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INFLUENCE OF OZONE ON THE ANTIOXIDANT CAPACITY AND ANTIOXIDANT LEVELS IN SEEDLINGS OF THREE CEREAL SPECIES AT EARLY STAGE OF DEVELOPMENT

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Key words: cereal leaves, ozone exposure, total phenolic compounds, glutathione, antioxidant capacity, superoxide dismutase-like activity

We studied effects of 7-h exposure of 70 ppb ozone during early developmental stage on three monocotyledonous crop species: oat (*Avena sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). After fumigation, levels of low molecular weight antioxidants, Trolox equivalent antioxidant capacity (TEAC) and superoxide dismutase-like activity (SOD) were examined. It has been concluded that ozone exposure caused negative effects on these cereals, although each cereal seemed to be affected differently. In contradiction to the generally held idea that young seedlings are not susceptible to ozone, we found effects of the short ozone episode on antioxidant capacity. There was an induction of TEAC in barley and of SOD-like activity in oat, while a decrease of TEAC was found in wheat and oat. Although no clear mechanistic scheme can be set up demystifying the results, it was proven that young seedlings can be influenced by low level ozone pollution. This information might be important when considering general effects of air pollution on monocot crop species.

INTRODUCTION

The potential impact of ozone on plants is of general interest to scientists and policy makers throughout the world. The generation of ozone (O_3) in the troposphere is known to pose a growing risk to vegetation in many industrialized regions [Chameides *et al*., 1994] and models suggest that ozone substantially reduces the yield of several crops and that the economic effect of these yield reductions may be important [Heck *et al.,* 1983]. It is well established that ozone has several effects on the biochemistry and physiology of terrestrial plants [Heath, 1994; De Temmerman *et al*., 2002]. The topic of ozone effects on antioxidant pools is one that has been studied in various plant systems for several decades. The phytotoxicity of O_3 originates from its powerful oxidising properties [Mehlhorn & Wellburn, 1988]. The gas enters leaves predominately through open stomata [Kerstiens & Lendzian, 1989], and dissolves into the aqueous phase of the mesophyll cell wall. Herein, the pollutant may generate additional reactive oxygen species (ROS) through reaction with biomolecules [Kanofsky & Sima, 1995] and the activation of plant responses reminiscent of those observed during incompatible plant-pathogen interaction [Schraudner *et al.,* 1998]. Acting in this manner, the defensive barrier in the leaf apoplast would prevent oxidative damage to the underlying membrane, thereby protecting against downstream effects of the pollutant on photosynthesis and, ultimately, plant productivity [Lyons *et al.,* 1999].

To prevent oxidative injury, plants possess a protective system composed of antioxidants, such as ascorbate and glutathione, and defence enzymes such as superoxide dismutases, peroxidases and catalases. In the chloroplasts and in the cytosol, antioxidative substrates and enzymes also interact in a series of reactions to remove the toxic oxidants and to regenerate the reduced, *i.e*. functional state of the antioxidants [Foyer & Halliwell, 1976]. Then, the efficiency of this integrated antioxidant system in plants has an important role on status of the main plant antioxidants [Arnao *et al.,* 1999]. Typically, plants show a large inter-specific and intra-specific variation in antioxidant levels, and the source of this variation is generally not recognized. Within a plant species, levels of low molecular weight antioxidants can be influenced by food-processing techniques, for example hydrothermal processing of whole grains [Nicoli *et al*., 1999; Zieliński & Troszyńska, 2000]. The most important, however, seems to be the impact of air pollution on plants during their early development. It can be suggested that any changes caused by air pollutants in plant at early stage of their development may result in lower antioxidant contents in the final crops being subjected to the food industry [Nicoli *et al*., 1999]. On the other hand, many plants have the capacity to compensate for early vegetative stress with no impact on yield and seed composition related to stress under final reproductive growth. The aim of this work was to find out the effect of a single-day ozone exposure on two-week old seedlings of three cereal species: oat, wheat and barley. The analysis of the effects of one-week after exposure was undertaken to evaluate higher plant responses to tropospheric ozone by measuring the content and redox status of some important antioxidants as well as antioxidant properties of three cereal species used in this study.

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MATERIAL AND METHODS

Chemicals

2,2' Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and myoglobin from horse heart, essentially salt free, were obtained from Sigma-Aldrich (St. Louis, Missouri). Bovine serum albumin, fraction V (BSA), reduced glutathione (GSH), N-ethylmaleimide (NEM), *o*-phthalaldehyde (OPA), (+) catechin and polyvinylpyrrolidone K 90 were obtained from Sigma (Sigma Chemical Co., St. Louis, MO). Superoxide dismutase determination kit (RANSOD, Cat No SD 125) was obtained from Randox Laboratories Ltd, UK. All other reagents of reagent-grade quality were from POCh, Gliwice, Poland.

Ozone exposure

Two-week old seedlings of three cereal species: oat (*Avena sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum Vulgare*) were subjected to 70 ppb of ozone or filtered air for 7 h on the fifth day after planting. The experiment was carried out in triplicate. The exposure conditions were 350 μmol/m^2/s photosynthetic active radiation, 24°C and 60% relative humidity. Ozone was generated by electric discharge passing pure oxygen through a Fisher ozone generator 500 (Fisher Labor und Verfahrenstechnik, Meckenheim, Germany). Ozone fumigation in the fumigation chamber was continuously monitored with an Environment S.A. Monitor operating on the principle of UV absorption and interfaced with a personal computer. After ozone exposure, the plants were then left to grow for another 7 days. Subsequently leaves were frozen in liquid nitrogen and lyophilized overnight.

Analytical methods

All lyophilized leaves were ground and then extracted in triplicate with phosphate buffered saline (PBS) pH 7.4 (4 mL per 0.5 g of dry leaf material) during 2 h of shaking at room temperature. They were then centrifuged at 12,000 × *g* for 10 min at 4°C in a Beckman GS-15 R centrifuge (Beckman Instruments, Inc., Palo Alto, CL., U.S.A.) and the fresh supernatants were used to determine their ability to scavenge superoxide anions, the content of soluble proteins and total phenolic compounds. In order to prepare methanol extracts, freeze-dried samples were ground and then extracted with 80% aqueous methanol (4 mL per 0.5 g of dry leaf material) on an electromagnetic stirrer with continuous mixing for 2 h at ambient temperature. The mixture was then centrifuged at 12,000 × *g* for 10 min at 4°C in a Beckman GS-15 R centrifuge and fresh supernatants were used to determine Trolox equivalent antioxidant capacity (TEAC) and total phenolic compounds.

The concentration of soluble protein in the extracts was measured using the Bradford protein microassay with bovine serum albumin (BSA) as a standard [Bradford, 1976]. The content of total phenolic compounds (TPC) was determined according to Shahidi & Naczk [1995]. Exactly 0.25 mL aliquots of the PBS or methanol extracts were mixed with 0.25 mL Folin-Ciocalteu's reagent (previously diluted with water 1:1 v/v) and 0.5 mL saturated sodium carbonate (Na_2CO_3) solution and 4 mL water. The mixture was allowed to stand at room temperature for 25 min and then was centrifuged at 2,000 × *g* for 10 min. Supernatant absorbance was measured at 725 nm using a spectrophotometer (UV-160 1PC, Shimadzu, Japan). The data were calculated as (±) catechin equivalents.

Reduced glutathione (GSH) and oxidised glutathione (GSSG) were determined according to the spectrofluorometric method [Hissin & Hilf, 1976]. This method was based on the reaction of o-phthalaldehyde (OPA) as a fluorescent reagent with GSH at pH 8.0 and with GSSG at pH 12.0; GSH was complexed to N-ethylmaleimide (NEM) to prevent interference of GSH with measurement of GSSG. Extraction was conducted according to Smith *et al*. [1988]. Freeze-dried samples (500 mg) were transferred to a centrifuge tube and mixed with 3 mL of 0.1 mol/L phosphate buffer (pH 7.5) containing 5 mmol/L EDTA and 1% (m/v) polyvinylpyrrolidone K 90. The detailed procedure was described previously [Zieliński *et al.,* 1999]. The assays were performed using a Perkin-Elmer LS 50 B Luminescence Spectrometer (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England).

The relative ability of antioxidants to scavenge radical cation $ABTS^{\bullet+}$ was measured by a spectrophotometric technique in comparison with the antioxidant potency of standard amounts of Trolox [Miller & Rice-Evans, 1996]. The determination of TEAC was carried out using the Randox kit (Randox Laboratories Ltd. U.K. Cat. No. NX2332). The antioxidant capacity (TEAC) was evaluated by spectrophotometric measurement of radical cation $ABTS^{\bullet+}$ generated from ABTS in the presence of metmyoglobin and hydrogen peroxide (UV-160 1PC with CPS-Controller, Shimadzu, Japan).

The superoxide dismutase-like activity of the extracts was measured using the superoxide dismutase kit. The assays were performed using a thermostated recording spectrophotometer (UV-160 1PC with CPS-Controller, Shimadzu, Japan) adjusted to 37°C inside the cuvettes. The test required 25 μL of sample, with a read time of 3 min. The results were finally recalculated on miligrams of soluble protein. A standard superoxide dismutase with an activity of 5.3 U/mL was supplied as part of the reagent kit. In general, one unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of reduced adenine nucleotides (NADH, NADPH) oxidation of the control by 50%. Extrapolation of 50% inhibition values in the samples allows calculating the enzyme activity. The percentage of the reaction inhibition was plotted against $\log_{10}$ of different SOD activities (SOD/mL) giving a standard curve and the SOD activity of the sample was then expressed as SOD units/mL of the investigated extract.

Statistical analysis

The results are given as the means and the standard deviation of three independent experiments in time. All analytical measurements were carried out in triplicate. Statistical analysis was performed using the Student's t-test at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

Trolox equivalent antioxidant capacity of both PBS and

methanol extracts ranged between 25 and 50 μmol of Trolox equivalents per g dry weight in control cereal species (Figure 1). The TEAC of control oat and control wheat leaves had the highest values whereas the one noted for barley leaves was lower by about 50%. The 7-h exposure to O_3 induced a decrease in TEAC values of PBS and methanol extracts of oat and wheat leaves, but the differences were statistically significant only in respect to the methanol extracts. In contrast, TEAC values increased significantly in the PBS extracts of barley leaves exposed to O_3.

The phenolic compounds found in methanol extracts of oat and wheat leaves were constitutively higher by 34% and 39% when compared to the extract originating from barley leaves (Figure 2A). The fumigation treatment of oat and wheat leaves induced a statistically insignificant decrease in phenolic contents by 3% and 15% when compared to those exposed to the filtered air. In contrast, an opposite response was noted in barley leaves exposed to O_3 where an increase of TPC content by 4% was observed in respect to the non-exposed leaves. The content of phenolic compounds found in PBS extracts was similar in oat and wheat leaves, but lower in barley leaves reaching only 43% of the average noted in the previous ones (Figure 2B). The fumigation treatment induced a decrease in phenolic contents of wheat leaves by 22% when compared to those exposed to the filtered air. In contrast, an opposite response was noted in oat and barley leaves exposed to O_3, respectively, where increases of TPC content by 13% and 18% were observed in respect to the non-exposed leaves. However, the changes caused by ozone fumigation in methanol and PBS extracts were not statistically significant.

The content of total glutathione found in untreated oat and wheat leaves was 8% and 27% higher than in barley leaves, respectively (Table 1). The reduced/oxidized ratio of glutathione was strikingly three times higher in oat leaves when compared to barley and wheat. The ozone exposure caused a 21% decrease of total glutathione in oat whereas the decrease in wheat was only 6%. In barley no ozone effects were observed in total glutathione content. The presence of ozone exposure-induced oxidative stress was confirmed by the increased content of glutathione disulfide (GSSG) by 8%, 14% and 9% in

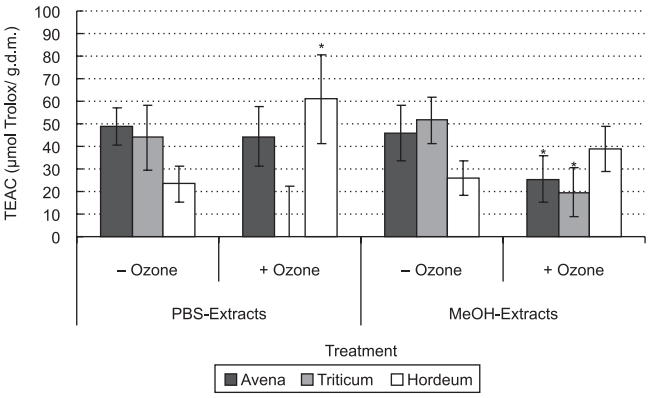

FIGURE 1. Trolox equivalent antioxidative capacity (TEAC) of three cereal species during early development stage exposed for 7 h to 70 ppb ozone (+ O_3) or filtered air (– O_3). Freeze-dried material was extracted in either PBS (left bars) or 80% methanol (right bars). Values represent means ± SD, n = 3 indicates the number of the independent experiments in time. Significance of differences for Student's t-test : *, $p<0.05$.

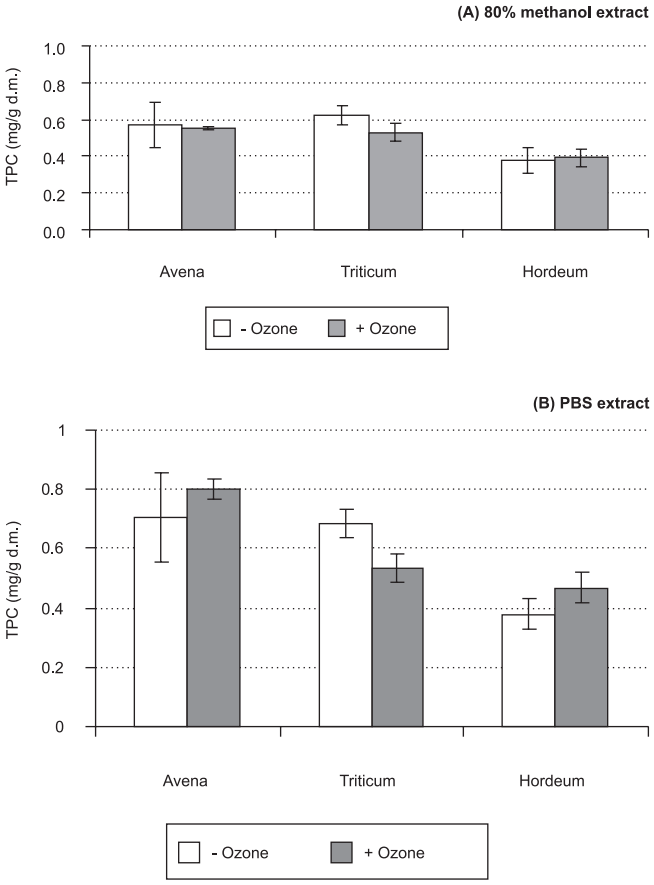

FIGURE 2. Total phenolic compounds (TPC) expressed as catechin equivalents in three cereal species during early development stage exposed for 7 h to 70 ppb ozone (+ O_3) or filtered air (– O_3). Freeze-dried material was extracted in 80% methanol (A) or in PBS (B). Values represent means ± SD, n = 3 indicates the number of the independent experiments in time. Significance of differences for Student's t-test as in Figure 1.

TABLE 1. Levels (nmol/g dry weight) of reduced (GSH), oxidized (GSSG) and total (GSH+GSSG) glutathione as well as the reduced/oxidized glutathione status (GSH/GSSG) in three cereal species exposed for 7 h to 70 ppb ozone (+ O_3) or filtered air (– O_3).

Treatment	GSH		GSSG		Total glutathione (GSH+GSSG)		GSH/GSSG ratio	
	$-O_3$	$+O_3$	$-O_3$	$+O_3$	$-O_3$	$+O_3$	$-O_3$	$+O_3$
Avena	290.5 ± 26.2	221.5 ± 2.1	31.1 ± 2.2	33.9 ± 5.3	32.6 ± 24.0	256.0 ± 6.5	9.4 ± 1.5	6.5 ± 0.8
Triticum	300.2 ± 12.6	259.2 ± 4.7	103.7 ± 3.5	121.1 ± 4.1	403.9 ± 16.1	380.4 ± 9.0	2.9 ± 0.1	2.1 ± 0.1
Hordeum	226.5 ± 4.9	221.5 ± 2.1	69.4 ± 5.6	76.1 ± 6.5	295.9 ± 10.6	297.6 ± 8.6	3.3 ± 0.2	2.9 ± 0.2

oat, wheat and barley leaves, respectively, when compared to those exposed to filtered air. The calculated GSH/GSSG ratio was also decreased in all three cereal species after fumigation.

In this study the superoxide dismutase-like activity (SOD--like activity) of phosphate buffered saline extracts was determined using the xanthine/xanthine oxidase (XA/XOD) system. The SOD-like activity of the investigated extracts is shown in Figure 3. A statistically significant increase of SOD-like activities after ozone exposure was found only in the extracts from wheat leaves whereas no changes were observed in oat and barley leaves.

Ozone has frequently been recognized as an abiotic stress factor and it has been generally accepted that sensitivity to O_3 was found to be positively correlated with leaf age [Guderian *et al*., 1985]. Young seedlings of monocot crops are believed to be fairly insensitive to ozone exposure but this conclusion is often drawn based on visual damage only. Changes in the concentration of metabolites in response to ozone often precede visual damage and form a more reliable indication as to whether a certain plant species is susceptible to ozone. Antioxidants and enzymes have been shown to be affected by O_3 [Bender *et al*., 1994; Machler *et al.,* 1995; Robinson & Britz, 2001; Wieser *et al*., 2001; De Temmerman *et al.,* 2002]. However, contradictory results have been obtained with respect to the direction and extent of these effects. Most of these studies have been conducted under laboratory conditions, and have involved short-term exposures to relatively high O_3 concentrations. Few studies have investigated the responses, and the role of antioxidants in the O_3 detoxification in plants grown under field condition is still uncertain [Chernikowa *et al.,* 2000; Robinson & Britz, 2000].

In this study, the effects of short term ozone exposure (1 day, 7 h, 70 ppb of O_3) at the early stages of three cereal species on their antioxidant capacity and antioxidant levels were investigated. The term "antioxidant capacity" corresponds to the measure of the moles of a given free radical scavenged by a test solution, independently of the antioxidant activity of any one antioxidant present in the mixture [Ghiselli *et al*., 2000; Zieliński, 2002; De Temmerman *et al*., 2002]. The results reported in this paper have indicated that oat and wheat leaves were sensitive to a short term ozone exposure (1 day, 7 h, 70 ppb of O_3) because of the lower TEAC values after fumigation. Under the same conditions barley leaves responded to fumigation with slightly higher TEAC values. It may be that the induced resistance is part of an adaptation process, but would have to be confirmed by applying repeated short-term ozone exposures. For this reason, it seems that the generally accepted view that high antioxidant capacity protects young seedling against oxidative stress does not hold here since the constitutive TEAC of barley leaves was lower by about 50% than the respective value for oat and wheat leaves. Previously, the results of the Open-Top Chamber Programme (Commission of the European Communities) [Weigel *et al*., 1988] indicated that spring wheat was one of the most sensitive species to ambient levels of O_3.

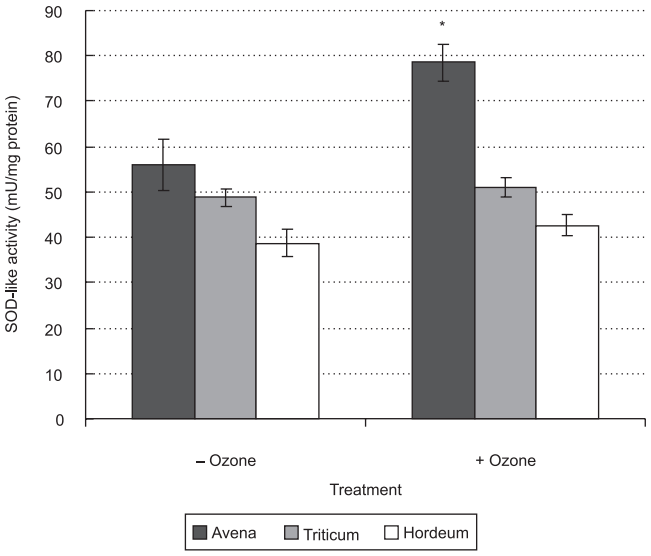

FIGURE 3. The superoxide dismutase-like activity (SOD-like-activity) of three cereal species during early development stage exposed for 7 h to 70 ppb ozone (+ O_3) or filtered air (– O_3). Freeze-dried material was extracted in PBS. Values represent means±SD, n=3 indicates the number of the independent experiments in time. Significance of differences for Student's t-test as in Figure 1.

Similarly as with the TEAC values, barley leaves, but not oat and wheat, showed an increased amount of PBS-soluble total phenolic compounds. It may indicate that induction in phenolic compounds in barley leaves can be a part of an adaptation process providing higher resistance or tolerance when compared to sensitive to ozone oat and wheat leaves. Previously, Ranieri *et al.* [1996] showed a decrease in phenolic compounds of both young and mature foliar tissues of pumpkin exposed to O_3. This was explained by the increased oxidation of these compounds at the cell wall level. In contrast, Roseman *et al*. [1991] found a small increase in phenolics in Scots pine (*Pinus sylvestris* L.) seedlings, and the increased synthesis of these compounds was suspected for this effect.

In this study, the glutathione levels following O_3 treatment showed decreases, although statistically not significant, in oat and wheat leaves. Thus, although the reduced/oxidized ratio of GSH seemed to be affected in all three cereal species, only barley was able to keep the total GSH level constant. Again, barley seemed to be more tolerant to ozone than oat or wheat. On the contrary, an increase of GSH was observed in two wheat cultivars of different sensitivities after continuous SO_2 fumigation [Soldatini *et al*., 1992].

Recently, it has been shown that apoplastic washing fluid isolated from foliage of *Avena sativa*, *Hordeum vulgare*, *Nicotiana tabacum*, *Picea abies, Pinus sylvestris* and *Plantago major* displays a SOD-like activity, suggesting that the observed activity is due to the presence of Cu/Zn-SOD isoforms [Streller & Wingsle, 1994; Vanacker *et al.,* 1998]. In our experiment, the SOD-like activity was clearly stimulated by the ozone episode only in oat leaves. This finding indicates a different response of oat leaves and barley and wheat leaves when O_3 or one of its degradation products diffuses through the cell. At this point, however, it was not possible to discriminate between enzymatic and non-enzymatic SOD-like activity.

CONCLUSIONS

The short and mild episode of ozone during early stage of development induced changes in barley, oat and wheat

seedlings. Not all antioxidant components determined here show similar responses to ozone and not all responses coincide between the three grass species analysed here. However, it was possible to demonstrate that young barley leaves responded to ozone with increased levels of TEAC. A part of this may be explained by the increase in PBS-soluble phenolics compounds in ozone fumigated leaves of barley. These compounds are antioxidants which are believed not to be continuously recycled like glutathione in the Halliwel-Asada cycle. Nevertheless, they may still play a considerable role in detoxifying ozone. The fact that barley was able to keep its total GSH level unaffected by ozone fumigation might indicate a higher tolerance towards ozone. However, further research is needed on the time-dependent accumulation of plant antioxidants during short-term plant exposure to ozone combined with more elaborated growth analysis on several crop species.

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