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Anoxic stress leads to hydrogen peroxide formation in plant cells

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Abstract

Hydrogen peroxide (H_2O_2) was detected cytochemically in plant tissues during anoxia and re-oxygenation by transmission electron microscopy using its reaction with cerium chloride to produce electron dense precipitates of cerium perhydroxides. Anoxia-tolerant yellow flag iris (*Iris pseudacorus*) and rice (*Oryza sativa*), and anoxia-intolerant wheat (*Triticum aestivum*) and garden iris (*Iris germanica*) were used in the experiments. In all plants tested, anoxia and re-oxygenation increased H_2O_2 in plasma membranes and the apoplast. In the anoxia-tolerant species the response was delayed in time, and in highly tolerant *I. pseudacorus* plasma membrane associated H_2O_2 was detected only after 45 d of oxygen deprivation. Quantification of cerium precipitates showed a statistically significant increase in the amount of H_2O_2 caused by anoxia in wheat root meristematic tissue, but not in the anoxia-tolerant *I. pseudacorus* rhizome parenchyma. Formation of H_2O_2 under anoxia is considered mainly an enzymatic process (confirmed by an enzyme inhibition analysis) and is due to the trace amount of dissolved oxygen (below 10^{-5} M) present in the experimental system. The data suggest oxidative stress is an integral part of oxygen deprivation stress, and emphasize the importance of the apoplast and plasma membrane in the development of the anoxic stress response.

Key words: Anoxia, apoplast, hydrogen peroxide, hypoxia, oxidative stress, reactive oxygen species.

Introduction

The recent hypothesis that a decrease in oxygen concentration and the formation of reactive oxygen species (ROS) may be recognized through the same sensing mechanism in the cell (Semenza, 1999) makes adaptation to anoxic stress an important model for the investigation of this mechanism. The experiments reported here are based on recent data on the generation of (ROS) under various stress conditions (Noctor and Foyer, 1998), their effects on metabolically active macromolecules and on cell structures, and their involvement in the regulation of adaptive response. These effects are all important for understanding stress physiology and the underlying signalling mechanisms.

Direct and indirect evidence has accumulated on the involvement of ROS in the anoxic stress response. A term 're-oxygenation injury' has been introduced to acknowledge the detrimental effect of ROS on anoxically pretreated tissue (Crawford *et al.*, 1994). Most of the studies in this field characterize the consequences of oxidative stress, i.e. membrane damage, lipid peroxidation (LP) and antioxidant status with respect to anoxia-tolerant and intolerant species. In previous studies evidence has been obtained of ROS engagement in the etiology of anoxic stress: enhanced formation of LP products—conjugated dienes, trienes and thiobarbituric acid reactive substances (TBARS), changes in superoxide dismutase (SOD) activity and a decrease in antioxidant content (Chirkova *et al.*, 1998; Blokhina *et al.*, 1999, 2000).

It has been shown earlier that anoxia leads to changes in lipid content and composition, membrane fluidity (Hetherington *et al.*, 1982; Chirkova *et al.*, 1989), decrease in the adenylate energy charge (Chirkova *et al.*,

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Abbreviations: LP; lipid peroxidation; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

1984; Hanhijärvi and Fagerstedt, 1994), cytoplasmic acidosis (Roberts *et al*., 1984), and changes in the patterns of protein synthesis (Chang *et al*., 2000). Most of these changes favour ROS production and the successive peroxidation of lipids (Crawford *et al*., 1994; Crawford and Braendle, 1996; Chirkova *et al*., 1998).

Formation of ROS occurs through several univalent reduction steps, yielding first the superoxide anion, then hydrogen peroxide and the hydroxyl radical, and finally water (Elstner, 1991). Of the above-mentioned chemical species only hydrogen peroxide is relatively stable and able to penetrate the plasma membrane as an uncharged molecule. Thus, it could act as a second messenger under stress conditions (Foyer *et al*., 1997). To achieve signal specificity three important components of the signalling pathway via ROS have to be considered: The source of the signal, target susceptibility and the antioxidant status of the cell (Alscher *et al*., 1997; Lander, 1997). Other than antioxidant-associated mechanisms can be involved in the fine adjustment of ROS action such as local elevations in Ca^{2+}-concentration (Subbaiah *et al*., 1998) and changes in the redox status and pH of the cell.

The role and place of ROS in the sequence of events leading to metabolic changes under oxygen deprivation stress is not yet clear. The aim of this study is to show direct ROS (e.g. hydrogen peroxide) generation under anoxia and re-oxygenation, and to localize the sites of H_2O_2 formation and reveal its possible enzymatic sources. Since the effect of ROS on the cell is non-specific, an attempt has been made to investigate any possible association between ROS accumulation, LP and redox state of the cell, and anoxia-induced metabolic changes.

In this investigation the anoxia-intolerant wheat and the more tolerant rice represent agriculturally important species. Their behaviour is compared with the naturally evolved anoxia-intolerant and tolerant species, *Iris germanica* and *Iris pseudacorus*, respectively. Study of native species capable of tolerance of anoxia for long periods, and the comparison of these with the intolerant agricultural plant cultivars, is important since it reveals the tolerance mechanisms which have appeared during evolution.

Materials and methods

Plant material and growth conditions

Seeds of wheat (*Triticum aestivum* L. cv. Leningradka) and 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 μmol s^{-1} m^{-2} for 7 d (wheat) or 10 d (rice). *Iris pseudacorus* L. rhizomes were collected by the Nupuri lake in southern Finland in a wet riverside meadow at the end of May and June in 1998. *Iris germanica* L. rhizomes were kindly supplied by the Botanical Garden of Helsinki University. Only current year rhizomes were used in the experiments. Before treatment the rhizomes were washed and roots and leaves removed. Experimental plants represent pairs of anoxia-intolerant–anoxia-tolerant plants: wheat–rice and *I. germanica*–*I. pseudacorus*, respectively. Plant organs which are susceptible to O_2 deprivation under natural conditions (the roots of cereals and the rhizomes of *Iris* spp.) were used in the experiments.

Anoxic stress treatment

Plants (rhizomes or whole seedlings) were placed in glass jars (1.5 l) on moist filter paper. Anoxic conditions were created with gas generating kits (Oxoid BR 10, Unipath Ltd., Basingstoke, UK) and anaerobic palladium catalysts (Oxoid BR 42). Hydrogen released by the kit immediately reacts with oxygen on the palladium catalyst to form water, and, therefore removes oxygen from the system. In addition, the kit causes carbon dioxide concentration inside the jars to rise to *c*. 7–10%. High CO_2 under anoxia is a natural condition in soil resulting from root and microbial anaerobic respiration. Absence of oxygen was checked with anaerobic indicators (Oxoid BR 55, sensitive down to 12 μM oxygen). Aerobic samples were placed into moistened quartz sand (rhizomes) or wrapped in several layers of moist filter paper (seedlings). Both anoxic and control samples were kept at room temperature in the dark. The duration of anoxia was chosen individually for each species based on their tolerance of anoxia to achieve physiologically equal treatment: wheat 1 and 3 d, rice 7 d, *I. germanica* 8 d, and *I. pseudacorus* 15 and 45 d. Investigations were undertaken earlier to establish the limit of anoxia tolerance and ultra-structural, physiological and biochemical changes in the experimental material in the time-course of anoxia (i.e. monitoring of cell plasmolysis as an integral parameter of cell damage under stress, measurement of electrolyte leakage and cytoplasmic pH changes) (Chirkova *et al*., 1991; Hanhijärvi and Fagerstedt, 1994).

Cytochemical localization of hydrogen peroxide

The assay is based on the reaction of H_2O_2 with $CeCl_3$, which produces electron-dense insoluble precipitates of cerium perhydroxides $Ce(OH)_2OOH$ and $Ce(OH)_3OOH$ (Bestwick *et al*., 1997). To visualize H_2O_2 accumulation under oxygen deprivation, vessels containing anoxic samples were transferred to a gas tight chamber and samples were treated with $CeCl_3$ under a continuous flow of nitrogen (hypoxia). To evaluate the impact of re-oxygenation on the intensity of H_2O_2 formation, similar specimens were kept in air at room temperature for 2 h in the dark after the anoxic period (anoxia + 2 h re-oxygenation). Plant tissues (whole seedlings of the cereals or fresh cuts of *Iris* rhizome core *c*. 2×2 mm) were treated with 5 mM $CeCl_3$ in 50 mM HEPES pH 7.3 for 1 h at room temperature. To test the effect of added H_2O_2 on cerium precipitate formation, some tissue samples were treated with 5 mM H_2O_2, rinsed several times and stained as were the other samples. After three washing steps (50 mM HEPES, 5–10 ml), tissue specimens were fixed in 2.5% glutaraldehyde in the same buffer for 1 h. After fixation *c*. 2 mm long root tips were removed. Both the root tips and the rhizome core samples were post-fixed in 1% osmium tetroxide (EMS, Washington, PA, USA). After dehydration in an ethanol series (50%, 70%, 96%, and 100%) tissue specimens were embedded in Epon LX 112 (Ladd Research Industries Inc., Williston, VT, USA) and polymerized. Root tips were sectioned starting from the tip. The first sections (60 nm) containing the root cap were discarded and only the meristematic cells were processed further. Both the rhizome and root specimen

sections were stained with uranyl acetate for 0.5 h (Ultrastain-1, Leica, Austria) and lead citrate for 1.2 min (Ultrastain-2, Leica, Austria) in Leica EM Staining automat. Another similar set of sections was left unstained for better visualization of the cerium perhydroxide deposits. The samples were viewed at an accelerating voltage 60 kV with a transmission electron microscope (Jeol JEM-1200EX, Jeol Ltd, Tokyo, Japan). The *I. pseudacorus* rhizome contains concentric amphivasal vascular bundles spread in parenchymal tissue. In the electron micrographs only parenchymal cells were examined to eliminate H_2O_2 associated with lignification of xylem elements.

Inhibition analysis

To verify H_2O_2 formation under anoxia and to confirm the reliability of the cerium perhydroxide precipitate method, one of the experiments (wheat 3 d under anoxia + 2 h aeration) was combined with the following 1 h treatments: 8 μM diphenyleneiodonium (DPI, an NADPH oxidase inhibitor), 3 mM KCN (superoxide dismutase and peroxidase inhibitor) and 1 mM NaN_3 (peroxidase inhibitor) and 25 μg ml^{-1} (1200 U ml^{-1}) of catalase (H_2O_2 removal). Each inhibitor treatment was given separately in 25 ml volume. After this treatment, the plant samples were washed and incubated with $CeCl_3$ as described above.

Quantification of cerium perhydroxide precipitates in electron micrographs by Image Pro® Plus

To compare the results on the amount of H_2O_2 (as judged by the cerium precipitates) in an anoxia-intolerant and an anoxia-tolerant plant species, wheat and *Iris pseudacorus* samples were processed further to quantify the amount of cerium precipitates in the electron micrographs. Original unprocessed scans from electron micrographs taken with the same magnification (×6000) were used for the quantification of H_2O_2. Micrographs were taken randomly of the sections; however, in *Iris* rhizome sections areas containing only parenchymal tissues were photographed and areas with vascular tissues were not used. The Image Pro® Plus programme differentiated cerium perhydroxide precipitates from the background by the difference in contrast. Threshold intensity values and area limits were chosen manually for each calculation, depending on the density of cerium perhydroxide accumulation and plant species. 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. To eliminate artefacts originating from uneven light distribution in the original micrographs, a 'flatten background' operation was performed. Statistical analyses were carried out to establish differences between aerated and anoxic material.

Results

Amount of hydrogen peroxide during anoxic stress and re-oxygenation

Comparison of samples treated (Figs 1b, e, f, 2b) or not treated (Fig. 2c) with cerium chloride (and not stained by uranyl acetate) indicated the formation of cerium precipitates. This result combined with the inhibition analysis (Fig. 5) confirmed the presence of cerium perhydroxides and hence H_2O_2. The cerium precipitates appeared in some samples as needle-like crystals (Figs 1f, 2f, 3e) possibly indicating a larger amount of H_2O_2. Treatment of the tissue samples with 5 mM H_2O_2 prior to cerium chloride staining resulted in intensive formation of precipitates at the plasma membrane (Fig. 1c).

In the aerated control plants H_2O_2 accumulation was observed as electron dense cerium precipitates mainly in the cell wall of the root tip meristematic cells and rhizome core parenchyma (Figs 1a, b, 2a, b, 3a, b, 4a). In some cases better visualization of cerium precipitates was achieved when the standard staining of thin sections with uranyl acetate and lead citrate was excluded from the procedure for electron microscopy (Figs 1b, c, e, f; 2a versus b, 3d, e, 4a–c, 5a–e). Cerium perhydroxides appear in the photographs as electron dense spots in association with the plasma membrane (Figs 1b–e, 2a, b, 3e, 4b, c) or as crystal-like matter when detected in the cell wall (Figs 1f, 2f) and intercellular spaces of *I. pseudacorus* (Fig. 3d, e). Some cerium perhydroxide deposits were detected in the cytoplasm of the anoxic samples (Figs 1f, 2f), which confirmed the penetration of $CeCl_3$ into the cells.

Inhibition analysis confirmed enzymatic origins of a large part of the H_2O_2 detected. The most efficient in H_2O_2 elimination were treatments with catalase (Fig. 5b) and diphenyleneiodonium (NADPH oxidase inhibitor, Fig. 5c) in comparison with anoxic tissue not treated with inhibitors (Fig. 5a). Peroxidase inhibitors KCN and NaN_3were less efficient (Fig. 5d, e, respectively), although they can act not only as enzyme inhibitors but also as direct reductants of H_2O_2 (Ros Barcelo, 1998). KCN also inhibits CuZn-SOD and Fe-SOD, which could have contributed to H_2O_2 production in the control sample.

In the cereals, imposition of anoxia increased H_2O_2 associated with the plasma membrane (Figs 1d, e, 2f). Both the enhancement of stress and the restoration of normoxic conditions caused plasmolysis and membrane degradation and an increase in the amount of H_2O_2 (Figs 1f, 2d–f). Figures 1e and f and 2e and f represent the result of re-oxygenation injury. Both wheat and rice roots showed deteriorated membrane structures and increased amount of H_2O_2 not only on the plasma membrane but inside the protoplast as well (Figs 1f, 2f). The difference in anoxia tolerance between the rhizomatous *Iris* species, estimated as the amount of H_2O_2, was more pronounced than that of the cereals (Figs 3, 4). In the cells of the anoxia-intolerant *I. germanica* intensive presence of H_2O_2 was detected on the plasma membrane and a less intensive presence in the cell wall after 8 d of oxygen deprivation (Fig. 4b, c). Anoxia-tolerant *I. pseudacorus* showed no increase in the H_2O_2 presence on the plasma membrane after 15 d of anoxia (Fig. 3b versus c). Re-oxygenation after 15 d of treatment did not cause any changes either in the cell ultrastructure or in H_2O_2

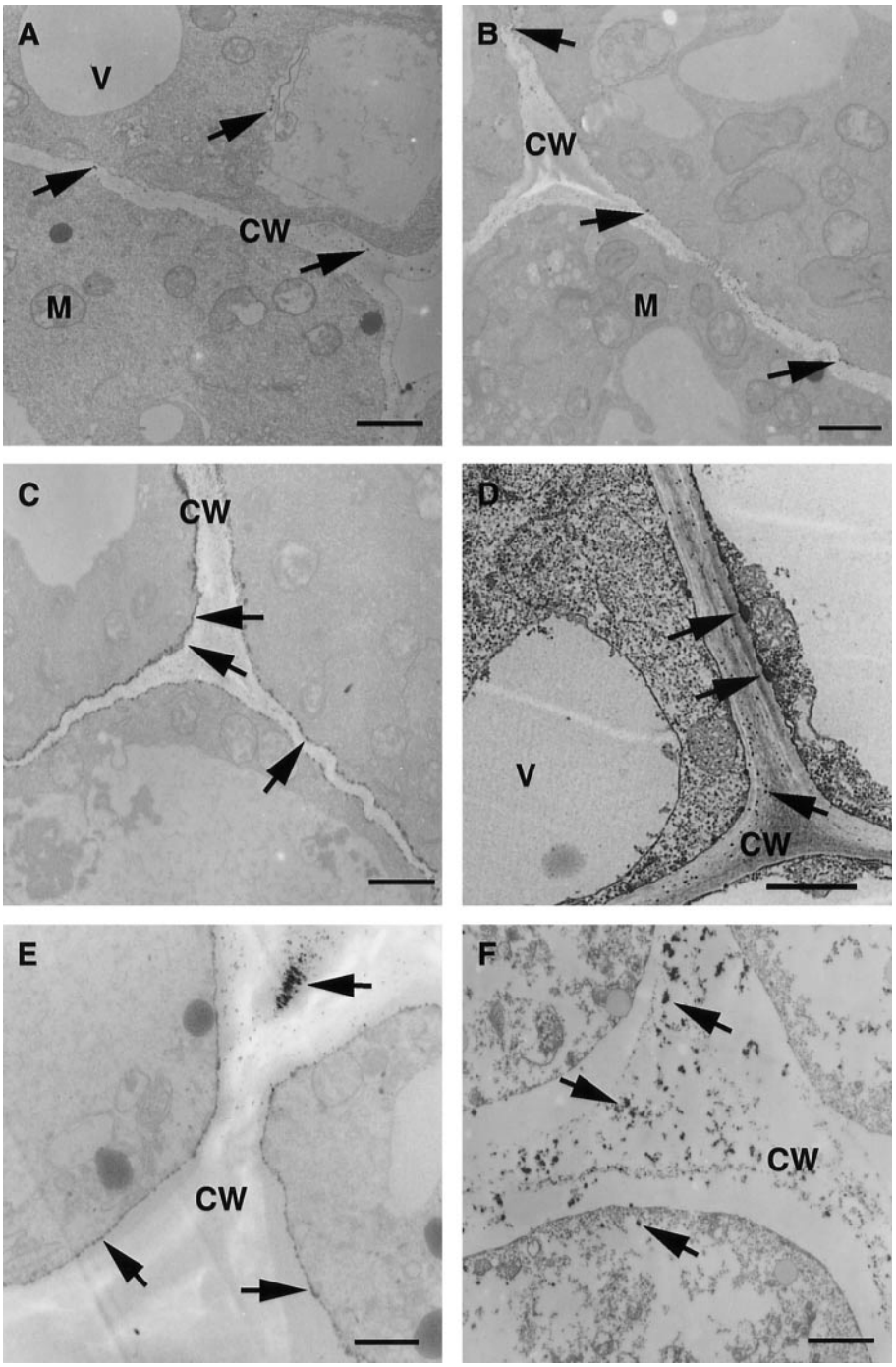

Fig. 1. Visualization of H_2O_2 by cerium precipitate formation in wheat root tip meristematic tissue under aeration (a–c) and anoxia (d–f). Arrows indicate cerium perhydroxide precipitates. In (f) the cerium precipitates form needle-like crystals. Plasmolysis has taken place in cells suffering from anoxia (e, f). Cellular organelles are damaged in (f). Control, days aerated plant material fixed at the beginning of the experiments (0 d) or plants under aeration in the dark; duration is the same as under anoxia. Anoxia, days plant material under anoxia in the dark. Anoxia, days + re-aeration = 2 h re-aeration period after anoxia prior to cerium chloride treatment. +/– URA = staining/no staining of plastic embedded plant material with uranyl acetate and lead citrate prior to transmission electron microscopy. +/– Ce = treatment with/without cerium chloride. CW, cell wall; M, mitochondrion; N, nucleus; PM, plasma membrane; V, vacuole. Bars represent 1 μm. (A) Control 1 d, +URA, +Ce; (B) control 1 d, –URA, +Ce; (C) control 1 d,–URA, +Ce, 5 mM H_2O_2 added; (D) anoxia 1 d, +URA, +Ce; (E) anoxia 1 d + 2 h re-aeration, –URA, +Ce; (F) anoxia 3 d + 2 h re-aeration, –URA, +Ce.

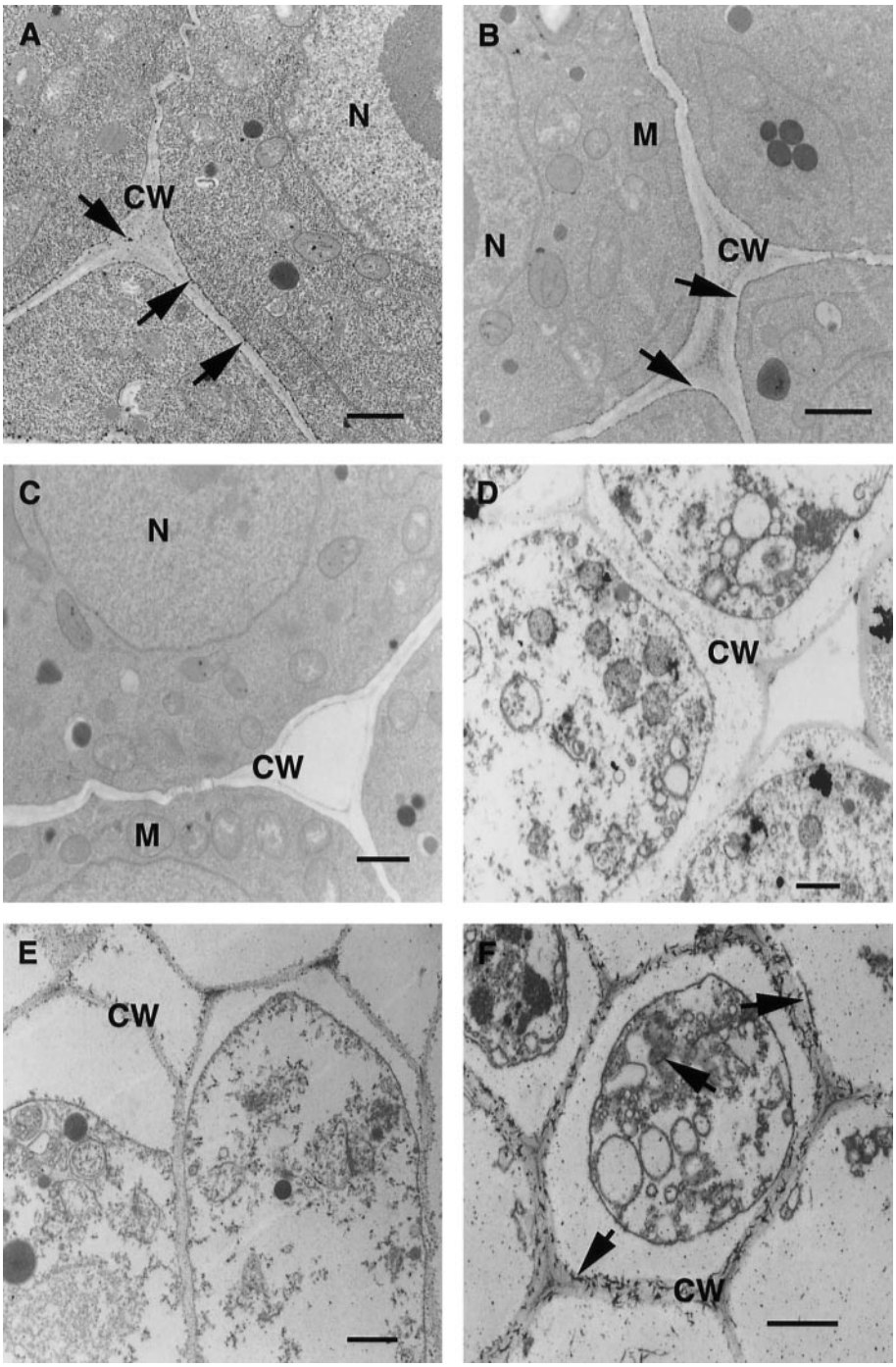

Fig. 2. Visualization of H_2O_2 by cerium precipitate formation in rice root tip meristematic tissue under aeration and anoxia. Note plasmolysis under anoxia in (d), (e) and (f) and the damage in cellular organelles in (e) and plasma membrane in (f) (the cell at the top of the micrograph). Arrows point to cerium precipitates. Other explanations as for Fig. 1. Bars represent 1 μm. (A) Control 7 d, +URA, +Ce; (B) control 7 d, –URA, +Ce; (C) control 7 d, –URA, –Ce; (D) anoxia 7 d, +URA, –Ce; (E) anoxia 7 d+2 h re-aeration, +URA, –Ce; (F) anoxia 7 d+2 h reaeration, +URA, +Ce.

amount (Fig. 3c). In the rhizomes of *I. pseudacorus* the intercellular space was a notable site of H_2O_2 presence both under aeration and anoxia (Fig. 3d, e). Anoxia for 45 d led to increased cerium precipitation in the cell wall (Fig. 3e).

Results on the quantification of cerium precipitates by Image Pro® Plus are presented in Table 1. The high values of deviation in the areas of the precipitates are due to the large magnification needed: in some pictures cell walls, containing most of the precipitates, covered

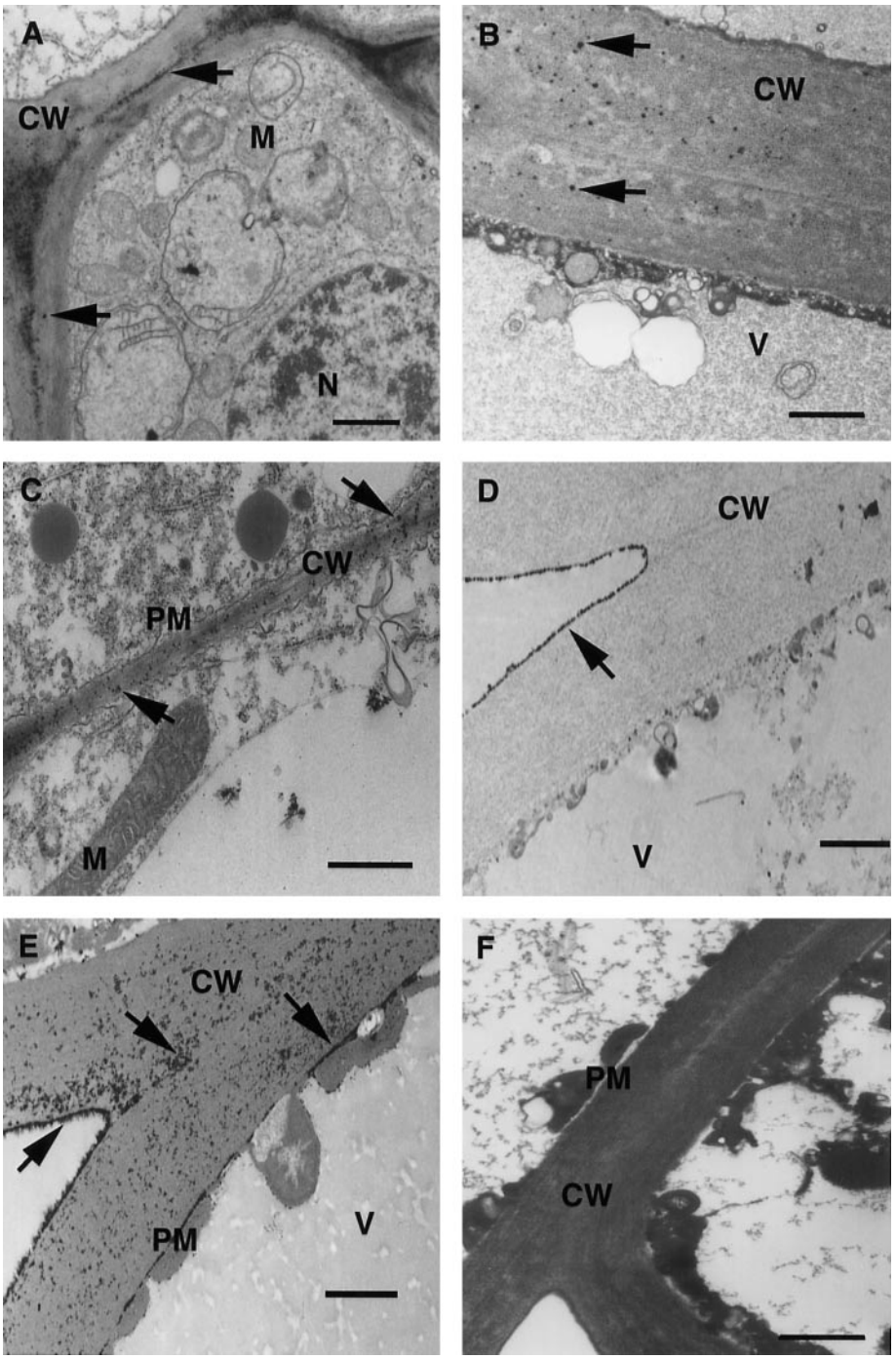

Fig. 3. Visualization of H_2O_2 by cerium precipitate formation in *Iris pseudacorus* rhizome core parenchymal storage tissue under aeration and anoxia. Arrows indicate cerium precipitates, which are seen in the cell walls of aerobic material (a, b), and similarly after 15 d of anoxia + 2 h re-oxygenation (c). The precipitates are more pronounced lining the intercellular spaces (d) after 45 d of anoxia and in the cell walls and at the plasma membrane (e) after 45 d of anoxia + 2 h re-oxygenation. The dark deposits seen in (e) and (f) are fructans, the vacuolar storage carbohydrates typical of this species (For further information see Hanhijärvi and Fagerstedt, 1994). Other explanations as for Fig. 1. Bars represent 1 μm. (A) Control 0 d, +URA, +Ce; (B) control 15 d, +URA, +Ce; (C) anoxia 15 d + 2 h re-aeration, +URA, +Ce; (D) control 45 d, −URA, +Ce; (E) anoxia 45 d + 2 h re-aeration, −URA, +Ce; (F) anoxia 45 d + aeration, +URA, −Ce.

more of the image area resulting in a larger area of the precipitates. Application of the Student's *t*-test revealed a statistically significant difference at the 1% level between aerated and anoxic wheat samples, while in the anoxia-tolerant *I. pseudacorus* the difference was not significant.

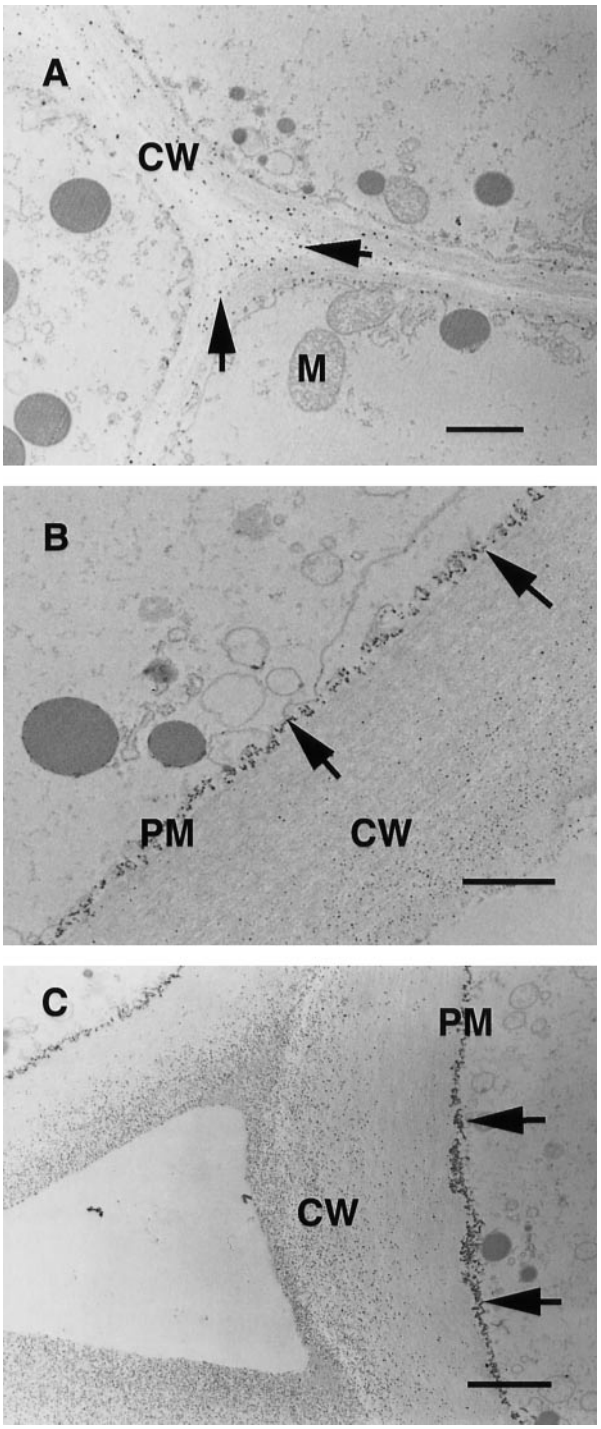

Fig. 4. Visualization of H_2O_2 by cerium precipitate formation in *Iris germanica* rhizome core parenchymal storage tissue under aeration and anoxia. Arrows indicate cerium precipitates, which are present in the cell wall under aerobic conditions (a) but are more pronounced in anoxic material (8 d) at the plasma membrane and in the cell wall (b, c). Other explanations as for Fig. 1. Bars represent 1 μm. (A) Control 8 d, −URA, +Ce; (B) anoxia 8 d + 2 h re-aeration, −URA, +Ce; (C) anoxia 8 d + 2 h re-aeration, −URA, +Ce.

Ultrastructural changes caused by anoxic stress

Anoxic treatment brought about both H_2O_2 accumulation and changes in cell ultrastructure. The ability to maintain cell intactness correlated positively with anoxia tolerance. *I. pseudacorus* rhizome tissue showed undisturbed structures after 15 d (Fig. 3c) and even after 45 d of anoxia (Fig. 3e, f) with thin layers of cytoplasm along the cell walls and amorphous fructan stores in the vacuoles (Fig. 3b, e, f). In the roots of the anoxia-intolerant wheat, plasmolysis could be observed after 1 d of oxygen deprivation (Fig. 1e). In rice, continued stress led to swelling of mitochondria (Fig. 2d, e) and to plasmolysis (Fig. 2d, e, f) and finally to the disintegration of the plasma membrane after 7 d of anoxia (Fig. 2f). Due to the large number of treatments, not all micrographs from every experiment can be shown.

Discussion

Anatomical and ultrastructural features

Incubation of the anoxia-intolerant plant tissues under oxygen deprivation caused plasmolysis and disintegration of organelles. Such changes have been described in detail earlier (Vartapetian and Zakhmilova, 1990). The anatomy and ultrastructure of *Iris pseudacorus* rhizome parenchyma containing much of storage fructan have been described earlier (Hanhijärvi and Fagerstedt, 1994). In this study, *I. pseudacorus* rhizome parenchymal cells showed no signs of damage at the ultrastructural level even after 45 d of anoxic incubation. According to this investigation and earlier biochemical studies (Chirkova, 1988; Hanhijärvi and Fagerstedt, 1994; Chirkova *et al.*, 1998; Blokhina *et al.*, 1999), the plant species used in the present paper can be placed in the following order of increasing anoxia tolerance: wheat, rice, *I. germanica*, *I. pseudacorus*.

Source of H_2O_2

The increased appearance of cerium perhydroxide deposits in the samples under anoxia can be due to the inhibition of H_2O_2 utilization by antioxidant enzymes, or to an enhancement of H_2O_2 production via an enzymatic or a chemical mechanism. Under severe stress conditions both processes can be present. The sensitivity of the oxygen indicators used in the anoxic jars, and hence the concentration of O_2 in the experiments, was below 12 μM. However, this concentration is still enough for the functioning of plant terminal oxidases (K_m 10^{-6} M for oxygen; Skulachev, 1997) and for the formation of ROS. Non-enzymatic one-electron O_2 reductions can occur at about 10^{-4} M and higher oxygen concentrations (Skulachev, 1997). H_2O_2 accumulation under anoxia employs the very low amount of dissolved oxygen present

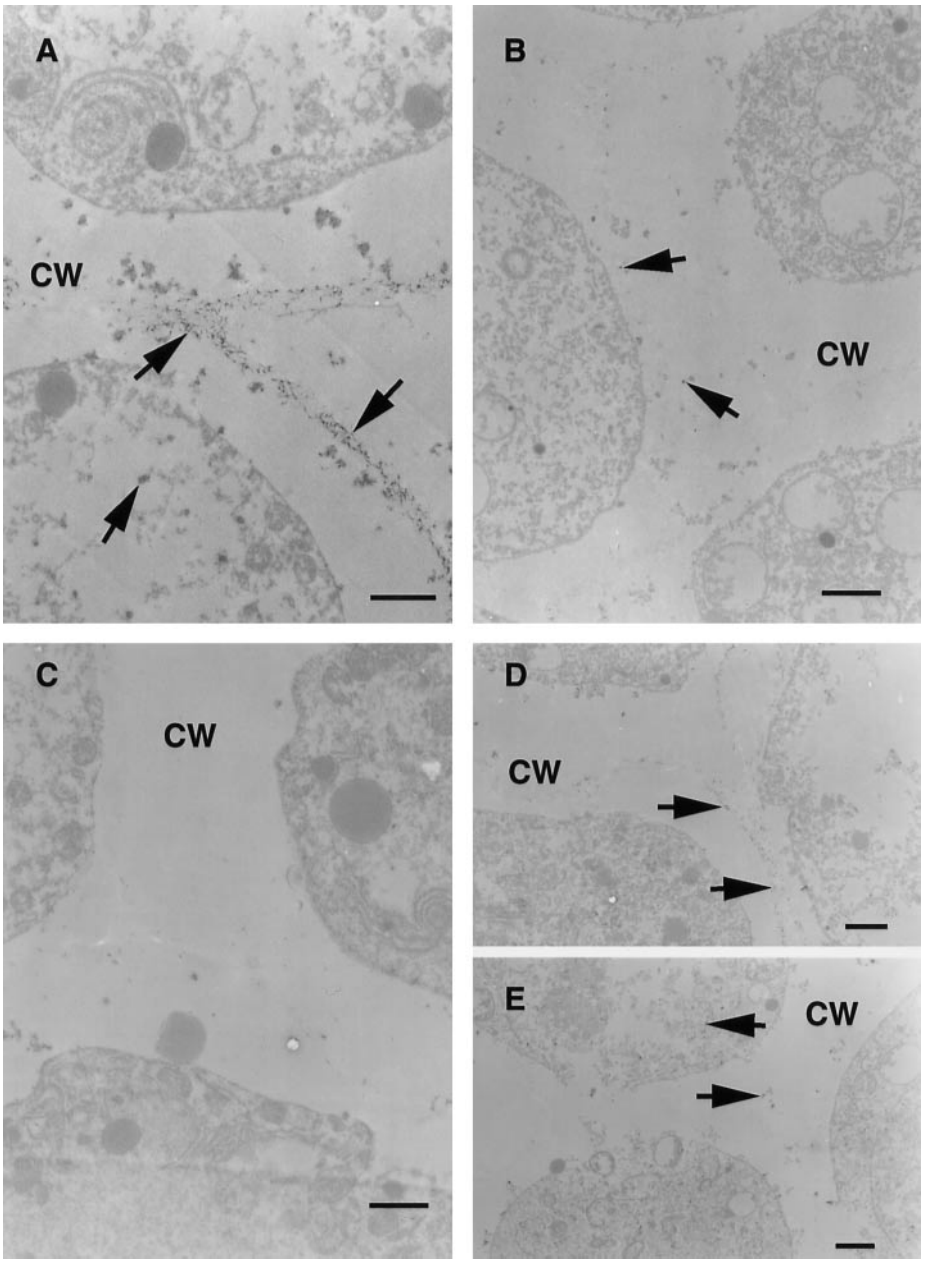

Fig. 5. An investigation on the enzymatic origin of H_2O_2 (and hence the formation of cerium precipitates) in wheat root tissue samples (subjected to 3 d anoxia and 2 h re-oxygenation) by inhibition with enzyme inhibitors. Catalase 1200 U ml^{-1} was used as a negative control (for H_2O_2 removal). The inhibitors used were: peroxidase inhibitors 3 mM KCN and 1 mM NaN_3 and NADPH oxidase inhibitor 8 μM diphenyleneiodonium. KCN also inhibits Cu,Zn-superoxide dismutase. Arrows indicate cerium precipitates, which are abundant in anoxic material (a), very few in catalase treatment (b), are not found after DPI treatment (c), while KCN and NaN_3 were less efficient in H_2O_2 elimination (d, e). Bars represent 1 μm. (A) anoxia 3 d + 2 h re-aeration, −URA, +Ce, no inhibitor; (B) anoxia 3 d + 2 h re-aeration, −URA, +Ce, + catalase; (C) anoxia 3 d + 2 h re-aeration, −URA, +Ce, + DPI; (D) anoxia 3 d + 2 h re-aeration, −URA, +Ce, +KCN; (E) anoxia 3 d + 2 h re-aeration, −URA, +Ce, +NaN_3.

in the experimental system presented here, and therefore, was mostly of enzymatic origin. The inhibition analysis of H_2O_2 production (Fig. 5) supports this fact.

Earlier investigations on H_2O_2 accumulation in barley and wheat have shown an increase in H_2O_2 in the roots suffering from hypoxia (Kalashnikov *et al*., 1994; Biemelt *et al*., 2000). In the present experiments the comparison between aerated and anoxic samples revealed the same tendency: accumulation of H_2O_2 in anoxic samples of the intolerant wheat (Table 1).

H_2O_2 is a constitutive metabolite in the cell wall. Some apoplastic enzymes may contribute to H_2O_2 production under normal and under stress conditions, for example, an extracellular germin-like oxalate oxidase and an amine oxidase capable of H_2O_2 production have been localized in the apoplast (Reggiani and Bertani, 1989; Hurkman

Table 1. *Quantification of H_2O_2 amount under anoxia and aeration*

The presence of H_2O_2 was estimated by counting the areas of cerium perhydroxide precipitates as a percentage of total image area in thin sections of wheat and *I. pseudacorus* samples studied with a transmission electron microscope (parenchymal cells in the rhizomes of *I. pseudacorus* and meristematic cells in the root tips of wheat). The figures are counts of the individual electron micrographs of the same magnification ($\times 6000$) taken randomly from the tissue sections. In each species aerated samples were compared with anoxic material and statistically analysed with the Student's *t*-test. A statistically significant difference between the groups at 1% level (**) was detected in wheat root tips.

	Aeration		Anoxia	
	Precipitate area (pixels $\times 10^3$)	Precipitates as % of total image area ($\times 10^{-3}$)	Precipitate area (pixels $\times 10^3$)	Precipitates as % of total image area ($\times 10^{-3}$)
Wheat	5.228	5.646	20.90	14.31
	1.991	2.810	10.78	7.28
	2.532	5.614	35.48	23.87
	0.425	0.980	18.42	12.46
	0.972	0.983	30.10	21.52
			30.10	21.52
			48.64	31.66
			2.07	12.16
			4.07	5.44
			24.98	25.54
			21.79	24.11
			33.27	
Average	2229.6	3.207	23.38	19.4
St. deviation	1.869	2.335	13.47	9.0
n	5		12	
Probability values, *t*-test		**0.0014****		
Iris pseudacorus	0.791	0.53	2.42	1.49
	9.216	6.13	4.94	5.26
	6.127	4.45	11.30	7.62
	6.063	6.79	19.00	12.68
	3.729	3.89	15.57	21.60
	13.328	13.19	1.75	1.96
	0.639	0.85	21.09	23.47
			12.42	14.17
			10.56	12.05
			12.95	14.54
			4.55	4.93
			3.44	3.23
Average	5.699	5.1	9.999	10.3
St. deviation	4.554	4.3	6.574	7.4
n	7		12	
Probability values, *t*-test		**0.197ns**		

and Tanaka, 1996). The presence of a stress-induced plasma membrane NADPH oxidase, capable of H_2O_2 formation, has also been indicated in plants (Dwyer *et al.*, 1996). According to this study's data, sites of hydrogen peroxide production in tissues suffering from oxygen deprivation are mainly associated with the plasma membrane (Figs 1d, e, 2f), and are possibly an indication of NADPH oxidase activity. This was confirmed by inhibition of H_2O_2 formation by diphenyleneiodonium (DPI) (Fig. 5c), an inhibitor of NADPH oxidase.

Disturbance of H_2O_2 decomposition by apoplastic peroxidases can lead to H_2O_2 appearing in the cell wall. However, some isozymes of cell wall peroxidases with an optimum in the neutral or alkaline pH-region can be responsible for H_2O_2 production under hypoxia through the reduction of Compound III of peroxidase (Dunford, 1993).

In these experiments, a decrease in cerium perhydroxide precipitates in the apoplast was observed upon treatment with peroxidase (NaN_3 and KCN) and superoxide dismutase (KCN) inhibitors suggesting the involvement of these enzymes in H_2O_2 production and decomposition under anoxia. According to the results of this study, the rise in H_2O_2 amount under anoxia is mainly due to the combined action of NADPH oxidase and peroxidases.

Antioxidant status of the cells

Reduced activity of the enzymes of the ascorbate-glutathione cycle can be responsible for H_2O_2 accumulation. Earlier results on the depletion of the reduced forms of ascorbate and glutathione suggest an imbalance in the glutathione-ascorbate cycle under anoxia (Blokhina

et al., 2000), a condition which affects the redox state of the cell and redox-dependent reactions. Enzymatic turnover of ascorbate and glutathione is problematic for plants under anoxic stress, and a decrease in enzyme activities has been observed under hypoxia and anoxia by several authors (Yan *et al*., 1996; Chirkova *et al*., 1998; Biemelt *et al*., 1998; Boo and Jung, 1999). 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. From that point of view, a decrease in ascorbic acid/dehydroascorbic acid and GSH/GSSG ratios in our experiments (Blokhina *et al*., 2000) represents one of the possible redox signals in anoxically damaged tissue undergoing oxidative stress.

Is H_2O_2 a stress signal under anoxia?

H_2O_2 is considered to act as a signalling molecule in plants in the pathogen defence reaction and wounding stress (Mehdy, 1994; Bowler and Fluhr, 2000). Recently, the participation and place of H_2O_2 in a particular signalling cascade has been demonstrated: H_2O_2 can act as a potent activator of a mitogen-activated protein kinase cascade in *Arabidopsis*, initiating a protein phosphorylation cascade, which induces stress-responsive genes (Kovtun *et al*., 2000). Also, it is known that oxidation of an exposed SH-group of the OxyR transcription factor of *Escherichia coli* activates transcription of genes involved in the detoxification of ROS (Lander, 1997). Recently, ozone-induced H_2O_2 production has been shown on the plasma membrane and cell wall of silver birch (*Betula pendula*) leaves. In that investigation an inhibition analysis suggested participation of both NADPH-dependent superoxide synthase and the cell wall peroxidases in H_2O_2 formation (Pellinen *et al*., 1999).

Under anoxia, H_2O_2 is localized mainly in the apoplast and may be responsible for the generation of the stress response together with the peroxidation products of the plasma membrane lipids. The importance of the apoplast as the site of environmental sensing has been shown earlier for light, submergence, hypergravity and water deficiency stresses (Hoson, 1998). Indeed, in these experiments cerium perhydroxides were detected not only in the anoxic material, but also in the apoplast of the aerated control samples (Fig. 1a, b, 2a, b, 3 d). The amount of precipitates detected under aeration was lower than that found in the anoxic samples. These results favour the suggestion that the apoplast is involved in the perception of different signals and acts in co-operation with the plasma membrane (Hoson, 1998). Signal specificity could be achieved with a particular balance of other anoxia-induced metabolic changes such as shifts in the reduction state of the key redox-active metabolites (i.e. ascorbate, glutathione, NAD(P)H, ions of transition metals), cytoplasmic acidification, elevation in cytosolic free Ca^{2+} concentration, disruption of the energy metabolism and generation of ROS (Fig. 6). The importance of a combination of factors has been shown for H_2O_2-induced apoptosis, where a certain intracellular superoxide anion concentration and an increase in the chemical reduction state and acidification of the intracellular environment are needed for the signal to proceed (Clement *et al*., 1998).

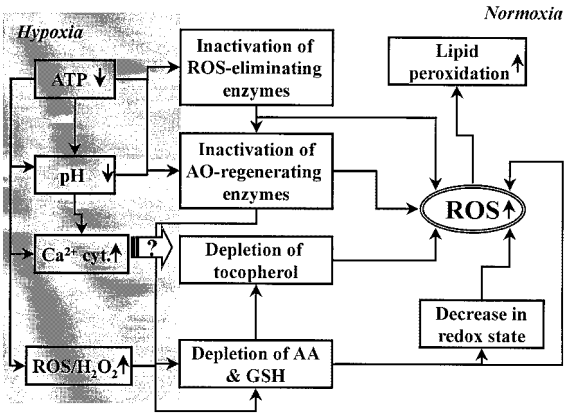

Fig. 6. Relationship between anoxia-induced metabolic changes, reactive oxygen species (ROS) and factors of oxidative stress under hypoxia and re-oxygenation. Under limited oxygen supply mitochondrial electron transport chain (ETC) becomes inhibited and the intracellular level of ATP decreases (Hanhijärvi and Fagerstedt, 1994). Upstream leakage of electrons from ETC favours the formation of superoxide and H_2O_2. Oxidative metabolism is switched to fermentation and accumulating lactic acid causes a pH decrease (Roberts *et al*., 1984). Lack of ATP also affects tonoplast H^+-ATPase and passive proton leakage from the vacuole adds to the cytoplasmic acidification (Roberts *et al*., 1984). Changes in ATP concentration and pH affect many cellular processes and lead to an increase in the cytosolic free Ca^{2+} (from the intracellular stores, particularly from mitochondria) (Subbaiah *et al*., 1998) and to the enzymatic formation of ROS (H_2O_2) via inhibition of ROS-eliminating and antioxidant-regenerating enzymes. The physiological role of free Ca^{2+}-elevation under anoxia is not well understood. Altogether, these changes cause subsequent depletion of the redox active antioxidants ascorbic acid and glutathione and membrane associated tocopherols (Blokhina *et al*., 2000). The resulting decrease in the redox state and low antioxidative status of the tissue fail to regulate ROS formation and manifest the initiation of oxidative stress. Upon restoration of normoxic conditions, excessive uncontrolled accumulation of ROS (mainly through the non-enzymatic pathway) leads to membrane lipid peroxidation, and eventually to cell death.

Under natural conditions anoxic stress necessarily includes several transition states (hypoxia, anoxia and subsequent oxidative stress) characterized by different O_2 concentrations and particular physiological states of the tissue. Excessive generation of ROS, the first sign of oxidative stress, is essential for the induction of a stress response. Changes in the steady-state equilibrium between ROS formation and utilization in the cell (Fig. 6) can induce both adaptive responses (ROS signalling, Lander, 1997) and detrimental changes in the cell structure and metabolism (LP). The sequence of events in 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. In plants undergoing anoxic stress factors, which can affect ROS formation and cell redox potential, have been described (Fig. 6). The exact place of H_2O_2 in anoxic signalling has yet to be identified, however, the localization of H_2O_2 in the apoplast and plasma membrane in the present study emphasizes the physiological importance of the apoplast in sensing/response to anoxic stress.

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References

Alscher RG, Donahue JL, Cramer CL. 1997. Reactive oxygen species and antioxidants: relationships in green cells. *Physiologia Plantarum* **100,** 224–233.

Bestwick CS, Brown IR, Bennett MHR, Mansfield JM. 1997. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolicola*. *The Plant Cell* **9,** 209–221.

Biemelt S, Keetman U, Albrecht G. 1998. Re-aeration following hypoxia or anoxia leads to activation of the antioxidative defense system in roots of wheat seedlings. *Plant Physiology* **116,** 651–658.

Biemelt S, Keetman U, Mock H-P, Grimm B. 2000. Expression and activity of isoenzymes of superoxide dismutase in wheat roots in response to hypoxia and anoxia. *Plant, Cell and Environment* **23,** 135–144.

Blokhina OB, Fagerstedt KV, Chirkova TV. 1999. Relationships between lipid peroxidation and anoxia tolerance in a range of species during post-anoxic reaeration. *Physiologia Plantarum* **105,** 625–632.

Blokhina OB, Virolainen E, Fagerstedt KV, Hoikkala A, Wähälä K, Chirkova TV. 2000. Antioxidant status of anoxia-tolerant and intolerant plant species under anoxia and reaeration. *Physiologia Plantarum* **109,** 396–403.

Boo YC, Jung J. 1999. Water deficit-induced oxidative stress and antioxidative defenses in rice plants. *Journal of Plant Physiology* **155,** 255–261.

Bowler C, Fluhr R. 2000. The role of calcium and activated oxygens as signals for controlling cross-tolerance. *Trends in Plant Science* **5,** 241–246.

Chang WWP, Huang L, Shen M, Webster C, Burlingame AL, Roberts JKM. 2000. Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment, and identification of proteins by mass spectrometry. *Plant Physiology* **122,** 295–317.

Chirkova TV. 1988. *Plant adaptation to hypoxia and anoxia*. Leningrad: Leningrad State University Press (in Russian), 245.

Chirkova TV, Novitskaya LO, Blokhina OB. 1998. Lipid peroxidation and antioxidant systems under anoxia in plants differing in their tolerance to oxygen deficiency. *Russian Journal of Plant Physiology* **45,** 55–62.

Chirkova TV, Sinyutina NF, Blyudzin YA, Barsky IE, Smetannikova SV. 1989. Phospholipid fatty acids of root mitochondria and microsomes from rice and wheat seedlings exposed to aeration or anaerobiosis. *Russian Journal of Plant Physiology* (in Russian) **36,** 126–134.

Chirkova TV, Zhukova TM, Goncharova NN. 1991. Method of determination of plant resistance to oxygen shortage. *Russian Journal of Plant Physiology* (in Russian) **38,** 359–364.

Chirkova TV, Zhukova TM, Tretiakov AL. 1984. Adenine nucleotides in wheat and rice seedlings under aeration and anoxia. *Vestnik LGU* (in Russian) **15,** 74–81.

Clement MV, Ponton A, Pervaiz S. 1998. Apoptosis induced by hydrogen peroxide is mediated by decreases superoxide anion concentration and reduction of intracellular milieu. *FEBS Letters* **440,** 13–18.

Crawford RMM, Braendle R. 1996. Oxygen deprivation stress in a changing environment. *Journal of Experimental Botany* **47,** 145–149.

Crawford RMM, Walton JC, Wollenweber-Ratzer B. 1994. Similarities between post-ischaemic injury to animal tissues and post anoxic injury in plants. *Proceedings of the Royal Society of Edinburgh* **102B,** 325–332.

Dunford B. 1993. Kinetics of peroxides reactions: horseradish, barley, *Corpinus cinereus*, lignin and manganese. In: Welinder KG, Rasmussen S, Penel C, Greppin H, eds. *Plant peroxidases: biochemistry and physiology*. University of Geneva, 113–124.

Dwyer SG, Legendre L, Low PS, Leto TL. 1996. Plant and human neutrophil oxidative burst complexes contain immunologically related proteins. *Biochimica et Biophysica Acta* **1289,** 231–237.

Elstner EF. 1991. Mechanisms of oxygen activation in different compartments of plant cells. In: Pell E, Steffen K, eds. *Active oxygen/oxidative stress and plant metabolism*. American Society of Plant Physiologists, 13–25.

Foyer CH, Lopez-Delgado H, Dat JF, Scott IM. 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. *Physiologia Plantarum* **100,** 241–254.

Hanhijärvi AM, Fagerstedt KV. 1994. Comparison of the effect of natural and experimental anoxia on carbohydrate and energy metabolism in *Iris pseudacorus* rhizomes. *Physiologia Plantarum* **90,** 437–444.

Hetherington AM, Hunter MI, Crawford RMM. 1982. Contrasting effects of anoxia on rhizome lipids of Iris species. *Phytochemistry* **21,** 1275–1278.

Hoson T. 1998. Apoplast as the site of response to environmental signals. *Journal of Plant Research* **111,** 167–177.

Hurkman WJ, Tanaka CK. 1996. Effect of salt stress on germin gene-expression in barley roots. *Plant Physiology* **110,** 971–977.

Kalashnikov JE, Balakhnina TI, Zakrzhevsky DA. 1994. Effect of soil hypoxia on activation of oxygen and the system of protection from oxidative destruction in roots and leaves of *Hordeum vulgare*. *Russian Journal of Plant Physiology* **41,** 583–588.

Kovtun Y, Chiu W-L, Tena G, Sheen J. 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proceedings of the National Academy of Sciences, USA* **97,** 2940–2945.

Lander HM. 1997. An essential role for free radicals and derived species in signal transduction. *FASEB Journal* **11,** 118–124.

Mehdy MC. 1994. Active oxygen species in plant defence against pathogens. *Plant Physiology* **105,** 467–472.

Noctor G, Foyer CH. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review on Plant Physiology and Plant Molecular Biology* **49,** 249–279.

Pellinen R, Palva T, Kangasjärvi J. 1999. Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. *The Plant Journal* **20,** 349–356.

Reggiani R, Bertani A. 1989. Effect of decreasing oxygen concentration on polyamine metabolism in rice and wheat shoots. *Journal of Plant Physiology* **135,** 375–377.

Roberts JKM, Callis J, Jardetzky O, Walbot V, Freeling M. 1984. Cytoplasmic acidosis as a determinant of flooding intolerance in plants. *Proceedings of the National Academy of Sciences, USA* **81,** 6029–6033.

Ros Barcelo A. 1998. Use and misuse of peroxidase inhibitors. *Trends in Plant Science* **11,** 418.

Semenza GL. 1999. Perspectives on oxygen sensing. *Cell* **98,** 281–284.

Skulachev VP. 1997. Membrane-linked systems preventing superoxide formation. *Bioscience reports* **17,** 347–366.

Subbaiah CC, Bush DS, Sachs MM. 1998. Mitochondrial contribution to the anoxic Ca^{2+} signal in maize suspension-cultured cells. *Plant Physiology* **118,** 759–771.

Vartapetian BB, Zakhmilova NA. 1990. Ultrastructure of wheat seedling mitochondria under anoxia and postanoxia. *Protoplasma* **156,** 39–44.

Yan B, Dai Q, Liu X, Huang S, Wang Z. 1996. Flooding-induced membrane damage, lipid oxidation and activated oxygen generation in corn leaves. *Plant and Soil* **179,** 261–268.