LONG-TERM ANOXIA TOLERANCE IN LEAVES OF THREE WETLAND SPECIES : (ACORUS CALAMUS L., IRIS PSEUDACORUS L., VACCINIUM MACROCARPON AIT)

Urte Schlüter

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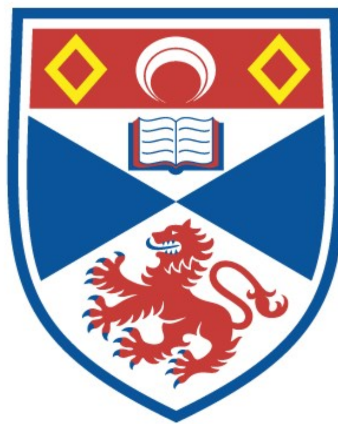

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Long-term Anoxia Tolerance in Leaves of Three Wetland Species

(*Acorus calamus* L., *Iris pseudacorus* L. *Vaccinium macrocarpon* Ait.)

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

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Abstract

Anoxia tolerance of *Acorus calamus*, *Iris pseudacorus* and *Vaccinium macrocarpon* has been investigated by incubating whole plants under anaerobic conditions in the dark. Long-term survival of rhizomes under anoxia has been described in previous studies, but this study has shown that green leaves can also endure anoxia for prolonged periods. Leaves of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* remained green and turgid under anoxia for up to 75d, 60d and 45d respectively.

All growth processes ceased in leaves under anoxia. Anaerobic energy production via ethanol fermentation was active in all investigated plant organs as shown by the accumulation of ethanol. Low rates of anaerobic CO_2 production indicated however, that the overall metabolic activity in the leaves was low under prolonged anoxia. The leaves seemed to adapt to the anaerobic conditions by an overall reduction of energy consumption rather than acceleration of the glycolytic rate. The demands for fermentable substrate were met by the mobilisation of internal carbohydrate reserves in leaves of *V.macrocarpon*. *A.calamus* and *I.pseudacorus* leaves contained only small amounts of carbohydrates, and these leaves possibly received carbohydrates from the stores in the rhizome.

Prolonged anoxia considerably affected the leaf capacity for respiration and photosynthesis. After 28d of anoxia, respiratory capacity was reduced in *A.calamus* and *V.macrocarpon* by 80%, and in *I.pseudacorus* by 90-95%; this corresponded with a decline in the activity of the cytochrome *c* oxidase. The photosynthetic capacity of leaves was decreased after 28d of anoxia by 83% in *A.calamus*, by 97% in *I.pseudacorus* and by 80% in *V.macrocarpon*. The reduction in the photosynthetic capacity was accompanied by alterations in the chlorophyll fluorescence pattern indicating damage to the PSII reaction centre and the subsequent electron transport; only minor changes occurred in the chlorophyll content of anaerobic leaves. On return to air and light, recovery of respiration and photosynthesis occurred in the leaves, but species-specific differences were observed in the speed of recovery.

Among the three investigated species, *A.calamus* leaves endured the anoxic conditions longer than leaves of the other two species; and on return to air, *A.calamus* leaves showed the most rapid recovery. *A.calamus* was characterised by efficient carbohydrate utilisation under anoxia. Cellular membranes and organelle ultrastructure appeared to be stable in *A.calamus* leaves for at least 28d of anoxia.

Table of Contents

Abstract II
Table of Contents III
Declarations VI
Acknowledgements VII
Abbreviations VIII

1. General Introduction

1.1. Life and Oxygen 2
1.2. Plants Under Oxygen Deprivation 2
1.2.1. Flooding 3
1.2.2. Ice-encasement 3
1.3. Adaptation to Oxygen Deprivation 4
1.3.1. Avoidance of Oxygen Deprivation 5
1.3.2. Tolerance to Oxygen Deprivation 6
1.4. Metabolism Under Oxygen Deprivation
1.4.1. Switch to Anaerobic Metabolism 8
1.4.2. Energy Metabolism Under Oxygen Deprivation 10
1.4.3. Carbohydrate Supply Under Oxygen Deprivation 13
1.4.4. Long-term Anoxia Tolerance 15
1.5. Post-anoxic Damage 16
1.6. Anoxia Tolerance and Other Environmental Influences
1.6.1. Influence of Seasons on Anoxia Tolerance 17
1.6.2. Soil Conditions Under Oxygen Deprivation 18
1.6.3. Light and Oxygen Deprivation 18
1.6.4. Temperature and Oxygen Deprivation........ 19
1.7. Description of the Plant Species Under Investigation
1.8.1. *Acorus calamus* L. 20
1.8.2. *Iris pseudacorus* L. 21
1.8.3. *Vaccinium macrocarpon* Ait. 22
1.8. Aim of the Thesis 23

2. Material and Methods

2.1. Material and Equipment 25
2.2. Origin of Plant Material and Growth Conditions 25
2.3. Anoxia and Dark Treatment 25
2.4. Sampling 26
2.5. Fresh and Dry Weight Determination 26
2.6. Carbohydrate Determination 26
2.6.1. Sample Preparation 27
2.6.2. Total Soluble Carbohydrates (TSC) 27
2.6.3. Total Non-soluble Carbohydrates (TNSC) 27

2.7. Determination of Ethanol Content by Gas Liquid Chromatography (GLC) 27
2.8. Analysis of Gas Exchange with the Warburg Respirometer 28
2.8.1. Oxygen Consumption 29
2.8.2. Aerobic Carbon Dioxide Production 29
2.8.3. Anaerobic Carbon Dioxide Production 29
2.9. Protein Analysis
2.9.1. Protein Determination 29
2.9.2. Total Soluble Protein Content 30
2.9.3. SDS-Polyacrylamide Gel Electrophoresis 30
2.9.4. Western Blotting (Rubisco) 31
2.9.5. α-Amylase Activity 31
2.9.6. Pyruvate Decarboxylase (PDC) Activity 32
2.9.7. Cytochrome *c* Oxidase (COX) Activity 33
2.10. Photosynthesis Measurements 34
2.11. Chlorophyll Determination 34
2.12. PSII Chlorophyll Fluorescence Emission 35
2.12.1. Fluorescence Analysis 35
2.12.2. Calculation of Fluorescence Parameter 36
2.13. Transmission Electron Microscopy (TEM)
2.13.1. Fixation 36
2.13.2. Dehydration 37
2.13.3. Embedding in Spurr's Resin 37
2.13.4. Ultrathin Sectioning 37
2.13.5. Heavy Metal Staining of Sections 37
2.13.6. Examination of Tissue Under TEM 38
2.13.7. Analysis of Micrographs 38
2.14. Statistical Analysis 39
Appendix 44

3. Effects of Long-term Anoxia on the Carbohydrate Metabolism of Leaves

3.1. Introduction
3.1.1. Previous Work on Anaerobic Carbohydrate Metabolism in Leaves 46
3.1.2. Oxygen Deprivation Under Laboratory Conditions 47
3.1.3. Aim of the Chapter 48
3.2. Results
3.2.1. Plant Survival Under Anoxia 48
3.2.2. Effects of Prolonged Darkness on Leaf Growth 51
3.2.3. Effects of Anoxia on Carbohydrate Content 51
3.2.4. Effects of Anoxia on Ethanol Content 53
3.2.5. Effects of Anoxia on Gas Exchange Capacity of Leaves 53
3.2.6. Effects of Anoxia on Enzymatic Activity of Leaves 54
3.3. Discussion
3.3.1. Species Survival Under Long-term Anoxia 70
3.3.2. Catabolism of Carbohydrates in Leaves Under Long-term Anoxia 72
3.3.3. Recovery of Carbohydrate Metabolism Under Post-anoxia 78
3.3.4. Comparison of the Effects of Anoxia and Darkness in Leaves 80

4. Effects of Long-term Anoxia on the Photosynthetic Apparatus

4.1. Introduction
4.1.1. Photosynthesis and Oxygen 84
4.1.2. Photosynthesis Under Oxygen Deprivation 86
4.1.3. Chlorophyll Fluorescence 88
4.1.4. Aim of the Chapter 92
4.2. Results.
4.2.1. Viability of Leaves 92
4.2.2. Effects of Anoxia on Photosynthetic Capacity 93
4.2.3. Effects of Anoxia on Chlorophyll Content 94
4.2.4. Effects of Anoxia on Chlorophyll Fluorescence of PSII 95
4.2.5. Effects of Anoxia on Rubisco Content of *A.calamus* Leaves 98
4.3. Discussion
4.3.1. Effects of Long-term Anoxia on the Photosynthetic Apparatus 110
4.3.2. Comparison of the Effects of Anoxia and Darkness on the Photosynthetic Apparatus 116

5. Effects of Long-term Anoxia on the Ultrastructure of Mesophyll Cells in *A.calamus*

5.1. Introduction
5.1.1. Metabolism and Mesophyll Cell Ultrastructure 120
5.1.2. Effects of Environmental Stress on Cell Ultrastructure 121
5.1.3. Quantitative Analysis of Subcellular Components 123
5.1.4. Aim of the Chapter 124
5.2. Results
5.2.1. Tissue Preservation 125
5.2.2. Effects of Anoxia and Darkness on Mesophyll Cell Ultrastructure 125
5.2.3. Effects of Anoxia and Darkness on Mitochondrial Ultrastructure 125
5.3.4. Effects of Anoxia and Darkness on Chloroplastic Ultrastructure 126
5.3. Discussion
5.3.1. Ultrastructure of Mesophyll Cells Under Long-term Anoxia 133
5.3.2. Comparison of the Effects of Anoxia and Darkness on Mesophyll Cell Ultrastructure 137

6. General Discussion

6.1. Leaf Anoxia Tolerance in *A.calamus*, *I.pseudacorus* and *V.macrocarpon* 140
6.2. Metabolic Adaptation to Anoxia in Leaves 141
6.3. Stamina of Leaves Under Long-term Anoxia 144
6.4. Post-Anoxia - Recovery after the Stress 146
6.5. Conclusions 147

References 150

Declarations

I, Urte Schlüter, hereby certify that this thesis, which is approximately 50,000 words in length, has been written by me, that this is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 22nd June 1999 Signature of Candidate ....

I was admitted as a research student to the School of Biological and Medical Sciences, University of St. Andrews, in October 1995; and as a candidate for the degree of Ph.D. in October 1996.

Date 22nd June 1999 Signature of Candidate ....

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date 22nd June 1999 Signature of Supervisor ....

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Abbreviations

A	absorbance
ACC	1-aminocyclopropane-1-carboxylic acid
ADH	alcohol dehydrogenase
Adh	mRNA for alcohol dehydrogenase
ADP	adenosine diphosphate
AEC	adenylate energy charge
AMP	adenosine monophosphate
ANOVA	analysis of variance
ANP	anaerobic proteins
ATP	adenosine triphosphate
ATPase	ATP synthase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
Chl	chlorophyll
COX	cytochrome *c* oxidase
DNA	desoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EM	electron microscope
ER	endoplasmic reticulum
F	fluorescence emission
Fm	maximum fluorescence
Fo	original fluorescence
Fv	variable fluorescence
FR	far red absorbing form of phytochrome
FW	fresh weight
g	relative centrifugal force
GLC	gas liquid chromatography
GR	glutathione reductase
I	absorbed light flux
k	rate constant
K_m	Michaelis constant
LDH	lactate dehydrogenase
LHC	light harvesting complex
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulforic acid
mRNA	messenger ribonucleic acid
n	number (e.g. of measurements)
NAD^+	oxidised nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	oxidised nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NBT	nitro blue tetrazolium
P680	phototrap pigment of PSII
P700	phototrap pigment of PSI
PAR	photosynthetically active radiation (400-700nm)

PDC	pyruvate decarboxylase
Pdc	mRNA for pyruvate decarboxylase
PFK	phosphofructokinase
PGM	phosphoglucomutase
pH	negative logarithm of hydrogen ion activity
PPi	pyrophosphate
PQ	Pasteur Quotient
PSI	photosystem I
PSII	photosystem II
PVPP	polyvinylpolypyrrolidone
q_P	photochemical quenching coefficient
q_{NP}	non-photochemical quenching coefficient
RbcL	large subunit of Rubisco
ROS	reactive oxygen species
rpm	revolutions per minute
RQ	respiratory quotient
Rubisco	ribulose-1,5-bisphosphate carboxylase oxygenase
SAG	senescence associated genes
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SE	standard error of the mean
SOD	superoxide dismutase
SuSy	sucrose synthase
TA	transverse area
TBS	Tris buffer saline
TCA cycle	tricarboxylic acid cycle
TEM	transmission electron microscopy
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLC	thin layer chromatography
TNSC	total non-soluble carbohydrates
TPP	thiamine pyrophosphate chloride
Tricine	N-tris(hydroxymethyl)methylglycine
Tris	2-amino-2(hydroxymethyl)-1,3-propandiol
TSC	total soluble carbohydrates
Tween-20	polyoxyethylenesorbittan monolaurate
UV-B	ultraviolet-B
V-PPi-ase	vacuolar H^+-translocating pyrophosphatase
V_v	volume fraction
v/v	volume per volume
w/v	weight per volume
ε	extinction coefficient
Φ_{PSII}	quantum efficiency of PSII

Chapter 1

General Introduction

1.1. Life and Oxygen

The photolysis of water evolved about 2.5 billion years ago and provided a new universally available hydrogen source. As a consequence, free oxygen accumulated in the atmosphere, nowadays stabilised at about 21%. The availability of this oxidant for extensive supply of free energy from metabolisable substances via respiration was one of the main preconditions for the evolution of highly organised complex biological systems (Hoffmann, 1998).

Compactly built organisms like animals, developed a highly specific oxygen transport system to supply every single cell with enough oxygen for their energy demands. In plants, cell walls and intercellular spaces allowed the diffusion of oxygen to the individual cells.

Although respiration accounts for the bulk of the oxygen utilised by the cells, oxygen assimilation is also involved in many other processes. In the majority of pathways oxygen is incorporated into organic compounds from water. Other processes involve direct incorporation of molecular oxygen, e.g. the addition of oxygen to unsaturated fatty acids via lipoxygenase, the synthesis of hydroxyproline - an important compound of cell wall proteins, the biosynthesis of ethylene from 1-aminocyclopropane-1-carboxylic acid (ACC) and photorespiration.

1.2. Plants Under Oxygen Deprivation

Oxygen consumption is particularly high in metabolically active plant tissue. Even under aerobic conditions, the oxygen demand of some cells can exceed its supply. Suboptimal oxygen conditions can be found in root tips, the cambium of several tree species, the bulky tissue of some fruits and in seeds before the rupture of testa (for review see: Crawford, 1992). Every plant cell has the potential to cope with short periods of oxygen deprivation and can generate energy by fermentative processes. Fermentation is independent of oxygen, but much less effective then aerobic mitochondrial respiration. Nevertheless, if the oxygen shortage is locally and temporarily limited, plants survive without serious damage.

While the plant shoots are usually in direct contact with the surrounding air, the subterranean organs often have to face fluctuations in oxygen pressure in their local environment. Well aerated soils can have oxygen concentrations of nearly 21%, but soil compaction and high water content often impair oxygen diffusion. Recent investigations showed that plant roots may possibly sense oxygen concentrations in the soil and orient their growth towards it (oxytropism) (Porterfield & Musgrave, 1998).

1.2.1. Flooding

The situation becomes critical when plants have to face long periods in waterlogged soils. Every year, during snow melt or after heavy rainfall, wide areas of land are covered for weeks or even months with stagnant water. During the past few decades, the frequency and dimension of floods have increased due to human activity such as deforestation, agricultural irrigation and canalisation of rivers.

The oxygen in the water is used rapidly by microbial and plant root respiration, but cannot be replaced due to the slow rate of oxygen diffusion in water (compared to air the diffusion is reduced by a factor of 10^4). Thus, the gaseous exchange of the root as an important plant organ is inhibited. When the plants are still small, high water levels, particularly in winter or spring, can submerge not only the lower parts of the plant, but subject the whole plant to oxygen deprived conditions.

Shoots are often not directly stressed by flooding, but alterations in hormone distribution within the plant can influence shoot growth. Leaves of flooded oak trees were 65% to 75% smaller than in non-flooded oaks (Angelov *et al.*, 1996). Shoot biomass retardation during flooding has been reported in many species (Limpinuntana & Greenway, 1979; Schlüter, 1994). The biosynthesis of ethylene is stimulated by oxygen deprivation. Auxin and abscisic acid concentrations increase due to reduced export into the root, forcing stomata closure and leaf wilting. The concentration of root-derived gibberellin and cytokinin decreases and promotes shoot growth retardation and leaf senescence (for review see: Jackson, 1990). The net photosynthetic rate often diminishes under flooding, probably due to restricted gas exchange and inhibition due to photosynthate accumulation (Huang *et al.*, 1994; Pezeshki & Santos, 1998). Due to reduced sink demands, assimilate transport into the root seems to be inhibited. Carbohydrate accumulation under flooding has been found in a number of species (Setter *et al.*, 1987; Albrecht *et al.*, 1993; Schlüter *et al.*, 1996; Albrecht *et al.*, 1997). Depressed root activity under flooding can furthermore impair nutrient uptake and transport in the plant.

1.2.2. Ice-encasement

In sub-continental or northern maritime climates, flooding may coincide with subzero temperatures resulting in the formation of an ice-blanket over the water surface. This is the condition of ice-encasement (Gudleifsson & Larsen, 1992; Andrews, 1996). It also takes place when layers of snow over the plants partially thaw, and water percolates downwards and then re-freezes. Rainfall may also contribute to an increase in the thickness of the ice layer and compaction of the snow layer. Solid ice is nearly impervious to oxygen and has the potential to completely interrupt the gaseous exchange of the ice-covered plants with

the atmosphere, inducing hypoxic (partial absence of oxygen) or even anoxic (complete absence of oxygen) conditions under the ice. However, conditions of this severity are not always realised in the fields. Ice can develop in irregular granulated forms that allow low levels of aeration. Sometimes aeration can even occur through air filled pores in the frozen soil. In contrast to flooding stress, the whole plant is usually exposed to oxygen deprivation during ice-encasement.

1.3. Adaptation to Oxygen Deprivation

Tolerance of oxygen deprivation varies greatly between the species of our current flora. Cotton roots and cyanogenic glucoside-containing roots of fruit trees are very sensitive, and can be seriously damaged in only a few hours without oxygen. Overwintering rhizomes of wetland species and some arctic species on the other hand survive up to several months under anoxic conditions (Armstrong *et al.*, 1994; Crawford, 1996). Significant differences in the tolerance to flooding and anoxia have also been detected in closely related species or even cultivars of the same species.

Because of their economic value, the flooding tolerance of crop plants has always been of special interest. The majority of crop plants are annual dryland species and prolonged waterlogging during the growing season leads to flooding injury and yield losses. In many parts of the world optimal plant production depends on drainage.

Potato tubers lose their viability after about 48h-72h of oxygen deprivation (Sieber & Brändle, 1991). Intact maize seedlings survive anaerobic conditions in their rooting environment for only 72h - 96h (Johnson *et al.*, 1989; Russell *et al.*, 1990). Wheat and barley seedlings can survive 10d-14d in a hypoxic nutrient solution, but show major reductions in the growth of roots and to lesser extent of shoots (Limpinuntana & Greenway, 1979; Barrett-Lennard *et al.*, 1988). If whole wheat or barley seedlings are exposed to anoxia they do not survive longer than 24h. Rice is the only major crop plant that is naturally adapted to wet habitats. In flood tolerant varieties, seedlings survive 10d-14d of complete submergence (Setter *et al.*, 1997). Mujer *et al.* (1993) report survival times of up to three weeks without oxygen for germinating rice. In suspension cultures, rice cells survive up to 52d of anoxia (Mohanty *et al.*, 1993).

Despite the complex problems for plant life under oxygen deprivation, a variety of plant species (about 4% of dicots and 33% of monocots; Crawford, 1992) can adapt to life in soils with permanently high water content; inhabiting bogs, wet marshland, pools, river banks and salt marshes. Some macrophytes ('aquatic species') have developed special

features that allow higher plant life under water; but the following sections concentrate on the effects of oxygen deprivation and submergence of normally emerged plants.

Two main strategies are involved in adaptations to flooding. Most wetland species overcome the oxygen shortage by a combination of both anatomical and morphological adaptations ('avoidance'-strategy) as well as physiological adaptations ('tolerance'-strategy).

1.3.1. Avoidance of Oxygen Deprivation

For long-term survival in waterlogged soils, plants have evolved a number of strategies to supply their flooded organs with oxygen. In standing water, oxygen concentration decreases with water depth; by exploitation of the aerobic surface layers roots avoid oxygen deprivation stress. Very characteristic are the 'breathing roots' (pneumatophores) of some tree species in tropical swamps and mangroves. In some species they take the form of 'knees' formed by lateral roots that emerge into the air and immediately bend down and re-enter the soil. Numerous lenticells and abundant intercellular spaces allow the aeration of the surrounding tissue. Surface rooting is also an important feature of non-wetland species under flooding. The seminal roots of wheat and maize roots stop growing under hypoxic conditions and instead produce adventivous roots at the water surface (Wiedenroth & Erdmann, 1989).

Large air spaces between the cells (aerenchyma) facilitate the downward diffusion of oxygen from the aerated shoot to the roots. This passive diffusion can be supplemented by internal pressurisation processes (thermo-osmosis), where differences between interior and exterior temperatures are used for additional air transport (Grosse & Schröder, 1986). In wetland species, the development of aerenchyma is genetically determined. The aeration system develops in stems, rhizomes and roots by cell separation during development (shizogeny) or by cell death and dissolution (lysogeny). Even under drained conditions, the root porosity in wetland species like *Juncus inflexus* was 52.3% (Justin & Armstrong, 1987).

Enlargement of intercellular space, mainly due to the lysis of cells, can also be initiated in dryland species when exposed to hypoxia. In rice, root porosity increases from 15.3% under drained conditions to 35.1% under flooding (Justin & Armstrong, 1987); in wheat it increases from 6% to 25-30% (Wiedenroth & Erdmann, 1989). What finally triggers the formation of aerenchyma in hypoxic tissue is still under debate. The accumulation of ethylene is supposedly involved in the initiation of selective cell death. In maize roots, high concentrations of ethylene and its precursor ACC are detected during hypoxia, and the activity of ACC synthase and ACC oxidase increases. On the contrary, anoxia alone fails to

induce aerenchyma formation. This could be explained by the fact that ethylene production is inhibited during anoxia, because the last step of ethylene synthesis from the precursor ACC requires oxygen. However, viable anoxic roots fail to respond to exogenous ethylene supply (for review see Drew, 1997). Recent studies show that the synthesis of some cell wall loosening enzymes is probably directly initiated by oxygen deprivation (Sachs *et al.*, 1996, He *et al.*, 1994).

All aeration mechanisms rely on only partial submergence, when the shoot is still exposed to the oxygen containing atmosphere. In well-adapted wetland plants, these mechanisms can prevent flooded organs from suffering oxygen deprivation. Flood tolerant species are therefore not necessarily anoxia tolerant as well. However, in most species anatomical and morphological adaptations are unable to compensate completely for the lack of oxygen in the rooting environment. Even during times of maximal leaf development, the oxygen concentrations in the rhizome of *Iris pseudacorus* are as low as 2.7%. In regions more distant from the leaf base, oxygen concentrations are much lower than in closer parts (Boulter *et al.*, 1963). Consequently, alterations in metabolism occur during prolonged flooding or total submergence and physiological adaptation is essential for survival.

1.3.2. Tolerance to Oxygen Deprivation

Because every plant cell has the potential for anaerobic metabolism, anoxia tolerance is relative. While some plants survive anoxia only for very short periods, other species show clear metabolic adaptation and remain viable for much longer under conditions where oxidative phosphorylation is suppressed by lack of oxygen.

Different plant organs differ in their sensitivity to oxygen deprivation. Roots are the most likely plant organ to be submerged by rising water levels. Nevertheless, in comparison with other plant organs, they are particularly sensitive to oxygen deprivation. This pertains for wetland species as well as non-wetland species. There are no records of root growth under oxygen deprivation. In maize roots, growth starts to be limited at a significantly higher oxygen pressure than respiration. This indicates that nonrespiratory oxidative processes with a low affinity to oxygen are involved in root elongation (Saglio *et al.*, 1984). The key enzyme for respiration, cytochrome oxidase, possesses a comparably high affinity to oxygen (cytochrome a3: $K_m = 0.1\text{-}1.0 mmol.m^{-3}$; Bonner, 1973) and can still work under lower oxygen concentrations than alternative oxidase. If respiration becomes restricted by decreasing oxygen levels, energy generation has to rely on anaerobic processes. What finally leads to the damage of cells which are not resistant to long-term anoxia is still under debate. Injury and death of root cells have been attributed to

imbalances in energy metabolism, acidification of cytoplasm, accumulation of toxic components or starvation of substances for energy production (for review see Drew, 1997). Because of the limited tolerance of roots to oxygen deprivation, the survival of anoxia tolerant species mostly resides in the shoots or rhizomes. In shoots, two different responses to anoxia can be observed. First, plants with sustained upward shoot elongation under anoxia and secondly, plants which survive anoxia without any shoot extension.

During total submergence, the advantage of the plants in the first group lies in the increased possibility of regaining contact with the atmosphere or well-oxygenated upper layers. Shoot elongation under anaerobic conditions has been studied in monocots such as rice or barnyard grass species (*Echinochloa* spp.) as well as some amphibious dicots (Barclay & Crawford, 1982; Fox *et al.*, 1994; Setter & Laureles, 1996). The shoot extension is a result of cell elongation while cell production is strongly inhibited. The aquatic plant *Potamogoton pecinatus* L. is until now the only example of enhanced growth in the absence of oxygen (Summers & Jackson, 1994). The mechanisms behind this phenomenon are not yet fully understood, but several plant hormones, as well as acidification of the surrounding media by carbon dioxide accumulation, are involved (Horton, 1992; Summers & Jackson, 1996). To compensate for the costs of shoot elongation, high levels of glycolysis and ethanol fermentation are necessary (Setter & Ella, 1994). If the elongated shoot cannot establish a connection with the atmosphere quickly, the plant soon dies of exhaustion. Setter and Laureles (1996) found a negative correlation between plant survival and elongation growth under anoxia.

The highest anoxia tolerance is found in plant organs which sustain periods without oxygen in a quasi-dormant state. This phenomenon is particularly common in the overwintering rhizomes of wetland species. Cessation of biosynthetic activity, stabilisation of the energy status of the tissue, reduction of metabolic activity to maintenance processes and the availability of large carbohydrate reserves apparently enables the rhizomes of wetland species (e.g. *Acorus calamus*, *Scirpus maritimus*) or even the leaves of some arctic species (e.g. *Dechampsia beringensis*, *Poa alpina*) to survive prolonged periods of anoxia (Crawford & Brändle, 1996).

Not all plant organs have to survive periods without oxygen for the whole plant to be considered anoxia tolerant. What is ecologically relevant in the end, is that those organs survive which enable the subsequent regeneration of the whole plant on return to air.

1.4. Metabolism Under Oxygen Deprivation

1.4.1. Switch to Anaerobic Metabolism

Without oxygen, the cell lacks the terminal electron acceptor in the respiratory chain, the reduced pyridine nucleotide NADH accumulates, and the TCA cycle slows down. The energy generation from one glucose molecule drops drastically from 36 ATP via glycolysis, TCA cycle and oxidative phosphorylation to only 2 ATP via glycolysis and fermentation. To allow cell survival, anaerobic processes must contribute to ATP regeneration in the short term. Specific regulation mechanisms must selectively promote certain processes critical for survival while inhibiting other less important processes.

Hypoxic Acclimatisation to Anoxia

Under aerobic conditions, most enzymes involved in anaerobic pathways are present, but only in trace amounts. The switch from aerobic to anaerobic processes is therefore a very critical phase for the cell's metabolism.

When anoxia sensitive tissue is faced with sudden anoxia, it can activate anaerobic pathways but fail to stabilise the metabolism, and the cells die within 24h. Slow acclimatisation to the anaerobiosis by a previous hypoxic phase of only a few hours to one day can extend cell viability significantly (Johnson *et al.*, 1989; Waters *et al.*, 1991b; Germain *et al.*, 1997). The tolerance of winter wheat to ice-encasement could be improved by a prior exposure to hypoxia during low temperature flooding. However, forage grass species which are much more tolerant to ice-encasement showed only a small response to hypoxic acclimatisation (Andrews, 1997).

During anoxic shock, glycolysis and ethanol fermentation run only at very low levels. The ATP content as well as the total adenylate content in the cell decrease quickly and can not be restored (Johnson *et al.*, 1994; Bouny & Saglio, 1996). The pH of the cytoplasm in non-acclimatised roots decreases to lower levels than in hypoxic pretreated ones. This can be attributed to the limited availability of ATP to energise the tonoplast H^+ pumps. Xia *et al.* (1995) showed that low ATP levels are not alone responsible, and lactic acid accumulation might be another important cause for the cytoplasmic acidosis. Low pH plus low ATP availability have inhibitory effects on hexokinase activity in cells and hence keep the glycolytic flux and energy generation down (Bouny & Saglio, 1996). After hypoxic acclimatisation, improved control of the cytoplasmic acidosis, maintenance of glycolytic flux and slowing down of ATP decline, seem to be responsible for the higher viability under the following anoxic treatment.

The hypoxic induced enhancement in sucrose synthase activity can further increase the glycolytic flux by the break down of sucrose (Germain *et al.*, 1997; Ricard *et al.*, 1998).

Besides sucrose synthase, many other enzymes involved in the anaerobic energy generation (see: ANP) show increased activity under anoxia after acclimatisation at low oxygen concentrations, while only minor changes can be detected under anoxic shock (Bouny & Saglio, 1996). Hypoxia leads to enhanced transcription of genes for most of these proteins, during the following anoxia mRNA levels decline only gradually (Andrews *et al.*, 1994). Under natural conditions plants are unlikely to be suddenly exposed to anoxia and a gradual decline of oxygen concentration provides the opportunity for metabolism to adapt to anaerobic processes.

Anaerobic Proteins (ANP) and Anaerobic Gene Expression

Sachs *et al.* (1980) showed that after 5h of anoxia only 20 proteins accounted for the majority of proteins synthesised in the cells of maize roots. These proteins are called 'Anaerobic proteins' or ANP. Since then, most of these proteins have been identified and characterised. Among them are the enzymes for fermentation [alcohol dehydrogenase (ADH), pyruvate decarboxylase (PDC)], and enzymes related to glycolysis [glucose-6-phospate isomerase, aldolase, enolase, glyceraldehyde-3-phosphate dehydrogenase]. Another anaerobic protein with a possible involvement in cell wall loosening in aerenchyma formation has been described (Sachs *et al.*, 1996). Similar investigations show different numbers of ANP in other species. In soybean roots, which are very anoxia sensitive, only four ANP were identified. In five barnyard grass species (*Echinocloa* spp.) and rice about 10-18 proteins were initiated or enhanced by anoxia (Mujer *et al.*, 1993).

The transcription of genes encoding proteins related to glycolysis and fermentation is induced after the onset of oxygen deprivation. However, variations in the time courses have been reported for different enzymes indicating differences in transcription rates and mRNA stability (Umeda & Uchimiya, 1994; Andrews *et al.*, 1994). Transcript levels are not directly correlated with enzyme levels. In maize roots, enolase is induced by hypoxia on the mRNA but not on the enzyme activity level (Fox *et al.*, 1995).

Beside the new synthesised transcripts, mRNAs encoding non-anoxia related ('aerobic') proteins are still available in the cells. The translational machinery of the cell is inhibited under oxygen limitation. In maize roots, polyribosomes dissociate within 1h of oxygen deprivation stress (Bailey-Serres & Freeling, 1990). Fennoy & Bailey-Serres (1995) showed, that the remaining polyribosomes preferably associate with newly synthesised, ANP encoding mRNA, while the loading with mRNA for an 'aerobic' protein is reduced. Hence, the synthesis of ANP results from a combination of transcriptional and post-transcriptional processes.

The selective synthesis of ANP usually continues in anoxia sensitive tissues until death (Sachs *et al.*, 1980). In more tolerant plants, the expression of anoxia related proteins also

peaks soon after the onset of oxygen deprivation. Under prolonged anoxia, transcription levels decline until they reach a constant level, and the plants return to a protein pattern similar to aerobic conditions (Kennedy *et al.*, 1992).

1.4.2. Energy Metabolism Under Oxygen Deprivation

The energy status of the cell deteriorates under anoxia. The ATP content and the adenylate energy charge [AEC = (ATP+½ADP)/(ATP+ADP+AMP)] reach very low levels in anoxia sensitive tissues soon after the onset of anoxia, e.g. in potato tubers, the AEC fell from 0.8 to only 0.3 after 6h of anoxia. Anoxia-tolerant tissue on the contrary, quickly regains control over their energy metabolism. In *Acorus calamus* rhizomes, the AEC drops only slightly at the start of the anoxia treatment, but re-equilibrates soon to nearly aerobic levels (Sieber & Brändle, 1991). In *Iris pseudacorus* rhizomes the AEC stays above 0.5 for at least 30 days (Hanhijärvi & Fagerstedt, 1995).

Cytosolic Acidosis and Lactic Fermentation

The main pathway of energy generation under anoxia is by glycolysis followed by NAD^+ regeneration via ethanol fermentation. An initiation of lactic fermentation has been observed at the start of anoxic treatments in some plants. A subsequent lowering of pH is thought to trigger high activity of pyruvate dehydrogase and ethanol fermentation. Hence, neutral ethanol is produced instead of acidic lactate (Davies, 1980). Cytoplasmic acidosis is still seen as one of the main causes of cell death under anoxia (Roberts *et al.*, 1985). An early inhibition of lactic fermentation seems to be of advantage, and in almost all of the tolerant tissues that have been investigated lactate production was neglectable during long-term anoxia. In relatively anoxia-tolerant rice shoots, the cytosolic pH drops only at the onset of anoxia from 7.5 to 7.0 and is followed by slow alkalisation (Menegus *et al.*, 1991). The acidosis might be counteracted by H^+-consuming processes like putrescine synthesis, glutamate decarboxylation to γ-aminobutyric acid and accumulation of amides or arginine. The additional induction of vacuolar H^+-translocating pyrophophatases (V-PPi-ase) under anoxia may further improve the maintenance of pH difference between cytoplasm and vacuole (Carystinos *et al.*, 1995).

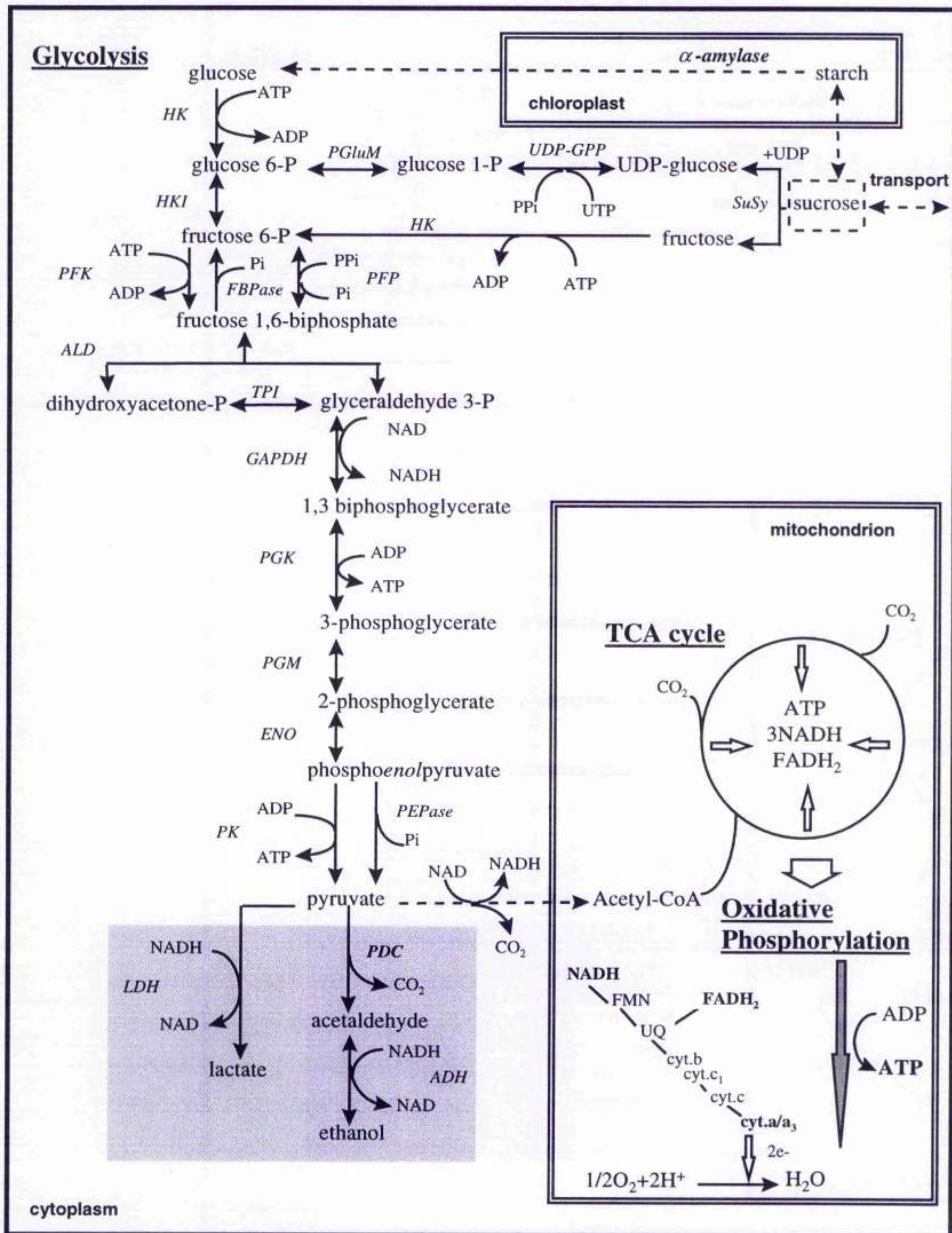


Figure 1.1. Catabolism of carbohydrates under aerobic and anaerobic (grey) conditions. (modified after Dennis *et al.*, 1997)

Abbreviations: HK-hexokinase, HPI-hexose-P isomerase, PFK-ATP-dependent phosphopructokinase, PFP-PPi-dependent phosphofructokinase, FBPase-fructose-1,6-biphosphatase, ALD-aldolase, TPI- triose-P-isomerase, GAPDH -glyceraldehyde-3-P dehydrogenase, PGK-phosphoglycerate kinase, PGM-phospho-glyceromutase, ENO-enolase, PK-pyruvate kinase, PEPase-phosphoenolpyruvate phosphatase, LDH-lactate dehydrogenase, PDC- pyruvate decarboxylase, ADH-alcohol dehydogenase; PGluM-Phosphoglucomutase, UDP-GPP - UDP-glucose pyrophosphatase, SuSy-Sucrose synthase; FMN-Flavine mononucleotide, UQ-Ubichinone, cyt.b-Cytochrome b, cyt.c_1-Cytochrome c_1, cyt.c-Cytochrome *c*, cyt.a/a_3-Cytochrome a/a_3 complex (cytochrome oxidase).

Glycolysis Under Anoxia

Glycolysis has evolved before the oxygen enrichment of our atmosphere and is still ubiquitously present in the plant kingdom. Glycolytic processes (Fig.1.1) take place in the cytosol and in the plastid of the plant cell. Unfortunately, the mechanism of control of the flow of carbon down the glycolytic pathway is still not understood (see: Dennis *et al.*, 1997).

Most measurements on glycolytic enzymes under oxygen deprivation have been made under short-term anoxia in sensitive maize roots (Bouny & Saglio, 1996). Only a few studies describe the alterations in the activity of glycolytic enzymes during longer lasting oxygen deprivation.

The reaction catalysed by PFK is generally seen as a major regulatory point of glycolysis. The activity of pyrophosphate-dependent phosphofructokinase (PPi-PFK) continues to increase in rice seedlings under anoxia, while the ATP-dependent phosphofructokinase (ATP-PFK) remains stable (Mertens *et al.*, 1990). Similar results have been obtained for catalytic enzyme activity in rice cell suspension cultures under anoxia (Mohanty *et al.*, 1993). The substitution of pyrophosphate for ATP as an energy source might be beneficial for the cell because the pyrophosphate levels were relatively insensitive to anoxia. Enhanced transcription and synthesis of pyrophosphate dependent enzymes was also found in other parts of the anaerobic metabolism, e.g. V-PPi.ase, SuSy; and there is considerable evidence for an increased importance of the PPi metabolism under anoxia (Stitt, 1998).

The activity of glucose-6-phosphate isomerase increases under anoxia in the embryo and shoot tissue of rice (Guglielminetti *et al.*, 1995a). Enolase activity is increased during a 10d anoxia treatment in seedlings of flood-tolerant *Echinocloa* species and rice (Fox *et al.*, 1995). However, other glycolytic enzymes such as phosphoglucomutase (PGM) fail to show increased activity under anoxia in rice and maize (Manjunath *et al.*, 1998; Guglieminetti *et al.*, 1995a).

The substrate flow through glycolysis and ethanol fermentation can be described by the Pasteur Quotient (PQ = 3 x [CO_2] production anaerobic / [CO_2] production aerobic). If the substrate consumption through glycolysis accelerates under anoxia (Pasteur effect), the PQ will be >1. In plants, a Pasteur effect is supposed to operate only in non-tolerant tissue or at the onset of oxygen deprivation (Albrecht & Wiedenroth, 1994b).

Ethanol Fermentation

Only two enzymes catalyse ethanol fermentation (Fig.1.1). Pyruvate is the substrate for pyruvate decarboxylase (PDC) yielding CO_2 and acetaldehyde, that is subsequently reduced to ethanol combined with NADH oxidation via alcohol dehydrogenase (ADH). Ethanol

fermentation is likely to be regulated by PDC activity rather then ADH activity. PDC is at the branch point and its levels are lower than that of ADH. Small levels of PDC and ADH activity are usually found under aerobic conditions, but hardly any accumulation of fermentation products occurs. Ethanol can be metabolised quickly under aerobic conditions by the catalase reaction and there seems to be a preference for carbon to enter the TCA cycle (Bucher *et al.*, 1994).

Many plants have more than one gene encoding for the two enzymes. In *Arabidopsis* roots, one set of genes seems to be expressed constitutively and thus may be used as an early pathway during the onset of anoxia; the other one is induced under oxygen deprivation and provides energy under longer periods without oxygen (Dolferus *et al*, 1997). A similar pattern has been observed for ADH in wild rice (Muench *et al.*, 1993). The induction pattern of several isoenzymes can differ between plant organs.

Gene expression and protein synthesis of fermentative enzymes is not initiated simply by the metabolic consequences of low oxygen concentrations such as low levels of ATP or adenylates. When oxidative respiration is hindered by the addition of inhibitors to the aerobic plant, ethanol is produced, but the treatment fails to initiate the transcription of the genes for ADH and PDC (Bucher *et al.*, 1994). This might indicate that other oxygen sensing systems are involved in cell response to anoxia. Haemoglobin as an oxygen binding protein or alterations in the Ca^{2+} concentrations have been discussed in this context (see: Drew, 1997).

The products of ethanol fermentation, ethanol and carbon dioxide, are usually removed from the plant tissue by diffusion. In anoxia tolerant tissues, high porosity and gaseous transport mechanisms such as aerenchyma facilitate their release into the environment. In the rhizomes of five wetland species, ethanol concentrations reached a plateau soon after the onset of anoxic conditions; but in the dryland species *Iris germanica* ethanol accumulation continued during the whole course of the experiment and achieved significantly higher concentrations (Monk *et al.*, 1984). It is still not clear which levels of ethanol may prove toxic for plant tissue, but at low concentration ethanol does not seem to be harmful to the plant. The binding of acetaldehyde to proteins is supposedly more dangerous to the plant tissue, but under oxygen deprivation, high ADH activity usually prevents the accumulation of acetaldehyde (Perata & Alpi, 1991).

1.4.3. Carbohydrate Supply Under Oxygen Deprivation

Carbohydrate starvation has always been seen as one of the major problems for plants under anoxia, particularly when there is a pronounced Pasteur effect. The exogenous feeding of anoxia sensitive tissue with glucose or sucrose prolongs survival under oxygen

deprivation, but in the end, anoxia-intolerant tissue dies in spite of sufficient sugar supply (Webb & Armstrong, 1983; Waters *et al.*, 1991a). This indicates that metabolic imbalances rather than carbohydrate starvation are directly responsible for cell death under anoxia. Nevertheless, the supply of carbohydrates is necessary to keep energy generation via glycolysis and ethanol fermentation running under anoxia. It is therefore not surprising that rhizomes with high carbohydrate stores are among the most anoxia tolerant tissues found. The presence of high amounts of reserves is an essential prerequisite, but is never the only cause of long-term tolerance to oxygen deprivation.

Breakdown of Sucrose

Two enzymes are involved in the cleavage of sucrose - alkaline invertase and sucrose synthase (SuSy). In anoxic rice seedlings, the invertase activity was reduced, while the SuSy activity was remarkably enhanced (Guglielminetti *et al.*, 1995a). Similar results have been obtained in tomato, maize, wheat and barley (Germain *et al.*, 1997; Guglielminetti *et al.*, 1997). The flow of carbon from sucrose to glycolysis via the SuSy pathway results in an ATP saving when compared to the invertase pathway. The preference of SuSy could therefore play a role in tolerance to anoxia. The quick death of SuSy double mutants of maize emphasises the importance of this enzyme under oxygen deprivation (Ricard *et al.*, 1998). In maize, two genes encode for sucrose synthase (*Shrunken1* and *Sus1*). Their transcription and subsequent protein synthesis seem to be regulated by changes in the sugar concentrations under hypoxia and anoxia (Zeng *et al.*, 1998).

Breakdown of Starch

Starch is the main storage carbohydrate in most higher plants, though some species also use fructans or sucrose for storage. The breakdown of starch is principally started by the activity of α-amylase and a range of enzymes (β-amylase, α-glucosidase, debranching enzyme) contribute to the complete hydrolysis of the polysaccharide chains. Under long-term anoxia, the availability of these enzymes is necessary to provide carbohydrates to sustain fermentative metabolism. In rice seedlings, starch mobilisation was accompanied by a 70-fold increase in amylolytic activity after three days of stress (Raskin & Kende, 1984).

In contrast to the majority of plants, rice can germinate under anaerobic conditions. Perata *et al.* (1997) showed that the whole set of starch degrading enzymes is active in rice seeds under anoxia. The inability of anoxia intolerant seeds like wheat or barley to germinate without oxygen correlates with their failure to induce and activate these enzymes under anoxia (Guglielminetti *et al.*, 1995b). The ability to germinate under anoxia is slightly improved in wheat, when fed with exogenous glucose or sucrose (Perata *et al.*,

1992). Nevertheless, the inability of many seeds to germinate under anoxia can be advantageous because dormant seeds are usually more resistant than seedlings.

1.4.4. Long-term Anoxia Tolerance

For long-term survival under oxygen deprivation, more enzymes than the few initially induced ANP are necessary. Besides glycolysis and fermentation, all essential cell functions must be maintained and stabilised with minimal energy consumption. Although protein synthesis is impaired under oxygen deprivation, *Acorus calamus* synthesises about 60-70 different proteins after 20h of anoxia (Armstrong *et al.*, 1994). The increase in gene expression and ANP activity occurs in tolerant plants only during the onset of the stress. In *Acorus calamus,* the transcription levels decrease slowly after an initial peak, and gene expression is still detectable after two months of anoxia (Bucher & Kuhlemeier, 1993). In anoxia tolerant *Echinochloa* species, a complete TCA cycle is present under anoxia suggesting that partial use of the cycle is possible. The formation of malate or succinate permits the reoxidation of pyridine nucleotides (see: Kennedy *et al.*, 1992). An accumulation of malate under anoxia has been reported from some flood-tolerant tree species (Armstrong *et al.*, 1994). Anaerobically germinated seedlings of *Echinochloa crus-galli* contain 70% of the cytochrome *c* oxidase (COX) activity found in air-grown seedlings (Kennedy *et al.*, 1987).

Ammonium is almost the only nitrogen source available to plants under flooding. In anoxia sensitive plants like barley, the nitrogen uptake declines shortly after the onset of flooding (see: Crawford, 1992). Anoxia tolerant plants such as *Acorus calamus* can detoxify ammonium and fix nitrogen in the form of alanine (Weber & Brändle, 1996). The pronounced accumulation of amino acids like alanine and γ-aminobutyric acid under anoxia has also been discussed as a mechanism to counteract cytoplasmic acidosis.

Another problem under long-term anoxia is the stabilisation of membrane lipids. The desaturating process is inhibited under oxygen deprivation, and the ability to preserve lipid molecules under oxygen deprivation is an important feature of anoxia tolerant plants. In *Acorus calamus*, the saturation level of membranes changes only slightly and no lipid breakdown occurs after 70d of anoxia. Therefore, organelle stability can be guaranteed even under prolonged anoxia. In the much less tolerant *Iris germanica*, lipids are fully saturated after 14d of anoxia, and an increase in the concentration of fatty acids refers to a pronounced lipid degradation (Henzi & Brändle, 1993).

1.5. Post-anoxic Damage

The ability of plants to survive longer periods without oxygen is ecologically only relevant if the plants can also adjust to the consequences of re-exposure to air. On return to an oxygen rich environment, the plant is also exposed to reactive oxygen species, e.g. superoxide radicals, iron-induced-hydroxyl radicals and hydrogen peroxide. Conditions such as low energy charge, high levels of reducing equivalents and saturated electron chains usually enhance the formation of reactive oxygen species (ROS) (VanToai & Bolles, 1991). An increased production of toxic oxygen radicals is therefore a common feature of plants under certain stress conditions and is supposedly responsible for post-anoxic injury in plants. The toxic radicals can produce oxidative damage on lipids or membranes and attack macromolecules like proteins and DNA. To counter the hazardous effects, all aerobic organisms have evolved a complex antioxidative defence system composed of both free radical scavengers and enzymatic constituents. Small molecules like glutathione or ascorbate are very common antioxidants in plants, antioxidative effects have also been reported for flavonoids, phenols and alkaloids. The enzymes dehydroascorbate reductase, monodehydrogenase and glutathione reductase (GR) are involved in cellular defence by maintenance of the reduced active form of glutathione and ascorbate (see: Wollenweber-Ratzer & Crawford, 1994). Superoxide dismutase (SOD) catalyses the disproportionation of the superoxide radical to hydrogen peroxide and dioxygen (Monk *et al.*, 1987).

In chickpea seedlings, treatment with anti-oxidant ascorbate before re-exposure to air after periods of anoxia improves the survival and growth during the post-anoxic recovery period (Crawford & Wollenweber-Ratzer, 1992). In wheat roots, the change from hypoxic to aerated conditions is accompanied by a shift in the ratios of reduced to oxidised, inactive forms of ascorbate and glutathione. The activities of antioxidant regenerating enzymes like GR are significantly reduced during anoxia and the plants suffer oxidative stress during re-aeration. An increased peroxidation of membranes is found directly after return to aerobic conditions (Albrecht & Wiedenroth, 1994a; Biemelt *et al.*, 1998). Depending on the duration of the previous hypoxic or anoxic period, the activity of antioxidative enzymes recovers after 16-24h in air (Ushimaro *et al.*, 1992).

An intact antioxidative defence system in anoxia treated plants is assumed to be advantageous for overcoming post-anoxic stress. In anoxia tolerant rhizomes of *Iris pseudacorus* a constant increase in the activity of SOD is observed during a period of imposed anoxia, and high levels are maintained in the post-anoxic recovery phase. Less tolerant species show a much less pronounced increase or even a decline in SOD activity (Monk *et al.*, 1987).

Another reason for post-anoxic injury might be the oxidation of anaerobically accumulated ethanol to acetaldehyde (see: Crawford, 1992). The peroxidation of ethanol by the catalase reaction is seemingly responsible for the rapid increase of acetaldehyde under post-anoxia (Zuckermann *et al.*, 1998). The avoidance of excessive ethanol accumulation under anoxia prevents the production of potentially dangerous amounts of acetaldehyde during post-anoxia (see: Crawford, 1992).

Post-anoxic damage has also been observed in plants after ice-encasement. The plants appear healthy for a short period after thawing, but show damage within 1-2d due to a major decrease in lipid unsaturation and an increase in free fatty acids (Andrews, 1996).

1.6. Anoxia Tolerance and Other Environmental Influences

1.6.1. Influence of Seasons on Anoxia Tolerance

The duration and frequency of flooding or ice-encasement usually differs between seasons. Ice-encasement is clearly connected with the frost period from late autumn until spring. The water levels in northern latitudes are often higher during winter and spring. A large proprtion of temperate plants are annuals and are adapted to these changes by surviving the winter as dormant seeds. The majority of plants, including many temperate and tropical species, germinate only under aerobic conditions. Perennial plants usually store great amounts of carbohydrates during summer in their overwintering organs and survive the cold season with hardly any growth and only minimal metabolic activity. Under these conditions, many rhizomatous wetland or arctic plants can survive periods of oxygen limitation. The underground organs of *Phragmitis australis* survive the winter in anaerobic mud with only very low oxygen requirements, and the dead culms often provide enough aeration (Crawford, 1992).

When the growing season starts in spring, carbohydrate stores are depleted and the metabolic activity in the plant tissue rises quickly, therefore oxygen deprivation can cause much greater damage. Remarkable seasonal variations in anoxia tolerance have been described for *Glyceria maxima*. Overwintering rhizomes survive in laboratory experiments for up to three weeks of anoxia (22°C), but during summer, the rhizomes die under anoxia (22°C) in less than one week (Crawford & Brändle, 1996). The developmental stages of temperate plants are usually closely related to the seasons. The differences in the submergence tolerance of two *Rumex* species during floods in early- and mid-summer are mainly attributed to their developmental stage at the time of submergence (van der Sman *et al.*, 1993).

1.6.2. Soil Conditions Under Oxygen Deprivation

Oxygen deprivation during flooding leads to major changes in microbial processes and chemical properties of the soil (see: Ponamperuna, 1984). These changes affect chemical mobility and plant availability of nutrients and can cause the formation of phytotoxic substances. Anaerobic soils accumulate low molecular weight organic acids, carbon dioxide, methane, hydrogen, ammonia, amines, mercaptans, hydrogen sulphide, reduced manganese and iron (Gambrell *et al.*, 1991).

Plants endure unfavourable conditions in anaerobic soil mainly by avoidance strategies. If the plant maintains a connection to the atmosphere, part of the oxygen that reaches the roots will diffuse into the soil, allowing the formation of an oxygenated layer around the roots. The oxygen enrichment of the rhizosphere encourages an aerobic microflora and prevents the formation of toxic components. Under anaerobic conditions, the nitrate levels decline quickly, but radial oxygen loss supports aerobic nitrifying bacteria or aerobic nitrogen fixing bacteria (Ernst, 1990). Highly developed aeration mechanisms can therefore improve plant survival under flooding not only by providing oxygen for roots metabolism, but also by oxygen release into the anaerobic soil (Burdick & Mendelssohn, 1990).

In anaerobic soils, the consequences of reducing conditions in the rhizosphere are probably more damaging to the plant than the effects on root metabolism. In the experiments of Brix & Sorrell (1996) growth of two wetland species was unaffected when placed in de-oxygenated solution, but were killed by reducing conditions in the rooting environment. In another experiment, the addition of low concentrations of sulphide severely affected root energy metabolism and growth of *Phragmitis australis* (see: Crawford & Brändle, 1996). Anoxia tolerant *Acorus calamus* on the contrary, are able to tolerate increased sulphide concentrations by detoxifying and binding them in the form of glutathione. The detoxification of ammonia followed a similar pattern, and is fixed into alanine in the roots and rhizomes (Weber & Brändle, 1996).

1.6.3. Light and Oxygen Deprivation

Under natural conditions, submerged plants or even plants under ice-encasement often obtain enough light for photosynthesis. Even low photosynthetic rates allow plants to produce oxygen and to improve their carbohydrate and energy metabolism. Hence, light can play an important role for plant survival under oxygen deprivation.

Photosynthesis under water is usually impaired compared to that in air. In stagnant water, low carbon dioxide levels decrease carbon assimilation. In contrast to aquatic species, occasionally submerged plants like rice can only utilise CO_2, but not HCO_3^-. The irradiance levels are often low and decrease quickly with water depth (Setter *et al.*, 1989a).

Under long-term flooding and submergence, the photosynthetic capacity of leaves decreases due to stomata closure and chlorosis (Jackson *et al.*, 1987). Additional shading before or during submergence can seriously affect carbohydrate metabolism and viability.

Illumination of plants under ice-encasement improves their survival due to oxygen generation by photosynthesis. Measurements of gaseous mixtures from ice blocks containing wheat seedlings at -5°C reveal oxygen concentrations up to 3%. On return to dark the oxygen is rapidly utilised (see: Andrews, 1996).

Anaerobic experiments are usually done in the dark to prevent oxygen production via photosynthesis.

1.6.4. Temperature and Oxygen Deprivation

Ambient temperature greatly influences the metabolic rate and carbohydrate consumption of all organisms. During periods of limited energy availability such as oxygen deprivation, the influence of temperature increases and can greatly affect the plant viability. Winter floods and ice-encasement are usually related to low temperatures. The American cranberry can survive for months under ice-encasement as long as the temperature is below zero, but prolonged flooding under higher temperatures can cause severe damage (Dana & Klingbeil, 1966). Two *Rumex* species showed better biomass preservation and viability after submergence at lower temperatures (van der Sman *et al.*, 1993). The rapid loss of elongation potential in wheat roots during anoxia at 25°C compared to 15°C also resembles the more adverse effects of oxygen deprivation at high rather than low temperatures. The reason for this might be different ATP requirements for maintenance at different temperatures (Waters *et al.*, 1991a). In rhizomes of *Schoenoplectus lacustris* glycolytic activity under anoxia (measured as PQ) doubles during a temperature rise from 10°C to 25°C (Brändle, 1980). For the assessment of anoxia tolerance, temperature influences should always be considered.

1.7. Description of the Plant Species Under Investigation

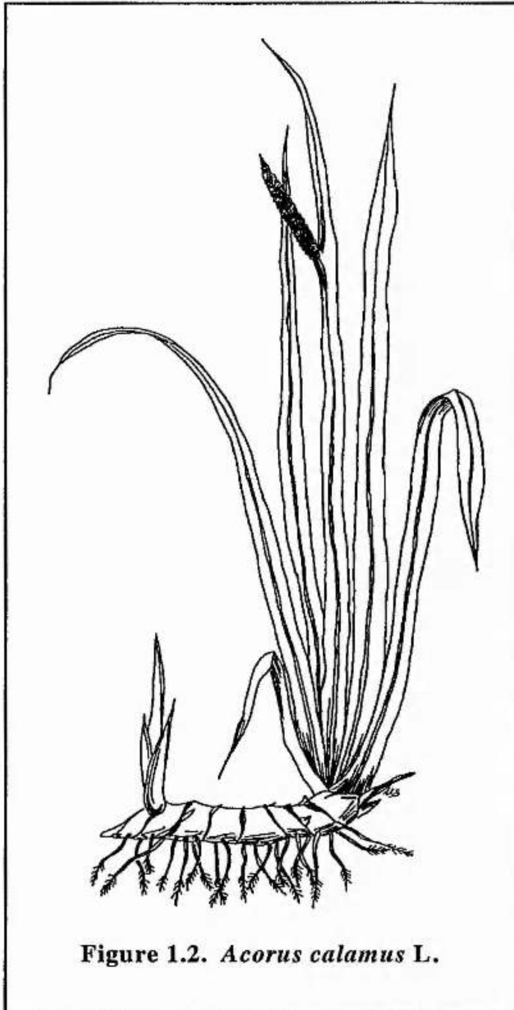


Figure 1.2. *Acorus calamus* **L.**

1.7.1. *Acorus calamus* L.

The Sweetflag or Sweet Sedge (*Acorus calamus* L.) (Fig.1.2.) is a monocotyledonous amphibious plant with long flat linear leaves, growing from a stout rhizome about 2-4cm thick. The leaves and rhizomes posses well developed aerenchyma. A major characteristic of this species is a strong aromatic smell from the ethereal oils in the spherical cells of the rhizome. The plant was mainly used for medical purposes because of its content of drugs, such as α- and β-asarone, pinene, camphene, eudenol and sesquiterpenes (Weber & Brändle, 1996).

Acorus calamus has its origin in the Himalayan region. The first plants were probably brought to Europe in 1557 as a gift from the Turkish court in Constantinople for Mathioloi in Prague (Hegi, 1935). Later Clusius (1525-1609) introduced more plants into his garden in Vienna. From there, it spread out into Central Europe and later on into England. Today it is most common on the banks of canals, pools and slow rivers in many places in England and Ireland. The European race is triploid (2n=3x=36) and sterile, so reproduction is dependent on vegetative propagation.

The rhizomes of *A. calamus* are positioned at the water-soil interphase and as a consequence are submerged for most of their lifetime. The rhizomes accumulate high amounts of starch over summer, and produce new green leaves in late autumn that stay small during winter. If the water table is high, rhizomes and shoots often overwinter totally submerged and thus experience prolonged periods of oxygen shortage (Weber & Brändle, 1994; Bucher *et al.*, 1996). *Acorus calamus* is highly adapted to this environmental stress, and under laboratory conditions the rhizomes survived periods of up to three months without any oxygen (Crawford, 1996; Crawford & Brändle, 1996). In spring, the shoots

grow rapidly and air can be transported via aerenchyma to the lower parts of the plant (Armstrong *et al.*, 1994).

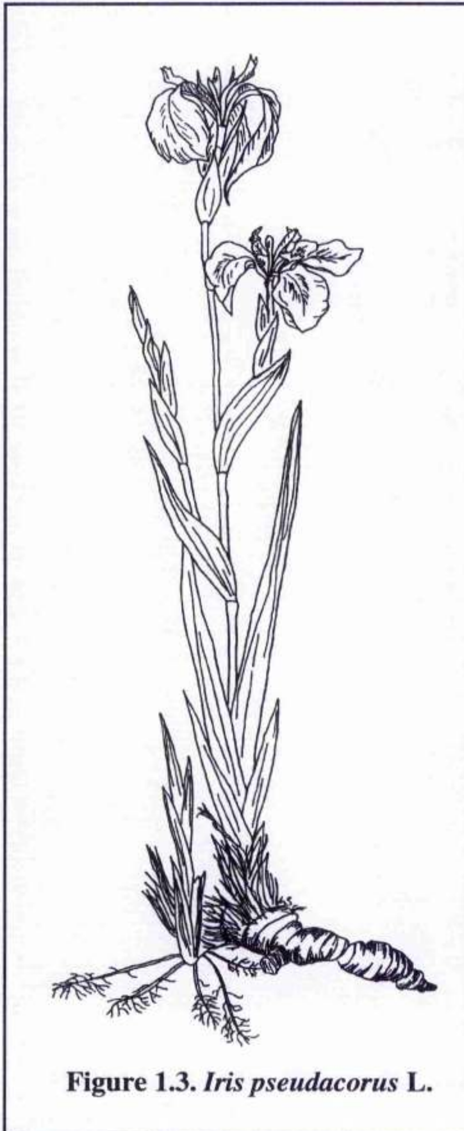


Figure 1.3. ***Iris pseudacorus*** **L.**

1.7.2. *Iris pseudacorus* L.

The Yellow-flag Iris or Common Yellow Iris (*Iris pseudacorus* L.) (Fig.1.3.) is a mono-cotyledonous amphibious perennial plant. The tall, flat and rather glaucous leaves have large lacunae and grow from rhizomes of about 1-4cm thickness. Leaves and rhizomes contain large amounts of glucosides and tannins. Extracts from the rhizome have been used for tanning and in connection with iron salts as black dye. The most characteristic feature of the species are the large yellow flowers, which reach a diameter of 8-10cm. Therefore, *I. pseudacorus* can be easily spotted every summer on the margins of pools, lakes, rivers and in marshy meadows throughout the British Isles. The light seed capsules are dispersed by wind and water.

Iris pseudacorus occurs in all European countries (except Iceland), in the Caucasus, Western Asia and Northern Africa and the north east of the United States. Usually it grows in sites with high water content, and grows on peat as well as on permanently submerged organic and inorganic soils. *I.pseudacorus* is capable of living amongst high levels of soluble organics and the metabolism of the plant seems to be well adapted to oxygen deprivation stress (Sutherland, 1990). Under laboratory conditions, rhizomes survived periods without oxygen for up to two months (Hanhijärvi & Fagerstedt, 1995). The rhizomes contain considerable amounts of non-structural carbohydrates throughout the year, and in contrast to the majority of higher plants *I. pseudacorus* stores no starch, but has instead the fructan irisin (Augem, 1928). Leaves are usually present throughout the year with peak growth occurring from April to June, and only during severe winters do they die back completely.

1.7.3. *Vaccinium macrocarpon* Ait.

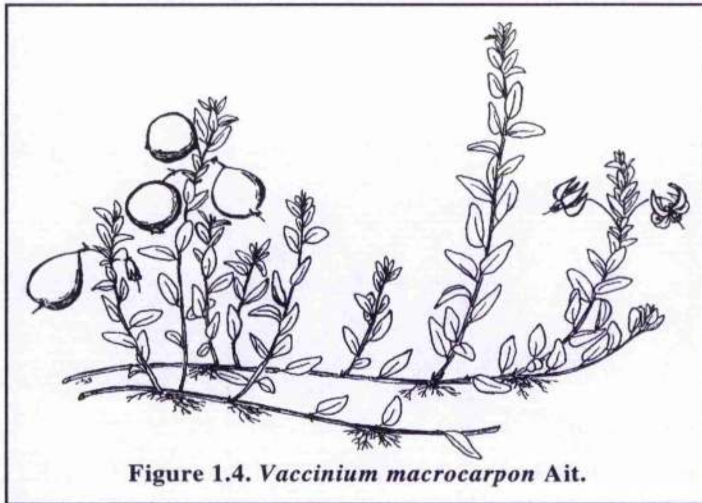


Figure 1.4. ***Vaccinium macrocarpon*** **Ait.**

The American cranberry (*Vaccinium macrocarpon* Ait.) (Fig. 1.4) is an evergreen, woody, perennial creeping vine. It belongs to the *Ericaceae* and grows naturally in acid peat bogs. The vines, called runners, spread over the soil surface to form a mat. Erect-growing shoots, called uprights, originate from the vines. The uprights have small, dark green, horizontal leaves, allowing maximal exposure to light. Individual leaves may remain on the plant for 2-3 seasons. The roots of the cranberry are not extensive. Only fine, fibrous roots develop in the surface layer of the soil, and at greater depth aeration is probably lacking. The pink flowers usually develop in early summer on the new elongated upright shoots, the fruits mature 60 - 120 days after fertilisation depending on cultivar characteristics and weather. The mature fruits are red with firm, crisp flesh and high acid content.

Vaccinium macrocarpon is indigenous to North America. The species was introduced in several places in Europe for their relatively large fruits. Some plants escaped and can be found today in the wild (Hegi,1935). In America some farmers started to grow cranberries commercially around 1830 by ditching of marshes. In 1965, 441,000 barrels of fresh fruit were harvested in the fields of Wisconsin (Dana & Klingbeil, 1966).

One of the main problems in cranberry production is frost damage. Because winter protection by snow cover is often unpredictable and insufficient, the farmers cover the vines during winter with water. The water freezes to form an ice blanket, which prevents drying of the vines and minimises fluctuations in temperature. *Vaccinium macrocarpon* is very flood tolerant and the dormant vines survive under ice for a few months. However, when photosynthesis under the ice is impaired or prevented by an additional snow cover, the oxygen level in the water drops quickly and the cranberry plants may suffer from oxygen deficiency injuries such as death of stems and terminal buds. Particularly dangerous are warmer periods during flooding, when respiration rises, and carbohydrate reserves in the plants are depleted quickly (Dana & Klingbeil, 1966). In the following growing season, the carbohydrate status of the vines directly influences fruit set (Marucci, 1966). Therefore,

high productivity in cranberry fields depends very much on the flooding management and requires a good understanding of use and damage of the flooding treatment by the grower.

1.8. Aim of the Thesis

Oxygen deprivation (hypoxia and anoxia) is a common environmental stress experienced by plants in poorly drained soils, during long-term flooding, during total submergence or under ice-encasement. The metabolic events that accompany the shift from aerobic to anaerobic processes have been studied particularly intensively in root tissue and germinating seedlings. However, the length of stress application was limited in most experiments by the short survival time of the plant material. Much less is known about the mechanisms behind the long-term anoxia tolerance found in a number of wetland and arctic species. Studies concentrated usually on experiments with the dormant rhizome. The aim of this thesis is to investigate the metabolic changes under long-term anoxia in leaves of three wetland species (*Acorus calamus*, *Iris pseudacorus*, *Vaccinium macrocarpon*).

The anoxia tolerance of leaves aroused special attention only more recently, when it was shown that some arctic species survive anoxia for longer periods without visible damage to the leaf tissue (Crawford *et al.*, 1994). The metabolic adaptation of leaves to anoxia appear to be particularly interesting. Leaves can endure anoxia much longer than roots. In comparison to rhizomes, that are adapted to withstand long periods of unfavourable environmental conditions in a dormant state, leaves possess higher metabolic activity including photosynthetic carbon fixation and access to carbohydrate stores is only limited. The metabolic consequences of anaerobic incubation are therefore supposed to be more drastic in leaves than in rhizomes.

The three species under investigation have been shown to be well adapted to oxygen deprivation under natural conditions; they show, nevertheless, some differences in their morphology and ecology. The maximal survival time of the plants was determined for all three species under anoxia. The effects of long-term anoxia on leaves have been investigated by estimating: fermentative activity and carbohydrate consumption under anoxia (Chapter 3); maintenance of respiratory capacity under anoxia (Chapter 3); maintenance of photosynthetic capacity under anoxia (Chapter 4); and ultrastructural modifications in mesophyll cells under anoxia (Chapter 5). Furthermore, the recovery of respiration and photosynthesis was studied in the leaves on return to aerobic conditions (Chapter 3 and 4). The effects of anoxia on the metabolism of leaves, especially on the photosynthetic apparatus, were related to the effects of long-term darkness (Chapter 3, 4 and 5).

Chapter 2

Material and Methods

2.1 Materials and Equipment

The majority of chemical agents used were from one of the following companies: Sigma Chemical Company Ltd, Poole, Dorset, UK; Boehringer Mannheim GmbH, Germany; BDH Chemicals Ltd, Poole Dorset, UK; Pharmacia LKB, Uppsala, Sweden; TAAB Laboratories Equipment Ltd, Reading, Berkshire, UK; Scotlab, Strathclyde, UK. All chemicals used were analytical grade, except those used for electron microscopy which were EM grade. Except where stated differently, the samples were centrifuged in a Biofuge A, Heraeus, Sepatech GmbH, Germany. All samples were vortexed with a Whirlmixer™ (Fisons Scientific Apparatus, Leicestershire, UK).

2.2 Origin of Plant Material and Growth Conditions

The *Acorus calamus* plants came originally from the Moossee, a lake in the vicinity of Berne, and were cultivated in a pond in the old botanic garden around the Sir Harold Mitchell Building of the University of St. Andrews. *Iris pseudacorus* was collected from natural sites at the shore of Loch Lindores, North East Fife, Scotland. The rhizomes of these plants were cleaned of soil, cut into pieces of about 5-7cm and potted separately in sand. The shoots of all plants were cut back to about 20-25cm above the soil. To allow the plants to establish in the pots, they were kept in the greenhouse for at least two weeks until new leaves developed.

Vaccinium macrocarpon plants, variety 'Steven', were supplied by the Ocean Spray Cranberry Company, Massachusetts, and had been checked for genetic purity by DNA fingerprinting (J. Davenport, personal communication). The plants were potted in peat of pH < 4. In summer, the plants were kept in the greenhouse, in winter they remained outside. Before any experiment, they were brought into the greenhouse for at least two weeks. Once a year, the *Vaccinium* plants were re-potted and fed with nutrient solution (Miracid® soil acidifier plant food, ICI Garden Products, Surrey, UK).

In the greenhouse, the plants received sufficient daylight in summer, and in winter artificial light was added to ensure a day length of at least 14h all year round. All plants were regularly watered and the temperature in the greenhouse was kept at 20-25 °C. The greenhouse adapted plants were used as a control representing day zero of the anoxia or dark treatment.

2.3. Anoxia and Dark Treatment

For the anoxia experiments, the plants were transferred directly from the greenhouse into the anaerobic chamber (Forma Scientific Anaerobic system, model 1024, Marietta, Ohio, USA). The atmosphere in the chamber consisted of 90% nitrogen and 10% hydrogen with a palladium catalyst to remove any traces of oxygen. The anaerobic atmosphere was checked

with methylene blue indicator strips (Dry Anaerobic Indicator strips, Becton Dickinson Microbiology Systems, Cockeysville, USA). To avoid the production of oxygen via photosynthesis, the plants were kept in the dark. The humidity in the chamber was nearly 100%, and plants were supplied with nitrogen bubbled water only when necessary. The temperature was kept at 20-25°C. After the anoxia treatment, the plants were returned to the greenhouse. Plant survival was checked by observing leaf development after return to aerobic conditions in the greenhouse.

For comparison, some experiments were repeated with plants that were kept in the dark but under aerobic conditions. The plants were transferred to a growth cabinet with the light switched off for 24h a day, the temperature was kept at 20°C and the plants were watered regularly.

2.4. Sampling

Leaf samples of *Acorus calamus* and *Iris pseudacorus* were harvested from the 3-4 youngest leaves about 10-15cm above the ground, except for the photosynthesis experiments, fluorescence measurements and electron microscopy, where the tissue about 5-15cm below the tips of the leaves was used. For rhizome and root sampling, the material was washed carefully with cold water. Rhizome samples were taken from about 2-3cm away from the base of the oldest green leaf. All *Vaccinium macrocarpon* samples were taken from dark green, one-year-old leaves.

2.5. Fresh and Dry Weight Determination

The fresh weight of the plant material was recorded using a fine balance. The leaves were then dried in paper bags at 100°C (Hotbox Oven, Gallenkamp, Germany) for 72 hours, after which dry weight was determined.

2.6. Carbohydrate Determination

The carbohydrate content was determined colorimetrically by the Anthrone Test (Morris, 1948). The test is based on the principle that under heat, the formed 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde reacts with anthronal, the enol form of anthrone, and the yellow anthrone reagent converts into a blue/green solution which absorbs at 623nm. The anthrone reagent (8.6mM anthrone in 80% v/v H_2SO_4) was always made up fresh and kept on ice. For calibration, glucose standard solutions of 10, 20, 40, 80 and 160μg.ml^{-1} in distilled water were used. To 1ml of each standard, 3ml of anthrone reagent were added and the mixture vortexed. The solutions were heated in a water bath at 80°C for 10min and then cooled on ice for 30min. The absorbance of each standard was read at 623nm against a blank, containing 1ml distilled water plus 3ml anthrone reagent, and treated as stated above. A Pye Unicam SP1800 Ultraviolet spectrophotometer (Pye Unicam, Cambridge, UK) was used. Fig. 2.2. shows the glucose calibration curve.

2.6.1 Sample Preparation

For carbohydrate assays, plant material of 0.5g fresh weight was cut into pieces, boiled for 30min in 10ml of 40% ethanol and filtered. The extraction was then repeated in 10ml distilled water for 45min and filtered again. The extracts were combined and dried under vacuum (Büchi Rotavapor R, Glasapparatefabrik Flawil, Switzerland). The residue was re-dissolved in 1ml distilled water and stored at -20°C for soluble carbohydrate assay. The boiled plant material was ground in liquid nitrogen, and freeze-dried (Edwards, Crawley, Sussex, UK) overnight. The dry material was stored at -20°C until used for insoluble carbohydrate assay. No material was kept in the freezer for longer than one month.

2.6.2. Total Soluble Carbohydrates (TSC)

Aliquots of 5-20µl of the concentrated soluble carbohydrate extract were made up to 1ml with distilled water, and 3ml of the anthrone reagent were added carefully. After vortexing, the samples were heated for 10min in a 80°C water bath, then cooled for 30min on ice. The absorbance of the solutions was recorded at 623nm and total soluble carbohydrate concentration calculated from the glucose calibration curve.

2.6.3 Total Non-soluble Carbohydrates (TSNC)

To hydrolyse the non-soluble carbohydrates in the freeze-dried plant material, 15mg of each sample were resuspended in 1.5ml of 1.6M perchloric acid and incubated in a water bath at 70°C for 2h, during which the samples were vortexed three times. Following this, samples were centrifuged at 10,000*g* for 10min. According to concentration, 40-80µl of the solution were made up to 1ml with distilled water, mixed with 3ml anthrone reagent, heated at 80°C for 10min and kept on ice for 30min. The absorbance of the samples was read at 623nm and total insoluble carbohydrate concentration calculated from the glucose calibration curve.

2.7. Determination of Ethanol Content by Gas Liquid Chromatography (GLC)

The plant material was cut into pieces and samples of 0.3g fresh weight were ground quickly with 2ml of ice-cold 6% (v/v) perchloric acid. Some acid washed sand was added to allow quick destruction of the material. After centrifuging the samples for 5min at 10,000*g*, the supernatant was decanted and a drop of methyl orange was added as an indicator. The solution was neutralised by the addition of a few drops of 5M K_2CO_3 until the colour changed from red to yellow. The samples were stored at 4°C for 15min to allow settlement of the precipitate.

Samples of 1µl extract were injected into a Pye Unicam series 104 model 64 Gas Liquid Chromatograph and flushed through a 1.75m glass column filled with Porapak Q, 100-120

mesh. Nitrogen was used as a carrier gas at 40ml/min. The investigation temperature was 150°C for the column and 160°C for the detector. The degree of ionisation as the ethanol burned in the detector was recorded, and the area of the ionisation peak produced calculated by a Hewlett Packard Integrator. The GLC system was calibrated daily with 1µl samples of 0.05%, 0.1% and 0.3% (v/v) ethanol standard solution (Sigma-Aldrich Company Ltd., Poole, UK). Ethanol content of the samples was calculated from the ethanol calibration curve (Fig.2.3). All samples were measured twice and the mean determined.

2.8. Analysis of Gas Exchange with the Warburg Respirometer

The Warburg respirometer (Warburg, Apparatebau B. Braun, Meisungen, Western Germany) was used to measure the O_2 consumption, and aerobic and anaerobic CO_2 production of leaf discs. Standard Warburg manometric technique is based on the principle that at constant temperature and constant gas volume, any changes in the amount of gas can be measured by changes in pressure (Umbreit *et al.*, 1957).

In all cases, 0.2g fresh weight of leaf material were sliced quickly into discs of no more then 1mm thickness and transferred into Warburg-flasks of known volume (about 15ml) containing 3ml distilled water. The flasks were connected to the manometer proper, and the manometer fluid adjusted to 150mm. The stopcock on the manometer and the stopper on the sidearm of the flasks were closed and the initial reading recorded. After 10min equilibration, readings were taken every 30min for 3h.

The temperature was kept at 20°C. Nine flasks were set up per experiment, three for O_2 consumption, aerobic and anaerobic CO_2 production respectively. A thermobarometer was set up to correct all measurements for variation in pressure and temperature in the room. The flasks were wrapped with aluminium foil to prevent any O_2 production via photosynthesis.

The amount of gas produced or consumed by the leaf tissue was calculated as follows:

$$k = \frac{V_g * \frac{273}{T} + V_f\,\alpha}{P_o}$$

$$x = h * k$$

k = flask constant
x = µl gas (0°C, 760mm.Hg)
V_g = Volume of flask + tubes
V_f = Volume of fluid in flask
P_o = standard pressure (760mm.Hg)
T = temperature in absolute degree
α = solubility in reaction of gas involved (ml_{gas}/ml_{liquid} at standard pressure and temperature T of measurement)
h = observed change in the manometer

2.8.1. Oxygen Consumption

For measuring the O_2 uptake, small filterpaper rolls soaked with 10% (w/v) KOH were inserted into the central wells of the flask to capture all produced CO_2. The amount of O_2 consumed by the leaf was calculated:

$$x_{O2}\ [\mu l] = h_{O2} * k_{O2}$$

2.8.2. Aerobic Carbon Dioxide Production

The amount of liberated CO_2 was estimated using flasks without added KOH so that the changes in the manometer resulted from decreasing pressure due to the consumption of O_2 and the increasing pressure due to the production of CO_2. Therefore, CO_2 production was calculated as follows:

$$x_{CO2}\ [\mu l] = k_{CO2} * \left[h - \frac{x_{O2}}{k_{O2}} \right]$$

2.8.3. Anaerobic Carbon Dioxide Production

The flasks with the leaf tissue were flushed with nitrogen for 5min to create an oxygen-free atmosphere. The anaerobically produced CO_2 was then calculated:

$$x_{CO2}\ [\mu l] = h_{CO2} * k_{CO2}$$

2.9. Protein Analysis

2.9.1. Protein Determination

Soluble protein content was measured after the method of Bradford (1976). The method is based on the shift in the absorption maximum of Coomassie Brilliant Blue G-250 from 465 to 595nm when bound to protein in an acidic environment. The increase in absorption at 595nm was monitored (Unicam UV-Visible Spectrometer Heλios α, Cambridge, UK). A standard curve was produced using bovine serum albumin (BSA) at concentrations of 0-100$\mu g.ml^{-1}$ each time an assay was carried out (Fig.2.4). Protein extracts were diluted with distilled water as necessary. In 1ml cuvettes, 0.1ml of the diluted extract was mixed with 1ml Bradford Reagent (0.01% w/v Coomassie Brilliant Blue G-250, 4.7% w/v ethanol, 8.5% w/v phosphoric acid) and stirred well. After 15min colour development, the absorbance was measured at 595nm against a reagent blank (0.1ml of the appropriate extraction buffer and 1ml of Bradford Reagent). All standard solutions were measured in duplicate.

2.9.2. Total Soluble Protein Content

The leaves of the three plant species used, *A. calamus*, *I. pseudacorus* and *V. macrocarpon*, are very rich in secondary compounds, e.g. phenols or polyphenols, which can interfere with buffer compounds and hinder or completely prevent a successful extraction. To obtain satisfactory results, several buffer compositions were checked for each species. The following protein extraction buffers gave the best results:

- *Acorus calamus*:	0.1M Na-phosphate buffer (pH 6.8)
	0.5mM EDTA
	0.01mM leupeptin
	1mM β-mercaptoethanol
- *Vaccinium macrocarpon*:	0.1M Na-acetate buffer (pH5.4)
	10mM $CaCl_2$
- *Iris pseudacorus*:	8M Urea
	1% (w/v) SDS
	5% (v/v) β-mercaptoethanol

Samples of 0.5g leaf material were ground in liquid nitrogen and 2ml extraction buffer and about 20mg PVPP were added. The samples were thawed during the process and then centrifuged at 10,000*g* for 15min at 4°C (MSE Micro Centaur; Scotlab, Coatbridge, UK). The supernatant was collected and used for protein determination (see Section 2.9.1.) All samples were measured in duplicate.

2.9.3. SDS-Polyacrylamide Gel Electrophoresis

Protein extracts from *A. calamus* leaves were prepared as described in Section 2.9.1. and analysed on the mini-Protean™ system (Bio-Rad, Hemel Hempsteat, UK). A separating gel (12.5% w/v acrylamide, 0.33% w/v bisacrylamide, 0.375M Tris-HCl pH 8.8, 0.1% w/v SDS, 0.05% w/v ammonium persulphate, 0.01% v/v TEMED) was poured and overlayed with a stacking gel (4% w/v acrylamide, 0.1% w/v bisacryamide, 0.125M Tris-HCl pH 6.8, 0.1% w/v SDS, 0.05% w/v ammonium persulfate, 0.01% v/v TEMED). The thickness of the gels was 1mm. The extracts were diluted with extraction buffer to give equal concentrations of soluble protein in each sample. Five parts of protein extract were mixed with one part of sample buffer (125mM Tris-HCl pH 6.8, 30% v/v glycerol, 10% w/v SDS, 0.6M DTT, 0.002% w/v bromphenol blue) and boiled for 3-5min. After centrifuging for 1min at 10,000*g*, the protein samples (30-50μg protein per lane) were loaded on the gel alongside 10μl of prestained molecular marker proteins (Prestained SDS-PAGE Standard Solution, molecular weight 25-127kDa, Sigma Aldrich Co. Ltd, Poole, UK, see Appendix I). Electrophoresis was carried out in SDS-running buffer (25mM Tris pH 8.3, 192mM glycine, 0.1% w/v SDS) at a constant voltage of 100V. Gels were stained in Coomassie blue solution (0.1% w/v Coomassie Brilliant Blue R-250 in water: methanol: acetic acid 5:5:2) for 2h, then destained overnight in 6.5% (v/v) acetic acid and 18.7% (v/v) methanol.

The gels were stored in 7% (v/v) acetic acid until dried in a gel-drier (GelAir System, BioRad, Hemel Hempstead, UK).

2.9.4 Western Blotting (Rubisco)

A monoclonal antibody raised in rat against the wheat Rubisco large subunit (RbcL), which was kindly donated by Dr. Martin A.J. Parry (IACR, Rothamsted, UK), was used for Western blotting.

Protein separation was carried out following the procedure described in the previous chapter, except that only 10μg protein were loaded per lane. Immediately after the electrophoresis, the gels were soaked in Towbin transfer buffer (25mM Tris-HCl pH 8.3, 192mM glycine, 0.1% w/v SDS, 20% v/v methanol) for 30min at 4°C. The gels were then placed against pre-wet nitrocellulose membranes (Trans-Blot® transfer medium 0.45 micron, Bio-Rad Laboratories, Hercules, USA) and assembled in the Western Transfer Unit (Hoefer TE 22 Tank Transfer Unit, Hoefer Scientific Instruments, San Francisco, USA) according to the manufacturers instructions. Protein transfer was performed for 3h at constant current of 50mA.

On completion of blotting, the nitrocellulose membranes were rinsed in Tris buffer saline (TBS: 25mM Tris-HCl pH 8.0, 140mM NaCl, 2.6mM KCl). To block all non-specific sites, the membranes were then incubated overnight in 10% (w/v) milk powder (Marvel™) in TBS buffer (pH 8.0) at room temperature. The membranes were rinsed with TBS buffer (pH 7.3) and transferred into primary antibody solution (TBS buffer pH 7.3, 1% w/v Marvel™ + RbcL primary antibody at a dilution of 1/100) for 2h at room temperature. All unreacted primary antibody was washed off sequentially in TBS (pH 8.0) containing, 0.1%, 0.5% and 0.1% (v/v) Tween-20 respectively for 15min each. The membranes were then incubated in secondary antibody solution (TBS buffer pH 7.3, 1% w/v Marvel™ + anti-rat antibody linked to alkaline phosphatase at a dilution of 1/1000) for 2h at room temperature. The secondary antibody was washed away sequentially in TBS (pH 8.0) containing 0.1% then 0.5% (v/v) Tween-20 respectively for 15min each and finally washed for 15min in sodium bicarbonate buffer (100mM $NaHCO_3$ pH 9.8, 10mM $MgCl_2$).

Colour development was carried out in a freshly prepared NBT/BCIP staining solution (0.4μM BCIP, 0.4μM NBT in sodium bicarbonate buffer pH 9.8). After 3-5min the reaction was stopped by rinsing the nitrocellulose with 20mM EDTA, and the stained membrane was air-dried.

2.9.5. α-Amylase Activity

Amylolytic activity was measured colorimetrically according to the method of Chrispeels & Varner (1967). The method is based on the principle that starch reacts with iodine

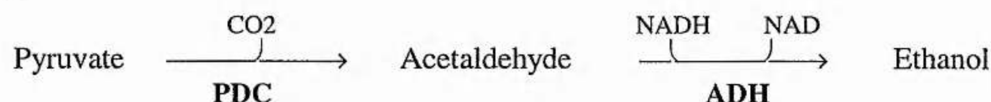
reagent, forming a dark blue dye that absorbs light at 620nm. Amylases break down the starch causing a decrease in the optical density of the iodine/starch solution at 620nm.

Enzyme extracts were prepared by grinding 0.5g leaf material in liquid nitrogen, and 20mg PVPP and 2ml extraction buffer (Na-acetate buffer pH 5.4, 10mM $CaCl_2$) were added while the material was still frozen. The extraction buffer for the *A. calamus* assay also contained 1% (v/v) Triton-X-100. The samples were then centrifuged at 10,000*g* (MSE Micro Centaur; Scotlab, Coatbridge, UK) for 15min at 4°C and the supernatant collected. Total soluble protein content was determined according to the method of Bradford (see Section 2.9.1).

For the amylase assay, 20-150µl enzyme extract were pipetted into 3ml cuvettes and adjusted with extraction buffer to 0.6ml. The reaction was started by the addition of 0.6ml starch solution and left at room temperature. A fresh starch solution was made up every day using 150mg potato starch, 600mg KH_2PO_4 and 30mg $CaCl_2$ in a final volume of 100ml distilled water. The suspension was boiled for 3min, cooled and centrifuged at 2,000*g* (MSE Minor Centrifuge; Scotlab, Coatbridge, UK) for 5min. The clear supernatant to be used for the assay was carefully separated from the unsolubilised starch in the bottom of the tube. After 15min, the reaction was stopped by the addition of 0.6ml iodine reagent (50mM HCl, 0.06% w/v I_2, 0.6% w/v KI). The solution was diluted with 2ml distilled water and the absorbance read at 620nm (Unicam UV-Visible Spectrometer Heλios α, Cambridge, UK). To determine the initial absorbance of the solution, comparative samples without the enzyme extract were prepared. The assay was calibrated from a standard curve (Fig.2.5) using solutions of 5-80µg α-amylase from barley malt (2.2Unit/mg solid; Sigma Aldrich Co. Ltd, Poole, Dorset, UK: one unit was defined as the liberation of 1.0mg maltose from starch in 3min at pH 6.9 at 20°C). Activity of α-amylase was measured in duplicate in all samples.

2.9.6. Pyruvate Decarboxylase (PDC) Activity

The Pyruvate Decarboxylase assay is based on the following reaction (Waters *et al.*, 1991b):



The consumption of NADH was measured colorimetrically at 340nm.

Leaf samples of 0.2g FW were ground in 1ml ice-cold extraction buffer (125mM MES-KOH pH 6.8, 100mM NaCl, 2.5mM $MgCl_2$, 1mM EDTA, 0.5mM TPP, 2mM DTT, 20mg PVPP). Acid-washed sand was used to accelerate rupture of plant material. The samples were centrifuged for 15min at 10.000*g* at 4°C (MSE Micro Centaur; Scotlab, Coatbridge, UK) and protein content determined (see Section 2.9.1.). For analysis of PDC activity, 50-100µl enzyme extracts were incubated in assay buffer (50mM MES-KOH pH 6.0, 25mM

NaCl, 1mM $MgCl_2$, 0.5mM TPP, 2mM DTT) at 25°C giving a final volume of 690µl. After 20-30min, NADH (final concentration in the assay 0.17mM), sodium oxamate (final concentration in the assay 50mM) and 10U ADH (from bakers yeast, 450 Unit/mg solid; Sigma Aldrich Co. Ltd, Poole, Dorset, UK) were added and the 'blind' reaction recorded for 5min at 340nm (Pye Unicam SP1800 Ultraviolet spectrophotometer; Cambridge, UK) connected to a chart recorder (Unicam AR 25 Linear Recorder; Cambridge, UK). ADH and NADH were prepared in stock solutions and stored at -20°C. During the experiment, the solutions were kept on ice. The sodium oxamate was included to inhibit lactate dehydrogenase (LDH) activity. The reaction was started by addition of Na-pyruvate (final concentration in the assay 10mM) and the absorbance recorded at 340nm for 10min. The final volume in the cuvette was 1ml and the temperature was kept at 25°C by connecting the spectrophotometer to a circulating water bath (RTE-9 refrigerating circulating bath, Neslab Instruments, Portsmouth, USA). For the calculation of PDC activity the 'blind' reaction was subtracted from the final result, and the extinction coefficient ε_{340}[NAD/NADH] used was 6.22 $mM^{-1}.cm^{-1}$. The PDC activity was measured in duplicate for all samples.

2.9.7. Cytochrome *c* Oxidase (COX) Activity

The Cytochrome *c* oxidase assay followed the method of Hodges & Leonard (1974), in which the oxidation of reduced cytochrome *c* by the COX is recorded in a spectrophotometer at 550nm.

For the enzyme extraction, 0.5g leaf material was ground in liquid nitrogen, 2ml of extraction buffer (MOPS-KOH pH 7.0, 100mM, 50mM $MgCl_2$, 1mM β-mercaptoethanol, 2mM EDTA) were added, and the samples were centrifuged for 20min at 10,000*g* at 4°C (MSE Micro Centaur; Scotlab, Coatbridge, UK). The cytochrome *c* (acid modified, from horse heart, purity 90%; Sigma Aldrich Co. Ltd, Poole, Dorset, UK) was made up in 100mM tricine buffer (pH 7.5) at 10mg/ml and reduced by adding a few crystals of Na-dithionine until a ratio of about 9:1 was obtained for A_{550}/A_{565}. The enzyme extract was diluted in a 1ml cuvette with tricine buffer (100mM, pH 7.5) to give a final volume of 850µl, and the final protein concentration in the assay was 0.1-0.3mg/ml. To force the lysis of membranes, the assay was incubated in the presence of 0.1% Triton-X-100 for 1min. The reaction was started by addition of 50µl reduced cytochrome *c* and decrease in absorbance was recorded at 550nm on a spectrophotometer (Pye Unicam SP1800 Ultraviolet spectrophotometer, Cambridge, UK) connected to a chart recorder (Unicam AR 25 Linear Recorder, Cambridge, UK). The temperature was kept constant at 25°C by connecting the spectrophotometer to a circulating water bath (RTE-9 refrigerating circulating bath, Neslab Instruments, Portsmouth, USA). Specific activity of COX was estimated by using an extinction coefficient ε_{550} for cytochrome *c* of 18.5$mM^{-1}.cm^{-1}$. The

COX activity was measured in duplicate in all samples. Protein content of the initial enzyme extract was measured by the method of Bradford (see Section 2.9.1).

2.10. Photosynthesis Measurements

Rates of oxygen evolution were measured using whole leaf sections in a Hansatech Leaf-Disc Electrode (Hansatech, Kings Lynn, Norfolk, UK) linked via a control box to a chart recorder. Oxygen evolution was measured under light intensities of 2,600μE.m^{-2}.s^{-1}. A constant temperature of 20°C was maintained in the leaf-disc chamber by circulating water from a temperature controlled water bath (Haake G, Gallenkamp, Germany) through the attached water jacket. The leaf-disc electrode was assembled and maintained according to the manufacturers instructions (Hansatech, Kings Lynn, UK; Walker, 1990).

The rate of CO_2-dependent O_2 evolution was measured in leaf sections taken about 5-10cm below the leaf tip of *A.calamus* and *I.pseudacorus* plants, while whole leaves were used from *V.macrocarpon.* The leaf material was placed into the leaf-disc chamber to which some drops of saturated $KHCO_3$ were added to generate sufficient CO_2 for the photosynthetic reaction in the chamber. The chamber was sealed and the leaf-disc electrode was calibrated before each measurement. A gas-tight syringe containing 1ml of air was attached to the leaf-disc chamber and the voltage output recorded from the control box. Air (1ml) was then injected into the leaf-disc chamber and the new voltage output recorded. The change in the voltage output refers to the change in oxygen concentration in the leaf-disc chamber. At standard pressure and 20°C, 1ml of air (ca. 21% O_2) contains 8.73μmol oxygen. After removing the syringe, the stopcock on the leaf-disc chamber was closed and the CO_2 dependent O_2 evolution measured by recording the voltage output at 20°C for 15min under light. On completion of measurements, the leaves were removed from the chamber and mounted on paper with clear tape. The leaf area of the material was determined using an AnalySIS™ image analyser equipped with a monochrome CCD camera (Norfolk Analytical Ltd, Hilgay UK).

2.11. Chlorophyll Determination

The chlorophyll content (Chl) of the leaves was measured according to the method of Arnon (1949). Leaf samples of 50mg were ground with acid washed sand in 2ml 80% (v/v) acetone and centrifuged for 5 min at 10,000*g*. The resulting supernatant was made up to 3ml with 80% acetone and the absorbance of chlorophyll measured against an acetone blank at 663nm and 645nm in a Pye Unicam SP1800 Ultraviolet spectrophotometer (Pye Unicam, Cambridge, UK).

Chlorophyll concentrations were calculated using the following equations:

$$\text{(I) Chl a } [\mu g/ml] = (12.7 \times A_{663}) - (2.69 \times A_{645})$$

$$\text{(II) Chl b } [\mu g/ml] = (22.9 \times A_{645}) - (4.68 \times A_{663})$$

2.12. PSII Chlorophyll Fluorescence Emission

The PSII chlorophyll fluorescence emission of intact leaves was measured with the Fluorescence Monitoring System (FMS) from Hansatech (Hansatech Instruments Ltd, Kings Lynn, Norfolk, UK).

When chlorophyll molecules absorb light energy, they change their electronic configuration. The 'excited' electronic configuration is inherently unstable and short lived as several photochemical processes (photosynthesis) and non-photochemical processes (e.g. infra-red radiation as heat, red/far-red radiation as fluorescence) compete to dissipate the absorbed energy. Therefore, measurements of changes in the extent of PSII fluorescence emission can be used to infer information about changes in the ability of a sample to utilise light energy for photochemistry.

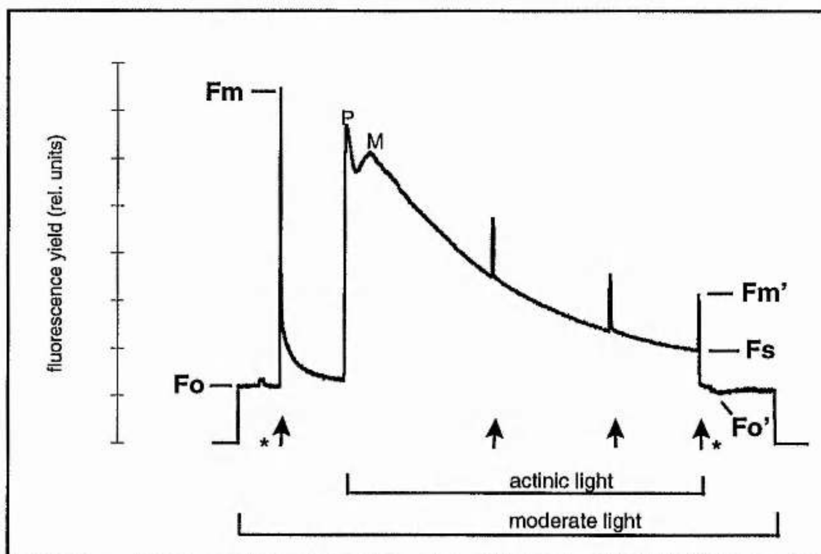


Figure 2.1. Variables measured for fluorescence analysis
(using the Fluorescence Monitoring System from Hansatech Instruments Ltd, Kings Lynn, UK). Fo-fluorescence origin at moderate light (6 PAR), Fm-fluorescence maximum obtained by a saturating pulse (arrow), Fm'-light-adapted fluorescence maximum, Fs -steady-state fluorescence yield (at actinic light: 180 PAR), Fo'-fluorescence after far-red pulse (star).

2.12.1. Fluorescence Analysis

Before the measurements, leaves were adapted to the dark for 30min using the provided leaf clips. The fluorescence monitoring system was assembled according to the manufacturers instructions, and the measuring program was written with the Hansatech FMS Windows® Software (see Appendix II). All measurements have been carried out at elevated CO_2.

First the fluorescence origin (Fo) was determined under moderate light (6 PAR). The maximal quantum yield of PSII photochemistry (Fv/Fm) in the dark adapted tissue was

estimated by exposing the leaf tissue to a saturating light flash (1740 PAR). Following this, the leaves were illuminated with actinic light (180 PAR). To separate photo-inhibitory damage from anoxia related damage, a relatively low intensity had been chosen for the actinic light. The sensitivity to photoinhibition was investigated in control and anoxia treated leaves by the exposure to higher actinic light intensities of 400 PAR. During the illumination, a saturating pulse (1740 PAR) was imposed every 60sec to determine Fs (steady state fluorescence signal) and Fm' (light adapted fluorescence maximum) until Fv'/Fm' was constant (after about 3min). After the exposure to light, Fo' was recorded and a flash of far-red light (735nm) was given to remove all electrons from the PSII reaction centre by stimulating PSI. Saturating pulses were given after the actinic light treatment for 4min 20sec to record the recovery of Fv/Fm values. The definition of fluorescence parameter is shown in Fig. 2.1.

2.12.2. Calculation of Fluorescence Parameter

The following signs and equations have been used for fluorescence analysis (see: Jones, 1992; Software by Hansatech Instruments Ltd, Kings Lynn, Norfolk, UK):

Fo	fluorescence origin	
Fm	fluorescence maximum	
Fv/Fm	maximum quantum efficiency of PSII	(Fm-Fo)/Fm
Fs	steady-state fluorescence yield	
Fm'	light-adapted fluorescence maximum	
Fv'/Fm'	antennae efficiency of PSII	(Fm'-Fo')/Fm'
Fo'	fluorescence after far-red beam	
Φ_{PSII}	quantum efficiency of PSII (Genty *et al.*, 1989)	(Fm'-Fs)/Fm'
q_P	photochemical quenching coefficient	(Fm'-Fs)/ (Fm'-Fo')
q_{NP}	non-photochemical quenching coefficient	[Fm-(Fm' * Fo/Fo')]/ (Fm-Fo')

2.13. Transmission Electron Microscopy (TEM)

2.13.1 Fixation

Transverse sections of 2x1mm were taken from *Acorus calamus* leaves about 5cm below the leaf tip. Sections were immediately transferred to primary fixative (2.5% v/v glutaraldehyde in 0.1M sodium cocadylate, pH 7.3). The sections were vacuum infiltrated

for 20-30min to aid penetration and therefore protein linkage in the tissue and left on a rotator (TAAB, Berkshire, UK) at 2rpm overnight at room temperature. After washing the sections in four changes of 0.1M Na-cacodylate (pH 7.3) they were incubated in secondary fixative (1% w/v osmium tetroxide in Na-cacodylate pH 7.3) on a rotator (2rpm) for 2h at room temperature to fix lipids in the tissue. The sections were then washed in Na-cacodylate (pH 7.3) for 2 x 5min.

2.13.2 . Dehydration

Fixed sections were dehydrated sequentially in 30, 50, 70, 90 and 100% (v/v) ethanol for a period of 1h in each dilution at room temperature, and then in fresh 100% ethanol for a further hour. The sections were en-bloc stained in the dark in 2% (w/v) uranyl acetate in 100% (v/v) ethanol on a rotator overnight at room temperature and then washed for 1h in 100% ethanol at room temperature to remove excess uranyl acetate.

2.13.3. Embedding in Spurr's Resin

The tissue sections were embedded in Spurr's medium grade epoxy resin (Spurr, 1969) by incubation in the following series at room temperature for a period of 1h each:

(i) 2:1 ethanol to Spurr's resin
(ii) 1:1ethanol to Spurr's resin
(iii) 1:2ethanol to Spurr's resin
(iv) Spurr's resin

Sections were then transferred to fresh resin and left overnight on a rotator (2rpm) at room temperature. The leaf sections were placed in flat green agar moulds (TAAB, Berkshire, UK), which allowed orientating the tissue. The moulds were filled up with fresh Spurr's resin and left for 1h at room temperature before being polymerised at 60°C for 24h.

2.13.4. Ultrathin Sectioning

Transverese tissue sections were cut from the polymerised blocks using glass knives (LKB Knifemaker 7801B, Leica UK Ltd, Milton Keynes, UK) on an ultramicrotome (Reichert-Jung Ultracut, Leica UK Ltd, Milton Keynes, UK) at a knife angle of 4° and a cutting speed of 2mm/s. Ultrathin sections (60-90nm) were cut onto distilled water, expanded with 100% chloroform vapour and mounted onto 200 mesh copper grids (AGAR Scientific, Essex, UK) that had been coated with 1% (w/v) formvar in chloroform and carbon (Carbon turbocoater, Bio-Rad Microscience Division, Herfordshire, UK).

2.13.5. Heavy Metal Staining of Sections

Staining was carried out on a wax bed in a petri dish by placing the grids (with tissue face down) onto droplets of the appropriate staining solution. All solutions were centrifuged at 10,000*g* (MSE Microcentaur, Loughborough, UK) for 10min and then filtered through a

2μm Milipore filter (Millipore Corporation, Bedford, UK) to minimise the incidence of artefacts on the grids. Grids were stained in 2% (w/v) uranyl acetate in 70% (v/v) ethanol for 20min in the dark, and then rinsed three times in distilled water. Grids were then stained with 0.3% (w/v) lead citrate in 0.1M sodium hydroxide in a CO_2 free environment (produced by the incorporation of NaOH pellets within the petri dish) for 5min. The grids were rinsed in distilled water, dried on filter paper and stored in a dust free environment.

2.13.6. Examining of Tissue Under TEM

Sections were examined on a Philips 301 TEM at 60kV (Philips, Cambridge, UK) and photographed with Agfa Scientia EM Film. The negatives were developed in Ilford Phensiol at 20°C for 3.5min, washed in running water for 2min, fixed in Ilford Hypham for 5min and washed again in running water for a further 20min, and left to air dry.

2.13.7. Analysis of Micrographs

The quantitative analysis of cell ultrastructure was based on the principles of stereology and morphometry (see Section 5.1.3). Leaf tissue from *A.calamus* was examined in control plants, after 28d under aerobic conditions in the dark, and after 28d under anaerobic conditions in the dark. Images of the micrographs were captured onto an AnalySIS image-analyser equipped with a mono-chrome CCD camera (Norfolk Analytical Ltd., Hilgay, UK), and the transverse area of the structures under investigation determined utilising the AnalySIS software programme.

The tissue was always examined at two different levels of magnification. TEM mircographs of transverse areas through mesophyll cells of the leaf were analysed at a magnification of x6250 to estimate the volume fraction of chloroplasts and mitochondria per volume cytoplasm (excluding vacuoles and nucleus). For more detailed analysis of chloroplast structure, micrographs at x15650 magnification were taken for estimation of volume proportion of stroma in the chloroplast and volume fraction of grana stacks per chloroplast volume, and for determination of number of grana sac per granum. The estimation of volume fractions was estimated using the following formula (see Section 5.1.3):

$$\mathbf{A}_A = \mathbf{V}_V$$

$$\mathbf{Area}_{compartment} / \mathbf{A}_{total} = \mathbf{Volume}_{compartment} / \mathbf{Volume}_{total}$$

e.g. chloroplast area/ cytoplasm area = V_V of cytoplasm occupied by chloroplasts

At each level of magnification, twenty micrographs were used for analysis. With this number of micrographs, standard errors (SE) were ≤ 10% of the mean value, and the results represent reliable estimates (see Section 5.1.3). The mircographs were taken from at least ten randomly selected blocks obtained from six leaves for each treatment.

2.14. Statistical Analysis

Statistical analysis was carried out using MINITAB software, Release 12 (MINITAB Inc, USA). Data sets were correlated to confirm normal distribution (Ryan-Joiner test for normality). Arithmetic mean and standard error of the mean (SE) were calculated. Data means were compared by one-way analysis of variance (ANOVA), using Tukey's multiple comparison test at a confidence limit of 95%.

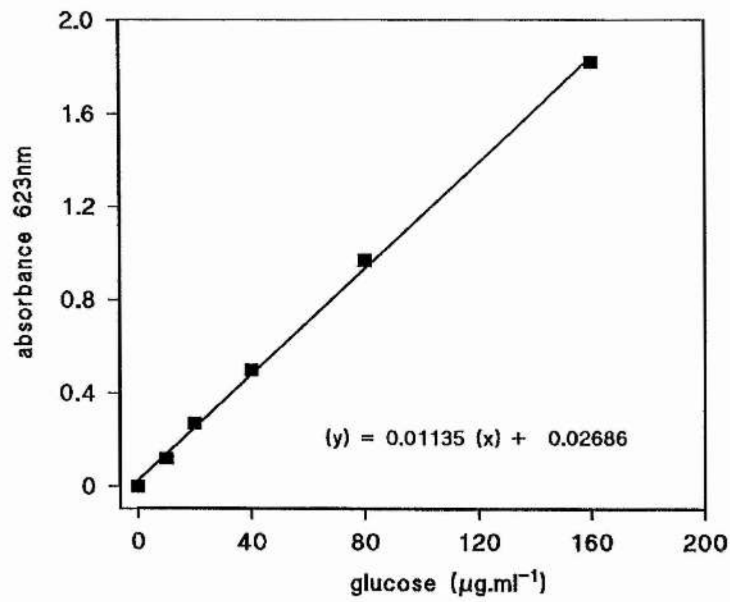


Figure 2.2. Standard glucose calibration curve
Glucose standard solutions of 10, 20, 40, 80 and 160µg.ml^{-1} were prepared in duplicate and measured as described in Section 2.6. Regression analysis was performed using the FigP software (BioSoft, Cambridge, UK).

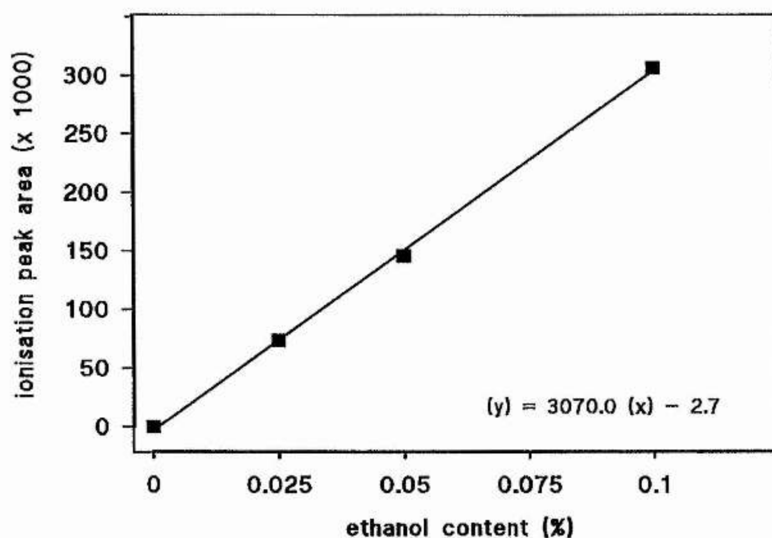


Figure 2.3. Standard ethanol calibration curve

Ethanol standard solutions (Sigma-Aldrich Company Ltd., Poole, UK) were used for calibration of the Gas liquid Chromatography System as described in Section 2.7. Regression analysis was performed using the FigP software (BioSoft, Cambridge, UK).

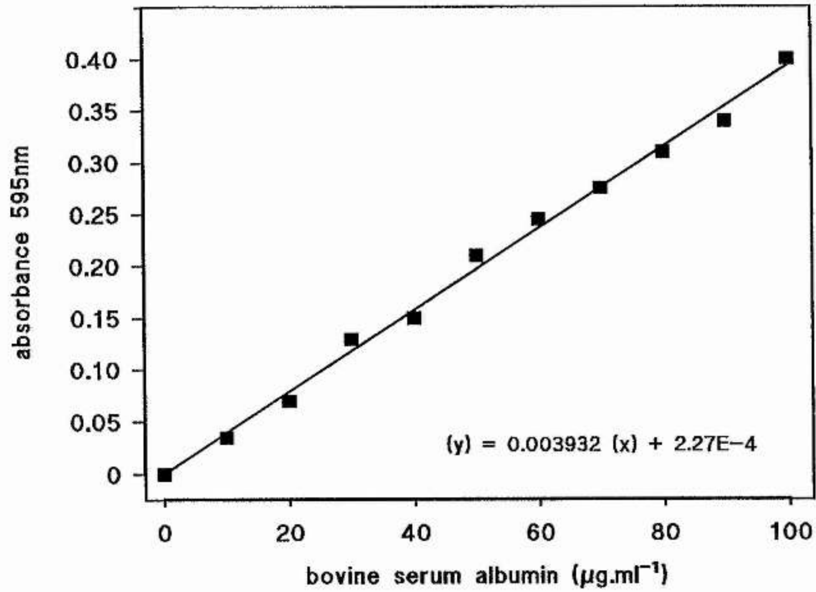


Figure 2.4. Standard protein calibration curve

Protein standard solutions were prepared using bovine serum albumin (BSA) at concentrations of 0-100μg.ml^{-1}, and assayed as described in Section 2.9. Regression analysis was performed using the FigP software (BioSoft, Cambridge, UK).

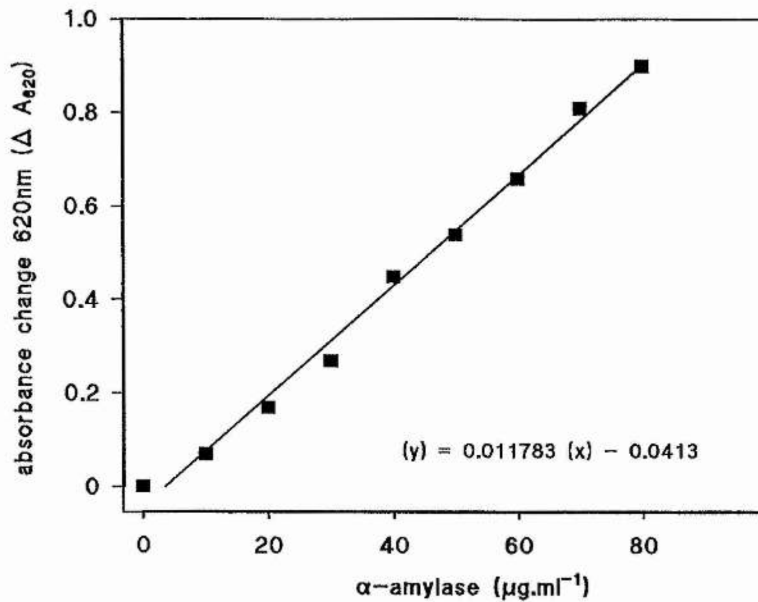


Figure 2.5. Standard α-amylase calibration curve
Standard solutions were prepared using 0-80μg α-amylase from barley malt (2.2Unit/mg solid; Sigma Aldrich Co. Ltd, Poole, UK), and assayed as described in Section 2.9.5. Regression analysis was performed using the FigP software (BioSoft, Cambridge, UK).

Appendix I: Molecular weight markers

Prestained protein markers (Sigma Aldrich Co. Ltd, Poole, UK) used for SDS-PAGE:

	kDa
Triosephosphate Isomerase	26.6
Lactic dehydrogenase	36.5
Ovalbumin	45.0
Pyruvate kinase	58.0
Fructose-6-phosphate kinase	84.0
β-Galactosidase	116.0

Appendix II: Protocol for measurements of PS II chlorophyll fluorescence emission.

The script was written using the FMS software by HANSATECH.

```
ID : 2,2
LOG : 1
WAIT : 10.0
Fo : 2.0
Fo' : 2.0
WAIT : 5.0
Fo : 2.0
FM : 0.7,45
WAIT : 30.0
ACT : 18
WAIT : 10.0
FS : 2.0
FM : 0.7,45
WAIT : 60.0
FS : 2.0
FM : 0.7,45
WAIT : 60.0
FS : 2.0
FM : 0.7,45
WAIT : 60.0
FS : 2.0
FM : 0.7,45
ACT : 0
WAIT : 5.0
Fo' : 2.0
WAIT : 30.0
Fo' : 2.0
WAIT : 5.0
Fo : 2.0
FM : 0.7,45
WAIT : 30.0
Fo : 2.0
FM : 0.7,45
WAIT : 60.0
Fo : 2.0
FM : 0.7,45
WAIT : 120.0
Fo' : 2.0
WAIT : 5.0
Fo : 2.0
FM : 0.7,45
WAIT : 5.0
```

Chapter 3

Effects of Long-term Anoxia on the Carbohydrate Metabolism of Leaves

3.1. Introduction

3.1.1. Previous Work on Anaerobic Carbohydrate Metabolism in Leaves

Studies concerning the effects of anoxia on shoot tissue are usually limited to experiments with either overwintering organs or coleoptiles and first leaves of young seedlings, while the responses of mature green leaves to anoxia arouse only little attention. Surprisingly, shoots of nearly all investigated species tolerate anoxic conditions longer than roots (Vatrapetian *et al.*, 1976; Fagerstedt & Crawford, 1987; Vartapetian, 1991; Bucher & Kuhlemeier, 1993; Ellis *et al.*, 1999). There do not seem to be any published findings of fundamental differences in the metabolic response of shoots and roots to anoxia. However, a number of studies revealed differences in the regulation of gene expression and protein synthesis in plant organs after the onset of anoxia (Cobb & Kennedy, 1987; Dolferus *et al.*, 1997; Tadege *et al.*, 1998). For assessment of the metabolic response of shoots, a differentiation should be made between (i) shoots of anoxia intolerant plants that die after only a few days under anoxia, (ii) shoots that continue to grow under anoxia and (iii) shoots that survive anoxia in a quasi-dormant state.

Leaves of anoxia intolerant dryland species such as *Arabidopsis* or barley outlive roots only by a few hours or days under anoxia (Ellis *et al.*, 1999; Fagerstedt & Crawford, 1987). In contrast to the immediate anaerobic response of roots, the leaves of these species show much less pronounced metabolic changes under anoxia. Anaerobic pea and maize seedlings express lower ADH activities in their shoots than in their roots (Cobb & Kennedy, 1987). In mature maize leaves, there was no evidence for the production of anaerobic proteins (Okimoto *et al.*, 1980). A comparative study of gene expression in *Arabidopsis* showed that the initiation of an additional set of anaerobic proteins such as PDC and ADH is restricted to the root (Dolferus *et al.*, 1997). Nevertheless, experiments with anoxia intolerant species are limited in time by the rapid death of the tissue and the investigations rarely exceeded 48h.

Shoot growth under anoxia occurs only in plants that are adapted to fluctuating water tables and has mainly been studied in seedlings of wetland grasses such as rice and *Echinochloa* species. Thus elongation growth takes place mainly in the stem tissue and not in the leaves (Summers & Jackson, 1994). The developing shoots show very high metabolic activity under anoxia. The protein synthesis is less inhibited in the leaves than in roots. The ADH activity increases in the first 4d under anoxia and is higher in the shoots than in the roots (Cobb & Kennedy, 1987). Mitochondrial enzymes are still synthesised in anaerobic shoots of rice. The activity of four mitochondria specific enzymes was low but still detectable after 15d of anoxia (Couée *et al.*, 1992). The high metabolic rate is dependent on access to carbohydrate stores in the seed tissue. If the seedlings fail to reach an aerobic environment they probably die from carbon starvation. Besides germinating

seedlings, shoot growth under anoxia has also been observed in some rhizomatous wetland plants in Spring, but nothing is known about the mechanisms which trigger the growth (Barclay & Crawford, 1982).

The survival of anaerobic conditions in a quasi-dormant state has been reported for rhizomes and leaves of wetland plants as well as for green leaves of some arctic species (Crawford & Brändle, 1996), but metabolic investigations are mainly restricted to the overwintering rhizome. The only long-term study on green leaf tissue was made in *Acorus calamus*, exposed to anoxia for up to two months (Bucher & Kuhlemeier, 1993). There was no visible damage in the leaves and they remained green for the course of the experiment. The gene expression of aldolase, PDC and ADH was monitored in leaves and rhizomes. Transcript levels of all enzymes peaked in the first three days after the onset of anoxia and then declined slowly, but were still detectable after two months of anoxia. The enzymatic activity of ADH was already high under aerobic conditions. It increased about 3- to 5-fold during the first week of anoxia, then remained constant for the rest of the experiment. For comparison, two 'housekeeping' enzymes were investigated as well, and their levels did not change throughout the anoxic treatment indicating that the translational machinery probably continued to work under anoxia. Leaves and rhizomes appeared to respond to anoxia in a similar fashion.

3.1.2. Oxygen Deprivation Under Laboratory Conditions

Generally, two levels of oxygen deprivation can be distinguished. Anoxia (anaerobiosis) describes the complete absence of oxygen (zero concentration of O_2), while hypoxia refers to partial oxygen deprivation (Crawford & Brändle, 1996). The use of these terms is now well established in literature. Nevertheless, the methods for the creation of oxygen free conditions during experiments can differ between research groups and this complicates the assessment and comparison of results. Especially in older publications, the terms anoxia or anaerobiosis refer to conditions where oxygen is not completely removed from the system. Hypoxic conditions are defined slightly differently by various authors: 5% (Huang & Johnson, 1995), 4% (Johnson *et al.*, 1989; Andrews *et al.*, 1994), 3% (Xia *et al.*, 1995; Bouny & Saglio, 1996; Zeng *et al.*, 1998) or 0.1% of oxygen (Ellis *et al.*, 1999).

However, these concentrations describe the situation in the plants' surroundings, but inside the plant tissue the conditions are more heterogeneous, with nearly normoxic, hypoxic and anoxic cells. The same problem exists for flooding experiments, where the root is exposed to an anoxic environment and the plant shoot is still in the air. Depending on the plants ability for oxygen transport, their metabolism will appear as a combination of anaerobic and partly aerobic processes.

The exposure of whole plants to complete anoxia probably occurs only seldom in nature, but during laboratory experiments an anoxic environment gives the advantage that plant metabolism can be studied under complete absence of oxygen.

3.1.3. Aim of the Chapter

To investigate long-term anoxia tolerance of selected wetland species (*A.calamus, I.pseudacorus, V.macrocarpon*), whole plants are exposed to anoxic conditions for prolonged periods, and survival is checked by observation of leaf development on return to air.

In this thesis, the metabolic response of leaves to anoxia is of special interest. Anoxia causes drastic alterations in the carbohydrate and energy metabolism. The availability of fermentable substrate and their economic utilisation are crucial for the survival under long-term anoxia, and the carbohydrate content is determined in the plant organs after prolonged periods of anoxia. The fermentative activity of leaves is estimated by measuring anaerobic gas exchange, ethanol content and pyruvate decarboxylase (PDC) activity.

Due to the altered energy metabolism and restricted biosynthetic activity under oxygen deprivation, the plant capacity for aerobic processes is also affected under long-term anoxia. The recovery of respiratory activity is investigated by measuring the leaf capacity for aerobic gas exchange and cytochrome *c* oxidase after the anoxia treatment.

Experiments with plants under anoxia are usually done in the dark to prevent any oxygen evolution by photosynthesis. Since most research concentrated on roots and rhizomes, the absence of light could be neglected. For leaves, light conditions are of major importance for carbohydrate balance, and therefore the impacts of prolonged darkness are compared under aerobic and anaerobic conditions.

3.2. Results

3.2.1. Plant Survival Under Anoxia

Table 3.1. Survival of leaves under anoxia in *A.calamus, I.pseudacorus* and *V.macrocarpon*

	Survival after 28d anoxia with the majority of leaves green and intact (n=50)	Maximal survival of overwintering leaves [days of anoxia]	Maximal survival of leaves during the growing season [days of anoxia]	
			old leaves	young leaves
A.calamus	96%	75d	40d	50d
I.pseudacorus	86%	60d	20d	35d
V.macrocarpon	80%	45d	30d	3-7d

Acorus calamus L.

The present experiment confirms the remarkable tolerance of *A.calamus* under long-term anoxia. Independent of the season, the majority of rhizomes (90%, n=10) survived up to 75d under anoxia and started re-growth of new leaves within 5d to 8d after return to aerobic conditions. Only the root of the plant was sensitive to anoxia. It was difficult to test their survival under anoxia, but the bulk of them were soft after only 7d to 14d of anoxia.

The behaviour of leaves under anoxia differed between (i) overwintering leaves, (ii) maximal expanded mature leaves and (iii) young growing leaves. Overwintering leaves are short and grow in late autumn. Under natural conditions, they survive often under ice or flooding, and show no growth or only retarded growth over winter. Before the anoxia experiments, the plants were brought into a growing condition in the greenhouse for about 2 weeks. Under these conditions the leaves started to grow slowly. The overwintering leaves endured anoxic conditions almost as long as the rhizomes. They survived up to 75d of anoxia, with only the very tip wilting in some leaves. No further damage was visible under post-anoxic conditions.

During the growing season (about April to September), the leaves of *A.calamus* grew much faster than during winter. The plant usually consisted of 2-3 growing inner leaves, about 2-4 fully expanded leaves and a few senescent outer leaves. Under anoxia, the older leaves lost their fresh green colour and turned soft, and on return to air these leaves wilted and yellowed rapidly (in the first 48h in air) and showed no sign of recovery. In the 4-5 youngest leaves, the damage was limited to the leaf tips and the bulk of the tissue remained green and turgid under anoxia and no further damage was visible on return to air. For the following metabolic experiments, only leaf tissue was used which had not been visibly damaged. Depending on their age, mature leaves died after about 35d to 40d under anoxia. The young immature leaves stopped their growth when exposed to anoxic conditions. Usually, they showed no or only slight injury for up to 50d under anoxia (Tab. 3.1). On return to aerobic conditions after a month of anoxia growth restarted within 3d to 4d.

After 28d of anoxia, the 4-5 youngest leaves *of A.calamus* survived without major damage in 96% (n=50) of the tested plants (Tab. 3.1; Plate 3.1a). If not stated otherwise these leaves were used for all the following experiments. There were no significant changes in the fresh/dry weight ratio after 28d of anoxia (Fig. 3.1).

Iris pseudacorus L.

The maximal survival time of *I.pseudacorus* rhizomes under anoxic conditions was about 65d in winter, and about 50d in summer. As with *A.calamus*, the roots were sensitive to anoxia and showed damage after 7d to 14d of anoxia.

The same leaf types as in *A.calamus* can be distinguished in *I.pseudacorus*. The overwintering leaves showed hardly any growth and one example tolerated anoxia for up to 60d. But on return to air, the leaves remained small. New leaves grew only slowly, and they

were much narrower and shorter than in untreated plants. Generally, the speed of leaf growth seemed to be negatively correlated with the survival under anoxia.

The fast grown mature summer leaves showed the first signs of damage after 7d of anoxia. Their tips became soft and brown, and often only the base of the older leaf remained green for longer than 20d of anoxia. On return to aerobic conditions the majority of mature leaves became yellow or brown within only a few days and died. The senescence of leaves seemed to be generally accelerated under post-anoxic conditions. The 2-3 youngest leaves, those which were still entirely or partly enclosed by older leaves, remained green and survived the anoxic treatment during the growing season for 35d to 40d. When the plants were re-exposed to air after 28d anoxia, their younger leaves restarted to grow slowly after about 10d.

After 28d under anoxic conditions, about 86% (n=50) of *I.pseudacorus* plants survived with at least 3-4 green leaves (Tab. 3.1; Plate 3.1b). The fresh/dry weight ratio of whole *I.pseudacorus* leaves did not change significantly under anoxia (Fig. 3.1).

***Vaccinium macrocarpon* Ait.**

V.macrocarpon expressed some seasonal differences in their anoxia tolerance. As with *I.pseudacorus* and *A.calamus*, the plant is more tolerant to anoxia during winter. The plant stopped the growth of new leaves in autumn and all leaves became dark green and hard. In this state, *V.macrocarpon* plants could tolerate anoxic conditions for up to 45d and about 60-70% of the leaves remained green and intact. The leaves from the previous growing season were usually most tolerant. These leaves were called 'one-year-old leaves' and were used in all the following experiments.

In spring the upright shoots start to grow new leaves at the terminal bud. The immature leaves are light green and much softer than the mature ones. A comparison between new leaves and one-year-old leaves revealed that the new leaves contained smaller amounts of soluble and non-soluble carbohydrates as well as proteins. In the young leaves the soluble and non-soluble carbohydrates were 70% and 80% respectively as compared with the mature leaves. The protein content in the young leaves was only 30% of that in mature leaves. In contrast to the anaerobic response in *A.calamus* and *I.pseudacorus*, these young leaves of *V.macrocarpon* were much more sensitive to anoxia than the older ones. Immature leaves first showed damage under anoxia after only 3d to 7d; then turned yellow and dry under anoxia (Plate 3.1c). In the following post-anoxia period they became brown and fell off. When plants had a high proportion of immature leaves, the anoxia tolerance of the whole plant was reduced, and after 25d to 30d under anaerobic conditions, all young leaves and a high proportion (often more than 50%) of mature leaves, were dead.

After 28d of anoxia, about 80% (n=50) of *V.macrocarpon* plants survived with the majority of their one-year-old leaves still dark green and intact (Tab. 3.1; Plate 3.1c). If the leaves survived the anoxic conditions, no post-anoxic injury could be detected and the

leaves remained on the plants for the whole of the next season. Re-growth of new leaves started under greenhouse conditions after about 10d of re-exposure to air. The fresh/dry weight ratio of *V.macrocarpon* leaves was stable under anoxia (Fig. 3.1)

***Hordeum vulgare* L.**

For comparison, the behaviour of anoxia sensitive 10d old barley seedlings was investigated under the same conditions as the three wetland species. About 3h after the onset of anoxic conditions, the leaf tips started to bend down. After 15h of anoxia the tips of the leaves were dry but still green with only the leaf bases still in an upright position. The whole plant finally lay flat on the ground after 21h of anoxia, and there was no recovery after return to aerobic conditions. The fresh/dry weight ratio decreased continuously during the anoxic treatment (data not shown).

3.2.2. Effects of Prolonged Darkness on Leaf Growth

The plants of all three investigated species continued to grow when kept in the absence of light under aerobic conditions. *A.calamus* and *I.pseudacorus* behaved similarly and developed new etiolated leaves which were usually thinner and softer than the light grown ones. At the same time, the oldest leaves wilted and changed their green colour to yellow. About 2-3 leaves remained green under prolonged darkness, and these leaves were used for the metabolic studies. *V.macrocarpon* growth continued in the terminal bud. The new leaves were also etiolated and smaller than the light grown ones. There was no visible colour change in the mature leaves. From all tested plants, 100% survived for 28d in the dark.

3.2.3. Effects of Anoxia on Carbohydrate Content

Total Soluble Carbohydrate Content (TSC)

The content of total soluble carbohydrates (TSC) was investigated in the leaves, rhizomes and roots *of A.calamus* and *I.pseudacorus* over nine weeks of anoxia in January/February 1996 (Fig. 3.2; 3.3) and in *V.macrocarpon* the TSC concentrations were measured in the leaves of anoxic plants over 6 weeks in March/April 1997 (Fig. 3.4). Before the experiment, the plants were brought into the greenhouse and showed slow growth. The mature leaves of *A.calamus* and *I.pseudacorus* were about 30-40cm tall. The duration of the anaerobic treatment was close to the maximal survival time for each species.

Generally, the main changes in the TSC concentration took place mostly in the first weeks under anoxia and remained stable thereafter. Similar amounts of TSC were found in leaves and rhizomes of *A.calamus* under control conditions. During the first two weeks of

anoxia, a significant decrease by about 33% compared with the initial value was detected in leaves. In the rhizomes however, TSC content increased slightly but significantly by 30% after one week of anoxia and declined slowly under the continuing anoxic conditions.

The leaves of *I.pseudacorus* contained comparably low concentrations of TSC (about 6mg/g FW) in winter 1996, and after the exposure to anoxia the TSC decreased even further by about 50% (Fig. 3.3). The rhizomes of untreated *I.pseudacorus* plants contained higher amounts of TSC (15mg/g FW). Besides glucose, fructose and sucrose, the soluble carbohydrate fraction contained short oligomers of fructose. Degrees of polymerisation up to 6 were revealed by Thin Layer Chromatography (TLC) stained with Thymol-Reagent (Heinze & Praznik, 1991). Under anoxic conditions, the TSC concentrations of the rhizome dropped rapidly down to 20% of the initial concentrations during the first 2 weeks and then remained at the lower level for the rest of the experiment.

The TSC content of the small but dense leaves in *V.macrocarpon* was generally higher than in the other two species. The anoxia treatment led to a slow decrease of TSC concentration, significantly different from the control level after 14d anoxia; but after 42d of the treatment, the leaves contained 14mg/g FW which was a higher concentration of TSC than in the other two species (Fig. 3.4).

During the anoxia experiment, all plants were kept in the dark, and as this would influence the carbohydrate metabolism of the plants, the effects of long-term darkness under aerobic conditions on the carbohydrate content of the leaves were investigated in May 1998. The results were compared with the effects of anoxia treatment for the same period of time. In *A.calamus* leaves, anoxia and dark treatment led to minor reductions in the TSC content. The leaves of *I.pseudacorus* contained generally higher contents of TSC during the measurements in May 1998 than in winter 1996. A 28d period of darkness had no effect on the TSC content of the leaves, but a significant decrease was detected after 28d of anoxia. In leaves of *V.macrocarpon*, TSC decreased significantly during the dark treatment by 13%, and anoxia initiated an even sharper drop of nearly 50% (Fig. 3.5).

Total Non-Soluble Carbohydrate Content (TNSC)

The total non-soluble carbohydrate (TNSC) content was always analysed from the same samples as the soluble carbohydrate content. As with the soluble carbohydrates, significant changes were mainly observed during the first weeks under anoxia. Leaves of *A.calamus* and *I.pseudacorus* contained similar amounts of TNSC under control conditions. A significant decrease of 25% was measured in *A.calamus* in the first week under anoxia, and after that the TNSC concentration was constant for the rest of the treatment.

In *I.pseudacorus,* TNSC concentration fell to 66% of the control value before a plateau was reached (Fig. 3.3). The two species differed greatly in the size of the carbohydrate store of the rhizome; *I.pseudacorus* contained double the amounts of TNSC of *A.calamus*. In the rhizomes of both species, anoxia resulted in a considerable decrease of TNSC. In

I.pseudacorus, a rapid drop in the TNSC level down to 20% of the initial value took place in only two weeks. In the rhizomes of *A.calamus*, TNSC reserves were mobilised more slowly and reached a minimum after about 42d at about 20% of the control value.

Vaccinium macrocarpon leaves contained higher amounts of TNSC than the other two species (about 100mg/g FW). After the onset of anoxia, a slow but steady and significant decrease occurred in the leaves for 21d, and the concentrations remained constant at about 40mg/g FW.

During the experiments in May 1998, no significant changes were found in the TNSC content of leaves of *A.calamus* and *I.pseudacorus* after extended periods of darkness or anoxia. In the *V.macrocarpon* leaves, TNSC levels declined significantly by 35% compared with the control after 28d of darkness, and they declined even more (by 65%) after 28d of anoxia (Fig. 3.5).

3.2.4. Effects of Anoxia on Ethanol Content

The ethanol content of leaves, rhizomes and roots was investigated over a period of 63d of anoxia in *A.calamus* and *I.pseudacorus*. In *V.macrocarpon* leaves, the ethanol content was measured over a period of 42d of anoxia (Fig. 3.6).

The ethanol concentrations increased significantly after the onset of anoxic conditions in all organs of the three investigated species. In *A.calamus* and *I.pseudacorus*, the ethanol accumulation was highest in the leaves reaching concentrations of about 80μmol/g FW. In rhizomes, maximal ethanol concentrations of 30μmol/g FW were found. Roots generally contained the smallest amounts of ethanol. The ethanol accumulation under anoxia followed a similar pattern in all three organs. After the initial increase, ethanol concentrations remained at a constant level for the rest of the experiment. In the leaves of *V.macrocarpon*, maximal ethanol concentrations of about 45μmol/g FW were found after 14d of anoxia, followed by a slow decrease until a constant level of about 20μmol/g FW was reached.

3.2.5. Effects of Anoxia on Gas Exchange Capacity of Leaves

Aerobic Gas Exchange

In the untreated control plants, O_2 uptake and CO_2 production were close to equality in the leaf tissue of all three species resulting in RQ[1] values of about one, and indicating that carbohydrates were the preferred respiratory substrate. Rates of oxygen uptake as well as

[1] Respiratory Quotient (RQ) = CO_2 production / O_2 uptake. The RQ was determined under aerobic conditions and in dark (see Section 2.8).

aerobic CO_2 evolution fell continuously when the leaves were exposed to anoxic conditions (Fig. 3.7 to 3.9). After 28d of anoxia, the capacity for aerobic gas exchange rates decreased by about 80% in *A.calamus* and *V.macrocarpon*, and even by 90-95% in *I.pseudacorus* when compared with the control. Generally, the aerobic release of CO_2 by the tissue was more inhibited than the O_2 uptake. When the plants returned to aerobic conditions, the respiratory activity increased rapidly in *A.calamus*, and after 3d of post-anoxia, rates of O_2 uptake and aerobic CO_2 release were back to the levels of the control. The aerobic gas exchange increased more slowly under post-anoxia in *V.macrocarpon* reaching about 70% of the control value after 10d post-anoxia, but there was no recovery in the leaves of *I.pseudacorus* and after 10 of post-anoxia, and O_2 uptake and CO_2 release were still reduced by 50% and 80% respectively.

Anaerobic Gas Exchange

When aerobically grown leaf tissue of the investigated plant species was exposed to anaerobic conditions, the CO_2 production decreased immediately (Fig. 3.7 to 3.9). In leaf tissue of control plants, the exposure to a 100% nitrogen atmosphere reduced the CO_2 production to about 30-40% of that in air.

Under prolonged anoxia, the anaerobic gas exchange decreased further in the leaf tissue of all three species. Very low levels were reached in *I.pseudacorus* after 28d anoxia treatment. Leaf tissue of the other two species produced about 80% less CO_2 than in the initial aerobic control. The capacity of anaerobic CO_2 evolution increased again after the plants returned to aerobic conditions.

3.2.6. Effects of Anoxia on Enzymatic Activity of Leaves

Total Protein Content of Leaves

The total protein content of leaves under anoxia and post-anoxia was analysed (Fig. 3.10a). In all three investigated species, the protein content was not significantly altered by the anoxia treatment. There were also no changes in the total protein content of *A.calamus* leaves when re-exposed to air after 28d of anoxia. The protein pattern for *A.calamus* leaf tissue after several periods of anoxia is shown in Plate 3.2 on a Coomassie blue stained SDS-PAGE. The protein separation and staining was repeated 10 times with independently extracted samples. Within the accuracy of the method, no visible changes could be detected in the protein composition of the leaf under anoxia. A comparison between the effects of prolonged darkness under aerobic and anaerobic conditions showed slightly decreased protein concentrations in the dark/air treated leaves of all three species, but only in *A.calamus* the changes were statistically significant (Fig. 3.10b).

α-Amylase Activity in Leaves

The activity of α-amylase was measured in *V.macrocarpon* leaves under anoxia for 28d, and in *A.calamus* leaves under anoxia and following post-anoxia (Fig. 3.11a). No significant changes occurred in the α-amylase activity of *V.macrocarpon* leaves. In *A.calamus*, the α-amylase activity increased significantly by 30% in the first day of the anaerobic incubation, but then remained at a steady level for the rest of the anoxia treatment. There were also no significant changes during the post-anoxia period. In contrast to the anoxia treatment, prolonged darkness initiated a small but significant increase in both species (Fig. 3.11b).

Pyruvate Decarboxylase Activity (PDC) in Leaves of A.calamus

PDC activity (Fig. 3.12a) was low in the leaves of the untreated control plants, but the transfer to an anaerobic environment initiated an immediate increase of about sevenfold after 24h. The PDC activity continued to rise under anoxia and reached a maximum level about 35 times higher than the control value after 21d, where it remained for the rest of the anoxia treatment. After re-exposure to air, the PDC activity remained high for at least 3d, and after 10d post-anoxia it was about eight times higher than in the control. The dark treatment did not induce any significant changes in the PDC activity of the leaf (Fig. 3.12b).

Cytochrome c Oxidase (COX) Activity in Leaves of A.calamus

In the first 24h of anoxia, the COX activity (Fig. 3.13) dropped by about 25%, but then was stable for 21d under anoxia. A further decrease took place after 28d of the anoxia treatment, and the COX activity was only 15% of the initial level. The re-exposure to aerobic condition led to a rapid recovery and after 24h post-anoxia the COX activity was about 50% of the control (significant increase). The activity continued to rise during post-anoxia, and was not significantly different from the control value after 7d of post-anoxia. Under prolonged darkness, the COX activity in the leaf was slightly but not significantly lower than in the control.

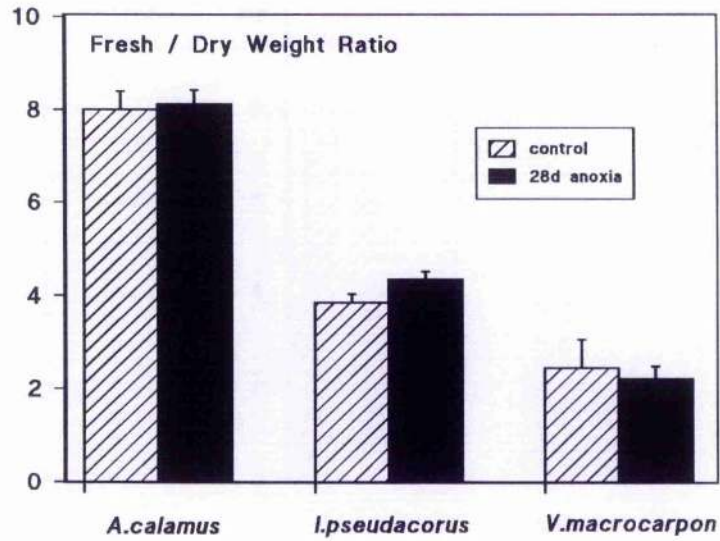


Figure 3.1. Fresh/dry weight ratio of leaves at day zero (control) and after 28d of anoxia.
Fresh and dry weight of leaves were determined as described in Section 2.5. Each datapoint represents the mean of 10 independent measurements, error bars show standard error of the mean.

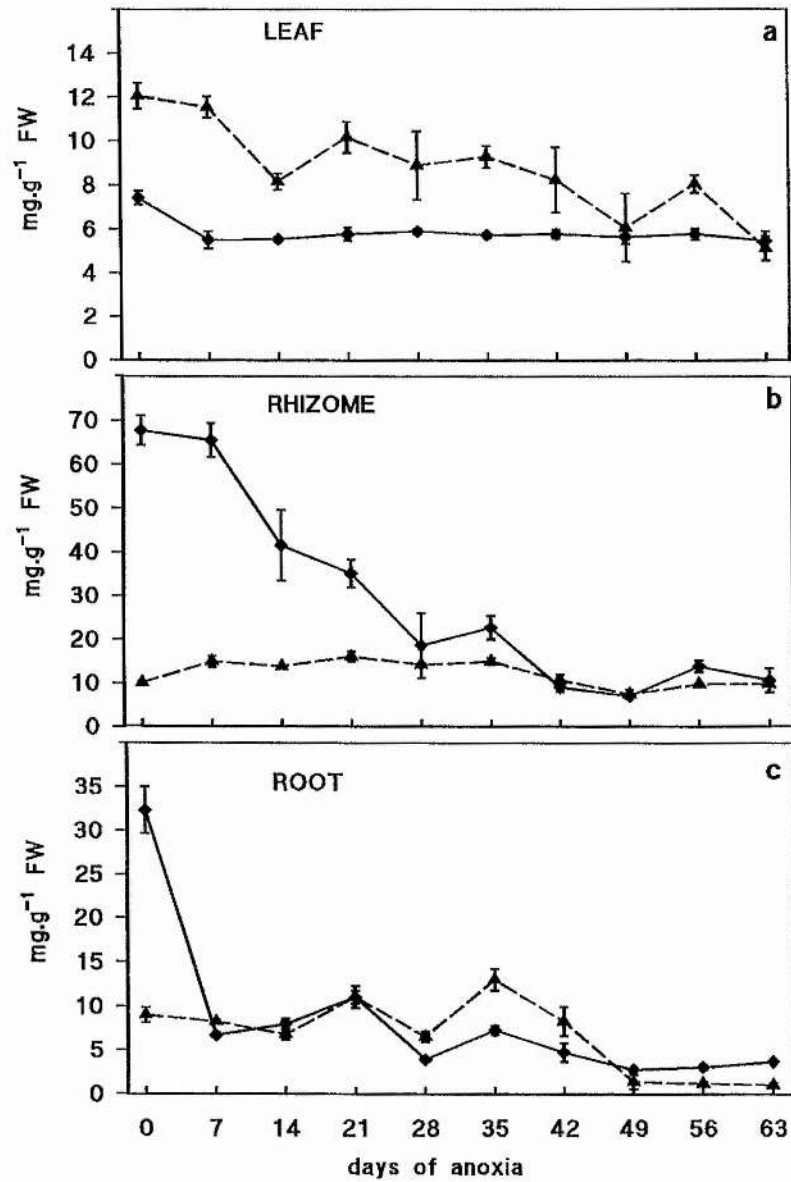


Figure 3.2. Carbohydrate content in leaves (a), rhizomes (b) and roots (c) of *Acorus calamus* under prolonged anoxia

Total soluble carbohydrates (--▲--) and total non-soluble carbohydrates (—◆—)were determined as described in Section 2.6. Each datapoint represents the mean of 6 independent measurements from 5 plants, error bars show standard error of the mean.

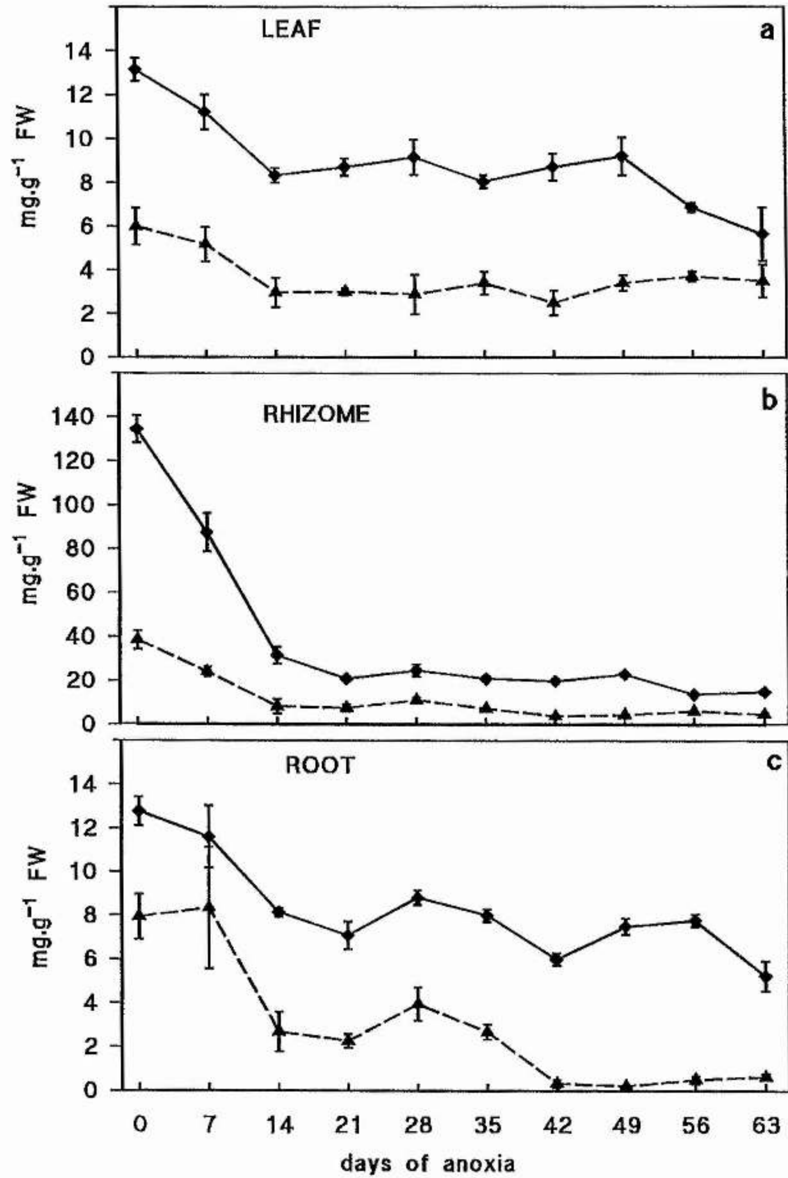


Figure 3.3. Carbohydrate content in leaves (a), rhizomes (b) and roots (c) of *Iris pseudacorus* under prolonged anoxia

Total soluble carbohydrates (--▲--) and total non-soluble carbohydrates (—◆—) were determined as described in Section 2.6. Each datapoint represents the mean of 6 independent measurements from 5 plants, error bars show standard error of the mean.

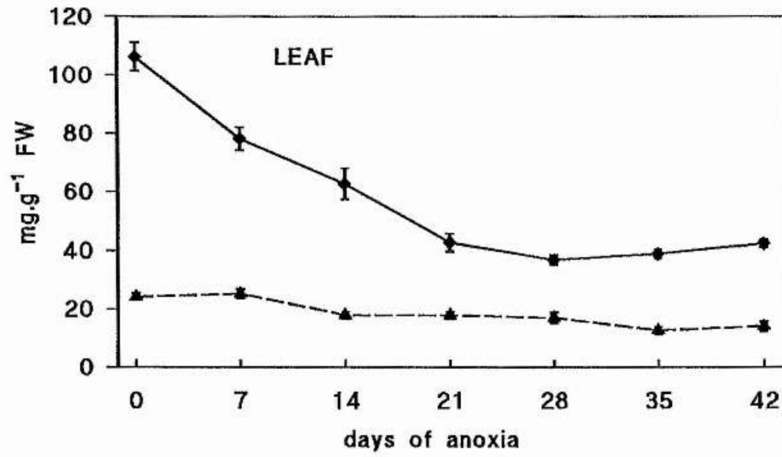


Figure 3.4. Carbohydrate content in leaves of *Vaccinium macrocarpon* under prolonged anoxia
Total soluble carbohydrates (--▲--) and total non-soluble carbohydrates (—◆—) were determined in one-year-old leaves as described in Section 2.6. Each datapoint represents the mean of 8 independent measurements from 5 plants, error bars show standard error of the mean.

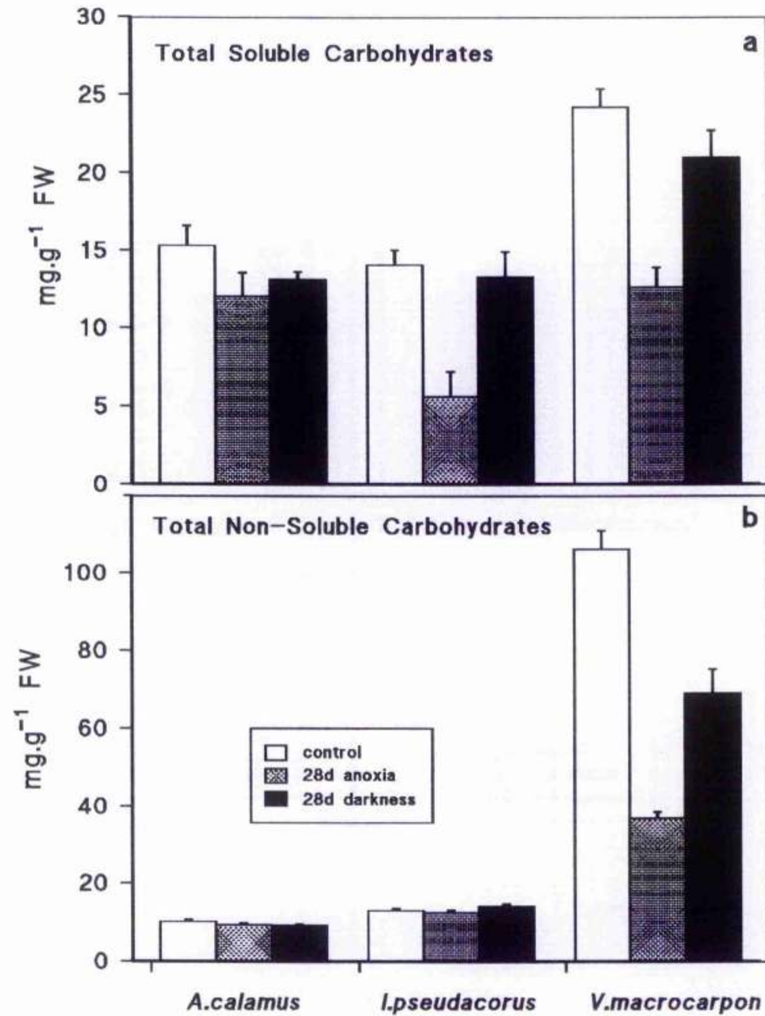


Figure 3.5. Carbohydrate content in leaves of *A. calamus*, *I. pseudacorus* and *V. macrocarpon* under prolonged anoxia and darkness.

Total soluble carbohydrates (a) and total non-soluble carbohydrates (b) were determined as described in Section 2.6 at day zero (control), after 28d of anoxia and after 28d of darkness. Each datapoint represents the mean of at least 6 independent measurements from 3 plants, error bars show standard error of the mean.

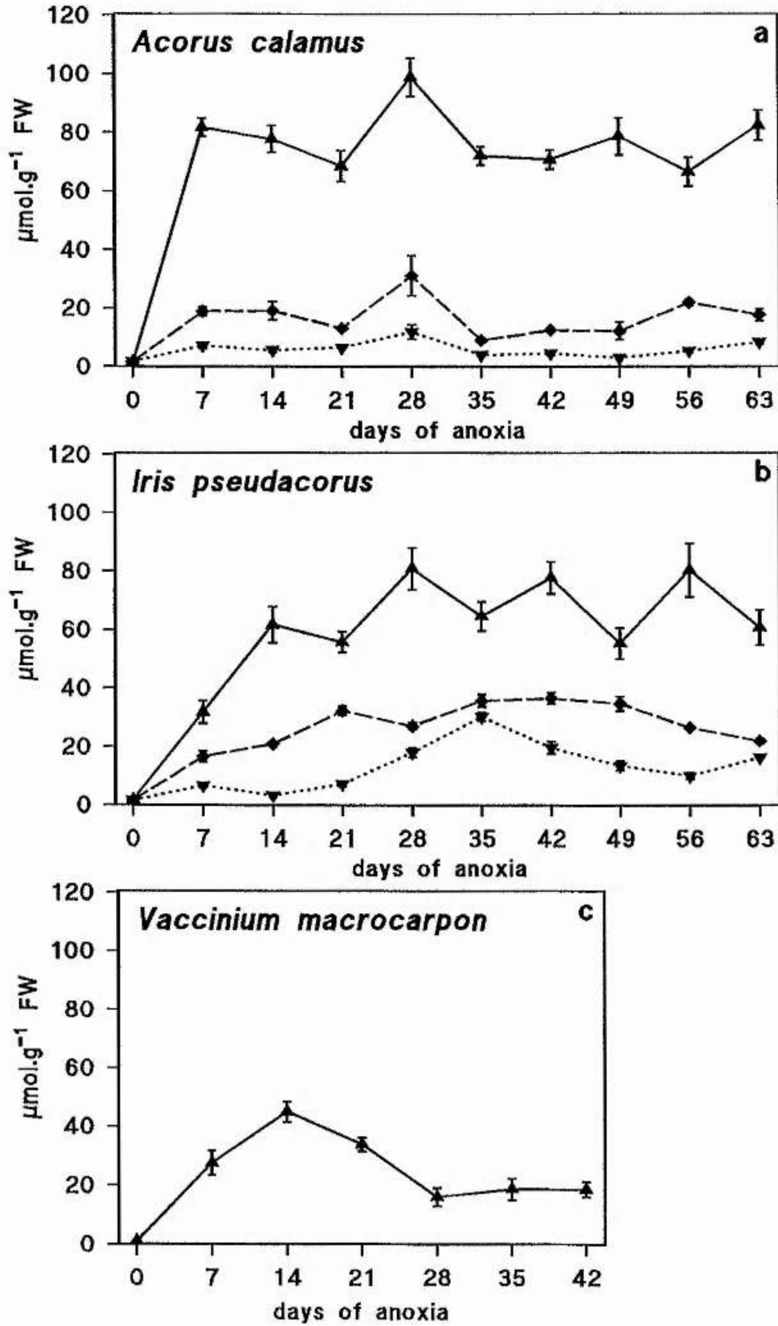


Figure 3.6. Ethanol content of *A. calamus* (a), *I. pseudacorus* (b) and *V. macrocarpon* (c) under prolonged anoxia.

Ethanol content was determined in leaves (—▲—), rhizome (--◆--) and roots (···▼···) as described in Section 2.7. Each datapoint represents the mean of 6 independent measurements from 5 plants, error bars show standard error of the mean.

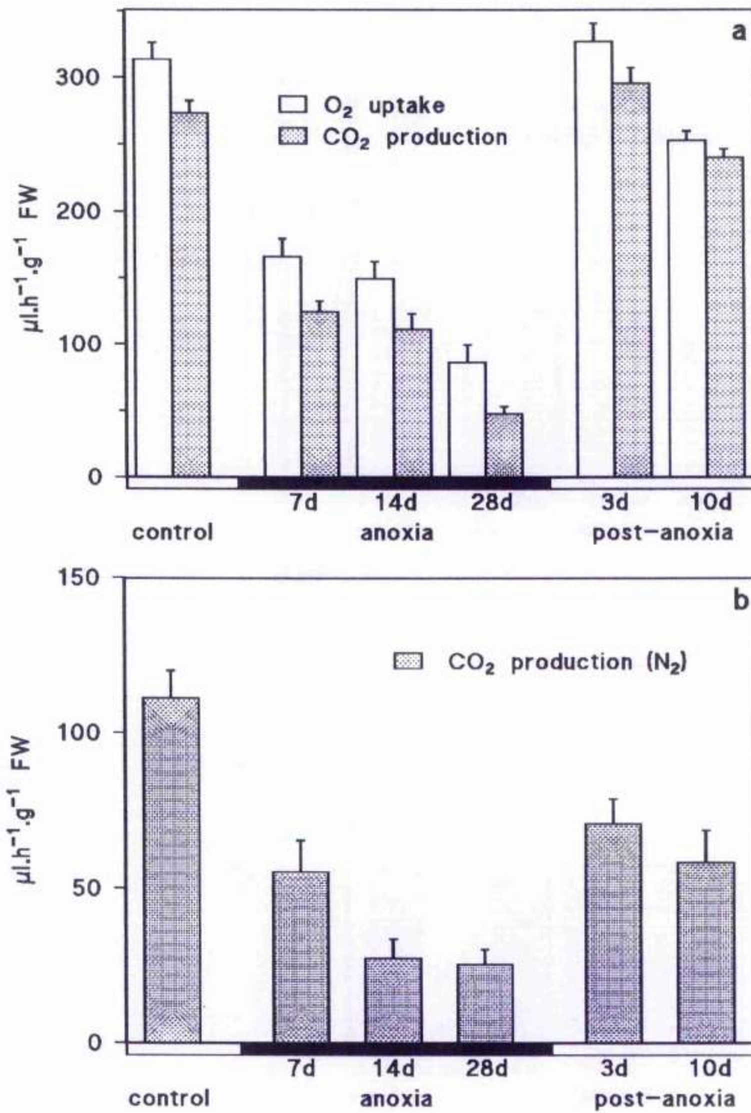


Figure 3.7. Gas exchange of *Acorus calamus* leaves under anoxia and post-anoxia
(a) O_2 uptake and CO_2 production under aerobic conditions; (b) CO_2 production under anaerobic conditions (N_2). Gas exchange of leaf tissue was determined as described in Section 2.8. at day zero (control), after 7, 14 and 28d of anoxia, and after 3 and 10d of post-anoxia (after 28d anoxia). Each datapoint represents the mean of 6 independent measurements from 3 plants, error bars show standard error of the mean.

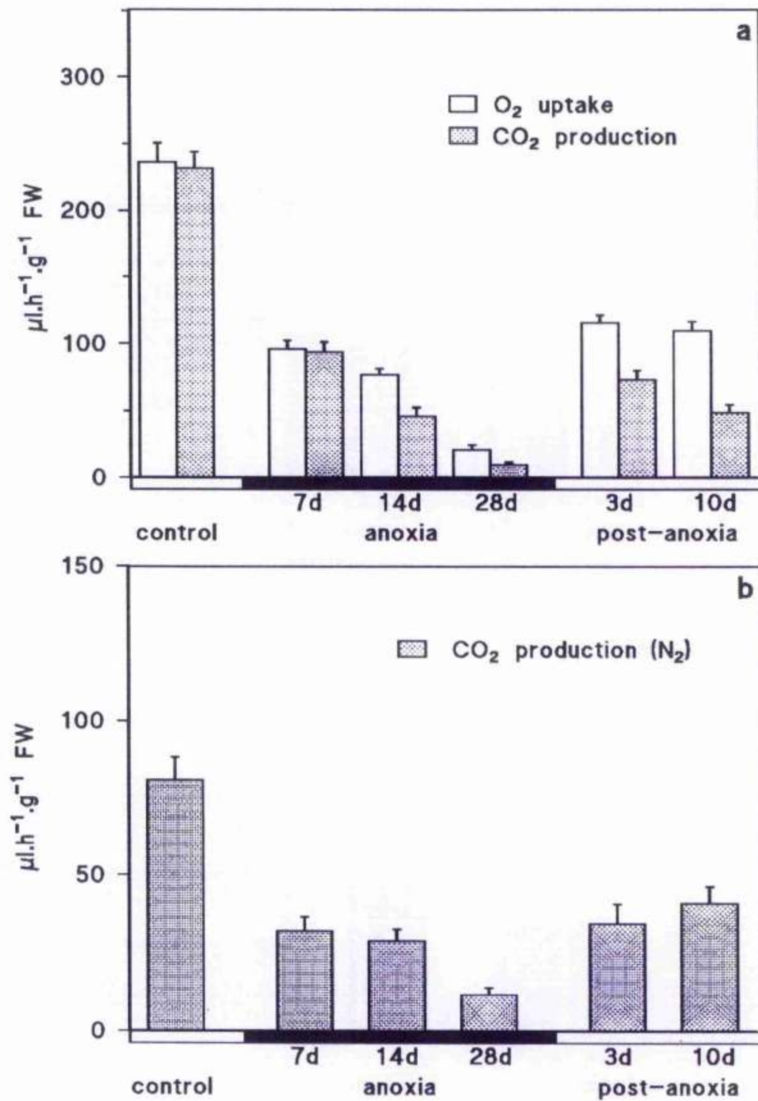


Figure 3.8. Gas exchange of *Iris pseudacorus* leaves under anoxia and post-anoxia
(a) O_2 uptake and CO_2 production under aerobic conditions; (b) CO_2 production under anaerobic conditions (N_2). Gas exchange of leaf tissue was determined as described in Section 2.8. at day zero (control), after 7, 14 and 28d of anoxia, and after 3 and 10d of post-anoxia (after 28d anoxia). Each datapoint represents the mean of 6 independent measurements from 3 plants, error bars show standard error of the mean.

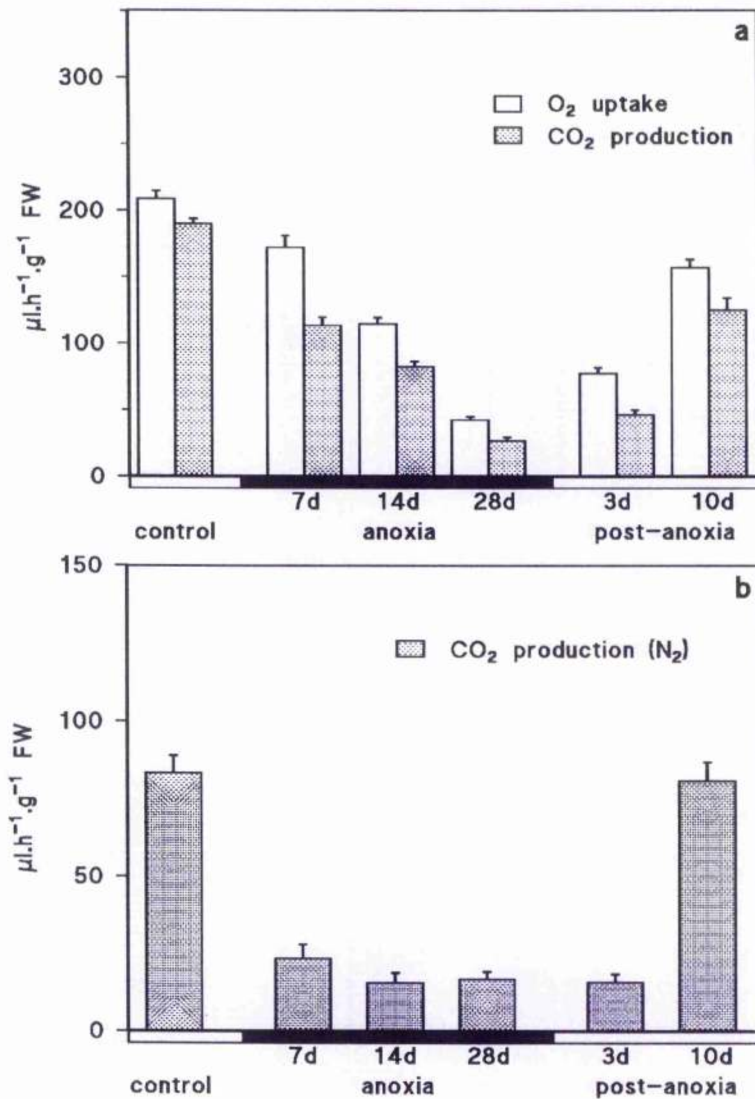
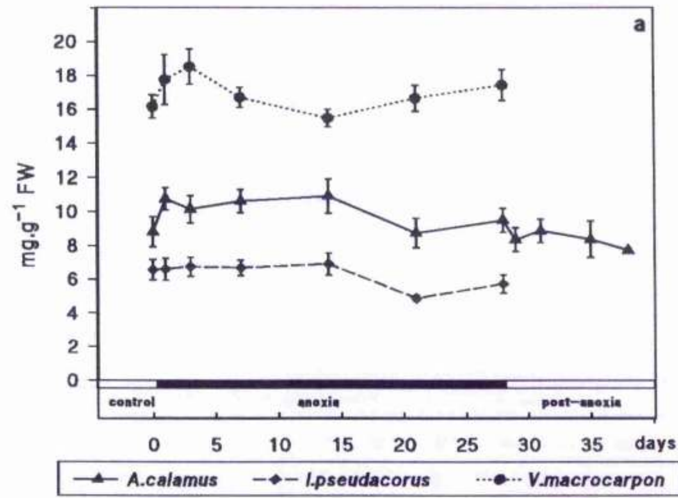


Figure 3.9. Gas exchange of *Vaccinium macrocarpon* leaves under anoxia and post-anoxia
(a) O_2 uptake and CO_2 production under aerobic conditions; (b) CO_2 production under anaerobic conditions (N_2). Gas exchange of leaf tissue was determined as described in Section 2.8. at day zero (control), after 7, 14 and 28d of anoxia, and after 3 and 10d of post-anoxia (after 28d anoxia). Each datapoint represents the mean of 6 independent measurements from 3 plants, error bars show standard error of the mean.



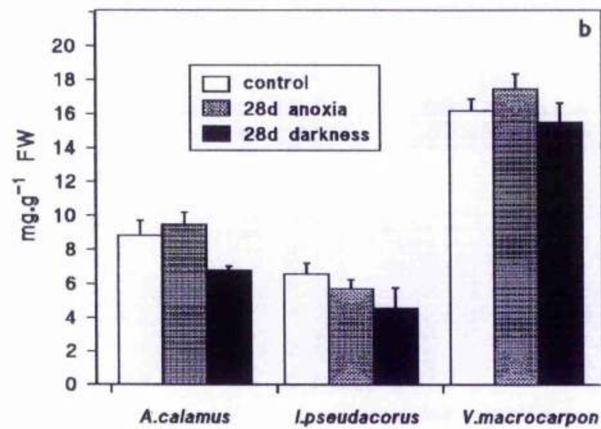


Figure 3.10. Total protein content in leaves of *A. calamus*, *I. pseudacorus* and *V. macrocarpon*
(a) under prolonged anoxia and post-anoxia; (b) at day zero (control), after 28d of anoxia and after 28d of darkness. Total protein content was determined as described in Section 2.9.Each datapoint represents the mean of 10 independent measurements from 4 plants, error bars show standard error of the mean.

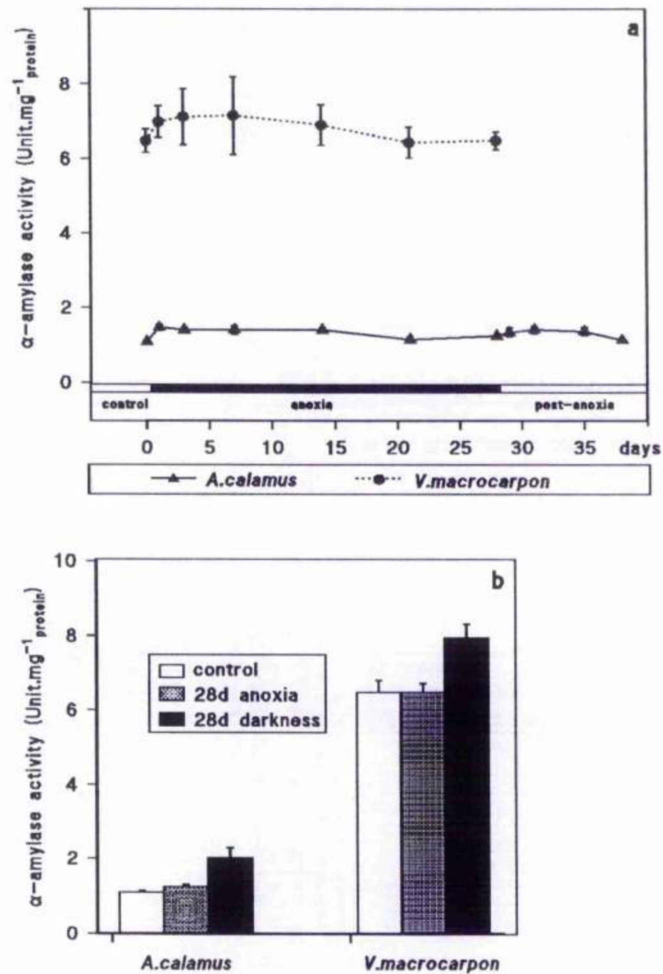


Figure 3.11. **α-amylase activity in leaves of *A. calamus* and *V. macrocarpon***

(a) α-amylase activity under prolonged anoxia and post-anoxia; (b) α-amylase activity at day zero (control), after 28d of anoxia and after 28d of darkness. α-amylase activity was determined as described in Section 2.9.5. [One unit will liberate 1.0mg of maltose from starch in 3min].Each datapoint represents the mean of 10 independent measurements from 4 plants, error bars show standard error of the mean.

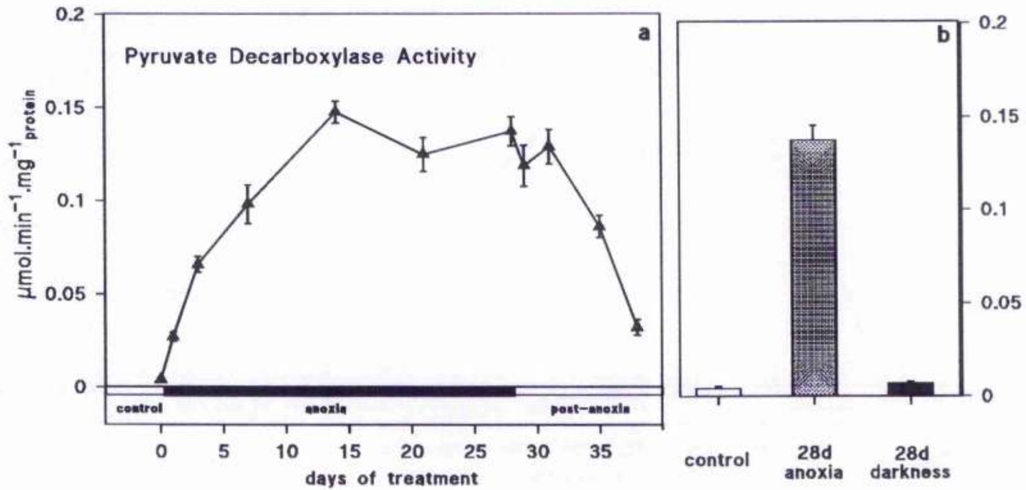


Figure 3.12. Pyruvate decarboxylase (PDC) activity in leaves of *A. calamus*
(a) PDC under prolonged anoxia and post-anoxia; (b) PDC at day zero (control), after 28d of anoxia and after 28d of darkness. PDC activity was determined as described in Section 2.9.6. Each datapoint represents the mean of 6 independent measurements from 3 plants, error bars show standard error of the mean.

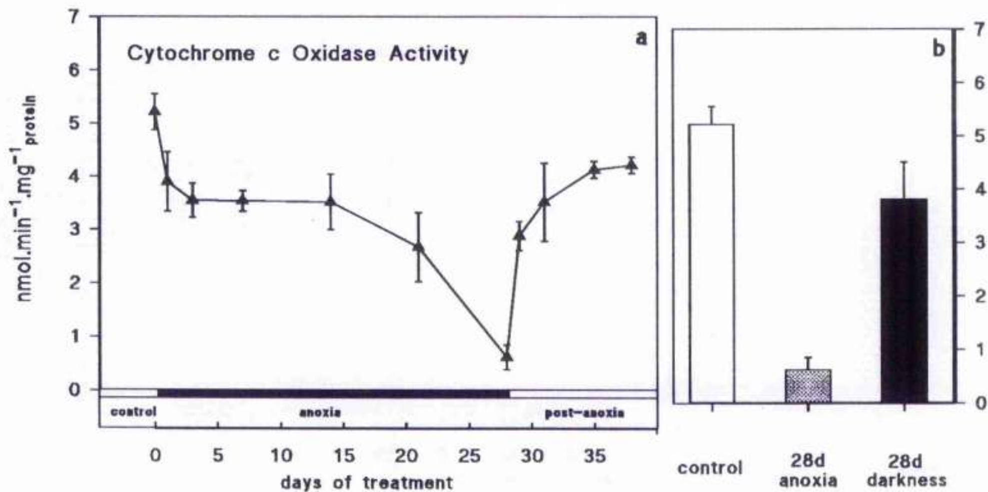


Figure 3.13. Cytochrome c oxidase (COX) activity in leaves of *A. calamus*
(a) COX under prolonged anoxia and post-anoxia; (b) COX at day zero (control), after 28d of anoxia and after 28d of darkness. COX activity was determined as described in Section 2.9.7. Each datapoint represents the mean of 6 independent measurements from 3 plants, error bars show standard error of the mean.

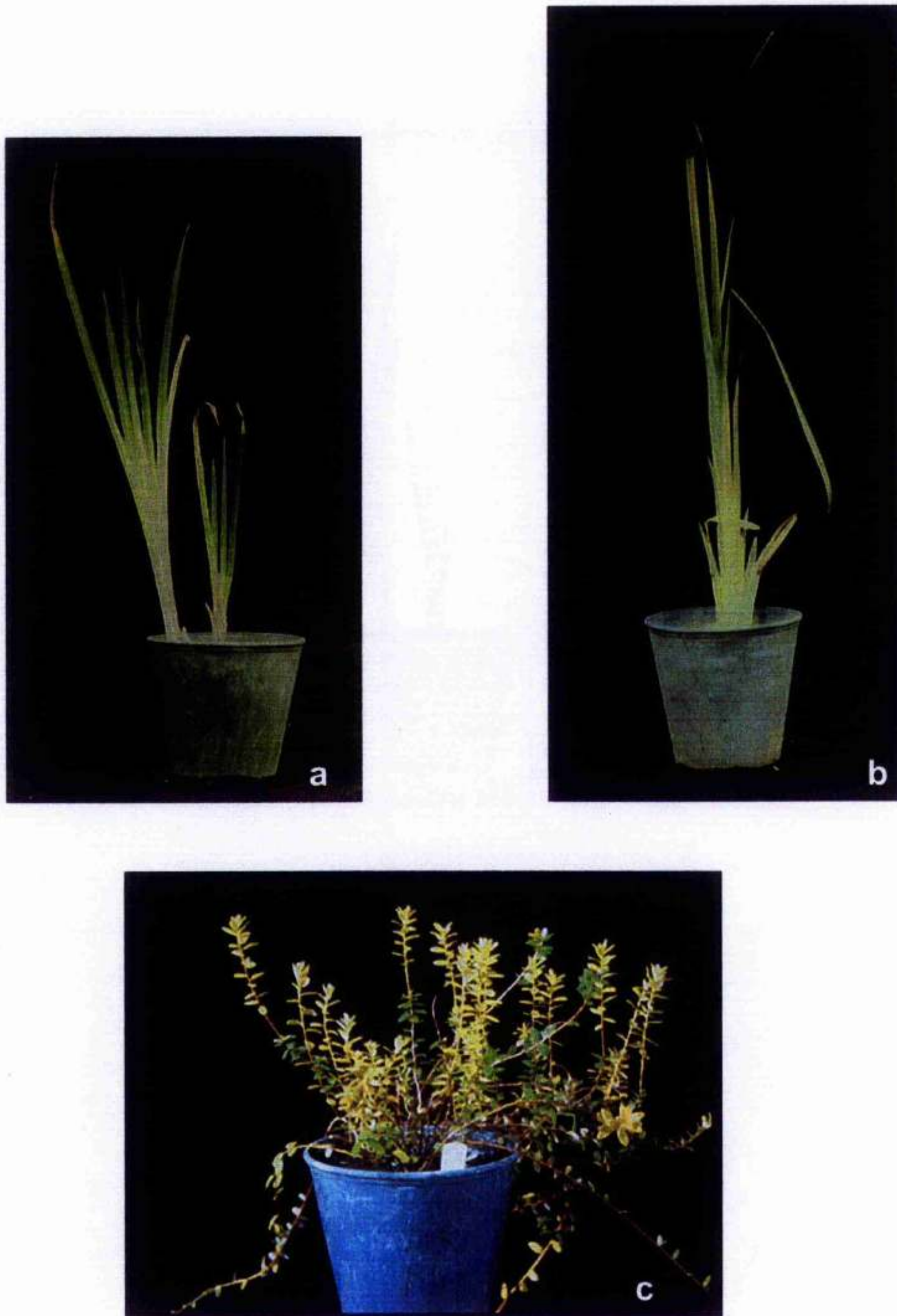


Plate 3.1. ***Acorus calamus*** **(a),** ***Iris pseudacorus*** **(b) and** ***Vaccinium macrocarpon*** **(c) after 28d of anoxia**
Plants were incubated under anoxic conditions as described in Section 2.3.
(Photo *V.macrocarpon*: R.M.M. Crawford)

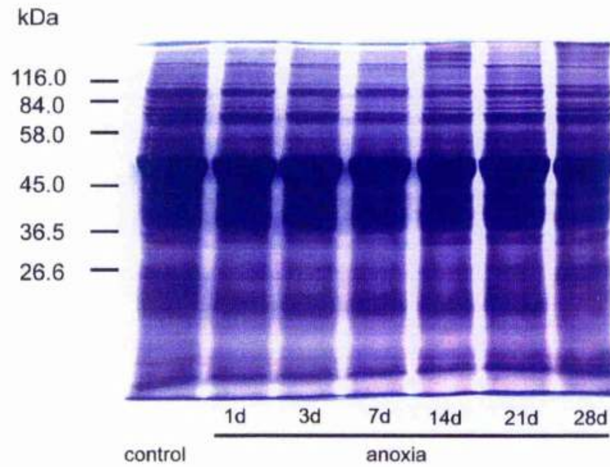


Plate 3.2. Analysis of protein content of *Acorus calamus* leaf by SDS-PAGE

Leaf samples were taken at day zero (control), and after 1d, 3d, 7d, 14d, 21d and 28d of anoxia. Protein extracts were prepared and analysed by SDS-Page as described in Section 2.9.

3.3. Discussion

3.3.1. Species Survival Under Long-term Anoxia

The majority of higher plants die rapidly under strict anoxia. The three investigated species however, *Acorus calamus*, *Iris pseudacorus* and *Vaccinium macrocarpon*, show very high survival rates after a month long period under anoxia, and anoxia tolerance is not restricted to the overwintering organs but includes mature green leaves. Up to now, leaves and roots have been seen as comparatively anoxia sensitive organs (Crawford, 1996) and metabolic studies of long-term anoxia tolerance have concentrated on leafless rhizomes. Long-term survival of mature leaves under anoxia has been described for a number of arctic species by Crawford *et al.* (1994). Seedlings of *Poa alpina* emerged with fully turgid green leaves and resumed growth without any visible sign of injury after 3 weeks of anoxia at 20°C. The only report concerning the metabolism of leaves that survive longer than a week under anoxia without any major damage exists for *A.calamus* (Bucher & Kuhlemeier, 1993).

The described experiments confirm the extreme anoxia tolerance in this species, but they also show that anoxia tolerance of leaves might be a more widespread phenomenon among wetland species than generally predicted. Leaves of *I.pseudacorus* and *V.macrocarpon* are also considerably well adapted to oxygen deprivation. The critical phase during the switch from aerobic to anaerobic processes has obviously been overcome in leaves of all three investigated species, and their continuous survival suggests that all essential metabolic processes can stabilise under anaerobic conditions. Maximal survival times appear in all three species in the overwintering leaves, where growth processes are generally impaired and the metabolic activity is low. Under natural conditions, overwintering leaves are the most likely to suffer oxygen deprivation stress.

In all three species, the green tissue survives in a quasi-dormant state and all growth processes stop after the onset of anaerobic conditions. Although the anoxia treated plants have been additionally kept in the dark, hardly any yellowing occurs. *A.calamus* leaves survive mainly undamaged. In *I.pseudacorus* leaf tips turn brown and soft, indicating partial destruction of the tissue. The unchanged fresh/dry weight ratio in the leaves of all three species also underlines that no dehydration occurrs. The exposure of anoxia intolerant barley seedlings on the other hand is accompanied by leaf wilting and a continuous decrease of the fresh/dry weight ratio. However, barley leaves also show no sign of yellowing under anoxia. Prolonged darkness under aerobic conditions on the contrary, initiates the yellowing of older leaves in the two monocots and the growth of new etiolated leaves. The lack of yellowing under anoxia shows that typical senescence processes such as chlorophyll breakdown and dehydration have been inhibited in the absence of oxygen (Rhodes, 1980). When the leaves were brought back to aerobic conditions their senescence appeared to be accelerated.

Marked differences were observed between the species in the anaerobic response of young and old leaves. In the two rhizomatous species, the younger leaves survive longer than the older ones, but the opposite reaction was found in *V.macrocarpon*, where the youngest leaves are the first to die. This could be explained by the different life cycle of leaves in these species. In all plants, new leaves initially require an input of nutrients for the synthesis of macromolecules, and later the leaves develop from a nutrient sink to a nutrient source as photosynthetic competence is achieved. The life span of the leaf is species specific. Besides leaf age, a number of external and internal factors are responsible for the initiation of leaf senescence (Bleecker & Patterson, 1997). *A.calamus* and *I.pseudacorus* continuously replace their old outer leaves by new ones. Leaf growth in monocots is concentrated in the basal region, where it receives maximal protection by older leaves. The average leaf remains with the plant for only a few months. Although oxygen deprivation inhibits some senescence processes, renewing and repairing mechanisms are impaired in older leaves, resulting in reduced stress tolerance.

Leaves of the dicot *V.macrocarpon* develop much more slowly, cell division clusters occur throughout the tissue. Young leaves are visibly softer and lighter green, and their carbohydrate and nutrient content is significantly lower. The long maturing period is marked by high metabolic activity and stress sensitivity. After the achievement of maturity the metabolism reaches high stability. The dark green leaves are also very tolerant to low temperatures and drought. The investments of the plant into the mature leaves of *V.macrocarpon* are high and under stress the maintenance of the mature leaves is probably less sumptuous than the replacement of immature leaves. The average leaf serves the plant as a carbohydrate source for up to three years, until senescence occurs (Dana & Klingbeil, 1967).

In contrast to the results for anoxia tolerance in leaves, the present study confirms the limited ability of roots to survive without oxygen (Crawford, 1996). The roots of both investigated monocots are already seriously damaged under anoxia, while the rest of the plant still appears intact and healthy. Bucher & Kuhlemeier (1993) also report root death in *A.calamus* after only 96h of anoxia. This shows, therefore, that roots even of generally anoxia tolerant plants are very sensitive to oxygen deprivation. Under natural conditions, the anaerobic root has to face additional stress due to changes in the chemical composition of the soil. Reducing and partly phytotoxic compounds are formed, and their uptake by the root can cause severe damage (see: General Introduction, Section 1.4.2). Weber & Brändle (1996) show that *A.calamus* roots are able to detoxify ammonium and sulphide, but their experiment did not exceed 72h of anoxia. The detoxification process requires additional energy, and under prolonged anaerobic conditions substrate demands for root maintenance might be high.

3.3.2. Catabolism of Carbohydrates in Leaves Under Long-term Anoxia

Survival of long-term anoxia requires the stabilisation of all maintenance processes with minimal energy consumption, and as the plants are also deprived of light, internal reserves are the only source for energy production. In higher plants, carbohydrates are the dominant storage substrate and their consumption has been studied under long-term anoxia.

Acorus calamus

A.calamus stores large amounts of starch in its rhizome. The gradual decline in the carbohydrate content proves that the plant is able to utilise the stored reserves under anoxia. Unlimited accessibility to storage carbohydrates has not been found in all species under anoxia. In *Glyceria maxima*, only the soluble carbohydrate content decreases under anoxia, but hardly any changes occur in the amount of starch (data not shown). In *Iris germanica* rhizomes, the starch breakdown also seems to be inhibited under anoxia and finally sugar starvation limits the metabolic activity (Hanhijärvi & Fagerstedt, 1995). In the *A.calamus* rhizome on the other hand, soluble carbohydrate levels are stable for nine weeks under anoxia.

The leaves of *A.calamus* contain only small amounts of non-soluble carbohydrates. Only immediately after the onset of anoxic conditions does the starch content of the leaf decrease, coincidental with a slight increase in the activity of α-amylase, but amylase activity is generally low in the leaves. This indicates that the leaves of *A.calamus* do not serve as a carbohydrate store, that the rhizome probably provides the bulk of substrate for leaf metabolism and that transport mechanisms are active under prolonged anoxia. A similar situation has been found in rice seedlings, where the substrate demands of the leaves under anoxia are dependent on the translocation of carbohydrates from the endosperm (Vartapetian *et al.*, 1976). In the present study, the carbohydrate pool of the *A.calamus* rhizome reaches a minimum after about 40d of anoxia and this coincides with the start of major leaf destruction. The costs for maintenance of the rhizomes are probably less after the death of the leaf tissue, and the rhizomes possibly continue to survive the anaerobic conditions with a further reduction of the metabolic activity. But in the end, the exhaustion of the carbohydrate reserves limits the viability under anoxia. In anoxia intolerant tissues on the contrary, death occurs in spite of sufficient carbohydrate availability, e.g. in potato tubers (Sieber & Brändle, 1993). However, once the tolerant plant manages to stabilise the anaerobic metabolism, limited substrate availability represents one of the major problems for survival. Therefore, the economic utilisation of carbohydrates is another essential feature for longevity under anoxia. Brändle (1991) could show the direct dependence of rhizome survival on carbohydrate content and daily carbohydrate loss in a number of species under anoxia. The importance of highly efficient fermentation processes under anoxia are also shown by Fox *et al.* (1994) in wetland grasses. In *Echinochloa phyllopogon* seedlings a significantly smaller proportion of seed carbohydrate reserves is required for the accumulation of shoot dry weight than in rice

seedlings, and despite their smaller carbohydrate reserves the *E.phyllopogon* seedlings tolerate the anaerobic conditions in the end longer than rice.

Under anoxia, the bulk of carbohydrates are converted into carbon dioxide and ethanol via fermentation. In *A.calamus,* ethanol accumulates in all plant organs immediately after the start of the anoxia treatment (Fig. 3.6). Leaves contain by far the highest amounts of ethanol, and this could be due to comparatively high ethanol production, but also due to its transport from the rhizomes towards the leaf and from there into the atmosphere. It has been shown that the removal of volatile fermentation products has a positive effect on survival under anoxia (Crawford *et al.*, 1987). Crawford (1992) assumed that under flooding the advantages of aerechymatous tissue lie not only in the improved downward movement of oxygen but also in the facilitated upwards movement of fermentation products. In the present experiment, the ethanol content of all organs stabilised after an initial rise indicating that in the plant an equilibrium between ethanol production and release was achieved. The results differ from the data reported by Joly & Brändle (1995), where the ethanol accumulation in the rhizome continued for 16d under anoxia, but their experiment was carried out on leafless rhizomes, and therefore the ethanol release might be reduced.

Measurements of the ethanol content in the plant organs give no quantitative information concerning the actual fermentation rate in the tissue. The respiratory activity of the leaf tissues has been determined via measurements of gas exchange. When leaf tissue from untreated control plants is exposed to anaerobic conditions, its CO_2 production decreases immediately to 40% of the initial value (Fig. 3.7). This is different to findings of Bertani *et al.* (1980) in rice seedlings, where the anaerobic CO_2 production is close to that in air. The calculation of the Pasteur Quotient[1] gives a value of 3 for rice seedlings, and this indicates that substrate consumption via glycolysis and fermentation accelerates in rice under anoxia (Pasteur effect). In the present experiment, the calculated Pasteur Quotient lies at 1.28. This indicates that no strong Pasteur effect operates in *A.calamus* leaves, and the glycolytic flux increases only slightly at the onset of anaerobic conditions. Nevertheless, the calculation of the PQ assumes that anaerobic CO_2 production occurs purely by ethanol fermentation, and it is very likely that other pathways (oxidative pentose phosphate pathway, TCA cycle) are still active after the onset of anoxia in *A.calamus.* A slight increase in the production of lactate and malate has been measured in the rhizomes of *A.calamus* under anoxia (Joly & Brändle, 1995). Therefore, the PQ values should be viewed with caution.

The only comparative studies concerning the gas exchange of plant tissue under long-term anoxia have been done in rice suspension cultures over a period of 52d (Mohanty *et al.*, 1993). Under prolonged anoxia the CO_2 production in these suspension cultures declines only

[1] Pasteur Quotient (PQ) = 3x CO_2 production (anaerobe)/ CO_2 production (aerobe) (see Section 1.4.2)

slightly, showing continuously high rates of fermentation. In the present experiment, the CO_2 production of the leaf tissue decreases remarkably under long-term anoxia. This suggests that the glycolytic activity slows down considerably in the leaves of *A.calamus* under anoxia. Interestingly, a very similar strategy is found in anoxia tolerant tissue of animals, where the glycolytic flux is remarkably reduced in the absence of oxygen. This phenomenon is called 'reverse Pasteur effect' or 'anaerobic arrest strategy'. Some animal tissues, for instance in brine shrimps embryos, can achieve a nearly ametabolic state (Hochachka, 1986). *A.calamus* probably does not reach such an extreme state, but the results suggest that the metabolic activity is not constant under long-term anoxia, but slows down continuously. The carbohydrate reserves decrease mainly in the first two weeks of anoxia, but the plant obviously continues to survive while drastically reducing the carbohydrate consumption and ATP turnover.

Nevertheless, a certain rate of energy generation is necessary under anoxia for the maintenance of essential cell functions. In the experiments of Joly & Brändle (1995), the total amount of nucleotides decreases by about 30% in *A.calamus* rhizomes during the first days of anoxia treatment, but then remains stable for the rest of the experiment (16d). A similar behaviour is found for the energy charge (AEC), which stabilises under anoxia at about 0.5; and in the experiments of Sieber & Brändle (1991), the AEC in *A.calamus* rhizomes under anoxia is maintained at an even higher level. Altogether, the low fermentation rate enables a stable energy metabolism at a slightly lower, but presumably equilibrated level in *Acorus calamus* under anaerobic conditions.

Energy generation under anoxia depends not only on the availability of the fermentable carbohydrates, but also on the functioning of the enzymes involved. The continued synthesis of nucleic acids, proteins and other macromolecules is essential for the cell's survival. Drastic changes have been described for gene expression and protein synthesis immediately after the transfer from aerobic to anaerobic conditions (see: General Introduction, Section 1.4.1), but only minimal information is available on the effects of long-term anoxia on protein turnover.

Generally, protein synthesis is impaired under anoxia. However, in the present experiment the total protein content of the leaves did not change significantly during the four weeks of anoxia treatment and no visible changes occur in the protein pattern on the SDS-PAGE (Plate 3.2). This can only be achieved by high persistence and/or continued synthesis of a whole range of proteins under long-term anoxia, and the bulk of 'aerobic' proteins is obviously present in the anaerobic cell as well. In a more detailed study of the protein synthesis in *Echinochloa* species under anoxia, it has been found that the synthesis of 'aerobic' protein resumes within 24h after the initial anaerobic response and fine regulatory mechanisms co-ordinate their activity (Mujer *et al.*, 1993). Although fermentation is still the predominant feature of anaerobic metabolism, the oxidative pentose phosphate pathway, TCA cycle and various aspects of the protein and lipid synthesis are also highly active (Kennedy *et al.*, 1991).

Brändle (1991) showed that the majority of labelled proteins in the rhizomes of *A.calamus* are identical in air and anoxia.

In the present experiment, the activity of three different enzymes (PDC, COX and α-amylase) was investigated over 28d of anoxia (see Fig.1.1). The activity of PDC is required for the anaerobic energy generation via ethanol fermentation, but it is usually not involved in metabolism under aerobic conditions. Cytochrome *c* oxidase however is not operating in the absence of oxygen, but its activity is essential for aerobic respiration. The activity of α-amylase is necessary for starch breakdown under aerobic and anaerobic conditions.

Under aerobic conditions, the PDC activity is low in the leaves of *Acorus calamus*. The exposure to anoxia leads to an immediate rise in PDC activity, and a maximum is reached after two weeks. Bucher & Kuhlemeier (1993) found a very similar pattern for the development of ADH activity in *A.calamus* leaves. It shows that the level of anoxia related enzymes is generally increased during oxygen deprivation. However, the activity of the fermentative enzymes does not correspond with the actual measured anaerobic CO_2 production. Although PDC activity was low in air, the CO_2 production of the leaf tissue was high after the transfer to an anaerobic environment. The CO_2 production might be caused by the activity of the existing and rapidly synthesised PDC, but as already suggested above, the contribution of other pathways is very likely. Under prolonged anoxia, the CO_2 production decreases in spite of very high levels of PDC activity. The fact that ADH activity usually exceeds PDC activity, led to the conclusion that PDC activity might play an important role in the regulation of the fermentation rate (Drew *et al.*, 1994). But the described results show that PDC activity is obviously not limiting for fermentation under long-term anoxia, and that the fermentative activity is more likely to be regulated by other glycolytic processes. Very similar results have been described by Zhang & Greenway (1994) in aged beetroot storage tissue.

The transcript levels of *Adh* and *Pdc* have been investigated in the rhizomes and leaves of *Acorus calamus* by Bucher & Kuhlemeier (1993). The transcript levels for both enzymes peaked in the first few days of anoxia and then declined gradually, but were still detectable after 52d of anoxia. When compared with the results presented here for the enzyme activity, this indicates high stability of the mRNAs and the proteins under anoxia.

High enzyme persistence under anoxia has also been detected for the oxygen requiring cytochrome *c* oxidase. The enzyme activity declines slightly at the beginning of the anoxia treatment, but then remained stable for up to three weeks, with a further drop occurring after four weeks of anoxia. A very similar picture was obtained for the aerobic gas exchange of the leaf, suggesting that COX activity is crucial for the leaf's respiratory capacity at re-exposure to air. The anaerobic synthesis of COX has been proven in *Echinochloa* seedlings by Kennedy *et al.* (1987). In *A.calamus* leaves, the synthesis seems to be suppressed during long-term anoxia.

The activity of the α-amylase is stable under anoxia, and this corresponds with the findings of Bucher & Kuhlemeier (1993) for two 'housekeeping' enzymes in leaves and rhizomes of *A.calamus* under long-term anoxia. The very constant protein content of *A.calamus* leaves indicates that a whole range of proteins are very stable or even synthesised under anoxia. Effective regulatory mechanisms for the enhancement or repression of certain proteins seem to be active in anoxia tolerant species, and enable the maintenance of the cell's metabolism under anaerobic conditions.

Iris pseudacorus

The anaerobic response in the two investigated rhizomatous plants is very similar, but some quantitative differences occur, and in the end *I.pseudacorus* fails to survive under anoxia for as long as *A.calamus*.

In contrast to *Acorus calamus*, the carbohydrate stores of *I.pseudacorus* consist of fructans (irisin) instead of starch (Augem, 1928; Hanhijärvi & Fagerstedt, 1994). Fructans have been found in about 15% of the current angiosperm flora (Hendry, 1993). They are oligomers of fructose with a great variability of branching out and degree of polymerisation. The research into the metabolism of a number of grasses and compositae has lately attracted enhanced attention (Nelson & Spollen, 1987; Pollock & Chatterton, 1988), but no detailed information is available about the fructan composition in *I.pseudacorus*. The accumulation of fructans takes place in the vacuole of the cells and increases usually when sucrose supply exceeds demand (Livingston *et al.*, 1994). A specific set of enzymes is necessary for the synthesis and degradation of fructans. While most plants rely on a combination of starch and fructan storage, a strong preference for fructan seems to exist in *I.pseudacorus*, and no starch could be detected in the rhizome by the I_2/KI-test (personal observation). There does not appear to be either a general advantage or disadvantage for fructan storage under long-term anoxia (Hanhijärvi & Fagerstedt, 1994).

In the present experiment, rhizomes of untreated *I.pseudacorus* plants contain about double the amount of soluble and non-soluble carbohydrates of *A.calamus*, but under anoxia the carbohydrate store depletes more rapidly than in *A.calamus*, indicating much less efficient fermentation processes. After only 14d under anoxia, the total carbohydrate content is lower in *I.pseudacorus* than in *A.calamus*. This shows that despite large stores, carbohydrate limitation occurs already after a shorter period under anoxia and might be responsible for the shorter survival times of the plant. As in *A.calamus*, the leaves contain only small carbohydrate reserves, and even a slight decrease takes place after the start of the anoxia treatment. The rhizome probably provides the bulk of substrate for leaf metabolism. The results described here for *I.pseudacorus* differ from the data presented by Hanhijärvi & Fagerstedt (1994; 1995) for the same species. Under aerobic control condition, the rhizomes contain similar amounts of carbohydrates in both experiments, while under anoxia the non-soluble carbohydrates are broken down rapidly. A simultaneous decline in soluble carbohydrates occured in the present

experiment, but Hanhijärvi & Fagerstedt (1994) observed a sugar increase. The only difference between the present study and those of Hanhijärvi & Fagerstedt (1994) was that they used leafless rhizomes for their experiment, and it is possible that in the present experiment, the soluble carbohydrates were preferably transported into the shoot.

Hanhijärvi & Fagerstedt (1994; 1995) also measured the AEC in the *I.pseudacorus* rhizomes over 60d of anoxia. Under anoxia, the AEC declined slowly for about 30d and then fell to near zero after 35d. The large drop in the AEC was judged as the time of tissue death, but unfortunately the recovering capacity of the rhizome in air was not checked. Nevertheless, in the recent experiments *Iris pseudacorus* rhizomes survived up to 65d under anoxia. As with the results from *A.calamus*, severe leaf injury occurred soon after the depletion of carbohydrate stores in the rhizome.

Under anaerobic conditions, *I.pseudacorus* accumulates ethanol in all organs. According to Monk *et al.* (1984), lactate production is negligible in this species under anoxia. As in *A.calamus* highest amounts of ethanol have been found in the leaves, supporting the theory that a large proportion of fermentative end products is released via the shoot (Crawford, 1992).

The gas exchange rate of the untreated *I.pseudacorus* leaves was slightly lower than in *A.calamus*. A comparison of the aerobically and anaerobically produced CO_2 would suggest that no acceleration occurs in the carbohydrate consumption via glycolysis under anoxia. This contrasts with the fast carbohydrate loss found in the plant. A possible explanation would be the increased production of certain organic acids under anoxia. An accumulation of shikimate and quinic acid in response to anoxia has been reported for *I.pseudacorus* by Davies (1980). The formation of these acids is associated with the pentose phosphate pathway and indicates its continued operation under anaerobic conditions. The accumulation of several metabolic end-products under anoxia has been described for animals (Hochachka, 1986) as well as plants (Crawford, 1982). Malate, oxalate, glycerol, lactate, succinate, γ-aminobutyrate, alanine and shikimate have been discussed in this context (see also: General Introduction, Section 1.4.2). McManmon & Crawford (1971) suggested that their production under anoxia could be advantageous for the plant, because it prevents the accumulation of other potentially toxic end products of fermentation. Nevertheless, the formation of these anaerobic end-products is not necessarily coupled with any ATP production and would not contribute to the energy generation in the cell (Davies, 1980). For the assessment of the efficiency of the anaerobic metabolism, more detailed studies about the carbohydrate utilisation are necessary.

Vaccinium macrocarpon

The habit of the dicot *V.macrocarpon* is very different from the two species discussed above. The plant consists of lignified stem tissue and small evergreen leaves. It does not possess any special storage organ comparable to the rhizome in *Acorus calamus* and *Iris pseudacorus*, and consequently, the carbohydrate distribution of the plant is different. Carbohydrate contribution

of below-ground tissue is of only minor importance for acropetal growth (Hagidimitriou & Roper, 1994). Leaves of untreated *Vaccinium macrocarpon* contain much higher amounts of carbohydrates than in *A.calamus* and *I.pseudacorus*, comparable to the concentrations in the investigated rhizomes. The main storage carbohydrate is starch. Roper & Klueh (1996) showed that one-year-old leaves store most of the assimilated carbohydrates in the tissue and that only a small proportion is translocated for new growth above.

Under anoxia, the accumulated carbohydrates are metabolised and the amounts of soluble and non-soluble carbohydrates decrease slowly. The activity of the α-amylase maintains stable in the *V.macrocarpon* leaves under anoxia (Fig. 3.11). The importance of α-amylase for the supply of fermentable substrates under anoxia has been shown by Perata *et al.* (1992). Enhanced α-amylase activities were made responsible for the mobilisation of carbohydrates which are needed to support internode elongation during submergence of deep-water rice (Raskin & Kende, 1984). However, increased α-amylase activity could be maladaptive under long-term anoxia, because it may facilitate the rapid depletion of carbohydrate stores. In comparison with *A.calamus* and *I.pseudacorus*, the carbohydrate content of *V.macrocarpon* leaves is still high after 42d of anoxia.

V.macrocarpon leaves generate energy under anoxia by ethanol fermentation. Ethanol accumulates in the leaves during the first two weeks of the treatment, but after that its content starts to decrease until it reaches an equilibrium on a lower level. After the initial decrease, the anaerobic CO_2 production is stable. Therefore, the ethanol decrease is probably due to facilitated release of the fermentative end-product rather than its reduced production. However, as in *A.calamus* the low anaerobic CO_2 production suggests reduced glycolytic flux in the anaerobic leaves of *Vaccinium macrocarpon*.

3.3.3. Recovery of Carbohydrate Metabolism Under Post-anoxia

When the plants return to air and light after 28d of anoxia, *A.calamus* definitely possesses the best recovery capacity. The younger leaves start to elongate almost immediately under post-anoxia. However, growth processes still seem to be impaired in the first week of post-anoxia in *I.pseudacorus*. Re-growth in *V.macrocarpon* is also delayed, but a direct comparison is problematic because of the differences in growth pattern between the species. It shows that all three species are able to grow new leaves despite the serious depletion of carbohydrate reserves.

The resumption of growth in *A.calamus* is probably closely related to the rapidly regained capacity for aerobic respiration. After 7d under anoxia, the leaves, particularly in *V.macrocarpon*, still maintain a considerably high potential for electron transport, but under prolonged anoxia, the leaf capacity for aerobic O_2 uptake and CO_2 production declines continuously. Only in *A.calamus* did the aerobic gas exchange rate return to the control level

after 3d post-anoxia. The immediate recovery of COX activity in the leaves confirms the fast re-installation of energy production via oxidative phosphorylation in this species. A fast recovery of mitochondrical enzymes has also been reported for other anoxia-tolerant species (Kennedy *et al.*, 1991). In anaerobically grown rice seedlings the COX activity reaches 70% of the value from aerobically grown ones (Öpik, 1973). In *V.macrocarpon* leaves the aerobic gas exchange increases more slowly under post-anoxia than in *A.calamus*, but since the leaves usually remain with the plant, it is very likely that respiration resumes full capacity. Among the investigated species, *I.pseudacorus* shows the poorest recovery of aerobic gas exchange, the oxygen consumption of the leaf is only 50% of the control value after 10d post-anoxia, the CO_2 production is even lower (about 20%). One reason for the discrepancy between O_2 uptake and CO_2 production could be the oxidation of accumulated metabolic intermediates. A part of the O_2 consumption under post-anoxia results probably from non-respiratory oxidation processes. However, leaves of all three species seem to have the potential to generate energy via oxidative phosphorylation after return to air. Fermentative processes probably stop soon under aerobic conditions, and in *A.calamus* PDC activity is greatly reduced after 10d post-anoxia.

The re-exposure to oxygen and light has not only positive effects for the plant, but also initiates the formation of potentially dangerous reactive oxygen species (ROS) (see Section 1.5). Without immediate detoxification, these species can cause irreparable damage by attacking macromolecules such as lipids or proteins and so impair metabolic recovery. The occurrence of oxidative stress has been proven in a number of papers (VanToai & Bolles, 1991; Albrecht & Wiedenroth, 1994a; Crawford, 1996). After periods under anoxia, the anti-oxidative defence mechanisms are often weakened. In *Glyceria maxima* for instance, the SOD activity decreases under anoxia and fails to be initiated during post-anoxia (Monk *et al.*, 1987). Other plants such as rice are able to activate their defence mechanisms rapidly on return to air (Ushimaru *et al.*, 1992). Rhizomes of *I.pseudacorus* on the contrary, are able to increase their SOD activity under prolonged anoxia, and thus are well equipped to counteract the potential hazards of superoxide generation (Monk *et al.*, 1987).

The above described investigations in the literature are all carried out on roots or rhizomes. But in the present experiment, leaves were faced with a sudden re-exposure of oxygen and light, and because the formation of many reactive oxygen species is associated with light reactions in the chloroplast (Elstner, 1987; Elstner & Osswald, 1994), the impact of oxidative stress would be even stronger in the leaf tissue. The fact that a high proportion of *A.calamus* and *V.macrocarpon* leaves show no visible damage in the first days after return to air, underlines their facility for adaptation to fluctuating oxygen pressures. In *I.pseudacorus,* the older leaves especially wilt and yellow more rapidly on return to air, but it is difficult to assess how much injury occurred under anoxia and how much occurred as a result of re-exposure to air. It would be interesting to see if the anaerobic initiation of detoxifying enzymes as described for rhizomes by Monk *et al.* (1987) works in the leaves in a similar way. In

A.calamus, leaf wilting appears after return to air more slowly than in *I.pseudacorus*, but in comparison with untreated control plants from the greenhouse, senescence progresses more rapidly, and the plant seems to rely more on the growth of new leaves instead of the repair of the old ones. Only in *V.macrocarpon*, do the slow grown leaves show no signs of accelerated ageing after they survived the anoxia treatment.

3.3.4. Comparison of the Effects of Anoxia and Darkness in Leaves

In the absence of light, plants have to satisfy their substrate demands completely by the mobilisation of internal resources; but under aerobic conditions the substrates can be utilised many times more efficiently than under anoxia. Comparative studies with sugar beet seedlings shows that the energy status (AEC) is stable in the dark for 12d, but drops immediately under anoxia and remains low (Elamarani *et al.*, 1994). The metabolic activity in the dark is not restricted to maintenance processes, and in all three species biosynthetic processes and leaf growth continue. Without the availability of large carbohydrate stores, prolonged darkness can cause severe damage through carbohydrate starvation, even under aerobic conditions. In maize leaves, the sugar level decreases after 48h in the dark down to 10%, and the cells start to metabolise proteins and lipids (Brouquisse *et al.*, 1998). However, in the leaves of *A.calamus* and *I.pseudacorus* only minor reductions occur in the soluble and non-soluble carbohydrate content of the darkened leaves, probably because, as discussed above, the leaves are likely to receive the bulk of their respiratory substrate from the stores in the rhizome. Leaves of *V.macrocarpon* on the other hand utilise internal carbohydrate reserves, and soluble as well as non-soluble carbohydrates decrease under prolonged darkness. As long as carbohydrates are available, the respiratory activity of the leaf is not affected in the dark (Brouquisse *et al.*, 1998). However, under the much more extreme conditions of anoxia the stores deplete about twice as fast.

Accelerated senescence processes in darkened leaves have been described for many species (Rhodes, 1980; Gan & Amasino, 1997). The initiation of several senescence-associated genes (SAG) and proceeding senescence-related processes are usually much more pronounced in older leaves under prolonged darkness (Weaver *et al.*, 1998). In the present experiments, the older leaves of *A.calamus* and *I.pseudacorus* wilted and yellowed rapidly in the dark, but at least 2-3 leaves maintained their green colour and showed no visible damage. In *V.macrocarpon* etiolation was limited to the young dark grown leaves, while the one-year-old leaves showed no visible sign of accelerated senescence.

The protein content of all three species in this study is slightly decreased in the dark, but not under anoxia. Since the leaves did not seem to suffer carbohydrate starvation, this could be due to the dark related initiation of proteolysis. An increase in the proteolytic activity and also an enhanced synthesis of proteolytic enzymes are common features of leaf senescence under prolonged darkness (Rhodes, 1980). It has been shown that activated degradative

processes are mainly responsible for protein loss during dark induced senescence (Lamattina *et al.*, 1985). The stable protein content of leaves under anoxia could be explained by a restriction in the synthesis of proteolytic enzymes. However, the measurements of the total protein content give no information concerning the actual availability of the enzymes in the cells, and many of them are possibly inactive under prolonged anoxia.

Another catabolytic enzyme, α-amylase is not significantly changed under anoxia, while under extended darkness a slight but significantly enhanced activity has been observed. The test used for α-amylase is based on the assumption that only endoamylases are able to initiate the cleavage of starch polymers and that the soluble oligosaccharides released in the reaction provide the substrate for the other enzymes of starch break down. This view is based on several reports which claim that α-amylase is the only enzyme capable of degrading native starch, and on the fact that starch phosphorylase has a much higher affinity for linear low molecular weight oligosaccharides than for branched polyglucans. Recently the validity of this view has been questioned and there is some evidence that starch phosphorylase is also able to attack starch granules (for review see Kruger, 1997). It is therefore possible that the plants could metabolise a part of their starch under anoxia via starch phosphorylase. In the rhizomes of the bulrush (*Schoenoplectus lacustris*), another anoxia tolerant wetland plant, starch mobilisation is paralleled by increases in the activity of both starch degrading enzymes. But compared to α-amylase, the activity of starch phosphorylase was about 10 times higher (Steinmamm & Brändle, 1984). The mobilisation by phosphorylase could be of advantage for the energy balance because it yields an additional ATP in glycolysis (3 instead of 2) (Brändle & Crawford, 1999). Only little is known about the regulation of starch breakdown in the leaves. In pea leaves, prolonged darkness also results in a marked increase of α-amylase activity, but a high proportion of the increase is due to enhanced activity of the apoplastic α-amylase and not directly related to the starch break down in the cells (Saeed & Duke, 1990). Nevertheless, it is likely that the amylolytic activity is influenced by the requirements of respiratory substrate, and soluble carbohydrate shortage might be responsible for the observed increase in the α-amylase under prolonged darkness. The absence of increased amylolytic activity under anoxia results possibly from the general impairment of protein synthesis.

Some anaerobic proteins such as ADH can be induced by environmental stresses other than anoxia, for instance during dehydration, low temperatures or wounding (Dolferus *et al.*, 1997), but the dark treatment in the present experiment did not initiate any changes in PDC activity.

In summary, leaves of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* survive prolonged anoxia with comparatively low energy requirements, and glycolysis and ethanol fermentation seem to perform on a moderate level under anoxia. *A.calamus* leaves appear to utilise their

carbohydrate reserves particularly efficiently. Carbohydrate consumption in *V.macrocarpon* leaves shows however, that substrate demands are doubled under prolonged anoxia when compared to plants under prolonged darkness. The leaf capacity for oxygen depending processes such as respiration is also considerably affected by anoxia, but on return to air leaves show partial (*I.pseudacorus*) or even complete recovery (*A.calamus*, *V.macrocarpon*) of respiratory activity.

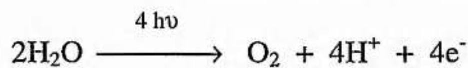
Chapter 4

Effects of Long-term Anoxia on the Photosynthetic Apparatus of Leaves

4.1. Introduction

4.1.1. Photosynthesis and Oxygen

Oxygen is released during the light reaction of photosynthesis. In the photosynthetic apparatus, antenna pigments absorb light, and the excitation energy is transferred to the reaction centres of the two photosystems (Fig. 4.1). In the reaction centre of PSII, the phototrap pigment P680 is promoted to a higher excited electronic state (first excited singlet state; Fig. 4.2), and one electron is transferred to phaeophytin a. From there, the electron is transferred to the primary quinone type acceptor Q_A. After the charge separation a manganese containing enzyme complex at the donor side of PSII catalyses the splitting of water into protons, electrons and oxygen (Hill-Reaction).



$$2H_2O \xrightarrow{4\,h\upsilon} O_2 + 4H^+ + 4e^-$$

This reaction donates the electrons that will be passed through a number of electron transport carriers and finally reduce $NADP^+$ to NADPH in the non-cyclic (linear) photophosphorylation (Fig. 4.1). Oxygen and protons are released into the intrathylakoid space. The proton accumulation in the intrathylakoid space forced by water splitting and plastoquinone oxidation, generates a transmembrane gradient in pH. Membrane bound ATPases equilibrate the proton gradient ('chemiosmotic theory', Mitchell-Hypothesis) and form ATP as another product of the light reaction of photosynthesis. The 'assimilatory power' of NADPH and ATP is utilised in the chloroplast for the fixation and reduction of CO_2. However, oxygen is not only a by-product of photosynthesis, it also has direct influence on the photosynthetic processes via photorespiration and the Mehler reaction (for review see Vacha, 1995).

Photorespiration describes the light dependent O_2 uptake and CO_2 production. The reaction is catalysed by the bifunctional ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) and involves coordinated activity in three cellular organelles, the chloroplast, the peroxisome and the mitochondrion, as well as the transport of the compounds through the cytoplasm between these organelles. The carboxylase activity of Rubisco permits the incorporation of atmospheric CO_2 into organic material and plays a crucial role for carbon assimilation in plants. The reaction represents the initial step of the Calvin cycle that provides triose phosphate molecules for further biosynthetic reactions, predominantly starch synthesis in the chloroplast. The oxygenase activity catalyses the oxygenation of ribulose-1,5-biphosphate during photorespiration. CO_2 and O_2 compete for the active site of Rubisco, and the reactions are controlled by the partial pressure of the two gasses in the atmosphere. Therefore, photosynthesis in C3 leaves is stimulated by 40% to 60% when the oxygen concentration is decreased from 21% to 2%, and photosynthesis is inhibited to a similar amount when oxygen concentration is increased from 21% to 50% (Canvin &

Salon, 1997). High temperatures also favour the oxygenase activity of Rubisco. In the process of photorespiration, ATP and NADPH are consumed and therefore it can serve as an alternative electron transport system. The pathway becomes particularly important under conditions with limiting CO_2 concentrations, e.g. during water deficiency when the stomata are closed. In such situations the capacity of photochemical energy dissipation is reduced and the resulting high energy state leads to damage in the photochemical system. This is called photoinhibition (Powel *et al.*, 1984). The draining off of electrons from the overreduced electron transport chain via utilisation of ATP and NADPH during photorespiration, possibly offers some protection against photoinhibition.

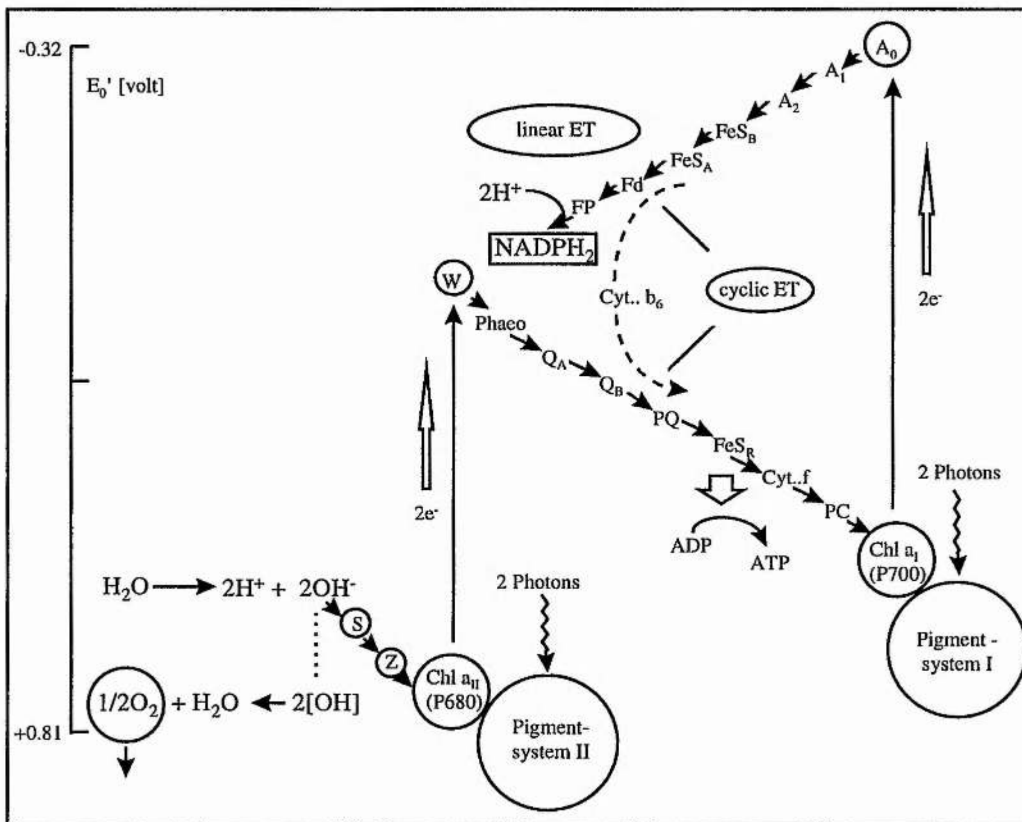


Figure 4.1. Linear and cyclic electron transport during photosynthesis in green plants (from Sitte *et al.*, 1997)
Abbreviations: P680-reaction centre of photosystemII, phaeo - pheophytin, PQ - plastoquinone pool, FeS_R-Rieske protein, cyt.f-cytochrome f, PC-plastocyanin, P700-reaction centre of photosystem I, Fd-ferredoxin, FP-ferredoxin-$NADP^+$-reductase, cyt.b_6-cytochrome b_6, ET-electron transport.

Another alternative manner of electron transport which involves oxygen is the 'pseudocyclic' electron transport or Mehler reaction. The reaction is characterised by the reduction of oxygen on the acceptor site of PSI, and operates probably when the natural electron acceptor $NADP^+$ is limited in supply. It has been proposed that the Mehler reaction is partly responsible for the non-photochemical fluorescence quenching and

zeaxanthin formation when CO_2 assimilation is limited (Neubauer & Yamamoto, 1992). The reaction provides ATP and initially forms the superoxide radical anion. The superoxide radical anion [$O_2 \cdot$] is usually rapidly converted into H_2O_2 by SOD, but in the presence of metal ions the highly reactive hydroxyl radical [HO·] could be formed (Fenton-Chemistry). These molecules with reduced oxygen may have destructive effects on biological systems. The extent of destruction by the oxygen radicals depends mainly on the efficiency of the protective scavenging mechanisms. Usually H_2O_2 reacts with ascorbate via ascorbate peroxidase to form monodehydroascorbate. In turn, monodehydroascorbate may be either re-reduced with NADPH or metabolised further to dehydroascorbate and re-reduced by glutathione followed by NADPH, completing a cycle for dissipation of both oxygen radicals and energy (see also General Introduction, Section 1.5). Another reactive oxygen species is singlet oxygen which is formed by the energy transfer from chlorophyll in the excited triplet state to the oxygen molecule in ground triplet state. The reaction takes place mainly in the antennae but also in the reaction centres of PSII. Singlet oxygen is very toxic due to its extreme reactivity. It can attack photosynthetic pigments and cause damage by pigment bleaching. Carotenoids and α-tocopherol can protect against the action of singlet oxygen molecules by trapping off their excited energy and returning the oxygen into the ground triplet state. The carotenoids then dissipate their energy as heat.

4.1.2. Photosynthesis Under Oxygen Deprivation

Photosynthesis is generally sensitive to environmental changes. Stomata closure for instance is an important adaptation to limited water supply, but it also restricts the CO_2 supply to the chloroplast and subsequently the photochemical reaction. Furthermore, environmental stress can alter the functioning of photosynthesis at the chloroplast level. Reduction of Calvin cycle and impairment of primary photochemistry associated with PSII have been reported under drought (Lu & Zhang, 1998), chilling (Kao *et al.*, 1997), heat (Yamane *et al.*, 1997), UV-B (Brandle *et al.*, 1977) and salt stress (Delfine *et al.*, 1999).

During flooding, the green aboveground organs are often not directly exposed to oxygen deprivation stress. Nevertheless, the photosynthetic activity in the leaves of flooded plants can be affected by alterations in the hormone constituents and the feedback from reduced sink activity (see General Introduction, Section 1.2). However, under total submergence illuminated plants usually continue to photosynthesise. Carbon assimilation and oxygen evolution, even at very low levels can considerably improve the energy status of the plant. The survival of rice cultivars during submergence is seriously affected by reduced irradiance in muddy water and the settling of silt on the leaves (Palada & Vergana, 1972).

The low oxygen concentrations in the surrounding solution favour the carboxylase activity of Rubisco and reduce photorespiration. But the stagnant water simultaneously impairs the diffusion of CO_2 and its concentration becomes crucial for carbon assimilation

in the submerged plant. Some aquatic plants appear to have adapted a strategy for taking up HCO_3^- (Smith & Walker, 1980), but submerged rice leaves could only utilise CO_2 (Setter *et al.*, 1989a). At low CO_2 concentrations in the surrounding solution, the submerged rice contained very low carbohydrate contents and little or no growth occurred. When gassed with 20 kPa CO_2 in air however, the submerged plants showed at most small decreases in carbohydrates and growth was up to 100% of the non-submerged plants. Under prolonged illumination of submerged plants, oxygen accumulates in the plant tissue due to the impaired release into the environment. The photosynthetic activity of submerged rice ceased when oxygen concentrations reached only 0.125 $mol.m^{-3}$ (Setter *et al.*, 1989a). The decrease was attributed to increased photorespiration. However, the accumulation of photosynthetically produced oxygen allows energy generation via respiration and improves the energy balance of the plant.

Under the extreme conditions of ice-encasement, survival is also increased when the plants are illuminated at low levels and there is evidence that this promotion of survival is a result of oxygen production by photosynthesis (Andrews, 1996). The survival of ice-encased cranberry plants is considerably reduced, when the ice-blanket is covered by an additional layer of snow, which interrupts illumination of the evergreen vines (Eck, 1990).

As discussed before, low oxygen concentrations can improve carbon fixation by the inhibition of photorespiration, but photorespiration may also provide a protective function. Under the complete absence of oxygen and high illumination, the plants suffer severe injurye from photoinhibition. Photoinhibition describes the reduction of photosynthetic capacity by excessive light (for review see: Long *et al.*, 1994). The damaging affects of illumination can be intensified by other stress factors like drought, nutrient deficiency or extreme temperatures. The part of the photochemical apparatus which is most susceptible to photoinhibition is PSII. Precisely which part of PSII is damaged, is still a matter of discussion, but there is no doubt that one component of the reaction centre undergoes more or less continuous replacement in the light and that inhibition of its synthesis can exaggerate photoinhibitory damage. The component which turns over rapidly is a 32-kDa protein (D1 protein) which is located in the reaction centre of PSII and binds the secondary electron acceptor Q_B. At high photoinhibitory light intensities, the degradation of the D1 proteins is faster than the resynthesis. Under strong illumination the over-reduction of Q_A leads to an inhibition of the electron transport, followed by the triggering of the D1 protein degradation. The presence of oxygen might be directly involved in photoinhibition, since oxygen-derived species such as singlet oxygen or oxygen free radicals are thought to be responsible for the degradation of the D1 protein (Jegerschöld & Styring, 1992). Under anaerobic conditions, the depletion of D1 was reduced (Gong *et al.*, 1993). According to studies of Barenyi & Krause (1985) with isolated thylakoid membranes, reaction systems that scavenge reactive products of O_2 significantly diminish but not totally prevent photoinhibition of electron transport. Damaged reaction centres are still capable of trapping

excitation energy and quenching of Q_A, but the energy is preferably dissipated as heat (Walker, 1990).

There are conflicting reports regarding the role of oxygen in photoinhibition. At low levels of oxygen corresponding to 1-2% oxygen in the atmosphere, the photoinhibitory effect was not different from that in air-saturated medium. But under anoxic conditions, a considerable enhancement of photoinhibition, measured as a decline in net carbon assimilation, occurs in spinach leaves (Krause *et al.*, 1985). High degrees of photoinhibition under anoxia have also been described by other authors (Jegerschöld & Styring, 1992; Gong *et al.*, 1993). The damage was found to constitute inactivation at multiple sites including reaction centre of PSII to Q_A, the Q_B-site and a component of PSI. It has been suggested that two mechanisms of photoinhibition exist, one which is suppressed and one which is promoted by oxygen (Krause *et al.*, 1985; Gong *et al.*, 1993). Under anoxia, the destructive action of oxygen radicals is prevented. But at the same time, the protective role of oxygen ceases and damage results from the over-reducing conditions and the depletion of bicarbonate. Differences in the mechanisms of photoinhibition in air and anoxia are also represented by fluorescence levels. The anaerobic conditions gave a strong increase in Fo. Much lower enhancement of Fo relative to loss of Fv is caused by aerobic photoinhibitory treatment (Satoh, 1971).

Krause *et al.* (1985) also reports strong inhibition of photosynthetic activity after exposure to anaerobic conditions in the dark. The damage appears to be of a different nature as indicated by different recovery kinetics. The present experiments concentrate on the effects of anoxia on the metabolism of leaves. The plants have therefore been exposed to anoxia in the dark. This prevents the release of any oxygen into the system via photosynthesis as well as the damage by high light intensities to the stressed leaves. The recovery of photosynthetic activity in the leaves after anoxia stress, was measured under aerobic conditions.

4.1.3. Chlorophyll Fluorescence

In the photosynthetic apparatus, light is absorbed by the antenna pigments and alters the electronic configuration of the pigments. The excitation energy is transferred to the reaction centres of the two photosystems by resonance transfer. Each quantum of red light which is absorbed by a chlorophyll molecule raises an electron from the ground state to an excited state (first excited singlet state). Absorption of blue light causes even greater excitation (upper or second excited singlet state) but the elevated electron then falls back into the lower singlet state by radiationless de-excitation as heat (Fig. 4.2). Thus, whatever the quality of light absorbed, the electron reaches the same energy level more or less immediately after excitation and all subsequent events derive from this common starting point. The energy released when the excited electrons return to the ground state can be

harnessed at the reaction centres to cause charge separation and hence to drive the electron transport which finally leads to the photochemical generation of ATP and NADPH.

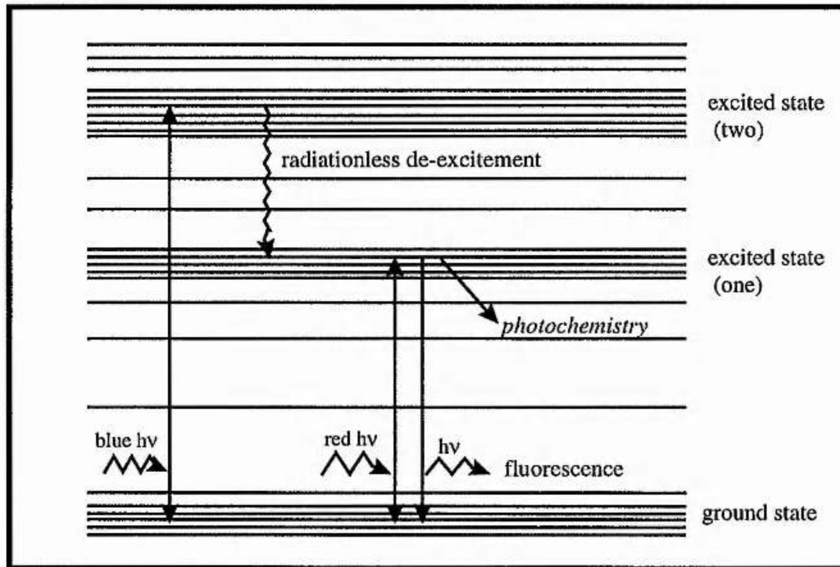


Figure 4.2. The excitement of chlorophyll by light (from: Walker, 1990)
The parallel lines represent sub-states or electronical orbitals. Energy delivered by the absorption of blue light raises an electron to the upper excited state ('excited state two') from where it rapidly returns to 'excited state one' by radiationless de-excitement. A photon of red light raises the electron only to 'excited state one', but this state is sufficiently stable to permit useful chemical work and represents the starting point of photosynthesis. 'Excited state one' can dissipate energy also by re-emitting light as (deep red) fluorescence.

In low light under optimal conditions, the primary photochemistry occurs with high efficiency and can account for more than 90% of absorbed light quanta (Björkman & Deming, 1987). A proportion of excited pigments that can not be 'usefully' employed is deactivated by the emission of chlorophyll fluorescence or infra-red radiation (heat). At room temperature, most fluorescence is emitted by Chl *a* of PSII, and exhibits a peak at 682nm and a broad shoulder at about 740nm (Krause & Weis, 1984).

Analysis of chlorophyll *a* fluorescence provides a powerful means of studying the functioning of the photosynthetic system (Krause & Weis, 1984; Walker, 1990; Krause & Weis, 1991; Jones, 1992). During recent years, remarkable progress in the interpretation of fluorescence signals and the continuously improving measurement techniques have made fluorescence analysis an important tool in basic and applied plant physiology. The bulk of studies have been carried out on isolated thylakoids and isolated chloroplasts, while the full understanding of complex signals emanating from intact organisms is still problematic. However, basic interpretations can - with care - be applied to the more complex situation in intact leaves (Krause & Weis, 1991).

The rate of fluorescence emission (F) is proportional to the absorbed light flux (I), and to the quotient of the rate constant of fluorescence (k_F) over the sum of rate constants (Σk_i)

of all competing reactions that result in a return of the chlorophyll molecule to the ground state (Krause & Weis, 1991). The most important of these are the photochemical reaction, thermal deactivation and excitation energy transfer to non-fluorescent pigments, e.g. to antennae of PSI.

$$F = I * k_F/\Sigma k_i \qquad \text{(Krause \& Weis, 1991)}$$

If the rate constant of photosynthesis decreases, for instance by the influence of external factors such as CO_2 availability or temperature, more energy will be dissipated by the competing reactions and an increase in chlorophyll fluorescence appears. Fluorescence analysis usually consists of two components, the fluorescence parameters associated with the dark-adapted tissue, and those made on tissue which is actively engaged in photosynthesis under ambient light.

Dark Adapted Measurements

The absence of photochemistry in the dark-adapted leaf allows the complete re-oxidation of PSII electron acceptor molecules, and all reaction centres are 'open'. In this state the probability that absorbed light can be used for photochemistry is maximal, while the fluorescence yield is minimal. The full oxidation of the primary electron acceptor, Q_A, is also achieved by pre-illumination with a far-red beam, that activates PSI. The ground level of fluorescence, Fo ('origin', 'minimal' or 'initial' fluorescence), is determined by illumination of the dark adapted leaf with an extremely weak measuring beam (modulated light). The Fo level of fluorescence is thought to represent an emission from the antennae Chl *a* molecules before the excitations have migrated to the reaction centres and thus the quantum yield of Fo is supposed to be independent of photochemical events.

Upon the illumination with a saturating pulse, Q_A will become fully reduced and all reaction centres are 'closed'. Under these conditions, the excitation of PSII cannot result in a stable charge separation and the maximal fluorescence yield, Fm is obtained. The difference between Fo and Fm represents the variable fluorescence, Fv. The origin of variable fluorescence emission is still controversial, but it is assumed to arise from a back-transfer of excitation energy from the closed reaction centres to the antennae (see: Krause & Weis, 1991). The ratio Fv/Fm gives the potential (or maximal) yield of the photochemical reaction of PSII or maximal efficiency of PSII (Krause & Weis, 1991). It has been established that the ratio Fv/Fm is very stable in the non-stressed leaves of many species and ecotypes and normally takes up values close to 0.80-0.83 (Björkman & Demming, 1987). Environmental stresses which affect PSII efficiency lead to a decrease in Fv/Fm.

Light Adapted Measurements

The illumination of a dark-adapted leaf with moderate light leads to an immediate rise in the fluorescence level ('Kautsky-effect'). The short-time fluorescence induction kinetics

follow a characteristic curve with distinctive phases (labelled OID) until a peak (P) is reached. The changes from O to P are completed in only one or two seconds. The decline from the peak (P) takes place more slowly (sec to min). The steady state fluorescence level under continuous illumination might be reached after a secondary maximum (M) (see Walker, 1990; Fig. 2.1).

The fluorescence decrease under continuous light is characterised by two quenching mechanisms. The first derives from the photochemical energy conversion at PSII reaction centres. Such photochemical quenching depends on the presence of Q_A in the oxidised or 'open' state. When Q_A is oxidised, it can except an electron before passing it on towards PSI, ferrodoxin, NADP and CO_2 (Fig. 4.1). When Q_A is reduced ('closed'), because it has been proffered more electrons than it can handle, or because there is no CO_2 to which electrons can be passed, an increasing amount of electrons fall back to ground level dissipating their energy as fluorescence. The coefficient for photochemical quenching (q_P) denotes the proportion of 'open' or oxidised reaction centres. The re-oxidation of Q_A^- causes the quenching. The value of q_P at the time *t* can be obtained by measuring of the maximal fluorescence by provision of a saturating pulse and thus momentarily removing the photochemical quenching (for calculation see Tab. 2.1; Fig. 2.1). According to Genty *et al.* (1989), the product of q_P and the photochemical efficiency of PSII in the light (Fv'/Fm') gives the quantum yield of non-cyclic electron transport through PSII (Φ_{PSII}) or quantum efficiency of PSII. The magnitude of this parameter has been shown to be closely related to the measurements by gas exchange (Genty *et al.*, 1989).

Apart from photochemistry, non-photochemical processes are also responsible for the fluorescence decline. A major proportion of non-photochemical quenching is supposed to originate in the LHC antennae. Three major mechanisms have been described as causes for the non-photochemical quenching, (i) energy dependent quenching, (ii) state transition quenching and (iii) 'photoinhibitory' quenching (Krause & Weis, 1991).

Energy dependent quenching depends on the energisation state of the thylakoids or pH gradient across the thylakoid membrane. The molecular mechanisms behind this are still not clear. It has been suggested that intrathylakoid acidification causes ultrastructural changes and initiates the energy dissipation as heat, thus lowering the amount of fluorescence. It is assumed to act synergistically with the xanthophyll cycle, i.e. the reversible conversion of violaxanthin via antheaxanthin to zeaxanthin upon illumination, because the presence of zeaxanthin may act as a quenching amplifier (Noctor *et al.*, 1991).

State transition quenching is related to the phosphorylation state of the LHC protein. It is supposed that the phosphorylation causes a detachment of the LHC from the core antennae of PSII, and as a consequence, some of the excitation energy from PSII is transferred to PSI. The third non-photochemical quenching mechanism is associated with the photoinhibitory effects of excessive light as described in Section 4.2. While the first

two quenching mechanisms are characterised by a rapid relaxation in the dark, recovery from photoinhibition occurs much more slowly.

The non-photochemical quenching coefficient (q_{NP}) compares the maximal fluorescence at the time *t* with the maximal fluorescence in the dark adapted leaf (for calculation see Section 2.12.2; Fig. 2.5). It has been demonstrated that enhanced non-photochemical quenching reduces the rate of photochemistry. The increased dissipation of excitation energy by non-radiative processes in the pigment matrices of PSII consequently results in a decreased delivery of excitation energy for PSII photochemistry (Genty *et al.*, 1990). In some situations, especially in stressed plants with strong energisation of the thylakoid membranes, it is also possible for Fo to be quenched (Bilger & Schreiber, 1989).

4.1.4. Aim of the Chapter

The previous chapter showed that leaves of some anoxia-tolerant species are able to survive prolonged periods without oxygen. However, anoxia severely affects central parts of cell metabolism such as energy generation, carbohydrate availability and biosynthetic activity. Therefore, prolonged anoxia influences not only those processes that are directly dependent on the availability of oxygen; but also alters the metabolism of all cell compartments. Because most research on anoxia has concerned non-photosynthetically active tissue, little is known about the effects of anoxia on chloroplasts. In this chapter, central features of the photosynthetic apparatus such as chlorophyll content, functionality of PSII and Rubisco content are investigated in leaves under anoxia.

Prolonged anoxia decreases carbohydrate availability, and after long periods without oxygen even anoxia-tolerant species can be damaged by carbohydrate starvation. The rapid onset of carbon assimilation on return to light could therefore secure plant survival. The recovery of photosynthesis on return to air and light is examined in the leaves after four weeks of anoxia.

The exposure to anaerobic conditions took place in the dark. Prolonged darkness, even under aerobic conditions, inhibits photosynthesis and considerably affects chloroplast metabolism. The effects of anoxia plus darkness are compared with the effects of darkness under aerobic conditions.

4.2. Results

4.2.1. Viability of Leaves

The effects of anoxia on the viability of whole plants of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* have been described in Chapter 3, Section 3.2.1, and show that a high proportion of leaf material survived under anoxia for up to four weeks. Anoxia related

damage occurred in *A.calamus* and *I.pseudacorus* in the older leaves and in the leaf tips of younger leaves; in *V.macrocarpon* damage occurred mainly in the younger leaves. Irreversible damage to the leaf tissue was visible by the colour change from fresh green to brownish green and loss of turgor. On return to air, the damaged tissue became dry and lost all green colour rapidly. In some leaves, particularly the older ones, visible damage appeared only on return to air. Leaf tissue that survived anoxia without any visible damage wilted under post-anoxia and turned yellow. This behaviour was most pronounced in *I.pseudacorus*. In *V.macrocarpon* the post-anoxia damage affected only a very small proportion of leaves. To investigate the recovery of photosynthetic activity after certain periods of anoxia[1], only leaf tissue that showed no visible damage, was used. The same applies for the dark experiments, where only leaf material that kept its green colour, was analysed.

4.2.2. Effects of Anoxia on the Photosynthetic Capacity

The exposure to anaerobic conditions decreased the photosynthetic capacity in the leaves, measured as oxygen evolution; but on return to aerobic conditions all three investigated species possessed the ability to recover (Fig. 4.3a-c). In *A.calamus,* the oxygen evolution declined continuously under anoxia reaching 17% of the initial level after 28days of anoxia. On return to air, recovery took place immediately and within three days of post-anoxia, the oxygen evolution was back to 50% of the control level. After seven days in air, the photosynthetic capacity of *A.calamus* leaves showed no significant differences from the control. *I.pseudacorus* leaves showed the fastest decline in photosynthetic capacity under anoxia, and after 21days of anoxia hardly any oxygen evolution was detectable. In comparison with *A.calamus,* the recovery on return to air was retarded. Within 10d of post-anoxia the oxygen evolution reached 80% of the control level. *V.macrocarpon* leaves showed only low initial levels of oxygen evolution. The main decrease in photosynthetic capacity took place during the first week of anoxia, and after that oxygen evolution was stable at about 20% of the initial level for up to four weeks of anoxia. On return to air, the oxygen evolution of the leaves recovered more slowly than in *A.calamus*. But after 10d of post-anoxia, no significant differences to the control were detected.

The exposure to extended darkness also decreased the photosynthetic capacity of the leaves significantly in all three investigated species, but to a lesser extent than the anoxia treatment. In *A.calamus* and *I.pseudacorus,* the oxygen evolution of leaves after four weeks in the dark was reduced to about 50% of the initial level (Fig. 4.3d, e); in *V.macrocarpon,*

[1] The term 'anoxia' always refers to anoxia treatment in the dark. The return to aerobic condition is always connected with the return to light. The term 'dark treatment' refers to the exposure to darkness under aerobic conditions.

the leaves still possessed 70% of their initial photosynthetic capacity after four weeks in the dark (Fig. 4.3f).

4.2.3. Effects of Anoxia on the Chlorophyll Content

The three investigated species showed slight differences in the effects of anoxia and post-anoxia on the chlorophyll content. The total chlorophyll content of *A.calamus* leaves decreased slightly under anoxia. The decrease was significant only during the first week of anoxia (Fig. 4.4a). After four weeks of anoxia, the chlorophyll content in the leaves was about 75% of the initial level. The reduction in the chlorophyll content was more pronounced for Chl *b* than Chl *a*, resulting in a slight increase of the Chl *a/b* ratio (Fig. 4.4b). On return to air, a further decline in the total chlorophyll content occurred and after 10d of post anoxia the chlorophyll content was down to 60% of the initial level, the post-anoxic decrease was however not statistically significant. The Chl *a/b* ratio in the leaves remained stable under post-anoxic conditions. Extended darkness also initiated a decrease in the total chlorophyll content of *A.calamus* leaves. The chlorophyll thereby reached even lower concentrations than during the anoxia treatment (Fig. 4.4c), and the Chl *a/b* ratio increased significantly from 2.3 in the control to 3.4 after four weeks in the dark (Fig. 4.4d).

In comparison with the other two species, *I.pseudacorus* leaves initially contained the highest amount of chlorophyll, but under anoxia *I.pseudacorus* showed the strongest decrease in total chlorophyll content (Fig. 4.5a). After four weeks of anoxia, total chlorophyll content was only 50% of the initial value. The re-exposure to an aerobic environment caused a further significant reduction in the chlorophyll content of the leaf, and after seven days of post-anoxia the leaves contained only 30% of the chlorophyll in the untreated control leaf. As in *A.calamus* the Chl *a/b* ratio increased slightly under anoxia, but these changes were not significant in *I.pseudacorus* (Fig. 4.5b). Anoxia and dark treatment caused similar reductions in the total chlorophyll content of the leaves (Fig. 4.5c), Chl *a* and Chl *b* seemed to be equally affected by the extended darkness as there were no significant differences in the Chl *a/b* ratio (Fig. 4.5d).

The chlorophyll content of *V.macrocarpon* leaves was hardly influenced by the anoxia treatment (Fig. 4.6a, c). A significant decline in the total chlorophyll content occurred only during the first week of anoxia, and the content remained stable for the rest of the treatment. Post-anoxia had no significant effects on the total chlorophyll content of *V.macrocarpon* leaves. In contrast to *A.calamus* and *I.pseudacorus*, the Chl *a/b* ratio decreased from 3.15 in the untreated plant to 2.5 after four weeks of anoxia (Fig. 4.6b). Prolonged darkness caused a stronger reduction in the total chlorophyll content than the anoxia treatment, but had no influence on the Chl *a/b* ratio (Fig. 4.6c, d).

4.2.4. Effects of Anoxia on the Chlorophyll Fluorescence of PSII

Chlorophyll Fluorescence in the Dark-adapted Leaf

In the untreated control plants the maximal efficiency of PSII (Fv/Fm) was about 0.8 in the leaves of *A.calamus*, *I.pseudacorus* and *V.macrocarpon*. The Fv/Fm value decreased in all three species when the plants were exposed to anoxic conditions (Fig. 4.10a), but the impact of Fo and Fm values to the calculated Fv/Fm varies between the species (Tab. 4.1). Upon re-exposure to aerobic conditions the maximal PSII efficiency recovered, with the fastest recovery observed in *A.calamus*. The anoxia-related decrease of the maximal PSII efficiency in *A.calamus* leaves was mainly caused by the increase in the initial fluorescence level Fo, while the maximal fluorescence Fm decreased slightly (Tab. 4.1; Fig. 4.7). When the plant returned to aerobic conditions the Fo level lowered rapidly, while Fm remained high. This allowed fast recovery of the maximal PSII efficiency, and the Fv/Fm ratio was back to the control level after seven days of post-anoxia (Fig. 4.10a).

In the leaves of *I.pseudacorus,* the anoxia treatment caused an even stronger increase in the Fo level than in *A.calamus* (Tab. 4.1; Fig. 4.8), and the high Fo level was also responsible for the low Fv/Fm ratios in this species. The Fm level decreased only slightly under anoxia. In contrast to *A.calamus,* the recovery of maximal PSII efficiency was impaired on return to air. The initial fluorescence Fo returned to a lower level under post-anoxia, but simultaneously a considerable decrease in Fm took place resulting in generally very low fluorescence in the post-anoxic leaves. The recovery of Fv/Fm was retarded and only started to increase again after seven days in air (Fig. 4.8c). After 10d of post-anoxia, the maximal efficiency of *I.pseudacorus* leaves was fully recovered (Fig. 4.10).

Table 4.1. Minimal and maximal chlorophyll fluorescence of leaves, and Fv/Fm ratios of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* under control condition and after 28d of anoxia.

Species		Fo [rel. Unit]	Fm [rel. Unit]	Fv/Fm
A.calamus	control	226	1334	0.830
	28d anoxia	580	1168	0.495
I.pseudacorus	control	251	1268	0.801
	28d anoxia	722	1073	0.331
V.macrocarpon	control	209	1008	0.791
	28d anoxia	296	507	0.416

In leaves of *V.macrocarpon*, anoxia caused a decrease in the Fv/Fm value which was very similar to the picture in the other two species. But in this species the decrease was mainly due to reduced Fm levels, while increase in the Fo level was less pronounced than in *A.calamus* and *I.pseudacorus* (Tab. 4.1). The recovery of Fv/Fm occurred slowly on return

of the plant to aerobic conditions, but was nearly complete after 10d of post-anoxia (Fig. 4.10a).

A comparison of the effects of prolonged darkness and prolonged anoxia shows that the decrease of the Fv/Fm ratio was caused mainly by anoxic condition, while the dark treatment had only minor influence on the maximal PSII efficiency of the leaves (Fig. 4.10b).

Fluorescence Quenching in the Light-adapted Leaf

Photochemical and non-photochemical quenching was studied in the leaves upon illumination with actinic light of relatively low intensity of 180 PAR to avoid photoinhibition. The photochemical quenching coefficient (q_P) decreased under anoxia in all three investigated species. Upon re-exposure to aerobic conditions, recovery of q_P occurred relatively rapidly in *A.calamus* and *V.macrocarpon* (Fig. 4.11). Due to the very low fluorescence levels on return to air in *I.pseudacorus*, q_P recovery appeared to be more slowly in this species. However, after 10d of post-anoxia no significant differences to the initial level could be detected in all tree species.

In *V.macrocarpon,* the decrease in photochemical quenching appeared mainly in the first weeks of the anoxia treatment and was accompanied by an increase in non-photochemical quenching (Fig. 4.12a). Subsequently, q_P remained stable, while the q_{NP} started to decline again under prolonged anoxia. Initially, the non-photochemical quenching was also enhanced in anoxia stressed *A.calamus* leaves, but after two weeks of anoxia the q_{NP} level dropped. In *I.pseudacorus,* a decrease in non-photochemical quenching was detected right from the start of the anoxia treatment. Re-exposure to air initiated an immediate short increase in the non-photochemical quenching of all three species (Fig. 4.12a). Bilger & Schreiber (1986) revealed that the exposure to light, especially in stressed tissue, is often accompanied by additional quenching of Fo (q_o) leading to a decrease in the level of Fo' when compared to the original Fo in the dark adapted leaf. A slightly enhanced level of q_o has been detected in *A.calamus* and *V.macrocarpon* under anoxia (Fig. 4.7b; 4.8b). In anoxia stressed *I.pseudacorus* leaves, the exposure to actinic light seemed to initiate a very strong decrease in the Fo level, which recovered only very slowly in the dark (Fig. 4.9b).

A decrease in photochemical quenching was also observed when the plants were kept under prolonged darkness (Fig. 4.11b). In *A.calamus,* anoxia and dark treatment resulted in a similar decline of q_P by about 80%. In *I.pseudacorus* and *V.macrocarpon* prolonged darkness caused a smaller reduction in q_P when compared with the anoxia treatment. The amount of non-photochemical quenching in the leaves treated with prolonged darkness differed between the species; in *A.calamus* and *I.pseudacorus* q_{NP} was reduced; in *V.macrocarpon* q_{NP} was slightly enhanced (Fig. 4.12b).

Efficiency of the Photosystem in the Light-adapted Leaf

The quantum efficiency of PSII (Φ_{PSII}) has been calculated according to Genty *et al.* (1989). In all three investigated species, Φ_{PSII} was decreased by the anoxia treatment. Very low levels of Φ_{PSII} were reached after 28d of anoxia in the leaves, and when compared with the control, the quantum efficiency of PSII was only 13%, 11% and 15% in *A.calamus*, *I.pseudacorus* and *V.macrocarpon* respectively (Fig. 4.13). On return to air, Φ_{PSII} increased immediately in *A.calamus*. *V.macrocarpon* showed medium speed of recovery and in *I.pseudacorus* the recovery seemed to be retarded. After 10d of post-anoxia, the quantum efficiency of *A.calamus* and *V.macrocarpon* was not significantly different from the initial value under control conditions. *I.pseudacorus* leaves regained 80% of its initial photosynthetic capacity.

Prolonged darkness also reduced the quantum efficiency of PSII in the leaves. However, in *I.pseudacorus* and *V.macrocarpon,* the Φ_{PSII} was much less affected by prolonged darkness than by prolonged anoxia, and only in *A.calamus* did the decrease in Φ_{PSII} reach nearly the same dimension during dark and anoxia treatment (Fig. 4.13b).

Relaxation of Fv/Fm in the Dark

All non-photochemical quenching mechanisms reduced the Fv/Fm ratio. The application of a saturating light pulse immediately after the switching off of actinic light therefore gave a lower Fv/Fm than in the dark-adapted leaf. The energy dependent quenching disappeared usually within one minute of darkening, while other non-photochemical quenching processes such as photoinhibition decayed much more slowly. The relaxation of Fv/Fm during the dark period following the exposure to actinic light was usually used as a measure of photoinhibition (Jones, 1992). To investigate the sensitivity of leaves towards photoinhibition, the leaves were exposed to actinic light of 400 PAR. The relaxation of Fv/Fm in the dark after the illumination is shown in Table 4.2.

In the untreated control plants, the relaxation of Fv/Fm was nearly complete after 4min of darkness. After the exposure to anoxia, leaves of *A.calamus* and *I.pseudacorus* seemed to be more sensitive to photoinhibition and the relaxation of Fv/Fm was slowed down. The exposure to prolonged darkness on the other hand had no influence on the dark recovery of Fv/Fm in these two species. The opposite picture was found in *V.macrocarpon*, where prolonged darkness but not anoxia caused the slowing down of Fv/Fm relaxation (Tab. 4.2)

Table 4.2. Relaxation of the Fv/Fm ratio in leaves after the illumination with actinic light of 400PAR for 2min; comparison between control plants and plants after 28d of anoxia and 28d of darkness.

Species		control	28d anoxia	28d darkness
A.calamus	dark-adapted leaf (leaf in the dark for at least 30min)	100%	100%	100%
	relaxation in the dark after illumination with actinic light			
	30s relaxation time	97.5%	74.4%	95.0%
	60s relaxation time	96.1%	72.5%	95.2%
	120s relaxation time	97.9%	80.6%	97.6%
	240s relaxation time	98.0%	87.1%	99.0%
I.pseudacorus	dark-adapted leaf (leaf in the dark for at least 30min)	100%	100%	100%
	relaxation in the dark after illumination with actinic light			
	30s relaxation time	93.8%	64.0%	82.5%
	60s relaxation time	95.6%	71.4%	88.9%
	120s relaxation time	97.1%	80.5%	94.5%
	240s relaxation time	98.1%	87.8%	97.7%
V.macrocarpon	dark-adapted leaf (leaf in the dark for at least 30min)	100%	100%	100%
	relaxation in the dark after illumination with actinic light			
	30s relaxation time	94.0%	92.7%	75.3%
	60s relaxation time	95.7%	92.7%	72.5%
	120s relaxation time	97.2%	94.6%	78.7%
	240s relaxation time	98.8%	97.4%	84.0%

4.2.5. Effects of Anoxia on the Rubisco Content of *A.calamus* Leaves

The Rubisco content of leaves was estimated using an antibody specific for the large subunit of the enzyme (RbcL). Six independent protein extracts were prepared and analysed for each sampling day. The immunoblotting produced a strong single band on the membrane. The molecular weight of RbcL has been estimated to be 54.5kDa.

Under anoxia, the RbcL content of leaves did not undergo very strong changes. On only four out of the six developed blots, the RbcL content tended to decline slightly (Plate 4.1). However, the response did not seem to be significant, because no changes were detectable on the other two blots. No notable changes were found for the RbcL content in the leaves under post-anoxia. Prolonged darkness had also no influence on the RbcL content of *A.calamus* leaves.

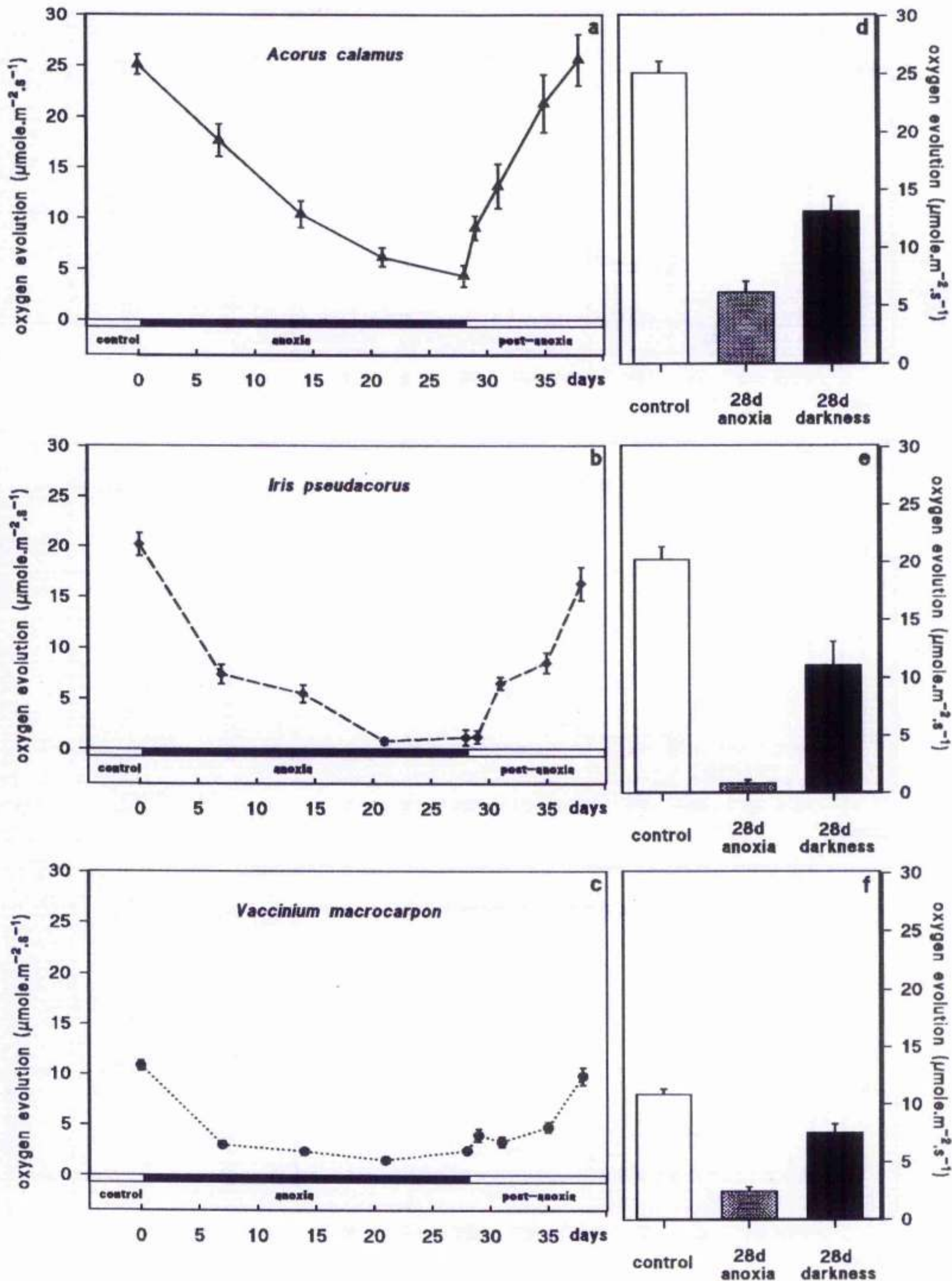


Figure 4.3. Photosynthetic capacity of leaves of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* under anoxia, post-anoxia and dark treatment

Oxygen evolution was determined at day zero (control), after 7d, 14d, 21d and 28d of anoxia; and after 1d, 3d, 7d and 10d of post-anoxia in *A.calamus* (a), *I.pseudacorus* (b) and *V.macrocarpon* (c). Oxygen evolution was compared in leaves at day zero (control), after 28d of anoxia and after 28d of darkness in *A.calamus* (d), *I.pseudacorus* (e) and *V.macrocarpon* (f). For method description see Section 2.10. Each datapoint represents the mean of 6 independent measurements, error bars show standard error of the mean.

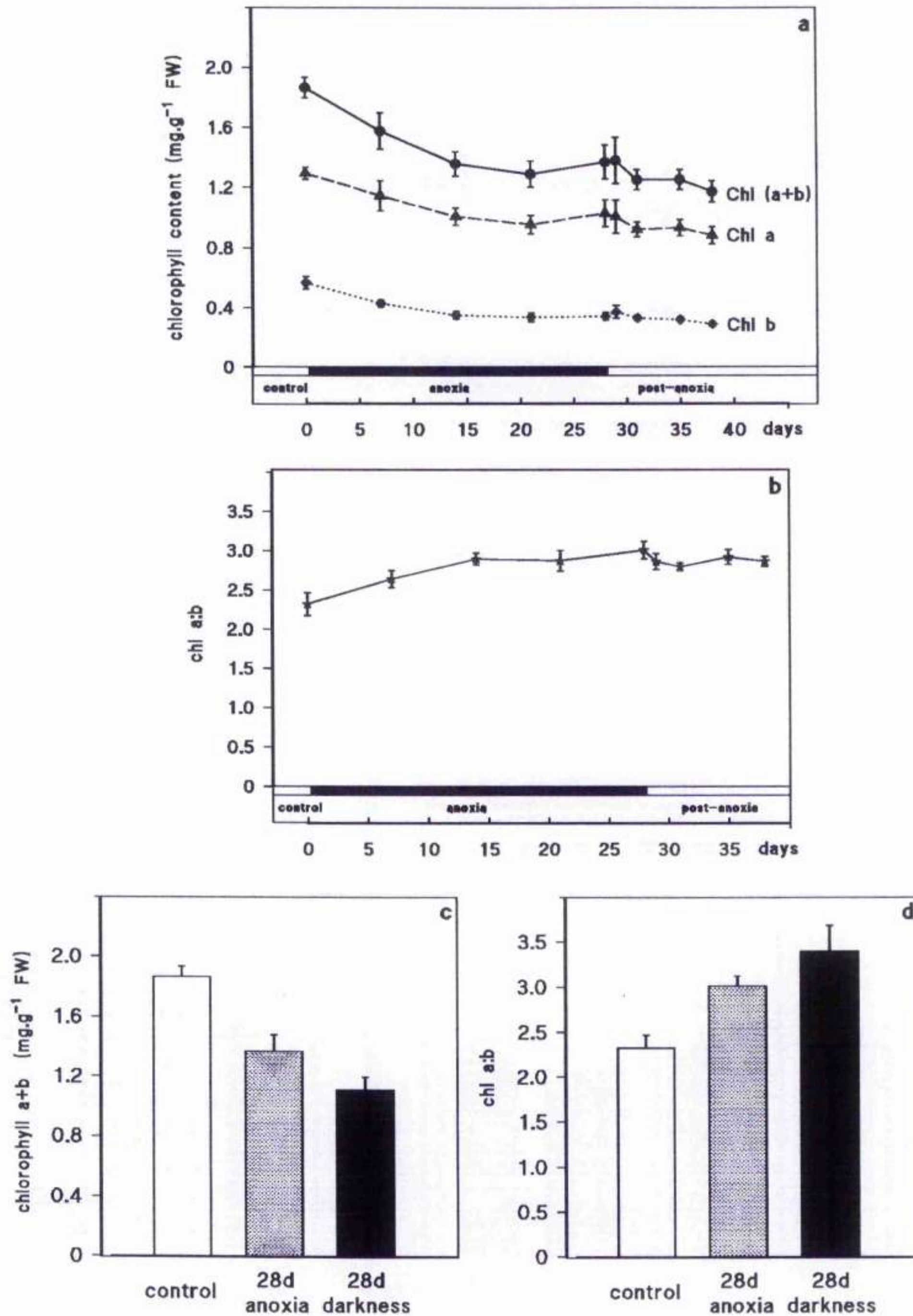


Figure 4.4. Chlorophyll content in *A.calamus* leaves under anoxia, post-anoxia and dark treatment

Chlorophyll content (a) and chlorophyll a/b ratio (b) were determined as described in Section 2.11. at day zero (control); after 7d, 14d, 21d and 28d of anoxia; and after 1d, 3d, 7d and 10d of post-anoxia. Chlorophyll content (c) and chlorophyll a/b ratio (d) were compared in leaves at day zero (control), after 28d of anoxia and after 28d of darkness. Each datapoint represents the mean of 10 independent measurements, error bars show standard error of the mean.

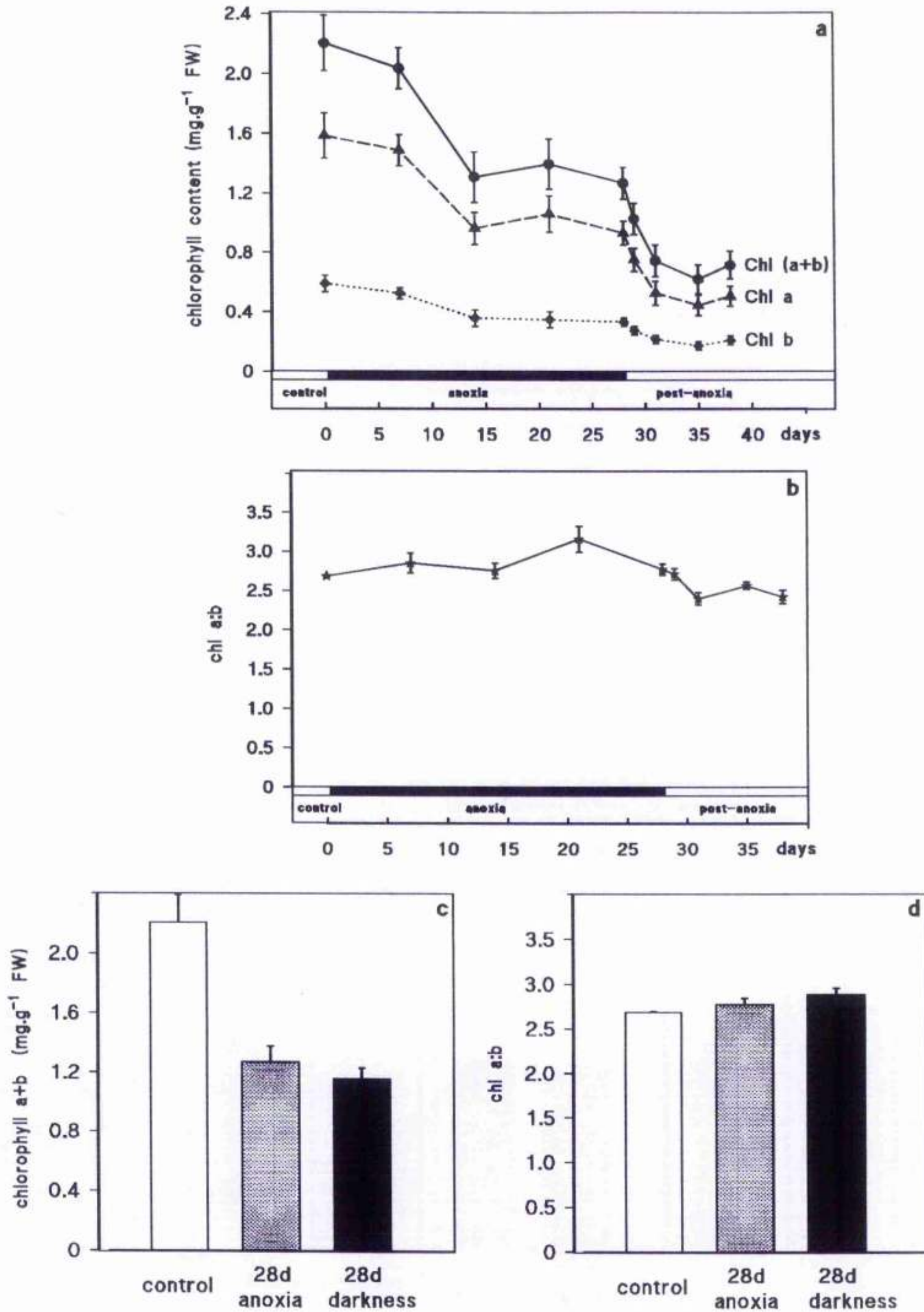


Figure 4.5. Chlorophyll content in *I.pseudacorus* leaves under anoxia, post-anoxia and dark treatment

Chlorophyll content (a) and chlorophyll a/b ratio (b) were determined as described in Section 2.11. at day zero (control); after 7d, 14d, 21d and 28d of anoxia; and after 1d, 3d, 7d and 10d of post-anoxia. Chlorophyll content (c) and chlorophyll a/b ratio (d) were compared in leaves at day zero (control), after 28d of anoxia and after 28d of darkness. Each datapoint represents the mean of 10 independent measurements, error bars show standard error of the mean.

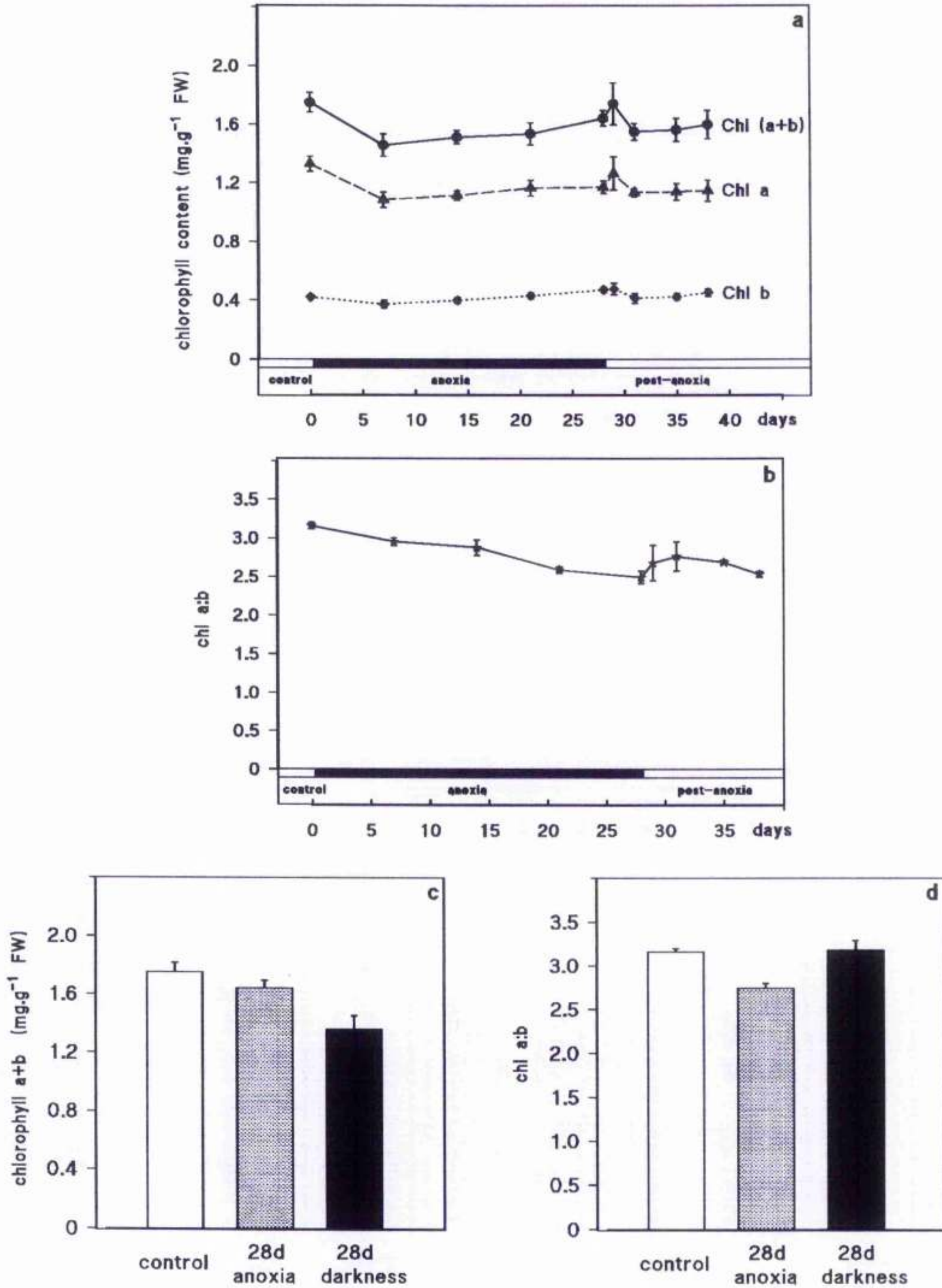


Figure 4.6. Chlorophyll content in *V.macrocarpon* leaves under anoxia, post-anoxia and dark treatment

Chlorophyll content (a) and chlorophyll a/b ratio (b) were determined as described in Section 2.11. at day zero (control); after 7d, 14d, 21d and 28d of anoxia; and after 1d, 3d, 7d and 10d of post-anoxia. Chlorophyll content (c) and chlorophyll a/b ratio (d) were compared in leaves at day zero (control), after 28d of anoxia and after 28d of darkness. Each datapoint represents the mean of 10 independent measurements, error bars show standard error of the mean.

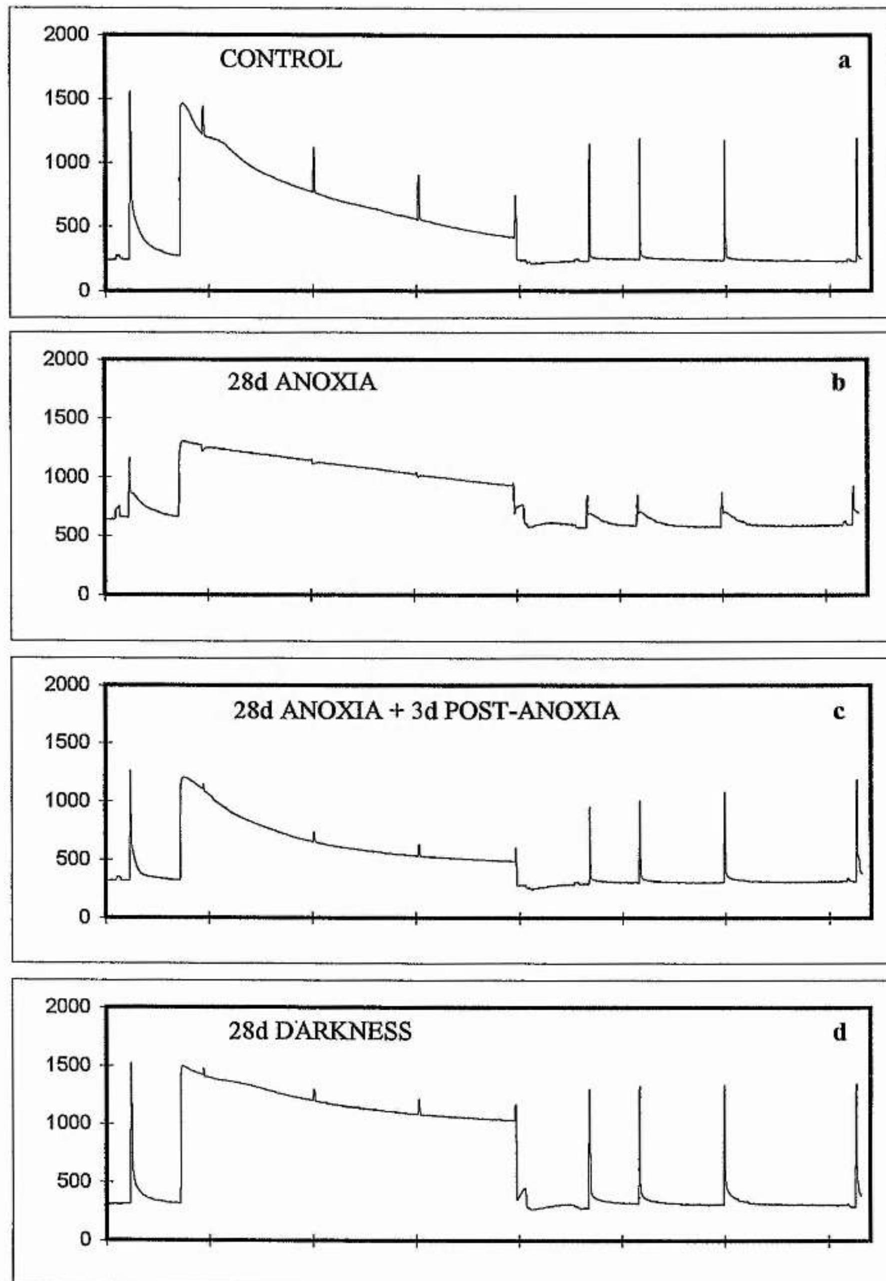


Figure 4.7. Chlorophyll fluorescence pattern in *A.calamus* leaves
Chlorophyll fluorescence (relative units) was measured as described in Section 2.12. at day zero (control) (a), after 28d of anoxia (b), after 3d of post-anoxia (c) and after 28d of darkness (d).

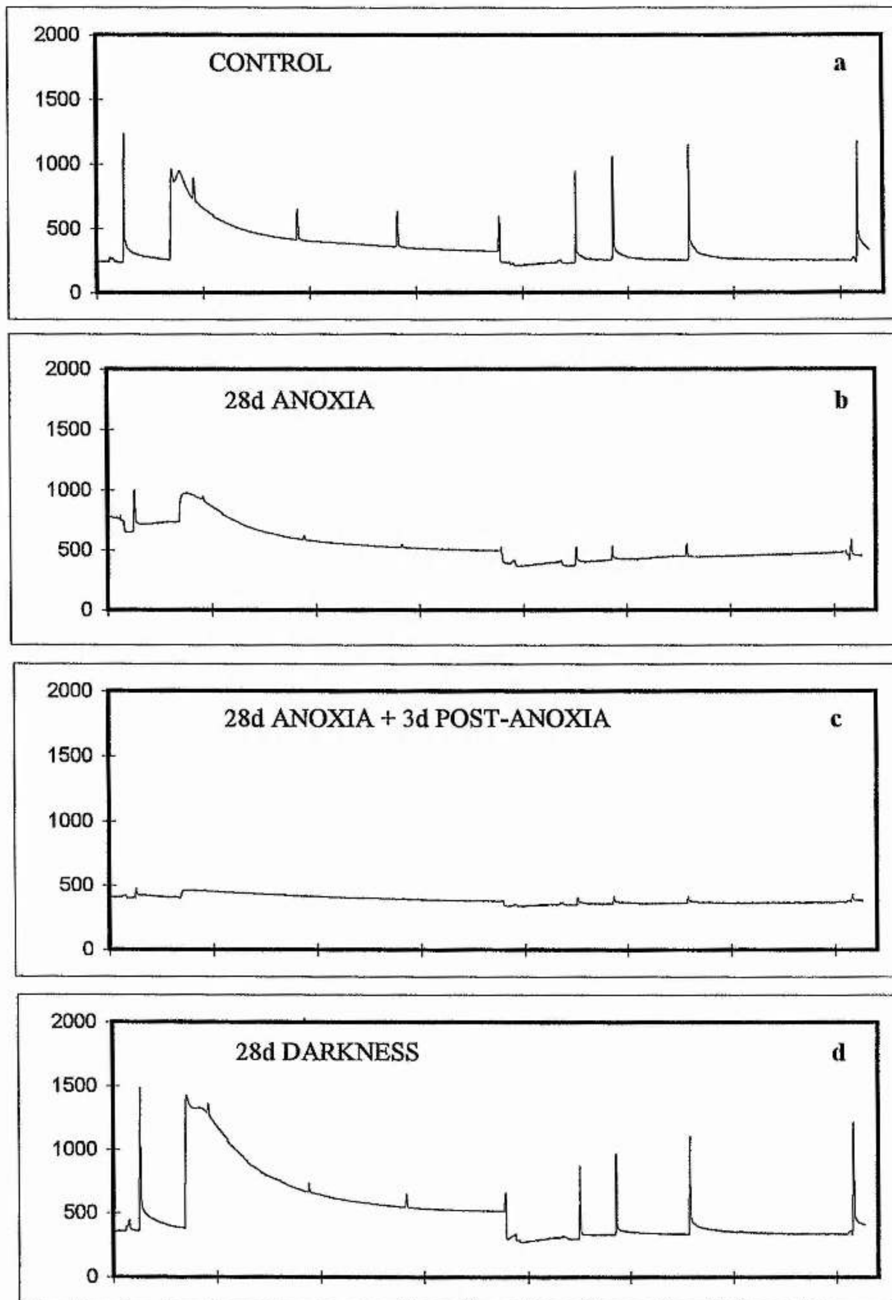


Figure 4.8. Chlorophyll fluorescence pattern in *I.pseudacorus* leaves
Chlorophyll fluorescence (relative units) was measured as described in Section 2.12. at day zero (control) (a), after 28d of anoxia (b), after 3d of post-anoxia (c) and after 28d of darkness (d).

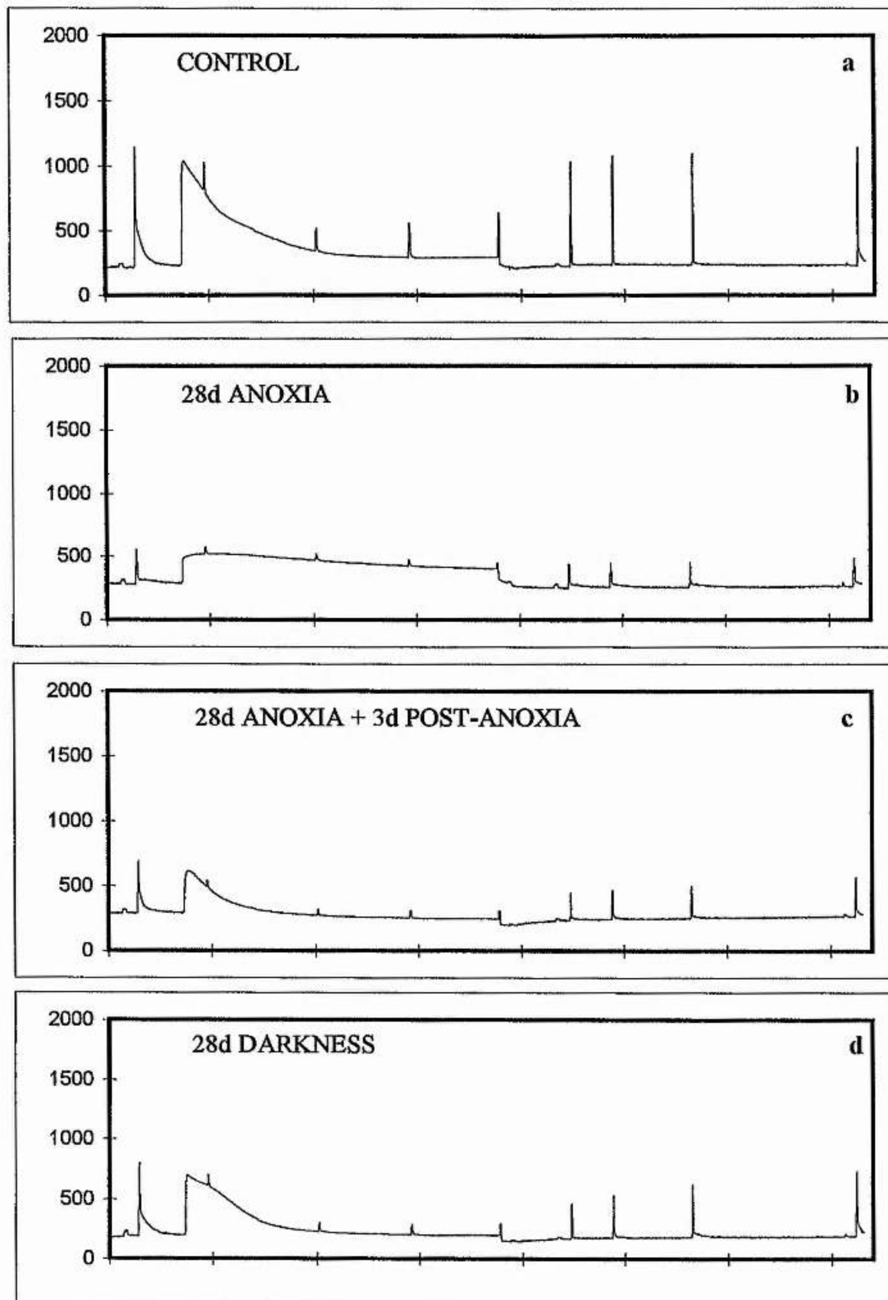


Figure 4.9. Chlorophyll fluorescence pattern in *V.macrocarpon* leaves
Chlorophyll fluorescence (relative units) was measured as described in Section 2.12. at day zero (control) (a), after 28d of anoxia (b), after 3d of post-anoxia (c) and after 28d of darkness (d).

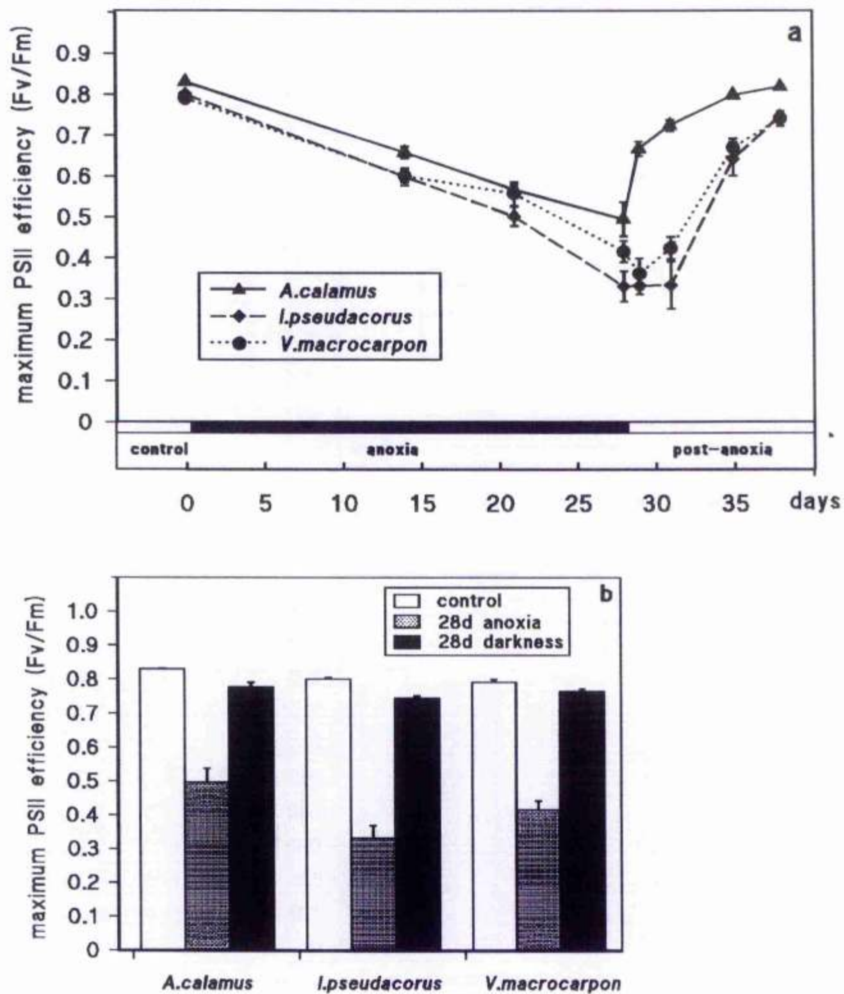


Figure 2.10. Maximal PSII efficiency (Fv/Fm) in leaves of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* under anoxia, post-anoxia and dark treatment

(a) Fv/Fm ratio was determined at day zero (control); after 7d, 14d, 21d and 28d of anoxia; and after 1d, 3d, 7d, and 10d of post-anoxia. (b) Fv/Fm was compared in leaves at day zero (control), after 28d of anoxia and after 28d of darkness. Calculation of Fv/Fm as described in Section 2.12.2. Each datapoint represents the mean of 5 independent measurements, error bars show standard error of the mean.

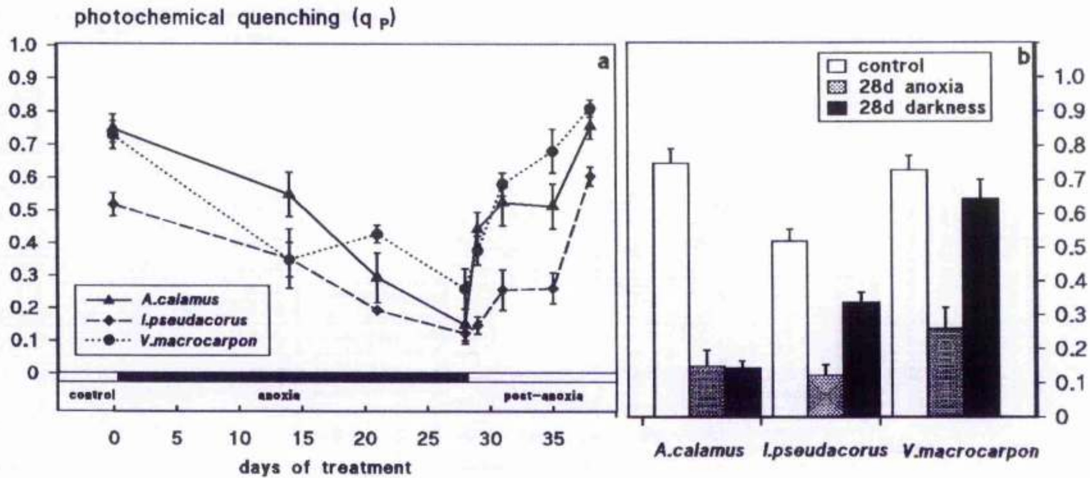


Figure 2.11. Photochemical quenching coefficient (q_P) in leaves of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* under anoxia, post-anoxia and dark treatment

(a) Coefficient q_P was determined at day zero (control); after 7d, 14d, 21d and 28d of anoxia; and after 1d, 3d, 7d, and 10d of post-anoxia. (b) Coefficient q_P was compared in leaves at day zero (control), after 28d of anoxia and after 28d of darkness. Calculation of q_P as described in Section 2.12.2. Each datapoint represents the mean of 5 independent measurements, error bars show standard error of the mean.

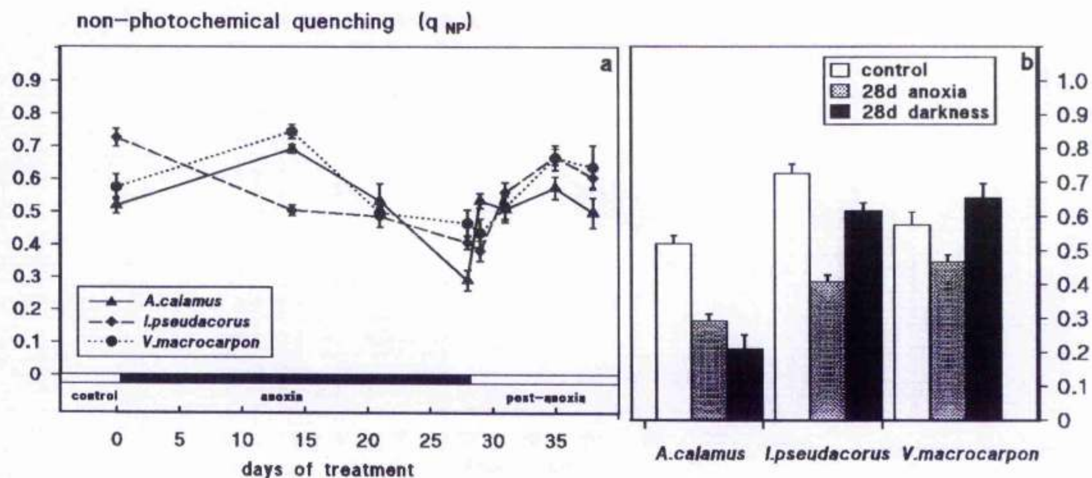


Figure 2.12. Non-photochemical quenching coefficient (q_{NP}) in leaves of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* under anoxia, post-anoxia and dark treatment

(a) Coefficient q_{NP} was determined at day zero (control); after 7d, 14d, 21d and 28d of anoxia; and after 1d, 3d, 7d, and 10d of post-anoxia. (b) Coefficient q_{NP} was compared in leaves at day zero (control), after 28d of anoxia and after 28d of darkness. Calculation of q_{NP} as described in Section 2.12.2. Each datapoint represents the mean of 5 independent measurements, error bars show standard error of the mean.

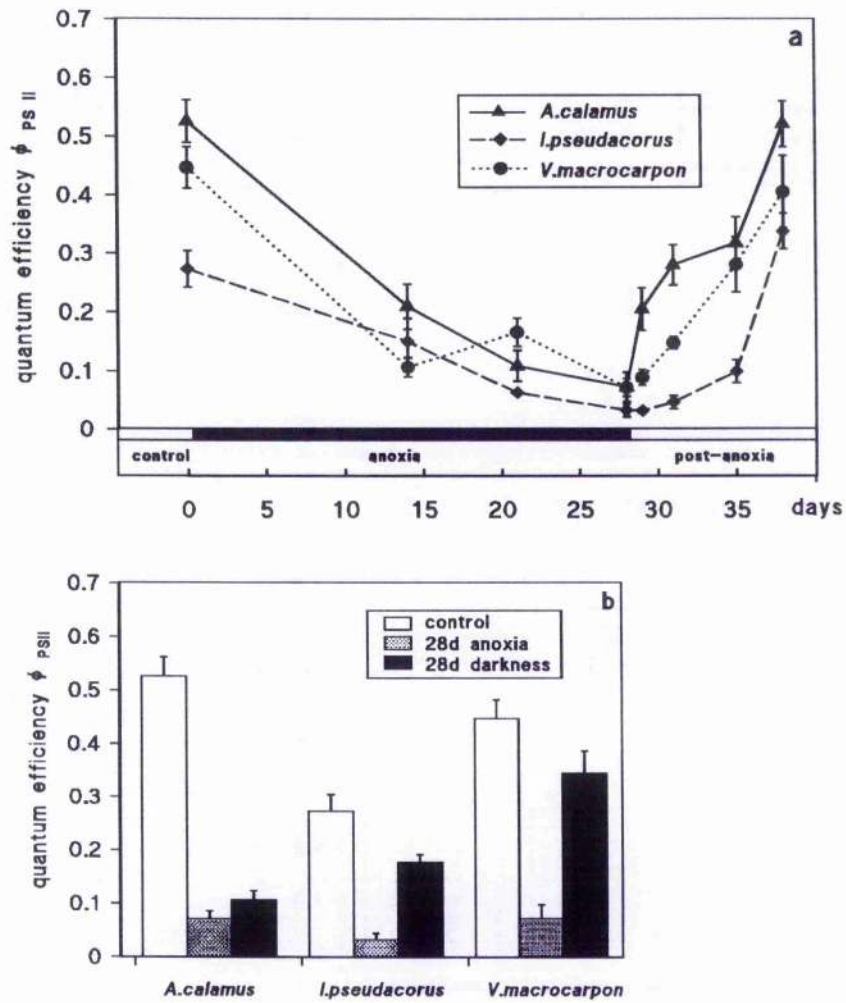


Figure 2.13. Quantum efficiency of PSII (Φ_{PSII}) in leaves of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* under anoxia, post-anoxia and dark treatment

(a) Φ_{PSII} was determined at day zero (control); after 7d, 14d, 21d and 28d of anoxia; and after 1d, 3d, 7d, and 10d of post-anoxia. (b) Φ_{PSII} was compared in leaves at day zero (control), after 28d of anoxia and after 28d of darkness. Calculation of Φ_{PSII} as described in Section 2.12.2. Each datapoint represents the mean of 5 independent measurements, error bars show standard error of the mean.

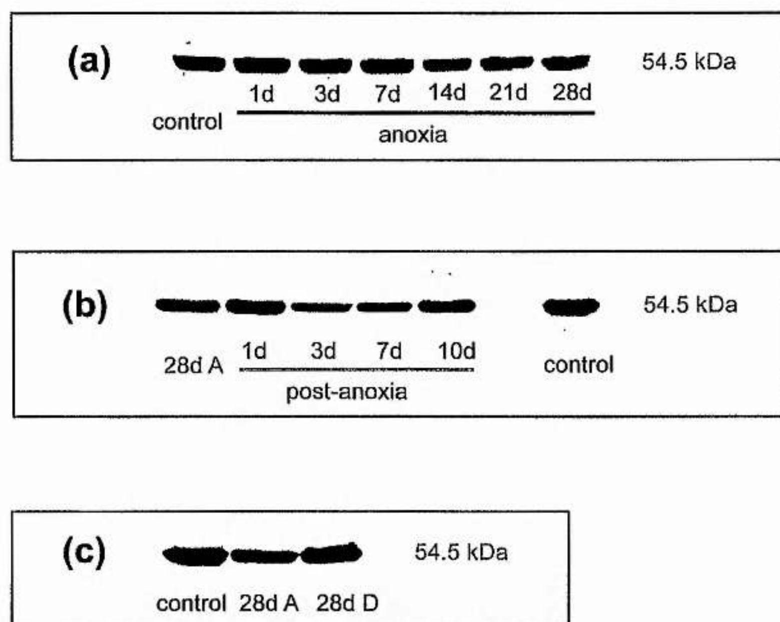


Plate 4.1. Analysis of Rubisco large subunit (RbcL) of *A.calamus* leaf by Western Blotting
Protein extracts were prepared and analysed as described in Section 2.9. Immunoblotting was carried out using a monoclonal antibody raised against the Rubisco large subunit (RbcL).

(a) Leaf samples were taken at day zero (control), after 1d, 3d, 7d, 14d, 21d and 28d of anoxia.
(b) Leaf samples were taken after 28d of anoxia (A), and after 1d, 3d, 7d and 10d of post-anoxia.
(c) Leaf samples were taken at day zero (control), after 28d of anoxia (A) and after 28d of darkness (D).

4.3. Discussion

4.3.1. Effects of Long-term Anoxia on the Photosynthetic Apparatus

In the previous chapter, it has been shown that the extreme conditions under oxygen deprivation have negative effects on the metabolism of cells; carbohydrate availability decreases, the energy budget is reduced, and biosynthetic activity is generally impaired. The present chapter shows that the photosynthetic activity of leaves is also strongly reduced by anoxia in all three investigated species. Comparison with the effects of prolonged darkness demonstrates than darkness under aerobic conditions decreases the photosynthetic capacity to a much lesser extent that darkness plus anoxia. Therefore, a considerable part of the inhibition can be attributed to the effects of anoxia.

Because the bulk of research on anoxia tolerance concentrates on non-photosynthetic tissue, only very little is known about its effects on the photosynthetic apparatus. In the present experiment, the photosynthetic capacity of the leaves is tested immediately after the re-exposure to air. In *I.pseudacorus* and *V.macrocarpon*, the strongest decrease occurs in the first week of anoxia, and very low levels are reached after three weeks in *I.pseudacorus*. In *A.calamus*, the photosynthetic capacity initially decreases more slowly, but the longer the anoxia treatment lasts, the greater seems the injury in the photosynthetic machinery. However, oxygen evolution is still detectable in all three species after four weeks of anoxia, showing that the photosynthetic apparatus in the leaves is not completely destroyed by the anoxia treatment. The picture is very similar to the development of the anaerobic gas exchange of the leaves as described in Chapter 3, indicating the importance of the general energy status of the cell for the maintenance of a functional photosynthetic apparatus.

During post-anoxia, the species show some differences in the speed of recovery. Only in *A.calamus*, does the photosynthetic activity start to increase immediately on return to air, and after 7d of post-anoxia the photosynthetic capacity is not significantly different from the control (Fig. 4.3). This coincides with a very fast recovery of respiratory activity in this species (see Chapter 3). The re-instalment of full respiratory activity occurs in *A.calamus* already after 3d of post-anoxia, thus securing the provision of energy for repair mechanisms. *A.calamus* does not seem to be seriously affected by post-anoxic injury. In the other two species, *I.pseudacorus* and *V.macrocarpon,* the recovery of respiratory activity is delayed on return to air, and the present experiments show that the repair of the photosynthetic apparatus only starts after a lag phase of about one to three days (Fig. 4.3). The occurrence of a similar lag phase before the onset of oxygen evolution has been described for anaerobically grown rice seedlings (Bozarth & Kennedy, 1987). After re-exposure to air and light, anaerobically grown seedlings have a 76h lag before net oxygen evolution occurs, compared to a only 6h lag in seedlings grown under aerobic conditions in the dark. In contrast to the present experiment with mature leaves, the seedlings initially

contained no pigments and the photosynthetic apparatus had to be newly synthesised. In the mature leaves, the recovery of photosynthesis under post-anoxia would depend on one hand on the amount of damage caused by anoxia, and on the other hand on the capacity for repair or neo-synthesis of the involved macromolecules.

The photosynthetic pigments in higher plants are Chl *a*, Chl *b* and carotenoids. Together with the conjugated proteins, they are localised in the thylakoid membrane of the chloroplast and represent basic components of the photosynthetic apparatus. Under anoxia treatment, the total leaf chlorophyll content decreases by 30% and 50% in *A.calamus* and *I.pseudacorus* respectively (Fig. 4.4; 4.5). The total chlorophyll content of *V.macrocarpon* leaves declines by 15% at the start of the treatment, but then remains more or less stable over four weeks of anoxia (Fig. 4.6). A minor reduction of the chlorophyll content is also found in the first leaves of rice and wheat seedlings under anoxia (Chirkova *et al.*, 1995). In the present experiment, the slight decrease in the chlorophyll content of the leaves does not correspond to the much larger decrease in the oxygen evolution under anoxia. Therefore, the reduction in the photosynthetic capacity seems to result only partially from the breakdown of the chlorophyll under anoxia.

The two photosystems of higher plants differ in their composition of chlorophyll pigments. Pigment system PSII which is predominantly found on the stacked, granal thylakoids, contains the bulk of accessory pigments and most, if not all, of the total Chl *b*. In addition, it contains several membrane-bound forms of Chl *a*. Photosystem PSI, which is predominantly located on the unstacked, stromal thylakoids, and consists mainly of Chl *a* pigments (Edwards & Walker, 1981). The Chl *a/b* ratio is therefore believed to provide information about the ratio of PSII/PSI. There is no uniform response in the changes of Chl *a/b* in anoxia treated leaves. In *A.calamus* and *I.pseudacorus* leaves, the Chl *a/b* ratio is about 2.5 under control conditions and a minor increase occurs under anoxia. In *V.macrocarpon* leaves, the initial Chl *a/b* ratio is higher (about 3.15), but decreases slightly under anoxia. Studies in anaerobic rice seedlings by Chirkova *et al.* (1995) suggested that PSII is more sensitive to oxygen deprivation than PSI. This would be in accordance with the present results for *A.calamus* and *I.pseudacorus*, where the increase in the Chl *a/b* ratio indicates a stronger decrease in the PSII pigments relative to PSI. However, the opposite development is found for *V.macrocarpon* leaves, where the PSII pigment content seems to be relatively stable under anoxia.

On return to air, no recovery is observed in chlorophyll content in anaerobically treated leaves, and even after 10d of post-anoxia the chlorophyll content in the leaves is significantly lower than the initial level in the control. In *I.pseudacorus*, the re-exposure to air even initiates a further decline in the total chlorophyll content of the leaf (Fig. 4.5). It is possible that the post-anoxic accumulation of reactive oxygen species (ROS) is involved in the destruction of chlorophyll. Re-exposure to air and light simultaneously, increases the

production of ROS in the leaves, because chloroplasts are especially prone to light-dependent oxygen activation, e.g. via Mehler reaction (see Section 4.1.1). Because anaerobic metabolism is mainly reduced to processes that are essential for survival, the antioxidative defence system of the plants is weakened on return to air (Biemelt *et al.*, 1996) and the plants are exposed to increased concentration of oxygen radicals (see: General Introduction, Section 1.5). An accumulation of ROS such as singlet oxygen is presumed to play a role in the initiation of chlorophyll breakdown (Pastori & del Rio, 1997; Elstener & Osswald, 1994), and it is possible that the enhanced production of ROS is involved in the post-anoxic chlorophyll degradation in *I.pseudacorus* leaves. Monk *et al.* (1984) shows that the activity of SOD increases in *I.pseudacorus* rhizomes under anoxia, but nothing is known about the antioxidative defence of anaerobically treated leaves.

In *A.calamus* and *V.macrocarpon*, the post-anoxic damage on the pigment content was less pronounced than in *I.pseudacorus.* It would be interesting to investigate the antioxidative defence system of the three species under anoxia and post-anoxia and to test, whether enhanced post-anoxic injury by ROS could be responsible for the comparatively slow metabolic recovery in *I.pseudacorus* leaves.

In spite of the reduction in chlorophyll content, the oxygen evolution in the leaves starts to increase again after re-exposure to air and light. The recovery of full photosynthetic activity does not seem to depend primarily on chlorophyll content. The same has been found for dark grown rice seedlings, where maximal values for oxygen evolution occurs far well before maximal values for the chlorophyll content are obtained on exposure to light (Bozarth & Kennedy, 1987).

To obtain further information regarding damage caused by anoxia and post-anoxia in the photosynthetic apparatus, especially about the stress sensitive PSII, chlorophyll fluorescence of dark- and light adapted leaves has been analysed. The maximal efficiency of PSII in the dark adapted leaf (Fv/Fm) is considerably reduced by anoxia in the three investigated species (Fig. 4.10). The Fv/Fm ratio gives information about the functioning of the PSII reaction centres and the observed decrease in the Fv/Fm value indicates the inactivation of reaction centres under oxygen deprivation stress. Decreased Fv/Fm values have been found in several other environmental stress situations such as extremely low or high temperatures (Yamane *et al.*, 1998), high light intensities (Yamane *et al.*, 1997), severe drought (Lu & Zhang, 1998) and prolonged salt stress (Delfine *et al.*, 1999). In *A.calamus* and *I.pseudacorus,* the decrease in Fv/Fm is mainly due to increased Fo levels in the anoxia stressed leaves (Tab. 4.1). The increase of Fo is a typical feature under stress, but the underlying mechanisms are still not fully understood. The Fo level depends on structural conditions that affect the probability of excitation energy transfer between antennae pigments and from these to the reaction centre of PSII (Krause & Weis, 1987). It is believed that under stress, perturbations in the thylakoid membranes cause a separation

of the light harvesting chlorophyll a/b protein complex of PSII from the PSII core complex. This theory is supported by experiments with an LHC II-less mutant in which the increase of Fo under high temperature stress was lost (Yamane *et al.*, 1997). Additionally, the blockage of PSII reaction centres seems to contribute to the Fo rise under stress.

Besides the Fo increase, anoxia also causes a decrease in the Fm level of anoxia treated leaves. The phenomenon is particularly pronounced in *V.macrocarpon*. The reduced Fm indicates a decrease in the energy trapping of PSII, and might be caused by the denaturation of proteins in the chlorophyll-protein complexes. Yamane *et al.* (1998) report that under high temperature stress, the manganese-stabilising 33kDa protein of PSII is possibly released from the PSII core complexes and causes the inactivation of the reaction centres. The loss of the 33kDa protein is partly reversible, when the plants return to ambient temperatures. Another stress sensitive protein is the D1 protein (see Section 4.1), which binds on the Q_B-site of the reaction centre. Even in low light, it shows fast turnover, and any environmental stress that impairs the D1 turnover, will also affect the functioning of PSII reaction centres (Long *et al.*, 1994). The deactivated PSII reaction centres become less fluorescent and cause a decrease in the Fm level. Destroyed reaction centres are supposed to migrate to non-appressed region and transfer an increased amount of energy to non-fluorescent PSI centres there (Yamane *et al.*, 1998). Anoxia reduces the efficiency of PSII of the dark-adapted leaf in all three investigated species, but changes in Fo and Fm contribute to a different proportion to the changes in Fv/Fm in the species. In *A.calamus* and *I.pseudacorus*, the separation of the LHC from the reaction core seems to be a major problem for the functioning of PSII under anoxia. In *V.macrocarpon,* the stability of reaction centres seems to be considerably reduced.

Generally, the drop in Fv/Fm under anoxia takes place to a lesser extent than the drop in the actual photosynthetic capacity of the leaf, indicating that oxygen evolution is more sensitive to anoxia than the maximal efficiency of PSII. A similar behaviour is also observed in other environmental stress situations, and it has been shown that the adaptation of the photosystem to light is impaired in certain stress situations (Lu & Zhang, 1998; Delfine *et al.*, 1998). The same is found for the anoxia stressed leaves of *A.calamus*, *I.pseudacorus* and *V.macrocarpon*. In all three species, the photochemical quenching (q_P) is considerably reduced after the anoxia treatment (Fig. 4.11), indicating an imbalance between the energy funnelled through PSII and the subsequent electron transport. The decrease in electron transport has been attributed for instance to an inhibition of the electron flow around PSI and impairments in the utilisation of ATP and NADPH in the Calvin cycle (Krause & Weis, 1987; Lu & Zhang, 1998). These disturbances would lead to the reduction of the plastoquinone pool in the illuminated chloroplast and an accumulation of reduced Q_A-. The amount of open reaction centres (q_P) decreases and the thylakoids become overexcited.

A number of non-photochemical quenching mechanisms (q_{NP}) are usually available in the chloroplast to overcome the overexcitation of the membrane (see Section 4.1.3). The dissipation of energy as heat instead of photochemistry decreases the quantum efficiency of the photosystem II, but at the same time the reduction in the membrane energisation also provides protection against damage of the photosystem (Genty *et al.*, 1989; Genty *et al.*, 1990). In *A.calamus* and *V.macrocarpon,* the decrease in photochemical quenching is initially accompanied by enhanced non-photochemical quenching (Fig. 4.12). A coincidental increase in non-photochemical energy dissipation is also observed in other stress situations, for instance when photochemical carbon assimilation was reduced by drought (Eastman & Camm, 1995). However, after two weeks under anoxia, non-photochemical quenching mechanisms also decline in the anoxia treated leaves. It has been suggested that xanthophylls might be involved in the quenching of the energised thylakoid membrane. The long-term acclimatisation of plants to excess light is usually accompanied by an increase in the size of the xanthophyll cycle pool (Horton *et al.*, 1996). Xanthophylls are oxygen containing carotenoid derivates in higher plants. Prolonged anoxia could possibly cause a decrease in the xanthophyll content of leaves and consequently diminish the capacity for energy-dependent quenching.

Besides the quenching of Fs and Fm', the illumination of anoxia treated leaves also causes changes in Fo. In *I.pseudacorus*, the fluorescence yield under actinic light decreased even below the Fo level indicating that not only Fm, but also Fo was quenched (Fig. 4.8). A suppression of Fo seems to be a common feature under conditions of strong energisation and has first been described by Bilger & Schreiber (1986). It has been suggested, that the Fo quenching is related to state transition quenching. The reduction of the plastoquinone pool under constant illumination initiates the phosphorylation of LHC II proteins. Subsequently, the energy transfer from the pigment complex is diverted towards the low fluorescent PSI in the margin region of the thylakoids. The re-oxidation of the plastoquinone pool in the dark allows the recovery of Fo (Bilger & Schreiber, 1986).

The quantum efficiency of PSII (Φ_{PSII}) depends on both photochemical and non-photochemical quenching mechanisms (Genty *et al.*, 1989). In the investigated leaves, the decreases in Φ_{PSII} coincide with the results for the oxygen evolution, proving that the calculated value for Φ_{PSII} after the anoxia treatment represent a useful parameter for carbon assimilation in the leaf (Fig. 4.3; 4.13). In the present experiment, non-photochemical quenching mechanisms are less affected by anoxia than the photochemical quenching. This indicates that disturbances in the electron transport after the charge separation are one of the main causes for the reduced photosynthetic capacity in the anaerobically treated leaves. The reduced non-photochemical quenching would lead to a permanent over-excitation of the thylakoid and enhance the danger of photoinhibitory damage. Photoinhibition is usually determined by analysing the relaxation of the Fv/Fm ratio after illumination in the dark. In *A.calamus* and *I.pseudacorus*, the dark relaxation of Fv/Fm is indeed impaired after the

anoxia treatment. The enhanced instability of PSII towards photoinhibition is also observed during drought (Lu & Zhang, 1998) and low temperatures (Long *et al.*, 1992). The impact of photoinhibition has also been shown to vary between species or between leaves grown under different light intensities (sun and shade leaves). Generally, photoinhibition seems to be inevitable as soon as over 40% of the PSII traps become continually closed (Öquist *et al.*, 1992). In the present experiment, *V.macrocarpon* leaves do not seem to suffer enhanced photoinhibition after the anoxia treatment. But fluorescence levels in these leaves are generally very low, and values for the Fv/Fm ratio should not be overestimated.

In contrast to chlorophyll content, the functioning of the photosystem II complex recovers upon re-exposure to air and light in the investigated plants. The repair of PSII therefore provides an explanation for the re-establishment of photosynthesis in the post-anoxic leaves. In *A.calamus*, both the maximal PSII efficiency of the dark adapted leaf and the quantum PSII efficiency in the light start to increase immediately after return to air. This coincides with the rapid recovery of oxygen evolution under post-anoxia in this species. In the other two species, the recovery of PSII efficiency and the capacity for oxygen evolution however are retarded. Particularly interesting is the decrease in the overall fluorescence found in *I.pseudacorus* leaves shortly after re-exposure to air and light. The high sensitivity of this species during post-anoxia has already been discussed above and the involvement of reactive oxygen species in the post-anoxic damage has been suggested. However, after 10d of post-anoxia the quantum efficiency of PSII recovered nearly completely in all three investigated species (Fig. 4.13). This coincides well with the recovery of the oxygen evolution in *A.calamus* (100% of the initial value), *I.pseudacorus* (80%) and *V.macrocarpon* (90%) after 10d anoxia (Fig. 4.3). The reduced chlorophyll content of post-anoxic *I.pseudacorus* leaves could be responsible for the incomplete recovery of oxygen evolution under post-anoxia.

A considerable proportion of the decrease in the PSII quantum efficiency of the anoxia treated leaves is shown to be due to the impaired re-oxidation of Q_A- (photochemical quenching). The redox state of Q_A is under control of the subsequent electron transport and the activity of the Calvin cycle. Any reduction in the activity of the Calvin cycle would result in the accumulation of ATP and the final electron acceptor of the electron chain NADPH in the illuminated leaf, and subsequently block the photochemical re-oxidation of Q_A-. This has been shown to be the case during drought stress, when CO_2 shortage due to the closure of stomata impairs the Calvin cycle (Lu & Zhang, 1998; Epron *et al.*, 1998). Reduced activity of the Calvin cycle also seems to be responsible for the decrease in photochemistry in senescent leaves (Bukhov, 1998).

The initial step of the Calvin cycle is catalysed by Rubisco. In all higher plants, Rubisco consists of eight large subunits and eight small subunits (Mott, 1997). On Western blots, leaf content of the large subunit (RbcL) decreases only slightly under anoxia in *A.calamus*,

with no significant differences observed (Plate 4.1). It is not very likely that Rubisco is synthesised under anoxia. First, because the biosynthesis of the bulk of proteins is limited under anoxia, and secondly because the synthesis of many chloroplast compounds, such as chlorophyll, carotenoids and enzymes, is regulated by phytochrome and impaired in the dark (for review see Smith, 1996). Phytochrome acts as a light sensor in the plant. Upon exposure to sunlight, it changes its conformation and initiates gene transcription. It has been proven that the transcription of mRNA for the nuclear encoded small subunit of Rubisco is activated by the FR form of phytochrome in the light (see Sitte *et al.*, 1992). In barley seedlings, the concentration of mRNA for the chloroplast encoded large subunit of Rubisco also decreases rapidly in the dark (Krause *et al.*, 1998). When rice seedlings are grown simultaneously under anoxia and in the dark, they show no Rubisco activity at all (Bozarth & Kennedy, 1987). It is therefore surprising, that Rubisco seems relatively stable in the anoxia treated *A.calamus* leaves. However, the shown Western Blot only reveals information about content of the large subunits of Rubisco in the leaf tissue, and gives no indication of the actual activity of the enzyme. In salt-stressed leaves of spinach, the Rubisco activity clearly declines before changes in the content of the enzyme are detectable (Delfine *et al.*, 1998). Because synthesis and repair of proteins are impaired under anoxia, it is likely that a large proportion of chloroplastic enzymes lose their activity under anoxia.

Return to air and light is not accompanied by significant changes in the Rubisco content of the *A.calamus* leaf, but the immediate increase in photosynthetic activity indicates that Rubisco and other enzymes of the chloroplast metabolism regain their activity rapidly.

4.3.2. Comparison of the Effects of Anoxia and Darkness on the Photosynthetic Apparatus

Prolonged darkness can induce physiological changes in the chloroplast, including a decline in chlorophyll content and photosynthetic capacity. Decreases in the transcript levels of genes related to photosynthesis can also occur (Krause *et al.*, 1998). The senescence process in older leaves is accelerated by dark incubation, and senescence related processes such as induction of SAG and chlorophyll, and protein degradation can also be initiated in younger leaves (Weaver *et al.*, 1998). The biochemical and physiological changes occurring during dark incubation of younger leaves are reversible by light (Krause *et al.*, 1998). In the present experiment, accelerated yellowing is observed in older leaves of *A.calamus* and *I.pseudacorus* during the four week dark incubation. The dark response and the age-mediated response of leaves seem to be additive for the induction of senescence (Weaver *et al.*, 1998). However, two to three younger leaves of *A.calamus* and *I.pseudacorus* maintain their green colour during dark incubation. No visible yellowing is found in the dark incubated mature *V.macrocarpon* leaves. The photosynthetic capacity of the green leaves is reduced after the dark treatment in air, but to

a lesser extent than after the anoxia/dark treatment. Oxygen evolution decreases by 50% in *A.calamus* and *I.pseudacorus* and by 30% in *V.macrocarpon* after 28d in the dark, compared with a reduction of 85%, 95% and 80% respectively, after anoxia incubation (Fig. 4.3).

The reduction in the photosynthetic capacity of leaves after dark incubation is accompanied by a decrease in chlorophyll content (Fig. 4.4-4.6). Interestingly, the decrease in the chlorophyll content is slightly more pronounced in the dark/air treated leaves than in the dark/anoxia treated leaves in the three investigated species. This proves again that the maximal photosynthetic activity of the leaves is not primarily depended on the chlorophyll content of the leaves. The steps of chlorophyll degradation are still not very clearly defined, but a number of oxygenases seem to be involved (Buchanan-Wollaston, 1997). The inhibition of oxygenase activity under anoxia would prevent the active degradation of chlorophyll. It is therefore possible that different processes are responsible for chlorophyll loss under aerobic and anaerobic conditions. In both experiments (the dark and the anoxia treatment), the analysed leaves show no visible signs of yellowing in spite of the reduction in the chlorophyll content. The phenomenon that chlorophyll loss is not necessarily accompanied by visible leaf yellowing has also been observed in detached *Arabidopsis* leaves in the light and in whole *Arabidopsis* plants under prolonged darkness (Weaver *et al.*, 1998).

In contrast to the anoxia treatment, the maximal PSII efficiency of the dark adapted leaves is hardly affected by prolonged darkness (Fig. 4.10). This applies for all three investigated species and indicates that the PSII reaction centre is not severely damaged in the dark. However, the PSII photochemistry of the light-adapted leaf is modified by the dark treatment. Photochemical quenching (q_P) is significantly lowered in all three species after the dark-treatment when compared with the control (Fig. 4.11). As discussed above, a decrease in q_P usually indicates disturbances in the electron transport beyond Q_A and is highly influenced by the activity of the Calvin cycle.

Because many chloroplastic proteins are only expressed in the light, repair and synthesis of proteins can be impaired in the dark, and this could explain the inhibition of the photochemical electron transport in the darkened leaf. However, in *I.pseudacorus* and *V.macrocarpon* the photochemical quenching is clearly less disturbed by dark incubation in air than under anoxia. Both species also maintain a higher capacity for non-photochemical energy dissipation in the dark (Fig. 4.12). In *V.macrocarpon*, the decline in photochemical quenching is accompanied even by an increase in non-photochemical quenching (q_{NP}). The enhanced q_{NP} could be due partly to photoinhibition as indicated by the slow relaxation of Fv/Fm in *V.macrocarpon* leaves after the dark treatment (Tab. 4.2). In the other two species, *A.calamus* and *I.pseudacorus* however, dark treatment does not cause photoinhibitory damage on return to light.

The results for Φ_{PSII}, calculated from the fluorescence data (Fig. 4.13), mirror the results for the capacity of oxygen evolution in *I.pseudacorus* and *V.macrocarpon*. The quantum efficiency of PSII is reduced after the dark treatment, but the reduction is not as severe as after the anoxia treatment. The decrease in Φ_{PSII} is mainly attributed to the limited availability of open reaction centres (q_P), while the efficiency of excitation energy capture by open PSII reaction centres is hardly affected by the dark incubation.

A discrepancy between the quantum efficiency and the capacity for oxygen evolution is only found for *A.calamus*. The dark treatment causes a surprisingly strong decrease in photochemical and non-photochemical quenching in this species. Subsequently, the quantum efficiency drops by 80% after the dark treatment (Fig. 4.13). In contrast to these results, oxygen evolution decreases by 50% (Fig. 4.3). In this context, it should be mentioned that oxygen evolution and fluorescence were measured in different plants at different times of the year. The general validity of these results should be verified by measurements in the same leaf.

Generally, fluorescence measurements prove to provide a good tool for the analysis of PSII functionality under stress. However, many aspects of the biochemical and biophysical processes behind the complex fluorescence modulation are still not completely understood. Stress related changes in the fluorescence pattern often vary between isolated thylakoids and intact organisms, plants and algae or different species, and general interpretations should be made with caution.

In summary, the present results prove that prolonged darkness impairs the photosynthetic metabolism of leaves under aerobic and anaerobic conditions. However, the damage is more severe under anoxia than in air. Under dark/air treatment, the decrease in photosynthetic capacity is caused by a reduction in the chlorophyll content and the inhibition of electron transport under illumination, but the PSII reaction centres seem to remain stable. Under anoxia/dark conditions, photosynthetic activity is limited by a slight reduction in the chlorophyll content, the inactivation of PSII complexes, and a strong inhibition of energy dissipation under illumination. The recovery of photosynthesis on return to light depends in both cases on the extent of damage and the capacity for repair.

Chapter 5

Effects of Long-term Anoxia on the Ultrastructure of Leaf Cells

5.1. Introduction

5.1.1. Metabolism and Mesophyll Cell Ultrastructure

Mesophyll cells form the ground tissue of a leaf and are the major site of photosynthesis in plants. The internal complexity of the cells is closely correlated with functional requirements. The major organelles found in plant cells are the nucleus, mitochondria, plastids, Golgi apparatus, microbodies, peroxisomes, glyoxysomes and vacuoles; and the cytoplasm also forms a compartment by itself (Plate 5.1). Cell organelles are enclosed by membranes which separate metabolic processes. The different cell compartments interact with each other via the controlled transfer of metabolites (Emes & Dennis, 1997).

The close relationship between structure and function is particularly well studied in chloroplasts and mitochondria. In leaf cell metabolism, these organelles play an essential role by providing energy in the form of ATP and metabolites for biosynthetic processes.

Mitochondria

Mitochondria are the sites of cellular respiration. By consumption of oxygen, mitochondria convert energy bound in organic compounds such as carbohydrates or lipids into ATP. Additionally they deliver numerous substances via specific carriers for biosynthetic reactions into the cytoplasm. Plant mitochondria vary in form, but are usually spherical or rod-shaped (0.5 x -2μm) (Newcomb, 1997a). They are characterised by two highly specialised membranes, an outer smooth membrane which forms the enclosing perimeter, and an inner membrane with numerous inwardly directed invaginations called cristae (Plate 5.3). The inner membrane contains a high proportion of integral components involved in electron transport and oxidative phosphorylation, e.g. cytochrome oxidase complex and ATPase. The compartment enclosed by the inner membrane is the mitochondrial matrix. The matrix consists of a ground substance of fine particles such as ribosomes, DNA and soluble proteins of the TCA cycle (Douce & Neuburger, 1989).

Mitochondria are self-replicating organelles and arise by division from pre-existing mitochondria. There is evidence to relate mitochondrial numbers and ultrastructure to the metabolic activity of the system. The enhanced formation of cristae enlarges the surface area of the inner membrane and is believed to increase the capacity of cellular respiration. During the development of the *Arum* spadix, the rise in enzyme activity parallels the increase in number of cristae per mitochondrion (Simon & Chapman, 1961). As the leaf ages, the respiration rate declines and this corresponds with a reduction in the number of cristae per mitochondrion (Geronimo & Beever, 1964).

Chloroplasts

Chloroplasts are the sites of photosynthesis, and absorb solar energy to drive the synthesis of organic compounds from carbon dioxide and water. The mature organelles are typically

lentiform with average dimensions of 5 x 2 x 1-2μm (Newcomb, 1997b). They are bound by a double membrane (envelope), and the interior of the chloroplasts is divided between the amorphous stroma medium and the highly orientated membrane system. The membrane phase shows a further differentiation into regions of stacked thylakoids (grana) and stroma thylakoids interconnecting the grana (Plate 5.4).

The light driven photosynthetic reactions are associated with the thylakoids and require the coordinated interaction of membrane bound electron carriers and enzymatic proteins that facilitate the electron transfer from dissociated water to $NADP^+$ (Fig. 4.2). Concomitantly a proton gradient develops across the thylakoid membrane. The gradient is discharged during ATP synthesis via ATPase. The concentration of the two photosystems differs in the thylakoid from the stroma (PSI) to the grana (PSII) (Glazer & Melias, 1987). The light independent reactions of photosynthesis occur in the stroma medium which also contains starch, plastoglobuli, numerous ribosomes and soluble proteins of the Calvin cycle, e.g. Rubisco.

Chloroplasts are not synthesised *de novo*, but arise from proplastid division in leaf meristematic cells. During ontogenesis the inner membrane of the proplastid envelope invaginates to form the first thylakoids. Stroma lamellae develop before grana appear. As the chloroplast matures, the size and number of grana increase. Generally, the development of the chloroplastic membrane system is closely related to increasing photosynthetic capacity during leaf maturation. The degree of grana stacking varies with physiological requirements. Extreme shade leaves for instance contain very large granal stacks, and the high density of light-harvesting assemblies can improve the efficiency of light quanta collection (Anderson *et al.*, 1973). As leaf senescence progresses, the decline in photosynthesis corresponds to the degradation of the thylakoids. Grana number and thylakoids per granum decrease during leaf senescence, while the number and size of plastoglobuli rises (Bondana & Oosterhuis, 1998).

5.1.2. Effects of Environmental Stress on Cell Ultrastructure

The exposure of plants to environmental stress, e.g. drought, enhanced UV-B, chilling or anoxia; can trigger alterations in cell ultrastructure. Many of these changes are interpreted as injury to the cell, but ultrastructural modifications can also be part of adaptation mechanisms. The break-up of the single original vacuole into numerous vesicular fractions for instance has been interpreted as part of the adaptation process in tolerant plants under drought and freezing stress (Quartacci *et al.*, 1997; Ristic & Ashworth, 1993).

For the maintenance of cell function and structure, the stability of membranes is crucial. Environmental stress such as water deficit can damage cell membranes through structural changes and by altering their composition and function. Desiccation tolerant plants are often characterised by their ability to change lipid metabolism and lipid composition of

membranes according to the water status of the tissue (Quartacci *et al.*, 1997). Cell membranes also play a key role in cold adaptation. During seasonal cold acclimation, the plasma membrane undergoes modification in lipid and protein composition as well as membrane behaviour and fluidity. In *Arabidopsis thaliana* plants, the plasma membrane shows signs of extensive turnover during cold adaptation, and membrane invagination and sequestering of membrane material occur (Ristic & Ashworth, 1993). The importance of lipid composition for the stability of membranes during low-temperature stress has been shown in the *fab*1 mutant of *A.thaliana* which contains increased levels of saturated fatty acids. When grown at 22°C or 12°C the mutants are indistinguishable from the wildtype, but after prolonged exposure to temperatures of 2°C the mutant show a marked decrease in photosynthetic capacity. This is accompanied by a rapid and extensive disruption of chloroplast membranes (Wu *et al.*, 1997).

The ultrastructure of chloroplasts seems to be particularly sensitive to environmental changes. Changes in the proportion of grana and stroma thylakoids have been found under enhanced UV-B (Hopkins, 1997; Fagerstedt & Bornman, 1997), salt stress (Locy *et al.*, 1996), nitrogen deficiency (Kutik *et al.*, 1995) and cold stress (Wu *et al.*, 1997). Damage to the membranes can occur rapidly under extreme stress such as high UV-B. In *Pisum sativum* leaves, first signs of damage are already found after a 15min exposure to UV-B. Membrane dilation is followed by disorientation of granal and stromal thylakoids, and finally the disruption of the chloroplastic envelope occurs (Brandle *et al.*, 1977). The ultrastructure of mitochondria is also effected by high UV-B levels, but damage occurs later than in chloroplasts. After 2d of UV-B exposure, mitochondria appear to have fewer cristae (Brandle *et al.*, 1977).

Effects of Anoxia on Cell Ultrastructure

The metabolism of mitochondria is strongly inhibited by the absence of oxygen, and the effects of anoxia on mitochondrial structure have been investigated in a number of plants. In anoxia-sensitive plants such as wheat, pea and maize, the exposure to anoxia results in swelling of mitochondria, reduction of cristae, and increased in the transparency of the matrix within a few hours (Vartapetian, 1991). At this stage, the structural changes are still reversible, but under prolonged anoxia all cristae disappear and the whole mitochondrion degrades. Similar results are obtained for roots of *Glyceria maxima*, *Lycopus europaeus* and *Alisma plantago-aquatica* (Vartapetian & Andreeva, 1986). Despite the fact that these plants grow naturally in flood prone soils, their roots seem to be anoxia sensitive and rely more on anoxia avoidance than on metabolic adaptation (see also General Introduction, Section 1.3.1)

Rice on the other hand is comparatively tolerant to anoxia, and this is also shown by a higher stability of mitochondrial ultrastructure. Four days after germination, Öpik (1973) found only very little difference in mitochondrial size and cristae density of aerobically and

anaerobically grown rice seedlings. Coueé *et al.* (1992) also investigated the development of mitochondria in germinating rice. In their study, anaerobic mitochondria were characterised by a matrix of lower density and more developed cristae. When aerobically germinated rice seedlings were exposed to anoxia, leaf mitochondria were slightly enlarged and contained a high amount of paralleled cristae (Vartapetian *et al.*, 1976). The other organelles of rice cells also seemed to possess high resistance against oxygen deprivation.

A comparison between the effects of anoxia on the coleoptiles, leaves and roots, reveals that the structure of mitochondria in roots is much more sensitive than in the other parts of the plants (Vartapetian *et al.*, 1976, Vartapetian, 1991). This underlines the hypothesis that roots generally appear to be the most sensitive organ of the plant as discussed in the introduction to Chapter 3 (Section 3.1.1).

5.1.3. Quantitative Analysis of Subcellular Components

Principles

The quantitative analysis of cell ultrastructure is based on the principle of stereology. Stereology generally describes the three-dimensional interpretation of two-dimensional images such as micrographs from light or transmission electron microscopy (Toth, 1982; Bolender, 1978; Briarty, 1975). The application of stereology to the quantitative study of different structures in a body, e.g. a cell, is usually called morphometry (Weibel, 1969).

The fundamental relations of stereology were developed by the French geologist Delesse in 1847. He showed that the volume density (V_V) of the various components of a rock (or similar composite solid) can be estimated on random sections by measuring the relative areas (or areal density A_A) of their profiles (see Briarty, 1975). The relationship can be expressed as:

$$\mathbf{Area_{compartment} / A_{total} = Volume_{compartment} / Volume_{total}}$$

$$\mathbf{A_A = V_V}$$

The application of these techniques allows the determination of the volume of chloroplasts or mitochondria in relation to the volume of the cytoplasm or the volume fraction of grana per chloroplastic volume.

Sampling

Stereological sampling is evaluated statistically. The number of micrographs to be included within a unit statistical sample depends on the size and distribution of the components in the compartment being quantitated. The number of micrographs examined should give a plateauing standard error of the mean of $\leq 10\%$ to achieve reliable estimates (Bolender, 1978).

Because stereology is based on estimates of probability, measurements will give satisfactory results only on an unbiased sample. Rigorous sampling procedures are therefore used to ensure randomness. Unfortunately, cell tissue is often not evenly distributed throughout the sample. It has been shown that a well conceived procedure of systematic sampling, in which micrographs are taken over selected areas, usually provides sufficient randomness and yields a smaller error than a simple random sampling (Weibel & Bolender, 1973).

For optimal results, the selection of an appropriate magnification is also important. Generally, the original micrograph should be at the lowest magnification which still permits the clear distinction of the structures of interest. This will allow sampling over a maximum amount of tissue. If structures under investigation differ strongly in size, it might be necessary to separate the analysis into a series of stages using micrographs of increasing magnification.

Sources of Error

Stereological principles are based on the utilisation of two-dimensional images. Biological sections, however, are of finite thickness (60nm) and opaque structures may be overestimated, whereas translucent structure may be covered by more dense components and therefore underestimated. The phenomenon is known as the Holmes effect, (Toth, 1982) and states that a correction factor is needed, if the section thickness is more than 1/10th of the object diameter.

Furthermore, the fresh tissue may experience some alterations during the preparation for TEM. It has been well documented that fixation and embedding may shrink the cells to some degree (Toth, 1982). During sectioning, the compression of the resin is usually an unavoidable problem. However, dimensionless parameters such as V_V are usually not affected, since it is assumed that uniform shrinkage and compression have occurred within the sample.

5.1.4. Aims of the Chapter

In the absence of oxygen, the metabolism of cell organelles such as mitochondria and chloroplasts is seriously affected (see Chapter 3 and 4). The functioning of the organelles is intimately related to its ultrastructure. In this chapter, the ultrastructure of mesophyll cells from *A.calamus* plants grown under control conditions is compared with the ultrastructure of cells from plants which were incubated under anoxia for 28d. The study mainly concentrates on the effects of anoxia on the fine structure of mitochondria and chloroplasts. To separate the effects of anoxia and darkness on the ultrastructure, comparative studies have been carried out with *A.calamus* plants that were exposed to darkness for 28d.

5.2. Results

5.2.1. Tissue Preservation

Initially, leaf tissue from *A.calamus* and *V.macrocarpon* was prepared for TEM. But only *A.calamus* sections showed satisfactory preservation. In *V.macrocarpon* tissue, no coherent cytoplasm could be found, and the membranes of most cell organelles were disrupted. It is possible that the fixative could not penetrate the tough *V.macrocarpon* leaves rapidly enough for preservation. Also, the addition of 2% paraformaldehyde to the primary fixative did not give satisfactory results, and ultrastructural studies were therefore limited to *A.calamus* leaf tissue.

For the analysis of *A.calamus* leaves, tissue samples were taken about 5cm below the leaf tip (see Section 2.13). To ensure that the leaf tissue was not irreversibly damaged, only fresh green leaves without any visible injury were chosen for the study. Leaf tissue was examined from leaves of control plants (day zero of the treatment), after 28d under aerobic conditions in the dark (dark treatment), and after 28d under anoxic conditions in the dark (anoxia treatment). The prepared tissue from plants of all three treatments showed good ultrastructural preservation, indicated by the lack of shrinkage of cell contents from the cell wall.

5.2.2. Effects of Anoxia and Darkness on Mesophyll Cell Ultrastructure

The bulk of *A.calamus* mesophyll cells from all three treatments (control, darkness, anoxia) were characterised by a large central vacuole within a peripheral layer of cytoplasm that was appressed to the cell wall. Cell organelles did not appear to be degraded after 28d exposure to darkness or anoxia. Nuclei, chloroplasts and mitochondria were surrounded by intact double membranes in control, dark and anoxia treated leaf tissue. Additionally, ER, Golgi apparatus, microbodies and plasmodesmata could also be observed (Plate 5.2). Tissue from control and dark treated plants contained dense cytoplasm, and the tonoplast was intact in the examined micrographs. Only in a small proportion of anoxia treated samples was the tonoplast disrupted. The cytosol also seemed to be less dense in these samples.

5.2.3. Effects of Anoxia and Darkness on Mitochondrial Ultrastructure

The ultrastructure of mitochondria seemed to be stable under prolonged darkness and anoxia. Micrographs from mesophyll cells of all three treatments showed an intact double membrane envelope and well developed cristae (Plate 5.3). In the anoxia treated plants however, the central matrix of some mitochondria seemed to be less dense than in the control tissue.

Volume Fraction of Mitochondria (V_V)

Mitochondria occupied 5.45% of the cytoplasm in mesophyll cells of control plants of *A.calamus*. The dark treatment resulted in a minor reduction of mitochodrial V_V. In mesophyll cells of anoxia treated plants, mitochondrial V_V deceased further reaching about 80% of the control (Fig. 5.1a). However, both decreases were not statistically significant.

Transverse Area of Mitochondria (TA)

In the mesophyll cells of control plants, the average mitochondrial TA was $0.445 \mu m^2$. Anoxia and dark treatment induced a slight but not significant increase in the TA by 10% (Fig. 5.1b).

5.2.4. Effects of Anoxia and Darkness on Chloroplastic Ultrastructure

The most obvious difference between the tissue from control plants and the dark and anoxia treated *A.calamus* plants was the shape of the chloroplasts. Tissue from control plants contained lens-shaped organelles, while the dark treated tissue (under aerobic and anaerobic conditions) developed more spherical chloroplasts (Plate 5.2).

Small starch granules were found only in a few chloroplasts in control tissue, but not in the anoxia and dark treated ones. Plastoglobili were observed in chloroplasts from all three treatments, but they seemed to appear more frequently in the dark treated tissue.

Volume Fraction of Chloroplasts (V_V)

In the mesophyll tissue of *A.calamus* control plants, 55.07% of the cytoplasm was occupied by chloroplasts. The V_V of chloroplasts decreased slightly, but not significantly after the exposure of leaves to darkness under aerobic and anaerobic conditions (Fig. 5.2a).

Transverse Area of Chloroplasts (TA)

The average transverse area (TA) of chloroplasts was $5.49 \mu m^2$ in the mesophyll tissue of control plants. The exposure to prolonged darkness resulted in a significant increase in the TA of chloroplasts; under aerobic conditions the TA of chloroplasts increased by 56.2%, and under anaerobic conditions the TA increased by 44.0% (Fig. 5.2b). There were no significant differences between the TA of dark and anoxia treated plants.

Characterisation of Chloroplastic Thylakoids

Chloroplasts from all three treatments contained well developed grana and stroma thylakoids (Plate 5.4). In the control leaf tissue, the thylakoids usually traversed the chloroplast along its longest axis, and the chloroplasts were nearly completely filled with thylakoid membranes. In the spherical chloroplasts of dark and anoxia treated plants, a proportion of thylakoids became distorted.

Under anoxia, the proportion of stroma area was considerably increased (by 36%); and in the chloroplasts of dark treated leaves the stroma proportion also increased, but to a lesser extent than under anoxia (Tab. 5.1).

The total TA of grana in the chloroplast increased when the plants were exposed to anoxia, and the increase was even more pronounced in the dark treatment. In the proportion of granal thylakoids per chloroplast however, a minor decrease occurred under prolonged darkness and anoxia. This discrepancy can be explained by the fact that the TA of the whole chloroplast was increased by the anoxia and dark treatment.

In the dark treated leaves, there was a reduction in the number of granal sacs per granum. This indicates that the chloroplasts were occupied by an increased number of smaller grana stacks after 28d under aerobic conditions in the dark when compared with chloroplasts from control or anoxia treated plants (Tab. 5.1).

Table 5.1. Characterisation of chloroplasts from mesophyll cells of *A.calamus* plants
Plants were grown under control conditions, after 28d of darkness under aerobic conditions, and after 28d of darkness under anoxic conditions. For each treatment, a total of 20 TEM micrographs was examined at a magnification of x 15650. (mean ± SE); TA - transverse area.

	CONTROL	28d DARKNESS	28d ANOXIA
proportion of stroma in chloroplast (%)	33.52 ± 1.56	39.66 ± 1.53	45.61 ± 1.35
proportion of grana thylakoids in chloroplast (%)	29.28 ± 1.17	25.98 ± 1.12	23.37 ± 0.66
total TA of grana in the chloroplast (μm^2)	1.48 ± 0.13	2.17 ± 0.19	1.85 ± 0.13
granal sac number per granum	14.85 ± 0.72	9.25 ± 0.53	13.11 ± 0.39

In the tissue from dark and anoxia treated plants, the stroma thylakoids were easily visible between the granal stacks. In many chloroplasts from anoxia treated plants, stroma fragments with no connection to the grana were found. In the control tissue, stroma thylakoids were less well noticeable and seemed to be dilated.

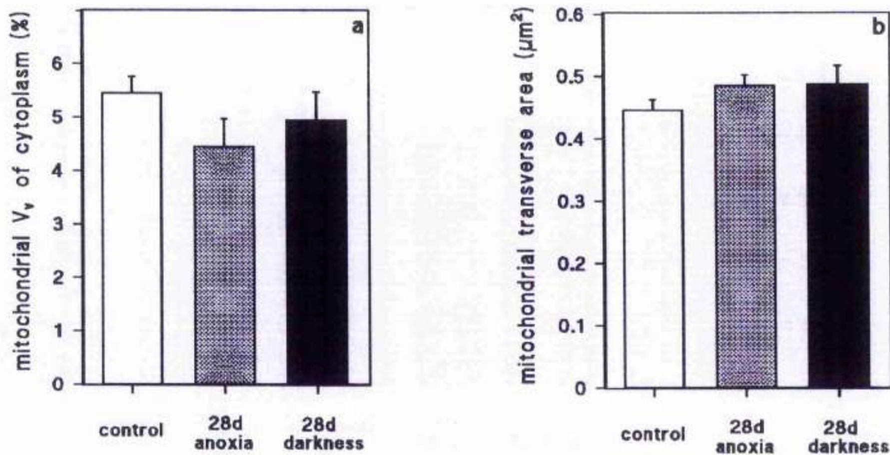


Figure 5.1. Mitochondrial volume fraction V_V (a), and transverse area (b) of mesophyll cells from *A.calamus* under long-term anoxia and darkness

Leaf samples were taken at day zero (control), after 28d of anoxia and after 28d of darkness. Tissue was fixed, embedded, sectioned, stained and analysed as described in Section 2.13. Each datapoint represents the mean of data obtained from the analysis of 20 TEM micrographs, error bars show standard error of the mean.

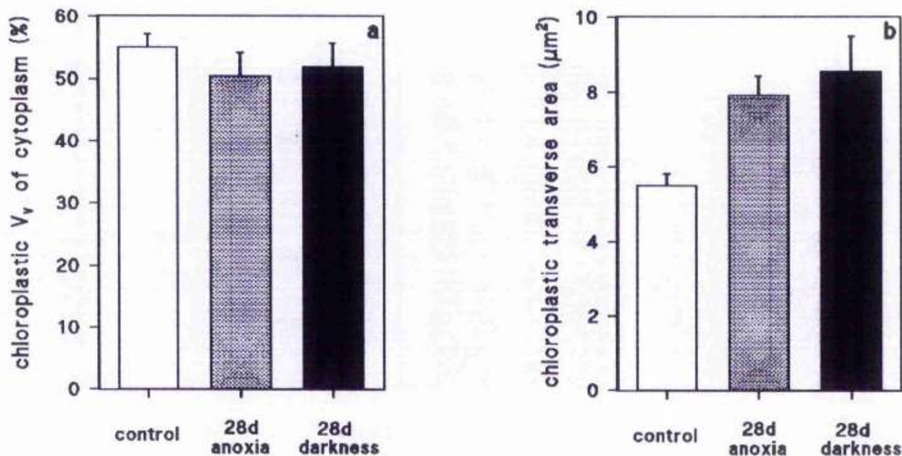


Figure 5.2. Chloroplastic volume fraction V_V (a), and transverse area (b) of mesophyll cells from *A.calamus* under long-term anoxia and darkness

Leaf samples were taken at day zero (control), after 28d of anoxia and after 28d of darkness. Tissue was fixed, embedded, sectioned, stained and analysed as described in Section 2.13. Each datapoint represents the mean of data obtained from the analysis of 20 TEM micrographs, error bars show standard error of the mean.

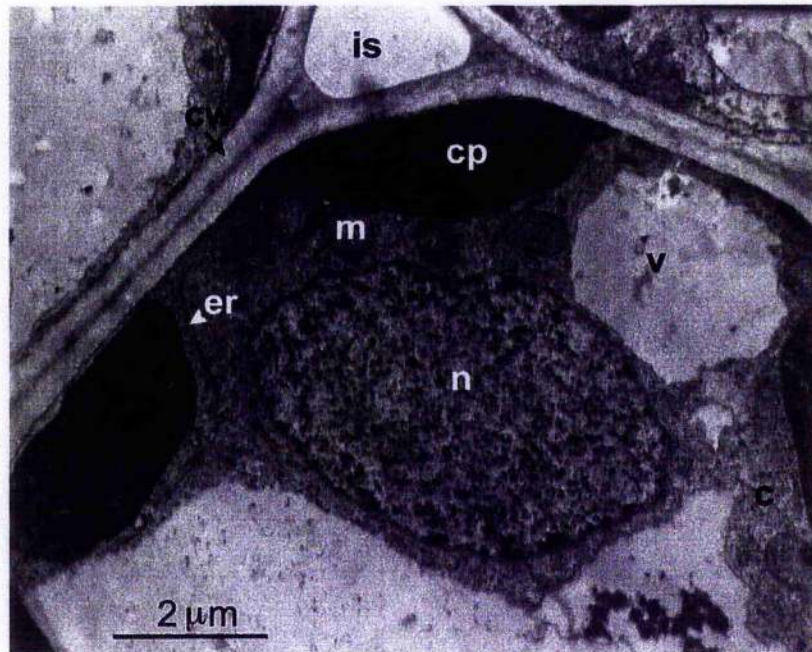


Plate 5.1. General mesophyll cell ultrastructure under the transmission electron microscope

Leaf tissue from *Acorus calamus* (control plant) was fixed, embedded, sectioned and stained as described in Section 2.13.

Abbreviations: c - cytoplasm, cp - chloroplast, cw - cell wall, er - endoplasmic reticulum, is - intercellular space, m - mitochondrion, n - nucleus, v - vacuole.

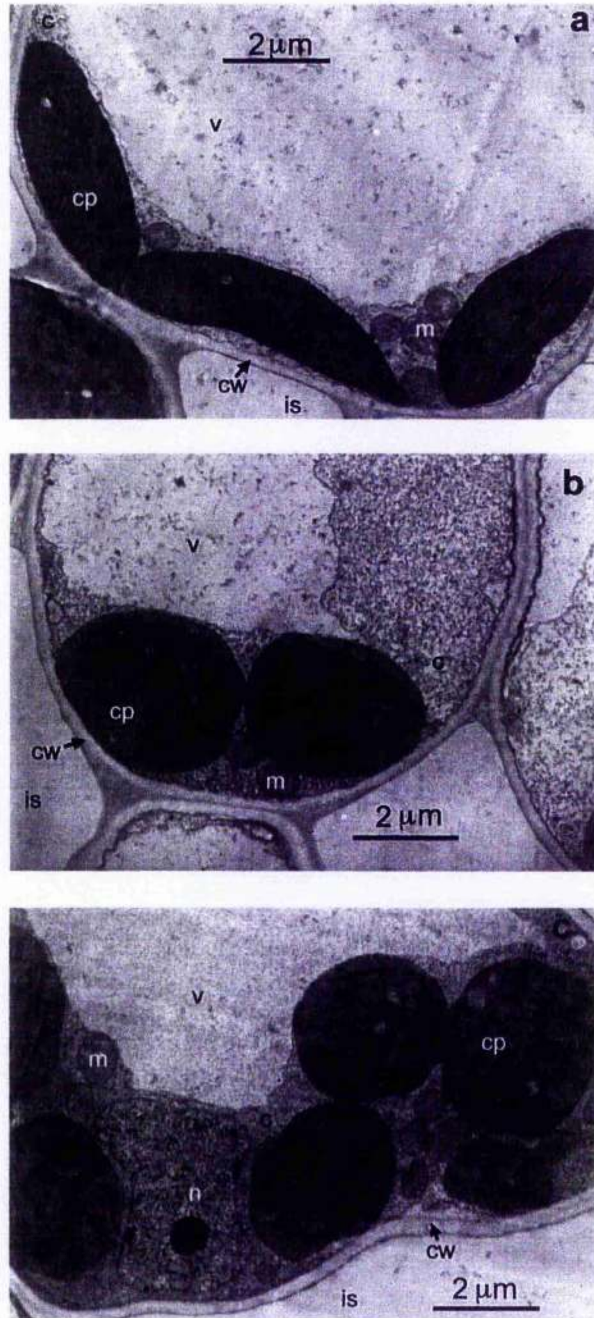


Plate 5.2. Transmission electron microscopy (TEM) of mesophyll cells from *A.calamus*

Leaf samples were taken at day zero (control) (a), after 28d of anoxia (b), and after 28d of darkness (c). Tissue was fixed, embedded, sectioned, stained and analysed as described in Section 2.13.

Abbreviations: c - cytoplasm, cp - chloroplast, cw - cell wall, is - intercellular space, m - mitochondrion, n - nucleus, v - vacuole.

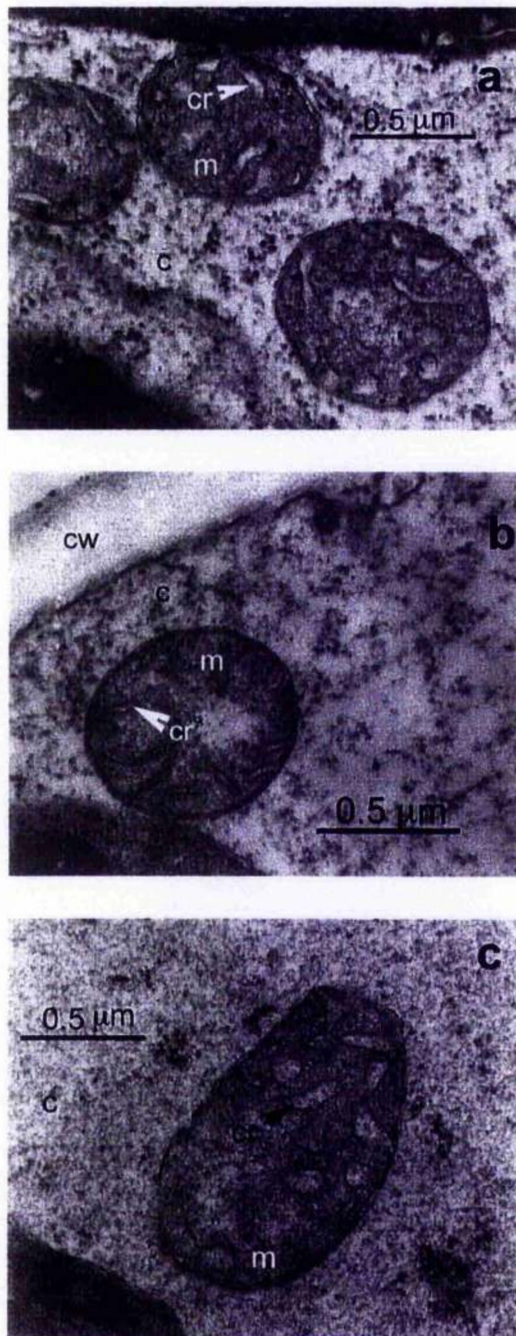


Plate 5.3. Transmission electron microscopy (TEM) of mitochondria from *A.calamus* leaf

Leaf samples were taken at day zero (control) (a), after 28d of anoxia (b), and after 28d of darkness (c). Tissue was fixed, embedded, sectioned and stained as described in Section 2.13.

Abbreviations: c - cytoplasm, cp - chloroplast, cr - cristae, cw - cell wall, m - mitochondrion.

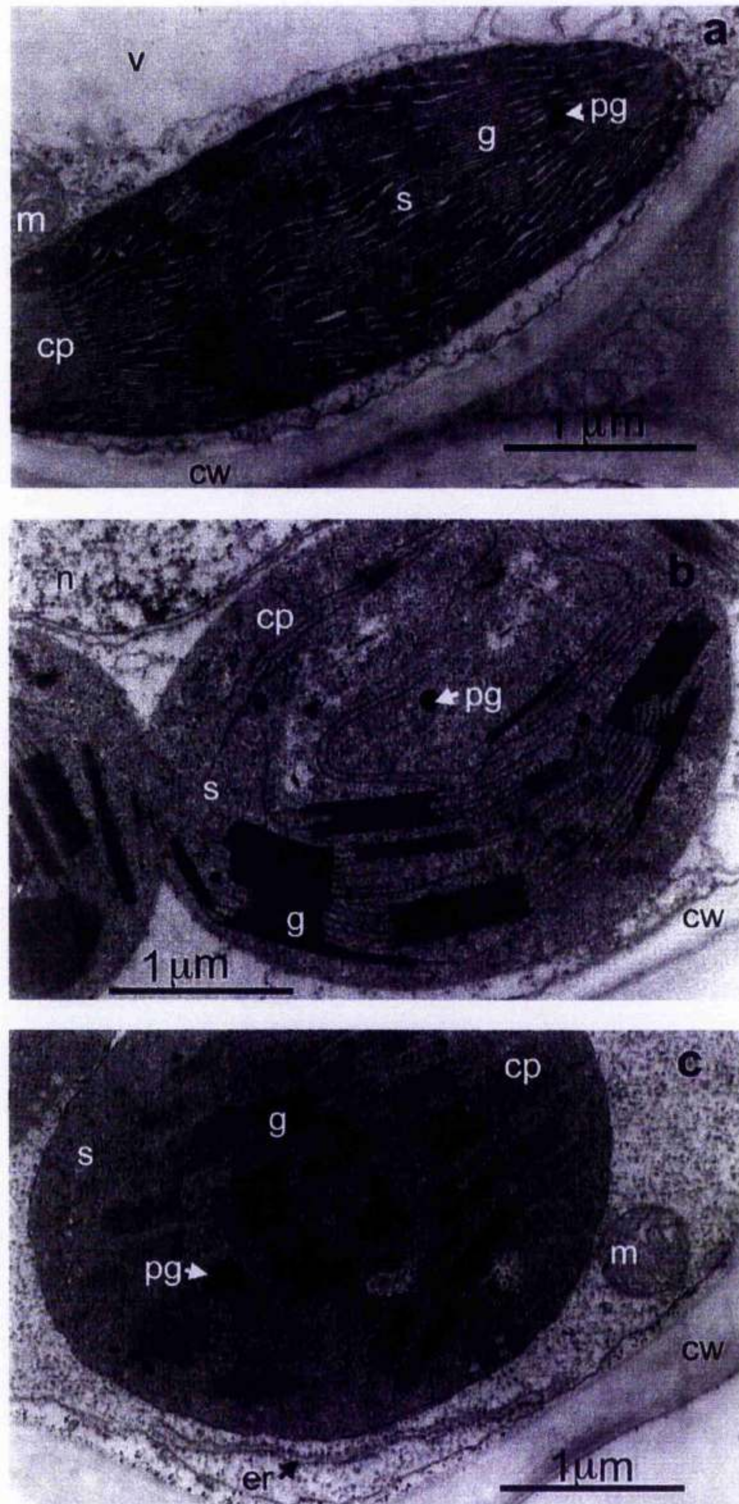


Plate 5.4. Transmission electron microscopy (TEM) of chloroplasts from *A.calamus* leaf
Leaf samples were taken at day zero (control) (a), after 28d of anoxia (b), and after 28d of darkness (c). Tissue was fixed, embedded, sectioned, stained and analysed as described in Section 2.13. Abbreviations: cp - chloroplast, cw - cell wall, er - endoplasmic reticulum, g - granal stack, m- mitochondrion, n - nucleus, pg - plastoglobulus, s - stroma, v - vacuole.

5.3. Discussion

5.3.1. Ultrastructure of Mesophyll Cells Under Long-term Anoxia

The ultrastructure of mesophyll cells in *A.calamus* experiences a number of modifications during long-term exposure to anoxia such as a decrease in the volume fraction of chloroplasts and mitochondria, swelling of the organelles, changes in the shape of chloroplasts, and modifications of suborganelle structure. However, most of the modifications are similar in dark treated plants under aerobic and anaerobic conditions indicating that prolonged darkness rather than the absence of oxygen itself is responsible for the ultrastructural changes.

Membranes

In the present experiment, the majority of cellular membranes remain intact even after 28d of anoxia. Considering the intensity and length of the applied anoxia stress, leaf cells of *A.calamus* seem to be characterised by extremely high membrane stability under anaerobic conditions. For comparison, membranes in anoxia sensitive potato cells start to degrade under anoxia after only 12h (Rawyler *et al.*, 1999).

In *A.calamus* leaves only a few cells showed disrupted tonoplasts after the anoxia treatment. From the extent of TEM analysis presented here however, it is not clear, if this disruption occurred in the anoxic plant or during the fixation process. In the tissue, the rupture of the cell vacuole would lead to acidification of the tissue. Because the tissue from the *A.calamus* leaf tip was examined close to its maximal survival time, it is possible that the destruction of the tonoplast indicated a first step of cell degradation under anoxia.

Membrane stability under environmental stress is highly dependent on their lipid and protein composition (see Section 5.1.2). Because desaturation requires oxygen (Schmid *et al*, 1997), anaerobic conditions usually lead to enhanced saturation levels of polar lipids. In rhizomes of the anoxia intolerant plant *Iris germanica*, the desaturation of membrane lipids declines rapidly under anoxia; and at the same time the content of gluco- and phospholipids decreases, and free fatty acid concentration increases indicating the degradation of cellular membranes (Henzi & Brändle, 1993). In *A.calamus* rhizomes on the contrary, a high proportion of unsaturated acids is preserved for up to 70d under anoxia, and little change occurs in the concentration of free fatty acids (Henzi & Brändle, 1993). High stability of lipid composition is possibly also responsible for the high anoxia tolerance of leaf cell ultrastructure in *A.calamus*.

The ability to preserve unsaturated fatty acids is also found in anoxia-tolerant *Echinochloa phyllopogon* seedlings (Kennedy *et al.*, 1991). Additionally, these seedlings continue to synthesise and incorporate lipids, thus providing the presumptions for membrane synthesis and cell growth under anoxia (Kennedy *et al.*, 1991). Therefore, lipids can also act as a hydrogen sink for the anaerobic metabolism.

Mitochondria

The metabolism of mitochondria as the site of oxidative phosphorylation is immediately affected by the absence of oxygen. It has been shown in Chapter 3 that the respiratory capacity of mitochondria experiences a considerable decrease by 80% after 28d of anoxia. In comparison with this, the effects of anoxia on the ultrastructure of mitochondria seem to be minor.

The TA of mitochondria increases only slightly under anoxia. A swelling of the organelle has also been described by a number of authors for anoxia tolerant and intolerant plants (Öpik, 1973; Vartapetian *et al.*, 1976; Vartapetian, 1991). Despite the increase in TA, the volume fraction of mitochondria is reduced in *A.calamus* under anoxia. This indicates a slight decrease in the number of mitochondria per cell and could result from the degradation of a small proportion of organelles under anoxia, e.g. by autophagy. The induction of autophagic processes is a common feature under conditions of carbon starvation and nutrient deficiency (Brouquisse *et al.*, 1998) and since the effects of anoxia and dark treatment on the V_V and TA of mitochondria are very similar, reduced sugar supply could be responsible for the degradation. However, the difference between the treatments are not significant and should not be overestimated.

Although quantitative morphometric analysis was not carried out on suborganelle structure, the micrographs indicate that there are no major differences in the organisation of cristae in mitochondria from control and anoxia treated leaves. The striking stability of mitochondrial ultrastructure under anoxia was also reported for anaerobic rice and *E.phyllopogon* seedlings (Kennedy *et al.*, 1980; Coueé *et al.*, 1992). Vartapetian *et al.* (1976) even found an increase in the cristae density of rice mitochondria under anoxia, and the cristae formed a characteristic parallel pattern. In the present study with *A.calamus*, such a parallel arrangement of cristae could not be found.

In contrast to the membrane stability found under anoxia in rice, *E.phyllopogon* and *A.calamus*, the degradation of mitochondrial ultrastructure is initiated rapidly in anoxia sensitive tissue. Mitochondria from wheat leaves are degraded completely after only 9h of anoxia (Vartapetian, 1991). The first structural changes become visible after a few hours of anoxia. The organelle swells, the number of cristae decreases and the matrix loses density. Prolonged anoxia leads to the formation of an electron-dense aggregation in the matrix. At this stage the damage is irreversible (Vartapetian *et al.*, 1986; Vartapetian, 1991).

Besides the lipids, mitochondrial membranes contain a high amount of structural and catalytic proteins. Despite the described stability of the mitochondrial membrane, the activity of most of the membrane bound enzymes is considerably reduced under anoxia even in tolerant species. In *A.calamus* leaves, cytochrome *c* oxidase lost 85% of its initial activity after 28d under anoxia (see Section 3.2.6). Marked decreases in the specific activity of membrane associated enzymes such as succinate dehydrogenase (Coueé *et al.*, 1992; Kennedy *et al.*, 1987) have also been found in anaerobic rice seedlings. This clearly

shows that the biochemistry of mitochondria is more affected by anoxia than its structure, and the decrease in the respiratory activity is caused by enzyme inactivation rather than structural damages. Generally, there is no obligatory relationship between cristae formation and the activity of associated enzymes (Öpik, 1973). Mitochondrial enzymes of the matrix (e.g. TCA cycle enzymes such as citrate synthase, 2-oxalglutarate dehydrogenase and fumarase), also express depressed activity in anaerobically grown *E.phyllopogon* (Kennedy *et al.*, 1991). However, TCA intermediates have been shown to be synthesised and accumulated under anoxia in a number tolerant species (see Kennedy *et al.*, 1992).

The experiments discussed above indicate that mitochondria of anoxia tolerant tissue possess high structural stability under anoxia, and that they are potentially functional although on a reduced level due to lowered enzyme activity. The high organelle stability would also explain the surprisingly fast recovery of respiratory activity of *A.calamus* leaves on return to air after long-term anoxia as described in Chapter 3.

Chloroplasts

It has been shown in Chapter 4 that the metabolism of chloroplasts is seriously affected by anoxia. After 28d of anoxia, the photosynthetic capacity of *A.calamus* leaves is only 15% of its original level. The presented micrographs reveal that the photosynthetic decline is accompanied by moderate structural modifications. Because chloroplast metabolism is highly dependent on illumination, the absence of light during the anoxia treatment is also likely to influence the ultrastructure of the organelle.

The most obvious difference is the change of chloroplast shape from lens-shape in the control to spherical in the anoxia sample. The same phenomenon however is found in the dark treated sample, suggesting that the absence of light rather than anoxia is responsible for the shape change. Concomitantly, the chloroplast TA of anoxia and dark treated plants increases, while the perimeter of the chloroplast cross sections remains more or less constant indicating that the envelope membrane did not experience any major changes and that the increase in TA is mainly caused by the modified shape.

The volume fraction of chloroplasts is only slightly reduced by the anoxia treatment. Considering that the TA of the organelle increased at the same time, it may indicate that the number of chloroplasts is reduced by the anoxia treatment. Comparisons however, are difficult to interpret because of the simultaneous change of shape. The possibility of autophagic processes initiated by limited sugar supply under anoxia stress (Elamrani *et al.*, 1994; Brouquisse *et al.*, 1998) has already been discussed for mitochondria.

The accumulation of starch granules in the chloroplasts is low, even in the control tissue of *A.calamus* leaves. In the anoxia or dark treated tissue, starch granules disappear completely. These results are in agreement with the generally low content of non-soluble carbohydrate measured in the *A.calamus* leaf (see Chapter 3, Section 3.2.3) and underline

the hypothesis that assimilated starch is preferably transported into the rhizome and not accumulated to a great extent in the leaf.

The suborganelle structure of chloroplasts seems to be particularly sensitive to environmental changes. Drought, enhanced UV-B, nutrient deficiency and extreme temperatures can cause very rapid changes in the proportion of grana and stroma thylakoids of chloroplasts (see Section 5.1.2). Protein phosphorylation has been shown to play an important role in the distribution and relative efficiency of PSI and PSII. But the mechanisms behind the formation of granal stacks are still under dispute (Stys, 1995). Two main theories exist. According to the surface-charge theory, the membrane lamellae are attracted by van der Waals' type forces and repulsed by electrostatic forces. Therefore, electrostatic repulsion is shielded by cations, and the surface charge is lower in appressed than in unappressed regions. The alternative theory is based on the molecular recognition and specific interaction between thylakoid proteins (for review see Stys, 1995). Additionally, lipid-lipid interactions and the lipid composition of thylakoids are supposed to be involved in the formation of granal stacks (McCourt *et al.*, 1987; Hugly *et al.*, 1989; Stys, 1995). Chloroplast membranes usually contain a very high proportion (80%) of polyunsatutated fatty acids. As shown before, the desaturation of lipid acids is inhibited in the absence of oxygen and long-term anoxia can lead to enhanced lipid saturation. Arabidopsis mutants with reduced levels of unsaturated lipid has been studied lately (McCourt *et al.*, 1987; Hugly *et al.*, 1989; Wu *et al.*, 1997), and the mutant has been shown to possess reduced chloroplastic TA and decreased amount of grana membranes, while the amount of stroma membranes remained constant (Hugly *et al.*, 1989). This does not mirror the changes observed in the anaerobically treated chloroplasts of *A.calamus,* and it is unlikely that the desaturation level of thylakoids was the main cause for the structural modifications.

In the present experiment, the amount of thylakoid membranes in the chloroplast seems to be more or less constant under anoxia. The increase in the chloroplast size is mainly caused by an increase in the stroma phase. The quantitative analysis of granal thylakoids suggests that the amount of appressed thylakoids increases slightly in the anoxic chloroplast; and the increase is similar under dark treatment. The stromal thylakoids are not estimated quantitatively, but the micrographs indicate that the amount of stromal thylakoids between the grana is reduced under anoxia. The enhancement of grana in relation to stroma thylakoids is usually a common phenomenon in leaves under low light conditions (Anderson *et al.*, 1973) but in has also been observed in other environmental stress situations such as enhanced UV-B (Fagerberg & Bornman, 1997). More detailed investigations however would be necessary to confirm the results about the effects of anoxia on thylakoid stacking.

One of the main components of chloroplast thylakoids is chlorophyll. *A.calamus* leaves lost 25% of their chlorophyll during the 28d anoxia treatment, but this did not seem to have

any major influence on the amount of thylakoids in the chloroplast. This is in agreement with the study of Fagerberg & Bornman (1997), who showed that there was no obligate relationship between the thylakoid surface area and the chlorophyll content in UV-B treated *Brassica napus* leaves. As discussed in Chapter 4, the chlorophyll concentration is also not primarily responsible for the marked decrease in the photosynthetic activity of leaves under anoxia. Considering this and the relative stability of chloroplast membranes under long-term anoxia in *A.calamus* leaves, the decrease in the photosynthetic capacity is most likely to be caused by damages to the protein part of the photosystems. On return to air, repair of the photosynthetic apparatus starts immediately and the photosynthetic capacity in *A.calamus* leaves is re-installed rapidly (see Chapter 4). The repair of chloroplasts with considerable damage to their membranes on the other hand, appears only slowly and is often not complete (Wu *et al.*, 1997).

5.3.2. Comparison of the Effects of Anoxia and Darkness on Mesophyll Cell Ultrastructure

In the present experiment, prolonged darkness and prolonged anoxia affect the mesophyll cell ultrastructure of *A.calamus* leaves in a similar manner. In both treatments, chloroplasts change their shape and their TA increases, mitochondria show only minor modifications in both treatments. This suggests that the ultrastructural modifications are not primarily caused by the absence of oxygen, but are influenced by concomitant circumstances such as lack of illumination and altered sugar concentrations.

The development and metabolism of chloroplasts is especially dependent on the quality and quantity of light. Higher plants contain at least four types of photoreceptors: blue light photoreceptors and three types of red light photoreceptors such as phytochrome, protochlorophyllide and chlorophyll (Anderson, 1986). These photoreceptors influence nearly all phases of chloroplast development, and as discussed for Rubisco in Chapter 4, the synthesis of many chloroplastic compounds is impaired in the dark. Light is also very likely to be responsible for the orientation of thylakoids along the longest chloroplastic axis in the control leaf, thus allowing maximal light absorption. In the absence of light, the thylakoids become disorientated, and the organelle changes its shape to a more spherical form. The light dependent orientation of chloroplasts in the cells has been investigated in a number of algae and plant species, but the underlying mechanisms are still under discussion (for review see Haupt, 1982).

One of the few differences in the suborganelle structure of chloroplasts from dark and anoxia treated leaves seems to be the distribution of granal stacks. Chloroplasts from dark treated leaves rearrange their thylakoids into an increased number of smaller granal stacks shown by the decrease in the number of sacs per granum while the total TA of grana per chloroplast increases. In the anoxia treated leaf on the contrary, thylakoids seem to maintain their organisation into few granal stacks with a higher number of granal sacs.

However, more detailed studies would be necessary to confirm the general validity of these findings.

Furthermore, chloroplasts from dark treated *A.calamus* leaves appear to contain an increased number of plastoglobuli. Plastoglobuli are aggregations of lipids or proteins in the chloroplast. Their number and size usually rises as soon as thylakoids start to become degraded, e.g. during leaf senescence (Bondada & Oosterhuis, 1998). As shown in the previous chapters, prolonged dark treatment of leaves initiates the breakdown of proteins and chlorophyll, and the enhanced appearance of plastoglobuli under prolonged darkness represents the accumulation of degradation products. In the anoxia treated leaves, plastoglobuli occur less frequently. This would be in agreement with the hypothesis discussed before that the synthesis of degrading enzymes might be impaired in the absence of oxygen.

Secondary effects of the prolonged dark and anoxia treatment are changes in the carbohydrate metabolism of the leaves. Carbon starvation affects all parts of the cell and lowers the stability and functioning of membranes (see Brouquisse *et al.*, 1998). It has been shown that the exogenous supply of glucose can improve the viability and membrane stability of plant cells under prolonged darkness (Elamrani *et al.*, 1994) and anoxia (Vartapetian & Andreeva, 1976). In *A.calamus* leaves, the content of total soluble carbohydrates declines only slightly under prolonged anoxia and darkness (see Chapter 3) and the stable sugar content is probably one important precaution for membrane stability under stress. Furthermore, it has been shown that a certain rate ATP synthesis is probably required for the preservation of cellular membrane integrity under anoxia (Rawyler *et al.*, 1999).

In summary, the results presented above show that *A.calamus* leaves are characterised by a very high structural organelle stability under anoxia. Although the biochemistry of mitochondria is strongly affected by the absence of oxygen, their membranes remains stable for at least 28d of anoxia. This phenomenon represents probably one of the great advantages of this species on return to air, when respiratory activity is rapidly regained.

The effects on the ultrastructure of chloroplasts seem to be more related to the lack of light than to the lack of oxygen. Chloroplastic membranes also maintain high stability under prolonged anoxia and darkness. Differences in the photosynthetic capacity of dark and anoxia treated leaves seem to be caused by changes in the biochemistry of the organelle rather than its structure.

Membranes are usually the susceptible target for damage by reactive oxygen species. Although the effects of post-anoxia are not investigated in this study, the immediate and constant increase in respiration and photosynthesis indicate that the *A.calamus* leaves possess a well developed defence system and post-anoxic damage to the membranes was only minor.

Chapter 6

General Discussion

6.1. Leaf Anoxia Tolerance in *A.calamus*, *I.pseudacorus* and *V.macrocarpon*

For the metabolic investigations, plant species were chosen which tolerate oxygen deficiency in their natural habitat (see Dana & Klingbeil, 1966; Hanhijärvi & Fagerstedt, 1994; Weber & Brändle, 1996). *A.calamus*, *I.pseudacorus* and *V.macrocarpon* proved to be very suitable objects for the study of leaf anoxia tolerance. Leaf material from all three species survives for at least 28d under anaerobic conditions, and on return to air, metabolic recovery occurs. Some species-specific differences could be detected in the metabolic responses and the maximal survival time under anoxia.

***Acorus calamus* L.** Among the investigated species, leaves of *A.calamus* clearly possess the greatest tolerance to anoxia. Leaves of this species sustain anaerobic conditions longer than the other two species. The anoxia tolerance of leaves is therefore very stable (96% survival with intact leaves after 28d anoxia). The anaerobic metabolism in *A.calamus* leaves is characterised by efficient utilisation of carbohydrates from the rhizome, maintenance of a certain level of respiratory and photosynthetic capacity, and stability of cellular membranes. On return to air after 28d anoxia, complete recovery of respiration and photosynthesis takes place rapidly.

***Iris pseudacorus* L.** The general appearance of *I.pseudacorus* is similar to *A.calamus*, but the leaves are less tolerant under long-term anoxia. The survival rate of leaves is still high (86% survival with intact leaves after 28d of anoxia), but damage is visible in the older leaves and the tips of the younger ones. Respiratory and photosynthetic capacity of the leaves decline constantly under anoxia and are close to the detection limit after 28d of anoxic incubation. Notably at the start of the treatment, carbohydrate consumption of the plant is high. When the plants return to air, leaf metabolism starts to recover only after a lag phase; recovery seems to be incomplete, and accelerated senescence occurs under post-anoxia.

***Vaccinium macrocarpon* Ait.** The habit of the dicot *V.macrocarpon* differs from the other two species, and the plant does not possess a storage organ similar to rhizomes. However, considerable amounts of carbohydrates are stored in the one-year-old leaves. Survival of the whole plant appears to be closely related to the anoxia tolerance of leaves, and eventually stems of *V.macrocarpon* sustain anoxia for only few days longer than the leaves. About 80% of the plants survive 28d of anoxia with intact leaves. The metabolic activity in the anaerobic leaves is low but stable, and internal carbohydrate reserves are mobilised. On return to air, the leaves regain their respiratory and photosynthetic capacity more slowly than *A.calamus*, but recovery is complete after about 10d post-anoxia.

6.2. Metabolic Adaptation to Anoxia in Leaves

In the absence of oxygen, the energy generation via oxidative phosphorylation is interrupted and has to be replaced by much less efficient anaerobic processes. The facility for the initiation of anaerobic energy production is present in all plant cells and no specific pathway associated with anoxia tolerant tissue has been detected. Differences in anoxia tolerance of plant species and plant organs have been mainly attributed to variations in their capacity for metabolic regulation under anoxia (Brändle, 1991). The present results underline the importance of metabolic regulation for the survival under anaerobic conditions. For nearly all investigated metabolic parameters, leaves from the three species react in a similar way, and differences appear to be mainly quantitative. In the end however, these differences in metabolic control could be crucial for extending the duration of survival under anoxia.

Ethanol fermentation. Ethanol fermentation has been shown to be the major pathway of anaerobic energy generation in anoxia tolerant and anoxia intolerant tissues (Monk *et al.*, 1984; Good & Muench, 1993; Drew, 1997). Ethanol fermentation is also active in all investigated plant organs of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* as indicated by the accumulation of ethanol under anoxia. This is in agreement with the results of Monk *et al.* (1984), who found ethanol fermentation to be the major source of energy production in the rhizomes of various species including *A.calamus* and *I.pseudacorus*. In the present experiment, highest amounts of ethanol are found in the shoots, suggesting that fermentative rate in this tissue is comparatively high. The release of volatile end-products of anaerobic metabolism via the shoot (Crawford, 1992) might also contribute to the ethanol accumulation in the leaves. After about one week of anoxia however, an equilibrium between ethanol production and release is reached in the plant organs preventing the accumulation of potentially damaging amounts of ethanol (Crawford *et al*, 1987; Monk *et al.*, 1984).

Energy metabolism. When oxygen concentration falls below the affinity level of cytochrome *c* oxidase, anaerobic energy production has to be available in the short-term to satisfy the requirements of cell maintenance. In some intolerant tissues, the greatly reduced ATP turnover fails to provide enough energy for the installation of anaerobic processes. Hypoxic acclimatisation therefore improves the adaptation of some intolerant tissues to anoxia (Johnson *et al.*, 1989; Hole *et al*, 1992; Andrews *et al.*, 1994; Johnson *et al.*, 1994).

In the present experiment, leaves from *A.calamus*, *I.pseudacorus* and *V.macrocarpon,* which can withstand anoxia without hypoxic acclimatisation, exhibit very low energy requirements, and these make minimal demands on the metabolic shift from aerobic to anaerobic processes. This is supported by low anaerobic CO_2 production in the leaf tissue

immediately after the transfer to anoxia, and the absence of a strong Pasteur effect. Low metabolic requirements are also found in other anoxia-tolerant tissues such as the rhizomes of wetland plant species (Crawford, 1978; Monk *et al.*, 1984; Weber & Brändle, 1996). The leaves studied here obviously re-equilibrated their metabolism under anoxia by an overall reduction of energy consumption rather than by acceleration of glycolytic energy production. In this way, anoxia tolerant tissue manages to stabilise the energy metabolism rapidly under anoxia, and carbohydrate consumption remains low.

It has been shown that the AEC in *A.calamus* rhizomes drops only moderately at the start of anoxia, but re-equilibrates soon to nearly aerobic levels. In anoxia-intolerant tissue on the contrary, the AEC fails to recover after the sudden drop under anoxia (Sieber & Brändle, 1991). Under long-term anoxia, the AEC in *A.calamus* and *I.pseudacorus* rhizomes stabilises at slightly reduced levels around 0.5-0.6 (Joly & Brändle, 1995; Hanhijärvi & Fragerstedt, 1995), what is demonstrably sufficient for the maintenance of all essential cell functions (e.g. prevention of cytoplasmic acidosis) and is achieved at lower AEC levels in anoxia tolerant tissue.

Protein synthesis. The reduction of energy consumption under anoxia is mainly achieved by a general decrease in biosynthetic activity and cessation of growth processes. Nevertheless, a certain amount of energy is required for the synthesis of enzymes and to maintain all essential cell functions. Enzymes involved in anaerobic energy production, e.g. PDC and ADH, are usually available only in trace amounts in the aerobic cell (see Section 3.2.6), and have to be newly synthesised under anoxia.

In the present experiment, the PDC activity in *A.calamus* leaves is low under control conditions, but synthesis is initiated immediately after the onset of anoxia, as shown by the sevenfold increase in the first 24h. PDC activity continues to rise for at least 14d under anoxia. These results are in accordance with the findings of Bucher & Kuhlemeier (1993) who report a rapid increase of mRNA for PDC in *A.calamus* leaves under anoxia as a prerequisite for the production of the enzyme.

An increase in the synthesis of proteins involved in anaerobic energy production has been described for anoxia tolerant and anoxia intolerant species. The protein synthesis under anoxia has been particularly well investigated in anoxia sensitive maize roots. After the onset of anoxia, only 20 proteins (ANP) account for the bulk of proteins synthesised, and continue to be synthesised in the same ratio until death of the roots after about 70h of anoxia (Sachs *et al.*, 1980). This is different to the anaerobic response in anoxia tolerant tissue such as *E.phyllopogon* seedlings. The synthesis of anoxia related proteins peaks only at the beginning of the anoxia treatment and returns thereafter to a protein pattern similar to aerobic conditions (Kennedy *et al.*, 1992; Mohanty *et al.*, 1993; Mujer *et al.*, 1993). It is likely that the leaves investigated here react in a similar way. This would offer some explanation for the absence of visible changes in the protein pattern with SDS-PAGE, and

the stability of COX activity and Rubisco content found in the anaerobic *A.calamus* leaf. The continuation of 'normoxic' protein synthesis - although on a reduced level - has already been shown for the *A.calamus* rhizome (Weber & Brändle, 1996).

Interestingly, the anoxia-related increase in the activity of anaerobic proteins such as ADH is often more pronounced in anoxia-intolerant plants than in tolerant ones. The phenomenon was initially described by McManmon & Crawford (1971). It has been shown since that there is no obligate relationship between ADH activity and ethanol production and that ADH activity often greatly exceeds its requirement in the anaerobic cell. It is possible that a considerable part of energy and substrate is 'wasted' in anoxia intolerant tissues for the overexpression of ADH, while the synthesis of ADH in the tolerant tissue is better adjusted to the actual requirements in the cell, thus also allowing the synthesis of a wider range of proteins under anoxia despite the low energy turnover.

Carbohydrate availability. To keep anaerobic energy production running, fermentable substrates have to be available. The internal sugar content of cells is usually limited and a continuous supply of sugar is necessary for maintenance of metabolism (Hanhijärvi & Fagerstedt, 1995). In leaves of *A.calamus* and *I.pseudacorus,* the soluble carbohydrate content remains relatively constant under anoxia. In contrast to rhizomes, leaves of *A.calamus* and *I.pseudacorus* do not possess large internal reserves. It has been reported that transport mechanisms and phloem unloading might be inhibited under anoxia (Saglio, 1985; Waters *et al.*, 1991a); but although carbohydrate transport from the rhizome to the shoot has not been investigated directly in the presented experiments, stable carbohydrate levels in the leaves of *A.calamus* and *I.pseudacorus* suggest that transport mechanisms for carbohydrates from the rhizome continued to work under anoxia, thus guaranteeing the stability of the sugar level in the leaf.

In *V.macrocarpon* internal starch resources are utilised under anoxia, and the non-soluble carbohydrate content of the leaves declines gradually under anoxia, with the level of soluble carbohydrates in the leaf remaining high. The functioning of carbohydrate breakdown in *V.macrocarpon* leaves is supported by the findings of stable α-amylase activity under anoxia.

Besides the provision of substrate for anaerobic energy generation, the maintenance of a certain sugar level is also important for the stability of cellular membranes (Vartapetian *et al.*, 1976; Brouquisse *et al.*, 1998). In *V.macrocarpon,* the younger leaves died more rapidly under anoxia than the older ones. One possible reason for this might be the imbalance between high metabolic activity and low carbohydrate content in the younger leaves. In the two monocot species on the contrary, the older leaves died first. This could also be due to impaired carbohydrate supply, as the younger leaves represented the stronger sink tissue under anoxia.

6.3. Stamina of Leaves Under Long-term Anoxia

Studies on anoxia tolerance in plants are mainly restricted to short-term experiments and as a consequence much less is known about long-term metabolic responses. Once anoxia tolerant plants have adjusted their metabolism to anaerobic conditions, a number of internal and external factors influence the stamina under anoxia. None of the investigated plants survived long-term anoxia entirely without damage. Access to stored carbohydrates is essential to keep fermentation running under long-term anoxia. The size of the carbohydrate store and the speed of its utilisation can dictate the plant survival limit under anoxia.

Two slightly different strategies seem to occur in anoxia tolerant shoots. Seedlings of rice, a few *Echinochloa* species (Kennedy *et al.*, 1992; 1993), and shoots of some rhizomatous wetland species (Barclay & Crawford, 1982; Brändle & Crawford, 1987), continue to elongate under anoxia. This is accompanied by relatively high rates of fermentation and substrate consumption, and in the end substrate availability limits survival under anoxia. Longer viability is found in plant shoots that sustain anoxic conditions in a quasi-dormant state characterised by cessation of all growth processes and a considerable decrease in overall metabolic activity. The remarkable anoxia tolerance in the leaves of *A.calamus*, *I.pseudacorus* and *V.macrocarpon* is achieved by the second strategy.

Efficiency of carbohydrate consumption. The three species investigated possess considerable amounts of stored carbohydrates and thus fulfil one precondition for long-term anoxia tolerance. Under anoxia, the carbohydrate content of the plants declines gradually. Noticeable is that *I.pseudacorus* mobilises its stored carbohydrates more rapidly than *A.calamus*, especially in the first weeks after the onset of anoxia. The more economic consumption of carbohydrates in *A.calamus* corresponds with its greater tolerance of anoxia. The importance of efficient carbohydrate utilisation is also demonstrated by the comparison of carbohydrate consumption in germinating rice and *E.phyllopogon*. Although *E.phyllopogon* seedlings are provided with smaller amounts of carbohydrates, their economic utilisation allows them in the end to survive anoxia longer than the less efficient rice seedlings (Fox *et al.*, 1994). The processes responsible for enhanced efficiency of substrate consumption under anoxia are still unknown; the operation of alternative pathways substituting PPi instead of ATP (see Stitt, 1998), the partial activity of the TCA cycle (Kennedy *et al.*, 1991; 1992) and the utilisation of NO_3^- as the terminal electron acceptor (Kennedy *et al.*, 1987; Fox *et al.*, 1994) have all been suggested. During the course of the present anoxia experiment, the carbohydrate consumption was not constant but decreased while the amount of damaged leaf material increased. After the death of the leaves the rhizome continued to survive with very low carbohydrate turnover, suggesting

that the maintenance of the rhizome required less energy than the maintenance of leaf material.

The mechanisms behind the regulation of anaerobic energy production under long-term anoxia are still not well understood. The fact that PDC catalyses the branch point reaction for ethanol fermentation and that PDC activity is usually lower than ADH activity, led to the conclusion that PDC plays a key role for the regulation of fermentation under anoxia (Waters *et al.*, 1991b; Drew *et al.*, 1994; Drew, 1997). This might be true for the first phase directly after the onset of anoxic conditions when PDC availability is limiting. The results presented here clearly demonstrate however that under long-term anoxia, CO_2 production in *A.calamus* leaves decreases in spite of high levels of PDC activity. This is in agreement with the results obtained for beetroot storage tissue by of Zhang & Greenway (1994). The fermentative activity under long-term anoxia is therefore more likely to be regulated by other glycolytic processes. Regulation of the glycolytic flux depends on complex control mechanisms (Dennis *et al.*, 1997), and maintenance of these fine control mechanisms could contribute to metabolic stability under long-term anoxia.

Maintenance of mitochondria. In the absence of oxygen, mitochondria are usually regarded as non-operating. More recently it has been shown by Kennedy *et al.* (1987; 1991) that mitochondrial enzymes are synthesised in anaerobic *Echinochloa* seedlings. Parts of the mitochondrial metabolism such as the TCA cycle may be active under anoxia and it is therefore assumed that mitochondria can play an active part in anaerobic metabolism (Kennedy *et al.*, 1991; 1992). A similar phenomenon has been found in anoxia tolerant animal tissue (Hochachka, 1991).

In *A.calamus* leaves, the structure of mitochondria is surprisingly well preserved under anoxia. The capacity of the respiratory machinery declines gradually under prolonged anoxia in leaf tissue of all three investigated species. This appears to be caused mainly by deactivation of the enzymes involved. The development of respiratory capacity in anaerobic leaves seemed to be closely related to COX activity. After 28d of anoxia, respiratory capacity is reduced in leaves of *A.calamus* and *V.macrocarpon* to 20%, and in *I.pseudacorus* to 5-10% of its initial value (Chapter 3). Considering the length of the anaerobic incubation, this suggests a high enzyme stability. It is also possible that COX synthesis continues under anoxia, but the generally reduced biosynthetic activity did not allow maintenance of full capacity. The synthesis of COX and other parts of the respiratory chain have been shown to be active in anaerobic rice and *E.phyllopogon* seedlings (Öpik,1973; Kennedy *et al*, 1987; Coueé *et al.*, 1993).

Maintenance of chloroplasts. The consequences of prolonged anoxia, such as energy limitation, changes in the sugar level and impaired biosynthetic activity, would have considerable effects also on cell organelles which are not as directly dependent on oxygen

as the mitochondrion. For the first time, the effects of long-term anoxia on the chloroplasts have been investigated. Because the plants were additionally kept in the dark, it was difficult to assess to what extent the alterations have been caused by anoxia or darkness. Photosynthetic capacity of the leaves is however significantly more affected by the anoxia than the dark treatment. After 28d of anoxia the photosynthetic capacity decreases in *A.calamus* to 17%, in *I.pseudacorus* to 3% and in *V.macrocarpon* to 20% of its initial level (Chapter 4). The reduction in photosynthetic capacity seems to be related only to a minor extent to modifications in the chlorophyll content of the leaves. However, damage does occur under long-term anoxia in the PSII reaction centre and in subsequent electron transport. The damage to PSII appears to be anoxia-related and is not observed under long-term darkness. Because chloroplastic proteins seem generally sensitive to environmental stress (Long *et al.*, 1994; Yamane *et al.*, 1997; 1998), it is assumed that instability of proteins in the PSII complex is responsible for the damage. Chloroplastic membranes are relatively stable under anoxia and only slight modifications have been detected in the organelle ultrastructure (Chapter 5).

Age and external factors. The stamina of individual leaves under anoxia is also greatly influenced by the age of the stressed leaves. In *A.calamus* and *I.pseudacorus*, older leaf tissue is significantly less tolerant to anoxia than that of the younger leaves. Enhanced sensitivity of older leaves under environmental stress is very common in fast growing plants (Weaver *et al.*, 1998). Death of leaf tissue under anoxia, did not follow the common pattern occurring during natural senescence. In slow growing *V.macrocarpon*, stress tolerance of immature leaves appears to be reduced.

Furthermore, seasonal changes and cultivation history of the individual plant considerably influences tolerance to anoxia (Waters *et al.*, 1991a). Every pre-anoxia condition that weakens the plant or lowers its carbohydrate content such as low light, high temperature, nutrient deficiency or infection, would reduce the duration of survival under anoxia. Because roots die quickly under anoxia, the nutrient uptake of the plant is impaired, and storage of N-rich components such as amino acids is advantageous for long-term tolerance to anoxia (Haldemann & Brändle, 1988).

6.3. Post-Anoxia - Recovery after the Stress

As shown before, leaf capacity for respiration and photosynthesis is considerably reduced by long-term anoxia. On return to air and light, the fast re-installation of these processes would be of advantage to the plant. Among the investigated species, *A.calamus* clearly shows the fastest recovery under post-anoxia. As discussed before, the metabolism of this species copes remarkably well with the extreme conditions of anoxia, and it is possible that

the comparatively low degree of damage enables the rapid metabolic recovery of the leaves. Although the biochemistry of the cells is altered under anoxia, cell membranes remain intact for 28d of anoxia. The leaves of *A.calamus* also appear to possess a well developed oxidative defence system, and no post-anoxic injury is visible in this species.

The metabolism of *I.pseudacorus* leaves is affected by long-term anoxia to a greater extent than in *A.calamus,* and the weakened condition on return to air could be responsible for the lag phase in the metabolic recovery. Post-anoxic damage in the PSII centre could be caused by the activity of oxygen reactive species. The enhanced sensitivity of plant material to oxygen stress after periods of anoxia has been demonstrated in roots and rhizomes (Wollenweber-Ratzer & Crawford, 1994; Albrecht & Wiedenroth, 1994a; Biemelt *et al.*, 1998). The impact of oxidative stress is thought to be even stronger in green leaf tissue as the formation of many ROS is associated with light reactions in the chloroplast (Elstner & Osswald, 1994). Further investigations are necessary to distinguish between anoxic and post-anoxic damages in the leaf. After the initial lag phase, the photosynthetic apparatus in the *I.pseudacorus* leaf regains activity reaching 80% of its original level after 10d post-anoxia (Chapter 4). The repair of the respiratory machinery occurs more slowly and is less complete under post-anoxia, indicating that *I.pseudacorus* mitochondria may have been damaged to a greater degree than the chloroplasts.

V.macrocarpon leaves recover slowly but completely under post-anoxia. In contrast to the two investigated monocot species which replace their damaged leaves relatively rapidly by new leaves, anoxia-stressed *V.macrocarpon* leaves remain with the plant and serve as a carbohydrate source for at least another year.

6.4. Conclusions

The metabolic survival strategy of mature leaves under anoxia appear to be similar to the anaerobic response found in anoxia tolerant rhizomes (see also Bucher & Kuhlemeier, 1993). Anoxia tolerant leaves are characterised by low energy requirements under oxygen deprivation. Anaerobic energy generation is initiated under anoxia, but carbohydrate reserves are utilised economically under long-term anoxia. Although leaves of all three investigated species (*A.calamus*, *I.pseudacorus*, *V.macrocarpon*) possess the ability to adjust their metabolism to the anaerobic environment, variations occur in survival under long-term anoxia. Differences in the metabolic responses of the species to long-term anoxia appear to be quantitative rather than qualitative, indicating that metabolic control plays a crucial role in anoxia tolerance. Anaerobic metabolism did not seem to be restricted to glycolysis and ethanol fermentation. Mobilisation of carbohydrate reserves, sugar transport, and preservation of mitochondria and chloroplasts also occurred in the anaerobic leaves. The maintenance of metabolic diversity - although on a lower level - could be an important

feature of anoxia tolerant tissue and might support the stabilisation of anaerobic metabolism. However, compared with anaerobic rhizomes, energy needs seem however higher in anaerobic leaves than in rhizomes, and subsequently leaf survival is limited under long-term anoxia.

This thesis presents only a first study of the anoxia tolerance of leaves, and takes a closer look at a few important parameters of leaf metabolism under anoxia. Further studies are necessary for a more detailed understanding of the metabolic responses in anoxia tolerant leaves to long-term oxygen deprivation and subsequent re-exposure to air. The study also shows that for investigation of the potential of higher plants to tolerate extreme environmental stress such as oxygen deprivation, wild species with highly developed adaptation mechanisms should be included in physiological research. These species play a significant role in occupying niches. The general importance of maintaining wetland habitats becomes particularly apparent during the annually recurring flooding of middle European streams such as the Rhine and Oder, where water meadows and riparian forests are needed as natural flood plains to prevent excessive damage to farmlands and towns.

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