Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury

V. Bermejo, B.S. Gimeno*, J. Sanz, D. de la Torre, J.M. Gil

Ecotoxicology of Air Pollution, CIEMAT (Ed. 70), Avda. Complutense 22, Madrid 28040, Spain

Abstract

Ozone (O_3) phytototoxicity has been reported on a wide range of plant species, inducing the appearance of specific foliar injury or increasing leaf senescence. No information regarding the sensitivity of plant species from dehesa Mediterranean grasslands has been provided in spite of their great biological diversity. A screening study was carried out in open-top chambers (OTCs) to assess the O_3-sensitivity of 22 representative therophytes of these ecosystems based on the appearance and extent of foliar injury. A distinction was made between specific O_3 injury and non-specific discolorations. Three O_3 treatments (charcoal-filtered air, non-filtered air and non-filtered air supplemented with $40\,nl\,l^{-1}$ O_3 during 5 days per week) and three OTCs per treatment were used. The Papilionaceae species were more sensitive to O_3 than the Poaceae species involved in the experiment since ambient levels induced foliar symptoms in 67% and 27%, respectively, of both plant families. An O_3-sensitivity ranking of the species involved in the assessment is provided, which could be useful for bioindication programmes in Mediterranean areas. The assessed *Trifolium* species were particularly sensitive since foliar symptoms were apparent in association with O_3 accumulated exposures well below the current critical level for the prevention of this kind of effect. The exposure indices involving lower cut-off values (i.e. $30\,nl\,l^{-1}$) were best related with the extent of O_3-induced injury on these species.

Keywords: Grasslands; Dehesa; Clover; Critical levels; Foliar damage

1. Introduction

Tropospheric ozone (O_3) has been reported to reach phytotoxic levels in European rural areas (EMEP, 2002). There is concern about the harm this pollutant might pose to semi-natural grassland vegetation, since it is expected that the different O_3 sensitivity of these plant species might induce changes in the floristic composition of plant communities (Fuhrer et al., 1994). Moreover, grassland ecosystems are particularly endangered by the interaction of other global-change components such as alterations in land-use, climate, nitrogen deposition, biotic exchange or atmospheric carbon dioxide (Sala et al., 2000). Therefore, O_3 effects on these ecosystems should be assessed to effectively reduce anthropogenic impacts on them.

Early experiments concerning O_3 effects on semi-natural plant species were focused on the detection of foliar visible injury following their exposure to elevated O_3 concentrations (Ashmore et al., 1987). Recent research has been carried out exposing herbaceous plants from different communities to realistic O_3 levels. This is the case of the studies carried out by Ashmore et al. (1995), Pleijel and Danielsson (1997), Bergmann et al. (1999) and Franzaring et al. (2000) on the most common herbaceous taxa of northern and central-European flora. However, no information exists on the O_3 sensitivity of herbaceous species from Mediterranean grasslands. This is particularly distressing since plant diversity in some of these ecosystems, such as the dehesa-type grasslands, is quite remarkable (Pineda et al., 1981), and also because ambient O_3 levels recorded in the Mediterranean region have been reported as phytotoxic (Fumagalli et al., 2001; Nali et al., 2002; Bermejo et al., 2002). Moreover, recent experimental work (Madkour and Laurence, 2002) has described the high sensitivity of a local Egyptian cultivar of *Trifolium alexandrinum*, driving attention to potential damage in the southern-Mediterranean area.

The dehesas represent an example of sustainable management of natural resources in the Iberian Peninsula, covering agricultural, timber and extensive livestock exploitation. The typical vegetation of dehesa ecosystems is composed of cleared oak woodlands with a grassland understorey. Annual species represents up to 71% of the herbaceous species from these ecosystems (Marañón, 1985). Large spatial and temporal fluctuations in floristic composition occur depending on tree coverage, slope–valley gradients, grazing pressure and meteorological conditions (Peco et al., 1998; San Miguel, 1994). As a result, plant species richness of these ecosystems is quite remarkable, 30 species per 400 cm^2 (Pineda et al., 1981). The most characteristic species of these grasslands belong to the Papilionaceae and Poaceae families. Several studies have reported a great sensitivity to O_3 of Papilionaceae plants in opposition to most of Poaceae species (see the review carried out by Davison and Barnes, 1998). Both taxa have different roles in the ecosystem, mainly in nitrogen cycling, and they also represent dissimilar nutritive sources for the livestock. Therefore, the assessment of the O_3 sensitivity of the plants from these communities is quite appealing since this pollutant might have an impact on the structure, diversity, function and human-utility of these ecosystems belonging to the Mediterranean basin, an area that has been considered as an "hyper-hot" candidate for conservation support (Myers et al., 2000).

When plants are exposed to realistic concentrations, screening studies aiming to assess the sensitivity of a wide range of plants to air pollutants are suitable for the determination of thresholds of injury and may have implications in risk assessment or legislative initiatives. The United Nations Economic Commission for Europe (UN ECE) is leading one of the most comprehensive efforts to control transboundary air pollution through the assessment of the areas, where O_3 levels exceed its phytotoxicity levels, also termed critical levels. *Critical level* has been defined as the pollutant concentration above which direct effects on receptors, such as plants, ecosystems or materials, may occur according to present knowledge (UN ECE, 1988). Ozone critical levels have been proposed for the protection of crops, semi-natural vegetation and forest-tree species (Fuhrer and Achermann, 1999).

The short-term O_3 critical level for the prevention of foliar injury on herbaceous plants was derived from the results of a pan-European experiment carried out under the framework of the International Cooperative Programme (ICP) on the effects of air pollution on natural vegetation and crops (ICP vegetation). This experiment involved the ambient exposure of *T. repens* and *T. subterraneum* following a standardised protocol (Benton et al., 2000). Ozone-induced foliar injury was found to be related to the O_3 exposure corresponding to the 5 days prior to the appearance of visible symptoms. Since air vapour pressure deficit (VPD) modulated O_3 phytotoxicity, the short-term critical level was established at AOT40 (accumulated exposure over a threshold of 40 $nl\,l^{-1}$) values of 200 and 500 $nl\,l^{-1}\,h$ when mean VPD (09:30–16:30) is below or above 1.5 kPa, respectively. Tonneijck and Van Dijk (2002) have reported injury on *T. subterraneum* with AOT40 values below 200 $nl\,l^{-1}\,h$. Therefore, it appears that this critical level needs further revision.

This work presents the results of an experiment that was carried out with the following objectives: (1) to rank O_3 sensitivity of therophytic plants from acidic dehesa pastures according to the appearance of foliar visible injury; (2) to assess whether differences in sensitivity could be attributed to certain plant genus or families, and (3) to evaluate the present short-term critical level for O_3-injury development.

2. Materials and methods

2.1. Experimental site

The experiment was carried out at Sant Jaume d'Enveija, Spain (40°41′N, 0°47′E). No major air pollutant sources that might have affected the results are located in the vicinity of the experimental site.

2.2. Plant material

All the species involved in this study are annual plants from dehesa acidic grasslands (Allué Andrade and Tella, 1986). Annual species are more predominant than perennials due to the extreme meteorological conditions occurring during summer and winter.

Most of the seeds were collected from a typical dehesa located North of 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 *T. striatum*, *T. subterraneum*, *T. 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 Papilionaceae species were immersed during 24 h in a Germinator© solution (Agro-Orgánicos Mediterráneos S.L., Granada, Spain) to ensure homogeneous germination. When seeds were swollen, they were sown in a 50% neutral peat and 50% vermiculite substrate. Poaceae seeds did not experience any pre-treatment and they were sown in the same substrate as Papilionaceae seeds. Seedlings were transplanted to 2.5 l pots with a 50% peat, 30% vermiculite and 20% perlite substrate and $2\,kg\,m^{-3}$ of a slow-release fertiliser (NPK:15/8/11). Plants were irrigated with a droplet system to ensure adequate and homogeneous water availability to plant material.

2.3. Ozone treatments

The same day the plants were transplanted into pots they were introduced in slightly modified NCLAN-type open-top chambers (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 $40\,nl\,l^{-1}$ O_3 from 07:00 to 17:00 (GMT) 5 days $week^{-1}$ (NFA+). Ozone concentrations in the NFA+ treatment were in the range of those reported by Plaza et al. (1997) in the areas where these plant communities are present. Four to six plants of each species were introduced in each chamber and three OTC replicates were used for each O_3 treatment. 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 active radiation (PAR). A complete description of the chambers and the operation of the system is provided in Pujadas et al. (1997) and Alonso et al. (2001). Details on the dates the plants were introduced in the chambers and the length of O_3 exposure in the different treatments can be found in Tables 1 and 2.

2.4. Visible injury assessment

Daily evaluations of O_3-induced visible injury were carried out on plant leaves from the different treatments until the first symptoms were observed in 90% of the plants of a given species. Weekly assessments were performed afterwards. In the last assessment, the number of total leaves, healthy leaves, leaves presenting O_3-specific symptoms and senescent leaves was recorded on three randomly selected stems or tillers per plant on Papilionaceae or Poaceae species, respectively. *T. striatum* and *T. hirtum* were not included in this last assessment. Injury intensity at the plant level was expressed as the percentage of affected to total assessed leaves.

2.5. Ozone exposure indices

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 the three O_3 treatments integrating different periods: (a) from the beginning of plant exposure in the chambers until the first visible injury was detected, (b) from the beginning of plant exposure until the last assessment of visible injury was carried out, and (c) integrating the exposure during the 5 days preceding the detection of O_3 injury and determining whether air VPD was below or above 1.5 kPa during this period. The AOT40 index was calculated as the sum of the differences between O_3 hourly concentrations in $nl\,l^{-1}$ and $40\,nl\,l^{-1}$ for each hour when concentration exceeds $40\,nl\,l^{-1}$ and solar radiation is above $50\,W\,m^{-2}$.

Ozone exposure throughout the experiment was also calculated using other indices such as AOT30 (accumulated exposure over the cut-off of $30\,nl\,l^{-1}$), AOT60 (accumulated exposure over the cut-off of $60\,nl\,l^{-1}$) and the number of hours presenting O_3 levels above 30, 40 and $60\,nl\,l^{-1}$ (NUM30, NUM40 and NUM60, respectively). In addition, O_3 10-h mean (07:00–17:00 GMT), the 24-h O_3 mean, the number of days where the O_3 daily mean exceeded $33\,nl\,l^{-1}$ (D33), the number of hourly averages exceeding $100\,nl\,l^{-1}$ (NUM100) and the maximum hourly levels (Max. hourly levels) were also determined.

2.6. Statistical analyses

Ozone effects on visible injury-related parameters were tested performing an ANOVA analysis for each species and variable. In addition, a two-way ANOVA analysis using O_3 treatment and family as factors was carried out to assess whether a given family could be associated with differential O_3 sensitivity. When significant differences ($p<0.05$) were detected, the differences between means were assessed using the least-significant difference (LSD) test. The validation process and the assumptions of analyses of variance followed using Shappiro–Wilk's W and Levenne tests to check the normal distribution and homogeneous variance, respectively. When non-compliance with ANOVA assumptions was observed, data transformation followed, using angular transformations ($\arcsin x^{0.5}$) of data expressed as percentages.

The relationship between visible injury records and the different O_3 exposure indices was analysed using Pearson's correlation index and the level of significance

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

Table 1
Experimental protocol and ozone exposure related with the induction of foliar visible injury on the Papilionaceae species involved in the screening experiment

Papilionaceae	Plant entrance in OTC	Total exposure length (days)	NFA				NFA+			
			Time length injury develop[a] (days)	AOT40 until injury[b] ($nl\,l^{-1}\,h$)	AOT40 VPD > 1.5[c] (5 days) ($nl\,l^{-1}\,h$)	AOT40 VPD < 1.5[d] (5 days) ($nl\,l^{-1}\,h$)	Time length injury develop[a] (days)	AOT40 until injury[b] ($nl\,l^{-1}\,h$)	AOT40 VPD > 1.5[c] (5 days) ($nl\,l^{-1}\,h$)	AOT40 VPD < 1.5[d] (5 days) ($nl\,l^{-1}\,h$)
Anthyllis cornicina L.	07/09/00	76	—	—	—	—	—	15,189[e]	—	—
Anthyllis lotoides L.	14/09/00	69	—	—	—	—	—	13,144[e]	—	—
Biserrula pelecinus L.	22/09/00	67	67	313	0	0	28	5698	0	1086
Medicago minima (L.) Bartal	11/10/00	43	—	—	—	—	28	3942	0	788
Ornithopus compressus L.	11/10/00	66	—	—	—	—	—	12,099[e]	—	—
Trifolium angustifolium L.	11/10/00	43	28	67	0	0	28	3942	0	788
Trifolium cherleri L.	08/09/00	77	5	359	17	342	5	1432	186	1247
Trifolium glomeratum L.	08/09/00	68	5	359	17	342	5	1432	186	1247
Trifolium hirtum All.	22/09/00	47	47	288	0	0	13	2956	108	711
Trifolium striatum L.	14/09/00	61	1	17	0	301	1	226	0	1776
Trifolium subterraneum L.	08/09/00	66	5	359	17	342	5	1432	186	1247

[a] Time length for the detection of the first visible injury.
[b] Accumulated ozone exposure over 40 $nl\,l^{-1}$ (AOT40) from the start of the experiment until the observation of the first foliar injury.
[c] Integrated ozone exposure 5 days prior to the detection of the first foliar injury when air vapour pressure deficit (VPD) was above 1.5 kPa, respectively, for the non-filtered air (NFA) or non-filtered air supplemented with 40 $nl\,l^{-1}$ O_3 treatments (NFA+).
[d] Integrated ozone exposure 5 days prior to the detection of the first foliar injury when air vapour pressure deficit (VPD) was below 1.5 kPa, respectively, for the non-filtered air (NFA) or non-filtered air supplemented with 40 $nl\,l^{-1}$ O_3 treatments (NFA+).
[e] Ozone exposure throughout the experiment, no visible injury was found in these species.

Table 2
Experimental protocol and ozone exposure related with the induction of foliar visible injury on the Poaceae species involved in the screening experiment

Poaceae	Plant entrance in OTC	Total exposure length (days)	NFA				NFA +			
			Time length injury develop[a] (days)	AOT40 until injury[b] ($nl\,l^{-1}\,h$)	AOT40 VPD > 1.5[c] (5 days) ($nl\,l^{-1}\,h$)	AOT40 VPD < 1.5[d] (5 days) ($nl\,l^{-1}\,h$)	Time length injury develop[a] (days)	AOT40 until injury[b] ($nl\,l^{-1}\,h$)	AOT40 VPD > 1.5[c] (5 days) ($nl\,l^{-1}\,h$)	AOT40 VPD < 1.5[d] (5 days) ($nl\,l^{-1}\,h$)
Aegilops geniculata Roth	07/09/00	71	43	776	0	47	28	7002	108	711
Aegilops triuncialis L.	07/09/00	72	—	—	—	—	28	7002	108	711
Avena barbata Link	11/10/00		28	67	0	0	9	1480	0	1086
Avena sterilis L.	03/10/00	49	35	160	0	0	17	3411	0	1086
Briza maxima L.	20/09/00	63	—	—	—	—	28	8934	0	788
Bromus hordeaceus L.	07/09/00	69	—	—	—	—	61	12,206	108	711
Bromus sterilis L.	14/09/00	67	—	—	—	—	54	10,160	0	788
Cynosurus echinatus L.	14/09/00	68	—	—	—	—	—	12,794[e]	—	—
Lolium rigidum Gaudin	11/10/00	43	—	—	—	—	9	1480	0	1086

[a] Time length for the detection of the first visible injury.
[b] Accumulated ozone exposure over $40\,nl\,l^{-1}$ (AOT40) from the start of the experiment until the observation of the first foliar injury.
[c] Integrated ozone exposure 5 days prior to the detection of the first foliar injury when air vapour pressure deficit (VPD) was above 1.5 kPa, respectively, for the non-filtered air (NFA) or non-filtered air supplemented with $40\,nl\,l^{-1}$ O_3 treatments (NFA+).
[d] Integrated ozone exposure 5 days prior to the detection of the first foliar injury when air vapour pressure deficit (VPD) was below 1.5 kPa, respectively, for the non-filtered air (NFA) or non-filtered air supplemented with $40\,nl\,l^{-1}$ O_3 treatments (NFA+).
[e] Ozone exposure throughout the experiment, no visible injury was found in these species.

was determined using the Student's *t*-test. All statistical analyses were carried out using Statistica 5.1. software.

3. Results and discussion

Ozone induced the appearance of specific and senescence-related foliar symptoms in 16 of the 22 assessed species. Both families (Poaceae and Papilionaceae) presented O_3-sensitive species, although differences in the type of injury, the number of affected species and the extent of visible injury on their foliage were found between them.

Ozone-induced injury on Papilionaceae species consisted of brown-reddish necrotic spots that were associated with foliar chlorosis in the case of *T. angustifolium* and *Medicago minima*. The *Trifolium* genus was the most O_3-sensitive taxa since all the assessed species showed visible symptoms following short O_3 exposures under ambient or close to ambient concentrations, in agreement with the results reported by Nebel and Fuhrer (1994). This is a finding of concern, since it is the most predominant genus in the therophytic and siliceous pastures of the Iberian Peninsula (Allué Andrade and Tella, 1986).

The O_3 sensitivity ranking of the assessed Papilionaceae species was: *T. striatum* > *T. cherleri, T. glomeratum, T. subterraneum, T. hirtum* > *T. angustifolium* > *Biserrula pelecinus, M. minima* > *Anthyllis lotoides, A. cornicina, O. compressus*.

T. striatum was the most sensitive species of this genus; O_3-induced injury was observed in the first fully expanded trifoliate even before the plants were introduced in the chambers, associated with a very low AOT40 value, 17 $nl\,l^{-1}\,h$ (Table 1). *T. cherleri, T. glomeratum, T. subterraneum* and *T. hirtum* were rather sensitive to O_3 exposure since the first O_3 visible injury was found with AOT40 values ranging 288–359 $nl\,l^{-1}\,h$ (Table 1). These values are much lower than those reported by Bergmann et al. (1999) for 25 species of German flora, ca. 2000 $nl\,l^{-1}\,h$. In fact, when the O_3 exposure corresponding to the 5 days prior to the first observation of injury was considered, the AOT40 values ranged from 0 to 17 when VPD > 1.5 kPa and from 0 to 342 when VPD < 1.5 kPa. These values are lower than the 200–500 $nl\,l^{-1}$ range reported for the induction of visible injury on a pan-European study involving *T. subterraneum* (Benton et al., 2000). A gradation in foliar injury (both O_3-induced visible injury and senescent leaves) was found in the Papilionaceae group when plants were exposed to the different treatments for 66–77 days (Fig. 1); the extent of visible injury increased ($p < 0.05$) in NFA and NFA+ plants by 45–60% and 75–85%, respectively, when compared with CFA plants.

The first foliar injury on *T. angustifolium* was found at a rather low AOT40 value (67 $nl\,l^{-1}\,h$); however, injury developed at a low rate after 43 days of exposure and only affected 10% of the foliage of NFA+ plants (Table 1, Fig. 1). Ozone exposure did not induce an increase in senescence-related symptoms. This behaviour would indicate a greater sensitivity of early stages of development when compared to late phenological stages. Franzaring et al. (2000) also reported more resistant and robust leaves in the later stages, while Carlsson et al. (1996) found an inverse relationship. However, in this species, O_3 exposure appears to be related with the induction and extension of interveinal chlorosis in the trifoliates (not quantified). This type of injury was not found in any other of the assessed *Trifolium* species and is considered as a non-specific O_3 injury.

B. pelecinus and *M. minima* could be considered as less sensitive than *Trifolium* species since ambient O_3 levels induced foliar injury *B. pelecinus* only at the end of the experiment and did not determine any effect on *M. minima*. Moreover, the first foliar injury was observed in the NFA+ plants of both species after 28 days of exposure in association with AOT40 values in the 3942–5698 $nl\,l^{-1}\,h$ range (Table 1). *B. pelecinus* would be slightly more sensitive than *M. minima* since after 67 days of exposure O_3-induced injury was found in 20% of the leaves of NFA and NFA+ plants and an increment of senescence in NFA+ leaves was also found ($p < 0.05$) (Fig. 1).

A. cornicina, A. lotoides and *O. compressus* can be considered as the most resistant species to O_3 exposure as no effects were detected on their leaves after an average exposure length of 70 days, with AOT40 values up to 12,000–15,000 $nl\,l^{-1}\,h$ in the NFA+ treatment (Table 1). Foliage was apparently healthy and O_3 exposure did not cause an increased senescence in these species (Table 1).

The O_3-induced foliar injury observed in the species of the Poaceae family was different from that observed in the Papilionaceae species. Apical necrosis and reddish-brown spots parallel to leaf's nerves were observed in *Avena sterilis* and *A. barbata*. Apical necrosis followed by a chlorotic banding parallel to leaf's nerves was found in *Aegilops geniculata* and *A. triuncialis*. Ozone exposure induced in 67% of Poaceae species an increase in all the senescent-related parameters assessed: number of senescent leaves to total leaves ratio, dry weight of total senescent foliage per plant or green biomass to senescent biomass ratio. Therefore, senescence appears to be a crucial parameter to determine the O_3 sensitivity of the dehesa therophytic species of the Poaceae family.

Also, the grass species were classified regarding their O_3 sensitivity considering the O_3 exposure needed for the induction of foliar visible injury symptoms: *A. barbata, A. sterilis, A. geniculata* > *Lolium rigidum* > *Briza maxima, A. triuncialis, Bromus hordeaceus, B. sterilis* > *Cynosurus echinatus, Micropyrum tenellum, Vulpia myuros*.

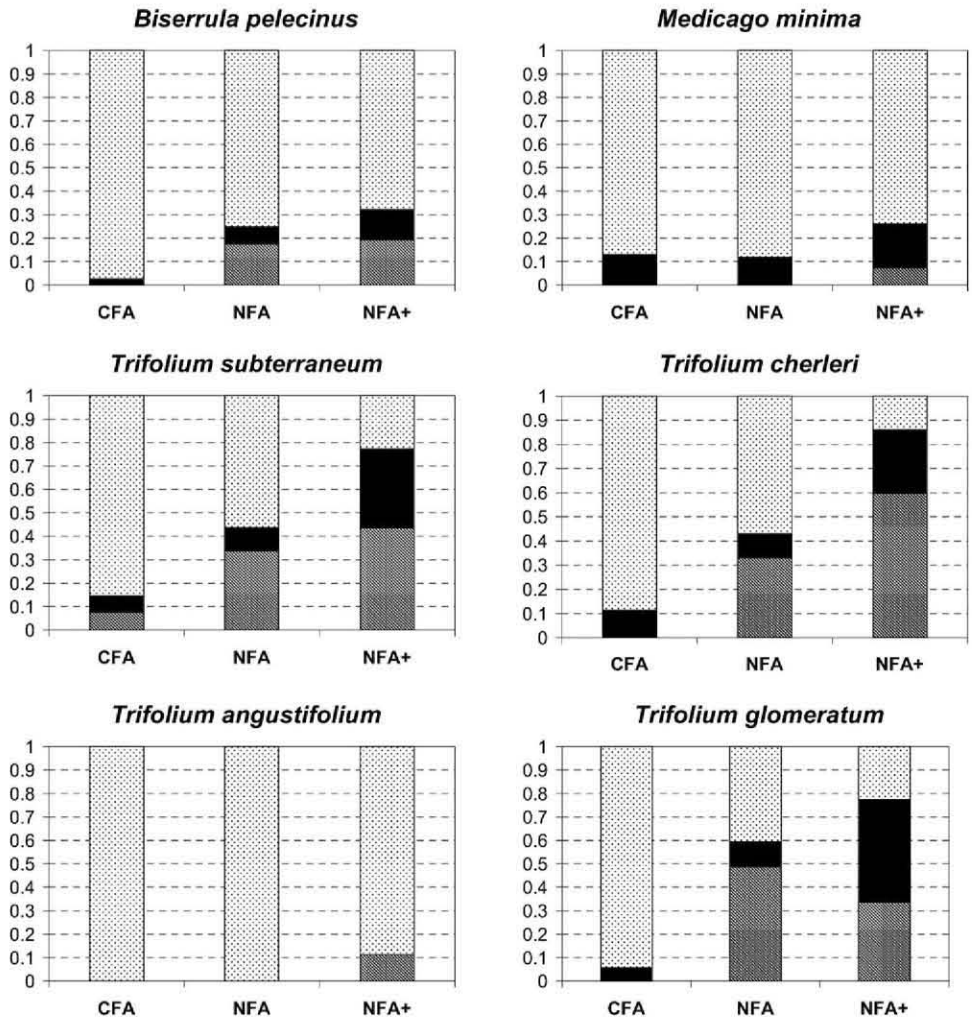

Fig. 1. Foliar injury (‰) of the Papilionaceae species involved in the experiment. = ozone-specific visible symptoms; = senescence-related injury; = healthy foliage. CFA = charcoal-filtered air; NFA = non-filtered air; NFA + = non-filtered air supplemented with 40 $nl\,l^{-1}$ ozone.

A. barbata, *A. sterilis* and *A. geniculata* would be the most sensitive grasses since O_3 foliar injury was observed after 28–43 days of ambient O_3 exposure corresponding to AOT40 values ranging 67–776 $nl\,l^{-1}\,h$ (Table 2). At the end of the experiment, ambient O_3 levels induced specific visible symptoms on about 20% of the leaves of *A. sterilis and A. geniculata* compared to control ($p<0.05$) (Fig. 2). In the case of *A. geniculata,* ambient O_3 levels also determined a 20% increase in foliar senescence ($p<0.01$).

Foliar injury was also observed in other species, but a greater accumulated O_3 exposure was needed to trigger visible injury on their foliage. For instance, the first foliar injury in *L. rigidum* was found in leaves of NFA + plants exposed to an AOT40 value of 1480 $nl\,l^{-1}\,h$ (Table 2). At the end of the experiment, O_3 foliar damage increased in NFA and NFA + compared to control ($p<0.01$), but no differences between NFA and NFA + plants with regard to the number of O_3-affected leaves were found. However, a significant gradation in the number of senescent leaves from the different O_3 treatments was observed in this species ($p<0.01$) (Fig. 2).

The group constituted by *A. triuncialis*, *B. hordeaceus*, *B. sterilis* and *B. maxima* could be considered as less sensitive since the first O_3-induced visible injury was found following AOT40 exposures ranging 7000–12,200 $nl\,l^{-1}\,h$ (Table 2). When the extension of visible injury was analysed at the end of the experiment, *B. maxima* was the most sensitive species within this group since the NFA and NFA + treatments induced a similar extent of injury on its foliage (Fig. 2). *A. triuncialis*

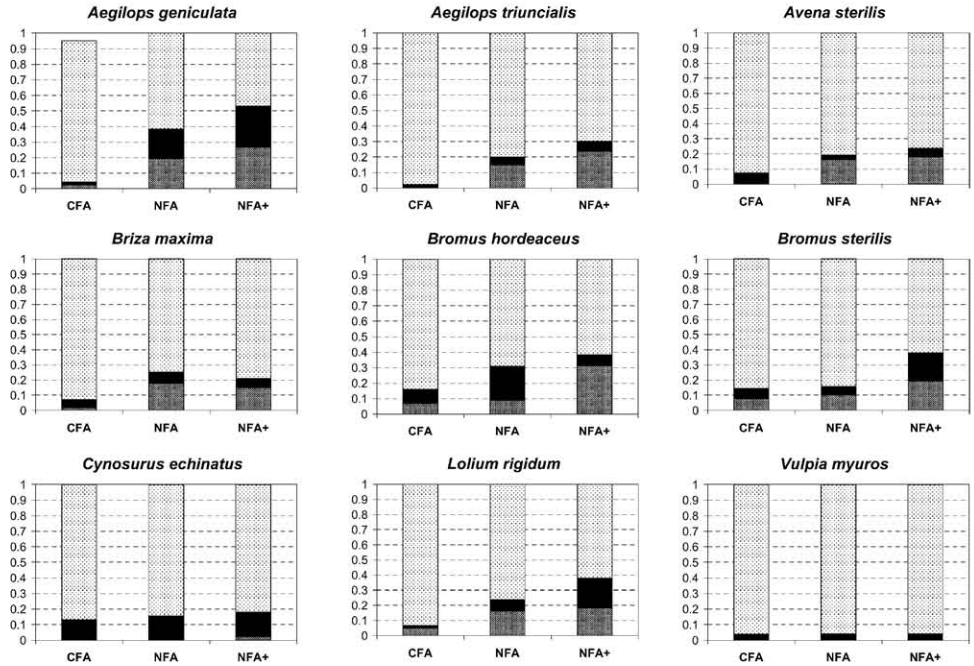

Fig. 2. Foliar injury (‰) of the Poaceae species involved in the experiment. ▒ = ozone-specific visible symptoms; ■ = senescence-related injury; ░ = healthy foliage. CFA = charcoal-filtered air; NFA = non-filtered air; NFA+ = non-filtered air supplemented with $40\,\mathrm{nl\,l^{-1}}$ ozone.

would be the next sensitive species with a marked gradation in the development of foliar injury between the different treatments ($p<0.05$). An increase in the extension of O_3 foliar injury was found only in those *B. hordeaceus* plants exposed to the NFA+ treatment when compared to control plants ($p<0.05$) (Fig. 2). An increase in senescence following O_3 exposure in NFA+ was observed in *B. sterilis* ($p<0.05$), an effect that was not observed in the other species of this group. The increase in the number of senescent leaves is an unspecific but characteristic effect of this pollutant on natural vegetation (Bergmann et al., 1999; Franzaring et al., 2000). In fact, the ratio between the number of senescent leaves to total leaves recorded at the end of the experiment was more sensitive than the induction of specific symptoms following the exposure of *B. sterilis* individuals to increasing O_3 concentrations.

The most O_3-resistant grass species regarding visible injury were *C. echinatus*, *M. tenellum* and *V. myuros*, species that did not show any effect on their foliage, either as specific O_3 symptoms or as senescence-related injury (Table 2).

The Papilionaceae species assessed in the experiment were more sensitive to O_3 than the Poaceae species. Ambient O_3 exposures involving AOT40 values close to $300\,\mathrm{nl\,l^{-1}\,h}$ and relatively short time lengths induced foliar visible symptoms in 67% of Papilionaceae species. By contrast, ambient O_3 exposures determined the appearance of foliar symptoms in only 27% of Poaceae plants, and usually O_3 exposures with AOT40 values over $1000\,\mathrm{nl\,l^{-1}\,h}$ were needed to induce foliar injury in the majority of the species of this family (Tables 1 and 2).

When the proportion of healthy, senescent and injured leaves was considered, O_3 impact was the greatest in the Papilionaceae family as revealed by the two-way ANOVA analysis considering family and ozone as factors (Table 3). The extent of visible injury was influenced by both family and O_3 treatment ($p<0.0001$). An O_3-family interaction ($p<0.05$) was found for the percentage of senescent leaves, since the NFA+ Papilionaceae plants presented an increase of 37% of senescent leaves when compared with the Poaceae plants of the same treatment.

When overall data were analysed, O_3-induced foliar injury was recorded in association with O_3 exposures well below current O_3 short-term critical levels (see Tables 1 and 2). This was especially true when air VPD was higher than 1.5 kPa. Under these conditions, O_3 visible injury was found associated with AOT40 values below the proposed $500\,\mathrm{nl\,l^{-1}\,h}$ O_3 threshold, in the ranges from 0–17 to $0–186\,\mathrm{nl\,l^{-1}\,h}$ for the most sensitive species when exposed to ambient or artificially supplemented O_3 levels respectively. Therefore, the present O_3 critical level for visible injury does not appear to protect the species from dehesa ecosystems. This conclusion is in agreement with the findings reported by Tonneijck and Van Dijk (2002) for *T. subterraneum*.

Table 3
Extent of foliar injury in Papilionaceae and Poaceae taxa at the end of the exposure period

Factor	% of leaves showing specific O_3 injury	% of leaves showing senescence-related injury	% of healthy leaves
O_3	<0.0001	ns	<0.0001
Family	<0.0001	<0.0001	<0.0001
O_3 × Family	ns	<0.05	<0.1
Papilionaceae			
CFA	0.09±0.04	0.06±0.01[a]	0.86±0.04
NFA	0.30±0.05	0.07±0.01[a]	0.63±0.05
NFA+	0.32±0.04	0.19±0.03[c]	0.48±0.06
Poaceae			
CFA	0.03±0.01	0.06±0.01[a]	0.90±0.01
NFA	0.11±0.01	0.09±0.01[ab]	0.79±0.02
NFA+	0.17±0.02	0.12±0.01[b]	0.71±0.03

Different letters indicate significant differences among ozone treatments. CFA = charcoal-filtered air; NFA = non-filtered air; NFA+ = non-filtered air supplemented with 40 nl l^{-1} O_3. Mean values ± standard errors.

The extent of O_3-induced injury on plant foliage was more related with plant performance than the appearance of the first visible injury. Correlation analyses between the extent of foliar injury in Papilionaceae or Poaceae plants and different O_3 exposure indices were carried out. Only significant relationships ($p<0.05$) are commented below. The average exposure indices, such as M24 or M10, and indices based on a cut-off of 30 nl l^{-1}, such as AOT30 or NUM30, performed better than AOT40 index in explaining the extent of visible injury on the assessed plant species. The performance of these indices depended on the type of injury. The extent of specific O_3 injury was best explained ($r = 0.85$) by NUM30 in the Papilionaceae plants, while it was almost equally explained by M10, M24, NUM30 or maximum hourly values ($0.62<r\leqslant 0.65$) in the Poaceae species.

Interestingly, the NUM30 ($r = 0.85$) and NUM40 ($r = 0.70$) indices performed better than the AOT30 ($r = 0.66$) and AOT40 ($r = 0.60$) indices, respectively, in explaining the extent of O_3-specific injury on the foliage of Papilionaceae plants. This would indicate that all concentrations above a given threshold are equally phytotoxic since the AOT-type indices implicitly provide more weight to the higher O_3 concentrations (Tuovinen, 2000) and would be related with the high O_3 sensitivity of this taxon.

The extent of leaf senescence in Papilionaceae plants was almost equally explained by AOT30, AOT40, NUM40, AOT60 or NUM60 indices ($0.74<r<0.76$). In the case of Poaceae plants, this parameter was also almost equally explained by NUM30, AOT30, M24, M10, maximum hourly values and NUM40 ($0.37<r<0.40$).

The number of hours where O_3 concentrations were above the cut-off of 30 nl l^{-1} (NUM30) was the index that best explained the total extent of O_3-induced foliar injury (specific O_3 injury plus senescent foliage) in both Papilionaceae ($r = 0.87$) and Poaceae species ($r = 0.68$). Regression equations were constructed separately to predict the response of both families to O_3: % affected leaves in Papilionaceae plants = 0.0911 NUM30 ($R^2 = 0.76$) and % affected leaves in Poaceae plants = 0.0369 NUM30 ($R^2 = 0.47$). Exposures to 55 and 110 h over 30 nl l^{-1} would be needed to determine 5% and 10% injury on the foliage of Papilionaceae species, respectively. Similarly, 5% and 10% foliage damage on Poaceae species would appear when exposed to 136 and 271 h over 30 nl l^{-1} of O_3.

Since visible injury was observed in association with zero AOT40 values accumulated during the 5 days preceding the detection of O_3 injury, our results suggest that a cut-off lower than 40 nl l^{-1} should be considered for adequate plant protection. This is in agreement with the findings of Pihl Karlsson et al. (1995) for *T. subterraneum* and Ribas and Peñuelas (2003) for tobacco cv. Bel-W3. A meta-analysis is envisaged to ascertain whether this proposal is valid when data from pan-European observations and experimentation are analysed.

4. Conclusions

The results of our experiments indicate the great O_3 sensitivity of the therophytic plants from dehesa ecosystems based on the induction of O_3 visible injury. The Papilionaceae plants, particularly those belonging to the *Trifolium* genus, were more sensitive than the Poaceae species. Ozone induced both symptomatic and asymptomatic (senescence-related) foliar injury on some species at exposures well below current short-term critical levels. Therefore, indices based on a cut-off of $30\,\mathrm{nl\,l^{-1}}$ instead of current $40\,\mathrm{nl\,l^{-1}}$ threshold are proposed. These species could be useful in future O_3 bioindication programmes focused in the Mediterranean area.

Acknowledgements

This research has been performed under the framework of the UN ECE ICP-Vegetation and funded by the EU BIOSTRESS project, Contract EVK2-C-1999-00040. Thanks are given to Modesto Mendoza, Sonia Sánchez and Rosa Inclán for their help during field work and to Dr. César Gómez Campos (ETSI Agrónomos, Madrid), Dr. Francisco González López (Servicio de Investigación y Desarrollo Tecnológico, DG. de Producción, Investigación y Formación Agraria, Consejería de Agricultura y Medio Ambiente, Comunidad de Extremadura) and José Cebolla (ETSI Montes, Madrid) for their co-operation in seed supply.

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