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Cell death behind invisible symptoms: early diagnosis of ozone injury

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Abstract

A simple histo-cytochemical method, combining Evans blue staining to assess cell death and *in vivo* 3,3'-diamino-benzidine uptake for H_2O_2 localisation, has been used to evaluate O_3 damages in leaf tissues of three *Phaseolus vulgaris* L. cultivars (Cannellino, BLF, Saxa) with different sensitivity to the pollutant. Bean plants were exposed to a single pulse of O_3 (150 ± 10 mm^3 m^{-3} $\times$ 3 h) and leaves were examined at different time-span after fumigation. Cannellino proved to be the most sensitive, showing chlorotic spots 2 h after fumigation and chlorotic lesions 24 h later. In BLF, necrotic spots appeared 4 h after fumigation and reddish necrotic lesions (bronzing) developed in further 24 h. Saxa remained symptomless up to 10 d of observation, thus appearing tolerant. The early appearance of symptoms in Cannellino correlated with H_2O_2 accumulation in leaf tissues and consequent extensive cell death, involving both palisade and spongy mesophyll. H_2O_2 accumulation was observed also in BLF, though to a lesser extent and dead cells were rare at 2 h after fumigation. However, they increased in number 24 h later, forming small groups in the palisade mesophyll. These groups further enlarged in the next 24 h, again involving only palisade mesophyll. In Saxa leaves, H_2O_2 accumulation was found only in the epidermal cells, though the number of dead cells was very similar to BLF, at least up to 24 h after fumigation. However, in Saxa, dead cells have been always found singly scattered through the palisade mesophyll, or forming very small groups around substomatal cavity, thus remaining invisible at a macroscopic level.

Additional key words: air pollution, hydrogen peroxide, *Phaseolus vulgaris*.

Introduction

Ozone (O_3) is one of the major phytotoxic pollutants both in urban and rural areas. In the lower atmospheric stratum (troposphere), it is formed by the interaction between the photochemical smog (composed by hydrocarbons, nitrogen, sulphur and carbon oxides) and the UV radiations (Kley *et al.* 1999).

O_3 enters the plant through stomata and, within the mesophyll, it induces both chronic and acute effects, depending on dose (Bergmann *et al.* 1999). Once in the apoplast, O_3 causes ozonolysis of alkenes, forming aldehydes and peroxides (Pryor and Church 1991). Among these, hydrogen peroxide (H_2O_2) can react with transition metals (Cu or Fe), according to Fenton or Haber-Weiss reactions, to form reactive oxygen species (ROS) (Byvoet *et al.* 1995). Plant cell scavenging systems, both enzymatic and non enzymatic, balance the ROS concentration, preserve the cellular redox homeostasis and represent the base of differences in pollutant tolerance among different species and cultivars (Asada 1992, Scandalios 1993, Creissen *et al.* 1994, Bernardi *et al.* 2004).

Long-term O_3 exposure at lower than critical concentrations, or chronic exposure (<100 ng g^{-1} for days to months), affects plant growth and productivity, inducing changes in biochemical and physiological processes, very often in absence of any visible symptom (Heath and Taylor 1997, Ambasht and Agrawal 2003). Cell membranes and enzymes are primary targets of this pollutant that, at first, inhibits Rubisco and alters tylakoid organization within the chloroplast (Heath 1987, Violini *et al.* 1992, Pell *et al.* 1997). In the case of acute exposure (>150 ng g^{-1} for 4 - 6 h) (Bergmann *et al.* 1999), macroscopic O_3 symptoms appear shortly after ozone exposure (from hours to a few days) and, in bean and

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cotton plants, may range from aspecific chlorosis to characteristic red necrotic lesions (bronzing) on the adaxial leaf surface.

Since many decades, it has been searching for biochemical and physiological changes that would allow a rapid and early detection of O_3 injury, particularly in the absence of visible symptoms (Saxe 1996). At this regard, the most reliable tool has proved to be chlorophyll fluorescence detection (Guidi *et al.* 1997, 2000). However, besides requiring specific equipments and a refined interpretation of measured data, this method implies a comparison with O_3-damaged and undamaged control leaves, which is not easy to be achieved in open field, especially in case of invisible symptoms (Bolhàr-Nordenkampf *et al.* 1989). Furthermore, in a comparative study on several bean cultivars, showing different sensitivity to O_3, authors found difficulties in studying the correlation between the appearance of visible symptoms and Chl fluorescence parameters (Guidi *et al.* 2000).

In this study, we have tested a simple histo-cytochemical method, combining Evans blue staining to assess cell death and *in vivo* 3,3'-diaminobenzidine (DAB) uptake for H_2O_2 localisation (Thordal-Christensen *et al.*, 1997), to evaluate O_3 damages in leaf tissues, early before the appearance of macroscopic symptoms. This method has been tested on *Phaseolus vulgaris* L. plants that respond very differently to ozone stress, depending on the different cultivar, ranging from high tolerance to extreme sensitivity (Tonneijck 1983). Furthermore, to better evaluate the reliability of the method itself, we used some of the bean cultivars previously screened for their response to ozone by leaf chlorophyll fluorescence (Guidi *et al.* 2000).

Materials and methods

Plant culture: *Phaseolus vulgaris* L. plants, cv. Borlotto Nano Lingua di Fuoco (BLF), Cannellino, and Saxa, being differently sensitive to ozone, as previously assessed by other authors (Stan and Schicker 1982, Guidi *et al.* 2000), were used in this study. Plants were sown in 12 cm pots in soil amended with 30 % of a blend of white and frozen through black sphagnum peat, at T 24 ± 2 °C, RH 60 ± 5 %, 16/8 h light/dark period. Ten to 12 d after seeding, when the primary leaves were almost completely expanded, plants were transferred to the growth chambers for fumigation experiments.

Ozone fumigation: Ozone fumigation was performed in air conditioned chambers (~0.50 m^3), with charcoal-filtered air, temperature 26 ± 1 °C, relative humidity 80 ± 5 %, photon fluence rate 250 μmol m^{-2} s^{-1}. Ozone was generated by electric discharge, passing pure oxygen through a *Fischer* ozone generator 500 MM (*Fischer Labor und Verfahrenstechnik GmbH*, Meckenheim, Germany). Ozone concentration, in the fumigation chambers, was continuously monitored with a *Photometric O_3 Analyzer* (model *400*; *Advanced Pollution Instrumentation, Inc.*, San Diego, USA), operating on the principle of UV absorption and interfaced with a personal computer. Control plants were kept in charcoal-filtered air chambers under the same conditions. Plants from greenhouse were pre-adapted to the chamber conditions for 48 h, and half of them (10 plants for each cultivar) were contemporarily exposed to 150 ± 10·mm^3 m^{-3} of ozone for 3 h which is considered an urban-like condition (Heath 1994), while the untreated ones were used as a control.

Symptom evaluation: Leaves were examined at 0, 2, 24 h, then daily up to 10 d after fumigation, and visual symptoms recorded according to Gumpertz *et al.* (1982).

Histo-cytochemistry: At 2, 24, 48 and 72 h after fumigation, 60 leaf discs (15 mm in diameter) were randomly punched with a cork-bore from primary leaf of 10 plants per bean cultivar, avoiding main veins. Half of the discs were infiltrated overnight with 1 mg dm^{-3} DAB-HCl, to detect H_2O_2 accumulation sites *in vivo*, according to Thordal-Christensen *et al.* (1997), with some modification (Faoro *et al.* 2001). After DAB uptake, discs were cleared in 96 % boiling ethanol and examined with a light microscope. H_2O_2 was visualized as a reddish-brown coloration. As negative control, DAB solution was supplemented with 10 mM ascorbate.

In order to asses cell death, Evans blue staining was carried out by boiling leaf discs for 1 min in a mixture of phenol, lactic acid, glycerol and distilled water containing 20 mg dm^{-3} Evans blue (1:1:1:1), prepared immediately before use. Tissues were then clarified overnight in a solution of 2.5 g dm^{-3} chloral hydrate in water (Keogh *et al.* 1980). Dead cells stained from dark to light blue, depending on the stage of cell membrane degradation (Keogh *et al.* 1980), while the undamaged ones appeared unstained. Alternatively, Evans blue was replaced with Trypan blue (10 mg dm^{-3}), using the same procedure as above and obtaining the same results. All samples were examined with an *Olympus BX50* light microscope (*Olympus*, Tokyo, Japan), equipped with differential interference contrast (DIC) and epi-polarization filters.

Cell death assessment: To quantify the number of dead cell, a micrograph was taken from each disc at 40 magnifications, using a *Pixera 120es* high-resolution digital camera (*Pixera Co.*, Los Gatos, CA, USA). Images were enlarged to a final 150×, thus allowing the observation of 1 cm^2 of leaf tissue, containing about 4500 - 5000 palisade mesophyll cells.

Micrographs (200 pixels per inch) were processed

with *Adobe Photoshop 6.0* selecting, in the "color range" option, the maximum value of blue, and adjusting fuzziness to 100 for each image. In this way, all dead cells were highlighted, including those only partially permeable to the stain (light blue) that can be considered irreversibly damaged (Keogh *et al.* 1980). From the image histogram it was possible to determine the corresponding number of blue pixels. The percentage of blue pixels in respect to the total image pixels was used to evaluate the number of dead cells and has been indicated as "cell death index". To avoid highlighting blue pixels of heavily stained veins, these were erased from the image before counting.

Presentation of results: Each experiment was repeated at least three times. As the repeated experiments showed similar tendencies, the results of one representative experiment are presented in the case of symptom evaluation and H_2O_2 localization. The statistical variation of the results regarding the number of dead cells is specified in the figure legend.

Results

Symptoms: Exposure to O_3 (150 mm^3 m^{-3} for 3 h) caused the appearance of visible symptoms only in the cultivars Cannellino and BLF, but not in Saxa (Fig. 1*a-f*). However, 2 h after the end of fumigation, only Cannellino showed early symptoms, consisting of small interveinal chlorotic spots, less than 1 mm diameter (Fig. 1*b*), which enlarged and flowed together to form chlorotic lesions in the next 24 h (Fig. 1*c*). These started to necrotize in further 24 - 48 h. The first symptoms in BLF appeared about 24 h after treatment as small interveinal necrotic spots, less than 1 mm diameter, surrounded by a chlorotic halo (Fig. 1*e*) that became reddish necrotic lesions (bronzing) in further 24 h (Fig. 1*f*). No apparent symptoms were observed in Saxa plants, up to 10 d after fumigation.

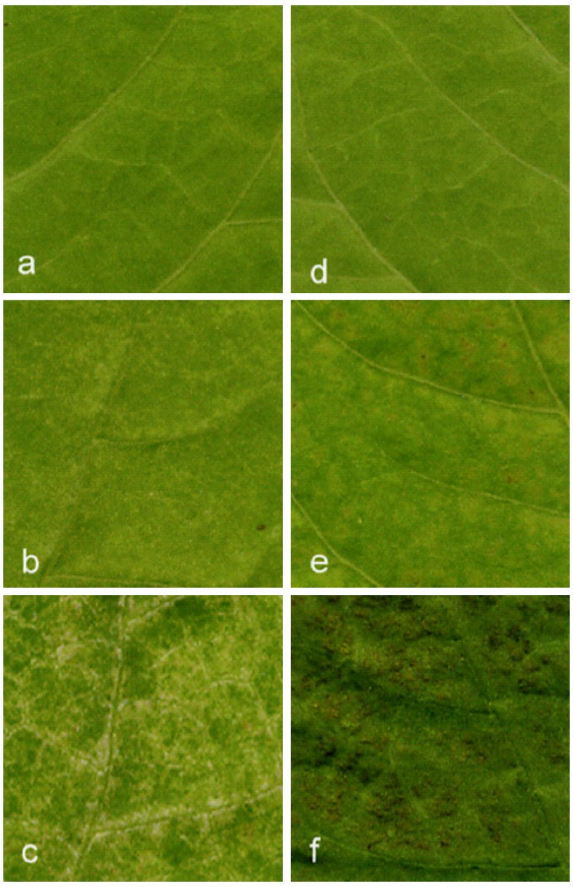

Fig. 1. Visible symptoms on leaves induced by a single pulse of O_3 (150 ± 10 mm^3 m^{-3} for 3 h) on *Phaseolus vulgaris* L., cvs. Cannellino (*a*, *b*, *c*) and BLF (*d*, *e*, *f*); *a*, *d* - leaves from non-fumigated control plants. Cannellino leaves have been photographed at 2 h (*b*) and 24 h (*c*) after fumigation, while BLF at 24 h (*e*) and 48 h (*f*).

H_2O_2 accumulation: DAB staining, for the localisation of H_2O_2 accumulation sites, was performed at 2 and 24 h after fumigation, with similar results as regards the distribution and localisation pattern of DAB precipitates in leaf tissues of all tested cultivars. However, in 24 h samples staining was generally more intense, thus all micrographs are referred to this time-span (Fig. 2*a-g*). DAB precipitates were heavier in Cannellino leaves, usually in small groups of cells randomly scattered in the interveinal areas, with a preferential localisation near 2nd and 3rd order veins (Fig. 2*b*). Focusing through the leaf tissues, it was possible to observe that DAB staining was localised, besides in the epidermal and palisade cells, even in the spongy mesophyll (Fig. 2*e*), thus accounting for the intensity of the staining itself when observing specimens at low magnifications. At the same low magnifications, DAB precipitates in BLF leaves were lighter and, apparently, involved a lower number of cell groups (Fig. 2*c*). However, at higher magnifications, the wall of a few epidermal cells, usually close to stomata, appeared intensely stained, as well as some of the palisade cells underneath (Fig. 2*f*). No H_2O_2 accumulation, in spongy mesophyll cells, was observed. In Saxa leaves (Fig. 2*d*), only epidermal cells, particularly those adjacent to stomata, showed, sometimes, localised H_2O_2 deposits (Fig. 2*g*).

In all cases, when ascorbate was added to the infiltration medium, DAB staining was almost completely reduced (not shown), indicating that DAB staining was actually due to local differences in H_2O_2

accumulation and not to peroxidase activity (Schraudner *et al.* 1998, Iriti and Faoro 2003).

Cell death: At 2 h after fumigation, the percentage of dead cells in cv. Cannellino was significant and consistent with the contemporaneous appearance of macroscopic symptoms. In contrast, the presence of dead cells was almost irrelevant in the other cultivars (Table 1). Furthermore, in Cannellino leaf tissues, dead cells were present both in palisade and spongy mesophyll (Figs. 3*b-d*), while in BLF (Figs. 3*e-h*) and Saxa (Figs. 3*i-n*), only palisade cells usually appeared damaged.

At 24 h after O_3 fumigation, large groups of dead cells were visible in the intervenal regions, often close to 3rd order veins, of the cv. Cannellino (Fig. 3*c*), while in BLF leaves only smaller groups of dead cells were detectable (Fig. 3*f*), and not necessarily adjacent to the veins. At the same time-span, Saxa leaves showed a similar percentage of dead cells (Table 1). However,

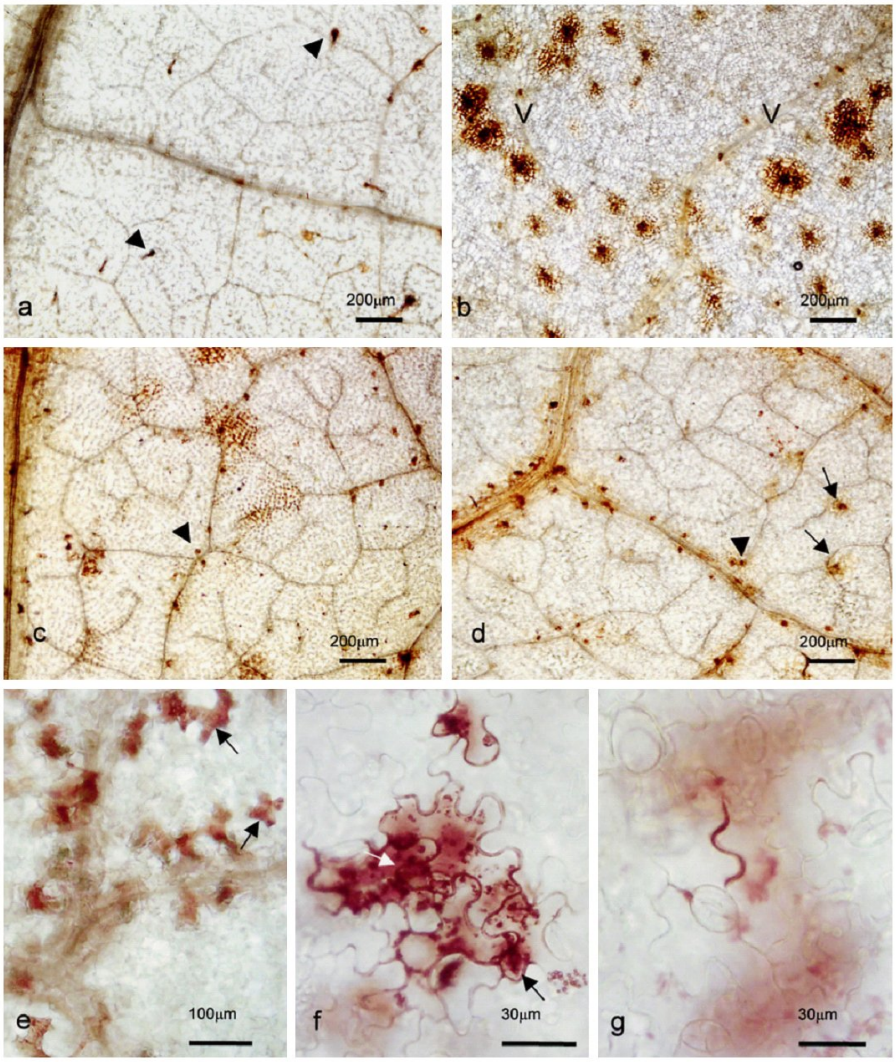

Fig. 2. Localisation of H_2O_2 in bean leaves, 24 h after fumigation, with the DAB uptake method: H_2O_2 deposits are visible as a dark brown staining of polymerised DAB; *arrowheads*, when present, point at epidermal hairs in which H_2O_2 is normally produced during sample manipulation. *a* - Non-fumigated Cannellino. *b* - Fumigated Cannellino showing numerous groups of densely stained cells: the most stained groups are mainly close to the veins (V). *c* - Fumigated BLF: stained cell groups are fewer and the staining is less intense. *d* - Fumigated Saxa: rare and small groups of cells (*arrows*) are stained. *e* - Enlargement of Fig. 2*b* showing that H_2O_2 accumulation occurs also in spongy mesophyll cells (*arrows*), particularly those adjacent to veins. *f* - Enlargement of Fig. 2*c*: DAB staining is localised in the wall of epidermal cells close to stomata (*arrows*); the intense staining observed in some of these cells is actually due to H_2O_2 accumulation in the palisade cells underneath. *g* - Enlargement of Fig. 1*d* showing that staining is restricted to portions of cell wall close to stomata.

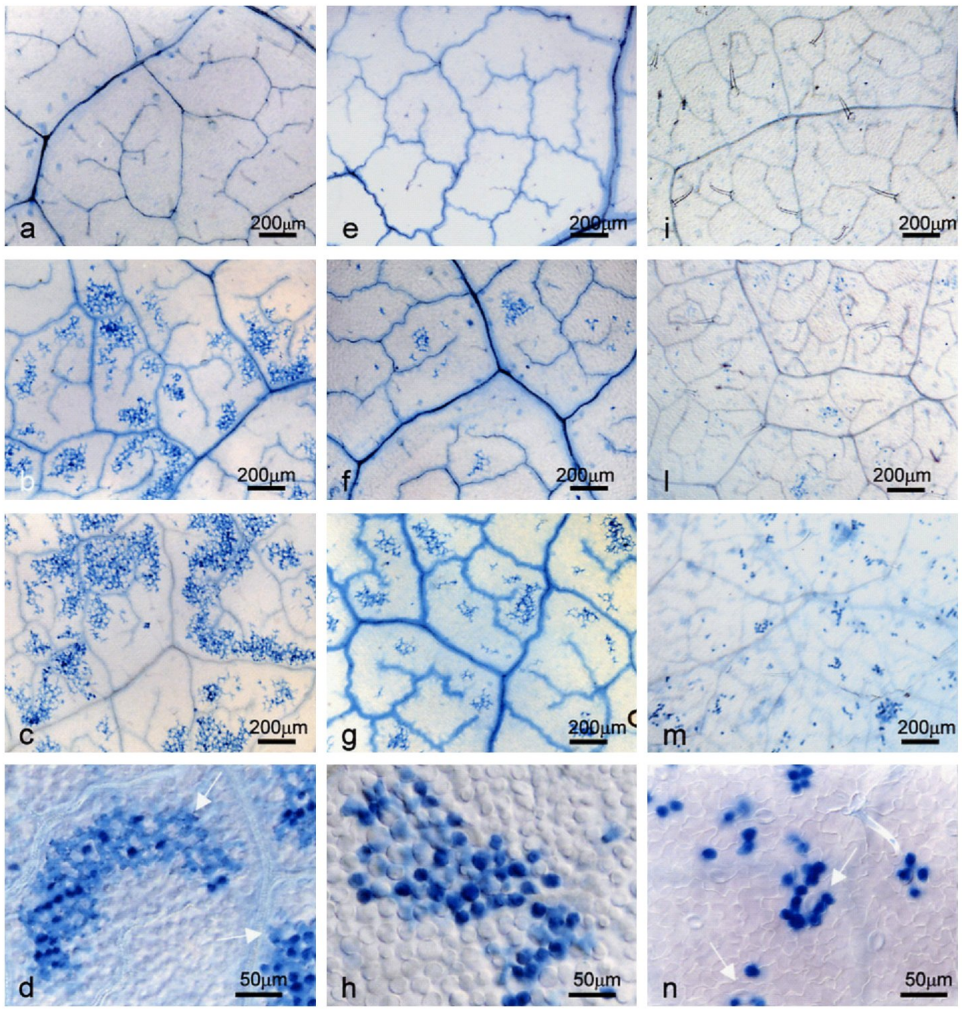

Fig. 3. Localisation of dead cells with Evans blue staining after fumigation with O_3 (150 ± 10 mm^3 m^{-3} for 3 h); the intensity of staining indicates different degrees of cell membrane degradation and/or different stages of cell death. (*a-d*) Cannellino leaves, either non-fumigated (*a*), or at 2 h (*b*), 24 h (*c*) and 48 h (*d*) after fumigation; note that numerous dead cells are already present at 2 h (*b*), preferably close to 2nd and 3rd order veins; also note that numerous spongy mesophyll cells (Fig. 3*d*, *arrows*) have died or are going to die. *e-h* - BLF leaves either non-fumigated (*e*) or at 24 h (*f*) and 48 h (*g*, *h*) after fumigation; small groups of dead cells are visible in the interveinal regions at 24 h (*f*); these groups increase in size and number at 48 h (*g*) and are entirely formed by palisade cells, as shown by the enlargement in Fig. 3*h*. *i-n* - Saxa leaves, either non-fumigated (*i*), or at 24 h (*l*) and 48 h (*m*, *n*) after fumigation: dead cells are singly scattered through the parenchyma tissues leaf and rarely they form small aggregates (*m*); either singly scattered or groups of dead palisade cells, are localised nearby the substomatal cavities (*arrows*).

Table 1. Cell death index, at different time after fumigation. Values are representative of dead cell percentage. Significant differences, following Duncan's test, are indicated by different letters ($P < 0.05$).

Cultivars	2 h	24 h	48 h
Cannellino	4.15^{c}	15.86^{d}	18.12^{d}
BLF	0.12^{a}	0.48^{ab}	2.48^{c}
Saxa	0.09^{a}	$0.42^{a\,b}$	1.13^{ab}

these were singly scattered through the tissues and rarely grouped to form small aggregates (Fig. 3*l*). Approximately 48 h after fumigation, the number of dead cells further increased in all the cultivars, in both palisade and mesophyll tissues of Cannellino leaves (Fig. 3*d*), and only in the palisade tissue of BLF (Fig. 3*g*) and Saxa (Fig. 3*m*). Again, the distribution pattern of dead cells was different in each cultivar. In particular, Cannellino leaves showed large aggregates of dead cells adjacent to the veins (Fig. 3*d*), BLF smaller aggregates in the interveinal regions and Saxa singly scattered cells, or

very small cell groups, usually around the substomatal chamber (Fig. 3*n*). No further increase in the number of dead cells was observed at 72 h after fumigation (data not shown). The correlation between the intensity and localization of H_2O_2 deposits at 24 h from fumigation and the percentage of dead cells was observed 48 h from fumigation (Table 2).

Table 2. Correlation between H_2O_2 localisation, at 24 h after fumigation, and cell death index 24 h later (48 h from fumigation). DAB staining: + - restricted to cell wall; ++ - involving the whole cell and up to 10 mesophyll cells per each stained group; +++ - involving more than 10 mesophyll cells per each stained group. Evans blue: + - 1 - 2 %; ++ - 3 - 5 %; +++ - over 5 % of dead cells evaluated as percentage of blue pixels (cell death index).

Cultivars	epidermis		palisade mesophyll		spongy mesophyll	
	H_2O_2 at 24 h	cell death at 48 h	H_2O_2 at 24 h	cell death at 48 h	H_2O_2 at 24 h	cell death at 48 h
Cannellino	+	-	+++	+++	+++	+++
BLF	+	-	+++	++	+	-
Saxa	+	-	+	+	-	-

Discussion

O_3 fumigation experiments confirmed that Cannellino is the most sensitive among the three bean cultivars tested, showing visible symptoms a few hours after exposure (Guidi *et al.* 2000). BLF appeared sensitive as well, but symptom appearance was delayed by 24 h after fumigation, while Saxa remained symptomless during the entire observation period. The latter can be, then, considered tolerant to O_3, at least under these experimental conditions.

Histo-cytochemical studies, carried out soon after fumigation, allowed to predict symptoms appearance and severity, and, consequently, O_3 sensitivity of the different cultivars. In fact, the large H_2O_2 deposits in both palisade and spongy mesophyll, combined with the discrete number of dead cells, already present in Cannellino tissues, indicated that the faint chlorotic spots, just starting to become visible, were possibly evolving in larger chlorotic lesions, involving both the adaxial and abaxial leaf surface, as it actually happened in the following 24 h. On the other hand, the localised heavy H_2O_2 deposits, scattered through BLF leaves at the end of fumigation, though in absence of a significant number of dead cell, allowed to predict that the cell groups showing H_2O_2 accumulation were destined to death, with the consequent appearance of visible symptoms later on. Again, the observation, in BLF leaves, of groups of dead cells restricted to the palisade tissue, explains the formation of necrotic spots, and the subsequent reddish necrotic lesions on the adaxial leaf surface. Interestingly enough, in the symptomless Saxa leaves, the number of dead cells, at 24 h after fumigation, was very similar to that found in BLF leaves with necrotic spots. This discrepancy could be due to the different distribution pattern of dead cells in the tissues, being those of BLF grouped in discrete aggregates of about 20-100 cells, thus visible at naked eyes. On the contrary, the singly scattered distribution of dead cells in Saxa leaves, accounts for their visibility only at microscopic level. In this view, the lack of relationships between percentage of dead cells and leaf area with visible ozone injury, previously found in five out of eight examined dicotyledonous plant species (Evans *et al.* 1996), could be attributed to the distribution pattern of dead cells in the leaf mesophyll. Unfortunately, no data are available at this regard, being that study carried out on sections of embedded material with the observation of a limited number of cells.

About cell death assessment, it was evaluated by the percentage of blue pixels out of the total image pixels, including those of veins and intercellular spaces. Thus, the reported values (Table 1) represent the extent of cell death in the different bean cultivars, and are referred as cell death index, though they are not the actual number of dead cells. In fact, by visual examination of 10 randomly selected micrographs at 150 magnifications per each cultivar, the real percentage of dead cells had been estimated to be 2 - 2.5 times the values reported in Table 1. However, as this correlation did not vary among cultivars, blue pixel values could be used to compare cell death extent in the different thesis with the advantage of being more objective and rapid than in direct cell counting.

A correlation between H_2O_2 accumulation and cell death, with consequent formation of necrotic lesions, was also found in Bel W3 O_3 sensitive tobacco cultivar, but not in Bel B tolerant one (Schraudner *et al.* 1998). In that case, authors provided evidences that Bel W3 sensitivity is due to a biphasic oxidative burst, similar to that induced during incompatible plant-pathogen interactions, and leading to localized accumulation of reactive oxygen species (Morel and Dangl 1997). The second burst, observed in Bel W3, 8 h after fumigation, would trigger programmed cell death and be then responsible for lesion formation (Schraudner *et al.* 1998). However, in our case,

cell death was observed also in tolerant Saxa, suggesting a different response mechanism, possibly similar to the "microburst" generated by a primary localised oxidative burst described in *Arabidopsis* leaves inoculated with *Pseudomonas syringae* (Alvarez *et al.* 1998).

The physiological significance of the different distribution pattern of dead cells in Cannellino, BLF and Saxa beans could be due to both anatomical and biochemical factors. Among the latter, the ability to detoxify superoxide anions plays a determinant role: in particular, the non-enzymatic scavenger ascorbate constitutes the first defensive barrier against ROS (Moldau 1999, Iriti *et al.* 2003). As regards bean plants, O_3 tolerance was recently found to be associated with genetic capacity to maintain extracellular high ratios of ascorbate/[ascorbate+dehydroascorbate] under O_3 stress (Burkey *et al.* 2003). It has also been demonstrated that in Saxa bean, a reduced ascorbate level, due to manganese deficiency, aggravates ozone injury (Wenzel and Mehlorn 1995). Therefore, it is possible that the localization of few dead cells around stomata observed in Saxa leaves, together with the limited H_2O_2 deposition, could be the consequence of a more efficient ROS scavenging system in comparison to that of BLF and Cannellino, which are, in fact, more sensitive to the pollutant. This view is also supported by the high H_2O_2 deposits found in these cultivars, though differently distributed in the leaf tissues. In BLF, they were localized only in the palisade mesophyll and, consequently, mainly palisade cells underwent death, developing typical adaxial lesions. In Cannellino leaves, H_2O_2 deposits were present also in spongy mesophyll, thus accounting for the development of chlorotic lesions in both adaxial and abaxial surface, and, possibly, explaining their earlier appearance.

In conclusion, the combined technique of DAB *in vivo* up-take and Evans blue vital staining, besides constituting a powerful tool in an early diagnosis of ozone injury, allow to gain some very useful insight into the mechanisms at the base of O_3 sensitivity or tolerance. Furthermore, the technique does not require sophisticated instrumentation and can be used in biomonitoring programs, when an early and correct diagnosis of O_3 injury, even before the appearance of visible symptoms, is particularly useful.

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