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Contrasting ozone sensitivity in related evergreen and deciduous shrubs

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Mediterranean evergreen shrubs have a constitutively higher capacity to tolerate ozone stress than their deciduous relatives.

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ABSTRACT

Plant responses to enhanced ozone levels have been studied in two pairs of evergreen-deciduous species (*Pistacia terebinthus* vs. *P. lentiscus*; *Viburnum lantana* vs. *V. tinus*) in Open Top Chambers. Ozone induced widespread visible injury, significantly reduced CO_2 assimilation and stomatal conductance (g_s), impaired Rubisco efficiency and regeneration capacity ($V_{c,max}$, J_{max}) and altered fluorescence parameters only in the deciduous species. Differences in stomatal conductance could not explain the observed differences in sensitivity. In control plants, deciduous species showed higher superoxide dismutase (SOD) activity than their evergreen counterparts, suggesting metabolic differences that could make them more prone to redox imbalances. Ozone induced increases in SOD and/or peroxidase activities in all the species, but only evergreens were able to cope with the oxidative stress. The relevancy of these results for the effective ozone flux approach and for the current ozone Critical Levels is also discussed.

1. Introduction

Tropospheric ozone is the most relevant pollutant both for crops and native vegetation. It causes a series of deleterious physiological and biochemical effects, visible injury and growth reductions, and may also interact with other biotic and abiotic stresses (Krupa and Manning, 1988; Krupa et al., 2000; Manning and von Tiedemann, 1995). Model predictions using IPCC scenarios suggest that background levels of this pollutant will increase worldwide in the future (Vingarzan, 2004). Furthermore, ozone is itself a greenhouse gas contributing to radiative forcing (IPCC, 2007).

In Europe, the Mediterranean area experiences relatively high ozone levels (Sanz et al., 2007) due to favorable conditions for ozone formation, and a combination of meso-scale re-circulatory processes and long-range transport (Millán et al., 1992, 1997, 2000; Sanz and Millán, 1998; Lelieveld et al., 2002). On forest sites, these levels regularly exceed the critical level of 5000 ppb h [AOT40, daytime hours, April to September] (e.g., Gerosa et al., 2007), established to protect sensitive species from growth losses (ICP Modelling and Mapping, 2004). While in the Mediterranean area there is field evidence of widespread injury in irrigated crops (Hayes et al., 2007), ozone-like symptoms in broadleaf evergreen species have only been reported sporadically (Skelly et al., 1999); for a number of species, they have been induced experimentally (Sanz et al., 2001). Both field and experimental evidence suggest that Mediterranean broadleaf evergreen species are comparatively ozone-tolerant (e.g., Bussotti and Gerosa, 2002; Nali et al., 2004). However, the sensitivity of evergreen species to this pollutant is largely under-investigated (Paoletti, 2006): not only have few species been studied, but also our knowledge on the mechanisms underlying their capacity to tolerate ozone stress is very fragmentary. While for many crops and deciduous plants exposure- or dose-response functions describing the effects of ozone have been established (ICP Modelling and Mapping, 2004), these functions are still lacking for typical Mediterranean species.

It is well-known that ozone effects on plants depend not only on the pollutant dose (concentration × exposure duration) entering the plant, i.e., on the ozone flux (Matyssek et al., 2007), but also

Abbreviations: AA, Ascorbate; A_{sat}, light saturated CO_2 assimilation; AOT40, accumulated exposure over threshold 40 ppb; CF, Charcoal Filtered Air; C_i, intercellular CO_2 concentration; DHA, dehydroascorbate; FRAP, Ferric Reducing Antioxidant Power assay; g_{max}, maximum stomatal conductance; g_s, stomatal conductance to water vapor; GPX, Peroxidase; HAC, Hydrosoluble Antioxidant Capacity; LAC, Liposoluble Antioxidant Capacity; NF+, Non Filtered Air + 30 ppb ozone; OTC, Open Top Chamber; PPFD, photosynthetic photon flux density; SOD, Superoxide dismutase; TAC, Total Antioxidant Capacity; T_r, transpiration rate.; F_0, minimal fluorescence; F_m, maximum fluorescence; F_v:F_m, maximum quantum efficiency of photosystem II (PSII) primary photochemistry; F_s, steady state fluorescence value; NPQ, non-photochemical quenching; F_{exc}, quantum efficiency of excitation capture by oxidized reaction centers of PSII; F_{PSII}, quantum yield of electron transfer at PSII.

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on the balance between the flux and effectiveness of the defence mechanisms to cope with oxidative stress (Paoletti et al., 2008; Castagna and Ranieri, 2009). Therefore, in order to better understand how this pollutant affects plants, both physiological (especially gas exchange) and biochemical (antioxidant systems) aspects have to be taken into account. While there is extensive literature on the effects of ozone on broadleaf deciduous species, as mentioned above, information for broadleaf evergreen shrubs is still scarce (e.g., Bussotti et al., 2003; Reig-Armiñana et al., 2004; Nali et al., 2004; Paoletti et al., 2009). As far as we know, a direct comparison between evergreen and deciduous shrub responses under the same ozone exposure conditions has not been carried out. In the present paper, we compare the responses of two pairs of deciduous-evergreen species in terms of visible injury, gas exchange, chlorophyll fluorescence, SPAD measurements, and antioxidant capacity. Comparability between both types of plants is assured by using two pairs of congeneric species. This is an important aspect, as some of the characteristics (e.g., constitutive antioxidant levels) and types of effects (e.g., enhanced senescence or not, type of visible injury, physiological responses) are expected to be genus-related. The experiment has been carried out under well-watered conditions, in order to achieve optimal conditions for ozone uptake. To the best of our knowledge, this is the first time in which antioxidant metabolites have been studied in any of the four species considered in relation to ozone stress.

Specifically, we test the following hypotheses: 1) Evergreen species are more tolerant to ozone that related deciduous species (Paoletti, 2006). 2) Stomatal conductance may explain differences in ozone sensitivity between evergreen and deciduous species (Reich, 1987). 3) Sensitivity of the species is related to its constitutive or induced antioxidant levels (Nali et al., 2004).

2. Materials and methods

2.1. Plant material

Plant seedlings were obtained from several Spanish nurseries. Plants of a similar size (1 or 2 years old) were selected. Ten-liter containers were filled with 70% peat, 15% sand, and 15% vermiculite, soil pH being close to 7.0. A slow release fertilizer was incorporated (Osmocote plus), with NPK 20:20:20 and additional micronutrients. Plants were irrigated twice a day using a droplet irrigation system.

2.2. Open top chambers and treatments

The experiment was conducted in an OTC experimental field ("La Peira") located in a rural area, in Benifaió (39°16′ 14.8″N, 00°26′59.6″W), at an altitude of 30 m. Air quality inside and outside the chambers was continuously monitored at regular intervals with an ozone monitor (Dasibi 1008-AH, Environmental Corp.); these monitors were calibrated periodically. Plants were placed in three Open Top Chambers (OTC), with two ozone treatments: Charcoal Filtered air (CF), and Non-Filtered air plus 30 ppb ozone (NF+). Plants were fumigated 8 h a day, from 10:00 to 18:00 CET, during the whole week. Ozone was generated from oxygen using a high-voltage electrical discharge generator (Sir sa). The experiment started on 18 May 2005, and ended on 16 September 2005. The critical level for ozone, accumulated exposure over a threshold of 40 ppb, was calculated according to the methods described by the EU 2002/3/EC Directive (EU, 2002), using mean hourly values from 8:00 CET to 20:00 CET. Ozone concentration data of from experimental site (ambient) and treatments are provided in Table 1. The AOT40 value of 35 755 ppb h of the NF+ treatment is slightly above the AOT40 values regularly measured in inner mountain areas of eastern Spain (e.g., up to 30 000 ppb h in Morella station some years, data of the regional Air Quality Network), although well above the values measured at the experimental site, which is placed in an area with particularly low ozone levels.

Table 1
Mean ozone concentrations for different daily time windows, maximum hourly value, and cumulative ozone exposures AOT00 and AOT40. The 8 h window, from 10 to 18 h, covered the 8 h in which plants of the NF + treatment were exposed to increased ozone levels. CF = Charcoal Filtered Air; NF+ = Non Filtered Air + 30 ppb ozone. Ambient air is not a treatment but refers to the ozone levels measured at the experimental site outside the Open Top Chambers.

	24 h mean (ppb)	12 h mean (ppb)	8 h mean (ppb)	hourly max (ppb)	AOT00 (ppb*h)	AOT40 (ppb*h)
CF	11	9	12	31	31 403	0
NF+	40	63	76	110	11 5293	35 733
Ambient	31	44	48	86	91865	10 664

2.3. Specific leaf area

At the beginning of the experiment, mature leaves were collected for calculation of dry weight and Specific Leaf Area (SLA); the latter was calculated as SLA = Leaf surface (mm^2)/Dry weight (mg). Leaf dry weight was determined by oven-drying leaves at 60 °C to stable weight, and leaf surface was assessed by image analysis.

2.4. Visible injury assessment

All the plants were examined once a week for ozone injury symptoms, recording the first date of symptom onset in each plant, the percentage of affected leaves per plant (LA), and the percentage of area affected for the symptomatic leaves (AAr). The latter was scored using a 5% step scale. To evaluate whole plant injury, a Plant Injury Index (PII) was calculated combining these two parameters: PII = (LA × AAr)/100.

2.5. Gas exchange measurements

Gas exchange was measured with an infrared gas analyzer (IRGA) (LICOR-6400, Li-cor Inc., Lincon, NE, USA) in 8 plants per species and treatment. Block temperature of the cuvette was fixed at 25 °C, and photon flux density (PPFD) was set at a saturating intensity of 1200 μmol / m^2 s. All measurements were taken during the morning. Tracking of gas exchange in the same marked leaves was carried out monthly (20 May, 21 June, 20 July and 23 August) after the starting of fumigation. The g_{max} for water value has been chosen by taking the 95th percentile of all the valid values of stomatal conductance.

2.6. A/C_i curves

The response of the assimilation rate to changing intercellular CO_2 partial pressure (i.e., A/C_i response curves, not shown) was measured on 29–30 August 2005 in 4 representative mature attached leaves per plant and ozone treatment. While these leaves showed no apparent visible injury, symptoms were sometimes present in other leaves of similar age. An infrared gas analyzer (IRGA) (LICOR-6400, Li-Cor Inc., Lincon, NE, USA) was used for the measurements. Maximum rate of Rubisco carboxylation ($V_{c,max}$), maximum RuBP regeneration capacity mediated by light harvesting and electron transport (J_{max}) were estimated by fitting the biochemical model of Farquhar et al., (1980), with modifications by Sharkey (1985), to A/C_i response curves, using non-linear least-square regression methods. Block temperature of the cuvette was fixed at 25 °C, and photon flux density (PPFD) at 1200 μmol /m^2s. Air relative humidity was kept at about 50%. Photosynthetic rates were taken into account when the coefficient of variation was lower than 1%.

2.7. Chlorophyll content

Chlorophyll content was measured non-destructively with a portable chlorophyll meter (SPAD-520, Minolta). 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 3 measurements was calculated for each leaf, 2 leaves were measured per plant and 8 plants per treatment.

2.8. Chlorophyll a fluorescence measurements

In the tracked leaves, modulated chlorophyll fluorescence measurements were taken at ambient temperature at the same time as gas exchange determinations (n = 8 leaves per species and treatment). Measurements were carried out 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; then, a subsequent application of a saturating flash of white light (0.8 s at 8000 μmol/m^2 s) raised fluorescence to its maximum value (F_m). This made it possible to determine the maximum quantum efficiency of photosystem II (PSII) primary photochemistry, given by $F_v{:}F_m = (F_m - F_o){:}F_m$.

At the end of August, 4 leaves per species and treatment were also selected for analysis of quenching components using the saturation pulse method (Schreiber et al., 1986). After $F_v{:}F_m$ determination, as described above, intermittent pulses of saturating strong white light (0.8 s at 8000 μmol /m^2s) were applied in the presence of white actinic light (PPFD at 1200 μmol/m^2s), so that both the steady-state fluorescence value (F_s) and the maximum fluorescence value (F_m') in the light-adapted state were determined; the minimum fluorescence in the light-adapted state (F_o') was also measured by applying a pulse of far red-light during a brief interruption in actinic illumination. At each saturating pulse, the quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined according to