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Ozone damage and tolerance in leaves of two poplar genotypes

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Abstract — The effects induced by an acute ozone exposure were investigated in two poplar hybrids differentially O_3 susceptible in terms of leaf injuries: *Populus deltoides x maximowiczii*, Eridano clone and *Populus x euramericana*, I-214 clone, the sensitive and the tolerant respectively. Both the leaf anatomy and the responses induced by ozone in the leaves were analysed, using a cyto-histochemical approach.
Morphoanatomical characters, such as amphistomatous lamina, higher stomatal density and relaxed mesophyll cell packing (evaluated by the palisadeness coefficient), observed in the sensitive clone leaves, may favour a greater O_3 uptake in the apoplast and increase the cumulative dose of pollutant per mesophyll cell, with respect to tolerant clone leaves. Mesophyll cells of sensitive plants were the main targets for O_3. After an acute ozone treatment, the palisade parenchyma cells showed a decrease in chloroplast number and size, resulting best suited both to perceive the stress by O_3 or reactive oxygen species and to activate several signal transduction pathways, in relation to their morphological, physiological and functional properties predisposing an efficient cell communication, signalling and stimuli sensing.
The quick and well localized pattern of cell death induced by O_3 in sensitive poplar leaves was accompanied by some hallmarks of programmed cell death: nuclear shrinkage, chromatin condensation and cell wall collapse.

Key words: Leaf injury, ozone stress, *Populus deltoides x maximowiczii*, Eridano clone, *Populus x euramericana*, I-214 clone, programmed cell death (PCD), reactive oxygen species (ROS).

INTRODUCTION

Current ozone ground level (45 ppb), in unpolluted areas of the world (Narsto 2000; Vingarzan 2004), has been hypothesized to increase globally by 1 to 2% per year (Olszyk *et al.* 2001). Also the peak episodes, reaching 150 ppb in the most polluted sites and occasionally up to 250 ppb (Sandermann 1998; Vahala *et al.* 2003), are predicted to increase in both duration and frequency (Meehl 2007), in spite of control measures for ambient ozone containment.

Ozone probably has the most negative impact of any air pollutant on tree vigour and growth (Reich 1987; Percy *et al.* 2003; Matyssek *et al.* 2007) and the effects of O_3 on plants are numerous, varying with the intensity and duration of exposure (Pasqualini *et al.* 2003).

In the most sensitive organisms, short high-peak concentration of ozone (150-300 ppb of O_3 for 4-6 hrs) may lead to visible injuries and cell death events, reminiscent of lesions induced during plant-pathogen interactions (Rao *et al.* 2000; Pasqualini *et al.* 2003).

Tree species and genotypes differ in their sensitivity to ozone: broadleaved fast-growing trees appear to be more susceptible to ozone rather than slow-growing ones and conifers (Reich *et al.* 1987; Skärby *et al.* 1998; Witting *et al.* 2007; Nikula *et al.* 2009). In particular, species belonging to the genus *Populus* are regarded as one of the most ozone sensitive ones (Nikula *et al.* 2009). At the present, it is still unclear what and how many different factors can determine

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an increased susceptibility to ozone of a species or populations and individuals of a certain species, nevertheless different experimental evidences propose that leaf anatomical traits, combined with other physiological, biochemical and environmental factors (PÄÄKKÖNEN *et al.* 1997; CHEN and GALLIE 2005; NIKULA *et al.* 2009; PAOLETTI *et al.* 2009) can play an important role in determining a more or less marked sensitivity to ozone increase.

The leaf represents the first target for phytotoxic action of O_3: this gaseous pollutant enters the leaf surface firstly via the open stomata and rapidly dissociates in apoplast, resulting in an excess of reactive oxygen species (SCHRAUDNER *et al.* 1998) that may result in a biphasic oxidative burst. The second oxidative peak is absent or reduced in ozone-resistant plants and can be considered a biochemical marker for ozone plant sensitivity (PELLINEN *et al.* 1999; WOHLGEMUTH *et al.* 2002).

The produced ROS can oxidize proteins and membrane phospholipids, increasing membrane permeability, and affecting photosynthesis (HEAT 1994).

ROS seem to act not only as damaging agents, but also as abiotic elicitors in redox-responsive pathways, determining a specific program of cell death and other reactions very similar to hypersensitive responses induced in plants by a wide range of biotic and abiotic stresses (OVERMYER *et al.* 2005; KANGASJÄRVI *et al.* 2005; GÜNTHARDT-GOERG and VOLLENWEIDER 2007).

In this study, two poplar clones differently susceptible in terms of visible leaf damage (the O_3-sensitive *Populus deltoides x maximowiczii*, Eridano clone and the O_3-resistant *Populus x euramericana*, I-214 clone) were subjected to an acute ozone stress, with the aim to increase the actual knowledge concerning the short-term responses of poplar trees, because of their high economic and ecological importance. Different research groups have carried out many studies on the biochemical, physiological and molecular effects induced by single pulse of acute ozone concentration in these two poplar clones (RANIERI *et al.* 1996; GUIDI *et al.* 1998; NALI *et al.* 1998; RANIERI *et al.* 1999; RANIERI *et al.* 2000; GUIDI *et al.* 2001; RANIERI *et al.* 2001; DIARA *et al.* 2005; RIZZO *et al.* 2007) but, no extensive data that relate morphoanatomical traits of the leaves from the two poplar clones and their differential ozone sensitivity are at the present available.

MATERIALS AND METHODS

Plant material - Rooted cuttings (length 10 cm) of two poplar hybrid clones (the O_3 sensitive Eridano clone of *Populus deltoides* × *P. maximowiczii*, and the O_3 resistant I-214 clone of *Populus x euramericana*) originating from plants growing in experimental field of the Department of Agricultural Plant Biology (University of Pisa), were grown for two months in a greenhouse in plastic pots containing a steam-sterilized substrate (soil:peat:perlite; 1:1:1, in vol.). During the plants growth, the average temperature in the greenhouse ranged from 15° (night) to 26°C (day), and the relative humidity (RH) ranged from a minimum of 55% to a maximum of 85%, in relation to the meteorological

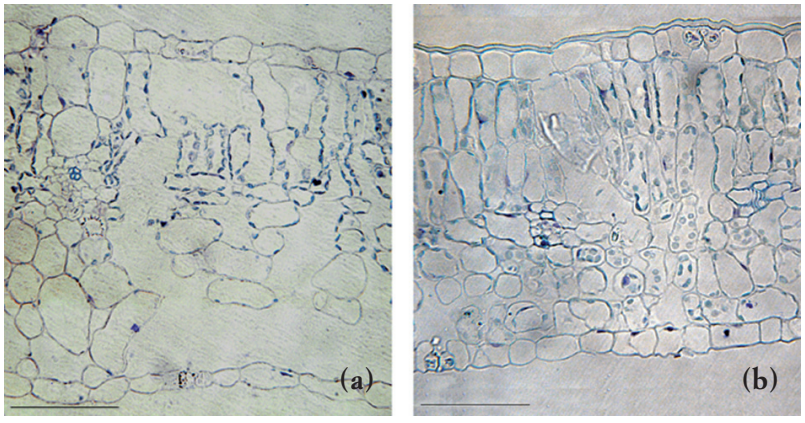

Fig. 1 — Transverse sections of leaves from the ozone sensitive *Populus deltoides x maximowiczii*, Eridano clone (a) and the ozone tolerant *Populus x euramericana*, I-214 clone (b). (TBO staining, bar = 50 µm).

conditions. Plants were watered every three days and fertilized every two weeks.

Uniform plants (about 50 cm high) from each poplar clone with at least ten fully expanded leaves were randomly selected and assigned to a control group and to the group of plants undergoing to O_3-fumigation.

Fumigation treatment - Ozone treatment, fumigation chamber and apparatus were as described in DIARA *et al*. (2005). O_3 fumigation was carried out in a fumigation apparatus located at the Department of Agricultural Chemistry and Biotechnology, University of Pisa. Ozone was generated by electrical discharge in pure oxygen through a Fisher 500 air-cooled generator (FISHER LABOR UND VERFAHRENSTECHNIK, Meckenheim, Germany). The O_3 concentration of the fumigation chamber was continuously monitored with a MONITOR LABORATORIES ANALYZER (MODEL 8810, Monitor Labs, San Diego, CA, USA) operating on the principle of ultraviolet (UV) adsorption and interfaced with a personal computer. During O_3 fumigation and the administration of charcoal-filtered air, the temperature in the growth chambers was maintained at 20±1°C, RH at 85±5%. The photosynthetic photon flux (PPF) at plant height was 530 µmol m^{-2} s^{-1} (photosynthetic active radiation: 400-700 nm) and was produced by an incandescent lamp.

Five watered and uniform plants for each poplar clone were placed in a fumigation chamber (0.48 m^3), pre-adapted to the chamber conditions for 48 hrs and then exposed to a single pulse of 150 ppb O_3 for five hours (from 09:00 to 14:00). At the same time, watered control plants (5 for each hybrid clone, from the control group) were kept in one charcoal-filtered air chamber (0.48 m^3), under the same conditions except for O_3 treatment. Mature leaves of O_3-treated and control plants were sampled before (0 hrs) the ozone exposure and at significantly responsive time-points during the ozone treatment (2 and 5 hrs from the beginning of the fumigation) or in the recovery time (24 and 48 hrs from the end of the fumigation), defined on the basis of the results reported in previous works (NALI *et al.* 1998; BERNARDI *et al.* 2004; DIARA *et al.* 2005; Rizzo *et al.* 2007) and then subjected to morpho-anatomical and histo-cytochemical investigations.

Morpho-anatomical investigations - Fresh strips of adaxial and abaxial leaf epidermis were performed using 5 fully-expanded leaves from each poplar clone (one leaf per tree); the stomata were counted under a light microscope from 3x3 mm^2 pieces (two pieces per leaf) randomly selected and averaged for each leaf, excluding stomata overlapping the frame margins. The average number of stomata obtained per considered piece was then converted in stomatal density referring this number to a piece having a surface area of 1 mm^2 (number of stomata per mm^2).

Leaf portions from O_3-treated and control samples were excised and fixed for 24 hrs in 4% formalin PBS-buffered (PBS, phosphate–buffered saline, pH 7.4), dehydrated in a graded ethanol series and embedded in LR-White medium Grade (LONDON RESIN COMPANY). Semi-thin sections (3 µm), were cut with an ultramicrotome Ultratome Nova LKB using glass knives. At least 100 leaf semi-thin sections for each clone were subjected to morpho-anatomical analysis. Fresh leaf strips and semi-thin sections were observed with a LEITZ DIAPLAN light microscope and captured using a LEICA DC300F digital camera.

Histochemistry - Semi-thin sections were stained with different techniques: Toluidine blue O staining for general cytological investigations

TABLE I — Morpho-anatomical leaf traits in the two poplar clones. Data are averaged from three independent experiments and values are shown as means±SD (n=100). (ns: not significant P value).

Characteristic	*Populus deltoides x maximowiczii*, Eridano clone	*Populus x euramericana*, I-214 clone	P-value Eridano clone vs I-214 clone
Upper epidermis thickness (mm)	0.01241± 0.0036	0.01364± 0.0022	ns
Palisade parenchyma thickness (mm)	0.05085± 0.0062	0.05248± 0.0064	ns
Spongy parenchyma thickness (mm)	0.0721± 0.01637	0.0509± 0.007	P<0.05
Lower epidermis thickness (mm)	0.01037± 0.0029	0.00976± 0.002	ns
Mesophyll thickness (mm)	0.13224± 0.0146	0.10834± 0.0136	P<0.05
Palisadeness coefficient (K%= palisade thickness/ mesophyll thickness)	39.5 %	49.1%	

(FEDER and O'BRIEN 1968); Feulgen staining (O' BRIEN and MC CULLY 1981) combined with Giemsa staining (30% in deionised water), for nuclear and cytoplasmic cell structures; Haematoxylin and Eosin double staining to reveal nuclei and cytoplasmic structures (adapted for semi-thin sections, from AL HAZZAA and BROWN 1998). The sections were air dried, mounted in DPX and then analyzed by optical microscope. Images of each slide were captured using a IMAGE LEICA DC300F digital camera.

Cell-viability assay - Mature leaf portions from each sample were excised, freshly stained with Evans Blue stain (2%, w/v, in water for 5 min, at room temperature) and washed: the dye enters only the cells with an altered membrane permeability, typical of dead cells (GAFF and OKONG'O-GOLA 1971). Microscopical analysis of staining pattern was performed immediately afterward. The images, observed with a LEICA DB LM light microscope, were captured using a IMAGE LEICA DC300F digital camera.

Statistical analyses - Statistical analysis was carried out using INSTAT TM (GRAPHPAD, SAN DIEGO, CA). All data from morpho-anatomical investigations were expressed as means ± SD and the means were subjected to a one-way analysis of variance (ANOVA) and Tukey–Kramer analysis. The reported anatomical microphotographs were representative of at least 20 samples yielding similar results.

RESULTS

Leaf anatomy in sensitive and tolerant poplar clones - We examined some key leaf morpho-anatomical characters that may differ between the two poplar clones and that could determine their different sensitivity to ozone.

Both *Populus deltoides x maximowiczii*, Eridano clone, and *Populus x euramericana*, I-214 clone, had dorsiventral amphistomatous leaves (Fig. 1 a-b; Fig. 2). Adaxial epidermal cells, in both the clones, showed straight anticlinal walls, while abaxial epidermal cells evidenced a lobed shape with sinuous anticlinal walls (Fig. 2). Cells displayed the same surface area between adaxial and abaxial epidermis in the same poplar clone, but the epidermal cells in Eridano clone were smaller in surface area than in I-214 one (Fig. 2). Outer walls of both adaxial and abaxial epidermal cells in I-214 clone were covered with a thicker cuticle than in the Eridano clone (Fig. 1). In both the sensitive and tolerant poplar clones, the upper mesophyll portion consisted in two layers of palisade parenchyma, rich in chloroplasts (Fig. 1).

In the sensitive clone the palisade cells showed larger intercellular spaces than in the tolerant clone (Fig. 1a). Between the palisade and spongy tissues, both the clones showed paraveinal mesophyll cells that extended horizontally between the veins. Most cells of the inner

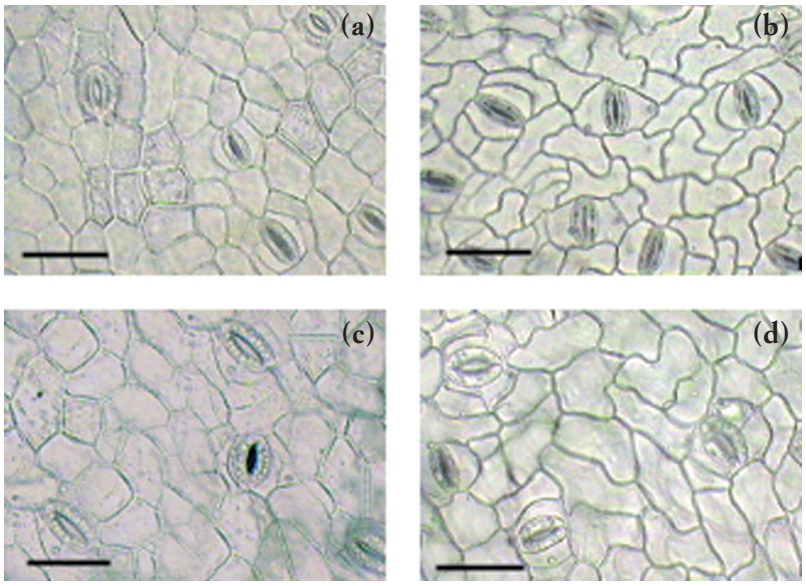

Fig. 2 — Stomata in epidermal strips taken from upper and lower surfaces of mature leaves of *Populus deltoides x maximowiczii*, Eridano clone (a; b) and *Populus x euramericana*, I-214 clone (c; d). (bar = 50 µm).

palisade layer were in direct contact with the paraveinal mesophyll and the remainders with bundle-sheath cells (Fig. 1). The spongy cells, with fewer chloroplasts than the palisade cells, were irregular in shape and size in the sensitive clone, that also showed more developed intercellular spaces than the tolerant. The resulting apoplast, in the sensitive clone, was more developed than the simplast (relaxed cell-packing, Fig. 1a) with a consequent increase in the internal gas circulation. The thickness of palisade parenchyma on the total mesophyll thickness (palisadeness coefficient K%; DINEVA, 2006) resulted 39% in the sensitive clone and 49% in the tolerant clone, that showed a more densely packed mesophyll with little and few air chambers in the spongy parenchyma (Fig.1b; Table I). In addition, the whole mesophyll thickness resulted higher in the sensitive clone leaf than in the tolerant one (Table I), mainly due to the higher thickness of the spongy tissue of the Eridano clone mesophyll with respect to the I-214 clone mesophyll.

In both the clones, the upper and the lower epidermis significantly differed in stomatal density, but, only in sensitive clone the stomatal density of the lower epidermis was about twofold with respect to the upper epidermis and resulted also about twice the lower epidermis of the tolerant clone. No significant difference has been detected between the stomatal density of the upper epidermis between the two clones (Table II).

Macro- and microscopical changes caused by ozone fumigation on sensitive and tolerant poplar leaves - The leaves of the sensitive clone, within 24-48 hrs after ozone fumigation, displayed typical macroscopical stippling (arrow head) and well developed dark-black necrotic spots (arrow) in the interveinal areas of the lamina (Fig. 3). On the contrary, no significant ozone induced responses, at macroscopic level, were detected in controls of sensitive clone and in samples from O_3 tolerant poplar hybrid.

The first manifestation of visible injuries induced by O_3 was at level of the adaxial side

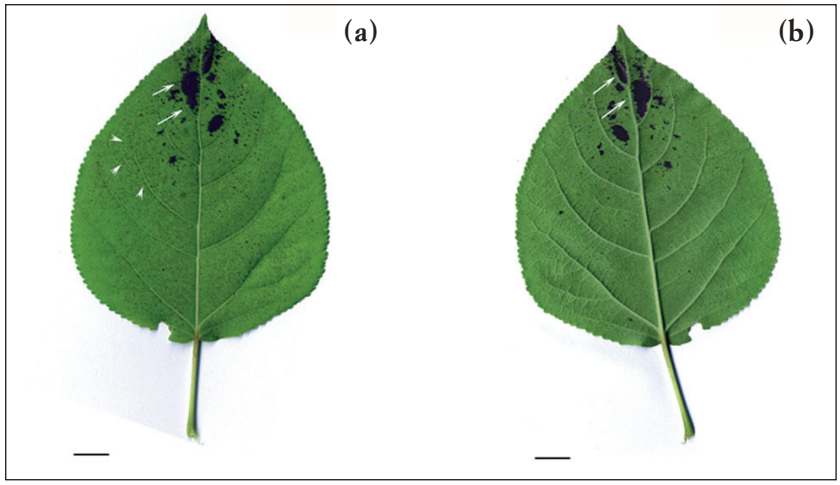

Fig. 3 — A leaf of *Populus deltoides x maximowiczii*, Eridano clone showing typical dark-black necrosis localized in the interveinal area of the adaxial (a) and abaxial (b) surfaces 24 hrs after O_3 fumigation. On the abaxial surface are detectable only necrotic spots at late level of degeneration (arrow) but not the initial stippling evidenced in adaxial surface (arrow head). (bar = 1 cm).

TABLE II — Stomatal density in ozone sensitive and tolerant poplar genotypes (mean±SD, n=10). (ns: not significant P value).

Stomatal density (number/mm²)	*Populus deltoides x maximowiczii*, Eridano clone	*Populus x euramericana*, I-214 clone	P-value			
			ue Eridano clone vs ue I-214 clone	le Eridano clone vs le I-214 clone	ue Eridano clone vs le Eridano clone	ue I-214 clone vs le I-214 clone
upper epidermis (ue)	153.9 ± 5.66	132.3± 4.37	ns	P<0.001	P<0.001	P<0.01
lower epidermis (le)	328.7± 6.24	176.5± 4.72				

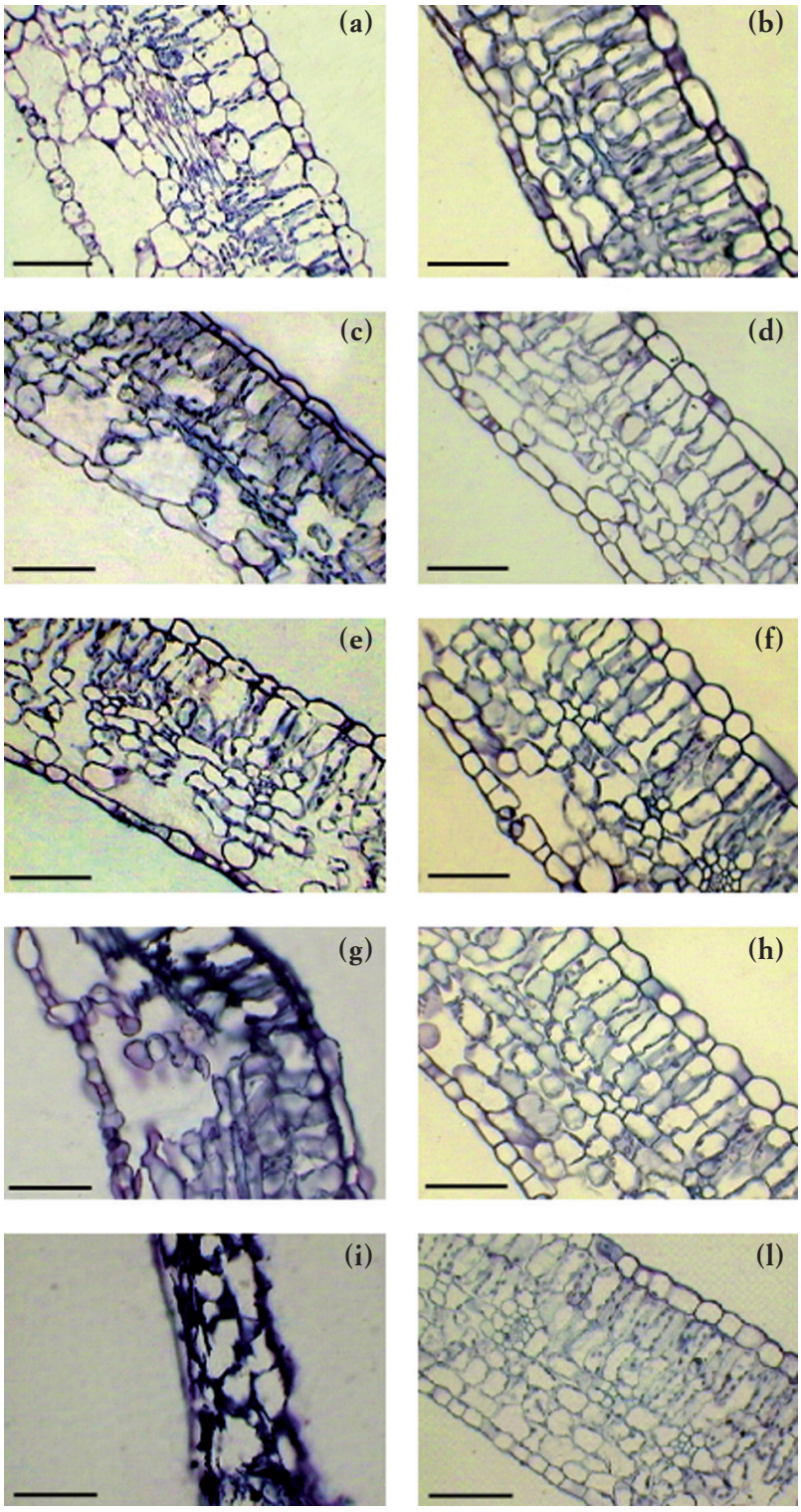

Fig. 4 — Transverse sections of leaves from sensitive (a, c, e, g, i) and tolerant (b, d, f, h, l) poplar clones, sampled before ozone fumigation (a, b), at 2 hrs from fumigation beginning (c, d), at the fumigation end (5 hrs; e, f) and during the recovery time, after 24 hrs (g, h) and 48 hrs (i, l). (TBO staining, bar = 50 μm).

of the leaf (Fig. 3 a); on the abaxial surface the necrosis was macroscopically detectable later (arrow), when a large part of mesophyll was degenerated (Fig. 3 b).

Light microscopy analyses, performed at the different time points, evidenced some distinctive changes in the O_3-treated sensitive clone samples, with respect to the unfumigated ones (Fig. 4 a). At the mid-treatment (Fig 4 c), palisade cells appeared shrunken and intensely Toluidine blue stained, indicating changes in vacuolar content, that foreshadow the final degeneration of vacuole by tonoplast disintegration during the following steps. At the fumigation end (Fig. 4 e), precocious signs of palisade cell wall disorganization took place. During the recovery times (24 and 48 hrs after ozone fumigation), conspicuous cell damage started from the inner layer of palisade parenchyma and then spread to the other mesophyll tissues; no chloroplasts were detectable in degenerating cells (Fig. 4 g and i). In damaged areas, cell walls became thickened and partially destroyed; large empty areas were detectable in mesophyll, surrounded by completely degraded cell walls. The changes in the spongy parenchyma were similar to those of the palisade parenchyma, although they appeared later (Fig. 4 g and i). In the O_3-affected areas of the palisade cells, the chloroplasts decreased both in size and number in the early phases of ozone stress (Fig. 5). No microscopic changes referable to the ozone exposition were evidenced in all the samples from tolerant poplar clone (Fig. 4 d, f, h and l), with respect to the untreated samples (Fig. 4 b).

Conspicuous nuclear changes were evidenced only in the sensitive clone tissues: in unfumigated leaves, the mesophyll cell nuclei appeared well-defined in shape, showing a homogenous chromatin and one or more nucleoli; the nuclear envelope appeared intact (Fig. 6 a). In leaf samples collected during the recovery (at 24-48 hrs) the nucleoli disappeared and the nuclei displayed an irregular shape, and a highly condensed chromatin, appearing as dark red blur spots, Feulgen staining positive (Fig. 6 b). In the same figure, the shrunk nuclei, with other degenerating organelles and cell remnants, were strictly associated to degenerating cell-walls.

The epidermal layers, mostly the lower, degenerated lately with respect to mesophyll tissues (Fig. 4 g, i). Tannins, only detected in the leaves from the sensitive clone, were homogeneously distributed within the vacuoles of parenchymatic cells around of veins (Fig. 7 a); after the cell break-down, tannins impregnated the partially degenerated cell-walls in the mesophyll (Fig. 7 b, c).

Just at precocious phases of O_3 fumigation, changes in membrane permeability in mesophyll cells were detected (evidenced by an intense Evans blue staining): single blue-stained cells, appeared at mid-treatment, evolved in large and well defined areas of dead cells with freely permeable plasma membrane (12 hrs; Fig. 8); these areas then progressed in "necrotic spots" (24 hrs). The epidermal cells appeared unstained by Evans Blue stain for many hours after the end of fumigation.

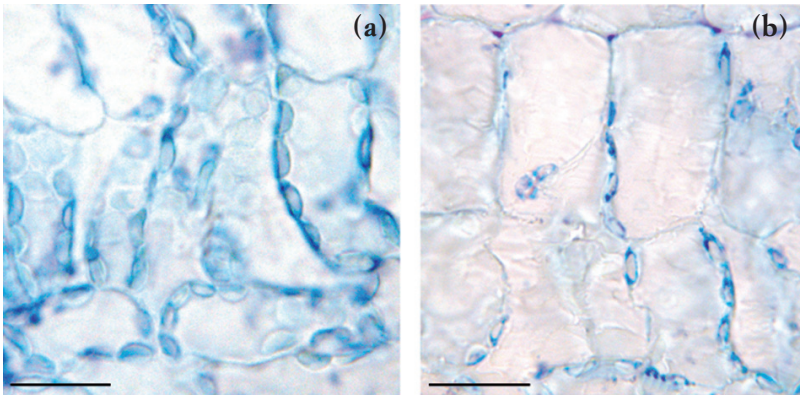

Fig. 5 — Details of transverse sections of leaves from the ozone sensitive *Populus deltoides x maximowiczii*, Eridano clone: palisade cells before ozone fumigation (a) and after ozone treatment (5 hrs; b), evidencing changes in number, shape and size of the chloroplasts. (TBO staining, bar = 50 µm).

DISCUSSION

An acute ozone exposure induced deleterious effects only in the mature leaves of *Populus deltoides x maximowiczii*, Eridano clone (the O_3-sensitive), that developed typical macroscopic symptoms clearly reflected in microscopic cell injuries. The evidenced adaxial stipples, related to the oxidant power of ozone, can be considered the main typical symptom induced by ozone in the most sensitive broadleaved species (ORENDOVICI *et al.* 2003).

A greater foliar O_3 injury has been positively related to a greater stomatal density (DEAN 1972; EVANS and TING 1974; BENNET *et al.* 1992; FERDINAND *et al.*, 2000), that potentially can lead both to greater rates of stomatal conductance and to greater ozone uptake into the leaf (REICH 1987; WIESER and HAVANEK 1993; WINNER 1994; EVANS *et al.* 1996 a and b).

Moreover, less densely packed mesophylls are proposed to increase the ozone sensitivity (BENNET *et al.* 1992; PÄÄKKÖNEN *et al.* 1997; LEE *et al.* 1999; FERDINAND *et al.* 2000) since, in relaxed mesophylls, the cells exhibit a larger contact surface exposed to apoplastic O_3 for the induction of ROS formation (Foyer et al. 1994). Accordingly, amphistomatous leaves, high stomatal frequency and low mesophyll density, observed in the O_3-sensitive poplar, may be important leaf characteristics influencing both gas uptake in apoplast and internal diffusivity among target cells and consequently ozone sensitivity.

In a previous study, NALI *et al.* (1998) evidenced a different stomatal conductance in *Populus deltoides x maximowiczii*, Eridano clone, and *Populus x euramericana*, I-214 clone, independently to ozone fumigation treatment. At the same experimental conditions (RH, temperature, photoperiod) the more sensitive genotype displayed an increased stomatal conductance than the more tolerant one. The higher stomatal conductance, as constitutionally displayed by the Eridano clone, in addition to the observed higher stomata number, could predispose the ozone sensitive clone to a massive ozone uptake than the tolerant poplar clone. This massive ozone uptake could not be efficiently detoxified by the antioxidative systems of the leaves, accordingly to BERNARDI *et al.* (2004) that evidenced a diminished activity of antioxidative enzymes in Eridano clone leaves than in I-214 ones. Consequently, the not scavenged reactive oxygen species could determine the observed behaviour in the more sensitive leaves consisting in the appearance of the typical injured areas. Morpho-anatomical characteristics of the tolerant poplar can be regarded as important traits that enable the more resistant genotypes to avoid or to oppose the adverse effects of gas pollutants, likely to xeromorphic plants or plants growing in polluted sites that limit the water loss or gaseous pollutants uptake, by reducing stomata density and intercellular air spaces.

In the ozone-exposed sensitive poplar leaves, the main contributions to the visible necrotic injuries derived from the mesophyll cells degeneration. In particular, as observed in deciduous trees and conifers subjected to ozone stress (EVANS and TING 1973; GUDERIAN 1985; EVANS *et al.* 1996 a and b), ozone preferentially affected palisade parenchyma cells, probably because of the higher surface to volume ratios displayed by these cells than spongy mesophyll ones: a

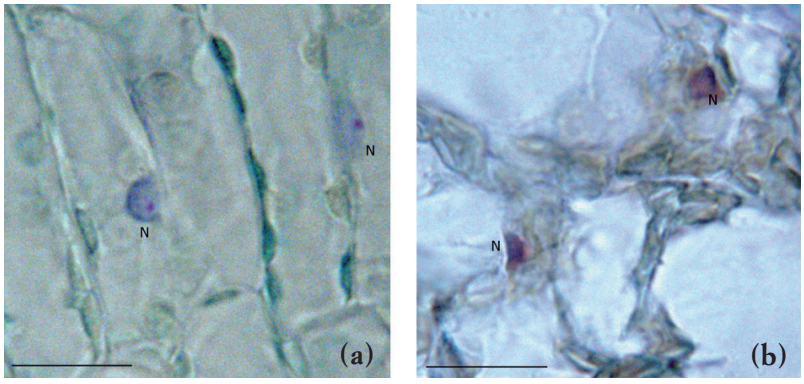

Fig 6 — Details of transverse sections of leaves from the sensitive poplar clone: mesophyll cells from a control leaf (a) and from an ozone treated leaf (24 hrs.; b), evidencing changes in nuclear structure (N, nucleus). (Feulgen-Giemsa double staining, bar = 50 µm).

larger palisade cell could represent a larger target for ozone damaging action at level of cell membranes. Later, also the spongy parenchyma cells degenerated. Epidermal cells, stomata and vascular tissues remained intact until a late stage when the whole mesophyll was degenerated. Epidermal cells resistance can be related to the well developed cuticular layer, more developed in the tolerant poplar clone, that provides an efficient diffusion barrier against the ozone spreading and limits the diffusion in the upper palisade cell layer except at stomata level. The protection role, affordable by the epidermis, becomes little effective by the intercellular spaces of the mesophyll and by the high stomata density of the abaxial epidermis in the sensitive poplar leaf. The inner palisade layer is more vulnerable to ozone damage than the upper, because of its direct contact with the large intercellular spaces of spongy parenchyma, which offer no appreciable resistance to gas exchange (EVANS and TING 1974; FERDINAND *et al.* 2000). Also the highly packed structure of palisade parenchyma may play an important role in conferring the ozone-sensitivity to this tissue, favouring the cell-to-cell communication by means of wide interchange surfaces that enhance the cell sensitivity to stimuli with a variable intensity, enabling a modulation in the responses. It may be hypothesized the coexistence of many and different short-distance signalling pathways involved in spatial progression of responses elicited by O_3 in the peripheral cells around the spreading lesion.

The high ozone sensitivity displayed by palisade parenchyma cells may also be due to their higher chloroplast number than the other leaf tissues. The chloroplasts are the privileged targets of phytotoxic action exerted by ozone (SUTINEN *et al.* 1990) and are believed the main sensors of O_3 or its breakdown products (KANGASJÄRVI *et al.* 1994). It is reasonable to suggest that the different chloroplast equipment in different leaf tissues may contribute to explain their different behaviour when subjected to O_3 exposure. Therefore, the palisade cells resulted the best candidates both to perceive an acute stress by ozone and to activate several signal transduction pathways, regulating the mesophyll responses to the increased oxidative load (KANGASJÄRVI *et al.* 2005), including the drastic changes in the global gene expression, as suggested by chromatin changes that we have observed in the sensitive poplar clone. Changes of gene expression have been reported in *Arabidopsis* (MAHALINGAM *et al.* 2003 and 2005) and in poplar hybrids (RIZZO *et al.* 2007) and these responses can be assimilated to the host defence mechanisms against infections (SANDERMANN *et al.* 1998), as programmed cell death (PCD) events, at the site of the attempted pathogen penetration (IRITI *et al.* 2006). In some plant systems an acute stress by O_3 (SCHRAUDNER *et al.* 1997; RAO *et al.* 2000; VOLLENWEIDER *et al.* 2003) or heavy metals exposure (PIQUERAS *et al.* 1999) can induce a defence PCD, named Hypersensitive Response Like, characterized by the restriction of PCD to a small group of cells, by the disruption of the cell contents, nuclear degeneration and high chromatin condensation, by the collapse of cell walls and the incomplete degeneration of cell remnants and their condensation in apoptotic-like bodies (LEVIN *et al.* 1996; FUKUDA 2000; VOLLENWEIDER *et al.* 2003). At late stages after ozone fumigation end, we observed a combination of these cytological markers in well defined areas in the most symptomatic sensitive poplar leaves. These features of the programmed cell death, induced by ozone stress in the sensitive clone mesophylls, are similar to the

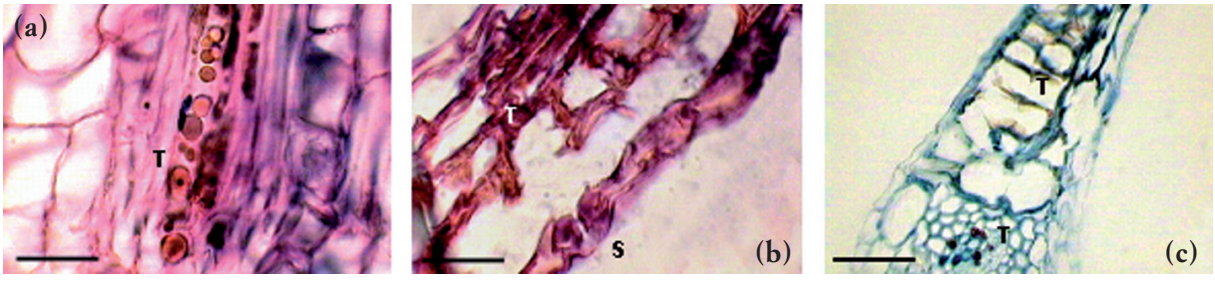

Fig. 7 — Transverse sections of sensitive clone leaves showing conductive bundles associated with vascular parenchymatic cells containing tannins (at 5 hrs; a). At 48 hrs, alterations of epidermal cells and stomata; also mesophyll shows severe structural injury in the form of collapsed and dead cells with thickening, unfolding and breaks of walls (b and c). Tannins are associated to xilem parenchymatic cells. T, tannins; S, stomata; (a, b: H&E staining, bar = 20 µm; c Giemsa staining, bar = 50 µm).

characters evidenced by light or fluorescence microscopy investigations in different plant systems, in which PCD has been confirmed with the classical approaches for programmed cell death validation *(*WANG *et al. 1996;* TAMAGNONE *et al. 1998;* CAO *et al. 2003;* DAT *et al. 2003;* GUNAWARDENA *et al. 2004;* MONTILLET *et al. 2005;* OVERMYER *et al. 2005;* PAPINI *et al. 2010 a and b).*

The changes evidenced in sensitive mesophylls in samples collected after the ozone fumigation end, during the recovery time, could be the cyto-histochemical manifestation of the dismantling phase of a cell death process evoked by ozone stress and that could be interpreted as the continuation and the consequence of the early molecular and physiological modifications triggered by ozone, at the beginning of the fumigation treatment. The highly defined cell death pattern was strongly underlined by the well localized stippling on the adaxial leaf surfaces and also by the pattern of Evans blue positive cells. Using this viable stain, we have also evidenced a direct correspondence between ozone fumigation, cell membrane damage and cell death events. These results are in agreement with previous findings (PELL *et al.* 1997), that described how, when stomata are open, there is a rapid loss of plasma membrane semi-permeability, followed by plasmolysis and, if the stress is maintained, by cell death. Other apoptotic hallmarks as the nuclear shrinkage and chromatin condensation, as evidenced by cyto-histochemical assays in sensitive poplar clone leaves after ozone treatment, suggest that ozone may induce PCD. Accordingly, our preliminary findings evidenced DNA fragmentation events, changes in cell-membrane asymmetry, abnormal callose deposition taking place only in mesophyll from sensitive poplar clones, after an acute ozone stress (BARTOLI *et al.* 2010).

In the most injured mesophylls, we evidenced also an increased production of tannins. The condensed tannins, as evidenced in the most injuried mesophylls of the sensitive poplar clone, are considered particularly remarkable markers of ozone stress (BOOKER *et al.* 1996; WULFF *et al.* 1996; PASQUALINI *et al.* 2003; GÜNTHARDT-GOERG and VOLLENWEIDER 2007). In several species, accumulation of secondary compounds,

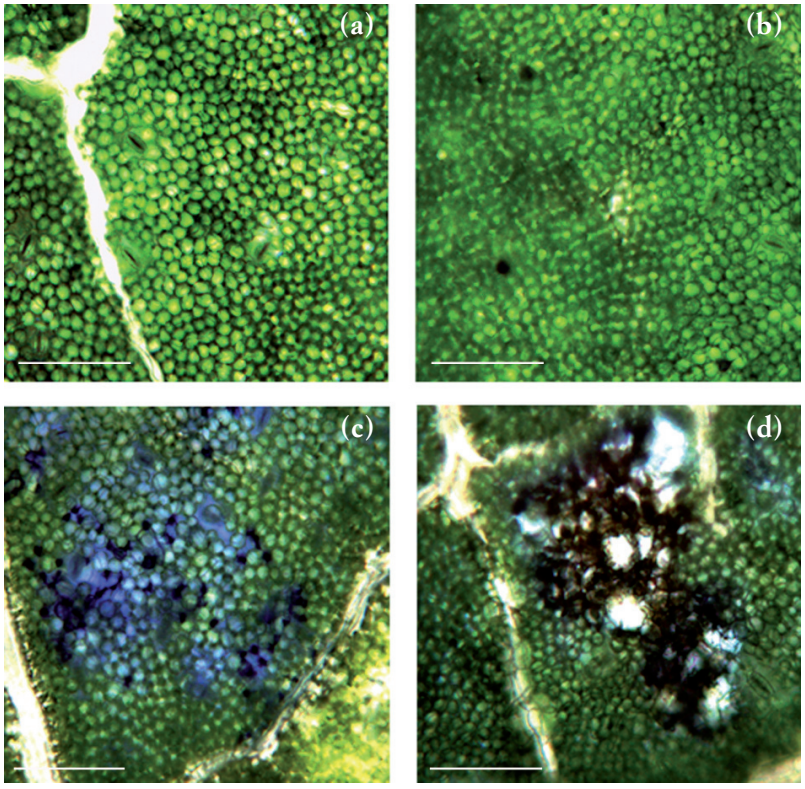

Fig. 8 — Cell viability in leaves of sensitive poplar clone (a, b, c, d) sampled at different time points (0, 2, 12, 24 hrs respectively). Not stained cells (green cells) are viable; blue, blue-dark cells evidence an altered plasma membrane permeability; brown, brown-dark cells are degenerated. (Evans Blue staining, Bar= 50 μm).

mainly phenols (including tannins), is considered an active response of the plant to differing environmental stresses and are believed to play an important role in defence responses, acting as powerful antioxidants (RIVERO 2001; PASQUALINI *et al.* 2003).

In a changing environment, the leaf visible symptoms, validated with microscopic analyses, may constitute a useful tool for the early diagnosis of stress induced by ozone and/or other pollutants and also to evaluate the adaptive capacity of different plant species to several stress factors.

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