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Research article

Oxidative stress in ryegrass growing under different air pollution levels and its likely effects on pollen allergenicity

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ABSTRACT

In the present work, for the first time in the literature, the relationship between the degree of air pollution, the physiological state of the plants and the allergenic capacity of the pollen they produce has been studied. The physiological state of *Lolium perenne* plants growing in two cities with a high degree of traffic, but with different levels of air pollution, Madrid and Ciudad Real, have been explored. The photosynthetic efficiency of the plants through the emission of fluorescence of PSII, the degree of oxidative stress (enzymatic activities related to the ascorbate-glutathione cycle), the redox state (reduced and oxidized forms of ascorbate and glutathione) and the concentration of malondialdehyde have been evaluated. During the development period of the plants, Madrid had higher levels of NO_2 and SO_2 than Ciudad Real. The greater degree of air pollution suffered by Madrid plants was reflected on a lower photosynthetic efficiency and a greater degree of oxidative stress. In addition, NADPH oxidase activity and H_2O_2 levels in pollen from Madrid were significantly higher, suggesting a likely higher allergenic capacity of this pollen associated to a higher air pollution.

1. Introduction

Plants are the main source of allergens that produce allergenic response in immunoglobulin E (IgE) mediated allergies. Allergens present in pollen are responsible for symptoms like rhinoconjunctivitis, asthma, edema, urticarial, and anaphylaxis (Sinha et al., 2014).

Nowadays, allergic diseases have become a pandemic health problem. In general, pollen allergens are considered a major risk factor for both seasonal allergic rhinitis and asthma. Studies showed that more than 50% of patients with perennial allergic rhinitis are sensitized to pollen allergens, and people affected by pollen allergy is on the increase worldwide (Bastl et al., 2018; Kmenta et al., 2017; Sedghy et al., 2018). Unfortunately, pollen allergens are difficult to avoid because of the extremely small size (< 2.5 μm) and high prevalence of pollen (Vieths et al., 2002; Reinmuth-Selzle et al., 2017).

Pollen from trees, grasses, and weeds all have been found to elicitate allergic reactions (Emberlin, 2009). To date, 11 groups of grass pollen allergens with the ability to elicit a specific IgE response have been identified (Hrabina et al., 2008).

Plants of the Poaceae family are the main source of grass pollen allergens, due to their worldwide distribution and their significant pollen producing capability. The most important species are *Lolium perenne* (perennial ryegrass), *Poa pratensis* (Kentucky bluegrass) and *Phleum pratense* (Taketomi et al., 2006). The pollen of perennial ryegrass is a widespread source of allergens and a major cause of allergic diseases (Hue Jung Park et al., 2016; Marsh, 1975; Subiza, 2003).

The genus *Lolium* has at least 5 species in the Iberian Peninsula, of which the most common is *Lolium perenne* L (perennial ryegrass). Its inflorescence is formed by two rows of compressed spikelets, which tend to be embedded in excavations of the axis and placed backwards, causing the inflorescence to be flattened; each spikelet has several flowers, up to 22, and only the terminal spikelet retains its two bracts or glumes (Subiza, 2003).

Allergies and hypersensitive responses initiated by specific immunologic mechanisms triggered by pollen and other allergens, constitute a major health issue in modern societies (Reinmuth-Selzle et al., 2017). The potential reasons discussed for this trend include climate change and anthropogenic air pollution (Kramer et al., 2000; Ring et al., 2001). Ozone and NO_2 are two important air pollutants that not only affect human health but also impact plants and their pollen (Frank

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and Ernst, 2016). Ground level ozone, which is a secondary pollutant, is the result of a photochemical reaction of volatile organic compounds and nitrogen oxides (NOx), which are mainly produced by combustion processes during energy production, industrial processing or car traffic (Frank and Ernst, 2016).

The increase of the green zones in the urban areas definitely improves quality of life in the cities; however, it also has some associated problems. The most serious issue might be the growing trend in sensitization to pollen, especially in urban settings (Bosch-Cano et al., 2011; Cariñanos and Casares-Porcel, 2011); in fact, people living near heavy traffic are affected with pollen-induced respiratory allergies to a greater extent than those in rural districts (D' Amato et al., 2010). Many works have evidenced the relationship between air pollution and a greater allergenic capacity of pollen from plants subjected to these pollutants (Alfaya Arias et al., 2014; Armentia et al., 2002; Feo Brito et al., 2007; Frank and Ernst, 2016; Ghiani et al., 2012; Mur Gimeno et al., 2007; Sénéchal et al., 2015).

Some recent reviews (Reinmuth-Selzle et al., 2017 Sedghy et al., 2018) reports the following mechanisms to explain this fact: a) cell wall damage. The interaction between air pollutants and pollen grains might damage the pollen cell wall, increasing the amount of allergens released into the environment (Lu et al., 2014); b) increasing allergenicity through interaction with air pollutants. Air pollutants can modify pollen allergenicity through interaction with allergens by various mechanisms, including transformations induced by post-translational modifications (Ghiani et al., 2012), or formation of nitrotyrosine residues in presence of NO_2, a major traffic-related air pollutant (Gruijthuijsen et al., 2006); c) adjuvant effects of air pollutants. Several air pollutants act as adjuvants through binding to allergens and stimulating IgE synthesis, resulting in exacerbation of asthma symptoms (Bartra et al., 2007; Kalbande et al., 2008). Diesel exhaust particles (DEPs) are one of the most important adjuvant in the environment (Diaz-Sanchez et al., 1999; Yoo and Perzanowski, 2014), and d) induction, in presence of air pollutants, of allergenic protein expression, expressed only in air contaminated areas (Bellanger et al., 2012). The effect of air pollution on allergens categorized as pathogenesis-related (PR) proteins, has attracted much attention (D'Amato, 2011).

In situations of stress such as air pollution, plants are forced to modify their physiological state, trying to adapt to that situation. The rapid generation of reactive oxygen species (ROS) represents a common plant response to different biotic and abiotic stresses (Cramer et al., 2011; Kotchoni and Gachomo, 2006; Kovtun et al., 2000; Lamb and Dixon, 1997; Mittler et al., 2011; Noctor et al., 2014; Orozco-Cardenas and Ryan, 1999; Petrov and Van Breusegem, 2012; Xia et al., 2015). A disturbance in the ROS/antioxidant homeostasis leads to a physiological situation called oxidative stress (Gill and Tuteja, 2010). Thus, oxidative stress (via unmetabolized and/or excess ROS and their reaction products within cells) can cause significant physiological challenges including cell death, and the arrest of plant growth and development, mainly by provoking oxidative modification of vital biomolecules including membrane lipids, cellular amino acids, proteins, and DNA (Anjum et al., 2012; Gill and Tuteja, 2010; Krasensky and Jonak, 2012; Mahajan and Tuteja, 2005). To maintain optimal levels of ROS, plants have a sophisticated system including enzymatic and non-enzymatic mechanisms.

In summary, there seems to be a relationship between the stress caused by environmental pollutants and the increase in pollen allergenicity associated to oxidative stress in plants. Therefore, an study was conducted in two locations in central Spain (Madrid and Ciudad Real), with high traffic and different pollution levels, especially in nitrogen and sulfur oxides, to explore the physiological changes in wild populations of *Lolium perenne* under both conditions, evaluating photosynthetic performance, oxidative stress and enzymes involved in ROS scavenging systems. The hypothesis of this work is that the different conditions of air pollution could determine different degree of oxidative stress and physiological state in both populations in the moment of pollen production, and this could impact on the pollen allergenic capacities. Altered allergenic capacity of the pollen was evaluated with NADPH oxidase activity and H_2O_2 levels, since it is demonstrated that NADPH oxidase–induced oxidative stress plays a critical role in facilitating pollen antigen–induced allergic airway inflammation (Boldogh, 2005; Dharajiya et al., 2008).

2. Material and methods

2.1. Air pollution data and meteorological conditions

Air pollution data were obtained from the air quality web portal of Madrid Town Hall (http://www.mambiente.munimadrid.es/opencms/opencms/calaire/consulta/boletin_mensual.html?_locale=es&CSRF_TOKEN=d5dc4c12c6361d3e22ec7248a565657b5636d8b2). Data from Ciudad Real were obtained from web portal of the Vice-Ministry of the Environment Monitoring and control of air quality of the Junta de Castilla la Mancha (http://www.castillalamancha.es/gobierno/agrimedambydesrur/estructura/vicmedamb/actuaciones/vigilancia-y-control-de-la-calidad-del-aire).

The data of the atmospheric pollutants shown in the work, SO_2 ($\mu g.m^{-3}$), NO_2 ($\mu g.m^{-3}$) and O_3 ($\mu g.m^{-3}$), are the monthly average of the period from October 2016 to May 2017. In both cases the data was collected from the nearest stations to the sampling point of pollen and plants.

Meteorological conditions data during the studied period were obtained from AEMET (Agencia Española de Meteorología) web portal (http://www.aemet.es).

2.2. Plant materials an experimental design

Lolium perenne plants growing in natural conditions in the cities of Ciudad Real and Madrid were used for all experiments. Plants were recollected at the moment when the dehiscence of the anthers began to take place (mid of May 2017), and photosynthesis efficiency was measured in this moment. Three populations of *Lolium perenne* separated by 50–100 m were selected. Ten plants from each population were harvested, constituting a replicate. To avoid the possible effect of the different state of plant development on results, all the plants sampled had the same size and the same stage of development and only plants in which anthers were already visible in some flowers (about 6 anthers per inflorescence) were sampled. Plants were brought to the lab in dry ice. In the lab, inflorescences were separated from the plants (leaves + stem), making the analysis of enzyme activities related to oxidative stress, ascorbate (ASC), glutathione (GSH) and malondialdehyde (MDA) determination in each one separately.

Pollen was recollected from the same populations and in the same day by the company Iberpolen S.L (Alcala la Real, Jaen, Spain). Briefly, once the plants are collected, they are transferred to a drying chamber with a dehumidifier that maintains an average temperature of 25 °C. After 170 h in the drying chamber the pollen is purified by mechanical sieving with vibration for 9 h. In this way the anthers are broken, and the pollen grains are separated from the rest. NADPH oxidase activity and H_2O_2 level were determined in this pollen.

2.3. Chlorophyll fluorescence. Photosynthetic efficiency

Photosynthetic efficiency was measured through the chlorophyll fluorescence emitted by photosystem II. Chlorophyll fluorescence was measured with a pulse amplitude modulated (PAM) fluorometer (Hansatech FM2, Hansatech, Inc, UK). After dark-adaptation of leaves, the minimal fluorescence (Fo; dark adapted minimum fluorescence) was measured with weak modulated irradiation ($1\ \mu mol\ m^{-2}\ s^{-1}$). Maximum fluorescence (Fm) was determined for the dark-adapted state by applying a 700 ms saturating flash ($9000\ \mu mol\ m^{-2}\ s^{-1}$). The variable fluorescence (Fv) was calculated as the difference between the

maximum fluorescence (Fm) and the minimum fluorescence (Fo). The maximum photosynthetic efficiency of photosystem II (maximal PSII quantum yield) was calculated as Fv/Fm. Immediately, the leaf was continuously irradiated with red-blue actinic beams (80 μmol m^{-2} s^{-1}) and equilibrated for 15 s to record Fs (steady-state fluorescence signal). Following this, another saturation flash (9000 μmol m^{-2} s^{-1}) was applied and then Fm' (maximum fluorescence under light adapted conditions) was determined. Other fluorescent parameters were calculated as follows: the effective PSII quantum yield ϕPSII = (Fm'-Fs)/Fm' (Genty et al., 1989); and the non-photochemical quenching coefficient NPQ = (Fm–Fm´)/Fm'. All measurements were carried out in 10 plants of each population.

2.4. Enzyme activities related to oxidative stress

Inflorescences and leaves + stem of each replicate separately were pooled and powdered in liquid nitrogen, and the powder obtained was used for the analyses of enzyme activities related to oxidative stress following the protocols described by Garcia-Limones et al. (2002) and Lucas et al. (2017).

Soluble proteins were extracted by resuspending 10 mg of powder in 1 mL of potassium phosphate buffer 0.1 M pH 7 containing 2 mM PMSF (Phenylmethylsulfonyl fluoride). These were sonicated 10 min and then centrifuged for 10 min at 18400*g*. The supernatant was divided into aliquots, frozen in liquid nitrogen and stored at −80 °C for further analysis. All above operations were carried out at 0-4 °C.

To measure the amount of total protein in plant extracts, 250 μL of Bradford reagent and 50 μL of sample and BSA dilutions were inoculated in ELISA 96 well plates and incubated for 15 min at room temperature and measured using a plate reader at absorbance of 595 nm. A calibration curve was constructed from commercial BSA dilutions expressed in milligrams. The units of protein were expressed as mg μL^{-1}.

Enzyme activities related to oxidative stress in plant extracts were measured spectrophotometrically: superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), guaiacol peroxidase (GPX, EC 1.11.1.7), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2). All were expressed as μmol mg $protein^{-1}$ min^{-1}, except SOD that was expressed as % inhibition mg $protein^{-1}$.

SOD activity was determined following the specifications of the SOD activity detection kit (SOD Assay Kit-WST, Sigma-Aldrich). With this method, the rate of the reduction with O_2 is linearly related to the xanthine oxidase (XO) activity and inhibited by SOD. Inhibition activity of SOD can be determined by colorimetric method.

CAT activity was measured by the method of Beers and Sizer (1952). The reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.0, 20 mM H_2O_2 and 120 μL of enzyme extract in a final volume of 1.2 mL. The reaction was started by adding H_2O_2 and the decrease in A_{240} produced by H_2O_2 breakdown was recorded. Extinction coefficient of 36 mM^{-1} cm^{-1} was used to calculate activity.

APX activity was measured by the method of Nakano and Asada (1981). The reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.0, 0.25 mM sodium ascorbate, 5 mM H_2O_2 and 100 μL of enzyme extract in a final volume of 1.2 mL. Adding H_2O_2 started the reaction and the oxidation of ascorbate was determined by the decrease in A_{290}. Extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate activity.

GPX activity was measured by the method of Srivastava and van Huystee (1977). The reaction mixture consisted of 100 mM potassium phosphate buffer, pH 6.5, 15 mM guaiacol, 0.05% (v/v) H_2O_2 and 120 μL enzyme extract in a final volume of 1.2 mL. Adding H_2O_2 started the reaction and the oxidation of guaiacol was determined by the increase in A_{470}. Extinction coefficient of 26.6 mM^{-1} cm^{-1} was used to calculate activity.

MDHAR activity was measured by the method of Xu et al. (2008). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.6), 0.2 mM NADH, 2.5 mM AsA, 1 unit of ascorbate oxidase and 100 μL enzyme extract in a final volume of 1.2 mL. Adding enzyme extract started the reaction and the reduction of monodehydro ascorbate was determined by the decrease in A_{340} Extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate activity.

DHAR activity was measured as described by Xu et al. (2008) at 265 nm. The reaction mixture consisted of potassium phosphate buffer 50 mM (pH 7.0), 2.5 mM reduced glutathione, 0.2 mM Dehydroascorbate, 0.1 mM EDTA and 100 μL enzyme extract in a final volume of 1.2 mL. Adding enzyme extract started the reaction and the reduction of dehydroascorbate was determined by the decrease in A_{265}. Extinction coefficient of 14 mM^{-1} cm^{-1} was used to calculate activity.

GR activity was measured by the method of Schaedle and Bassham. (1977). The assay mixture consisted of 50 mM potassium phosphate buffer, pH 7.5, 3.5 mM $MgCl_2$, 0.15 mM NADPH, 0.5 mM oxidized glutathione and 180 μL of enzyme extract in a final volume of 1.2 mL. Adding NADPH started the reaction and oxidation of this compound was determined by the increase in A_{340}. Extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate activity.

In all assays the blank consisted of the components of the reaction mixture except for the enzyme extract, which was replaced by an equal volume of the assay buffer. In the case of the GR assay, an additional blank without oxidized glutathione was included in order to account for the presence in the extracts of other enzyme activities able to oxidize NADPH.

2.5. Ascorbate (ASC) and glutathione (GSH) analysis

Fifty mg of powder was homogenized with 1.5 mL of cold 5% metaphosphoric acid at 4 °C in a porcelain mortar. The homogenate was centrifuged at 20,000*g* for 15 min at 4 °C and the supernatant was collected for analysis of ascorbate and glutathione.

ASC and its oxidized form dehydroascorbate (DHA) were measured according to Kampfenkel et al. (1995) with minor modifications. Briefly, total ascorbate was determined after reduction of DHA to ASC with DTT, and the concentration of DHA was estimated from the difference between total ascorbate pool (ASC plus DHA) and ASC The reaction mixture for total ascorbate pool contained a 0.1 mL aliquot of the supernatant, 0.25 mL of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, and 0.05 mL of 10 mM DTT. After incubation for 10 min at room temperature, 0.05 mL of 0.5% N-ethylmaleimide were added to remove excess DTI. ASC was determined in a similar reaction mixture except that 0.1 mL of H_2O was added rather than DTT and N-ethylmaleimide. Color was developed in both reaction mixtures after addition of the following reagents: 0.2 mL of 10% trichloroacetic acid, 0.2 mL of 44% orthophosphoric acid, 0.2 mL of 4% α,α'-dipyridyl in 70% ethanol and 0.3% (w/v) $FeCl_3$. After vortexing, the mixture was incubated at 40 °C for 40 min and the A_{525} was read. A standard curve was developed based on ASC in the range of 0–100 μg mL^{-1}. Data were expressed as μg mg^{-1} FW (fresh weight).

The glutathione pool was assayed according to Zhang et al. (1996) utilizing 0.4 mL aliquots of supernatant neutralized with 0.6 mL of 0.5 M phosphate buffer (pH 75). For GSSG (oxidized form) assay, the GSH (reduced form) was masked by adding 20 μL of 2-vinylpyridine to the neutralized supernatant, whereas 20 μL of H_2O was added in the aliquots utilized for total glutathione pool (GSH plus GSSG) assay. Tubes were mixed until an emulsion was formed. Glutathione content was measured in 1 mL of reaction mixture containing 0.2 mM NADPH, 100 mM phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM 5,5′- dithiobis (2-nitrobenzoic acid), and 0.1 mL of sample obtained as described above. The reaction was started by adding 3 units of glutathione reductase and was monitored by measuring the change in absorbance at 412 nm for 1 min. GSH was estimated as the difference between the

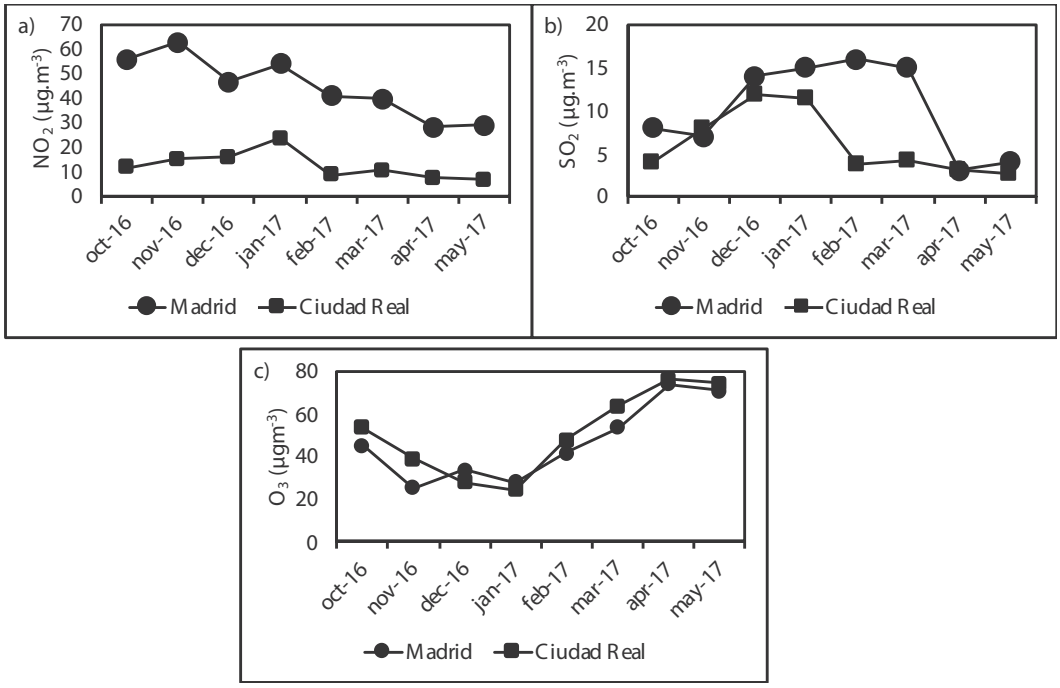

Fig. 1. Air pollution data from Madrid and Ciudad Real during the period of plants development until the pollen collection.

amount of total glutathione and that of GSSG. A standard curve for GSH in the range in the range of 0–100 µg mL^{-1} was prepared. Data were expressed as µg mg^{-1} FW (fresh weight).

2.6. Malondialdehyde (MDA) determination on plants

The MDA content was determined by the method of Hu et al. (2016) with modifications. Briefly, 0.25 g of powder was mixed with 2 mL of reaction solution containing 0.5% (v/v) thiobarbituric acid (TBA) and 20% (v/v) trichloroacetic acid (TCA). The mixture was heated at 95 °C for 30 min, then quickly cooled down to room temperature, treated to eliminate air bubbles, and centrifuged at 6030*g* for 20 min. Then, absorbance of the supernatant was determined by a spectrophotometer at 532 and 600 nm. The MDA content was calculated using the formula: MDA (nmol g FW^{-1}) = [(OD532-OD600)]/(ε*FW), where FW is the fresh weight and ε the extinction coefficient (155 mM $^{-1}cm^{-1}$). Data were expressed as µmol g FW^{-1} (fresh weight).

2.7. Pollen NADPH oxidase activity

Pollen grains (100 mg $assay^{-1}$) were hydrated in PBS for 10 min and mixed with 2 mM nitroblue tetrazolium (NBT) with and without nicotinamide adenine dinucleotide (reduced) (NADH) (100 mM) or nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) (100 mM). After 15 min at 37 °C of incubation, NBT was completely removed centrifuging for 3 min at 7300*g*. Two washing steps with PBS were carried out. Formazan precipitate was dissolved in methanol. Absorbance was determined at 530 nm. Absorbance with NADH or NADPH minus absorbance without NADH or NADPH was used to calculate NADPH oxidase activity. Formazan concentrations were calculated using an extinction coefficient of 12.8 L mM^{-1} cm^{-1}. Data were expressed as nmol mg $pollen^{-1}$ min^{-1}.

2.8. Measurement of hydrogen peroxide (H_2O_2) level in pollen

The H_2O_2 level was measured spectrophotometrically at 560 nm following the protocol of Bacsi et al. (2005) with an Amplex® Red assay kit (Molecular Probes®, Invitrogen™). One hundred µg of pollen were mixed with 200 µL of PBS and 100 µL Amplex Red reagent/horseradish peroxidase solution. Mix was incubated at 37 °C for 30 min prior to measure spectrophotometrically at 560 nm. The H_2O_2 concentrations were calculated by comparison with assays of standard serial dilutions of H_2O_2 (0–10 µM). Data were expressed as µM mg $pollen^{-1}$.

2.9. Statistical analysis

To determine the statistical differences between the results obtained, *t*-test and analysis of variance (ANOVA) were used.

In those cases, in which there was only one independent variable (a single source of variation) and two populations were compared (Ciudad Real and Madrid) *t*-test was used. T-test was performed to chlorophyll fluorescence parameters (Fo, Fv/Fm, ΦPSII, and NPQ), NADPH oxidase and H_2O_2 levels in pollen.

In those data in which there were two independent variables (two sources of variation) the two-way ANOVA was used. Two-way ANOVA (also known as a factorial ANOVA, with two factors) is used when one dependent variable and two independent variables (sources of variation) are analyzed. In our experiment, the dependent variables that were analyzed with this ANOVA were: enzymes activities related with oxidative stress, ascorbate (ASC, DHA and redox state), glutathione (GSH, GSSG and redox state) and malondialdehide (MDA) content. The two independent variables were sampling place (Madrid and Ciudad Real) and the part of the plant (Inflorescence or leaves + stem). The significant differences between cities (Madrid and Ciudad Real) taking into account the results of both parts of the plant (inflorescence and leaves + stem) at the same time can be revealed with this analysis. Also, combinations between independent variables can be analyzed: Madrid-inflorescence, Madrid-leaves + stem, Ciudad Real-inflorescence and Ciudad Real-leaves + stem.

In both cases, prior to analysis homoscedasticity and normality of the variance was checked, meeting requirements for analysis. When significant differences appeared ($P < 0.05$) a Fisher test was used (Sokal and Rohlf, 1980). Analysis were performed with Statgraphics plus 5.1 for Windows.

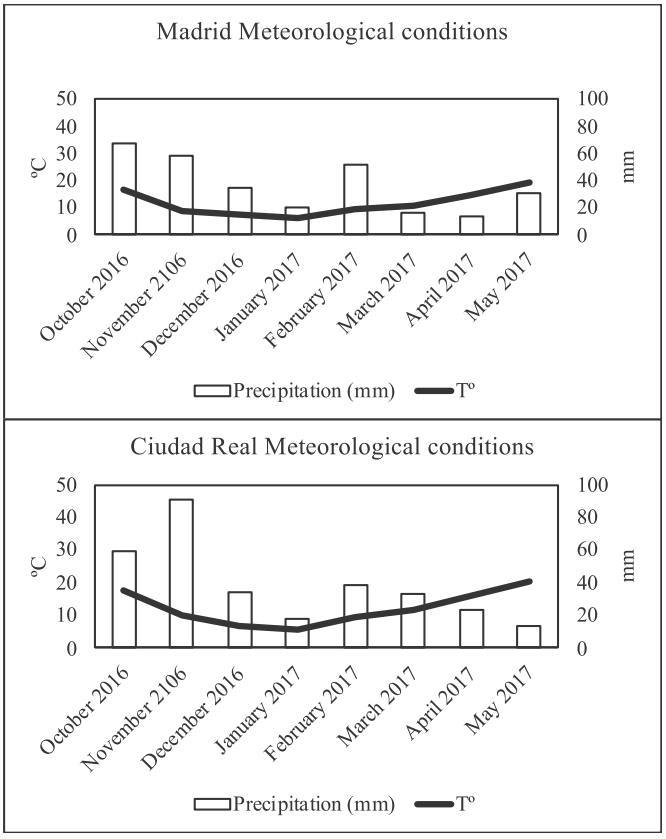

Fig. 2. Climatological diagrams of Madrid and Ciudad Real showing the meteorological conditions during the studied period.

3. Results

Fig. 1 shows air pollution data: nitrogen dioxide (NO_2), ozone (O_3) and sulfur dioxide (SO_2), in both cities. Nitrogen dioxide and sulfur dioxide (Fig. 1a and b) were always higher in Madrid. Also, NO_2 values in Madrid were above the annual limit value for the protection of human health (40 μg m^{-3}). Ozone values were very similar in both cities (Fig. 1c), and in any case values were above values for the protection of human health (120 μg m^{-3}).

In Fig. 2, the meteorological conditions during the studied period in both cities are shown. Climatological diagrams of both cities show the typical meteorological conditions of Mediterranean climate, with a dry period from march-april where the line of temperature is above of precipitation bar. Temperatures were very similar from march to may in both cities, with some more precipitation in Ciudad Real than in Madrid.

In Fig. 3, data of photosynthetic efficiency measured through the chlorophyll fluorescence emitted by photosystem II is shown. Minimal fluorescence (Fo; Fig. 3b) was significantly lower in plants of Ciudad Real than plants of Madrid. However, The maximum photosynthetic efficiency of photosystem II (Fv/Fm; Fig. 3a) and effective PSII quantum yield (ϕPSII; Fig. 3c) were significantly higher in *L. perenne* from Ciudad Real. Non-photochemical quenching (NPQ; Fig. 3d) did not show significant differences. These data indicate a greater photosynthetic efficiency in the plants of Ciudad Real than the plants of Madrid.

In Fig. 4, data from enzyme activities related with oxidative stress are shown. In all cases except catalase activity (Fig. 4b) and DHAR (Fig. 4f), there were significant differences between the two cities (Madrid and Ciudad Real) considering both parts of the plant. SOD (Fig. 4a), APX (Fig. 4c) and GPX (Fig. 4d) activities in Ciudad Real plants, were significantly higher in both the inflorescence and the leaves + stem. The same behavior had the catalase (Fig. 4b) and DHAR (Fig. 4f) activities, but in this case, differences were found only in the leaves + stem. In the MDAR activity (Fig. 4e), plants from Ciudad Real showed significantly higher values in inflorescences, showing contrary results in the leaves + stem. GR activity (Fig. 4g) showed the same behaviour, but in this case, differences between the inflorescences of the plants of both cities were non-significant.

Ascorbate (ASC), dihydroascorbate (DHA; oxidized form of ASC), gluthathione (GSH), its oxidized form (GSSG) and redox state of ASC and GSH calculated as the relation between ASC or GSH and total ASC and GSH (ASC/ASC + DHA and GSH/GSH + GSSG) are shown in Tables

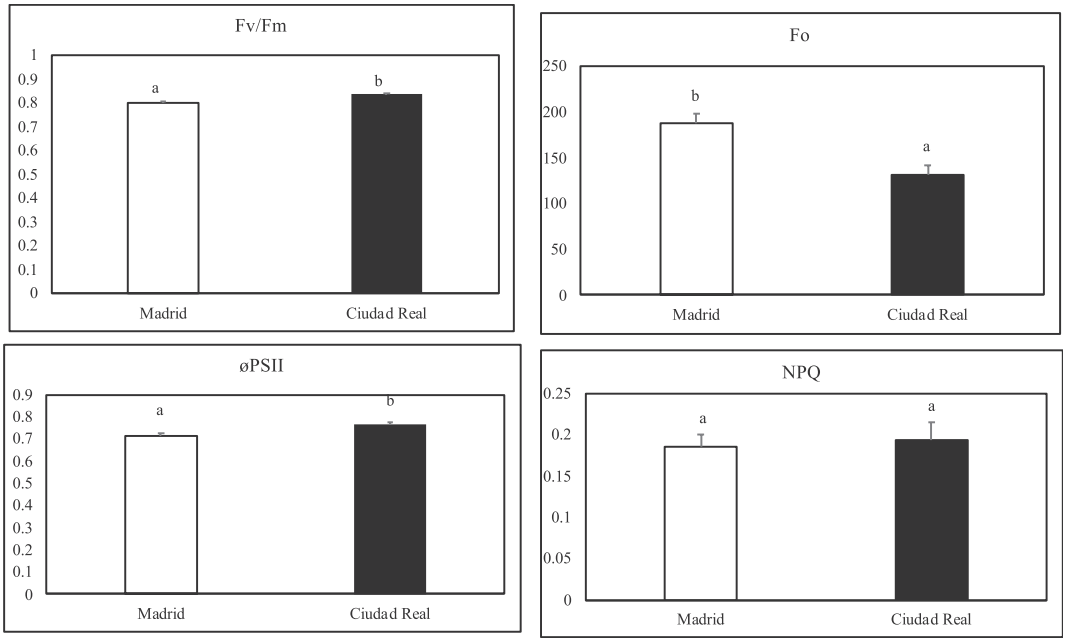

Fig. 3. Fluorescence emission from dark adapted leaves (Fo), maximum photosynthetic efficiency of photosystem II (Fv/Fm), effective PSII quantum yield (ϕPSII) and non-photochemical quenching coefficient (NPQ) in plants of *Lolium pernne* growing in Madrid and Ciudad Real. Different letters show significant differences (*t*-test, $P < 0.05$).

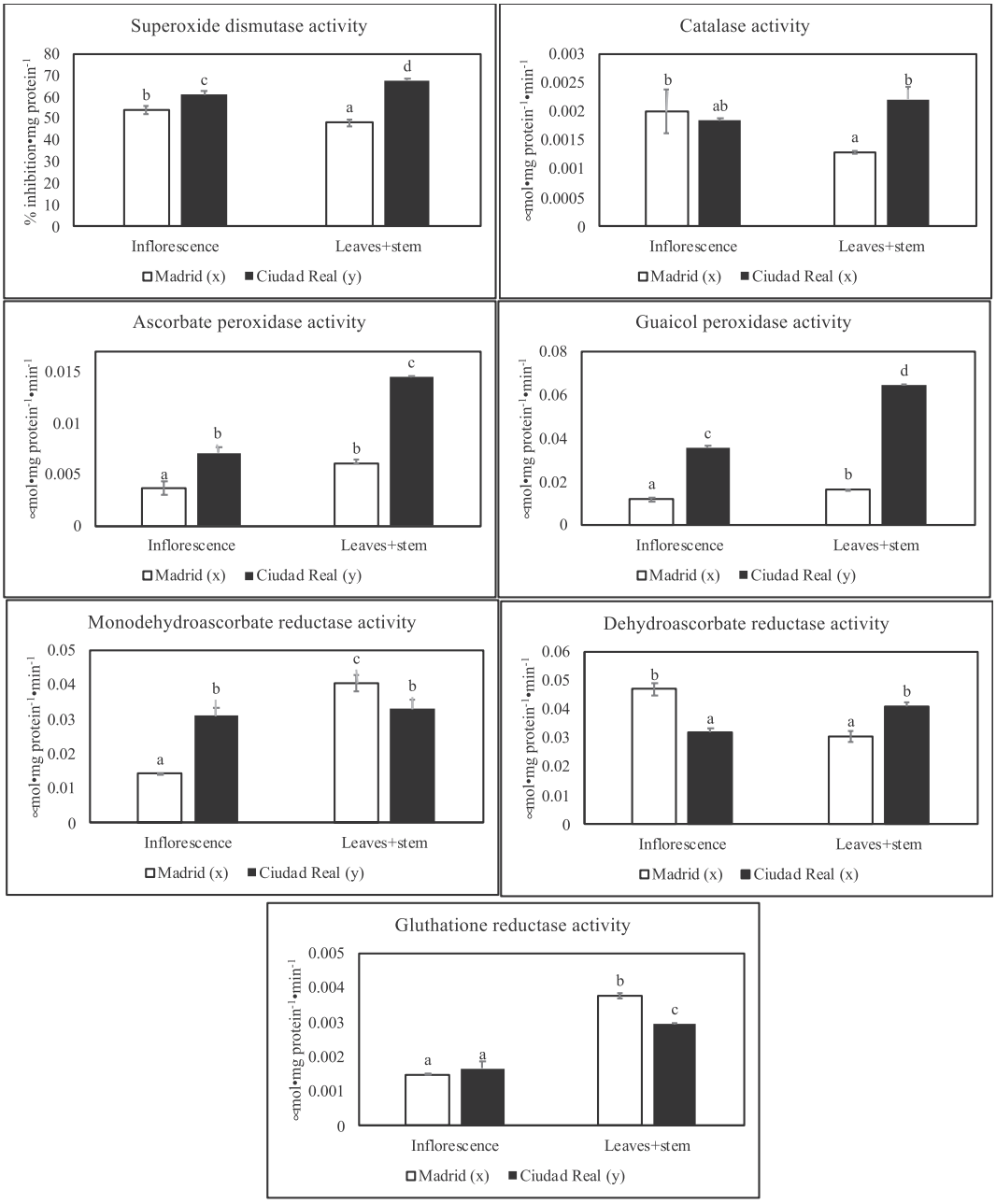

Fig. 4. Enzyme activities related with oxidative stress in plants of *Lolium perenne* growing in Madrid and Ciudad Real: (a) superoxide dismutase (SOD); (b) catalase; (c) ascorbate peroxidase (APX); (d) guaiacol peroxidase (GPX); (e) monodehydroascorbate reductase (MDHAR); (f) dehydroascorbate reductase (DHAR) and (g) gluthatione reductase (GR). SOD is expressed as % inhibition mg protein^{-1}, all other enzymes are expressed as mmol mg protein^{-1} min^{-1}. Different letters indicate significant differences ($P < 0.05$). Letters x and y indicate significant differences, detected by two-way ANOVA, between Madrid and Ciudad taking into account the results of both parts of the plant (inflorescence and leaves + stem). All measured were done separately in inflorescence and in the leaves + stem of the plants.

Table 1
ASC: ascorbate, DHA: dehydroascorbate and redox state of ASC calculated as the relation between ASC and total ASC (ASC/ASC + DHA) in plants of *Lolium perenne* growing in Madrid and Ciudad Real. Data are expressed as μg mg^{-1} FW (fresh weight). Two-way ANOVA was performed in each parameter (ASC, DHA and redox state). Different letters indicate significant differences ($P < 0.05$).

		ASA	DHA	Redox state (ASA/ASA + DHA)
Madrid	Inflorescence	0.074 ± 0.005 a	0.365 ± 0.007 c	0.168 ± 0.012 a
	Leaves + stem	0.106 ± 0.007 a	0,224 ± 0.006 a	0.321 ± 0.020 b
Ciudad Real	Inflorescence	0.111 ± 0.015 a	0.271 ± 0.015 b	0.291 ± 0.039 b
	Leaves + stem	0.095 ± 0.012 a	0.263 ± 0.021 ab	0.266 ± 0.040 b

Table 2
GSH: gluthatione, GSSG: reduced gluthatione and redox state of GSH calculated as the relation between GSH and total GSH (GSH/GSH + GSSG) in plants of *Lolium perenne* growing in Madrid and Ciudad Real. Data are expressed as μg mg^{-1} FW (fresh weight). Two-way ANOVA was performed in each parameter (GSH, GSSG and redox state). Different letters indicate significant differences ($P < 0.05$).

		GSH	GSSG	Redox state (GSH/GSH + GSSG)
Madrid	Inflorescence	2.185 ± 0.0.280 a	1.092 ± 0.025 b	0.661 ± 0.033 c
	Leaves + stem	2.371 ± 0.0.081 a	1.167 ± 0.099 b	0.670 ± 0.206 c
Ciudad Real	Inflorescence	0.546 ± 0.025 b	0.720 ± 0.049 a	0.432 ± 0.024 a
	Leaves + stem	0.621 ± 0.180 b	1.912 ± 0.194 c	0.248 ± 0.071 b

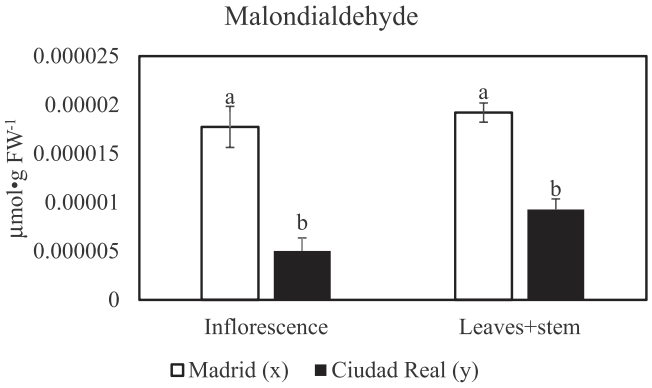

Fig. 5. Concentration of Malondialdehyde (MDA) in plants of *Lolium perenne* growing in Madrid and Ciudad Real. MDA was measured as μmol g FW^{-1} (fresh weight). Different letters indicate significant differences ($P < 0.05$) according to LSD.

Table 3
NADPH oxidase activity and H_2O_2 concentration in pollen of *Lolium perenne* plants growing in Madrid and Ciudad Real. Different letters indicate significant differences (*t*-test, $P < 0.05$).

	NADPH oxidase (nmol mg $pollen^{-1}$ min^{-1})	H_2O_2 (μM mg $pollen^{-1}$)
Madrid	9.432 ± 0.322 a	2.013 ± 0.009 a
Ciudad Real	3.245 ± 0.112 b	0.312 ± 0.006 b

1 and 2. With regards to ASC, there were non-significant differences between Madrid and Ciudad Real, and in all cases DHA was higher than ASC. Redox state of ASC was low in all cases, and differences between inflorescences of the both cities were significant, being higher in Ciudad Real. GSH showed significant differences between plants of both cities. In Madrid, GSH was higher than GSSG in both parts of the plants. However, in Ciudad Real was the contrary, but only in the leaves + stem of the plants had significant differences. Redox state of GSH was significantly higher in Madrid in both parts of the plants.

The oxidative stress marker (malondialdehyde; MDA; Fig. 5) was significantly higher in Madrid for both parts of the plants. These results were similar to those in 2016 (data not shown).

NADH oxidase and H_2O_2 level of pollen from plants of Ciudad Real and Madrid were significantly higher in Madrid (Table 3).

4. Discussion

Photosynthesis is the first physiological function to be affected by a stress situation, even before symptoms are noticed on the leaves. Therefore, effects of stress can be detected by measuring parameters related with photosynthetic efficiency as Fv/Fm, ØPSII and NPQ (Baker, 2008; Kummerová et al., 2008; Lucas García et al., 2013; Nwugo and Huerta, 2008; Pérez-Bueno et al., 2007; Rodríguez-Moreno et al., 2008). A reduction in Fv/Fm ratio, especially under stress conditions, is often an indicator of photoinhibition or other kind of injury to PSII components (Kummerova et al., 2008; Nwugo and Huerta, 2008; Rohacek, 2002).

The results obtained indicate that Madrid plants have a lower photosynthetic efficiency than those from Ciudad Real (Fig. 3), which could be related to the different levels of nitrogen oxides and sulfur dioxide between both cities (Fig. 1), although this must be taken with great caution as to be able to affirm this with some security, a statistical correlation analysis between the photosynthetic efficiency measured throughout the entire plant development period until flowering and the monthly levels of pollutants it should be done. Both nitrogen oxides and sulfur dioxide are air pollutants that have been shown to negatively affect the photosynthetic efficiency valued by measuring the fluorescence emission of photosystem II (Chung et al., 2013; Darrall, 1989; Schmidt et al., 1990; Wellburn, 1990; Zhao et al., 2017).

SO_2 causes damage at the level of photosystem II and inhibition of Calvin cycle activity. These effects could be due to cytoplasmic and especially stromal acidification (Pfanz et al., 1987a, b). Similarly, NO_2 could act as an acidifying agent, because it produces the strong acids HNO_2 and HNO_3 when dissolving in the liquid phase of the leaf (Schmidt et al., 1990). The mentioned works carry out experiments in laboratory conditions with short and very severe exposures of plants to high pollutant concentrations (between 100 and 1000 times). Conversely, our study reports results from field conditions, which represents a different situation, lower pollutants concentrations for much longer periods (months), a chronic situation.

Stress conditions, as indicated above, are also linked to oxidative stress as a consequence of the generation of reactive oxygen species (ROS), such as superoxide ion, hydrogen peroxide and hydroxyl radicals, which are detrimental to plant survival (Sewelam et al., 2016). Level of MDA (Fig. 5), a marker of oxidative stress, (AbdElgawad et al., 2016; Lucas et al., 2017) is consistent with air pollution and ROS generation stresses; as MDA levels were higher in plants of Madrid, indicating higher oxidative stress in the area with higher levels of air contaminants.

Stressed plants display a complex enzyme oxidative defense strategy involved in scavenging of these ROS in response to oxidative stress (Kohler et al., 2009; Lucas et al., 2017). This strategy efficiently maintains ROS at a safe level (Anjum et al., 2012; Gill and Tuteja, 2010; Mittler, 2002). However, a disturbance in the ROS/antioxidant homeostasis leads to a situation of oxidative stress (Gill and Tuteja, 2010), causing significant physiological challenges confirmed by photosynthesis data in the present study; also modification of vital biomolecules as membrane lipids (as confirmed by MDA levels), cellular amino acids, proteins, and DNA (Anjum et al., 2012; Gill and Tuteja, 2010; Krasensky and Jonak, 2012; Mahajan and Tuteja, 2005).

The activity of most enzymes involved in ROS scavenging (SOD, APX, GPX, MDAR, and GR) was higher in Ciudad Real than in Madrid, and the same happens when observing the results analyzing the part of the plant. All enzymes analyzed have been cited in the bibliography relating its higher activity with a greater resistance of plants against different stress situations (Hong et al., 2009; Lucas et al., 2017; Moradi and Ismail, 2007; Wang et al., 2011). These results clearly indicate that the higher stress suffered by Madrid plants is generating greater quantity of ROS than plants of Ciudad Real. The greater degree of

oxidative stress in Madrid plants could be provoking oxidative modification of vital biomolecules with the consequent failure in generating new molecules like ROS detoxifying enzymes. All these data agree with the degree of oxidative stress evidenced by MDA levels.

In addition to enzymatic systems, plants have other non-enzymatic systems to avoid oxidative stress produced by ROS. These system comprising of ascorbic acid (ASC), glutathione (GSH), α-tocopherol, carotenoids, phenolics, flavonoids, and amino acid and the osmolyte proline (Das and Roychoudhury, 2014).

ASC is the most abundant and the most extensively studied antioxidant compound, which is the substrate of the APX enzyme. It is considered powerful antioxidant compound as it can donate electrons to a wide range of enzymatic and non-enzymatic reactions (Foyer and Noctor, 2011; Jaspers and Kangasjarvi, 2010; Smirnoff, 2000). It is the best-known molecule for detoxifying H_2O_2, especially as a substrate of ascorbate peroxidase (APX), an essential enzyme of the ascorbate-glutathione cycle, present in most compartments of the plant cell (Smirnoff and Wheeler, 2000). Reduced glutathione (GSH) is also an important antioxidant molecule. GSH scavenges H_2O_2 and other ROS by forming adducts (glutathiolated) or by reducing them and generating oxidized glutathione (GSSG) (Das and Roychoudhury, 2014). GSH also plays a vital role in regenerating ASC to yield GSSG. The GSSG thus generated is converted back to GSH, either by de novo synthesis or enzymatically by GR. GSH also helps in the formation of phytochelatins, via phytochelatin synthase (Roychoudhury et al., 2012a), which helps to chelate heavy metal ions and thus scavenges another potential source of ROS formation in plants (Roy Choudhury et al., 2012b). Therefore, the delicate balance between GSH and GSSG is necessary for maintaining the redox state of the cell. The behavior of the redox state of ASC pool might support the relevant role involvement of ASC in the redox reactions triggered by local stress conditions, since ASC oxidation is very high in both plants, which could be related with the type and intensity of stress (Paradiso et al., 2008; Rodriguez-Serrano et al., 2006). The conversion of the ASC to DHA is higher in plants of Ciudad Real than in Madrid, matching results of APX analysis. Something similar happens with the conversion of GSH to GSSH, the GSSH values are much higher in Ciudad Real plants matching results with the much higher GR activity of the Ciudad Real plants.

Pollen from Madrid plants had a higher activity NADPH oxidase. NADPH oxidases, called respiratory burst oxidase homologs [Rbohs] in plants (Lamb and Dixon, 1997), are transmembrane enzymes which catalyze the generation of superoxide radical $O_2^{\cdot-}$ in the apoplast (Lambeth, 2004), leading to extracellular ROS increase (Lamb and Dixon, 1997), especially H_2O_2. Several authors have demonstrated that NADPH oxidase–induced ROS vigorously augments specific IgE production and allergic airway inflammation induced by the major pollen antigens, and that oxidative stress generated by NADPH oxidase in pollen grains augments immediate-type hypersensitivity reactions and pollen antigen-driven allergic conjunctivitis (Boldogh, 2005; Bacsi et al., 2005; Wang et al., 2009; Speranza and Scoccianti, 2014). Dharajiya et al. (2008) discovered that these oxidases induce reactive oxygen species in mucosal cells (signal 1) independent of adaptive immunity. This reactive oxygen species facilitates antigen (signal 2)-induced allergic inflammation. These results could justify, at least in part, the fact known that pollen of plants growing in contaminated cities with a high air contaminated levels is more allergenic than plants growing in places less contaminated.

5. Conclusions

Our work reports for the first time in the literature, the possible relationship between the pollution degree, the physiological state of the plants and the allergenic capacity of the pollen they produce. The plants studied have developed and have produced pollen in cities with different levels of air pollution, Madrid and Ciudad Real. As a consequence, its physiological state is different. Plants from Madrid (with higher air pollution than Ciudad real) have a lower photosynthetic efficiency and differ in the status of the mechanisms involved in metabolism related to oxidative stress, both enzymatic and non-enzymatic. As ROS scavenging systems in plants from Madrid are altered, it seems not to be able to maintain ROS homeostasis, which is probably responsible for the greater degree of oxidative stress. All this is also reflected in a greater NADPH oxidase activity and H_2O_2 level in pollen from Madrid plants, which is shown to be a relevant factor in the increase of the allergenic capacity of pollen.

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