Anoxia and Oxidative Stress: Lipid Peroxidation, Antioxidant Status and Mitochondrial Functions in Plants

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Academic Dissertation

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Front cover: Flowering *Iris germanica* (upper picture) and *Iris pseudacorus*.
Corresponding electron micrographs show the formation of hydrogen peroxide under hypoxia on the plasma membrane and in the cell wall.

TABLE OF CONTENTS

Abbreviations **5**
Original publications **6**
Summary **7**
Preface **9**
1. Introduction **11**
1.1. Physiology of anoxic stress 11
1.2. Structural adaptations to anoxia 11
1.3. Anoxia induced metabolic changes 12
1.4. Anoxia and gene expression 14
1.5. Membrane function and structure under anoxia 15
1.6. Role of ROS in the stress response 17
1.7. Chemistry of reactive oxygen species: Types of ROS 19
1.8. Lipid peroxidation 23
1.9. Antioxidant system 26
1.9.1. Superoxide dismutase (SOD) 26
1.9.2. Catalase and peroxidases 27
1.9.3. Phospholipid hydroperoxide glutathione peroxidase 27
1.9.4. Enzymes regenerating active forms of ascorbate and glutathione 27
1.9.5. Redox active compounds: ascorbate and glutathione 28
1.9.6. Phenolic compounds 28
1.9.7. Tocopherols 29
1.10. Antioxidative network 31
1.11. Antioxidant status of the cell under stress conditions 32
1.12. Role of mitochondria in stress response 33
1.13. Aims of the present study 34
2. Materials and methods **36**
2.1. Experimental design 36
2.2. Plant material 37
2.3. Growth conditions 37
2.4. Anoxic stress treatment 37
2.5. Cytochemical visualisation of hydrogen peroxide 38
2.6. Extraction of lipids 38
2.7. Detection of lipid-conjugated dienes and trienes 39
2.8. Second derivative spectrophotometry of conjugated dienes 39
2.9. Thiobarbituric acid reactive substances (TBARS) assay 40
2.10. Superoxide dismutase activity determination 40
2.11. Extraction and analysis of tocopherols by HPLC and mass spectrometry 40
2.12. Ascorbic acid assay 41
2.13. Determination of reduced and oxidised forms of glutathione 41

2.14. Isolation of mitochondria 41
2.15. Measurement of oxygen consumption 42
2.16. Observation of the swelling of mitochondria 42
2.17. Measurement of Ca^{2+} transport across inner mitochondrial membrane 43
2.18. Determination of membrane potential with Safranine O 43

3. Results 44

3.1. Formation of reactive oxygen species (H_2O_2) and lipid peroxidation 44
3.1.1. Ultrastructural changes caused by anoxic stress 44
3.1.2. Visualisation of H_2O_2 under anoxia and reaeration 44
3.1.3. Formation of conjugated dienes and trienes 45
3.1.4. Accumulation of TBARS 47

3.2. Antioxidant status under oxygen deprivation 48
3.2.1. Superoxide dismutase activity 48
3.2.2. Ascorbic and dehydroascorbic acid content 48
3.2.3. Changes in glutathione concentration 49
3.2.4. Tocopherols under anoxia and aeration 49

3.3. Characterisation of mitochondrial functions 50
3.3.1. Ca^{2+} uptake by plant mitochondria 51
3.3.2. Swelling of mitochondria 52
3.3.3. Inner membrane potential 53

4. Discussion 56

4.1. Correlation between ROS formation, lipid peroxidation and anoxia tolerance 56
4.1.1. Anatomical and ultrastructural features 56
4.1.2. H_2O_2 formation 57
4.1.3. Anoxia-induced lipid peroxidation: CD, CT and TBARS 58

4.2. Antioxidant status under anoxia and reoxygenation 61
4.2.1. Superoxide dismutase 61
4.2.2. Ascorbate and glutathione pools under anoxia and reoxygenation 62
4.2.3. Changes in the tocopherol content 65

4.3. Characterisation of mitochondrial functions and PTP 69

5. Conclusions and future prospects 71

6. References 74

ABBREVIATIONS

AA	ascorbic acid
ADH	alcohol dehydrogenase
ANP	anaerobically induced proteins
CD	conjugated dienes
CT	conjugated rienes
CsA	cyclosporin A
DHA	dehydroascorbic acid
DHAR	dehydroascorbate reductase
DNP	dinitrophenol
ETC	electron transport chain
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulphide
HIF1	hypoxia–inducible factor1
FCCP	*p*-trifluoro-metoxyphenylhydr-azone
LDH	lactate dehydrogenase
LP	lipid peroxidation
MDA	malon dialdehyde
MDHA	monodehydro-ascorbic acid
MDHAR	monodehydro-ascorbate-reductase
MS	mass spectrometry
NEM	N-ethyl-maleimide
PDC	pyruvate decarboxylase
Pi	inorganic phosphorus
PTP	permeability transition pore
PUFA	polyunsaturated fatty acid
PUFA–O$^{\bullet}$	lipid alkoxyl radical
PUFA–OO$^{\bullet}$	lipid peroxyl radical
PUFA–OOH	lipid hydroperoxide
ROS	reactive oxygen species
SD	second derivative
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TLC	thin layer chromatography
TOH	reduced tocopherol
XO	xanthine oxidase

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals.

I Chirkova T.V., Novitskaya L.O., Blokhina O.B. 1998. Lipid peroxidation and antioxidant systems under anoxia in plants differing in their tolerance to oxygen deficiency. Russ. J. Plant Physiol. 45(1): 55-62.

II Blokhina O.B., Fagerstedt K.V., Chirkova T.V. 1999. Relationships between lipid peroxidation and anoxia tolerance in a range of species during post-anoxic reaeration. Physiol. Plantarum 105(4): 625-632.

III Blokhina O.B., Virolainen E., Fagerstedt K.V., Hoikkala A.,Wähälä K., Chirkova T.V. 2000. Antioxidant status of anoxia tolerant and intolerant plant species under anoxia and reaeration. Physiol. Plantarum 109: 396-403.

IV Blokhina O.B., Fagerstedt K.V., Chirkova T.V. 2000. Anoxic stress leads to hydrogen peroxide formation and lipid peroxidation in plant cells. Manuscript submitted for publication.

SUMMARY

Research on the formation of reactive oxygen species (ROS) and the consequences in the cell under anoxia is of great importance in the elucidation of essential questions in stress physiology. Oxygen deprivation stress, and particularly transient hypoxia, has been suggested recently as a convenient model for the investigation of O_2/ROS sensing. Hence, it is of importance to show direct ROS formation under oxygen deprivation in plant tissues with respect to anoxia tolerance. Another problem, which is of great practical importance, includes physiological processes underlying anoxia tolerance. In the present study emphasis has been placed on the differences between anoxia-tolerant (*Iris pseudacorus, Oryza sativa* or *Ovena sativa*) and anoxia-intolerant (*Iris germanica, Triticum aestivum*) plant species in ROS production, development of lipid peroxidation (LP) during propagation and termination phases and antioxidant status of the cells under anoxic stress. In addition, the consequences of re-admission of oxygen (reoxygenation injury) have been studied. The above mentioned parameters can be affected by the metabolic changes brought about by anoxic stress: a decrease in adenylate energy charge, acidification of cytoplasm, elevation of cytosolic Ca^{2+} concentration, changes in the redox state and alterations in membrane structure and functions. Possible correlations between the parameters representative of oxidative stress and anoxia induced metabolic changes are discussed.

As further evidence for ROS formation, anoxia and especially post-anoxic reoxygenation caused cell wall and plasma membrane associated H_2O_2 accumulation, visualised by $CeCl_3$ detection and transmission electron microscopy. The results suggest that anoxic stress together with other stresses shares a common mechanism of induction, i.e. generation of ROS. In addition, the peroxidation of lipids was more intensive in anoxia-intolerant plants (*Triticum aestivum* and *Iris germanica*), as measured by conjugated diene and triene formation during the propagation phase. The same tendency was observed on the termination stage of LP, characterised by thiobarbituric acid reactive substances (TBARS) accumulation (with the exception of the extremely anoxia-tolerant *I. pseudacorus*).

Different responses of antioxidant systems in anoxia-tolerant and -intolerant plants suggest that there is no universal mechanism incorporating all the antioxidants and leading to ROS detoxification. The plants studied here differed significantly in initial antioxidant content, which did not correlate with anoxia tolerance. The most important characteristic of anoxia tolerance was the ability to maintain a high ratio of reduced to oxidised forms of antioxidants, rather than their absolute levels. However, prolonged anoxia and subsequent

reoxygenation led to a decrease in all antioxidants studied (ascorbate, glutathione, tocopherol, superoxide dismutase) indicating oxidative stress and revealing a decrease in the redox state of the cell.

An important feature determining stress tolerance is the ability to preserve energy resources and/or to efficiently terminate ROS formation. Mitochondria are responsible not only for energy conservation, but also for the regulation of Ca^{2+} fluxes. They produce ROS and are an essential component in the signalling pathway leading to programmed cell death. A tighly regulated inner membrane channel − a permeability transition pore (PTP) − is induced in animal mitochondria by high matrix Ca^{2+}, dissipation of the inner membrane potential, redox changes, oxidation of GSH and an elevation in ROS level. Consideration of the role of PTP in animal tissues and metabolic changes under anoxia in plant cells imply the possibility of PTP induction in plant mitochondria under stress conditions, although the phenomenon of permeability transition has not been described in plants. In the present study mitochondrial functions under anoxia were studied with respect to PTP induction. High-amplitude mitochondrial swelling indicative of the PTP opening (in mammalian mitochondria) was observed in wheat root mitochondria after Ca^{2+} uptake and energisation by a respiratory substrate. However, this process was insensitive to cyclosporin A, a specific inhibitor of the permeability transition in mammalian mitochondria and, hence, the results are not conclusive on the presence of the PTP in plant mitochondria and require further investigation.

In general, the formation of ROS under oxygen deprivation stress represents a common mechanism of stress response initiation. It was shown that low amounts of oxygen present in the system were sufficient for H_2O_2 accumulation. Restoration of normoxic conditions caused secondary oxidative stress and led to an increase in LP, membrane damage and exhaustion of antioxidant resources. Lower intensity of oxidative damage in anoxia-tolerant plants demonstrated the higher stability of their membranes. The mechanisms responsible for such stability probably incorporate structural properties of the membranes as well as the antioxidative capacity and the ability to control metabolic functions for a longer time under stress conditions.

PREFACE

This work was carried out at the Department of Plant Physiology and Biochemistry of St. Petersburg University and at the Division of Plant Physiology at the Department of Biosciences, Viikki Biocenter, University of Helsinki. This research was supported by the Center of International Mobility (CIMO) and the Academy of Finland, which are gratefully acknowledged.

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Olga Blokhina

1. INTRODUCTION

1.1. Physiology of anoxic stress

Oxygen status of cells and tissues varies significantly during organism ontogenesis, and depends on environmental conditions of oxygen supply. Under flooding the root system is the most susceptible plant organ to suffer from oxygen deprivation. Since under natural conditions transient hypoxia occurs prior to strict anoxia, and because of accumulating evidence that low oxygen concentration may trigger metabolic responses, it is necessary to define anoxia here. According to Drew (1997) tissues or cells are hypoxic when the O_2 partial pressure limits the production of ATP by mitochondria. Anoxia occurs when the production of ATP by oxidative phosphorylation is negligible relative to that generated by glycolysis and fermentation. To prolong survival under unfavourable conditions plants develop structural and metabolic adaptations, which are genetically controlled. Since the problem of tolerance to flooding and anoxia is of great economical importance, numerous investigations have been undertaken to elucidate the mechanisms underlying high resistance to oxygen deprivation. Despite the fact that the response of a particular organism can be very specific, some general changes brought about by anoxic stress can be described.

1.2. Structural adaptations to anoxia

Constitutive aerenchyma formation in the stems and roots of aquatic and flooding tolerant species provides long distance oxygenation of hypoxic tissues. Two distinct pathways lead to the formation of interconnected and gas-filled spaces: Cell separation during development and programmed cell death (and/or necrosis), the latter being widespread even among dry-land species (Drew 1997). Non-constitutive induction of aerenchyma via cell death implies ethylene signalling in sunflower stems (Jackson 1985). Low concentrations of ethylene in air (0.1 - 1.0 $\mu l/L^{-1}$) promote cell death selectively in normoxic roots, while hypoxic roots have been shown to contain higher concentrations of ethylene and its precursor, and increased activity of enzymes responsible for ethylene biosynthesis (He et al. 1994). Anoxic stress also causes changes in cell ultrastructure, most of them leading to cell injury and death. Plasmolysis of the

cells, mitochondrial elongation and swelling are detectable at early stages of anoxia (Aldrich 1985, Andreev et al. 1991).

1.3. Anoxia induced metabolic changes

No fundamental differences in metabolic pathways have been detected between anoxia tolerant and intolerant plant species. Probably a complex of minor adaptations underlies better survival of anoxia-resistant plants (Pfister-Sieber and Braendle 1994).

Under oxygen deprivation mitochondrial electron transport chain (ETC) is suppressed and, hence, the synthesis of ATP is inhibited. Cells overcome energy shortage by switching to anaerobic glycolytic ATP production, and the availability of sugars as respirable substrates becomes extremely important. However, roots have been shown to suffer from sugar starvation under anoxia, because of inhibition of phloem transport, where the unloading step is affected (Saglio 1985). Mobilisation of starch is also affected under anoxia: The anoxia-intolerant wheat seeds fail to degrade starch of the endosperm under anoxia, while in the more tolerant rice α-amylases, responsible for starch breakdown, are induced under anaerobiosis (Perata et al. 1992). In general, anoxia causes a significant decrease in ATP levels especially in the anoxia-intolerant species but also in the tolerant species, and the ability of the tolerant plant species to maintain their energy supply for a longer time is considered to be the key factor for survival under anoxia (Chirkova et al. 1984, Hanhijärvi and Fagerstedt 1994, 1995, Crawford 1992).

Another important feature of anoxic metabolism is acidification of the cytoplasm and accumulation of fermentation products. A decrease in cytoplasmic pH from 7.3 − 7.4 to 6.8 is attributed to lactic acid production. Inhibition of lactate dehydrogenase and activation of pyruvate decarboxylase by the acidic pH leads to accumulation of acetaldehyde, which is converted to ethanol (by alcohol dehydrogenase, ADH, EC 1.1.1.1.) − one of the end products of anaerobic metabolism. However, the degree of acidification cannot be attributed solely to lactic acid accumulation. Another possibility is passive H^+ leakage from the vacuole under limited ATP availability and inhibition of vacuolar H^+-ATPase (Ratcliffe 1995).

Under prolonged anoxia ethanol is the most abundant end product of fermentation, even in anoxia-tolerant plants, which are able to release it into the surrounding medium. It has been shown that the ability to remove volatile end products of fermentation (i.e. ethanol and acetaldehyde) leads into increased anoxia tolerance in the anoxia sensitive chickpea (*Cicer arietinum* L.)

(Crawford and Zochowski 1984). Under anaerobic fermentation pyruvate can be converted into products other than ethanol and lactate. Increased amounts of alanine (by transamination of aspartate), malate and succinate can be detected at the beginning of anoxia. It seems that diversification of the glycolytic end products may be helpful in anoxia-tolerance, however the exact physiological mechanism to avoid injury is not fully known (Pfister-Sieber and Braendle 1994).

One of the most important consequences of energy limitation under anoxia is altered redox state of the cell. When oxygen – the terminal electron acceptor of ETC – is unavailable, the intermediate e^- carriers become reduced. This process in turn affects redox active metabolic reactions. Indeed, the ability to maintain redox characteristics of the cell (i.e. $NADH/NAD^+$-ratio) unaltered for a prolonged period has been shown for the anoxia-tolerant species rice and *Glyceria* (Chirkova 1992) and is considered important for plant survival under anoxia. A decrease in $NADH/NAD^+$ has been observed in the anoxia-intolerant wheat and bean (Chirkova et al. 1992). Ascorbic acid (AA) and glutathione (GSH) metabolism (reduction to an active antioxidant form) may provide an additional sink mechanism for excess protons and NADH production during the first stages of anaerobiosis. Participation of NADH in monodehydroascorbic acid (MDHA) reduction to ascorbate has been demonstrated (Noctor and Foyer 1998) as a process, which can play an adaptive role supplying reducing equivalents for antioxidant turnover. The redox changes can affect other redox-dependent reactions i.e. the oxidation state of ferrous ions – the promoters of ROS generation (through the Fenton reaction, eq. 4) and peroxidation of lipids. If oxygen deprivation persists, the need for oxidised NAD^+ and ATP leads to the fermentation pathway, where both LDH and ADH can regenerate NAD^+.

The signalling mechanism leading to anoxia-specific metabolic responses is not yet understood. Several investigations indicate that cytosolic Ca^{2+} may play the role of a second messenger. A rapid biphasic elevation of cytosolic Ca^{2+} has been observed in *Arabidopsis thaliana* seedlings (Sedbrook et al. 1996) and the inhibition of organellar Ca^{2+} channels by ruthenium red has been reported to cause inhibition of anoxic gene expression and post-stress survival of plants. Anoxia is known to induce a rapid elevation of cytosolic Ca^{2+} in maize suspension-cultured cells (Subbaiah et al. 1998). This rise was attributed to the release of the ion from intracellular stores, particularly from the ER and mitochondria. Together with the experiments performed on animal cells, these data suggest Ca^{2+} participation in anoxic signalling (Sedbrook et al. 1996).

1.4. Anoxia and gene expression

Anoxia-induced metabolic changes are associated with changes in gene expression: A decrease in general mRNA translation and an activation of expression of 'anoxic genes' has been observed. Normal protein synthesis is inhibited under anoxia, and only 10-20 anaerobically induced proteins (ANPs) appear. However, they account for more than 70% of total translation (Sachs et al. 1980, 1996). The majority of the genes induced code for enzymes involved in starch and glucose mobilisation, glycolysis and ethanol fermentation (Russel and Sachs 1991, Chirkova and Voitzekovskaya 1999). E.g. anaerobic induction of enolase (2-phospho-D-glycerate hydratase, EC 4.2.1.11), an integral enzyme in glycolysis, which catalyses the interconversion of 2-phosphoglycerate to PEP, has been reported in maize (Lal et al. 1998). Some other glycolytic and fermentation pathway enzymes, such as alcohol gehydrogenase, glucose phosphate isomerase, pyruvate decarboxylase (PDC) and sucrose synthase have been characterised as ANPs in maize. However, two genes not related to sugar metabolism and inducible under oxygen deprivation have been found. They may be involved in aerenchyma formation (Sachs et al. 1996).

The patterns of ADH and PDC expression in *Arabidopsis thaliana* includes two sets of alcoholic fermentation pathway genes, each of which may play a different role in the adaptive response to anoxia. One set is strongly induced in roots, while the other is expressed constitutively in both roots and leaves (Dolferus et al. 1997). Differential transcript levels of genes associated with glycolysis and alcohol fermentation suggest that corresponding genes may be differently regulated under submergence stress. A stimulating effect of low oxygen concentration (0 to 4%) on the induction of adh1 transcripts and ADH activity has been observed in roots and shoots of maize (Andrews et al. 1993). Anaerobiosis-specific transcriptional and translational changes have been detected in rice coleoptiles and roots: Plants responded to anoxia within 1 hour by synthesising low molecular weight proteins (c. 33 kD) (Breviario et al. 1994), the transition peptides. However, the early induced genes, those responding after 1-2 h of anoxia, have not been studied extensively. A novel gene family aie (anaerobically inducible early) has been identified recently in a very flooding-tolerant variety of rice. mRNA levels of aie genes peaked after 1.5 to 3 h of anoxia and were at high levels after 72 h of anoxia (Huq and Hodges 1999). Sequence analysis of one of the aie genes did not show any significant homology to any known genes or proteins.

1.5. Membrane function and structure under anoxia

Changes in the physical properties of membranes and their function and composition under oxygen deprivation are non-specific stress reactions, which have been reported in a number of other stresses (Chirkova 1988, Shewfelt and Purvis 1995). Under anoxia a decrease in membrane integrity is a symptom of injury, measured as changes in lipid content and composition (Chirkova et al. 1989, Hetherington 1982), activation of lipid peroxidation (Crawford 1994, Crawford and Braendle 1996, Chirkova et al. 1998, Blokhina et al. 1999), enhanced electrolyte leakage (Chirkova et al. 1991a, 1991b) and as a decrease in adenylate energy charge (Hanhijärvi and Fagerstedt 1994, 1995, Chirkova et al. 1984). Since *de novo* lipid synthesis is energy dependent, and could hardly occur under anoxia, the preservation of membrane lipids is the most efficient way to maintain functional membranes. A decrease in unsaturated to saturated fatty acid ratio under anoxia may represent a result of lipid peroxidation (LP), and at the same time sets limits for substrates of LP, the polyunsaturated fatty acids (PUFA). In the anoxia tolerant *A. calamus* a decrease in linolenic acid (18:3) is compensated by linoleic (18:2) and oleic (18:0) acids under oxygen deprivation. The original lipid composition is recovered during two days of re-aeration (Pfister-Sieber and Brändle 1994). In general, lipids of anoxia-tolerant plants are more preserved during oxygen deprivation in respect to composition and the degree of unsaturation. Similar results have been obtained for the anoxia tolerant and intolerant cereals rice and wheat, respectively (Chirkova et al. 1989). In the rhizomes of the anoxia resistant *Iris pseudacorus* imposition of anoxia is known to result in a significant decrease in the ratio of saturated to unsaturated fatty acids in polar lipids. In contrast, no changes in either lipid classes or fatty acid composition have been observed in *Iris germanica* rhizomes, although these *Iris*-species have been shown to possess a highly similar lipid profile (Hetherington 1982). On the other hand, there are no significant qualitative and quantitative changes in the composition of fatty acids in anaerobically treated rice seedlings (Generosova et al. 1998). In that study it was postulated that the reduction of unsaturated fatty acids esterified in lipids was of no significance as a mechanism of plant adaptation to anaerobic conditions. The key role in survival was assigned to the energy metabolism (Generosova et al. 1998). However, during the recent years evidence has accumulated on the importance of lipid metabolism, and especially on unsaturated fatty acids, in the induction of defence reactions under biotic and abiotic stresses. Linolenic acid (18:3) has been shown to be a precursor of jasmonic acid, the latter being a signal transducer in defence reactions in plant-pathogen interactions (Rickauer et al. 1997). Free fatty acids, liberated during

membrane breakdown under stress conditions, are not only the substrates for LP, but also can act as uncouplers in mitochondrial ETC (Skulachev 1998).

Alterations in the degree of fatty acid unsaturation have been observed also under abiotic stress conditions: A four-fold increase in the ratio of unsaturated to saturated fatty acids in a cold-tolerant cultivar of bermudagrass has been observed under low temperature (Samala et al. 1998). Also low irradiance and spectral composition of light are known to affect C-18 fatty acid desaturation in soybean leaves (Burkey et al. 1997). Lipid hydroperoxides, formed as a result of LP, can affect the membrane properties, i.e. increase hydrophilicity of the internal side of the bilayer (Frenkel 1991). This phenomenon is very important for the termination of LP, since increased hydrophilicity of the membrane favours the regeneration of tocopherol by ascorbate.

Hence, membrane lipids undergo changes under anoxia, which may be considered as adaptive, and which may result in the acceleration of lipid peroxidation after restoration of the oxygen supply. However, preliminary steps may occur under anoxic conditions, i.e. maintenance of the high level of fatty acid unsaturation, appearance of free fatty acids and the production of low amounts of ROS due to membrane associated electron transport.

Reoxygenation injury is a well-documented fact for both animal and plant tissues. Indeed, under anoxia saturated electron transport components, the highly reduced intracellular environment (including ions of transition metals) and low energy supply are factors favourable for ROS generation. Formation of free radicals within minutes after restoration of the oxygen supply has been shown by electron paramagnetic resonance spectroscopy (EPR) in the rhizodermis of the anoxia-intolerant *I. germanica*, while in the tolerant *I. pseudacorus* no signal was detected (Crawford et al. 1994). Readmission of oxygen most probably causes the generation of superoxide radicals measured as an induction of SOD activity during re-aeration (Monk et al. 1989). Accumulation of various products of LP as a result of reoxygenation has been observed in the roots of the anoxia-intolerant wheat and tolerant rice, the latter showing higher membrane stability and lower level of LP (Chirkova et al. 1998, Blokhina et al. 1999).

The existence of anoxia inducible changes in plant metabolism implies that plant cells sense anoxic conditions and respond to them quickly by glycolytic production of ATP and the regeneration of $NAD(P)^+$ (Richard et al. 1994). Anoxic and especially postanoxic injury is the result of the formation of ROS. This causes peroxidation of lipid membranes, depletion of reduced glutathione, an increase in cytosolic Ca^{2+} concentration, oxidation of protein thiol groups and membrane depolarisation.

1.6. Role of ROS in the stress response

The generation of reactive oxygen species (ROS) is considered to be a primary event under a variety of stress conditions (Noctor and Foyer 1998). The consequences of ROS formation depend on the intensity of the stress and on the physicochemical conditions in the cell (i.e. antioxidant status, redox state and pH). It has been generally accepted that active oxygen produced under stress is a detrimental factor, which causes lipid peroxidation, enzyme inactivation, and oxidative damage to DNA (Shewfelt and Purvis 1995). However, during the recent years evidence has accumulated on the participation of ROS and their oxygenated products in a signal transduction cascade (Tarchevskii 1992, Lander 1997). Antioxidant status and redox state of the cell are the main components in the fine regulatory mechanism of ROS signal specificity (Lander 1997). ROS seem to affect the cell through a combination of the following factors: the amount of ROS produced (correlates with the severity of the stress) and biochemical status of the cell (i.e. activity of antioxidative and other enzymes, antioxidant content, pH, energy resources, integrity of membranes, redox characteristics etc.). The particular mechanisms and the place of ROS in the signal transduction cascade are not yet known. Recently, it has been proposed that sensing of the O_2-concentration and of ROS shares the same mechanism (Semenza 1999). Several models of sensing oxygen concentration have been proposed for animal cells and bacteria.

A. Direct oxygen sensing:

The sensor, heme, binds O_2 directly and adopts an inactive oxygenated state; when oxygen concentration declines, deoxygenation of heme occurs. This process is considered as the first step in oxygen sensing. There is an increasing amount of evidence that low concentrations of haemoglobin are present in roots of many plant species, although the physiological function is described only for the roots of plants capable of nodulation, i.e. facilitation of the oxygen supply. An alternative function for plant haemoglobin may be the indication of O_2-concentration and switching of metabolism from oxidative to fermentative pathway (Crawford 1992). Two haemoglobin genes have been identified in *Arabidopsis*

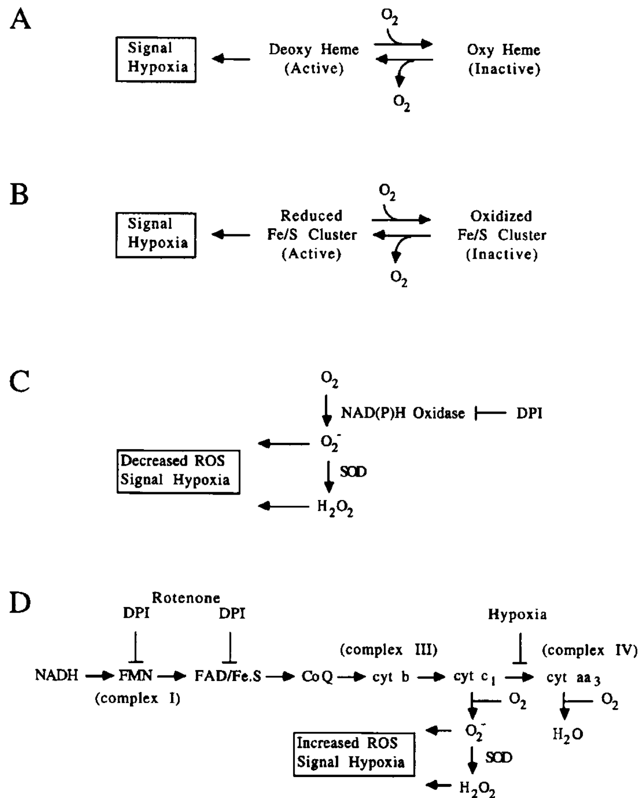


Figure 1. Models of O_2 sensing (from Semenza 1999). See explanations in the text

thaliana, one of which is induced by hypoxia (Dolferus et al. 1997). In flooding-stressed barley roots and in isolated barley aleurone layers exposed to anaerobic conditions, induction of the haemoglobin gene has been observed and was considered an integral part of the normal anaerobic response (Taylor et al. 1994). Besides its possible function as an oxygen sensor, another two physiological functions have been suggested for haemoglobin under low oxygen tension: Oxygen carrier and terminal oxidase or oxygenase (Hill 1998).

B. Redox cycling of iron-sulphur clusters represents another possibility for oxygen signalling.

Sensing via O_2-metabolites (ROS) suggests several mechanisms:

C. NAD(P)H oxidase converts O_2 to superoxide, which is dismutated by SOD to hydrogen peroxide. This model predicts that under oxygen deprivation the production of ROS declines, thus providing a redox signal for hypoxia.

D. Complex IV (cytochrome c oxidase) of the mitochondrial electron transport chain (ETC) is inhibited under hypoxic conditions and upstream electron leakage leads to ROS formation at complex III. (from Semenza 1999).

The experimental data obtained until now are contradictory and partly support all proposed models. Evaluation of the models in animal cells mostly employs the effect of O_2/ROS on inhibition or induction of expression of the hypoxia-inducible factor 1 (HIF-1), a transcription factor stimulating O_2 delivery, glucose transporters and glycolytic enzymes, which facilitate ATP production (Semenza 1998). In plants the involvement of ROS in signalling and in the induction of the stress response is well characterized for the hypersensitive response, although the sensor of oxygen metabolites is unclear (Goodman 1994, Lamb and Dixon 1997). Participation of ROS in the stress response has been described phenomenologically under a wide range of environmental conditions: dehydration, high salinity, low temperature, anoxia, senescence and high ozone concentrations. Altogether these observations suggest a fundamental role for reactive oxygen species in non-specific stress adaptation and signalling.

1.7. Chemistry of reactive oxygen species: Types of ROS

Four-electron reduction of oxygen in the respiratory chain is always accompanied with a partial one- to three-electron reduction, yielding the formation of ROS. This term includes not only free radicals ($O_2^{-\bullet}$, $HO^\bullet$), but also the molecules H_2O_2, singlet oxygen $^1O_2$ and ozone O_3.
Molecular oxygen is relatively unreactive (Elstner 1987) due to its electron configuration (two unpaired electrons with parallel spins). Activation of oxygen (i.e. first univalent reduction step) is energy dependent and requires an electron donation. The following one- electron reduction steps are not energy dependent and can occur spontaneously or require appropriate e^-/H^+ donors. In biological systems transition metal ions (Fe^{2+}, Cu^+) and

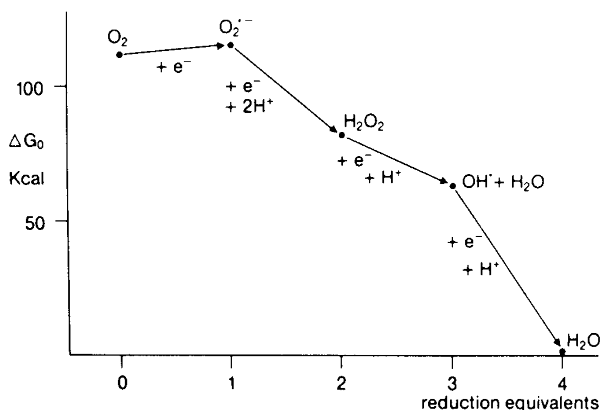


Figure 2. Energetics of oxygen reduction at 25° C and pH 7.0 (from Elstner 1987).

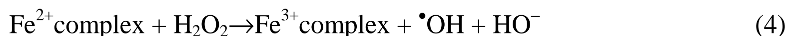
semiquinones can act as e^- donors. The superoxide anion $O_2^{-\bullet}$ can be protonated at a low pH to yield the perhydroperoxyl radical $^{\bullet}HO_2$ (1). Both O_2^- and $^{\bullet}HO_2$ undergo spontaneous dismutation to produce H_2O_2 (2, 3).

$$H^+ + O_2^{-\bullet} \leftrightarrow {}^{\bullet}HO_2 \quad (1)$$

$${}^{\bullet}HO_2 + {}^{\bullet}HO_2 \rightarrow H_2O_2 + O_2 \quad (2)$$

$${}^{\bullet}HO_2 + O_2^{-\bullet} + H_2O \rightarrow H_2O_2 + O_2 + OH^- \quad (3)$$

Although H_2O_2 is less reactive than $O_2^{-\bullet}$ in the presence of reduced transition metals such as Fe^{2+} in a chelated form (which is the case in biological systems), the formation of $OH^{\bullet}$ can occur in the Fenton reaction:



$$Fe^{2+}complex + H_2O_2 \rightarrow Fe^{3+}complex + {}^{\bullet}OH + HO^- \quad (4)$$

Fe^{3+}complex can be efficiently reduced by $O_2^{-\bullet}$ (5), the product of one-electron oxygen reduction, thus providing the cycling of Fenton reaction:

$$O_2^{-\bullet} + Fe^{3+}complex \rightarrow O_2 + Fe^{2+}complex \quad (5)$$

Mechanisms for the generation of ROS in biological systems are represented by both non-enzymatic and enzymatic reactions. The partition between two pathways under oxygen deprivation stress can be regulated by oxygen

concentration in the system. Non-enzymatic one electron O_2 reduction can occur at about 10^{-4} M and higher oxygen concentrations (Skulachev 1997). Even in very low O_2 concentrations plant terminal oxidases (K_m 10^{-6} M for oxygen) and the formation of ROS via mitochondrial ETC still remain functional.

Among other enzymatic sources of ROS, xanthine oxidase (XO), an enzyme responsible for initial activation of dioxygen should be mentioned. As electron donors XO can use xanthine, hypoxanthine or acetaldehyde (Bolwell and Wojtaszek 1997). The latter has been shown to accumulate under oxygen deprivation (Pfister-Sieber and Braendle 1994) and can represent a possible source for hypoxia-stimulated ROS production.

$$O_2 \xrightarrow{\text{Xanthine oxidase}} O_2^{-\bullet} \quad (6)$$

The next enzymatic step is the dismutation of the superoxide anion by SOD:

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow 2H_2O_2 \quad (7)$$

The reaction catalysed by SOD has a 10 000-fold faster rate than spontaneous dismutation (Bowler et al. 1992). The intracellular level of H_2O_2 is regulated by a wide range of enzymatic reactions and those catalysed by catalase and peroxidases are the most important ones. Catalase functions through an intermediate catalase-H_2O_2 complex (Compound I) and produces water and dioxygen (catalase action), or can decay to the inactive Compound II. In the presence of an appropriate substrate compound I drives the perioxidatic reaction. Compound I is a much more effective oxidant than H_2O_2 itself, thus the reaction of Compound I with another H_2O_2 molecule (catalase action) represents a one-electron transfer, which splits peroxide and produces another strong oxidant, the hydroxyl radical $OH^{\bullet}$ (Elstner 1987). $OH^{\bullet}$ is a very strong oxidant and can initiate radical chain reactions with organic molecules, particularly with polyunsaturated fatty acids (PUFA) in membrane lipids.

Lipoxygenase (LOX, linoleate:oxygen oxidoreductase, EC 1.13.11.12) reaction is another possible source of ROS and other radicals. It catalases the hydroperoxidation of PUFA (Rosahl 1995). The hydroperoxyderivatives of PUFA can undergo autocatalytic degradation, producing radicals and thus initiating a chain reaction of LP. In addition LOX-mediated formation of singlet oxygen (Kanofsky and Axelrod 1986) or superoxide (Lynch and Thompson 1984) has been shown.

Peroxidases, besides their main function in H_2O_2 elimination, can also catalyse $O_2^{-\bullet}$ and H_2O_2 formation by a complex reaction in which NADH is

oxidized using trace amounts of H_2O_2 first produced by non-enzymatic breakdown of NADH. Next the $NAD^•$ radical formed reduces O_2 to $O_2^{-•}$, some of which dismutates to H_2O_2 and O_2 (Lamb and Dixon 1997). Thus, peroxidases and catalase play an important role in the fine regulation of ROS concentration in the cell through activating and deactivating H_2O_2 (Elstner 1987).

Several other apoplastic enzymes may lead to ROS production under normal and stress conditions. Other oxidases, responsible for the two-electron transfer to dioxygen (amino acid oxidases and glucose oxidase) can contribute to H_2O_2 accumulation. Also an extracellular germin-like oxalate oxidase catalyses the formation of H_2O_2 and CO_2 from oxalate in the presence of oxygen (Bolwell and Wojtaszek 1997). In barley roots the induction of the germin gene expression has been shown to occur in response to salt stress and after treatments with salicylate, methyl jasmonate and plant growth regulators (Hurkman and Tanaka 1996). Amine oxidases catalyse the oxidation of biogenic amines to the corresponding aldehyde with a release of NH_3 and H_2O_2. Data on polyamine (putrescine) accumulation under anoxia in rice and wheat shoots (Reggiani and Bertani 1989) and predominant localisation of amine oxidase in the apoplast, suggest amine oxidase participation in H_2O_2 production under oxygen deprivation.

H_2O_2 is the first stable compound among ROS produced in the plant cell under normal conditions and as a result of stress. Hence, it is the most probable candidate for ROS-mediated signal transduction. This compound is relatively stable, is able to penetrate the plasma membrane as an uncharged molecule, and, therefore can be transported to the site of action (Foyer et al. 1997). Until now it is not clear how the organism senses ROS (see section 1.6.), but all models proposed discuss redox modification of cellular components and consider changes in O_2/ROS concentration as a primary event in the signalling cascade. To achieve signal specificity three important components of the signalling pathway via ROS have to be considered: 1. The source of the signal, 2. Target susceptibility (e.g. exposed SH groups of a protein or orientation of unsaturated fatty acids in the membrane), and 3. The antioxidant status of the cell (terminates the signal or allows it to proceed) (Alscher et al. 1997, Lander 1997). Thus, ROS action and the development of the stress response (adaptation) rely on a dynamic equilibrium between the rate of ROS production (concentration) and their utilisation. When the concentration of ROS exceeds the antioxidative capacity of the system oxidative stress occurs. The imposition of stress results in the elevation of ROS levels (Foyer et al. 1994, Alscher et al. 1997) and causes changes in the redox balance through the oxidation of metabolically active compounds leading to lipid peroxidation and degradation.

1.8. Lipid peroxidation

LP is a natural metabolic process under normal conditions. It can be divided into three stages: initiation, propagation and termination (Shewfelt and Purvis 1995). The initiation phase includes activation of O_2 (see section 1.7.) and is rate limiting. Polyunsaturated fatty acids (PUFA, the main components of membrane lipids) are susceptible to peroxidation. LP is one of the most investigated consequences of ROS action on the membrane structure and function. The idea of LP as a solely destructive process has changed during the last decade. It has been shown that lipid hydroperoxides and oxygenated products of lipid degradation as well as LP initiators (i.e. ROS) can participate in the signal transduction cascade (Tarchevskii 1992).

Hydroxyl radicals and singlet oxygen can react with the methylene groups of PUFA forming conjugated dienes, lipid peroxy radicals and hydroperoxides (Smirnoff 1995):

$$PUFA{-}H + X^{\bullet} \rightarrow PUFA^{\bullet} + X{-}H \quad (8)$$

$$PUFA^{\bullet} + O_2 \rightarrow PUFA{-}OO^{\bullet} \quad (9)$$

The peroxyl radical formed is highly reactive and is able to propagate the chain reaction:

$$PUFA{-}OO^{\bullet} + PUFA{-}H \rightarrow PUFA{-}OOH + PUFA^{\bullet} \quad (10)$$

The formation of conjugated dienes occurs when free radicals attack the hydrogens of methylene groups separating double bonds and leading to rearrangement of the bonds (Fig. 3) (Recknagel 1984). The lipid hydroperoxides produced (PUFA−OOH) can undergo reductive cleavage by reduced metals, such as Fe^{2+}, according to the following equation:

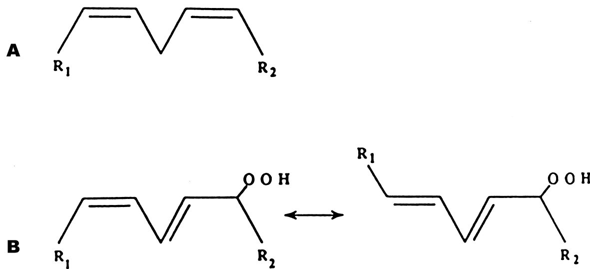
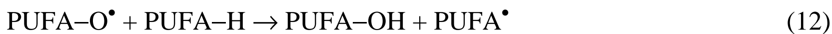


Figure 3. Structure of a conjugated diene. A, polyunsaturated fatty acid; B, fatty acid hydroperoxide with conjugated diene.

$$Fe^{2+}\text{complex} + \text{PUFA–OOH} \rightarrow Fe^{3+}\text{complex} + OH^{-} + \text{PUFA–O}^{\bullet} \qquad (11)$$

The lipid alkoxyl radical produced, PUFA–O$^{\bullet}$, can initiate additional chain reactions (Buettner 1993).



$$\text{PUFA–O}^{\bullet} + \text{PUFA–H} \rightarrow \text{PUFA–OH} + \text{PUFA}^{\bullet} \qquad (12)$$

However, until now it is not quite clear whether peroxidation can be considered a cause of membrane damage and metabolic disorders, or a secondary effect of these processes. This problem arises from controversial observations concerning the mechanisms and products of LP in plant tissues. A comprehensive model for lipid peroxidation in plant tissues (Shewfelt and Purvis 1995) emphasizes the importance of chemical, rather than biochemical, processes in the oxidative stress. However, phospholipid hydroperoxides can be formed enzymatically via LOX reaction (Rosahl 1995). The multistage character of the process i.e. branching of chain reactions, allows several ways of regulation (Schwelt and Purvis 1995).

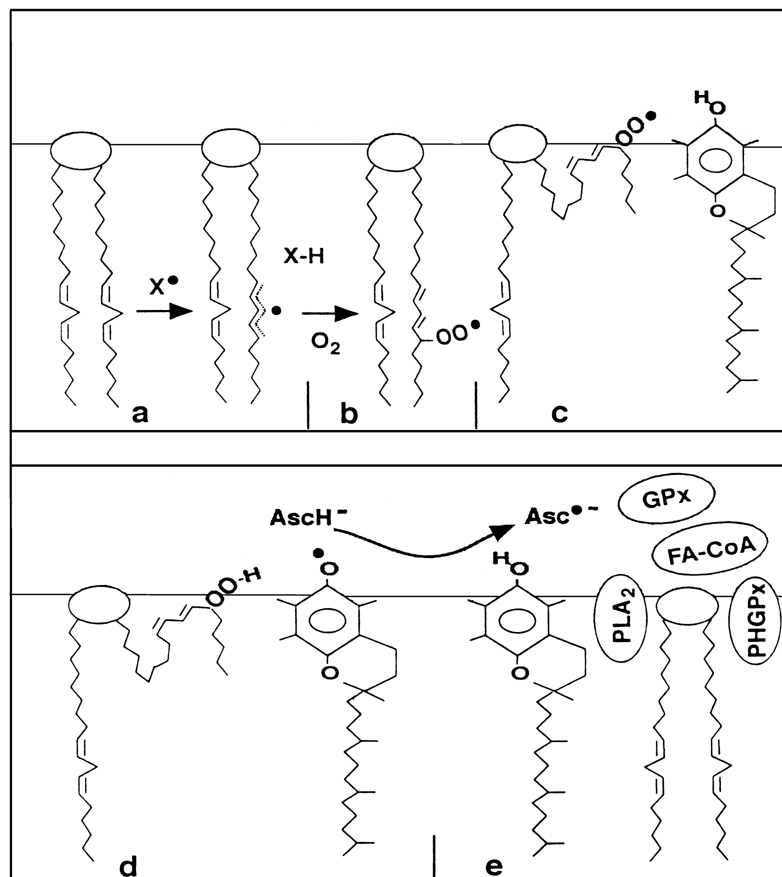


Figure 4. Membrane lipid peroxidation. (a) Initiation of the peroxidation process by an oxidizing radical $X^{\bullet}$, by abstraction of a hydrogen atom, thereby forming a pentadienyl radical. (b) Oxygenation to form a peroxyl radical and a conjugated diene. (c) Peroxyl radical moiety partitions to the water-membrane interface where it is poised for repair by tocopherol. (d) Peroxyl radical is converted to a lipid hydroperoxide, and the resulting tocopherol radical can be repaired by ascorbate. (e) Tocopherol has been recycled by ascorbate; the resulting ascorbate radical can be recycled by enzyme systems. The enzymes phospholipase A2 (PLA2), phospholipid hydroperoxide glutathione peroxidase (PH-GPx), glutathione peroxidase (GPx) and fatty acyl-coenzyme A (FA-CoA) cooperate to detoxify and repair the oxidized fatty acid chain of the phospholipid. (from Buettner 1993).

Among the regulated properties are the constitutive properties of the membranes (composition and organisation of lipids inside the bilayer in a way which prevents LP (Merzlyak 1989), the degree of PUFA unsaturation, mobility of lipids within a bilayer, localization of the peroxidative process in a particular membrane and the preventive antioxidant system (ROS scavenging and LP product detoxification) (Fig. 4).

1.9. Antioxidant system

To control the level of ROS and to protect cells under stress conditions, plant tissues contain several enzymes scavenging ROS (SOD, catalase, peroxidases) and a network of low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds, tocopherols). In addition, a whole array of enzymes is needed for the regeneration of the active forms of the antioxidants (ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase). In the following chapters they are all presented separately.

1.9.1. Superoxide dismutase (SOD)

Because of high reactivity of $OH^{\bullet}$ radicals (the main cause of cellular damage under oxidative stress), it is difficult to control their concentration enzymatically. Living organisms avoid the presence of this radical by controlling the upstream reaction of superoxide dismutation via SOD (eq. 7). The enzyme is present in all aerobic organisms and in all subcellular compartments susceptible of oxidative stress (Bowler et al. 1992). The three types of this enzyme can be identified by their metal cofactor: The structurally similar FeSOD (procaryotic organisms, chloroplast stroma) and MnSOD (procaryotic organisms and the mitochondrion of eucaryots); and structurally unrelated Cu/ZnSOD (cytosolic and chloroplast enzyme). Apart from their localisation, these isoenzymes differ in their sensitivity to H_2O_2 and KCN (Bannister et al. 1987). All three enzymes are nuclear encoded, and SOD genes have been shown to be sensitive to environmental stresses, presumably as a consequence of increased ROS formation. This has been shown in an experiment with corn (*Zea mays*), where a 7-day flooding treatment resulted in a significant increase in TBARS content, membrane permeability and the production of superoxide anion-radical and hydrogen peroxide in the leaves (Yan et al. 1996). An excessive accumulation of superoxide due to the reduced activity of SOD under flooding stress was shown also (Yan et al. 1996). Similar results on SOD activity have been obtained for wheat and rice roots under anoxia (Chirkova et al. 1998).

1.9.2. Catalase and peroxidases

These enzymes execute the next step in the detoxification of ROS: The elimination of excess H_2O_2, and, as discussed above (see section 1.7), participate in the fine regulation of the H_2O_2-concentration in the cell.

1.9.3. Phospholipid hydroperoxide glutathione peroxidase

Phospholipid hydroperoxide glutathione peroxidase (PXGPX) is a key enzyme in the protection of the membranes exposed to oxidative stress and is inducible under various stress conditions. The enzyme catalyses the regeneration of phospholipid hydroperoxides at the expense of GSH (13) and is localised in the cytosol and the inner membrane of mitochondria of animal cells. PXGPX can also react with H_2O_2 but this is a very slow process.

$$2GSH + \text{lipid hydroperoxide} \rightarrow GSSG + \text{lipid} + 2\ H_2O \qquad (13)$$

Until know, most of the investigations have been performed on animal tissues. Recently, a cDNA clone homologous to PHGPX has been isolated from tobacco, maize, soybean, and *Arabidopsis* (Sugimoto et al. 1997). The PHGPX protein and its encoding gene *csa* have been isolated and characterised in citrus. It has been shown that *csa* is directly induced by the substrate of PHGPX under the conditions of heat, cold and salt stresses, and that this induction occurs mainly via the production of ROS (Avsian-Kretchmer et al. 1999).

1.9.4. Enzymes regenerating active forms of ascorbate and glutathione

To prevent LP, H_2O_2 can also be removed by the Halliwell-Asada pathway (Fig. 5), originally described in the chloroplasts (Nakano and Asada 1980). In a recent investigation on the relationship between H_2O_2 metabolism and the senescence process in mitochondria and peroxisomes Jumenez et al. (1998) have indicated the presence of ascorbate-glutathione cycle components in both organelles. Ascorbate-specific peroxidase, which actually reacts with H_2O_2, works in cooperation with dehydroascorbate reductase, glutathione and glutathione reductase with NADPH as a donor of reducing equivalents. The reaction may be of particular importance under hypoxic conditions, since it allows $NADP^+$ regeneration. The latter is implicated in ATP production via glycolysis.

1.9.5. Redox active compounds: ascorbate and glutathione

Ascorbic acid has an ability to scavenge a wide range of ROS: Superoxide anion, singlet oxygen and H_2O_2, and acts as a chain-breaking antioxidant (Beyer 1994). However, in the presence of metal ions and at high concentrations of ascorbate, it can act as a pro-oxidant (Foyer et al. 1991). Under these conditions hydroperoxyl and superoxide radicals can be formed *in vitro* (Halliwell and Gutteridge 1989). The autooxidation of ascorbate proceeds with the formation of an intermediate superoxide anion:

$$AH^{-} + O_2 \rightarrow A^{-\bullet} + O_2^{-\bullet} + H^{+} \qquad (14)$$

As a water soluble compound ascorbic acid functions most efficiently in the aqueous phase of the cell, and is able to carry out the non-enzymatic regeneration of α-tocopherol (TOH) from the α-tocopheroxyl radical ($TO^{\bullet}$) in the hydrophobic surroundings (Beyer 1994). Besides, ascorbate takes part in the regulation of the cell cycle by affecting the progression from G1 to S phase, and it has been implicated in the regulation of cell elongation (Smirnoff 1995).

Glutathione is a potent cellular reductant with a broad redox potential. It acts as a scavenger of peroxides and serves as a storage and transport form of reduced sulphur (May et al. 1998). It has been shown also that glutathione acts as a regulator of gene expression (Alscher 1989, Baier and Dietz 1997), and is a precursor of phytochelatins (Grill et al. 1989). Due to the redox active thiol group GSH may be involved in the regulation of the cell cycle and can act as a defence compound against oxidative stress. GSH has been shown to participate in the regeneration of the reduced form of ascorbate through non-enzymatic reduction of DHA at an alkaline pH (Noctor et al. 1998).

1.9.6. Phenolic compounds

Polyphenols possess ideal structural chemistry for free radical scavenging activity, and they have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilize and delocalise the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions (termination of the Fenton reaction) (Rice-Evans et al. 1997). Another

mechanism underlying the antioxidative properties of phenolics is the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes (Arora et al. 2000). These changes could sterically hinder diffusion of free radicals and restrict peroxidative reactions. Moreover, it has been shown recently that phenolic compounds can be involved in the hydrogen peroxide scavenging cascade in plant cells (Takahama and Oniki 1997). According to our unpublished results (R. Tegelberg) the content of condensed tannins (flavonols) as measured by HPLC, was 100 times higher in *I. pseudacorus* rhizomes in comparison with that of *I. germanica*. The effect of anoxia on the flavonol content (a decrease after 35 days of treatment) suggests their participation in the antioxidative defence in *I. pseudacorus* rhizomes.

1.9.7. Tocopherols

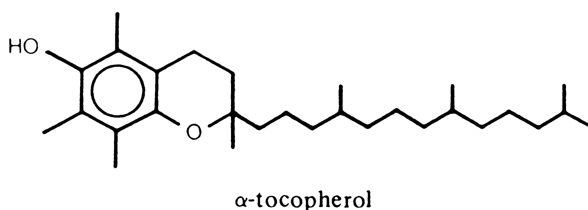


Figure 5. Schematic structure of α-tocopherol.

Tocopherols (Fig. 5) are unique antioxidants in carrying antioxidant functions for the detoxification of several types of ROS: quenching of singlet oxygen, direct reaction with $OH^{\bullet}$ radical, interaction with other free radicals i.e. termination of the chain via production of the unreactive tocopheryl radical (Kamal-Eldin and Appelqvist 1996, Kagan 1989). Due to the low rate constant of chain propagation (equation 10), tocopherol can compete with this reaction to "repair" PUFA–$OO^{\bullet}$, forming lipid hydroperoxides, PUFA–OOH:

$$\mathrm{TOH + PUFA{-}OO^{\bullet} \rightarrow PUFA{-}OOH + TO^{\bullet}} \qquad (15)$$

The optimal concentration ratio of TOH to PUFA–H in the membranes is estimated at 1:1000, thus one tocopherol molecule is able to protect 1000 lipid molecules from the chain propagation step in equations 10 and 14 (Buettner

1993). Since tocopherol functions in the lipid phase of the cell protecting membrane structures; its ability to synergetically interact with the water-soluble ascorbate provides the basis for overall cellular defence (Fig. 4). Ascorbate recycles tocopherol via $TO^{\bullet}$, producing the ascorbate radical:

$$AA{-}H^{-} + TO^{\bullet} \rightarrow AA^{-\bullet} + TOH \quad (16)$$

$AA^{-\bullet}$ can be removed by dismutation, yielding $AA{-}H^{-}$ and dehydroascorbate (DHA). Both DHA and $AA^{-\bullet}$ can be reduced by enzyme systems, which use NADH or NADPH as sources of reducing equivalents (Buettner 1993).

Tocopherols are not evenly distributed in cell membranes. There are membrane domains in biological membranes where lipid composition and fluidity differ from the other parts of the membrane. It has been suggested that tocopherols are accumulated in the most fluid membrane domains containing most of the unsaturated fatty acids of the membrane. There are two proposed mechanisms, which compensate the low tocopherol concentration in cell membranes: First, the accumulation of tocopherols into the most fluid membrane domains supports the antioxidant function of tocopherols in protecting the PUFAs against lipid peroxidation. Secondly, studies have revealed that tocopherols move rapidly in the lateral plane of the lipid bilayer, hence being able to move to parts of the membrane where they are needed (Gomez-Fernandez et al. 1989).

In addition, tocopherols have non-antioxidant functions in cell membranes which are less known. They seem to regulate membrane structures by modifying membrane permeability and phase transition (Wassal et al. 1986). It has been demonstrated that tocopherols can protect biological membranes against phospholipases and their hydrolysis products, free fatty acids and lysophospholipids, which are characteristically produced in large amounts in several stress situations such as hypoxia and ischemia (Kagan 1989). α-Tocopherol forms stable complexes with free fatty acids and lysophospholipids which stabilize the membrane structure. There are differences in complex-formation efficiency between tocopherol isomers. The efficiency in complex-formation is in order of $\alpha > \beta > \gamma > \delta$, which is suggested to explain the differing biological activities of tocopherol-isomers *in vivo* (Fryer 1992). It is noteworthy that α-tocopherol is a more lipophilic tocopherol isomer than β-, γ- or δ-tocopherol due to its three methyl substituents attached to the phenolic ring (Kamal-Eldin and Appelqvist 1996). α-Tocopherol is localised deeper in the membrane core than other tocopherol isomers, and it is possible that the different localisation in membrane has some role in the efficiency of α-tocopherol to form complexes with free fatty acids or lysophospholipids. It has

been suggested that α-tocopherol can prevent or even abolish the disordering effects of free fatty acids and lysophospholipids due to the formation of complexes within the membrane. Presence of the double bonds in the acyl chain of free fatty acids or lysophospholipids enhanced the interaction between the chromanol head group of α-tocopherol and the acyl chain.

Membrane hydrolysis products disturb also membrane proteins. Studies have revealed that α-tocopherol is also able to eliminate the modifying effects of free fatty acids on intrinsic membrane proteins (Kagan 1989). It has been suggested that a fundamental part of the biological action of tocopherols is due to their ability to physically stabilise membrane structures (Fryer 1992).

1.10. Antioxidative network

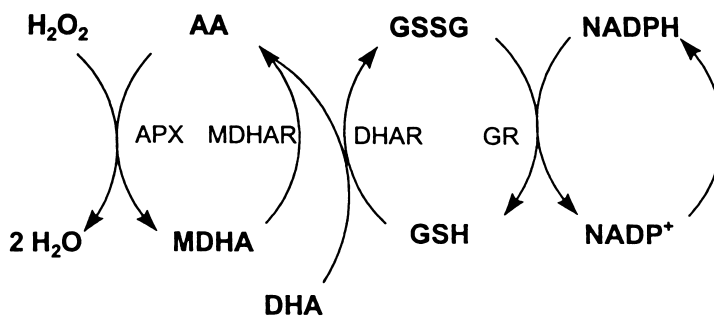


Figure 6. Halliwell-Asada pathway or ascorbate-glutathione cycle. APX, ascorbate-peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase. From May et al. 1998.

It is very important for plant survival under stress conditions that antioxidants can work in co-operation, thus providing better defence and regeneration of the active reduced forms. Ascorbate and glutathione remove H_2O_2 via the Halliwell-Asada pathway (Fig. 6). Ascorbate works in co-operation not only with glutathione, but also maintains the regeneration of α-tocopherol, providing synergetic protection of the membranes (Thomas et al. 1992). Recently, redox coupling of plant phenolics with ascorbate in the H_2O_2-peroxidase system has been shown. This acts in the vacuole, where H_2O_2 diffuses and can be reduced by peroxidases using phenolics as primary electron donors. Phenoxyl radicals

generated by this oxidation can be reduced by both AA and the monodehydroascorbic acid radical. If regeneration of AA is performed in the cytosol and AA is supplied back to the vacuole, a peroxidase/phenolics/AA system in vacuoles could function to scavenge H_2O_2 (Yamasaki and Grace 1998). This mechanism is specific for plant tissues and can improve stress tolerance under oxidative stress.

1.11. Antioxidant status of the cell under stress conditions

Data on antioxidant levels and the activity of antioxidant enzymes are somewhat contradictory, both decreases and increases in antioxidative capacity of the tissues have been reported. Such diversification partly arises from the response specificity of a particular plant species and from different experimental conditions (stress treatment, duration of stress, assay procedure and parameters measured). A large-scale investigation on monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) activities, and AA and GSH contents in 11 species with contrasting tolerance to anoxia has revealed an increase in MDHAR and/or DHAR in the anoxia tolerant plants after several days of anoxic treatment. In the intolerant plants activities were very low or without any changes. GSH decreased significantly during the post-anoxic period, while AA showed increased values in the tolerant species (Wollenweber-Ratzer and Crawford 1994). In anaerobically germinated rice seedlings a 3-fold increase in tocopherol and low TBARS formation has been observed (Ushimaru et al. 1994). However, an anoxia-induced elevation in the tocopherol level observed in the anoxia-intolerant wheat seedlings could not be detected in rice seedlings subjected to anoxia (**I**). In *Iris* spp. both α- and β-tocopherol levels decreased only after long-term anoxia (**III**). An investigation on the antioxidative defence system in the roots of wheat seedlings under root hypoxia or whole plant anoxia (Biemelt et al. 1998) has revealed a significant increase in the reduced forms of ascorbate and glutathione. Nevertheless, a rapid decrease in the redox state of both antioxidants was observed during reaeration. The activities of monodehydroascorbate reductase, dehydroascorbate reductase and GR decreased slightly or remained unaltered under hypoxia, while anoxia caused a significant inhibition of enzyme activities (Biemelt et al. 1998). Inhibition of glutathione reductase (GR), ascorbate peroxidase, catalase and superoxide dismutase (SOD) activities has been shown also by Yan et al. (1996) in corn leaves under prolonged flooding, while a short term treatment led to an increase in the activities. Considering the above-mentioned data, it is difficult to delineate a universal mechanism for the whole antioxidant system response to anoxia. Of course, it may be probable that there is no such

mechanism, and other factors are involved in the protective machinery of plants. Metabolic changes specifically induced by anoxia may alter the antioxidant status of the tissue.

1.12. Role of mitochondria in stress response

Dependence of plant survival on energy metabolism under environmental stress and the central role of mitochondria are well established. However, mitochondrial functions in the stress response are not limited only by the energy supply. The phenomenon of permeability transition in the inner membrane of mitochondria may be a possible link between the perception of the stress signal and the adaptive response.

Mammalian mitochondria contain an inner membrane channel, the permeability transition pore (PTP), that, when fully open, permits free diffusion of solutes with a molecular mass of up to 1500 Da (Bernardi et al. 1994, Zoratti and Szabo 1991). The PTP is controlled by several ligands as well as by the electrical potential across the membrane; high values favour the closed state, while dissipation of the potential increases pore opening probability (Bernardi 1992). The same effect on PTP has been observed under alkaline pH and elevated levels of reactive oxygen species (ROS). High matrix Ca^{2+}-concentrations, inorganic phosphate and oxidation of intramitochondrial pyridine nucleotides promote pore opening, whereas ADP, H^+ and cyclosporin A (a cyclic immunosuppressant peptide) cause inhibition. In mammalian mitochondria oxygen depletion has been shown to open the PTP. Such factors as a decrease in the inner membrane potential, formation of ROS in ETC and Ca^{2+} uptake by mitochondria have been shown to promote pore opening. Anoxia-induced metabolic changes in plant cells create similar conditions, and hence provide an opportunity for PTP induction.

The physiological function of the permeability transition pore is not fully understood, but circumstantial evidence suggests that it is involved in Ca^{2+} homeostasis (Bernardi and Petronilli 1996, Ichas et al. 1997) and linked to stress sensing through the programmed cell death. There is some evidence that a similar pore, which is regulated partly in a different manner, is to be found in yeast cell mitochondria (Jung et al. 1997). There is also some evidence that a similar pore is present in plant mitochondria (Vianello et al. 1995).

1.13. Aims of the present study

Data accumulated on the generation of ROS under various stress conditions, and their involvement in the regulation of the adaptive response and effects on metabolically active macromolecules and cell structures, are important for understanding stress physiology and the fine mechanisms underlying stress tolerance. Oxygen deprivation, by its nature, seems to occupy a special place in the general scheme for ROS participation in the stress response. However, changes in membrane lipid structure and function, disturbances in membrane integrity and other anoxia induced changes suggest peroxidative damage (LP), especially after re-admission of oxygen. The hypothesis that oxygen concentration and ROS may be recognised by the cell through the same sensing mechanism (Semenza 1999), makes anoxic stress an important model for the investigation of this mechanism.

The main goals of the present study are to delineate the development of stress reaction in the time-course of anoxia and during reoxygenation, and to elucidate the protective mechanisms underlying higher tolerance to anoxia. Special emphasis is placed on the evidence of ROS generation under oxygen deprivation and under reoxygenation, on the development of membrane LP and on the role of antioxidant systems in a range of plant species with different tolerance to anoxia. An attempt is undertaken to characterise mitochondrial functions during the transition to anoxia and to identify a permeability transition pore in the inner mitochondrial membrane. To complete the study the following experimental approaches were chosen:

– direct visualisation of H_2O_2 under oxygen deprivation and reoxygenation by means of transmission electron microscopy for the localisation of the sites of ROS generation.

– investigation of membrane LP at different stages of the process: Propagation phase (conjugated diene and triene formation) and termination phase (TBARS accumulation) in order to characterise the functional state of the membranes.

– estimation of the impact of post–anoxic reaeration on peroxidative processes by the determination of TBARS content in anoxically treated samples under a nitrogen atmosphere in an air-tight chamber; H_2O_2 visualisation under the same experimental conditions.

– evaluation of the role of the antioxidant system in LP regulation and anoxia-tolerance by the measurement of SOD activity, changes in the redox state and

content of the hydrophilic antioxidants ascorbate and glutathione, and by determination of the hydrophobic tocopherol concentration.

– characterisation of mitochondrial functions under anoxic conditions, and of the possible involvement of PTP in the stress response by measurement of O_2 consumption, Ca^{2+} transport, changes in the membrane potential and alterations in mitochondrial volume.

The parameters studied are discussed in a framework of anoxia-induced metabolic changes in order to integrate the data into a general sequence of events representing stress response to provide better understanding of anoxia tolerance.

2. MATERIALS AND METHODS

2.1. Experimental design

Oxygen sensitivity		Anoxia tolerant			Anoxia intolerant	
Plant species		***I. pseud.***	***Oryza sativa***	***Avena sativa***	***I.germ.***	***Triticum aestivum***
Duration of anoxia, days		**0, 15, 30, 45**	**0, 3, 7**	**0, 3, 5**	**0, 4, 8, 12**	**0, 1, 3**
Experiments		**Methods employed** * Performed with marked species				
1. ROS & lipid peroxidation	**H_2O_2 visualisa-tion**	- $CeCl_3$ staining; transmission electron microscopy - Quantification of H_2O_2 with Image Pro Plus				
		*	*		*	*
	Conjugated dienes/ trienes formation	Extraction and purification of membrane lipids Second derivative spectrophotometry of conjugated double bonds Inorganic phosphorus determination				
		*		*	*	*
	TBARS accumulati-on	Measurement of specific absorption of the complex:TBA-aldehyde product of lipid peroxidation				
		*	*	*	*	*
	TBARS acc. in mito-chondria	Isolation of mitochondria from heterotrophic tissue				
			*			*
2. Anti-oxidant status	**SOD**	Spectrophotometric assay in artificial O_2^- generating system. Competition of SOD and nitroblue tetrazolium.				
			*			**
	Ascorbate, Dehydro-ascorbate	Spectrophotometric assay with dipyridyl and FeCl3				
		*	*		*	*
	GSH; GSSG	Kinetic determination with glutathione reductase and DTNB				
		*	*		*	*
	Tocopherol	TLC, HPLC				
		HPLC	TLC	TLC	HPLC	TLC

3. Mito–chondrial functions	**O_2 consump-tion**	Clark–type oxygen electrode
	M. swelling	Decrease in light scattering of mitochondria at 540 nm
	Ca^{2+} uptake	Spectral shift of metallochromic indicator due to Ca^{2+} binding 665–685 nm
	Membra-ne potential assay	recording of spectral shift of the lipophilic cationic dye, safranine O, 511–533 nm

2.2. Plant material

The plant species used in the experiments can be arranged in a descending order of anoxia tolerance: *I .pseudacorus* > *I. germanica* > *Oryza sativa* > *Avena sativa* > *Triticum aestivum*. Their relative tolerance to anoxia has been estimated in a range of previous investigations according to viability, electrolyte leakage and adenylate energy charge (Chirkova et al. 1991, Hanhijärvi and Fagerstedt 1994, 1995). Only roots of cereals and rhizomes of *Iris* spp. were used for antioxidant determinations, because under natural conditions these organs are the first to suffer under flooding. Only current year rhizomes were used in the experiments. Before anoxic treatment roots and leaves were removed from the rhizomes**.**

2.3. Growth conditions

Seeds of wheat (*Triticum aestivum* L. cv. Leningradka), oat (*Avena sativa* L. cv. Borrus) or rice (*Oryza sativa* L. - cv. VNIIR) were planted in plastic trays and grown at 23°/20°C (day/night) with a 16-h photoperiod and illumination at 40 $\mu molm^{-2}s^{-1}$ for 7 days (wheat, oat and rice). *Iris pseudacorus* rhizomes were collected locally in a wet riverside meadow. *Iris germanica* rhizomes were kindly supplied by the Botanical garden of Helsinki University.

2.4. Anoxic stress treatment

Plants (rhizomes or whole seedlings) were placed in glass jars (1.5 L) on moistened filter paper. Anoxic conditions were created with gas generating kits (Oxoid BR 10, Unipath Ltd., Basingstoke, UK) and anaerobic palladium catalysts (Oxoid BR 42). Absence of oxygen was checked with anaerobic indicators (Oxoid BR 55). In addition to releasing hydrogen to remove oxygen,

the kit causes carbon dioxide concentration inside the jars to rise to ca. 7- 10%. High CO_2 under anoxia can be considered as a natural condition in soil and is due to root and microbial anaerobic respiration. The oxygen indicators used are sensitive to oxygen concentrations in the aqueous phase only down to 12 µM, which is still enough for the functioning of terminal oxidases and enzymatic production of ROS (Skulachev 1997).
Aerobic control samples were placed into moistened quartz sand (rhizomes) or wrapped in several layers of filter paper (seedlings). Both the anoxic and control samples were kept at room temperature in the dark. In some experiments a two-hour reoxygenation period after the anoxic treatment preceded extractions. Reaeration period (the same for all plant species) was chosen to allow the development the peroxidative reaction, which occurs very quickly after re-admission of oxygen.

2.5. Cytochemical visualisation of hydrogen peroxide

This assay is based on the reaction of H_2O_2 with $CeCl_3$ to produce electron dense insoluble precipitates of cerium perhydroxides $Ce(OH)_2OOH$ and $Ce(OH)_3OOH$ (Bestwick et al. 1997). Experimental plants were treated with 5 mM $CeCl_3$ and/or inhibitors of H_2O_2 formation, i.e. 8 µM DPI (diphenyleneiodonium for NADPH oxidase inhibition), 3 mM KCN (peroxidase inhibitor) and 1 mM NaN_3 (catalase inhibitor) and 25 µg/ml (1200 Uml^{-1}) of catalase (H_2O_2 removal). After three washing steps, plants were fixed in 2.5% glutaraldehyde, and H_2O_2 deposition was visualised by transmission electron microscopy.

Quantification of cerium perhydroxides in electron micrographs was performed by Image Pro® Plus. The programme differentiated cerium perhydroxide precipitates from the background by the difference in contrast. Calculation of precipitates was based on area and percentage of total area parameters, measured in pixels and as a percentage of the whole image area, respectively. Threshold intensity values and area limits were chosen manually for each calculation, depending on density of cerium perhydroxide accumulation, plant species and electron micrograph quality.

2.6. Extraction of lipids

Lipids were extracted by the classical method of Folch et al. (1957) with the modification of Kates (1972) for the extraction of plant tissues. Plant material

was homogenized with methanol in a glass Potter homogenizer for 5 min on ice and extracted with two volumes of chloroform (to achieve a chloroform:methanol ratio of 2:1 (v/v)). Contamination with water- and methanol-soluble compounds was avoided by co-extraction with 0.1% (w/v) NaCl followed by centrifugation at 600 g x 10 min. The upper water-methanol phase was aspirated and the procedure repeated three times with 0.1% NaCl:methanol:chloroform (47:48:3, v/v/v) mixture. The final lipid extract was evaporated (+40°C) under a stream of oxygen-free nitrogen, redissolved immediately in chloroform:methanol (2:1, v/v) and used for conjugated diene and triene assay, second derivative spectrophotometry and inorganic phosphorus determination.

2.7. Detection of lipid-conjugated dienes and trienes

Spectrophotometric detection of conjugated dienes and trienes reflects the presence of fatty acid hydroperoxides in lipid extracts. The procedure was carried out according to Recknagel and Glende (1984). An aliquot of the lipid extract was evaporated under a stream of oxygen-free nitrogen and redissolved in cyclohexane (spectrophotometric grade). UV spectra of lipids were monitored with Shimadzu UV 2100 spectrophotometer (Shimadzu, Kyoto, Japan). The characteristic absorption maxima at 232 nm (conjugated dienes, CD) and 274 nm (conjugates trienes, CT) were measured. Intensity of CD and CT formation was quantified per μg of inorganic phosphorus (P_i) determined by the method of Bartlett modified by Gerlach and Deutike (1963) and expressed as relative units (RU). RU=$Abs_{233nm\ (274\ nm)}$/ μg P_i.

2.8. Second derivative spectrophotometry of conjugated dienes

Non-peroxidized lipids exhibit strong absorption in the region of 200-220 nm, and therefore their absorption maximum masks the characteristic absorption maximum of conjugated double bonds. The SD method allows us to extract distinct signals out of shoulders on absorption slopes and to achieve better resolution. In SD spectra of peroxidized lipids signals with a minimum at 233 nm and another minimum at 242 nm have been detected and attributed to *trans-trans* and *cis-trans* conjugated dienes, respectively (Corongiu et al. 1986). Lipid extracts were prepared as described above. The following instrument settings were used for SD measurements: bandpass 1 nm; delta wavelength 5 nm; scan speed 11.5 nmmin^{-1}.

2.9. Thiobarbituric acid reactive substances (TBARS) assay

Plant material was extracted with TRIS-HCl buffer (pH 7.4) in the presence of 1.5% Polyclar-AT [w/v] to eliminate polyphenols, which were found to interfere with the assay. After filtration and centrifugation (20 min x 10 000 g, Sorvall) thiobarbituric acid (0.5% [w/v] in 20% [w/v] TCA) was added to an aliquot of the supernatant and the mixture heated in a boiling water bath for 30 min. After cooling and centrifugation (20 min x 10 000 g) absorbance of the supernatant was measured at 532 nm. Malone dialdehyde extinction coefficient (0.156 $\mu M^{-1} cm^{-1}$) was used for calculations of the TBARS content (Rubin et al. 1976).

2.10. Superoxide dismutase activity determination

The assay was performed in a photochemical system, containing 1.3 mM riboflavin, 13 mM methionine, 63 μM nitroblue tetrazolium (NBT) and enzyme extract in phosphate buffer, pH 7.6. The method is based on the reduction of NBT by superoxide radicals, produced by photochemistry under constant illumination, and the formation of purple formazan. SOD competes with the photochemical system for superoxide and decreases the amount of NBT reduced. The amount of enzyme, which inhibited NBT reduction by 50% was taken as an activity unit (Giannopolitis et al. 1977).

2.11. Extraction and analysis of tocopherols by HPLC and mass spectrometry

Vitamin E compounds were extracted by a modified solvent extraction method (Thompson and Hatina 1979). α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols were determined by normal phase liquid chromatography (NP-HPLC) using the LiChrosorb Si 60 column (5 μm, 250 × 4 mm, Merck), an isocratic mobile phase (99.8% hexane and 0.2% 2-propanol), flow rate 1.9 ml/min and column temperature +38°C. Sample fluorescence (20 μl injection volume) was detected at 292 nm excitation and 324 nm emission wavelengths. A mixture was prepared from commercial α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols where the concentration of each isomer was 10 μg/ml hexane. Tocopherol contents were calculated according to the corresponding peak areas.

Identification of the peaks corresponding with tocopherol isomers was verified by preparative HPLC and mass spectrometry (MS). An analytical column (Lichrospher® Si 60, 5 µm, 250 × 4 mm, Merck), the flow rate of 1.9 ml/min and the injection volume 20 µl and the column temperature of +38°C were used during the analysis. The fractions containing probable α-, β- and γ-tocopherol were collected and analysed with a semi-preparative column (LiChrospher® Si 60, 5 µm, 250 × 10 mm, Merck) with the flow rate 11.9 ml/min and the injection volume 200 µl. The fractions containing probable α-, β- and γ-tocopherol were concentrated in gaseous argon for MS analysis.

2.12. Ascorbic acid assay

Ascorbic (AA) and dehydroascorbic (DHA) acid content was determined according to Okamura (1980) with the modification of Knörzer et al. (1996). The absorbance at 525 nm was recorded and total ascorbate and AA contents were calculated on the basis of standard curves (AA in the range of 2-16 µg/ml in 5% metaphosphoric acid). DHA content was calculated as the difference between total ascorbate and AA levels.

2.13. Determination of reduced and oxidised forms of glutathione

Glutathione was determined under the following conditions: 100 mM sodium phosphate buffer (pH 7.5), 2.5 mM EDTA, 1 mM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.25 units of GR (from bakers yeast, type III, Sigma; 1 unit = 1 µmol GSSG reduced min^{-1} at pH 7.6), 0.2 mM NADPH and 20 µl or 50(100) µl of sample (metaphosphoric acid extract) for the GSH and GSSG determinations, respectively (Law et al. 1983). The reaction was initiated by the addition of GR and an increase in the absorbance was followed for 4 min at 412 nm. GSH and GSSG content were calculated from the linear part of the line on the GSH basis, since the reduced and oxidised forms of glutathione have been shown to produce the same standard curves under these assay conditions (Griffith 1980). Standard curves were prepared in the range of 20-200 ng $GSHml^{-1.}$

2.14. Isolation of mitochondria

Since the existing methods, e.g. the one originally designed for potato tubers (Douce et al. 1987), for the separation of mitochondria by fractional

centrifugation from wheat roots did not produce required results, the following method was developed. Mitochondria were isolated from the roots of 6–7 day old etiolated wheat seedlings by means of differential centrifugation as follows: Plant material was gently homogenized in 2 volumes of ice-cold extraction medium (Sucrose 0.25 M, EDTA 5 mM, EGTA 1 mM, dithioerythritol 1 mM, BSA 0.1%, Polyclar AT 0.6 % in HEPES-TRIS 10 mM pH 7.4) The homogenate was filtered and squeezed through Miracloth and the mitochondria were immediately separated from the cytoplasmic fraction by centrifugation at 10000 g x 10 min. The resulting crude mitochondrial pellet was resuspended in medium I (Sucrose 0.25 M, EDTA 5 mM, EGTA 1 mM, BSA 0.1% in HEPES-TRIS 10 mM pH 7.4) and centrifuged at 600 g x 5 min to remove nuclei and heavy cell debris. This washing procedure was repeated two times. Washed mitochondria were resuspended in medium II (Sucrose 0.25 M, EGTA 30 μM in HEPES-TRIS 10 mM pH 7.4) and stored on ice. Mitochondrial protein was determined according to Bradford (1976) using BSA as a standard.

2.15. Measurement of oxygen consumption

O_2-consumption was monitored at +25°C by a Clark-type oxygen electrode in 2 ml of continuously stirred medium. The incubation mixture contained 10 mM Hepes/TRIS pH 7.3, 0.25 M sucrose and 30 μM EGTA (the medium was essentially Ca^{2+}-free, as checked by EGTA titration in the presence of Arsenazo III. Mitochondrial protein concentration was 0.4 mg/ml, and 5 mM succinate or 1 mM NADH were used as respiratory substrates.

2.16. Observation of the swelling of mitochondria

An increase in mitochondrial (or intercristal) volume, caused by permeability transition (PT) or by other physiological reasons, results in diminished light scattering of the mitochondrial suspension at 540 nm, due to osmotic equilibration of solutes and water. The decrease in the light scattering of a suspension of mitochondria was followed at 540 nm (or 570 nm when measured simultaneously with Ca^{2+} uptake, since 570 nm is an isosbestic point for Arsenazo III) at +25°C as a function of time. Assay conditions are indicated in the figure legends. In all the experiments an inhibitor (rotenone) of the electron transport chain complex I was used to avoid the effect of pyridine nucleotides on the process studied. A range of factors inducing permeability transition were used in the experiments: high matrix Ca^{2+}-concentration, depolarisation of inner

mitochondrial membrane (induced by uncouplers: FCCP, DNP) and SH-modifying reagents (phenylarsine oxide). Also inhibitors of mitochondrial ETC and ATP synthesis were used: rotenone (complex I), KCN (cytochrome *c* oxidase) and oligomycin (ATPase).

2.17. Measurement of Ca^{2+} transport across inner mitochondrial membrane

A metallochromic indicator of Ca^{2+}-concentration, Arsenazo III, was used (10 μM) (Scarpa 1979). Differential absorbance changes (665-685 nm) of Arsenazo III as a function of Ca^{2+}-concentration were recorded with a dual beam UV-Visible spectrophotometer (Hewlet-Packard 8452A). Arsenazo III calibration was performed in an incubation medium (Sucrose 0.25 M, EGTA 30 μM in a range of Ca^{2+}-concentrations (5-30 μM).

2.18. Determination of membrane potential with Safranine O

Membrane potential of the inner mitochondrial membrane was measured by recording the spectral shift of the lipophilic cationic dye, safranine O (10 nmol ml^{-1}) (Moore et al. 1982) with a dual beam UV-Visible spectrophotometer (HP 8452A). The dye accumulates in mitochondria in response to the generation of a potential (negative inside). The wavelength pair 511-533 nm was used. An increase in absorbance (511-533 nm) correlates positively with the generation of a membrane potential.

3. RESULTS

The results of the present study are described in more detail in original publications. Here we present data, which reflect the main tendencies in the plant response to anoxia and reoxygenation, and are of crucial importance for discussion and conclusions. Data on control samples (before anoxic treatment) and aerated controls (during the treatment) are given when necessary. The experiments on mitochondrial characteristics under anoxia and identification of the PTP in plant mitochondria have not been published yet and, therefore, are described here in details.

3.1. Formation of reactive oxygen species (H_2O_2) and lipid peroxidation

3.1.1. Ultrastructural changes caused by anoxic stress

Anoxic treatment brought about both H_2O_2 accumulation and changes in the cell ultrastructure. The ability to maintain cell intactness correlated positively with anoxia tolerance. *I. pseudacorus* rhizome tissue showed undisturbed structures after 15 days and even after 45 days of anoxia (**IV**) with thin layers of cytoplasm along the cell walls and amorphous fructan stores in the vacuoles (**IV**). In the roots of the anoxia intolerant wheat plasmolysis could be observed already after 1 day of oxygen deprivation.
In rice continued stress led to swelling of mitochondria and to plasmolysis and finally to the disintegration of the plasma membrane after 7 days of anoxia (**IV**).

3.1.2. Visualisation of H_2O_2 under anoxia and reaeration

In the cereals imposition of anoxia resulted in the accumulation of H_2O_2 associated with the plasma membrane and the cell wall (**IV**)**.** Both the enhancement of stress and the restoration of normoxic conditions caused rapid membrane degradation and an increase in H_2O_2 formation. Reoxygenation injury caused by a 2 h reaeration period resulted in deteriorated membrane structures, and formation of H_2O_2 not only on the plasma membrane but inside the protoplasts of both wheat and rice. In rhizomatous species the difference in anoxia tolerance, estimated as H_2O_2 production, was more pronounced in comparison to that of the cereals. In the cells of the anoxia intolerant *I. germanica* intensive accumulation of H_2O_2 was detected on the plasma

membrane and less intensive in the cell wall after 8 days of oxygen deprivation. Anoxia tolerant *I. pseudacorus* showed no H_2O_2 formation on the plasma membrane after up to 45 days of anoxia. Reoxygenation after 15 days of treatment did not cause any changes neither in the cell ultrastructure nor in H_2O_2 accumulation in the rhizomes of *I. pseudacorus* (**IV**).

3.1.3. Formation of conjugated dienes and trienes

The degree of membrane peroxidation was measured as conjugated diene (CD) and conjugated triene (CT) formation (**II**). This process (i.e. free radical attack on the double bonds of polyunsaturated fatty acids) is characteristic of the propagation phase of LP (Recknagel and Glende 1984). The initial content of CD in the roots of cereals was several times lower than that of *Iris* rhizomes, and this difference was preserved during the anoxic treatment. In general, varying lengths of anoxia, which preceeded the 2 h reaeration period, resulted in enchanced LP in both cereal roots and *Iris* rhizomes. The intensity of the peroxidative reaction was different between species and correlated with anoxia tolerance. In anoxia tolerant species the content of both dienes and trienes was lower after long-term exposure as compared with intolerant species. The highest CD and CT content and degree of increase were detected in the rhizomes of the intolerant *I. germanica*. The increase was 1300% of the initial CD value after 12 days of anoxia. In *I. pseudacorus* the same parameter was only 144% of the initial value after 15 days of treatment. In wheat and oat roots the accumulation of CD and CT had a similar pattern as in *Iris* spp. However, the difference between the absolute levels of dienes in the tolerant oat and intolerant wheat was not so pronounced as in the *Iris* spp.

Second derivative (SD) spectrophotometry of lipid extracts from oxygen-deprived plants confirmed the formation of conjugated double bonds with specific absorption minima, and hence the presence of peroxidized lipids. The minima in SD spectra corresponded with the maxima in the original UV spectra of lipid extracts and allowed the quantification of

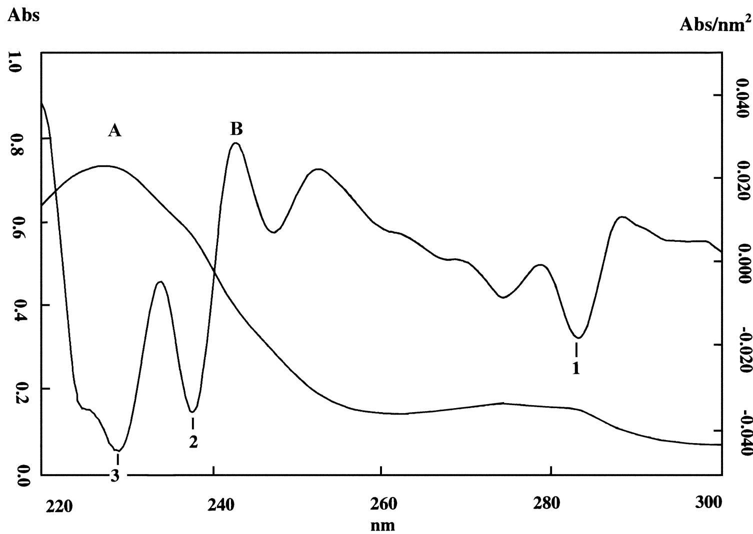


Figure 7. Simple UV (A) and second derivative (SD) spectra (B) of lipid extracts from anoxically treated (1 day) wheat roots. Minima in SD spectra correspond with: 1, conjugated trienes; 2, *cis-trans* conjugated dienes; 3, *trans-trans* conjugated dienes.

conjugated bonds via the peak heights. Two characteristic minima in SD spectra could be detected and ascribed to two stereoisomers of CD: *trans-trans* with a minimum at a shorter wavelength region (228-230 nm) and *cis-trans* shifted to longer wavelengths (237-240 nm) (Fig 7). There were no significant differences between the peak heights of *trans-trans* and *cis-trans* dienes, a fact which suggests an equal probability for stereoisomer formation. The additional minima in the SD spectrum, clearly seen at about 280 nm, probably corresponded with conjugated triene stereoisomers. Two peaks resolved in this region were at about 274 and 283 nm. In general, anoxia was shown to result in an increase in the peaks in the SD spectra corresponding with CD and CT stereoisomers especially in anoxia-intolerant plants. However, in the anoxia-tolerant *I. pseudacorus* no CD signal was observed in the SD spectra even after 45 days of anoxia. A strong signal was detected in this species at about 280 nm with two minima (271 and 282 nm), which probably corresponded with conjugated triene isomers.

3.1.4. Accumulation of TBARS

Formation of substances, which react with thiobarbituric acid (TBARS), is characteristic of the terminal stage of LP and indicates the breakdown of peroxidised lipids. The anoxia-intolerant wheat contained initially (before the treatment) a higher amount of TBARS as compared with the tolerant rice. Monitoring of the degradative stage of LP in connection with anoxia indicated a general increase in TBARS with the exception of the anoxia-tolerant *I. pseudacorus* (**I**, **II**). The dynamics of this process in the roots of cereals and *I. germanica* rhizomes correlated positively with that of CD and CT formation. Determination of LP level in mitochondria, isolated from the roots of wheat and rice (**I**), revealed the same tendency, i.e. accumulation of peroxidative products in response to anoxic stress and higher intensity of this process in anoxia intolerant species.

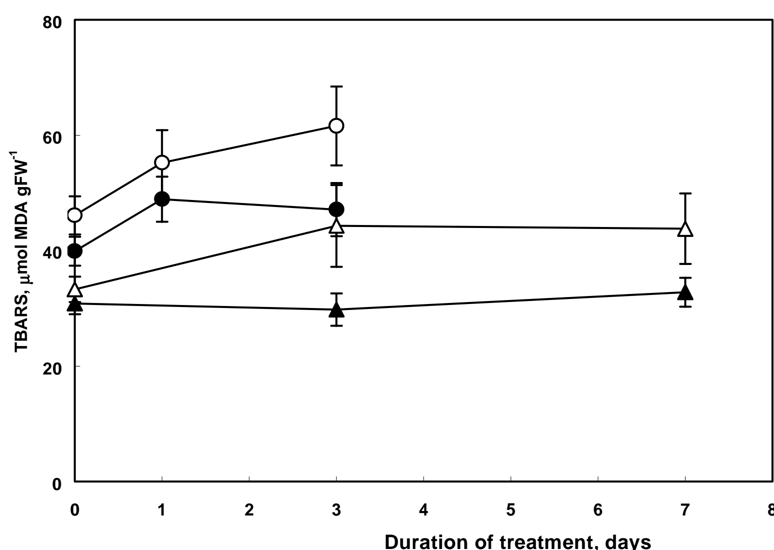


Figure 8. **Determination of lipid peroxidation products (TBARS) under normoxia and hypoxia in wheat and rice roots after anoxic treatment.**
Circles = wheat, triangles = rice; open labels = normoxia, closed labels = hypoxia. Bars represent standard errors n = 5-7.

Extremely high levels of TBARS were found in the untreated rhizomes of *I. pseudacorus*. These levels dropped significantly during anoxic treatment, while in the aerated controls only a slight decrease was observed. According to the TBA test, no lipid peroxidation had occurred in the rhizomes of anoxia tolerant

I. pseudacorus. (This is discussed further in **II**: Probably LP was terminated at the earlier stages or polyphenols interfered with the assay.)

In order to evaluate the impact of re-admission of oxygen into the samples during the assay procedure and the role of low oxygen concentration on LP, an experimental series on TBARS accumulation in cereal roots was performed in an air-tight chamber under hypoxic conditions (ca. 2% of O_2) (Fig. 8). In the controls (anoxically treated plants, in which TBARS content was determined under normal aeration) the same, as described above, increase in LP products was detected. Under the hypoxic TBARS assay, only in the anoxia intolerant wheat a significant increase was observed, while in rice roots there were no detectable changes in LP.

3.2. Antioxidant status under oxygen deprivation

3.2.1. Superoxide dismutase activity

In the present study the activity of SOD was determined without a reoxygenation period, immediately after termination of the anoxic treatment in the roots of cereals with different tolerance to anoxia. To elucidate the stress response early under anoxia, the experiments were performed after 5, 14, 24, 72, 168 hours (**I**). In the roots of the control wheat seedlings SOD activity was two times higher than in rice. In the course of the experiment the activity decreased in wheat under both aeration and anoxia, however, in the anoxic samples this decline was slower. As a result, after 3 days of anoxia the activity was by 65% higher than in the control roots. In the more anoxia tolerant rice, anoxia did not affect SOD activity. The determination of SOD activity in the rhizomes of *Iris* spp. has been done earlier (Monk et al. 1989).

3.2.2. Ascorbic and dehydroascorbic acid content

Cereals and *Iris* spp. were different from each other both in absolute levels of ascorbate and in its reduction state. Oxidised dehydroascorbic (DHA) acid was the main form in wheat and rice roots in the initial control plants and under anoxia and aeration (58.4% of total ascorbate for wheat and 78% for rice, with AA/DHA ratio of 0.7 and 0.3 for wheat and rice, respectively). On the contrary, the rhizomes of *Iris* spp. were characterised by elevated levels of reduced ascorbic acid (AA), which was the predominant form in the ascorbate pool. In

the rhizomes of the anoxia tolerant *I. pseudacorus* the initial level of total ascorbate and the degree of ascorbate reduction were higher than in the intolerant *I. germanica*. AA/DHA ratio was 4.8 for *I. pseudacorus* and 2.7 for *I. germanica*. The imposition of anoxia and subsequent reoxygenation caused a decrease both in the content of ascorbate and in its reduction state. However, no consequent changes in DHA content were detected. Prolongation of the anoxic treatment led to a decline in the antioxidant level, both reduced and oxidised forms, in all plants tested. A decrease in AA/DHA ratio indicated a shift in the reduction state of the ascorbate pool under oxygen deprivation.

3.2.3. Changes in glutathione concentration

In the roots of wheat and rice the initial level of glutathione (under aeration, before treatment) was approximately the same, while the reduction state (measured as GSH/GSSG ratio) appeared to be higher in the roots of the anoxia tolerant rice (III). The highest content of glutathione in the control plants was detected in the anoxia intolerant rhizomes of *I. germanica*, while in *I. pseudacorus* the amount of glutathione was three times lower. A remarkable drop in the level of reduced glutathione was detected after anoxia and reoxygenation in all plant species studied. The most rapid decrease was observed after short periods (considering anoxia tolerance of the species) of oxygen deprivation. The GSH content in the plant tissues after short term oxygen deprivation was only 13% and 55% of the initial level in wheat and rice roots, while in the rhizomes it was 36% and 11% for *I. germanica* and *I. pseudacorus*, respectively. These changes were not associated with a corresponding increase in GSSG, as the same tendency for decline was observed for the changes in GSSG content.

3.2.4. Tocopherols under anoxia and aeration

Tocopherol levels in the cereals and in the *Iris* spp. were measured by two different methods: TLC and HPLC, respectively (**I, III**). Both methods gave similar results considering the order of tocopherol content in the species studied. The HPLC-method allowed the detection of alterations in both α- and β-tocopherols under anoxia.

Tocopherol content (**I**) in the wheat control plants at the beginning of the experiment was similar to that of rice (0.5 - 0.8 $\mu g g^{-1} FW$). Short-term anoxic

treatment revealed a different response of the tocopherol system in anoxia tolerant and intolerant plants: A significant increase in wheat and preservation of the initial tocopherol level in rice roots were recorded. Continuation of anoxia up to 3 days led to the decline of the tocopherol content in wheat roots back to the control level, while in rice this parameter remained unaltered. In the aerated control plants of both wheat and rice, the tocopherol level was not affected.

HPLC of tocopherols in rhizomes of *Iris* spp. confirmed the presence of both α- and β-tocopherols (**III**). β-Tocopherol appeared to be the predominant compound in rhizomatous tissue. In *I. pseudacorus* however, the difference in α- and β-tocopherol levels was less pronounced. *I. germanica* rhizomes were characterised by a very high content of β-tocopherol (about 7 $\mu g g^{-1}$FW, in comparison to 1.5 $\mu g g^{-1}$FW in *I. pseudacorus*). To our knowledge, there are no previous reports of as high a β-tocopherol content in plant tissues. Under experimental conditions (anoxia and aeration) the same pattern of changes was shown in *Iris* spp. for both α- and β-tocopherols, i.e. the preservation of the tocopherol content during the first days of anoxia. Prolonged anoxia (45 days for the tolerant *I. pseudacorus* and 12 days for the intolerant *I. germanica*) caused a decrease in α- and β-tocopherols in the rhizomes of both species. At the end of the anoxic treatment 43% of the initial α-tocopherol content in the rhizomes of *I. pseudacorus* remained, while for *I. germanica* this value was 62%.

Although the retention times of the HPLC-peaks suggested the presence of a few tocopherol isomers, the existence of only α-tocopherol could be confirmed by a mass spectrometry (MS) analysis. Another tocopherol isomer with a molecular mass of 414 was found and this was most probably β-tocopherol (MW= 416.7).

3.3. Characterisation of mitochondrial functions

The main aims of the experiments were to characterise any alterations in particular mitochondrial functions caused by oxygen deprivation, and to identify the permeability transition pore in the inner membrane of plant mitochondria. The choice of the parameters measured was, therefore, determined by the factors important for PTP induction in animal mitochondria: high amplitude swelling, Ca^{2+} overload and membrane depolarisation. Oxygen consumption by isolated mitochondria was used as an integral parameter for evaluation of mitochondrial viability and the degree of coupling.

3.3.1. Ca^{2+} uptake by plant mitochondria

Since a high matrix Ca^{2+}-concentration has been reported as a crucial condition for PTP induction in animal mitochondria, it was necessary to show the ability of plant mitochondria (and particular mitochondrial preparation) to take up external Ca^{2+}. The choice of incubation medium was determined by the facts that Ca^{2+} uptake is facilitated in the presence of inorganic phosphorus (Pi) and is dependent on membrane potential.

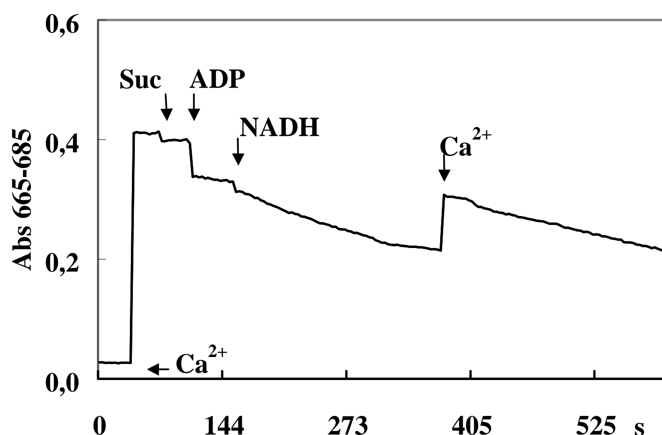


Figure 9. Ca^{2+} uptake by isolated plant mitochondria. Incubation medium: 0.25 M sucrose in 0.010 M TRIS-HEPES pH 7.4, 30 µM EGTA, Arsenazo III, 10 µM Pi, 5 mM; rotenone, 1 µM;mitochondria 0.4 mg protein ml^{-1}. Additions: 100 nM Ca^{2+} was added each time, Suc = 5 mM succinate, 1 mM ADP, 1 mM NADH.

Addition of 100 nmol of Ca^{2+} to the incubation medium and subsequent additions of a respiratory substrate (5mM succinate or 1 mM NADH) and 5 mM Pi induced Ca^{2+} uptake by isolated plant mitochondria (Fig. 9).

It was observed that NADH was more effective than succinate in the induction of Ca^{2+} uptake. It should be taken into consideration, that only qualitative evaluation of Ca^{2+} uptake is given, since plant mitochondria can, as it seems, slowly metabolise the Ca^{2+}-specific dye Arsenazo III used. This results in gradual fading of the indicator colour and hence introduces an error, which is difficult to take in consideration in the calculation of Ca^{2+} uptake. Nevertheless, high sensitivity of Arsenazo III to Ca^{2+} allows the use of the indicator in the detection of changes in Ca^{2+}-concentrations below 100 µM. However, we were not able to detect any Ca^{2+}-induced Ca^{2+}-release from plant mitochondria - a

feature which is characteristic of permeability transition and the resulting diffusion of solutes according to the concentration gradient.

3.3.2. Swelling of mitochondria

In our experiment high-amplitude mitochondrial swelling was observed under physiological conditions. The effects of different PTP inducers and inhibitors were examined. Employment of cyclosporin A (CsA), a specific inhibitor of PT in mammalian mitochondria, permits the distinction between PT and other processes. In all the cases studied here swelling was observed in actively respiring mitochondria after the addition of a respiratory substrate (succinate, NADH). In contrast to facts known about animal mitochondria, succinate-driven swelling of mitochondria was inhibited by 100 µM Ca^{2+}. Addition of Pi and KCl prior to succinate greatly enhanced the amplitude of swelling (Fig. 10). Application of CsA did not affect plant mitochondria, however in a few experiments some CsA sensitivity was observed (data not shown). According to our results, the high-amplitude swelling of mitochondria observed, was not caused by PTP operation in the inner mitochondrial membrane, and can be attributed to the 'high energy' swelling, as defined by Bernardi (1999). The next step was to characterise this type of swelling by other parameters (i.e. membrane potential measurements) and to monitor changes in mitochondrial volume under anoxia.

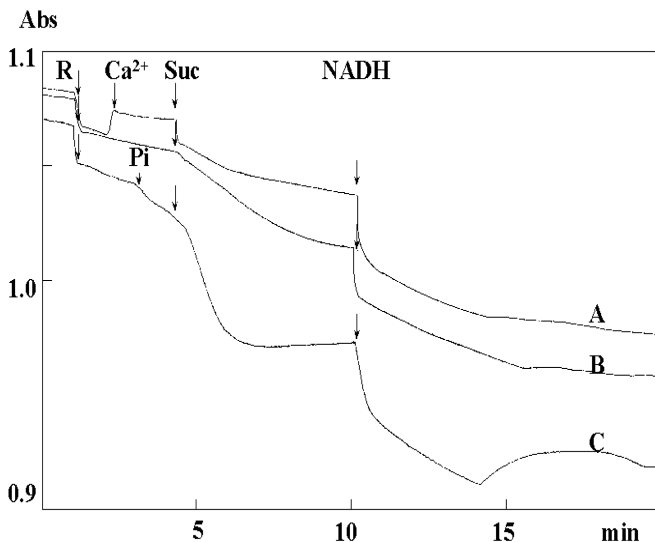


Figure 10. Dependence of the amplitude of the mitochondrial swelling on Ca^{2+}, Pi and respiratory substrates. Incubation medium:

0.25 M sucrose in 0.010 M TRIS-HEPES pH 7.4, 30 μM EGTA, mitochondria 0.4 mg protein ml^{-1}. Additions: R= 1 μM rotenone, 100 μM Ca^{2+}, 5 mM Pi, Suc = 5mM Succinate, 1 mM NADH. Trace A: effect of Ca^{2+} ions and respiratory substrates on the amplitude of mitochondrial swelling; trace B: effect of the respiratory substrates; trace C: effect of Pi

Among many factors and compounds that increase PTP opening probability, anoxia takes an important place. Anoxia itself is an integral factor, which induces several PTP-triggering conditions: alterations in membrane potential, pH shift, GSH depletion, cytosolic Ca^{2+} increase and release of free fatty acids (have been shown to act as uncouplers). Monitoring of anoxia induced changes in mitochondrial swelling revealed that upon the depletion of oxygen from the medium due to respiration, swelling stopped immediately and the opposite process – shrinkage of mitochondria took place. These changes were detected as an increase in light scattering at 540 nm, indicating increased matrix density (Fig.11). Restoration of the oxygen supply reversed the response. This fact indicates an active physiological state of mitochondria and that the changes observed cannot be considered degradative. Oxygen consumption measurement under the same conditions confirmed mitochondrial viability and the timing of anoxic conditions in the spectrophotometer cuvette: All oxygen was consumed after 8-13 min from the addition of 0.4 mg mitochondrial protein ml^{-1} into 1 ml of incubation medium at +25°C. This time was dependent on the quality of the mitochondrial preparation and number of additions to the cuvette (since each time some oxygen is introduced).

3.3.3. Inner membrane potential

The magnitude of the inner membrane potential is an important parameter for PTP regulation and under oxygen deprivation. Moreover, it was necessary to characterise basic electrophysiology of plant mitochondria and in our particular mitochondrial preparation, since such experiments have not been executed with plant mitochondria earlier.

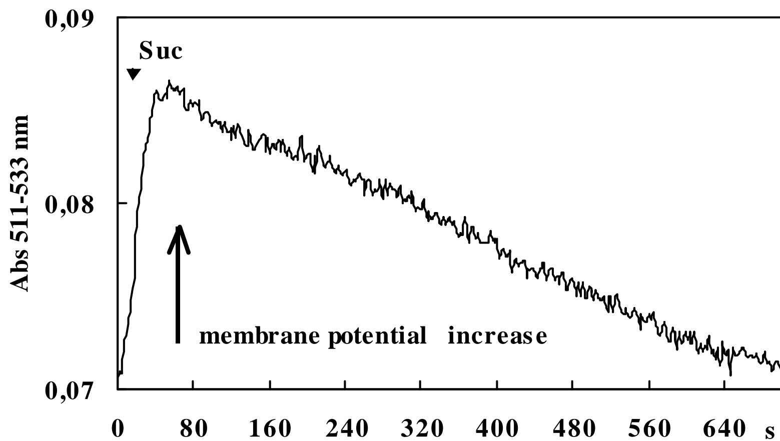


Figure 11. Time-dependent decrease in the inner mitochondrial membrane potential of NEM-treated mitochondria. Mitochondria were loaded with 2mM Pi, then treated with 0.8 mM NEM (an inhibitor of Pi uniporter) and washed. Addition of 5 mM succinate caused the generation of $\Delta\Psi$, which slowly decreased because of the inhibition of Succinate/Pi exchange. Incubation medium: 0.25 M sucrose in 0.010 M TRIS-HEPES pH 7.4, 30 µM EGTA, 10 nmol ml^{-1} safranine O, 0.4 mg mitochondrial protein ml^{-1}, Suc= 5mM succinate.

Qualitative estimation of membrane potential with safranine O revealed the absolute necessity of Pi (when succinate, but not when NADH was used as a substrate) for the generation of a membrane potential. Together with the observation that Pi enhanced succinate-induced mitochondrial swelling, and the fact that Complex II succinate dehydrogenase active sites face mitochondrial matrix, these observations suggest the existence of a Pi/succinate exchanger on the inner mitochondrial membrane. To check this hypothesis mitochondria were loaded with 2mM Pi, then treated with N-ethylmaleimide (an inhibitor of the Pi uniporter), washed and treated with 5mM succinate. As predicted, an initial generation of membrane potential was followed by a linear decrease, because of Pi exhaustion inside mitochondria and, therefore, inhibition of succinate uptake by mitochondria (Fig 11). This finding is of particular importance for PTP investigation, since Pi also facilitates Ca^{2+} uptake, which requires a high membrane potential.

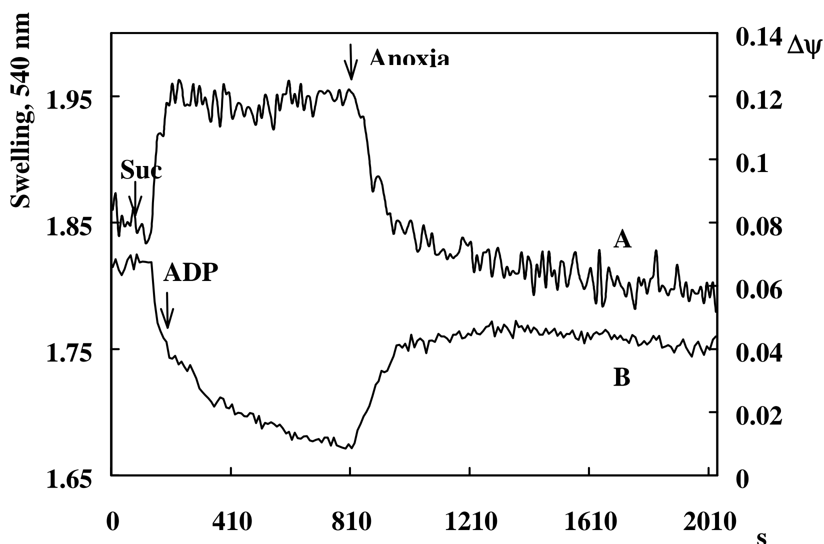


Figure 12. Changes in mitochondrial volume and inner membrane potential induced by anoxia. Incubation medium: 0.25 M sucrose in 0.010 M TRIS-HEPES pH 7.4, 30 μM EGTA, 0.4 mg mitochondrial protein ml^{-1}. Suc= 5mM succinate, 1 mM ADP, Trace A, inner membrane potential; trace B, swelling of mitochondria. Both parameters were recorded simultaneously.

Changes in the membrane potential brought about by anoxia were monitored simultaneously with swelling. The results show a clear negative correlation between the magnitude of the inner membrane potential and the degree of mitochondrial shrinkage (Fig.12). The same tendency was observed in the experiments on PTP identification: As soon as oxygen was consumed by mitochondrial respiration, an increase in light scattering at 540 nm (swelling) was detected. The amplitude of the increase was lower, probably because of the different additions of chemicals and a different incubation system.

4. DISCUSSION

4.1. Correlation between ROS formation, lipid peroxidation and anoxia tolerance

During the last decade the role of ROS in tissue disorders and later in signalling cascades has been intensively studied in connection with various environmental and biotic stresses. The fundamental role of activated oxygen as a non-specific messenger in stress physiology has been widely accepted. Under natural conditions anoxia-induced changes represent a combined effect of two stresses: Anoxic stress itself and oxidative stress, which occurs after the restoration of normoxic conditions. Experimental determination of anoxia-induced changes necessarily implies a reoxygenation period, and hence oxidative stress. Indications of ROS formation under oxygen deprivation (**IV**) lead to the question of interaction and priority between the physiological processes taking place under hypoxia and during reaeration. The investigation of ROS formation (H_2O_2) and LP (TBARS) under hypoxic conditions (10^{-5} M of oxygen), suggests that ROS concentration increases already under a very low oxygen concentration, and subsequent normoxia affects only the rate of the process**.** Indeed, the functioning of plant terminal oxidases (K_m 10^{-6} M for oxygen) (Skulachev 1997) is not limited by oxygen concentration in the experimental system and the formation of ROS is possible. Non-enzymatic one electron O_2 reduction can also occur at about 10^{-4} M and higher oxygen concentrations (Skulachev 1997).

4.1.1. Anatomical and ultrastructural features

Incubation of the anoxia intolerant plant tissues under oxygen deprivation caused changes in the cell ultrastructure such as plasmolysis and disintegration of organelles. Such changes have been described in detail earlier by Vartapetian and Zakhmilova (1990). The anatomy and ultrastructure of *Iris pseudacorus* rhizome parenchyma containing masses of storage fructan have been described earlier by Hanhijärvi and Fagerstedt (1994). In this study *I. pseudacorus* rhizome parenchymal cells did not show any signs of damage on the ultrastructural level even after 45 days of anoxic incubation. According to our investigation and earlier biochemical studies (Chirkova, 1988; Hanhijärvi and Fagerstedt, 1994; Chirkova et al., 1998; Blokhina et al., 1999), the plant species used can be placed in the following order of increasing anoxia tolerance: wheat –.oat – rice - *I. germanica* – *I. pseudacorus*.

4.1.2. H_2O_2 formation

Under normal metabolic conditions the formation of ROS and peroxidation of membrane lipids are in dynamic equilibrium with the activity of the antioxidant systems. Any changes in environmental conditions may alter this equilibrium and lead to oxidative stress. Such alteration may be achieved either by enhanced ROS formation, or by slowing down its decomposition, i.e. inhibition of antioxidant systems.

H_2O_2 visualised by transmission electron microscopy (**IV**) is probably of enzymatic origin, considering oxygen concentration in our experiments and the positive effects of the various inhibitors of H_2O_2 producing enzymes on the amount of H_2O_2. An investigation on H_2O_2 accumulation under root hypoxia in the roots and leaves of *Hordeum vulgare* has shown two times higher levels of H_2O_2 in the roots of hypoxic plants as compared with the controls, however, in leaves this parameter was only 18% higher under stress conditions (Kalashnikov et al. 1994). In contrast to the chemical reduction of oxygen, an enzymatic process implies a high level of metabolic regulation, which can be accomplished, in the case of oxygen deprivation stress, by anoxia-specific metabolic changes. The latter can inhibit or stimulate the formation of ROS via indirect mechanisms. A decline in ATP content observed under anoxia (Chirkova et al. 1984, Hanhijärvi and Fagerstedt 1995) increases the probability of ROS formation in the ETC of mitochondria and, at the same time inhibits the energy-dependent step of GSH biosynthesis. The key enzyme in GSH biosynthesis, γ-glutamylcysteine synthase (EC 6.3.2.2), requires ATP as a cofactor and has an alkaline pH optimum 8-8.4 (Noctor et al. 1998). The regulatory role of ATP concentration and a threshold dependence of membrane integrity on ATP concentration have been shown (Rawyler et al. 1999). If the rate of ATP synthesis falls below 10 $\mu mol g^{-1} FW h^{-1}$ membrane lipid hydrolysis occurs, thus providing free fatty acids as the substrates for LP. Possible involvement of metabolic parameters in sensing the lack of oxygen (or ROS) and in the induction of the stress response has been proposed (Clement et al. 1998). The intracellular superoxide anion concentration and an increase in the chemical reduction state and acidification of the intracellular environment have been considered as signals for H_2O_2-mediated apoptosis (Clement et al. 1998). This particular combination of conditions constitutes a signal and is an indication of reductive as opposed to oxidative stress (Clement et al. 1998).

A decrease in ATP availability during anoxia can slow down ATP-dependent plasma membrane H+-ATPase and lead to the acidification of the cytoplasm and possibly to the alkalinisation of the cell wall. Localisation of H_2O_2 predominantly in the cell wall under anoxia and reoxygenation (**IV**) (taking into consideration the control treatments with inhibitors) suggests disturbance of the

enzymatic decomposition of H_2O_2 in this compartment. Another reason is the exhaustion of the reductants, especially ascorbate, under anoxia and reoxygenation (**III**), which are necessary for H_2O_2 utilisation by peroxidases. Acidification of the cytoplasm under anoxia can affect the activity of enzymes involved in the antioxidative defence system. Thus the very first step in antioxidative defence – detoxification of ROS − can be affected by anoxia-induced metabolic changes.

4.1.3. Anoxia-induced lipid peroxidation: CD, CT and TBARS

In respect to anoxia tolerance the formation of ROS (H_2O_2) appearance was less pronounced and postponed in time in anoxia-tolerant in comparison to anoxia-intolerant species. In the rhizomes of *I. pseudacorus*, an extremely anoxia-tolerant plant, H_2O_2 was detected only after 45 days of anoxia, indicating that no damage to the membranes had occurred. This observation correlated positively with conjugated diene formation measured under the same experimental conditions: There are no minima at characteristic wavelengths in *I. pseudacorus* rhizome samples (**I**)**.** Only after 45 days of anoxia both *cis-trans* and *trans-trans* diene minima appeared in the SD spectrum. Similar results have been obtained for *I. pseudacorus* rhizomes using EPR (electron paramagnetic resonance) (Crawford et al. 1994). In this species the signal corresponding to free radicals could not be observed in the spectrum, while in the anoxia-intolerant *I. germanica* the signal was clear. The data suggests that there was no detectable free radical formation, and hence neither diene nor TBARS production emerged in the rhizomes of the anoxia tolerant *I. pseudacorus*. In our experiments the CD and CT content corresponded with resistance to anoxia. The anoxia-tolerant oat and *I. pseudacorus* both exhibited very low accumulation of dienes and the highest tolerance to oxidative stress as compared with wheat and *I. germanica*, respectively.

Data gained by SD spectrophotometry on the formation of different stereoisomers permit us to evaluate the impact of chemical and enzymatic processes in LP development. Hydroperoxides, formed as a result of the autooxidation of lipids, represent a mixed population of stereoisomers with the *cis-trans/trans-trans* ratio depending on the temperature and substrate concentration (Porter et al. 1980). Another source of hydroperoxides is the lipoxygenase reaction, which leads to specific positional and configurational isomers. The enzyme converts the *cis,cis*-1,4-pentadiene structure, present in PUFA, to *cis, trans* conjugated hydroperoxydiene (Vick and Zimmerman 1987). Also tocopherols have been shown to affect the isomeric composition of

hydroperoxides. The addition of 5% α-tocopherol led to an absolute formation of *cis, trans* isomers in the autooxidation of methyl linolenate (Kamal-Eldin and Appelqvist 1996). In our experiments the absence of peaks corresponding to *cis, trans* dienes in SD spectra of aerated controls in the dark, can be explained by the slowing down of constitutive LP under the lack of light (Tremolieres 1985). According to our calculations from the peak heights in the SD spectrum (Corongiu and Banni 1994), *cis, trans* dienes are formed in a larger amount under anoxia in wheat and rice than under aeration. It is known that anoxia leads to cytoplasmic acidification (Richard et al. 1994). The pH optimum for many characterized lipoxygenases is acidic (Vick and Zimmerman 1987, Reddy 1991), and hence the probability of *cis, trans* diene formation increases.

Conditions favouring free radical generation such as low energy charge, high reducing equivalent levels and saturated ETC components, typically exist in anoxic tissue (Van Toai and Bolles 1991). A burst of ROS occurs when aerobic conditions are restored. In terms of membrane damage the post-anoxic burst of oxidative reactions have a more pronounced effect than anoxia itself. In our experiments the degree of such damage correlated positively with the duration of anoxic exposure (Table 1) and negatively with the plant's tolerance to anoxia as estimated by other means than LP parameters i.e. maintenance of AEC and electrolyte leakage (Chirkova et al. 1991). Hence, anoxically treated membranes were more susceptible to ROS attack and lipid peroxidation than aerobic controls (**I, IV**). Since *de novo* synthesis of lipids needs molecular oxygen, desaturases and large amounts of ATP, it stops under anoxia (Crawford and Brändle 1996). Therefore, the most efficient way to maintain functional membranes under anoxia is to preserve PUFA and lipids instead of their synthesis (Pfister-Sieber and Brändle 1994).

It is a common feature that anoxia induces the formation of free fatty acids. It has been suggested that an increased amount of free fatty acids and a simultaneous decrease in polar lipids may be a common feature to all anoxia-intolerant species under oxygen deprivation stress (Hetherington et al. 1982). It has also been suggested that long-term anoxia-tolerance is dependent on the ability to maintain membrane function and membrane integrity (Chirkova, 1988). In the anoxia-sensitive *I. germanica* large amounts of free fatty acids are formed under anoxia and simultaneously the amount of polar lipids decreases due to membrane collapse. In contrast, in the anoxia-tolerant *Acorus calamus* formation of free fatty acids does not increase and the amount of polar lipids does not decrease in long-term anoxia (Henzi and Brändle 1993).

The factors underlying higher membrane stability of anoxia-tolerant plants can include both structural properties of the membranes (the degree of PUFA unsaturation, fluidity, permeability, incorporation of tocopherols) and anoxia-

induced metabolic changes such as cytoplasmic acidification, lowered energy charge and elevation of cytosolic Ca^{2+}.

Effect of LP on membrane properties and the reasons underlying membrane stability can be approached through a variety of methods: Biochemical characterisation of LP products (**I**), measurements of membrane function, such as barrier and transport properties and direct investigation of membrane structure. LP products have a pronounced effect on membrane orientational order, packing and interaction of phospholipids (Van Ginkel and Sevanian 1994). The formation of hydroperoxides causes the migration of the affected acyl chain from the membrane and tends to increase the area occupied by the phospholipid. Among other effects of lipid peroxides the decrease in molecular orientation order and a tendency for the bilayer structure to collapse should be named. Interestingly, LP products do not affect the fluidity of the membranes (Van Ginkel and Sevanian 1994), but membrane fluidity has been shown to affect the rate of LP propagation stage (Shewfelt and Purvis 1995). Both lipids and proteins are the targets of peroxidative processes, although LP is kinetically favoured. Thus, if the process is regulated strictly by kinetics, peroxidation of PUFA may serve a protective function to prevent a direct attack on proteins (Shewfelt and Purvis 1995). Lipid hydroperoxides increase the hydrophilic properties of the lipid bilayer (Frenkel 1991). A similar change in hydrophilicity has been shown under anoxia in the mitochondrial proteins. The transition of phenylalanyl residues to tyrosyl, providing an increase in hydrophilicity, has been detected under anoxia (Chirkova et al. 1993). This transition could affect synergetic action of tocopherol and ascorbate (Beyer 1994), which occurs on the hydrophobic-hydrophilic interface.

Still another aspect of LP role in stress metabolism should be discussed as the generally accepted approach of LP as a detrimental process is not complete. A role in stress signalling has been recently suggested not only for ROS but also for the oxygenated derivatives of LP (Tarchevskii 1992). 13-hydroperoxylinolenic acid, which is formed by lipoxygenase, is a precursor of jasmonic acid. Jasmonic acid has phytohormone-like activities and is involved in the regulation of developmental processes and in the plant's response to wounding and pathogens (Rosahl 1996). Peroxidation products have been proposed also to act as primary mediators in plants during heat stress (Kurganova et al. 1999).

Examples of cross-resistance can be found between other environmental stresses and oxidative stress. These observations have been used to propose a common basis for resistance against ROS (Alsher et al. 1997). According to Alsher et al. (1997) a first hypothesis could be that cross-resistance occurs between stresses, which originate in the same subcellular compartment, but not between stresses, which have disparate sites of action. However, cell

membranes are usually on the first line of defence, and hence they form the most probable compartment for both sensing and resisting the stress.

4.2. Antioxidant status under anoxia and reoxygenation

The antioxidant status of the cells is probably species and tissue specific. The differential response of the antioxidant systems in the plants studied here, indicates the importance of interaction between multiple parameters for the provision of protection: The rate of turnover of redox active ascorbate and glutathione, anoxia-induced metabolic changes and structural and functional properties of the membranes. Superimposed factors such as the duration of the anoxic treatment and the reoxygenation period are significant for the estimation of the defense efficiency, and hence anoxia tolerance. With respect to antioxidant systems, different rates of ROS formation in anoxia-tolerant and -intolerant species should be noted.

4.2.1. Superoxide dismutase

The investigation of SOD activity in our experimental plants resulted in contradictory observations (**I** and Monk et al. 1989), which can be explained by the different anoxia-tolerance of the species studied and experimental setup. In cereals the activity of SOD declined and was dependent on the duration of the anoxic treatment, while in *Iris pseudacorus*. a 14-fold increase was observed. The reason for the difference in results possibly originates from the particular experimental conditions, e.g. a prolonged reoxygenation period in the case of *Iris* spp. In cereal roots activity of the enzyme was determined immediately after anoxia. The formation of ROS already under hypoxic conditions and during the oxidative burst after re-admission of oxygen caused rapid substrate overload of constitutive resources of SOD, while induction was hindered by other factors (e.g. time, activity of downstream enzymes in the ROS-detoxification cascade, inhibition by the product (H_2O_2) and consequences of anoxic metabolism). Observations on SOD activity in different plant species under another stress conditions (drought and salinity) suggest that different mechanisms may be involved in oxidative stress injury (Yu and Rengel 1999). Activation of oxygen may proceed through different mechanisms, not necessarily producing a substrate for SOD. Changes in O_2 electronic configuration can lead to the formation of highly reactive singlet oxygen $^1O_2$. Comparison of drought and water stress effects on tolerant and intolerant wheat genotypes suggests that different mechanisms can participate in ROS

detoxification. Water stress did not affect SOD activity, while under drought conditions a significant increase was detected (Sairam et al. 1998). Under oxidative stress conditions combined with cold acclimation of cold-resistant and unresistant wheat cultivars, SOD activity was unaffected by low temperature treatment both in the leaves and in the roots, but plants exhibited higher guaiacol peroxidase activity (Scebba et al. 1998). Inefficiency of ROS detoxificating enzymes (SOD, catalase, ascorbate peroxidase and non-specific peroxidase) has been shown under water deficit-induced oxidative stress in rice (Boo and Jung 1999). A decrease in enzymatic activity was accompanied by LP, chlorophyll bleaching, loss of AA, GSH, α-tocopherol and carotenoids in stressed plants. The authors suggest the formation of a certain strong pro-oxidant, which is neither superoxide nor H_2O_2 under the conditions of water deficit (Boo and Jung 1999). However, in the roots and leaves of *Hordeum vulgare* under root hypoxia the activity of SOD increased by 40-60% (Kalashnikov et al. 1994). The ability of plants to overcome oxidative stress only partly relies on the induction of SOD activity because of the following reasons: Diversification of the pathways of ROS formation, compartmentalisation of oxidative processes (charged ROS can not penetrate the membrane) and compartmentalization of SOD isozymes. It is also possible that in different plant species and tissues different mechanisms are involved in the protection against oxidative stress.

4.2.2. Ascorbate and glutathione pools under anoxia and reoxygenation

The intensity of the oxidative stress, as mentioned above, relies on the interaction of several factors. One of them, antioxidant status of the tissue, is in turn regulated by the following parameters: Constitutive levels of antioxidants, redox state of the cell metabolites, the activity of antioxidant-regenerating enzymes and the conditions for successful antioxidant interaction (e.g. ascorbate-glutathione cycle).

Initial levels of AA and GSH, measured in the experimental plants before treatment did not correlate with the tolerance to anoxia. In cereal roots the differences were minor, while the rhizomes of *Iris* spp. differed significantly in their constitutive antioxidant levels (**III**). AA content in the rhizomes of the anoxia-tolerant *I. pseudacorus* was 7 times higher than that in the intolerant *I. germanica*, while the comparison of GSH content revealed the opposite situation: Anoxia-intolerant *I. germanica* contained 3 times more GSH than *I. pseudacorus*. However, these differences did not affect the initial level of LP in

these species (**I**). In the roots of cereals the main form of ascorbic acid was DHA, which is probably due to its transport role in these species (discussed in **III**). However, the higher content of DHA than AA in the roots affects the redox state of the tissue, antioxidant properties and, probably, anoxia tolerance of cereals. In order to elucidate the uniform relationship between antioxidant status and the intensity of LP the data were calculated as per cent of the control level in plants before treatment (Table I). During the time course of anoxia increased LP, measured as CD, CT and TBARS formation, resulted in a significant decrease of both reduced and oxidised forms of ascorbic acid and glutathione, despite the differences in the initial antioxidant content. The situation reveals severe oxidative damage to anoxically treated membranes and utilisation of antioxidants for ROS scavenging and disturbance of AA and GSH turnover via enzymatic mechanisms (discussed below). Calculation of the correlation coefficients between the parameters of LP and reduced and oxidised forms of the antioxidants was performed only for the treatments with identical experimental setup (anoxia and 2h reoxygenation) (Table 2). A negative correlation has been shown between LP levels and antioxidant content for all anoxic treatments with the exception of the anoxia-tolerant *I. pseudacorus*. In this species the correlation coefficient between the data sets on TBARS and ascorbate, and TBARS and glutathione was positive, and revealed the suppression of peroxidative processes before the termination stage of LP (TBARS accumulation) (**I**). In the roots of wheat, despite of the relatively low AA and GSH content in comparison with *Iris* spp., the correlation coefficients were close to -1 in the case of conjugated TBARS/AA (-0.999) and TBARS/GSH (-0.905), emphasising the importance of this redox couple for protection against LP. Similarly, the correlation coefficients calculated for *I. pseudacorus* between Dienes/GSH (-0.171) and Trienes/GSH (-0.414), probably indicate that these parameters do not significantly depend on each other and that another factor (e.g. high polyphenol content) is more important for the regulation of LP in *I. pseudacorus* rhizomes.

Imposition of anoxic stress causes several non-specific and anoxia-specific metabolic changes, some of which can interfere with the antioxidant turnover and synthesis, as well as affect non-antioxidant functions of the compounds studied. Consequences of ATP depletion on antioxidant system and LP are discussed later

An increase in the $NADH/NAD^+$-ratio during the first hours of anoxia can play an adaptative role by supplying reducing equivalents for the antioxidant turnover. The participation of NADH in MDHA reduction to ascorbate has been demonstrated (Noctor and Foyer 1998). AA and GSH metabolism (reduction to an active antioxidant form) may provide an additional sink mechanism for excess protons and NADH produced during the first stages of anaerobiosis

(Chirkova et al. 1992), therefore explaining the increase in AA and GSH contents observed under anoxia by other authors (Biemelt et al. 1996, 1998). Our results on the depletion of the reduced forms of ascorbate and glutathione suggest an imbalance in the glutathione-ascorbate cycle under anoxia, a condition which affects the redox state of the cell and redox-dependent reactions. A decrease in oxidised forms, DHA and GSSG, suggests that there is no turnover of antioxidants under these experimental conditions, despite the fact that tocopherol (necessary for AA regeneration) was maintained (**III** *Iris* spp. and **I** rice roots) or even enhanced (**I** wheat and oat roots). AA can be eliminated from the Halliwell-Asada cycle via oxidation to DHA and further decomposition to diketogulonic acid (Smirnoff 1995). Enzymatic turnover of ascorbate and glutathione is problematic for the plants under anoxic stress, since a decrease in corresponding enzymatic activities has been observed under prolonged flooding (anoxia) by several authors. Monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) activities decreased slightly or remained unaltered under hypoxia, while anoxia caused a significant inhibition of enzyme activities (Biemelt et al. 1998). Upon the restoration of normoxic conditions, however, the activity of antioxidative enzymes returned to the control level, which was interpreted by the authors as activation of the antioxidative defence. Inhibition of GR, ascorbate peroxidase, catalase and SOD activities has been shown also by Yan et al. (1996) in corn leaves under prolonged flooding. In contrast, many investigators report the induction of active oxygen-processing enzymes under oxygen deprivation stress. In an investigation on the effect of root hypoxia on H_2O_2 accumulation and the activity of the antioxidative enzymes the induction of SOD, GR, peroxidase and catalase has been shown in the roots and leaves of *Hordeum vulgare* (Kalashnikov et al. 1994). In these experiments higher levels of enzymatic activity have been detected under hypoxia (120-160% of the control) as compared with the same activities measured after the restoration of normoxic conditions (85-106 % of the control). A systematic study to characterise the specific effects of various environmental stressors (high temperature, enhanced light intensity, water deficiency, chilling and UV-B radiation) on the activities of several antioxidant enzymes in *Arabidopsis thaliana* under comparable experimental conditions has been undertaken by Kubo et al. (1999). The results reveal differential response of the antioxidant enzymes, although all of the above mentioned stressors are known to induce the production of ROS. The authors suggest that particular antioxidative enzymes have different threshold values of stress needed to increase their activity. This phenomenon may be explained by assuming that there are differences in major ROS produced under specific stress conditions, or there are differences in the cellular sites where ROS are formed (Kubo et al. 1999).

The activity of the antioxidant enzymes and the antioxidant turnover depends to a great extent on experimental conditions, especially in the case of oxygen deprivation stress. Indeed, O_2 availability is a necessary condition for ROS formation and for the induction of protective reactions i.e. ROS-scavenging and antioxidant-regenerating enzymes. Such induction is probably achieved through redox changes and is sensed by an unknown sensor. From that point of view, a decrease in AA/DHA and GSH/GSSG ratios in out experiments represents one of the possible redox signals in anoxically damaged tissue, undergoing oxidative stress. Another reason for the diversification of data on antioxidant content and corresponding enzymatic activity may originate from species- and tissue-specific factors underlying stress tolerance. These factors can include the concentration and compartmentalisation of a particular antioxidant, its functioning at the expense of other antioxidants, properties of the membranes and metabolic parameters, which can vary to a great extent under the same stress situation in different tissues and species.

4.2.3. Changes in the tocopherol content

Under anoxic conditions *de novo* synthesis of lipids is abolished and the protection against ROS is maintained via preservation of membrane structures and of antioxidant resources. The concentration of α-tocopherol, which has numerous functions in stress protection (i.e. quenching and scavenging of ROS, stabilisation of membrane structures and formation of complexes with free fatty acids), is extremely important for plant survival. High efficiency of the free-radical scavenging function of tocopherols should be supported by its turnover via reduction by ascorbate. During short-term anoxia in our experiments tocopherol levels were maintained (**III** *Iris* spp. and **I** rice roots) or enhanced (**I** wheat and oat roots). At the same time rapid utilisation of AA and GSH was observed (**III**). The formation of LP products detected after anoxia (**II,** Table 1) suggests intensive involvement of all antioxidant resources in membrane protection. A clear negative correlation between levels of LP products (CD, CT and TBARS) and antioxidants' reduced forms has been shown in anoxic, but not in control samples (Table 2). Therefore, tocopherol as an efficient and polyfunctional compound was maintained at the expense of AA and GSH, the latter at the same time being involved in radical scavenging in the aqueous phase of the cell. A similar temporal pattern of antioxidant interaction has been shown under ischemia-reperfusion in an isolated rat heart: Ascorbate and glutathione were depleted selectively prior to vitamin E (Haramaki et al. 1998). These results were considered as evidence of the antioxidant network under ischemia-reperfusion.

Lipid peroxidation reactions are dependent on molecular oxygen by definition. The reaction between a tocopherol molecule and PUFA is inhibited in the absence of oxygen (Saucy et al. 1990). A significant decrease in the tocopherol content observed after prolonged anoxia in *Iris* spp. (**III**) could be attributed to the membrane breakdown rather than antioxidative reactions *per se*. Indeed, the liberation of free fatty acids observed by Henzi and Brändle (1993) in *I. germanica* rhizomes under anoxia was interpreted as breakdown of membrane structures. It is possible that α-tocopherol molecules localised in membranes could be destroyed simultaneously with the collapse of the membranes. The absence of the same effect on β-tocopherol content could be partly explained by different localisation of α- and β-tocopherols. The significance of tocopherols for stress protection, and an indirect indication of the possibility of tocopherol synthesis under anoxic conditions, have been demonstrated in the experiments on anaerobically grown rice seedlings (Ushimaru et al. 1994). Their tocopherol level was 3 times higher than that of the aerobic controls and LP products (TBARS) were present at one third of aerobic levels.

However, other than oxygen-centered radicals can be formed and interact with unsaturated lipids. Under anaerobic conditions carbon-centered radicals (pentadienyl or epoxyallylic) can undergo radical-radical coupling with tocopheryl radical ($TO^{\bullet}$), thus forming adducts and terminating the reaction (Kamal-Eldin and Appelqvist 1996). When oxygen is present in trace amounts and hydroperoxides are present in negligible concentrations tocopherols can react directly with alkyl radicals ($L^{\bullet}$):

$TOH + L^{\bullet} \rightarrow TO^{\bullet} + LH$ (Kamal-Eldin and Appelqvist 1996).

Hence, under hypoxic conditions the possibility of free radical reactions exists, and tocopherol can carry out its antioxidant function. α-Tocopherol has been shown also to form stable complexes with free fatty acids (increasing under stress conditions) and lysophospholipids, thus stabilising the membrane structure (Kagan 1989). Therefore, the decrease in tocopherols under anoxia has a detrimental effect on the membranes through a dual mechanism: The deprivation of antioxidant protection and destabilisation of membranes. Oxidative stress causes a decrease in α-tocopherol concentration in a cell because of its radical scavenging function. The decrease in α-tocopherol concentration in turn acts as a signal of the cell redox state in sensitive cells. Thus, α-tocopherol can act as a sensor of the oxidant status of the cell and a transducer of the stress signal (Azzi et al. 1995).

Plant	Treatment	Days	Dienes	Trienes	TBARS	SOD	AA	DHA	GSH	GSSG	a-toc	b-toc
Wheat	Control	1	132	97	113	58	121	92	70	130	53	
		3	110	139	87	37	92	105	62	68	93	
	Anoxia	1	197	189	120	68	45	99	13	49	365	
		3	1252	788	135	60	25	63	12	23	151	
Oat	Control	3	129	133								
		5	166	186								
	Anoxia	3	1093	1124								
		5	1096	1000								
Rice	Control	3			91	60	257	115	129	190	53	
		7			70	59	107	78	119	161	174	
	Anoxia	3			133	50	138	85	55	117	109	
		7			127	60	68	55	38	106	217	
I. germ.	Control	4	242	1252	87		94	92	78	150	63	84
		8	259	1296	84		128	139	104	265	74	95
		12	334	1523	55		133	164	89	226	122	158
	Anoxia	4	628	2212	135		85	85	35	262	79	94
		8	730	2765	166		90	150	7	117	70	105
		12	1382	4986	236		89	125	18	93	61	98
I. pseud.	Control	15	37	44	100		72	60	61	110	96	282
		30	73	65	96		99	263	47	33	47	210
		45	92	111	86		79	140	184	45	64	187
	Anoxia	15	144	248	26		45	62.2	11	29	114	131
		30	180	255	70		59	230	13	16	57	115
		45	815	604	29		38	94.5	34	8	38	62

Table 1. Effect of anoxia on the content of lipid peroxidation products and antioxidant levels in anoxia-tolerant and -intolerant plant species. Data presented as per cent of initial value (0 days control)

Correlation between		Dienes/AA	Dienes/DHA	Dienes/GSH	Dienes/GSSG	Trienes/AA	Trienes/DHA	Trienes/GSH	Trienes/GSSG	TBARS/AA	TBARS/DHA	TBARS/GSH	TBARS/GSSG
Plant	**Treatment**												
Wheat	**Control**	0,832	-0,754	-0,596	0,648	-0,760	0,837	-0,611	-0,909	0,967	-0,992	0,205	1,000
	Anoxia	-0,759	-0,999	-0,575	-0,806	-0,786	-0,996	-0,609	-0,831	-0,982	-0,844	-0,905	-0,994
I. germ.	**Control**	0,693	0,737	-0,343	0,791	0,582	0,598	-0,387	0,794	-0,754	-0,852	0,274	-0,632
	Anoxia	-0,602	0,420	-0,789	-0,141	-0,625	0,456	-0,825	-0,131	-0,464	0,524	-0,745	-0,313
I. pseud.	**Control**	0,610	0,236	0,592	-0,349	0,280	0,023	0,842	-0,297	0,237	-0,307	-0,807	0,724
	Anoxia	-0,601	-0,178	-0,171	-0,559	-0,781	-0,120	-0,414	-0,746	0,944	0,394	0,740	0,787

Table 2. Correlation coefficients between the parameters of lipid peroxidation and antioxidant content

4.3. Characterisation of mitochondrial functions and PTP

In general, close consideration of anoxia-induced metabolic changes and PTP-triggering factors suggest PTP induction under anoxia. This phenomenon has been described for mammalian mitochondria (Kuzminova et al. 1998) but it has not been shown in any plant mitochondria.

Permeability transition, which was not dependent on a high Ca^{2+}- and Pi-concentration, has been observed in yeast mitochondria, and it was cyclosporin A (CsA) insensitive (Jung et al. 1997). High matrix Ca^{2+} is generally considered vital for PTP induction and the ability of mitochondria to take up this cation from the surrounding medium can be crucial for pore functioning. Our results on Ca^{2+}-uptake by plant mitochondria are in accordance with other observations obtained on plant mitochondria. In purified mitochondria isolated from pea (*Pisum sativum* L.) and Jerusalem artichoke (*Heliantus tuberosus* L.) accumulation of external free Ca^{2+} by means of an electrophoretic uniporter with a K_m of approximately 150 nM was observed. This uptake was Pi dependent in the Jerusalem artichoke, but was Pi independent in pea stem mitochondria (Zottini and Zannoni 1993). Mitochondrial Ca^{2+}-fluxes have been detected also under anoxia in maize suspension culture cells: A majority of mitochondria released Ca^{2+} in response to anoxia and took it up upon reoxygenation. These changes were prevented by ruthenium red, an inhibitor of intracellular Ca^{2+} channels (Subbaiah et al. 1998). However, these investigations were not aimed at the Ca^{2+} fluxes in connection with the possible induction of PTP, and hence other important mitochondrial parameters were not measured.

The swelling of mitochondria observed in our experiments after Ca^{2+}-uptake and energization of the mitochondria with a respiratory substrate (succinate or NADH) was not inhibited by CsA. The Ca^{2+}-dependent Ca^{2+}-release, characteristic of the pore opening in animal mitochondria, was not detected. These two parameters routinely used to detect permeability transition in animal physiology, failed to reveal the same phenomenon in plant mitochondria. In a similar investigation on yeast mitochondria and the CsA-insensitive, Ca^{2+}- and Pi-independent permeability transition, the pore was characterised by means of size calibration (Jung et al. 1997). The changes observed in yeast mitochondria were considered a result of PTP functioning, which was regulated in a different manner from the mammalian PTP. To our knowledge there are no reports on the functioning of PTP in plant mitochondria, however some sensitivity of membrane potential to CsA has been demonstrated in pea stem mitochondria (Petrussa et al. 1992). According to our results PTP was not induced in wheat

root mitochondria under the conditions studied here. Swelling occurred after the energisation of mitochondria but Ca^{2+}-uptake was not accompanied by membrane potential loss and Ca^{2+}-release. This type of swelling can be defined as ‘high energy swelling’ and is due to electrophoretic uptake of monovalent cations, K^+ in particular. It is characterised by the preservation of the membrane potential and low inner membrane permeability (Bernardi et al. 1999).

Changes in the mitochondrial volume according to recent findings on mitochondrial ultrastructure (Mannella et al. 1997) and the role of mitochondria in programmed cell death via cytochrome c and apoptogenic protein release (Skulachev 1998), can be considered a signalling event. Passage of water and solutes through the PTP is one of the possible mechanisms of mitochondrial volume regulation. However, other mechanisms can be involved in this process. The contraction of mitochondria, observed in our experiments upon the onset of anoxic conditions, which was preceeded by swelling (i.e. osmotic influx of water and electrophoretic uptake of ions), was probably due to passive extrusion of osmotically active compounds, accumulated during high energy swelling. This process is energy independent, since mitochondrial contraction occurred simultaneously with the membrane depolarisation. However, the physiological role of mitochondrial volume changes under anoxia is unclear and requires further investigation.

5. CONCLUSIONS AND FUTURE PROSPECTS

Under natural conditions anoxic stress necessarily includes several transition states (hypoxia, anoxia and subsequent oxidative stress) characterised by different O_2 concentrations and particular physiological states of the tissue. Excessive generation of ROS, one of the possible signs of oxidative stress, is an integral part of many stress situations (Noctor and Foyer 1998). However, any condition under which redox homeostasis is disturbed can be defined as oxidative stress (Alscher 1997). Changes in the steady-state equilibrium between ROS formation and utilisation in the cell can induce both adaptive responses (ROS signalling, Lander 1997) and detrimental changes in the cell structure and metabolism. The mechanism of ROS sensing is not known, but several existing models (Semenza 1999) employ redox changes and suggest that hypoxia (low oxygen concentration) and ROS could be sensed through the same mechanism. The consequences of post-anoxic oxidative stress depend on several factors: Duration of the oxygen deprivation, reoxygenation period and plant's tolerance to anoxia (an integrative parameter, which implies metabolic and structural adaptations to anoxia). Anoxia leads to modifications in cell metabolism and the functional state of the membranes in a way, which creates the basis for ROS generation. E.g. changes in the lipid content and composition, membrane fluidity (Chirkova et al. 1989, Hetherington et al. 1982), decreased adenylate energy charge (Hanhijärvi and Fagerstedt 1994, 1995, Chirkova et al. 1984), and cytoplasmic acidosis can be considered favourable for ROS production and the successive peroxidation of lipids (Crawford et al. 1994; Crawford and Brändle 1996, Chirkova et al. 1998). Hence, the sequence of events representing the stress reaction such as generation of ROS, lipid peroxidation and antioxidant responses is affected by metabolic changes under oxygen deprivation.

The initial non-specific step in the stress response is the generation of ROS, a phenomenon, which has been shown to be the primary event in a large number of stresses (Foyer et al. 1997). Since the process has been started and the signal produced, the differences between plants in stress tolerance are mainly due to the ability of the membranes to maintain their functional integrity, and to initiate metabolic adaptations (the oxygenated products of PUFA have been shown to act as endogenous regulators of metabolic processes (Tarchevskii 1992)). At that point stress specific reactions could be observed. Therefore, such membrane properties as phospholipid composition and the degree of fatty acid unsaturation together with energy metabolism play an important role in stress resistance. The investigation of LP process on the primary (CD and CT

formation) and secondary (TBARS accumulation) steps under anoxic stress allows us to conclude that the rate of LP and, hence, the extent of membrane damage correlate negatively with anoxic stress tolerance.

In general, resistance to stress implies the ability of a plant to respond to the stressor in a way which maintains homeostasis (unresistant plant) or, vice versa, changes homeostasis in an adaptive direction (resistant plant). These two strategies allow unresistant plants to survive for a short period of time and enable resistant plants to overcome the stress. To obtain an adaptive response the plant has to produce a signal, which is impossible without changes in homeostasis. Changes in pH, redox state, ROS levels and adenylate energy charge are likely to participate in signalling.

Imposition of anoxia with subsequent reoxygenation results in an overall depletion of AA and GSH and lowers the redox status of the cell in all of the species studied except in the very anoxia tolerant *I. pseudacorus*. ROS generation has been shown to be a compartmentalised process; hence, antioxidant protection must be targeted to a particular place (PM, chloroplast, mitochondria, peroxisome). The membrane-integrated tocopherols are preserved as the last resort to maintain membrane integrity under extreme stress conditions, when the hydrophilic antioxidants have been depleted already. Under these conditions the main protective function of tocopherols is the stabilisation of the peroxidised membrane, rather than free radical scavenging. Different responses of antioxidant systems in anoxia-tolerant and -intolerant plants suggest that there is no universal mechanism incorporating all the antioxidants and leading to ROS detoxification in the plant species studied here. No detailed causal links can be shown between the extent of damage and antioxidant content. It is suggested that tolerance to oxidative stress, occurring upon reoxygenation, requires integration between the antioxidant system and anoxia specific metabolic changes.

Numerous attempts have been made to enhance plants' resistance to oxidative stress by over-expression of ROS-scavenging elements. Positive effects of SOD and APX overexpression have been reported. However, the protective effects were marginal and were observed under strictly controlled conditions (Bohnert and Sheveleva 1998). Elevated GSH biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically caused increased oxidative stress because of the failure of the redox sensing process in the chloroplast (Creissen et al. 1999).

Elevated ROS levels are a general attribute of disturbed metabolic balance. During evolution it probably originated from a redox sensing process and developed into a system employing O_2 as a main trigger of redox changes. From this point of view an investigation into O_2 sensing via Fe-S-clusters seems to be the most promising approach. The proposed mechanism also covers oxygen

sensing through the mitochondrial ETC, since its modification by O_2/ROS includes redox transitions in the Fe-S-clusters of ETC components. Another important direction in the identification of the stress signalling components is the investigation of mitochondrial functions. Induction of PTP in animal mitochondria is mediated by enhanced production of ROS, redox changes etc., and is considered one of the possible mechanisms of stress signalling (i.e. elimination of ROS producing mitochondria, generation of a nuclear-targeted signal leading to programmed cell death), and, probably also involves sensing of the O_2 concentration.

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