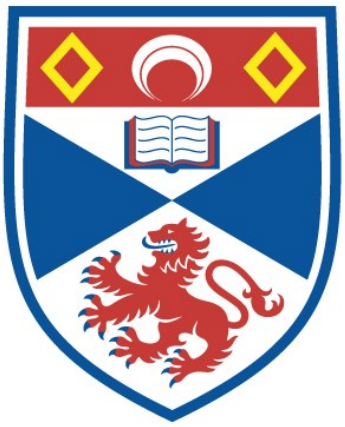
POST-ANOXIC INJURY IN HIGHER PLANTS

Lorna Sophia Monk

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



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ABSTRACT

The perennating organ, the rhizome, was chosen for examination of response to anoxia in monocotyledonous species known to differ in tolerance of flooding. Survival of prolonged anoxia was monitored in the wetland species (Acorus calamus, Iris pseudacorus, Phragmites australis, Schoenoplectus lacustris and Typha latifolia), and the dryland species (Iris germanica). Fermentation capacity was estimated in the anoxic and post-anoxic phases together with ethanol and lactate accumulation. Under N_2 an accumulation of ethanol took place in all species; lactic fermentation was of less importance. There was a steady-state condition of low ethanol accumulation in the wetland species, where an apparent equilibrium of production and elimination from rhizome tissue was reached. In contrast, the dryland species showed a continuous increase in ethanol accumulation during oxygen deprivation.

Catalase and superoxide dismutase activities were measured during the post-anoxic recovery phase in their role as enzymatic defences against oxygen toxicity. In rhizomes of the anoxia tolerant species A.calamus, S.lacustris and I.pseudacorus, no changes in catalase activity were observed. However, the relatively anoxia intolerant Glyceria maxima, Juncus effusus and I.germanica exhibited significant increases in catalase activity. To investigate whether catalase (acting in its 'peroxidatic' mode) was perhaps here involved in an ethanol oxidation reaction, anaerobic rhizomes of G.maxima were exposed to ethanol vapour: considerable increases in catalase activity were recorded. It is proposed that acetaldehyde thus produced may be a source of post-anoxic injury. Superoxide dismutase activity in

I.pseudacorus rhizomes rose significantly during anoxia, while small increases were found in the less anoxia tolerant I.germanica and a drop in activity was recorded in G.maxima. It is suggested that I.pseudacorus may be less susceptible to post-anoxic injury by means of oxygen toxicity, while in the other species oxidative damage on return to air may contribute to anoxia intolerance.

Declaration

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

Signed

Lorna S. Monk

St Andrews

December 1986

Statement

I was admitted to the Faculty of Science of the University of St Andrews under Ordinance General No. 12 in October 1982 and as a candidate for the degree of Doctor of Philosophy in November 1984.

Signed

Lorna S. Monk

St Andrews

December 1986

Certificate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Doctor of Philosophy.

Signed

R.M.M. Crawford

St Andrews

December 1986

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Ecological aspects of the wetland niche

Wetland plants occupy an ecological niche that poses definite physiological problems to unadapted plants. One of these problems is the relative unavailability of molecular oxygen in standing water. When a soil becomes waterlogged, existing plant root systems and microbial activity rapidly use up any dissolved oxygen, and since diffusion of oxygen - and carbon dioxide - through an aqueous medium is c. 10^{-4} times slower than in air (Grable, 1966), oxygen supply from the soil is cut off and gases formed by soil metabolism accumulate. Unless the plant possesses a well-developed aerenchymatous system throughout the shoot, which can be assumed to be in contact with the atmosphere thus providing a gaseous diffusion pathway to submerged parts of the plant, these tissues will switch over to anaerobic respiration.

Under these hypoxic conditions, which appear within hours of flooding, the redox potential (E_h) of the soil will simultaneously fall and continue to decline over a number of days or weeks (Ponnamperuma, 1984). Facultative and obligate anaerobic bacteria, which proliferate in waterlogged soils use oxidized soil components and dissimilation products of organic matter as electron acceptors. Low redox potentials are thus brought about through denitrification and the sequential reduction of manganese, iron and sulphate and finally the formation of methane (Gambrell and Patrick, 1978). Therefore, plants rooted in such an environment may be subject to nutrient toxicity by uptake of the increasingly large amounts of

soluble ferrous and manganous ions available. In addition, toxins such as hydrogen sulphide, may also prove injurious to plant tissues (Vámos and Köves, 1972).

Tolerance of flooding, as seen in wetland plants as a group, may then be constituted of adaptations to more than one physiological stress. Within this group it is clear that many species possess anatomical characteristics such as aerenchyma which circumvent or at least reduce the impact of the anoxic stress (Smirnoff and Crawford, 1983), rather than being truly physiologically adapted. Reed-grass (Glyceria maxima) appears to be one of these species (Barclay and Crawford, 1982; ap Rees and Wilson, 1984). Other species show both physiological and morphological adaptation e.g. the bulrush (Schoenoplectus lacustris), (Steinmann and Brändle, 1981; Monk et al., 1982).

A lacunar system extending from the shoot to the rhizome where applicable, and the root is a morphological adaptation predominantly found in monocotyledonous wetland plants (Crawford, 1982), which may not only provide efficient supplies of oxygen to the roots, but also allows some escape of the gas, creating an oxidized rhizosphere (Armstrong, 1979). In this way the root is protected against the toxic effects, for instance, of soluble ferrous ions by their oxidation to the insoluble ferric form. Release of oxygen into the surrounding medium has also been shown in rhizomes of Schoenoplectus lacustris (Haldemann and Brändle, 1983).

Recently, Schröder et al. (1986) confirmed that the aquatic dicot Nuphar lutea possesses a mechanism for pressurized ventilation of rhizomes rooted in anaerobic mud driven by thermo-osmotically active leaves, first reported by Dacey (1980). This, it was suggested, would not only provide oxygen but would also remove carbon dioxide which might be produced by anaerobic respiration in any hypoxic tissues.

Physiological adaptations to anoxia come into play when plants not reliant on any aerenchymatous system are flooded, or when silting occurs, or winter die-back takes place in wetland plants, cutting off the oxygen supply to submerged organs. Severe oxygen shortage will appear in a short space of time and physiological responses have been found to vary according to tissue, length of oxygen deprivation and the particular species. Under anoxic experimental conditions, where there is complete deprivation of oxygen, roots have been shown to survive only hours or days (review by Jackson and Drew, 1984). Whether hypoxic or anoxic damage to roots is lethal or not may depend on the developmental stage of the plant (Stanley et al., 1980). Alcoholic fermentation as a means of anaerobic respiration, has been proposed to be more efficient in the hypoxic adventitious roots of barley than in the seminal roots, leading to a more favourable energetic status within the more mature roots (Fagerstedt and Crawford, 1986). Therefore, for a given limited period of anoxic stress younger plants of this barley cultivar could be expected to be more susceptible to injury.

In a year long study of physiological responses to flooding in swamp tupelo (Nyssa sylvatica), Keeley (1979) found that the original root system died very quickly (within days of flooding) to be replaced by new more succulent roots in the first month in flood tolerant ecotypes. At the end of the year a root system similar to the initial roots present was found. Thus, roots appear to have limited anoxic survival capacity in vivo and in vitro, and seem relatively expendable even in flood tolerant plants, older roots giving way to better adapted new ones.

In contrast to roots, rhizomes in general have plentiful carbohydrate reserves, which may be a limiting factor in root anoxic survival (Webb and Armstrong, 1983). Rhizomes of some wetland taxa are able to remain healthy during periods of imposed anoxia of weeks or months, regenerating shoots and roots on re-introduction to air (Brändle and Crawford, in press). In the over-wintering perennating organs of wetland plants such an adaptation in rhizomes is of vital ecological importance. The physiological adaptations allowing this extended survival are not fully understood. A study featuring the pre-formed buds on rhizomes of the Lesser Reedmace (Typha angustifolia) has revealed appreciable capacity for anoxic and hypoxic biosyntheses (Jenkin and ap Rees, 1986), not found in roots of the relatively anoxia intolerant Glyceria maxima (ap Rees and Wilson, 1984), although no biochemical explanation for the phenomenon was put forward.

Parameters of anoxia tolerance to be investigated

The problems of adaptation to prolonged periods of oxygen deficiency, as seen by the author fall into two areas, that of survival of the anoxic period and that of recovery from the stress. Some plants appear to suffer post-anoxic injury rather than succumbing to the actual anoxic stress (Hunter et al., 1983). In the wetland niche some degree of physiological tolerance of oxygen deprivation may be advantageous. The extent of physiological adaptation in the rhizomes of wetland taxa may be related to the habitat specialization within the niche (Brändle and Crawford, in press): best adapted will be those found in permanently anaerobic muds, next those growing at the interface zone between lake mud and free water, and least adapted will be those from only seasonally flooded soils. Species from each of these categories, as well as one dryland species were chosen for the study in hand, and rhizomes were monitored during a post-anoxic recovery phase in addition to during the actual anoxic treatment.

Although the experimental imposition of, and removal from, anoxia will be more sudden than the stress as it occurs in rhizomes in the field, it is assumed that this will provide a clear physiological response. Previous experiments comparing post-anoxic recovery in the rhizomes of a wetland and a dryland Iris species have revealed that protection from post-anoxic injury may be a contributing factor to anoxia tolerance in the wetland species (Hunter et al., 1983). Therefore, while in Part I of this thesis anaerobic metabolism, and in particular alcoholic fermentation and accumulation of potentially toxic anaerobic metabolites is investigated, in Part II post-anoxic catalase and superoxide dismutase activities are determined with

regard to the efficiency of the cellular defence system against oxygen toxicity after the prolonged imposed absence of oxygen. Catalase activity is also examined in relation to a possible role in post-anoxic ethanol metabolism. Catalase-aided ethanol oxidation has been well documented in animals (Oshino et al., 1973), but not so far in plants. From the data gathered new features of anoxia intolerance are identified for the rhizomes of species unable to survive long-term oxygen deprivation.

PART I

SURVIVAL OF ANAEROBIOSIS

CHAPTER 2

FERMENTATION CAPACITY IN RHIZOMES OF SIX SPECIES

INTRODUCTION

Roots and other underground organs of plants require a supply of oxygen for the support of aerobic respiratory metabolism, and this comes mainly from the root environment. For the majority of plants the distribution of roots through soil may be influenced by oxygen availability. Huck (1970) has shown that the apical meristems of cotton and soybean taproots are killed within hours of oxygen deprivation, and subsequently lateral roots develop in an upper zone where oxygen is not limiting. Wetland plants constitute a group of plants that do not rely on an aerobic rooting medium, but are morphologically or metabolically adapted to standing water or waterlogged soil. Within this group of plants monocots commonly provide efficient oxygen transport to underground organs via a lacunar system, consisting of gas spaces continuous throughout the shoot, rhizome if present, and roots (Smirnoff and Crawford, 1983; Studer and Brändle, 1984). This morphological adaptation may, or may not be, accompanied by metabolic adaptation to lack of oxygen (Crawford, 1982; Jenkin and ap Rees, 1986).

The point at which root respiration first begins to suffer from oxygen shortage is termed the critical oxygen pressure (COP), and as Crawford (1982) indicates in a review on physiological responses to flooding, may be species specific. Oryza sativa roots showed an *in vitro* COP of 0.21 atmospheres oxygen (Luxmoore et al., 1970), while Eriophorum angustifolium roots, again *in vitro*, showed a COP of 0.14

(Armstrong and Gaynard, 1976).

Limiting oxygen concentrations equivalent to the COP have also been defined for a range of other root physiological properties (Hopkins et al., 1950), including ion uptake and root extension. Bertani and Brambilla (1982a,b) have shown the different points of initiation of progressive increase in alcohol dehydrogenase (E.C. 1.1.1.1) activity in root tips of wheat and rice in response to decreasing - 10% to 1% and 5% to 0% respectively - oxygen concentrations.

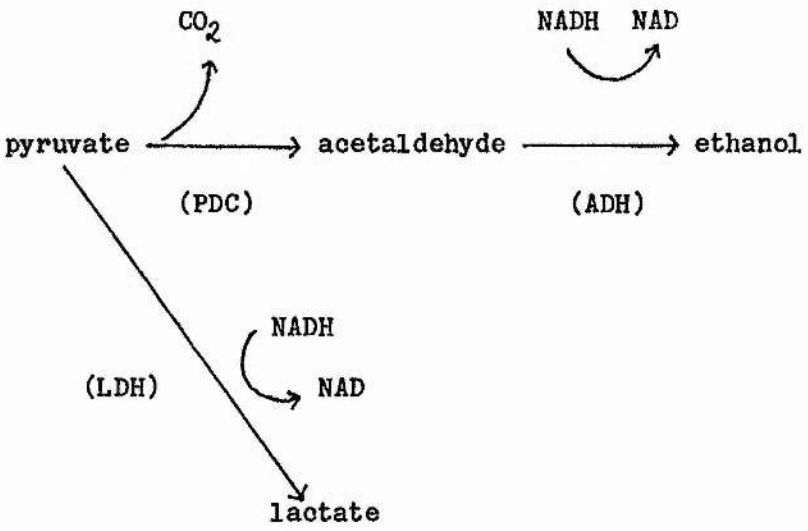
The appearance of high activities of alcohol dehydrogenase (ADH) in response to oxygen deprivation in maize roots has been attributed to the de novo synthesis of new ADH isozymes (Sachs et al., 1980). These workers identified a new pattern of polypeptide synthesis under anaerobiosis, which they interpreted as the expression of metabolic adaptation to anoxia. The ADH isozymes were among the major polypeptides detected. In rice embryo, Mocquot et al. (1981) observed a correlation between the appearance of a modified pattern of polypeptide synthesis under anoxia and a high value for energy charge (EC), a measure of adenine nucleotide ratios in the cell: ATP + 0.5 ADP / ATP + ADP + AMP , (Atkinson, 1969; Pradet and Bomsel, 1978). On transfer to anoxia EC dropped sharply, to recover once the anaerobic polypeptides were manifested. Monk and Brändle (1982) have found a correlation between increases in ADH activity after three days anoxia and a simultaneous return of EC to levels approaching that of aerobic controls in Schoenoplectus lacustris rhizomes.

In the absence of oxygen, the electron transport chain is blocked, and synthesis of ATP via this system ceases. In addition, NADH and reduced flavoproteins formed during the Krebs cycle can also no longer be oxidized via the electron transport chain, and the cycle is thus halted. Glycolysis however continues, as the oxidation of NADH generated by this pathway may be achieved by the production of reduced compounds such as ethanol or lactate. The oxidation of one mol of glucose under these conditions gives a net yield of two mol ATP.

This does not compare favourably with ATP production under fully aerobic conditions where 38 mol are formed per mol of glucose oxidized. In order to adapt to a period of anoxic stress, the energy status of the cell must be adequate to allow at least key physiological processes to take place. According to Jackson and Drew (1984), ethanol glycolysis constitutes the main pathway that operates in plants to furnish this energy supply, but this is disputed by Crawford (1982), where more than one metabolic solution to the deprivation of oxygen is argued for, such as those occurring in facultative anaerobes, diving reptiles and mammals.

Accelerated rates of substrate utilization, termed the Pasteur effect (Turner and Turner, 1980) commonly occur under anoxia, due to a reduction in citrate and ATP which removes the allosteric regulation of the glycolytic pathway. Faster glycolysis may provide sufficient concentrations of ATP for cellular processes, but has also been correlated with excessive accumulation of the end product, ethanol, and subsequent death in various species of germinating seedlings (Crawford, 1977; Barclay and Crawford, 1981).

The putative self-poisoning effect in plant tissues by ethanol accumulation will be the subject of the following chapter. There has been much debate as to whether there is significant diversification of the end products of glycolysis in plants (Davies, 1980; Crawford, 1983; Jackson and Drew, 1984), and this will be discussed later in relation to ethanol production. Much work on anaerobic respiration has been centred on the alcholic and lactic fermentation reactions themselves and their possible control:



It has been proposed that lactate dehydrogenase (E.C. 1.1.1.27.), which forms part of the investigation described in this chapter, and pyruvic decarboxylase (PDC) together represent a cellular pH stat (Davies et al., 1974). Lactate dehydrogenase (LDH) has an alkaline pH optimum, and as lactate builds up in the cell pH will tend to fall, inactivating the enzyme. PDC, which competes with LDH for pyruvate, has an acidic pH optimum, so that as LDH activity decreases, PDC activity increases. Provided ADH has the capacity, there is an

increase in ethanol production, while rates of lactate production remain low. At acid pH values ATP has been shown to exert an allosteric effect on LDH from potato tubers (Davies and Davies, 1972). Control of the two fermentative reactions has been further investigated: Hanson et al. (1984) have suggested that the higher affinities for NADH and acetaldehyde found in the ADH isozymes induced under anoxia in barley roots (Mayne and Lea, 1984) could increase the ability of ethanol glycolysis to compete with lactate glycolysis.

The second enzyme featuring in the present investigation into fermentation capacity of anaerobic rhizomes is ADH. It is noted at this point that PDC is likely play an important regulatory role in alcoholic fermentation in view of the main control mechanism mentioned above, and the fact that it is located at the branch point of pyruvate metabolism. Parallel induction and synthesis of both PDC and ADH have been described in anoxic rice and maize roots (John and Greenway, 1976; Laszlo and St Lawrence, 1983). However, ADH catalyses the conversion of acetaldehyde to ethanol in the absence of oxygen, and changes in its activity will constitute "coarse" control (Turner and Turner, 1980) in the production of ethanol, and so activities of this enzyme were analysed.

MATERIALS AND METHODS

Plants of six different species were collected in the canton of Berne, Switzerland, as this piece of work was carried out at the Pflanzenphysiologisches Institut, University of Berne, Switzerland. Plants were grown until needed for experimentation in a glasshouse where the ambient temperature was kept between 14 °C and 20 °C. The wetland species were Acorus calamus L., Iris pseudacorus L., Phragmites australis Trin. ex Steud., Schoenoplectus lacustris (L.) Palla and Typha latifolia L. The typical dryland plant Iris germanica L. was included for comparison of response to anoxic stress.

All plants were harvested for experimentation in the winter months, between October and March, when metabolism is sustained at a more or less steady level, in contrast to marked seasonal changes during the rest of the year (Steinmann and Brändle, 1984). Rhizomes were first surface sterilized by bathing for 1 minute in 0.1% $HgCl_2$, and next underwent a 72 hour aerobic pretreatment. Pieces of rhizome were then incubated in the dark at 22-25 °C. A row of airtight glass jars, connected with tubing, served as incubation containers; rhizomes, with shoots cut off at 3-5 cm, were gassed with either atmospheric air or oxygen-free nitrogen. The anaerobic incubation lasted 15 days, with 16 days for the aerobic controls, and eight days nitrogen followed by nine days air for rhizomes monitored for recovery from anoxic stress.

At specific intervals, rhizomes were removed from the appropriate treatments and the rate of oxygen uptake was determined in pieces of 1 g in weight, in 100% humidity on a Gilson respirometer (GRP 14, Gilson Medical Electronics, France). Rhizomes for the determination of alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) were removed simultaneously, and 1-2 g of tissue was finely sliced, homogenized in 5 ml buffer, pH 7.4 (0.1×10^3 mol m^{-3} glycylglycine, 20 mol m^{-3} EDTA, 20 mol m^{-3} DIECA, 20 mol m^{-3} mercaptoethanol, plus 100 mg soluble PVP-40 per 5 ml buffer). The extract was then centrifuged at 5000 g for 15 minutes and subsequently passed through a Sephadex G 25 column for desalting. This final step in the preparation of the extract, all of which was carried out at 0-4 °C, improved the stability of the enzymes during the spectrophotometric assay, which was performed at 25 °C.

Soluble protein content of the extract was used as the basis for calculation of enzymatic activity, and was assayed colorimetrically by the Coomassie Blue method (Bio-Rad, Richmond, Calif., U.S.A.). Globulin served as a standard.

Preparation of extracts for the determination of sugars was carried out at 0-4 °C, as follows: rhizomes were homogenized in 6% (w/v) perchloric acid and thus deproteinized, then centrifuged at 3500 g and subsequently neutralized with 5.0×10^3 mol m^{-3} K_2CO_3. Total reducing sugars (glucose, fructose and maltose) were estimated colorimetrically with alkaline copper and arseno-molybdate reagents according to Somogyi (1952), using glucose as standard. Glucose, fructose and sucrose were determined enzymatically after Bergmeyer (1974), using test kits (Boehringer, Mannheim).

RESULTS

Rhizomes of all species showed depressed rates of oxygen uptake when removed from anoxia (Fig. 2.1). Lowest rates of oxygen consumption (less than 20 $\mu g\ O_2\ g^{-1}$ fr wt h^{-1}) were found in the dryland species, I.germanica. Nevertheless, when rhizomes were removed from the nitrogen treatment after eight days, and further incubated in air, all species showed an increase in oxygen uptake approaching that found in the controls. In I.germanica the rate of oxygen uptake during the recovery period was seen to overshoot the rate found in the controls by 100%. This high rate fell back to control levels approximately 72 hours after removal from anoxia.

Alcoholic fermentation, as estimated by alcohol dehydrogenase (ADH) activity, was found to operate on different levels in the various species tested (Fig. 2.2). In A.calamus rhizomes a relatively high ADH activity of between 500-600 nmol mg^{-1} protein min^{-1} occurred under all treatments. In contrast, I.germanica and I.pseudacorus both showed considerably lower activities in the region of 100 nmol mg^{-1} protein min^{-1} under all treatments. P.australis and S.lacustris, on the other hand, exhibited an activation or possibly induction of ADH under anoxia, where activity increased during the whole course of the treatment. Levels reached c. 350 nmol mg^{-1} protein min^{-1} in P.australis and c. 400 nmol mg^{-1} protein min^{-1} in S.lacustris. Activities decreased again during the post-anoxic recovery phase. Rhizomes of T.latifolia showed an increase of 100% to c. 330 nmol mg^{-1} protein min^{-1} by the eighth day of anoxic treatment, after which a decrease in activity, to a level comparable to that of the aerobic controls was observed. During the aerobic recovery phase

a similar fall in activity, to levels as found in controls, was seen.

Compared to alcoholic fermentation, lactic fermentation appeared to operate at considerably lower levels in all species (Table 2.1). After 15 days nitrogen treatment S.lacustris showed the greatest lactate dehydrogenase (LDH) activity, a six-fold increase compared to controls, to 54.5 nmol mg^{-1} protein min^{-1}. A two-fold increase in LDH activity under anoxia was recorded in A.calamus to 18.3 nmol mg^{-1} protein min^{-1}. Slight increases were observed in P.australis and T.latifolia, while no differences in LDH activity were seen between air and nitrogen treatments in rhizomes of either of the Iris species.

Figure 2.3 shows that rhizomes of all species possessed adequate amounts of carbohydrates to survive 15 days anoxia. Amounts of reducing sugars (glucose, fructose and maltose) did not differ significantly between the nitrogen and air treatments. However, levels varied between different species. S.lacustris rhizomes contained the largest amounts, c. 30 mg g^{-1} fr wt. When concentrations of sucrose were determined in the same rhizomes, no significant differences between anaerobic and aerobic treatments were found, with one exception. A.calamus showed a slight accumulation of sucrose during anaerobiosis, to c. 15 mg g^{-1} fr wt. Again, levels of this sugar varied between species.

FIGURE 2.1 Oxygen uptake in rhizomes of six species at 25°C, incubated in aerobic conditions (●—●), gassed with nitrogen (■—■), and undergoing further incubation in air subsequent to anoxia (▲--▲). Mean values from 2-4 rhizomes.

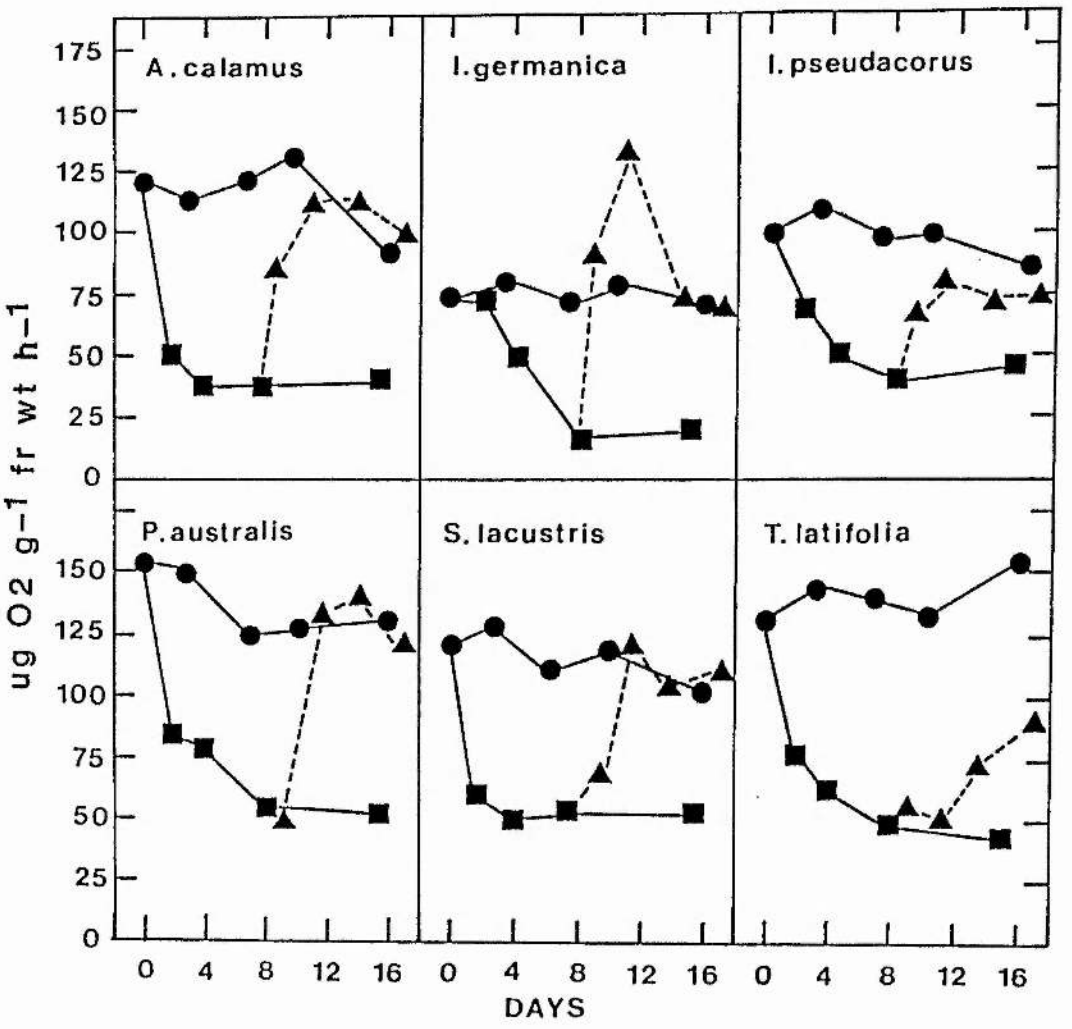

A. calamus
I. germanica
I. pseudacorus
P. australis
S. lacustris
T. latifolia
ug O2 g-1 fr wt h-1
DAYS
0
25
50
75
100
125
150
175
0
4
8
12
16

FIGURE 2.2 ADH activity in rhizomes of six species incubated in aerobic conditions (●—●), gassed with nitrogen (■—■), and undergoing further incubation in air subsequent to anoxia (▲--▲). Mean values from 2-5 rhizomes.

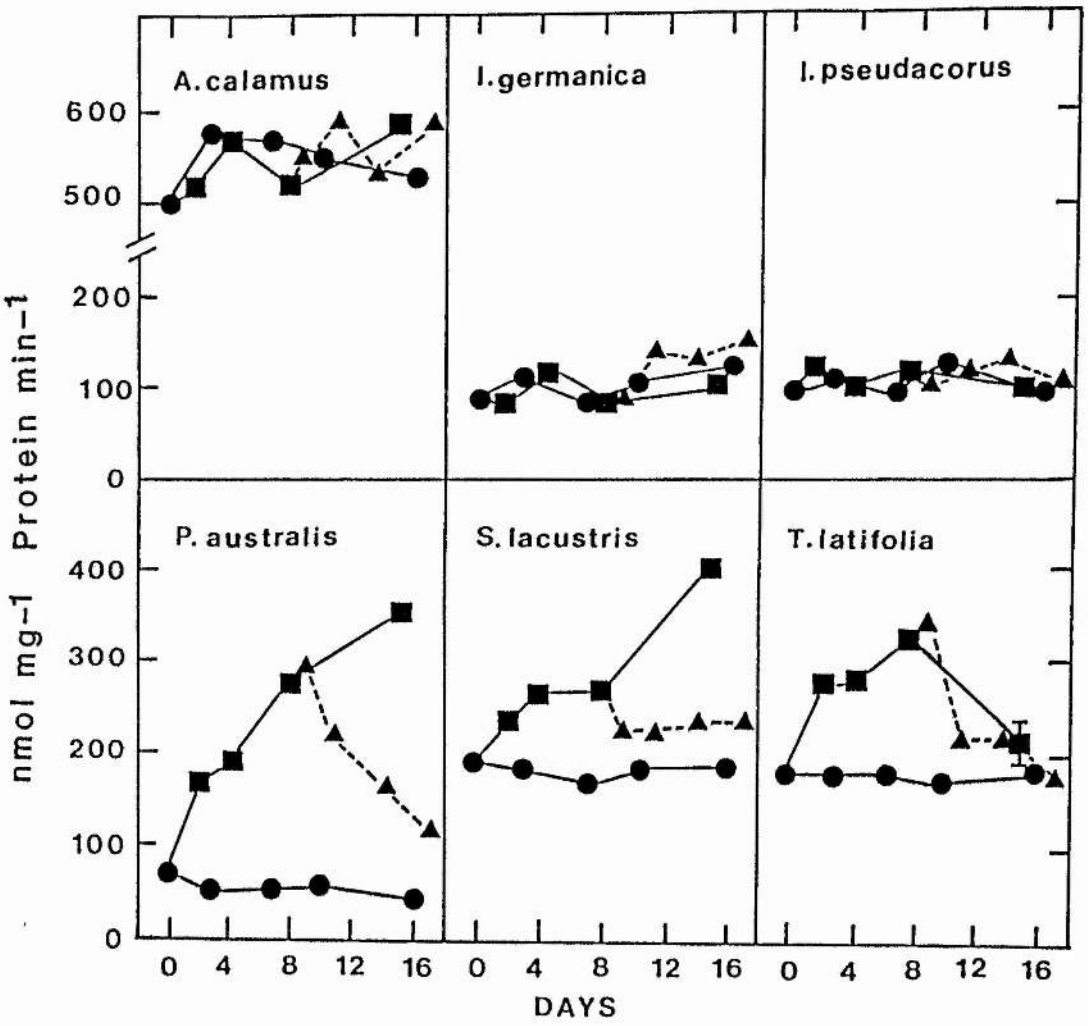

A. calamus
I. germanica
I. pseudacorus
P. australis
S. lacustris
T. latifolia
nmol mg-1 Protein min-1
DAYS
0
100
200
400
500
600
0 4 8 12 16

FIGURE 2.3 Concentrations of various sugars in the rhizomes of six species after 16 days in air/15 days in N_2. Blank column = total reducing sugars, shaded area = glucose, lined area = fructose. Mean values of 6-12 rhizomes ± S.D.

1 = *A. calamus*, 2 = *I. germanica*,

3 = *I. pseudacorus*, 4 = *P. australis*,

5 = *S. lacustris*, 6 = *T. latifolia*.

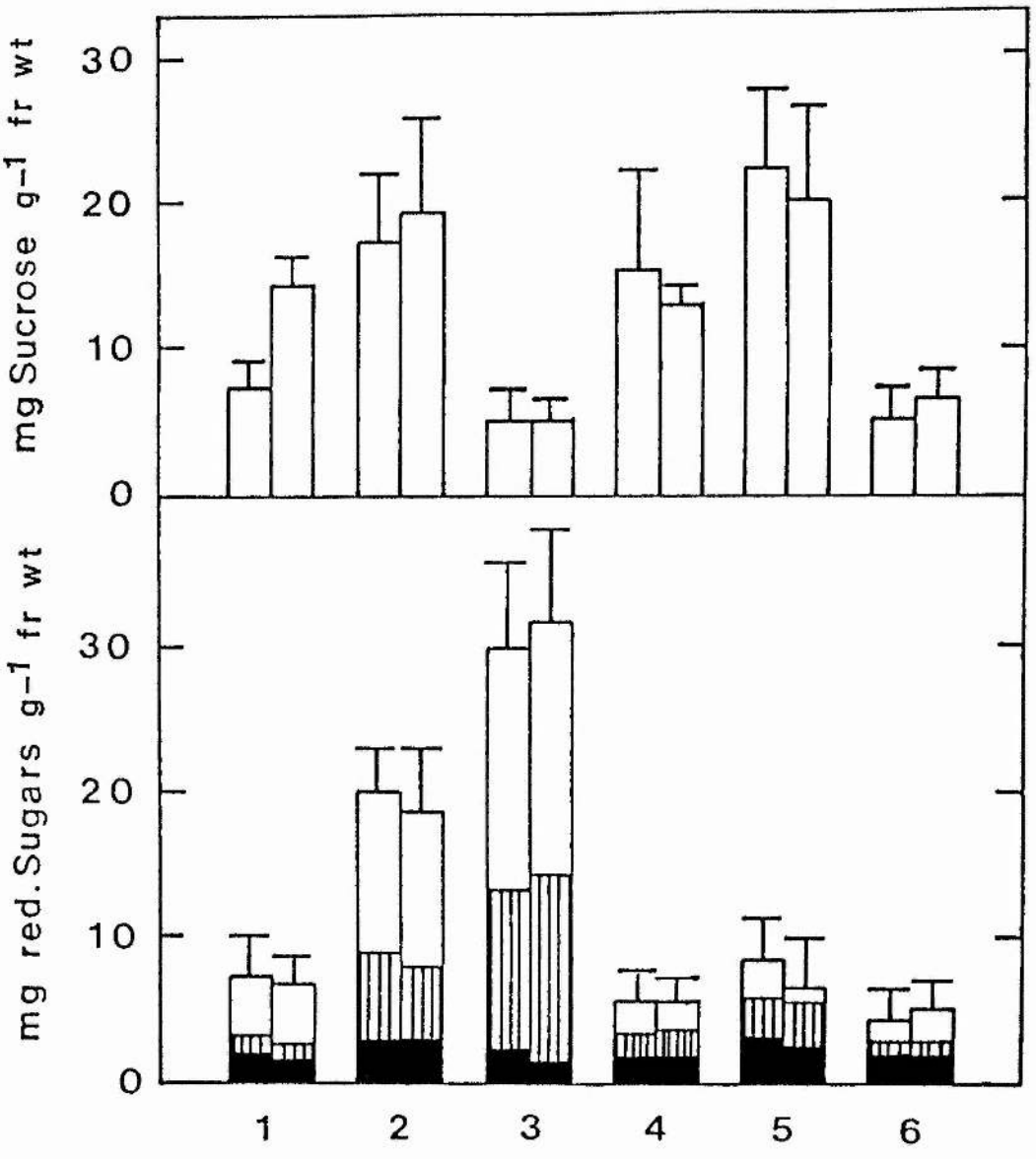

mg Sucrose g-1 fr wt
30
20
10
0
mg red. Sugars g-1 fr wt
30
20
10
0
1
2
3
4
5
6

TABLE 2.1 Lactate dehydrogenase activity in rhizomes of six species after periods of 15/16 days N_2/air. Mean values from 6-12 rhizomes ± standard deviation.

Species	Air	N_2
	LDH (nmol mg^{-1} protein min^{-1})	
A. calamus	9.5 ± 2.8	18.3 ± 4.0
I. germanica	4.4 ± 1.8	6.5 ± 2.2
I. pseudacorus	6.9 ± 2.5	5.7 ± 1.4
P. australis	17.5 ± 2.6	28.4 ± 7.8
S. lacustris	9.6 ± 4.3	54.5 ± 2.6
T. latifolia	8.7 ± 2.5	13.4 ± 2.3

DISCUSSION

The significance of changes in fermentation capacity

The present experiments were designed to examine metabolic adaptation to prolonged anoxia. All species showed recovery of aerobic respiration rates after eight days of oxygen deprivation (Fig. 2.1), so that it may be surmised that no irreparable damage at the ultrastructural level (Vartapetian et al., 1977) had taken place. Rhizomes of all species exhibited active rates of fermentation, as represented by ADH activity (Fig. 2.2) during periods of up to 15 days anoxia. It is known that the single dryland species, Iris germanica, survives only 14 days anoxia, where the criterion of survival is regrowth of shoots and roots after anoxic stress (Hetherington et al., 1983). However, collapse of the tissue and subsequent death apparently occurs on return to air (Hunter et al., 1983; Chapter 6). The large overshoot in rate of oxygen uptake during the recovery period in air after eight days anoxia may derive from non-respiratory as well as respiratory processes. This phenomenon may be more pronounced after longer periods of anaerobiosis and signal death of the tissue; the issue will be discussed further in Chapters 4 and 6 in the light of possible oxidative damage.

In three of the species analysed - I.germanica, I.pseudacorus and A.calamus - no increases in ADH activity were recorded during the course of the 15 day anoxic treatment. The capacity to catalyse the conversion of acetaldehyde to ethanol at greater rates was apparently already sufficient in the rhizomes of these taxa. From data which is presented in Chapter 3, it can be seen that significant ethanol

accumulation occurs in rhizomes of these species. It is clear that although the constitutive level of ADH does not change, there are almost certainly considerable increases in PDC activity. Since PDC evidently regulates alcholic fermentation in these cases, PDC would here have been superior to ADH as an indicator of fermentation capacity. John and Greenway (1976) are of the opinion that both enzymes should feature in investigations into alcoholic fermentation.

In rhizomes of S.lacustris and P.australis, large increases (two-fold and five-fold, respectively) in ADH activity are seen in response to gassing with nitrogen for 15 days. T.latifolia rhizomes show a two-fold increase, which drops back to aerobic levels during the course of the treatment, probably due to some regulatory mechanism. Increases in ADH activity in these wetland plants are modest, but indicate that a metabolic pathway is being followed that will yield an energy supply in terms of ATP (Saglio et al., 1980; Monk and Brändle, 1982). Some anoxia tolerant wetland plants are not only able to maintain cellular processes under strict anoxia, but can also show shoot extension. Of the species in the present investigation, this has been observed in A.calamus (Brändle, pers. comm.), S.lacustris (Barclay and Crawford, 1982) and T.latifolia (Monk, 1982).

Coleoptile extension under complete anoxia has been observed in germinating rice seedlings (Vartapetian et al., 1978), and seedlings of the flood tolerant weed of rice, Echinochloa crus-galli (Kennedy et al., 1980). The ecological advantage of this costly (in terms of lost formation of ATP via glycolysis) behaviour, which is analogous to the shoot extension observed from the perennating organ of wetland plants, is that once the shoot breaks the surface of the water under which it

is flooded, an oxygen supply to other parts of the plant can be provided. In a recent report (Mayne and Kende, 1986), which followed glucose metabolism in anaerobic rice seedlings, the pentose phosphate pathway was found to be operative, and it was suggested that this reflected the capacity for coleoptile elongation and synthesis of new cell wall material. Similarly, Rumpho and Kennedy (1983) have detected increased activity of the pentose phosphate pathway in anaerobic seedlings of E.crus-galli, and have proposed that important metabolic intermediates are thus formed, as well as NADPH. It was suggested that this reducing power was used in the synthesis of fatty acids for membrane formation.

Metabolic adaptation of this nature may be present in the rhizomes of the three wetland species in this piece of work that send up shoots during anoxia. However, only alcoholic fermentation has been examined here, along with lactic fermentation (Table 2.1). LDH was found to be approximately an order of magnitude less abundant than ADH after prolonged anaerobiosis in all species. This is consistent with the proposed control of this enzyme, where accumulation of the product, an organic acid which acidifies the cytosol, increases the competitive ability of PDC and alcoholic fermentation (Davies et al., 1974), keeping lactic fermentation at low levels. More recently, Hanson and Jacobsen (1984) reported optimal LDH activities at low oxygen concentrations in barley aleurone layers, which declined markedly at 0% oxygen, and inferred that the LDH and ADH systems were independently regulated.

It is not known for certain whether there is de novo synthesis of enzymes for either of these fermentation reactions, or if the balance of synthesis and degradation is altered to produce changes in activity. There was no increase in LDH activity in either of the Iris species, and it is possible that ethanol glycolysis represents the main anaerobic respiratory pathway in these taxa. It should be noted that there have been reports of diversification of the end products of glycolysis in I.pseudacorus (Crawford, 1972), and in S.lacustris (Duss and Brändle, 1982), but alcholic fermentation appears to predominate. Lactic fermentation may be of significance in anaerobic S.lacustris rhizomes, as the largest increases in LDH activity are seen here. Nevertheless, lactic fermentation appears to be in general of secondary importance, if at all, during strict anoxia. Perhaps in view of Hanson and Jacobsen's findings (1984), lactic fermentation has a definite function in some plants under hypoxic conditions.

Availability of substrate for fermentation

A prerequisite for survival of long-term anoxia is the possession of carbohydrate reserves, and possibly conservation of those reserves. If a strong Pasteur effect is not at some point regulated, plentiful reserves such as those found in rhizomes will quickly be depleted (Barclay and Crawford, 1983). In a review on habitat specialization in wetland plants, different patterns of seasonal fluctuation of the carbohydrate content in rhizomes of various species were identified (Brändle and Crawford, 1986). There may be considerable seasonal variation of carbohydrate content: S.lacustris rhizomes show a peak in autumn, but are severely depleted by early

summer (Steinmann and Brändle, 1984). Carbohydrate content of the rhizome has been correlated with survival of anoxia: S.lacustris survives approximately 94 days oxygen deprivation in the winter, but only 28 days in the summer (Brändle, pers. comm.). T.latifolia also exhibits a marked seasonal fluctuation in rhizome carbohydrate content (Kausch et al., 1981). The metabolic cost of resuming growth in spring from rhizomes buried deep in anaerobic mud, the habitat of these wetland species, is high.

In contrast, A.calamus rhizomes show much shallower fluctuations in rhizome carbohydrate reserves (Haldemann and Brändle, 1986). Rhizomes of this species are typically found at the interface between anaerobic mud and free water, so that less reserves may be used in sending up new shoots in spring; in addition rhizome tissue is often green and may be actively photosynthesizing all year round.

In short-term anoxic experiments, lack of substrate has been associated with death of rice, pea and pumpkin roots within hours (Webb and Armstrong, 1983). Anoxia has also been observed to bring about (over four days) the progressive incapacity of rice roots to metabolize sucrose (Bertani et al., 1981). Translocation of soluble sugar has also been found to be halted by anoxia in saxifrage stolons (Qureshi and Spanner, 1973). These do not appear to be problems which occur in any of six species investigated here for capacity of fermentation reactions during prolonged anaerobiosis. Rhizomes of all taxa possessed adequate supplies of carbohydrate even after 15 days anoxia (Fig. 2.3). T.latifolia rhizomes showed a decline in alcoholic fermentation after seven days anoxia which may be the result of a regulatory mechanism conserving storage carbohydrate.

The ability to mobilize starch reserves during a period of oxygen deprivation has been reported for P.australis, S.lacustris and T.latifolia (Brändle, 1985). In addition, it has been suggested that in S.lacustris rhizomes subjected to anoxic conditions, synthesis of maltose as a transport substance from the rhizome to developing parts of the plant may be favoured, since synthesis of sucrose is more energy consuming (Duss and Brändle, 1982).

It can then be said that increased fermentation capacity, supported by adequate carbohydrate reserves, is correlated with survival of up to 15 days anoxia in rhizomes of the six selected species here. Other metabolic pathways, such as the pentose phosphate pathway, may be operative in some of the taxa; there may also be other end products of glycolysis than ethanol and lactate, with differing yields of ATP. However, it is not known to what extent these various reactions occur. In a population study of flood tolerant and intolerant ecotypes of Nyssa sylvatica, tolerant plants were on flooding initially found to develop roots with a higher capacity for alcoholic fermentation (Keeley, 1979). The energy supply thus provided apparently served to form another root system that had better aeration, which obviated the need for ethanol glycolysis, and was seen to be the long-term response to flooding.

In a recent study on fermentation reactions in roots of various marsh plants, Smith et al. (1986) observed a correlation between oxygen tension and alcoholic fermentation. When flooded, Filipendula ulmaria showed an increase in ADH activity with depth of root, which was consistent with the fact that roots of this plant possess little aerenchyma (Smirnoff and Crawford, 1983). ADH activity was 14 times

higher below ten cm depth than near the surface. It can be concluded that increased fermentation capacity in general provides a valuable energy supply as a response to anoxia. In the six species examined here different patterns of ADH and LDH activity were identified, reflecting differing control of ethanol/lactate glycolysis. Since the experiment involved responses to relatively long-term anoxia, some regulation of fermentation rates might be expected with respect to conservation of substrate, and perhaps also the avoidance of accumulation of ethanol/lactate. Whether there is metabolic or morphological adaptation to avoid the accumulation of potentially phytotoxic metabolites is the subject of the next chapter.

CHAPTER 3

FERMENTATIVE METABOLITE ACCUMULATION IN RHIZOMES OF SIX SPECIES

INTRODUCTION

During anaerobic respiration organisms throughout the plant and animal kingdoms have been observed to accumulate various fermentative metabolites (Crawford, 1982; Hochachka and Mommsen, 1983). The pathways leading to the accumulation of any of these substances may vary in energetic efficiency, and it will be discussed later whether the energetic cost confers a physiological advantage in particular cases. Lactate and ethanol are the most studied end products of fermentation in higher plants, although malate and alanine, to name but two alternatives, may well be of importance in certain groups of plants.

Of the known end products of fermentation, ethanol has been found in the highest concentrations in plant tissues. Barclay and Crawford (1981) reported that the internal ethanol concentration of 60 mol m^{-3} appeared to be the threshold value for seedling survival in peas. This concentration signalled anoxic death when achieved by any combination of temperature and length of anaerobic incubation. Intraspecific differences in the tolerance of flooding have been associated with the induction of an active vs. less active ADH isozyme. Susceptible cultivars possessed the active form, presumably leading to greater accumulations of ethanol, in the dicots Lupinus angustifolius (Marshall et al., 1974) and Trifolium subterraneum (Francis et al., 1974), and in the monocots Zea mays (Marshall et al., 1973) and Bromus mollis (Brown et al., 1976). Hence the debate as to

whether there is a self-poisoning effect by excessive accumulation of ethanol as an end product of fermentation.

Changes in the fluidity of the phospholipid bilayer of membrane systems can be brought about by high concentrations of ethanol (Kiyosawa, 1975; Ingram, 1976). However, it is not known whether physiological concentrations are sufficient to cause these changes, and if they are, whether the phenomenon is deleterious. In a review on the possible role of ethanol toxicity in flooding injury, Jackson et al. (1982) concluded that exogenously applied ethanol (in concentrations of up to 0.39×10^3 mol m^{-3}) had no significant adverse effects on pea plants. However, it has previously been shown that endogenously produced ethanol is considerably more toxic than ethanol applied exogenously (Nagodawithana and Steinkraus , 1976).

For every mol of ethanol produced one mol of carbon dioxide is formed through the decarboxylation of pyruvate. In a review by Bown (1985) on the impact of high CO_2 concentrations on intracellular pH, attention was drawn to the fact that bulky tissues and flooded root systems will commonly be exposed to a high presence of CO_2, which may cause a drop in pH within the cell. Dissolved CO_2 diffuses away very slowly compared to gaseous CO_2, resulting in accumulation within tissues. Stolzy and Sojka (1984) have stated that the concentration of CO_2 in flooded soils can exceed 12%, and Ponnamperuma (1976) has reported that concentrations of up to 50% CO_2 can persist for several weeks in cold and acid, waterlogged soils. According to Bown (1985) 5% CO_2 is sufficient to lower intracellular pH, with possible adverse effects on cellular metabolism, since pH is normally regulated within narrow limits.

In a study on toxicity of anaerobic metabolites by Andrews and Pomeroy (1979), ethanol was not found to exert toxicity when applied on its own to wheat seedlings, but when both CO_2 and ethanol were applied together there appeared to be an additive toxic effect. CO_2 was found to be injurious when applied in very high concentrations alone. In experiments subjecting bean roots to different gas mixtures Williamson (1968) found that high CO_2 concentrations rendered more damage than zero O_2. There may therefore be a physiological advantage to be gained in limiting the production of ethanol, or at least avoiding the accumulation of this metabolite, especially in conditions where CO_2 is likely to accumulate too (Crawford et al., in press).

The frequently investigated alternative to ethanol glycolysis is lactate glycolysis. The energetic yield from either of these pathways is the same, but lactate production is apparently strictly limited. A cytoplasmic pH stat that switches on production of ethanol as the pH of the cytosol drops due to formation of lactic acid (Davies et al., 1974), may be the main form of control of accumulation of this anaerobic metabolite. Roberts et al. (1984, 1985) have cited lactic fermentation as a contributor to cytoplasmic acidosis (along with CO_2 produced concomitantly with ethanol), which they argue is a determinant of flooding intolerance in plants.

Malate may be of importance as an alternative to the potentially toxic anaerobic metabolites mentioned above. Accumulations of malate have been found in a number of flood tolerant species, but not in flood intolerant taxa, when flooded (Crawford and Tyler, 1969). Recently, Smith et al. (1986) reported a significant accumulation of malate, equal to 30% of the amount of ethanol produced, in flood

tolerant Carex riparia roots after two hours anoxia. In a study on long-term flooding tolerance Keeley (1978) has shown that during the first month of inundation of swamp tupelo trees, ethanol fermentation predominated in the roots but later fell away, while malate production reached a peak after this and remained at this level throughout the year's experiment. The occurrence of malate as an anaerobic metabolite does not however appear to be widespread, and one reason for this may be that there is no net yield of ATP by its formation.

On the other hand, alanine production during anaerobiosis yields two mol ATP per mol glucose metabolized. Alanine accumulation as a product of fermentation has been found in appreciable quantities in roots of some marsh plants (Smith and ap Rees, 1979), and maize seedling roots (Kohl et al., 1978). The formation of glycerol as an end product of fermentation also provides a net gain of ATP, and small accumulations have been found in anoxia-treated rhizomes of S.lacustris, alongside greater quantities of ethanol and lactate (Duss and Brändle, 1982). Other metabolites that have been observed to accumulate during anaerobiosis include shikimate in I.pseudacorus rhizomes (Boulter et al., 1963) and proline and glutamate in roots of Senecio aquatica (Lambers, 1976).

However, it is generally thought that ethanol glycolysis is the main pathway that operates under anaerobic conditions in most plants (Smith and ap Rees, 1979; Davies, 1980; Jackson and Drew, 1984), and it is still open to debate whether diversification of the end products of fermentation contributes significantly to tolerance of anoxia, or indeed flooding. During prolonged anoxia or hypoxia such as experienced by wetland plants for at least part of the year, accumulation of fermentative metabolites will occur in the submerged

parts of the plant. It is known that some of the compounds in question, in particular ethanol, can be eliminated from the tissue (Rumpho and Kennedy, 1981; Duss and Brändle, 1982). Therefore accumulation, as opposed to total production, of the two most widely occurring metabolites, ethanol and lactate, was examined in various wetland species and one dryland species in the following series of experiments, in order to determine whether there is any relation between anoxia intolerance and the excessive accumulation of one or both of these compounds.

MATERIALS AND METHODS

Plant species, pretreatments and mode of incubation in this investigation were the same as in the previous chapter. Removal of rhizome material from particular treatments for estimation of ethanol and lactate concentration was carried out simultaneously to the assays for ADH and LDH activity for each species. Where possible the same rhizome was used for determination of the enzyme activity and accumulation of the product.

Preparation of the extracts for determination of ethanol and lactate content of the rhizome were performed at 0-4 °C. 1-2 g rhizome tissue was finely sliced and homogenized in 5 ml 6% (w/v) perchloric acid for deproteinization. Following centrifugation at 3500 g for 15 minutes, the supernatant was neutralized with 5×10^{3} mol m^{-3} K_2CO_3. Ethanol and lactate concentrations were then determined enzymatically (Bergmeyer, 1974) with test kits (Boehringer, Mannheim).

RESULTS

Accumulation of ethanol, rather than total production, was determined in the various species during anoxia and is presented in Figure 3.1. In A.calamus and the two Iris species no correlation was observed between ethanol accumulation and ADH activity (Fig. 2.2, previous chapter). In A.calamus rhizomes quantities of no more than 6.0 μmol g^{-1} fr wt ethanol were found during anaerobiosis, this amount being accumulated by day four and thereafter not exceeded. In contrast, I.germanica exhibited a steady increase in ethanol accumulated in the rhizomes during the whole course of the anoxic treatment, where the relatively large quantity of 71 μmol g^{-1} fr wt was recorded at day 15. In the related I.pseudacorus a more modest 26 μmol g^{-1} fr wt was accumulated over the same time period.

In P.australis, an accumulation of approximately 20 μmol g^{-1} fr wt ethanol was observed under anoxia. This level was reached by day four, and was kept more or less stable for the rest of the treatment, representing an equilibrium of production and release of ethanol in the tissue. Similarly, in S.lacustris ethanol levels rose to roughly 20 μmol g^{-1} fr wt within two days of nitrogen gassing, and no further accumulation was recorded. In T.latifolia there was an accumulation of ethanol to 10 μmol g^{-1} fr wt, which figure was not exceeded and in fact fell, showing a correlation with the decreased ADH activity during the anoxic treatment (Fig. 2.2, previous chapter). Rhizomes of all species undergoing a recovery phase in air subsequent to eight days anoxia showed decreases in ethanol concentrations in rhizome tissue, approaching levels found in aerobic rhizomes. It was noted that there was some ethanol accumulation even under aerobic

conditions, particularly in P.australis and S.lacustris rhizomes.

Quantities of lactate in anaerobic rhizomes of all species, shown in Table 3.1, were low relative to the ethanol accumulations recorded. There were slight accumulations in every species during the 15 day anoxic period, except in I.pseudacorus where negligible amounts were found. The highest quantities were seen in S.lacustris rhizomes, where 5.3 μmol g^{-1} fr wt was measured. Only I.germanica, S.lacustris and T.latifolia exhibited measurable amounts during the control period of aerobic incubation.

FIGURE 3.1 Ethanol accumulation in the rhizomes of six species incubated in aerobic conditions (●—●), gassed with nitrogen (■—■), and undergoing further incubation in air subsequent to anoxia (▲--▲). Mean values from 2-4 rhizomes.

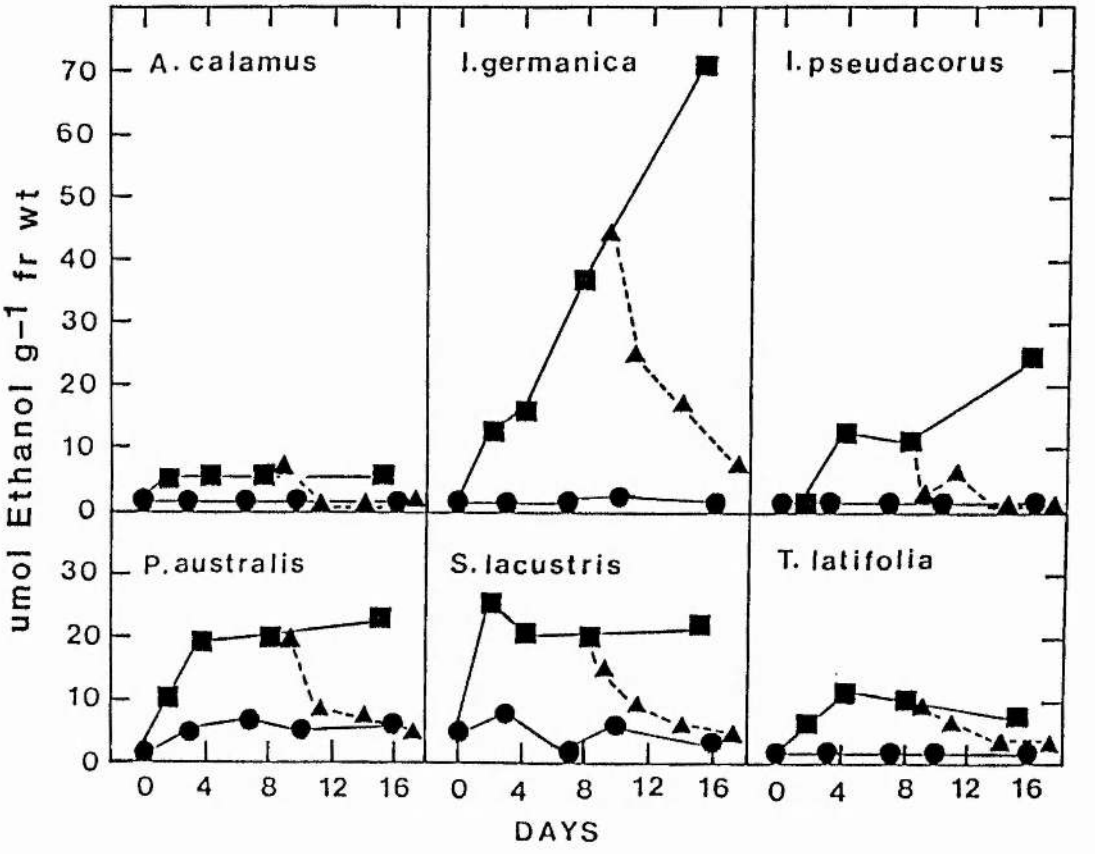

A. calamus
I. germanica
I. pseudacorus
P. australis
S. lacustris
T. latifolia
umol Ethanol g-1 fr wt
DAYS
0
4
8
12
16
10
20
30
40
50
60
70

TABLE 3.1 Lactate accumulation in rhizomes of six species after periods of 15/16 days N_2/air. Mean values from 6–12 rhizomes ± standard deviation.

Species	Air	N_2
	Lactate (μmol g^{-1} fr wt)	
A. calamus	<1	1.6 ± 0.5
I. germanica	1.1 ± 0.7	5.2 ± 0.7
I. pseudacorus	<1	<1
P. australis	<1	2.4 ± 0.6
S. lacustris	2.0 ± 0.6	5.3 ± 0.7
T. latifolia	2.4 ± 0.8	4.2 ± 0.7

DISCUSSION

The significance of ethanol accumulation

All five wetland species that featured in this investigation (A.calamus, I.pseudacorus, P.australis, S.lacustris and T.latifolia) appeared to reach a 'plateau' of ethanol accumulation during anoxia, representing an equilibrium of production and release of ethanol. In contrast, I.germanica rhizomes, the single dryland species showed a steady increase in accumulation of this metabolite throughout the treatment (Fig. 3.1). Under the experimental conditions used - a constant flow of nitrogen over the rhizomes - some ethanol could be expected to be lost to the gaseous phase, as well as to the aqueous phase. It was, however, of interest here to determine actual accumulation of ethanol in the rhizome tissue, in relation to anoxia tolerance.

Although not correlated with increased ADH activity (Chapter 2), ethanol concentrations as high as 70 mol m^{-3} were observed in anaerobic I.germanica rhizomes. The tissue of this storage organ consists of densely packed cells in I.germanica, while that of its wetland relative I.pseudacorus possesses well-developed gas spaces both in the cortex and the central cylinder (Monk et al., in press). Therefore, in vivo and in this experimental system ethanol, being a volatile product of fermentation, may easily be eliminated from the rhizome of I.pseudacorus. The situation is the same in the remaining wetland taxa: all possess lacunar systems, which to a greater or lesser extent may provide a pathway of escape for ethanol, leading to relatively small accumulations (Monk et al., 1984).

Both ethanol and lactate have been found to be eliminated from the rhizome tissue of S.lacustris during anoxia (Duss and Brändle, 1982), and from the thin roots of rice (Bertani et al., 1980) and the weed of rice, Echinochloa crus-galli (Rumpho and Kennedy, 1981). In the case of rice roots 98% of the ethanol produced was found in the surrounding medium. It is not known how much ethanol diffuses from the bulky storage organs typical of wetland plants. It is possible that some of the ethanol produced may enter the transpiration stream, as has been found in tomatoes (Fulton and Erikson, 1964) and escapes via the shoot. Many wetland taxa do in fact possess a continuous lacunar system from shoot to rhizome and root, which has been implicated in oxygen transport to submerged parts of the plant (Studer and Brändle, 1984), but which could readily function as a ventilation system to prevent potentially toxic accumulation of metabolites (Crawford, 1982).

It appears then that much ethanol, and at least in some cases lactate, may commonly be eliminated or lost from the plant. It should be noted that production of lactate during anoxia appeared to be strictly limited in the rhizomes of plants used in the present investigation (Table 3.1), probably regulated by mechanisms discussed in Chapter 2. Therefore excessive acidification of the cytoplasm, which has been cited as a determinant of flooding intolerance in roots of various seedlings (Roberts et al., 1985) may be avoided. These small accumulations of lactate may be oxidized and further metabolized when the oxygen supply is restored (Davies and Davies, 1972).

Likewise, many plants and plant tissues are able to utilize ethanol (Cossins, 1978). It is possible that the oxygen debt as represented by ethanol production, is in part repaid when some of the ethanol is further metabolized in the aerobic shoots of a flooded plant. This may be the case in some woody flooding tolerant and intolerant plants where there appears to be appreciable constitutive levels of ADH in the leaves (MacDonald and Kimmerer, pers. comm.). Certainly, various cereal seedlings have been shown to metabolize a proportion of the ethanol produced under hypoxic stress experienced during germination (Cossins and Turner, 1962).

When the leaf and root activities of ADH were estimated in two subspecies of Echinochloa crus-galli, one less flood tolerant than the other, it was found that the intolerant variety possessed twice as much ADH activity in the root than in the shoot, while the tolerant subspecies showed the opposite ratio (Kennedy, pers. comm.). It may be advantageous to an oxygen-stressed plant to produce a limited amount of ethanol, a significant proportion of which can later be further utilized.

Estimations of ethanol concentrations in rhizomes of wetland plants grown under natural conditions have revealed seasonal variation, which was correlated with fluctuations in ADH activity throughout the year (Haldemann and Brändle, 1986). The species tested were Acorus calamus, Glyceria maxima, Phragmites australis and Typha latifolia: all showed limited ethanol accumulation in the rhizome, not exceeding c. 30 mol m^{-3} concentrations, with levels for the two species possessing hollow rhizomes (G.maxima and P.australis) considerably lower than this figure. This set of data serves as an

interesting comparison for the results from the present experiments, as the species examined (excepting G.maxima) feature in both investigations. It appears that in vivo, and under the experimental conditions used, wetland taxa reach a plateau of ethanol accumulation in the rhizomes (less than c. 30 mol m^{-3} in both situations), eliminating excess of this metabolite perhaps by more than one means. If a gaseous route of escape is present, as it is in all five wetland species in the present experiment, then CO_2 formed concomitantly with ethanol may also escape.

Ethanol - a toxic metabolite ?

Thus, accumulation of large quantities of ethanol, as found in the dryland species I.germanica, is here associated with intolerance of anoxia. This species may not be able to eliminate significant amounts of ethanol produced within the dense rhizome tissue. Ethanol can, as mentioned earlier, have a direct effect on the permeability of cellular membranes, and one study has shown that the metabolite irreversibly denatures membrane Na+ and K+ ATP-ases by disruption of the phospholipid bilayer in the immediate vicinity (Grisham and Barnett, 1972). However, it is not known whether physiological concentrations, such as these reported here, can bring about this type of damage. Another study has shown that high concentrations of ethanol inhibit calcium uptake (Kondo and Kasai, 1973), which has a function in membrane stabilisation. Hunter et al. (1983) have reported increased rates of lipid peroxidation of membranes on restoration of oxygen supply in I.germanica rhizomes, while no changes in level of peroxidation were observed in I.pseudacorus. Therefore, later work by the author investigates the role of the

'oxygen-protecting' enzymes catalase and superoxide dismutase (Chapters 4 and 6).

If ethanol, and possibly CO_2 in its own right or in an additive manner (Andrews and Pomeroy, 1979; Roberts et al., 1985), exert a toxic effect on plant tissues, then limitation of accumulation and/or diversification of fermentative metabolites could provide a solution. In S.lacustris (Steinmann and Brändle, 1984), P.australis and T.latifolia, though not in A.calamus (Haldemann and Brändle, 1986) total amino acid content was shown to rise in flooded, hypoxic rhizomes, in parallel with ethanol accumulation, one or more amino acids possibly acting as alternative end products of glycolysis. Lactate and glycerol production may also contribute to some extent to diversification, especially during periods of prolonged anoxia, in S.lacustris (Duss and Brändle, 1982). In some marsh plants malate could perhaps be added to the list (Crawford, 1982), although there is no net yield of ATP by its production. If there is sufficient energetic yield from other pathways, then there may be a physiological function for the formation this organic acid, in that it may prevent a build-up of other potentially injurious metabolites, thus improving chances of recovery from anoxia.

Morphological adaptation providing oxygen transport from the shoot has been confirmed for S.lacustris (Haldemann and Brändle, 1983), A.calamus, P.australis and T.latifolia (Studer and Brändle, 1984). For these species it has to be said that ethanol appears to be the main fermentative metabolite. It is conceivable that the lacunar systems identified then may serve a dual purpose by allowing the escape of potentially toxic volatile products, such as ethanol, CO_2 and also acetaldehyde. The possibility that ethanol exerts a toxic

effect indirectly, through oxidation to acetaldehyde on restoration of oxygen supply, will be the subject of Chapter 5. In conclusion, it seems that the wetland plants in question show metabolic and morphological adaptations to long-term anoxic stress, which include limitation of accumulation, if not production of the glycolytic end product ethanol.

PART II

RECOVERY FROM ANAEROBIOSIS

CHAPTER 4

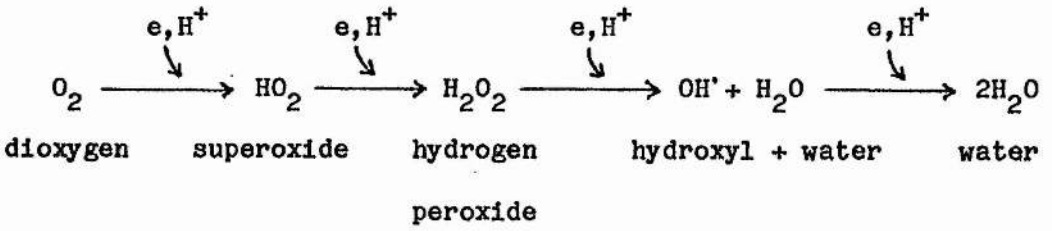
CATALASE ACTIVITY IN THE RHIZOMES OF ANOXIA TOLERANT AND INTOLERANT SPECIES

INTRODUCTION

Oxidative damage and the role of catalase

First of all, catalase will be considered in the role that it is better known, namely protection against oxygen toxicity. However, it should be emphasized that workers have for some time been sceptical that this is the major role for the enzyme (Keilin and Hartree, 1936; Nicholls and Schonbaum, 1963; Halliwell, 1974b). The 'peroxidatic' mode of action of catalase, with ethanol as a cosubstrate (Oshino et al., 1973), may be more relevant to the subject under investigation here, as in some cases large accumulations of ethanol occur during anoxia (Chapter 3), but this role will be considered later.

Oxygen supports a multitude of marvellous aerobic life forms. However, oxygen can cause lethal damage to these same organisms since utilization of molecular oxygen may proceed by a series of single electron transfers, which generate very reactive intermediates:



Most reactions avoid the successive univalent reduction of dioxygen, through catalysis by certain enzymes which mediate divalent reduction (to yield H_2O_2) or tetravalent reduction (to produce water), with no detectable formation of radical intermediates (Fridovich, 1976; Elstner, 1982). An oxygen free radical may be defined as any species that has one or more unpaired electrons (see Table 4a).

Hydrogen peroxide (H_2O_2) and the superoxide radical (HO_2, in its protonated form, possesses a pK_a of 4.8; O_2^- in its ionized form) are however produced in a number of biological systems, and oxygen toxicity may be mediated by uncontrolled production of, or inefficient scavenging of these intermediates. Singlet oxygen ($^1O_2$), the excited form of the rather unreactive "triplet" ground state oxygen, is not a free radical but may be damaging, and commonly occurs in chloroplasts of photosynthetic organisms (Halliwell, 1984).

Table 4a Principal oxygen free radicals occurring in biological systems:

Dioxygen (O_2)	Two unpaired electrons with parallel spins, ("triplet" ground state).
Superoxide (O_2^-)	Single unpaired electron, as a result of univalent reduction of dioxygen.
Hydroxyl ($OH^\cdot$)	Single unpaired electron, formed by homolytic fission of the O-O bond in H_2O_2.

Oxidative intermediates may take part in various reactions together in which reactive oxygen species are generated. The hydroxyl radical ($OH^{\cdot}$) can be formed in mixtures of hydrogen peroxide and ferrous iron (Fenton reaction) or from a reaction between superoxide and hydrogen peroxide (Haber-Weiss reaction), (Halliwell and Gutteridge, 1984). Thus, $OH^{\cdot}$ may be formed in biological systems. Hydroxyl radicals have extremely high rate constants for almost every type of molecule found in living cells and so cause indiscriminate damage.

The cell components damaged by the various reactive oxygen species are: lipids (peroxidation of unsaturated fatty acids in organelle or plasma membranes); proteins (oxidation of sulfhydryl-containing enzymes, leading to inactivation); carbohydrate (polysaccarharide depolymerization); nucleic acids (base hydroxy lations, "nicking", cross-linkage, scission of DNA strands, i.e. mutations (Frank, 1985). In addition there may be an inhibitory effect on protein, nucleotide and fatty acid biosynthesis.

A fuller account of oxygen toxicity will be given in a later chapter in relation to superoxide dismutase (SOD), scavenger of the superoxide radical which is produced in some enzymic reactions. SOD perhaps plays a more central role than catalase in the defences against oxidative damage (Halliwell, 1974). Here attention will be paid to the role of catalase, which along with peroxidases decomposes hydrogen peroxide, also produced regularly in cellular metabolism.

Catalase (E.C. 1.11.1.6) is found uniformly distributed in the matrix of peroxisomes in animals, but both in the core or nucleoid and in the matrix in plant peroxisomes (Tolbert, 1978). Various oxidases, such as D-amino acid oxidase, xanthine oxidase and uricase generate hydrogen peroxide and like catalase itself are compartmentalized in peroxisomes. In fact, de Duve and Baudhuin (1966) have suggested that a peroxisome is defined by the presence of at least one H_2O_2-producing flavin oxidase and catalase. In contrast, peroxidases do not appear to be associated with either mitochondria or peroxisomes, but according to specific metabolic function have been found in the intercellular regions of cortical cell walls, cytoplasm and vacuole of stelar cells and the chloroplasts in green cells (Butt, 1980). Decomposition of hydrogen peroxide is in these cases coincidental with a peroxide-dependent oxidation of the substrate (SH_2):

$$SH_2 + H_2O_2 \longrightarrow S + 2H_2O$$

Hydrogen peroxide can act both as an oxidant and a reductant, but in the absence of metal catalysts or enzymes has a low reactivity towards organic molecules, and therefore is the most stable of the intermediates of the reduction of oxygen. Indeed, Fridovich (1976) points out that several publications demonstrating deleterious oxidations of cellular compounds by peroxide were carried out with concentrations far higher than levels attainable *in vivo*. It appears that the toxicity of peroxide lies in its accumulation and subsequent reaction with superoxide to produce hydroxyl radicals. It is at high concentrations of peroxide that catalase catalyses the decomposition

reaction very rapidly, serving the defence mechanism against oxidative damage well:

$$Cat + H_2O_2 \longrightarrow Cat\text{-}H_2O_2 \text{ (Compound I)}$$

$$Cat\text{-}H_2O_2 + H_2O_2 \longrightarrow Cat + 2H_2O + O_2$$

However, at low concentrations of substrate catalase is not so efficient due to a high Km, as measured by Nicholls and Schonbaum (1963) of 1.1×10^3 mol m^{-3} at 30 °C, pH 7.0.

There are in fact examples in the literature where workers have found catalase to be absent from the enzymatic defences against oxygen toxicity. A recent example for non-prokaryote organisms is found in Riftia pachyptila (a tube worm) and Calyptogena magnifica (a clam), reported by Blum and Fridovich (1984), where hydrogen peroxide removal is apparently mediated by dianisidine peroxidase and glutathione peroxidase. Kendall et al. (1983) have isolated a catalase-deficient mutant of barley, whose leaves contained only 10% of the catalase activity found in the wild-type. However, plants from this line eventually died, presumably because peroxide generated inside the peroxisomes by photorespiration diffused throughout the cell and over time built up to a high level. As peroxidases and other compounds such as ascorbate and glutathione which destroy hydrogen peroxide, were not able to limit the accumulation death of the leaves was brought about, probably due to lipid peroxidation of membranes.

Thus, there appears to be an indispensable role for catalase protection against oxidative damage in peroxisomes where generation of high concentrations of peroxide may occur, posing an immediate threat to membranes, especially in the case of photosynthetic tissues. At lower concentrations of peroxide various peroxidases or other compounds may fill this role. On this background it is of interest to investigate whether intolerance of anoxia is correlated with level of catalase activity, as during recovery from complete deprivation of oxygen indications of some lipid peroxidation were reported in the anoxia sensitive Iris germanica, but not in the related species Iris pseudacorus which is tolerant of anoxia (Hunter et al.,1983). It is possible that levels of catalase drop during the total absence of oxygen as aerobic cellular metabolism is disrupted, so that on re-exposure to air oxidative injury may occur.

Where steady-state levels of hydrogen peroxide are low the efficiency of catalase acting in its 'catalatic' mode to remove peroxide has been questioned (Boveris et al. ,1972; Halliwell,1974). A proportion of the peroxide produced in peroxisomal metabolism was shown to have escaped to the surrounding medium or in the latter case to be taking part in other reactions. However, at this concentration of substrate it is known that catalase acts in a 'peroxidatic' mode using methanol, ethanol, formate, nitrite and phenols as hydrogen donors (Bergmeyer,1974). In the next section the possibility that there is a definite physiological role for the peroxidatic activity of catalase in plants is considered.

Catalase - a dual function in higher plants?

Properties of the peroxidatic reaction of catalase were investigated by Oshino et al. (1973), where the first direct measurements of ethanol oxidation by catalase were made for rat liver. It was found that when the ratio of peroxide to catalase was low then the reaction was predominantly peroxidatic. When the ratio of peroxide to catalase was high, however, a significant decrease in the rate of ethanol oxidation as compared with the total H_2O_2-generation rate was observed, and this was due to an increase in the proportion of catalatic decomposition of peroxide. In later work (Oshino et al.,1975), it was reported that hepatic rat catalase accounted for 10% of total ethanol oxidation (ADH catalysing the remaining proportion), and 30% of the reaction when the hydrogen peroxide source was enhanced.

For plants that have undergone a period of flooding and therefore at least partial anaerobiosis, excessive concentrations of ethanol may be accumulated in the roots or rhizomes (Crawford, 1982), and in direct parallel to the zoological situation it is possible that this ethanol is oxidized on restoration of adequate supplies of oxygen not only by ADH, but also by catalase. Ethanol would not be the only cosubstrate known to take part in the peroxidatic reaction of catalase that has been ascribed a definite physiological function: formic acid oxidation has described in animals (Oro and Rappoport, 1959) as well as in plants (Leek et al., 1972; Halliwell, 1974) and methanol metabolism has been reported in animals (Mannering et al.,1969).

Thus, the protective role against oxidative damage may not be the sole function of catalase. However, Halliwell (1974) interprets the situation in the following way: an accumulation of the H_2O_2-catalase complex (compound I) will take place at slow, but continuous rates of H_2O_2-generation (due to an extremely fast reaction rate constant) and compound I is readily converted to compound II, an inactive complex. Loss of most of the catalase activity in the peroxisome is the result, unless peroxidatic hydrogen donors are present, which produce free catalase again. Therefore, in order to maintain sufficient levels of catalase activity and hence control of cellular concentrations of peroxide the peroxidatic reaction may be essential. In any case it appears to be of great interest to investigate levels of catalase activity during a post-anoxic phase in anoxia tolerant species that tend not to accumulate ethanol during anaerobiosis, and in anoxia intolerant species that accumulate large amounts of ethanol.

MATERIALS AND METHODS

Supplies of plants for experimentation were obtained from various sources. The relatively anoxia intolerant Glyceria maxima (Hartm.) Holmberg and Juncus effusus L. were collected locally at Tentsmuir, Fife, while the third anoxia sensitive species Iris germanica L. (var. Quechei) was provided by the University Botanic Garden. The related, but anoxia tolerant Iris pseudacorus L. was collected from Dura Den, Fife; also tolerant, Schoenoplectus lacustris (L.) Palla was sent from Orkney and Acorus calamus L. from Berne, Switzerland. All species were allowed to grow on in the greenhouse for several months before harvesting for experiments. The plants were kept on a 16 hour/day light regime at 20 °C, and since they were planted in coarse gravel only, were fed weekly with a modified Hoagland's solution (Epstein, 1972).

Prior to experimentation rhizomes were surface-sterilized with 0.16 mol m^{-3} chloramphenicol and underwent a 48-72 hour aerobic pretreatment, wrapped in moist filter paper at 20 °C. Anoxic incubation of rhizome material was accomplished by filling anaerobe jars (Gaspak, Becton Dickinson and Co., Cockeysville, U.S.A.), containing a palladium catalyst to remove traces of oxygen, in the anaerobic workbench (Forma Scientific, Ohio, U.S.A.), and subsequently placing them in incubators at 20 °C. The anoxic atmosphere thus consisted of 90% nitrogen and 10% hydrogen. The jars also contained methylene blue indicator strips which remain colourless in the absence of oxygen. During the post-anoxic phase in air rhizomes were kept on moist filter paper at 20 °C.

A modified Clarke-type inverted oxygen electrode (Rank Bros., Bottisham, Cambridge) was used with a Spectro-Plus (M.S.E. Scientific Instruments, Crawley, Sussex) for measurement of dissolved oxygen for the catalase assay. The sample chamber had a built-in water jacket connected to an external constant temperature water bath, and was mounted on a base containing the electrode and a magnetic stirrer.

The catalase activity of 1mm thick transverse rhizome slices was estimated as rate of oxygen evolution in Sorensen's buffer (pH 7.0) when hydrogen peroxide (7 mol m^{-3} final concentration) was added to the solution. The buffer was always allowed to saturate with air at 25 °C before rhizome slices were added, and the tissue allowed to respire, so that rate of oxygen evolution was taken between zero and the saturation point of the solution.

Since H_2O_2 decomposes spontaneously to O_2 and H_2O, it was necessary to check the concentration of the commercial preparation before stock solutions were made up. This was done by titration against potassuim permanganate in the presence of dilute sulphuric acid. Titrations were carried out regularly, and fresh solutions of peroxide made up daily. Spontaneous rate of H_2O_2 decay in 3 ml buffer (the amount used with slices of rhizome) in the oxygen electrode sample chamber was also checked.

Under these conditions catalase activity was proportional to the number of slices placed in the sample chamber and remained linear for approximately 30 seconds. In additional experiments using rhizome slices ground in Sorensen's buffer, and centrifuged for five minutes at 8730 g, 7 mol m^{-3} hydrogen peroxide gave a proportional response

with the amount of extract added. Recovery experiments adding commercially purified bovine liver catalase (Boehringer, Mannheim) to the supernatant of ground rhizome slices of G.maxima gave recovery rates of 87.3% ± 4.9 S.E. (n=3) from aerobic tissue and 81.4% ± 2.0 in rhizomes from post-anoxic treatments. Rates of oxygen uptake and evolution were calculated from a chart recorder trace and expressed as nanomoles of oxygen evolved per minute per cm^2.

Recovery rates for catalase in extracts (supernatants) of the remaining five species for the two respective treatments were: J.effusus, 85.3% ± 3.6 and 95.5% ± 1.9; I.germanica, 91.9% ± 2.9 and 83.9% ± 4.1; S.lacustris, 95.9% ± 1.2 and 94.3% ± 2.8; A.calamus, 85.5% ± 1.3 and 89.3% ± 5.5; I.pseudacorus, 85.2% ± 3.4 and 90.8% ± 4.6. Due to the release of phenols on homogenization 3% (w/v) soluble PVP-40 was added to the I.pseudacorus extracts to remove the complete inhibition of catalase activity.

The high recovery rates found in all species preclude the possibility that peroxidases are interfering with the assay, producing an underestimation of catalase activity. At the concentration of substrate used peroxidases may react as rapidly as catalase (10^7 1 mol^{-1} sec^{-1}), and as reported in the case of glutathione peroxidase, the metabolic route of hydrogen peroxide will depend predominantly on the molarities of the enzymes competing (Flohé et al., 1972). The good recovery rates also rule out the possibility that an endogenous inhibitor of catalase, such as that described by Sorenson and Scandalios (1976) in maize seedlings, is in operation in any of the species tested.

RESULTS

Catalase activity was monitored during a three day aerobic recovery period in six species (three anoxia tolerant shown in Figure 4.1 and three anoxia intolerant shown in Figure 4.2) where rhizomes had previously been subjected to 3 (two species only), 7, 14, 21, and 28 days of oxygen deprivation. Only very slight increases were found in S.lacustris, A.calamus and I.pseudacorus, where amounts between c. 100-200 nmol cm^{-2} min^{-1} were measured (Fig. 4.1). However, in the anoxia intolerant J.effusus (Barclay and Crawford, 1982) a three- to four-fold increase in activity, up to 1012 nmol cm^{-2} min^{-1}, occurred on return to air after three and seven days without oxygen (Fig. 4.2). The longer periods of anoxia resulted in death of the tissue in this species before re-exposure to air.

Similarly, in the relatively anoxia-intolerant I.germanica (Hetherington et al., 1983) two- to three-fold increases in activity up to 570 nmol cm^{-2} min^{-1} were observed during the post-anoxic period. Levels remained fairly constant during the absence of oxygen. On removal from 28 days anoxia rhizomes exhibited catalase activities which after 24 hours showed a drop to below that of control levels, indicating imminent death of the tissue.

The least anoxia tolerant species investigated in this series of experiments was G.maxima. After seven days of anaerobiosis catalase was found to be six times as active as at the beginning of the experiment. This level of catalase activity under anoxia was not exceeded in the longer periods of oxygen deprivation. Monitoring the 72 hour post-anoxic phase in air, however, revealed a 12- to 19-fold

rise in the enzyme activity, up to 1651 nmol cm^{-2} min^{-1}, compared to initial levels. Again on removal from 28 days anoxia rhizomes showed catalase activities which during the post-anoxic period fell sharply indicating tissue death. A three day anoxic treatment produced only a two-fold increase in activity, with a further two-fold increase on return to air. In this, the most responsive species, the longer the anoxic treatment given the greater the activation/induction of catalase in the post-anoxic phase. However, in the longer treatments it becomes obvious that severity of the stress produces considerable variability of activity and large standard errors.

FIGURE 4.1 The development of catalase activity on return to air in rhizomes of three anoxia tolerant species that were exposed to anoxia for 28 days. The column sets represent 0-3 days post-anoxic treatment, and the different columns within the sets represent 0 (), 7 (), 14 (), 21 () and 28 days () previous anaerobic incubation (n = 3-6, with standard error bars).

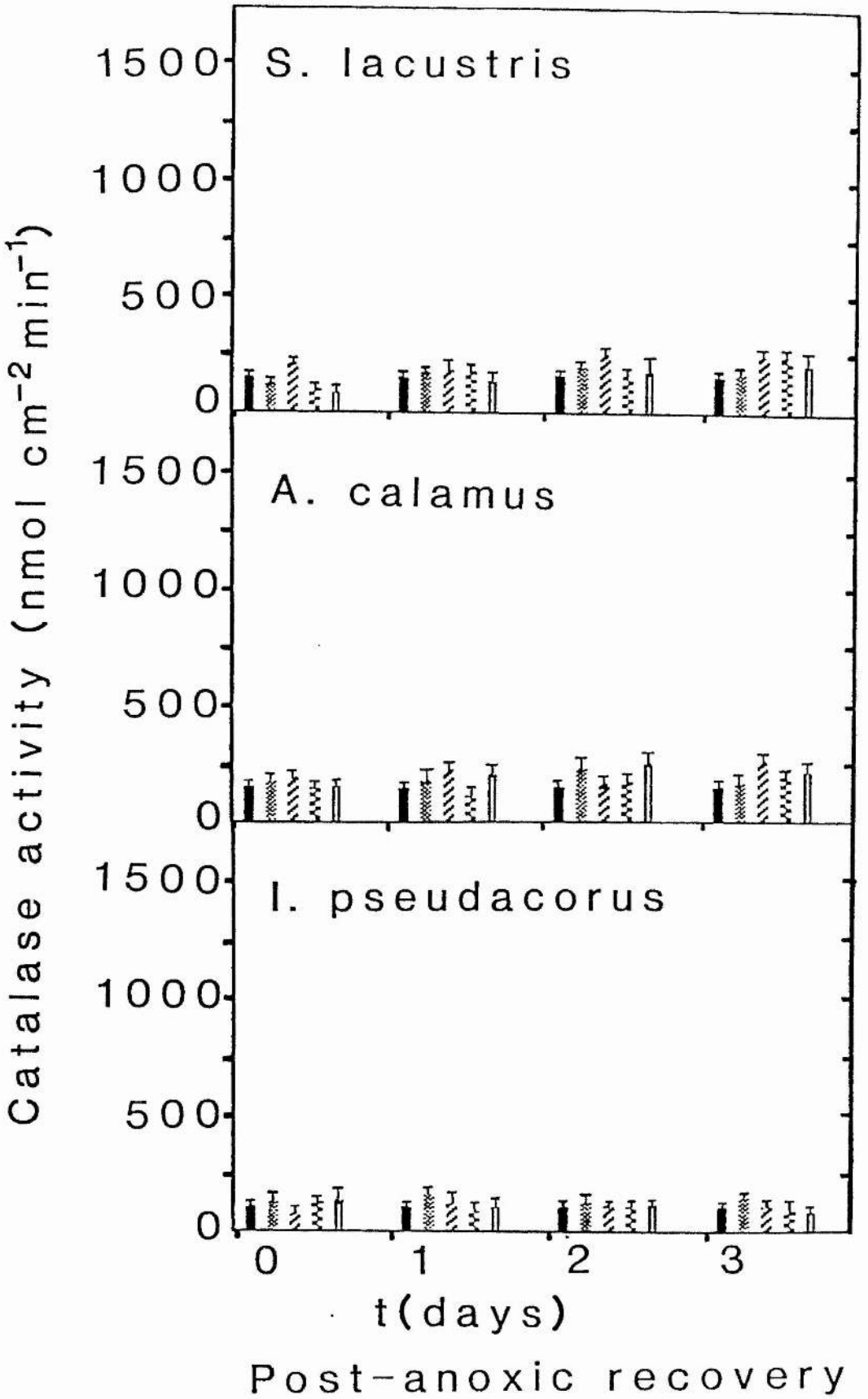
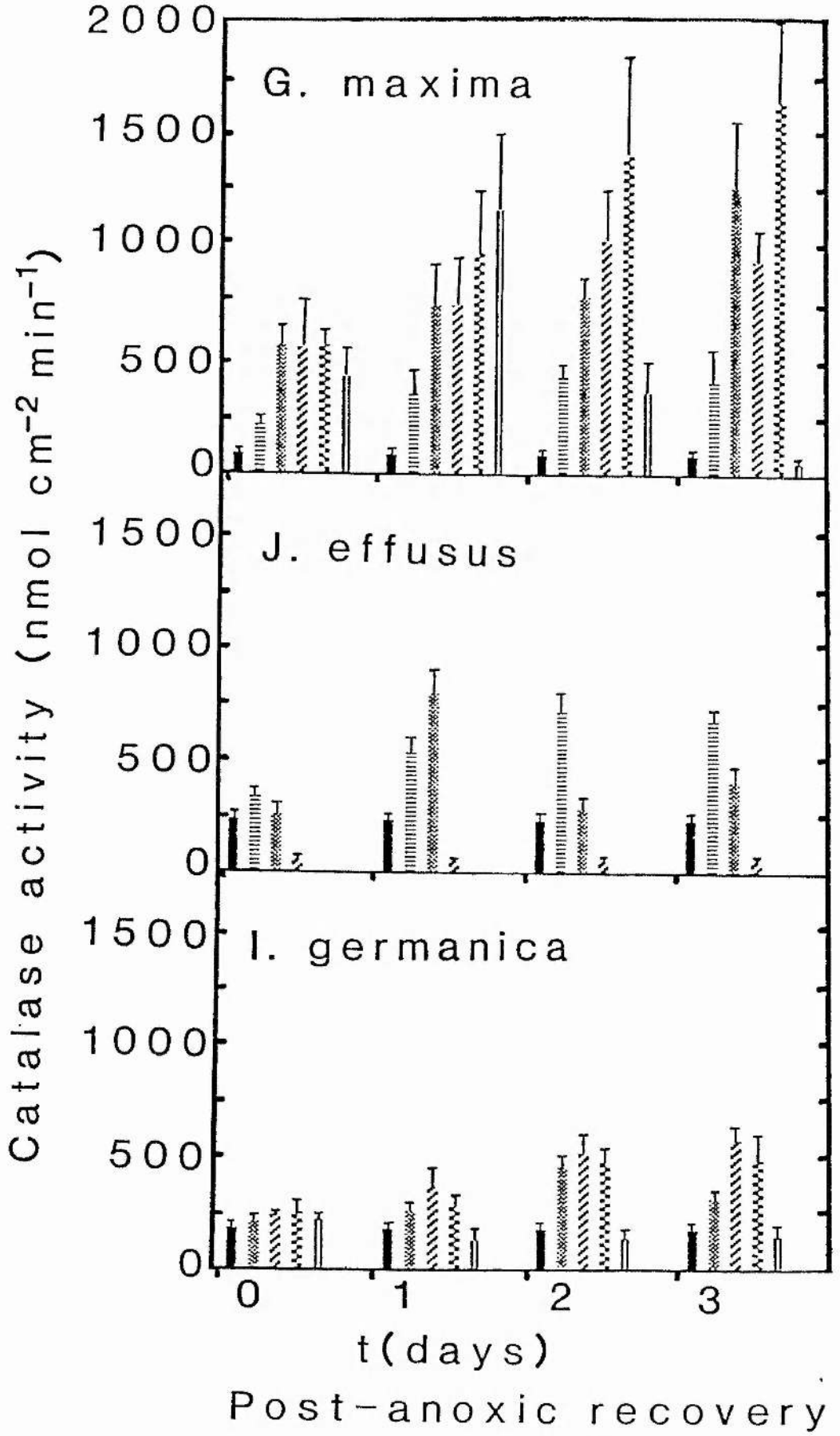

S. lacustris
A. calamus
I. pseudacorus
1500
1000
500
0
Catalase activity (nmol cm^{-2} min^{-1})
0
1
2
3
t(days)
Post-anoxic recovery

FIGURE 4.2 The development of catalase activity on return to air in rhizomes of three anoxia intolerant species that were exposed to anoxia for up to 28 days. The column sets represent 0-3 days post-anoxic treatment, and the different columns within the sets represent 0 (■), 3 (≡) - only two species, 7 (▩), 14 (▨), 21 (▧) and 28 days (‖) previous anaerobic incubation (n = 3-6, with standard error bars).


G. maxima
J. effusus
I. germanica
2000
1500
1000
500
0
1500
1000
500
0
1500
1000
500
0
0
1
2
3
Catalase activity (nmol cm^{-2} min^{-1})
t(days)
Post-anoxic recovery

DISCUSSION

The significance of changes in level of catalase activity

The three species intolerant of anoxia in this series of experiments showed similar responses to anoxic stress. Large increases in catalase activity occurred in a three day aerobic recovery phase after each period of anaerobic treatment, and the most anoxia sensitive species, G.maxima, also showed modest increases during the treatment. In contrast, the three anoxia tolerant species exhibited levels of catalase much the same as that found in aerobic rhizomes at the beginning of the experiment. It is difficult to determine whether the activations/inductions of catalase observed here are still reflecting protection or already indicating damage. It should be noted that data on catalase activities alone will not provide conclusive evidence of the capacity of the tissue in question to protect against oxidative injury.

Other mechanisms of protection include superoxide dismutase, which removes the superoxide radical in a reaction that produces hydrogen peroxide and dioxygen, and so is relevant to this part of the investigation of defences against oxygen. A later chapter will deal with the relationship between SOD and catalase, where levels of SOD in the rhizomes of three species reported. Ascorbic acid plays an important role in scavenging superoxide, hydroxyl radicals and singlet oxygen; reduced glutathione scavenges hydroxyl radicals and singlet oxygen, protects enzyme -SH groups and helps regenerate ascorbate from dehydroascorbate; α-tocopherol inhibits the chain reaction of lipid peroxidation (membrane damage) and scavenges singlet oxygen. A

complete picture is not gained without comprehensive data on these systems of protection. Nevertheless, certain features of the changing levels of catalase activity observed can be discussed here as they stand.

Of the three species intolerant of anoxia examined in these experiments, G.maxima and J.effusus are killed by only seven days anoxia (Barclay and Crawford, 1982). The third species, I.germanica survives up to 14 days anoxia (Hetherington et al., 1983). Yet rhizomes of G.maxima and I.germanica possess active catalase and indeed appear turgid and healthy on removal from anoxic treatments of up to 28 days.

Deprivation of oxygen per se does not appear to be lethal, but the tissues collapse during a post-anoxic phase in air. Survival of strict anoxia as determined for these species in the investigations cited above, was defined as the ability to regrow shoots and roots after a period of anoxic stress. Death of the G.maxima and I.germanica rhizomes from the present experiments occurred some days after removal from anoxia; catalase activity was observed to fall significantly during the designated three day recovery phase in rhizomes from the 28 day treatment, indicating imminent death.

In the case of J.effusus high catalase activities were observed after re-exposure to air following three and seven days anoxia, but rhizomes from the 14 day anoxic treatment were already found to be dead. The capacity to survive during anoxia was not so pronounced in this species, but again the same post-anoxic increases in catalase were seen, indicating oxidative damage or perhaps a response to accumulated ethanol.

The post-anoxic increases in catalase enzyme in these three anoxia sensitive taxa may then indicate that some intracellular oxidative damage is taking place. In the shorter anoxic treatments activation or induction of catalase may aid recovery from the stress, but in the longer treatments the more severe stress may bring other factors into play, such as accumulation of potentially toxic metabolites like ethanol.

Catalase has been specifically implicated in the oxygen defences of plants, but is not always a key enzyme. Harper and Harvey (1978) showed that elevated superoxide dismutase and catalase activities (and in some cases, peroxidase too) in four cultivars of Lolium perenne was correlated with a tolerance of the herbicide paraquat. The herbicidal action of this substance resides in its ability to produce oxygen free radicals, mainly in the chloroplast. On the other hand, Foster and Hess (1980) found no differences in catalase activity, but increases in glutathione reductase when leaf tissue from intact cotton plants was exposed to 75% oxygen. Levels of SOD were high under air and enriched O_2 treatments. In non-green material catalase may, along with SOD, play a more central role.

During complete deprivation of oxygen interestingly no drop in catalase activity was recorded in any of the species tested in the present series of experiments. Nevertheless, in the sensitive species existing levels may not have been adequate for protection, although without direct measurement of H_2O_2-production rates this can only be speculated upon. Certainly, the three anoxia tolerant species, S.lacustris, A.calamus and I.pseudacorus showed no increases on return to air. In the latter situation, it is possible that other

peroxide-scavenging systems are contributing to defence.

The increase of catalase activity during the course of the anoxic treatments in G.maxima may be in response to the accumulation of ethanol, which can act as cosubstrate with peroxide (Oshino et al., 1973,1975). This will be further discussed in the next chapter, where catalase is investigated in relation to endogenous ethanol accumulation.

Increased catalase activity in relation to the peroxidatic function

Catalase isozyme patterns undergo changes during development and differentiation, much as other enzymes such as alcohol dehydrogenase have been shown to do. In a review of this subject Scandalios (1974) noted that maize catalase varied in isozyme complement according to particular tissue or organ. In addition, as well as being linked to developmental state, specific isozymes at any time were observed to show different rates of synthesis and degradation, and thus activities. Therefore, if the genetic machinery were there, as it is in the much studied monocot maize, catalase could show distinct responses, given the appropriate environmental cue.

Responses in the form of increases in catalase enzyme in the rhizomes have been found in three monocots in the present investigation. It is possible that a particular isozyme or group of isozymes has been activated or induced to take part in the peroxidatic oxidation of ethanol on return to air. The metabolism of ethanol involving oxidation to acetaldehyde as the first step, has been reported for widely differing species and tissues (Cossins and

Beevers, 1963; Cossins, 1978), and a catalase-mediated oxidation of ethanol may contribute to the reaction known to be catalysed by ADH. The dense, bulky rhizome of I.germanica accumulates large amounts of ethanol during anoxia (Chapter 2), so that the environmental cue, if this is the one which triggers the increases in catalase, is there.

Although found growing in wet places, G.maxima is not metabolically adapted to anoxia (Barclay and Crawford, 1982), and studies comparing pea and Glyceria roots have endorsed this original finding (Jenkin and ap Rees, 1983; ap Rees and Wilson, 1984). Ethanol production may be very high, (c. 135 mol m^{-3} concentrations were measured after five days anoxia, Chapter 5), and although the rhizome is hollow this metabolite will accumulate in a closed system such as that used here for anoxic treatments. J.effusus grows in only periodically flooded areas and possesses a dense, partially lignified rhizome, so that ethanol is also likely to accumulate in this species.

It is in those species that do not accumulate excessive concentrations of ethanol under anaerobiosis (Monk et al.,1984), that levels of catalase remain the same. A certain amount of ethanol is of course accumulated, but may not reach the threshold needed to trigger the response to increase activity. Whether the cue is peroxide or ethanol or both (and whether the main function is to remove H_2O_2 or oxidize ethanol) higher catalase activity during the post-anoxic phase is correlated with the inability to survive anoxia.

CHAPTER 5

CATALASE ACTIVITY IN RELATION TO ETHANOL ACCUMULATION IN GLYCERIA MAXIMA RHIZOMES

INTRODUCTION

Catalase is localised in peroxisomes, where duplicate metabolic pathways of reactions catalysed in other parts of the cell are typically present. Tolbert (1978, 1981) has reviewed peroxisomal metabolism, and noted apparently wasteful reactions such as that catalysed by α-hydroxy acid oxidase (oxidation of lactate to pyruvate with concomitant production of H_2O_2, which is decomposed by catalase to O_2 and H_2O), while lactate dehydrogenase in the cytosol conserves the energy as NADH. In conditions of excess ethanol it is conceivable that a parallel oxidation of this metabolite, by the peroxidatic reaction of catalase (cf. ADH in the cytosol), should take place in the unspecialised peroxisome or microbody of rhizomes.

No specific physiological function has been ascribed to microbodies found in the roots/storage organs of plants (Kausch, 1984; Ruis, 1973). Microbodies of lipid-storing seeds are involved in fatty acid β-oxidation, and are termed glyoxysomes, while those of green tissues, called peroxisomes are the site of photorespiratory reactions. Vigil (1973) stated that microbodies in general probably participate in several other aspects of cellular metabolism. These suggestions include generation and/or elimination of excessive reducing power, regulation of various metabolic pools by selective oxidation of uric acid, amino acids etc., synthesis of required amino acids for proteins or membrane lipids, and formation of carbon

precursors essential for the synthesis of sucrose and other carbohydrates.

In a recent paper (Donaldson et al., 1985), the contribution of glyoxysomal catalase to the oxidation of ethanol, accumulated during a period of imposed anoxia in germinating castor beans, was evaluated. These workers argued that the rate of NADH generation by ADH may exceed the capacity of mitochondrial oxidation (where a marker enzyme showed diminished activity), and therefore that the amount of free NAD could limit oxidation by ADH, and increase the proportions of ethanol metabolised in the glyoxysomes, especially at high concentrations of this substrate. Rate of ethanol oxidation by ADH may also be determined by the substrate affinities for ethanol/acetaldehyde and the relative activities of the isozyme complement involved (Felder et al., 1973).

Many plants and plant tissues have been shown to have the ability to utilize ethanol as Cossins (1978) has noted in a review. An exception is constituted by barley seedlings, although other cereals such as wheat and maize were able to oxidize ethanol (Cossins and Turner, 1962); apple slices also showed very small incorporation of ^{14}C when incubated with labelled ethanol, and potato tubers metabolized ethanol only very slowly (Cossins and Beevers, 1963). In work thus far, the initial oxidation to acetaldehyde in ethanol metabolism in higher plants is taken to be mediated by ADH, excluding the single citation above (Donaldson et al., 1985).

Therefore, G.maxima, a species which accumulates ethanol rapidly under anaerobic conditions and also shows very large increases in catalase activity in a post-anoxic phase (Monk et al., in press) was chosen for investigation into this issue. The possibility that a toxic concentration of acetaldehyde is accumulated in this first step in ethanol metabolism is also examined.

MATERIALS AND METHODS

The anoxia intolerant species, Glyceria maxima (Hartm.) Holmberg, collected locally at Tentsmuir, Fife and grown on as described earlier, was used in this series of experiments. Pretreatments and estimation of catalase activity in the rhizomes were carried out as in the previous chapter, using an inverted oxygen electrode.

For the studies on rhizomes in anoxic static vs. anoxic circulating atmospheres, anaerobe jars with a volume of 2.4 l provided the static environment. A moving atmosphere for rhizomes for the catalase assays was created by circulating the atmosphere in the anaerobic workbench (volume of 0.613 m^3, 21 ± 3 °C). In an alternative method, a constant flow of nitrogen (45 ml min^{-1}) was passed over the rhizomes, which were wrapped in moist filter paper, and this provided the environment for the survival experiments. In this last case both static and moving treatments were placed in a growth cabinet at 20 ± 3 °C.

Quantitative estimation of ethanol accumulation in rhizome tissue after anoxic incubation in static vs. moving environments was determined by gas-liquid chromatography. A column 1.4 m long and 7.0 mm in diameter, filled with 100 mesh Porapak Q was used; carrier gas flow was 40 ml min^{-1} and main and detector oven temperatures were 150 °C. Ethanol concentrations were based on peak area as measured by an integrating recorder (Hewlett Packard - reporting integrator 3390). Extracts of rhizomes were made by grinding (pestle and mortar, then all-glass homogenizer) slices of tissue (1 g in 6 ml) in ice-cold 6% (w/v) perchloric acid, followed by two minutes centrifugation at 8730

g. 0.5 µl aliquots were then immediately injected into the GLC for analysis. A standard curve was obtained from progressive dilutions of analytical ethanol preparations (Sigma).

For experiments which involved the application of ethanol or acetaldehyde to the head space, 500 ml of a series of different concentrations of each volatile compound was placed with a wick in the anaerobe jar in turn, and allowed to equilibrate over three days anoxia at 20 °C.

Ethanol and acetaldehyde evolved during anoxic and post-anoxic treatments were detected in the head space over rhizomes incubated at 20 °C on the GLC. For the aerobic post-anoxic treatment rhizomes were transferred to clean, dry test tubes, 16 ml in volume and sealed with a Suba-seal stopper, which were then placed in a water bath at 20 °C. At intervals 0.5 ml aliquots were withdrawn by means of a gas syringe and injected into the GLC for analysis. Standard curves were made from analytical grade ethanol (Sigma) and acetaldehyde (BDH), using aqueous samples.

For the survival experiment (static vs. moving anoxic incubation), rhizomes with well-developed buds attached were washed and green shoots and roots cut away. After surface-sterilization with 0.16 mol m^{-3} chloramphenicol, rhizomes were placed in the appropriate treatment in the growth cabinet in the dark at 20 ± 3 °C. For recovery from the anoxic stress, rhizomes were planted out in the greenhouse in a mixture of peat and coarse gravel, and survival, i.e. regrowth of shoots and roots, was scored after two weeks.

RESULTS

Figure 5.1 shows catalase activity in G.maxima during and after anoxic treatments in static and circulating atmospheres. A four-fold rise in active catalase was found in rhizomes subjected to five days in a static anoxic atmosphere, while rhizomes in a circulating anoxic atmosphere showed only a two-fold increase over the same time. During the course of the post-anoxic period in air, a catalase activity of 960 nmol cm^{-2} min^{-1} (11-fold increase on initial levels) was found in rhizomes which had been in static anoxic conditions, while a level of 352 nmol cm^{-2} min^{-1} (only a four-fold rise) was reached in the plants from the circulated anoxic atmosphere. The concentrations of active catalase found in the latter treatment (five days anoxia) are comparable to those in rhizomes that had been subjected to only three days anaerobiosis, but under static conditions (Fig. 4.2).

Levels of ethanol were measured in rhizomes from static and circulating anoxic treatments. The moving stream of nitrogen removed considerable amounts of ethanol produced by alcoholic fermentation within the plants. Quantities of ethanol in the rhizomes after five days anoxia in a moving atmosphere were only one sixth (22.7 μmol g^{-1} fr wt $\pm$ 2.6 S.E.) of that found in rhizomes from the static atmosphere (134.7 μmol g^{-1} fr wt $\pm$ 8.1 S.E.).

Table 5.1 records the survival probabilities for G.maxima rhizomes from static and circulating anoxic conditions. Plants from both treatments showed 100% survival after three days oxygen deprivation. Four days without oxygen produced 20-30% mortalities in rhizomes from both conditions, while five days appeared to be critical

for plants subjected to static anoxic conditions. In this treatment only 30% regrew shoots and roots, compared to 80% from the moving (constant stream of nitrogen) anoxic environment (see also photos). Six days anoxia was lethal for plants from the static anoxic atmosphere, with only 10% survival produced from the moving anoxic environment. The latter treatment enhances survival under anoxic stress by a period of at least 24 hours.

Figure 5.2 shows catalase activity in rhizomes subjected to three days anoxia with different concentrations of ethanol applied to the head space. Ethanol (2.5 mol m^{-3} - 1 x 10^3 mol m^{-3}) applied during anaerobiosis resulted in increased catalase activities during anoxia, but most markedly in a post-anoxic phase. The increases noted were greater than those which occurred over the same time span when no volatile compound was added; the 2.5 mol m^{-3} added ethanol treatment produced activities similar to these 'control' levels. The higher the concentration applied, roughly the greater and more rapid the increase in catalase activity, the greatest activity measured being 1818 nmol cm^{-2} min^{-1} (c. 20-fold increase). A five-fold increase in catalase activity whether produced by incubation in a static anaerobic atmosphere or by the addition of ethanol always signalled the eventual death of the rhizomes.

Figure 5.3 shows catalase activity in rhizomes subjected to three days anoxia with different concentrations of acetaldehyde applied to the head space. Glyceria appeared to be more sensitive to acetaldehyde (lower concentrations between 0.5 mol m^{-3} - 0.1 x 10^3 mol m^{-3} were used) than ethanol. Increased catalase activities were found in treatments with acetaldehyde concentrations between only 2.5 mol m^{-3} and 50 mol m^{-3}. Again the higher the concentration applied, the

greater and more rapid the increase. The 0.5 mol m^{-3} added acetaldehyde treatment resulted in activities comparable to 'control' levels. The 0.1 x 10^{3} mol m^{-3} treatment was enough to render the rhizomes moribund on return to air, with lower activities of catalase than those found in aerobic rhizomes. The highest activity measured was 1071 nmol cm^{-2} min^{-1} (c. 12-fold compared to initial levels) in response to 2.5 mol m^{-3} acetaldehyde. As with the ethanol experiments any acetaldehyde treatment that gave rise to catalase activities greater than c. five-fold the amount found under air, signalled the eventual death of the rhizome in the post-anoxic phase.

Table 5.2 records the ethanol and acetaldehyde concentrations and their ratio in the head space above G.maxima rhizomes, at the end of four days anaerobic incubation and during a subsequent post-anoxic recovery phase. Immediately before removal from anoxia, the ratio of ethanol to acetaldehyde in the gas phase above incubated rhizomes stood at 65:1 (0.613 µmol g^{-1} fr wt: 9.459 nmol g^{-1} fr wt). On removal from strict anoxia some ethanol and acetaldehyde was lost before rhizomes were placed in the second closed system containing air, hence the obviously large fall in ethanol concentration at this stage.

Thirty minutes after restoration of the oxygen supply the ratio was found to be 7.5:1 (0.386 µmol g^{-1} fr wt: 51.67 nmol g^{-1} fr wt). Thereafter a further, though less significant, change in ratio took place to 4.9:1 by six hours of the post-anoxic phase. It appears that as soon as the rhizomes are returned to air, ethanol is oxidized at a rapid rate, and during the first minutes and hours of recovery from the anoxic stress, acetaldehyde is accumulated as the first step in the metabolism of ethanol.

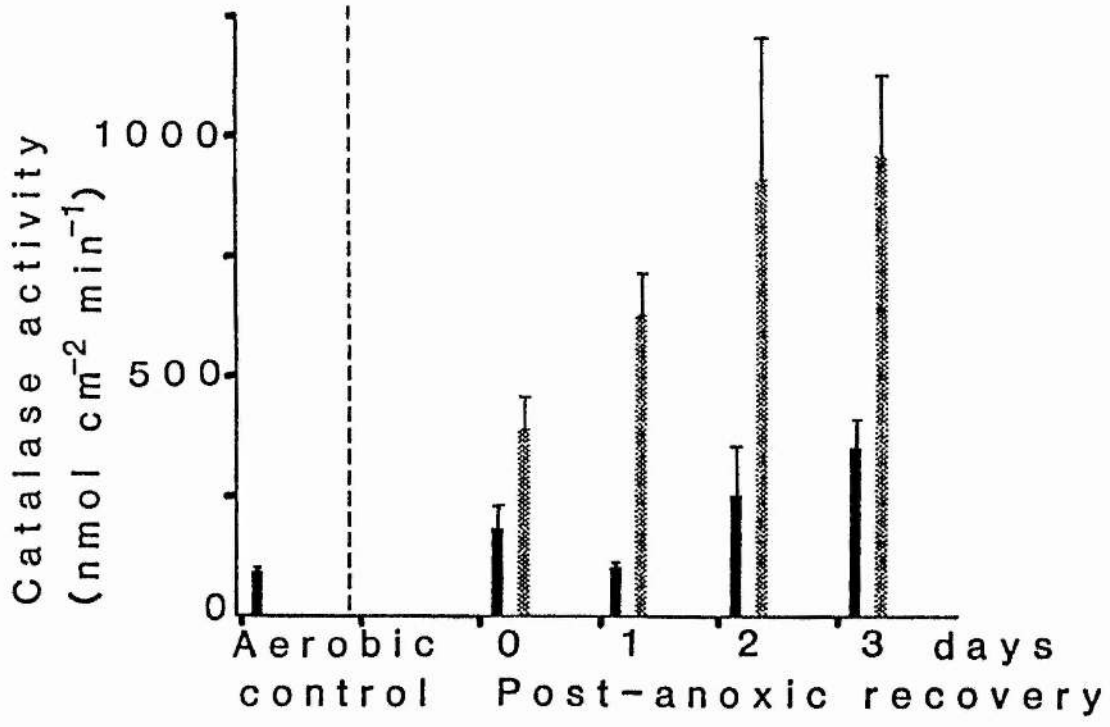


FIGURE 5.1 Catalase activity in rhizomes of Glyceria maxima after 5 days in a static (▒) as opposed to a moving (■) anaerobic atmosphere (n = 3-6, with standard error bars).

FIGURE 5.2 The development of catalase activity on return to air in rhizomes of Glyceria maxima, after 3 days previous anaerobic incubation with varying concentrations of ethanol added to the head space (n = 3–6). No ethanol applied (■), 2.5 mol m^{-3} (□), 10 mol m^{-3} (●), 50 mol m^{-3} (○), 0.1 x 10^3 mol m^{-3} (▲), 0.25 x 10^3 mol m^{-3} (△), 0.5 x 10^3 mol m^{-3} (✪), 1 x 10^3 mol m^{-3} (☆). Standard error values on the final day of the experiment, indicative of values during the course of the experiment, were: (■), ± 154; (□), ± 32; (●), ± 153; (○), ± 81; (▲), ± 231; (△) ± 173; (✪), ± 256; (☆) ± 104 nmol cm^{-2} min^{-1}.

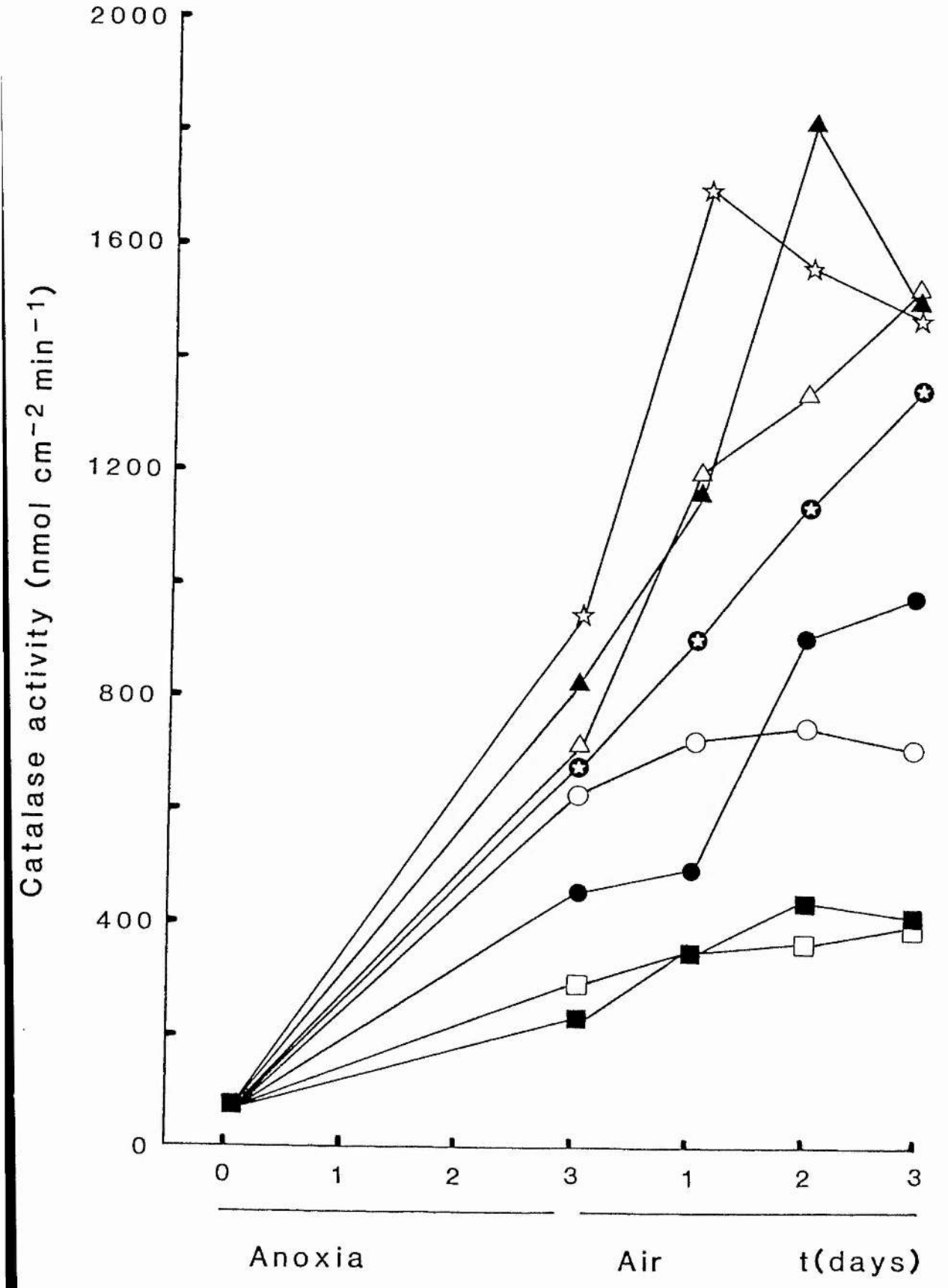

2000
1600
1200
800
400
0
Catalase activity (nmol cm^{-2} min^{-1})
0
1
2
3
1
2
3
Anoxia
Air
t(days)

FIGURE 5.3 The development of catalase activity on return to air in rhizomes of Glyceria maxima, after 3 days previous anaerobic incubation with varying concentrations of acetaldehyde added to the head space (n = 3-6). No acetaldehyde applied (■), 0.5 mol m^{-3} (□), 2.5 mol m^{-3} (●), 10 mol m^{-3} (○), 50 mol m^{-3} (▲), 0.1 x 10^3 mol m^{-3} (△). Standard error values on the final day of the experiment, indicative of values during the course of the experiment, were: (■), ± 154; (□), ± 188; (●), ± 179; (○), ± 72; (▲), ± 14 nmol cm^{-2} min^{-1}; (△), plants had perished.

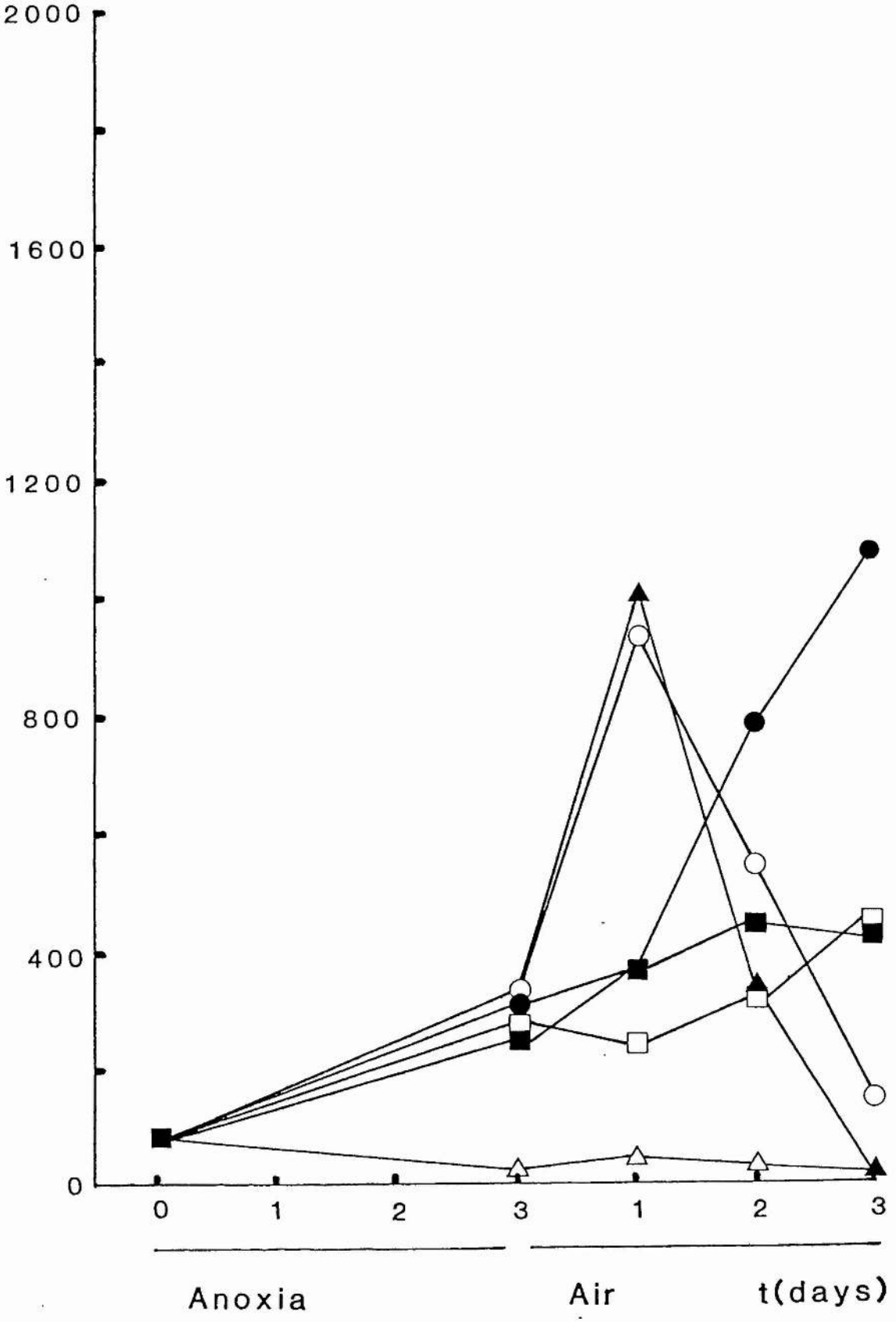
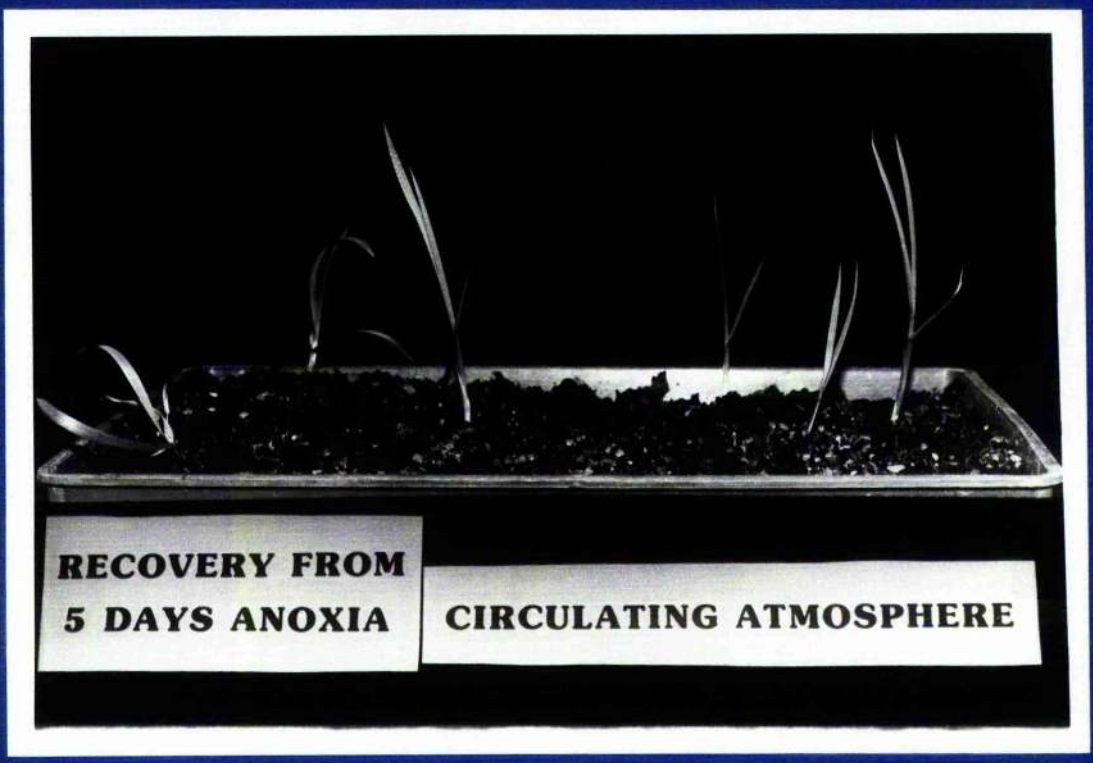

2000
1600
1200
800
400
0
0
1
2
3
1
2
3
Anoxia
Air
t(days)

TABLE 5.1 The effect of a moving versus a static anaerobic environment on the survival probability (p) of rhizomes of Glyceria maxima tested by growing on for 14 d in air after the anaerobic treatment. (n = 10, two replicate experiments).

Length of treatment	Static	Moving	Significance
3 d	p = 1.0	1.0	n.s.
4 d	0.7	0.8	n.s.
5 d	0.3	0.8	<0.05
6 d	0.0	0.1	n.s.

PHOTOGRAPHS A & B Survival and regrowth of *Glyceria maxima* rhizomes after a period of two weeks, subsequent to differing (static vs. circulating) anoxic treatments - ten rhizomes per treatment.

A.


RECOVERY FROM
5 DAYS ANOXIA
CIRCULATING ATMOSPHERE

B.

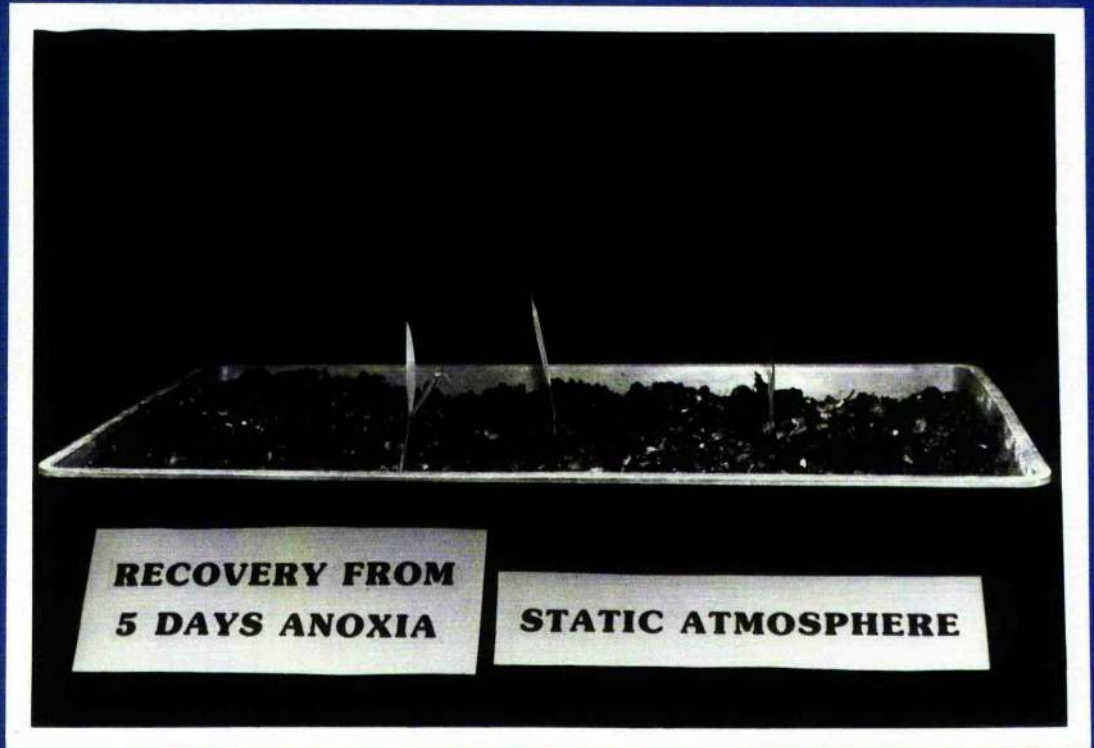

RECOVERY FROM
5 DAYS ANOXIA
STATIC ATMOSPHERE

TABLE 5.2 Ethanol and acetaldehyde in the head space above G. maxima rhizomes after four days anoxia and during a post-anoxic phase.

Mean values from 4-9 rhizomes ± S.E.

Treatment	Ethanol nmol g^{-1} fr wt (in gas phase)	Acetaldehyde nmol g^{-1} fr wt (in gas phase)	Ratio of Ethanol: Acetaldehyde
4 d anoxia	613.2 ± 114.0	9.5 ± 2.4	65
vessel changed, aerobic post-anoxic phase			
+ 30 min	385.7 ± 62.2	51.7 ± 8.0	7.5
+ 60 min	381.8 ± 71.2	49.8 ± 4.0	7.7
+ 120 min	356.4 ± 16.1	69.6 ± 12.3	5.1
+ 240 min	313.9 ± 27.3	66.4 ± 8.2	4.7
+ 360 min	327.8 ± 28.5	67.3 ± 4.8	4.9

DISCUSSION

Low levels of catalase and ethanol, and survival

When the anoxic atmosphere was circulated rather than kept static, levels of ethanol were found to be reduced to one sixth (c. 23 μmol g^{-1} fr wt) in G.maxima rhizomes. This reduction in accumulated ethanol in a moving anoxic atmosphere is correlated with better survival (i.e. regrowth after complete deprivation of oxygen) of rhizomes (Table 5.1). It is likely that this species does not accumulate ethanol in vivo by virtue of its aerenchymatous morphology, which not only provides efficient transport of oxygen from the shoot but probably also furnishes an escape route volatile products of any fermentation when the plant is flooded. Concentrations of ethanol in the rhizome tissue, which was tested at intervals throughout the year never exceeded 5 mol m^{-3} (Studer and Brändle, 1984). Glyceria, although flood tolerant, appears not to be metabolically adapted to anoxia (ap Rees and Wilson, 1984). The results here implicate the accumulation of excess ethanol as a factor in the intolerance of anoxia.

Crawford and Zochowski (1984) have shown that a circulating anoxic atmosphere reduces accumulation of ethanol in chickpea seedlings to one thirteenth of that found in static conditions, and postpones death due to anoxic stress by 48 hours. Carbon dioxide produced during alcoholic fermentation will also have been removed, but although there is some evidence that high concentrations of carbon dioxide exert a toxic effect on hypoxic tissues (Williamson, 1968; Andrews and Pomeroy, 1979; Roberts et al., 1984) this will not be

discussed here (see Chapter 3).

Under static anoxic conditions catalase exhibited greater increases in activity during anoxia and a post-anoxic phase than under moving (constant stream of nitrogen) anoxic conditions (Fig. 5.1). Thus, catalase appears to be induced or activated in response to accumulation of ethanol in this tissue, suggesting a more significant contribution to the ethanol oxidation reaction than the 3% that Donaldson et al. (1985) propose, ADH catalysing the major proportion of the reaction.

When varying concentrations of ethanol were applied to the head space above anaerobic rhizomes, clear increases in catalase were observed in response (Fig.5.2). Few inducible enzyme systems have been shown in plants (Stewart and Rhodes, 1977), but positive responses have been found in this system probably because the substrate ethanol diffuses easily into the aerenchymatous tissue of the hollow rhizomes of Glyceria. Most substrates are not so easily supplied. At the higher concentrations added (up to 1×10^3 mol m^{-3}) large standard errors were apparent, due to the extreme sensitivity of G.maxima rhizomes to ethanol and the time it took individual plants to achieve an appropriate level of catalase activation/induction.

Acetaldehyde applied to the head space also appeared to be effective in increasing catalase activities in the rhizomes (Fig. 5.2). However, the increases here are probably due to the fact that during anoxia the rhizomes will convert acetaldehyde to ethanol by means of ADH, and this endogenously produced ethanol would induce catalase in the same manner as in the ethanol treatment experiments.

It is noteworthy that the 0.1 x 10^3 mol m^{-3} acetaldehyde treatment rendered rhizomes moribund already during anoxia, whereas rhizomes exposed to ten-fold higher ethanol treatments all survived anoxia, dying only in the post-anoxic phase in air. It is possible then, that ethanol may be only indirectly toxic to plants through a post-anoxic oxidation to acetaldehyde, which on accumulation may be deleterious to the cell.

Acetaldehyde - a lethal accumulation in G. maxima?

It has been established that ethanol is often further metabolized after a period of natural anaerobiosis, such as that occurring in germinating seeds (Cossins and Turner, 1962). In bean seedlings, Doireau (1972) showed that as soon as radicle emergence occurs, the cotyledons and embryonic axis oxidize accumulated ethanol. Labelling experiments revealed that ethanol was further metabolized via the TCA cycle and then used in the biosynthesis of amino and fatty acids. A wide variety of plants can oxidize ethanol, but as Cossins and Beevers (1963) have stated, plant tissues may not always have the ability to further metabolize this product, and rates and pathways of utilization may differ considerably.

A slow rate in the further metabolism of ethanol may be the situation in G.maxima, where a steep fall in the ratio of ethanol:acetaldehyde in the gas space above rhizomes in an aerobic post-anoxic phase was observed (Table 5.2). Rhizome tissue in this species may not have the capacity to utilize high concentrations of ethanol; acetaldehyde dehydrogenase, which oxidizes acetaldehyde

produced by the initial oxidative step to acetate, may be present at only low levels, perhaps due to decreased rates of synthesis in the absence of oxygen (Verma and Marcus, 1974).

According to Grunnet et al. (1974) acetaldehyde may be oxidized both in the mitochondrial and cytosolic compartments of rat liver cells, although Lindros et al. (1974) have proposed that acetaldehyde oxidation in the same organism normally (i.e. when acetaldehyde is present at physiological concentrations - less than 0.2 mol m^{-3}) takes place in the mitochondria. The acetaldehyde dehydrogenase of peanut cotyledons has been reported to have a pH optimum of 8.6 (Oppenheim and Castelfranco, 1967), so that if cell damage has occurred during anoxia, involving some cytoplasmic acidosis as envisaged by Roberts et al. (1985), then the enzyme, if it is cytosolic, could be expected to be less active.

It is under acidic conditions that the peroxidatic reaction of catalase in formate metabolism is predominant (Halliwell, 1974a). Thus, assuming that some acidosis has taken place, catalase may be very active in the parallel reaction, the oxidation of ethanol, given a continuous source of hydrogen peroxide:

$$Cat + H_2O_2 \longrightarrow Cat\text{-}H_2O_2 \text{ (Compound I)}$$

$$Cat\text{-}H_2O_2 + CH_3CH_2OH \longrightarrow Cat + 2H_2O + CH_3CHO$$

As well as flavin oxidases in the microbody itself, peroxide sources have been reported in the cytosol, mitochondria and endoplasmic reticulum (Boveris et al., 1972). If the peroxide:ethanol ratio

remains low, then the higher the concentrations of ethanol present the more ethanol is oxidized (Oshino et al., 1973).

The surge of acetaldehyde production observed in G.maxima rhizomes on return to air (Table 5.2), may represent concentrations of this intermediate which are toxic to the plant. Temporary accumulations of acetaldehyde in ethanol metabolism have been reported in germinating buckwheat seedlings (Effer and Ranson, 1967) and pea seedlings (Cossins and Turner, 1963). The amounts of acetaldehyde being produced in Glyceria rhizomes could be relatively large, as great quantities of ethanol, c. 135 μmol g^{-1} fr wt were found in this tissue after only five days anoxia. It is very likely that catalase will increase the rates of oxidation by ADH significantly.

At a temperature of 20.8 °C acetaldehyde boils and will rapidly escape from plant tissues, and any toxic effects will be averted. However, at lower temperatures the hydrated aldehyde is known to produce cross-linkages in proteins (Schauenstein et al., 1977); free acetaldehyde also reacts with amino groups of amino acids and primary amines to give carbinolamines and Schiff's bases. If acetaldehyde is present in significant amounts, such reactions will profoundly affect energy metabolism within the cell.

A screening method for the selection of haploid embryos of maize, used by Dhaliwal and King (1979) illustrates the toxicity of aldehydes in comparison to ethanol. Mutants lacking ADH survived exposure to allyl alcohol, but wild-type embryos oxidized the compound to acrylaldehyde and were killed. Therefore, it is suggested that post-anoxic oxidation of excessive accumulations of ethanol in Glyceria rhizomes produces lethal concentrations of acetaldehyde, and

contributes significantly to the failure to survive anoxia in this species. Although flood tolerant by virtue of morphological adaptations (Studer and Brändle, 1984), G.maxima is not an anoxia tolerant species (Barclay and Crawford, 1982).

CHAPTER 6

SUPEROXIDE DISMUTASE ACTIVITY IN RHIZOMES OF THREE SPECIES OF DIFFERING TOLERANCE OF ANOXIA

INTRODUCTION

Oxidative damage in biological systems may be brought about by various oxygen species (see Chapter 4), and the superoxide radical (O_2^-) represents one potential source of toxicity. There are a number of cellular reactions 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 among those that produce superoxide in plant tissues. Superoxide dismutase (E.C. 1.15.1.1.) catalyses the disproportionation of superoxide to peroxide and dioxygen:

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Therefore, superoxide dismutase (SOD) forms part of the defences against oxygen toxicity in aerobic organisms. In higher plants SOD has been isolated in many tissues (Giannopolitis and Ries, 1977; Matkovics, 1977), and recently a survey was carried out in 43 families to determine the distribution of the three distinct known forms of the enzyme (Bridges and Salin, 1981). The SOD enzyme contains a metal prosthetic group, which may be either copper-zinc, manganese or iron.

The Cu,Zn-SOD isozyme was the first to be discovered (McCord and Fridovich, 1969), and in plants is characteristically found in the cytosol, and occasionally in chloroplasts and mitochondria. Mn-SOD is commonly associated with mitochondria and on occasion with chloroplasts, and there has been a report of its occurrence in the microbodies of pea (Del Rio et al., 1983). Fe-SOD is predominantly found in prokaryotes, but has been found to occur sporadically in some higher plant families, and is associated with the chloroplasts (Kwiatowski et al., 1985). The Cu,Zn-SOD and Mn-SOD forms of the enzyme could then be expected to be present in rhizomes, the tissue which is the subject of this investigation.

The main site of superoxide radical production is the mitochondrion (Rich and Bonner, 1978; Boveris and Cadenas, 1982), although there is some O_2^- formation in endoplasmic reticulum (Pederson and Aust, 1972) and chloroplasts (Asada and Kiso, 1973). According to Flohé et al. (1977), most if not all the hydrogen peroxide formed in mitochondria is derived from O_2^- which has undergone disproportionation by SOD. This is a very rapid reaction, and the Mn-SOD (of pea leaves) rate constant has been found to be 1.61×10^9 l mol^{-1} sec^{-1} at 25 °C, pH 7.8. Nevertheless, Nohl and Hegner (1978) have proposed that 20% of the superoxide produced escapes quenching by intramitochondrial SOD. Hirata and Hayaishi (1977) have proposed that some superoxide may be utilized for oxidizing organic compounds, analogous to the utilization of hydrogen peroxide by peroxidases. It is interesting to note that Boveris and Cadenas (1982) estimate steady-state levels of superoxide in mitochondria to be regulated at approximately 10^{-8} mol m^{-3}, compared with levels of about 10^{-5} mol m^{-3} for peroxide.

Superoxide is a more reactive product of oxygen utilization than hydrogen peroxide, but in general has not been shown to mediate intracellular damage per se. However, one report of superoxide as an active oxidizing agent was made by Bielski and Chan (1977): O_2^- was found to react slowly with free NADH, but four orders of magnitude faster when the nucleotide was bound to lactate dehydrogenase. Subsequently the enzyme-bound nucleotide radical intermediate reacted rapidly with molecular oxygen to produce NAD^+ and another O_2^-, thus creating a chain reaction. Another report has described the oxidizing action of superoxide on the interior of membranes (Niehaus, 1978): phospholipids were destroyed by a nucleophilic attack upon the carbonyl groups of the ester bonds linking fatty acid side chains to glycerol. It has been suggested (Halliwell and Gutteridge, 1985) that as much of the superoxide generated within cells comes from membrane-bound systems, it is possible that some is formed on the membrane interior.

In the absence of a suitable reactant, superoxide reacts with itself, as in the dismutation reaction outlined above. However, the oxygen produced is in the excited "singlet" state ($^1O_2$), which is far more reactive than normal ground state "triplet" that is formed when the reaction is catalysed by SOD. Singlet oxygen is quenched rapidly by water, but survives longer in the hydrophobic environment on the interior of membranes. Here singlet oxygen may attack polyunsaturated fatty acid side chains, forming lipid peroxides and accelerating lipid peroxidation which is a chain reaction. When uncontrolled, lipid peroxidation effects a loss of membrane integrity, as lipid peroxides and some of their degradation products cause extensive damage to enzymes and to membranes, producing a decrease in electrical

resistance and membrane fluidity (Hicks and Gebicki, 1978; Pauls and Thompson, 1980). Loss of membrane integrity will affect cellular metabolism deleteriously. SOD may be able to scavenge $^1O_2$ as well as O_2^- (Paschen and Weser, 1973), and according to subcellular location, antioxidants such as ascorbate, reduced glutathione and α-tocopherol also remove this oxygen species.

If superoxide is allowed to accumulate, the following reactions may take place, leading to the formation of the most reactive oxygen species, the hydroxyl radical:

$$Fe^{3+}\text{-salt} + O_2^- \longrightarrow Fe^{2+}\text{-salt} + O_2$$

$$Fe^{2+}\text{-salt} + H_2O_2 \longrightarrow OH^\cdot + OH^- + Fe^{3+}\text{-salt (Fenton reaction)}$$

$$\text{Net: } H_2O_2 + O_2^- \xrightarrow[\text{catalyst}]{\text{Fe-salt}} O_2 + OH^\cdot + OH^-$$

This reaction has been termed the iron-catalysed Haber-Weiss reaction (Halliwell and Gutteridge, 1984). Prerequisites for the reaction are traces of a transition metal ion, and accumulations of superoxide and hydrogen peroxide. Hydroxyl radicals thus formed react indiscriminately with cellular compounds. Examples of such potentially deleterious reactions are found in the hydroxylation of purine and pyrimidine bases present in DNA, which will give rise to mutations, and the abstraction of hydrogen radicals from membrane lipids, which will trigger lipid peroxidation.

Kellogg and Fridovich (1975) have proposed that mixtures of superoxide and hydrogen peroxide react together, in the absence of iron salts, to produce singlet oxygen and the hydroxyl radical:

$$H_2O_2 + O_2^- \longrightarrow OH^- + OH^{\cdot} + {}^1O_2 \quad \text{(Haber-Weiss reaction, modified)}$$

Moreover, they argued that hydroxyl radicals were more likely to be scavenged indiscriminately, and that singlet oxygen would tend to survive and attack macromolecules at critical sites. However, it has been shown that the rate constant for the Haber-Weiss reaction (producing ground state, triplet oxygen) in aqueous solution is virtually zero, so that the significance of this reaction in vivo is not known (Halliwell and Gutteridge, 1984).

It has been noticed that plants completely deprived of oxygen survive a period of imposed anoxia, only to die on re-exposure to air (Chapter 4). Therefore, experiments to examine SOD levels in three species of differing tolerance of anoxia were carried out, subsequent to the investigation of catalase, where the picture is clouded due to the probable peroxidatic reaction of that enzyme with ethanol (Chapter 5). SOD is thought to play a central role in protection against oxygen toxicity (Fridovich, 1974), and a study with microorganisms (aerobes, aerotolerant anaerobes and strict anaerobes) has shown that SOD is essential to survive in the presence of oxygen, while the possession of catalase was less well correlated with the ability to grow in oxygen, and indeed the aerotolerant anaerobes contained no catalase at all (McCord et al., 1971).

In the higher plant rice, rates of protein synthesis under anoxia have been reported to be slower than under air (Bertani et al., 1981) with some new polypeptides appearing possibly as an adaptation to the new environmental conditions (Mocquot et al., 1981). If a particular species is intolerant of anoxia, with slower rates of protein synthesis a fall in activity in certain enzymes may be seen. If there is a drop in activity in the key enzyme in protection against oxygen, SOD, it could be expected that some oxidative damage will take place on return to air. Indeed, a significant rise in the lipid peroxidation product, malondialdehyde, has previously been found in rhizomes of Iris germanica on return to air after prolonged anoxia, while levels remained the same in the related Iris pseudacorus (Hunter et al., 1983). Therefore these two species, and the relatively anoxia intolerant Glyceria maxima, were chosen for the series of experiments investigating levels of SOD during recovery from anoxia.

MATERIALS AND METHODS

The three species in this series of experiments were: Glyceria maxima, Iris germanica (var. Quechei) and Iris pseudacorus. Glyceria is the least tolerant of anoxia (less than one week, Chapter 5; Barclay and Crawford, 1982), while I.germanica is of intermediate tolerance and I.pseudacorus is very tolerant (two weeks and eight weeks respectively, Hetherington et al., 1983). Plants were harvested from the greenhouse, prepared and incubated as for the catalase experiments, and also followed a similar time-scale to that investigation.

Superoxide dismutase was assayed spectrophotometrically, based on the method by McCord and Fridovich (1969). A Unicam SP 1800 Ultraviolet Spectrophotometer was used attached to a waterbath at 25 ± 1 °C, which kept the temperature of the cuvettes constant. The reaction mixture (total volume, 3 ml) contained: 50 mol m^{-3} K-phosphate buffer pH 7.8, 1.0 mol m^{-3} NaN_3, 0.10 mol m^{-3} EDTA 0.01 mol m^{-3} ferricytochrome c. 0.10 mol m^{-3} xanthine and xanthine oxidase (XOD), which was diluted approximately ten times to produce an increase in absorbance at 550 nm of 0.025 units min^{-1}. The substrate for SOD is the unstable superoxide free radical, which in this indirect assay is continuously generated at a controlled rate by XOD. Cytochrome c in the assay mixture will thus be reduced by superoxide at a known rate, which on the addition of SOD will decrease. Under these conditions, the amount of SOD required to inhibit the rate of reduction of cytochrome c by 50% is defined as one unit of activity.

Calibration curves (0.1 - 0.5 µg SOD/ml) were made daily with bovine liver SOD (Sigma). The curves were linear up to 30-35% inhibition. Buffer containing cytochrome c and xanthine was made up weekly and stored at -20 °C. Each commercial preparation of horse heart cytochrome c (Sigma) was first checked spectrophotometrically to ascertain the true concentration of ferricytochrome c. Dilutions of buttermilk xanthine oxidase (Sigma) were made daily, and the known rate of reduction of cytochrome c was checked regularly throughout the assay.

Peroxidases and cytochrome oxidase are known to interfere with this assay, by using cytochrome c as a substrate (Flohé and Otting, 1984), so 1.0 mol m^{-3} azide was added to the buffer to block these possible reactions (Keilin, 1936). At this concentration Fe-SOD, an isozyme not ubiquitously found in higher plants (Bridges and Salin, 1981) and then only associated with chloroplasts, is significantly inhibited (Asada et al., 1975). Non-green material was used in these experiments. Cyanide could not be used as the Cu,Zn-SOD of the cytosol is cyanide-sensitive.

Rhizome extracts were prepared firstly by grinding with a pestle and mortar 0.5 g slices in 3 ml phosphate buffer (pH 7.8), containing 0.1 mol m^{-3} EDTA and 1.0 mol m^{-3} NaN_3. After 15 minutes centrifugation at 8730 g at + 5 °C, the supernatant was put through a filter, pore size 1.2 µm (Millipore). The extract was then desalted by running through a Sephadex G-25 column (Pharmacia), which had been equilibrated with the extraction buffer. Some dilution of the extract occurred at this step, and in the case of G.maxima and I.germanica the extracts were then concentrated in Centricon centrifugal

microconcentrators (Amicon). Due to the large amounts of SOD found in I.pseudacorus, however, the extract was often diluted two to four-fold. The extraction procedure was carried out as far as possible at temperatures below 5 °C. 3% (w/v) soluble PVP was added to the extraction buffer in the case of I.germanica and I.pseudacorus in order to give maximal catalytic activities.

Soluble protein content of the extracts was taken as a reference value for SOD activity. Soluble proteins were measured by the binding of bromophenol blue to proteins under acidic conditions, the bound form absorbing light at 610 nm wavelength (Flores, 1978). Bovine serum albumin was used as a standard. It was noted that PVP interferes with this protein assay, causing some overestimation of protein content so that the specific activity of SOD in the Iris species may be consistently slightly underestimated.

Enzyme recovery experiments with mixtures of samples and standards, including boiled (10 minutes) plant samples were carried out for each species. A recovery value of 88.1% ± 3.0 S.E. was found for Glyceria (n=3); 104.0% ± 10.6, for I.germanica (n=3); 94.2% ± 2.8, for I.pseudacorus (n=3). Boiled plant samples yielded no inhibition of the base rate of the reaction.

RESULTS

Superoxide dismutase activity was determined in rhizomes of three species after 3 (G.maxima only), 7, 14, 21 and 28 days anoxia, and during a 48 hour post-anoxic recovery phase. Each of the species examined showed a different pattern of development of SOD as a result of anoxic stress.

Figure 6.1 shows SOD activity in I.pseudacorus rhizomes. Initially, aerobic rhizomes exhibited an activity of 119.4 U SOD mg^{-1} protein. Remarkable rises in levels of SOD were found during the course of the anoxic treatments. Seven days anoxia resulted in a three and a half-fold increase in activity to 427.8 U mg^{-1} protein. At the end of 14 days anoxia an eight-fold rise was apparent; while 21 days anaerobiosis produced an 11-fold increase. 28 days anoxia brought about a 13-fold increase in levels of SOD to 1576 U mg^{-1} protein. The longer the period of imposed oxygen deprivation, the greater the increase in SOD activity seen. These high activities were maintained in every case during the post-anoxic recovery phase.

Figure 6.2 shows SOD activity in I.germanica rhizomes. Initial levels were similar to that measured in the related I.pseudacorus (Fig. 6.1). At the beginning of the experiment 110.3 U SOD mg^{-1} protein was recorded. No significant change in level was found at the end of seven days anoxia, but 14 days oxygen deprivation resulted in a 170% increase to 271.6 U mg^{-1} protein; 21 days anoxia brought about a 120% increase. However, 28 days produced a slight drop in activity compared to initial levels.

During the 48 hour recovery phase after seven days anoxic stress, SOD increases up to 197.3 U mg^{-1} protein were noted. During recovery phases after 14 and 21 days anaerobiosis, the relatively high levels found during anoxia are approximately maintained. In contrast, there is a post-anoxic increase in SOD activity to 159.4 U mg^{-1} protein in rhizomes from the 28 day treatment, which falls sharply again at 48 hours.

Figure 6.3 shows superoxide dismutase activity in Glyceria rhizomes. Higher levels than found in the Iris species, of 273.5 U SOD mg^{-1} protein were found in rhizomes at the beginning of the experiment. After three days anoxia, however, a drop of c. 50% to 147.4 U mg^{-1} protein was recorded. Decreases in activity of similar proportion were also seen in the longer periods of anoxia. In rhizomes exposed to three and seven days anaerobiosis, SOD activities increased during the 48 hour post-anoxic phase to levels approaching those at the beginning of the experiment. After the 14, 21, and 28 day anoxic treatments SOD activity fell away during the post-anoxic phase to levels only 30% of the initial measurements.

FIGURE 6.1 The development of superoxide dismutase activity on return to air in rhizomes of Iris pseudacorus that were exposed to anoxia for up to 28 days. The column sets represent 0–2 days post-anoxic treatment, and the different columns within the sets represent 0 (□), 7 (▨), 14 (▩), 21 (▥) and 28 days (▤) previous incubation (n = 3–6, with standard error bars).

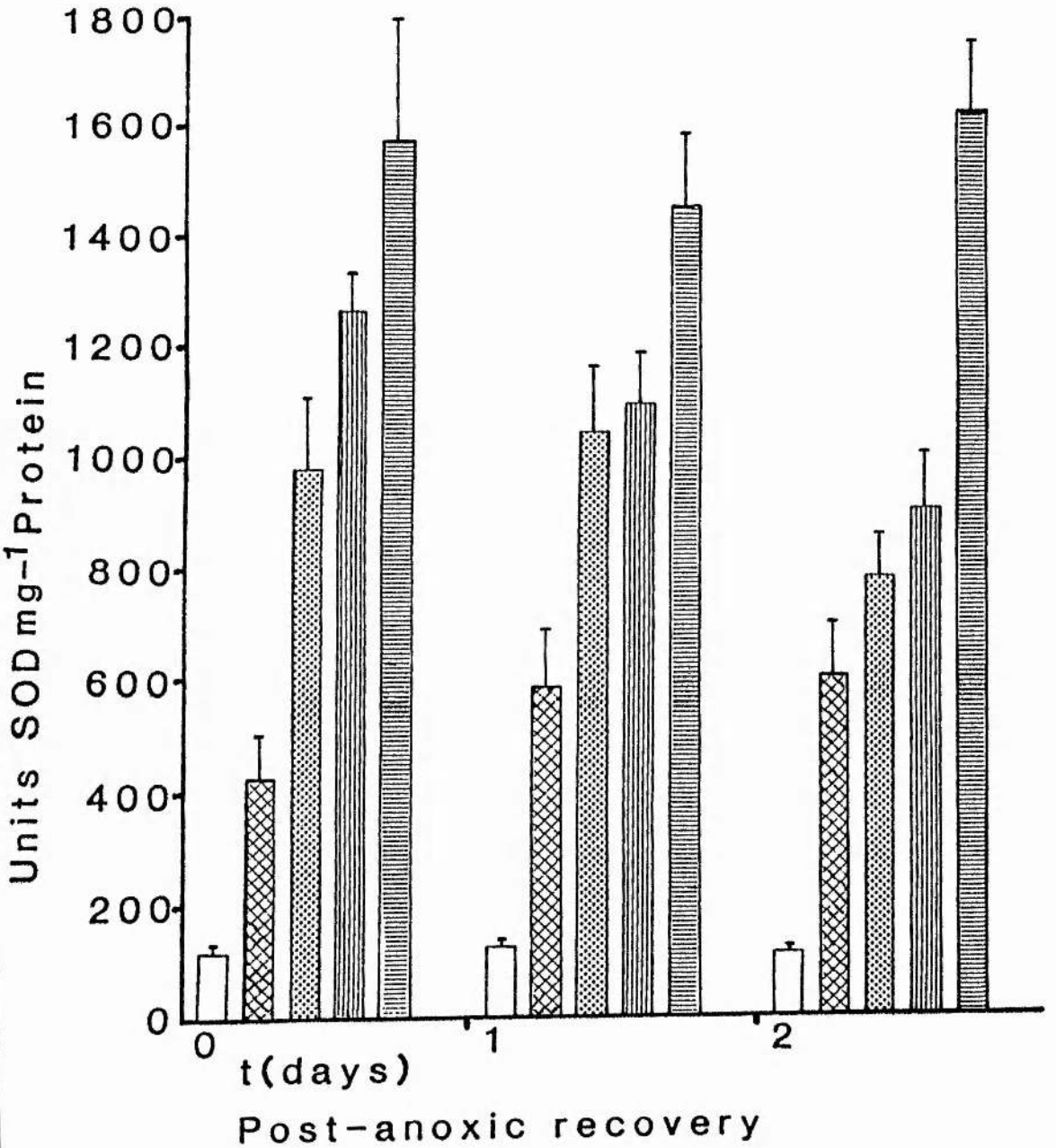
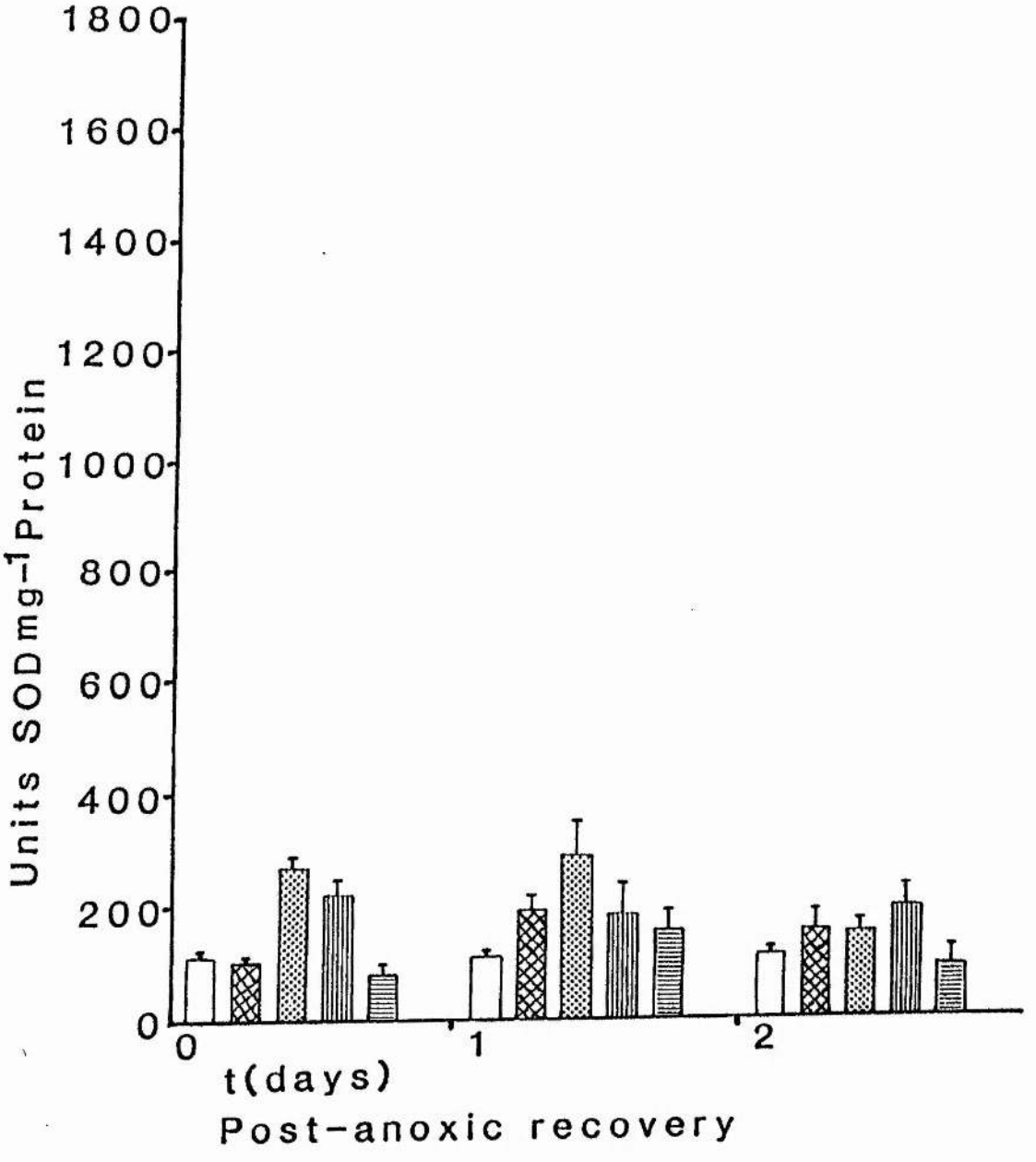

1800
1600
1400
1200
1000
800
600
400
200
0
Units SOD mg-1 Protein
0
1
2
t(days)
Post-anoxic recovery

FIGURE 6.2 The development of superoxide dismutase activity on return to air in rhizomes of Iris germanica that were exposed to anoxia for up to 28 days. The column sets represent 0-2 days post-anoxic treatment, and the different columns within the sets represent 0 (□), 7 (▨), 14 (▩), 21 (▥) and 28 days (▤) previous incubation (n = 3-6, with standard error bars).


1800
1600
1400
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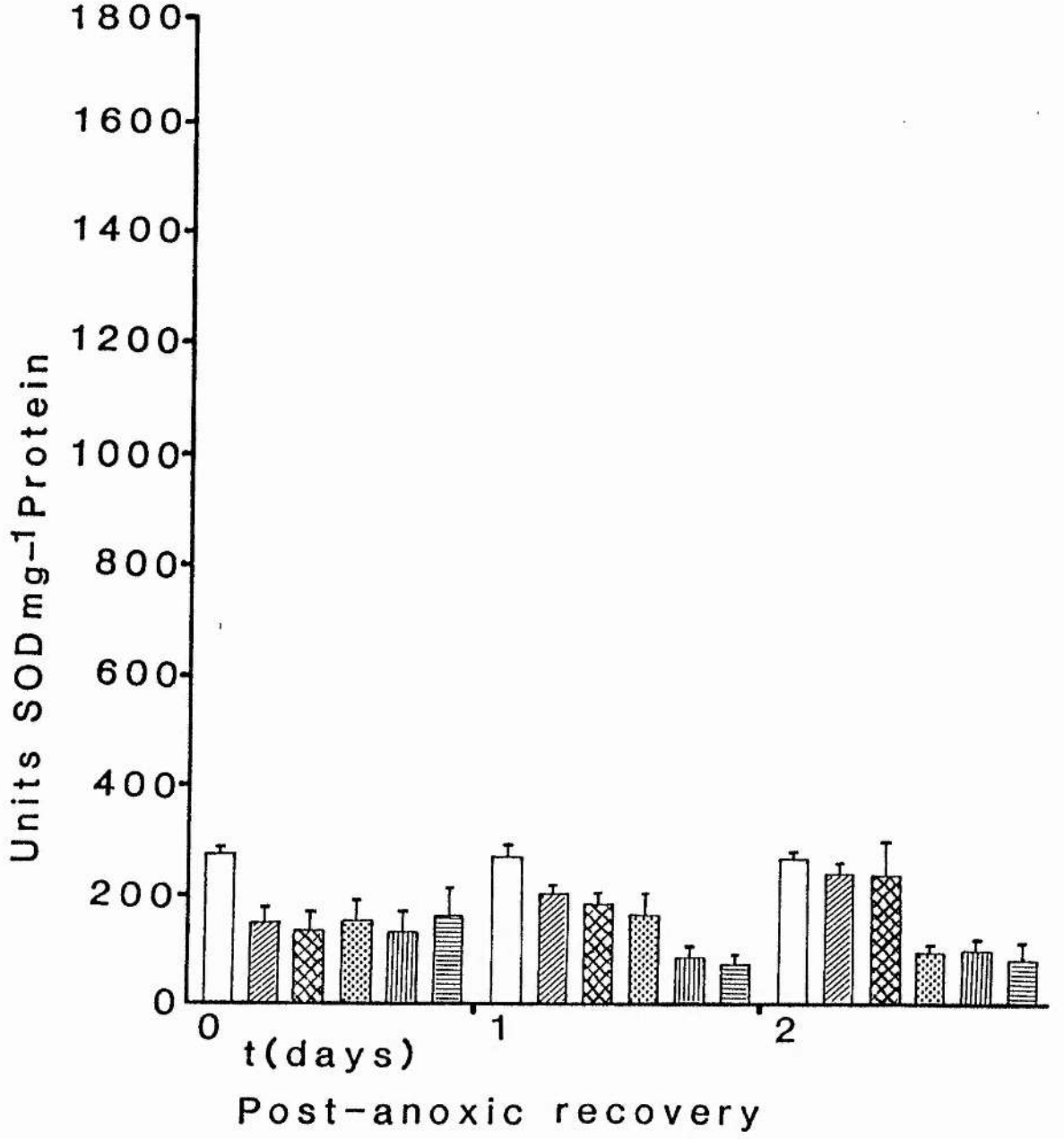
200
0
Units SOD mg-1 Protein
0
1
2
t(days)
Post-anoxic recovery

FIGURE 6.3 The development of superoxide dismutase activity on return to air in rhizomes of Glyceria maxima that were exposed to anoxia for up to 28 days. The column sets represent 0-2 days post-anoxic treatment, and the different columns within the sets represent 0 (□), 3 (▨), 7 (▩), 14 (▦), 21 (▥) and 28 days (▤) previous anaerobic incubation (n = 3-6, with standard error bars).


1800
1600
1400
1200
1000
800
600
400
200
0
Units SOD mg-1 Protein
0
1
2
t(days)
Post-anoxic recovery

DISCUSSION

The significance of changes in SOD activity

The level of SOD found in rhizomes of the three chosen species in this series of experiments, was closely associated with the ability of the particular species to survive anoxia. 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 (Fig. 6.1). 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 in this species, 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 (Fig. 6.2) are not large enough, and oxidative damage may be a determining factor in the post-anoxic death of rhizomes of this plant.

The least anoxia tolerant species in this series of experiments, with a limit of seven days, (Barclay and Crawford, 1982) was G.maxima. A fall in SOD concentrations during anoxia, such as that found in Glyceria (Fig. 6.3), might expose the plant to oxidative damage on return to air. Levels of SOD may not be sufficient to scavenge superoxide being produced in the course of aerobic respiration, and in fact increases in SOD are seen during the aerobic post-anoxic phase from the shorter anoxic treatments, indicating that there is a need for greater protection against deleterious oxidative reactions.

There may be parallels here with the clinical phenomenon in animals known as post-ischemic injury. When the normal flow of blood to a tissue is restricted or blocked, a state of partial or complete ischemia results, where the tissue becomes hypoxic or completely anoxic. However, most of the injury occurs not during the hypoxic period, but on restoration of the oxygen supply (Haglung 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 re-perfusion with oxygen decreased the incidence of post-ischemic injury.

A biochemical mechanism for the uncontrolled production of O_2^- radicals on return to air, causing this type of tissue damage was proposed by McCord: when energy charge drops below a certain level in hypoxic tissue, a calcium-triggered proteolytic attack on cellular xanthine dehydrogenase converts the enzyme to O_2^--producing xanthine oxidase, which becomes active on contact with molecular oxygen. Thus, oxygen toxicity appears to be the main cause of post-ischemic injury in many animal tissues and may be the case in some plant tissues.

Presuming that the high levels of SOD are actively dismutating superoxide to hydrogen peroxide and molecular oxygen in I.pseudacorus, during the post-anoxic phase, there may be a relative increase in peroxide levels, compared to amounts produced normally. Oshino et al. (1973) showed that when the rat liver cellular peroxide:catalase ratios are low, the peroxidatic action of catalase (with ethanol as a cosubstrate) predominates. However, when the peroxide:catalase ratio is high then the proportion of catalatic action of catalase is considerably increased. It is likely that the latter condition will

be the case in I.pseudacorus. Therefore, as well as being protected from oxygen toxicity, it could be postulated that unregulated oxidation of ethanol by catalase is also avoided. This suggestion gains support from the fact that catalase levels remain low during anoxic and post-anoxic treatments (Chapter 4), and ethanol accumulation within the rhizome is limited (Chapter 3).

The control of biosynthesis of SOD has been stated to be complex (Fridovich, 1986). Why SOD activities are increased in the complete absence of oxygen in I.pseudacorus, and to a certain extent in I.germanica, is not clear. In view of the biological role of SOD, the enzyme should be induced by oxygen. Manipulation of Mn-SOD levels, at constant pO2, have demonstrated that oxygen is not itself responsible for derepression of SOD biosynthesis. For instance, glucose as an energy source, depressed Mn-SOD in E.coli (Hassan and Fridovich, 1977), while enrichment of the culture medium with manganese or iron salts elevated Mn-SOD and Fe-SOD respectively, in P.ovalis (Yamakura, 1976).

SOD and other aspects of anoxic and waterlogging stress

A recent study on nutrient toxicity and anoxia has implicated lipid peroxidation as an effect of iron toxicity in the iron sensitive wetland plant Epilobium hirsutum (Hendry and Brocklebank, 1985). An iron-induced oxygen radical metabolism was proposed when substantial increases in SOD activity were observed in the roots of this plant in response to applications of varying amounts of iron to hypoxic cultures. In older plants (eight to ten weeks) there was a parallel increase in levels of peroxidase (catalase activity was neglible),

which together with SOD appeared to prevent oxidative damage. In younger plants oxygen toxicity took its effect since only an increase in SOD took place and extensive tissue damage was recorded, presumably from deleterious oxidative reactions with accumulated H_2O_2.

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 too, 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 monocots that form the subject of the present 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.

The relatively anoxia intolerant I.germanica exhibited high rates of oxygen uptake after removal from eight days anoxia (Fig. 2.1). However, some of this oxygen was probably being used in non-respiratory reactions such lipid peroxidation: the rate of oxygen consumption dropped back to control levels after 48 hours. It could be speculated here that SOD concentrations are sufficient to quench oxidative damage, but that after 14 days anoxia, which is lethal to this species (Hetherington et al., 1983), lipid peroxidation contributes to the death of the tissue, perhaps along with toxic accumulations of acetaldehyde from unregulated oxidation of ethanol (Chapter 5). Certainly, Hunter et al. (1983) noted a significant increase in the lipid peroxidation product, malondialdehyde, on

re-exposure to air after 14 days anoxia in the same Iris species, indicating that some membrane damage was taking place.

In a study on the same two Iris species that feature in the present investigation, 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 in rice and wheat (Khoang et al., 1979) and 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, an antioxidant associated specifically with membranes. Both these mechanisms may form an important second line of

defence against oxygen toxicity, after SOD.

Ethanol, which is the main end product of anaerobic respiration in plants, has long been known to radiation chemists as an efficient scavenger of the hydroxyl radical. Neta and Dorfman (1968) reported a rate constant of 1.85×10^9 l mol^{-1} sec^{-1} at 25 °C. It is possible that the metabolite, which is accumulated in particularly large concentrations in precisely those species (G.maxima and I.germanica) that show lower SOD activities, will remove $OH^{\cdot}$ as it forms perhaps preventing some cellular damage. It is an ironic twist of fate that oxidation of this ethanol to acetaldehyde, which may be taking place at an enhanced rate due to the peroxidatic activity of catalase, could be mediating equally deleterious effects in the same species. Ethanol could also be scavenging hydroxyl radicals in the anoxia tolerant species, where it is accumulated to lesser concentrations, although it is not known how efficient the compound is in vivo at a particular concentration.

It can be seen then that the extraordinarily high SOD activities in rhizomes of one (I.pseudacorus) of the three monocots in question, although correlated with the ability to prolonged survive anoxia, may only partially account for its tolerance of this environmental stress. Ethanol accumulation, catalase activity, and antioxidant status of the cell in general, may all be contributing factors.

CHAPTER 7

GENERAL DISCUSSION

Flooding tolerance vs. anoxia tolerance

Under flooded conditions, whether completely submerged or not, plants may experience some degree of oxygen stress. It has been emphasised by certain workers (Hook and Scholtens, 1978; Crawford, 1982; Jackson and Drew, 1984) that the potentially deleterious effects of flooding in woody and non-woody plants is not entirely due to lack of oxygen for respiration. Reduced soil components, such as manganous and ferrous ions, may give rise to nutrient toxicity; microbial metabolites can accumulate to toxic levels with regard to plant tissues; leaching and denitrification may deplete soil nitrogen. The relative importance of each of these stresses is not known. General symptoms of injury due to flooding in whole plants include wilting, leaf epinasty and leaf shedding which will precede death.

In the present investigation only the underground storage organ, the rhizome, of various wetland plants, and one dryland plant, was examined for metabolic response to the single stress of anoxia. Certain biochemical parameters were chosen in order to attempt to elucidate the nature of injury - or protection from injury - due to complete deprivation of oxygen in this plant tissue. Species were selected that were known to occupy different micro-sites within the wetland habitat and and had been shown to exhibit related levels of anoxia tolerance (Brändle and Crawford, in press), e.g. *S.lacustris*, rhizomes are buried in permanently saturated or water covered muds, and survive more than 90 days experimentally imposed anoxia; *A.calamus*, rhizomes occur at the interface between anaerobic mud and

free water, and survive more than 14 days imposed anoxia; J.effusus, rhizomes are buried in soils subject only to seasonal flooding, and survive a mere 4-7 days imposed anoxia.

Survival of anoxic stress: anaerobic respiration capacity

The initial experiments recorded in this thesis investigated anaerobic respiration in the rhizomes of various monocots (A.calamus, I.germanica, I.pseudacorus, P.australis, S.lacustris and T.latifolia). In Chapter 2 it was established that there was sufficient respiratory capacity in these plants to ensure survival of prolonged - up to 15 days - anoxic stress, and that ethanolic glycolysis appeared to be the major pathway operative in all species (Fig. 2.2). Lactate glycolysis appeared to be of strictly secondary importance in S.lacustris, and was insignificant in the remaining species (Table 2.1). Only these two pathways of fermentation were considered, but it is not impossible that some diversification in anaerobic pathways was taking place, since no carbon balance sheet was made.

All species possessed adequate carbohydrate reserves in the rhizome to ensure survival of 15 days of relatively inefficient, in terms of ATP production, anaerobic respiration (Fig. 2.3). Vartapetian and workers (1977) have shown that supplying glucose to excised roots preserved the ultrastructure of mitochondria subjected to anoxia - when structural changes would normally take place in this organelle - and conferred greater tolerance of anaerobiosis. Carbohydrate metabolism must provide an energy supply to meet the needs of a particular tissue in order to survive.

Energy charge, which is a measure of the energy status of a given tissue (Atkinson, 1969; Pradet and Bomsel, 1978), has been observed to fall when rhizomes of S.lacustris were made anaerobic, and recover once alcohol dehydrogenase (ADH) activity increased to a certain level (Monk and Brändle, 1982). Recently, Barta (1986) reported a correlation between the higher rate of anaerobic respiration and the more favourable energy status in the hypoxic roots of flood tolerant trefoil, than in the roots of flood intolerant alfalfa that underwent the same stress. As no significant differences were found in carbohydrate availability under anoxia and in air (Fig. 2.3) in the rhizomes of the six species investigated here over 15 days anoxia, it is concluded that the levels of ADH found - taken to represent alcoholic fermentation - provide sufficient energy to ensure the anoxic survival of these plant organs.

It was noticed that there was some residual fermentation in the rhizomes of all of the species tested during the control incubation in air (Fig. 2.2, Table 2.1). Areas of the rhizomes of these different taxa may consist of densely packed cells, and probably undergo fermentative reactions more or less continuously. This phenomenon is known to occur not uncommonly in the closely packed cells of meristematic areas such as the leaf primordia of lilac by Öpik (1980).

Survival of anoxic stress: anaerobic metabolite accumulation

There were noteworthy interspecific differences in the concentration of the end product of alcoholic fermentation, ethanol, accumulated within the rhizome tissue (Fig. 3.1) in the six species tested above for anaerobic respiration capacity. It is suggested that there is a close association between the possession of a ventilation mechanism, such as the lacunar system typically found in monocotyledonous wetland species (Crawford, 1982), and the limitation of ethanol accumulation. The five wetland taxa are known to possess 25-60% gas spaces within the rhizomes (Studer and Brändle, 1984; Monk et al., in press), and appear from data presented in Chapter 3 to reach a 'plateau' of ethanol accumulation of c. 30 mol m^{-3} or less, representing an equilibrium of production and release. In contrast, I.germanica, the single dryland species tested, exhibited a steady rise in ethanol concentration within the rhizome to c. 70 mol m^{-3}; the rhizomes of this plant conspicuously lack aerenchyma of any description (Monk et al., in press).

In one species, T.latifolia, a drop in alcoholic fermentation was observed after seven days anoxia: both ADH activity and ethanol accumulation fell, approaching levels found in aerobic controls. This phenomenon may represent a strategy for conservation of carbohydrate reserves during long-term anoxia, as well as avoidance of excessive ethanol, and of course carbon dioxide, accumulation. In periods of short-term oxygen deficiency, Roberts et al. (1984, 1985) have proposed that the avoidance of cytoplasmic acidosis will enhance survival of the hypoxic or anoxic stress. Contributing factors to the acidification of the cytosol may be a leaky tonoplast membrane, lactic

fermentation and carbon dioxide accumulation from alcoholic fermentation. Assuming that the tolerant plants in the present investigation have mechanisms to guard the integrity of membranes for long periods of anoxia, the non-accumulation of certain anaerobic metabolites may serve to largely avoid or at least postpone the apparently lethal syndrome.

The elimination of anaerobic metabolites, such as ethanol, carbon dioxide and lactate from the organ of production, into the surrounding environment or other parts of the plant to be further metabolized, may constitute a significant adaptation to long-term anoxia tolerance. New evidence with regard to the toxicity of physiological concentrations of ethanol in plant cells and tissues has been reported by Perata et al. (1986) and supports this suggestion. Cell division in Helianthus tuberosus discs cultured in vitro was inhibited more than 50% by 8.5 mol m^{-3} ethanol; induction of α-amylase by GA_3 in barley aleurone layers was strongly reduced by 34 mol m^{-3}; the viability of single protoplasts from carrot cell cultures was only slightly affected by 10 mol m^{-3} ethanol, but embryogenesis was arrested at this concentration.

A previous publication by the same group of workers (Alpi et al., 1985), where concentrations of ethanol 10-100 fold higher than the above amounts were applied to cereal seedlings under aerobic conditions, came to the contrary conclusion that the adverse effects of anoxia could not be ascribed to ethanol accumulation. The time-scale of experimentation here was relatively short and the species examined are unable to survive prolonged oxygen deprivation. Therefore, other factors such as the onset of cytoplasmic acidosis may play a large part in the intolerance of anoxia in these plants.

Thus, it is still a matter of debate how toxic the high concentrations of ethanol often found in anaerobic plant tissues really are, and whether excessive ethanol accumulation is lethal by itself. In Part II (Chapter 5) the following possibility was investigated: that ethanol is indirectly toxic through the production of large amounts acetaldehyde, when the anaerobic metabolite is oxidized on return to air. Certainly, survival of anoxia treated rhizomes of G.maxima was improved by circulating the anaerobic environment (Table 5.1), thus reducing the tissue accumulation of volatile products such as ethanol and carbon dioxide.

Although a wetland plant, G.maxima inhabits a niche which is only periodically flooded, and appears to rely on morphological adaptation to minimize ethanol accumulation during hypoxic stress (Haldemann and Brändle, 1986). It had previously been noticed that G.maxima rhizomes can in fact endure up to 28 days anoxia, only to collapse and die on return to aerobic conditions, when it was known that the plant is capable of surviving (growing new shoots and roots during a subsequent recovery period) a mere 4-7 days without oxygen (Barclay and Crawford, 1982). In the present investigation, head space analysis of the volatile compounds above post-anoxic rhizomes showed a steep decrease in the ethanol:acetaldehyde ratio (Table 5.2), indicating that oxidation of the large concentrations of ethanol that accumulate in the rhizome tissue of this species (c. 135 mol m^{-3} over five days anoxia) was taking place. Relatively low concentrations of acetaldehyde are phytotoxic, when compared to the effects of ethanol (Takijima, 1963; Schauenstein et al., 1977; Jackson et al., 1982). Thus, it would appear that limitation of the accumulation of ethanol enhances survival of prolonged anoxia and possibly more importantly

post-anoxia.

Post-anoxic events: the role of catalase in relation to ethanol

The rate of oxidation of ethanol catalysed by ADH in rhizomes undergoing an aerobic post-anoxic phase, will be dependent on the affinities of the ADH isozyme complement present in the particular species. Williamson et al. (1974) have shown that the rate of reoxidation of NADH may determine the rate of this first step in ethanol metabolism, so that if aerobic metabolism has been damaged by the period of oxygen deprivation acetaldehyde production may not be optimal. In animals, catalase acting in its peroxidatic mode, has been shown to contribute significantly to the oxidation of ethanol in the liver (Oshino et al., 1973, 1975). When ethanol was applied to anoxic rhizomes of G.maxima (Fig. 5.2), catalase activity increased in response and it is suggested that there may be considerable production of acetaldehyde by this 'alternative' reaction in this plant tissue.

G.maxima rhizomes that underwent anoxic incubation under circulated (where ethanol accumulation has been shown to be reduced to one sixth of the concentration expected) rather than static conditions exhibited considerably lower activations of catalase, both in the anoxic and post-anoxic phases (Fig. 5.1). Therefore, when high concentrations of ethanol are endogenously produced in the anoxia-treated rhizomes of G.maxima, it may be that on restoration of the oxygen supply catalase aids oxidation of ethanol to generate lethal concentrations of acetaldehyde.

Post-anoxic events: catalase in the oxygen defence system

With regard to other possible forms of post-anoxic injury catalase and superoxide dismutase activities were determined in their capacity as part of the cellular system of defence against oxygen toxicity (Part II, Chapters 4 and 6). In the relatively anoxia intolerant species (J.effusus, I.germanica and G.maxima) significant post-anoxic increases in catalase activity were observed (Fig. 4.2). In the latter case, the enzyme increased in activity during anaerobiosis, which is an unusual occurrence for an 'aerobic' polypeptide (Sachs et al., 1980). Increases in catalase may in these species constitute a response to large accumulations of ethanol, as appears to be the case in G.maxima (Chapter 5).

On the other hand the increased catalase activities may be in response to higher levels of hydrogen peroxide, the better known substrate for catalase, which can arise as a product of reactions between oxygen free radicals. Superoxide dismutase (SOD) catalyses a reaction between two superoxide radical molecules that produces one molecule of dioxygen and one of peroxide. Hydrogen peroxide is converted to oxygen and water by catalase acting in its catalatic mode. If allowed to accumulate, peroxide may undergo further potentially deleterious reactions with other oxygen radicals, escalating the damaging reactions (Hendry and Brocklebank, 1985). That catalase increases in the aerobic post-anoxic phase indicates that some oxidative damage may be taking place on return to air in rhizomes of the three species, J.effusus, I.germanica and G.maxima.

The extremely anoxia tolerant species (S.lacustris, A.calamus and I.pseudacorus) that typically limit ethanol accumulation (Chapter 3), and that underwent the same anoxic stress showed no increases in the activity of this enzyme (Fig. 4.1). Whether this was because the concentrations of ethanol were too low to trigger increases such as those seen in G.maxima (Fig. 5.1), or because defences against deleterious oxidative reactions were adequate, is not clear.

Post-anoxic events: SOD in the oxygen defence system

Superoxide dismutase (SOD) is thought to play a key role in the defence against oxygen toxicity in aerobic organisms (Fridovich, 1974). Of the three species analysed in the present investigation the least anoxia tolerant, G.maxima, showed a drop in SOD during anaerobiosis, with some recovery in air (Fig. 6.3); I.germanica, of intermediate tolerance, exhibited small increases in SOD activity in the post-anoxic phase (Fig. 6.2). In contrast, the extremely anoxia tolerant rhizomes of I.pseudacorus showed significant increases in SOD during anoxia, these levels being maintained in the aerobic recovery phase (Fig. 6.1).

If as McCord (1985) suggests, certain changes in elements of aerobic metabolism take place under anoxia, bringing about increased production of deleterious superoxide radicals on return to air, then the post-anoxic recovery of I.pseudacorus rhizomes is safeguarded. Rhizomes of G.maxima and I.germanica however may be susceptible to the effects of oxygen toxicity. It should be said that this will be the case only if other antioxidants do not replace the function of SOD in

scavenging certain oxygen free radicals. A report identifying several non-enzymatic compounds which exhibit strong antioxidant qualities in rhizomes of turmeric (Curcuma longa) has provided scientific justification to the wide use of this spice in food in Eastern countries (Toda et al., 1985).

If SOD is induced during anoxia as an enzymatic antioxidant for protection against oxygen toxicity on return to air, as it appears to be in I.pseudacorus (Fig. 6.1), then this enzyme could be termed an anaerobic polypeptide (ANP), (Sachs et al., 1980). These authors designated the ADH isozymes induced during anoxia in maize ANPs as they featured in the new polypeptide pattern in plants deprived of oxygen, representing adaptation to this environmental stress.

Future work and the wider significance of tolerance of anoxia

In conclusion, the following new features of intolerance of prolonged anoxia may be proposed: post-anoxic oxidation of high concentrations of ethanol accumulated during anaerobiosis, possibly catalase-aided, leading to a lethal build-up of acetaldehyde within the tissue; normal or below normal levels of SOD activity and possibly other antioxidants on entering the aerobic post-anoxic phase, exposing the recovering tissue to oxygen toxicity.

Future work on anoxia intolerance might include a study of the fate of the fermentation product, ethanol, including its further metabolism in the rhizome or other parts of the plant. It would be interesting to ascertain what proportion of the first oxidation step, if any, is catalysed by catalase in a series of anoxia

tolerant/intolerant plants. Also, tissue estimations of possible acetaldehyde accumulation in the same species should be done. In addition, it would be interesting to investigate whether carbon dioxide accumulates to any extent in hypoxic/anoxic tissues, and what role, if any this anaerobic metabolite plays in anoxic injury.

In order to establish if post-anoxic injury is due at least in part to oxidative damage ethane and/or malondialdehyde - measures of membrane damage - could be determined in rhizomes of various species that had previously undergone prolonged anoxia. In addition, a concomitant study of the antioxidant status of the tissue under analysis, including determination of such substances as glutathione, ascorbate, α-tocopherol as well as SOD, should be carried out.

Rhizome homogenates of ginger, when added to meat products have been shown to confer improved storage stability, since this plant organ exhibits strong antioxidant activity (Lee et al., 1986). Precisely what proportion of this oxygen defence system is enzymatic is not known, but earlier reports have shown that this plant and other Eastern spice plants typically possess potent antioxidant properties (Toda et al., 1984). The European wetland plant, I.pseudacorus, appears to be able to induce increases in the antioxidant enzyme SOD in the rhizomes during anoxia in preparation for the recovery phase in air. If this remarkable phenomenon can be confirmed as a key adaptation to the tolerance of anoxia, by following up some of the lines of investigation suggested above, then the finding could have significance in agriculture. If certain species have evolved adaptations, perhaps at the level of gene expression to the adverse conditions of anoxia, which normally retards growth and development, then to identify those areas of adaptation could be advantageous in

the cultivation of commercial crops, where hypoxia is part of the problem of waterlogged soils.

In addition, the storage of bulky fruits and vegetables poses a problem of premature deterioration due to hypoxic conditions or non-recovery from the same. If more light were then thrown on the nature of post-anoxic injury and its role in the intolerance of anoxia, altered gene expression could perhaps be identified, and as Sachs and Ho (1986) suggest in a recent review of the subject, genetic manipulation to produce crop plants more resistant to the not uncommon environmental stress of anoxia, could take place.

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