Appendix A. Urban air quality in Europe – results of three years of standardised biomonitoring studies

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Abstract

Supported by the LIFE Environment Programme of the European Commission, *EuroBionet*, the 'European Network for the Assessment of Air Quality by the Use of Bioindicator Plants' was implemented in 1999 as a cooperative project of local authorities and scientific institutes in twelve urban agglomerations of eight EU member states. It aimed at using standardised bioindication methods in air quality monitoring and environmental awareness raising. During three years, air quality was assessed by means of accumulative and sensitive bioindicator plants at more than 100 monitoring sites. The experiments provided numerous data on the spatial and temporal distribution of air pollution impacts within the local networks and at the European level.

By the use of tobacco plants, a clear gradient of ozone-induced effects with increasing plant injury levels from the north and northwest of Europe to central and southern regions became evident. The analysis of data on ambient ozone concentrations documented that current threshold and target values for the protection of the vegetation were exceeded in most of the cities. The strongest ozone-induced leaf injuries were observed at sites in Lyon, Barcelona and Hohenheim/Ditzingen, whereas only weak to moderate ozone impact occurred at Edinburgh, Sheffield, Copenhagen, and Düsseldorf.

Keywords: air quality, air pollution, bioindicators, biomonitoring, urban areas, city network, standardisation, ozone, tobacco, poplar

Introduction

The general goal of the Air Quality Framework Directive adopted by the European Council (EC 1996) as outlined in its Article 1 is to develop a common strategy that among other objectives aims at defining and establishing objectives for ambient air quality in the Community designed *to avoid, prevent or reduce harmful effects on human health and the environment as a whole*. Thus, in its first article, the directive is pointing out that the avoidance of adverse effects on man and ecosystems constitutes the ultimate goal of all environmental legislation. Further on, in Annex II, the same directive recommends to consider the sensitivity of flora and fauna and their habitats when setting limit values of air pollution. In general, however, the emphasis of this basic directive concerning air quality control is clearly placed on the measurement of ambient levels of air pollutants by means of chemical and physical methods in automated monitoring stations, possibly accomplished by modelling techniques.

The data on ambient air pollutant concentrations obtained by such measurements permit control of compliance with the limit and target values established by the so-called Daughter Directives. They do, however, by no means allow to infer whether harmful effects on the environment are being avoided or are actually occurring. Such effect-related monitoring is requested by the CAFE ('Clean Air for Europe') Programme established in 2001 where measures to develop, collect and validate scientific information on the effects of air pollution is cited as one of the programme's objectives.

Biomonitoring of air pollution effects by the use of bioindicator plants has a long history dating back to the 19th century or even earlier. Since the 1950s, numerous studies on the suitability of a large number of plant (and animal) species as sensitive or accumulative bioindicators of air pollution have been conducted in both North America and Europe. In most cases, the studies have been performed independently from each other and with a low degree of standardisation thus reducing their reproducibility. Just in a few countries such as Germany, Austria or the Netherlands, some of these methods have also been applied by environmental authorities and private enterprises for routine monitoring of ambient air quality near industrial installations and in urban agglomerations or even regional monitoring grids. At the European level, however, the use of bioindicator plants to assess air pollution effects is not very well established. The insufficient standardisation of the techniques and consequently the low comparability of the results are among the major reasons for the poor acceptance of

this effect-related methodology of air quality monitoring by policy makers, public administration and the private sector. To date, only isolated efforts have been made to standardise the methodology, although normalised procedures are already available in some countries.

The only existing pan-European biomonitoring programme, performed in the framework of ICP Vegetation under the CLRTAP (Convention on Long-Range Transboundary Air Pollution), aims at assessing the effects of ozone and some other air pollutants mostly in *rural* areas of the UNECE member states applying moderately standardised biomonitoring techniques (Benton et al. 2000). Apart from a pilot project (Stabentheiner et al. 2004), no coordinated multi-lateral bioindicator studies have been conducted in *urban* agglomerations of the European Union yet. However, this would be particularly important as the city centres present considerably elevated levels of various air pollutants due to the large number of stationary and mobile emission sources concentrated on a relatively small area and the frequently bad dispersion conditions as a consequence of the high-density building development. Concomitantly, the risk for pollution-induced health effects is increased due to the high population density typical for large urban agglomerations.

Besides, bioindicator plants feature several properties that qualify them not only for the effect-oriented monitoring of air quality but especially for environmental communication and education. Thus, they may be potentially useful in communicating information on the state of the environment and on the options for mitigation measures to the broad public and in performing environmental education programmes aiming at switching to more environmentally sound attitudes. Such properties are particularly important in view of the principles adopted by the Earth Summit held at Rio de Janeiro in 1992 which ensured the citizens' right of appropriate access to information concerning the environment. This right has been confirmed by the Århus Convention (UNECE 1998) and by a recent EC Directive (EC 2003). As a consequence, the demand for efficient and attractive communication strategies in the environmental sector is obviously increasing.

This was the background for the initiation of the EuroBionet project under the umbrella of the LIFE Environment Programme of the European Commission in 1999.

Material and methods

The network

Under the scientific and technical coordination of the University of Hohenheim (Germany), participants in this project included the cities of Copenhagen (DK), Düsseldorf (D), Edinburgh (GB), Glyfada / GreaterAthens (GR), Klagenfurt (A), Sheffield (GB), Valencia (E), Verona (I) and the regions of Grand Nancy (F) and Grand Lyon (F) as well as the Autonomy of Catalonia / Barcelona (E) (Figure 1). Most of the municipal authorities established specific cooperation agreements with local research institutes or private organisations in order to execute the technical and scientific programme.

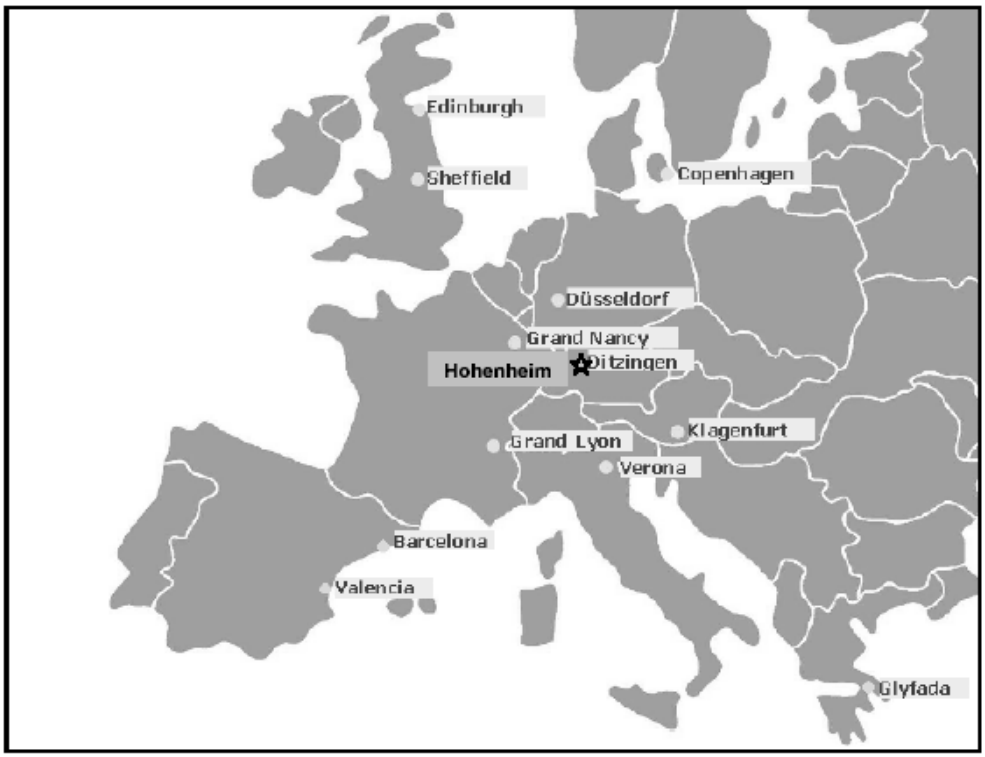

Figure 1. Map of Europe showing the partner cities of EuroBionet.

In each of the cities, local bioindicator grids consisting of 8–10 exposure sites characterised by different pollution loads were installed summing up to more than 100 bioindicator stations in twelve cities which were operated during three years. The locations included urban, suburban, rural, traffic and industrial sites. Rural sites or stations located in urban green

areas with low concentrations of primary air pollutants served as local reference. Further criteria for the selection of the exposure sites included the proximity to automated air monitoring stations, urban planning aspects, protection from vandalism as well as communicative considerations (cp. Ansel et al. 2004). The microscale siting of the exposure equipment followed the recommendations given by the VDI-Guideline 3957 Part 1 (VDI 1999). The local network of Ditzingen consisted of only six stations five of them being installed on school yards and one at the edge of the highway A81. Four additional sites were maintained by the University of Hohenheim, located on the university campus southeast of Stuttgart and near to a coal-fired power plant in the Neckar Valley (three sites) close to the city of Plochingen, which is characterised by intense industrialisation and traffic.

Tobacco (Nicotiana tabacum Bel–W3)

The methods of cultivation and exposure of the ozone-sensitive tobacco cultivar Bel–W3 followed the procedure described in a draft version of the corresponding VDI-Guideline (VDI 2003). The plants were grown from seeds to the six-leaf stage in the local greenhouses. At each bioindicator site, 4–6 plants were exposed to ambient air during eight consecutive periods of two weeks each between May and September. At the end of exposure, the percentage of ozone-induced injuries on three predetermined leaves was estimated visually in steps of 5 % of the leaf area using a photo catalogue as a reference. Local staff in charge of the visual estimation was regularly trained by the coordination team to insure a high degree of reproducibility. Mean per cent injury for each station and each exposure period, and afterwards mean annual injury degrees were calculated and classified according to a system of five damage classes frequently used in biomonitoring programmes (Nobel and Maier-Reiter, 1992).

Poplar (Populus nigra 'Brandaris')

Plants of the ozone-sensitive poplar clone 'Brandaris' were grown from cuttings and exposed to ambient air during 14 weeks between May and September. The criteria assessed in biweekly intervals included the percentage of damaged leaves, total number of leaves, leaf drop, and shoot length. At the end of the exposure period 2001, ten elder, undamaged leaves per plant were sampled, joined to form a mixed sample of the four plants per site and analysed for their heavy metal concentrations.

Cultivation and exposure methods

For the plant cultivation, a mixture of standardised soil type ED73 (containing peat, clay and slow-release fertiliser) and river sand (8:1) was used. Nutrient solutions made from reagent-grade chemicals were taken. All plants were grown in plastic pots in the greenhouse, using a semi-automatic watering system (glass fibre wicks). The exposure of tobacco and poplar in the field was done on metal racks at a height of about 1–1.2 m above ground, with plastic basins as water reservoirs, styrofoam plates with drillings to accommodate the pots and shading fabric (except for curly kale), according to the system originally proposed by Arndt et al. (1985).

Results and discussion

The presentation of the most significant outcomes of the three years' project is mainly based on the results obtained in 2001 as the data sets of that year are rather complete whereas in 2000 and 2002 data of some cities are missing.

Ozone pollution and its effects on tobacco plants

Today, due to the widespread occurrence of elevated ambient levels and to its ecological and economic consequences, ozone is considered one of the most important air pollutants in Europe and probably also on a global scale (EEA 2003). Consequently, EuroBionet has placed special emphasis on the description of the ozone pollution characteristics in the participating cities and on the assessment of ozone-induced effects on bioindicator plants. Using data from urban ozone monitoring stations in the participating cities, diurnal cycles of ozone concentrations and the number of exceedances of various European threshold and target values were calculated.

On the basis of the AOT40 values for the period May–July, a clear North-South gradient of the ozone load became evident, with the only exception of the two Spanish cities where the specific topographic conditions and the higher levels of primary air pollutants were probably responsible for the low to intermediate ozone concentrations in both conurbations (Figure 2). The WHO threshold value (WHO 2000) and EC long-term objective for the protection of the vegetation of 3 000 ppb * h (EC 2002) was exceeded in most cities, only Edinburgh, Sheffield, Copenhagen and Valencia met this standard. The ozone levels

measured at the monitoring sites in Klagenfurt, Lyon and Verona exceeded also the EC target value of 9 000 ppb * h between May and July and the EC target value for the protection of forests of 10 000 ppb * h between April and September. The same holds true for the measuring sites at Hohenheim and Plochingen in southern Germany (data not shown).

Figure 3 shows the mean diurnal cycles of ozone concentrations measured at urban and suburban monitoring sites. In most cities, a typical daily course of ozone levels with a minimum during early morning hours, increasing concentrations after sunrise with a maximum in the afternoon, and a reduction of ozone levels in the evening was observed

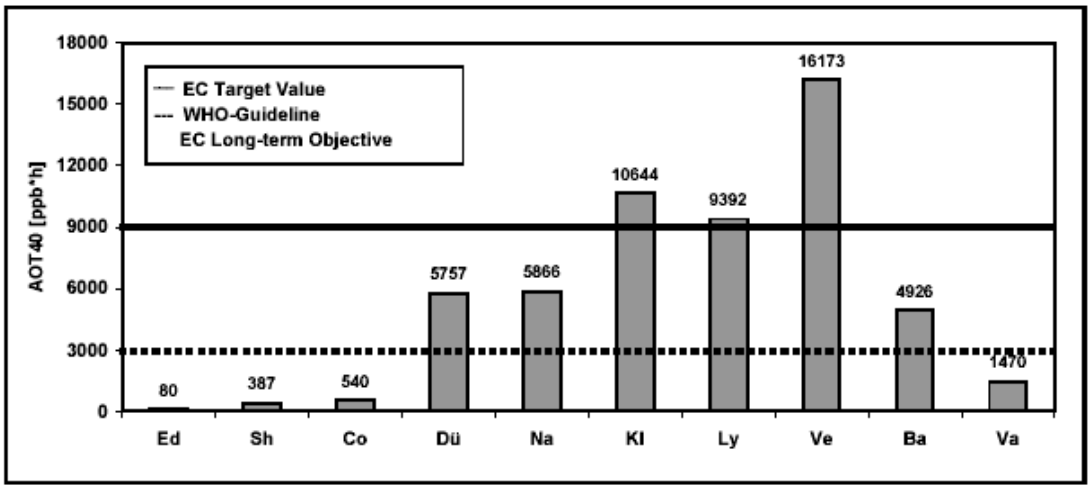

Figure 2. AOT40 values at urban and suburban monitoring sites of the EuroBionet during May – July 2001. Ed corresponds to the city of Edinburg, Sh to Sheffield, Co to Copenhagen, Du to Düsseldorf, Na to Nancy, Kl to Klagenfurt, Ly to Lyon, Ve to Verona, Ba to Barcelona and Va to Valencia

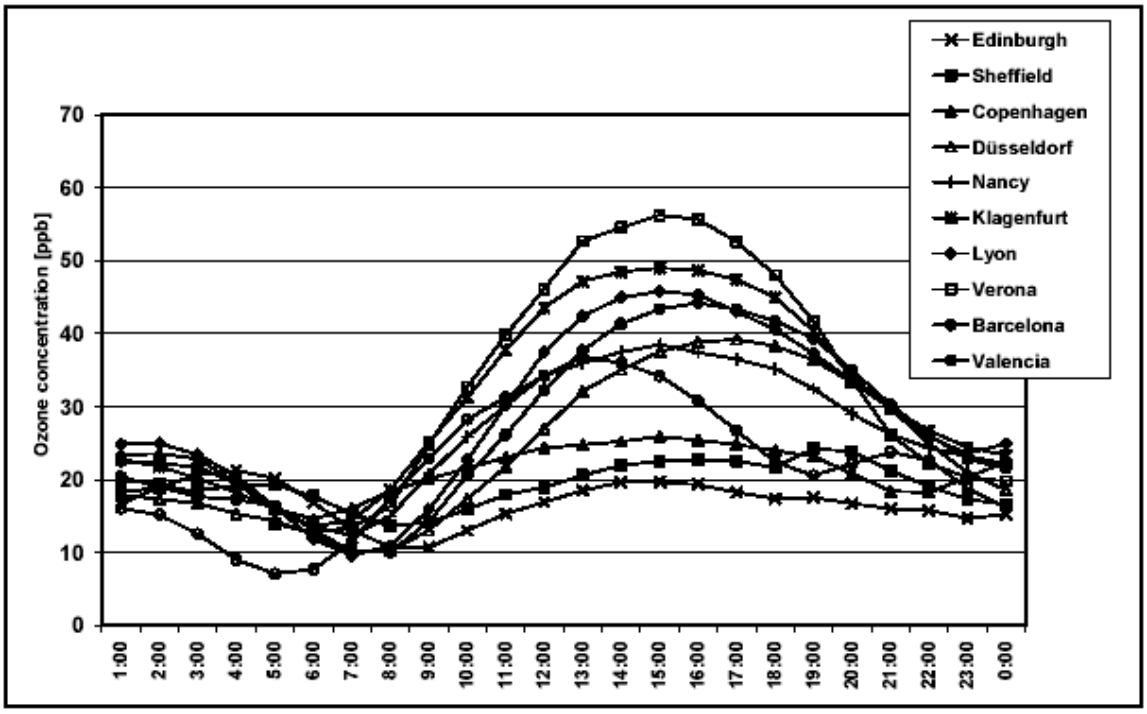

Figure 3. Mean diurnal cycle of ozone concentrations at urban and suburban monitoring sites during April to September 2001.

as a consequence of photochemical ozone formation. Highest amplitudes between maximum and minimum values were observed in the south and central European cities like Verona, Klagenfurt or Lyon. In northern European cities like Edinburgh and Copenhagen, ozone levels were generally lower than in southern regions, but elevated concentrations were also found in the early morning hours, probably due to the greater relevance of vertical mixing processes during night time in comparison to photochemical ozone production during the day (Coyle et al., 2002).

Typical ozone-induced leaf lesions were observed in all local networks and in all three experimental years. Applying the five-stage classification system (per cent leaf injury / ozone impact: < 5 % / very low; 6–15 % / low; 16–30 % / medium; 31–60 % strong; > 60 % / very strong), most of the injury assessments performed in UK cities and Copenhagen resulted in "very low" to "medium" injury degrees, whereas the classifications "strong" and "very strong" dominated in Lyon and Barcelona. The bioindicator networks in Düsseldorf, Nancy, Klagenfurt, Verona, Ditzingen and Hohenheim occupied an intermediate position. The data basis of Glyfada and Valencia was not sufficient for such a comparative classification.

The Box-Whisker-Plot in Figure 4 gives an overview of the variability of the annual mean injury values of the exposure sites within the local networks. This diagram reinforces the already mentioned gradient of increasing ozone pollution from the north and northwest of Europe to the central and southern regions. Particularly Lyon and Barcelona (together with the measuring grid in Ditzingen/Hohenheim) showed high levels of ozone-induced tobacco damage. The site Feyzin in Lyon presented the highest annual mean (83 %) and the site Tinsley in Sheffield the lowest mean value (7 %) of the whole European network. The variation between the sites of a given network was in general relatively small (Figure 5) with somewhat lower median values at urban and traffic sites and higher values at suburban and rural stations.

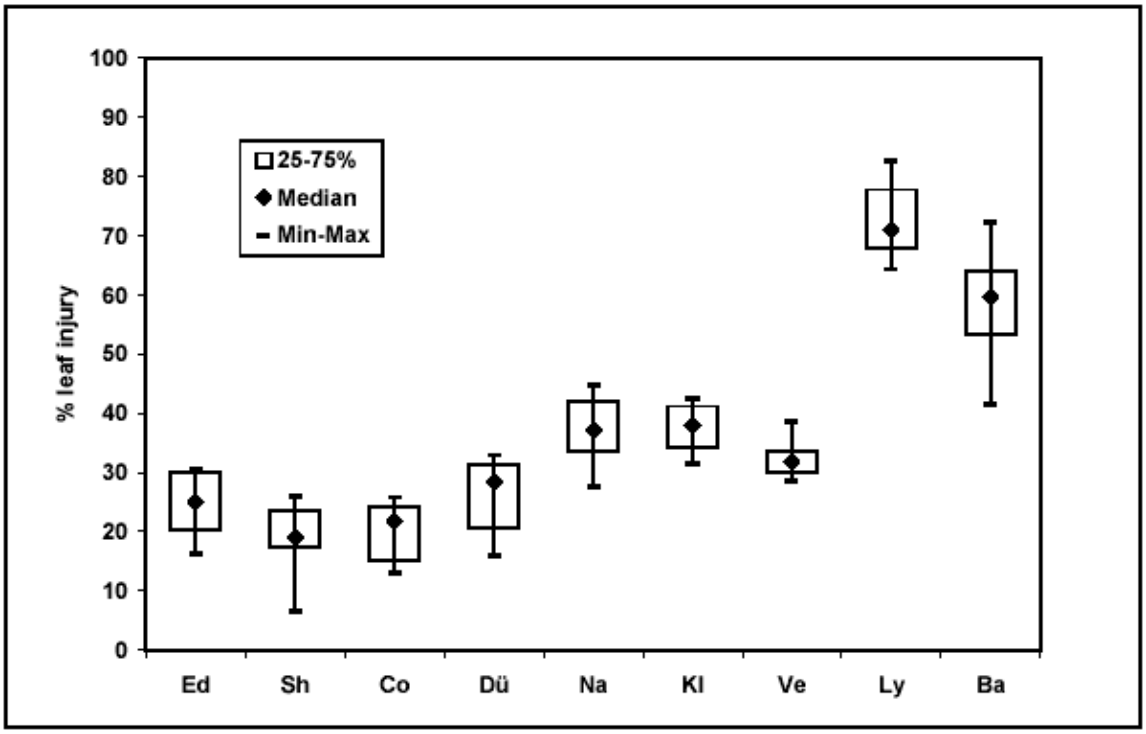

Figure 4. Percentage of ozone-induced injury on tobacco leaves (mean values of 8 exposure periods between May and September 2001). Ed corresponds to the city of Edinburg, Sh to Sheffield, Co to Copenhagen, Du to Düsseldorf, Na to Nancy, Kl to Klagenfurt, Ly to Lyon, Ve to Verona, Ba to Barcelona and Va to Valencia.

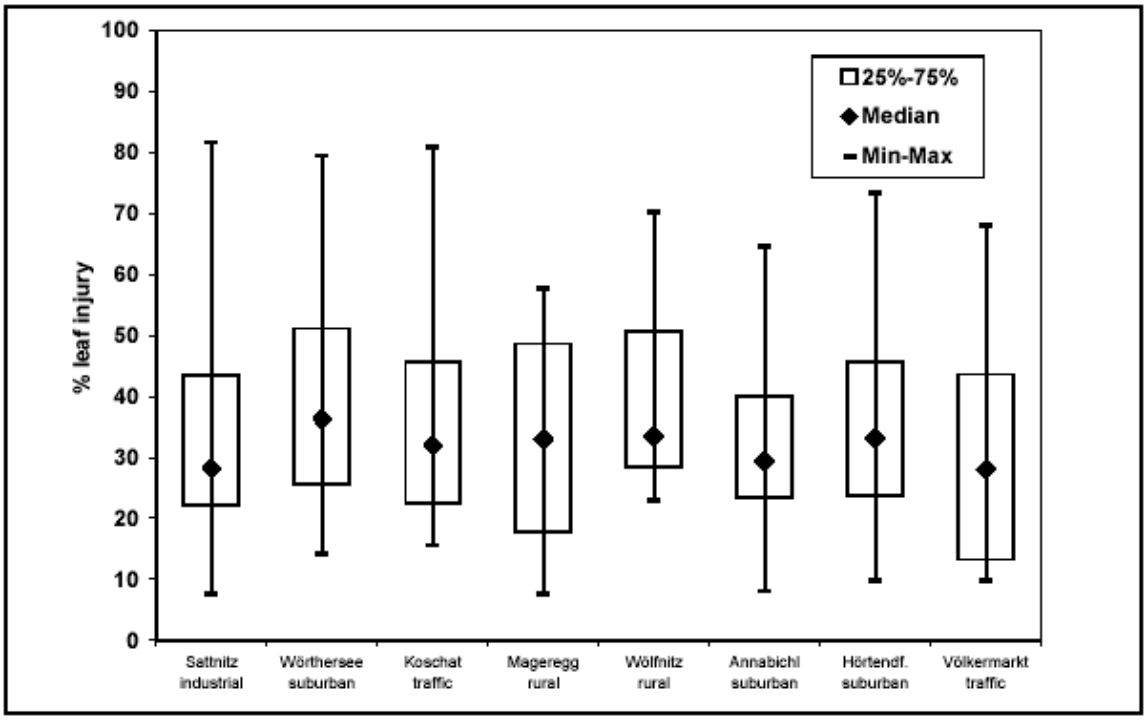

Figure 5. Variation of ozone-induced injury on tobacco leaves in the monitoring network of Klagenfurt during 2000 – 2002.

There were no clear and statistically significant correlations between ozone effects on tobacco plants on one hand and ozone pollution characteristics like AOT20, AOT40 or ozone concentrations on the other hand. Such relationship, however, had not been expected as several external meteorological factors like temperature, water vapour deficit, and global radiation as well as internal plant factors like the water balance are known to exert strong influence on the stomata regulation and thus on ozone uptake. Ozone flux into the leaf rather than ambient ozone concentrations are thus considered to be determining for the development of leaf injury. However, when data sets of single networks were separately investigated, particularly under stable atmospheric conditions linear relationships could be observed, as e.g. in Düsseldorf, similar to the findings of Ribas and Peñuelas (2002) in Barcelona and other authors.

Conclusions

Up to now, EuroBionet has been the major urban biomonitoring network comprising more than 100 bioindicator stations installed at urban, suburban, traffic-exposed and rural sites in twelve European conurbations. Placing strong emphasis on the application of highly standardised methods and on quality control concerning cultivation, exposure and assessment procedures, the project served as a test campaign for the Europe-wide use of bioindicators in air quality monitoring. In the following, the major conclusions concerning the applied methodology and the main results are summarised.

Evaluation of the bioindication methods applied in EuroBionet

Most of the bioindication techniques utilised by the EuroBionet project proved to be very suitable means to provide evidence of the spatial and temporal extension of air pollution impacts as well as short-term alterations of the pollution load. They may at relatively low cost and operating expense be used for air quality monitoring in local and regional networks as well as for source-related monitoring in and around industrial plants. Additionally, they enable the localisation of local 'hot spots' of air pollution load, the verification of the success of mitigation measures and the control of substances released into the environment following the introduction of new technical production processes. Thus, biomonitoring techniques provide information on really occurring effects on living organisms that complement the data obtained by physico-chemical measurements and modelling of emissions and ambient pollutant concentrations.

Depending on the climatic conditions and the actual weather situation during cultivation and exposure, only minor adaptations of the procedures described in the respective guidelines were necessary when plants were exposed in different geographical regions of Europe. The exposure of the ozone-sensitive tobacco cultivar Bel–W3 proved to be very suitable for the effect-related monitoring of phytotoxic ozone concentrations as well as for the demonstration of the harmful effects of ozone in environmental education and communication campaigns. The sensitivity of the species towards bad weather conditions reduces the time period in which the method may be applied particularly in northern Europe during spring, whereas its high ozone sensitivity may limit the applicability in southern European regions during episodes with extremely elevated ozone levels. Simultaneous exposure of less sensitive cultivars like Bel–B and specific technical requirements concerning

the cultivation period (e.g., climatisation and air-filtering facilities in the greenhouse) and other modifications of the proposed method may help to circumvent the mentioned difficulties. As a next step towards a Europe-wide establishment of these biomonitoring techniques, the development and subsequent field validation of European bioindication guidelines are now recommended.

Contrary to the methods mentioned above, the exposure of the ozone-sensitive poplar clone did not produce reliable results as for the long-term ozone impact. More studies including poplar clones with a higher ozone sensitivity like the system proposed by Bücker et al. (2004) and lower susceptibility to pests and insect attack will be necessary to develop an effective and standardised method for monitoring chronic ozone effects on woody plant species. The methodological approach, in particular the cultivation and exposure methods applied, proved to be effective and cost-saving and may serve as a basis for further studies investigating the influence of different environmental factors on the response to air contaminants.

Assessment of urban air quality by the use of bioindicator plants

By the exposure of a sensitive tobacco cultivar, a clear geographical pattern of ozone pollution and ozone effects on plants with increasing levels from northern Europe to central and southern Europe was demonstrated, locally and regionally modified by specific topographical conditions influencing the photochemical ozone formation and the atmospheric dispersion. The highest ozone concentrations and the strongest ozone damages to leaves were observed at rural and suburban sites which are characterised by low levels of primary air pollutants, especially nitrogen oxides, but ozone impact reached strongly elevated levels also at urban sites and even at stations close to heavily-trafficked roads.

EuroBionet may be considered an example for the utilisation of bioindicator plants in the fields of environmental monitoring and environmental education not only in the EU member states but also in the accession and candidate countries. The high degree in standardisation and quality control achieved in the frame of this project demonstrated that a Europe-wide use of these procedures is feasible and useful, particularly if European standards will soon be established. Moreover, taking into account the continuously growing pollution problems in many developing countries and the easy implementation of biomonitoring techniques, this methodology should play an important role in air quality monitoring and

control in urban agglomerations and industrial zones of Africa, Asia, and Latin America in the future. In fact, some of the methods used by EuroBionet have already
been adopted by bioindicator networks in Hungary (Várbíró et al. 2004) and Poland (Zbierska and Karolewicz-Borowiak 2004) and in similar projects in developing countries like Bolivia, Brazil, China and Ethiopia.

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Chapter 3. The ecophysiological basis of O_3 phytotoxicity

3.1. Ozone exposure induces the activation of senescence-related processes and morphological changes in Mediterranean tree species.

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Abstract

Four Mediterranean tree taxa: *Quercus ilex ssp. ilex*, *Quercus ilex ssp. ballota*, *Olea europaea* and *Ceratonia siliqua*, were exposed to different ozone (O_3) concentrations in open top chambers (OTCs) during two years. Three treatments were applied: charcoal-filtered air (FI), non-filtered air (NF) and non-filtered air plus 40 ppb_v of O_3 (NF+). The photochemical maximal efficiency, Fv/Fm, decreased in NF+ plants during the second year of exposure, especially during the most stressful Mediterranean seasons (winter and summer). However, there were no significant effects on instantaneous measurements of net photosynthetic rates (A) or stomatal conductances (g_s) in any of the studied species. Although there was not either significant change in instantaneous measurements of WUE, there was an increase of $\delta^{13}C$ in three of the four studied species during the first year of exposure. This finding was only maintained in *C. siliqua* during the second year. Decreases in the chlorophyll content were detected during the first year of fumigations in all the studied species, but not during the second year. The NF+ treatment induced changes in foliar anatomical characteristics, especially in LMA (leaf mass area) and spongy parenchyma thickness, which increased in some species. A reduction in N content and an increase in $\delta^{15}N$ were found in all species during the second year when exposed in the NF+ OTCs, suggesting a change in their retranslocation pattern linked to an acceleration of leaf senescence, as also indicated by the above mentioned biochemical and anatomical foliar changes. The two *Q. ilex* subspecies were the most sensitive species since the changes in N concentration, $\delta^{15}N$, chlorophyll, leaf area, LMA and biomass occurred already at ambient O_3 levels. However, *C. siliqua* was the most responsive species (24% biomass reduction) when exposed to the NF+ treatment, followed by the two *Q. ilex* subspecies (12-17%) and *O. europaea* (no significant reduction). Ozone resistance was linked to some plant traits such as chlorophyll concentrations, or spongy parenchyma thickness.

Keywords: Biomass reduction, *Ceratonia siliqua, Olea europaea*, Ozone exposure, *Quercus ilex*.

Introduction

Ozone (O_3) is a phytotoxic air pollutant widely distributed in the Mediterranean region where adverse effects on different plant tree species have been reported. Probably the most studied species has been Aleppo pine (Alonso et al., 2001; Elvira et al., 1998; Peñuelas et la., 1995; Sanz et al., 2000). Recently, a screening study carried out by Inclán et al. (1999) indicated that ozone exposure induced adverse effects on the biomass of holm oak (*Quercus ilex*) and olive trees (*Olea europaea*). However, some studies have highlighted the great intraspecific variability of Mediterranean vegetation to ozone (Elvira et al., 2003; Minnocci et al., 1999). As a result, the information regarding the response of Mediterranean tree species is still scarce, increasing the uncertainties derived from the definition of O_3 critical levels. Critical level is defined as the concentration of the pollutant above which adverse effects are likely to occur (UNECE, 1988). The UN-ECE Convention on Long-Range Transboundary Air Pollution has promoted the definition of ozone critical levels for different plant receptors and its activities have largely influenced present European ozone directive. Present critical level for forest tree species is defined as a six months AOT40 (ozone accumulated exposure over a cut-off of 40 nl l^{-1}) value of 10 ppm.h. The suitability of this critical level is controversial since many uncertainties still remain to be solved. Most of them are derived from the lack of experimental or field studies addressing this issue. This is especially the case for the Mediterranean area, making it difficult to establish an adequate environmental protection policy for the protection of Mediterranean forests (Bussotti and Gerosa, 2002).

Due to the great diversity of tree species and populations occurring in the Mediterranean area, it would be useful to define those plant traits that could be used as indicators of their potential sensitivity to ozone exposure. Such an approach has been recently adopted by Paoletti et al. (2003) for Mediterranean vegetation, using antioxidant levels as biomarkers of this sensitivity. Although their results are controversial, since the combination of ozone exposure, drought stress and low VPD values could induce an activation of these molecules; the conceptual scheme may be still valid. The selection of these key parameters is still an open issue.

The factors that determine plant sensitivity or tolerance are not clearly understood but it is thought to be related to many underlying physiological, anatomical, biochemical and environmental factors (Alonso et al., 2001; Pääkkönen et al., 1998). Ozone effects on tree biomass are the result of several processes occurring at the cellular and physiological levels. Acceleration of leaf senescence has been widely reported as one of the most

characteristic processes derived from ozone exposure. These processes involve chlorophyll degradation and reductions in CO_2 assimilation (Elvira et al., 1998; Zheng et al., 2002). Leaf senescence can also be characterized by reductions in N foliar levels. Therefore, carbon and nitrogen isotopic discrimination may be useful tools to describe the integrative responses of tree species to O_3 exposure. The fractionation of ^{13}C and ^{12}C is caused by diffusion of CO_2 through the leaf, boundary layer and stomata, dissolution of CO_2 in the apoplastic fluid and enzymatic reactions involved in carbon fixation (Farquhar et al., 1989). In general, the processes of N loss (NH_4 volatilization, nitrification, denitrification and lixiviation) enrich the system with the heavy isotope ^{15}N (Peñuelas and Estiarte, 1997).

Ozone effects on leaf morphology have also been reported. For instance, the early exposure of birch to O_3 resulted in a reduction in leaf size, with an increase in the density of stomata, hairs and veins (Gunthardt-Goerg et al., 1993). Similarly, the pectinaceous layer of spongy parenchyma cells was found to swell and protrude (Gunthardt-Goerg et al., 1997) following the exposure of birch to ozone. Many of these defence mechanisms are similar to those found in the schlerophyllous Mediterranean vegetation to prevent damages derived from drought, low VPD values and great solar radiation intensities.

This paper is focused on the response of four Mediterranean taxa presenting different physiological and anatomical traits: *Quercus ilex ilex*, *Quercus ilex ballota*, *Ceratonia siliqua* and *Olea europaea* cv. *sylvestris*, to assess interspecific differences in sensitivity to ozone. The response of two subspecies of Q. ilex was also studied to assess the intraspecific sensitivity to O_3. The following hypotheses were tested: 1) ozone exposure enhances plant senescence-related processes, 2) plant responses in biomass are related to alterations in integrated carbon assimilation and 3) plant traits can be associated with a differential sensitivity to ozone exposure.

Material and Methods

Growth conditions and ozone treatments

Plants of *Quercus ilex* ssp. *ilex, Quercus ilex* ssp. *ballota, Olea europaea* cv. *sylvestris,* and *Ceratonia siliqua* were raised from seeds. One-year old homogeneous seedlings of each species were transplanted in July 1998 into 3 dm^3 containers filled with Universal substrate (peat and bark pine) with a 34% of organic material. Soil pH was 6.6, and 9 g per pot of a slow-release fertilizer (NPK 15:8:11; Osmocote plus) were supplied.

This experiment was conducted in an experimental field of slightly modified NCLAN-type open top chambers (OTCs) (Gimeno et al., 1999), located at the Ebro Delta (NE Spain, 40º 41.5' North, 0º 48' East), 10 m above sea level. Three O_3 treatments were established: charcoal filtered air (FI), non-filtered air (NF) with close to ambient O_3 levels, and non-filtered air supplemented with 40 nl l^{-1} O_3 from 7:00 to 17:00 GMT 5 days to week (NF+). Ozone concentrations in the NF+ treatment were in the range of those reported by Millán et al. (2000). Three OTC replicates were used for each O_3 treatment. Three open air plots (AA) were established to check for possible chamber effects. An automatic system provided a continuous monitoring of O_3, sulphur dioxide and nitrogen oxides concentrations in the different treatments along with meteorological parameters such as wind speed and direction, air temperature and relative humidity, and photosynthetic radiation active (PAR). Complete descriptions of the chambers and the operation of the system are provided in Alonso et al. (2001). We introduced eight individual plants of each species per plot (8x4x3).

Plants were irrigated with a droplet system to ensure adequate and homogeneous plant water availability. Soil volumetric water content was measured using Time Domain Reflectometry (TRIME, IMKO, Micromodultechnick, Germany). The experiment lasted two years (July 1998-August 2000).

Chlorophyll fluorescence and gas exchange measurements

Chlorophyll fluorescence and gas exchange measurements were conducted during 2-4 consecutive days each season of the year during 2 years. Well-developed leaves were measured under clear-sky conditions.

The maximum photochemical efficiency of PSII (Fv/Fm), yield (ΔFv/F'm) and the apparent photosynthetic electron transport rate (ETR) were measured with a PAM-2000 fluorometer (Walz, Effeltrich, Germany). ETR was estimated as

$$ETR = \Delta F/F'm \times PPFD \times 0.84 \times 0.5,$$

where ΔF/F'm (actual photochemical efficiency of PSII) was calculated according to Genty et al. (1989), 0.84 is the coefficient of absorption of the leaves, and 0.5 is the fraction of electron involved in the photoexcitation produced by one quanta, since two photosystems are involved.

For measurements of the maximum PSII photochemical efficiencies (Fv/Fm), leaves were kept in the dark for 20 min (dark adaptation) prior to fluorescence measurements. We previously checked that the coefficient of fluorescence reached constant values after this 20 minute dark period. Chlorophyll fluorescence was measured

on three well developed leaves of three plants per chamber and species (3x3x3= 27 leaves in each treatment and species) from 07:00 to 11:00 h (solar time).

Net photosynthetic rates (A), transpiration rates (E) and stomatal conductances (g_s) were measured with a protable gs exchange system ADC LCA4, with a PLC4B chamber (ADC Inc., Hoddesdon, Hertfordshire, UK) inside the open top growth chambers. Water use efficiency (WUE) was calculated as (A/E) in μmol fixed CO_2 per mmol transpired H_2O. Two well developed leaves of one individual plant per chamber and species were measured from 7:00 to 11:00 h (solar time). A, E, and gs values were expressed on a projected leaf area basis. This leaf area was measured with a LiCor 3100 Area Meter (Li-Cor Inc., Lincoln, Nebraska, USA).

SPAD measurements

Chlorophyll content was determined non-destructively using a SPAD-502 meter (Minolta Co, LTD, Osaka, Japan). This instrument uses measurements of transmitted radiation in the red and near-infrared wavelengths to provide numerical values related to leaf chlorophyll content. Close linear correlations between SPAD values and extractable chlorophyll concentration have been reported for a wide range of species including plants exposed to elevated O_3 (Tenga et al., 1990). SPAD measurements were conducted at the beginning of the experiment (July 1998, data not shown), five months later (December 1998), seventeen months later (November 1999), and twenty one months later (March 2000). Four plants per chamber and species were sampled (each plant measure corresponded to a mean of four leaves per plant).

Isotope and elemental analyses

To analyse C and N isotopic composition, 4-6 well-developed leaves from different plants were pooled for each chamber and species after being collected in summer 1999 and summer 2000, just before biomass harvest was carried out. The foliar $\delta^{15}N$ and $\delta^{13}C$ were measured on a SIRA Series II isotope ratio mass spectrometer (VG Isotech, Middlewich, UK) operated in direct inlet continuous flow mode after combustion of the samples in an elemental analyser (NA1500, Series 1, Carlo Erba Instrumentazione, Milan, Italy). The reference CO_2, calibrated against standard Pee Dee belemnite (PDB) was obtained from Oztech (Dallas, TX, USA). A system check of analysis was achieved with interspersed working standards of cellulose, atropine and urea (Sigma, St. Louis, MO, USA). The accuracy of the measurement was ± 0.1‰ for $\delta^{13}C$ and ± 0.2 ‰ for $\delta^{15}N$.

Carbon (C) and nitrogen (N) leaf concentrations were analysed with a Carlo Erba NA 1500 Analyser (Milan, Italy), using a standard configuration for those determinations. The samples were previously dried at 70ºC to constant weight. They were weighed with a Mettler UM3 microbalance enclosed in tin containers.

Leaf morphology and anatomy and plant aerial biomass

Leaf morphology and anatomical variables were assessed every season throughout the two experimental years. At the same time trees were visually inspected for foliar symptoms of chronic O_3 injury. Foliar samples were also collected to determine leaf area and leaf mass area (n=3 per chamber). Leaf area was measured before drying using a LICOR LI-3100 area meter (LICOR, Licoln, Nebraska). Thereafter the leaves were dried at 70ºC until constant weight.

Three leaves of each chamber and species were sampled in summer 1999 to measure their parenchyma thickness. The leaf petioles were introduced in a container with distilled water to hydrate them. The leaf cuttings were performed with a manual microtom (EN-0101, ENOSA, Barcelona, Spain). The observations were carried out with a microscope with graduated ocular (Model CHS, OLYMPUS OPTICAL Co., LTD., Japan). The thickness of the palisade and spongy parenchyma was determined in 5 leaf cuttings of each individual leaf for. The parenchyma ratio value of an individual leaf was obtained as the mean of the five leaf cuttings.

At the end of the exposure experiment, in summer 2000, all aboveground biomass was harvested and dried out in an oven at 70ºC until a constant weight was reached.

Statistical analyses

The main design was a randomised complete block with the mentioned three ozone treatments and three chamber replications per O_3 treatment. An ambient outside chambers (AA) treatment was used to additionally check for possible chamber effects. ANOVAs were used to compare tree responses and to detect potential differences between AA and NF treatments.

Fluorescence (Fv/Fm, ETR and yield) and gas exchange (A, g_s, ci, and WUE) measurements were analysed using a two way factorial ANOVA (season and treatment). One way ANOVA was used to analyse the data involving morphological and anatomical parameters (leaf area, leaf dry weight, leaf thickness, LMA, parenchyma thickness) using treatment as a factor. All these analyses were conducted for each individual species. Data collected on particular dates during the study were also analysed by two-way ANOVAs.

They were conducted with isotopic composition and C and N values as dependent variables and ozone treatment and species as independent factors for each year (1999, 2000). The same analysis was used to test differences for chlorophyll measurements in December 1998 and November 1999, and for biomass differences when the final harvest was carried out. When significant effects of any factor were detected, a post-hoc Tukey test was used to test within groups differences. All mentioned analyses and additional correlation analyses were performed with the software package Statistica 6.0 (StatSoft Inc., Tulsa, USA).

Results

Ozone exposure and meteorological variables

In this experiment, AOT40 in the NF+ treatment ranged from 6,142 (for the three months December 1998 to February 1999) to 30,147 ppb_v (for the three months May 1999 - July 1999). In the NF treatment the AOT40 ranged between 4 ppb_v and 2,877 respectively for the same periods. AOT40 was always 0 in the FI treatment (Fig. 1). Typical seasonal coastal Mediterranean trends were observed in temperature, radiation and ozone concentrations, with mild winters and warm and dry summers. Minimum temperatures occurred from January to March, between 5-7 ºC, and the maximum temperatures occurred in summer, between 28 and 33ºC as monthly mean values (Fig. 1). Humidity values were very high throughout all the year (between 70 and 90 % as monthly mean values).

Chlorophyll fluorescence

No significant differences in potential photochemical efficiency, estimated as dark-adapted Fv/Fm ratio, were found, but a generalized decline was observed for all species during the second year of O_3 exposure in the fumigated treatment (Fig. 2) indicating more photoinhibition (Krause, 1988). Maximum differences occurred during winter of the second exposure year. The decrease in Fv/Fm produced by O_3 fumigation in winter 1999 - 2000 was 7.33 % in *C. siliqua*, 3.97 % in *O. europaea,* 1.59 % in *Q. ilex* ssp. *ilex* and 0.94% in *Q. ilex* ssp. *ballota*. In *O. europaea* there was a marginally significant decrease ($p=0.09$) of Fv/Fm throughout all the exposure period in the fumigation treatment. During fall and winter *O. europaea* and *C. siliqua* presented lower Fv/Fm values than the *Q. ilex*

subspecies. Fluorescence yield and ETR did not show any significant difference among treatments (data not shown).

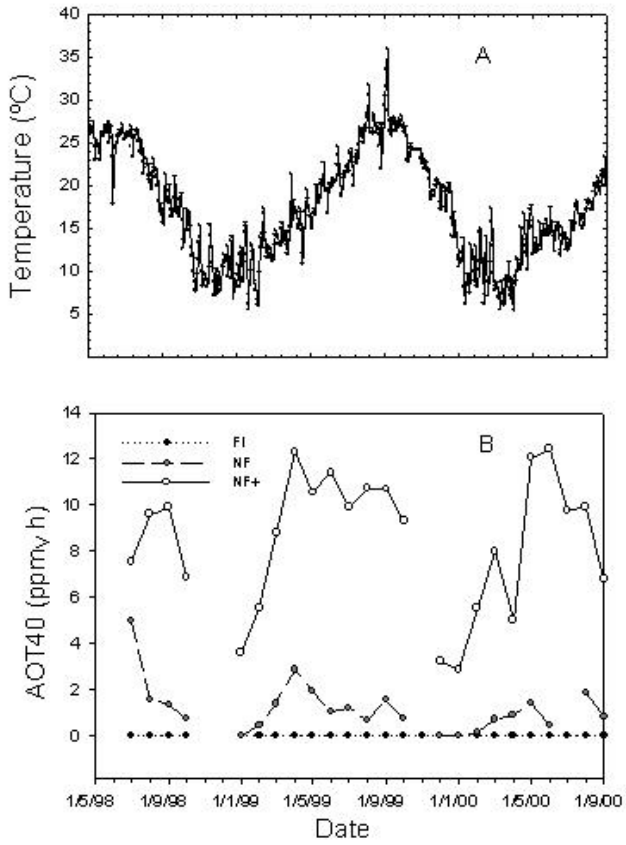

Figure 1. A) Field site daily mean Temperature (ºC), and B) monthly AOT40 during the experimental period, from July 1998 to September 2000, in the different O_3 treatments. FI, charcoal-filtered air; NF, non-filtered air; NF+, non-filtered air plus 40 ppb_v ozone. The white arrows show the sampling dates after irrigation treatment started.

Leaf gas exchange responses

A seasonal pattern was not always observed for all the species, but in general we found highest values in assimilation rates in fall and spring (equinoctial maxima), an minimum values for summer and winter periods as it is characteristic for Mediterranean ecosystems (Fig. 3). Among the studied species, *O. europaea* presented the highest assimilation rates (Fig. 3).

No significant changes for instantaneous net photosynthetic rates were detected between control and fumigated treatments, with the exception of a significant interaction between O_3 and time of exposure in *C. siliqua* ($p<0.05$) (Fig. 3). There was a trend towards lower net photosynthetic rates for NF+ plants in the second year of exposure, whereas in the first year the trend had been towards higher rates.

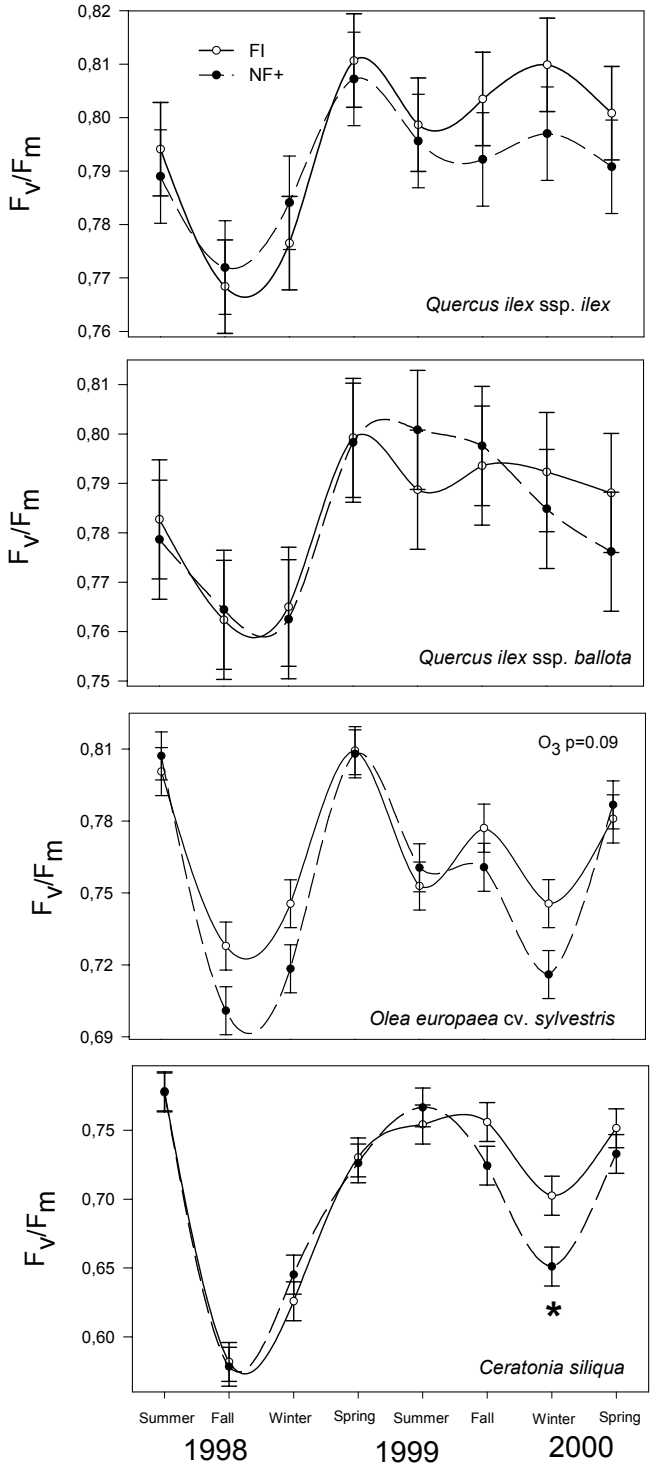

Figure 2. Maximum potential photochemical efficiency (Fv/Fm) of *Q. ilex ilex*, *Q. ilex ballota*, *O. europaea*, *C. siliqua* through the study period. Only FI (Charcoal-filtered air) and NF+ (Non-filtered air + 40 ppv of O_3) treatments are depicted for sake of clarity of the figure. Error bars indicate the standard error of the mean (n=3 chamber means of n=6-9 plant measurements). Significant differences in each particular date are indicated by asterisks when $p < 0.05$. Statistical significant factor effects (depicted in the panels) were calculated including the third treatment NF (NF=Non-filtered air) and for each species individually.

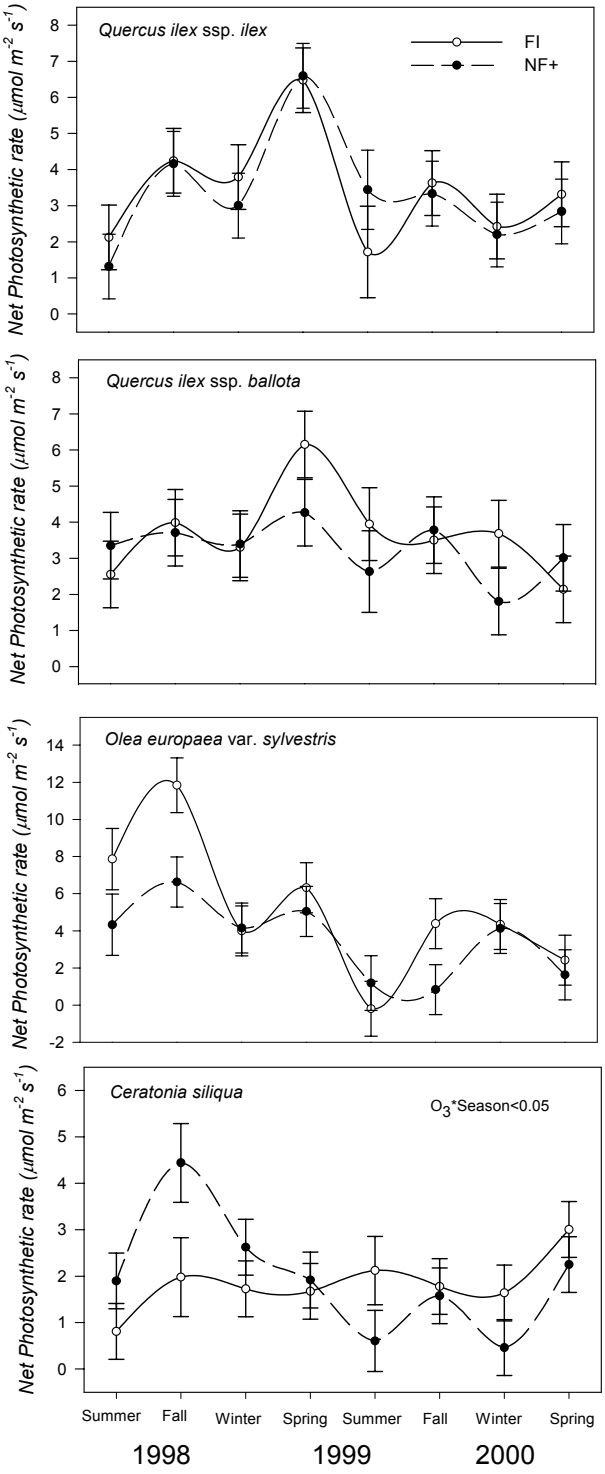

Figure 3. Net photosynthetic rates (μmol CO_2 m^{-2} s^{-1}) of *Q. ilex ilex*, *Q. ilex ballota*, *O. europaea*, *C. siliqua* exposed to ozone in open-top chambers through the study period. Only FI (Charcoal-filtered air) and NF+ (Non-filtered air + 40 ppv of O_3) treatments are depicted for sake of clarity of the figure. Error bars indicate the standard error of the mean (n=3 chamber means of n=2 plant measurements per chamber). Significant differences in each particular date are indicated by asterisks when $p < 0.05$. Statistical significant factor effects (depicted in the panels) were calculated including the third treatment NF (NF=Non-filtered air) and for each species individually.

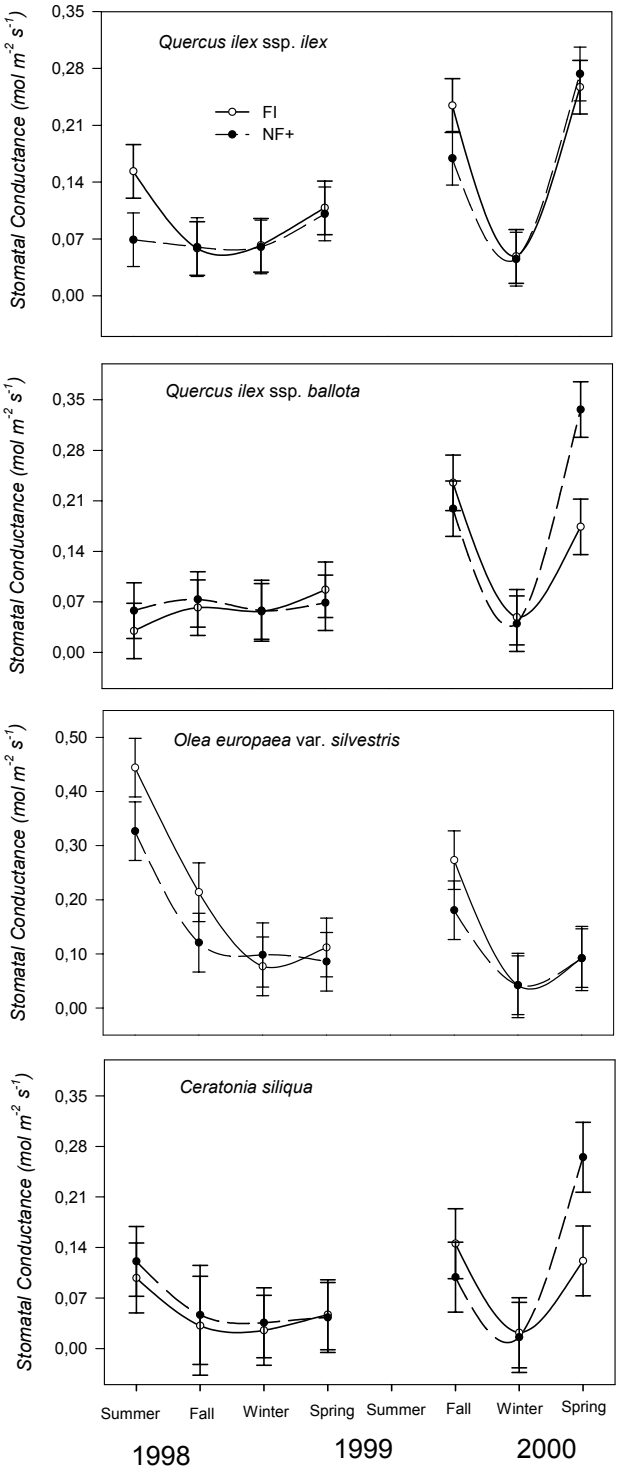

Figure 4. Stomatal conductance (mmol CO_2 m^{-2} s^{-1}) of *Q. ilex ilex*, *Q. ilex ballota*, *O. europaea*, *C. siliqua* exposed to ozone in open-top chambers through the study period. Error bars indicate the standard error of the mean (n=3 chambers means of n=2 plant measurements per chamber). Only FI (Charcoal-filtered air) and NF+ (Non-filtered air + 40 ppv of O_3) treatments are depicted for sake of clarity of the figure. Significant differences in each particular date are indicated by asterisks when $p < 0.05$. Statistical significant factor effects (depicted in the panels) were calculated including the third treatment NF (NF=Non-filtered air) and for each species individually.

Stomatal conductances (g_s) did not either show any significant difference between treatments. However, at the end of the experiment, in spring 2000 there was a trend towards higher stomatal conductances (statistically not significant, p=0.152 and p=0.155 respectively) in fumigated plants of *Q. ilex* ssp. *ballota* and *C. siliqua* (Fig. 4). No significant differences were either found for the intercellular CO_2 concentration (ci) or the WUE in any of the studied species (data not shown).

Isotopic composition and N and Chl leaf concentrations

We analysed the $\delta^{13}C$ values of well-developed leaves as an integrative measure of plant water use efficiency over the entire growing season (Farquhar et al., 1989; Peñuelas et al., 2000). There was a significant (p=0.007) overall species increase in the $\delta^{13}C$ values of O_3-fumigated seedlings during the first year of exposure (Fig. 5); only *O. europaea* did not show significant responses. Significant differences were also found among species and there was a significant effect of the interaction between species and O_3 concentrations (sp*O_3). During the second year of exposure, no significant overall species differences were found among treatments although greater $\delta^{13}C$ values were still found in NF and NF+ *C. siliqua* seedlings (Fig. 5).

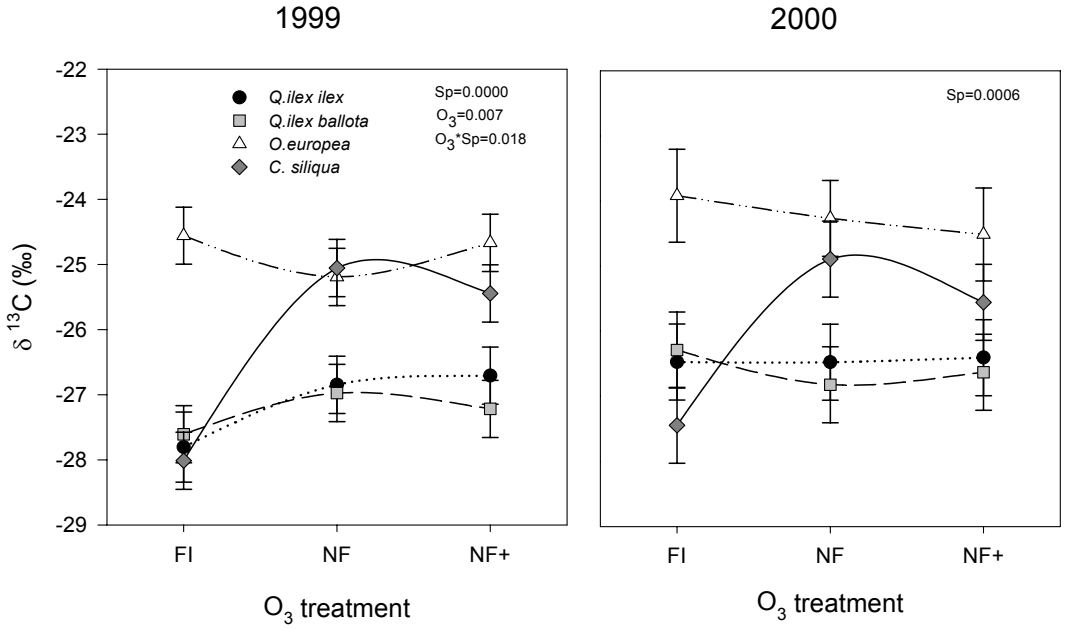

Figure 5. Foliar $\delta^{13}C$ (‰) of *Q. ilex ilex*, *Q. ilex ballota*, *O. europaea*, *C. siliqua* leaves in summer 1999 and 2000. Statistically significant effects are depicted inside the panels. FI (Charcoal-filtered air treatment), NF (Non-filtered air treatment), and NF+ (Non-filtered air + 40 ppb_v O_3 treatment). Error bars indicate the standard errors of the mean (n=3 chambers; 6-9 leaves were pooled for each chamber measure).

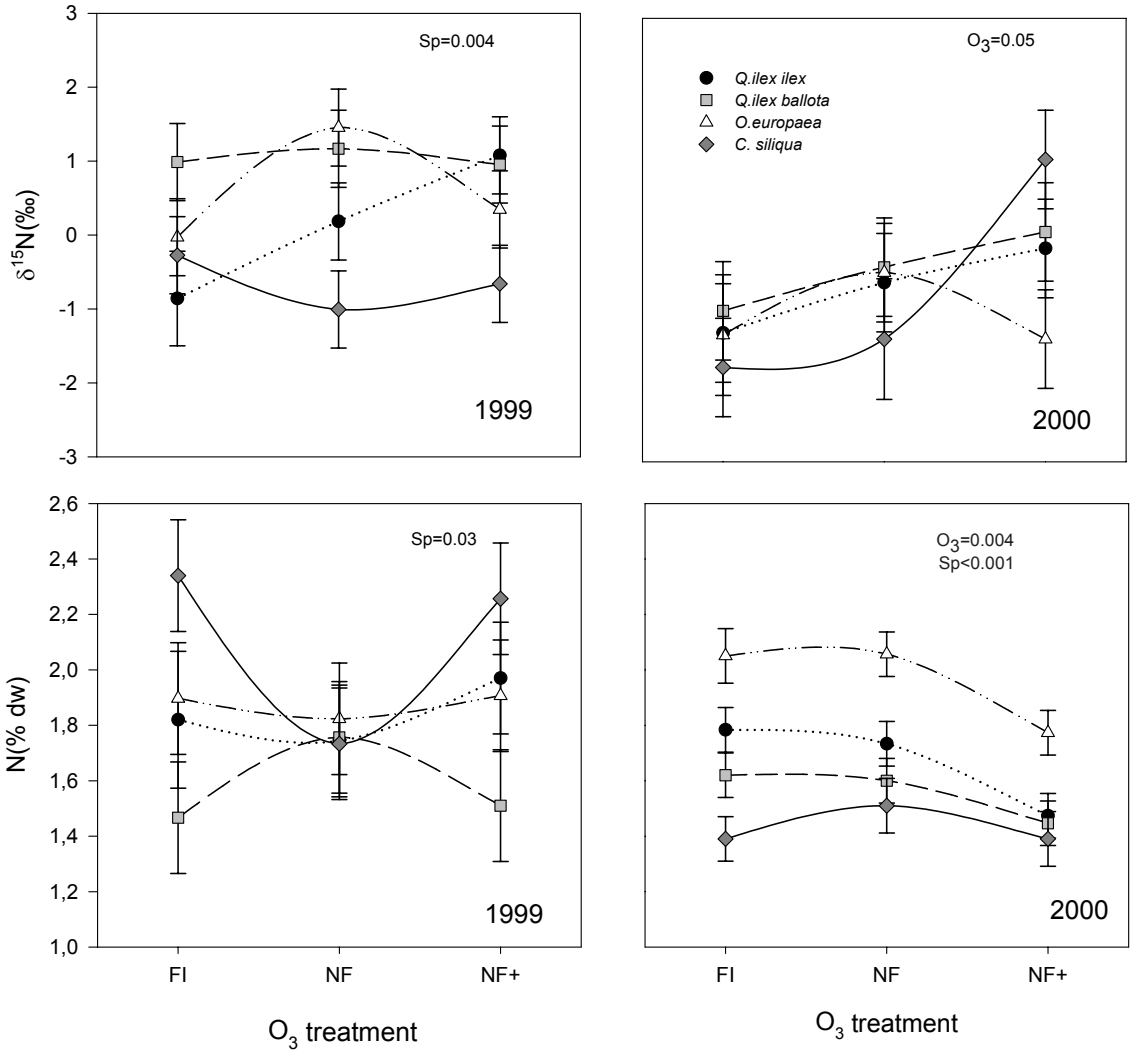

Figure 6. Foliar δ^{15}N (‰) and nitrogen concentrations (% dry mass) of *Q. ilex ilex*, *Q. ilex ballota*, *O. europaea*, *C. siliqua* leaves in summer 1999 and 2000. Statistically significant effects are depicted in the panels. FI (Charcoal-filtered air treatment), NF (Non-filtered air treatment), and NF+ (Non-filtered air + 40 ppb_v O_3 treatment). Error bars indicate the standard errors of the mean (n=3 chambers; 6-9 leaves were pooled for each chamber measure).

All species, except *O. europaea,* tended to present increased $\delta^{15}N$ values with increasing ozone exposures in the second year of the experiment (overall species effects p=0.05) (Fig. 6). For the first year this increasing trend was only present in *Q. ilex ilex* (Fig. 6).

After two years of exposure, above-ambient O_3 levels induced a decrease in N foliar concentration when compared to the NF treatment (15% in *Q. ilex* ssp. *ilex*, 14% in *O. europaea,* 9.6% in *Q. ilex* ssp. *ballota,* and 7.95% in *C. siliqua*). As a result, there was an overall species significant N depletion with increasing ozone concentrations (p=0.07) (Fig. 6). During the first year of exposure, no significant changes were detected (Fig. 6). The lowest N concentrations were found in the two *Q. ilex* subspecies (Fig. 6).

This general trend of depleted foliar N concentrations with increasing O_3 levels, was associate with a depletion in foliar chlorophyll concentrations of the NF+ plants of each species (overall effect, p<0.001). This effect was statistically significant in *Q. ilex ilex* and Q*. ilex ballota* (6.5% lower in the NF+ treatment than in the FI treatment) (Fig. 7). Higher SPAD values were found in *O. europaea* than in the other species (Fig. 7). But after eighteen months of fumigation, this pattern of decreasing chl concentrations with increasing O_3 concentrations was maintained only in *O. europaea* (Fig. 7).

Leaf morphology, visible injury and aerial biomass

Increasing ozone concentrations increased the spongy parenchyma thickness in *O. europaea* (p=0.12) and in *C. siliqua* (p=0.06) (Fig. 8). Increasing O_3 concentrations decreased leaf area in *Q. ilex ilex* (p=0.08) and in *O. europaea* (p=0.08) during the second year of exposure. These effects were already evident at ambient O_3 concentrations (data not shown). Throughout the experimental period, O_3 induced an increase of the average leaf mass area (LMA) in all the studied species but this increase was statistically significant only for *Q. ilex ilex* (Fig. 9).

After two years of O_3 fumigation, no clear visible leaf injuries occurred in any of the studied species. Only in the two *Quercus ilex* spp., and mostly in *Quercus ilex* ssp. *ballota*, a slight mottle stipple bronzing appeared on a few old leaves that had been exposed for two years (data not shown).

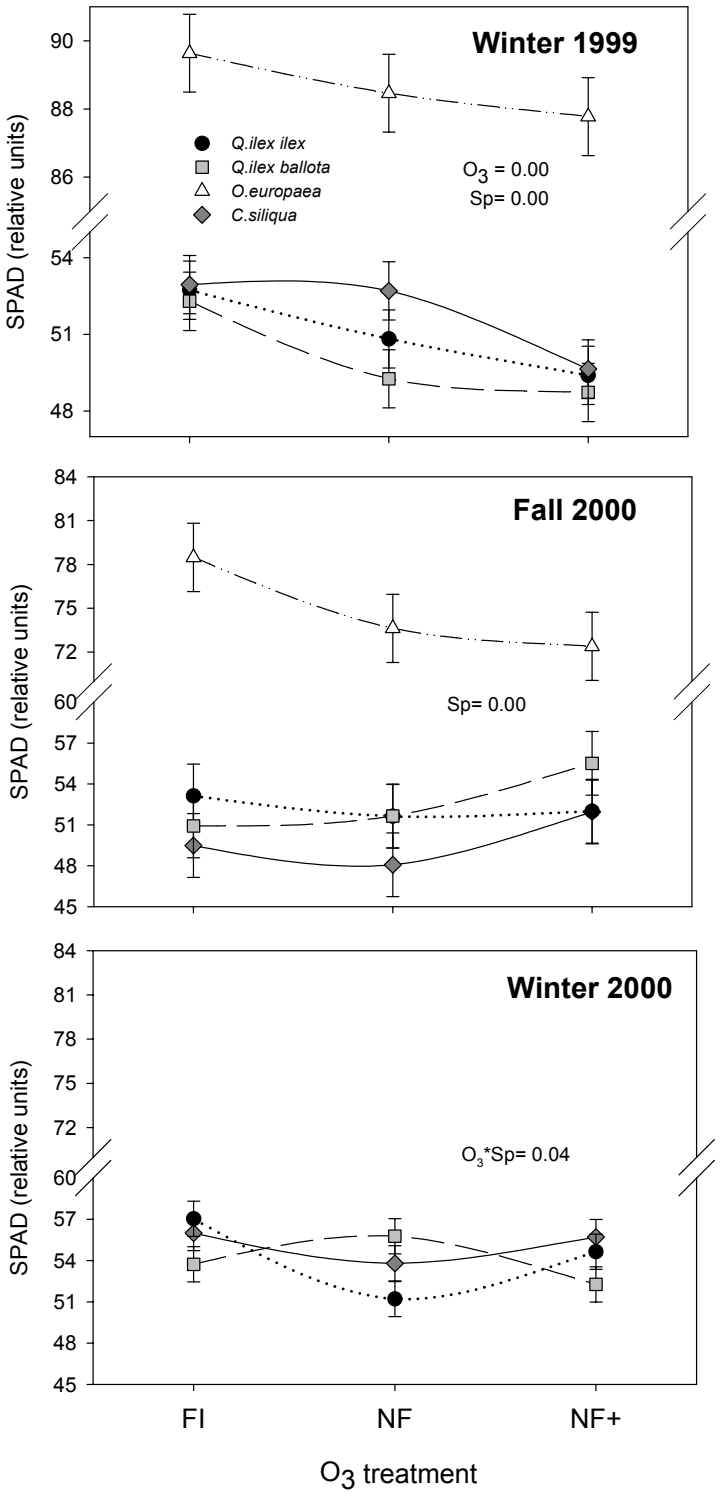

Figure 7. Leaf chlorophyll concentrations of *Q. ilex ilex*, *Q. ilex ballota*, *O. europaea*, *C. siliqua* in December 1998 and in November 1999, after six and eighteen months of O_3 exposure. Statistically significant effects are depicted in the panels. FI (Charcoal-filtered air treatment), NF (Non-filtered air treatment), and NF+ (Non-filtered air + 40 ppb_v O_3 treatment). Error bars indicate the standard errors of the mean (n=3 chamber means of n=4 plant measurements per chamber; each plant value was the mean of three leaf measurements).

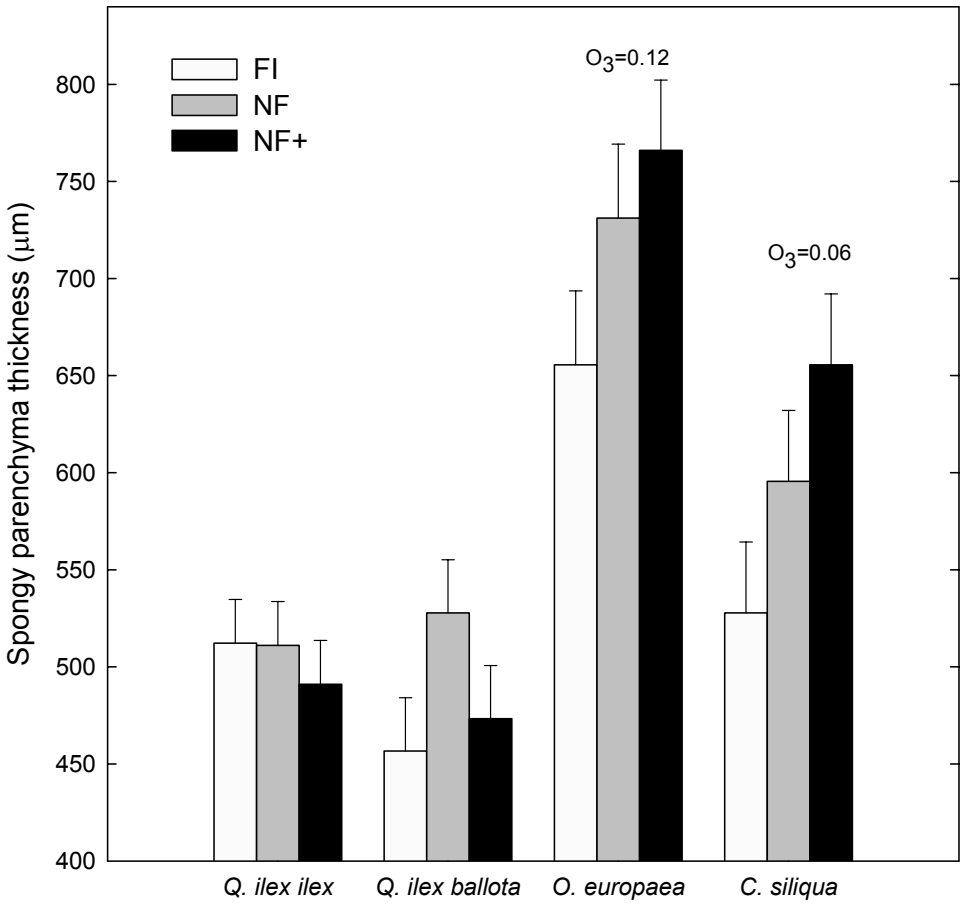

Figure 8. Spongy parenchyma thickness (μm) in the four studied species, *Q. ilex ilex*, *Q. ilex ballota*, *O. europaea* and *C. siliqua*, in summer 1999 after twelve months of O_3 exposure. Statistically significant effects are depicted in the panels. FI (Charcoal-filtered air treatment), NF (Non-filtered air treatment), and NF+ (Non-filtered air + 40 ppb_v O_3 treatment). Error bars indicate the standard errors of the mean (n=3 chamber means of n=3 leaves per chamber; each leaf value was the mean of five leaf cuttings).

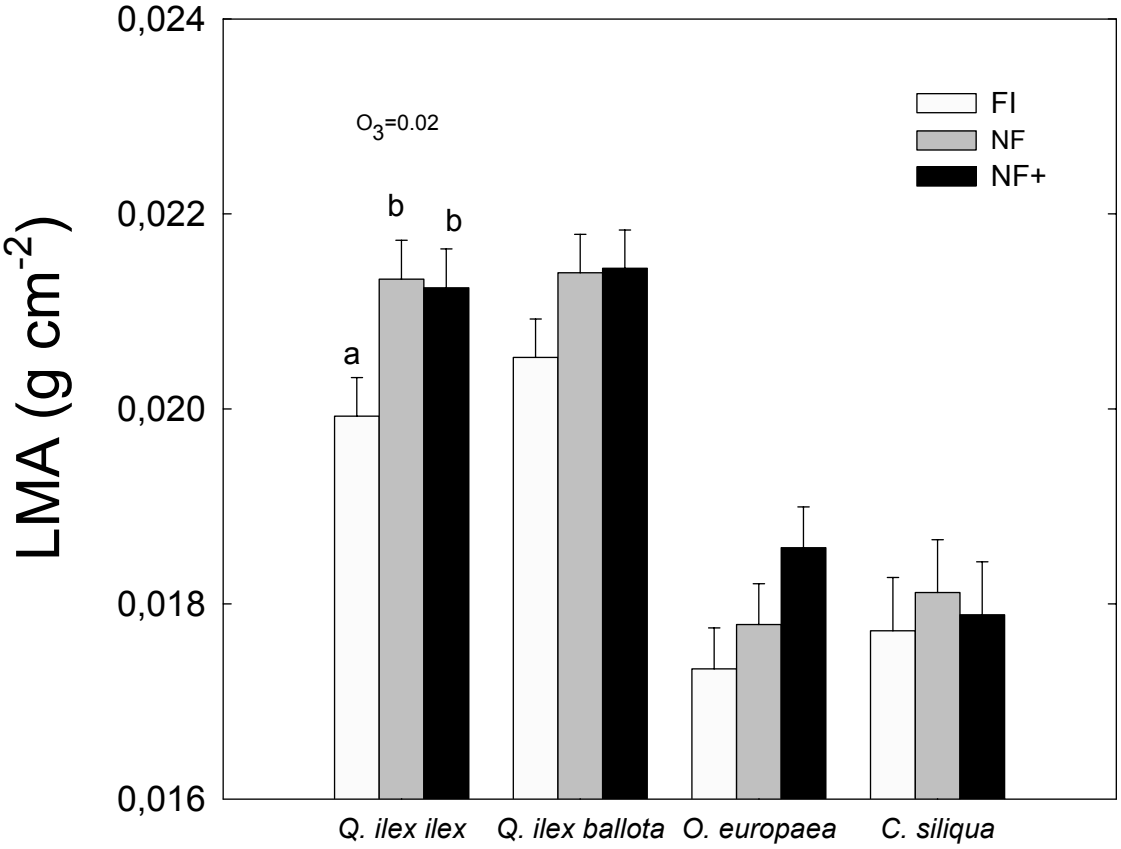

Figure 9. Average Leaf mass area (LMA) of *Q. ilex ilex*, *Q. ilex ballota*, *O. europaea*, *C. siliqua* throughout the experimental period. Statistically significant effects are depicted in the panels. FI (Charcoal-filtered air treatment), NF (Non-filtered air treatment), and NF+ (Non-filtered air + 40 ppb_v O_3 treatment). Error bars indicate the standard errors of the mean (n=3 chamber means of n=12 plant measurements per chamber). Different letters indicate significantly ($p<0.05$) different values.

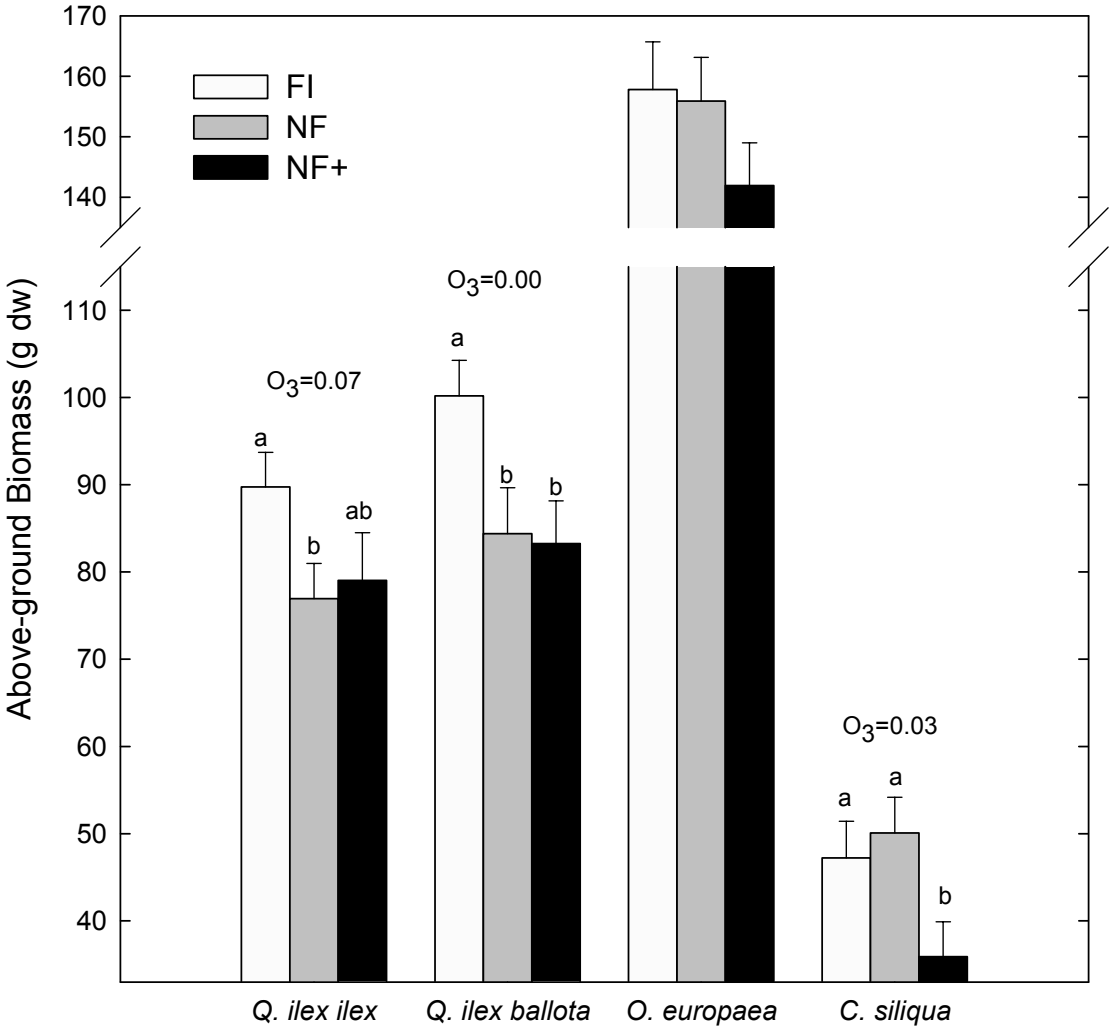

Figure 10. Final above ground biomass (g dw) of *Q. ilex ilex*, *Q. ilex ballota*, *O. europaea*, *C. siliqua* exposed to ozone in open-top chambers from summer 1998 to summer 2000. Statistically significant effects are depicted in the panels. FI, charcoal-filtered air; NF, non-filtered air; NF+, non-filtered air plus 40 ppb_v ozone. Error bars indicate the standard errors of the mean (n=3 chamber means of n=6-8). Different letters indicate significantly ($p<0.05$) different values.

Ozone fumigation reduced the total plant biomass of *Q. ilex ilex* (11.9%, p=0.07), *Q. ilex ballota* (16.9%, p<0.0001), and *C. siliqua* (23.9%, p=0.03) (Fig. 10). The NF plants of *Q. ilex ilex* and *Q. ilex ballota* also presented smaller final biomass than the FI plants (14.3% and 15.8% respectively) (Fig. 10). Exposure to ambient O_3 concentrations did not induce significant effects in *O.* europaea and *C. siliqua* plants (Fig. 10).

Potential chamber effects

PAR values inside the chambers were significantly (p=0.007) smaller than those of AA plots, c.a. 17%, due to the shading effect of the chamber frame and walls. Seasonal mean saturation vapour pressure deficit and mean, minimum, and maximum air temperatures were slightly higher in the OTC treatments than in the chamberless plots.

No significant differences were found for net in photosynthetic rates, stomatal conductances, foliar N concentrations, $\delta^{15}N$, or $\delta^{13}C$ between AA and NF treatments. The only significant difference was found for fluorescence variables. Fv/Fm values significantly decreased in AA treatment when compared to NF chambers, especially in winter 1999, when differences between them were the greatest for all species (data not shown). The decreased irradiance in the chambers decreases the possibility of photoinhibition and the higher temperature protected seedlings from winter stress (Oliveira and Peñuelas, 2000). Therefore, regardless of this effect on fluorescence, the OTCs apparently had little effect on the overall physiology of our studied species.

Discussion

A gradation in O_3 sensitivity was found for the species involved in this experiment. Biomass responses could be considered as an integration of O_3-induced physiological alterations and therefore they could be used as a valuable criterion to rank the sensitivity of these species to the pollutant. Both *Q. ilex* subspecies were the most sensitive species since detrimental effects on their biomass were detected already when exposed to ambient O_3 levels, which are below the present critical level of 10000 $ppb_v \cdot h$ for six months. However, *C. siliqua* was the species showing the greatest adverse effects when exposed to above-ambient ozone levels for two years (24 %), followed by the two holm oak subspecies (12-17%). Therefore these three species presented a greater sensitivity than another widespread Mediterranean tree species, Aleppo pine, which has been reported to present biomass depletions of 5-10% when exposed to similar or higher

AOT40 values (AOT40 values of 60000 $ppb_v \cdot h$ from April to September) (Karlsson et al., 2003).

However, no detrimental significant effects where found in *O. europea* after two years of exposure, contrasting with the results of Inclán et al (1999) that indicated biomass reductions of this species when exposed to a short-term exposure to above-ambient levels. These contrasting results could be explained by the different length of the exposure and also by the intraspecific sensitivity associated with different populations, such as Elvira et al. (2003) have recently reported for *Q. coccifera*. The differences in response between the species could also be related with the system induction of cellular oxidative defence and repair systems (Rao et al., 2000; Zheng et al., 2002).

The processes activated by ozone exposure are quite complex and no complete knowledge exists of the reaction events that take place from plant exposure until plant injury or damage is detected (Heath and Taylor, 1997). Ozone induces an acceleration of foliar senescence in many plants (Pell et al, 1997; Miller et al., 1999). Leaf senescence is the sequence of degradation processes leading to the remobilization of nutrients and eventual leaf death. Senescent processes are highly regulated, involving chlorophyll degradation, photosynthetic decline, protein degradation and lipid peroxidation (Pell et al., 1994; Elvira et al., 1998; Nali et al., 1998, Alonso et al., 2001; Noormets et al., 2001; Bielenberg et al., 2002).

These senescent-related processes could induce the changes in overall carbon assimilation indicated by the $\delta^{13}C$ changes reported here. However, this parameter is a complex resultant of both stomatal conductance and the activity of the photosynthetic system, both of which may be independently affected by O_3 (Heath and Taylor, 1997; Robinson et al., 1998; Zheng et al, 2002). Recent studies indicate that conductance may rise or fall in response to O_3 damage, dependent on genotype, O_3 concentration, and the sensitivity of stomatal guard cell (Robinson et al., 1998).

The activation of senescent-related processes by O_3 exposure would also modify nitrogen foliar content (Bielenberg et al., 2002). Several authors have reported reductions of N foliar content following plant exposure to ozone (Elvira et al, 1995; Wellburn et al., 1996; Kopper and Lindroth, 2003) as we found here. All major pathways of nitrogen loss (denitrification, ammonia volatilisation, and nitrate leaching) are thought to cause a $\square^{15}N$ enrichment of the remaining nitrogen (Durka et al., 1994; Peñuelas and Estiarte 1997; Peñuelas et al., 2000) as also found here. Several authors (Gebauer and Shulze 1991; Gebauer et al. 1994) explain the enrichment of foliar $\delta^{15}N$ by the reallocation of the N stored in the plant to the newly formed leaves, since older leaves are enriched relative to

younger leaves. However, the interpretation of these values is complex because of the existence of multiple processes and factors that influence them (Nadelhoffer and Fry 1994; Kolb and Evans, 2002).

The gradation in the ozone sensitivity of the studied species appears to be related to the activation of these senescence-related processes. The two holm oak species showed the greatest O_3 sensitivity according to their biomass response to ambient O_3 concentrations. A depletion in chlorophyll content was found in the first winter along with an increase in $\delta^{13}C$ during the first year of exposure to above-ambient ozone levels. These alterations in C metabolism induced a reduction of foliar N content and an increase of the $\delta^{15}N$ of those holm oaks exposed to the NF+ treatment.

The sensitivity of *C. siliqua* to ozone was also related with alterations in carbon metabolism. For instance, the plants of this species that were exposed to above-ambient ozone levels showed a depletion of leaf chlorophyll content in the first winter, while decreases in Fv/Fm ratio and A were found during the second winter. These findings were consistent with the increment in $\delta^{13}C$ that was found in this species in summer. These alterations of C metabolism would induce changes in the foliar N content of the older leaves since depletions in foliar N and increments in $\delta^{15}N$ were found in this species at the end of the second year of exposure. All these processes are related with senescence and would be supported by the observed increases in leaf spongy parenchyma.

The low O_3 sensitivity of *O. europea* derived from its biomass response could be related to the absence of long-term alterations in C metabolism, since no effects were found in its $\delta^{13}C$ values. However, transient adverse effects were observed in its chlorophyll content during the first winter and in the Fv/Fm ratio during two consecutive winters. This effect in chlorophyll content was very small and probably had a slight influence since this species presented an overall chlorophyll content that almost doubled that of the other studied species. Nitrogen metabolism was also affected since reductions in %N were found although no increases in $\delta^{15}N$ were detected. Increases in spongy parenchyma were also found although this species showed a greater (34%) thickness than the rest of species.

In summary, ozone exposure determined three types of responses: induction of senescence-related nutrient processes, alterations of overall carbon assimilation and morphological changes. Ozone exposure activated several senescent-related processes in all the species. These effects appeared to induce a retranslocation of foliar nitrogen; in fact ozone fumigation reduced by 8-15% the N concentrations of the well developed leaves of all the studied species. In most cases, the activation of senescence-related

processes was translated into reductions in integrated carbon assimilation and therefore reductions in plant biomass. The only exception to this pattern was *O. europaea*. No overall effects on carbon assimilation were detected in this species and its plant biomass remained unaffected when exposed to above-ambient ozone concentrations. The resistance of this species could be related to intrinsic characteristics such as greater foliar chlorophyll content (Fig. 7), thicker spongy parenchyma (Fig. 8) or higher photosynthetic rates (Fig. 3).

Interestingly, O_3 exposure induced modifications in the leaf morphology of these species with a trend to favour those traits related with leaf sclerophylly such as increased LMA, although this increase was statistically significant only significantly for *Q. ilex ilex* (Fig. 9), the most sensitive species in this study.

Therefore, in conclusion, the results of this experiment support the three tested hypotheses. Ozone exposure enhanced leaf senescence-related processes and induced a depletion of integrated carbon assimilation and aerial biomass accumulation. Some plant traits such as foliar chlorophyll levels and spongy parenchyma thickness appear to be associated to plant resistance to ozone.

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3.2. Contrasting interactive effects of ozone and water supply in two Mediterranean tree species

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Abstract

Interactions between ozone (O_3) exposure and water availability were studied in two Mediterranean tree species: *Quercus ilex* and *Ceratonia siliqua*. Plants were exposed to different O_3 concentrations in open top chambers (charcoal-filtered air (FI), non-filtered air (NF) and non-filtered air plus 40 ppb_v of O_3 ((7:00-16:00 solar time) (NF+)) during two years, and to different water regimes (IR, ample irrigation, and WS, reduced water dose to 50 %) through the last one of the those two years. AOT40 in the NF+ treatment ranged from 6,142 (for December 1998 - February 1999) to 30,147 ppb_v (for May 1999 - July 1999). In the NF treatment, the AOT40 ranged between 4 and 2,877 ppb_v for the same periods. AOT40 was always 0 in the FI treatment. WS plants presented lower stomatal conductances and net photosynthetic rates, and higher foliar N concentrations than IR plants in both species. The irrigation treatment did not change the response trends to ozone in *Q. ilex*, the most sensitive species, but it changed those of *C. siliqua*, the least sensitive species, since its fumigated WS plants did not decrease their net photosynthetic rates nor their biomass accumulation as it happened to its IR plants. These results show the complex species-specific interactive responses between ozone and water availability. We hypothesize that a more severe water stress is needed to affect the ozone responses in the most O_3-sensitive species, including reversing the O_3-induced stomatal aperture, and that higher O_3 concentrations would be needed to affect the WS plants of the least O_3-sensitive species.

Keywords: Biomass reduction, *Ceratonia siliqua,* Chlorophyll fluorescence, $\delta^{15}N$, gas exchange, N content, Ozone exposure, *Quercus ilex*, low water supply, species-specific ozone response, water-ozone interactions.

Introduction

The AOT40 (sum of 1-h mean ozone concentrations above a threshold of 40 ppb_v) of 10 ppm_v calculated during the 6-month growing season, that was established as critical level of ozone (O_3) damage for forest trees (Kärenlampi and Skärby, 1996) is exceeded every year over USA and Europe (Stockwell et al., 1997), exposing trees to a potential phytotoxicity.

In effect, it has been demonstrated that O_3 exposure can negatively impact physiological characteristics associated with carbon acquisition such as foliar pigmentation, stomatal function and photosynthesis (Miller et al., 1963; Anderson et al., 1997). However, phytotoxicity will depend on the flux within the leaf cells, which in turn will depend on stomatal opening, a process linked to various climatic factors. Thus, stomatal activity is considered the key element determining the sensitivity of a particular species to ozone (Emberson et al., 2000).

The Mediterranean climate favours the generation of high ozone concentrations but on the other hand, soil water shortage during summer causes partial stomatal closure for lengthy periods each day and limits plant growth. As a result, Mediterranean vegetation may avoid or diminish ozone uptake when concentrations are at their highest levels (Bussotti and Gerosa, 2002).

Stomatal activity is thus determinant of ozone phytotoxicity, but moderately elevated ozone concentrations have been shown to interfere with stomatal closure during drought (Pearson and Mansfield, 1993; Broadmeadow and Jackson, 2000). However, this interaction between ozone and water availability is very complex and can go in more than only one direction. Whereas many studies report stomata opening following exposure to air pollution, many others report stomata closure in response to air pollutants, or also absence of any response to air pollutants (see review by Darrall 1989). The pollution-induced changes in stomatal aperture (Mansfield and Majernik 1970, Biscoe et al., 1973, LeThiec et al. 1994) have been explained in terms of pollutant concentration, duration of exposure or external conditions during the measurements. However, the precise mechanisms underlying these stomatal responses have not been identified.

Maier-Maercker (1999) proposed that air pollutants, especially SO_2 and ozone, directly attack the walls of guard and subsidiary cells, so that the stomata lose their capacity to "sense" small changes in either atmospheric or cellular hydration. The author concluded ozone exacerbates the effects of water deficits. On the contrary, there are several studies showing that drought stress can protect trees against ozone-induced

visible injury and growth reductions. This has been shown when the ozone and drought stresses were applied simultaneously, presumably due to a reduction in the ozone uptake (Davidson et al., 1992; Temple et al., 1993; Van der Driessche and Langebartels, 1994).

Thus, ozone and drought stress can interact in several ways; ozone might disturb the stomatal regulation during drought potentially reducing the water use efficiency and favouring drought stress, and drought stress might instead protect against ozone injury by decreasing conductance and ozone uptake. We aimed to study the effects of these two stresses and whether there is an interaction, synergic or antagonic, between them. We tested the effects of different O_3 concentrations and irrigation regimes on the physiology and growth of two Mediterranean tree species: *Quercus ilex* and *Ceratonia siliqua.* We hypothesized that the interaction would be stronger in the less O_3 sensitive species, *C. siliqua* (see chapter 3.1), since in that species a moderate drought would be able to revert ozone effects on stomatal conductance, O_3 uptake and O_3 damage. In order to test this hypothesis, we measured the seasonal responses of their gas exchange rates, fluorescence, foliar $\delta^{15}N$ and $\delta^{13}C$, leaf morphology, foliar N concentration, and growth in response to ozone fumigation (ambient plus 40 ppb_v) and decreased water supply.

Material and Methods

Growth conditions. Ozone and water treatments

Plants of *Quercus ilex* ssp. *ilex* and *Ceratonia siliqua* were raised from seeds. In July 1998, one-year old homogeneous seedlings of each species were transplanted into 3 dm^3 containers filled with Universal substrate (peat and bark pine) with a 34% of organic material. Soil pH was 6.6, and 9 g of slow-release fertilizer (NPK 15:8:11; Osmocote plus) were added to each pot.

This experiment was conducted in an experimental field of slightly modified NCLAN-type open top chambers (OTCs) (Gimeno et al., 1999), located at the Ebro Delta (NE Spain, 40º 41.5' North, 0º 48' East), 10 m above sea level. Three O_3 treatments were established: charcoal filtered air (FI), non-filtered air (NF) with ambient O_3 levels, and non-filtered air supplemented with 40 nl l^{-1} O_3 from 7:00 to 17:00 GMT 5 days every week (NF+). Three OTC replicates were used for each O_3 treatment. Three open air plots (AA) were established to check for possible chamber effects (for more details see chapter 3.1, material and methods). An automatic system provided a continuous monitoring of O_3, sulphur dioxide and nitrogen oxides concentrations in the different treatments along with meteorological parameters such as wind speed and direction, air temperature and relative

humidity, and photosynthetic radiation active (PAR). Complete descriptions of the chambers and the operation of the system are provided in Alonso et al. (2001). We introduced eight individual plants of each one of the two studied species in each one of the three chambers of each ozone treatment and water treatment (a total of 8x2x3x2=96).

Plants were irrigated with a droplet system to ensure adequate and homogeneous water availability to plant material. Soil volumetric water content was measured using Time Domain Reflectometry (TRIME, IMKO, Micromodultechnick, Germany). Well-watered plants (irrigated plants, IR from now on) were irrigated twice a week. Plants under low water supply treatment (water-stressed, WS from now on) were irrigated once a week. The ozone experiment lasted two years (July 1998-June 2000), while the restrictions in the water availability started in plants after one year of ozone exposure (since fall 1999 to spring/summer 2000). Shoot water potential was determined using a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR, USA) in fall 1999, winter and spring 2000. On each sampling date, shoots of three plants (one shoot per plant, chamber and water treatment) of *Q. ilex* and *C. siliqua* were measured at predawn (02:30- 04:30h solar time in spring and 04:30-06:30h solar time in autumn and winter) and midday (11:00-13:00 h, solar time).

Chlorophyll fluorescence, gas exchange, and chlorophyll measurements

Chlorophyll fluorescence and gas exchange measurements were conducted during 2-4 consecutive days in spring 2000 (after two years of ozone treatment). Well developed leaves were measured under clear-sky conditions. The maximum photochemical efficiency of PSII (Fv/Fm), yield (ΔFv/F'm) and the apparent photosynthetic electron transport rate (ETR) were measured with a PAM-2000 fluorometer (Walz, Effeltrich, Germany). The maximum photochemical efficiency of PSII (Fv/Fm) was measured on leaves kept in the dark for 20 min (dark adaptation) prior to fluorescence measurements. We previously checked that this fluorescence coefficient reached constant values after this 20 minute dark period. Chlorophyll fluorescence was measured on three well developed leaves of three plants per chamber treatment and species (3x3x3x2= 54 leaves) from 07:00 to 11:00 h (solar time). ETR was estimated as ETR= ΔF/F'm x PPFD x 0.84 x 0.5, where ΔF/F'm (fluorescence yield or actual photochemical efficiency of PSII) was calculated according to Genty et al. (1989), 0.84 is the coefficient of absorption of the leaves, and 0.5 is the fraction of electron involved in the photoexcitation produced by one quanta, since two photosystems are involved.

Net photosynthetic rates (A) and stomatal conductances (g_s) were measured with a portable gas exchange system ADC LCA4, with a PLC4B chamber (ADC Inc., Hoddesdon, Hertfordshire, UK) inside the open-top growth chambers. Two well developed leaves of two different plants per treatment, chamber and species were measured from 07:00 to 11:00 h (solar time). A and g_s values were expressed on a projected leaf area basis. The leaf area was measured with a Li-Cor 3100 Area Meter (Li-Cor Inc., Lincoln, Nebraska, USA).

Chlorophyll content was determined non-destructively using a SPAD-502 meter (Minolta Co, LTD, Osaka, Japan). This instrument uses measurements of transmitted radiation in the red and near-infrared wavelengths to provide numerical values related to leaf chlorophyll content. Close linear correlations between SPAD values and extractable chlorophyll concentration have been reported for a wide range of species including plants exposed to elevated O_3 (Tenga et al., 1990). SPAD measurements were conducted in March 2000. We measured four plants per chamber, treatment and species (each plant measure as a mean of four leaves per plant).

Isotope and elemental analyses

To analyse C and N isotopic composition, 4-6 well developed leaves from different plants were collected and pooled for each chamber, treatment and species in summer 2000 before harvesting. The foliar $\delta^{15}N$ and $\delta^{13}C$ were measured in a SIRA Series II isotope ratio mass spectrometer (VG Isotech, Middlewich, UK) operated in direct inlet continuous flow mode after combustion of the samples in an elemental analyser (NA1500, Series 1, Carlo Erba Instrumentazione, Milan, Italy). The reference CO_2, calibrated against standard Pee Dee belemnite (PDB) was obtained from Oztech (Dallas, TX, USA). A system check of analysis was achieved with interspersed working standards of cellulose, atropine and urea (Sigma, St. Louis, MO, USA). The accuracy of the measurement was ± 0.1‰ for $\delta^{13}C$ and ± 0.2 ‰ for $\delta^{15}N$.

Carbon (C) and nitrogen (N) leaf concentrations were analysed with a Carlo Erba NA 1500 Analyser (Milan, Italy) by using a standard configuration for those determinations. The samples were previously dried at 70ºC to constant weight. They were weighed by using a Mettler UM3 microbalance in tin containers.

Leaf morphological and anatomical characteristics and final biomass harvest

Trees were visually inspected for foliar symptoms of chronic ozone injury seasonally throughout the two years of exposure. Foliar samplings were also collected to measure

leaf area and leaf mass area (n=3 per chamber and water regime). The leaf area was measured with a LICOR LI-3100 area meter (LICOR, Licoln, Nebraska). Thereafter the leaves were dried at 70ºC until constant weight.

At the end of the exposure experiment, after spring 2000 ecophysiological measurements, all aboveground biomass was harvested and placed in a forced-air oven at 70ºC. Final dry weight was determined when dry biomass reached constant weight.

Statistical analyses

The main design was a randomised complete block with the mentioned three ozone and two irrigation treatments, and there were three chamber replications per interaction treatment. Data for fluorescence (Fv/Fm, yield and ETR), gas exchange measurements (A, g_s), chlorophyll concentrations, $\delta^{13}C$, N concentrations, $\delta^{15}N$, morphological parameters (leaf area, LMA) and above-ground biomass were analysed as dependent variables using two way factorial ANOVAs with ozone and water regime as independent variables. One-way ANOVAs for ozone effects in each water regime were also conducted and always considering as replicate only the mean value of the dependent variable in each chamber (n=3). Analyses were conducted for each individual species. All mentioned analyses were performed with the software package Statistica 6.0 (StatSoft Inc., Tulsa, USA).

Results

Ozone exposure and meteorological data

The AOT40 ranged from 6,142 (for the three months period between December 1998 and February 1999) to 30,147 ppb_v (for the three months between May 1999 and July 1999) in the NF+ treatment (see Fig. 1). Therefore, the suggested daylight AOT40 critical level of 10 $ppm_v \cdot h$ in six months (Kärenlampi and Skärby, 1996) was exceeded all years in NF+. In the NF treatment, the AOT40 ranged between 4 ppb_v and 2,877 respectively for the same periods. AOT40 was always 0 in the FI treatment. Typical seasonal coastal Mediterranean trends were observed in temperature, radiation and ozone concentrations (Fig. 1), with soft winters and warm and dry summers. Minimum temperatures occurred from January to March, between 5-6 ºC, and the maximum temperatures occurred in summer, between 28 and 33ºC as monthly means (Fig. 1). Humidity values were very high throughout all the year (between 70 and 90 % as monthly mean values) (data not shown).

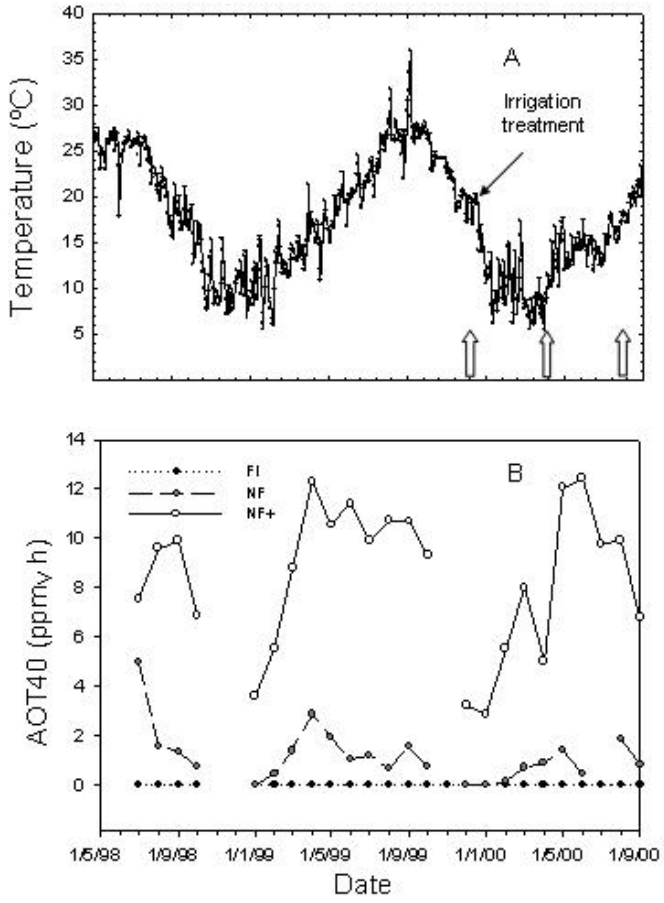

Figure 1. A) Field site daily mean Temperature (ºC), and B) monthly AOT40 during the experimental period, from July 1998 to September 2000, in the different O_3 treatments. FI, charcoal-filtered air; NF, non-filtered air; NF+, non-filtered air plus 40 ppb_v ozone. The white arrows show the sampling dates after irrigation treatment started.

Effects on water potential, Fv/Fm, stomatal conductance and net photosynthesis

Measurements showed that the "drought" treatment did not affect water potential, stomatal conductance, or net photosynthetic rates neither in fall nor in winter (data not shown) as expected from the low evaporative demands of these seasons that precluded any clear symptom of drought stress. However, in spring 2000, a reduction in the pre-dawn and midday water potential in response to the WS treatment was detected in the two species (Fig 2). The WS treatment reduced the water potential of the *Q. ilex* plants ca. 0.7 MPa both at predawn and midday (Fig. 2) and those of *C. siliqua* ca. 0.2 MPa at predawn and ca. 0.5 MPa at midday (Fig. 2). There were no significant differences in

shoot water potentials among the ozone treatments. There was not either any significant interaction effect of irrigation and ozone treatments in any of the two species.

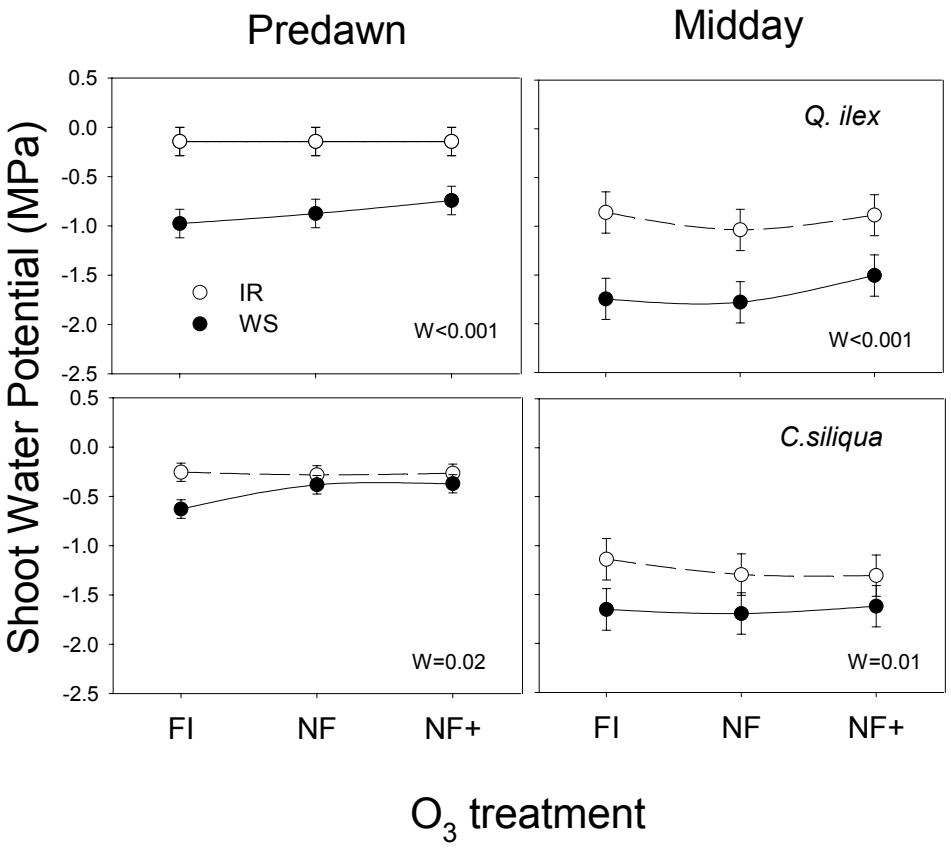

Figure 2. Shoot water potential (MPa) of *Q. ilex* and *C. siliqua* at predawn and midday points in spring 2000. Error bars indicate the standard error of the mean (n=3 plant measurements, one per chamber). Statistically significant effects are depicted in the panels. FI, charcoal-filtered air; NF, non-filtered air; NF+, non-filtered air plus 40 ppb_v ozone. IR, well-watered and WS, reduced irrigation (50 % of IR).

Table1. Mean ± standard error of the mean values of Fv/Fm, yield, ETR, $\delta^{13}C$, LMA, leaf area, SPAD, foliar N concentration and $\delta^{15}N$ of *Q. ilex* and *C. siliqua* leaves in March 2000 for each one of the ozone and irrigation treatments. For fluorescence variables (Fv/Fm, yield and ETR) standard error corresponds to n=3 chamber means of n=6-9 plant measurements. For $\delta^{13}C$, $\delta^{15}N$ and N concentration standard error corresponds to n=3 chamber measurements of n=6-9 pooled leaves. For LMA and leaf area, standard error corresponds to n=3 chamber means of n=6-9 leaves. For SPAD, standard error corresponds to n=3 chamber means of n=4 plants (mean of 4 leaves for plant). Statistically significant effects are depicted in the last column.

	Filtered		*Non-Filtered*		*Non-Filtered + 40 ppbv*		
Quercus ilex	IR	WS	IR	WS	IR	WS	Significant effects
Fv/Fm	0.802±0.005	0.797±0.005	0.799±0.005	0.795±0.005	0.788±0.005	0.797±0.005	_
Yield	0.60±0.05	0.48±0.05	0.60±0.05	0.56±0.05	0.56±0.05	0.53±0.05	_
ETR (μmolelectron m^{-2} s^{-1})	48.09±8.38	41.02±8.38	46.04±8.38	48.47±8.38	35.38±8.38	50.16±8.38	_
$\delta^{13}C$ (‰)	-26.47±0.36	-26.52±0.36	-26.48±0.44	-25.78±0.36	-26.41±0.41	-26.57±0.36	_
LMA (g cm^{-2})	0.021±0.001	0.025±0.001	0.021±0.001	0.022±0.001	0.022±0.002	0.020±0.001	_
Leaf area (cm^{-2})	6.59±0.54	5.68±0.54	4.64±0.54	6.55±0.54	4.61±0.54	5.44±0.54	O_3=0.09, int=0.02
SPAD (relative units)	53.7±1.4	56.7±1.4	55.8±1.4	51.8±1.4	52.3±1.4	53.4±1.4	O_3=0.02
N concentration	1.78±0.08	1.94±0.08	1.73±0.08	1.79±0.08	1.47±0.08	1.78±0.08	O_3=0.04, W=0.02
$\delta^{15}N$ (‰)	-1.26±0.3	-1.07±0.3	-0.58±0.3	0.003±0.3	-0.11±0.3	-0.34±0.3	O_3=0.03

	Filtered		*Non-Filtered*		*Non-Filtered + 40 ppbv*		
Ceratonia siliqua	IR	WS	IR	WS	IR	WS	Significant effects
Fv/Fm	0.746±0.012	0.745±0.012	0.747±0.012	0.727±0.012	0.725±0.012	0.731±0.012	_
Yield	0.48±0.05	0.43±0.05	0.44±0.05	0.56±0.06	0.47±0.05	0.39±0.05	W=0.08
ETR (μmolelectron m^{-2} s^{-1})	35.69±7.03	42.84±7.03	39.11±7.03	37.77±7.03	31.31±7.03	47.37±7.03	_
$\delta^{13}C$ (‰)	-27.45±0.80	-23.93±0.80	-24.89±0.80	-25.77±0.80	-25.56±0.80	-24.20±0.80	W=0.07, int=0.05
LMA (g cm^{-2})	0.019±0.001	0.022±0.001	0.021±0.001	0.020±0.001	0.019±0.001	0.019±0.001	_
Leaf area (cm^{-2})	12.16±1.22	10.86±1.22	11.36±1.22	10.34±1.22	10.66±1.22	10.09±1.22	_
SPAD (relative units)	56.0±2.1	60.7±2.1	53.8±2.1	57.6±2.1	55.7±2.1	52.4±2.1	_
N concentration	1.39±0.3	3.01±0.3	1.51±0.4	1.97±0.4	1.39±0.3	3.19±0.3	W<0.001
$\delta^{15}N$ (‰)	-1.72±1.3	-2.83±1.3	-1.34±1.6	-2.08±1.3	-1.09±1.3	-0.17±1.3	O_3=0.10

The dark-adapted maximum photochemical efficiency of photosystem II (Fv/Fm) tended to decrease under higher ozone concentrations, especially in well-watered plants, but no statistically significant differences were found in any of the two species (Table 1). Neither significant interactions (irrigation*O_3) were detected. Fluorescence yield and ETR did not either show any significant difference among treatments except for a slight decrease of yield in response to reduced irrigation (Table 1).

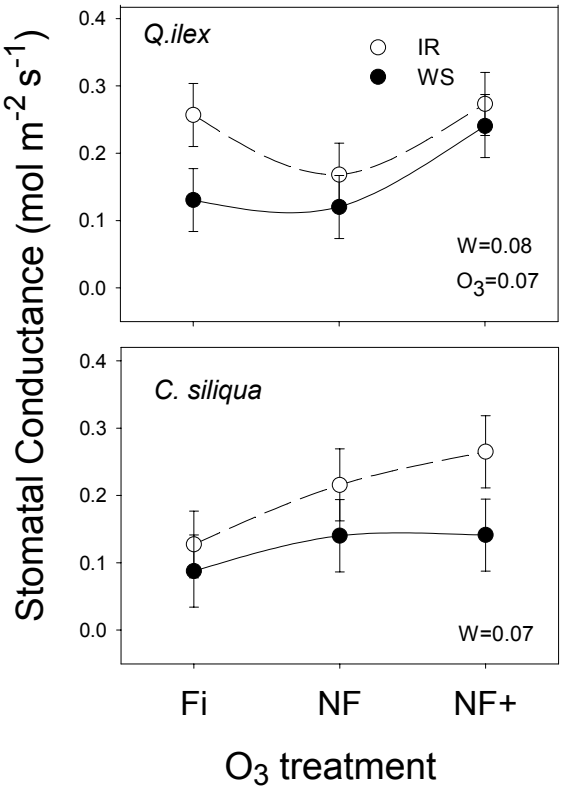

Figure 3. Stomatal conductances (mmol CO_2 m^{-2} s^{-1}) of *Q. ilex* and *C. siliqua* in spring 2000. Error bars indicate the standard error of the mean (n=3 chamber means of n=2 plant measurements). FI, charcoal-filtered air; NF, non-filtered air; NF+, non-filtered air plus 40 ppb_v ozone. IR, well-watered and WS, reduced irrigation (50 % of IR). Statistically significant effects are depicted in the panels. Different letters indicate statistically significant differences among ozone treatments (lower case for IR plants, upper case for WS plants).

Reduced water supply produced lower stomatal conductance in both species (Fig. 3). Stomatal conductance tended to be higher under NF+ conditions in both species, but this trend was only significant in *Q. ilex* (Fig. 3). This trend disappeared in WS *C. siliqua* plants.

Lower net photosynthetic rates were found in WS plants, being the decrease statistically significant in *C. siliqua* (Fig. 4). While *Q. ilex* plants did not change their photosynthetic response to ozone fumigation because of reduced irrigation dose, *C. siliqua* plants showed a significant reduction in net photosynthetic rates when well watered but not when half irrigated (Fig. 4).

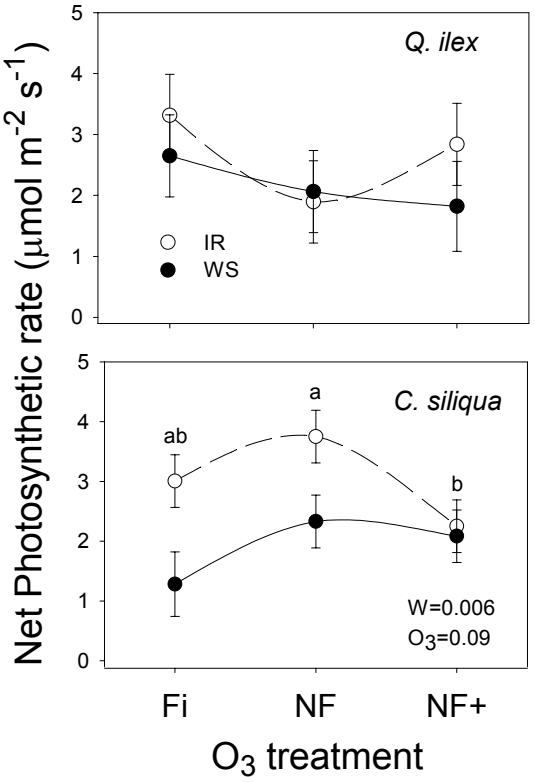

Figure 4. Net photosynthetic rates (μmol CO_2 m^{-2} s^{-1}) of *Q. ilex* and *C. siliqua* exposed to ozone in spring 2000. Error bars indicate the standard error of the mean (n=3 chambers means of n=2 plant measurements per chamber). FI, charcoal-filtered air; NF, non-filtered air; NF+, non-filtered air plus 40 ppb_v ozone. IR, well-watered and WS, reduced irrigation (50 % of IR). Statistically significant effects are depicted in the panels. Different letters indicate statistically significant differences among ozone treatments (lower case for IR plants, upper case for WS plants).

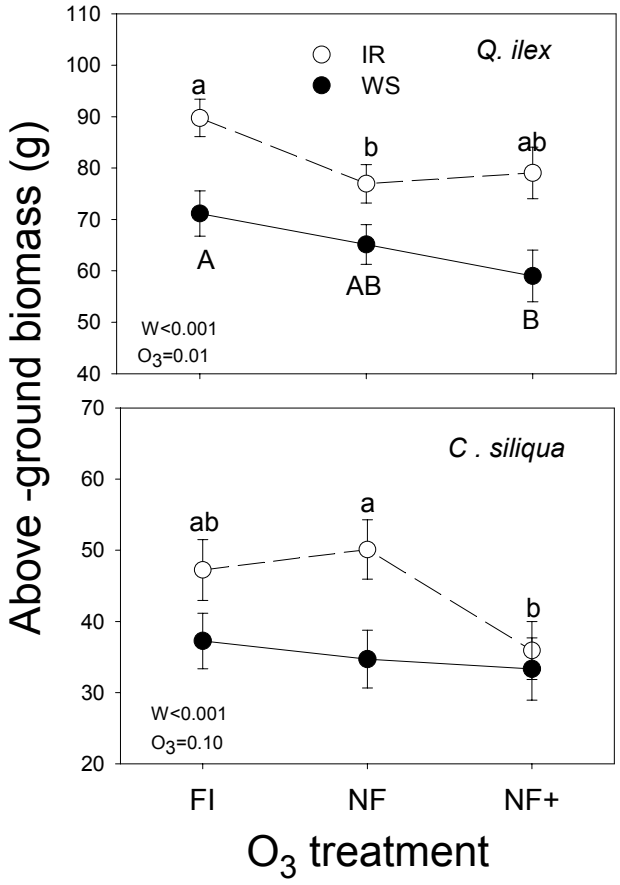

Figure 5. Final above ground biomass (g dw) of *Q. ilex* and *C. siliqua* exposed to both ozone in open-top chambers from summer 1998 to summer 2000 and to irrigation treatment from fall 1999. Error bars indicate the standard errors of the mean (n=3 chamber mean of 6-8 plants per chamber). FI, charcoal-filtered air; NF, non-filtered air; NF+, non-filtered air plus 40 ppb_v ozone. IR, well-watered and WS, reduced irrigation (50 % of IR). Statistically significant effects are depicted in the panels. Different letters indicate statistically significant differences among ozone treatments (lower case for IR plants, upper case for WS plants).

Effects on biomass, N, $\delta^{15}N$ and $\delta^{13}C$

The irrigation treatment significantly affected the increase in total biomass of both studied species (Fig. 5). The reduction in final above-ground biomass ranged from a not significant 7.2% (NF+ *C.siliqua* plants) to a significant 30.7% ($p<0.01$) (NF *C. siliqua* plants) (Fig.5). However, while in *Q. ilex* ozone fumigation similarly reduced final biomass in IR and WS plants (11 % and 17% respectively) (Fig. 5), in *C. siliqua* the ozone

reduction of biomass was statistically significant only in IR plants (23.9%). In WS plants there was a not significant reduction of 10.5% (Fig. 5).

After two years of exposure, elevated O_3 caused a significant decrease in the *Q. ilex* foliar concentrations of nitrogen (17%) but not in *C. siliqua* (Table 1). WS plants presented higher N foliar concentrations in both species but there was no interaction with O_3 fumigation. Both species increased their $\delta^{15}N$ values with increasing ozone exposures after two years of fumigation (Table 1). No significant effect of irrigation or of the interaction irrigation-ozone fumigation on $\delta^{15}N$ was found in any of the studied species (Table 1). No changes were either found in $\delta^{13}C$ either due to ozone or irrigation except for an irrigation effect on filtered *C. siliqua* plants (Table 1).

Leaf morphology, chlorophyll and visible injury

Increasing O_3 concentrations tended to decrease the leaf area in both species but only in a statistically significant way in *Q. ilex* (Table 1) (p= 0.09). This effect occurred in IR plants. No significant effects were found in WS plants. Therefore, there was a significant interaction effect between ozone and irrigation (p=0.02). Similar trends were found in *C. siliqua* (Table 1). There were no significant responses of LMA to increased ozone concentrations or irrigation (Table 1). Significant decreases in response to elevated O_3 concentrations were observed in chlorophyll SPAD values for *Q. ilex* (Table 1). After two years of O_3 fumigation no clear visual leaf injuries occurred in any of the studied species. Only in *Q. ilex* a slight mottle stipple bronzing appeared on a few old leaves that had been exposed for two years. The bronzing frequency seemed to be related to the irrigation treatment. It was found only in one NF+ chamber for the 12% of WS plants, while it was found in two of NF+ chambers for the 38 and 25 % of IR plants.

Discussion

The increase in stomatal conductance, the slight trend to decrease in net photosynthetic rates and the decrease in accumulated final biomass in response to high ozone concentrations occurred with similar intensities in IR and WS *Q. ilex* plants. However, in *C. siliqua* these effects of elevated O_3 concentrations were only found in IR plants. Therefore, there was a species-specific interaction between O_3 and irrigation treatments.

The differences of behaviour of these two studied species are not surprising. They already were found to present different sensitivity to high O_3 concentrations in a previous

study (see chapter 3.1). *Q. ilex* decreased their accumulated biomass already at ambient O_3 concentrations, whereas *C. siliqua* only presented a significant response under the fumigation (NF+) treatment (Fig. 5). In this study, whereas the negative effect of O_3 fumigation remained in *Q. ilex* WS plants it disappeared in *C. siliqua* WS plants. *Q. ilex* has thus been found again to be more sensitive to ozone exposure. The moderate water stress induced by our irrigation treatment did not change its ecophysiological responses to ozone, whereas the less sensitive *C. siliqua* changed its response to ozone when we applied our moderate water stress treatment.

Changes on the normal stomatal behaviour under high ozone concentrations may be a likely explanation for this variety of responses in plants submitted to stress interactions. More evident O_3 effect on biomass for well-watered plants compared with drought-stressed plants, as we found in *C. siliqua*, had already been observed in other species (Broadmeadow and Jackson, 2000; Temple et al., 1993; Karlsson et al., 1997). For example, several studies have reported a reduction in visible injury in plants grown in drier soil (Tingey et al., 1985; Fuhrer, 1995). The reduced effects of ozone when combined with drought stress could be explained by assuming that the process that is affected by ozone, for example an increase of stomatal conductance such the one found in this study or reported in several previous ones (Pearson and Mansfield 1994), is greatly suppressed by the drought stress, and consequently the ozone effect is not expressed to the same extent. In fact, the most widely accepted explanation to the protective response of drought on O_3 effects is assumed to be a decrease in ozone uptake due to a stomatal closure effect under low water availability (Cannon and Roberts, 1995; Panek and Goldstein, 2001). In other studies where ozone and drought stress were also applied simultaneously (Davidson et al., 1992; Temple et al., 1993), a reduced ozone sensitivity has been previously explained by reduced ozone uptake through stomata. The greater sensitivity of *C. siliqua* O_3 responses to the water reduction found here was further supported by the $\delta^{13}C$ data which showed an increment of water use efficiency for *C. siliqua* species whereas *Q. ilex* did not show any response.

There are also several studies reporting no interaction between water stress and O_3 (Pearson and Mansfield, 1994; Karlsson et al., 2002; Khan and Soja, 2003) as it occurred to *Q. ilex*. These studies also found that the combination of the two stresses caused additional reduction in growth. However, we did not found synergic reductions in carbon gain as modelled in other studies (Retzlaff et al., 2000) that predict that moderate amounts of atmospheric O_3 and drought could be more detrimental than either stress singly on the simple addition of both for another species such as white fir.

So, in summary, we have found species - specific response to the interaction between O_3 and water availability when comparing this two Mediterranean species. The most O_3 sensitive species, *Q. ilex* presented an additive response to both stressors. The less sensitive species *C. siliqua*, did not respond to O_3 increase when water available was low. We hypothesize that a more severe water stress is needed to affect the ozone responses in the most O_3-sensitive species, including reversing the O_3-induced stomatal aperture, and that higher O_3 concentrations would be needed to affect the WS plants of the least O_3-sensitive species. Further research to test these hypotheses and advance in our knowledge of ozone-drought interactions is warranted.

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Resultats i Conclusions

Les concentracions d'O_3 a Catalunya

L'estudi de diferents tipologies d'estació (urbana, de costa, de muntanya, i d'interior) mostra diferents pautes en les concentracions de l'ozó. L'evolució de les mitjanes anuals en l'ultima dècada mostra una disminució del 22% a la costa i, en canvi, un increment del 14% en l'estació muntanyenca, mentre que en la zona urbana (Barcelona) i a l'estació d'interior (Juneda) no hi van haver canvis. L'únic patró significatiu dels estudiats correspon al descens dels valors de fons a l'estació de costa, probablement degut a un decreixement en les concentracions de precursors a Europa, de forma coetània als descrits en altres països Europeus. El cicle anual d'ozó a l'estació de costa, amb màxims a la primavera, és típic de zones amb forta influència dels valors de fons d'ozó, mentre que als altres llocs, la presència de màxims a principis d'estiu indica el domini dels fenòmens de producció fotoquímica locals. També trobem diferències en el cicle diari segons el lloc. Aquests resultats mostren la existència de diferents patrons per localitats amb condicions ambientals diferents, de manera similar com s'ha trobat en altres punts d'Europa. El llindar de protecció per humans va ser sobrepassat un promig de 54 dies per any en l'última dècada a la costa i només 3 dies en l'estació urbana. Mentre que el llindar de protecció per a la vegetació va ser excedit en un terme mig 297 dies per any a la costa, i només ho va ser 14 dies a Barcelona. Segons aquestes dades i les últimes referències en la literatura, els valors observats a Catalunya en els darrers anys podrien estar tenint un impacte negatiu tant en la salut com en l'ambient.

La fitotoxicitat de l'O_3 a Catalunya

Estudis metodològics

Durant el 1995 es van dura terme una sèrie d'estudis amb diferents metodologies i variables associades a aquestes metodologies per determinar quin era el millor indicador per realitzar estudis sobre el seguiment de la fitotoxicitat O_3 a Catalunya. D'aquests estudis es va despendre que la millor variable per avaluar les concentracions d'O_3 era el percentatge d'àrea foliar danyada en la varietat més sensible (Bel-W3) de la planta de tabac. La capacitat de bioindicació mitjançant la taxa de dany va presentar una gran variabilitat en funció depenent de les condicions locals, i es va trobar una relació negativa entre la taxa de dany i les condicions que afavorien la resistència estomàtica.

Dels resultats obtinguts amb altres sistemes combinats com ara l'ús d'antiozonants (EDU) per excloure l'efecte de l'ozó es va despendre que les concentracions ambientals d'ozó a Catalunya comporten reduccions en la producció d'espècies sensibles com la mongetera (*Phaseolus vulgaris*). Els resultats d'aquest estudi també varen mostrar que la utilització de l'antiozonat EDU protegeix de l'efecte de l'ozó en aquesta espècie, però que la intensitat en la protecció difereix segons les condicions de creixement i les concentracions d'ozó.

Seguiment de la fitotoxicitat de l'O_3 en zones rurals de Catalunya

En el posterior seguiment de la fitotoxicitat de l'O_3 a Catalunya que es va dur a terme es van estudiar les variacions geogràfiques, estacionals i anuals en la taxa de dany i les seves relacions amb les condicions meteorològiques. Tant les concentracions d'ozó com els danys foliars van ser màxims al final de la primavera/principis d'estiu. Les concentracions mes altes del contaminant es van detectar a la costa, però es van detectar majors sensibilitats a la toxicitat de l'O_3 a les estacions de muntanya. Les concentracions d'O_3 presentaven bones correlacions amb el dany foliar. Malgrat això els danys observats presentaven una amplia variança en la resposta a escala local, de manera que només un 11 % era explicat per l'O_3. La resposta de les plantes de tabac a les concentracions d'O_3 i per tant la seva capacitat com a bioindicadors depenia de les condicions ambientals, principalment d'aquelles que afecten al comportament estomàtic com ara el VPD.

La integració i categorització dels valors de dany foliar obtinguts arreu del territori i dels anys de seguiment va mostrar una forta relació (99 % de la variança) amb les concentracions d'O_3 expressades com valors d'AOT20. Així, i a escala regional *Nicotiana tabacum* cv. Bel-W3 es va mostrar com un bon bioindicador de les concentracions d'O_3.

Tenint en compte els resultats obtinguts de la resposta d'aquesta espècie, proposem el valor d' AOT40 (concentració d'O_3 acumulada per sobre del llindar de 40 ppb_v en condicions diürnes) de 1.28 $ppm_v \cdot h$ acumulat per períodes quinzenals, com un dintell a partir del qual podrien produir-se danys en espècies sensibles a Catalunya.

Fitotoxicitat versus dosi efectiva

De l'estudi comparatiu que vàrem dur a terme entre l'ús de les dades de bioindicació amb tabac var. Bel-W3 i de la modelització del flux d'O_3 mitjançant el model WINDEP va resultar que els valors

Els valor de dany foliar estaven mes relacionats amb OAD (dosi absorbida d'O_3) que amb l'AOT40 en les tres estacions estudiades. El llindar de producció de dany en les espècies més sensibles en aquesta regió sota bones condicions de rec s'estableix en una OAD de 180000 μg m^{-2} acumulada en 15 dies. Els resultats d'aquest estudi ens mostraren a) l'aplicabilitat del flux real d'O_3 pel càlcul del risc a escala local; b) la millora en l'estimació del dany quan utilitzem la dosi adsorbida, en aquest cas utilitzant el WINDEP, en lloc de l'O_3 exposat, i finalment c) les possibilitats obertes per l'ús de les xarxes de bioindicació.

Seguiment de la fitotoxicitat de l'O_3 en zones urbanes: gradient europeu

En l'estudi de bioindicació dut a terme a diferents ciutats europees es va comprovar com a moltes d'elles les concentracions ambientals d'O_3 excedeixen els nivells crítics per a la protecció de la vegetació. La utilització del tabac com a bioindicador dels nivells de fitotoxicitat en zones urbanes arreu d'Europa va permetre la detecció d'un clar gradient creixent en la producció de danys de nord i nord-oest d'Europa cap a les regions centrals i del sud. Ells valors més elevats de danys observats en les plantes de tabac van ser observats a Lyon (França), Barcelona (Espanya) i Hohenheim/Ditzingen (Sud Alemanya), mentre que els valors més moderats de fitotoxicitat es van veure a Edinburgh (Escòcia), Sheffield (Anglaterra), Copenhagen (Suècia), i Düsseldorf (Nord Alemanya).

Respostes ecofisiològiques a l'O_3

Sensibilitat de les espècies mediterrànies

En l'estudi de les dues subespècies *Quercus ilex* ssp. *ilex* i *Quercus ilex* ssp. *ballota*, i de les espècies *Olea europaea* cv. *sylvestris* and *Ceratonia siliqua* exposades a diferents concentracions d'O_3 en condicions controlades (en cambres de cel obert) es varen observar diferents efectes de l'O_3 sobre l'ecofisiologia d'aquestes espècies. Durant el segon any d'exposició a l'O_3 la eficiència fotoquímica màxima, Fv/Fm, va decréixer en les plantes fumigades especialment en els períodes més estressants en els sistemes

mediterranis (hivern i estiu). Les mesures instantànies d'intercanvi gasós (A, g_s i E) no van presentar un efecte clar de la fumigació de l'O_3, ni tampoc el WUE calculat a partir d'aquestes variables (A/E). En canvi durant el primer any d'exposició sí que es va observar un increment en el $\delta^{13}C$ en dos de les espècies estudiades, un increment que es va mantenir en el segon any només en *C. siliqua*. Totes les espècies estudiades van presentar reduccions en el contingut de clorofil·la durant el primer any d'exposició, però aquest efecte no es va mantenir durant el segon any. Les elevades concentracions d'O_3 van produir canvis en les característiques anatòmiques i morfològiques de les fulles d'algunes espècies, especialment en el LMA (massa foliar per unitat d'àrea específica) i el gruix del mesòfil. Durant el segon any d'exposició es van detectar disminucions en el contingut de N i un increment en $\delta^{15}N$ en totes les espècies, segurament provocat per un canvi en el patró de retranslocació lligat a una acceleració en la senescència indicada pels anteriorment mencionats canvis bioquímics i anatòmics.

Els resultats mostren una gradació en la sensibilitat a l' O_3 segons l'espècie. Les dues subespècies de *Q. ilex* van presentar la major sensibilitat a l'O_3, ja que van presentar canvis en les concentracions de N, de $\delta^{15}N$, de clorofil·la, en l'àrea foliar, en LMA i la biomassa ja a concentracions ambientals d'O_3. Tot i així, *C. siliqua* es va mostrar com a l'espècie més sensible a les concentracions més elevades del tractament amb fumigació dons va presentar una disminució del 24%, seguida de les dues subespècies de *Q. ilex* (12-17%) i d'*O. europaea* (amb una reducció del ca. 10% estadísticament no significativa). La resistència a l'O_3 d'aquestes espècies sembla anar lligada a característiques bioquímiques i morfològiques com la concentració de clorofil·la o el gruix del mesòfil.

En aquestes espècies l'O_3 sembla actuar a tres nivells: inducció dels processos lligats a senescència, alteracions en l'assimilació de carboni i canvis morfològics. L'exposició a l'O_3 va activar processos relacionats amb la senescència en totes les espècies. L'O_3 sembla induir processos de retranslocació del N foliar, produint-se una disminució generalitzada en les concentracions de N foliar de fulles ben desenvolupades d'entre un 8-15% en totes les espècies estudiades. En alguns casos, la activació d'aquests processos d'acceleració de la senescència es tradueixen en reduccions en l'assimilació integrada de carboni i en la conseqüent reducció de la biomassa accumulada. L'única excepció a aquest patró va ser l'olivera borda (*Olea europaea cv. sylvestris) en* la que no es van detectar efectes en el l'assimilació de carboni. La resistència d'aquesta espècie podria estar associada a característiques intrínseques com un contingut de clorofil·la major o un mesòfil més gruixut. De fet, l'ozó va produir modificacions en la morfologia de

les espècies estudiades, accentuant trets característics de l'esclerofília, com ara una major LMA o una menor àrea foliar (en el cas de l'olivera o l'alzina litoral).

Interaccions de l'O_3 amb disponibilitat hídrica

Les baixes dosis de rec en les espècies *Q. ilex* i *C. siliqua* varen resultar en disminucions de la màxima eficiència fotoquímica (Fv/Fm), de la conductància estomàtica i de les taxes fotosintètiques, i en increments de les concentracions de N. La disminució en la disponibilitat d'aigua no va canviar el patró de resposta a l'O_3 de *Q. ilex*, però sí que ho va fer en *C. siliqua* que no va presentar disminucions en la seva taxa fotosintètica, i per tant en la seva biomassa en resposta a la fumigació amb O_3. Aquests resultats són una mostra de les complexes respostes d'interacció entre l'ozó i la disponibilitat hídrica, específiques per l'espècie. Els efectes interactius de l'estrès hídric moderat aplicat en aquest estudi es varen fer palesos en l'espècie menys sensible davant de l'ozó, però no en la més sensible, que va respondre additivament als dos estressos, l'O_3 i la restricció de rec.

Conclusions

Capítol 1. Concentracions d'O_3 a Catalunya

- L'evolució de les mitjanes anuals de les concentracions d'O_3 mostra diferents tendències en l'ultima dècada segons el lloc. Al territori hi ha hagut des de disminucions al punt de la costa fins a increments en el punt de muntanya, o absència de canvis a l'interior i a la zona urbana. Per tant, l'evolució de l'O_3 en els punts estudiats esta lligada a dinàmiques locals.

- El llindar de protecció per humans (110 $\mu g\ m^{-3}$, valor promig en 8 h) va ser sobrepassat per terme mig des de només 3 dies a l'estació urbana de Barcelona fins a 54 dies a l'estació de costa a Begur.

- El llindar de protecció per vegetals (65 $\mu g\ m^{-3}$, valor promig en 8 h) va ser excedit des de 14 dies a l'any a Barcelona fins a 297 a Begur.

- Les concentracions mesurades a Catalunya representen un problema potencial pels organismes vius més sensibles.

Capítol 2. Fitotoxicitat de l'O_3 a Catalunya

- L'EDU (etilè diurea) té un efecte protector o antiozonant davant diferents concentracions ambientals d'O_3.

- Les mongeteres presenten disminucions en la producció i creixement a concentracions ambientals d'O_3.

- En l'ús de les mongeteres com a biomonitors es destaca la variable producció de fruits com la que va presentar les millors relacions amb les concentracions ambientals d'O_3.

- Tant els efectes de l'O_3 com de l'EDU es van veure influïts per les condicions ambientals de creixement.

- Els productes antiozonant com l'EDU semblen una possible opció en el desenvolupament de noves metodologies en l'estudi dels efectes de l'O_3 en condicions naturals.

- Els nivells fitotòxics definits utilitzant les 3 varietats de tabac (Bel-B, Bel-C i Bel-W3) no van ser uns bons estimadors de les concentracions d'O_3.

- L'ús de l'àrea foliar danyada en la varietat Bel-W3 va ser, en canvi, millor indicador de les variacions d'O_3.

- La planta del tabac var. Bel-W3 es va mostrar com un molt bon bioindicador en l'estudi dels nivells de fitotoxicitat de l'O_3 a escala regional, després d'integrar la variabilitat temporal i espacial.

- Els seguiments dels nivells de fitotoxicitat duts a terme en aquesta tesi en zones rurals permeten establir un llindar de producció de dany per a espècies sensibles a Catalunya d'una AOT40 (concentracions O_3 per sobre de 40 ppb_v) de 1.28 $ppm_v \cdot h$ acumulades en 15 dies.

- A escala local la resposta de les plantes de tabac a les concentracions d'O_3 i per tant la seva capacitat com a bioindicadors depèn de les condicions ambientals, principalment d'aquelles lligades al comportament estomàtic com per exemple el dèficit de pressió de vapor i el vent.

- La modelització de la dosi absorbida d'O_3 millora l'estima del dany a escala local.

- El llindar de producció de dany en les espècies més sensibles en aquesta regió sense restricció en la humitat del sòl s'estableix en una dosi absorbida d'O_3 de 180000 $\mu g\ m^{-2}$ en 15 dies.

- De forma similar a l'observat a Catalunya, zones urbanes arreu d'Europa, sobre tot la meridional, s'assoleixen i excedeixen els nivells crítics de protecció a la vegetació.

- Amb la utilització del tabac com a bioindicador dels nivells de fitotoxicitat en zones urbanes arreu d'Europa, es va apreciar la detecció d'un clar gradient creixent en la producció de danys des del nord i nord-oest d'Europa cap a les regions centrals i del sud. Els nivells més alts es varen detectar a Barcelona i Lyon.

Capítol 3. Respostes ecofisiològiques a l'O_3

- Els estudis de les variables ecofisiològiques d'espècies mediterrànies sotmeses a diferents concentracions d'O_3 mostren una gradació en la sensibilitat segons l'espècie.

- A les espècies mediterrànies (*Quercus ilex*, *Ceratonia siliqua* i *Olea europaea*) s'ha vist que l'increment d'O_3 estudiades actua a tres nivells: indueix processos lligats a la senescència, altera l'assimilació de carboni i produeix canvis morfològics. Finalment, acaba reduint la producció de biomassa.

- Aquests efectes semblen induir processos de retranslocació del N foliar, produint-se en el nostre cas una disminució generalitzada en les concentracions de N foliar de fulles ben desenvolupades per totes les espècies estudiades d'entre un 8 i un 15%.

- De les tres espècies estudiades va ser *Quercus ilex* la que va presentar la major sensibilitat a l'O_3, ja que es van detectar canvis en les concentracions de N, de $\delta^{15}N$, de clorofil·la, de l'àrea foliar, de LMA i de la biomassa ja en resposta a les concentracions ambientals d'O_3.

- A majors concentracions d'O_3 (quan hi afegíem 40 ppb_v a les concentracions ambientals) es va produir una disminució en la biomassa d'entre 24 % a *C. siliqua* i un 12-17 % en dues les subespècies de *Q. Ilex*. *O. europaea* va presentar una reducció encara menor i estadísticament no significativa.

- La resistència a l'O_3 sembla anar lligada a algunes característiques com la concentració de clorofil·la o el gruix del mesòfil.

- L'O_3 produeix modificacions en la morfologia de les espècies estudiades, que tendeixen a accentuar trets característics de l'esclerofília, com increments en la massa per àrea (LMA) en l'alzina, o reduccions en l'àrea foliar en l'olivera i l'alzina.

- Les respostes a les concentracions elevades d'O_3 són modulades per altres factors ambientals com ara la disponibilitat d'aigua, de manera més o menys intensa depenent de l'espècie (i és clar, de les concentracions d'O_3 i de la severitat de la sequera).

- Així, la disminució en la disponibilitat d'aigua no va canviar el patró de resposta a l'O_3 de *Q. ilex*, però si que ho va fer en *C. siliqua*, que no va presentar disminucions en la seva taxa fotosintètica, i per tant en la seva producció de biomassa, en resposta a l'augment d'O_3.

- Els efectes interactius de l'estrès hídric moderat aplicat en aquest estudi es varen fer palesos en l'espècie menys sensible davant de l'ozó, però no en la més sensible, que va respondre additivament als dos estressos, l'O_3 i la restricció de rec.

Conclussió final

- Tots aquests resultats mostren que les altes concentracions d'O_3 a força punts de la regió mediterrània afecten la productivitat de les espècies vegetals amb major o menor grau depenen de la seva sensibilitat i de la interacció amb els factors ambientals que determinen la dosi real d'O_3 i l'estat fisiològic de la planta.

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Cortina de cel per dibuixar
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forjats de cada espurna,
guspires dels nostres ulls
dels sentiments, vivències
companys de la lluna
al cor restaran

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¿Qui sabrà mai aquest camí
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I és camí incert cada matí,
N'és cada vida
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