ACTA BIOLOGICA CRACOVIENSIA Series Botanica 51/2: 37–44, 2009

EFFECT OF TWO DIFFERENT AMBIENT OZONE CONCENTRATIONS ON ANTIOXIDATIVE ENZYMES IN LEAVES OF TWO TOBACCO CULTIVARS WITH CONTRASTING OZONE SENSITIVITY

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Received February 4, 2009; revision accepted September 2, 2009

Eight-week-old tobacco (*Nicotiana tabacum* L.) Bel W3 (ozone sensitive) and Bel B (ozone resistant) cultivars were exposed to ozone for two weeks at two sites with differing tropospheric ozone levels in five independent series from May 27 to July 25, 2004. After each exposition, the degree of ozone-caused visible leaf damage and the activity of APX, GuPX, and SOD were examined. Visible leaf damage was observed only in the sensitive cultivar; the resistant one did not exhibit any external symptoms. Three-way ANOVA revealed that the activity of all enzymes varied by exposure site, series and cultivar effects. Significant correlations between GuPX activity in the two cultivars and with the degree of leaf damage to the sensitive cultivar were found. This indicates that GuPX activity in the sensitive as well as in the resistant cultivars track changes in tropospheric ozone levels. The positive correlation between ozone level and APX activity in the resistant cv. Bel B, which did not reveal visible symptoms, indicates that this enzyme may contribute to detoxication of H_2O_2 and alleviation of oxidative damage caused by O_3.

Key words: Tobacco, tropospheric ozone, ascorbate peroxidase, guaiacol peroxidase, superoxide dismutase.

INTRODUCTION

Tropospheric ozone (O_3) has been recognized as the main phytotoxic air pollutant in urban, suburban and rural areas in developed countries (Stanners and Bourdeau, 1995; Ribas and Penuelas, 2003; Blum and Didyk, 2007). Ambient ozone concentrations in industrial countries may reach levels that exceed the tolerance threshold of many plants. Recognition of the importance of ozone as a stress factor which impacts plant health and productivity has been embodied in European Union law as a separate Ozone Directive (Directive 2002/2/EC) and, at international level, in a Geneva Convention – the 1999 Gothenburg Protocol to Abate Acidification, Eutrophication and Ground-level Ozone.

Ground-level ozone causes a decrease in yields of crop plants and accounts for a decrease of wooded areas (Chevone and Linzon, 1988; Ashmore and Ainsworth, 1995). Visible damage occurring in selected plant species (varieties) has become the basis for their use as bioindicators for tropospheric ozone. One of these plants is tobacco and its sensitive (Bel W3) and resistant (Bel B) cultivars (Heggestad, 1991). The first symptoms become apparent in sensitive cultivars on the upper side of the leaf as small necrotic spots. Later they enlarge and occur on both leaf sides (Krupa et al., 1998). Earlier investigations revealed a positive correlation between ozone concentration and visible leaf damage (Borowiak, 2005; Klumpp et al., 2006). This became the basis for using bioindication to complement continuous monitoring as a way of increasing the database without additional, costly measurements.

As a powerful oxidant, ozone is thought to affect plants by generating reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\cdot-}$) and hydrogen radicals ($OH\cdot$) (Pell et al., 1997; Langebartels et al., 2002). Accumulation of these ROS is probably the real cause of cellular and foliar damage (Langebartels

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PL ISSN 0001-5296

et al., 2002). Plants have evolved a number of antioxidative enzymatic and non-enzymatic defense mechanisms which efficiently remove these toxic ROS to alleviate cellular damage (Sharma and Davis, 1997; Calatayud et al., 2003; Apel and Hirt, 2004; Castagna and Ranieri, 2009). Among these enzymatic antioxidative systems are superoxide dismutase (SOD) and peroxidases, enzymes that prevent generation and accumulation of $O_2^{\cdot-}$ and H_2O_2, respectively. Among the peroxidases, it is known that ascorbate peroxidase (APX) uses ascorbate and guaiacol peroxidase (GuPX) as electron donors. Other authors have shown that higher activity of antioxidant enzymes protects plants against oxidative stress and leaf damage under conditions of increased ambient ozone levels (Chernikova et al., 2000; Scebba et al., 2003).

The aim of this study was to determine the activity of these antioxidative enzymes in ozone-sensitive (Bel W3) and ozone-resistant (Bel B) tobacco cultivars exposed at two sites of Poznan city, characterized by different ambient ozone concentrations. Another objective was to determine any correlations between leaf damage in the sensitive cultivar and the activity of the antioxidative enzymes.

MATERIAL AND METHODS

PLANT MATERIAL

Tobacco (*Nicotiana tabacum* L.) cv. Bel W3 (ozone sensitive) and cv. Bel B (ozone resistant) plants were grown and exposed to ozone according to the standard method of the EuroBionet project (Klumpp et al., 1999) and the German Engineering Association (VDI, 2000). Seeds were germinated under greenhouse conditions in a 1:8 (w/w) mixture of sand and standardized peat. Three-week-old seedlings were transplanted to large pots (1.5 dm^3) containing this soil mixture with fertilizer added. Each pot contained one plant. The plants were watered daily during the growth period. Eight-week-old plants were transported to two exposure sites differing in tropospheric ozone levels. One of the sites was in the center of Poznan city (Site 1) near a monitoring station for automatic measurements of air pollution. The second site was in Świerczewo, a suburb of Poznań (Site 2). These two exposure sites were chosen on the basis of previous work in this area showing visible differences in tobacco plant responses to tropospheric ozone (Borowiak, 2005). The daily mean value of ozone concentrations measured at Site 1 during the whole experimental period in 2004 was 52.1 $\mu g \cdot m^{-3}$; for comparison, the value in the corresponding period two years earlier reached 87.4 $\mu g \cdot m^{-3}$. Plants were exposed to ozone for 2 weeks under ambient conditions. Five exposure series were conducted from May 27 to July 25, 2004. After each exposition the plants were transported to the laboratory, where the degree of ozone-caused visible leaf damage and the activity of antioxidant enzymes were assessed. All measurements used the fifth leaf up from the bottom of the plant. Previous work showed the fifth leaf to be best for determining physiological responses, mainly because it is sufficiently mature (Borowiak et al., 2009). For SOD activity measurements, samples were frozen in liquid nitrogen and stored at -20°C until estimation the next day. Immediately after collection the samples for APX and GuPX activity were pulverized in liquid nitrogen and then homogenized in the appropriate buffer.

OZONE-CAUSED LEAF DAMAGE ASSESSMENT

Visible ozone damage occurred as bifacial necrosis. The degree of ozone damage, that is, the necrotic area, was assessed as a percentage of total leaf area, and employed EuroBionet pictorial atlas methodology (Klumpp et al., 1999), which recommends assessing percentages in 2% steps for the range of small damage (0, 2, 4, 6, 8, 10%) and 5% steps for higher leaf damage (15, 20, 25, 100%).

ENZYME EXTRACTION AND ASSAY

Ascorbate (APX) and guaiacol (GuPX) peroxidase

Samples (500 mg) were homogenized in a chilled mortar with 4 cm^3 0.1 M potassium phosphate buffer (pH 7.0) with 20 mg Polyclar AT added, and centrifuged at 16,000 g for 30 min. at 4°C. The supernatant was used for enzyme assays and protein measurements.

APX activity was determined according to Nakano and Asada (1987). The reaction mixture contained 2.3 cm^3 0.1 M potassium phosphate buffer (pH 7.0), 0.2 cm^3 5 mM L-ascorbate (As), 0.3 cm^3 1 mM H_2O_2 and 0.2 cm^3 enzyme extract. The hydrogen peroxide-dependent oxidation of As was followed by a decrease in absorbance at 290 nm (absorption coefficient 2.8 $mM^{-1} \cdot cm^{-1}$). APX activity was expressed as nkat $\cdot$ mg^{-1} protein.

GuPX activity was estimated according to Hammerschmidt et al. (1982). The reaction mixture contained 0.5 cm^3 enzyme extract, 0.5 cm^3 3.4 mM guaiacol and 0.5 cm^3 0.9 mM H_2O_2. The oxidation of guaiacol to tetraguaiacol in the presence of H_2O_2 was measured as the increase in absorbance recorded at 470 nm. Enzyme activity was calculated using the absorption coefficient for tetraguaiacol (absorption coefficient 26.6 $mM^{-1} \cdot cm^{-1}$), and expressed as nkat $\cdot$ mg^{-1} protein.

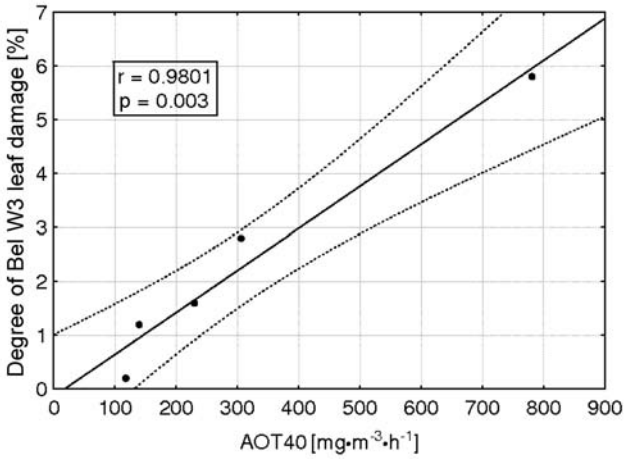

Fig. 1. Linear correlation between degree of leaf damage in ozone-sensitive tobacco cultivar (Bel W3) and ozone concentration measured at Site 1 (dotted lines represent 95% confidence interval).

Superoxide dismutase (SOD) (III)

Plant samples (500 mg) were homogenized in a chilled mortar with 4 cm^3 buffer (50 mM sodium phosphate buffer, pH 7.0, containing 1% polyvinylpolypyrrolidone, 1 mM EDTA-Na and 0.5 M NaCl) and centrifuged at 16,000 g for 25 min at 4°C. The supernatant was used for estimation of enzyme activity according to Beauchamp and Fridovich (1971). The incubation mixture contained 2.35 cm^3 50 mM sodium phosphate buffer (pH 7.8) with 0.1 mM EDTA-Na, 0.4 cm^3 97 mM methionine, 0.1 cm^3 2 mM NBT (nitro blue tetrazolium) and 0.05 cm^3 enzyme extract. Finally, 0.1 cm^3 120 μM riboflavin was added and the samples were placed under fluorescent lamps for 10 min. A blank without the enzyme extract was run at the same time. Absorbance was measured at 560 nm and the unit of activity was taken as the quantity of enzyme reducing absorbance to 50% of the blank.

Protein was determined according to Bradford (1976), with bovine serum albumin as the standard.

STATISTICAL ANALYSIS

Enzyme analyses for each combination were made in three determinations derived from three different samples of leaves from one plant. Three-way ANOVA was used to verify the effects of exposure site, exposure series and cultivar on the investigated parameters (Seber, 1982).

ANOVA provides no information about significant differences between selected pairs of populations, so we applied Tukey's multiple range test and presented those results graphically (Ott, 1984). The statistical analyses employed STATISTICA 8 (edition 0608c – PStatSoft Polska).

RESULTS

VISIBLE LEAF DAMAGE

Ozone-caused damage was visible in the sensitive tobacco cultivar, and not in the resistant cultivar. In the majority of exposure series, leaf damage was higher in samples from the site in the city center (Site 1). The extent of visible leaf damage in the sensitive cultivar at Site 1 ranged from 0.2% to 5.8%, and at Site 2 from 0.1% to 4.0%. Leaf damage at Site 1 was highest in the fifth exposure series (12–25.07.2004), when the ozone concentration was highest. At Site 2, on the other hand, ozone damage to leaves of the sensitive cultivar was highest (4.0%) during the third exposure series (14.–27.06.2004). Visible ozone symptoms were least extensive at both exposure sites during the first exposure series (17–30.05.2004), when measured ozone concentrations were the lowest.

Analysis of the correlations between visible ozone damage to the sensitive cultivar and the measured ozone concentrations at Site 1 revealed a positive and significant relationship (Fig. 1). This means that this damage can be used as an indicator of tropospheric ozone levels. We therefore used it to estimate the correlation with the antioxidative enzyme activity in both cultivars.

ENZYME ACTIVITY

On the basis of three-way ANOVA the null hypothesis was rejected for first-order effects, indicating that the effects of exposure site, exposure series and cultivar resulted in variation of all enzyme activities. The highest variability of enzyme activity was correlated with the effect of exposure series. The second- and third-order interactions were also significant (Tab. 1).

Tukey's test revealed significant differences in APX activity between the sensitive and resistant cultivars ($\alpha = 0.05$). APX activity varied between exposure series and site. In the sensitive cultivar it was highest in the first exposure series at Site 1, and in the fourth exposure series at Site 2 (Fig. 2a). In the resistant cultivar it was highest in the fourth and fifth exposure series at Site 1, and in the third exposure series at Site 2 (Fig. 2b). In both cultivars, APX activity was lowest at both exposure sites in the second exposure series (Fig. 2a,b). The sensitive Bel W3 cultivar was characterized by lower APX activity at Site 2, where visible leaf damage was lower (Fig. 3a). There was no correlation between visible leaf damage and APX activity in that cultivar (Tab. 2). APX activity in Bel B was positively correlated ($\alpha = 0.05$) with leaf damage in Bel W3 (Tab. 2).

TABLE 1. Three-way ANOVA of APX, GuPX and SOD activity in tobacco leaves

Source of variability	Degree of freedom	Sum of squares	Mean square	*F* ratio	Significance level p
		APX			
Series	4	890.186	222.547	157.039	<0.001
Site	1	6.911	6.911	4.877	0.033
Cultivar	1	10.611	10.611	7.487	0.009
Series*cultivar	4	396.801	99.200	70.000	<0.001
Series*site	4	279.598	69.900	49.324	<0.001
Cultivar*site	1	223.452	223.452	157.678	<0.001
Series*site*cultivar	4	398.928	99.732	70.375	<0.001
Error	40	56.686	1.417		
		GuPX			
Series	4	147.1733	76.7752	374.116	<0.001
Site	1	20.9168	36.7933	179.289	<0.001
Cultivar	1	76.7752	20.9168	101.925	<0.001
Series*cultivar	4	46.8465	11.7116	57.069	<0.001
Series*site	4	10.0007	2.5002	12.183	<0.001
Cultivar*site	1	24.7238	24.7238	120.476	<0.001
Series*site*cultivar	4	18.4517	4.6129	22.478	<0.001
Error	40	8.2087	0.2052		
		SOD			
Series	4	16376.94	4094.23	426.642	<0.001
Site	1	1395.73	1395.73	145.443	<0.001
Cultivar	1	310.85	310.85	32.392	<0.001
Series*cultivar	4	941.27	235.32	24.521	<0.001
Series*site	4	1834.86	458.71	47.801	<0.001
Cultivar*site	1	48.07	48.07	5.009	0.031
Series*site*cultivar	4	1441.54	360.38	37.554	<0.001
Error	40	383.86	9.60		

TABLE 2. Correlation coefficients and significance levels of relationship between enzyme activity levels in both tobacco cultivars and visible leaf damage in Bel W3 cultivar

Parameter	APX Bel W3	APX Bel B	GuPX Bel W3	GuPX Bel B	SOD Bel W3	SOD Bel B
Degree of visible leaf damage Bel W3	r = -0.085	r = 0.396	r = 0.741	r = 0.752	r = 0.188	r = -0.058
	p = 0.656	p = 0.030	p < 0.001	p < 0.001	p = 0.319	p = 0.762

In the sensitive cultivar, GuPX activity was highest in the second and fifth exposure series at Site 1, and in the second series at Site 2 (Fig. 2c). For the resistant cultivar, GuPX activity was highest in the fourth and fifth exposure series at Site 1 and in the fifth exposure series at Site 2 (Fig. 2d). GuPX activity at both exposure sites was significantly lower in Bel B than in Bel W3 (Fig. 3b). GuPX activity in the two cultivars was positively correlated (α <0.001) with leaf damage in Bel W3 (Tab. 2).

SOD activity in the Bel W3 tobacco cultivar was highest in the third exposure series at both sites (Fig. 2e). In Bel B, SOD activity was highest in the third exposure series at Site 1; at Site 2, in contrast, there were no significant differences in SOD activity between the series (Fig. 2f). At Site 2, SOD activity values in both cultivars were lower than at Site 1, and significantly lower in Bel B than in Bel W3 (Fig. 3c). There was no correlation between visible leaf damage in Bel W3 and SOD activity in either cultivar (Tab. 2).

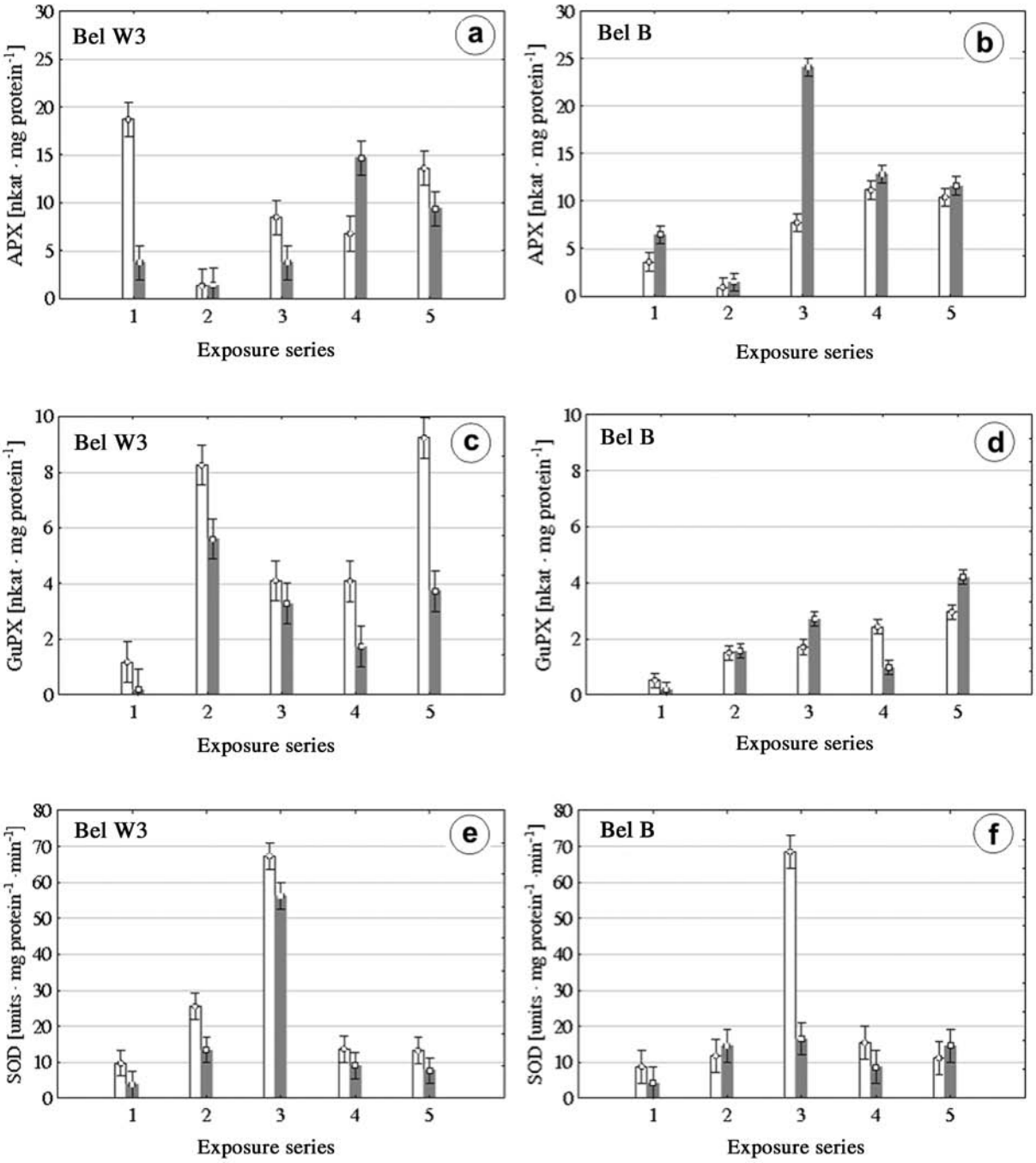

Fig. 2. APX, GuPX and SOD activity in ozone-sensitive (Bel W3) and ozone-resistant (Bel B) tobacco cultivars, by exposure series (whiskers represent standard deviations). White bars – Site 1; grey bars – Site 2.

DISCUSSION

The automatic air quality monitoring station recorded low and medium levels of ozone in the center of Poznań during the period of this study, and the results of our bioindication experiments were in accord with those data. We found visible leaf damage caused by ozone only in the sensitive cultivar, while the resistant one showed no gross symptoms. Visible ozone damage in the sensitive cultivar was positively and highly correlated with the measured tropospheric ozone concentrations (Fig. 1). These results are in keeping with those from similar studies conducted earlier in Poznań and other places,

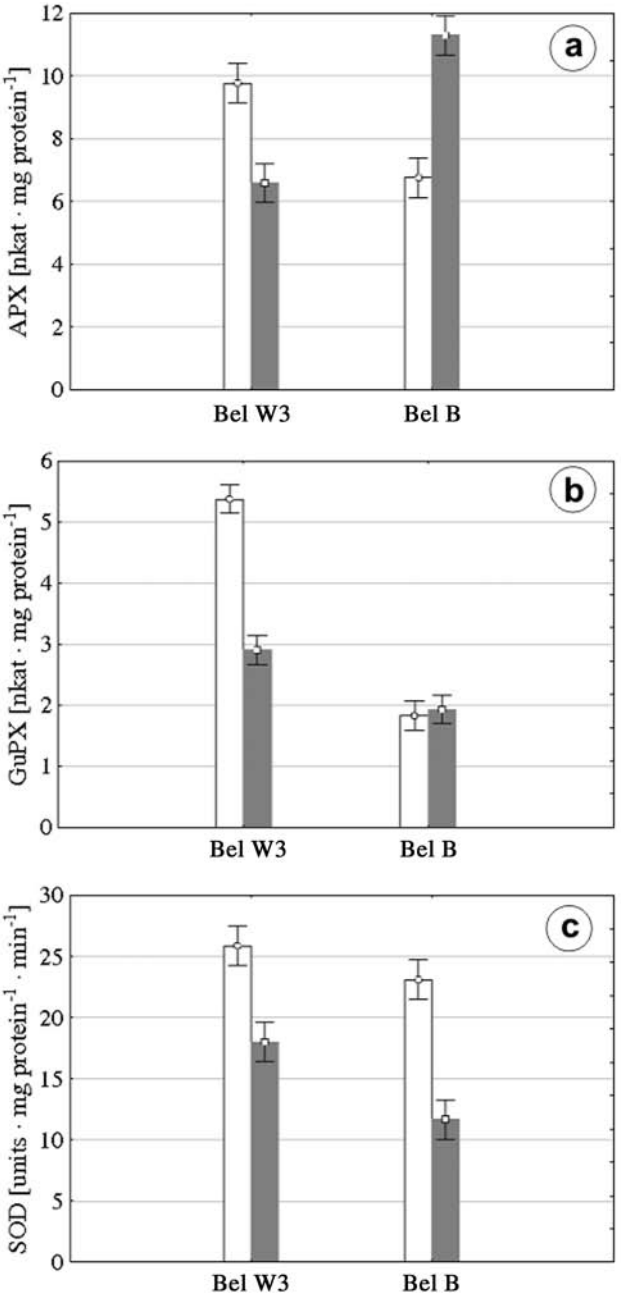

Fig. 3. APX, GuPX and SOD activity in ozone-sensitive (Bel W3) and ozone-resistant (Bel B) tobacco cultivars at exposure sites (whiskers represent standard deviations). White bars – Site 1; grey bars – Site 2.2

reporting high correlations between ozone concentration and visible leaf damage in ozone-sensitive tobacco (Godzik, 1997; Nali et al., 2001; Borowiak, 2005; Klumpp et al., 2006). Other bioindication studies have also suggested that the degree of leaf damage in the sensitive cultivar can be used as an indicator of ozone level (Borowiak et al., 2005, 2007, 2009). A high degree of leaf damage is typically observed in tobacco plants in many European suburban and/or rural areas, while in city centers this damage is low (Godzik, 2000; Klumpp et al., 2004, 2006). Ozone precursors normally are transported far from their emission sources, and the ozone is formed in favorable meteorological conditions in suburban and rural areas with high insolation (Jeffries and Tonnesen, 1994). During 2004, rainy weather atypical for the Wielkopolska region distorted the usual ozone concentration characteristics for urban, suburban and rural areas.

Ozone enters leaves through stomata, diffuses into the substomatal chamber and reacts with a number of compounds present in cell walls, apoplastic fluid and plasma membranes to generate reactive oxygen species (Castagna and Ranieri, 2008). Rapid H_2O_2 production accompanied by the development of necrotic lesions has been observed in O_3-treated ozone-sensitive tobacco, tomato cultivars and poplar clones. Schraudner et al., 1998; Langebartels et al., 2002). Wohlgemuth et al. (2002) reported that blocking of hydrogen peroxide and superoxide accumulation markedly reduced ozone-induced cell death in various plants. APX is recognized as one of the most efficient ROS-scavenging enzymes; this capacity is attributed to its presence in different cell compartments and its high affinity for H_2O_2 (Foyer et al., 1994; Castagna and Rannieri et al., 2008). Many authors have indicated that stimulation of APX in ozone-stressed plants plays a fundamental role in reducing O_3-derived toxic H_2O_2 concentrations (Ranieri et al., 2003; Diara et al., 2005; Nali et. al., 2005). According to Pasqualini et al. (2001), the higher ozone tolerance of cv. Bel B is associated with greater antioxidant enzyme activity. Our work showed a significant correlation between APX activity in the resistant cultivar and the degree of leaf damage in the sensitive one. This result attests to the high sensitivity of this enzyme to changes in ground-level ozone concentrations; the concurrence of that correlation with the lack of ozone-caused symptoms in the resistant cultivar suggests activation of the plant's antioxidative defense system, effectively preventing visible necrotic damage to the leaves. Pasqualini et al. (2001) showed that only ozone-tolerant cv. Bel B triggered activation of APX under ozone stress conditions.

Other authors have found GuPX to play a regulatory role in scavenging H_2O_2 under ozone and other stress conditions (Pukacka and Pukacki, 2000; Bandurska, 2002). Both resistant and sensitive cultivars show a positive and highly significant correlation between GuPX activity and the degree of leaf damage in sensitive cv. Bel W3. In our study, changes in GuPX activity in both cultivars tracked changes in tropospheric ozone levels, consistent with earlier investigations showing that ozone-sensi-

tive and ozone-tolerant snap bean genotypes did not differ in their GuPX activity under ozone stress conditions (Burkey et al., 2000). The data suggest that screening for ozone tolerance based on GuPX activity is not a reliable approach.

Wohlgemuth et al. (2002) showed that ozone-induced cell death and leaf damage in *Arabidopsis thaliana* accessions and *Malva sylvestris* is preceded by the formation of $O_2^{\cdot-}$. An important role in removing $O_2^{\cdot-}$ is played by SOD, which scavenges toxic superoxide radicals from various cellular compartments (Van Camp et al., 1994; Pitcher and Zilinskas, 1996; Scebba et al., 2003). In our work, SOD activity differed by series, site and cultivar effect, but there was no significant correlation between SOD activity and leaf damage in the sensitive cv. Bel W3 (Tab. 2). Other studies, in contrast, showed SOD activity to increase with the increase of ozone concentrations in the sensitive as well as the resistant cultivar (Borowiak et al., 2009). However, ozone-sensitive and ozone-resistant poplar clones exhibited different patterns of ozone-induced SOD activity (Bernardi et al., 2004).

Our experiment showed that GuPX activity can serve as a good biochemical marker of changes in ambient ozone levels, but it was not a reliable indicator of ozone tolerance. The positive correlation between ozone levels and APX activity in the resistant cv. Bel B, which did not exhibit visible symptoms, suggests that this enzyme may contribute to detoxication of H_2O_2 and alleviation of oxidative damage caused by O_3.

ACKNOWLEDGEMENT

We thank Stewart Brown for helpful comments on English usage in this paper.

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