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Research Article

VISIBLE FOLIAR INJURY AND RESPONSE OF ANTIOXIDANT DEFENSE SYSTEM IN BEECH (*FAGUS SYLVATICA* L.) TO ACUTE OZONE EXPOSURE UNDER CONTROLLED CONDITIONS

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Summary: Tropospheric ozone (O_3) is one of the most widespread atmospheric pollutants in many parts of the world. Elevated ambient O_3 concentrations have deleterious effects on the physiological status and productivity of a wide range of plant species. Experiments with controlled O_3 fumigation are often performed in order to distinguish O_3-induced injury from a wide range of injury symptoms caused by other environmental stress agents. In the present study, branches of common beech (*Fagus sylvatica* L.) were subjected to O_3 fumigation for three days under controlled conditions. Ozone was provided at a concentration of 600 ppb in the course of 6 h during the day. The visible foliar symptoms of injury appeared as small pale green dots on the adaxial leaf surface. By the end of the first day, these dots were spread on the entire leaf surface. The acute O_3-induced symptoms developed rapidly as reddish-brown stipple which enlarged covering the entire leaf surface on the third day of O_3 exposure. These typical visible symptoms could serve as indicators of the presence of phytotoxic levels of O_3 in natural conditions. Ozone fumigation treatment caused a significant decrease in the amount of total chlorophyll as well as metabolic changes in the antioxidant defense system of beech leaves. Elevated total antioxidant and free radical scavenging activities indicated enhanced capacity of the non-enzymatic plant defense system in response to oxidative stress caused by O_3-induced generation of reactive oxygen species (ROS). Total flavonoid and anthocyanin contents were also increased. The enhanced activities of the antioxidant enzymes superoxide dismutase (SOD) and peroxidase (PO) can serve as indicators of oxidative burst caused by O_3 fumigation in beech leaves.

Keywords: Ozone; beech (*Fagus sylvatica* L.); visual foliar injury; antioxidant defense system; anthocyanins; superoxid dismutase; peroxidase.

Abbreviations: CAT – catalase; O_3 – ozone; PO – peroxidase; ROS – reactive oxygen species; SOD – superoxide dismutase.

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INTRODUCTION

Tropospheric ozone (O_3) is one of the most widespread atmospheric pollutants in many parts of the world (Nolle et al., 2002; Ferretti et al., 2007; Bussotti and Ferretti, 2009). It is formed in air polluted with volatile organic compounds (VOC) and nitrogen oxides (defined as ozone precursors) as a result of the sun radiation activity (Krupa et al., 2001; Ferretti et al., 2007). Ozone plays a substantial role in the chemistry of troposphere which is due to its high oxidation activity, ability to absorb sun radiation in the infrared and ultraviolet spectra as well as to exhibit properties of greenhouse gases (Jacobson et al., 2002).

Ozone levels have been gradually increasing worldwide (Colette et al., 2011; Wilson et al., 2012). Particularly, the southern part of Europe is known to be affected by high O_3 concentrations (Nolle et al., 2002; Sanz et al., 2007). According to Percy et al. (2003) by 2100, approximately 50% or a total of 17 million km^2 of forest areas in the world will be exposed to increased O_3 concentrations.

The deleterious effects of tropospheric O_3 on the physiological function and productivity of a wide range of plant species, including agricultural crops, trees, shrubs, herbaceous perennials, and lichens in different parts of Europe are well documented (Skelly et al., 1999; Sanz and Millan, 2000; Manning et al., 2002; Gerosa et al., 2007). Leaf damages caused by O_3 can be defined as visible and physiological. Visible O_3-induced injury on needles and leaves is the only detectable indication of the presence of phytotoxic levels of O_3 and its detection is used for monitoring the effects of O_3 (Bergmann et al, 1999). The occurrence of visible leaf damages, their color, density, and spread on the leaves depends on plant species and genotype, the position of leaves, their age, exposure, meteorological factors, O_3 concentration and duration of exposure, as well as other habitat conditions (Bussotti and Ferretti, 2009; Schaub et al., 2010). Typical visible foliar O_3-induced symptoms include brown or purple stipple on the upper surface between the veins of leaves, chlorosis, tip or margin burn, necrosis, premature abscission of leaves and flowers (Brace et al., 1999; Krupa et al., 1998; Kohut, 2005). Bifacial necrosis is the final stage of leaf damage. It is the result of cell death in the epidermal tissue, palisade and spongy mesophyll and can be visible on both sides of the leaves. The presence of dots, spots and bilateral necrosis as well as the degree of their manifestation is a reliable diagnostic criterion for assessment of the negative impact of ambient O_3 (Kohut, 2005; Bussotti and Ferretti, 2009; Schaub et al., 2010).

Visible foliar O_3-induced symptoms are often similar in appearance to damage caused by other biotic and abiotic stress agents (e.g., viruses, insects, fungi, drought, herbicides) (Treshow and Anderson, 1991; Smith, 2012). That is why assessment of O_3 impact on plants should also cover the pre-existing physiological and biochemical changes triggered by O_3. The negative impact of ambient O_3 on forests has been studied at morphological, histological, ultra-structural and biochemical levels (Bussotti and Gerosa, 2002).

Ozone is absorbed by plants through the stomata. Species with high stomatal

conductivity are susceptible to O_3-induced damage due to increased gas exchange rate resulting in the uptake of higher amounts of air pollutants (Matyssek et al., 2007). In addition to stomatal conductivity, another factor that determines the sensitivity of plants to O_3 is their metabolic capacity to cope with O_3-generated oxidative stress (Kangasjärvi et al., 1994; Matyssek et al., 2004; 2007). Oxidative stress occurs as a result of the interaction of O_3 with water and apoplast solutes, thus leading to the formation and accumulation of ROS in plant cells, which are more toxic and reactive than molecular O_2 (Mehlhorn et al., 1990; Wohlgemuth et al., 2002). ROS react directly with cellular organelles and macromolecules, resulting in damage of cell membranes, oxidation of proteins, lipids, carbohydrates, damage of cell structure, ultimately leading to the activation of programmed cell death (PCD) pathways. On the other hand, ROS can induce antioxidant defense system as a major mechanism protecting plants from oxidative damage to cellular components under stress conditions. Due to the dual function of ROS, cells possess mechanisms to strictly control their level (Mittler, 2002; Blokhina et al., 2003; Sytar et al., 2013).

Experiments with O_3 fumigation performed under controlled conditions represent an important approach to distinguish O_3-induced injury from other environmental stress conditions. Controlling the concentration of O_3 and other environmental factors (light, temperature and humidity) allows monitoring of visible injury symptoms, physiological and biochemical reactions in a relatively short period of time, especially when the exposure-response relationships are assessed as indicators of species sensitivity to O_3 exposure (Manning, 2005). Depending on plant material (branches, saplings or adult individuals) as well as the purposes of the study, different types of chambers for controlled O_3 fumigation have been used (Brace et al., 1999; Wittmann et al., 2007; Pellegrini et al., 2011).

Branches of adult forest trees are often used as surrogates for examining the crown-level response of trees to elevated O_3 regimes as the defense metabolism of branches seems to be relatively autonomous from neighboring crown sections (Wieser et al., 2012). Although the branch-level approach does not substitute whole-tree O_3 fumigation and related flux assessments, O_3-exposed branches may be used to assess dose-response reactions as measures of O_3 sensitivity of tree species (Wieser et al., 2012).

Common beech (*Fagus sylvatica* L.) is an important broad-leaved species, widely spread in Europe. In Bulgaria it is found in almost all mountains from 700 to 1700 m above sea level. Most often it forms pure stands and less often mixed with common hornbeam (*Carpinus* betulus *L.*), Norway spruce (*Picea abies* (*L.*) *Karst.*) and silver fir (*Abies alba* Mill.) The species has an important water-regulating and anti-erosion role and is economically valuable since it is a source of quality wood that is widely used in construction, furniture, pulp and paper industries.

In the present study, branches of common beech (*Fagus sylvatica* L.) were subjected to O_3 fumigation under controlled conditions with the aim to

assess the range of damage at levels above typical ambient condition. The analysis included visible foliar O_3-induced symptoms and changes in the antioxidant capacity including antioxidant enzymes and low molecular weight non-enzymatic antioxidants.

MATERIALS AND METHODS

Plant material and conditions for O_3 fumigation treatment

Branches of common beech (*Fagus sylvatica* L.) were taken from sample trees in the Level II Forest monitoring station of Vitinya situated in the Western Balkan mountain range (Bulgaria) at 950 m above sea level. The average age of sample trees was 140 years. They were selected from the forest edge closest to the O_3 measuring equipment within a maximum radius of 500 m. The branches were taken from the sun exposed part of the tree crown in the summer. Each branch had at least 30 well-developed leaves with healthy appearance and lacking any visible injury. Fumigation of beech branches was done in glass chambers with dimensions 30x20x50 cm (LxWxH) having movable lids with two small apertures, one for ventilation and the other for the O_3 flow provided by an ozonator (Onnic ES 215 A, UK), (Fig. 1). The beech branches were kept in the glass chambers for three days at a photon flux density of 120 μmol m^{-2} s^{-1} provided by luminescent lamps, 16/8 photoperiod, 22°C and relative humidity 80%. Ozone was provided at an average concentration of 600 ppb in the course of 6 h during the day. In comparison, the average O_3 background concentration measured at the Level II Forest monitoring station of Vitinya was 80 ppb. The concentration of O_3 in the chambers was calculated on the basis of the passport data for the flow rate of the ozonator and the capacity of the chambers. Fumigation with high concentrations of O_3 for short periods aimed to stimulate the appearance of leaf damages during the day when O_3 concentration is normally high in natural conditions. Samples for biochemical

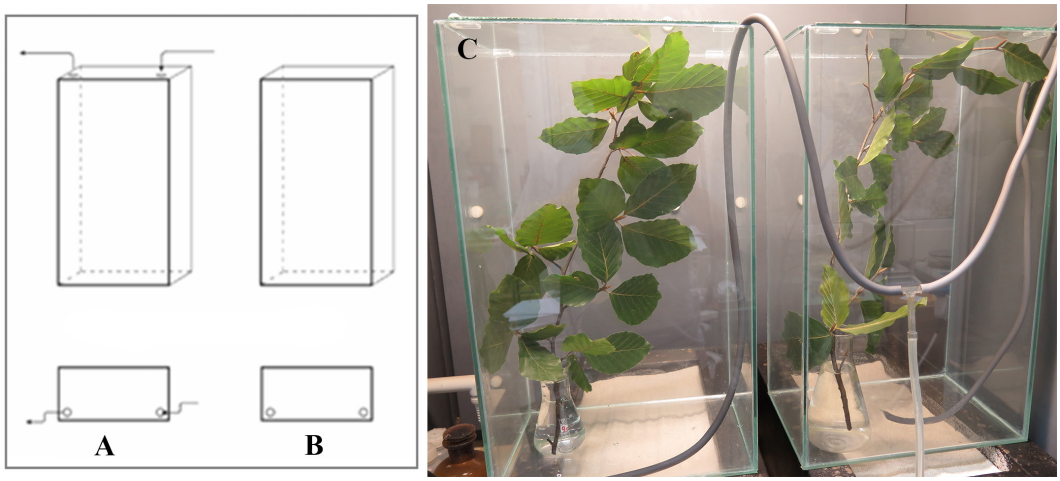

Figure 1. Controlled fumigation of beech branches with O_3 provided at a concentration of 600 ppb. A) scheme of the experimental glass chamber for O_3 fumigation. Arrows indicate the direction of the O_3 flow provided by an ozonator; B) control chamber; C) photograph of the experimental chambers.

analysis were taken on the third day of O_3 exposure.

Determination of chlorothyll content

Chlorophyll was extracted in N, N-dimethyl-formamide (5%, w/v) as described by Moran and Porath (1980). Chlorophyll content was analyzed using a Cary 50 Conc UV-Visible spectrophotometer (Varian, Australia Pty Ltd, Mulgrave, Victoria) and calculations were based on the extinction coefficients proposed by Inskeep and Bloom (1985). Chl content is expressed as (mg g^{-1} FW).

Determination of total anthocyanin content

Total anthocyanin content was determined according to Hodges and Nozzolillo (1996). Fresh plant material (0.1 g) was extracted by grinding with 7 ml 1% (w/v) HCl in methanol. The homogenate was filtered through red filter (medium porosity) into a 10 ml tube. After rinsing with ca. 3 ml of the solvent, the volume was adjusted to 10 ml. The extinction (E) of the solution was measured at 530 nm and 600 nm against the solvent. The value of E_{600} was subtracted from the E_{530} value (correction for pheophytin). The content of anthocyanins is expressed as μmoles per 1 g fresh weight on the basis of the molar extinction coefficient for anthocyanins $\varepsilon_M = 33{,}000$.

Analysis of antioxidant enzymes

Extract preparation

One gram of frozen leaf samples were ground and extracted (1:3, w/v) with an ice-cold 0.1 M K-phosphate buffer (pH 7.8) containing EDTA (2 mM), glycerol (10%) and PMSF (1mM). Insoluble PVP (Polyclar AT) was added directly to the plant sample in the mortar (0.02 g PVP per 1 ml of extraction medium). After centrifugation at 15,000 x *g* for 30 min the supernatant was used for the assay of peroxidase activity, superoxide dismutase activity as well as protein content determination.

Activity of superoxide dismutase (SOD; E.C. 1.15.1.1)

SOD activity was measured spectrophotometrically at 560 nm based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) as described by Beauchamp and Fridovich (1971). The enzyme activity is expressed as enzyme units (EU). One unit of SOD activity is defined as the quantity of the enzyme required to cause 50% inhibition of the initial NBT reduction under the assay conditions.

Peroxidase activity (PO; E.C.1.11.1.7)

Peroxidase activity was measured spectrophotometrically at 25°C by following the H_2O_2-dependent oxidation of the substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Herzog and Fahimi, 1973). The reaction mixture contained 3,3'-diaminobenzidinetetrahydrochloride dihydrate (DAB) solution containing 0.1% (w/v) gelatine, 150 mM Na-phosphate-citrate buffer (pH 4.4), and 0.6% H_2O_2. The increase in the absorbance at 465 nm was monitored for 3 min. The enzyme activity is expressed as enzyme units (EU). This is the change in the absorption per 1 min and per 1 mg protein.

Protein content was determined according to the method of Lowry et al. (1951).

Biochemical analysis of cellular non-enzymatic antioxidant capacity

Preparation of extracts

Leaf material (1 g) was ground in an ice bath with 10 ml 80% ethanol (v/v) and the homogenate was immediately centrifuged at 10,000 x *g* for 20 min at 4°C. The clear supernatant was decanted and used in the analysis of total antioxidant activity, antiradical activity and total flavonoid content.

Determination of total antioxidant activity (FRAP method)

Total antioxidant activity was determined using the ferric reducing antioxidant power (FRAP) assay according to Benzie and Strain (1999). This procedure involves the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in a blue colored complex in the presence of bioactive compounds (antioxidants). Briefly, freshly prepared FRAP reagent (1.5 ml) and an aliquot of the extract solution (0.05 ml) were mixed, and after 15 min the absorbance was measured at 593 nm using FRAP solution as a blank. The anioxidant potential of samples was calculated from a standard curve plotted using known concentrations of $FeSO_4.7H_2O$. The antioxidant activity is expressed as (μmoles FRAP g^{-1} FW).

Determination of antiradical activity

The free radical scavenging activity was measured according to Brand-Williams et al. (1995) using 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) as the source of free radicals. The method is based on the reduction of the stable radical $DPPH^{\cdot}$ by antioxidants and other radicals in the sample. Freshly prepared $DPPH^{\cdot}$ reagent (1.99 ml) and extract solution (0.01 ml) were mixed, and the decrease of the absorbance during the reaction was measured at 515 nm. For the blank, methanol was used. The free radical scavenging activity is expressed as *trolox* equivalents using a standard curve made with known concentrations of Trolox.

Analysis of total flavonoids

The total flavonoid content was estimated using the aluminum chloride colorimetric assay (Zhishen et al., 1999). An aliquot of the sample solution (0.5 ml) in ethanol (5mg/100ml) was mixed with 2ml dH_2O and 150 μl of 5% sodium nitrate. After 6 min, 150 μl of 10% aluminum chloride and 2 ml of 1 M sodium hydroxide were added and left at room temperature for 30 min. Absorbance of the mixtures was measured at 510 nm and total flavonoid content was calculated as rutin equivalents from a calibration curve of rutin. Total flavonoid content is expressed as (mg rutin g^{-1} FW).

The results of the biochemical analyses represent the mean values ± SE (n=4).

RESULTS AND DISCUSSION

Visible foliar symptoms resulting from O_3 exposure can be considered as indicators of either acute or chronic injury (Brace et al., 1999). Acute injury, caused by cell death of mesophyll cells, develops within a few hours to days following exposure and is normally induced by relatively high O_3 concentrations. These symptoms can appear visually as stipple (many small red, purple, or other pigmented dots), fleck (many small yellow spots), bleaching (loss of green color), or bifacial necrosis (areas of dead

cells on both sides of a leaf). Chronic injury develops more slowly (days to weeks) and can lead to premature senescence resulting in leaf abscission. It is normally induced by long-term, low O_3 concentrations (Brace et al., 1999).

In the present study, the first visible foliar symptoms of acute injury caused by controlled fumigation of beech branches with a high O_3 concentration appeared after 25 min of exposure as small pale green dots on the adaxial leaf surface. By the end of the first day, these dots were spread on the entire leaf surface (Fig. 2). The visible O_3-induced symptoms developed rapidly as reddish-brown spots which enlarged with time covering the entire leaf surface on the third day of O_3 exposure. These typical visible injury symptoms in beech could serve as indicators of the presence of phytotoxic levels of O_3 in natural conditions.

Visible O_3-induced symptoms are species-specific, although some variability could occur depending on plant genotype, leaf age, O_3 exposure as well as habitat conditions. Calatayud et al. (2007) observed the formation of large brown to dark brown spots on leaves of *Acer pseudoplatanus* L. and pale yellow to yellowish brown spots in *Acer campestre* L. and *Acer monspessulanum* L. in response to controlled O_3 fumigation. In experiments with adult individuals of common beech (*Fagus sylvatica* L.) fumigated with O_3 at concentrations twice the background, Nunn et al. (2002) described the formation of unevenly distributed yellowish-brown spots on the leaf surface. In a monitoring study on deciduous and coniferous tree species Parvanova et al. (2008) observed typical O_3 injury such as redness on the upper leaf surface of the broad-leaved species *Prunus avium* L. and *Rubus idaeus* L. as well as the appearance of purple-red dots on beech leaves (*Fagus sylvativa* L.).

Many authors have observed that increased ambient O_3 concentrations reduce the photosynthetic function in different tree species (Calatayud and Barreno, 2004; Wittmann et al., 2007; Pellegrini et al., 2011). The membranes of cell organelles, and especially those of chloroplasts, are most vulnerable to O_3 exposure. The damage of a cell or a group of mesophyll cells, accompanied in some cases with a collapse of epidermal cells reduces total chlorophyll content in leaves (Schraudner et al., 1997; Saitanis

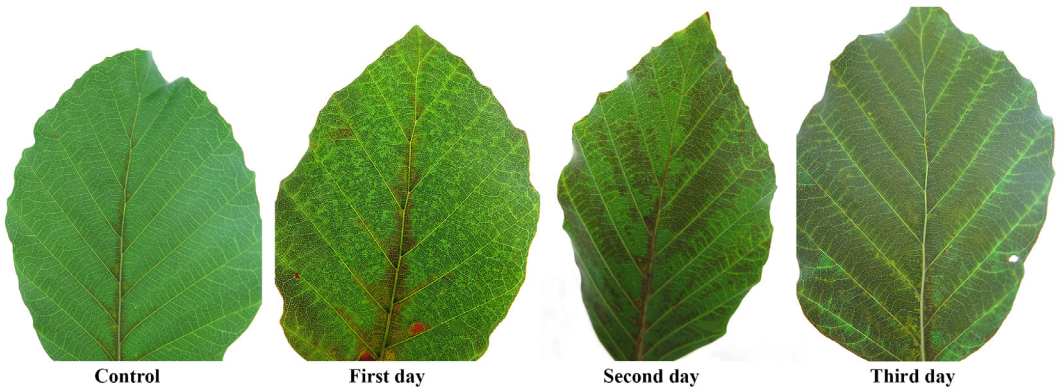

Figure 2. Visible O_3-induced injury symptoms in leaves of common beech (*Fagus sylvatica* L.) after O_3 fumigation treatment of beech branches conducted for three days under controlled conditions.

et al. 2001; Castagna et al, 2001). Our results showed a significantly reduced amount of total chlorophyll (by 44%) due to O_3 fumigation treatment compared with control (Fig. 3A). Similar results showing that O_3 significantly decreased chlorophyll content in the leaves of beech saplings were reported by Lütz et al. (2000). The decrease in chlorophyll concentration in response to O_3 may be due to chlorophyll breakdown initiated by O_3-induced ROS (Castagna et al., 2001). According to Calatayud and Barreno (2001) reduced chlorophyll content is usually observed under conditions of oxidative stress.

Plant's productivity in environment with increased O_3 concentration is closely related to its ability for scavenging the highly toxic free radicals formed. The antioxidant defense system represents a major mechanism for neutralizing ROS through enzymatic and non-enzymatic antioxidants (Mittler, 2002; Blokhina et al., 2003; Sytar et al. 2013). Metabolic changes in antioxidant defense system contribute to cellular repair processes, which alleviate the initial oxidative damage caused by O_3-induced generation of ROS (Castagna and Ranjeri, 2009). Anthocyanins in the vacuole as well as tocopherol in the membrane are low molecular weight non-enzymatic antioxidants, which play an important role in plant defense mechanisms against oxidative stress (Araceli et al., 2009). Anthocyanin and flavonoid contents increase with increasing the amount of ROS (Yamasaki et al., 1997; Hernandez et al., 2009). In the present study, anthocyanin content in beech leaves was significantly enhanced (by 46%) compared with control (Fig. 3B) thus indicating increased generation of ROS due to O_3 exposure.

Altered antioxidant metabolic activity is a crucial component in defense mechanisms against oxidative damage.

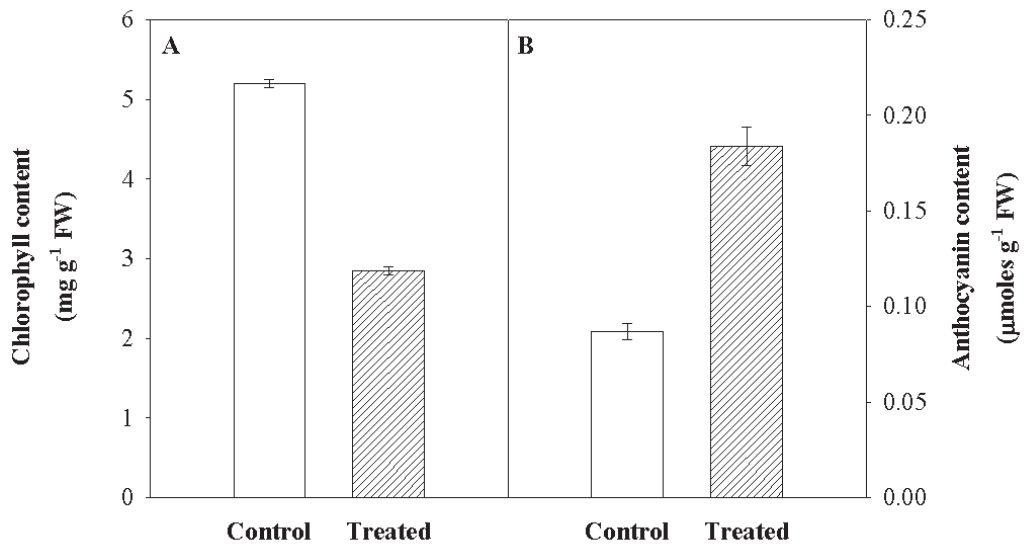

Figure 3. Chlorophyll content (A) and anthocyanin content (B) in leaves of common beech (*Fagus sylvatica* L.) after O_3 fumigation treatment of beech branches conducted for three days under controlled conditions. The results represent the mean values ± SE (n=4).

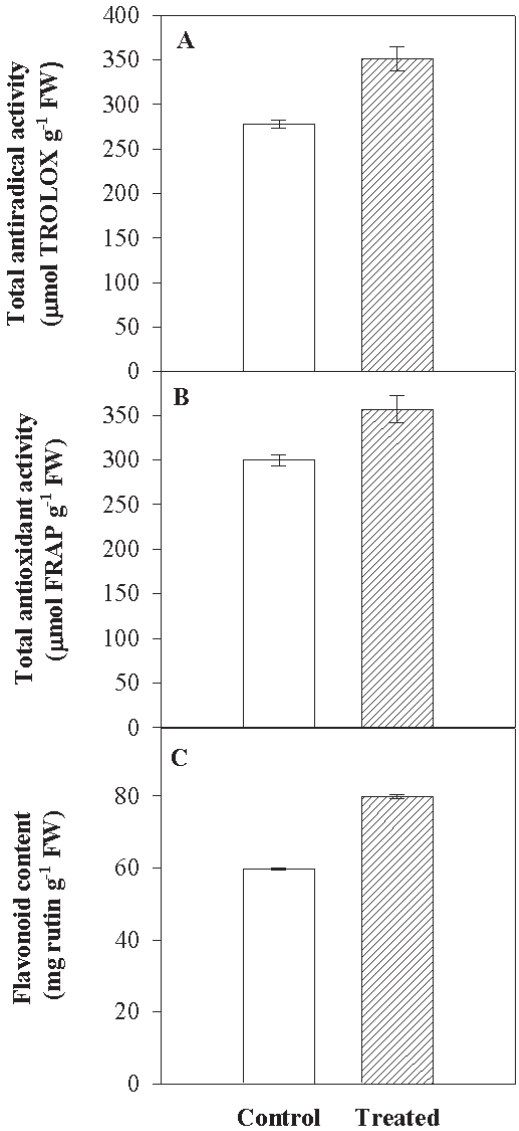

Figure 4. Total antiradical activity (A), total antioxidant activity (B) and flavonoid content (C) in leaves of common beech (*Fagus sylvatica* L.) after O_3 fumigation treatment of beech branches conducted for three days under controlled conditions. The results represent the mean values ± SE (n=4).

Our results showed that both free radical scavenging activity and antioxidant activity were elevated (by 20% and 15%, respectively), thus suggesting increased capacity of the non-enzymatic plant defense system in beech leaves in response to oxidative stress caused by O_3-induced generation of ROS (Fig. 4A, B).

Highly affected by O_3 fumigation treatment was total flavonoid content, which increased by 30% compared with control (Fig. 4C). Flavonoids represent a large family of secondary metabolites that have been found to directly scavenge free radical ions (e.g. hydroxyl and superoxide anion) due to their ability to donate electrons or hydrogen atoms thus protecting plant cells from the adverse effects of abiotic stresses (Hernandez et al., 2009). Flavonoids are one of the major components of the non-enzymatic antioxidant defense system in plants that suppress formation and lower levels of newly formed ROS (Agate and Tattini, 2010).

Ozone fumigation treatment enhanced the activities of the two investigated antioxidant enzymes SOD and PO in beech leaves by 29% and 38%, respectively compared with control (Fig. 5A, B). SOD is the most effective antioxidant enzyme found in all aerobic organisms and all subcellular components. There are controversial views on the relationship between ambient O_3 concentration and SOD activity. Bowler et al. (1992) observed strong enhancement of SOD activity in tree species during the summer which was associated with enhanced concentration of O_3 and its precursors. Elevated SOD and CAT activities in *Populus* leaves due to O_3-induced oxidative stress were reported (Sen Gupta et al., 1991). On the other hand, Parvanova et al. (2013) did not show a significant correlation between SOD activity and O_3 levels in common

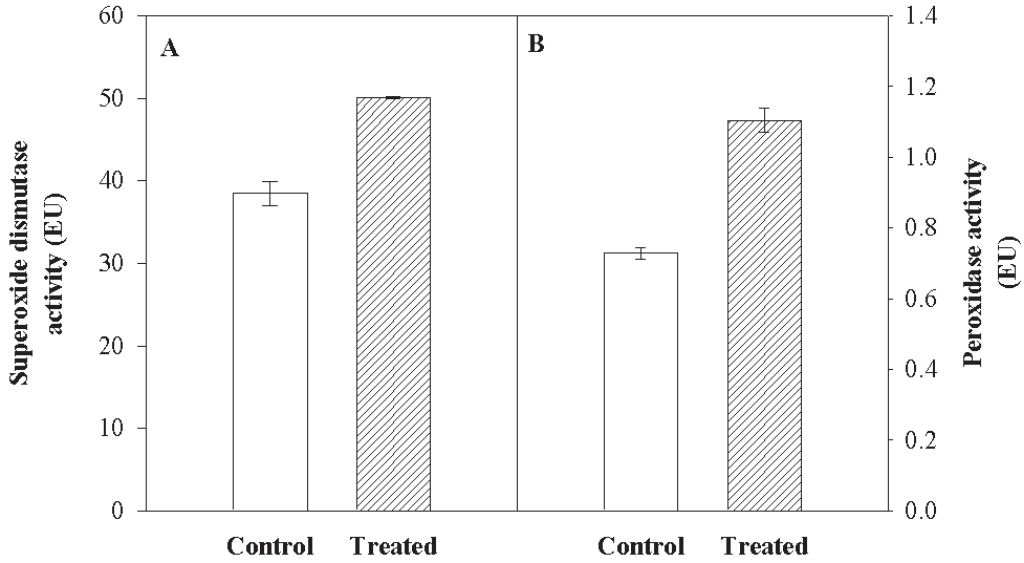

Figure 5. Activity of SOD (A) and PO (B) in leaves of common beech (*Fagus sylvatica* L.) after O_3 fumigation treatment of beech branches conducted for three days under controlled conditions. The results represent the mean values ± SE (n=4).

ash (*Fraxinus excelsior* L.) seedlings in response to ambient O_3 exposure in urban and mountain areas. To neutralize the harmful effects of H_2O_2, plants use a wide variety of enzymes, including PO (Blokhina et al., 2003). Enhanced PO activity was observed in a sensitive poplar species (Bernardi et al., 2004) as well as in a susceptible birch variety (*Betula pendula Roth*) after exposure to O_3 (Toumainen et al., 1996).

CONCLUSION

Altered antioxidant metabolic activity in response to O_3 stress reflects the ability of plants to modify metabolic processes in order to alleviate the oxidative damage caused by O_3-induced generation of ROS. In the present study, O_3 fumigation treatment of beech branches caused typical visible foliar injury accompanied with elevated total antioxidant and free radical scavenging activities, increased accumulation of low-molecular antioxidants such as anthocyanins and flavonoids as well as enhanced activities of the antioxidant enzymes SOD and PO. The observed metabolic changes indicate activation of antioxidant defense mechanisms to limit ROS-mediated damage in beech leaves.

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REFERENCES

Agate G, M Tattini, 2010. Multiple functional roles of flavonoids in photoprotection. New Phytol, 186: 786–793.

Araceli CO, PH Ma de Lourdes, PH Ma Elena, JA Rodriguez, GV Carlos

Andrés, 2009. Chemical studies of anthocyanins. Rev Food Chem, 13: 859–871.

Beauchamp C and Fridovich I, 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287

Benzie IF, J-J Strain, 1999. Ferric reducing antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version of simultaneous measurement of total antioxidant power and ascorbic acid concentration. In: Methods in Enzymology, 299: 15–27.

Bergmann E, J Bender, H-J Weigel, 1999. Ozone threshold doses and exposure-response relationships for the development of ozone injury symptoms in wild plant species. The New Phytol, 144 (3): 423–435.

Bernardi R, C Nali, P Ginestri, C Pugliesi, G Lorenzini, M Durante, 2004. Antioxidant enzyme izoforms on gels in two poplar clones differing in sensitivity after exposure to ozone. Biol Plant, 48: 41–48.

Blokhina O, E Virolainen, KV Fagerstedt, 2003. Antioxidants, oxidative damage and oxygen deprivation stress: A review. Ann Bot, 91: 179–194.

Bowler C, MV Montagu, D Inze, 1992. Superoxide dismutase and stress tolerance. Annu Rev Plant Physiol Plant Mol Biol, 43: 83–116.

Brace S, DL Peterson, D Horner, 1999. A guide to ozone injury in vascular plants of the Pacific Northwest. Gen Tech Rep PNW-GTR-446. Portland, OR: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station. 63 p.

Brand-Williams W, ME Cuvelier, C Berset, 1995. Use of a free radical method to evaluate antioxidant activity. Lebensm Wiss Technol, 28: 25–30.

Bussotti F, G Gerosa, 2002. Are the Mediterranean forests in southern Europe threatened from ozone? J Mediterr Ecol, 3: 23–24.

Bussotti F, M Ferretti, 2009. Visible injury, crown condition, and growth responses of selected Italian forests in relation to ozone exposure. Environ Pollut, 157(5):1427–1437.

Calatayud A, E Barreno, 2001. Chlorophyll a fluorescence, antioxidant enzymes and lipid peroxidation in tomato in response to ozone and benomyl. Environ Pollut, 115: 283–289.

Calatayud A, E Barreno, 2004. Response to ozone in two lettuce varieties on chlorophyll a fluorescence, photosynthetic pigments and lipid peroxidation. Plant Physiol Biochem, 42: 549–555.

Calatayud V, J Cerveró, M José Sanz, 2007. Foliar, physiological and growth responses of four maple species exposed to ozone. Water, Air and Soil Pollut, 185: 239–254.

Castagna, A., Nali, C., Ciompi, S., Lorenzini, G., Soldatini, G.F., Ranieri, A., 2001. Ozone exposure affects photosynthesis of pumpkin (*Cucurbita pepo)* plants. New Phytol, 152: 223–229.

Castagna A, A Ranieri, 2009. Detoxification and repair process of ozone injury: from ozone uptake to

gene expression adjustment. Environ Pollut 157(5): 1461–1469.

Colette A, C Granier, Ø Hodnebrog, H Jacobs, A Maurizi, A Nyiri, B Bessagnet, A D'Angiola, M D'Isidoro, M Gauss, F Meleux, M Memmesheimer, A Mieville, L Rouil, F Russo, S Solberg, F Stordal, F Tampieri, 2011. Air quality trends in Europe over the past devade: a first multi-model assessment. Atmos Chem Phys, 11: 11657–11678.

Ferretti M, M Fagnano, T Amoriello, M Badiani, A Ballarin-Denti, A Buffoni, F Bussotti, A Castanga, S Cieslik, A Costantini, A De Marco, G Gerosa, G Lorenzini, F Manes, G Merola, C Nali, E Paoletti, B Petriccione, S Racalbuto, G Rana, A Ranieri, A Tagliaferri, G Vialetto, M Vitale, 2007. Measuring, modelling and testing ozone exposure, flux and effects on vegetation in southern Europe conditions – What does not work? A review from Italy. Environ Pollut, 146: 648–658.

Gerosa G, M Ferretti, F Bussotti, D Rocchini, 2007. Estimates of ozone AOT40 from passive sampling in forest sites in South-Western Europe. Environ Pollut, 145: 629–635.

Hernandez I, L Alegre, FV Breusegem, S Munne–Bosch, 2009. How relevant are flavonoids as antioxidants in plants? Trends Plant Sci, 14: 125–132.

Herzog V, H Fahimi, 1973. A new sensitive colorimetric assay for peroxidase using 3,3′-diaminobenzidine as hydrogen donor. Anal Biochem, 55: 554–562.

Hodges DM, C Nozzolillo, 1996. Anthocyanin and anthocyanoplast content of Cruciferous seedlings subjected to mineral nutrient deficiency. J Plant Physiol, 147, 6: 749–754.

Inskeep WP, PR Bloom, 1985. Extinction coefficients of chlorophyll a and b in N, N-dimethylformamide and 80% acetone. Plant Physiol, 77: 483 – 485.

Jacobson MZ, 2002. Control of fossil-fuel particulate black carbon and organic matter, possibly the most effective method of slowing global warming. J Geophys Res, 107: doi:10.1029/2001JD001376.

Kangasjärvi J, J Talvinen, M Utriainen, R Karjalainen, 1994. Plant defense systems induced by ozone: Commissioned Review. Plant, Cell Environ, 17: 783–794.

Kohut R, 2005. Handbook for assessment of foliar ozone injury on vegetation in the national parks. Boyce Thompson Institute, Cornell University.

Krupa SV, AEG Tonneijck, WJ Manning, 1998. Ozone. In: Flagler RB, (Ed.), Recognition of Air Pollution Injury to Vegetation: A Pictorial Atlas, 2nd Edition. Air and Waste Manage Assoc, Pittsburg, PA, pp. 1–28.

Krupa SV, MT McGrath, CP Andersen, FL Booker, KO Burkey, AH Chappelka, BI Chevone, EJ Pell, BA Zilinskas, 2001. Ambient ozone and plant health. Plant Disease, 85: 4–12.

Lütz C, S Anegg, D Gerant, B Alaoui-Soussé, J Gérard, P Dizengremel, 2000. Beech trees exposed to high CO_2 and to simulated summer ozone levels: effects on photosynthesis, chloroplast components and leaf enzyme activity. Physiol Plant, 109: 252–259.

Manning WJ, B Godzik, R Musselman,

2002. Potential bio indicator plant species for ambient ozone in the forested mountain areas of central Europe. Environ Pollut, 119(3): 283–290.

Matyssek R, G Wieser, AJ Nunn, AR Kozovits, IM Reiter, C Heerdt, JB Winkler, M Baumgarten, K-H Häberle, TEE Grams, H Werner, P Fabian, WM Havranek, 2004. Comparison between AOT40 and ozone uptake in forest trees of different species, age and site conditions. Atmos Environ, 38: 2271–2281.

Matyssek R, A Bytnerowicz, P-E Karlsson, E Paoletti, M Sanz, M Schaub, G Wieser, 2007. Promoting the O_3 flux concept for European forest trees. Environ Pollut, 146: 587–607.

Mehlhorn H, BJ Tabner, AR Wellburn, 1990. Electron spin resonance: Evidence for the formation of free radicals in plants exposed to ozone. Physiol Plant, 79: 377–383.

Mittler R, 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci, 7: 405–410.

Moran R, D Porath, 1980. Chlorophyll determination in intact tissues using N, N-dimethylformamide. Plant Physiol, 65: 478–479.

Nolle M, R Ellul, G Heinrich, H Güsten, 2002. A long term study of background ozone concentrations in the Central Mediterranean – diurnal and seasonal variations on the island of Gozo. Atmos Environ, 36:1391–1402.

Nunn A, I Reiter, K-H Häberle, H Werner, C Langebartels, H Sandermann, C Heerdt, P Fabian, R Matyssek, 2002. "Free – Air" Ozone canopy fumigation in an old – growth mixed forest: Concept and observations in beech. Phyton (Austria) Special issue: "Global change" 42: 105–119.

Parvanova P, R Fikova, S Bratanova, M Lyubenova, 2008. Tropospheric ozone pollution in the city of Sofia and its vicinity - data from passive biomonitoring. Proceedings of the III Congress of Ecologists of the Republic of Macedonia with international Participation, 06-09.10.2007, Struga. Special issues of Macedonian Ecological Society 8: 361–366.

Parvanova P, N Tzvetkova, Sv Bratanova-Doncheva, N Chipev, R Fikova, E Donev, 2013. Responses of *Fraxinus excelsior* L. seedlings to ambient ozone exposure in urban and mountain areas based on physiological characteristics and antioxidant activity. Environ Sci: Processes and impacts, 15: 1452–1458.

Pellegrini E, A Francini, G Lorenzini, C Nali, 2011. PSII photochemistry and carboxylation efficiency in *Liriodendron tulipifera* under ozone exposure. Environ Exp Bot, 70: 217–226.

Percy KE, AH Legge, SV Krupa, 2003. Tropospheric ozone: a continuing threat to global forests? In: Karnosky DF, KE Percy, AH Chappelka, C Simpson, J Pikkarainen (Eds.), Air pollution, global change and forests in the new millennium. Elsevier, Amsterdam, 85–118.

Pollastri S, M Tattini, 2011. Flavonols: old compound for old roles. Ann Bot, 108: 1225–1233.

Saitanis CJ, AN Riga-Karandinos, MG Karandinos, 2001. Effects of ozone on chlorophyll and quantum yield of tobacco (*Nicotiana tabacum* L.) varieties. Chemosphere, 42: 909–917.

Sanz MJ, MM Millán, 2000. Ozone in the Mediterranean region: evidence of injury to vegitation. In: Inns JL, J Oleksyn, (Eds), Forest dynamics in heavily polluted regions. IUFRO Research Series, 1, CABI Publishing: 165–192.

Sanz MJ, V Calatayud, G Sanchez, 2007. Measures of ozone concentrations using passive sampling in forests of South Western Europe. Environ Pollut, 145: 620–628.

Schaub M, V Calatayud, M Ferretti, G Brunialti, G Lövblad, G Krause, MJ Sanz, 2010. Assessment of ozone injury. International Co – operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests).

Schraudner M, C Langebartles, H Sandermann, 1997. Changes in the biochemical status of plant cells induced by environmental pollutatnt ozone. Physiol Plant, 100: 274–280.

Sen Gupta A, RG Alscher, and D McCune, 1991. Response of photosynthesis and cellular antioxidants to ozone in Populus leaves. Plant Physiol, 96: 650–655.

Skelly JM, JL Innes, KR Snyder, D Vanderheyden, J Zhang, MJ Sanz, 1999. Observation and confirmation of foliar ozone symptoms of native plant species of Switzerland and Southern Spain. Water Air and Soil Pollut, 116: 227–234.

Smith WH, 2012. Air pollution and forests: interactions between air contaminants and forest ecosystems. Springer-Verlag, New York, Heidelberg, Berlin.

Sytar O, A Kumar, D Latowski, P Kuczynska, K Strzalka, MNV Prasad, 2013. Heavy metal – induced oxidative damage, defense reactions and detoxification mechanisms in plants. Acta Physiol Plant, 35: 985–999.

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

Treshow M, FK Anderson, 1991. Plant stress from air pollution. The Bath Press, Bath, UK: pp. 283.

Wieser G, R Matzssek, B Götz, L Grünhage, 2012. Branch cuvettes as means of ozone risk assessment in adult forest tree crowns: combining experimental and modelling capacities. Trees, 26 (6): 1703–1712.

Wilson RC, ZL Fleming, PS Monks, G Clain, S Henne, IB Konovalov, S Szopa, L Menut, 2012. Have primary emission reduction measures reduced ozone across Europe? Analysis of European rural background ozone trends 1996-2005. Atmos Chem Phys, 12: 437–454.

Wittmann C, R Matyssek, H Pfanz, M Humar, 2007. Effects of ozone impact on the gas exchange and chlorophyll fluorescence of juvenile birch stems (*Betula pendula* Roth.). Environ Pollut, 150: 258–266.

Wohlgemuth H, K Mittelstrass, S Kschieschan, J Bender, HJ Weigel, K Overmyer, J Kangasja¨rvi, C Langebartels, H Sandermann, 2002. Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. Plant Cell Environ, 25: 717–726.

Yamasaki H, Y Sakihama, N Ikehara, 1997. Flavonoid–peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2. Plant Physol, 115: 1405–1412.

Zhishen J, T Mengcheng, W Jianming, 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem, 64: 555–559.