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Antioxidant enzyme isoforms on gels in two poplar clones differing in sensitivity after exposure to ozone

R. BERNARDI*, C. NALI**, P. GINESTRI*, C. PUGLIESI*,[1], G. LORENZINI** and M. DURANTE*

*Dipartimento di Biologia delle Piante Agrarie, Sezione di Genetica, Università degli Studi, Via Matteotti 1/B, I-56124 Pisa, Italy**
*Dipartimento di Coltivazione e Difesa delle Specie Legnose "Giovanni Scaramuzzi", Università degli Studi, Via del Borghetto 80, I-56124 Pisa, Italy***

Abstract

The effect of acute ozone (O_3) fumigation on isozyme patterns of superoxide dismutase (SOD), peroxidase (POD) and ascorbate peroxidase (APX) in mature (ML) and young leaves (YL) of two poplar clones, contrasting in O_3-sensitivity was analysed. Untreated leaves of both the O_3-sensitive (O_3-S) clone Eridano of *Populus deltoides* × *P. maximowiczii* and the O_3-resistant (O_3-R) clone I-214 of *P.* × *euramericana* showed four distinct SOD isoforms with a relative mobility (R_f) of 0.54 (MnSOD), 0.60 (Cu/ZnSOD), 0.65 (unidentified), and 0.71 (Cu/ZnSOD). After O_3-fumigation the activity of the SOD isoforms showed only quantitative variations with respect to control plants. In ML of untreated O_3-R plants seven POD isoforms (R_f = 0.13, 0.19, 0.34, 0.59, 0.64, 0.70 and 0.75) were found, while in YL one isoform (R_f = 0.34) was undetected. Only three POD isoforms in both ML and YL of untreated O_3-S plants were resolved. The electrophoretic pattern of POD in O_3-S leaves was greatly modified by acute O_3-fumigation with the appearance of new isoforms in both YL and ML and the disappearance of an isoform (R_f = 0.13) in YL. Additionally, O_3-exposure induced the appearance of two APX isoforms in YL (R_f = 0.66 and 0.70), and one isoform in ML (R_f = 0.70) of the O_3-S clone. By contrast, the activity of the three APX isoformes (R_f = 0.64, 0.70 and 0.76) detected in O_3-R leaves showed only quantitative variation with respect to untreated plants. From these data it is concluded that: *1*) in these poplar hybrids antioxidant enzyme activity is developmentally regulated and greatly affected by acute O_3 stress treatments and *2*) the different enzymes activity displayed by the two poplar clones, especially for POD and APX isoformes, could partly explain their distinct O_3-sensitivity.

Additional key words: air pollution, antioxidant systems, oxidative stress, *Populus deltoides* × *P. maximowiczii*, *Populus* × *euramericana*.

Introduction

During the last three decades, concentration of ozone (O_3) in the lower troposphere of the Northern hemisphere has increased considerably, mainly due to vehicular traffic (Barnes *et al.* 1999). Ozone has been demonstrated to alter basic metabolic processes of trees, including reduction of photosynthetic rate (Coleman *et al.* 1995, Wieser 1997), a decrease in Rubisco quantity and activity (Pell *et al.* 1999), a reduction of foliar conductance (Paakkonen *et al.* 1996), an increase of the de-epoxidation state of the xanthophyll cycle pigments (Wieser *et al.* 2001) and an acceleration of leaf senescence (Pell *et al.* 1999).

Ozone, entering plant leaves through stomata, decomposes spontaneously in the aqueous solution of the apoplast into a number of reactive molecular oxygen species (ROS), which are harmful for plant cells (Moldau 1999). A number of enzymes (*e.g.*, superoxide dismutase, catalase, peroxidase, and enzymes of the ascorbate-

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Abbreviations: APX - ascorbate peroxidase; ML - mature leaves; O_3-R - ozone resistant; O_3-S - ozone sensitive; POD - peroxidase; R_f - relative mobility; ROS - reactive oxygen species; Rubisco - ribulose-1,5-bisphosphatecarboxylase/oxygenase; SOD - superoxide dismutase; YL - young leaves.
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[1] Author for correspondence; fax: (+39) 5057 6750, e-mail: cpuglies@agr.unipi.it

glutathione cycle) and non-enzymatic antioxidant metabolites (*e.g.*, α-tocopherol, β-carotene, glutathione and ascorbate) have been documented to protect plants against the damage caused by ROS (for review, see Kangasjärvi *et al.* 1994, Pell *et al.* 1997, Noctor and Foyer 1998, Sandermann *et al.* 1998).

The superoxide dismutases (SOD, EC. 1.15.1.1) catalyze the dismutation reaction of superoxide radical anions into molecular oxygen and H_2O_2 (Scandalios 1993). Three forms of SOD exist with respect to different ions bound in the active reaction centre of the enzyme. There are copper/zinc containing SOD (Cu/ZnSOD), manganese containing SOD (MnSOD) and iron containing SOD (FeSOD), which are all localized in different cellular compartments (Bowler *et al.* 1994).

Removing of the highly toxic H_2O_2, produced during the above-mentioned dismutation, is essential for the plant cell to avoid inhibition of such enzymes controlling the Calvin cycle in the chloroplast (Creissen *et al.* 1994). Chloroplasts eliminate H_2O_2 in the ascorbate-glutathione cycle (Asada 1994), by the ascorbate peroxidase enzyme (APX, EC. 1.11.1.11). The enzyme reduces H_2O_2 to water and dehydroascorbate by utilizing the electron donor ascorbate (Creissen *et al.* 1994).

Although the catalase enzyme, performing H_2O_2 degradation via disproportionation, is rather specific for H_2O_2, it reacts with a limited number of organic hydroperoxides (Scandalios 1993). Peroxidases (POD, EC. 1.11.1.7), also function in this mode. Induction of peroxidase is thought to be an important component of O_3-elicited adaptive mechanisms (Kronfuss *et al.* 1996, Schwanz *et al.* 1996, Tuomainen *et al.* 1996, Noctor and Foyer 1998). In addition to their role in scavenging systems, peroxidases have been recognized to be involved in several cellular processes, including the control of development, lignification, pathogen defense and the catabolism of growth regulators (Van Huystee 1987, Lewis *et al.* 1999).

There is currently great interest in the mechanism and the signal transduction pathway responsible for the gene expression induced by oxidative stress. In this study in order to better understand the mechanism underlying O_3 injury, the electrophoretic patterns of three enzymatic complexes (SOD, POD and APX) in two poplar hybrids contrasting in O_3-sensitivity exposed to acute O_3 treatments were analyzed. Ozone response is under genetic control, as there are differences among species and cultivars (Gressel and Galun 1994, Barnes *et al.* 1999). Poplar clones from O_3-polluted areas had much higher tolerance to an acute O_3 treatment than those from a pristine area, suggesting that long-term natural selection can work in this fast-growing tree species (Berrang *et al.* 1991). Results concerning the differential O_3-sensitivity of the clones Eridano of *Populus deltoides* × *P. maximowiczii* (O_3-sensitive: O_3-S) and I-214 of *P.* × *euramericana* (O_3-resistant: O_3-R), used in this study, have been recently reported (Nali *et al.* 1998). As the phytoxicology of O_3 is very complex and many factors such as leaf age play important roles in determining the overall response (Guidi *et al.* 1997, Strohm *et al.* 1999), the enzymatic antioxidant activities were analysed both in young expanding (YL) and fully mature (ML) leaves. Results pertinent to peroxidase activities, measured spectrophotometrically in the apoplastic (IWF) and in the intercellular matrix (RCM), have been already reported elsewhere (Ranieri *et al.* 1996).

Materials and methods

Plants: Rooted cuttings of two poplar hybrid clones (Eridano of *Populus deltoides* × *P. maximowiczii*, O_3-S, and I-214 of *P.* × *euramericana*, O_3-R) were grown for two months in a greenhouse in plastic pots containing a steam-sterilized substrate (soil:peat:perlite; 1:1:1, in vol.) at a temperature ranging between 15 and 26 °C and relative humidity (RH) between 55 and 85 %. Uniform plants with ten fully expanded leaves were selected.

Exposure chambers and fumigation techniques: Gas treatments were performed in a *Perspex* fumigation apparatus (Lorenzini *et al.* 1994) placed inside a walk-in growth chamber. Temperature was maintained at 20 ± 1 °C and RH at 85 ± 5 %; a photon flux density at plant height of 530 μmol(photons) m^{-2} s^{-1} was provided by incandescent lamps (*Sylvania*, Wembley, England) for a 14-h photoperiod. Ozone was generated by electrical discharge in pure oxygen. Plants were pre-adapted to the chamber conditions for 48-h and exposed to a single pulse of 150 mm^3 m^{-3} O_3 for five hours, in the form of a square wave. Another group of plants, used as controls, was exposed to charcoal-filtered air. Portions of ML and YL (3 - 4 cm long) from each plant were excised at the end of the fumigation, rapidly frozen in liquid nitrogen and stored at -80 °C.

Total soluble leaf protein extraction and gel electrophoresis: Soluble proteins were extracted by grinding 1 - 2 g leaf tissue in 2 - 4 cm^3 of 0.22 M Tris HCl buffer, pH 7.4, 250 mM sucrose, 1 mM $MgCl_2$, 50 mM KCl, 50 g dm^{-3} phenylmethyl sulfonyl fluoride (PMSF), 10 mM β-mercaptoethanol, 0.1 % (m/v) of ascorbic acid and 0.1 % (m/v) of insoluble polyvinyl-polypyrrolidone (PVP) (Bernardi *et al.* 2001a). The homogenate was centrifuged twice at 15 000 *g* for 15 min and the clear supernatant was assayed for protein content (Bradford reagent: *Bio-Rad* protein assay Kit II, *Bio-Rad Laboratories GmbH*, München, Germany). For native

polyacrylamide gels (PAGE), the samples were dissolved in 62.5 mM Tris-HCl buffer, pH 6.8 and 1 % (v/v) glycerol. Aliquots (80 μg per lane) were loaded on 1.5 mm thick 7 % non-denaturing polyacrylamide gels (Bernardi *et al.* 2001b) and run at 4 °C at 20 mA per gel, using a vertical slab gel apparatus (*Mini-PROTEAN II*^{TM}, *Bio-Rad Laboratories Ltd*, Hercules, CA).

Staining for antioxidant enzymes activity: After PAGE, the gels were stained for SOD, POD and APX activity. The SOD activity was analysed using the riboflavin-nitroblue tetrazolium reaction (Beauchamp and Fridovich 1971). Individual SOD isozymes were identified, according to Sandalio *et al.* (1987), by specific inhibition assays. Cu/Zn-containing enzymes are inhibited by KCN and inactivated by H_2O_2, whereas FeSOD is resistant to KCN and inhibited by H_2O_2, and MnSOD is resistant to both KCN and H_2O_2. Gels were preincubated for 30 min at 25 °C in 50 mM phosphate buffer (pH 7.8), containing either 2 mM KCN or 5 mM H_2O_2 and then stained for SOD activity.

Zones of POD activity were detected according to Brewbaker *et al.* (1968), immerging the gels for 15 min at 25 °C in 0.2 M acetate buffer (pH 6.0) containing 1 mM H_2O_2 and 100 mM *o*-dianisidine as a hydrogen donor; staining was blocked by washing in 50 % (v/v) methanol and 7 % (v/v) acetic acid.

For APX isoforms determination, gels were pre-run using Tris-glycine buffer to which 2 mM ascorbate was added (Scebba *et al.* 2001) After electrophoretic separation, gels were incubated for 15 min in the dark at 25 °C in 0.1 M phosphate buffer (pH 6.2) containing 4 mM ascorbic acid and 4 mM H_2O_2. The gels were subsequently washed with distilled water and stained in a solution containing 0.125 M HCl, 0.1 % (m/v) potassium ferricyanide and 0.1 % (m/v) ferric chloride. Gels were fixed in 50 % (v/v) glycerol after staining.

Bromophenol-blue was used as the marker for estimating R_f values, indicating the mobility of enzyme bands relative to the mobility of the bromophenol-blue front. Densitometry of the gels was performed with a *UVP Image Store 5000* (*Ultra Violet Product Ltd*, Cambridge, England) scanner and employing the *UVP GelBase TM Windows* Software. The area, expressed as arbitrary unit (a.u.), corresponding to the activity on gel of each isoform were calculated. Each value is the mean of four independent experiments. The data were analysed using analysis of variance and means were separated by Tukey's test ($P = 0.05$).

Results

Within 24 - 48 h after the fumigation, ML of the O_3 sensitive poplar clone Eridano developed round dark-black necrosis, localized in the interveinal area of the adaxial surface, while only slight marginal lesions were apparent in young apical leaves. In contrast, on both ML and YL of the O_3 resistant poplar clone I-214, visible leaf symptoms were absent.

Untreated control leaves of both clones showed four distinct SOD isoforms with R_f values of 0.54, 0.60, 0.65, and 0.71 (Fig. 1). The isoforms with R_f values of 0.60 and 0.71 were sensitive to both H_2O_2 and KCN (data not shown), indicating that they were Cu/ZnSODs. Insensitivity of the most cathionic isoform ($R_f = 0.54$) to both H_2O_2 and KCN (data not shown), suggested that it was the MnSOD. The isoform with R_f value of 0.65 was not identified until now, because low activity was still detected after KCN treatment. For the cathionic ($R_f = 0.60$) Cu/ZnSOD isozyme, a higher activity was observed in untreated O_3-S leaves in contrast to the resistant clone (Fig. 1, Table 1). In addition, control ML of O_3-S plants also showed, with respect to YL and O_3-R leaves, a higher activity of the anionic ($R_f = 0.71$)

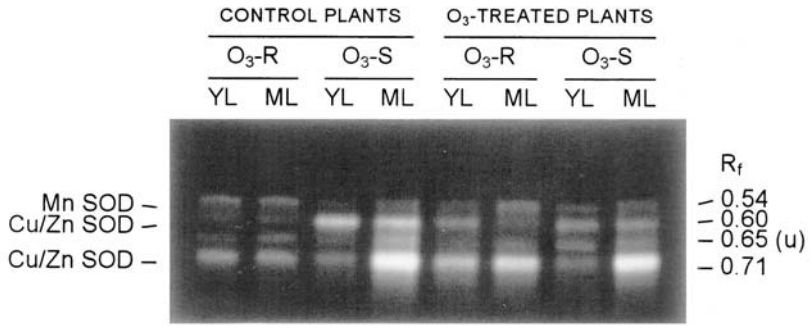

Fig. 1. SOD isozyme patterns of soluble leaf protein extracts from two poplar clones exposed to acute fumigation with 150 $mm^3\ m^{-3}$ of ozone for five hours. O_3-S = O_3-sensitive clone (Eridano, *Populus deltoides* × *P. maximowiczii*); O_3-R - O_3-resistant clone (I-214, *P.* × *euramericana*); ML - mature leaves; YL - young leaves; u - unidentified SOD isoform.

Table 1. Superoxide dismutase (SOD), peroxidase (POD) and ascorbate peroxidase (APX) isoforms of two poplar clones exposed to acute O_3-fumigation (150 mm^3 m^{-3} O_3 for five hours). The area, expressed as arbitrary unit, corresponding to the activity on gel of each isoforms were calculated by densitometry as described in Materials and methods. Each determination was done four times and the value represents the mean. Statistical analysis was done separately for every isoform; values within a row followed by a common letter are not significantly different at $P = 0.05$, according to Tukey's test. O_3-S - O_3-sensitive clone (Eridano, *Populus deltoides* × *P. maximowiczii*); O_3-R - O_3-resistant clone (I-214, *P.* × *euramericana*); ML - mature leaves; YL - young leaves; nd - not detected.

Enzyme	R_f	Controls				O_3-treated			
		O_3-R		O_3-S		O_3-R		O_3-R	
		YL	ML	YL	ML	YL	ML	YL	ML
SOD	0.54	165 a	163 a	101 a	145 a	139 a	189 a	102 a	148 a
	0.60	101 a	102 a	490 c	381 bc	193 a	114 a	278 b	257 ab
	0.65	134 a	153 a	112 a	189 a	102 a	103 a	122 a	143 a
	0.71	220 a	221 a	198 a	695 c	255 a	510 b	220 a	552 bc
POD	0.13	69 a	98 a	88 a	nd	64 a	67 a	nd	nd
	0.19	152 a	165 a	nd	154 a	115 a	230 a	156 a	224 a
	0.34	nd	220 b	nd	83 a	nd	221 b	nd	180 ab
	0.59	80 a	83 a	nd	nd	111 ab	153 ab	165 b	169 b
	0.64	192 a	295 ab	nd	nd	208 a	346 b	nd	nd
	0.66	nd	nd	98 a	nd	nd	nd	187 ab	294 b
	0.70	368 b	504 c	198 a	257 ab	447 bc	708 d	364 b	387 b
	0.75	109 a	283 b	nd	nd	193 ab	428 c	151 a	203 ab
APX	0.64	253 a	298 a	nd	nd	239 a	451 b	nd	nd
	0.66	nd	nd	nd	nd	nd	nd	180	nd
	0.70	305 b	326 b	nd	nd	330 b	590 c	212 ab	150 a
	0.76	230 a	289 a	nd	nd	249 a	247 a	nd	nd

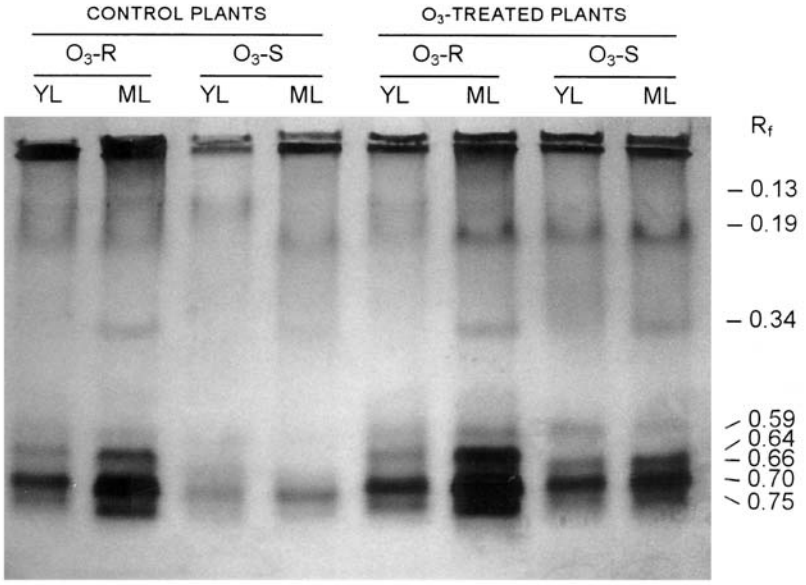

Fig. 2. POD isozyme patterns of soluble leaf protein extracts from two poplar clones exposed to acute fumigation with ozone; same conditions and legend as Fig. 1.

Cu/ZnSOD (Table 1). In O_3-R plants the O_3-exposure induced a rise in the intensity of the anionic (R_f = 0.71) Cu/ZnSOD band (Fig. 1, Table 1). By contrast, O_3-S leaves showed a reduced activity of the cathionic (R_f = 0.60) Cu/ZnSOD isoform in comparison to the control. After fumigation, ML also exhibited a minor intensity of the anionic (R_f = 0.71) Cu/ZnSOD isozyme (Fig. 1, Table 1).

Seven zones of POD activity were resolved in ML of untreated O_3-R plants (R_f = 0.13, 0.19, 0.34, 0.59, 0.64, 0.70 and 0.75) (Fig. 2). By contrast, in YL the isoform with R_f value of 0.34 was undetected. In untreated O_3-S

leaves a reduction in staining intensity and number of POD isoforms was observed, in comparison to the O_3-R clone (Fig. 2, Table 1). In fact, only three zones of POD activity in YL (R_f = 0.13, 0.66 and 0.70) and ML (R_f = 0.19, 0.34 and 0.70) were resolved. After fumigation, only negligible changes of POD isoforms were observed in YL of the O_3-R clone, while in ML the activity of all POD isozymes were clearly increased (Fig. 2, Table 1). In the O_3-S clone the pattern of peroxidases was greatly modified by O_3 exposure (Fig. 2). In fact, treated YL were characterized by the appearance of three isoforms (R_f = 0.19, 0.59 and 0.75) undetected in control plants, and by the disappearance of the POD isozyme with R_f of 0.13. Also in ML, along with the enhanced activity of POD isozymes with R_f values of 0.34 and 0.70, O_3-exposure induced the appearance of three new POD isoforms (R_f = 0.59, 0.66 and 0.75).

Three well-resolved bands (R_f = 0.64, 0.70 and 0.76) of APX were detected in both YL and ML of untreated O_3-R plants (Fig. 3). By contrast, zones of APX activity were not detected in untreated O_3-S leaves. After acute O_3-fumigation the activity of the three APX isoforms in YL of the O_3-R clone remained almost unaltered (Fig. 3, Table 1), while in ML a rise in activity was observed especially for the APX isoforms with R_f of 0.64 and 0.70. In YL of the O_3-S clone, O_3-exposure induced the appearance of two APX isozymes (R_f = 0.66 and 0.70), while a single faint band of APX activity (R_f = 0.70) was detected in fumigated ML (Fig. 3).

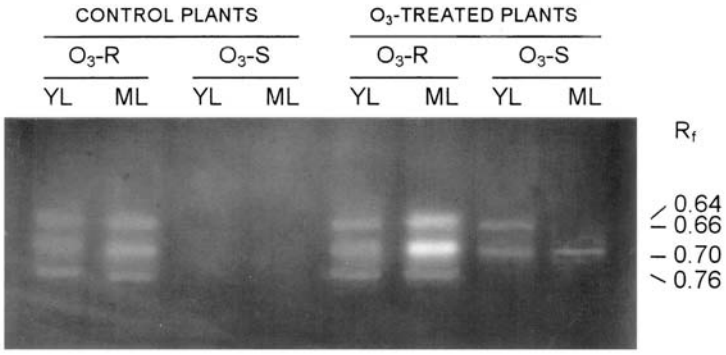

Fig. 3. APX isozyme patterns of soluble leaf protein extracts from two poplar clones exposed to acute fumigation with ozone; same conditions and legend as Fig. 1.

Discussion

In the present study, we have shown that, in poplar SOD, POD and APX isozymes exhibited differential patterns of activity in relation to genotype, leaf development and O_3 exposure. Generally, in both poplar hybrids tested the contents of all isoforms of the three-enzymes were highest in fully expanded leaves compared to those of young leaves. It has been shown that several enzymatic systems (*e.g.*, POD, catalase, SOD) are developmentally regulated (Kuroda *et al.* 1990, Acevedo and Scandalios 1991, Zhu and Scandalios 1993, Pramarik *et al.* 1996, Kurepa *et al.* 1997, Alonso *et al.* 1999). In particular, in both poplar clones, profound differences between YL and ML were observed for POD isozymes. An increased ROS generation due to aging could account for these differences (Bestwick *et al.* 1997, Pellinen *et al.* 1999). In addition, the increase of the peroxidase activity during leaf maturation, could be associated with the biosynthesis of cell wall and lignin (Van Huystee 1987).

Ozone toxicity is thought to result from direct damage by this oxidizing compound, as well as from the generation of O_3-derived ROS (Sandermann *et al.* 1998). The metabolism of ROS is dependent on various functionally interrelated antioxidant enzymes, such as catalase, POD, SOD, APX and glutathione reductase (Tanaka 1994). Many studies have shown that plants with high levels of antioxidants, either constitutive or induced, are more resistant to oxidative damage (Tanaka *et al.* 1985, Shaaltiel *et al.* 1988, Perl-Treves and Galun 1991, Perl *et al.* 1993, Van Camp *et al.* 1994, Benes *et al.* 1995, Pitcher and Zilinskas 1996, Chernikova *et al.* 2000, Bernardi *et al.* 2001a). In poplar, increased activity of both Cu/ZnSOD isoforms and MnSOD after 90 min of O_3 treatment has been reported (Sen Gupta *et al.* 1991). In experimental conditions here described, only negligible differences in MnSOD activity of both clones were observed between controls and O_3-treated plants. By contrast, O_3-exposure induced an increased activity of the anionic Cu/ZnSOD in the O_3-R clone. Although untreated O_3-S plants had a higher activity of Cu/ZnSOD isozymes compared to the resistant clone, O_3-exposure induced a reduction in staining intensity especially for the cathionic isoform. Likewise, Cu/ZnSOD appeared to be increased by O_3-exposure in leaves of the O_3-tolerant cv. Essex of soybean, but not in those of the O_3-sensitive

cv. Forrest (Chernikova *et al.* 2000). The decreased activity of the cathionic Cu/ZnSOD may be closely associated with the cellular damages that characterized the clone O_3-S after O_3 fumigation. In O_3-treated plants of the O_3-sensitive cv. PBD6 of *Nicotiana tabacum* none of the SODs showed any major alteration in mRNA levels before the onset of visible damage, FeSOD mRNA levels only decreased dramatically in the damaged leaf (Willekens *et al.* 1994).

Profound differences in POD and APX patterns were observed between the two poplar clones. Untreated O_3-S leaves showed a reduction in staining intensity and number of POD isoforms in comparison to the O_3-R clone. Moreover, in the sensitive clone zones of APX activity were only detected after O_3-exposure.

The first compartment of leaves that pollutants reach, after passing through the stomata and before reacting with the plasma membrane, is the apoplast. Extracellular PODs can play an important role in protecting plasma membranes from injury through air pollutants or secondary products (Weil and Schaub 1999, Ranieri *et al.* 2000). In our previous experiments (Ranieri *et al.* 1996), the presence of peroxidase activity has been observed in IWF of both O_3-R and O_3-S poplar clones. After O_3-exposure, this activity was stimulated when compared to untreated controls. Analogously, in this work we shown that, acute O_3-fumigation induced a general rise in the activity of POD isozymes in both YL and ML of the O_3-S clone and in ML of the resistant one. Moreover, O_3 treatments were effective to generate in O_3-S leaves (both YL and ML) the appearance of new POD isoforms, undetected in unexposed leaves, and the disappearance in YL of a cathionic POD isoform resolved in untreated plants.

Although after O_3 fumigation the activity of the three APX isoforms in YL of the O_3-R poplar clone remained almost unaltered, in ML a rise in activity for two APX isoforms (R_f = 0.64 and 0.70) was evidenced. An increased APX and glutathione reductase activity has been frequently observed after exposure to O_3 (Tanaka *et al.* 1985, Castillo and Greppin 1987, Mehlhorn *et al.* 1987, Kubo *et al.* 1995, Rao *et al.* 1996, Pell *et al.* 1999, Yoshimura *et al.* 2000). It is a reasonable assumption that if ascorbate in higher plant leaves is oxidized by O_3, the increases in the APX activity as a result of fumigation may contribute to the regeneration of the reductant. Ascorbate can serve as a scavenger, as well as indirectly by regenerating the membrane-bound scavanger α-tocopherol to its reduced form. Spinach cultivars with higher ascorbate content had a higher tolerance to O_3 (Tanaka *et al.* 1985, Tanaka 1994). Ranieri *et al.* (1998) reported an increase of APX activity, associated with a rise of ascorbic acid, in IWF and RCM of Eridano and I-214 clones exposed to O_3. Probably, these results indicate the action of apoplastic APX (and of ascorbic acid, as specific substrate for APX and as antioxidant able to scavenge directly ROS) in depressing H_2O_2, produced as a consequence of O_3 fumigation. The stimulation of the enzymatic and non enzymatic scavengers in RCM suggests that apoplastic defence alone is not sufficient to detoxify ROS produced following O_3 treatment.

The different activities of POD and APX isoforms displayed by the two poplar clones, O_3-S and O_3-R, could partly explain their differential sensitivity against acute injury. Further studies examining the expression of genes encoding antioxidant enzymes, recently isolated in these clones (Durante *et al.* unpublished results), will provide new insights into the role of these enzymes on poplar response to O_3.

References

Acevedo, A., Scandalios, J.G.: Catalase and superoxide dismutase gene expression and distribution during stem development in maize. - Dev. Genet. **12**: 423-430, 1991.

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S.: Antioxidative defense and photoprotection in *Pinus halepensis* induced by Mediterranean conditions and ozone exposure. - Free Rad. Res. **31**: 59-65, 1999.

Asada, K.: Production and action of active oxygen species in photosynthetic tissues. - In: Foyer, C.H., Mullineaux, P.M. (ed.): Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. Pp. 77-104. CRC Press, Boca Raton 1994.

Barnes, J., Bender, J., Lyons, T., Borland, A.: Natural and man-made selection for air pollution resistance. - J. exp. Bot. **50**: 1423-1435, 1999.

Beauchamp, C.O., Fridovich, I.: Superoxide dismutase: improved assays and an assays applicable to acrylamide gels. - Anal. Biochem. **44**: 276-287, 1971.

Benes, S.E., Murphy, T.M., Anderson, P.D., Houpis, J.L.: Relationship of antioxidant enzymes to ozone tolerance in branches of mature ponderosa pine (*Pinus ponderosa*) trees exposed to long-term, low concentration, ozone fumigation and acid precipitation. - Physiol. Plant. **94**: 124-134, 1995.

Bernardi, R., Nali, C., Gargiulo, R., Pugliesi, C., Lorenzini, G., Durante, M.: Changes in protein synthesis and in superoxide dismutase activity in *Phaseolus* bean plants under ozone stress. - J. Plant Pathol. **83**: 91-100, 2001a.

Bernardi, R., Nali, C., Gargiulo, R., Pugliesi, C., Lorenzini, G., Durante, M.: Protein pattern and Fe-superoxide dismutase activity of bean plants under sulphur dioxide stress. - J. Phytopathol. **149**: 477-480, 2001b.

Berrang, P., Karnosky, D.F., Bennet, J.P.: Natural selection for ozone tolerance in *Populus tremuloides*: an evaluation of nationwide trend. - Can. J. Forest Res. **21**: 1091-1097, 1991.

Bestwick, C.S., Brown, I.R., Benneth, M.H.R., Mansfield, J.W.: Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas*

syringae pv *phaseolicola*. - Plant Cell **9**: 209-221, 1997.

Bowler, C., Van Camp, W., Van Montagu, M., Inzé, D.: Superoxide dismutase in plants. - Crit. Rev. Plant Sci. **13**: 199-218, 1994.

Brewbaker, J.L., Upadhya, M.D., Mäkinen, Y., MacDonald, T.: Isoenzyme polymorphism in flowering plants. III Gel electrophoretic methods and applications. - Physiol. Plant. **21**: 930-940, 1968.

Castillo, F.J., Greppin, H.: Extracellular ascorbic acid and enzyme activities related to ascorbic acid metabolism in *Sedum album* L. leaves after ozone exposure. - Environ. exp. Bot. **28**: 231-238, 1997.

Chernikova, T., Robinson, J.M., Lee, E.H., Mulchi, C.L.: Ozone tolerance and antioxidant enzyme activity in soybean cultivars. - Photosyn. Res. **64**: 15-26, 2000.

Coleman, M.D., Isebrands, J.G., Dickson, R.E., Karnosky, D.F.: Photosynthetic productivity of aspen clones varying in sensitivity to tropospheric ozone. - Tree Physiol. **15**: 585-592, 1995.

Creissen, G.P., Edwards, A.E., Mullineaux, P.M.: Glutathione reductase and ascorbate peroxidase. - In: Foyer, C.H., Mullineaux, P.M. (ed.): Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. Pp. 343-364. CRC Press, Boca Raton 1994.

Gressel, J., Galun, E.: Genetic control of photooxidant tolerance. - In: Foyer, C.H., Mullineaux, P.M. (ed.): Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. Pp. 237-273. CRC Press, Boca Raton 1994.

Guidi, L., Nali, C., Ciompi, S., Lorenzini, G., Soldatini, G.F.: The use of chlorophyll fluorescence and leaf gas exchange as methods for studying the different responses to ozone of two bean cultivars. - J. exp. Bot. **48**: 173-179, 1997.

Kangasjärvi, J., Talvinen, J., Utrianen, M., Karjalainen, R.: Plant defense systems induced by ozone. - Plant Cell Environ. **17**: 783-794, 1994.

Kronfuss, G., Wieser, G., Havranek, W.M., Polle, A.: Effects of ozone and mild drought stress on total and apoplastic guaiacol peroxidase and lipid peroxidation in current-year needles of young Norway spruce (*Picea abies* L., Karst.). - Plant Physiol. **148**: 203-206, 1996.

Kubo, A., Saji, H., Tanaka, R., Kondo, N.: Expression of arabidopsis cytosolic ascorbate peroxidase gene in response to ozone or sulfur dioxide. - Plant mol. Biol. **29**: 479-489. 1995.

Kurepa, J., Hérouart, D, Van Montagu, M., Inzé, D.: Differential expression of CuZn- and Fe-superoxide dismutase genes of tobacco during development, oxidative stress, and hormonal treatments. - Plant Cell Physiol. **38**: 463-470, 1997.

Kuroda, M., Ozawa, T., Imagawa, H.: Changes in chloroplast peroxidase activities in relation to chlorophyll loss in barley leaf segments. - Physiol. Plant. **80**: 555-560, 1990.

Lewis, N.G., Davin, L.B., Sarkanen, S.: The nature and function of lignins. - In: Pinto, P.M. (ed.): Comprehensive Natural Product Chemistry. Vol. 3. Pp. 617-745. Elsevier, Amsterdam 1999.

Lorenzini, G., Medeghini Bonatti, P., Nali, C., Baroni Fornasiero, R.: The protective effect of rust infection against ozone, sulphur dioxide and paraquat toxicity symptoms in broad bean. - Physiol. mol. Plant Pathol. **45**: 263-279, 1994.

Mehlhorn, H., Cottam, D.A., Lucas, P.W., Wellburn, A.R.: Induction of ascorbate peroxidase and glutathione reductase activities by interactions of mixtures of air pollutants. - Free Rad. Res. Commun. **3**: 193-198, 1987.

Moldau, H.: Ozone detoxification in the mesophyll cell wall during a simulated oxidative burst. - Free Rad. Res. **31**: 19-24, 1999.

Nali, C., Guidi, L., Filippi, F., Soldatini, G.F., Lorenzini, G.: Photosynthesis of two poplar clones contrasting in O_3 sensitivity. - Trees **12**: 196-200, 1998.

Noctor, G., Foyer, C.H.: Ascorbate and glutathione: keeping active oxygen under control. - Annu. Rev. Plant Physiol. Plant mol. Biol. **49**: 249-279, 1998.

Paakkonen, E., Vahala, J., Holopainen, T., Karjalainen, R., Kärenlampi, L.: Growth responses and related biochemical and ultrastructural changes in the photosynthetic apparatus in birch (*Betula pendula*) sampling exposed to low concentration of ozone. - Tree Physiol. **16**: 597-605, 1996.

Pell, E.J., Schlagnhaufer, C.D., Arteca, R.N.: Ozone-induced oxidative stress: Mechanism of action and reaction. - Physiol. Plant. **100**: 264-273, 1997.

Pell, E.J., Sinn, J.P., Brendley, B.W., Samuelson, L., Vinten-Johansen, C., Tien, M., Skillman, J.: Differential response of four tree species to ozone-induced acceleration of foliar senescence. - Plant Cell Environ. **22**: 779-790, 1999.

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

Perl, A., Perl-Treves, R., Galili, S., Aviv, D., Shalgi, E., Malkin, S., Galun, E.: Enhanced oxidative-stress defense in transgenic potato expressing tomato Cu,Zn superoxide dismutases. - Theor. appl. Genet. **85**: 568-576, 1993.

Perl-Treves, R., Galun, E.: The tomato Cu,Zn superoxide dismutase genes are developmentally regulated and respond to light and stress. - Plant mol. Biol. **17**: 745-760, 1991.

Pitcher, L.H., Zilinskas, B.A.: Overexpression of copper/zinc superoxide dismutase in the cytosol of transgenic tobacco confers partial resistance to ozone-induced foliar necrosis. - Plant Physiol. **110**: 583-588, 1996.

Pramarik, S., Raychaudhuri, S.S., Chakraborty, S.: Changes in esterase and superoxide dismutase isoenzymes during *in vitro* morphogenesis in *Plantago ovata* Forsk. - Plant Cell Tissue Organ Cult. **44**: 123-127, 1996.

Ranieri, A., Nali, C., Amoroso, S., Lorenzini, G., Soldatini, G.F.: Are peroxidase systems related to different ozone resistance of poplar clones? - In: Obinger, C., Burner, U., Ebermann, R., Penel, C., Greppin, H. (ed.): Plant Peroxidase: Biochemistry and Physiology. Pp. 352-357. University of Geneva, Geneva 1996.

Ranieri, A., Castagna, A., Amoroso, S., Nali, C., Lorenzini, G., Soldatini, G.F.: Ascorbate levels and ascorbate peroxidase activation in two differently sensitive poplar clones as a results of ozone fumigation. - In: De Kok, L.J., Stulen, I. (ed.): Responses of Plant Metabolism to Air Pollution and Global Change. Pp. 435-438. Backhuys Publishers, Leiden 1998.

Ranieri, A., Petacco, F., Castagna, A., Soldatini, G.F.: Redox state and peroxidase system in sunflower plants exposed to ozone. - Plant Sci. **159**: 159-167, 2000.

Rao, M.V., Paliyath, G., Ormrod, D.P.: Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. - Plant Physiol. **110**: 125-136, 1996.

Sandalio, L.M., Palma, J.M., del Río, L.A.: Localization of

manganese superoxide dismutase in peroxisomes isolated from *Pisum sativum* L. - Plant Sci. **51**: 1-8, 1987.

Sandermann Jr., H., Ernst, D., Heller, W., Langebartels, C.: Ozone: an abiotic elicitor of plant defence reactions. - Trends Plant Sci. **3**: 47-50, 1998.

Scandalios, J.G.: Oxygen stress and superoxide dismutases. - Plant Physiol. **101**: 7-12, 1993.

Scebba, F., Sebastiani, L., Vitagliano, C.: Activities of antioxidant enzymes during senescence of *Prunus armenica* leaves. - Biol. Plant. **44**: 41-46, 2001.

Schwanz, P., Häberle, K.-H., Polle, A.: Interactive effects of elevated CO_2, ozone and drought stress on the activities of antioxidative enzymes in needles of Norway spruce trees (*Picea abies* [L.] Karsten) grown with luxurious N-supply. - J. Plant Physiol. **148**: 351-355, 1996.

Sen Gupta, A., Alsher, R., McCune, C.: Response of photosynthesis and cellular antioxidants to ozone in *Populus* leaves. - Plant Physiol. **96**: 650-655, 1991.

Shaaltiel, Y., Glazer, A., Bocion, P.F., Gressel, J.: Cross tolerance to herbicidal and environmental oxidants of plant biotypes tolerant to paraquat, sulfur dioxide, and ozone. - Pesticide Biochem. Physiol. **31**: 13-23, 1988.

Strohm, M., Eiblmeier, M., Langebartels, C., Jouanin J., Polle, A., Sandermann, H., Jr., Renneberg, H.: Responses of transgenic poplar (*Populus tremula* × *P. alba*) overexpressing glutathione synthetase or glutathione reductase to acute ozone stress: visible injury and leaf gas exchange. - J. exp. Bot. **50**: 365-374, 1999.

Tanaka, K.: Tolerance to herbicides and air pollutants. - In: Foyer, C.H., Mullineaux, P.M. (ed.): Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. Pp. 365-378. CRC Press, Boca Raton 1994.

Tanaka, K., Suda, Y., Kondo, N., Sugahara, K.: O_3 tolerance and the ascorbate-dependent H_2O_2 decomposing system in chloroplasts. - Plant Cell Physiol. **26**: 1425-1430, 1985.

Tuomainen, J., Pellinen, R., Roy, S., Kiiskinen, M., Eloranta, T., Karjalainen, R., Kangasjärvi, J.: Ozone effects birch (*Betula pendula* Roth) phenylpropanoid, polyamine and active oxygen detoxifying pathways at biochemical and gene expression level. - J. Plant Physiol. **148**: 179-188, 1996.

Van Camp, W., Van Montagu, M., Inzé, D.: Superoxide dismutases. - In: Foyer, C.H., Mullineaux, P.M. (ed.): Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. Pp. 317-341. CRC Press, Boca Raton 1994.

Van Huystee, R.B.: Some molecular aspects of plant peroxidase biosynthetic studies. - Annu. Rev. Plant Physiol. **38**: 205-219, 1987.

Weil, M., Schaub, H.: Influence of exhaust gas and ozone on extracellular peroxidase activity of *Helianthus annuus* L. leaves. - J. Plant Physiol. **154**: 523-528, 1999.

Wieser, G.: Ozone impact on photosynthetic capacity of mature and young Norway spruce (*Picea abies* (L.) Karst.): external versus internal exposure. - Phyton **37**: 297-302, 1997.

Wieser, G., Tausz, M., Wonisch, A., Havranek, W.M.: Free radical scavengers and photosynthetic pigments in *Pinus cembra* L. needles as affected by ozone exposure. - Biol. Plant. **44**: 225-232, 2001.

Willekens, H., Van Camp, W., Van Montagu, M., Inzé, D., Langebartels, C., Sandermann H., Jr.: Ozone, sulfur dioxide, and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumbaginifolia* L. - Plant Physiol. **106**: 1007-1014, 1994.

Yoshimura, K., Yabuta, Y., Ishikawa, T., Shigeoka, S.: Expression of spinach ascorbate peroxidase isoenzymes in response to oxidative stresses. - Plant Physiol. **123**: 223-233, 2000.

Zhu, D., Scandalios, J.G.: Maize mitochondrial manganese superoxide dismutases are encoded by a differential expressed multigene family. - Proc. nat. Acad. Sci. USA **90**: 9310-9314, 1993.