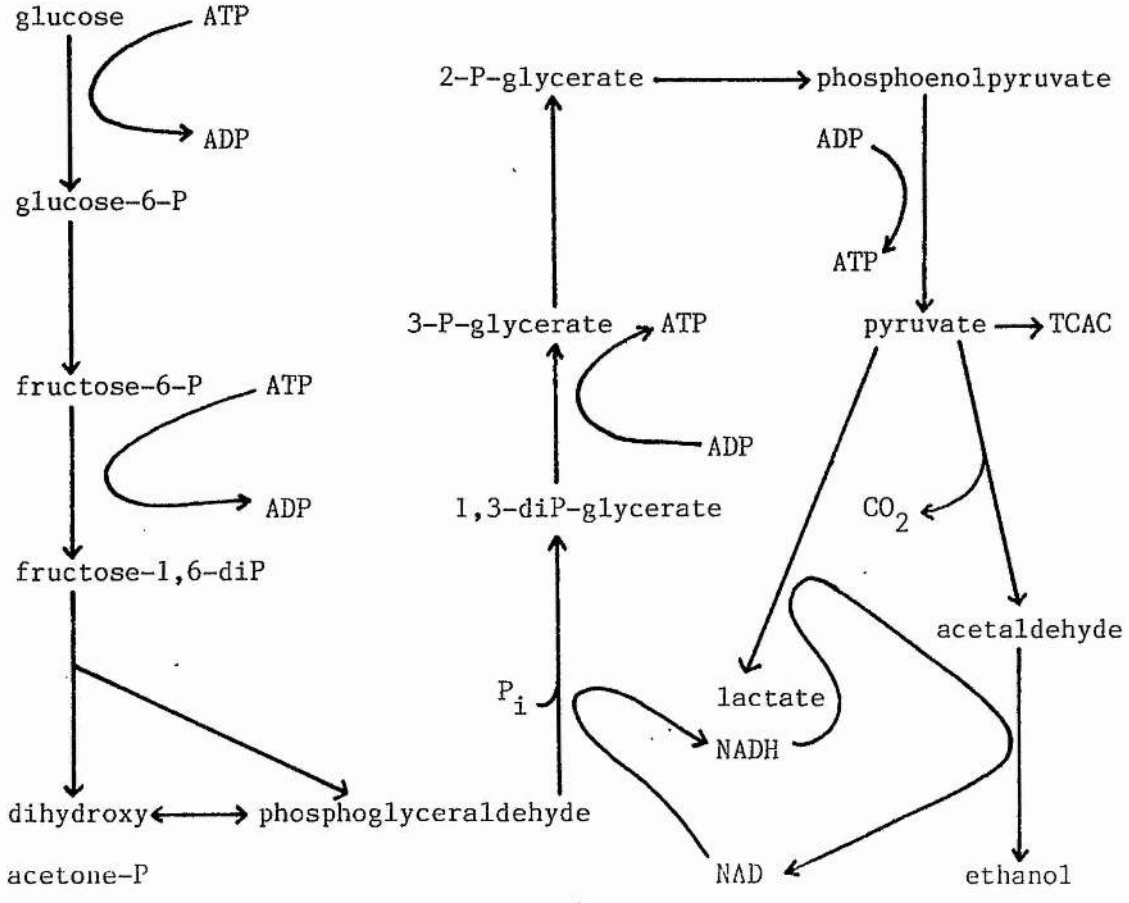
similar large variation in the ADH response in plant roots (Jenkin and ap Rees, 1983), and they attributed this to the extreme sensitivity of their plant to flooding (Pisum sativum). However, it has been stated further, that in flooded roots there is a much larger amount of ADH than is needed for fermentation (Smith et al., 1986). Consequently, no correlation can be expected to be found in the amount of ADH and ethanol production, and ADH and flooding tolerance in these plants. In maize it has been shown, though, that a certain amount of activity or certain isozymes are needed for better tolerance of flooding (Schwartz, 1969). But it is obvious that minor differences in the level of ADH activity cannot account for variability in tolerance of oxygen stress, but a change in the isozyme content may have an effect on flooding tolerance (see chapter 4).

A number of organic acids have been proposed as being possible end products of glycolysis under oxygen deprivation. Therefore, a High Performance Liquid Chromatography -study (HPLC) was carried out to examine any changes in the organic acid content of the barley seedlings during anoxic stress.

The environment for this experiment was planned to be similar to the barley anoxia tolerance experiment (see chapter 3), to allow comparisons between these two investigations. Hence the test was carried out in the dark at +5°C (conditions similar to the flooded field in the spring when the seeds are germinating). After a

two week anoxic period fresh and dry weight of the seedlings were measured as well as the concentration of ethanol, CO_2 and organic acids. Furthermore, to observe possible differences in the rate of aerobic metabolism in the barley seedlings, the respiration of the seedlings of these cultivars was estimated at +5°C in the dark before any anoxic experiments.

FIGURE 5.1. The glycolytic pathway for oxidation of glucose and production of ethanol and lactate by fermentation. Note regeneration of NAD and production of ATP in the various steps.



5.1. Methods and Results

Before the anaerobic incubation begun, respiration (production of CO_2) of seedlings of the three barley cultivars were recorded with an infra-red CO_2 analyser ADC-225-MK3, after the plants had been kept in +5°C waterbath in open bottles on moist filter paper for two hours to allow the respiration rates to stabilize in this temperature. Production of carbon dioxide was then measured by connecting the bottle to the closed loop of the infra-red gas analyser (Table 5.1). No statistically significant differences were recorded between the cultivars. Atmospheric pressure was 763.76 mmHg and the analyser was calibrated with known amounts of CO_2. The respiration of the cultivars was measured to observe whether there are any differences in their respiration rates (and hence in metabolic rate) which could have affected their production of CO_2 and ethanol during the anaerobic incubation.

The following experimental was used for estimations of CO_2 and ethanol production under anoxic conditions. After germination at +20°C for three days the seedlings were placed in McCartney flasks ten seedlings in each, and the atmosphere in the flasks changed to 90% N_2 and 10% H_2 in the anaerobic workbench with three

evacuations in the airlock under nitrogen. An oxygen indicator was placed in one flask to test the tightness of the seal in the stopper. One control flask had only the filter paper and distilled water for measurements of the concentration of ethanol and CO_2 present in the anaerobic workbench at the beginning of the experiment. The flasks were allowed to stand in the workbench for half an hour to eliminate all remaining oxygen in the flasks. They were then stoppered and placed in a waterbath at +5°C in the dark for two weeks. The fresh weight of the ten seedlings in the flasks was measured before the atmosphere was changed and the dry weight of three baches of ten seedlings of each cultivar were determined. It was also noticed that the volume of the McCartney flasks varied a little ($28.67 \pm 0.14 \times 10^{-6}$ m^3), so their volume was measured and each bottle marked for calculations of CO_2 and ethanol content of the the gas space. After the anaerobic incubation the fresh and dry weight of the seedlings were measured as well as the concentration of ethanol in the tissue and ethanol and CO_2 in the gas space. Also, and HPLC-study of the organic acid content of the seedlings was conducted.

For estimations of ethanol content the seedlings were dropped in cold 6% (w/v) perchloric acid and homogenized in a cold room (+5°C) with an Ultra-Turrax apparatus, centrifuged, and neutralised with 79% K_2CO_3. To remove the precipitated salt the samples were centrifuged again at 8730 g prior to injection to the gas-liquid chromatographer (PYE Series 104). Gas samples from the McCartney

flasks were injected to the GLC with pre-heated syringes to avoid any condensation inside the syringe. The column was 1.4 m long and 7.0 $x10^{-3}$ m in diameter packed with 100 mesh Porapak Q (oven temperature 140°C), attached to a hydrogen flame detector the carrier gas being nitrogen (flow rate 40 $x10^{-6}$ m^3/min). Estimates of ethanol concentration were based on peak area as measured by a Hewlett Packard integrator 3390A. A standard curve was obtained with progressive dilutions of analytical ethanol preparations (Sigma). The ethanol peak was identified by retention time. Results of ethanol and CO_2 measurements and dry weight of the material are presented in table 5.2.

The same samples from which ethanol was measured, were used for organic acid estimations. The HPLC-method was modified from Ashoor and Knox (1984) and Bushway et al., (1984); especially the sample preparation had to be changed to suit the plant material. Samples of 20 $x10^{-9}$ m^3 were injected to an LKB High Performance Liquid Chromatograph with an Aminex HPX-87H column (ambient temperature) especially designed for organic acids and equipped with an UV-detector at 254 nm wavelength. The column included a Mirco-Guard ion-exclusion cartridge which filtrated all positively charged material from the samples including amino acids. The mobile phase was 0.01 N H_2SO_4 and the flow rate 0.60 $x10^{-6}$ m^3/min. Organic acid standards (Table 5.3) were created by dissolving analytical grade compounds in the mobile phase in several concentrations and running them through the column.

Peaks in the barley samples were identified by retention time. Only one of the peaks in the barley material could be positively identified with standard compounds, namely lactate, shikimate or succinate, which all had very similar retention times, however, in anaerobic barley material one would expect to find lactate. In aerobic samples this peak could not be detected and even in anoxic seedlings the amount was so small that accurate estimation was impossible with this method. Only in some Hankkija-673 samples there was enough lactate (or shikimate or succinate) for it to be detected (119 $x10^{-6}$ g/g fresh weight). This figure is too large which is possibly due to an overestimation of the peak area by the integrator since another peak was overlapping with the lactate/shikimate/succinate peak. In other cultivars a slight alteration in the basic line of the recorder was visible at the retention time for lactate, but the amount was too small for the integrator to distinguish from the background. Also, malate standards were run with the HPLC but no peak was detected in the samples at this retention time either in aerobic or in anaerobic barley material. All the standard compounds and the major peaks in the barley samples and their retention times are presented in table 5.3 and a typical chromatogram of a barley sample and a standard run are shown in figure 5.2. Also all additives in the plant samples (EDTA, PVP, K_2CO_3, perchloric acid and methyl orange indicator) were run individually through the column to observe any possible interference with standard peaks. Methyl orange and K_2CO_3 did not produce a peak (absorbed by the guard column) whereas EDTA, PVP and perchloric

acid eluted with the void volume of the column thus interfering with some acids such as phosphoenolpyruvate and oxalate, which eluted very near the void volume.

The concentration of carbon dioxide at the end of anoxic incubation was estimated with an infra-red gas analyser as stated above by injecting samples from the anoxic McCartney flasks to the closed loop of the analyser. The apparatus was calibrated with commercial 5% carbon dioxide (Table 5.2).

TABLE 5.1. Respiration (production of CO_2) of barley seedlings Hordeum vulgare L. cv. Kustaa, Hankkija-673 and Pokko under aerobic conditions at +5°C in the dark. Figures are means and standard errors of five separate samples of 10 barley seedlings. The differences are statistically not significant.

Cultivar	Production of CO_2 $x10^{-9}$ m^3 g^{-1} min^{-1} x±S.E.
Kustaa	1.32±0.06
Hankkija-673	1.23±0.06
Pokko	1.40±0.06

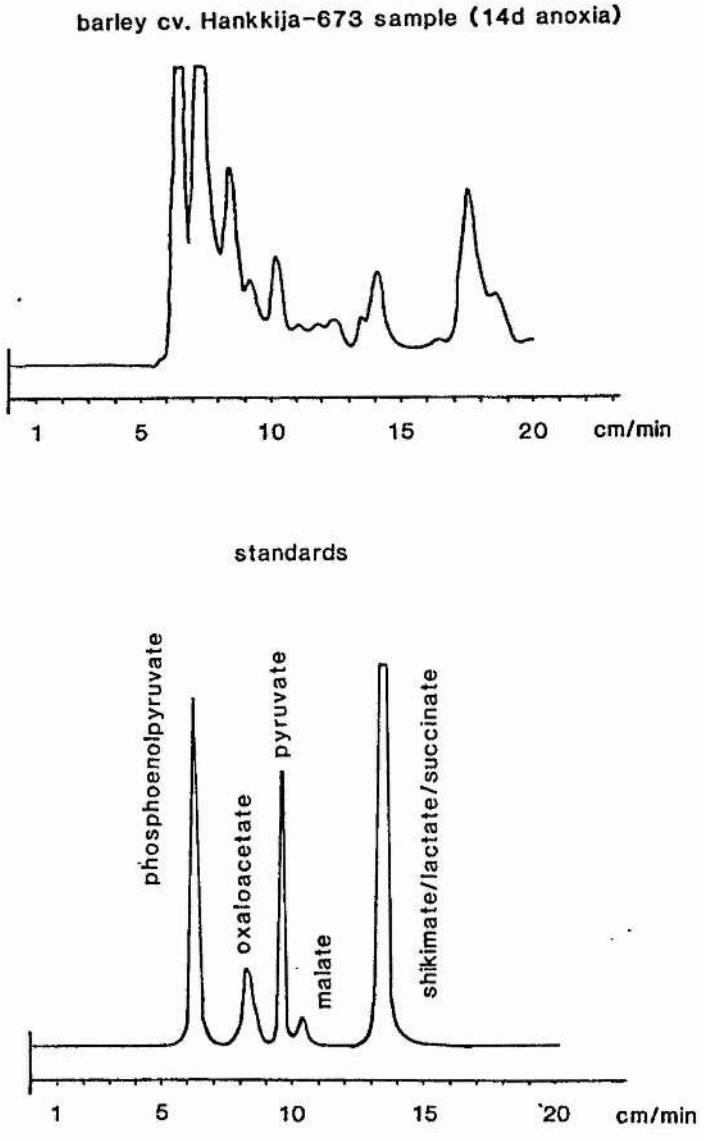
TABLE 5.2. Amount of ethanol and CO_2 produced by three barley cultivars (Hordeum vulgare L. cv. Kustaa, Hankkija-673 and Pokko) during a 14 day anaerobic incubation at +5°C in the dark. The figures are given as g/g fresh weight and as mol/g fresh weight to enable comparisons between production of CO_2 and ethanol and the loss of dry weight during the incubation. Student's T-test was used to compare the differences between cultivars. N.S. = not significant, * = p 0.05, ** = p 0.01, and *** = P 0.001.

Cultivar	Kustaa		Hankkija-673		Pokko
Ethanol in tissue g/g		←	*	→	
$*10^{-3}$	2.565±0.130	← * →	3.051±0.037	← N.S. →	3.023±0.073
mol/g $*10^{-5}$	5.568±0.282		6.623±0.080		6.562±0.158
Ethanol in gas space		←	N.S.	→	
g/g $*10^{-6}$	5.148±0.255	← ** →	7.121±0.036	← ** →	5.515±0.318
mol/g $*10^{-7}$	1.117±0.055		1.546±0.008		1.197±0.069
CO_2 in gas space		←	*	→	
g/g $*10^{-3}$	8.063±0.173	← ** →	9.368±0.058	← *** →	8.628±0.053
mol/g $*10^{-4}$	1.833±0.039		2.129±0.013		1.961±0.012
Ratio of ethanol and CO_2 production		←	N.S.	→	
EtOH:CO_2	0.304	← N.S. →	0.312	← N.S. →	0.335
Dry weight lost during incubation		←	*	→	
g/g fresh weight	0.050±0.004	← N.S. →	0.052±0.008	← N.S. →	0.070±0.003
Percentage of dry weight lost accountable by ethanol and CO_2		←	N.S.	→	
	21.7±2.2%	← N.S. →	25.2±3.5%	← N.S. →	16.7±0.7%

TABLE 5.3. The standard compounds and their retention times (RT) in the HPLC-runs with Aminex HPX-87H column. On the right the retention times of the major peaks in the barley samples. Underlining shows the corresponding peaks in samples and standards. (Eluent 0.01 N H_2SO_4, ambient temperature, flow 0.6 ml min^{-1}, chart speed 0.5 cm min^{-1}.)

Standard compound	RT min	Peaks in barley samples (RT) min
Phosphoenolpyruvate	6.40	8.50
Oxalate	7.06	9.15
Oxaloacetate	8.34	10.20
Citrate	8.64	11.15
-ketoglutarate	9.03	12.45
pyruvate	9.79	<u>13.48</u>
malate	10.43	14.05
lactate	<u>13.31</u>	17.18
Succinate	<u>13.45</u>	18.48
shikimate	<u>13.47</u>	

FIGURE 5.2. A chromatogram of the separation of barley cv. Hankkija-673 seedling organic acids with an Aminex HPX-87H column. (Eluent 0.01 N H_2SO_4, ambient temperature, flow 0.6 ml min^{-1}, chart speed 0.5 cm min^{-1}.) The first peaks in the barley material probably contain several small molecular organic acids as well as the remains of the perchloric acid, PVP and EDTA used in the extraction of the material, which elute with the void volume of the column. On the right a chromatogram of several organic acid standards.



5.2. Importance of ethanol and organic acids in flooding tolerance

Probably the most important factor in flooding tolerance in the plants point of view is how to provide the energy for the maintenance of cellular structures and metabolism in the absence of oxygen. During anaerobiosis ethanolic fermentation provides for only 5% of the ATP the cell gains through glycolysis and the TCA cycle in fully aerated conditions. During short term oxygen deficiency energy-rich compounds are produced by increasing the rate of glycolysis (Pasteur-effect), but this brings about more problems: What to do with the end products and how to maintain regeneration of NAD? Several compounds other than ethanol have been suggested as being possible end products for anaerobic respiration, icluding glycerol, shikimate, lactate, malate, alanine and aspartate. When the value of each of these is weighed in the scales of metabolism there are at least three things to bear in mind. First, whether sufficient energy can be produced for maintenance of cellular metabolism; second, whether the end product is toxic to the life of the plant cell and third, whether the regeneration of NAD is possible through the synthesis of the end product.

1. PRODUCTION OF ETHANOL AND LACTATE. Evidently the most common

way of producing energy in higher plants under anoxia is by ethanolic fermentation (James, 1953). Ethanol is a substance in flooding tolerance the importance of which has been under considerable debate for a number of years. In the early experiments in the 1960's ethanol was thought of being a highly toxic compound which would cause the death of plant cells that were not capable of adjusting their metabolism under waterlogged conditions (McManmon and Crawford, 1971), and produce some less harmful compounds like malate, alanine, glycerol, shikimate or others (Crawford, 1978). However, since these early experiments a number of researchers have concentrated on flooding tolerance and ethanol toxicity, and hence our knowledge has increased considerably.

By producing ethanol a net gain of 2 mol of ATP from each mol of glucose is achieved. At the same time the NAD needed for the running of glycolysis is regenerated. Lactate production allows the same, the only difference being that lactate has an influence on the pH of the cytoplasm (Roberts et al., 1984a,b). Davies et al. (1974a) have plausibly suggested some factors controlling the production of ethanol and lactate in cell free extracts prepared from pea seeds (Pisum sativum) and parsnip roots (Pastinaca sativa), both flood-intolerant species. Their results suggest that under aerobic conditions pyruvate decarboxylase (PDC) is inactive. With the onset of anaerobiosis glycolysis leads to an accumulation of lactate with a

corresponding fall in pH, which activates PDC and initiates competition between lactate dehydrogenase (LDH) and PDC for pyruvate. The pH optimum for LDH is alkaline whilst that of PDC is acidic, thus the two enzymes act as a pH stat. The work by Roberts et al. (1984a,b) is slightly contrary to this theory, since in their nuclear magnetic resonance experiments with maize root tissue, production of lactate and the consequent lowering of the cytoplasmic pH was pinpointed as the lethal factor. The discrepancy may be explicable by the fact that Davies et al. (1974a) used tissue extracts whereas Roberts et al. (1984a,b) were working with whole root tips and measured the products in vivo by nuclear magnetic resonance spectroscopy. Also alcohol dehydrogenase works in creating the balance between ethanol and lactate production under oxygen deprivation (see chapter 4), by having more affinity for acetaldehyde and NADH under anoxic conditions than in air.

The possible toxicity of ethanol has been the subject of a great number of scientific studies. Some early work showed a clear correlation of accumulation of ethanol with sensitivity to anoxia (Fulton and Erickson, 1964; Crawford, 1966; Crawford, 1977 and Crawford and Baines, 1977), but these observations failed to provide any direct evidence that ethanol was toxic to plant tissues. In an extensive study Jackson et al. (1982) showed that no injury resulted from supplying ethanol in aerobic or anaerobic nutrient solution at similar concentrations to those

found in flooded soil. Only at relatively high concentrations has ethanol caused injury in pea (434 mol m^{-3}, Jackson et al., 1982), 100 mol m^{-3} in willow roots (100 mol m^{-3}, Chirkova, 1978) and in marigold and sunflower callus (347 mol m^{-3}, Hildebrandt and Riker, 1955). However, very recently, Perata et al. (1986) published some experiments in which ethanol was applied to sterile cultures of plant tissues in physiological concentrations. This reduced the growth of sunflower (Helianthus tuberosus) discs, affected the embryogenesis of carrot (Daucus carota) protoplasts and decreased α-amylase inducibility by GA_3 in barley (Hordeum vulgare) aleurone layers. These experiments show that ethanol does have a toxic effect on plant tissues in physiological concentrations, but still it remains to be seen whether ethanol is the most important of the many hazards of anaerobic metabolism. Regulation of the amount of ethanol in the tissue during anaerobic conditions has been observed in some flood tolerant species. Monk et al. (1984) have shown in rhizomes of a number of monocotyledonous species that even though they were active fermentors under oxygen deficiency, they did not accumulate ethanol in concentrations above of 30 $x10^{-6}$ mol g^{-1}. In these flood tolerant wetland species an equilibrium was reached between ethanol production and its removal from the rhizomes by diffusion. This contrasted with the non-flood tolerant Iris germanica which reached no such equilibrium and continued to accumulate ethanol.

The experiments on ethanol and CO_2 production presented in this thesis (Fig. 5.2) as well as the dry weight data, do not give a very clear indication why the cultivars were different in their tolerance of anoxia. However, the more anoxia-intolerant cultivars, Hankkija-673 and Pokko, produced significantly more ethanol and carbon dioxide and lost more of their dry weight (especially Pokko) during the anoxic period than the more anoxia-tolerant Kustaa. Since it has been noted earlier that e.g. rice seedlings do not mobilize their carbohydrate reserves during oxygen deprivation (Bertani et al., 1981), the intolerant cultivars may have consumed their cytoplasmic sugar reserves at a faster rate than Kustaa and hence died from lack of soluble carbohydrates. It has been shown that addition of sugars to excised roots lengthens their anaerobic life (Vartapetian et al., 1978). Also, the possible accumulation of lactate, though in small amounts, could have caused severe cytoplasmic acidosis and lead to cell death.

2. PRODUCTION OF MALATE. The theory which claims that production of malate under anoxia is an alternative to ethanol in wetland plants (McManmon and Crawford, 1971), has not been widely accepted by plant scientists. There is, however, some proof that the concentration of malate does increase in some flood tolerant plants during flooding (Linhart and Baker, 1973;

Keeley, 1978; Joly and Crawford, 1982), although the evidence is still conflicting with the experiments of Smith et al. (1979b) and Davies et al. (1974b). The amount of accumulating malate has, however, been too small to account for all end products of glycolysis. Also, the formation of malate through oxaloacetate does not allow net ATP production, but it does regenerate NAD for glycolysis. In our HPLC-experiments the amount of malate in barley seedlings was too low to allow proper calculations but there was no detectable increase in this trace amount of malate in the anoxic barley material.

3. PRODUCTION OF GLYCEROL. The original proposal by Crawford (1972) of glycerol accumulating in Alnus incana roots during flooding was srongly opposed to by Smith et al. (1984) who pointed out by labelling studies that glycerol did not accumulate in Alnus root tissue nor did it have any connection to glycolysis under oxygen deprivation, since no label was found in glycerol in the feeding experiments. Furthermore, contrary to Crawford's (1972) original diagram, the production of glycerol from glycerophosphate does not bring about any ATP for cellular maintenance. The reaction from glycerol to glycerophosphate requires ATP but the reverse reaction does not produce any ATP (Figure 5.1). Synthesis of glycerol does, however, allow the regeneration of NAD. On the whole the reaction sequence from glucose to glycerol would not produce any ATP, it

would consume 2 mol ATP per each mol glucose. An increase in glycerol in cells of Fagopyrum esculentum under oxygen deprivation has been recorded earlier by Effer and Ranson (1967), but they calculated that the amount of accumulated glycerol was only 8% of ethanol produced during the same time.

4. PRODUCTION OF AMINO ACIDS. The production of alanine and aspartate by a transamination step from pyruvate and oxaloacetate, respectively, has been proposed as being one more way for the disposal of the end products of glycolysis (Crawford, 1978). However, only the production of alanine would end up in a net synthesis of ATP, while NAD would not be regenerated (Fig. 5.1). In the formation of aspartate and alanine, glutamate is synthesized from NH_4+ and α-ketoglutarate by the action of glutamate dehydrogenase. This enzyme also works in the degradation of amino acids, but in the biosynthetic direction NADPH is the reductant, whereas NAD is the oxidant in the catabolic reaction (Stryer, 1981).

Several investigations have revealed significant increases in the concentration of alanine in root tissue of wetland species (Bertani et al., 1981; Bertani and Brambilla, 1982 ; Smith and ap Rees, 1979b), but also in flood intolerant species such as pea (Pisum sativum) (Smith and ap Rees, 1979a) and maize (Zea mays) (Kohl et al., 1978; Saglio et al., 1980), but

still the amount synthesized does not outweigh that of ethanol produced during the same time (Davies et al., 1974a; Avadhani et al., 1978; Smith and ap Rees, 1979a,b ; Bertani et al., 1980; Saglio et al., 1980).

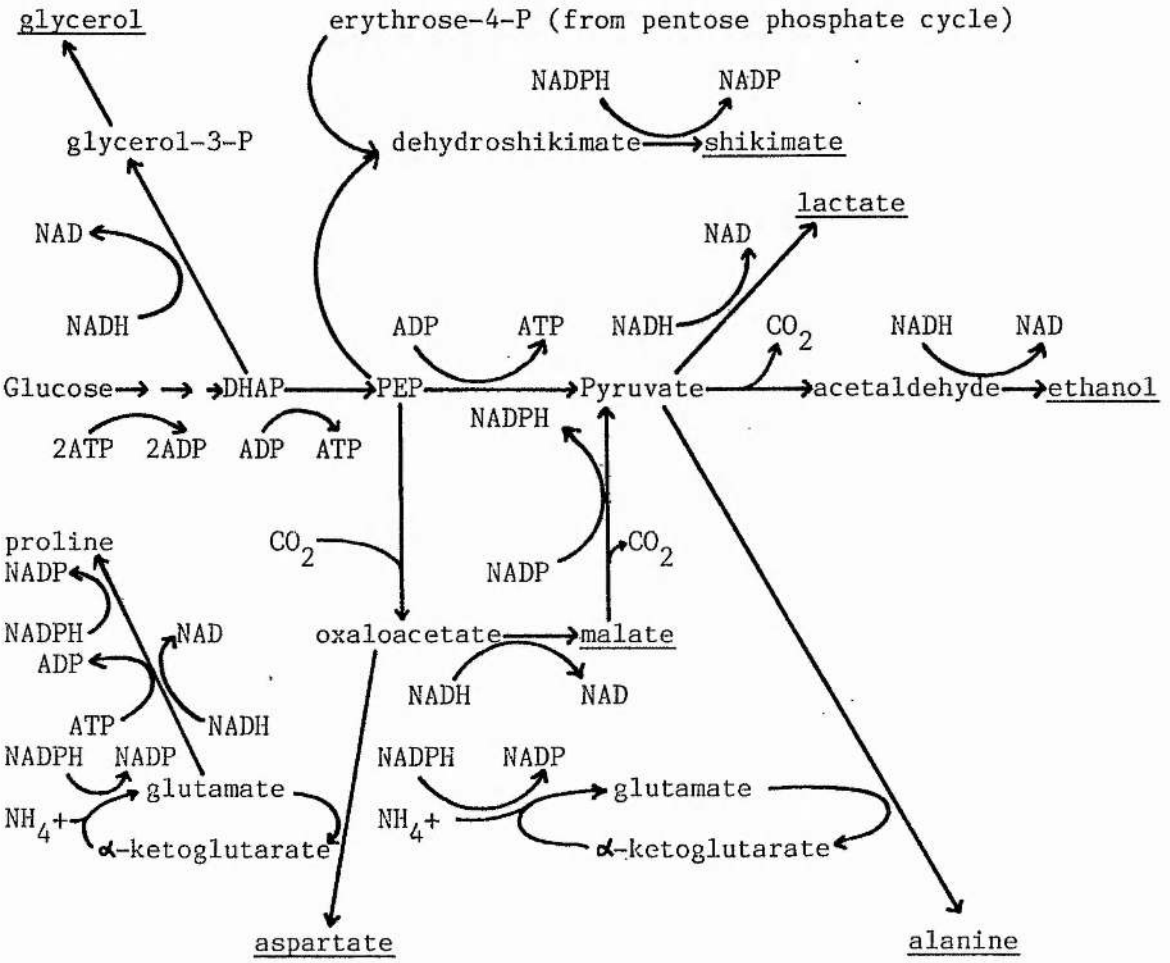
Also, accumulation of proline has been recorded to take place in flooded tomato (Lycopersicon esculentum) roots (Aloni and Rosenstein, 1982), but the cultivars which accumulated the highest levels of proline where those which showed the most severe injury. Furthermore, the synthesis of proline from glutamate uses ATP, but also regenerates NAD and NADP (Stryer, 1981).

It seems that a large part of the increase in amino acid content of the plant cells is due to the increased availability of puryvate during oxygen deprivation when the citric acid cycle is suppressed (Effer and Ranson, 1967; Jackson and Drew, 1984).

One possible factor influencing the length of anoxia tolerance is the depletion of carbohydrate reserves. However, it has been noticed that large amount of carbohydrates do not ensure survival after the anoxic period; there must also be regulation of the use of the reserves (Barclay and Crawford, 1983). The most anoxia tolerant wetland plants seem to be able to conserve some of their carbon reserves under long term anoxia and still manage to keep the concentration of free sugars in the cytoplasm constant (Braendle,

1985). In rice the amount of starch does not change significantly during four days anoxia (Bertani et al., 1981). If the glucose for respiration is derived from starch by starch phosphorylase, the resulting glycolysis would actually produce 3 mol of ATP for each mol of glucose-6-phosphate used. There is some contrasting evidence that starch is not readily mobilised for respiration in roots of maize and rice (Saglio and Pradet, 1980; Saglio et al., 1980; Massimino et al., 1981; Bertani et al., 1981), but in Schoenoplectus lacustris, Phragmites australis and Typha latifolia mobilisation of starch during anaerobiosis has been recorded (Braendle, 1985). In our experiments the loss of dry weight of the barley seedlings during anoxia correlated positively with the flooding and anoxia tolerance of the seedlings. The most intolerant cultivar, Pokko, lost 40% more of its dry weight than Kustaa, the most anoxia tolerant cultivar, during the two week long anoxic period. Where this reduction in dry weight disappeared is not revealed in this experiment. The amount of ethanol and CO_2 produced during the same period was not considerably higher than in Kustaa. It may have been possible though, that lactate production was greater in Pokko than in the other cultivars and lactate leaked from the roots to the filter paper in the flasks. It has been shown that leakage of lactate in roots takes place under oxygen deprivation (Hiatt et al., 1967). However, it has been noted earlier that in buckwheat (Fagopyrum esculentum) seedlings CO_2, ethanol and lactate production did not account for all the carbohydrates consumed under anoxia (Effer and Ranson, 1967).

FIGURE 5.3. A composite model of fermentation with all possible reactants which could accumulate under oxygen stress in plant tissue. Modified from several authors including Conn et al., 1949; Balinsky and Davies, 1961; Dubinina, 1961; Fowden, 1965 and Crawford, 1978. Attention should be focussed on the coenzyme balance and production of energy acquired by accumulation of certain substances. In transaminations pyridoxal phosphate is the prosthetic group and NADP the coenzyme for glutamate dehydrogenase. NAD would be the coenzyme in the catabolic amino acid metabolism (oxidation).



6. SUPEROXIDE DISMUTASE (SOD) ACTIVITY IN BARLEY, RICE AND IRIS UNDER HYPOXIC AND ANOXIC CONDITIONS

Superoxide dismutase (SOD) (E.C. 1.15.1.1.) plays a central role in the protection of plant and animal tisues against oxygen toxicity (Fridovich, 1974; Frank, 1985; Halliwell and Gutteridge, 1984a), catalysing the disproportionation of superoxide radical to hydrogen and dioxygen. The reaction consumes protons and produces hydrogen peroxide and molecular oxygen:

$$O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Three types of this isozyme with different metal prosthetic groups have been characterized. The Cu, Zn-SOD is localized mainly in the cytosol of eucaryotes (Asada et al., 1980), but it has also been found in mitochondria (Arron et al., 1976) and higher plant chloroplasts (Lumsden and Hall, 1974; Jackson, Dench et al., 1978; Foster and Edwards, 1980). Mn-SOD is present commonly in bacteria and mitochondria (Asada et al., 1980). Formerly, Fe-SOD was believed to to be confined to procaryotes but more recently it has been isolated from the green alga Euglena gracilis (Kanematsu and Asada, 1979) and, subsequently, from higher plants Brassica

campestris (Salin and Bridges, 1980), Nuphar luteum (Salin and Bridges, 1982), Lycopersicon esculentum (Kwiatowski et al., 1985) and Phaseolus vulgaris (Kwiatowski and Kaniuga, 1984). The Fe form appears to be connected only with the chloroplast (Bridges and Salin, 1981). Hence, the Cu,Zn-SOD and Mn-SOD forms of the enzyme could be expected to be present in non-photosynthetic plant material such as root and rhizome tissue, which are the subject of this investigation (see also paragraph 6.3).

There are a number of cellular rections which proceed by a single electron transfer from the substrate onto each molecule of oxygen used, producing superoxide. Halliwell (1984) has cited the enzymes nitropropane dioxygenase, galactose oxidase and xanthine oxidase as being those that produce superoxide in plant tissues. If there is sufficient superoxide production, reactions such as the iron-catalysed Haber-Weiss reaction may take place leading to the generation of hydroxyl radicals (Halliwell and Gutteridge, 1984b):

$$\begin{array}{l} Fe^{3+}\text{-salt} + O_2^{\cdot -} \longrightarrow Fe^{2+}\text{-salt} + O_2 \\ Fe^{2+}\text{-salt} + H_2O_2 \longrightarrow OH^{\cdot} + OH^{-} \quad \text{(Fenton reaction)} \\ \hline \text{net: } H_2O_2 + O_2 \xrightarrow[\text{Fe-salt as catalyst}]{} O_2 + OH^{\cdot} + OH^{-} \end{array}$$

Prerequisites for the reaction are traces of a transition metal ion. Hydroxyl radicals will react indiscriminately with cellular compounds, and are capable of abstracting hydrogen from membrane lipids, which triggers the chain reaction of lipid peroxidation. If uncontrolled, the lipid peroxidation process (autocatalytic once started) may affect

membrane integrity, since lipid peroxides and some of their degradation products cause extensive damage to membrane-bound enzymes and to the lipid bilayer itself, producing a decrease in electrical resistance and membrane fluidity (Hicks and Gebicki, 1978; Pauls and Thompson, 1980).

The nonenzymatic defence against such oxidative injury has been the subject of recent studies (Finckh and Kunert, 1985) and includes small molecular compounds such as ascorbate, α-tocopherol (vitamin E), uric acid and phenols. Phenols especially may be of great importance in plant tissues against oxidative damage. Vitamin E is the only known fat-soluble antioxidant in plant tissues.

Recently, some evidence of oxidative damage taking place in plant tissue after anoxic stress has been recorded. Plants which have been completely deprived of oxygen maintain healthy tissue during the period of imposed anoxia, only to degenerate rapidly on re-exposure to air (Monk et al., 1987), suggesting oxidative damage during the recovery phase. Indeed, a significant rise in malonylaldehyde, a lipid perioxidation product, has been reported in rhizomes of the anoxia and flooding insensitive species Iris germanica on return to air after oxygen deprivation. In contrast, levels remained unaltered in the related anoxia-tolerant wetland species I.pseudacorus (Hunter et al., 1983). Recently, increased production of ethane, another lipid peroxidation product, has been observed after anoxia in hyperoxic conditions in intolerant species

(Monk, personal communication). Ethane is considered as a better indicator of lipid peroxidation, since the malonylaldehyde method is subject to many interferences by various compounds in tissue homogenates (Kappus, 1985). Since anoxic stress is a major factor in the flooded environment (Crawford, 1982; see also chapter 3), lipid peroxidation could be expected to take place in a plant which has undergone waterlogging.

Very recently, massive increases in SOD activity have been recorded in the flood- and anoxia-tolerant I.pseudacorus during anoxia and post-anoxia, whereas in the less anoxia-tolerant I.germanica hardly any increase occurred in similar conditions (Monk, 1987). Consequently, SOD activity in barley cultivars compared with that of rice and yellow flag was of considerable interest. SOD activity was measured in barley and rice under hypoxic conditions, in a similar experimental set-up as in the earlier ADH activity experiments to allow comparisons, as well as under total anoxia for comparisons with the existing data on SOD activity in I.pseudacorus. Also, a further study on the inducibility of SOD in I.pseudacorus was carried out to establish whether SOD is a true anaerobic polypeptide in this species.

6.1. Optimisation of SOD activity determination

The photochemical method (Fridovich, 1974) was chosen for the

estimation of SOD activity in roots of barley and rice and rhizomes of yellow flag for the reason that it is independent of other enzymes and proteins and, therefore, more reliable in the case of crude extracts than enzymic assay systems (McCord and Fridovich, 1969; Giannopolitis and Ries, 1977). The photochemical method is relatively simple to control and the results are very reproducible.

6.1.1. Production of superoxide radicals

The photochemical determination of SOD activity is based on the photoreduction of nitro blue tetrazolium (NBT) in the presence of riboflavin and methionine (Fig. 6.1). NBT is reduced to blue formazan, which has a strong absorbance at 560 nm wavelength. Under aerobic assay conditions SOD inhibits the formation of blue formazan. The reaction mixture (3×10^{-6} m^3) contained:

1.3 $\times 10^{-3}$ mol m^{-3} riboflavin
13 mol m^{-3} methionine
63 $\times 10^{-3}$ mol m^{-3} NBT
0.05 $\times 10^{-3}$ mol m^{-3} K-phosphate buffer with
0.01 mol m^{-3} EDTA
enzyme extract appropriately diluted

The production of superoxide radicals as measured at 560 nm wavelength by the colour change in the reaction mixture is presented in figure 6.2. Since the production of superoxide radicals was linear up to 15 min incubation in the light at +25°C, this length of time was used in the subsequent SOD activity determinations. Two 6 W fluorescent Phillips tubes were used as a light source at a distance of 0.1 m from the test tubes, which were placed in a glass water bath at +25°C on a rotating circular tube rack.

FIGURE 6.1. A diagram of the basic reactions which take place in the photochemical method of SOD activity determinations. Superoxide radicals are produced by reduced riboflavin reacting with molecular oxygen. Superoxide reduces colourless NBT to blue formazan, which absorbs light at 560 nm. SOD scavenges superoxide radicals and hence less reduced NBT is formed. (Adapted from Beauchamp and Fridovich, 1971).

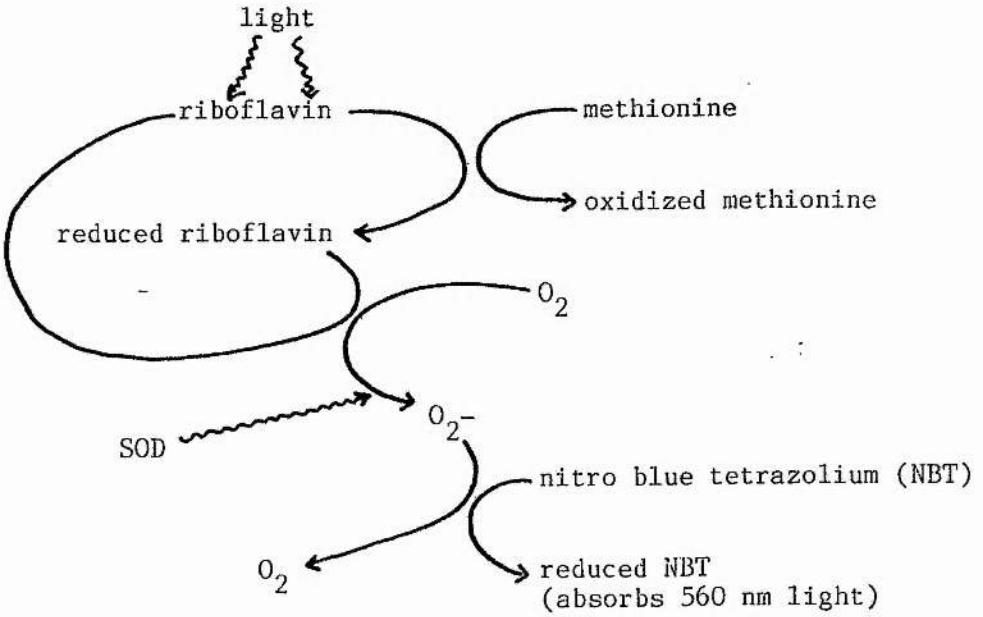
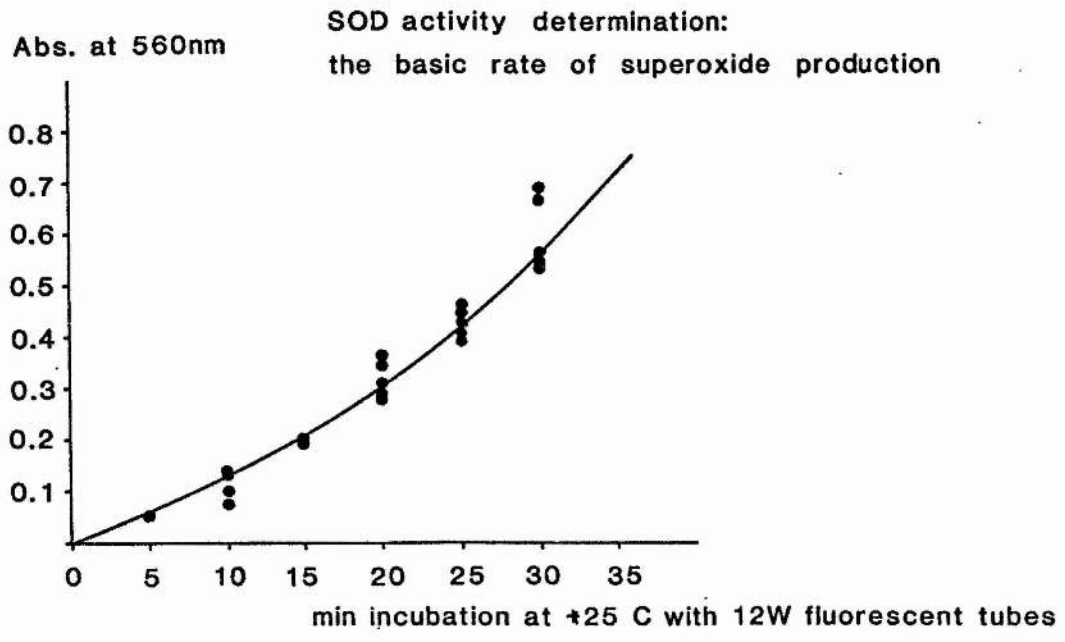


FIGURE 6.2. The production of superoxide radicals in the photochemical method of SOD activity determination. The reaction mixture consisted of 1.3 $x10^{-3}$ mol m^{-3} riboflavine, 13 mol m^{-3} methionine 63 $x10^{-3}$ mol m^{-3} nitro blue tetrazolium, 0.1 mol m^{-3} EDTA and 0.05 $x10^{-3}$ mol m^{-3} potassium phosphate buffer pH 7.8.



6.1.2. Optimisation of the method for barley, rice and yellow flag

To optimise the method of SOD activity determination in root tissue of barley, the effects of PVP, EDTA and DTT on SOD activity were tested. The addition of 1.7% of insoluble PVP into the root samples did not bring about any higher SOD activity than in the control samples, so it was not used in the final extraction medium. Dithiotreitol, (DTT 5 mol m^{-3}) increased the basic reaction rate of NBT reduction so much that it could not be used in the samples, whereas EDTA (0.1 mol m^{-3}) had a beneficial effect on barley SOD activity, which is understandable, since EDTA stabilizes metal ions and SOD is a metalloprotein. The final extraction medium for barley root extract was 0.068 $x10^{-3}$ mol m^{-3} potassium phosphate buffer pH 7.8 containing 0.1 mol m^{-3} EDTA. To ensure that the measured SOD activity was truly proteinaceous in origin, a boiling experiment was conducted with barley root extracts. It is known that SOD is very heat stable, hence the extracts were placed in a boiling water bath for two hours before activity determination. After the boiling 85.6% of the activity had disappeared. Rice root tissue was extracted in a similar manner to that of barley. The results of a recovery experiment with barley root SOD and commercial bovine SOD are shown in table 6.1.

Since the concentration (production) of superoxide radicals in the reaction mixture is very difficult - if not possible - to measure or calculate due to the very nature of the radicals, a standard line of superoxide dismutase activity and an arbitrary system of enzyme units had to be created. To achieve this, SOD activity in ten different dilutions of barley and rice root extracts were determined and plotted against inhibition of the basic rate of production of reduced NBT. One SOD unit was defined as 50% inhibition of the basic rate of reaction according to McCord and Fridovich (1969). However, to establish a linear relationship between SOD activity and inhibition, V/v transformation (V= basic reaction rate without root extract, v= reaction rate with extract) was used (Giannopolitis and Ries, 1977). Linear correlation gave the following equations:

BARLEY SOD: SOD U $x10^{6}$ m^{-3}=(0.806 V/v - 0.613) x dilution factor (Fig. 6.3)

RICE SOD: SOD U $x10^{6}$ m^{-3}=(0.946 V/v - 0.915) x dilution factor (Fig. 6.4)

The correlation coefficients for these lines were 0.997 and 0.987, respectively.

FIGURE 6.3. Inhibition of the photoreduction of nitro blue tetrazolium by barley root SOD. Since inhibition is not linear with SOD concentration, a V/v transformation was used to obtain linearity (V= basic reaction rate without barley extract, v= reaction rate with extract). When 50% inhibition is considered as one unit of SOD activity, equation for the line was SOD U $x10^{6}$ m^{-3}= (0.806 V/v - 0.613) x dilution factor. Correlation coefficient for this line was 0.997.

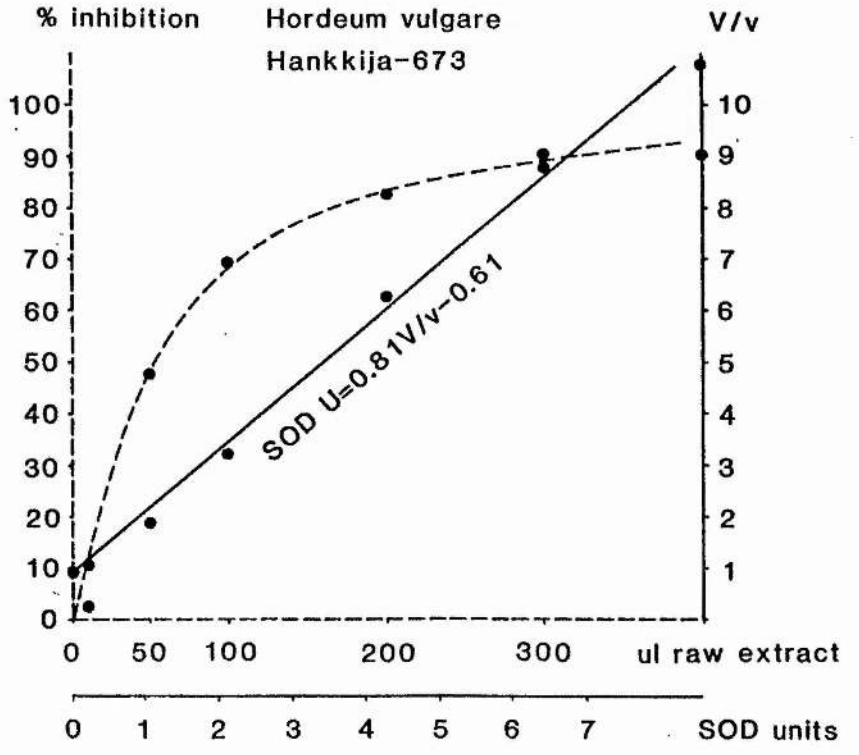


FIGURE 6.4. Inhibition of the photoreduction of nitro blue tetrazolium by rice root SOD. Since inhibition is not linear with SOD concentration, a V/v transformation was used to obtain linearity (V= basic reaction rate without rice extract, v= reaction rate with extract). When 50% inhibition is considered as one unit of SOD activity, equation for the line was SOD U $x10^6$ m^{-3}= (0.946 V/v - 0.915) x dilution factor. Correlation coefficient for this line was 0.987.

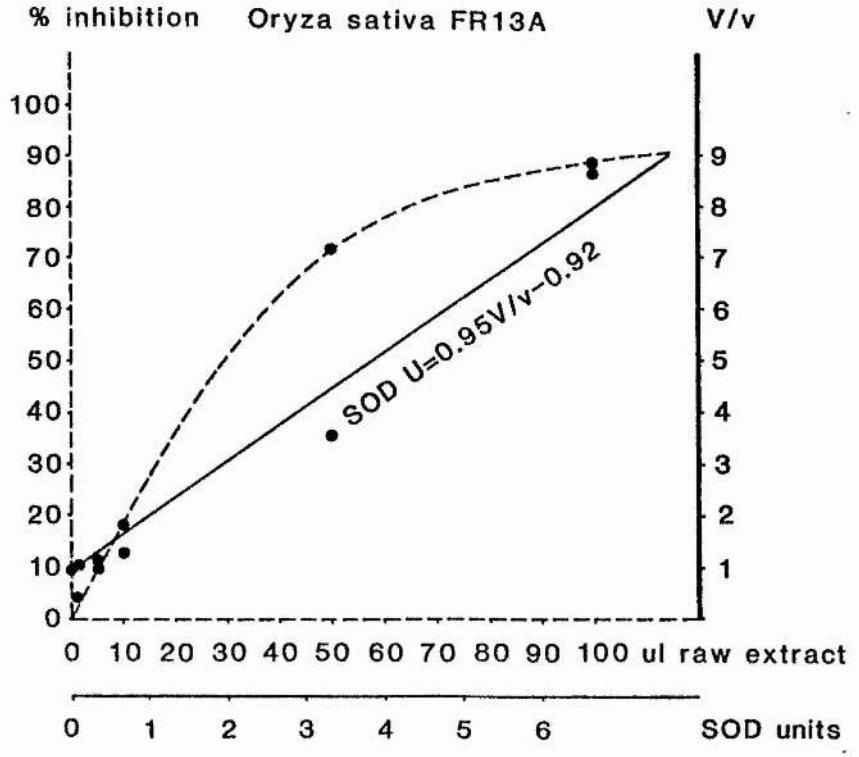


TABLE 6.1. A recovery test to observe the effect of a crude barley root extract on commercial pure Bovine Liver SOD. First, preparates of barley root extracts and commercial bovine liver SOD were assayed separately and then a mixture of these was assayed and recovery calculated. The experiment was replicated thrice.

	Absorbance at 560 nm wavelength			
	Barley root extract 1.00 g root tissue in 6.00 $x10^{-6}$ m^3 buffer	Bovine Liver SOD 0.33 gm^{-3} in reaction mixture	Mixture of the previous in 1:1	Blank
rpl. I	0.132 0.133 0.135	0.205 0.220 0.209	0.167 0.160	0.371 0.410 0.405 0.403
rpl. II	0.149 0.147 0.146	0.240 0.231 0.218	0.180 0.185 0.185	0.429 0.430 0.425
rpl.III	0.142 0.137		0.165 0.170	0.422 0.423 0.440
x	0.140	0.221	0.173	0.416
S.E.	0.002	0.005	0.004	0.006
SOD units in 0.1 $x10^{-6}$ m^3	1.80	0.91	1.34	

$$\frac{\text{Barley root extract + BLSOD}}{2} = 1.36 \text{ U } x10^{7} \text{ } m^{-3}$$

$$\text{Percent Recovery} = \frac{1.34 \times 100\%}{1.36} = 98.53 \pm 2.70\%$$

The same procedure as with the barley root extracts was followed when optimising the extraction of Iris pseudacorus rhizome material. The method for SOD activity measurements was the same as for barley root extracts, viz. photochemical method, which is based on the photo-oxidation of NBT in presence of riboflavin and methionine. The additives which were tested were polyclar AT (insoluble polyvinylpyrrolidone) and soluble polyvinylpyrrolidone. EDTA (0.1 mol m^{-3}) was added to the buffer to stabilize metal ions. Also, the presence of small molecular antioxidants in the extract was ruled out with Sephadex G-25 prepacked columns. Since the activity of the small molecular fraction is impossible to measure with these columns, a parallel study with molecular filters (Centricon tubes with filters for M.W. of 10 000 and 30 000) was carried out. Both the proteinaceous and the small molecular fractions can be recovered and analysed for SOD activity with this device.

First, the effect of insoluble and soluble PVP on extraction of SOD from Iris pseudacorus rhizome material was tested. Addition of 3% insoluble PVP resulted in a 50% drop in activity compared with control extracts without any PVP, whereas soluble PVP (3%) increased the activity over four-fold compared with control samples extracted with a plain buffer. Also 1.5% soluble PVP brought about similar increases in the extractable SOD. Consequently, this concentration of soluble PVP was used in the subsequent SOD activity determinations from yellow

flag rhizomes. Sephadex G-25 gel filtration columns decreased SOD activity in spite of the dilution effect being taken into account in the calculations. These columns were not used in the final experiments, since it was also of vital importance that the small molecular antioxidative capacity of the rhizomes was determined during anoxia. This was tested by measuring SOD activity in aerobic rhizomes as well as in rhizomes which had been kept under total anoxia for seven days. An increase in SOD activity was clearly noticeable and it was due to an increase in the large molecular (proteinaceous, MW over 30 000) fraction as judged by Centricon centrifuge tubes. The small molecular fraction was calculated to cover 3-7% of the total activity in raw rhizome extracts; and, furthermore this fraction did not increase during the anoxic stress. Sephadex G-25 gel filtration columns caused a very similar decrease in the activity as did Centricon tubes.

Hence, in the subsequent experiments Iris pseudacorus rhizomes were extracted with 0.068 $x10^{3}$ mol m^{-3} potassium phosphate buffer pH 7.8 with 0.1 mol m^{-3} EDTA and 1.5% soluble PVP.

After optimisation a standard line was created by diluting raw rhizome extract to several concentrations and plotting these against V/v (as explained previously for barley SOD). The calculations brought about the following standard line for SOD activity in Iris rhizomes. The correlation coefficient for this line was 0.990.

IRIS SOD: SOD U $x10^6$ m^{-3}=(0.953 V/v - 0.907) x dilution factor (Fig. 6.5)

Since Iris rhizome material contains large amounts of phenolic compounds the protein concentration estimations could not be carried out with the bromophenol blue method by Flores (1978, see chapter 4). Also, the soluble PVP in the extracts interfered with the dye in the protein estimations. Consequently, the total protein content of the rhizome extracts was estimated using a kit supplied by Sigma based on Peterson's modification (1977) of the micro-Lowry method with precipitation of proteins. A standard line for this method with bovine serum albumin is presented in figure 6.6.

FIGURE 6.5. Inhibition of the photoreduction of nitro blue tetrazolium by yellow flag (Iris pseudacorus) rhizome extract. Since inhibition is not linear with SOD concentration, a V/v transformation was used to obtain linearity (V= basic reaction rate without rhizome extract, v= reaction rate with extract). When 50% inhibition is considered as one unit of SOD activity, equation for the line is SOD U $x10^{6}$ m^{-3}= (0.953V/v - 0.907) x dilution factor. Correlation coefficient for this line was 0.990.

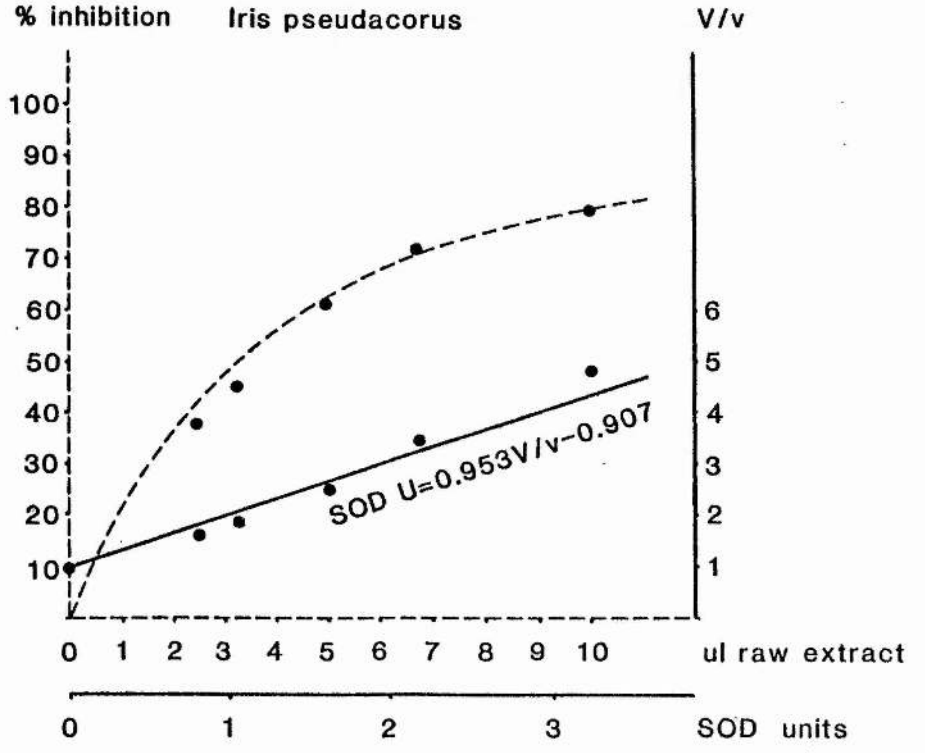
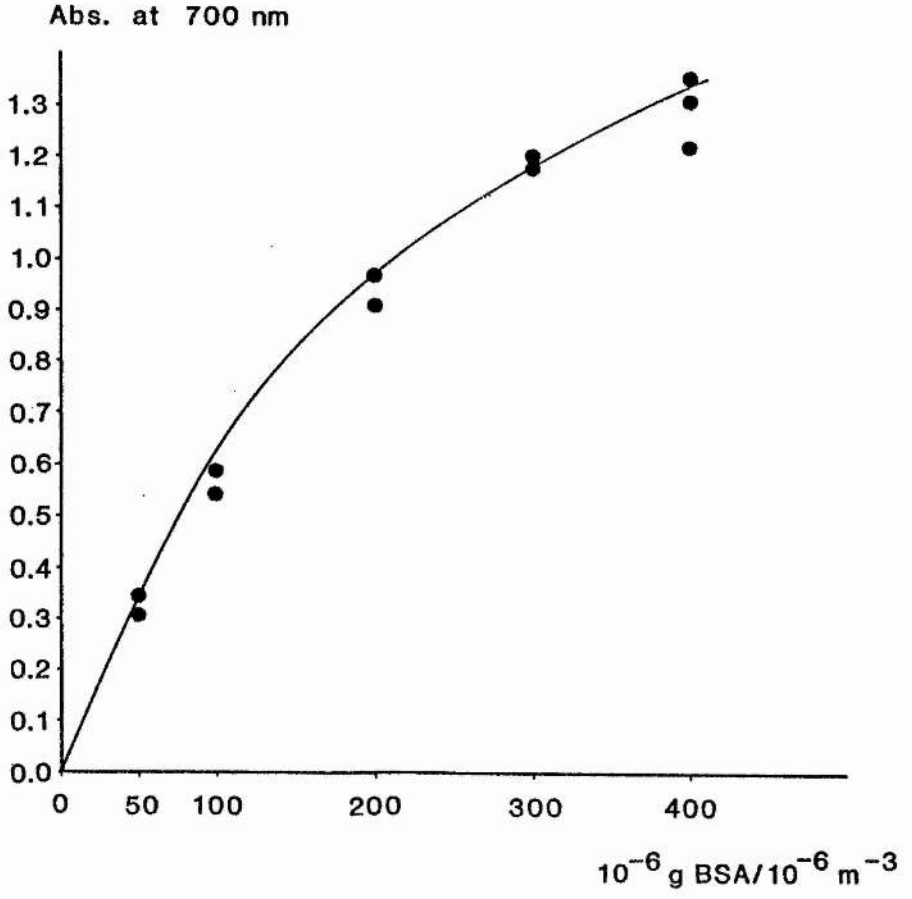


FIGURE 6.6. A standard line for the protein content estimations from Iris pseudacorus rhizome extracts. The total protein content of the extracts was estimated using a kit supplied by Sigma based on Peterson's modification (1977) of the micro-Lowry method with precipitation of proteins. The standard line for this method was obtained by dissolving bovine serum albumin (Sigma) in the extraction buffer and plotting this against absorbance of the protein-dye mixture.



6.2. SOD activity in barley and rice during hypoxia and anoxia

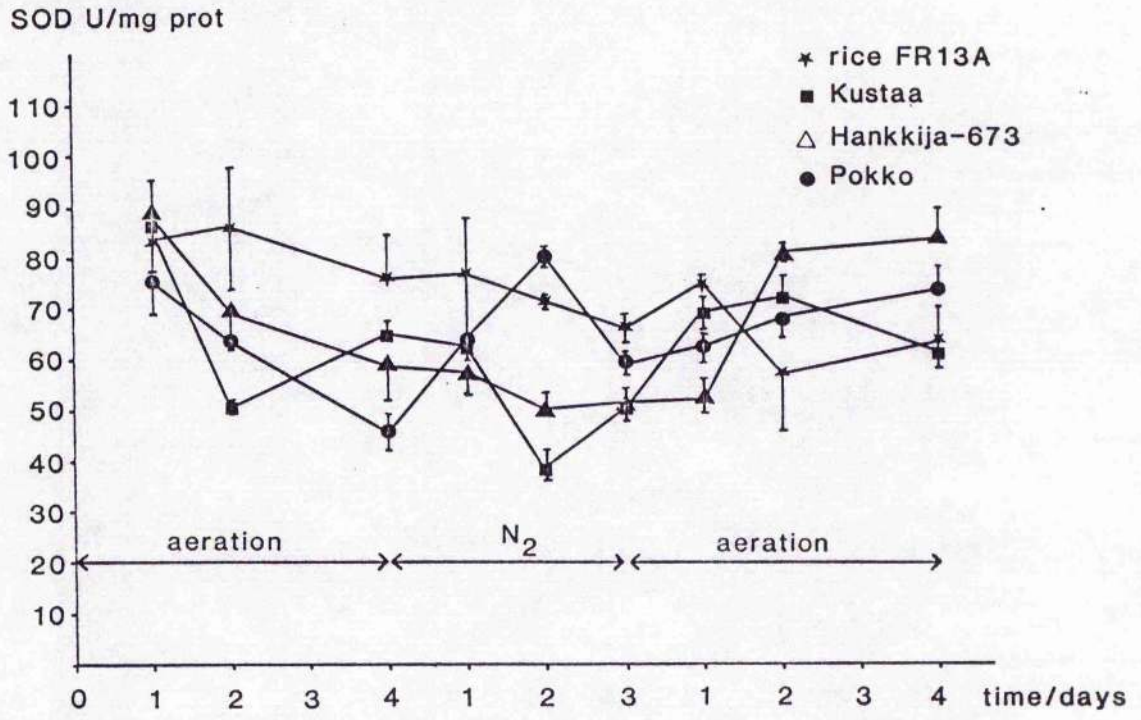
The experiment on the inducibility of SOD in three barley cultivars and rice consisted of two parts. SOD activity in roots was first measured during aeration of solution cultures for four days, then during hypoxic conditions created by bubbling nitrogen through the medium (Ruakura nutrient solution) for three days, and subsequently, during four days of aeration to observe recovery from the anoxic stress (Fig. 6.7). SOD activity remained stable during the whole period in all barley cultivars as well as in rice and no major changes occurred.

The second part of the experiments consisted of an incubation of the most anoxia-tolerant barley cultivar (Kustaa) and rice seedlings under total anoxia for six days at room temperature in the dark with subsequent SOD activity determinations. After the anoxic period, which was of sufficient length to kill most of the barley seedlings, SOD activity had decreased by 73% in the roots of the barley seedlings, but by only 25% in rice (Table 6.2).

TABLE 6.2. SOD activity in the root tissue of barley cultivar (Hordeum vulgare cv. Kustaa) and rice cultivar (Oryza sativa cv. FR13A) seedlings under total anoxia for six days. Student's T-test was used to calculate the statistical significance of the differences in SOD activity between the beginning and the end of the anoxic incubation. *** = P 0.001, ** = P 0.01. n=4.

	Incubation under anoxia for six days SOD activity in Units $x10^6$ m^{-3} ± S.E.		
cultivar	SOD activity at start	SOD activity at the end	reduction in SOD activity
Hordeum vulgare cv. Kustaa	37.8±0.49	10.4±0.42***	73%
Oryza sativa cv. FR13A	50.1±0.77	37.7±0.0**	25%

FIGURE 6.7. Superoxide dismutase activity in barley (Hordeum vulgare) cv. Kustaa, Hankkija-673 and Pokko and rice (Oryza sativa) cv. FR13A in aerobic and hypoxic conditions and during a recovery period in solution cultures. n=3.



6.3. Induction of SOD activity in Iris pseudacorus under anoxia

To observe whether the induced SOD activity is synthesized de novo or activated during anaerobiosis in Iris pseudacorus, an experiment with the protein synthesis inhibitors cycloheximide and actinomycin D was carried out. Slices of rhizome tissue were incubated anaerobically in 1 $x10^{-4}$ m^3 flasks on a shaker at +20 C in the dark (Figure 6.9). The flasks contained 0.068 M K-phosphate buffer pH 7.8, 0.1 mol m^{-3} EDTA, 100 g m^{-3} cycloheximide and/or 50 g m^{-3} actinomycin D. To exclude bacterial contamination during the incubation 0.16 mol m^{-3} (50 g m^{-3}) chloramphenicol was added to all samples. This also inhibits mitochondrial protein synthesis. SOD activity in the tissue was determined at 0, 4, 7, and 11 days anoxia (Fig. 6.8). Control material was incubated under the same conditions but without actinomycin D and cycloheximide. Chloramphenicol was still added to inhibit the growth of bacteria.

Furthermore, in order to find out what proportion of the Cu,Zn-SOD and Mn-SOD was present in aerobic and anaerobic rhizome material, an inhibition experiment with KCN was performed (McCord and Fridovich, 1969). KCN (3 mol m^{-3}) was added to the cuvette before illumination. It inhibited 84.0% and 75.5% of the SOD activity present in aerobic and anaerobic material, respectively, indicating

that Cu,Zn-SOD is the prevalent form of the enzyme in both environments (see introduction of chapter 6). Boiling the rhizome extracts for 20 min resulted in c. 64% decrease in activity.

FIGURE 6.8. The effect of protein synthesis inhibitors actinomycin D (50 g m^{-3}) and cycloheximide (100 g m^{-3}) on induction of superoxide dismutase in rhizomes of Iris pseudacorus. The tissue slices were incubated under anoxia in the dark at +20°C in a shaker in 5 $x10^{-6}$ m^3 of 0.068 $x10^3$ mol m^{-3} K-phosphate buffer which contained 0.1 mol m^{-3} EDTA and 0.16 mol m^{-3} chloramphenicol. n=4-6.

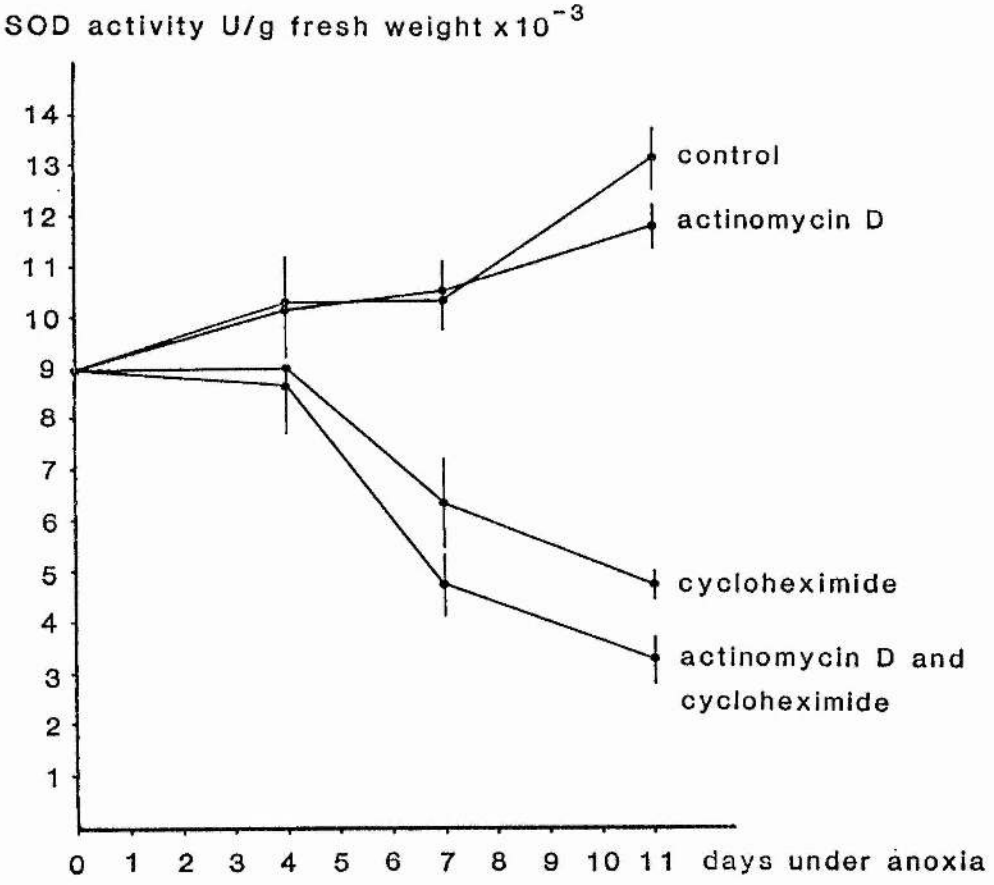


FIGURE 6.9. A photograph of the experimental set-up for the protein synthesis inhibitor experiment. Slices of I.pseudacorus rhizomes were incubated under total anoxia with protein synthesis inhibitors actinomycin D and cycloheximide.

6.4. The importance of superoxide dismutase as an antioxidant in plant tissues under and after oxygen deprivation

Plants as well as animals live under a constrant threat of growing rancid. Most of the compounds found in living tissues would interact with oxygen more or less instantly, if there were no defence systems to prevent this uncontrolled oxidization. It is rather a paradox that oxygen - a vital element for all aerobic orgasnisms - should have such toxic potential if provided in excess or under conditions where cells are very susceptible to oxidative damage. It has been stated that superoxide dismutase (SOD) has a key role in the antioxidant defense system of all organisms which enjoy the benefits of an aerobic lifestyle (Frank, 1985). Naturally, the antioxidative defense includes other compounds; both enzymatic and non-enzymatic substances exist to scavenge oxidative free radicals (see chapter 6.5).

Interestingly, the physiological role of SOD in plants has been the subject of very few studies, even though the clinical background of SOD is well known in animals. Since the discovery of the enzymatic activity of erythrocuprein and other cupreins by McCord and Fridovich (1969), SODs have been characterized from plant tissues, but no work has as yet been published on anoxia or hypoxia tolerance of plants in

connection with SOD and oxidative damage to tissues, even though this area has been widely studied within medicine. Oxygen radicals have been of especial interest in a condition of animal tissues known as post-ischemic injury. When the normal flow of blood is restricted or blocked, a state of ischemia results where the tissue becomes severely hypoxic. However, most of the injury occurs not during the hypoxic period, but on restoration of the oxygen supply (Haglund and Lundgren, 1978). Moreover, in a review of the subject by McCord (1985), many instances were cited where applications of SOD to the experimental tissue prior to reperfusion with oxygen decreased the incidence of post-ischemic injury. Recently, some evidence of oxidative damage taking place in plants after anoxic stress has been recorded by Hunter et al. (1983) and Monk (personal communication) in studies comparing a flood sensitive Iris germanica and a flood tolerant Iris pseudacorus. The flood tolerant species suffered much less oxidative damage than the flood intolerant after a period of anoxic stress.

A biochemical mechanism for the uncontrolled production of superoxide radicals on return to air, causing this type of tissue damage has been proposed (Battelli et al., 1972; DellaCorte and Stirpe, 1972; McCord,1985): when energy charge falls below a certain level in hypoxic tissue, a calcium-triggered sulphhydryl oxidation or limited proteolytic attack on cytosolic xanthine dehydrogenase converts the enzyme to superoxide-producing xanthine oxidase, which becomes active on contact with molecular oxygen (Fig.

6.10). In normal conditions 90% of the enzyme is in the dehydrogenase form and catalyses the reaction:

$$\text{xanthine} + H_2O + \text{NAD} \longrightarrow \text{uric acid} + \text{NADH}$$

After the transformation xanthine oxidase produces superoxide radicals:

$$\text{xanthine} + H_2 + 2O_2 \longrightarrow \text{uric acid} + 2O_2^{-} + 2H^{+}$$

These reactions were originally recorded from homogenized tissue, but more recently they have also been noted to take place *in vivo* (McCord, 1985). Another possibility for the increased production of reactive oxygen species is spontaneous oxidation, on restoration of oxygen supply, of reduced compounds accumulated in the tissue during anoxia (Meerson *et al.*, 1982). Other intracellular sources of superoxide radical are aldehyde oxidase, tryptophan dioxygenase, indoleamine dioxygenase and flavoprotein dehydrogenase (Frank, 1985). Autoxidation of reduced ferredoxin and certain flavins, thiols and hydroquinones can also produce superoxide radicals (Frank, 1985).

Oxidative damage takes place in every component of the cell, the major targets being lipids (peroxidation of unsaturated fatty acids), proteins (oxidation of sulfhydryl-containing enzymes leading to enzyme inactivation), carbohydrates (polysaccharide depolymerization), nucleic acids (base hydroxylations, nicking, cross-linkage, scission

of DNA strands). These effects have been extensively studied and reviewed within medicine (e.g. Fridovich, 1978; Lewis and Del Maestro, 1980; Brawn and Fridovich, 1980).

Very recently, in a study by (Monk, 1987) I.pseudacorus showed remarkable increases in SOD activity during anoxia, and these high levels (up to 13-fold rises, compared to initial levels) were maintained during the recovery phase in air. This species has been observed to survive a noteworthy eight weeks oxygen deprivation (Hetherington et al. 1983). High SOD activities may contribute to the tolerance of anoxia by providing adequate defences against oxygen toxicity on restoration of oxygen supply. It is possible that the modest increases seen in the related I.germanica are not large enough, and hence oxidative damage could be a determining factor in the post-anoxic death of rhizomes of this monocotyledonous species. Indeed, increases in the lipid peroxidation products, malonylaldehyde and ethane, have been shown in I.germanica after anoxic stress (Hunter et al., 1983; Monk, 1987). On the basis of these results and since oxygen toxicity is the main cause of post-ischemic injury in many animal tissues (McCord, 1985), it is suggested that oxygen radicals cause significant post-anoxic damage in seedlings of barley cultivars, especially since SOD activity dropped drastically in the seedlings during anoxic incubation, whereas in the more tolerant rice the activity was maintained at a much higher level under oxygen deprivation. A fall in SOD activity during anoxia, such as that found in barley seedlings, would expose

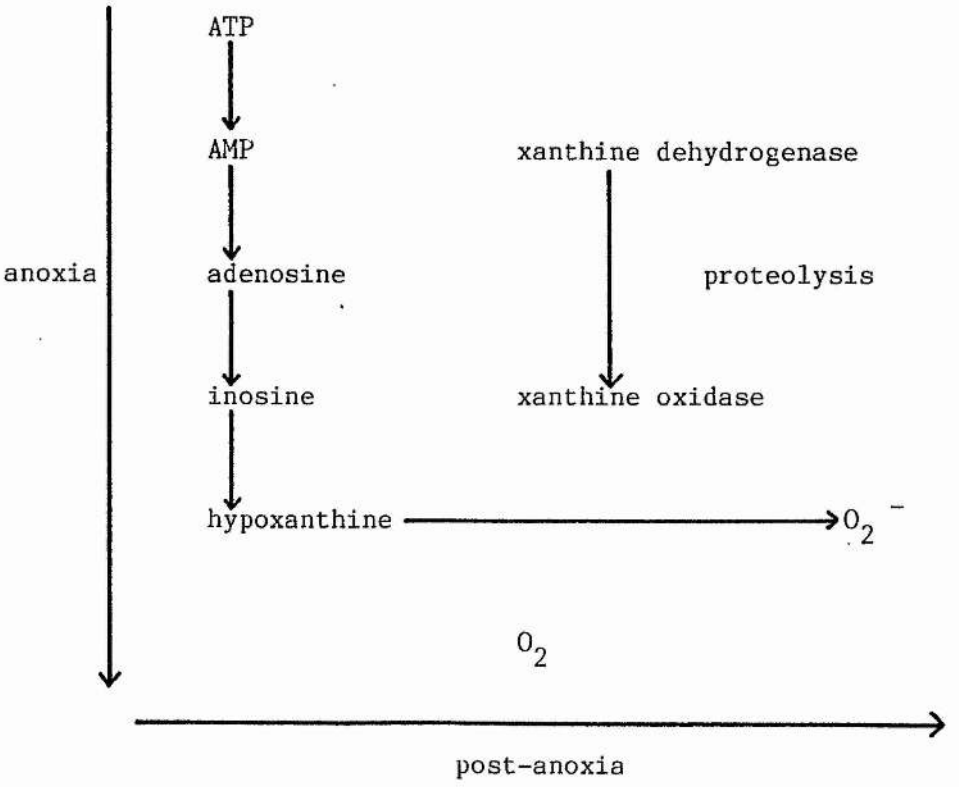
the plants to oxidative damage on return to air, since the levels of SOD are most probably not sufficient to scavenge superoxide being produced after the sudden onset of aerobic conditions.

It is not clear what triggers the induction of SOD activity in complete absence of oxygen in I.pseudacorus, and to a certain extent in I.germanica. In view of the biological role of SOD, the enzyme should be induced by reactive oxygen species, which indeed is the case in E.coli (Hassan and Fridovich, 1977a, b) and in rats exposed to hyperoxia (Crapo and McCord, 1976). However, in terms of evolution, an increase in SOD level during hypoxia is of advantage to the plant when aerobic conditions are restored.

The inhibition of protein synthesis by actinomycin D and cycloheximide in I.pseudacorus suggested SOD activity to be induced by increased translation of SOD messenger-RNA. Similar results on induction of SOD in rats by elevated oxygen concentrations have been recorded (Crapo and McCord, 1976). Inhibition of transcription by actinomycin D did not affect the induction, although the measured activities were slightly lower than in the control material. On the whole the protein concentrations decreased during the treatment in tissue slices incubated with the protein inhibitors. Calculating the SOD results on a protein basis was considered inappropriate, since protein synthesis itself was being inhibited. Since the tissue is non-photosynthetic it can be assumed that Fe-SOD was not present in the rhizomes. Also, Mn-SOD, which is possibly mitochondrial, may have

been inhibited by the chloramphenicol added to the incubation medium (0.16 mol m^{-3}). Hence, the isozyme which could have been induced was Cu,Zn-SOD. On the basis of this experiment it is suggested that SOD is one of the 20 anaerobic polypeptides (Sachs and Ho, 1986) synthesized under anoxic stress. Boiling raw I.pseudacorus extract reduced SOD activity by c. 64 per cent. SOD has previously been reported as being very heat stable in raw extracts (Giannopolitis and Ries, 1977).

FIGURE 6.10. A proposed mechanism for the production of superoxide by hypoxanthine and xanthine oxidase (adapted from McCord, 1985).



Mocquot et al. (1981) investigated protein synthesis under anoxic conditions in rice embryos, and concluded that oxidative capabilities were conserved during anoxia, so that the tissue was immediately able to resume efficient energy metabolism on return to air. Species physiologically equipped to survive waterlogging could be expected to show this phenomenon, since rapid and efficient recovery after environmental stress will secure a place in that particular ecological niche. Such metabolic adaptation is probably present at least to some extent in the rice and yellow flag which are subjected to this study. Among the enzymes conserved one would expect to find SOD, since this enzyme serves aerobic respiration by scavenging oxygen free radicals generated by oxygen utilization.

In a study on Iris pseudacorus and Iris germanica Hetherington et al. (1982) showed that a 14 day period of anaerobiosis resulted in an increased ratio of unsaturated:saturated fatty acids in rhizome tissue in the tolerant I.pseudacorus, but no change in the intolerant I.germanica. Similar changes have been detected earlier in rice and wheat (Khoang et al., 1979) and they were interpreted as adaptations to anoxic conditions. More recently, Misra et al. (1986) have reported higher ratios of unsaturated:saturated fatty acids in the leaves of three species of mangrove that were grown under periodically flooded conditions, compared with plants that were grown normally. The significance of these apparently adaptive changes may lie in the fact that

polyunsaturated fatty acids, if oxidized by radicals, can easily be removed and subsequently replaced by new lipid molecules. Thus, if oxygen free radicals are produced in significant amounts on return to air, less disruption to membrane function would occur if they were to react with a lipid constituent than with a protein constituent, since replacement of a membrane-bound protein is likely to be a much more complicated and protracted process (Quinn and Williams, 1978). In a study on ufasome membranes (prepared vesicles of oleic and linoleic acids entrapping glucose), Hicks and Gebicki (1978) reported that a considerable degree of peroxidation can be tolerated in membranes before permeability changes could be detected. In this way polyunsaturated fatty acids may supplement the protection of α-tocopherol (vitamin E), an antioxidant associated specifically with membranes. Both these mechanisms may form an important second line of defence against oxygen toxicity, after SOD.

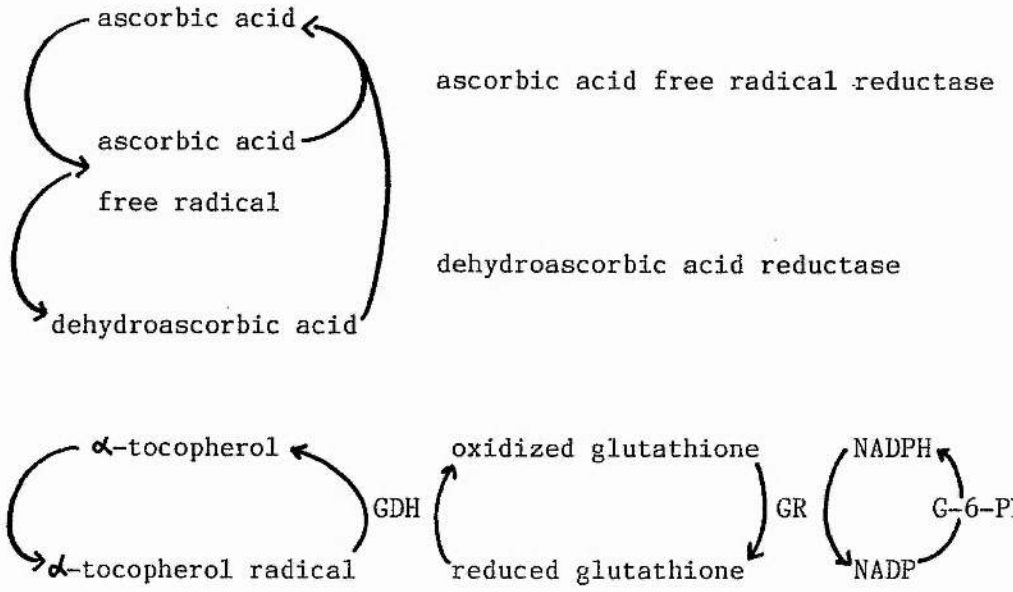
It can be seen that the extraordinarily high anoxic SOD activity in rhizomes of I.pseudacorus in comparison to that of barley and rice, is correlated with the ability to survive prolonged anoxia, which is the main stress factor in waterlogging (see chapter 3). The data presented here is supported by earlier studies of anoxia tolerance both in plant and animal species, and it suggests that SOD is of some importance in protection against oxygen toxicity during recovery from oxygen deprivation in anoxia-tolerant plants.

6.5. Antioxidant protection of plant tissues

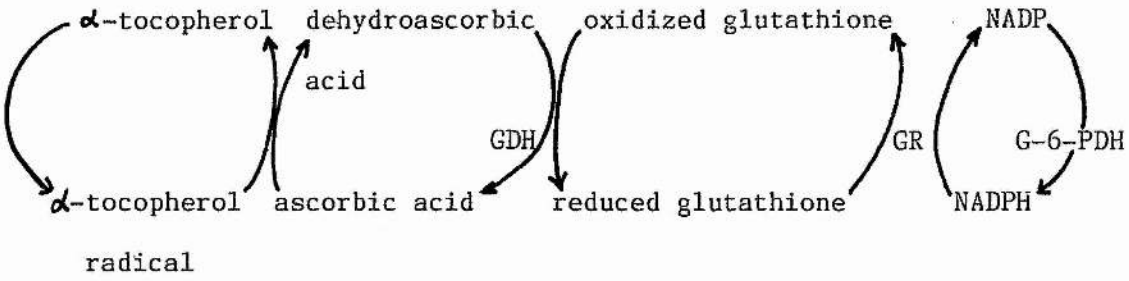
It is rather extraordinary that so very little has been written on plant antioxidants or oxidative stress of plant tissues. This is possibly due to the fact that oxidative damage is very seldom obvious in plants. This indicates the antioxidative defence system being so effective, that damage does not take place in a normal environment. A situation contrary to this occurs, however, when plants or plant tissues are incubated under anoxic or severely hypoxic conditions. After the stress period the more intolerant plants show signs of oxidative damage (see chapter 6.4) and eventually die, depending on the tolerance of the individuals and the length and severity of the stress period.

Plant antioxidative status consists of a number of antioxidative compounds, both enzymatic and small molecular, each of which takes care of one or a few reactions which produce possibly harmful oxidative radicals. One of the most important antioxidants in animal tissues is superoxide dismutase, which catalyses the disproportionation of superoxide to oxygen and hydrogen peroxide. Judging by the similarity of the antioxidative system of plants and animals, we can assume that SOD is of equal importance in plant tissues.

Other antioxidative agents found in plants are listed in table 6.3. They include some well known protective agents such as catalase, ascorbic acid and vitamin E (the only known fat soluble antioxidant, important for membrane integrity), but also some less well known antioxidants such as uric acid, β-carotene and phenols, of which especially the last are abundant in many plant tissues. Regeneration of ascorbic acid and α-tocopherol can take place in the following ways:



or alternatively:



Where GDH is glutathione dehydrogenase, GR glutathione reductase and G-6-PDH glucose-6-phosphate dehydrogenase.

Plant and animal antioxidants can be divided into two groups: class I contains compounds, which inhibit the initiation of autoxidation i.e. formation of the first radical. Class II consists of antioxidants which prevent the propagation of autoxidation. Catalase and glutathione peroxidase belong to class I, whereas SOD, ascorbic acid, uric acid and vitamin E belong to class II. Thus, the potentially hazardous reactions are counteracted on three levels of protection: prevention of radical formation, interception of autoxidation and repair of possible damage which has taken place. It can be seen that the antioxidative defense of plant tissues is a powerful means of retaining redox-reactions under control and directing oxidations to respiration.

TABLE 6.3. Antioxidant defenses of plant tissues (collected from: Frank, 1985; Sies, 1985; Dalton *et al.*, 1987; Ingold, 1987, personal communication).

Enzymatic	Comments
superoxide dismutase (SOD)	Several isozymes: Mn-SOD mitochondrial, Fe-SOD in chloroplasts, Cu,Zn-SOD cytoplasmic
catalase (CAT)	peroxisomal?
(glutathione peroxidase (GPX)	selenoenzyme in animals)
glutathione reductase (GR)	regeneration of α-tocopherol
ascorbate peroxidase	removal of H_2O_2
glucose-6-phosphate dehydrogenase (G-6PDH)	NADPH supply for previous
NADPH-quinone oxidoreductase	two-electron reductions
Nonenzymatic	
ascorbic acid	water-soluble
uric acid	singlet oxygen quencher
β-carotene	singlet oxygen quencher
glutathione	regeneration of α-tocopherol
vitamin E	the only lipid soluble natural antioxidant
cysteamine	
cysteine	
thiols	
nonessential polyunsaturated fatty acids	
flavonoids	plant antioxidants (e.g. rutin, quercetin, etc.)

7. SCANNING ELECTRON MICROSCOPY OF ROOTS OF BARLEY AND ROOTS AND RHIZOMES OF BEAKED SEDGE

The aim of this study was to examine root anatomy of both the barley cultivar Hankkija-673 and beaked sedge under fully aerated and hypoxic conditions created in solution cultures. Also, it was of botanical interest to investigate the root anatomy of beaked sedge, which has not been studied in detail before, although the root anatomy within Cyperaceae is known to be very uniform (Plowman, 1906). Scanning electron microscopy was used in the study since the extensive depth of the field in focus lends it to anatomical and morphological studies.

Root anatomy greatly affects the flooding tolerance and growth of roots in an anaerobic or hypoxic environment. However, it is still under debate whether aerenchyma development is enough to ventilate roots and root tips that grow deep into anaerobic soil, especially since some root tips do not possess aerenchyma. The root tips have been noticed to be the most anoxia intolerant organs (see chapter 3). Also, respiratory activity in plant tissues usually declines with age; consequently, roots naturally exhibit their greatest oxygen demand at the apex followed by a basipetal decline (Armstrong, 1978). Hence, the demand for oxygen is largest where the concentration is at its lowest due to resistance to

diffusion. McKee and Mendelssohn (1987) have measured oxygen concentrations in the aerenchymatous roots of Avicennia germinans and stated that oxygen concentrations decreased from 16% to less than 2% within one hour after exposure to hypoxic conditions in solution cultures. This indicates that the aerenchyma was not very efficient in maintaining aeration of the tissue. However, in marshlands it may well be that an early season flush of water temporarily provides a well-aerated environment for rapid root growth, so that when conditions become stagnant and anaerobic, little further growth occurs (Jackson and Drew, 1984). Hence, under anaerobic conditions aerenchyma would only provide enough oxygen to maintain the existing root tissue. McKee and Mendelssohn (1987) did not, however, determine the critical oxygen pressure (COP) of the roots in question, and hence the effect of the low oxygen concentration inside the roots on respiration remains unclear i.e. we do not know whether the root cells experience oxygen deprivation or not.

In some wetland plants it has been shown, that ventilation of rhizomes is possible through floating leaves (Dacey and Klug, 1982a, b). It remains to be seen whether this kind of aeration occurs in other wetland plants.

7.1. Material and methods

The plants were grown in solution cultures in Ruakura nutrient solution with vigorous aeration. For the aerated root samples the barley were grown for 4 weeks and for the hypoxic samples for 10 days more with N_2-bubbling. The Carex rostrata samples were also grown in similar solution cultures but the plants were grown from material collected in the nature. Before the samples were taken these plants had grown in solution culture for a year.

Samples for scanning electron microscopy were fixed with 1% glutaraldehyde in 0.025 $x10^{3}$ mol m^{-3} Na-K-phosphate buffer pH 6.8 for 3 hours and then in 2% glutaraldehyde in the same buffer overnight. At the beginning infiltration was accelerated with a vacuum pump until the samples ceased to float. After fixation glutaraldehyde was washed with the buffer and the samples dehydrated with an ethanol and acetone series (20, 40, 60, 80, 90 and twice 100% ethanol, ethanol:acetone 2:1, 1:1, 1:2 and twice pure acetone). The samples were then dried in a critical point apparatus with carbon dioxide and coated with an ion sputter until the layer of gold was about 12 $x10^{-6}$ m thick. The samples were then examined with a JSM-35CF scanning electron microscope and subsequently photographed.

The samples for the SEM-study were collected as follows (numbers refer to photographs):

Barley (Hordeum vulgare L. cv. Hankkija-673)

Aerobic solution culture	Hypoxic solution culture
Mature seminal roots (7.1)	Mature seminal roots
Adventitious root tips (7.2)	Adventitious root tips
Mature adventitious roots	Mature adventitious roots (7.3)

Beaked sedge (Carex rostrata Stokes)

Aerobic solution cultures	Hypoxic solution cultures
Mature roots (7.4)	Mature roots (7.5)
Rhizome (not root tissue)(7.6)	Rhizome (7.6)

7.2. Results

The rigid but flexible roots of Carex rostrata lended themselves to this kind of a study with the scanning electron microscope. The roots and rhizomes were easy to prepare for critical point drying and they stood well the coating with gold and the examination under the electron beam. The same cannot be said of the thin and fragile roots of the barley cultivar. Some of the structure was already lost while the roots were excised and more damage took place during critical point drying and coating with gold. However, since an earlier light microscopical study did not reveal any

differences between the cultivars in the formation of air spaces, the spending of more time on developing the technique was not justifiable.

The roots (Figs. 7.4 and 7.5) and rhizomes (Fig. 7.6) of Carex rostrata had well developed aerenchyma under both aerated and hypoxic conditions and no differences could be detected in the micrographs between the two treatments. However, there may have been some quantitative differences in the amount of air spaces in the roots and rhizomes. In the roots, sheets of cortical cells were arranged in tangential or circular layers, whereas in the rhizomes similar formations were organised radially.

In figure 7.1 it can be seen that the cortex of an aerobically grown seminal root is full of cells which appear round in cross section. No lysigenous development is evident. In figure 7.3, however, it can be seen that some lysigenous or schizogenous activity has taken place in the cortex developing to cavities (even though the root has been damaged by cutting). The root cap and massive production of root hairs (Fig. 7.2) seems to have been unaffected by hypoxic conditions.

FIGURE 7.1. A SEM photograph of a mature seminal root of barley grown under aerobic conditions. No visible formation of lysigenous spaces has taken place in the cortex. The bar represents 10 $x10^{-6}$ m.

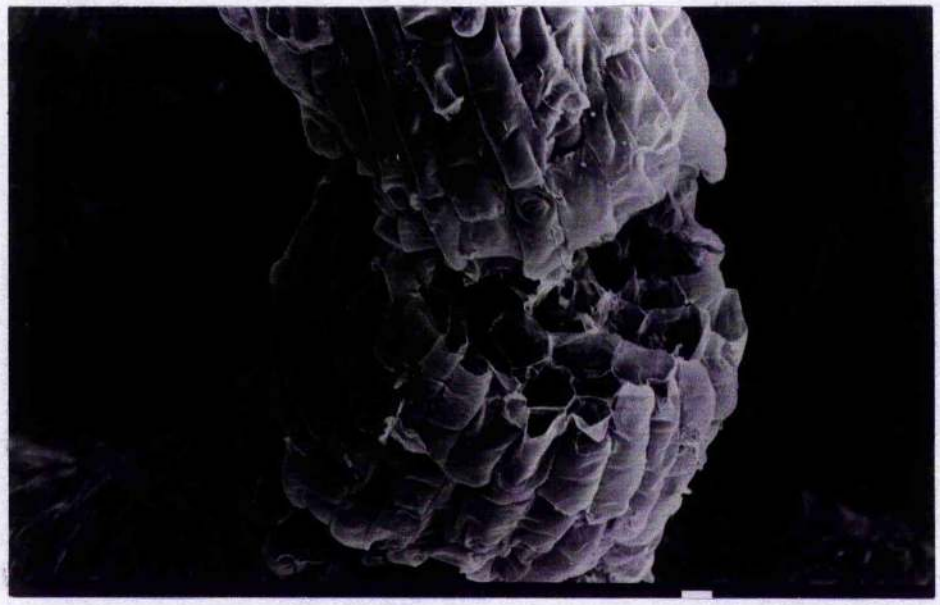


FIGURE 7.2. A SEM photograph of a root tip of an adventitious root of barley grown under aerobic conditions. The bar represents 1 $x10^{-3}$ m.

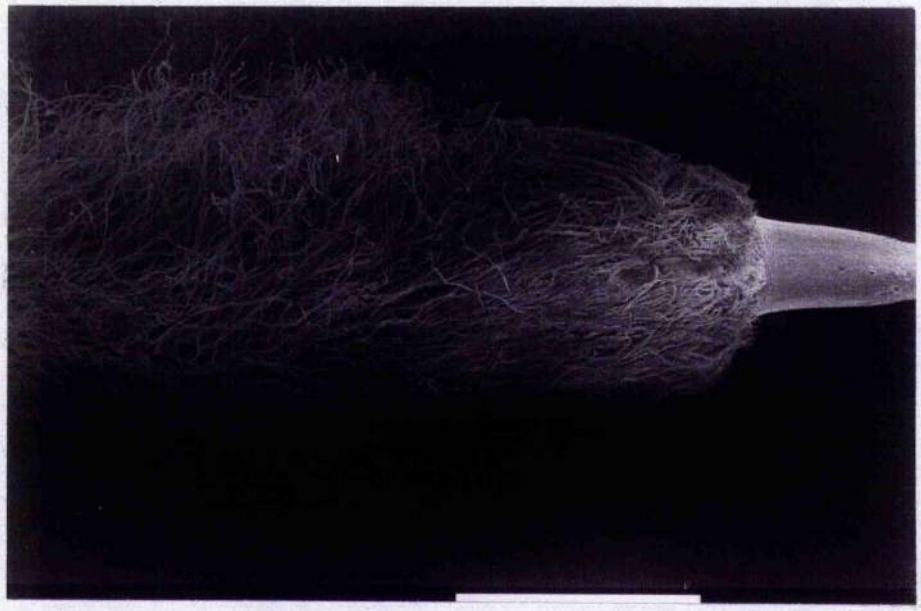


FIGURE 7.3. A SEM photograph of an adventitious barley root grown under hypoxic conditions. Formation of lysigenous spaces has taken place in the cortex. The bar represents 100 $x10^{-6}$ m.

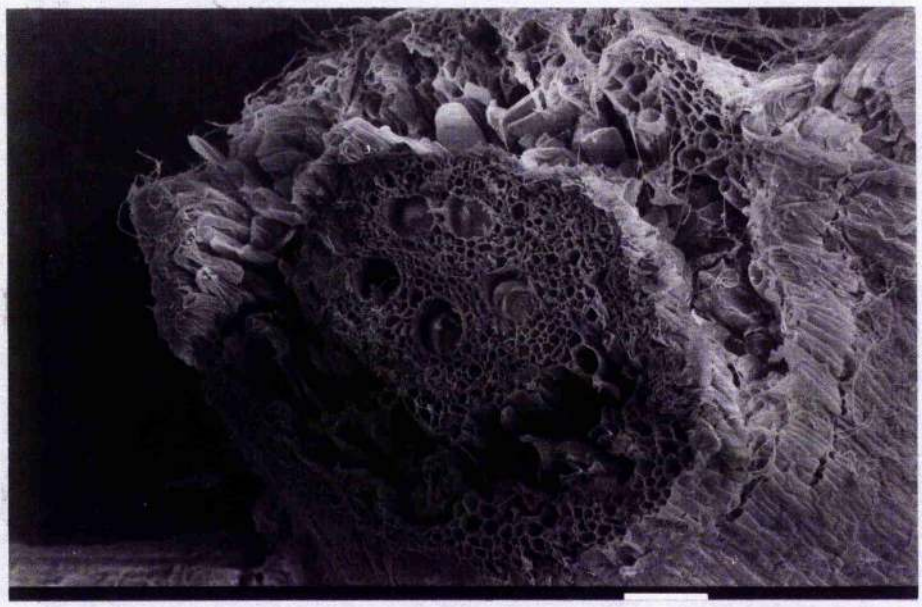


FIGURE 7.4. A SEM photograph of the cortex of a root of beaked sedge grown under aerobic conditions. The cortex is very aerenchymatous. The bar represents 10 $x10^{-6}$ m.

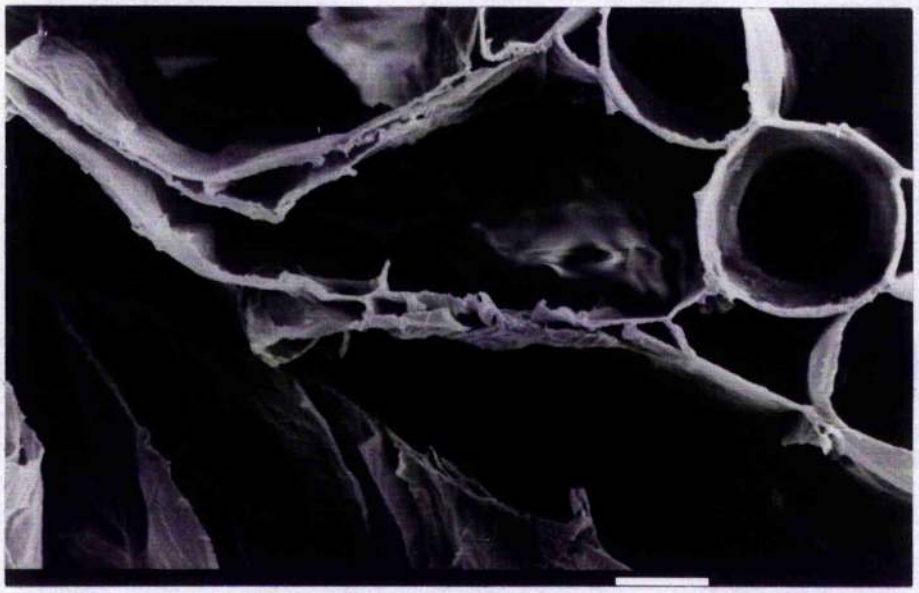


FIGURE 7.5. Two SEM photographs of beaked sedge root grown under hypoxic conditions. The cortex is very aerenchymatous. The bar represents 100 $x10^{-6}$ m (above) and 10 $x10^{-6}$m (below).

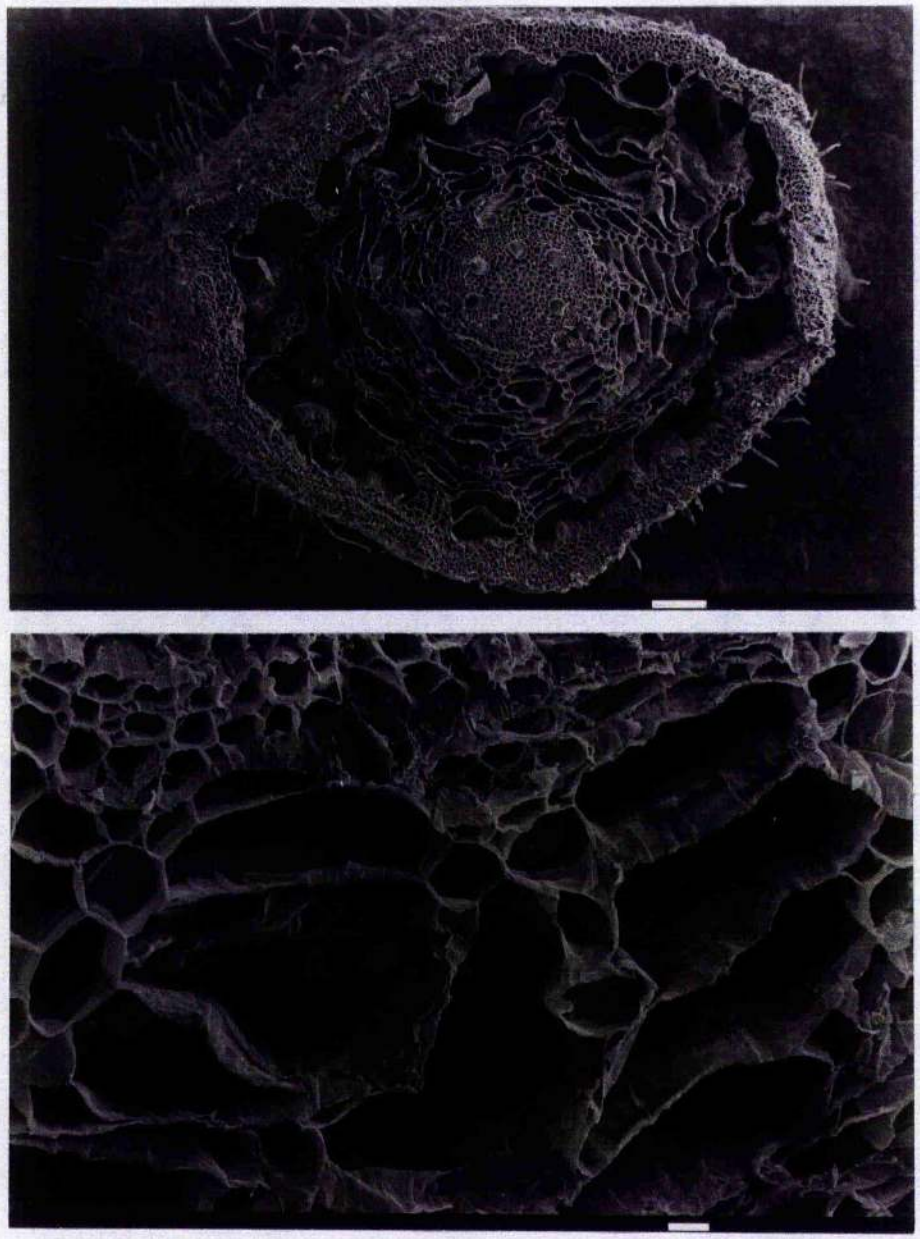
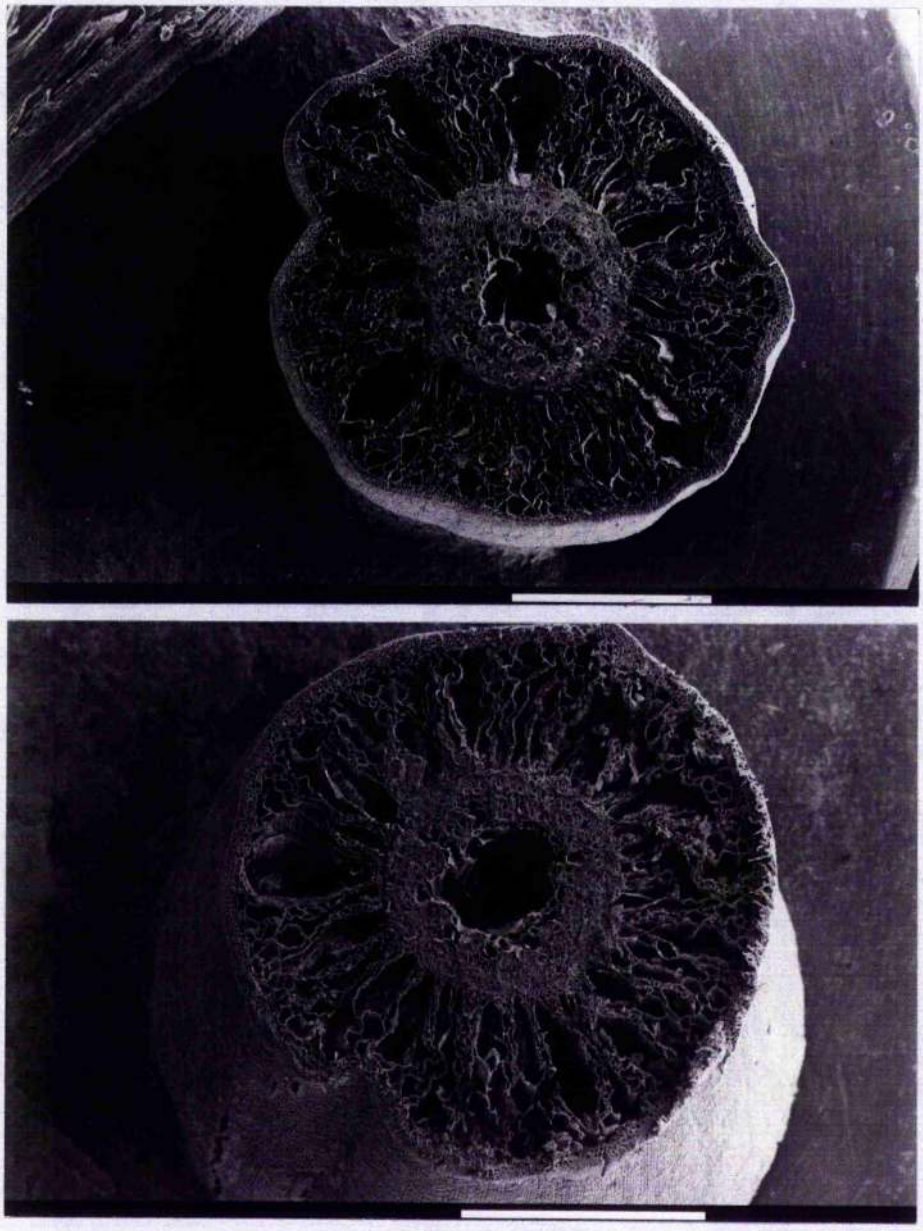


FIGURE 7.6. Two SEM photographs of beaked sedge rhizomes grown under aerobic (above) and hypoxic (below) conditions. Radial sheets of cell walls are clearly visible in the cortex. The bar represents 1 $x10^{-3}$ m.



7.3. Discussion

Since the studied barley seedlings differed in their anoxia tolerance (see chapter 3), when the whole plants were under anoxia, the dissimilarities in their root anatomy (aerenchyma formation) could not have affected their survival. This evidently shows the existense of metabolic differences between the cultivars. In the flooding tolerance experiment (see chapter 3) where only the roots where flooded, aerenchyma formation could have affected the survival and productivity of the plants. Therefore, it was of interest to examine the structure of the roots grown under aerobic and hypoxic conditions. A similar study with the three barley cultivars was carried out earlier by the author (M.Sc. project) on a light microscopical level and lysigenous development was noticed to take place in the cortex of the roots. However, the scanning electron microscope used in the present study gives a three dimensional picture of the roots which helps to give a better understanding of the continuity of the cavities formed. From the results it was clear that a development of cavities takes place in the cortex of the barley cultivar studied; however, it cannot be concluded from SEM studies alone whether these cavities were filled with air or with liquid. With the beaked sedge roots and rhizomes, however, this

problem was resolved, since air bubbles could be readily detected by pressing some submerged roots and rhizomes.

The significance of aerenchyma in aeration has for long been of considerable debate (Armstrong etc.). However, it is well known that many marsh plants develop aerenchyma which seems to contribute to the survival of many wetland plant species (Kawase, 1981a). Aerenchyma in the roots is formed either schizogenously or lysigenously and since it is present even under well oxygenated conditions its development seems to be under genetic control in wetland species. Hypoxic conditions can enhance the formation of aerenchyma still further and increase the internal porosity of the roots (Armstrong, 1971; Das and Jat, 1977). Lysigenous aerenchyma can also develop in newly formed adventitious roots that emerge from the base of the shoot in many nonwetland species, including barley and other crops, in response to waterlogging or low O_2 concentration (Bryant, 1934; Arikado, 1955a,b; Arikado and Adachi, 1955; Drew and Sisworo, 1979; Benjamin and Greenway, 1979). The formation of lysigenous aerenchyma in response to a shortage of oxygen takes place in the cortex, external to the endodermis, and becomes well developed in the zone 3-4 $x10^{-2}$ m behind the root tip in elongating roots of maize (Drew et al., 1979), rice (Armstrong, 1971), and wheat (Trought and Drew, 1980b), where cell extension growth is complete. The formation of such gas spaces has been attributed to the death and dissolution of cells caused by anoxia (McPherson, 1939).

The growth of roots in wet soil is probably a good indicator of the insufficiency of internal aeration in the nonwetland plant; recorded observations also demonstrate some degree of adaptation to the wetland environment among nonwetland species, although the plasticity of response found in wetland species is never realised. Yu et al. (1969) observed the root growth of several nonwetland crop species under a whole range of soil treatments, which included full flooded, half flooded and drained conditions. With the exception of barley, the roots produced in the drained treatments were invariably of lower porosity than those that grew in the fully flooded soil. Maize showed the greatest degree of adaptation to waterlogged conditions, but notably poor penetration occurred in tomato (5 $x10^{-2}$ m), wheat (4-10 $x10^{-2}$ m) and barley (12 $x10^{-2}$ m). Penetration by barley was nevertheless more than expected with its low root porosity of 2.4%. This could have been accounted for by a particularly low respiratory rate. Yu et al. (1969) were thus able to explain their observations and the variations discovered in terms of the factors likely to control the internal ventilation of the roots. Similarly, others have related the depth of wet soil penetration by nonwetland species to the oxidizing activities of their roots. However, lysigenous aerenchyma formation has been noted to take place in barley roots under flooding stress (Bryant, 1934; Arikado and Adachi, 1955). A detailed account of the ventilating structure of wetland plants can be found elsewhere (Sifton, 1945, 1957; Sculthorpe, 1967; Arber, 1920).

As early as in 1921 Dunn noted that maize roots grown under normal conditions did not develop aerenchyma or lacunae, but in hydroponic cultures the roots of intact plants developed air spaces in the cortex of root tissue. Also in barley seedlings similar changes have been recorded (Bryant, 1934). However, anatomical preparates do not show whether the lysigenous spaces are filled with air or water; the experiments have to include measurements of porosity. This has been achieved by Benjamin and Greenway (1979), who measured increased porosity of barley root tissue in a hydroponic culture deficient of oxygen.

In 1939 McPherson proposed a theory on the development of air spaces in maize. According to this the cortical cells loose turgidity and fall apart, which is due to oxygen deprivation starting a reaction which leads to the disintegration of proteins and the cytoplasm. Air spaces did not develop if the nutrient solution contained enough calcium, which was said to turn the pectic acid of the cell walls to calcium pectate. It has also been suggested that the synthesis of polysacharides, the essential building blocks of cell walls, is hindered by oxygen deprivation, which again would affect air space formation (van der Heide et al., 1963).

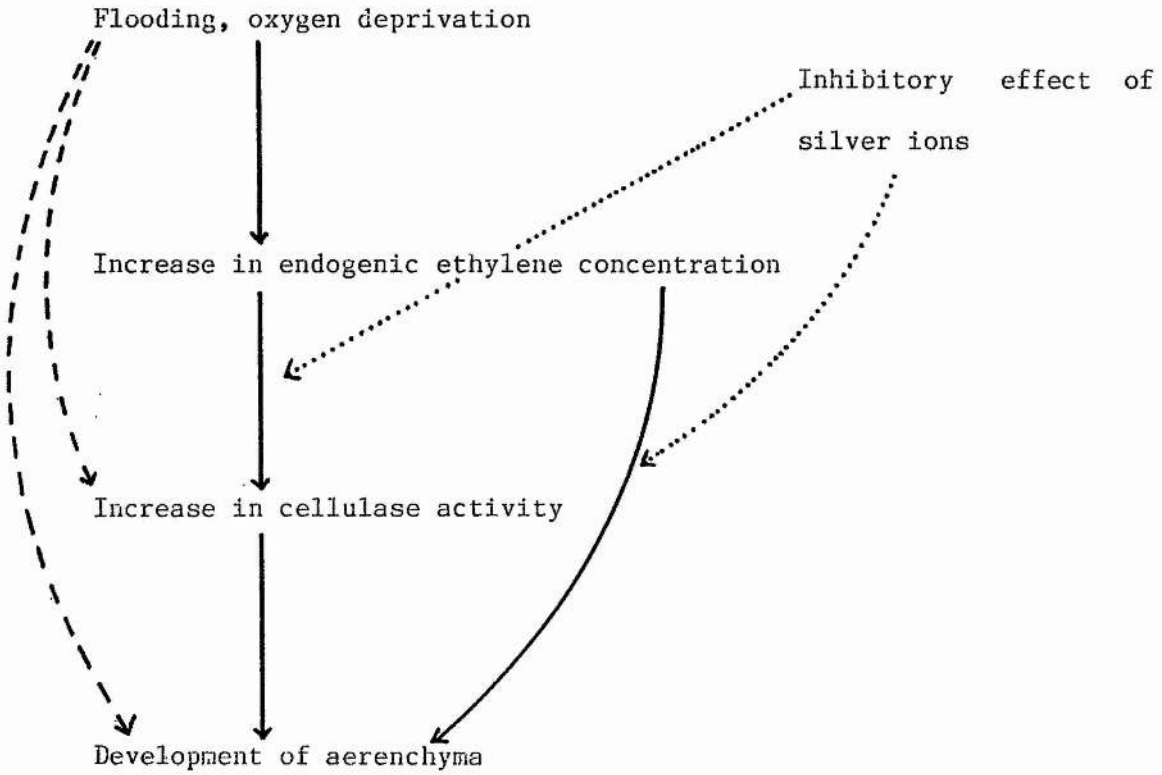
The effect of indoleacetic acid (IAA) on aerenchyma formation has proved to be of some interest. IAA treatment induces the softening of cell walls (if calcium concentration is low) (Grable, 1966) by causing the methylation of pectic acid (van Overbeek, 1959). It has been

noticed that in a non-aerated $CaSO_4$-solution barley roots develop air-spaces and the Ca-concentration of these roots is lower than in control roots (Pitman, 1969). Therefore, an IAA induced softening of the cell walls could have taken place. Also, the enlargening of cells by IAA suggests that IAA does have a softening action on cell walls. Datko and Lahlan (1968) have indeed shown that IAA induces glucanase-1,4-activity in Pisum sativum, and that sections of tissue developed lacunae if treated with an IAA solution. Because inactivation of IAA by peroxidase uses oxygen, it seems possible that flooding or oxygen deprivation results in greater IAA concentrations in the roots provided that the transport of IAA from the shoots to the roots is not hindered.

Furthermore, ethylene has also been found to be of importance in the development of aerenchyma. Kawase (1974, 1976), Kawase and Whitmoyer (1980) and Drew et al. (1979, 1981) have noticed a connection between ethylene and flooding damage in roots. Flooding stress causes an increase in ethylene concentration in root tissue, which possibly induces cellulase activity leading to the development of aerenchyma (Kawase, 1979, 1981a,b). This theory is supported by the fact that silver ions, which inhibit ethylene action (Beyer, 1976, 1979), also suppress the development of aerenchyma in maize (Drew et al., 1981) (Figure 7.7). The following generalisation of aerenchyma formation in flood intolerant species can be made: Oxygen deprivation induces a biochemical process which leads to the death of cells in the cortex of root tissue. The biochemical background is not well known

as yet, but ethylene, IAA and calcium seem to have an important role in the development of air spaces in root tissue.

FIGURE 7.7. The physiological background of aerenchyma development as collected from the following publications: Beyer 1976, 1979; Kawase, 1979; Drew et al., 1981.



8. MAJOR CONCLUSIONS

The first part of the thesis, flooding and anoxia tolerance of barley cultivars, showed clearly that there are marked differences between barley cultivars both in their anoxia and flooding tolerance and that these differences could be of value to the commercial farmer. Even the smallest differences between cultivars are of importance in large scale cultivation of barley. It is well known both to the breeder and the farmer that the introduction of a new cultivar commonly increases the yield by only 1%, but this is enough to warrant the decade-long breeding of that particular cultivar. Furthermore, the anoxia and flooding experiments confirmed the earlier assumptions of a positive correlation between anoxia and flooding tolerance.

Also, the morphological study of the barley seedlings with a TTC-test showed the most anoxia-intolerant organs being the root tips. It is pointed out here that the differences between cultivars may have been seen at this stage, since in the most tolerant cultivar, Kustaa, the bases of the roots remained alive longer than in the more intolerant cultivars.

The alcohol dehydrogenase activity and K_M value determinations of barley and beaked sedge root tissue indicated that the increase in ADH activity during hypoxic periods was accompanied by

a change in the ADH isozyme content of the tissues. In adventitious roots of barley the K_M of ADH for acetaldehyde decreased considerably indicating greater affinity for the substrate. Similar, but smaller changes could be detected in seminal roots of barley and also in beaked sedge root ADH. It is suggested that the changes in the activity and kinetics of barley root ADH are an adaptation to short-term oxygen deficiency. The increase in ADH activity during oxygen deprivation reflects the overall increase in the rate of glycolysis to produce energy for the demands of metabolism while citric acid cycle is blocked. Why the induction of activity is so great while ADH is not one of the pacemaker reactions of glycolysis, is not yet known.

The determination of ethanol and CO_2 production and dry weight loss under anoxic conditions revealed the greater loss of dry weight in the most anoxia-intolerant cultivar, Pokko, compared with that of the most tolerant, Kustaa. Only c. 20% of the loss in dry weight could be accounted for by ethanol and CO_2 production during the same period. It is pointed out, though, that lactate production may have been greater in the intolerant cultivar, since leakage of lactate has been shown to take place in roots under oxygen deprivation (Hiatt et al., 1967).

The most recent experiments into the antioxidant protection of plant tissues and post-anoxic damage proved to be very interesting. It seems that damage after an anoxic period takes place when the

reduced compounds which have accumulated during anoxia are quickly oxidised. This uncontrolled oxidation has been detected by measurements of malonylaldehyde and ethane production, both of which are products of lipid peroxidation. In anoxia-intolerant plants such as barley and Iris germanica a surge of malonylaldehyde production takes place after an anoxic period, whereas in the very anoxia tolerant Iris pseudacorus oxidative damage does not seem to occur, and, furthermore, the activity of the key enzyme in antioxidative protection, superoxide dismutase (SOD), increases significantly. However, it remains to be shown that the anoxia tolerance and increased SOD activity are in a cause-effect relationship, and not a mere correlation which happens to take place under anoxic conditions in this plant species. In animal tissues SOD has been shown earlier to be the most important enzymatic defence against oxidative injury.

The examination of the root tissue of barley and beaked sedge showed large differences in root anatomy between these species. Some lysigenous development in the cortex of adventitious barley roots was recognizable.

9. FUTURE PERSPECTIVES

It seems that it is difficult to assess the significance of the induction of alcohol dehydrogenase activity on the background of the physiological data available. Future research in the line of plant molecular biology will eventually reveal the mechanism and causes behind induction of ADH and, hence, the "meaning" of increased levels of ADH activity under flooded conditions. Chang and Meyerowitz (1986), Ellis *et al.* (1987) and Ferl *et al.* (1987) have initiated such research but it seems that a considerable effort in basic research is still needed to allow the gap between plant physiology and gene technology to be filled in. If the promoter sites could be isolated and the signal component identified, the promoter could be used in the synthesis of other proteins as well and not only with ADH.

The author himself was astonished that there were considerable differences in the anoxia and flooding tolerance of the three studied barley cultivars. Naturally, field experiments are needed to ascertain that the cultivars truly differ in their tolerance of waterlogged conditions in an agricultural environment, which can be quite variable and different from the experimental conditions imposed on the plants in this thesis. However, even on the basis of these simple experiments, we can say that most probably the cultivars would

behave in a similar manner in the open field. Hence, we could go and suggest to the farmer that if his field is prone to waterlogging, to sow this cultivar for a maximum crop in the event of an unfavourable summer.

Superoxide dismutase activity and inducibility of superoxide dismutase has proved to be a new and exciting difference between the flood tolerant Iris pseudacorus and intolerant Iris germanica. It remains to be seen whether this difference extends to any other species. Future research with more exact methods will reveal whether the induction is truly de novo synthesis of the enzyme or some form of activation of dormant proenzyme. This could be achieved by isopycnic equilibrium centrifugation with density labeling of the enzyme by adding deuterium oxide to the growth medium. SOD produced under oxygen deprivation could then be detected as a shift in the density of the enzyme in the CsCl-gradient (Filner and Varner, 1967).

Naturally, it would be of interest to investigate the genetic background of the induction of SOD by first purifying the enzyme and then creating antibodies for immunoelectrophoresis, and gradually extending the work to finally reveal the promoter sequence etc. In addition, antibodies can be used in immunogold labeling of TEM thin sections to localize the enzyme in question.

There is another possibility of examining the role of SOD in protection of plant tissues from oxidative damage, which has been

earlier used in experiments with animal tissues. External SOD can be applied to tissues which are suffering from oxidative damage. If this addition of SOD (in physiological concentrations) has an effect on the survival of the tissue it can be concluded that induction of SOD in similar conditions would have a similar effect. Experiments of this nature have been conducted with animal tissues (Woodward and Zakaria, 1985), but it is doubtful whether supplied external SOD would enter plant cells.

Furthermore, experiments with specific inhibitors of SOD activity would be useful in evaluating the importance of SOD in I.pseudacorus rhizomes. Some inhibitors of SOD activity are known, but they are not very specific.

10. ACKNOWLEDGEMENTS

My thanks are due to my supervisors Prof. R.M.M. Crawford of St Andrews University and Prof. L.K. Simola of Helsinki University and the research staff of both the Department of Plant Biology and Ecology of St Andrews University and the Department of Botany of Helsinki University. I am especially grateful to Dr. Lorna Monk, Dr. Bill Good, Ms. Jenny Crawford and Mr. Donal Finegan for critical comments and companionship during the work. It has been a tremendous experience to have been able to work with such a wide range of excellent researchers.

The first year of this research work was supported by grants from the British Foreign and Commomwealth Office through the British Council, and from the Academy of Finland. The subsequent two years of research were funded by the Academy of Finland, the University of St Andrews and the Overseas Research Student scheme. All the grants are gratefully acknowledged.

11. PUBLICATIONS

The following articles based on this work have been written during the course of the three years of research for this thesis:

FAGERSTEDT, K.V. and CRAWFORD, R.M.M. 1986: Changing kinetic properties of barley alcohol dehydrogenase during hypoxic conditions. - Journal of Experimental Botany, 37: 857-864.

FAGERSTEDT, K.V. and CRAWFORD, R.M.M. 1987: Is anoxia tolerance related to flooding tolerance? - Functional Ecology 1: 49-55.

MONK, L.S., FAGERSTEDT, K.V. and CRAWFORD, R.M.M. 1987: Superoxide dismutase as an anaerobic polypeptide - a key factor in recovery from oxygen deprivation in Iris pseudacorus? - Plant Physiology 85 (in press).

MONK, L.S., FAGERSTEDT, K.V. and CRAWFORD, R.M.M. 1987: The paradox of oxygen toxicity and the role of superoxide dismutase in physiological stress. (Under consideration in Physiologia Plantarum).

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