Functional Plant Science and Biotechnology ©2007 Global Science Books

Localization of Reactive Oxygen Species and Lignification in Leaves of Young Sulphate-Deprived Maize Plants

Dimitris L. Bouranis[1*] • Styliani N. Chorianopoulou[1] • Vassilis E. Protonotarios[1] • Vassilis F. Siyiannis[1] • Malcolm J. Hawkesford[2]

[1] Plant Physiology Laboratory, Department of Plant Biology, Faculty of Agricultural Biotechnology, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece
[2] Plant Sciences Department, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, United Kingdom

Corresponding author: * **bouranis@aua.gr**

ABSTRACT

Young maize (*Zea mays* L., Poaceae) plants were grown in a complete, well-oxygenated nutrient solution and after 15 days were subjected to sulphate deprivation for 12 days. The lamina of the fully expanded 2nd leaves in sulphate-deprived plants (–S) presented more developed lower sclerenchyma and an intense lignification. Vascular bundles (VB) were more developed in the lamina of the expanding 4th leaves of –S plants, possessing more and larger xylem vessels compared with the control. Sulphate deprivation also affected the spatial and temporal distribution of reactive oxygen species. Superoxide anions and hydrogen peroxide in 2nd leaf of control plants were located mainly in the mesophyll, whilst in the 2nd leaf of –S plants they were located in the parenchymatic sheath of the VB. Addition of the superoxide dismutase inhibitor, N,N-diethyldithiocarbamate (DDC), resulted in an increased level of superoxide anions in –S leaves. The presence of superoxide anions was particularly evident in the cell walls between the VB of leaf 2 of –S plants. In contrast, there was a higher concentration of hydrogen peroxide in control leaves at day 12. Leaf 4 of the –S plants, had less superoxide anions than control plants, and there was no detectable superoxide anion in the VB. Conversely, in control leaves intense presence of superoxide anion was observed inside and around the VB (in the area where the midvein VB develops). There was no appearance of hydrogen peroxide, either in the treatment with DDC or in the treatments with ascorbate or ascorbate plus DDC, for both control and –S leaves.

Keywords: hydrogen peroxide, *in situ* localization, superoxide anion, vascular bundle, *Zea mays*
Abbreviations: BS, bundle sheath; **BSC**, bundle sheath cells; **DDC**, N,N-diethyldithio carbamate; **MC**, mesophyll cells; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase; **VB**, vascular bundles

INTRODUCTION

The bundle sheath cells (BSC) and mesophyll cells (MC) of maize leaves have differentiated metabolic functions, as the initial steps of CO_2 assimilation in C4 plants are spatially separated from the enzymes of the Benson-Calvin cycle (Furbank and Taylor 1995). The maize MC contains chloroplasts with both PSI and PSII. In contrast, the bundle sheath (BS) chloroplasts are deficient in PSII and exhibit very low net O_2 evolution. Hence, the BS chloroplasts are restricted in their capacity for noncyclic electron flow and NADPH formation. The capacity of each cell type to scavenge reactive oxygen species (ROS) and to maintain a highly reduced state of the antioxidants, ascorbate and glutathione, contribute to the stress tolerance of maize (Wise 1995; Prasad 1996). According to Doulis *et al*. (1997), the majority of the superoxide dismutase (SOD) and ascorbate peroxidase activities are localized in the BSC, whereas the glutathione oxidoreductase and dehydroascorbate reductase activities are almost exclusively localized in the MC. Monodehydroascorbate reductase and catalase were found to be equally distributed between the two tissue types. Ascorbate was predominantly localized in the BSC, whereas glutathione was found in both BSC and MC.

The enzymes of the Benson-Calvin cycle are very sensitive to inhibition by H_2O_2 (Foyer *et al*. 1994), and are located in the BS chloroplasts. Most of the H_2O_2 is localized in the MC, while very little is present in the BSC. Whereas the capacity for O_2 evolution by PSII is severely restricted in the BS, the presence of SOD activity in this compartment would indicate H_2O_2 production from the enzymatic dismutation of superoxide. H_2O_2 destruction is rapid in the BSC. Superoxide produced in the MC spontaneously dismutates to H_2O_2 or this reaction is catalyzed by ferredoxin or other cellular electron-transfer components of a suitable electrochemical potential. Since ascorbate peroxidase is largely absent from this compartment, H_2O_2 must be destroyed by peroxisomal catalase, which is energetically much less costly than ascorbate-dependent reduction via APX (Doulis *et al*. 1997).

There is also cooperation between BSC and MC for sulphate reduction and glutathione synthesis (Burgener *et al*. 1998; Kopriva and Koprivova 2005; Kopriva 2006). Sulphate is a macronutrient required for cell growth and development and sulphur deficiency causes retarded and chlorotic growth of plants, significantly reducing crop performance (Maruyama-Nakashita *et al* 2003). Plants utilize sulphate for synthesis of various organic compounds (such as cysteine, cystine, methionine, lipoic acid, co-enzyme A, thiamine pyrophosphate, glutathione, biotin, adenosine-5′-phosphosulfate, 3-phosphoadenosine and proteins) through a complex metabolic network (Leustek and Saito 1999; Leustek *et al* 2000; Grossman and Takahashi 2001). Many of the symptoms of sulphur deficiency are similar to those of nitrogen deficiency, including chlorosis and stunting of growth. This similarity is not surprising, since sulphur and nitrogen are both constituents of proteins (Marschner 1995). The concentration of glutathione, a key component of the antioxidant system, is dependent upon S-nutrition (Blake-Kalff *et al* 1998).

In the present work, 15-days-old maize plants (*Zea mays* L., Poaceae) grown in a complete and well oxygenated nutrient solution, were subjected to sulphate deprivation for 12 days, and leaf anatomy was examined. Hypothesizing that the occurrence and/or ability to deal with ROS are affected by sulphur nutrition, ROS distribution were examined in

Received: 17 September, 2007. Accepted: 30 October, 2007.

Original Research Paper

space and time, focusing on the tissue localization of superoxide anion and H_2O_2.

MATERIALS AND METHODS

Plant material and growth conditions

Maize hybrid LG-2447 was germinated in pots containing a commercial peat-based growing medium in a growth chamber at 22°C, 65% relative humidity, 16 h light/8 h dark and 250 µmol photon m^{-2} s^{-1}. When the 3rd leaf appeared (after 10 days), plants were removed from pots, roots were washed with water to remove soil particles and plants were transferred to hydroponic batch culture system under the same conditions. The complete nutrient solution (control) contained: 7 mM KNO_3, 0.9 mM $MgCl_2$, 1 mM KH_2PO_4, 1.71 mM $Mg(NO_3)_2$, 0.1 mM NaCl, 1 mM $MgSO_4$, 0.06 mM EDTA FeNa, 5 mM $Ca(NO_3)_2$, 0.5 mM $CaCl_2$, 0.095 µM Zn acetate, 2.458 µM H_3BO_3, 0.05 µM $Cu(NO_3)_2$, 0.082 µM $(NH_4)_6Mo_7O_{24}$, 0.984 µM $Mn(NO_3)_2$. Plants were maintained for 1 d on 0.1 strength nutrient solution followed by a further day on 0.5 strength solution and thereafter full-strength nutrient solution (control) was used for three days. Following this (day 0), the plants were maintained in complete nutrient solution (C) or in nutrient solution without SO_4^{2-} (–S) for 12 days. Nutrient solutions were changed every day, and were continuously aerated.

Sampling and observations

Free hand transverse sections were made in the 2nd true leaf at days 6 and 12 and in the 4th true leaf at day 12, taken from the middle of the lamina for both leaves. The 2nd and 4th leaf were selected in order to compare a fully developed and an expanding leaf, the 2nd leaf already being developed at day 0, while the 4th leaf was the youngest leaf present at day 12. Sections were first observed in distilled H_2O in the light microscope. All sections were viewed and photographed using a Zeiss Axiolab HBO 50 light microscope, equipped with a G-365 (UV) excitation filter (barrier filter at 420 nm) and a G-450-490 (blue) excitation filter (barrier filter at 520 nm) for fluorescence microscopy.

Detection of lignins

Lignins were detected using the Wiesner test (specific for cinnamaldehyde groups). For this, leaf sections were soaked in 1% (w/v) phloroglucinol in 25:75 (v/v) HCl:ethanol for 15-30 min at room temperature (Ros Barceló 1998). This dye gave an orange to deep purple colour in the lignified areas, depending on the degree of lignification.

UV-excited autofluorescence

Sections were viewed in distilled H_2O in the fluorescent microscope. The excitation wavelength was 365 nm.

Blue-excited autofluorescence

Sections were viewed in distilled H_2O in the fluorescent microscope. The excitation wavelength was 450-490 nm.

Detection of Reactive Oxygen Species

Intracellular production of ROS was measured using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes). This non-polar compound is converted to the membrane-impermeant polar derivative H_2DCF by esterases following entry into the cell. H_2DCF is non-fluorescent, but is rapidly oxidized to the highly fluorescent DCF by intracellular H_2O_2 and other peroxides. Stocks of H_2DCF-DA (5 mM) were made in absolute ethanol. Sections were incubated in H_2DCF-DA at a final concentration of 5 µM (in distilled H_2O) for 1 h at 25°C, rinsed with distilled H_2O and observed in distilled H_2O by means of fluorescence microscopy. The excitation and the emission wavelengths were 450-490 and 520 (green) nm respectively (Maxwell *et al*. 1999, with modifications).

Detection of superoxide anion

Areas of superoxide anion production in leaf sections were monitored on the basis of SOD-sensitive reduction of nitroblue tetrazolium (NBT). Cross-sections were incubated directly in a reaction medium containing 0.25 mM NBT in 50 mM K-phosphate buffer (pH 7.8) for 1h at 25°C. The superoxide anion was detected under the light microscope by the formation of blue formazan. To examine the effects of inhibitors on the superoxide-depended reduction of NBT, another set of sections was pre-incubated in a inhibitor of CuZn-SOD (1 mM N,N-diethyldithiocarbamate; DDC) for 10 min and then incubated in NBT reaction medium for 1 h (Ros Barceló 1998, with modifications).

Detection of hydrogen peroxide

H_2O_2 localization was monitored by staining the sections with KI/starch reagent, composed of 4% (w/v) starch and 0.1 M KI, adjusted to pH 5.0 with KOH. This reagent was boiled and then cooled to room temperature. Sections were incubated for 1 h at 25°C, and the areas of H_2O_2 production were monitored by observing the development of a dark stain on the cut surface under the light microscope. Another set of sections was pre-incubated for 10 min in 1 mM ascorbic acid prior to addition of the starch/KI reagent and incubation for 1 h (Ros Barceló 1998, with modifications).

RESULTS

Effects of sulphate deprivation on leaf anatomy and lignification

All of the VB in the lamina of –S 2nd leaves were well developed by d 6 (**Fig. 1**, compare **A** and **B** with **D** and **E**). Compared with the control, chloroplasts were yellow in –S leaves at d 12 (**Fig. 1**, compare **C** and **F**). In the lamina of the 2nd –S leaf at d 6 and 12, greater breadthwise and lengthwise development of lower sclerenchyma (fibers) was observed, the total length being 520 ± 36 µm in –S leaves, compared to a 435 ± 28 µm length of lower sclerenchyma in control leaves. Two layers of cells were present, the inner sclerenchymatic and the outer parenchymatic sheaths of the VB, between the phloem of the midvein VB and the small cells of the lower sclerenchyma in control leaves. Additional cells were observed between these two layers in –S leaves, and all of these cells were lignified. Except for the larger lower sclerenchyma, lignification of –S leaves was more intense, even in the cells of the epidermis close to the lower sclerenchyma (**Fig. 1**, compare **G-I** to **J-L**).

Lignification was also observed in the VB and particularly in the midvein VB of –S leaves. After 6 d of S-deprivation, the inner sclerenchymatic sheath was already lignified, including the xylem vessels and the cells of xylem parenchyma. This pattern was observed only at d 12 in the control control leaves (**Fig. 1G-L**).

Using the UV filter, an intense blue/white fluorescence was observed in the cells of the lower epidermis (and the cuticle) at d 6 and 12 (**Fig. 2A-F**). The epidermal cells close to the lower sclerenchymatic fibers of the midvein VB did not fluoresce. All VB fluoresced similarly to the midvein VB. Generally the walls of the cells containing chloroplasts fluoresced green. In contrast, –S leaf cross sections showed less intense fluorescence, with the epidermal cells having reduced fluorescence, whilst the VB (phloem and xylem) continued to fluoresce. Reduced fluorescence was evident also in the cells with chloroplasts.

With the blue filter, the walls of the cells of the midvein VB fluoresced intensely green, except the phloem and the walls of the cells of the lower sclerenchyma (**Fig. 2G-L**). Less intense fluorescence characterized the walls of the cells in the developing VB and the MC. Chloroplasts gave an intense red fluorescence and the intensity of the fluorescence was reduced in –S leaves relative to control. Particularly at d 12, the chloroplasts fluoresced only slightly in contrast to the cells of the midvein VB (except the phloem) and the lower sclerenchyma.

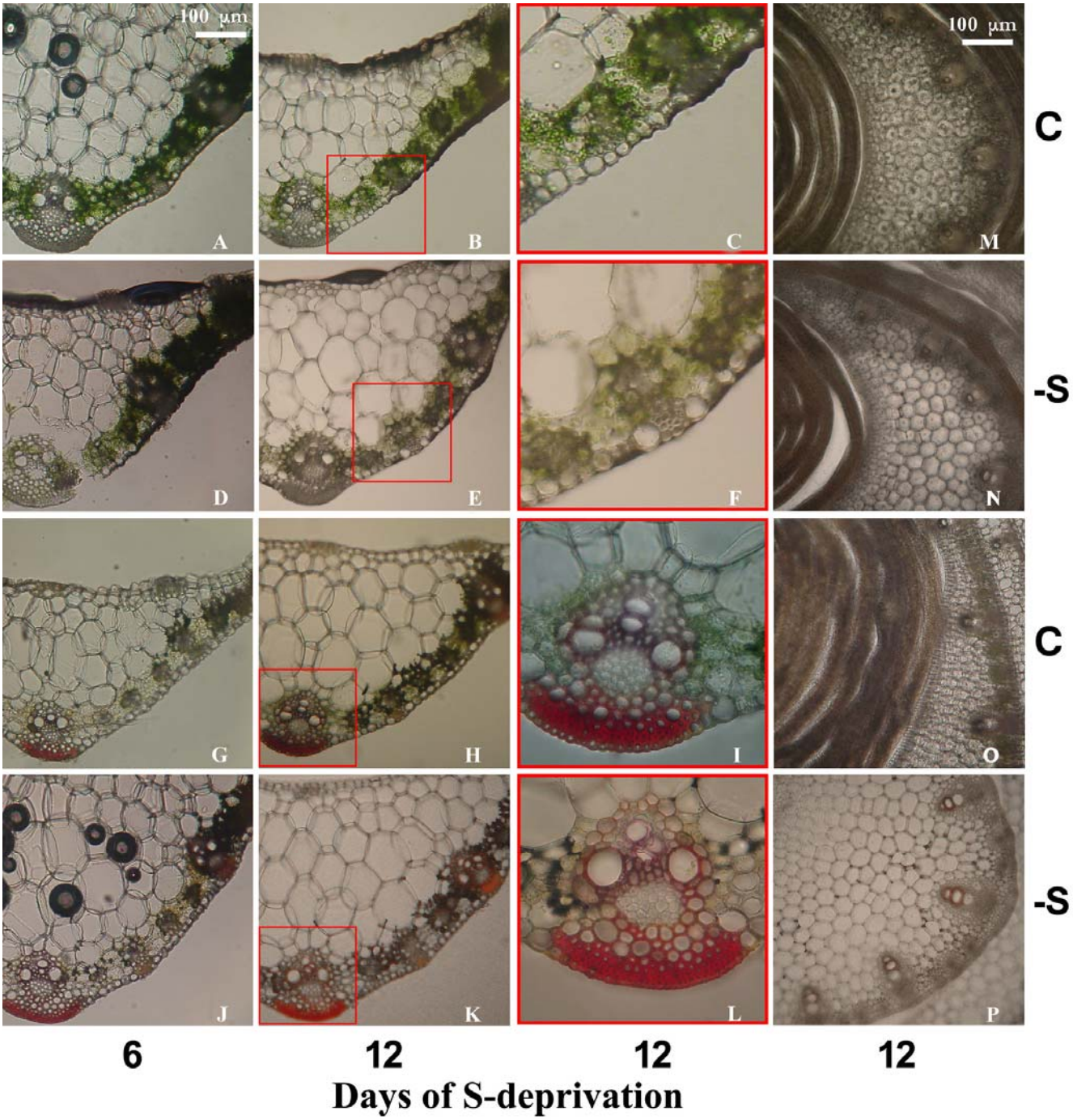

Fig. 1 Free hand transverse sections of control (C) and S-deprived (-S) maize 2nd (**A-L**) and 4th (**M-P**) leaves. (**A-F**)**:** Anatomy of the 2nd leaf as seen in the light microscope, for controls, and 6 and 12 days after S-deprivation. Sections were viewed in distilled water. Photographs **C** and **F** are magnifications of **B** and **E**, respectively. (**G-L**)**:** Lignification of the 2nd leaf as seen in the light microscope in controls and 6 and 12 days after S-deprivation. Sections were treated with phloroglucinol. Photographs **I** and **L** are magnifications of **H** and **K** respectively. (**M-N**)**:** Anatomy of the 4th leaf as seen in light microscope 12 days after S-deprivation. Sections were viewed in distilled water. (**O-P**)**:** Lignification of the 4th leaf as seen in light microscope 12 days after S-deprivation. Sections were treated with phloroglucinol.

The areas of the leaf cross section that were stained with phloroglucinol were those that fluoresced under the blue filter, in complete contrast to those that fluoresced under the UV filter (**Figs. 1I, L**, **2C, F, I, L**). More extended lignification (indicated by phloroglucinol dye and more intense green fluorescence with the blue filter) was observed in –S leaves compared with the control leaves. The midvein VB of control leaves fluoresced more intensely with the UV filter, with a light blue colour to characterize the phloem and the epidermal cells.

More rapid development of VB was found where the main vein will develop in the –S leaf 4 (larger, with more xylem vessels) compared with the control (**Fig. 1M, 1N**). No red colour was observed in the control leaf 4, while in the main vein of –S leaves, two vessels of every developed VB were red (**Fig. 1O, 1P**). With the UV filter, the developing xylem vessels fluoresced intensely blue. A small xylem vessel fluoresced in every VB of control leaves, while two xylem vessels (a smaller and a larger one) fluoresced in –S leaves (**Fig. 2M, 2N**). With the blue filter, chloroplasts of –S leaves fluoresced intensely (**Fig. 2O, 2P**).

Effect of sulphate deprivation on leaf tissue localization of reactive oxygen species

Intense fluorescence indicating a substantial presence of ROS was located mainly in the cells which have chloroplasts of the 2nd control leaf at d 6 (**Fig. 3A**). This was not true for BSC and MC close to the midvein VB. A similar pattern was apparent at d 12, but with less intense fluores-

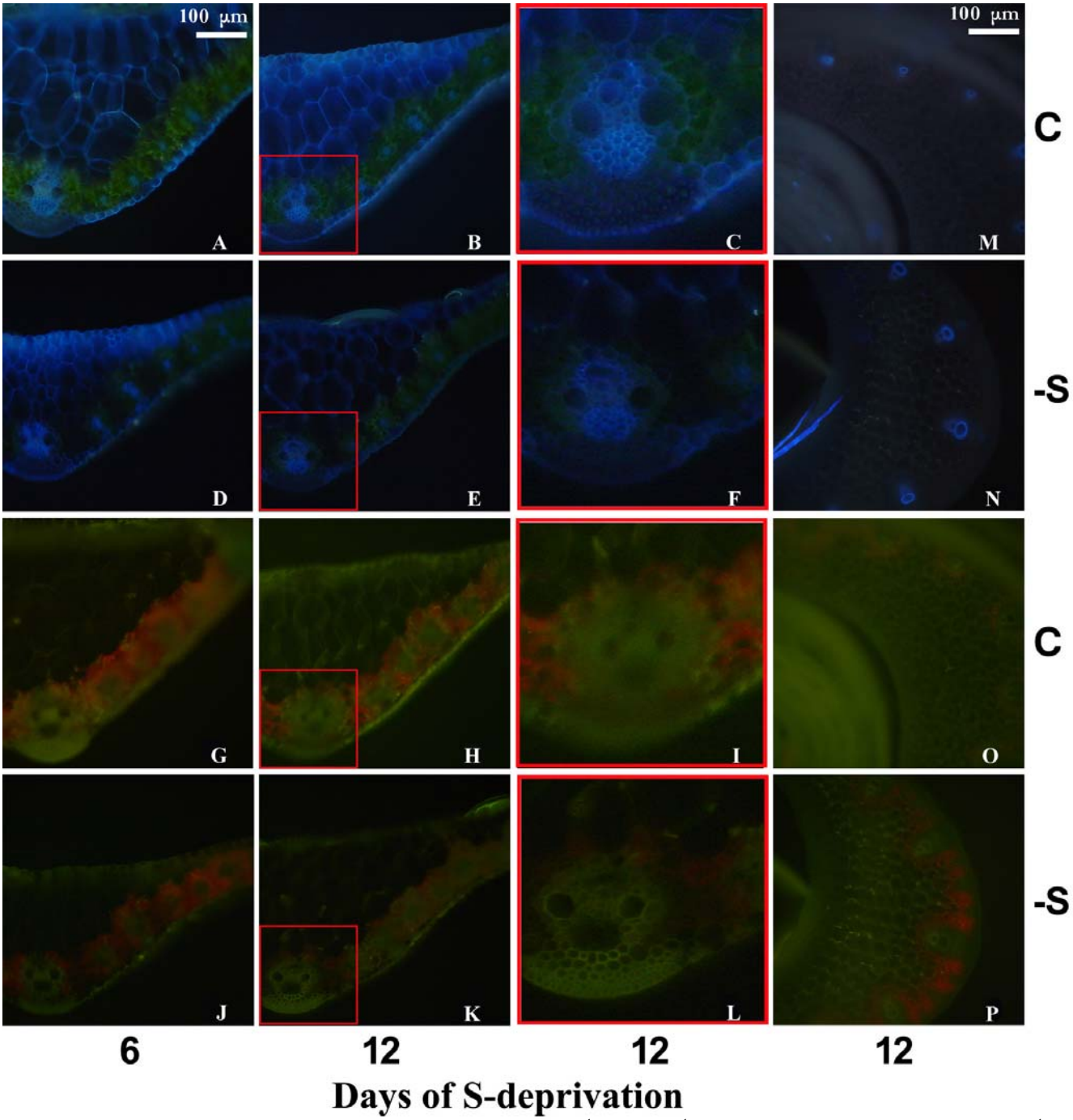

Fig. 2 Free hand transverse sections of control (C) and S-deprived (-S) maize 2nd (**A-L**) and 4th (**M-P**) leaves. (**A-F**)**:** Autofluorescence of the 2nd leaf cross sections as viewed in a fluorescence microscope (UV filter, excitation wavelength 365 nm) for controls, and 6 and 12 days after S-deprivation. Sections were viewed in distilled water. Photographs **C** and **F** are magnifications of **B** and **E**, respectively. (**G-L**)**:** Autofluorescence of the 2nd leaf cross sections as viewed in a fluorescence microscope (blue filter, excitation wavelength 450-490 nm) 6 and 12 days after S-deprivation. Sections were viewed in distilled water. Photographs **I** and **L** are magnifications of **H** and **K** respectively. (**M-N**)**:** Autofluorescence of the 4th leaf cross sections as viewed in a fluorescence microscope (UV filter, excitation wavelength 365 nm) 12 days after S-deprivation. Sections were viewed in distilled water. (**O-P**)**:** Autofluorescence of the 4th leaf cross sections as viewed in a fluorescence microscope (blue filter, excitation wavelength 450-490 nm) 12 days after S-deprivation.

cence. Almost all cells fluoresced intensely in –S leaves at d 6, except those of the midvein VB. Even BSC of the midvein VB fluoresced in this case. A similar situation but with less intense fluorescence, was observed at d 12 (**Fig. 3A-D**). The whole of leaf 4 of control plants fluoresced intensely at d 12, in contrast with leaf 4 of –S plants which showed negligible fluorescence (**Fig. 3E, 3F**).

Localization of superoxide anion

Presence of superoxide anion was observed in the BSC of the midvein VB of control leaf 2 at d 6 and 12, and was more intense at d 12. In –S leaves at d 12, superoxide anions were located in the walls of the cells which were between the VB, and the phenomenon was weaker compared to d 6 (**Fig. 4A-D**).

Treatment with *N*,*N*-diethyldithiocarbamate (DDC) resulted in a more intense presence of superoxide anion at all timepoints and treatments (**Fig. 4E-H**). Much more superoxide anion compared to that of the treatment without DDC appeared at d 6 in both control and –S leaves, however in –S leaves higher concentration of superoxide anion was observed. At d 12, both control and –S leaves seemed to produce the same amount of superoxide anion in the presence of DDC. In control leaves at d 6 and 12 the same amount of superoxide anion was produced in the presence of DDC,

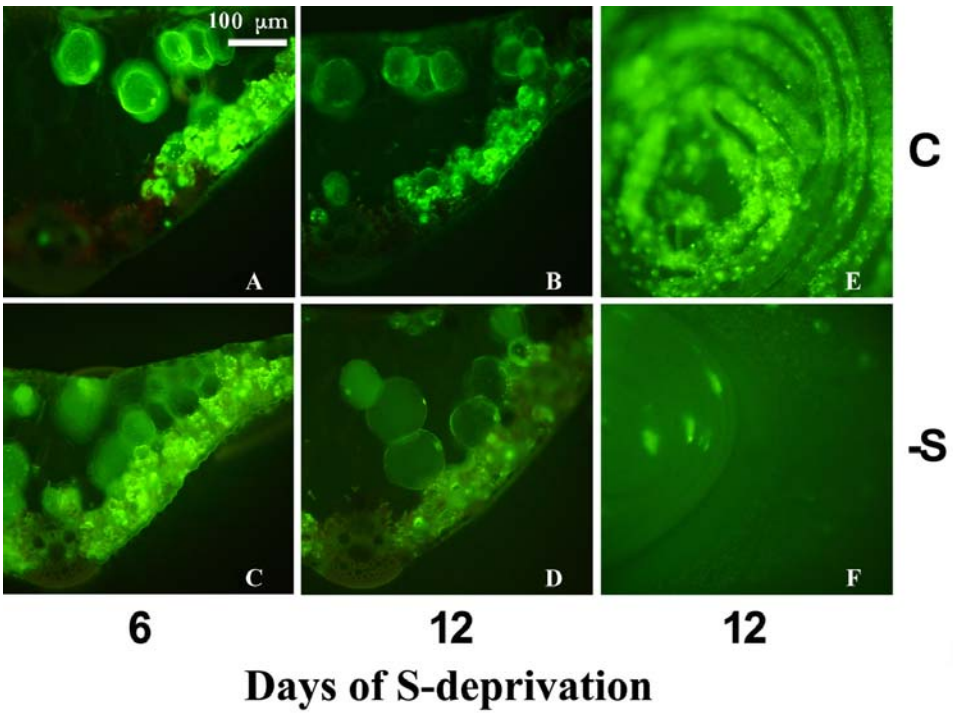

Fig. 3 Free hand transverse sections of control (C) and S-deprived (-S) maize 2nd (**A-D**) and 4th (**E-F**) leaves. Root sections treated with H_2-DCF-DA for the detection of reactive oxygen species (ROS), as viewed in the fluorescence microscope (excitation wavelength 450-490 nm). Presence of ROS is indicated by green fluorescence emission.

while in –S leaves more superoxide anion was produced at d 6 compared with d 12.

An intense presence of superoxide anion was observed in the control leaf 4, both in and around the VB, in the area where the midvein VB of leaf will develop (**Fig. 5A-D**) and intense presence of superoxide anion was also observed elsewhere, away from the midvein VB. In –S leaves, intense presence of superoxide anion was observed in the VB and the walls of the MC of the midvein VB. Intense presence of superoxide anion was observed in the presence of DDC at the same locations, as described for the treatment without DDC in both control and –S leaves (**Fig. 5E-H**). As a rule, –S leaves produced less superoxide anion compared with the control control leaves, with the exception of the walls of MC, where in –S leaves more superoxide anion was observed.

Localization of hydrogen peroxide

The presence of hydrogen peroxide decreased in the lamina of leaf 2 of both control and –S plants between the 6th to the 12th day. The appearance of hydrogen peroxide seems to be located mainly in the cell walls. On the other hand, there was a higher hydrogen peroxide concentration in control control leaves at d 12 compared with the –S ones (**Fig. 4I-L**). Undoubtly in ascorbate-treated cross sections, a much lower hydrogen peroxide concentration was observed as a result at all days (**Fig. 4M-P**). No presence of hydrogen peroxide was detected in leaf 4 of control plants. In contrast, the presence of hydrogen peroxide was observed in the walls of the MC of –S leaves, where the midvein VB will develop (**Fig. 5I-L**). No hydrogen peroxide was found in ascorbate-treated control and –S leaves (**Fig. 5M-P**).

DISCUSSION

Several classes of phenolic compounds (hydroxycinnamic acids, coumarins, stilbenes and styrylpyrones) are strongly autofluorescent when irradiated with UV or blue light (Ibrahim and Barron 1989; Hutzler *et al*. 1998). Ferulic acid and p-coumaric acid absorb at around 310 nm and emit at 415-455 nm, whereas lignin shows a maximum emission at 358 nm. Using a standard Zeiss filter combination (exciter filter at 365 nm and a longwave pass filter at 418 nm), ferulic acid and associated monomeric phenylpropanoids appear white-blue, whereas walls containing lignin appear dark purple-black. In the Poaceae family, ferulic acid is bound to all cell walls (Harris and Hartley 1976) and can be recognized early in the development by its characteristic fluorescence spectrum. Lignin on the other hand is deposited specifically in vascular tissue and in adjacent sclerenchyma cells later in development when cell growth has slowed. The presented data show that sulphate deprivation affects leaf anatomy. It appeared that the –S leaf by d 6 is at the age of a control leaf at d 12. The lamina of the fully expanded 2nd –S leaf presented more developed lower sclerenchyma and intense lignification mainly in the epidermal cells close to the lower sclerenchyma as well as in the VB. In the lamina of the expanding 4th –S leaf, there were more developed VB, larger with more xylem vessels compared with the control. These observations coincide with the assumption made by Astolfi *et al*. (2001) that S-starvation probably induces premature senescence within the leaves of maize plants. Under the blue filter, the walls of the cells of the midvein VB (except of the phloem), the cells of the lower sclerenchyma and the chloroplasts fluoresce intensely. In general, fluorescence intensity is reduced in –S leaves compared with the control. At d 12, chloroplasts of –S leaves fluoresce only slightly, while the cells of the VB (except of the phloem) and the lower sclerenchyma fluoresce intensely.

The presence of ROS may be an inductive signal or a stress, and the data indicate that sulphate deprivation affects ROS metabolism spatially and temporally. Moreover, it seems that sites of ROS presence do not coincide with sites of lignification. In particular, the appearance of ROS, and especially superoxide anions and hydrogen peroxide in control 2nd leaf, is located mainly in the mesophyll, while in –S 2nd leaf it is located in the parenchymatic sheath of the VB. This is not surprising, as ROS are found in the parenchymatic sheath only under stress (Noctor *et al*. 2002). The presence of superoxide anion in the 2nd leaf at d 6 is similar in both control and –S leaves in the absence of DDC. Addition of this inhibitor of SOD activity resulted in increased superoxide anion in –S leaves, indicating that plants might have increased SOD activity in –S leaves, in order to handle the increased production of superoxide anion. The result is that finally the superoxide anion concentration is the same in both control and –S leaves. Presence of superoxide anions was observed in the walls of the cells located between the VB of –S leaf 2. The presence of hydrogen peroxide decreased in the lamina of the 2nd leaf of both control and –S from d 6 to 12. There was higher hydrogen peroxide con-

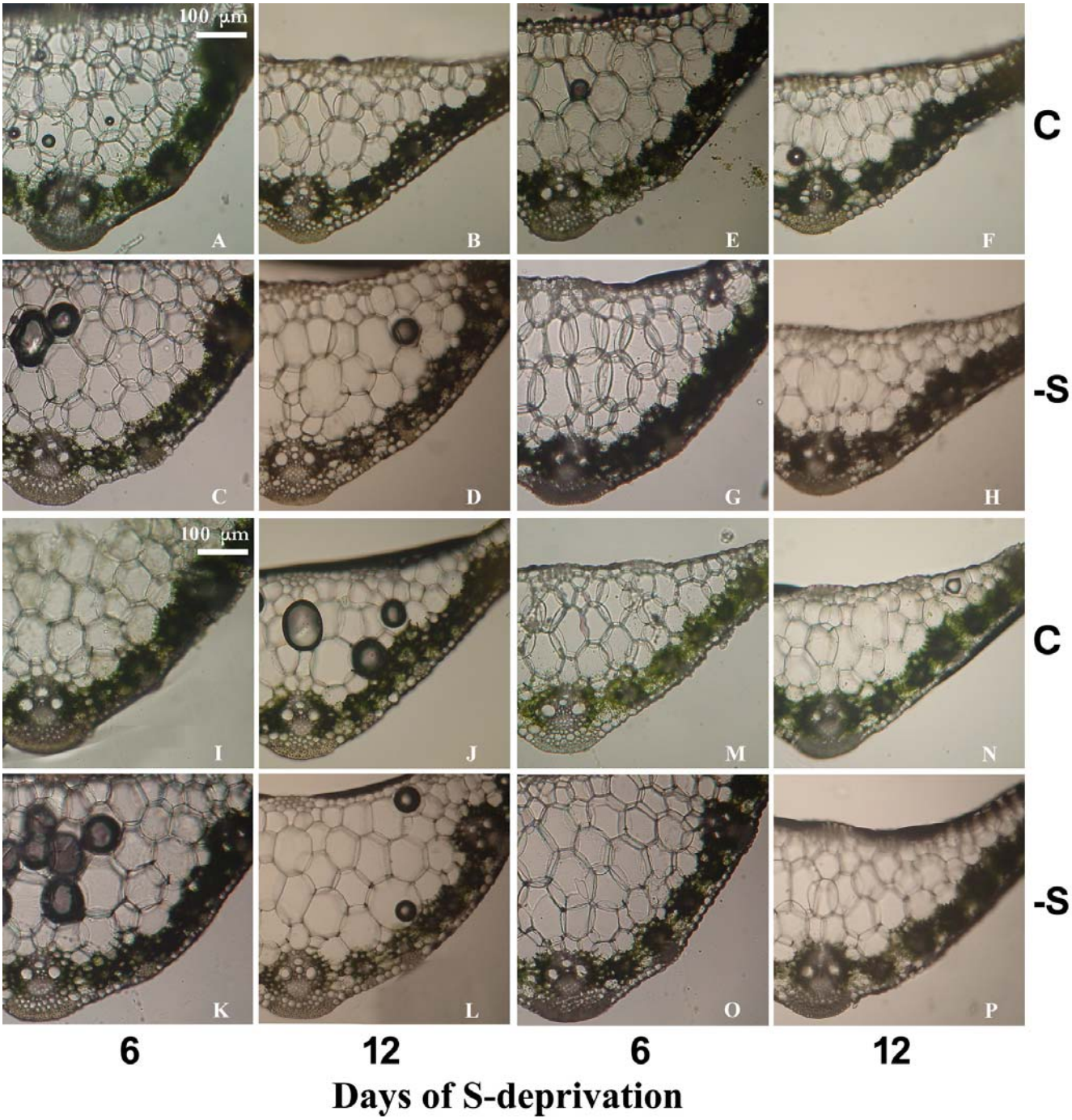

Fig. 4 Free hand transverse sections of control (C) and S-deprived (-S) maize 2nd leaves 6 and 12 days after S-deprivation for the detection of superoxide anion (**A-H**) and hydrogen peroxide (**I-P**). Sections were viewed in light microscope. For the detection of superoxide anions, cross sections were incubated in a NBT reaction medium (**A-D**), or pre-incubated in DDC and then incubated in the NBT reaction medium (**E-H**). The superoxide anion was detected by the formation of blue formazan. For the detection of hydrogen peroxide, cross sections were incubated in the KI/starch reagent (**I-L**), or pre-incubated in ascorbate (**M-P**) and then incubated in the KI/starch reagent. H_2O_2 production was monitored by observing the development of a dark stain.

centration in control control leaves at d 12.

In the –S leaf 4, less ROS and superoxide anion were observed in general compared with control and there was no superoxide anion in the VB. In control leaves intense presence of ROS and superoxide anion was observed in both the VB and in the surrounding area, where the midvein VB will develop. In contrast, in –S leaves particularly intense presence of superoxide anion was observed in the phloem and not around the VB. There was no appearance of hydrogen peroxide in control leaves, while presence of hydrogen peroxide was only observed in MC walls of –S leaves. In monocotyledonous species, leaf growth is restricted to the leaf base where cell division and expansion occur. Cell growth is controlled by water uptake and the rheological properties of the cell walls, and cell expansion requires first the loosening of cell walls, which thus can yield to the pressure exerted by the symplasm (Carpita and McCann 2000). Cell wall loosening has been regarded as a process mainly catalyzed by expansins, hydrolases such as endoglucanases, and xyloglucan endotransglycosylase (McQueen-Mason 1995; Cosgrove 1999). Several *in vitro* studies have shown that nonenzymatic processes involving ROS cause wall polysaccharide scission (Fry 1998; Schweikert *et al*. 2000). For this process to occur *in vivo*, ROS must be present in the apoplast, and apoplastic ROS accumulation has been shown in many plant tissues (Schopfer 1994). Rodriguez *et al*. (2002) studied the production and the role of ROS in elongation growth in the expanding zone of maize leaf blades. Their results indicated that ROS production, and extrusion, were high in the expanding region, and almost nil

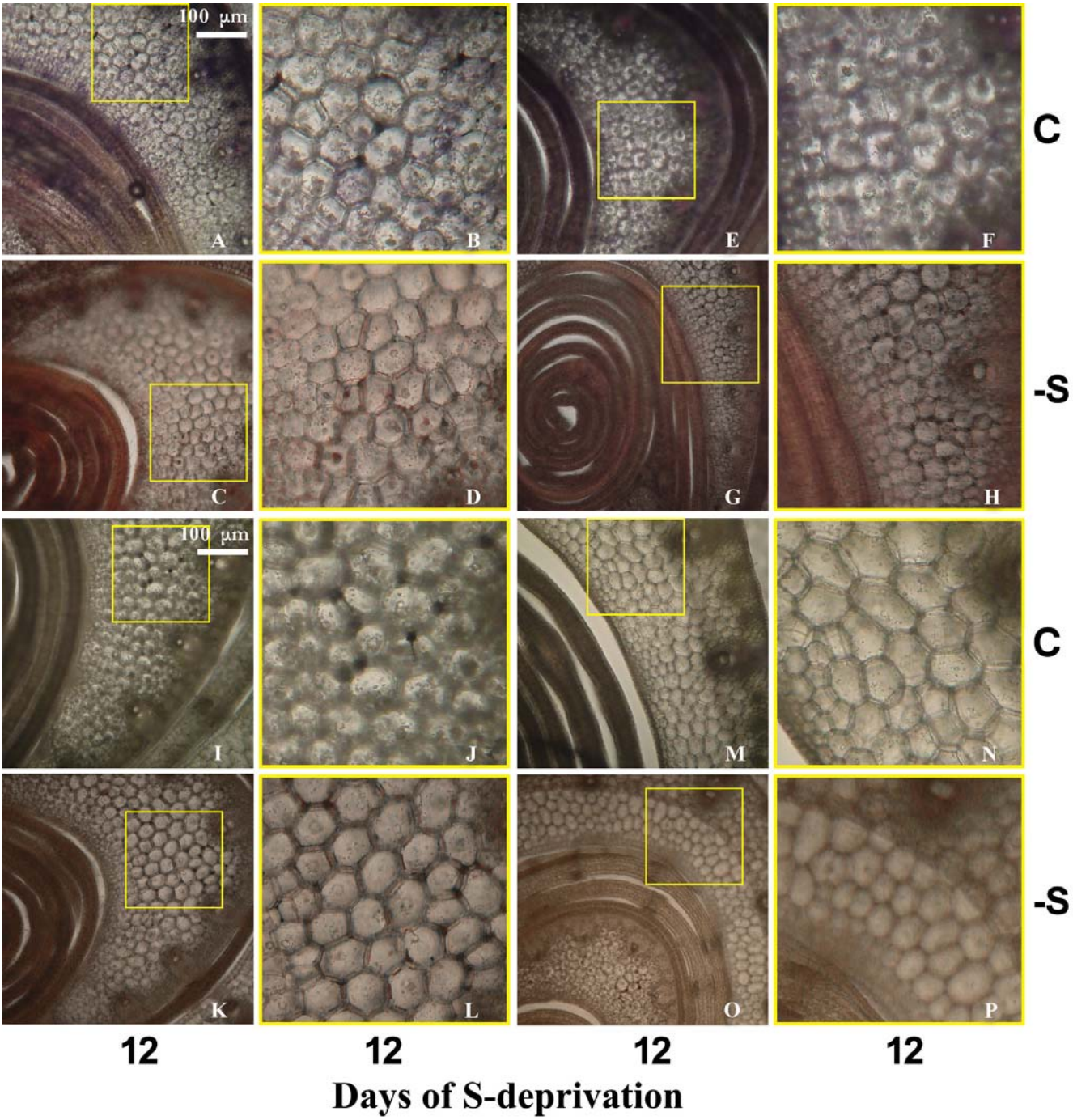

Fig. 5 Free hand transverse sections of control (C) and S-deprived (-S) maize 4th leaves 12 days after S-deprivation for the detection of superoxide anion (**A-H**) and hydrogen peroxide (**I-P**). Sections were viewed in light microscope. For the detection of superoxide anions, cross sections were incubated in a NBT reaction medium (**A-D**), or pre-incubated in DDC and then incubated in the NBT reaction medium (**E-H**). Photographs **B**, **D**, **F** and **H** are magnifications of **A**, **C**, **E** and **G**, respectively. The superoxide anion was detected by the formation of blue formazan. For the detection of hydrogen peroxide, cross sections were incubated in the KI/starch reagent (**I-L**), or pre-incubated in ascorbate (**M-P**) and then incubated in the KI/starch reagent. Photographs **J**, **L**, **N** and **P** are magnifications of **I**, **K**, **M** and **O,** respectively. H_2O_2 production was monitored by observing the development of a dark stain.

in the expanded one. Observations of electron micrographs showed ROS in the apoplast of the leaf expansion zone. In the lamina of control leaf 4, intense presence of ROS was observed at d 12, in contrast with –S leaves, where there was no fluorescence. This is in accordance with the findings of Rodriguez *et al.* (2002), suggesting that in the control leaves ROS contribute to the normal development of the young leaf, while in –S leaves, decreased ROS resulted in the reduced leaf growth rate, which has been described as a symptom of sulphate deficiency in maize leaves (Bouranis *et al*. 2003).

ACKNOWLEDGEMENTS

Rothamsted Research receives grant-aided support from the Biotechnology and Biological Science Research Council (BBSRC) of the UK.

REFERENCES

Astolfi S, De Biasi MG, Passera C (2001) Effects of irradiance-sulphur interactions on enzymes of carbon, nitrogen, and sulphur metabolism in maize plants. *Photosynthetica* **39 (2)**, 177-181

Blake-Kalff MMA, Harrison KR, Hawkesford MJ, Zhao FJ, McGrath SP (1998) Allocation of sulfur within oilseed rape (*Brassica napus* L.) leaves in response to sulfur-deficiency. *Plant Physiology* **118**, 1337-1344

Bouranis DL, Chorianopoulou SN, Protonotarios VE, Siyiannis VF, Hop-

kins L, Hawkesford MJ (2003) Leaf responses of young iron-inefficient maize plants to sulfur deprivation. *Journal of Plant Nutrition* **26 (6)**, 1189-1202

Burgener M, Suter M, Jones S, Brunold C (1998) Cyst(e)ine is the transport metabolite of assimilated sulfur from bundle-sheath to mesophyll cells in maize leaves. *Plant Physiology* **116**, 1315-1322

Carpita N, McCann M (2000) The cell wall. In: Buchanan BB, Gruissem W, Jones RL (Eds) *Biochemistry and Molecular Biology of Plants*, American Society of Plant Physiologists, Rockville, MD, pp 52-108

Cosgrove DJ (1999) Enzymes and other agents that enhance cell wall extensibility. *Annual Review of Plant Physiology and Molecular Plant Biology* **50**, 391-417

Doulis AG, Debian N, Kingston-Smith A, Foyer CH (1997) Differential localization of antioxidants in maize leaves. *Plant Physiology* **114**, 1031-1037

Foyer CH, Lelandais M, Kunert KJ (1994) Photooxidative stress in plants. *Physiologia Plantarum* **92**, 696-717

Fry S (1998) Oxidative scission in plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochemical Journal* **332**, 507-515

Furbank RT, Taylor WC (1995) Regulation of photosynthesis in C3 and C4 plants: a molecular approach. *The Plant Cell* **7**, 797-807

Grossman A, Takahashi H (2001) Macronutrient utilization by photosynthetic eukaryotes and the fabric of interactions. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 163-210

Harris P, Hartley R (1976) Detection of bound ferulic acid in cell walls of the Gramineae by ultraviolet fluorescence microscopy. *Nature* **258**, 508-510

Hutzler P, Fischbach R, Heller W, Jungblut TP, Reuber S, Schmitz R, Veit M, Weissenbock G, Schnitzler JP (1998) Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. *Journal of Experimental Botany* **49**, 953-965

Ibrahim RK, Barron D (1989) Phenylpropanoids. In: Dey PM, Harborne JB (Eds) *Methods in Plant Biochemistry* (Vol 1) *Plant Phenolics*, Academic Press, New York, pp 197-235

Kopriva S (2006) Regulation of sulfate assimilation in *Arabidopsis* and beyond. *Annals of Botany* **97**, 479-495

Kopriva S, Koprivova A (2005) Sulfate assimilation and glutathione synthesis in C_4 plants. *Photosynthesis Research* **86**, 363-372

Leustek T, Saito K (1999) Sulfate transport and assimilation in plants. *Plant Physiology* **120**, 637-643

Leustek T, Martin MN, Bick J, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular genetic studies. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**, 141-166

Marschner H (1995) *Mineral Nutrition of Higher Plants* (2^{nd} Edn), Academic Press, London, 889 pp

Maruyama-Nakashita A, Inoue E, Watanabe-Takahashi A, Yamaya T, Takahashi H (2003) Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways. *Plant Physiology* **132**, 597-605

Maxwell D, Wang Y, McIntosh L (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proceedings of the National Academy of Sciences USA* **96**, 8271-8276

McQueen-Mason SJ (1995) Expansins and cell wall expansion. *Journal of Experimental Botany* **46**, 1639-1650

Noctor G, Gomez L, Vanacker H, Foyer C (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. *Journal of Experimental Botany* **53**, 1283-1304

Prasad TK (1996) Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: changes in antioxidant system, oxidation of proteins and lipids, and protease activities. *The Plant Journal* **10**, 1017-1026

Rodriguez A, Grunberg K, Taleisnik E (2002) Reactive oxygen species in the elongation zone of maize leaves are necessary for leaf extension. *Plant Physiology* **129**, 1627-1632

Ros Barceló A (1998) The generation of H_2O_2 in the xylem of *Zinnia elegans* is mediated by an NADPH-oxidase-like enzyme. *Planta* **207**, 207-216

Schopfer P (1994) Histochemical demonstration and localization of H_2O_2 in organs of higher plants by tissue printing on nitrocellulose paper. *Plant Physiology* **104**, 1269-1275

Schweikert C, Liszkay A, Schopfer P (2000) Scission of polysaccharides and peroxidase-generated hydroxyl radicals. *Phytochemistry* **53**, 565-570

Wise RR (1995) Chilling-enhanced photooxidation. The production, action and study of reactive oxygen species produced during chilling in the light. *Photosynthesis Research* **45**, 79-97