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Physiological, anatomical and biomass partitioning responses to ozone in the Mediterranean endemic plant *Lamottea dianae*

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ABSTRACT

Ozone effects on the perennial forb *Lamottea dianae* were studied in an open-top chamber experiment. Ozone was found to induce reductions in CO_2 assimilation and water use efficiency in the leaves of this species. These reductions were mainly related to a decline in the *in vivo* CO_2 fixation capacity of Rubisco ($V_{c,max}$), rather than to stomatal limitations or photoinhibitory damage (F_v:F_m). In addition to chloroplast degeneration, other observed effects were callose accumulation, formation of pectinaceous wart-like cell wall exudates and phloem alterations. Moreover, ozone exposure significantly reduced root dry biomass. The possible relevance of these adverse effects for Mediterranean forbs is commented. These results show that endemic plants can be very sensitive to ozone, suggesting that risks associated with this pollutant should be taken into account for conservation purposes.

1. Introduction

Tropospheric ozone is the most important air pollutant toxic to plants worldwide, causing crop losses and inducing a series of adverse effects on native vegetation (Krupa et al., 2000). In Europe, some of the highest ozone levels occur in the Mediterranean Region (Sanz et al., 2007). This area can be regarded as a "large natural photochemical reactor" (Millán et al., 1996), with the polluted air masses undergoing complex long-range transport processes (Lelieveld et al., 2002). Under some of the conditions described by the IPCC, ozone levels are expected to increase worldwide (Vingarzan, 2004) which, in addition to the continuous increase in population, industry and traffic in the Mediterranean area, will probably favor a higher frequency of ozone episodes in this region.

Up to now, very little work has been done on the effects of pollutants in endemic or rare plant species. Species with relatively small populations may be more vulnerable to pollution and other threats not only because of their reduced distribution areas or low number of individuals, but also due to their lower genetic variability, which may limit their possibilities of adaptation to changes in their habitat. From a botanical point of view, the Mediterranean Region is a major center of diversity in Europe, hosting many plants that are either endemic or/and of restricted distribution. As an example, of the 9000 species in the Spanish flora, 1414 taxa are included in the Red List (Domínguez-Lozano, 2000) and more than 400 are endangered. *Lamottea dianae* (Webb) G. López (=*Carduncellus dianius* Webb) (Fam. Asteraceae) is an endemism from Spain, classified as "Vulnerable" on the Red List of Spanish Vascular Flora (Domínguez-Lozano, 2000). Efforts to protect this species have led to preserving its habitat through micro-reserves, in vitro micro-propagation and re-introduction (Laguna et al., 1998).

Abbreviations: A_{max}, net CO_2 assimilation per unit area under light and CO_2 saturation; A_{sat}, net CO_2 assimilation per unit area under light saturation; AOT40, accumulated ozone exposure over a threshold of 40 ppb (=80 μg m^{-3}) calculated for daylight hours; C_i, intercellular CO_2 concentrations; CF, charcoal-filtered treatment; Φ_{exc}, quantum efficiency of excitation capture by oxidized reaction centers of PSII; Φ_{PSII}, quantum yield of electron transfer at PSII; F_m, maximum fluorescence; F_m', maximum fluorescence in the light adapted state; F_o, minimal fluorescence; F_o', minimum fluorescence in the light-adapted state; F_s, fluorescence yield at this steady state; F_v:F_m, maximum quantum efficiency of photosystem II (PSII) primary photochemistry; g_s, stomatal conductance to water vapor; J_{max}, maximum RuBP regeneration capacity mediated by light harvesting and electron transport; NF+30, Non-filtered+30 ppb of ozone treatment; NPQ, quenching due to non-photochemical dissipation of absorbed light energy; PII, Plant Injury Index; PPFD, photosynthetic photon flux density; RSL, relative stomatal limitation; qP, coefficient for photochemical quenching; R_d, daytime respiration; TPU, rate of triose phosphate utilization; T_r, transpiration rate; $V_{c,max}$, maximum rate of Rubisco carboxylation

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The main objective of the present paper is to determine the vulnerability of *L. dianae* to increasing ozone levels by studying its physiological, anatomical and biomass responses against this pollutant. A complementary study showed that ozone produced both a significant reduction in the number of flowers produced per plant and an increase in altered pollen cell production in this species (García-Breijo et al., 2005).

2. Materials and methods

2.1. Plant material

Plant seedlings were obtained from the Vivero de Quart nursery, Banc de Llavors Forestals (Valencia). The origin of the seeds was El Montgó (Alicante, Comunidad Valenciana). The 7 l containers were filled with 50% coconut-peat, 30% peat, 10% sand and 10% vermiculite, soil pH being close to 7.0. A slow-release fertilizer was incorporated (Osmocote plus), with NPK 20:10:20. Plants were irrigated twice a day until field capacity using a droplet irrigation system. Twelve plants were kept in filtered air, and another 12 were ozone-exposed.

2.2. Open-top chambers and ozone exposure

The experiment was conducted in the OTC experimental field that the Instituto Universitario CEAM-UMH operates in Benifaió (Comunidad Valenciana, 39°16′14.8″N, 00°26′59.6″W, 30 m altitude). Plants were distributed in six Open-top chambers (OTC), 3-m diameter (NCLAN model, Heagle et al., 1973), with two ozone treatments (three chambers per treatment): Charcoal filtered air (CF) and non-filtered air plus 30 ppb ozone (NF+30). As ozone levels are relatively low at the experimental plot, the latter regime is more representative of the higher ozone concentrations measured in mountain areas of eastern Spain (Millán et al., 2000). Ozone was generated from oxygen using a high-voltage electrical discharge generator (Sir sa), and air quality inside and outside the chambers was continuously monitored at regular intervals with an ozone monitor (Dasibi 1008-AH, Environmental Corp.). The experiment started on 18 May 2005, and ended on 8 September 2005, when the plants were harvested. All week long, the plants were ozone-exposed 8 h a day, from 10:00 to 18:00 CET. For the exposure period, the 24, 12 and 8 h means were calculated, as well as the AOT40, i.e., accumulated ozone exposure over a threshold of 40 ppb (80 $\mu g\ m^{-3}$), based on hourly averages, during daylight hours from 8:00 to 20:00, as described by the EU 2002/3/EC Directive (EU, 2002). Plants were grown together with other species and regularly moved inside chambers and among chambers of the same treatment in order to avoid positional effects.

2.3. Assessment of visible symptoms

All plants were examined daily to detect the first symptom in each plant. The intensity of the visible ozone symptoms was also scored weekly by recording both the percentage of affected leaves per plant (LA), and the mean percentage of area affected for the symptomatic leaves (AA) in each plant, using a 5% step scale. To evaluate the overall plant injury, a Plant Injury Index (PII) was calculated by combining these two parameters: PII=(LA*AA)/100.

2.4. Microscopy methods

Samples of mature leaves were randomly selected from control and from fumigated plants (five plants per treatment, one leaf per plant) on 8 June 2005. Some portions of these leaves were fixed *in situ* with formyl acetic alcohol (FAA). After washing them with a 0.1 M phosphate buffer (pH 7.4), they were dehydrated by means of an ethanol series. Some samples were cut in sections of approximately 20 μm with a freezing microtome (CM 1325, Leica, Wetzlar, Germany). Callose was detected by fluorescence microscopy after aniline blue staining. Other portions of the collected leaves were embedded in LR-White medium-grade acrylic resin (London Resin Co.). The sectioning of these latter blocks was performed with a Sorvall MT 5000 Ultramicrotome (Knifemaker, Reichert-Jung) provided with special glasscutters (45°) (Leica 6.4 mm glass strips). This microtome enabled semi-thin sections (1.5 μm). These samples were stained with toluidine blue (1%) (TB). Sections were observed and photographed with an Olympus Provis AX 70 brightfield microscope fitted with an Olympus Camedia C-2000 Z camera.

2.5. Gas exchange, chlorophyll content and chlorophyll a fluorescence measurements

Eight of the twelve plants per treatment were selected for the gas exchange, chlorophyll content and chlorophyll fluorescence measurements. Measurements were carried out in asymptomatic parts of leaves (one leaf per plant for each treatment), on 4 June 2005, 3 weeks after starting the ozone exposure, from 8:00 to 11:00 CET. Complementary measurements on symptomatic leaves were also carried out, but not shown.

Gas exchange measurements were taken with an infrared gas analyzer (IRGA) (LICOR-6400, Li-cor Inc., Lincon, NE, USA). In order to reduce the possible effects of changes in environmental variables during the measurements, cuvette block temperature was fixed at 25 °C, photon flux density (PPFD) was at a saturating value of 1200 $\mu mol\ m^{-2}\ s^{-1}$, and the reference CO_2 concentration was 370 $\mu mol\ mol^{-1}$. Relative humidity during the measurements was 46.9% ± 2.7%. Chlorophyll fluorescence measurements were taken with a portable fluorometer (PAM-2000, Walz, Effeltrich, Germany). Leaves were dark-adapted for at least 30 min prior to the measurements. After dark adaptation, the minimal fluorescence (F_o) was determined using the measuring light. Subsequent application of a saturating flash of white light (0.8 s at 8000 $\mu mol\ m^{-2}\ s^{-1}$) raises fluorescence to its maximum value (F_m). This permits the determination of the F_v:F_m parameter, maximum quantum efficiency of photosystem II (PSII) primary photochemistry, given by $F_v:F_m=(F_m-F_o):F_m$. The saturation pulse method was subsequently applied for the analysis of quenching components (Schreiber et al., 1986). After F_v:F_m determination, 15 intermittent pulses of saturating strong white light (0.8 s at 8000 $\mu mol\ m^{-2}\ s^{-1}$) were applied in the presence of actinic red light (118 $\mu mol\ m^{-2}\ s^{-1}$). This enables the determination of the maximum fluorescence in the light adapted state ($F_{m'}$) after each saturating pulse, and the actinic light allows steady-state photosynthesis and modulated fluorescence yield at this steady state (F_s). The minimum fluorescence in the light-adapted state ($F_{o'}$) is also measured by applying a pulse of far-red light during a brief interruption of actinic illumination. At each saturating pulse, the quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined according to the equation $NPQ=(F_m-F_{m'}):F_{m'}$. The coefficient for photochemical quenching (qP), which represents the redox state of the primary electron acceptor of PSII, Q_A, was calculated as $(F_{m'}-F_s):(F_{m'}-F_{o'})$. The quantum yield of electron transfer at PSII (Φ_{PSII}) was estimated as $\Phi_{PSII}= F_{v'}:F_{m'}=(F_{m'}-F_s):F_{m'}$ (Genty et al., 1989), and the quantum efficiency of excitation capture by oxidized reaction centers of PSII was calculated from the equation $\Phi_{exc}=(F_{m'}-F_{o'}):F_{m'}$. For statistical analysis, we considered the values of the last pulse, when conditions under actinic illumination were steady.

Chlorophyll content was estimated non-destructively with a portable chlorophyll meter (SPAD-520, Minolta) in the same leaves used for gas exchange and fluorescence analysis. This instrument uses measurements of transmitted radiation in the red and near-infrared wavelengths to provide numerical values related to leaf chlorophyll content. The average of three measurements was calculated for each leaf.

2.6. A/C_i curves

Leaf assimilation rate responses to changes in the intercellular partial pressure of CO_2 (i.e., A/C_i response curves) were measured in 4–5 attached leaves per ozone treatment. These measurements were taken on 5 June 2005 (3 weeks after starting fumigation), in leaves randomly selected from those measured the day before. An infrared gas analyzer (IRGA) (LICOR-6400, Li-Cor Inc., Lincon, NE, USA) was also used for these measurements. Curves were constructed by varying the reference CO_2 air concentration in the following steps: 50, 100, 200, 300, 370, 400, 600, 800, 1000, 1400 and 1700 $\mu mol\ mol^{-1}$. Block temperature of the cuvette was fixed at 25 °C, and photon flux density (PPFD) at 1200 $\mu mol\ m^{-2}\ s^{-1}$. Maximum rate of Rubisco carboxylation ($V_{c,max}$), maximum RuBP regeneration capacity mediated by light harvesting and electron transport (J_{max}), rate of triose phosphate utilization (TPU) and rate of non-photorespiratory CO_2 evolution in light (R_d) were calculated from these curves using the Photosyn Assistant 1.2 software (Dundee Scientific, Dundee, USA). This program uses an iterative procedure to calculate the parameter values that represent the best fit for the data, based on the model proposed by Farquhar et al. (1980), with improvements. The relative stomatal limitation of photosynthesis (RSL) was calculated according to Farquhar and Sharkey (1982) as $RLS=((A_o-A)/A_o)*100$, where A_o is the potential assimilation rate if there was no stomatal limitation and A is the actual assimilation rate at an ambient air CO_2 concentration of $C_a=370\ \mu mol\ CO_2\ mol^{-1}$. The maximal photosynthetic rate under light and CO_2 saturation (A_{max}) was calculated at $C_a=1400\ \mu mol\ CO_2\ mol^{-1}$, in the plateau phase of A/C_i response curves, and before a possible reduction by TPU limitation.

2.7. Senescence and biomass partitioning

Senescent leaves were regularly collected throughout the experiment, and the accumulated dry weight of senescent leaves per plant was calculated on a 2-week basis. At the end of the experiments (5–8 September) the 24 plants were collected, and the above-ground (leaves, stems, flowers) and below-ground (roots) biomass was calculated separately after oven-drying at 60 °C to stable weight. Roots were separated from the soil by washing them onto sieves.