Long-term anoxia tolerance in leaves of
Acorus calamus L. and Iris pseudacorus L.

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Abstract

Mature green leaves of Acorus calamus and Iris pseudacorus have been shown to survive at least 28 d of total anoxia in the dark during the growing season, increasing up to 75 d and 60 d in overwintering leaves in A. calamus and I. pseudacorus, respectively. During the period of anaerobic incubation the glycolytic rate is reduced, carbohydrate reserves are conserved and ethanol levels in the tissues reached an equilibrium. Prolonged anoxia significantly suppressed leaf capacity for respiration and photosynthesis. After 28 d of anoxia, respiratory capacity was reduced in A. calamus and I. pseudacorus by 80% and 90%, respectively. The photosynthetic capacity of leaves decreased by 83% in A. calamus and by 97% in I. pseudacorus after 28 d of anoxia. This reduction in photosynthetic capacity was accompanied by a modification of the chlorophyll fluorescence pattern indicating damage to the PSII reaction centre and subsequent electron transport. Chlorophyll content was only slightly reduced after 28 d under anoxia and darkness in A. calamus, whereas there was a 50% reduction in I. pseudacorus. On return to air A. calamus leaves that endured 28 d of anoxia recovered full photosynthetic activity within 7 d while those of I. pseudacorus had a lag phase of 3–10 d. This well-developed ability to endure prolonged periods of oxygen deprivation in both these species is associated with a down-regulation in metabolic activity in response to the imposition of anaerobiosis. It is suggested that when leaf damage eventually does take place in these species after protracted anoxic stress, it is anoxic rather than post-anoxic stress that is responsible.

Key words: Acorus calamus, Iris pseudacorus, anoxia, carbohydrate, metabolic down-regulation, photosynthesis, chlorophyll fluorescence.

Introduction

Oxygen deprivation resulting in either temporary or prolonged hypoxia or even anoxia is a common environmental stress experienced by plants in poorly drained soils, during long-term flooding, total submergence or ice-encasement. The metabolic events which accompany the shift from aerobic to anaerobic processes have been intensively studied in root tissues and germinating seedlings of crop plants. However, the length of experimental stress application in these species is always limited due to their short survival time under anoxia. The majority of crop plants are annual dryland species and prolonged waterlogging during the growing season usually leads rapidly to flooding injury and yield loss. Despite the complex problems for plant life under anoxia, a number of plant species are well adapted to prolonged oxygen deprivation. Mostly these long-term anoxia-tolerant species inhabit bogs, wet marshlands, pools, river banks, and salt marshes or else are accustomed to survive long periods under a closed ice-layer (Crawford and Braendle, 1996). As yet there is little information concerning the metabolic mechanisms involved in long-term anoxia tolerance.

For a species to be considered anoxia-tolerant it is not necessary that every organ or tissue has to survive oxygen deprivation. Ecologically, all that is required is the survival of those organs that are essential for subsequent regeneration of the whole plant on return to air. Roots are relatively sensitive to oxygen deprivation, and the

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survival capacity of most tolerant species resides in the shoots or rhizomes. The longest survival times for plant tissues under anoxia have been found in the overwintering rhizomes of some wetland species (Monk et al., 1984). In a quasi-dormant state rhizomes can withstand oxygen deprivation for up to 3 months. During flooding, green shoots are often only partly submerged and maintain a connection to atmospheric oxygen. In many wetland species flooding-enhanced aerenchyma develops in roots allowing the diffusion of oxygen to the flooded plant organs. Consequently, green plant tissues have been largely ignored in oxygen-deprivation studies. However, in temperate climates prolonged waterlogging often takes place in winter or spring, when shoots are still small and liable to submergence. Total submergence or ice-encasement can also impose oxygen deprivation on green shoots and, in the absence of photosynthesis or contact with the atmosphere, survival depends on physiological adaptation to anoxia or at least hypoxia.

The anoxia tolerance of leaves has recently claimed attention when it was shown that intact plants of some arctic species survived lengthy periods of total anoxia without suffering any visible damage to the leaf tissues (Crawford et al., 1994). The possibility that leaves can adapt metabolically to anoxia is especially interesting as in comparison to rhizomes, leaves possess higher metabolic activities in addition to photosynthetic carbon fixation. The metabolic consequences of anaerobic deprivation for leaves are therefore likely to be more severe than in dormant rhizomes. Two different growth responses of shoots to anoxia have been observed. First, it has been shown that there are some species which are able to sustain upward shoot elongation under anoxia and secondly, there are species where the shoot can survive anoxia but does not grow in the absence of oxygen (Barclay and Crawford, 1982). The advantage of the first group lies in the increased possibility for the plant to regain contact with air. If the elongated shoot, however, cannot establish a connection with the atmosphere quickly, the plants soon die of exhaustion. In the second group, green shoots seem to outlast anoxia by reducing metabolic activity to a minimum until environmental changes allow re-aeration.

The two species under investigation, *Acorus calamus* L. and *Iris pseudacorus* L., naturally inhabit sites with high water content and high risk of oxygen deprivation stress. The high anoxia tolerance of the Sweet Flag (*A. calamus*) shoots has already been proven (Bucher and Kuhlmeier, 1993). This monocotyledonous amphibious species is native to south and eastern Asia and was introduced as a triploid into Europe in 1557 where it has never been observed to set fruit and is entirely dependent on vegetative propagation. The rhizomes are positioned at the water-soil interphase and as a consequence are submerged for most of their lifetime. If the water table is high, rhizomes and shoots often overwinter totally submerged and are thus liable to experience prolonged periods of oxygen deprivation. The second species investigated, the Yellow Flag or Yellow Iris (*I. pseudacorus*), is native to Europe and is comparable to *A. calamus* with respect to its morphology and life style. Leaves are usually present throughout the year with peak growth occurring from April to June. While the carbohydrate content of *A. calamus* consists mainly of starch, *I. pseudacorus* stores carbohydrate reserves in the form of fructans (Augem, 1928).

In this paper, some central features of anaerobic metabolism including ethanol fermentation are reported from leaves that were kept under prolonged anoxia. After the switch from aerobic to anaerobic metabolism, the main problems of long-term anoxia lie in the maintenance of those cell functions that are necessary to guarantee metabolic recovery. On return to an oxygen-rich environment, the plants have to contend with problems of post-anoxic injury caused by the generation of reactive oxygen species or the oxidation of anaerobically accumulated substances (Biemelt et al., 1998). An intact anti-oxidative defence system is therefore assumed to be advantageous for the prevention of further damage to membrane lipids and other cellular macromolecules under post-anoxia.

The functionality of the respiratory pathway and the photosynthetic apparatus was therefore studied in the leaves on return to aerobic conditions.

**Materials and methods**

**Plant material and anoxic incubation**

*Acorus calamus* plants originated from the Moossee (Bern), and were cultivated in a pond at the University of St Andrews. *Iris pseudacorus* was collected from semi-natural sites, such as overgrown curling ponds, in North East Fife, Scotland. The rhizomes of these plants were cleaned from soil, cut into pieces of about 5–7 cm and potted separately in sand. The shoots of all plants were cut back to about 20–25 cm above the soil. To allow the plants to establish in the pots, they were kept in the greenhouse for at least 2 weeks until new leaves developed. The greenhouse-conditioned plants were used as controls representing day zero of the anoxia and dark treatment.

For the anoxia experiments, plants were transferred directly from the greenhouse into an anaerobic chamber (Forma Scientific Anaerobic systems, model 1024, Marietta, Ohio, USA). The atmosphere in the chamber consisted of 90% nitrogen and 10% hydrogen with a palladium catalyst to remove any traces of oxygen. The anaerobic atmosphere was checked with methylene blue indicator strips (Dry Anaerobic Indicator strips, Becton Dickinson Microbiology systems, Cockeysville, USA). To avoid the production of oxygen via photosynthesis, plants were kept in the dark. Humidity in the chamber was nearly 100% and plants were supplied with nitrogen-bubbled water if necessary. The temperature was kept at 20–25 °C. After the anoxia treatment, plants were returned to the greenhouse. Plant survival was checked by observing leaf development after the return to aerobic conditions in the
greenhouse. For comparison, experiments were repeated with plants which were kept in the dark but under aerobic conditions.

Leaf samples were harvested from the 3–4 youngest leaves about 10–15 cm above the ground, except for photosynthesis experiments and fluorescence measurements, where tissues about 5–15 cm below the leaf tips were used. For rhizome and root sampling, the material was washed carefully with cold water. Rhizome samples were taken from about 2–3 cm below the base of the oldest green leaf.

**Carbohydrate assay**

For carbohydrate assays, plant material of about 0.5 g fresh weight was cut into pieces, boiled for 30 min in 10 ml of 40% ethanol and filtered. The extraction was then repeated in distilled water and filtered again. The extracts were combined and dried under vacuum ( Büchi Rotavapor, R, Glasapparatefabrik Flawil, Switzerland). The residue was redissolved in 1 ml distilled water and used for total soluble carbohydrate determination (TSC). Aliquots of the concentrated soluble carbohydrate extract were carefully mixed with anthrone reagent (8.6 mM anthrone in 80% v/v H₂SO₄) and heated for 10 min in an 80 °C water bath, then cooled for 30 min on ice. Absorbance was recorded at 623 nm and total soluble carbohydrate concentration calculated from a glucose calibration curve. For the determination of total non-soluble carbohydrate content (TNSC), the boiled plant material was ground in liquid nitrogen and freeze-dried overnight. To hydrolyse the non-soluble carbohydrates the freeze-dried material was resuspended in 1.6 M perchloric acid and incubated in a water bath at 70 °C for 2 h. Samples were centrifuged at 10 000 g for 10 min and the carbohydrate concentration in the supernatant was determined via the anthrone method as described above.

**Ethanol determination**

Ethanol content of the plant material was determined by gas liquid chromatography. The plant material (about 0.3 g fresh weight) was ground quickly with 2 ml of ice-cold 6% (v/v) perchloric acid. Some acid-washed sand was added to allow rapid destruction of the material. After centrifuging the samples for 5 min at 10 000 g, the supernatant was decanted and a drop of methyl orange added as an indicator. The solution was neutralized by the addition of 5 M K₂CO₃ until the colour changed from red to yellow. The samples were stored at 4 °C for 15 min to allow settlement of the precipitate.

Samples of 1 ml extract were injected into a Pye Unicam series 104 model 64 Gas Liquid chromatograph and flushed through a 1.75 m glass column filled with Porapak Q, 100–120 mesh. Nitrogen was used as a carrier gas at 40 ml min⁻¹. The investigation temperature was 150 °C for the column and 160 °C for the detector. The degree of ionization as the ethanol burned was recorded, and the area of the ionization peak calculated by a Hewlett Packard Integrator. The GLC system was calibrated by linear correlation with ethanol standard solutions in the range of 0.05% to 0.3% (Sigma-Aldrich Company Ltd., Poole, UK).

**Gas exchange analysis**

Oxygen uptake, and aerobic and anaerobic CO₂ production of leaf discs were determined with a Warburg respirometer. About 0.2 g of leaf material were sliced quickly into discs of no more than 1 mm thickness and transferred into Warburg flasks containing 3 ml of distilled water. The flasks were connected to the manometer proper, and the manometer fluid adjusted to 150 mm. After 10 min equilibration, readings were taken every 30 min for 3 h. For measuring the O₂ uptake, small filter-paper rolls soaked with 10% KOH were inserted into the flask to capture all CO₂ produced. Anaerobic CO₂ production was measured in flasks flushed with nitrogen for 5 min to remove all oxygen. The temperature was kept at 20 °C. A thermo-barometer was set up to correct all measurements for variation in pressure and temperature in the room. The flasks were wrapped with tin foil to prevent any O₂ production via photosynthesis. Calculation of gas exchange parameters was carried out according to Umbreit et al. (Umbreit et al., 1957).

**Enzyme assay**

For the determination of pyruvate decarboxylase (PDC) activity, leaf material of 0.2 g fresh weight was ground in ice-cold extraction buffer (125 mM MES–KOH pH 6.8, 100 mM NaCl, 2.5 mM MgCl₂, 1 mM EDTA, 0.5 mM thiamine pyrophosphate chloride, 2 mM DTT, and 20 mg PVPP). The samples were centrifuged for 15 min at 10 000 g at 4 °C. Depending on PDC activity, 50–100 μl enzyme extracts were incubated in an assay buffer (50 mM MES–KOH pH 6.0, 25 mM NaCl, 1 mM MgCl₂, 0.5 mM thiamine pyrophosphate chloride, and 2 mM DTT) at 25 °C. After 20–30 min, NADH (final concentration 0.17 mM), sodium oxamate (final concentration 50 mM) and 10 U ADH (from baker’s yeast, 450 U mg⁻¹ solid; Sigma-Aldrich Co. Ltd., Poole, UK) were added and the ‘blind’ reaction recorded for 5 min at 340 nm. The reaction was started by the addition of Na-pyruvate (final concentration 10 mM) and the absorbance was recorded at 340 nm for 10 min. Temperature was kept at 25 °C.

For determination of cytochrome c oxidase (COX) activity, 0.5 g leaf material was ground in liquid nitrogen. Extraction buffer (100 mM MOPS–KOH pH 7.0, 50 mM MgCl₂, 1 mM β-mercaptoethanol, and 2 mM EDTA) was added and the samples centrifuged for 20 min at 10 000 g at 4 °C. The cytochrome c (acid modified, from horse heart, Sigma-Aldrich Co. Ltd., Poole, UK) was made up in 100 mM tricine buffer (pH 7.5) at 10 mg ml⁻¹ and reduced by adding a few crystals of Na-dithionite. Enzyme extracts were incubated in 100 mM tricine buffer (pH 7.5) in the presence of 0.1% Triton-X-100 for 1 min. The reaction was started by the addition of reduced cytochrome c and the decrease in absorbance was recorded at 550 nm for 10–15 min. The temperature was kept constant at 25 °C. Protein contents of the enzyme extracts were determined according to Bradford (Bradford, 1976). Enzyme activity measurements were carried out only on A. calamus leaves because large amounts of phenol in the rhizomes interfered with the assay in L. pseudocorus protein extracts.

**Photosynthesis measurements**

Rates of oxygen evolution were measured using whole leaf sections in a Hansatech Leaf disc Electrode (Hansatech, Kings Lynn, Norfolk, UK) linked to a chart recorder. The leaf material was placed into the leaf disc chamber to which a few drops of saturated KHCO₃ were added to generate sufficient CO₂ for the photosynthetic reaction. The leaf disc electrode was assembled according to the manufacturer’s instructions and oxygen evolution was measured for 15 min under saturating
light intensities (2600 μE m⁻² s⁻¹). A constant temperature of 20 °C was maintained in the chamber by circulating water from a temperature-controlled water bath. On completion of the measurements, the leaves were mounted on paper with clear tape and leaf area was determined using an AnalySIS® image analyser equipped with a monochrome CCD camera (Norfolk Analytical LTD, Hilgay, UK). The chlorophyll content of leaves was measured by acetone extraction according to Arnon (Arnon, 1949).

The PSII chlorophyll fluorescence emission of intact leaves was measured with the Fluorescence Monitoring System (FMS) from Hansatech (Hansatech Instruments Ltd., Kings Lynn, UK). Before the measurements, leaves were adapted to the dark for 30 min and \( F_{m} \) was determined after exposure to modulated light. \( F_{m} \) was detected by application of a saturating light pulse. Following this, leaves were illuminated with actinic light (180 PAR) until a steady-state photosynthetic rate was reached. Saturating pulses (1740 PAR) were imposed every 60 s. All measurements were carried out at ambient CO₂. The parameter for maximum efficiency of PSII (\( F_{v}/F_{m} \)), photochemical (\( q_{P} \)) and non-photochemical quenching (\( q_{NP} \)) were calculated according to Jones (Jones, 1992). The quantum efficiency of PSII (\( \Phi_{PSII} \)) was estimated according to Genty et al. (Genty et al., 1989).

### Results

**Plant survival under anoxia**

The present experiments confirm the remarkable tolerance of \( A. \) calamus under long-term anoxia (Table 1). The majority of rhizomes (90%; \( n = 10 \)) survived up to 75 d under anoxia and started regrowth within 5–8 d after return to aerobic conditions. This agrees with the finding of Bucher and Kuhlemeier who reported the survival of anoxia by leaves and rhizomes for up to 2 months (Bucher and Kuhlemeier, 1993). \( I. \) pseudacorus proved to be less tolerant than \( A. \) calamus. The maximal survival time of individual rhizomes of \( I. \) pseudacorus under anoxic conditions was about 65 d in winter, and about 50 d in summer. The survival was checked by the ability of the plants to regrow new shoots on return to air. Hanhijärvi and Fagerstedt estimated the survival time of about 30 d under anoxia in \( I. \) pseudacorus rhizomes, but in their experiment this was judged solely from the drastic drop at this time in adenylate energy charge (AEC) (Hanhijärvi and Fagerstedt, 1995). Roots in both species tested were sensitive to anoxic conditions and became soft after only 7–14 d of anoxia.

The behaviour of leaves under anoxia differed between (i) overwintering leaves, (ii) maximal expanded mature leaves and (iii) young growing leaves. Maximal survival times of leaves from individual plants are presented in Table 1. Generally, overwintering leaves are short and grow in late autumn. They survived anoxia almost as long as the rhizomes with only the tips wilting. Regrowth of new leaves started within 2–3 d in \( A. \) calamus, but was retarded in \( I. \) pseudacorus. During the growing season, plants consisted of 2–3 growing inner leaves, about 2–4 fully expanded leaves and a few senescent outer ones. Under anoxia, the older leaves lost their green colour and turned soft in both the tested species. On return to air these leaves wilted and yellowed rapidly. In the 4–5 youngest leaves of \( A. \) calamus, damage was limited to the leaf tips and the bulk of the tissue remained green and turgid up to 40–50 d of anoxia (Table 1). On return to air no further damage was visible and the youngest leaves started to regrow after 3–4 d of post-anoxia. The outer leaves of \( I. \) pseudacorus showed damage after 1–2 weeks of anoxia with the tips turning soft and brown. On return to air the tissue became yellow and showed no sign of recovery. Only the 2–3 mature leaves of \( I. \) pseudacorus were able to survive up to 35 d under anoxia (Table 1) and remain green when returned to aerobic conditions.

The aim of the research reported in this paper was to investigate the metabolic responses in tissues tolerant of anoxia. As only plant material without any visible damage was to be used, the above results on anoxia survival capacity determined the conditions for the metabolic studies. After 28 d of anoxia, the bulk of leaf tissue survived without major damage in 96% of the tested \( A. \) calamus plants and in 86% of the tested \( I. \) pseudacorus plants (Table 1). Most of the metabolic experiments were therefore done on leaf tissue which experienced up to 28 d of anoxia. This period was judged to be long enough for the plants to respond metabolically to long-term anoxia, and also ensured that only living leaf tissue was used for metabolic tests. For comparison, plants of both species were kept in the dark under aerobic conditions. These plants continued to grow without light

| Table 1. Survival of leaves under anoxia in \( A. \) calamus and \( I. \) pseudacorus |
|---------------------------------|---------------------------------|---------------------------------|
| Survival after 28 d anoxia       |
| with the majority of leaves      |
| green and intact (\( n = 50 \))  |
| Maximal survival of overwintering |
| leaves (days of anoxia)          |
| Maximal survival of leaves       |
| during the growing season        |
| (days of anoxia)                 |
| (i) overwintering leaves         |
| (ii) maximal expanded mature     |
| leaves                          |
| (iii) young growing leaves       |
| \( A. \) calamus \%              | 96%                             | 75 d                            |
| \( I. \) pseudacorus \%          | 86%                             | 60 d                            |
| Old leaves                       | 40 d                            | 50 d                            |
| Young leaves                     | 20 d                            | 35 d                            |
and developed new etiolated leaves. Older leaves wilted and yellowed and only 2–3 leaves remained green under prolonged darkness.

**Effects of anoxia on carbohydrate content**

After 28 d under anoxia the total soluble carbohydrate (TSC) content of leaves was reduced in *I. pseudacorus* leaves by 60% and in *A. calamus* by 15% while the dark treatment led only to minor changes in the TSC content (Fig. 1c, d). The total non-soluble carbohydrate (TNSC) content of leaves was comparably low and did not decline significantly when plants were kept in the dark either under anoxia or in air.

The carbohydrate content in the rhizomes was therefore monitored over 9 weeks of anoxia (Fig. 1a, b). The two species differed greatly in the size of the carbohydrate store of the rhizome; *I. pseudacorus* contained double the amounts of TNSC of *A. calamus*. In the rhizomes of both species, anoxia resulted in a considerable decrease of TNSC. In *I. pseudacorus*, a rapid drop in TSC and TNSC level to 20% of the initial value took place in only 2 weeks. In the rhizomes of *A. calamus*, TNSC reserves were mobilized more slowly and reached a minimum after about 6 weeks at about 20% of the control value. TSC content, however, increased slightly but significantly in *A. calamus* during the first week of anoxia and declined only slowly under continuing anoxia.

**Effects of anoxia on ethanol content**

Energy production via oxidative phosphorylation is hindered under anoxia, and energy needs have to be provided by anaerobic processes. In plants, ethanol fermentation appears to be the major pathway. The ethanol content of leaves, rhizome and roots was investigated over a period of 9 weeks under anoxia in both species (Fig. 2). Ethanol concentrations increased significantly after the onset of anoxic conditions in all organs. Highest accumulations of ethanol occurred in leaves, reaching levels up to 80 μmol g⁻¹ FW. In rhizomes ethanol concentrations of up to 30 μmol g⁻¹ FW were found. The ethanol accumulation under anoxia followed a similar pattern in both species. After the initial increase, ethanol concentrations remained at a constant level for the remainder of the experiment. At this stage both species reached an apparent equilibrium between ethanol production (reduced—see below) and dissipation by diffusion aided by the presence of intact leaf tissue.
Effects of anoxia on gas exchange capacity of leaves

In the untreated control plants, O₂ uptake and CO₂ production were close to equality in the leaf tissue of both species, indicating that carbohydrates were the preferred respiratory substrate (Table 2). Oxygen uptake as well as aerobic CO₂ evolution fell continuously when leaves were exposed to anoxic conditions. After 28 d of anoxia, the capacity for aerobic gas exchange rates decreased by about 80% in *A. calamus*, and by 90–95% in *I. pseudacorus* when compared to the control. Generally, the aerobic release of CO₂ by the tissue was more inhibited than O₂ uptake. When plants returned to aerobic conditions, the respiratory activity increased rapidly in *A. calamus*, and rates of O₂ uptake and CO₂ production were back to control levels after 3 d post-anoxia. Recovery of aerobic gas exchange capacity was considerably impaired in *I. pseudacorus*. After 10 d of post-anoxia, O₂ uptake and CO₂ production were still reduced by 50% and 80%, respectively.

When the aerobically grown leaf tissue was exposed to anaerobic conditions, CO₂ emission decreased immediately. In leaf tissues of control plants, the exposure to 100% nitrogen atmosphere reduced the CO₂ emission to 30–40% of that in air. Under prolonged anoxia, the anaerobic CO₂ production decreased still further in leaf tissue of both species. Very low levels of 11.7 μl h⁻¹ g⁻¹ FW (14% of the controls) were reached in *I. pseudacorus* after 28 d of anoxia (Table 2).

Effects of anoxia on enzyme activity of leaves

The translational machinery of the cell is inhibited under oxygen limitation and therefore regulation of protein synthesis plays a major role for survival under anoxia. Pyruvate decarboxylase (PDC) is usually not involved in metabolism under aerobic conditions, and low PDC activity was observed in untreated leaves of *A. calamus* (Fig. 3a). However, the enzyme is required for energy production via ethanol fermentation and needs to be initiated under anoxia. The transfer of *A. calamus* plants to anoxia triggered an immediate 7-fold increase of the activity during the first 24 h of the treatment. The PDC activity continued to rise under anoxia and reached a maximum level about 35 times higher than the control value after 21 d, where it remained for the rest of the anoxia treatment. After re-exposure to air, the PDC activity remained high for at least 3 d, and after 10 d post-anoxia it was still about eight times higher than in the control. The dark treatment did not induce significant changes in the PDC activity of the leaf (Fig. 3c).

Cytochrome c oxidase (COX), on the contrary, is essential for aerobic respiration, but can not operate under anoxia. The COX activity time-course under anoxia and post-anoxia gives an opposite picture to that obtained for PDC. The activity of the enzyme was investigated in leaf tissue of *A. calamus* after exposure to prolonged anoxia and post-anoxia. In the first 24 h of anoxia, the COX activity (Fig. 3b) dropped by about 25%, but then was stable for 21 d under anoxia. A further decrease took place after 28 d of the anoxia treatment and the COX activity was only 15% of the initial level. Re-exposure to air led to a rapid recovery and after 24 h post-anoxia when the COX activity was about 50% of the control. The activity continued to rise during post-anoxia, and was not significantly different from the control value after 7 d of post-anoxia. Prolonged darkness did not alter the COX activity significantly (Fig. 3d).

Effects of anoxia on photosynthetic capacity

In the present experiments, plants were kept in the dark during the anoxia treatment. The ability to maintain photosynthetic oxygen evolution was tested therefore, in leaves of *A. calamus* and *I. pseudacorus* after anoxic incubation in the dark (Fig. 4). Surprisingly, the bulk of leaf tissue remained green and without visible etiolation under anoxia. The total chlorophyll content of *A. calamus*...
Table 2. Gas exchange of leaves under anoxia and post-anoxia

Oxygen uptake and CO₂ production under aerobic conditions as well as CO₂ production under anaerobic conditions were measured in leaf discs at day zero (control); after 7, 14 and 28 d of anoxia, and after 3 and 10 d of post-anoxia after 28 d anoxia treatment. The data represents the mean of six independent measurements from three plants ± standard error of the mean.

<table>
<thead>
<tr>
<th>Species</th>
<th>Oxygen uptake (µl h⁻¹ g⁻¹ FW)</th>
<th>Aerobic CO₂ production (µl h⁻¹ g⁻¹ FW)</th>
<th>Anaerobic CO₂ production (µl h⁻¹ g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acorus calamus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>313.6 ± 12.7</td>
<td>273.2 ± 9.4</td>
<td>110.9 ± 8.9</td>
</tr>
<tr>
<td>Anoxia 7 d</td>
<td>166.0 ± 13.5</td>
<td>124.7 ± 8.0</td>
<td>55.2 ± 10.2</td>
</tr>
<tr>
<td>Anoxia 14 d</td>
<td>149.8 ± 12.6</td>
<td>111.5 ± 11.9</td>
<td>27.5 ± 6.2</td>
</tr>
<tr>
<td>Anoxia 28 d</td>
<td>86.3 ± 13.5</td>
<td>47.7 ± 5.4</td>
<td>25.4 ± 4.9</td>
</tr>
<tr>
<td>Post-anoxia 3 d</td>
<td>326.9 ± 13.8</td>
<td>295.7 ± 11.9</td>
<td>70.7 ± 8.1</td>
</tr>
<tr>
<td>Post-anoxia 10 d</td>
<td>252.8 ± 7.1</td>
<td>239.9 ± 6.4</td>
<td>58.3 ± 10.3</td>
</tr>
<tr>
<td>Iris pseudacorus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>236.5 ± 14.2</td>
<td>231.6 ± 12.3</td>
<td>80.8 ± 7.4</td>
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<tr>
<td>Anoxia 7 d</td>
<td>95.9 ± 6.3</td>
<td>93.7 ± 7.4</td>
<td>32.2 ± 4.5</td>
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<tr>
<td>Anoxia 14 d</td>
<td>76.7 ± 4.6</td>
<td>45.9 ± 6.5</td>
<td>28.9 ± 3.7</td>
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<tr>
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<td>9.2 ± 2.1</td>
<td>11.7 ± 2.2</td>
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<tr>
<td>Post-anoxia 3 d</td>
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<td>73.4 ± 6.6</td>
<td>34.5 ± 6.2</td>
</tr>
<tr>
<td>Post-anoxia 10 d</td>
<td>109.6 ± 6.7</td>
<td>48.7 ± 5.6</td>
<td>41.0 ± 5.3</td>
</tr>
</tbody>
</table>

Fig. 3. Enzyme activity in leaves of A. calamus. Pyruvate decarboxylase (PDC) and cytochrome c oxidase (COX) activities were determined in A. calamus leaves. The time-course of PDC (a) and COX (b) activity are shown under prolonged anoxia (28 d) and following post-anoxia (10 d). Enzyme activities for PDC (c) and COX (d) under control conditions are compared to the enzyme activities after 28 d under anoxia, and after 28 d in the dark. Each datapoint represents the mean of six independent measurements from three plants ± SE.
leaves was only slightly reduced after 28 d anoxia treatment (Fig. 4c). The potential for photosynthetic capacity declined in *A. calamus* continuously under anoxia reaching 17% of the initial level after 28 d (Fig. 4a). On return to air, recovery began immediately and within 3 d of post-anoxia, the oxygen evolution was back to 50% of the control level. After 7 d in air, the oxygen evolution of *A. calamus* leaves showed no significant differences from the control (Fig. 4a). In *I. pseudacorus* leaves, photosynthetic capacity reached even lower levels and after 21 d under anoxia hardly any oxygen evolution was detectable. The total chlorophyll content in *I. pseudacorus* leaves declined to about 50% of the initial value after 28 d anoxia (Fig. 4c). In comparison with *A. calamus*, recovery on return to air was retarded. Within 10 d of post-anoxia, the oxygen evolution reached 80% of the control level (Fig. 4a). Because photosynthesis is light-dependent, chloroplast metabolism is affected by prolonged darkness even under aerobic conditions. For comparison, the photosynthetic capacity of leaves kept in air was also tested after exposure to continuous darkness for over 28 d (Fig. 4b). On return to light after 28 d of darkness, photosynthetic capacity in non-etiolated leaves had decreased, but to a lesser extent than after the 28 d anoxia treatment (Fig. 4b). The chlorophyll content of leaves was reduced to a similar degree by anoxia and dark treatment in both investigated species, an indication that the absence of light and not anoxia was responsible (Fig. 4c). In *I. pseudacorus* a further reduction in the chlorophyll content was detected during the post-anoxia recovery phase.

**Effects of anoxia on chlorophyll fluorescence of PSII**

To obtain further information on the degree of anoxic damage to the photosynthetic apparatus under anoxia as well as the repair capacity under post-anoxia, measurements were made of chlorophyll fluorescence of PSII in tolerant leaves (Fig. 5). The maximal efficiency of PSII in the dark-adapted leaf (*Fv*/*Fm*) was considerably reduced by anoxia in both plant species (Fig. 5a). Upon re-exposure to aerobic conditions, the maximal PSII efficiency recovered rapidly in *A. calamus*. The anoxia-related decrease in *Fv*/*Fm* was caused mainly by the increase in the initial fluorescence level *Fo*, while the maximal fluorescence *Fm* decreased only slightly (data not shown). Upon return to air the *Fv*/*Fm* ratio recovered rapidly and returned to the control level after 7 d post-anoxia. In the leaves of *I. pseudacorus*, the anoxia treatment caused an even greater decrease in *Fv*/*Fm*. In contrast to *A. calamus*, the recovery of maximal PSII efficiency was impaired on return to air and only started to increase again after 7 d in air. The dark treatment had only minor effects on the maximal PSII efficiency of the leaves (data not shown).

Photochemical and non-photochemical quenching was studied in illuminated leaves. The photochemical quenching coefficient (*qP*) decreased in both species under anoxia. Again, recovery under post-anoxia was more rapid in *A. calamus* than *I. pseudacorus* leaves (Fig. 5c). Non-photochemical quenching was initially enhanced in anoxia-treated *A. calamus* leaves, but after 14 d of anoxia the *qNP* level dropped. In *I. pseudacorus*, a decrease in non-photochemical quenching was detected from the very beginning of the anoxia treatment. Re-exposure to air initiated an immediate short increase in the non-photochemical quenching of both species (Fig. 5d). Bilger and Schreiber showed that the exposure to light, especially in stressed leaf tissue, is often accompanied by quenching of *Fo* leading to a decrease in the level of *Fo* after the light treatment when compared to the original *Fo* in the dark-adapted leaf (Bilger and Schreiber, 1986).
Fo levels were only slightly changed in *A. calamus* under anoxia. In anoxia-stressed *I. pseudacorus*, however, the exposure to light seemed to initiate a very strong decrease in the Fo level (data not shown).

The calculation of the quantum PSII efficiency gives a parameter for the proportion of light absorbed by chlorophyll associated with PSII that is used in photochemistry and should show a strong linear relationship to the efficiency of the carbon fixation. Considering that these experiments were carried out on different leaves, a remarkably regular response is found, indicating strong similarity between photosynthetic rate (Fig. 4) and efficiency of PSII in the fluorescence experiment (Fig. 5b).

**Discussion**

**Anoxia tolerance**

Under natural conditions leaves may be less frequently affected by oxygen deprivation than underground organs, but once the whole plant loses a connection to an oxygen-rich atmosphere, leaf survival can be crucial for recovery on return to air. The phenomenon of long-term anoxia tolerance of green leaves was first described for a number of arctic species (Crawford et al., 1994), where rapid onset of respiration and photosynthesis after many months deprivation through ice-encasement enables maximal exploitation of the remainder of the short vegetative period. The two species investigated in this study, *A. calamus* and *I. pseudacorus*, also showed tolerance to anoxia in green leaves for surprisingly long periods. The bulk of green leaf tissue survived 28 d of anoxia without any major visible damage in *A. calamus* and with damage limited to older leaves and leaf tips in *I. pseudacorus*. The small overwintering leaves showed particularly high tolerance to anoxia and individual leaves remained green and turgid for 75 d or 60 d without oxygen in *A. calamus* and *I. pseudacorus*, respectively. For comparison, anoxia-intolerant plants, such as maize, wheat, barley, *Arabidopsis thaliana*, and potato survive without oxygen for no longer than 72–96 h (Johnson et al., 1989; Sieber and Braendle, 1991; Ellis et al., 1999; U Schlüter and RMM Crawford, personal observations). More tolerant plants such as rice and *Echinochloa* spp. survive up to

![Fig. 5. Chlorophyll fluorescence of PSII in leaves under anoxia and post-anoxia. Chlorophyll fluorescence parameters were determined in leaves of *A. calamus* and *I. pseudacorus* immediately on removal from prolonged anoxia (28 d) and following post-anoxia (10 d). (a) Maximal PSII fluorescence Fv/Fm in the dark-adapted leaf. (b) Quantum efficiency of PSII (ΦPSII) (calculated according to Genty et al., 1987). (c) Photochemical quenching coefficient qP and (d) non-photochemical quenching coefficient qNP calculated according to Jones (Jones, 1992). Each data point represents the mean of five independent measurements ± SE.](image-url)
3 weeks of anoxia (Mujer et al., 1993). In both these currently investigated species, all growth stopped after the onset of anoxic conditions. Although the anoxia-treated plants had the additional potential stress of being kept in continuous darkness hardly any yellowing occurred. However, dark treatment under aerobic conditions resulted in etiolation and wilting of large parts of leaf tissue. Leaf senescence is usually an orderly process where first degradation and then transportation of cellular compounds assures the recycling of nutrients. It is possible that certain senescence processes such as breakdown of plastids or dehydration are inhibited under anoxia.

**Anaerobic metabolism**

The metabolic switch from aerobic to anaerobic processes is regarded as a critical moment for cell metabolism and has been intensively studied (Drew, 1997; Chang et al., 2000). Energy generation declines drastically and enzymes for anaerobic pathways frequently show increased activity. The present experiments were concerned with the investigation of long-term effects of anoxia. One of the main problems in the absence of oxygen is the maintenance of an adequate energy status in the cell. In contrast to anoxia-intolerant potato tubers, the adenylate energy charge (AEC) in *A. calamus* rhizomes drops only moderately at the onset of anoxia, but stabilizes soon to aerobic levels (Sieber and Braendle, 1991). Ethanol fermentation has been shown to be the major energy-generating pathway under anoxia in both investigated species (Monk et al., 1984) and ethanol accumulation was observed in all plant organs of *A. calamus* and *I. pseudacorus* under anoxia. The highest amounts of ethanol were found in the shoots, suggesting either that ethanol fermentation in this tissue is comparably high or else the release of volatile end-products of anaerobic metabolism via the shoot is contributing to the ethanol accumulation in the leaves. After about a week of anoxia however, an equilibrium between ethanol production and release is reached, preventing the accumulation of potentially damaging amounts of ethanol (Crawford et al., 1987; Monk et al., 1984).

Further information on the fermentative activity in anoxic leaves was obtained from gas exchange measurements. Leaves of *A. calamus* and *I. pseudacorus* seem to be generally well prepared for the drop in energy generation when metabolism switches from aerobic to anaerobic processes. The calculation of the Pasteur Quotient \( PQ = \frac{3 \times CO_2}{CO_2} \) production (anaerobe)/CO_2 production (aerobe); see Summers et al., 2000) gives values of 1.28 for *A. calamus* and 1.05 for *I. pseudacorus*. This indicates that there was no accelerated substrate consumption via glycolysis (Pasteur effect) when the leaves were suddenly transferred to anoxia. This is in contrast to the strong Pasteur effect \( (PQ \approx 3) \) found in anaerobic rice seedlings (Bertani et al., 1980) and *Potamogeton pectinatus* tubers (Summers et al., 2000). High fermentation rates may be necessary for the realization of anaerobic growth. However, *A. calamus* and *I. pseudacorus* stopped all growth processes under anoxia and re-equilibrated their anaerobic metabolism by an overall reduction of energy consumption rather than by acceleration of glycolytic energy production. This strategy seems to be very successful and maximal survival times are found in leaves, which under anoxia enter a quasi-dormant state. Under prolonged anoxia anaerobic CO_2 production decreases even further suggesting that glycolytic activity slows down considerably under anoxia. Interestingly, a very similar strategy is found in anoxia-tolerant animal tissues, where glycolytic flux is remarkably reduced in the absence of oxygen. The phenomenon is called the ‘reverse Pasteur effect’ or ‘anaerobic arrest strategy’ (Hochachka, 1986). The similarities between animals and plants capable of enduring long-term tolerance of anoxia had already been discussed (Crawford, 1978), and later refined, pointing out differences between long-term and short-term tolerance of anoxia (Crawford and Braendle, 1996; Braendle and Crawford, 1999).

Even under comparably low substrate consumption by glycolysis, fermentable substrates need to be available to keep anaerobic energy production running. Carbohydrate starvation has long been regarded as one of the main causes of cell death under anoxia. As well as sugar depletion in the cell, other effects have been shown to limit substrate supply under prolonged anoxia. These include inhibition of transport mechanisms and phloem unloading (Saglio, 1985), and impaired starch breakdown due to reduced amylolytic activity (Guglielminetti et al., 1995; Arpagaus and Braendle, 2000). Leaves of *A. calamus* and *I. pseudacorus* contained only limited amounts of carbohydrates at the beginning of the experiment. Under anoxia total soluble and non-soluble carbohydrate contents were relatively stable while the large carbohydrate reserves in the rhizomes declined rapidly after the onset of anoxia. This suggests that transport mechanisms were active in *A. calamus* and *I. pseudacorus* guaranteeing substrate supply in the leaves.

Similarly, carbohydrate feeding of the shoot has also been found to prolong anaerobic survival in tubers of *P. pectinatus* (Summers et al., 2000). The continual breakdown of non-soluble carbohydrates in the rhizome indicates that all necessary catabolic enzymes were active under anoxia. The functionality of \( \alpha \)-amylase has already been demonstrated in *A. calamus* rhizomes under anoxia (Arpagaus and Braendle, 2000). It is noteworthy that *I. pseudacorus* mobilizes its stored carbohydrates more rapidly than *A. calamus*, especially in the first weeks after the onset of anoxia. The more economic consumption of carbohydrates in *A. calamus* corresponds with its greater
tolerance of anoxia and underlines the importance of the down-regulation of metabolism for survival under oxygen deprivation.

Enzymes which are especially required for the anaerobic metabolism such as ADH and PDC need to be initiated when oxygen availability ceases. In *A. calamus* leaves, the PDC activity increases immediately after the onset of anoxia, and remains high for the duration of the anoxic treatment. The fact that ADH activity usually exceeds PDC activity, led to the conclusion that PDC activity might play an important role in the regulation of the fermentative rate (Drew, 1997). In the present experiment, however, anaerobic CO₂ production decreased despite high PDC activity, indicating that under long-term anoxia neither of the two enzymes involved limits ethanol fermentation. Regulation of fermentative rate is more likely to be regulated by other glycolytic processes such as phosphofructokinase (Gibbs et al., 2000).

**Maintenance of respiratory and photosynthetic capacity under anoxia**

The necessity for ongoing synthesis of glycolytic and fermentative enzymes under long-term anoxia is readily apparent. The functionality of pathways regarded as non-operating under anoxia, however, have aroused only little attention. In the present study, the capacity of leaves for aerobic gas exchange decreased considerably under anoxia. This appears to be mainly caused by deactivation of the enzymes involved. The activity of COX as an important enzyme of the oxidative phosphorylation pathway decreased in *A. calamus* leaves at the beginning of the anoxia treatment, then remained stable for up to 3 weeks, with a further drop occurring after 4 weeks of anoxia. It is also possible that COX synthesis continues under anoxia, but the generally reduced biosynthetic activity did not allow maintenance of full capacity. The synthesis of COX and other parts of the respiratory chain have been shown to be active in anaerobic rice and *Echinochloa phyllopogon* seedlings (Couée et al., 1992).

The consequences of prolonged anoxia, such as energy limitation and impaired biosynthetic activity, would have considerable effects also on cell organelles which are not as directly dependent on oxygen as the mitochondrion. For the first time, the effects of long-term anoxia on the chloroplast have been investigated. Because the plants were kept in the dark in the anaerobic treatments, it was difficult to assess to what extent the alterations have been caused by anoxia or darkness. Photosynthetic capacity of the leaves is, however, significantly more affected by anoxia than by the dark treatment. After 28 d of anoxia the photosynthetic capacity decreased in *A. calamus* to 17% and in *I. pseudacorus* to 3% of its initial level. The reduction in photosynthetic capacity does not seem to be due to any reduction in chlorophyll which remained remarkably stable under anoxia.

The PSII system is generally very stress sensitive and measurement of chlorophyll fluorescence has become a powerful tool for the assessment of stress-induced damage to the photosynthetic apparatus (Maxwell and Johnson, 2000). The *Fₐ/Fm* ratios measured in the dark-adapted leaf assess the functioning of the PSII reaction centres. The observed decrease in *Fₐ/Fm* in the leaves of *A. calamus* and *I. pseudacorus* indicated the inactivation of these reaction centres under anoxia. Similar decreases in *Fₐ/Fm* ratios have also been found in other stress situations such as extremely low or high temperatures (Yamane et al., 1997), severe drought (Lu and Zhang, 1998) and prolonged salt stress (Deline et al., 1999). In both investigated species the decrease in *Fₐ/Fm* is due mainly to increased *F₀* levels in the anoxia-stressed leaf. The *F₀* level depends on structural conditions that affect the probability of excitation energy transfer between the antennae pigments and from these to the reaction centre of PSII (Krause and Weis, 1984). It is believed that under stress, the separation of the light-harvesting complex from the reaction core is responsible for the rise in *F₀* and causes the reduction in the functioning of PSII. Furthermore, the adaptation of the photosystem to light is impaired in anoxia-treated leaves from *A. calamus* and *I. pseudacorus*. Photochemical quenching (*qP*), which is also considerably reduced after the anaerobic treatment, indicating an imbalance between the energy channelled through PSII and the subsequent electron transport system. It is very likely that the activity of the Calvin cycle is considerably impaired by long-term anoxia. In *A. calamus* the decrease in photochemical quenching is initially accompanied by an increase in non-photochemical quenching (*qNP*), which indicates that an increasing proportion of absorbed light is dissipated as heat. This process is regarded as a protection mechanism preventing further damage to the photosystem. In *I. pseudacorus* and later on in *A. calamus* leaves, anoxia also seems to affect non-photochemical quenching mechanisms. Reduced *qNP* would lead to a permanent over-excitation of the thylakoids and enhance the danger of photo-inhibitory damage. Considering all photochemical and non-photochemical quenching mechanisms, the calculation of quantum efficiency *Φₚ₅₁₈₈* gives a tool to estimate the rate of linear electron transport in the leaf (Genty et al., 1989). In the investigated leaves, the decrease in *Φₚ₅₁₈₈* coincides with the results obtained for oxygen evolution, suggesting that the calculated *Φₚ₅₁₈₈* value represented a useful parameter for assessing potential carbon assimilation in the leaf. Generally, instability of proteins in the PSII complex and disturbances in the subsequent electron transport seem to be responsible for the decrease in photosynthetic capacity of anoxia-stressed leaves.
Recovery of metabolism under post-anoxia

Leaves of both the species investigated tolerated anoxia for prolonged periods. *A. calamus*, however, showed a significantly faster recovery on re-exposure to air and light after 28 d of anoxia. Leaf growth, as well as respiratory and photosynthetic activity recovered in *A. calamus* almost immediately on return to air, while a lag phase of 3–10 d was observed for *I. pseudacorus*. There were no qualitative differences in the reaction of the measured parameters between the two investigated species, but maintenance of respiratory and photosynthetic capacity was reduced in *I. pseudacorus* when compared to *A. calamus*. When plants return to an oxygen-rich atmosphere they suffer not only from weakening by anoxia stress, but they also have to endure the formation of reactive oxygen species (ROS). The enhanced sensitivity of plant material to oxidative stress after periods of anoxia has been demonstrated in roots and rhizomes (Wollenweber-Ratzer and Crawford, 1994; Biemelt et al., 1998). The impact of oxidative stress is thought to be even stronger in green leaf tissue as the formation of many ROS is associated with light reactions in the chloroplast (Elstner and Osswald, 1994). The lag phase in the recovery of photosynthetic activity in *I. pseudacorus* is possibly related to oxidative damage. In the first 24 h after re-exposure to air a considerable decrease in the overall fluorescence of PSII and a further decrease in the chlorophyll content of the leaf was found. The involvement of singlet oxygen on initiation of chlorophyll breakdown has already been demonstrated (Pastori and del Rio, 1997). In *A. calamus*, however, a functioning anti-oxidative defence system seems to be rapidly available on return to air allowing the reinstallation of aerobic metabolism and repair of the photosynthetic apparatus. It has already been shown that synthesis of enzymes involved in the anti-oxidative processes (e.g. superoxide dismutase) increase under anoxia in certain tolerant tissues (Monk et al., 1987) thus providing protection against the burst of ROS on return to air.

Although oxidative stress might have contributed to the delayed recovery in *I. pseudacorus* leaves on return to air, whatever problems it may have created were overcome after a few days repair of photosynthetic and respiratory pathways. Except for the chlorophyll content, no further damage was detected for the studied parameters under post-anoxia. Altogether, the results presented indicate that in these highly tolerant species anoxia rather than post-anoxia stress is eventually responsible for any damage to leaf tissues. This is in agreement with the findings of Pavelic et al., who demonstrated that post-anoxic damage to membrane lipids by ROS is negligible in comparison to the extensive lipid hydrolysis taking place under anoxia (Pavelic et al., 2000).

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References


