Growth response to ozone of annual species from Mediterranean pastures

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"Capsule": *The therophytes from dehesa acidic pastures of central of the Iberian peninsula present a great sensitivity to ozone, as derived from growth- and biomass-related variables.*

Abstract

Ozone (O_3) phytotoxicity has been reported on a wide range of plant species. However, scarce information has been provided regarding the sensitivity of semi-natural grassland species, especially those from dehesa Mediterranean grasslands, in spite of their great biological diversity and the high O_3 levels recorded in the region. A screening study was carried out in open-top chambers (OTCs) to assess the O_3-sensitivity of representative therophytes of these ecosystems based on the response of selected growth-related parameters. Three O_3 treatments and 3 OTCs per treatment were used. Legume species were very sensitive to O_3, because 78% of the tested species showed detrimental effects on their total biomass relative growth rate (RGR) following their exposure to O_3. The *Trifolium* genus was particularly sensitive showing O_3-induced adverse effects on most of the assessed parameters. Gramineae plants were less sensitive than Leguminosae species because detrimental effects on total biomass RGR were only observed in 14% of the assessed species. No relationship was found between relative growth rates when growing in clean air and O_3 susceptibility. The implications of these effects on the performance of dehesa acidic grasslands and on the definition of ozone critical levels for the protection of semi-natural vegetation are discussed.

Keywords: Relative growth rate; Dehesa; Screening; Therophytes; Critical levels

1. Introduction

Semi-natural vegetation is frequently exposed to elevated ozone (O_3) concentrations throughout Europe (EMEP, 2002) which can adversely affect the most sensitive species, inducing changes in the floristic composition of plant communities (Ashmore and Ainsworth, 1995; Barbo et al., 1998; Fuhrer et al., 1994). However, limited information has been provided regarding the sensitivity of these ecosystems to O_3 as has been recently reported in the last UN/ECE (United Nations Economic Commission for Europe) workshop held to define ozone critical levels for plant protection (Ashmore and Franzaring, 2003). As a result, O_3 critical levels for semi-natural vegetation are poorly defined, especially for the Mediterranean area where high O_3 concentrations have been recorded.

Few studies have been carried out aiming to evaluate the O_3 sensitivity of Mediterranean grassland species. For instance, Velissariou and Davison (1994), Madkour and Laurence (2002) and El-Khatib (2003) reported the great sensitivity of several local Greek and Egyptian cultivars of fodder crops, the Leguminosae species *Medicago sativa* and *Trifolium alexandrinum* being the most sensitive ones of those assessed. More recently, Bermejo et al. (2003) and Gimeno et al. (2004) have carried out experiments focused on the therophytic pastures from dehesa acidic grasslands of the central Iberian peninsula. These are valuable ecosystems presenting a great biodiversity (Pineda et al., 2002). In these pastures therophytes are more predominant than perennial species, representing up to 72% of the species (Azcarate et al., 2002). The screening experiment carried out by Bermejo et al. (2003) to assess the sensitivity of 22 annual species from these ecosystems based on the detection of foliar visible injury indicated that the legumes, especially the assessed *Trifolium* species, were more sensitive to O_3 than grasses. This differential sensitivity might affect the structure and function of these ecosystems and also could modify the nutritive quality of the pastures for the herbivores. Several experiments have also indicated a greater O_3 sensitivity of Leguminosae species when compared with Gramineae plants (Nussbaum et al., 1995; Warwick and Taylor, 1995).

Plant sensitivity to this pollutant is usually related to the induction of foliar visible injury. However, the use of foliage health as the main criterion to assess potential O_3 adverse effects may not be the best indicator of plant performance due to the weak association between visible injury and other important parameters, such as plant growth, which are more fundamental for plant survival and reproduction (Davison and Barnes, 1998; Reiling and Davison, 1992). Nevertheless, the immense number of wild species makes it essential that species can be ranked and the most sensitive taxa identified for the definition of O_3 critical levels (Davison and Barnes, 1998).

To overcome the above-mentioned gaps in knowledge a screening study involving 19 characteristic therophytes of the dehesa acidic grasslands was carried out to falsify the hypothesis that no differences in the growth responses of annual legumes and grasses to ozone exposure should be expected. The following specific objectives of the study were defined: (1) to assess their O_3-sensitivity based on plant growth-related parameters, (2) to rank the sensitivity of the selected species and (3) to test whether plant O_3 sensitivity can be linked to its classification within a given taxon.

2. Materials and methods

2.1. Plant material

The 19 species involved in this study are characteristic therophytes of dehesa acidic grasslands of central Iberia peninsula (Montoya et al., 1988; San Miguel 1994). Most of the seeds were collected from a typical dehesa located northwards from Madrid (Dehesa de Moncalvillo, Guadalix de la Sierra, Madrid; 40°40′N 03°46′W). The germoplasm bank of the Agriculture and Environment Council from the Extremadura Autonomous Community supplied the *Trifolium striatum*, *Trifolium subterraneum*, *Trifolium angustifolium* and *Ornithopus compressus* seeds, collected at different sites from central-western areas of the Iberian peninsula. The Spanish *T. subterraneum* cv. Zujar was used in the experiments.

All seeds of Leguminosae species were immersed during 24 h in a Germinator© solution (Agro-Orgánicos Mediterráneos S.L., Granada, Spain) to ensure a homogeneous germination. When seeds were swollen they were sown in a 50% neutral peat and 50% vermiculite substrate. Gramineae seeds did not experience any pretreatment and they were sown in the same substrate as Leguminosae seeds. The seedlings were transplanted to 2.5 l pots with a 50% peat, 30% vermiculite and 20% perlite substrate; $2\,\mathrm{kg\,m^{-3}}$ of a slow-release fertilizer (NPK:15/8/11) was applied. Plants were irrigated with a droplet system to ensure adequate and homogeneous water availability to plant material. The species involved in the study and their sowing dates are presented in Table 1.

2.2. Ozone treatments

The assay was performed in an open-top Chamber (OTC) experimental field located at Sant Jaume d'Enveija, Spain (40°41′N, 0°47′E). The same day the plants were transplanted into pots they were introduced in slightly modified NCLAN-type OTCs (see Gimeno et al., 1999). Three O_3 treatments were used: charcoal filtered air (CFA) presenting subphytotoxic O_3 levels, non-filtered air (NFA) with close to ambient O_3 levels and non-filtered air supplemented with 40 ppb O_3 from 07:00 to 17:00 (GMT) 5 days week^{-1} (NFA+). Realistic O_3 levels were recorded in the latter treatment, similar to those experienced in the field by the selected species (Palacios et al., 2002; Plaza et al., 1997). Four to six plants from each species were introduced in the chambers and three OTC replicates were used for each O_3 treatment. An automatic system was used to provide 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 active radiation (PAR). A complete description of the chambers and the operation of the system is provided in Alonso et al. (2001).

The O_3 exposure index AOT40 currently used by both the UN/ECE CLRTAP[1] and the European daughter O_3 Directive (2002/3/EC) was calculated for each

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[1] UN/ECE CLRTAP. United Nations/Economic Commission for Europe Convention on Long-Range Transboundary Air Pollution.

Table 1
Time-table and ozone exposure corresponding to the assessment of the ozone sensitivity of dehesa therophytic species

	Sowing date	Plant entrance in OTC	First harvest date (day/month)	Last harvest date (day/month)	Total Exposure length (days)	AOT40 (ppb h)	
						NFA	NFA+
Leguminosae (legumes)							
Anthyllis cornicina L.	01/08/00	07/09/00	22/09	22/11	76	821	15,189
Anthyllis lotoides L.	01/08/00	14/09/00	15/09	22/11	69	415	13,144
Biserrula pelecinus L.	01/08/00	22/09/00	22/09	28/11	67	313	12,236
Medicago minima (L.) Bartal	02/09/00	11/10/00	09/09	23/11	43	92	7222
Ornithopus compressus L.	02/09/00	11/10/00	09/10	27/11	47	313	12,099
Trifolium angustifolium L.	02/09/00	11/10/00	09/10	23/11	43	92	7222
Trifolium cherleri L.	01/08/00	08/09/00	16/09	9/11	62	783	15,493
Trifolium glomeratum L.	01/08/00	08/09/00	20/09	8/11	61	780	13,578
Trifolium striatum L.	01/08/00	14/09/00	15/09	7/11	54	412	11,479
Trifolium subterraneum L.	01/08/00	08/09/00	15/09	13/11	66	775	12,988
Gramineae (grasses)							
Aegilops geniculata Roth	01/08/00	07/09/00	15/09	10/11	64	818	14,183
Aegilops triuncialis L.	01/08/00	07/09/00	16/09	11/11	65	818	14,570
Avena sterilis L.	02/09/00	03/10/00	05/10	21/11	49	185	8503
Briza maxima L.	01/08/00	20/09/00	21/09	22/11	63	327	11,917
Bromus hordeaceus L.	01/08/00	07/09/00	15/09	16/11	69	818	13,865
Bromus sterilis L.	01/08/00	14/09/00	15/09	20/11	67	415	12,550
Cynosurus echinatus L.	01/08/00	14/09/00	15/09	21/11	68	415	12,791
Lolium rigidum Gaudin	02/09/00	11/10/00	09/10	23/11	43	92	7222
Vulpia myuros (L.) C.C.Gmelin	01/08/00	20/09/00	21/09	22/11	63	327	11,917

species and O_3 treatment as the sum of the differences between O_3 hourly concentrations in ppb and 40 ppb for each hour when the concentration exceeds 40 ppb and solar radiation is above 50 $\mathrm{W\,m^{-2}}$.

2.3. Growth-related parameters

Plant height, diameter, aerial and root biomass of plant material were determined when plants were exposed to the different O_3 treatments during 43–77 days, mostly depending on the timing of seed germination.

Plant height and diameter were used to estimate the canopy volume and surface of the plant species involved in the experiment. Two diameter estimations were carried out per plant, their mean value was used for subsequent statistical analyses. The aboveground biomass was harvested by excising the plants at the surface of the substrate. Leaves and stems of Leguminosae plants were separated and dried at 60 °C until constant weight and subsequently weighed to determine their foliage/stem dry weight ratio. A similar procedure was followed for Gramineae species. Similarly, roots were cautiously extracted, washed, dried at 60 °C and weighed afterwards. The shoot/root ratios were also calculated to evaluate potential shifts in photosynthate distribution. Prior to the entrance of plant material in the OTCs the mean of the initial weights of the root and shoot biomass of an extra-set of 10 plants per species was used to derive the relative growth rates (RGR, $\mathrm{g\,week^{-1}}$) of the experimental plants. The RGRs for aerial, root, and total biomass were calculated using the formula:

$$\mathrm{RGR} = \ln W_2 \ln W_1/t_2\ t_1$$

where W_2 and W_1 are the dry weights at the final harvest and at the harvest performed prior to the entrance of plant material in the chamber, respectively, and t_2 and t_1 are the number of days of both harvests (Hunt, 1990). Details of the dates when the plants were introduced in the chambers and the length of O_3 exposure in the different treatments can be found in Table 1.

2.4. Statistical analyses

An ANOVA analysis for each parameter and species was performed to evaluate O_3 effects on plant growth-related parameters. Also, a combined analysis involving all the assessed species was performed to assess whether plant sensitivity to O_3 exposure could be related to plant family; therefore a two-way ANOVA analyses were carried out considering ozone exposure and family as factors. When significant differences ($p < 0.05$) were detected, the differences between means were assessed using the least significant difference (LSD) test. Normal probability plots and scatter plots of residuals were used to test data normality. Levenne tests (Milliken and Johnson, 1994) were applied to check the variance homocedasticity. When necessary logarithmic or square-root transformations were used. The relationship between growth parameters and the different O_3 exposure indices was analysed using Pearson's correlation index and the level of significance was determined using the student *t*-test. All statistical analyses were carried out using Statistica 97 software.

3. Results and discussion

Of the 19 species assessed in this study, only *Anthilllis cornicina* could be considered as resistant to O_3 exposure since it was not significantly affected by any of the assessed parameters on this species. The growth or the foliar morphology of the remaining species was adversely affected by those concentrations of the pollutant which are actually recorded in the dehesas of the central Iberian peninsula (Palacios et al., 2002; Plaza et al., 1997). However, the nature of the effect differed depending on species and family.

In general, the Leguminosae species were more sensitive to O_3 than Gramineae species regarding the response of the assessed growth-related parameters as shown in Tables 2–4. Ozone exposure induced significant detrimental effects on the aerial and root RGRs of 70%

Table 2
Plant growth-related parameters of the legume grown in the different O_3 treatments

	Foliage dw (g)	Stem dw (g)	Root RGR	Aerial RGR	Total RGR	Foliage/stem	Shoot/root
Anthyllis cornicina L.							
CFA	1.91	0.26	0.26	0.40	0.38	8.88	10.27
NFA	1.82	0.26	0.28	0.39	0.37	6.93	7.17
NFA+	2.44	0.50	0.25	0.43	0.40	5.50	12.02
Anthyllis lotoides L.							
CFA	9.07	2.32	–	0.54	–	4.25	–
NFA	9.52	2.25	–	0.55	–	4.43	–
NFA+	8.42	2.01	–	0.54	–	5.07	–
Biserrula pelecinus L.							
CFA	9.15^{a}	4.53	0.34	0.36^{b}	0.36^{a}	2.07	4.76
NFA	7.00^{ab}	3.76	0.33	0.34^{ab}	0.34^{ab}	1.86	3.20
NFA+	5.16^{b}	2.39	0.31	0.29^{a}	0.29^{b}	2.13	2.66
Medicago minima (L.) Bartal							
CFA	0.95^{a}	0.47^{a}	0.41^{a}	0.46^{b}	0.44^{a}	2.07^{a}	2.27
NFA	0.56^{b}	0.30^{b}	0.39^{a}	0.44^{b}	0.42^{a}	1.87^{ab}	2.41
NFA+	0.45^{b}	0.27^{b}	0.32^{b}	0.37^{a}	0.35^{b}	1.64^{b}	2.33
Ornithopus compressus L.							
CFA	3.07	0.52	0.51	0.75^{a}	0.65	5.99	3.06^{a}
NFA	2.52	0.39	0.50	0.72	0.61	6.58	2.72^{a}
NFAA+	2.44	0.38	0.52	0.72	0.62	6.61	2.18^{b}
Trifolium angustifolium L.							
CFA	1.43^{a}	0.17^{a}	0.54^{a}	0.59^{b}	0.56^{a}	8.98	1.86
NFA	1.32^{a}	0.15^{a}	0.53^{a}	0.57^{b}	0.56^{a}	9.23	1.73
NFA+	0.88^{b}	0.10^{b}	0.46^{b}	0.51^{a}	0.49^{b}	10.57	1.89
Trifolium cherleri L.							
CFA	6.16^{a}	0.87	0.36^{a}	0.51^{b}	0.48^{a}	5.88	7.66^{a}
NFA	3.19^{ab}	1.03	0.36^{a}	0.46^{ab}	0.43^{ab}	3.22	4.93^{b}
NFA+	2.30^{b}	0.70	0.31^{b}	0.42^{a}	0.39^{b}	3.66	5.35^{b}
Trifolium glomeratum L.							
CFA	10.66^{a}	1.99	0.50^{a}	0.66^{b}	0.63^{a}	5.63	6.72^{a}
NFA	10.68^{a}	2.01	0.49^{a}	0.65^{ab}	0.62^{ab}	5.56	6.66^{a}
NFA+	8.65^{b}	1.35	0.41^{b}	0.62^{a}	0.57^{b}	7.53	9.97^{b}
Trifolium striatum L.							
CFA	6.87^{a}	1.85^{a}	0.65^{a}	0.71^{b}	0.69^{a}	3.75	3.42^{a}
NFA	4.92^{b}	1.39^{ab}	0.60^{b}	0.67^{b}	0.66^{a}	3.71	4.11^{ab}
NFA+	3.36^{b}	0.95^{b}	0.49^{c}	0.57^{a}	0.55^{b}	3.63	4.55^{b}
Trifolium subterraneum L.							
CFA	13.79	2.70^{a}	0.27^{a}	0.37^{b}	0.35^{a}	5.20	6.68^{a}
NFA	13.17	1.83^{b}	0.23^{b}	0.39^{b}	0.36^{a}	7.53	9.28^{ab}
NFA+	9.98	1.34^{b}	0.17^{c}	0.34^{a}	0.29^{b}	7.58	10.75^{b}

Different letters indicate significant effects between O_3 treatments ($p < 0.05$). CFA, charcoal filtered air; NFA, non-filtered air, NFA+, non-filtered air supplemented with 40 ppb of O_3.

Table 3
Plant growth-related parameters of the grasses grown in the different O_3 treatments

	Root RGR	Aerial RGR	Total RGR	Shoot/root
Aegilops geniculata Roth				
CFA	0.35^{a}	0.35	0.36	1.53
NFA	0.34^{a}	0.34	0.33	1.19
NFA+	0.27^{b}	0.31	0.29	1.72
Aegilops triuncialis L.				
CFA	0.37	0.41	0.38	1.59
NFA	0.39	0.42	0.40	1.66
NFA+	0.37	0.42	0.39	1.90
Avena sterilis L.				
CFA	0.61	0.57	0.59	0.25
NFA	0.61	0.57	0.59	0.22
NFA+	0.59	0.55	0.58	0.41
Briza maxima L.				
CFA	0.61	0.59	0.60	2.49
NFA	0.66	0.60	0.63	1.85
NFA+	0.61	0.57	0.59	2.71
Bromus hordeaceus L.				
CFA	0.60	0.48	0.53	0.63
NFA	0.58	0.49	0.54	0.64
NFA+	0.52	0.48	0.50	1.06
Bromus sterilis L.				
CFA	–	0.53	–	–
NFA	–	0.52	–	–
NFA+	–	0.51	–	–
Lolium rigidum Gaudin				
CFA	0.83	0.72	0.75	1.56
NFA	0.83	0.69	0.73	1.33
NFA+	0.81	0.68	0.72	1.51
Cynosurus echinatus L.				
CFA	0.62^{a}	0.73	0.69^{a}	2.98^{a}
NFA	0.70^{b}	0.74	0.73^{b}	1.12^{b}
NFA+	0.65^{ab}	0.74	0.70^{a}	2.26^{a}
Vulpia myuros (L.) C. C. Gmelin				
CFA	–	0.55	–	–
NFA	–	0.52	–	–
NFA+	–	0.55	–	–

Different letters indicate significant effects between O_3 treatments ($p < 0.05$). CFA, charcoal filtered air; NFA, non-filtered air, NFA+, non-filtered air supplemented with 40 ppb of O_3.

and 67%, respectively, of the assessed Leguminosae species and caused a reduction in the total RGR of 78% of the species of this family. The root RGR was only affected in 29% of the Gramineae species involved in the experiment, while no detrimental effects were found on their aerial biomass RGR and a single species of this family showed a significant effect on its total RGR.

A combined analysis using a two-way ANOVA was carried out to assess whether total RGR was affected by ozone exposure and/or plant family (see Fig. 1). Ozone significantly ($p < 0.05$) affected RGRs, inducing lower rates in the NFA+ treatment when compared to CFA or NFA plants. Similarly, grass species significantly ($p < 0.0001$) presented greater RGRs than legumes while a trend ($p = 0.07$) towards a family-O_3 interaction was found. Grasses and legumes presented similar RGRs in the CFA treatment while the total RGR of grasses was greater than for legumes when both taxa were exposed to the NFA or NFA+ treatments. This pattern of sensitivity for both plant families based on growth responses to O_3 is in agreement with that found when foliar visible injury was considered (Bermejo et al., 2003), although in this experiment a significant O_3–family interaction was detected. These results match with other studies reporting a greater sensitivity of the Leguminosae family when compared with Gramineae species (Bungener et al., 1999; Warwick and Taylor, 1995). Therefore a separate analysis of the results was carried out for both families.

3.1. Ozone effects on species of the Leguminosae family

Ambient O_3 levels (NF treatment) corresponding to AOT40 values ranging 92–775 ppb h (Table 1) caused significant reductions in the aerial biomass (foliage or stem biomass) of *T. striatum*, *T. subterraneum* and *Medicago minima* (Table 2). Ozone affected both the foliage and the stem of *Medicago,* that showed reductions of 41 and 36%, respectively, when compared with the control treatment. A similar pattern was found in *T. striatum*, however, ambient O_3 levels only significantly affected its foliage dry weight (by 28%), while shoot biomass was the most affected parameter in *T. subterraneum* (32%). Ambient O_3 exposure also induced adverse effects on root RGR of *T. striatum* and *T. subterraneum*, with reductions ranging 8–15% when compared with control plants.

Above ambient O_3 levels (NF+ treatment) corresponding to AOT40 values ranging 7222–15,493 ppb h (Table 1) induced detrimental effects in the range of 6–14% in the aerial RGR of *T. angustifolium*, *Trifolium glomeratum* and *T. subterraneum* and of ca. 20% in *Biserrula pelecinus*, *M. minima*, *Trifolium cherleri* and *T. striatum*. These O_3 levels determined reductions (147–37%) in the root RGR of these species except for *Biserrula*, where no effects were detected. The total RGR was adversely affected in these seven species following their exposure to the NFA+ treatment, reductions in the 10–21% range were found when compared with control plants, *Medicago* and *T. striatum* being the most affected species. The intensity of the O_3-induced adverse effects are in the range of those reported by Warwick and Taylor (1995) regarding the sensitivity of calcareous herbaceous species, where the most sensitive species showed 20% depletions in its root and shoot RGRs. However, in our study the greatest adverse effect was found in the root RGR of *T. subterraneum* (37%). Ozone exposure did not determine any effect on the root, aerial or total RGR of *Anthyllis cornicina*, *Anthyllis lotoides* and *O. compressus*.

Table 4
Ozone-induced effects on plant canopy-related parameters of the dehesa therophytes involved in the experiment

Exposure	*Leguminosae*				*Gramineae*			
	Diameter (cm)	Height (cm)	Surface (m^2)	Vol. (dm^3)	Diameter (cm)	Height (cm)	Surface (m^2)	Vol. (dm^3)
	Anthyllis cornicina L.				*Aegilops geniculata* Roth			
CFA	26.66	7.16	0.072	5.32	46.53	14.18^{a}	0.22	31
NFA	26.08	7.50	0.069	5.40	46.46	15.92^{a}	0.22	35
NFA+	23.91	7.17	0.058	4.13	47.78	12.00^{b}	0.23	29
	Anthyllis lotoides L.				*Aegilops triuncialis*			
CFA	48.88	8.44^{a}	0.24	21.26	57.03	14.43	0.33	48
NFA	49.5	8.77^{a}	0.25	22.77	59.08	14.16	0.37	51
NFA+	45.33	7.33^{b}	0.23	16.49	57.96	14.07	0.35	48
	Biserrula pelecinus L.				*Avena sterilis* L.			
CFA	62.3^{a}	6.6	0.39^{a}	25.72^{a}	103.29^{a}	18.91	1.08	199^{a}
NFA	59.2^{a}	–	0.37^{a}	–	100.54^{a}	17.5	1.02	174^{a}
NFA+	40.4^{b}	6.2	0.17^{b}	10.63^{b}	87.16^{b}	16.92	0.77	128^{b}
	Medicago minima (L.) Bartal				*Briza maxima* L.			
CFA	19.04^{a}	4.41^{a}	0.037^{a}	1.66^{a}	55.13	19.81	0.31	61^{a}
NFA	17.66^{ab}	3.91^{ab}	0.032	1.31^{ab}	56.83	20.08	0.33	68^{a}
NFA+	16.25^{b}	3.33^{b}	0.027^{b}	0.91^{b}	49.29	18.00	0.25	45^{b}
	Ornithopus compressus L.				*Bromus hordeaceus* L.			
CFA	38.44^{a}	8.00	0.15^{a}	12.07	67.95	34.08^{a}	0.46	161
NFA	32.92^{b}	8.28	0.11^{b}	9.17	66.87	28.25^{b}	0.46	132
NFA+	35.00^{b}	8.41	0.12^{b}	11.04	64.16	28.00^{b}	0.42	119
	Trifolium angustifolium L.				*Bromus sterilis* L.			
CFA	28.09^{b}	12.18	0.079^{a}	9.71^{a}	61.83	24.44^{a}	0.38	94
NFA	25.62^{b}	11.83	0.066^{b}	7.94^{a}	63.36	22.42^{b}	0.40	89
NFA+	22.20^{a}	10.58	0.049^{c}	5.52b	64.33	23.00^{b}	0.42	96
	Trifolium cherleri L.				*Lolium rigidum* Gaudin			
CFA	20.46	8.33	0.046	3.44	65.66	19.88	0.44	87
NFA	19.53	6.50	0.040	2.69	68.05	20.66	0.47	98
NFA+	18.54	7.33	0.036	2.37	71.25	20.13	0.52	106
	Trifolium glomeratum L.				*Cynosurus echinatus* L.			
CFA	30.60	7.38^{a}	0.13	9.76	49.5^{a}	25.55^{a}	0.24	62^{a}
NFA	34.71	6.07^{b}	0.12	7.57	50.38^{a}	22.66^{b}	0.27	61^{ab}
NFA+	28.80	5.69^{b}	0.11	7.08	46.56^{b}	23.44^{b}	0.22	51^{b}
	Trifolium striatum L.				*Vulpia myuros* (L.) C. C. Gmelin			
CFA	30.10	12.90^{a}	0.090^{a}	11.72^{a}	60.58^{a}	27.5^{a}	0.37	102^{a}
NFA	26.30	11.10^{b}	0.069^{b}	7.80^{b}	54.77^{b}	22.72^{b}	0.30	71^{b}
NFA+	22.13	9.92^{b}	0.050^{c}	5.22^{c}	57.25	24.70^{ab}	0.33	81^{ab}
	Trifolium subterraneum L.							
CFA	38.12^{a}	14.00^{a}	0.150^{a}	22.10^{a}				
NFA	31.08^{b}	10.66^{b}	0.100^{a}	11.50^{b}				
NFA+	29.41^{b}	11.08^{b}	0.088^{b}	10.13^{b}				

Different letters indicate significant effects between O_3 treatments ($p < 0.05$). CFA, charcoal filtered air; NFA, non-filtered air, NFA+, non-filtered air supplemented with 40 ppb of O_3.

Ozone exposure determined shifts in the shoot/root dry weight biomass ratio of 55% of the legumes involved in the experiment. Ambient (AOT40 = 783 ppb h) and above ambient O_3 levels (AOT40 = 15,493 ppb h) induced average reductions of 33% in the shoot/root ratio of *T. cherleri*, when compared to CFA plants. Similarly, the *O. compressus* plants grown in the NFA+ chambers presented a 29% lower shoot/root ratio than CFA plants, in association with AOT40 values of 12,099 ppb h. On the contrary, increases in the shoot/root ratio, influenced by a greater O_3 adverse effect on the root systems, were found in the NFA+ plants (AOT40 values over 11400 ppb h) of *T. subterraneum*, *T. striatum* and *T. glomeratum* (33–60%) when compared with those grown in the CFA treatment. The largest O_3-induced increase in the shoot/root ratio was found in *T. subterraneum* (38%). The alteration of carbon allocation is a common effect induced by O_3 (Cooley and Manning, 1987) and has also been reported in similar experiments involving the exposure of herbaceous

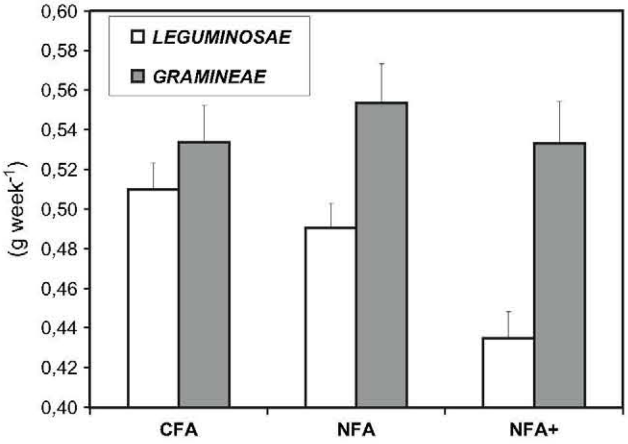

Fig. 1. Average total relative growth rate for Leguminosae (○) and Gramineae (●) plants grown in the different O_3 treatments. CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non-filtered air supplemented with 40 ppb O_3. Mean ± SE.

species. However, shoot or root partitioning was species-dependent (Franzaring et al., 2000; Reiling and Davison, 1992; Warwick and Taylor, 1995).

The observed changes in shoot/root dry weight biomass ratio or in the root RGR might determine important ecological implications in the dehesa Mediterranean systems that usually experience water and nutrient limitations. A significant amount of the photosynthates of these species is usually accumulated in the root system to overcome stressful periods when soil moisture and nutrient availability are limited. Therefore the observed shifts induced by O_3 on shoot/root ratio may affect the competitive ability of these species (Aers et al., 1991; Franzaring et al., 2000).

Changes in plant morphology were observed in all the species following their exposure to O_3 except for *A. cornicina* and *T. cherleri* (see Table 4). These effects did not necessarily correspond with the observed responses in aerial or subterranean biomass or RGR, as was the case for *O. compressus*.

A ranking of decreasing O_3 sensitivity is proposed for the Leguminosae species involved in the experiment, based on the changes induced by ambient and above ambient levels of this pollutant on the aerial, root and total RGRs and the shoot/root ratio. The responses of the morphological parameters were used to fine-tune the O_3 sensitivity of the different species. The first criterion was to select those species showing adverse effects on their RGRs following their exposure to ambient O_3 levels (*T. striatum*, *T. subterraneum*). The second criterion was to choose those species where above ambient O_3 levels induced detrimental effects on their RGRs and were simultaneously affected by ambient levels on morphological parameters (*T. cherleri*, *T. angustifolium*). A third sensitivity group was defined by those species that only showed adverse effects when exposed to the NF+ treatment (*M. minima*, *T. glomeratum*, *Biserrula*). The group encompassing the least sensitive species was defined by the lack of response of their RGR even when exposed to the maximum O_3 levels or just showing adverse effects on a single morphological parameter (the two *Anthyllis* species). *O. compressus* was considered slightly more sensitive than this group since O_3 exposure altered its shoot/root ratio. As a result the following ranking is proposed:

> *T. striatum*, *T. subterraneum* > *T. cherleri*, *T. angustifolium* > *M. minima*, *T. glomeratum*, *B. pelecinus* > *O. compressus* > *A. cornicina*, *A. lotoides*.

This sensitivity ranking based on growth-related parameters showed a great similarity with that based on the induction of foliar injury reported by Bermejo et al. (2003). However, *T. glomeratum* was more resistant than expected from visible injury responses while the opposite was true for *M. minima*. These types of disagreements in the sensitivity classifications when visible injury or growth-related parameters have been reported by many authors in the literature (see review by Davison and Barnes, 1998). It is worth noting the high sensitivity of the *Trifolium* species involved in the study, in agreement with Nebel and Fuhrer (1994), Nussbaum et al. (1995), Karlsson et al. (1995), Balls et al. (1996), Ashmore et al. (1996), Bergmann et al. (1999) and Gimeno et al. (2004). Since the ecology of these *Trifolium* species is quite different, Bergmann et al. (1999) suggested that their great sensitivity could be related to evolutionary and genetic features.

3.2. *Ozone effects on species of the Gramineae family*

Significant O_3 effects on the aerial, root or total RGRs were found in only two of the Gramineae species involved in the experiment (see Table 3). Above ambient O_3 levels (AOT40 = 14,183 ppb h) caused a 23% reduction in the root RGR of *Aegilops geniculata*. Ambient O_3 levels (AOT40 = 415 ppb h) induced a 13% increase in this parameter on *Cynosurus echinatus* when compared with CFA plants. The aerial RGR was not affected on any of the assessed grass species. Ambient O_3 (415 ppb h) levels induced a 6% increase in the total biomass RGR of *Cynosurus echinatus*; no effects on this parameter were found for the remaining grass species.

Regarding O_3 effects on plant structure and canopy architecture (see Tables 3 and 4), ambient O_3 levels determined a reduction in the shoot/root ratio of *C. echinatus*. No effects on this parameter were found in any of the remaining species. Ambient O_3 levels with AOT40 values ranging from 327–818 ppb h induced 87–17% reductions in the height of *Bromus hordeaceus*, *B. sterilis*, *C. echinatus* and *Vulpia myuros*. Above ambient O_3 levels (AOT40 levels in the 12,500–14,200 ppb h range) determined 6–18% reductions in the height of *A. geniculata*, *B. hordeaceus*, *B. sterilis* and *C. echinatus*. No effects on plant surface were detected in any grass species when exposed to the NFA or NFA+ treatment. A 21% reduction was induced by ambient O_3 exposure (AOT40 = 327 ppb h) in the plant canopy volume of *V. myuros* plants, when compared to CFA-treated plants. Above ambient O_3 levels corresponding to AOT40 values in the 8500–12,800 ppb h range were associated with reductions of 36, 26 and 18% in the plant volume of *A. sterilis*, *B. maxima* and *C. echinatus*, respectively.

Although the grass species involved in the experiment were rather insensitive to O_3, they were ranked according to the AOT40 levels that determined effects on biomass RGR and alterations in plant morphology. The first criterion followed to classify these species was the detection of an effect on any RGR (*Cynosurus echinatus* and *Aegylops geniculata*). *Cynosurus echinatus* was classified as a sensitive species although O_3 exposure induced increases and reductions in some of the assessed parameters. The second criterion adopted was that any alteration in plant performance would be indicative of plant sensitivity. Three groups were defined according to this criterion. The first group involved those species that showed alterations in morphological parameters when exposed to ambient O_3 levels (*V. myuros*, *B. hordeaceus* and *B. sterilis*). The second group encompassed those species showing morphological changes following their exposure to above ambient O_3 levels (*Avena sterilis* and *B. maxima*). Ozone exposure did not induce effects on any of the assessed parameters of *A. triuncialis* and *L. rigidum*, thus forming the least sensitive group. As a result, the Gramineae species are ranked as follows:

C. echinatus, *A. geniculata* > *V. myuros*, *B. hordeaceus*, *B. sterilis* > *Avena sterilis*, *B. maxima* > *A. Triuncialis*, *L. rigidum*

As was the case for legumes, discrepancies were found between this sensitivity ranking and that proposed in Bermejo et al. (2003) for the same species based on foliar injury. The most remarkable disagreement is observed in *C. echinatus* and *V. myuros*, that would be most sensitive according to growth but insensitive regarding visible injury.

Some authors have attempted to evaluate whether the C-SR model of plant strategies defined by Grime (1979) could be associated with O_3 susceptibility. Reiling and Davison (1992), Selldén and Pleijel (1995), Bungener et al. (1999) and Franzaring et al. (2000) have found that the most sensitive species of their studies presented a high component of C or R strategies (R/CR or CR) and were the fastest-growing species, showing the greatest RGR in clean air. However, in our experiment plant sensitivity to O_3 was neither explained by its RGR values in clean air (Table 2 and 3) nor by its CSR classification (data not shown), in agreement with the reports from other authors (Pleijel and Danielsson, 1997; Warwick and Taylor, 1995) that did not find a clear association between these plant traits and O_3 sensitivity.

The AOT40 was the exposure index that best explained the observed effects of O_3 on the aerial, subterranean or total RGR of grasses and legumes of our experiment, when compared with other indices based on mean, maximum values or accumulated indices using other cut-offs (data not shown). AOT40 was selected to derive equations relating the aerial, subterranean or total RGR of grasses and legumes to O_3 exposure (see Fig. 2). In these equations the observed responses were expressed relative to the CFA treatment (100% growth). Because Leguminosae and Gramineae species showed a great disparity regarding their O_3 sensitivity, separate equations were also carried for both taxa. According to the suggestions provided by Ashmore and Franzaring (2003) the two most resistant Leguminosae species were not included in the analysis

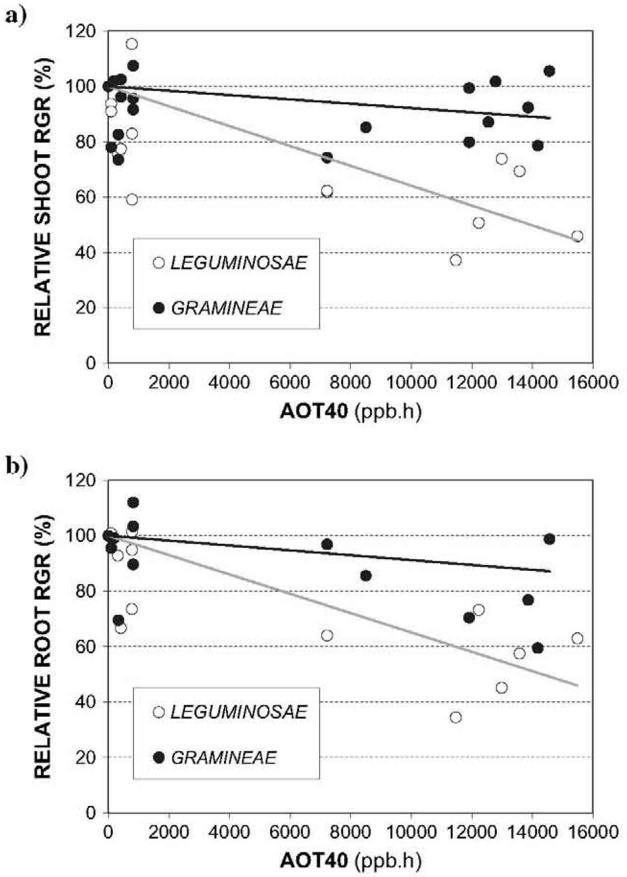

Fig. 2. Growth response of the plants involved in the experiment in relation to ozone exposure (AOT40, ppb h). Models were constructed for Leguminosae plants (lighter line) and Gramineae plants (darker line). (a) Relative shoot RGR; Gramineae: $y = -0.0008x + 100$ ($r^2 = -0.08$, ns); Leguminosae: $y = -0.0036x + 100$ ($r^2 = 0.51$, $p < 0.01$). (b) Relative root RGR; Gramineae $y = -0.0009x + 100$ ($r^2 = 0.02$, ns); Leguminosae: $y = -0.0035x + 100$ ($r^2 = 0.64$, $p < 0.01$).

(see Fig. 2). The best fit was found for the subterranean biomass RGR of legumes plants ($r^2 = 0.64$), followed by the total and aerial biomass RGR of this group (r^2 values of 0.57 and 0.51, respectively). Non-significant relationships between O_3 exposure and the performance of Gramineae species were found. Ten per cent reductions in the RGRs of the Leguminosae plants would be expected following exposures to AOT40 values close to 2800 ppb accumulated in a range of 43–67days, in agreement with present critical level for the protection of semi-natural grasslands, 3000 ppb h over a period of up to three months.

In summary, the legumes involved in this screening experiment were more sensitive to O_3 than the grass species. Ozone caused detrimental effects on the aerial, subterranean or total biomass of most of the Leguminosae species. Changes in plant morphology were also observed in many legumes following their exposure to O_3 levels. Further studies will be carried out to evaluate whether these changes could affect plant O_3 uptake. The O_3 exposure that induced detrimental effects on the most sensitive species was in the range of the current critical level (Fuhrer et al., 2003). The results of this study indicate that O_3 exposure might induce changes in the composition of dehesa grasslands by decreasing the performance of legumes when compared to grasses, and thus affecting the structure and function of these ecosystems and their forage quality. More research is needed to assess the impact of O_3 on the competitive ability of these species and its interactive effects with nitrogen and water availability.

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