J. Plant Biol. (2009) 52:340–347
DOI 10.1007/s12374-009-9044-8

ORIGINAL RESEARCH

In Situ Localisation of Superoxide Generated in Leaves of *Alocasia macrorrhiza* (L.) Shott under Various Stresses

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Received: 13 March 2009 / Revised: 15 May 2009 / Accepted: 18 May 2009 / Published online: 16 June 2009

Abstract Leaf discs of *Alocasia macrorrhiza* were treated with various stress factors, including two photo-oxidants, methyl viologen (MV) or riboflavin (RB); three pollutants, sodium bisulphite ($NaHSO_3$), or the heavy metals lead or cadmium; or an osmotic medium, polyethylene glycol 6000. The in situ localisation sites for O_2^- generation were identified using specific dye nitro blue tetrazolium as a probe. The level of superoxide production was determined by scanning the blue-stained formazan area and was defined as the percentage of pixels from the stained portion versus the total number of pixels in the entire leaf disc area. All stress factors induced the generation of O_2^- in a time- or concentration-dependent pattern. Although superoxide production also was enhanced by longer time periods in untreated discs (control), the degree to which this occurred was less than that measured in leaves treated with either MV or RB. Generation sites were primarily found in the chloroplasts of stomatal guard cells and in the plasma membrane of the epidermis and mesophyll cells, indicating that they were most responsive to stress conditions. Nevertheless, the site of O_2^- generation varied among these stress factors.

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Keywords *Alocasia macrorrhiza* · Heavy metal · Histochemical localisation · Osmotic stress · Oxidants · Superoxide radical

Abbreviations

DDC	diethyldithiocarbamic acid
MV	methyl viologen
NBT	nitroblue tetrazolium
O_2^-	superoxide anion
RB	riboflavin
Tiron	4,5-dihydroxy-1,3-benzene disulfonic acid

Reactive oxygen species (ROS) are produced in cells via aerobic pathways. They is a by-product of normal metabolism or in response to various stresses (Murphy et al. 1998). ROS have dual functions: (1) intrinsic signals in plant growth and development processes, and (2) stress signal molecules (Vranová et al. 2002). One important ROS, the superoxide anion (O_2^-), is the direct single-electron reduction product of oxygen. It plays an enigmatic role that has been widely debated (Ort and Baker 2002). This anion is an indicator of cell damage or toxicity and a protective reaction under various stress conditions. Methods for measuring its level in plants have involved ESR spin-trapping with superoxide-specific spin probes, as well as chemiluminescence, fluorescence, and spectrophotometer assays (Shulaev and Oliver 2006). However, because of its short life (approximate 2 to 4 μs) and with moderate reactivity, direct measurement of O_2^- in intact materials is difficult, and in situ observations of the site for its production are still uncertain (Vranová et al. 2002).

In general, increases in O_2^- generation are found in preparations of plant extracts and isolated organelles that have previously been subjected to biotic and abiotic stresses, such as highlight (Fryer et al. 2002), heavy metals (Karuppanapandian and Manoharan 2008) or challenges from pathogens (Doke 1983; Huckelhoven and Kogel 1998; Romero et al. 2008). However, little information is available concerning the in situ localisation of O_2^- in cells. Techniques for directly measuring those sites in vivo can provide spatial information about the effects of stress at tissue, cellular and sub-cellular levels (Shulaev and Oliver 2006); ROS tracer dyes make it possible to localise stress at the latter two. The reaction of superoxide with an exogenous dye, nitro blue tetrazolium (NBT), leads to the formation of a dark-blue insoluble formazan compound that can be detected microscopically (Flohe and Otting 1984). An NBT assay has been used to demonstrate O_2^- generation in single cells undergoing a hypersensitive response (Doke 1983; Adam et al. 1989), in *Arabidopsis* leaves subjected to light stress (Fryer et al. 2002), in tobacco leaves exposed to ozone (Schraudner et al. 1998) and in pea leaves under salinity stress (Hernández et al. 2001).

Nevertheless, little is known about its sub-cellular localisation, and few quantification analyses have been conducted. Therefore, we used NBT staining as a tracer dye in the leaves of *Alocasia macrorrhiza* to address the following: (1) finding the site of direct generation at the cellular or sub-cellular level, (2) determining whether O_2^- production is common under different stresses and (3) using pixel calculations to relatively quantify this phenomenon in intact plant tissues. *A. macrorrhiza* is a stem-succulent herbaceous species that originated in the understory of a natural subtropical forest in Guangdong, China. These fast-growing plants are usually grown in urban shade gardens. Their very large leaves with fine veins make them suitable for such a model study because bigger, flat leaf discs are easier to manipulate than those from leaves with more prominent veins.

Materials and Methods

Plant Material

Six-month-old seedlings of *A. macrorrhiza* L. Scott were grown in the campus field of South China Botanical Garden, Guangdong, China. Uniform plants with four fully expanded leaves were selected, and discs (15 mm diam.) were punched from leaves that were second from the top of each plant.

Stress Treatments

The discs were rinsed with distilled water and wiped dry. They were then infiltrated under vacuum with either treatment solutions or distilled water as our control. Treatments (6 to 12 discs each) included 20 μM methyl viologen (MV) or 20 μM riboflavin (RB) for 0 to 4 days; PEG-6000 (0%, 5%, 10%, 15% or 20%) for 3 days; $NaHSO_3$ (0.0, 0.5, 1.0, or 1.5 mM) for 2 days; or one of two heavy metals, Pb ($PbHC_2$; 100 or 150 μM) or Cd ($CdCl_2$; 100 or 150 μM) for 3 days. After infiltration, all discs were incubated in the light (20 μmol m^{-2} s^{-1} PFD) at 28°C.

Histochemical and Cytochemical Localisation of O_2^-

Stress-induced generation of O_2^- in situ was detected by monitoring the reduction of NBT as described by Adam et al. (1989) and Schraudner et al. (1998) with minor modification. The reaction of NBT with this superoxide anion radical is a two-electron reduction event (Abugo and Rifkind 1994): NBT+2 $O_2^- \rightarrow NBT^{\bullet -} + O_2 + O_2^- \rightarrow 2O_2 +$ NBTr (a dark-blue formazan). Detection was accomplished by infiltrating the discs under vacuum with 0.05% (*w*/*v*) NBT, 10 mM sodium azide (NaN_3) and 50 mM HEPES buffer (pH 7.6) for 30 min. The discs were then held at room temperature until the blue colour became visible. To determine that this staining was attributable to the formation of O_2^-, $MnCl_2$ (10 mM), an effective inhibitor (Hernández et al. 2001) and Tiron (4,5-dihydroxy-1, 3-benzene disulfonic acid, 30 mM), an O_2^- trap, as well as diethyldithiocarbamic acid (DDC; 3 mM), which inhibits SOD activity, were added together with NBT as another control. After staining, the chlorophyll was removed by boiling the discs in a 9:1 solution of ethanol and glycerin. Photographs of NBT-stained samples were taken with a digital camera, and the histochemical localisation site of O_2^- was observed via light microscopy (Axioplan, Zeiss, Germany). For determining sub-cellular localisation, semi-thin transverse sections were prepared from stained leaf segments and observed microscopically.

O_2^- Quantitative Analysis of Leaf Discs

Quantitative analysis was conducted by scanning the pixels of blue-stained spots and the entire disc area with Photoshop 8.0 (Adobe System). Generation of O_2^- was expressed as the percentage of pixels in the stained area versus total number of pixels from the disc. Because the cut edge of the discs had obvious staining, it was subtracted from the stained area. Hence, the actual percentage of pixels in that area related to stress-induced O_2^- production was calculated as:

$$\%\ \text{of}\ O_2^-\ \text{staining area} = 100 \times (\text{TPLD} - \text{PUS} - \text{PSC})/\text{TPLD}$$

where TPLD was the total pixels for the entire disc, PUS was the pixel count in the unstained area and PSC was the number of pixels within the stained cut edge.

Data obtained from this quantification were analysed statistically with a Student's *t* test.

Results

Tissue Localisation of O_2^- Generation under Various Stresses

A blue formazan precipitate resulted from the reaction between NBT and O_2^- in leaf discs from *A. macrorrhiza* (Fig. 1). Clear blue rings around the cut edges of those discs appeared in all control and treated samples, indicating that the formation of O_2^- was a wound-inducible response. When Mn^{2+} was combined with NBT, this strong staining of formazan at the edges of our control discs was completely abolished. Treatment with 20 μM MV caused O_2^- production to increase, but this effect was either reduced by Tiron or enhanced by DDC (Fig. 1A). Therefore, we could confirm that this staining was not an artefact but was due to the specific reaction product between O_2^- and NBT. In control discs, i.e., those infiltrated with distilled water, this blue staining was increased over 4 days (Fig. 1B), indicating that leaf senescence also could lead to a rise in O_2^- generation. Furthermore, after 4 days of MV treatment, the number and area of blue spots in leaf discs away from the wound edge increased significantly over that in the control as the treatment period was lengthened. Heavy staining was observed on day 4, whereas only a few blue spots were found on day 1 (Fig. 1C). This implied that O_2^- production was enhanced temporally. When we applied another O_2^--generating agent, riboflavin, the status of O_2^- followed a trend similar to that found with our MV treatment, except that the blue colouring was more intense with RB exposure (Fig. 1D).

The generation of O_2^- also was induced by PEG osmotic stress (Fig. 1E), following a concentration-dependent pattern. A slight track of blue spotting was found in the control discs. Although this developed slowly at PEG concentrations of 5% and 10%, a greater concentration (20%) caused a remarkable increase in the formation of blue formazan precipitates. In fact, 25% of the chlorophyll content was degraded by this highest level of PEG (data not shown).

Bisulphite ions (HSO_3^-) are the main hydrate state of gaseous SO_2 in cells. At greater concentrations, bisulphite can lead to ROS formation. Here, our comparison with the untreated control revealed no obvious differences at a low concentration of $NaHSO_3$ (0.5 mM); however, the area and intensity of blue formazan increased greatly as the level of $NaHSO_3$ rose to 1.5 mM. These results demonstrated a dose-dependent response for O_2^- generation after bisulphite exposure.

Two heavy metals, lead and cadmium, are important sources of environmental pollution. Their toxic mechanism in plants may involve metal-induced oxidative damage (Ercal et al. 2001). However, because both are redox-inactive, further direct evidence is necessary to confirm that ROS is really induced in cells by Pb and Cd. Our in situ NBT-staining showed a pronounced accumulation of O_2^- in leaves exposed to 150 μM Pb or Cd for 3 days (Fig. 1G), a trend that was consistent with that for redox-active iron (150 μM $FeSO_4$ for 1 day). Therefore, we could conclude that both redox-active and redox-inactive metals caused O_2^- production to increase. Here, staining and total blue surface areas were more intensive in samples treated with Cd than with Pb at identical concentrations.

Cellular and Sub-cellular Localisations of O_2^- Generation

Upon treatment with MV or RB, microscopic observations of the abaxial leaf surfaces showed that O_2^- generation occurred mostly in the chloroplasts of the stomatal guard cells (Fig. 2A, B); after PEG, Pb or Cd applications, those sites were the plasma membrane of the epidermal cells and the chloroplasts of the guard cells (Fig. 2D–F). Following $NaHSO_3$ treatment, superoxide production was observed only in the plasma membrane of the lower epidermal cells (Fig. 2C). Hence, it seems that these sites varied according to specific stress conditions.

In the transverse sections, small, numerous blue formazan precipitates were frequently distributed in the chloroplasts of all green mesophyll cells under MV (Fig. 3A), RB (Fig. 3B) or PEG (Fig. 3C) stresses versus both the chloroplasts and the plasma membrane of chlorenchyma cells because of Pb (Fig. 3E). However, the site of formazan precipitates in mesophyll cells following induction by $NaHSO_3$ (Fig. 3D) or Cd (Fig. 3F) occurred only in the chloroplasts of spongy tissue cells. This suggests that both chloroplasts and the plasma membrane were the primary sites of O_2^- generation in those cell types.

Quantification of In Situ O_2^- Accumulations

Accumulations of O_2^- were calculated as the pixel ratio of stained formazan/total leaf disc areas (Fig. 4). Senescence by detached leaves caused O_2^- generation to rise from 18.9% to 52.9% of all pixels in 4 days (Fig. 4A). Treatment with 20 μM MV or RB also rapidly triggered the production of O_2^- over that same period. For example, MV application for 1, 2, 3 or 4 days was correlated with

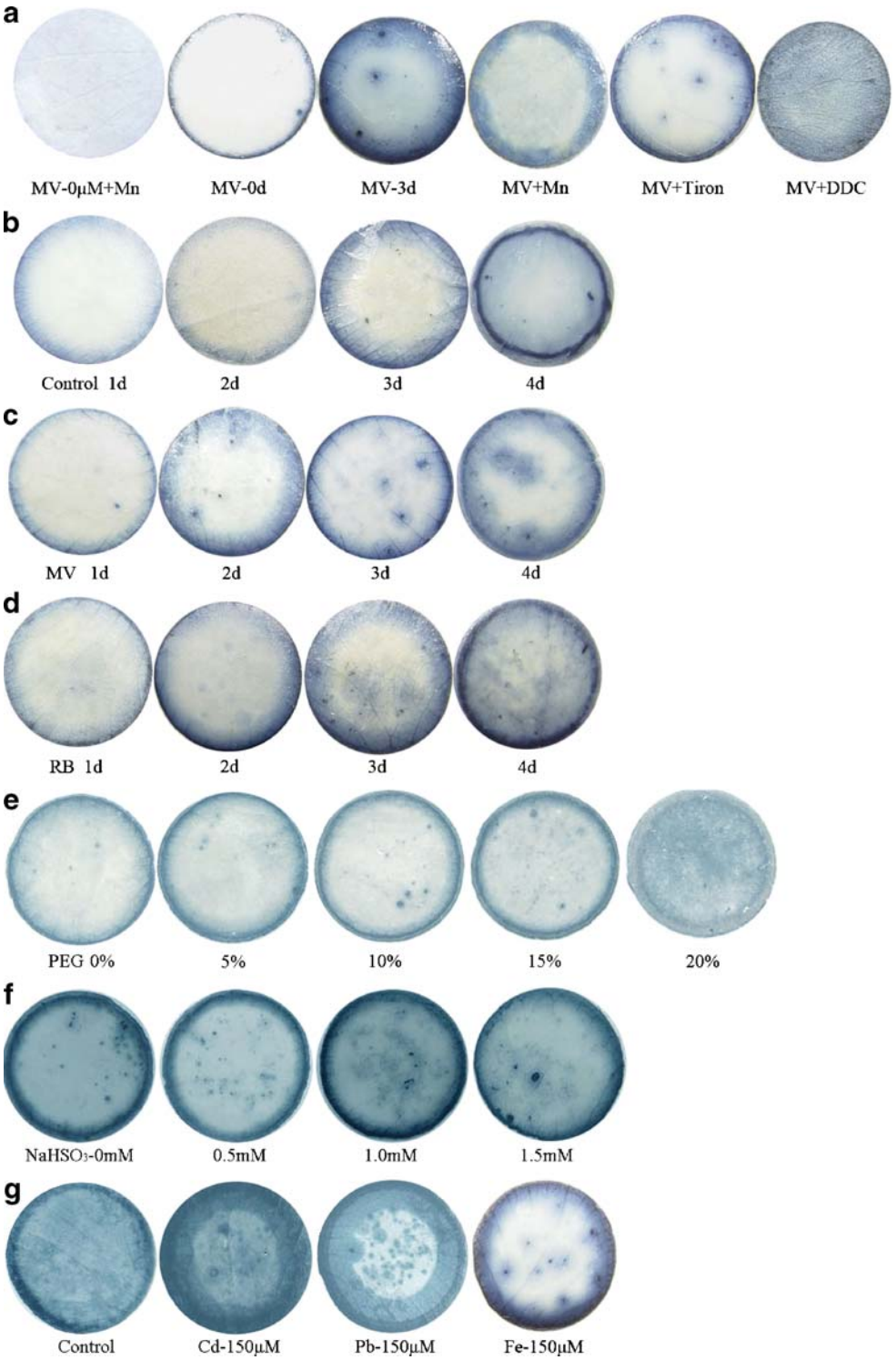

Fig. 1 Tissue localisation of O_2^- generation by NBT-staining under various stress conditions: **a** *left to right*, treatment with 10 mM Mn^{2+} but no MV pre-treatment (day 0), 20 μM MV at day 0, MV for 3 days, MV + Mn for 3 days, MV+30 mM Tiron for 3 days, MV+3 mM DDC for 2 days. Mn, Tiron or DDC was applied with MV to reduce or enhance NBT staining area and intensity; **b** control (in water), 1–4 days; **c** 20 μM MV, 1–4 days; **d** 20 μM RB, 1–4 days; **e** 0–20% PEG, 3 days; **f** 0.0–1.5 mM $NaHSO_3$, 42 h; **g** 150 μM Pb or Cd (3 days) or 150 μM Fe (1 day). Blue precipitates are O_2^- and NBT reaction product (formazan). *Numbers* below leaf discs represent treatment concentration and duration

specific pixel percentages for formazan of 27%, 46%, 54% or 71%, respectively, of the entire disc area. Furthermore, values at those time points were 1.74-, 2.99-, 3.47- and 4.20-fold greater than amounts measured prior to treatment (i.e., day 0; Fig. 1) and 123% to 154% higher than the control level (Fig. 4B). In contrast, the accumulation of O_2^- in leaves exposed to RB accounted for 32%, 40%, 51% or 57% of the entire disc area at day 1, 2, 3 or 4, respectively, which was 8% to 72% greater than that measured in the control (Fig. 4C).

Fewer formazan precipitates were found after exposure to either 10% PEG or 0.5 mM $NaHSO_3$ but was

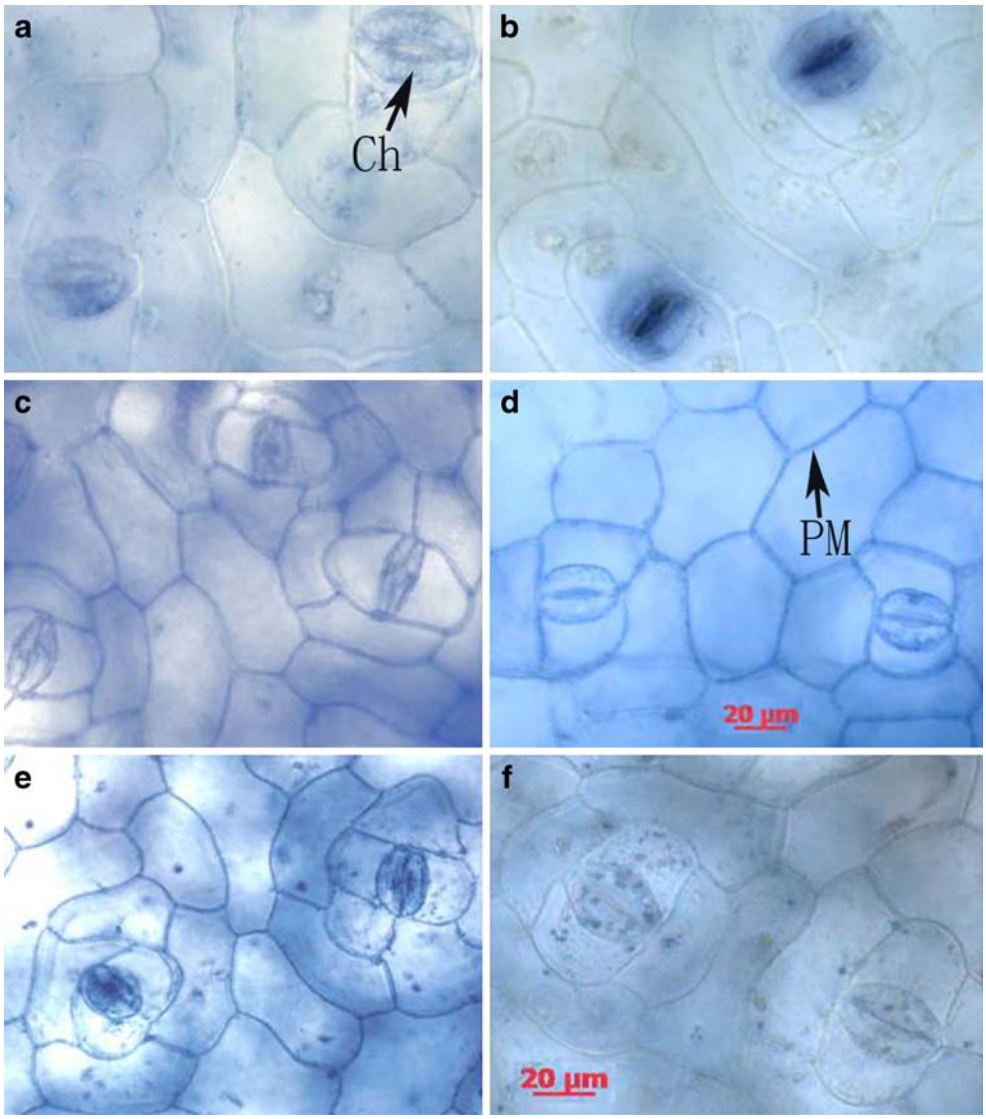

Fig. 2 In situ cellular localisation of O_2^- in abaxial epidermal cells in leaves treated with MV (**a**), RB (**b**), $NaHSO_3$ (**c**), PEG (**d**), Pb (**e**) or Cd (**f**). *PM* plasma membrane; *Ch* chloroplasts

rapidly elevated at higher concentrations. For example, the percentage of formazan pixels was 146% or 187% higher than the control value following treatment with 20% PEG or 2 mM $NaHSO_3$, respectively (Fig. 4D, E). Likewise, O_2^- generation was increased by both the redox-active iron and the redox-inactive lead and cadmium. Compared with the untreated control, this enhancement in the relative proportion of pixels was 86.9% or 162% after 3 days of treatment with 150 μM Cd or Pb but was 113.5% after 1 day of exposure to 150 μM Fe (Fig. 1G). Moreover, MV-induced O_2^- accumulation (20 μM for 3 days) resulted in 52.8% of pixels being stained. That level, however, was diminished to 38.2% by 10 mM Mn or to 35.1% by 30 mM Tiron, while being increased to 57.8% after the addition of 3 mM DDC.

Discussion

Generation of superoxides is the first step in ROS production. Localisation and the extent of its production can provide a dynamic imaging pattern for understanding the mechanism by which plant cells respond to oxidative stress. NBT reduction reacts preferentially with O_2^- but not with H_2O_2 (Jabs et al. 1996). Using NBT as a probe here, we found that all tested stress factors—MV, RB, $NaHSO_3$, PEG, Pb and Cd—induced the active generation of O_2^- in leaves of *A. macrorrhiza*. Such induction was time- or dose-dependent, with the principal generation sites being the chloroplasts and the plasma membrane. To our knowledge, this is the first report of tissue and cellular localisation, with our information having been obtained through in situ NBT

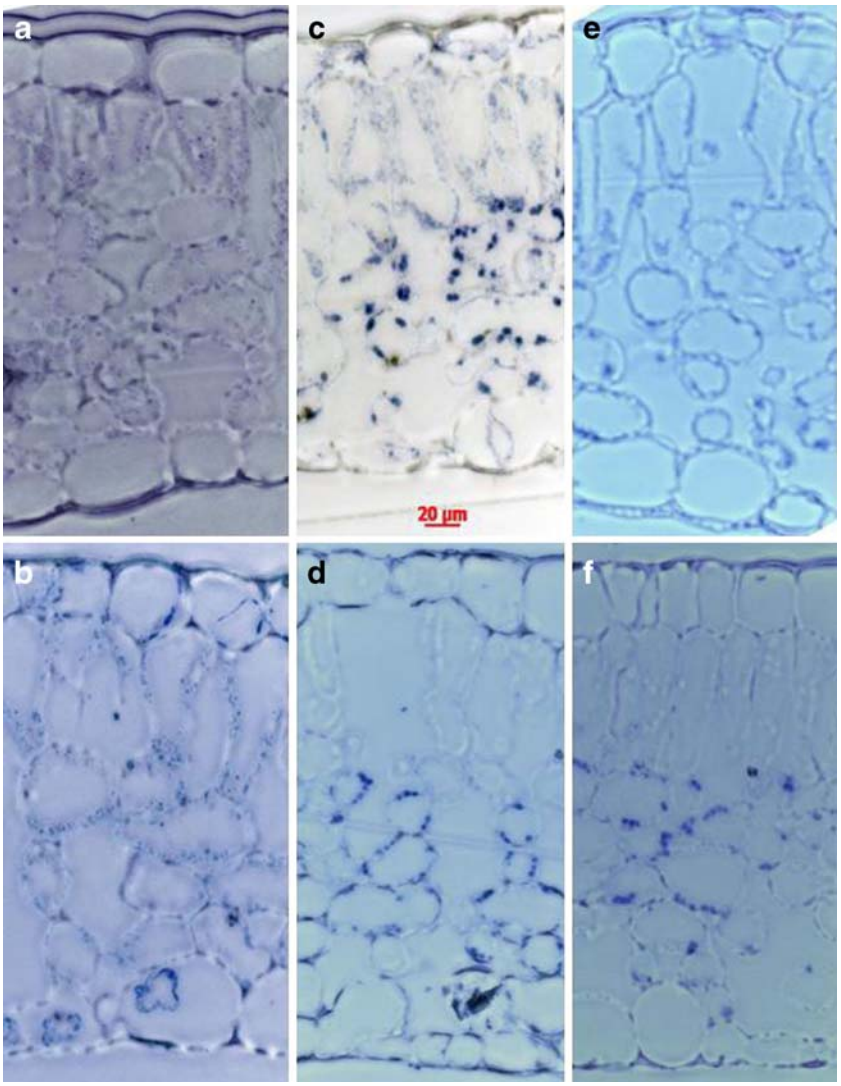

Fig. 3 Sub-cellular localisation of O_2^- in mesophyll cells of leaf transverse section treated with MV (**a**), RB (**b**), PEG (**c**), $NaHSO_3$ (**d**), Pb (**e**) or Cd (**f**)

staining in plants under $NaHSO_3$, PEG, MV or Pb stress. Moreover, we have now demonstrated that NBT is a suitable and valuable tool for such monitoring of O_2^- generation in plants.

Monovalent oxygen reduction yields superoxide during the electron transport processes of photosynthesis and respiration. Chloroplasts with high photo-oxidative potential are the most likely organelle to form ROS in response to unfavourable environment conditions (Salin 1991). In the photosynthetic apparatus, ROS are produced from three sites: the light-harvesting complex associated with PSII, the PSII reaction centre and the PSI acceptor (Niyogi 1999). Methyl viologen can act as a PSI electron acceptor and then transfer the electron to an oxygen molecule to form the superoxide radical. Cells in vivo are more permeable to MV than are isolated chloroplasts (Powles and Cornic 1987). Because our leaf epidermal tissue was first exposed to an MV solution, it is apparent that O_2^- is mainly present in the chloroplasts of stomatal guard cells and in mesophyll cells. Nevertheless, the increase in O_2^- generation by redox-inactive Pb and Cd may involve multiple factors. This was discovered because oxidative stress arises indirectly through the disturbance of photosynthetic electron transport and the depletion of major antioxidants (particularly the thiol containing compounds, such as glutathione), leading to ROS formation in the chloroplasts (Ercal et al. 2001; Choudhury and Panda 2004). Although the specific mechanism is still unclear, our results provide better evidence for Pb- and Cd-induced generation of O_2^- in the chloroplasts.

Lead and cadmium are retained at the surface of the plasmalemma and cell walls, and the former is able to bind strongly to the carboxyl groups of carbohydrates in

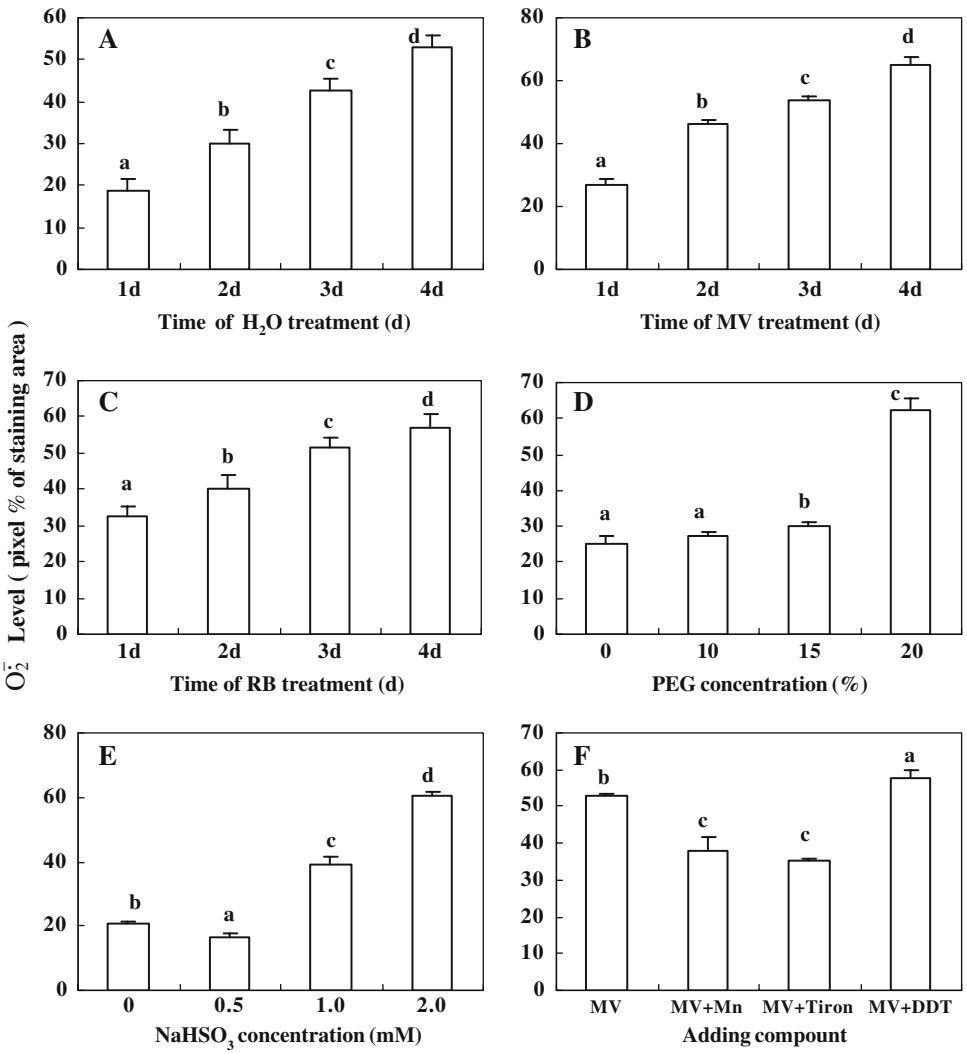

Fig. 4 Levels of O_2^- generation (percentage of pixels in staining area compared to total pixels from entire disc) under various stress conditions: **a** control, 1–4 days; **b** 20 μM MV, 1–4 days; **c** 20 μM RB, 1–4 days; **d** 0–20% PEG, 3 days; **e** $NaHSO_3$, 42 h or **f** addition of Mn, Tiron or DDC. Values are means ± SE from five determinations. *Different letters* represent significance at $P<0.05$

those walls (Sharma and Dubey 2005). Researchers have proposed that a plasma membrane-localised NADPH oxidase, which transfers electrons from cytosolic NADPH to O_2 to form O_2^- (i.e. $O_2 + NADPH \rightarrow O_2^- + NADP^+ + H^+$), is responsible for the production of O_2^- and, subsequently, H_2O_2 in plants responding to SO_2 (Li et al. 2007), nickel (Hao et al. 2006), NaCl (Hernández et al. 2001) and Cd (Olmos et al. 2003, H_2O_2 burst). Furthermore, Ogawa et al. (1997) have reported that the development of formazan in the vascular tissue of spinach hypocotyls could be diminished by inhibitors of NADPH oxidase. Li et al. (2007) have shown that the bisulphite-induced enhancement of ROS (revealed by DCF fluorescence) in spinach leaves originates mainly from plasma membrane NADPH oxidase. A signal transduction pathway has been used to demonstrate the stimulatory effect of NADPH oxidase on human neutrophils, producing active oxygen radicals by sulfite, one of the hydrate derivatives of SO_2 in cells (Beck-Speier et al. 1993). We have previously found pronounced blue formazan in rice leaves under $NaHSO_3$ stress (unpublished data). Monitoring O_2^- accumulation inside the cells might be a better indicator of NADPH oxidase activity (Song et al. 2006). The plasma membrane is apparently an important site for generating O_2^- via NADPH oxidase. Because the plasma membrane-binding NADPH oxidase is azide-insensitive, we performed NBT-staining in the presence of 10 mM sodium azide. The obvious formazan precipitate in the plasma membrane of epidermal cells observed here could be attributed to the activation of NADPH oxidase that was induced by heavy metals, PEG or $NaHSO_3$. However, further experiments are required that will focus on generation sites and mechanisms in different organs, cell types and plant species in response to an alteration of environmental factors.

Acknowledgements We thank Prof. Fred Chow (Research School of Biological Sciences, the Australian National University) for critical reading of the manuscript and helpful suggestions and Ms. X.L. Xu for preparation of semi-thin leaf sections. This research was financially supported by the National Natural Science Foundation of China (30770173).

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