

## Article

# Evaluation of Ambient Ozone Effect in Bean and Petunia at Two Different Sites under Natural Conditions: Impact on Antioxidant Enzymes and Stress Injury

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**Abstract:** Tropospheric ozone is a harmful air pollutant and greenhouse gas that adversely affects living organisms. The effect of long-term ozone stress on the activity of SOD, APX, and GuPX, as well as lipid peroxidation and membrane injury in bean and petunia growing at a city site and in a forest, characterised by different ozone concentrations, was examined. The experiments were conducted in three growing seasons with different tropospheric ozone concentrations and meteorological conditions. Plants' exposition to increased ozone concentration resulted in enhanced activity of antioxidant enzymes, level of lipid peroxidation, and membrane injury. In all years, higher ozone levels and solar radiation were observed at the forest site. The pattern of the changes in enzyme activity was dependent on ozone concentrations as well as on environmental conditions and varied from year to year. In the second year with the highest ozone concentration, the activity of GuPX and SOD increased the most. However, despite higher ozone concentration in the forest, a larger increase in APX and SOD activity in both species and GuPX activity in bean was recorded at the city site. The present results revealed that plant response to ozone might vary in different locations not only due to differences in ozone concentration but also because of the impact of other environmental factors, such as solar radiation and temperature.

**Keywords:** peroxidases; superoxide dismutase; lipid peroxidation; membrane injury



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## 1. Introduction

Ozone is a naturally occurring component in the atmosphere. It is present in the stratosphere (10–40 km above the Earth) and the troposphere (0–10 km above the Earth). The stratospheric ozone layer is essential for protecting life on Earth from the harmful effect of UV-B radiation. However, tropospheric ozone is a toxic air pollutant and greenhouse gas that adversely affects living organisms, including plants [1,2]. This pollutant is not directly emitted from various sources but is formed in the presence of sunlight and high temperature from such precursors as nitrogen oxides, carbon monoxides, volatile organic compounds, and methane. The primary sources of ozone precursors are anthropogenic activities (fossil fuel combustion, industrial and traffic pollution) leading to the release of these harmful compounds [3,4]. Using motor vehicle catalysts led to decreased ozone precursor emissions, but unfortunately, this has not led to a significant reduction of tropospheric ozone levels. In the early 21st century, the level of tropospheric ozone increased by 23% compared with that in 1960, and a 31% increase is projected by 2060 [2]. The abundance of tropospheric ozone in the 21st century is affected by its enhanced flux from the stratosphere, climate changes leading to the increase of temperature and solar radiation, and anthropogenic emission of NO<sub>x</sub> and methane [2]. A substantially high trend of ozone concentration increase is observed in the northern hemisphere [5]. In the areas of temperate climate, an increased concentration of tropospheric ozone is present from the end of April and persists

during the summer when temperature and sunlight are higher. What is more, ozone formed in the cities and its precursors originating from various anthropogenic sources are easily transported over long distances, and elevated tropospheric ozone concentrations are expected in non-urban areas, including forests [5,6]. The ozone concentration during the growing season often exceeds the critical level for plant life in natural and crop ecosystems, adversely affecting vegetation [1,7]. Plants may suffer from acute stress when the ozone level is high for several consecutive days or chronic stress when there is a low dose of ozone over extended periods (during the growing season) [6,8]. Acute ozone stress usually results in visible leaf injury (leaf stipple, chlorotic or necrotic lesions), which can alter the economic value of ornamental plants and leafy vegetables [9].

Long-term exposure to ozone is associated with earlier leaf senescence and the reduction of photosynthesis, growth, and yield [10,11]. Ozone toxicity is caused by its high redox potential and capacity to generate reactive oxygen species (ROS), leading to changes in the structure and function of plasma membranes. Furthermore, because of its phytotoxic properties, ozone directly affects the outer surfaces of the leaves, causing damage to the leaf blades [1,12]. Ozone diffuses into the plant via open stomata. It dissolves in apoplastic fluid and can accumulate to phytotoxic concentrations inside living cells, rapidly reacts with many molecules, and affects the generation of harmful ROS [1,3]. Stomatal closure is considered to be the first line of defence against ozone (O<sub>3</sub>) stress, but once it enters the leaf through the stomata, ROS detoxification in the apoplast and plant cells is thought to be the second line of defence [3]. Many antioxidant molecules and enzymes are present in diverse cellular compartments, which likely play a role in the detoxification of ROS [3,8,13]. The enzymatic antioxidants include, among others, ascorbate peroxidase (APX), guaiacol peroxidase (GuPX), and superoxide dismutase (SOD), scavenging hydrogen peroxide and superoxide ion. Increased activity of these enzymes protects cells against the increased production of ROS and oxidative damage caused by ozone [13]. Lipids and proteins in plant cells are the major targets of oxidative damage [13,14]. Oxidative decomposition of polyunsaturated lipids in the plasma membrane, known as lipid peroxidation, is often considered a sign of stress-induced damage [15].

Literature data have shown that elevated tropospheric ozone causes yield reduction in various vegetable plants such as tomato, bean, soya, cabbage, grapes, rape, spinach, and parsley grass [3,12,16]. Ornamental plants affected by tropospheric ozone include, among others, petunia, poinsettia, tuber nasturtium, *Buddleia davidii*, and geranium [17,18]. Several researchers have studied the phytotoxic effect of ozone stress on crops and model plants. However, most studies were conducted in glasshouses, growth rooms, or open-top chambers and examined the short-term ozone effect [3]. In recent years, the free-air controlled enrichment (FACE) system was developed to investigate plant responses to ozone, which provides more reliable results, but its installation and running costs are relatively high [5]. To date, few studies have been conducted under natural environmental conditions and examining the effects of elevated ozone on plant responses. Moreover, information about the effects of tropospheric ozone on crops is also crucial for future practical aspects of cultivation.

Thus, the present study aimed to fill this gap and was performed to evaluate the accumulative tropospheric ozone effect on the activity of antioxidant enzymes and oxidative injury in leaves of bean and petunia growing at city and forest sites. The experiments were conducted during three vegetation seasons with various meteorological conditions and ozone concentrations. The obtained results provide information about the 28-day tropospheric ozone effect in representatives of the horticultural crop (bean) and ornamental (petunia) plants in ambient air conditions. What is more, the estimation of antioxidant enzymes was performed several times and simultaneously. Such experiments allowed us to track the impact of ozone on the antioxidant activity in the leaves of examined plants in changing environmental conditions.

## 2. Materials and Methods

### 2.1. Plant Cultivation and Exposure to Ozone

Bean (*Phaseolus vulgaris* L. ‘Nerina’) and petunia (*Petunia* × *hybrid*, ‘White cascade’) were used in this study. The seeds of bean and petunia were purchased from certificated companies professionally growing seeds, Fratelli Ingegnoli il Vivaio (Italy, Milano) and Legutko W. (Poland, Jutrosin), respectively. The selection of certain cultivars was related to their potential to reveal ozone-sensitivity features [19,20]. Three independent experiments were conducted during three vegetation seasons differing in ozone concentrations, solar radiation, and temperature. The seeds of bean and petunia were sown into a 15 cm<sup>3</sup> pot filled with a standard mixture of peat and sand enriched with the addition of slowly released fertiliser. In each pot, one plant was grown. Plants were grown in a control condition of 21–23 °C and PAR at level 800–1000  $\mu\text{moles m}^{-2} \text{s}^{-1}$  of photon flux density during natural daylight hours in a greenhouse located at Poznan University of Life Sciences (N 52°25′50″, E 16°55′01″). Twenty pots of six-week-old petunia and four-week-old bean plants were transported at the end of June to two exposure sites characterised by varied ozone concentrations and meteorological conditions and left there for four weeks (Tables 1 and 2).

**Table 1.** Ozone concentrations, solar radiation, and temperature measured in forest and city during exposure periods in three years of research (data obtained by automatic air monitoring system of National Environmental Monitoring; presented data are calculated based on mean hour values). Different capital letters show statistically significant differences between sites, and different small letters present differences between years at city and forest.

| Parameter                                       | Exposure Day | City<br>(N 52°25′13″, E16°52′40″)<br>Located in Poznan City (Central-Western Poland)<br>with a Population of about 550,000 Inhabitants,<br>Surrounded by Buildings, Near the Road. |                       |                       | Forest<br>(N 52°30′00″ E 17°46′26″)<br>Located about 80 km from Poznan City<br>in a Forestry Area. The Nearest Village<br>Is about 5 km from the Site. |                       |                       |
|-------------------------------------------------|--------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|-----------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|-----------------------|
|                                                 |              | 1st Year<br>2009                                                                                                                                                                   | 2nd Year<br>2010      | 3rd Year<br>2011      | 1st Year<br>2009                                                                                                                                       | 2nd Year<br>2010      | 3rd Year<br>2011      |
|                                                 |              |                                                                                                                                                                                    |                       |                       |                                                                                                                                                        |                       |                       |
| Mean O <sub>3</sub><br>( $\mu\text{g m}^{-3}$ ) | 7            | 53.9                                                                                                                                                                               | 59.6                  | 55.7                  | 64.1                                                                                                                                                   | 76.3                  | 63.5                  |
|                                                 | 14           | 47.2                                                                                                                                                                               | 58.0                  | 47.2                  | 50.5                                                                                                                                                   | 74.2                  | 65.1                  |
|                                                 | 21           | 49.6                                                                                                                                                                               | 78.1                  | 57.9                  | 67.4                                                                                                                                                   | 110.1                 | 68.5                  |
|                                                 | 28           | 44.4                                                                                                                                                                               | 65.4                  | 49.5                  | 57.7                                                                                                                                                   | 84.6                  | 58.0                  |
|                                                 | Means        | 48.78 <sup>b,B</sup>                                                                                                                                                               | 65.28 <sup>a,B</sup>  | 52.58 <sup>ab,B</sup> | 59.93 <sup>b,A</sup>                                                                                                                                   | 86.3 <sup>a,A</sup>   | 63.77 <sup>b,A</sup>  |
| Solar<br>radiation<br>(W m <sup>−2</sup> )      | 7            | 239.2                                                                                                                                                                              | 305.0                 | 185.5                 | 347.4                                                                                                                                                  | 440.9                 | 255.5                 |
|                                                 | 14           | 196.5                                                                                                                                                                              | 271.5                 | 228.9                 | 251.5                                                                                                                                                  | 369.1                 | 317.7                 |
|                                                 | 21           | 201.8                                                                                                                                                                              | 272.3                 | 216.5                 | 316.7                                                                                                                                                  | 334.4                 | 343.2                 |
|                                                 | 28           | 208.5                                                                                                                                                                              | 178.9                 | 148.2                 | 326.6                                                                                                                                                  | 215.5                 | 239.1                 |
|                                                 | Means        | 211.50 <sup>a,B</sup>                                                                                                                                                              | 256.93 <sup>a,B</sup> | 194.78 <sup>a,B</sup> | 310.55 <sup>a,A</sup>                                                                                                                                  | 339.98 <sup>a,A</sup> | 288.88 <sup>a,A</sup> |
| Temperature<br>(°C)                             | 7            | 20.4                                                                                                                                                                               | 20.0                  | 24.7                  | 19.2                                                                                                                                                   | 18.8                  | 14.5                  |
|                                                 | 14           | 15.9                                                                                                                                                                               | 20.3                  | 20.1                  | 14.3                                                                                                                                                   | 19.3                  | 17.0                  |
|                                                 | 21           | 19.5                                                                                                                                                                               | 23.8                  | 17.7                  | 18.8                                                                                                                                                   | 23.7                  | 17.5                  |
|                                                 | 28           | 17.3                                                                                                                                                                               | 19.9                  | 14.7                  | 16.6                                                                                                                                                   | 19.8                  | 15.2                  |
|                                                 | Means        | 18.28 <sup>a,A</sup>                                                                                                                                                               | 21.00 <sup>a,A</sup>  | 19.30 <sup>a,A</sup>  | 17.23 <sup>ab,A</sup>                                                                                                                                  | 20.40 <sup>a,A</sup>  | 16.05 <sup>b,B</sup>  |

Pots with plants were placed on special aluminium racks covered by shadow fabric to protect plants against excessive solar radiation (Figure 1). Continuous water supply was provided by glass-fibre wicks placed in pots and water trays located below the Styrofoam covering pots. Plants were exposed to ozone for four weeks. A similar set of plants was used as a control combination under ozone-free conditions (remained at a stable level of 25–40 ppb, which is treated as a background for the northern hemisphere), but meteorological conditions were maintained similar to the ambient ones (the temperature

was at a constant level of 23–25 °C and PAR at 800–1000  $\mu\text{moles m}^{-2} \text{s}^{-1}$  of photon flux density). Healthy plants without visible mechanical injuries were taken to examine the membrane injury index (*MI*), lipid peroxidation, and the activity of antioxidant enzymes.

**Table 2.** One-way ANOVA results for effect of site (city, forest) and year on level of ozone concentration, radiation, and temperature. Numbers are F-values with level of significance given as superscript. \*\*  $\alpha \leq 0.01$ , \*\*\*  $\alpha \leq 0.05$ , <sup>ns</sup>—not significant).

| Source of Variation | Year   | Ozone      | Radiation           | Temperature          |
|---------------------|--------|------------|---------------------|----------------------|
| Site                | 1      | 6.918 ***  | 18.880 **           | 0.4736 <sup>ns</sup> |
|                     | 2      | 98.036 *** | 402.193 ***         | 2.980 <sup>ns</sup>  |
|                     | 3      | 49.432 *** | 31.401 ***          | 136.020 ***          |
| Source of Variation | Site   | Ozone      | Radiation           | Temperature          |
| Year                | City   | 7.174 ***  | 2.691 <sup>ns</sup> | 0.889 <sup>ns</sup>  |
|                     | Forest | 7.024 ***  | 0.606 <sup>ns</sup> | 4.998 ***            |



**Figure 1.** Exposure sites structure of pots with plants placed on Styrofoam and water trays within a special aluminium construction.

## 2.2. Sample Collection

Three plants of each species were randomly chosen from both ozone exposure and control sites and were transported to the laboratory after 7, 14, 21, and 28 days of exposure. Fully matured leaves were collected and used for the estimation. The values of *MI*, lipid peroxidation, and the activity of ascorbate peroxidase (APX) and guaiacol peroxidase (GuPX) were immediately determined after sampling. The plant material for the estimation of superoxide dismutase (SOD) activity was frozen in liquid nitrogen and stored at  $-20\text{ }^{\circ}\text{C}$  until analysis. The analyses were carried out using three independent biological replicates. Each replicate was a sample of plant material derived from a different plant.

## 2.3. Cell Membrane Injury Index

Five discs (1.5 cm diameter) were cut from leaves and washed three times in 10  $\text{cm}^3$  of deionised water to remove electrolytes adhering to the surface. Then the discs were put in a 50  $\text{cm}^3$  flask, submerged in 10  $\text{cm}^3$  of deionised water, and kept at  $10\text{ }^{\circ}\text{C}$  for 24 h. After that time, the electrical conductivity of effusates was measured using a microcomputer conductometer CC-317 (ELMETRON, Zabrze, Poland). Next, tissues were killed by autoclaving for 15 min, and electrical conductivity was measured once again. In each case, the

measurement of electrical conductivity was performed after the temperature stabilised at 25 °C. Cell membrane injury (CMI) was evaluated according to the formula [21]

$$CMI = 1 - \frac{1 - (T1/T2)}{1 - C1/C2} \times 100\%$$

where C1 and C2 represent the conductivity values of samples from control plants before and after autoclaving, respectively; T1 and T2 represent the conductivity values for samples from ozone-treated plants before and after autoclaving, respectively.

#### 2.4. Enzyme Extraction and Assay

Leaf samples (500 mg) were homogenised in a chilled mortar with 4 cm<sup>3</sup> of 0.1 M potassium phosphate buffer (pH 7.0) and 20 mg of Polyclar AT. Homogenates were centrifuged at 16,000 × g for 30 min at 4 °C. The obtained supernatants were used to determine the activity of APX [22], GuPX [23], and lipid peroxidation.

The activity of APX was determined in a reaction mixture containing 2.3 cm<sup>3</sup> of 0.1 M potassium phosphate buffer (pH 7.0), 0.2 cm<sup>3</sup> of 5 mM L-ascorbate, 0.3 cm<sup>3</sup> of 1 mM H<sub>2</sub>O<sub>2</sub>, and 0.2 cm<sup>3</sup> of enzyme extract. The hydrogen-peroxide-dependent oxidation of ASC was followed by a decrease in absorbance at 290 nm (absorption coefficient 2.8 mM<sup>-1</sup>·cm<sup>-1</sup>). APX activity was expressed in nkat·mg<sup>-1</sup> protein.

The activity of GuPX was estimated in a reaction mixture containing 0.5 cm<sup>3</sup> of enzyme extract, 0.5 cm<sup>3</sup> of 3.4 mM guaiacol, and 0.5 cm<sup>3</sup> of 0.9 mM H<sub>2</sub>O<sub>2</sub>. The oxidation of guaiacol to tetraguaiacol in the presence of H<sub>2</sub>O<sub>2</sub> was measured as an increase in absorbance recorded at 470 nm. The enzyme activity was calculated using the absorption coefficient for tetraguaiacol (absorption coefficient 2.66 mM<sup>-1</sup>·cm<sup>-1</sup>), and it was expressed in nkat·mg<sup>-1</sup> protein.

SOD activity was estimated by the method of [24]. Leaf samples (500 mg) were homogenised in a chilled mortar with 4 cm<sup>3</sup> of buffer (50 mM sodium phosphate buffer pH 7.0 containing 1% polyvinylpyrrolidone, 1 mM EDTA-Na, and 0.5M NaCl). Homogenates were centrifuged at 16,000 × g for 25 min at 4 °C, and supernatants were used for the assay of enzyme activity. The incubation mixture contained 2.35 cm<sup>3</sup> of 50 mM sodium phosphate buffer (pH 7.8) with the addition of 0.1 mM EDTA-Na, 0.4 cm<sup>3</sup> of 97 mM methionine, 0.1 cm<sup>3</sup> of 2 mM NBT (nitro blue tetrazolium), and 0.05 cm<sup>3</sup> of enzyme extract. Finally, 0.1 cm<sup>3</sup> of 120 µM riboflavin was added, and the samples were placed under fluorescent lamps for 10 min. At the same time, a blank without the enzyme extract was prepared. 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 and it was expressed in U·mg<sup>-1</sup> protein. The concentration of protein in the supernatant was estimated according to [25]. All spectrophotometric measurements were made using a Jasco V-530 UV-VIS spectrophotometer (Japan).

#### 2.5. Lipid Peroxidation

The level of lipid peroxidation was estimated by quantifying the malondialdehyde (MDA) content using thiobarbituric acid assay (TBA test) according to [26]. For the assessment of the MDA content, 2 cm<sup>3</sup> of 20% trichloroacetic acid containing 0.5% TBA was added to 0.5 cm<sup>3</sup> of the supernatant. The mixture was heated at 95 °C for 30 min then was cooled in an ice bath and centrifuged at 10,000 × g for 10 min. The absorbance of the supernatant was measured at 532 nm and 600 nm (Jasco V-530 UV-VIS, Tokyo, Japan). The value of nonspecific absorption at 600 nm was subtracted from the reading at 532 nm. The MDA content was calculated using the MDA's absorption coefficient (155 mM<sup>-1</sup>·cm<sup>-1</sup>), and it was expressed in µmol·g<sup>-1</sup> fresh matter.

#### 2.6. Statistical Analysis

Statistical analysis of the data (separately for each year) was performed using STATISTICA 13.3 package (StatSoft, Inc., Tulsa, OK, USA). One-way analysis of variance (ANOVA) was used to determine whether year and site of exposure had a significant effect on radiation temperature and ozone concentration. Two-way ANOVA was used to determine

whether time and sites of exposure had a significant effect on plant responses. The post hoc Tukey's test was employed to identify significant differences between individual means of measured parameters. The significance level was set at  $\alpha \leq 0.05$ . The differences were considered statistically significant when  $p$  values were less than or equal to  $\alpha$ . For the determination of structure and rules in relations between variates (analysed parameters and site characteristics parameters), principal component analysis (PCA) was used. The orthogonal transformation of variates to a new set of noncorrelated variates (components) was obtained in this analysis.

### 3. Results

The results of the two-way ANOVA revealed significant effects of site and days of exposure to ozone and their interaction on the activity of antioxidant enzymes, lipid peroxidation, and membrane injury in almost all analysed combinations (Table 3). However, the pattern of changes was different at tested exposure sites and years of research. Some differences in responses were also observed between bean and petunia (Figures 2–6).

**Table 3.** Two-way ANOVA results for effect of site (city, forest, control) and days of exposure on activity of APX, GuPX, SOD, level of lipid peroxidation (MDA), and membrane injury (MI) in bean and petunia in three experimental years. Numbers are F-values with level of significance given as superscript. \*  $\alpha \leq 0.001$ ; \*\*  $\alpha \leq 0.01$ , \*\*\*  $\alpha \leq 0.05$ , <sup>ns</sup>—not significant).

| Source of Variation Bean       | Year | APX       | GuPX      | SOD       | MDA               | MI         |
|--------------------------------|------|-----------|-----------|-----------|-------------------|------------|
| Site                           | 1    | 147.3 *** | 23.2 ***  | 37.0 ***  | 19.5 ***          | 19.1 ***   |
|                                | 2    | 4.9 ***   | 118.8 *** | 6.9 *     | 23.7 ***          | 7.2 *      |
|                                | 3    | 67.6 ***  | 7.3 **    | 57.5 ***  | 33.6 ***          | 4.5 *      |
| Days of exposure               | 1    | 154.0 *** | 91.6 ***  | 41.2 ***  | 109.7 ***         | 243.7 ***  |
|                                | 2    | 131.9 *** | 451.7 *** | 32.8 ***  | 8.9 ***           | 21.5 *     |
|                                | 3    | 25.4 ***  | 36.2 ***  | 140.2 *** | 19.6 ***          | 18.4 ***   |
| Site $\times$ days of exposure | 1    | 140.3 *** | 70.0 ***  | 30.6 ***  | 10.2 ***          | 4.3 *      |
|                                | 2    | 10.5 ***  | 40.9 ***  | 22.2 ***  | 5.2 ***           | 3.7 *      |
|                                | 3    | 17.3 ***  | 5.1 ***   | 54.7 ***  | 5.4 ***           | 2.9 *      |
| Source of Variation Petunia    | Year | APX       | GuPX      | SOD       | MDA               | MI         |
| Site                           | 1    | 238.8 *** | 68.0 ***  | 62.6 ***  | 31.7 *            | 49.048 *** |
|                                | 2    | 5.1 *     | 65.0 ***  | 8.9 ***   | 36.5 ***          | 13.002 *   |
|                                | 3    | 32.2 ***  | 10.0 **   | 15.3 ***  | 63.5 ***          | 7.468 *    |
| Days of exposure               | 1    | 27.5 ***  | 68.0 ***  | 62.6 ***  | 16.0 ***          | 43.755 *** |
|                                | 2    | 195.6 *** | 393.9 *** | 36.4 ***  | 402.7 ***         | 11.941 *   |
|                                | 3    | 128.7 *** | 8.0 **    | 22.8 ***  | 4.5 <sup>ns</sup> | 6.421 *    |
| Site $\times$ days of exposure | 1    | 63.6 ***  | 21.3 ***  | 126.8 *** | 11.3 *            | 54.889 *** |
|                                | 2    | 24.1 ***  | 34.0 ***  | 12.8 ***  | 15.5 ***          | 12.751 *   |
|                                | 3    | 3.9 *     | 12.6 *    | 27.4 ***  | 11.4 *            | 7.208 *    |

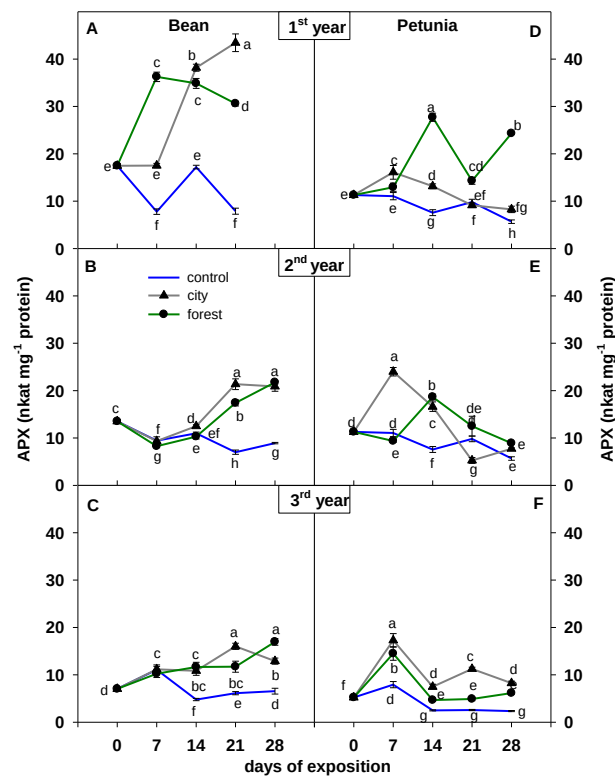


**Figure 2.** Effect of long-term exposure to ozone at different sites and years on activity of APX in leaves of bean (A–C) and petunia (D–F). Data are shown as mean  $\pm$  standard error. Different letters indicate significant differences between means ( $p \leq 0.05$ ) within years for petunia and bean.

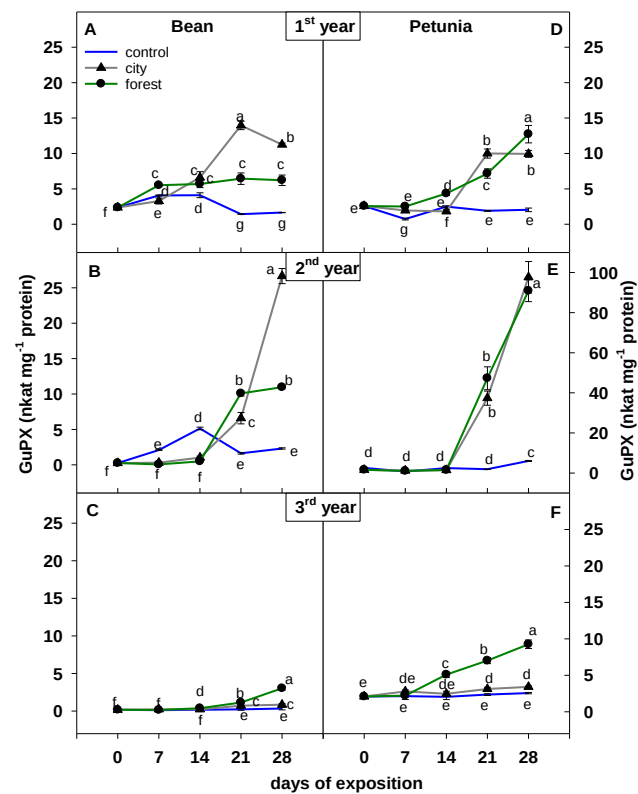


**Figure 3.** Effect of long-term exposure to ozone at different sites and years on activity of GuPX in leaves of bean (A–C) and petunia (D–F). Data are shown as mean  $\pm$  standard error. Different letters indicate significant differences between means ( $p \leq 0.05$ ) within years for petunia and bean.

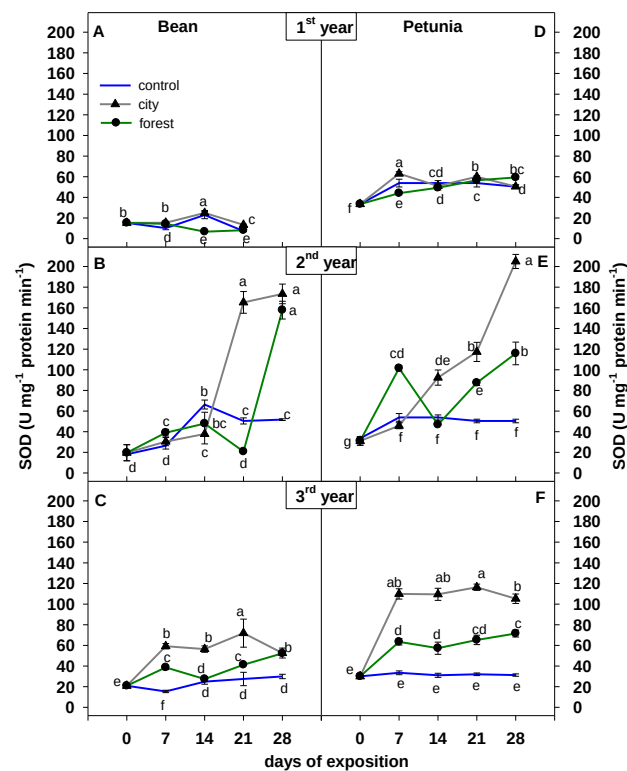


**Figure 4.** Effect of long-term exposure to ozone at different sites and years on activity of GuPX in leaves of bean (A–C) and petunia (D–F). Data are shown as mean  $\pm$  standard error. Different letters indicate significant differences between means ( $p \leq 0.05$ ) within years for petunia and bean.

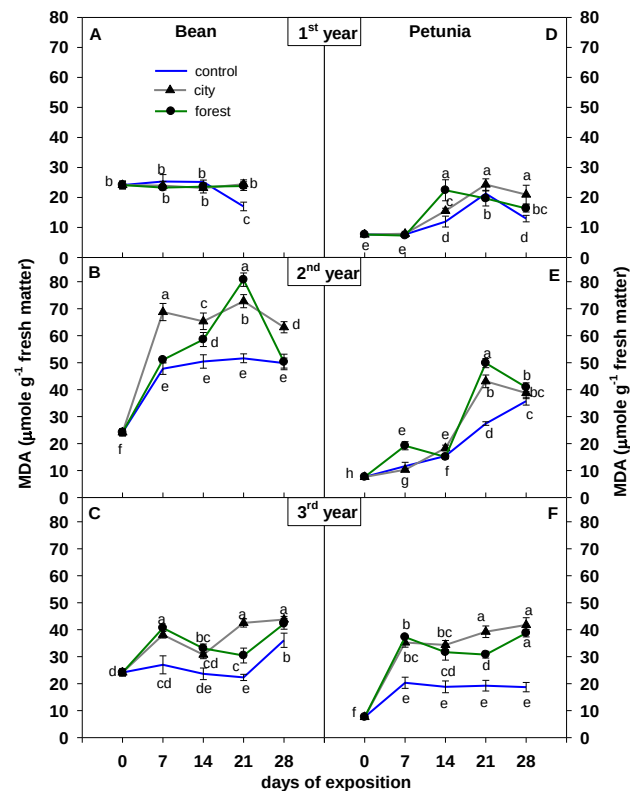


**Figure 5.** Effect of long-term exposure to ozone at different sites and years on level of lipid peroxidation (MDA) in leaves of bean (A–C) and petunia (D–F). Data are shown as mean  $\pm$  standard error. Different letters indicate significant differences between means ( $p \leq 0.05$ ) within years for petunia and bean.

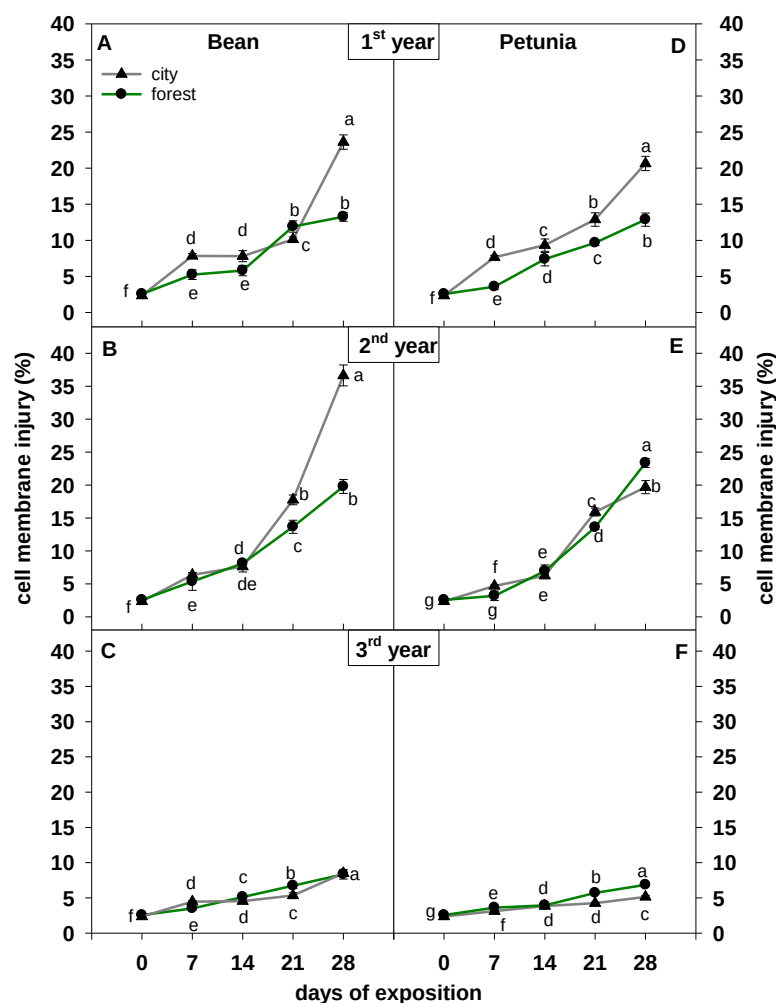


**Figure 6.** Effect of long-term exposure to ozone at different sites and years on cell membrane injury (CMI) in leaves of bean (A–C) and petunia (D–F). Data are shown as mean  $\pm$  standard error. Different letters indicate significant differences between means ( $p \leq 0.05$ ) within years for petunia and bean.

### 3.1. Antioxidant Enzymes

In each year of the study, the activity of APX before exposure to the city and forest sites was similar in bean and petunia (Figure 2A–F). However, slightly higher activity in both species was recorded in the first two years. The activity of this enzyme in the leaves of the plants grown under the control conditions was different on the subsequent sampling days, but no clear upward or downward trend was observed. A clear and gradual increase in the activity of APX during the exposure period was found in the bean at both exposure sites in all years, but the lowest level was observed in the last year (Figure 2A–C). In the first year, the increase in the activity of this enzyme was earlier in the forest than in the city (Figure 2A). However, in the last two years, the pattern of changes in APX activity was similar in both sites (Figure 2B,C). In all the years of the study, petunia showed a significant increase in APX activity after 7 or 14 days of exposure, followed by a decrease in the activity at most subsequent dates (Figure 2D–F). The increase in APX activity in the first year was greater in the forest; in the second year, it was earlier and greater in the city; and in the third year, it was similar in both sites.

The activity of GuPX in both species before exposure was similar in the first two years of the study (Figure 3A,B,D,E). However, in the last year, it was slightly higher in petunia (Figure 3C,F). Under control conditions, the activity of this enzyme in the leaves of both species remained at a similar level and slightly changed at some sampling dates. A gradual increase in GuPX activity during exposure in the city and forest in all years

of the study was observed in bean (Figure 3A–C). In the first two years, the activity of GuPX after 28 days of exposure increased more in the city (Figure 3A,B). In the last year, it was higher in the forest (Figure 2C). In petunia, the increase in GuPX activity in all years, especially at forest site, was greater than that in bean. In the first two years, the activity of this enzyme increased similarly at both sites, but much more in the second year of the study (Figure 3D,E). However, in the last year, an earlier and greater increase in the activity of this enzyme was found in the forest (Figure 3F).

In the first year of the study, the activity of SOD before exposure was greater in petunia than in bean (Figure 4A,D). In the following years, it was similar in both species (Figure 4B,C,E,F). In the first year of the study, the activity of this enzyme in bean similarly changed under control conditions and in the city, i.e., it increased after 21 days of exposure and then it decreased. On the other hand, in the forest, a decrease in the activity of this enzyme was observed after 21 days of exposure and no changes on the last date (Figure 4A). However, the activity of SOD in petunia showed a gradual but slight increase in the first year of the study at all sites (Figure 4D). In the following two years, the activity of this enzyme before exposure was similar in both species and remained at a comparable level at subsequent time points in the control combination (Figure 4B,C,E,F). A gradual increase in SOD was found in both species growing at both exposure sites. In bean, the activity of SOD increased much more after 21 days in the city than in the forest, but it reached the same level in both sites after 28 days of exposure (Figure 4B,C). In petunia, a greater increase in SOD activity was observed in the city than in the forest until the end of the experiment (Figure 4E,F). In both species, a greater increase in SOD activity in the second and third years of research at most time points was found in the city than in the forest site.

### 3.2. Lipid Peroxidation

In all the years of the study, the level of lipid peroxidation (MDA) before the exposure in the city and forest was higher in bean than in petunia (Figure 4A–F). In the first year, the level of MDA in bean did not change during 28 days of the experiment, both at forest and city sites. It was almost the same at all exposure sites during 21 days of the experiment and decreased in control plants after 28 days (Figure 5A). On the other hand, in petunia, a significant increase in MDA at all sites was found after 14 days of exposure and slight fluctuations in subsequent dates (Figure 5D). In the following year, the level of MDA was maintained at an increased level in bean, starting from 7 days of exposure, in all experimental combinations. At most time points, the level of lipid peroxidation was higher in the city and forest than in the control (Figure 5B). In the second year of the study, petunia showed a gradual and similar increase in the level of MDA in all combinations (Figure 5E). In the last year, the level of lipid peroxidation significantly increased just after 7 days of exposure in all experimental combinations (Figure 5C,F). A considerably higher and similar increase in MDA was found in the city and forest than in the control. At subsequent dates, the level of MDA did not change in the control combination and slightly increased at both exposure sites, reaching the same level after 28 days.

### 3.3. Cell Membrane Injury

A gradual increase in the level of cell membrane damage was observed in bean and petunia during 28 days of plant exposure at both sites (Figure 6A–F). After 28 days of exposure, the level of damage was greater in the first two years of the study than in the last year. In the first year, greater membrane damages and similar in both species were found in the city (Figure 6A,D). In the following year, in bean, greater membrane damage was found in the city than in the forest, and it was greater than in petunia (Figure 6B,E). In the last year, similar membrane damage was found at both sites (Figure 6C). Membrane damage in petunia in the last two years after 28 days of exposure was higher in the forest than in the city (Figure 6E,F).

### 3.4. Principal Component Analysis

The graphical presentation of data by principal component analysis for physiological parameters, mean ozone concentration, and meteorological parameters in both sites for three years explained approximately 60% of the data variability. The results in the first year revealed a negative relation between ozone and all analysed parameters (Figure 7A,D). However, in the case of petunia, a positive relationship between temperature and SOD was noted, whereas for bean, a positive relation of cell membrane injury (CMI) to temperature was also observed. In the second year, a positive relation of ozone to temperature as well as to MDA was recorded in both analysed plant species.

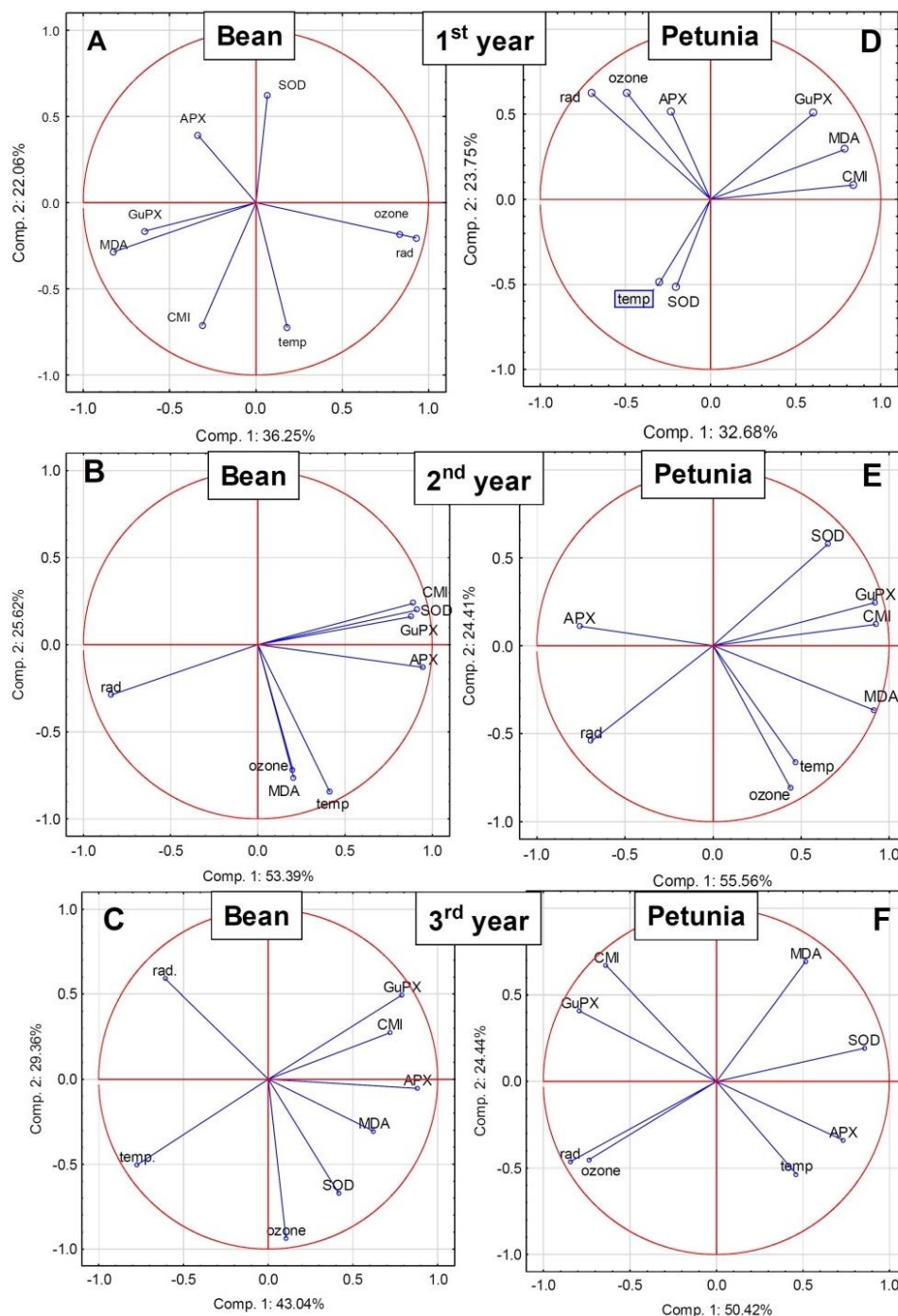


**Figure 7.** Principal component analysis of bean (A–C) and petunia (D–F). Abbreviations: rad—solar radiation; tem—temperature; ozone—mean ozone concentration.

The enzymes revealed a slightly positive or no relation to ozone (Figure 7B,E). In the last year, variation in relations between parameters was observed. In the case of bean, a positive relation of ozone to SOD and MDA was especially notable, whereas for petunia, a positive relation of ozone to GuPX was recorded (Figure 7C,F).

#### 4. Discussion

Plant responses to ozone are modified by multiple environmental factors, such as solar radiation, temperature, and air humidity, which affect its formation from their precursors and influx into the leaves through stomata [7,27–29]. Antioxidant enzymes play an important role in the plant response to elevated ozone, and their activity is affected by the changes in ambient ozone concentration as well as by meteorological conditions [1,3]. There is a small amount of data in the literature regarding the effect of varied ozone concentrations in ambient air on antioxidant enzyme activity obtained from experiments conducted under natural conditions during the vegetation season. The results of experiments carried out under controlled air conditions with only one stress factor affecting the plants (ozone concentration) may lead to misleading conclusions because they do not reflect the real plants' response to this air pollutant under a natural environment characterised by variable weather conditions. Plant responses to long-term elevated ozone concentration under natural environmental conditions with variable levels of meteorological factors are different than the responses under controlled conditions [30]. The differences in the responses are mainly due to changes in temperature and solar radiation, which are found to affect ozone formation and modulate stomata opening and ozone fluxes into the leaves leading to changes at the cellular level including enzyme activity [18,31,32].

Our study revealed that 28-day exposure to elevated tropospheric ozone concentration under natural conditions triggered changes in antioxidant enzyme activities both in bean and petunia. The pattern of observed changes was dependent on exposure sites, the year of study, and species. In year 2, higher solar radiation and tropospheric ozone concentration were observed, and the activity of GuPX and SOD increased the most in both species and exposition sites. On the other hand, greater increases in SOD and APX in both species and GuPX in bean have been recorded in the city than in the forest with higher ozone concentrations. This might be an effect of higher air temperature at the city on the first dates of exposure in this second year of research. The results of the analysis of time-dependent variations in the activity of studied enzymes revealed differences between examined species in the course of changes in APX activity. The activity of APX increased in bean during the following days of exposure, but differently in particular years, whereas in petunia, APX activity rapidly increased at the beginning and then decreased at the end of the exposure. However, in year 2, a larger increase at the beginning of the experiment was noted at the forest site, where the highest ozone concentration and solar radiation were recorded. In all years, the activity of GuPX systematically increased during the growing season in both species in the forest and city. However, in years 1 and 2, this increase was larger in bean at the city characterised by lower ozone concentration and solar radiation. In these years, similar increase in the activity of GuPX was shown at both sites in petunia. It may indicate that the bean is more sensitive to ozone. Otherwise, in year 3, a larger increase in GuPX was shown in both species at the forest site with higher ozone concentrations and solar radiation than those in the city site. However, in bean, the increase was lower than in petunia. This also shows the species-dependent effect of ozone on the activity of examined enzymes. A temperature-dependent effect of ozone on the activity of SOD was observed in both species in year 3 with a higher ozone concentration in the forest, but a larger SOD increase was shown in the city where the higher air temperature was noted. The effect of temperature on the responses of *Brassica juncea* to ozone was reported by [33]. Higher temperature affects the increase in the level of ROS, decrease in the chlorophyll content, and leaf physiological activity due to the increase in the stomatal density, which allows ozone flux into the leaf. Our studies pointed to the role of temperature in relation to ozone and the plant response. Year 2 was characterised by higher solar

radiation and temperature as well as higher tropospheric ozone concentrations than the other two years. It seems that some parameter combinations, such as ozone, temperature, and solar radiation, determined the plant response. Environmental factors such as solar radiation or temperature cause ozone formation and also modify ozone flux and the plant response [34].

Our study revealed that ambient ozone concentration was higher at the forest than at the city site despite the fact that the air temperature, especially in year 3, was lower in the forest. Higher ozone concentrations in the forest site may be caused by the transport of ozone precursors from the pollution sources occurring in the city site. Solar radiation, which was higher in the forest area than in the city, was effective in ozone formation from precursors coming from the city and results in its greater concentration [35,36]. Moreover, volatile organic compounds (VOCs) emitted from trees in the forest could also be ozone precursors involved in ozone formation at this site [37,38]. The obtained results provide evidence that environmental factors modify the plants' response to elevated ozone concentration under natural conditions. It seems that meteorological conditions, especially temperature, could have a greater effect on the activity of antioxidant enzymes under conditions of the lower level of ambient ozone concentrations.

Antioxidant enzymes prevent the occurrence of oxidative damage, which is shown by the level of MDA [39]. One of the effects of lipid peroxidation is cell membrane damage manifested by the loss of selective permeability [1,14]. However, our results revealed an increased level of MDA as well as the membrane injury index during exposure to elevated ozone concentrations. The level of MDA also increased in control plants, which might be caused by leaf senescence, but in most cases, this increase was smaller than in plants exposed to the site with increased ozone concentration. Therefore, the obtained results show that the increased activity of antioxidant enzymes did not reduce oxidative damage (MDA) or prevent membrane damage. What is more, the significant negative correlation between the activity of APX and the level of MDA in the second year of research in petunia, which resulted from a decrease in the activity of this enzyme at the last exposure date, along with the increase in the level of MDA, indicates the important role of APX in the mitigation of oxidative stress and prevention of lipid peroxidation. This was also apparent from the PCA results, where a strong positive relation between ozone concentration and MDA was recorded, whereas there was a negative relation to APX. In this study, we also found a systematic increase in *CMI* in the leaves of both species under exposure to ozone. We recorded the lowest level of membrane injury in year 3 when the level of lipid peroxidation was the lowest. PCA analyses confirmed a positive tendency in the relation between antioxidant enzymes and *CMI* for all years in both species, except bean in the second year, when no relation was noted.

## 5. Conclusions

Overall, the main finding of the presented research is to show that several weeks of plant exposure to the increased ozone concentration under natural conditions increase the activity of the antioxidant enzyme. Moreover, it has also been found that the plants' final response depends on meteorological conditions. Higher ozone concentration along with higher solar radiation affected a higher increase in antioxidant enzyme activity. On the other hand, the obtained results also indicate the modifying effect of temperature on plants' response to ozone stress. Hence, it is crucial to relate the observed biochemical and physiological changes to ozone concentration and general environmental conditions, especially solar radiation and air temperature.

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