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White clover clones as a cost-effective indicator of phytotoxic ozone: 10 years of experience from central Italy

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White clover clones can be successfully used to detect the presence of phytotoxic levels of ozone in the Mediterranean area, completing the data from analysers.

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ABSTRACT

Data collected at one site in central Italy using the NC-S/NC-R clover (*Trifolium repens*) biotype system during 1997–2007 were analysed in order to assess: (a) its performance under Mediterranean conditions; (b) variations of ozone damage linked with meteorological conditions; (c) if critical level approach is a good predictor of ozone risk on vegetation. NC-S dry biomasses were systematically lower than those of NC-R, the mean ratio being 0.7. Relevant relationship between ozone visible injury and cumulated values of AOT40 were also reported. Temperature and number of rainy days were the most important factors associated with ozone presence and, as a consequence, with leaf injury index. Photosynthetic gas exchange properties indicate that NC-S has higher values of stomatal conductance.

1. Introduction

Bioindicators have been used to detect the presence of toxic ambient levels of ozone (O_3) on a large scale at least since the early 1950s (Karlsson et al., 2003) and studies aimed at identifying suitable bioindicator species for areas which lack or cannot afford conventional monitoring networks continue. The sensitivity/resistance of plants to this pollutant is an important consideration underpinning the concept of critical levels, establishment of exposure indices (such as AOT40, Accumulated exposure Over the Threshold of 40 ppb, sensu Kärenlampi and Skärby, 1996), more recent flux-related exposure indices and the development of effective contamination-control legislation. Models developed to predict the impact of future increases in atmospheric O_3 concentration and their impact on vegetation have become increasingly sophisticated to take into account complex interactions between factors influencing O_3 fluxes and effects (Black et al., 2007).

Recently, Ferretti et al. (2007) have reviewed O_3 levels, uptake and plant response to O_3 in Italy and have concluded that O_3 exposure at remote sites exceeds concentration-based critical levels of United Nation Economic Commission for Europe (UN/ECE), if expressed in terms of AOT40. Among possible reasons, there are establishments of current critical levels in terms of cut-off value and accumulation level, environmental limitations to O_3 uptake and inherent characteristics of Mediterranean vegetation. Thus, it is necessary to provide data on long-term temporal and spatial trends of air pollutants and their effects to establish better biomonitoring relationships and their temporal evolution in spatially heterogeneous regions, such as the Mediterranean one (Ribas and Peñuelas, 2003).

A field experiment conducted in USA in the late '80s, revealed a broad range of variability in sensitivity to O_3 in the Regal commercial line of *Trifolium repens* L. (Heagle et al., 1989). The vegetative propagation of the plants selected on the basis of their field response determined the identification of two clones with opposite behaviour: NC-S (O_3-sensitive) and NC-R (O_3-resistant). Although this system has the peculiarity to show a detectable difference in biomass production at ambient O_3 levels (Heagle et al., 1994, 1995), the reason for the difference in O_3 sensitivity is not yet extensively fully understood. Only few authors studied metabolic changes induced by O_3 on these clones at the physiological level or the biochemical background or a combination of both (as for instance, Tang et al., 1999; Postiglione et al., 2000; Vandermeiren et al., 2002; D'Haese et al., 2005; Nali et al., 2005; Bermejo et al., 2006; Crous et al., 2006; Francini et al., 2007; Severino et al., 2007).

The clover clone system has been used successfully in the USA (Heagle and Stefanski, 2000), but when the UN/ECE led to establish with several experimental groups a common protocol to investigate the system usability in Europe, poorer correlations between O_3

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exposure and NC-S/NC-R biomass ratio were found. This was probably due to the lower O_3 concentrations that were recorded at the European sites when compared with the USA ones (Bermejo et al., 2002). Furthermore, when an artificial neural network was used to analyse the European data, the NC-S/NC-R biomass ratio was shown to be also affected by other variables, such as nitrogen oxide concentration and air temperature (Ball et al., 2000; Mills et al., 2000).

In Europe, variations in, and interactions between, a range of environmental factors make the O_3 dose responses of plants under uncontrolled conditions very "noisy" (Balls et al., 1995). This is true especially in Italy, where similar studies carried out in Rome, Naples, Milan and Pisa on sensitive and resistant clones of white clover have proved the importance of environmental factors as a modifying element of response to O_3 (Postiglione et al., 2000; Manes et al., 2003; Nali et al., 2004, 2007; Fagnano and Merola, 2007). The performance of this system could be assessed using an extensive data set from Mediterranean sites exposed to lower O_3 concentrations than those recorded in the USA, and showing a wide range of environmental variability.

In this paper, data collected in central Italy using the NC-S/NC-R clover biotype system during 1997–2007 are analysed to assess: (a) its performance under Mediterranean conditions; (b) variations of O_3 visible damage linked with meteorological conditions; (c) if a critical level approach is a good predictor of O_3 risk on vegetation.

2. Materials and methods

2.1. Experimental site, ozone exposure indices and climate parameters

Experiments were performed at San Piero a Grado (latitude 43° 40′ N, longitude 10° 19′ E, 3 m a.s.l.), a suburban village 8 km away on the SW of Pisa (Italy), well away from traffic and not directly influenced by any specific pollutant source. Meteorological parameters were monitored in a nearby station (300 m); they included: air temperature (minimum, mean and maximum, °C), total rain (mm and number of days) and total solar radiation (W m^{-2}).

An automatic photometric O_3 analyser was operating at Giardino Scotto in central Pisa; it belonged to the local environmental authorities (A.R.P.A.T.) and was run routinely (1 scan min^{-1}, one daily zero-span-zero cycle calibration procedure). The instrument was thoroughly checked twice a year. The height of the measuring point was 2 m. This instrument operated at an efficiency rate of 98%. O_3 concentrations are expressed in ppb in volume (1 ppb is 1.96 μg m^{-3}, at standard temperature and pressure). The following cumulative indexes of O_3 exposure were calculated in order to evaluate biological impact: AOT0, AOT10, AOT20, AOT30, AOT40, AOT60, AOT80, which represent the sum of the differences between the hourly O_3 concentrations (in ppb) and a threshold value of 0, 10, 20, 30, 40, 60 or 80 ppb, respectively, for each hour in the interval 08:00–20:00 h (solar time) (*Directive 2002/3/EC, in Official Journal of European Communities, L 67, March 9, 2002*).

The maximum daily hourly means (M1), the "daily highest consecutive 7-h concentration" (M7) and the 12-h mean from 09:00 to 21:00 (M12) were computed, together with the 24-h mean (M24) (sensu Karlsson et al., 2003).

2.2. Plant cultivation and harvest procedure

The white clover clone system was used in the experiments performed in 10 years (1997–2007, with exception of 1998). Each year, cuttings of NC-S and NC-R clones were received from the Coordination Centre of UNECE ICP-Vegetation at the Centre for Ecology and Hydrology (Bangor, UK) and were processed as described by the ICP-Vegetation European network protocol (http://icpvegetation.ceh.ac.uk/Reports/manual_PDF/ProtocolICPVEG2006%20with%20photosl.pdf) Following the methodology, every 28 days, above ground portions were cut and weighed (fresh weight), oven-dried at 105 °C and weighed again (dry weight). Twenty plants per clone were arranged in a completely randomized way and exposed to ambient air conditions at the experimental site. The exposure intervals were different in the different years, with the start of the exposure period varying from 8 May to 5 July and the date of final harvest varying from 30 August to 10 November.

Leaf greenness was assessed on mature leaflets with a chlorophyll meter (Minolta SPAD 502) at each harvest (20 measurements per plant) according to Ribas et al. (2005). Photosynthetic gas exchange was measured at each harvest with an open infrared gas analyser (CIRAS-1 PP-Systems). Measurements of stomatal conductance (Gw) and CO_2 assimilation rate (*A*) were carried out on the third fully expanded trifoliate leaves of stolons in three randomly selected plants (i.e. pots) of both clones. Data from leaves of each plant/pot were combined, as no significant differences were found among them. Subsequently, water use efficiency (WUE) was calculated as the A:Gw ratio. The calculation of intercellular CO_2 concentration (Ci) was based on the equations described in von Caemmerer and Farquhar (1981).

Plants were also examined for the occurrence of O_3 leaf injury, calculating the intensity of visible damage per pot, using a score from 0 to 5 (0: no lesions; 5: >50% of the leaves with very heavy injury) (Nali et al., 2004).

2.3. Statistical analysis

A temporal trend analysis was carried out by subjecting AOT40 values to Spearman's rank correlation procedure. The relationship among O_3 and meteorological parameters has been formulated as a regression equation, considering a multiple linear model. Two-way analysis of variance (factors being clone and time of harvest) was performed on the epigeous biomass data for the entire period. For each year and harvest, the means of NC-S vs. NC-R were compared with paired Student's *t*-test. Correlation analysis and determination coefficient were used to assess the relationships between variables (meteorological and O_3 parameters and biological data). All these analyses were performed using NCSS 2000 Statistical Analysis System.

3. Results

3.1. Meteorological characterization

Since 1997 to 2007 the weather was characterized by a maximum hourly mean temperature of 39.8 °C (Table 1), recorded in 2002; average temperature of the entire period was 21.4 °C. The total daily solar radiation was between 137 and 272 W m^{-2}. Compared with the historical data (temporal window from May 1 to October 31 in the 1942–1998), this period may be regarded as quite typical of the investigated area. A total rainfall of 506 mm was the maximum recorded (2002) and 7 mm was the minimum (2005). The mean of rainfall of the 10-year period was lower than those of the historical series (224 vs. 408 mm).

3.2. Instrumental O_3 monitoring

Ozone hourly means were analysed and averaged, and cumulated descriptors were derived (Table 2). Correlations between these descriptors were highly significant (*data not shown*), confirming previous observations (Nali et al., 2007). In 2001 mean index values were higher, showing maximum data of 84 and 89 ppb, for M12 and M7, respectively. The hourly maximum O_3 value reached 117 ppb on 5 September 1997. In 1997 and 2000, AOT40 and AOT60 levels were higher in comparison with the other years (Table 2). The trend in AOT40 (*data not shown*) showed that the average weekly O_3 load was 799 ppb h, with a peak of 2232 ppb h (week of June 10–16). A temporal trend analysis was carried out by subjecting AOT40 values to Spearman's rank correlation procedure: no linear temporal trend is identifiable in the

Table 1
Climate data in the period 1997–2007 at San Piero a Grado (Pisa) (temporal windows related to exposure times).

Year	Temperature (°C)		Rainfall (mm)	Solar radiation (W m^{-2})
	Max	Mean	Total	Mean
1997	34.3	21.6	154	256
1999	36.0	20.8	449	185
2000	35.2	20.1	365	210
2001	36.5	20.8	169	272
2002	39.8	20.2	505	229
2003	38.3	22.3	124	200
2004	33.9	20.4	166	192
2005	30.0	20.5	7	137
2006	34.6	25.5	132	188
2007	36.4	21.9	171	186

Table 2
Actual ozone levels in Pisa during the 10 years of biomonitoring campaigns. M7: daily highest consecutive 7-h mean concentrations (ppb); M1: maximum hourly peak (ppb); AOT: accumulated exposure over 0, 10, 20, 30, 40, 60 ppb (in the interval 08:00–20:00 h, solar time) for the duration of the exposure period (all in ppm h).

Year	M7		M1		AOT					
	Mean	Max	Mean	Max	0	10	20	30	40	60
1997	41	67	64	117	108	83	59	39	22	4
1999	32	73	45	99	73	49	31	18	9	1
2000	49	89	59	105	109	81	56	37	22	4
2001	53	89	59	93	69	53	38	24	13	2
2002	43	78	53	91	78	56	38	23	8	1
2003	52	79	62	99	93	72	53	36	21	3
2004	33	63	53	72	65	49	34	21	10	0
2005	48	66	57	81	77	61	45	29	15	0
2006	48	71	58	82	52	40	28	18	9	2
2007	40	69	51	91	62	45	31	18	8	1

series analysed ($r_s = -0.30$, $P > 0.05$). The "week-end effect" did not discriminate AOT40 value measures from Monday to Friday from those observed on Saturday and Sunday (107 ± 31.1 ppb h vs. 118 ± 40.0 ppb h, $P > 0.05$, Student's *t*-test).

The significant relationship (F-ratio = 10.766, $P < 0.001$) between O_3 and meteorological parameters is described in the following equation: $AOT40 = -10011.56 + 123.62Rf + 271.49Sr + 220.15T_{max}$, where maximum temperature (F-ratio = 5.034, $P < 0.05$), solar radiation (F-ratio = 16.998, $P < 0.001$) and the days of rainfall (F-ratio = 4.385, $P < 0.05$) are important factors.

3.3. *Biomonitoring of O_3 with clover clones and relationships with chemical and meteorological parameters*

Two-way analysis of variance of data showed a significant ($P < 0.001$) F-ratio for the sources of variability "clone" (150.1), "harvest" (99.9) and their interaction (6.1). At each harvest, the biomass of the NC-S was systematically lower than that of NC-R, the mean dry weight ratio NC-S/NC-R during the entire period being 0.7. The stronger percentages of difference in dry biomass production was observed in 2000 and 2002 (55 and 45%, respectively) (Table 3). Fresh weights show similar trends (*data not shown*). Besides the total epigeous biomass, the number of flowers produced by NC-S was significantly lower than those produced by NC-R (Table 3). NC-S usually had lower leaf greenness values in comparison to NC-R (Table 3). In all the campaigns, O_3 foliar injuries were observed on both clones (Fig. 1).

The ratio NC-S/NC-R showed an inverse proportionality with the AOT40 cumulative index ($y = 0.9884 - 3 \times 10^{-5}x$, $R^2 = 0.41$) (Fig. 2). It was also interesting to note that AOT40 at each harvest was not correlated with NC-S/NC-R ratio when the AOT40 values ranged between 0 and 3000 ppb h ($y = 0.4695 + 9 \times 10^{-5}x$, $R^2 = 0.26$, $P < 0.05$); on the contrary, it became significantly negative in the range of 3000–7000 ppb h ($y = 1.3993 - 0.0001x$, $R^2 = 0.44$, $P < 0.001$) (Fig. 3). The relevant relationships between O_3 visible injury and cumulated values of AOT40 are reported in Fig. 4. Negative significant correlations (Fig. 5) were also found between cumulated AOT40 and leaf greenness.

The association between leaf injury index and meteorological parameters is described in Table 4: mean temperature and the number of days of rainfall are the most important factors; solar radiation is not statistically significant.

Table 5 shows a synthesis of photosynthetic gas exchange data of the 2000–2001 campaigns. Photosynthetic activity of NC-S was significantly lower in comparison with NC-R. Results regarding Gw showed interesting differences between clones which were

Table 3
Epigeous biomass (dry weight, DW, g), flowers (mean number per plant, #) and leaf greenness (SPAD values, arbitrary units) of NC-S and NC-R white clover clones during the 1997–2007 period. Δ% is the percentage of reduction of NC-S in comparison to NC-R. The asterisks indicate the following: *** = $P \le 0.001$; ** = $P \le 0.01$; * = $P \le 0.05$.

Year	Clone	Epigeous biomass		Flowers		SPAD	
		DW	Δ%	#	Δ%	Arbitrary units	Δ%
1997	NC-S	25.9	−33.9**	43	−55.2***		
	NC-R	39.2		96			
1999	NC-S	11.1	−28.4**	70	−27.8**	45.8	−9.7
	NC-R	15.5		97		50.7	
2000	NC-S	10.4	−55.4***	44	−70.3***	42.3	−15.1
	NC-R	23.3		148		49.8	
2001	NC-S	18.8	−34.5***				
	NC-R	28.7					
2002	NC-S	6.7	−44.6***			40.2	−20.5**
	NC-R	12.1				50.6	
2003	NC-S	9.4	−19.6*				
	NC-R	11.7					
2004	NC-S	19.2	−21.3*				
	NC-R	24.4					
2005	NC-S	9.4	−28.2*			38.4	−21.0*
	NC-R	13.1				48.6	
2006	NC-S	23.9	−20.1**			42.4	−15.9*
	NC-R	29.9				50.4	
2007	NC-S	9.5	−44.8***			46.6	−2.7
	NC-R	17.2				47.9	

statistically significant especially in the year 2001, the NC-S Gw being higher than those of NC-R (+23%).

4. Discussion

In this study, a combination of data collected in 10 years involving the exposure of NC-S and NC-R white clover clones to ambient O_3 has been analysed. As known from the literature (Ferretti et al., 2007), the O_3 levels observed in the 10-year period (1997–2007) regarding this experience are typical in the Mediterranean area. Even if absolute maximum hourly values (M1) were not exceptionally high (117 ppb being the top score), the results highlight intense photochemical activity in central Italy. Although the situation is not particularly alarming, the information threshold

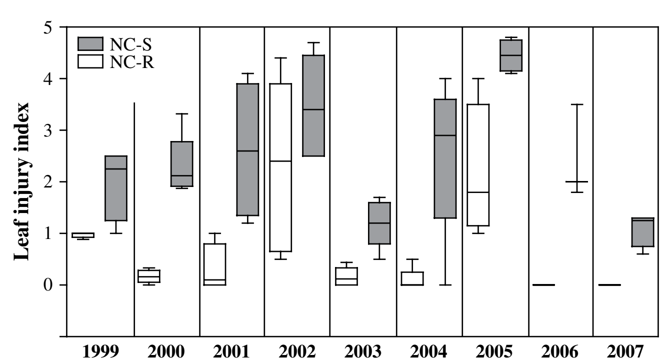

Fig. 1. Box-and-whiskers representation of the temporal trend of mean values of the leaf injury index (scale 0–5) observed on NC-R and NC-S clones of white clover exposed to ambient air in 1997–2007. For each year, the top line represents the 90th percentile, the bottom line represents the 10th percentile and the box represents the 75th percentile (upper side), the 25th percentile (lower side) and the median (50th percentile, central line), respectively.

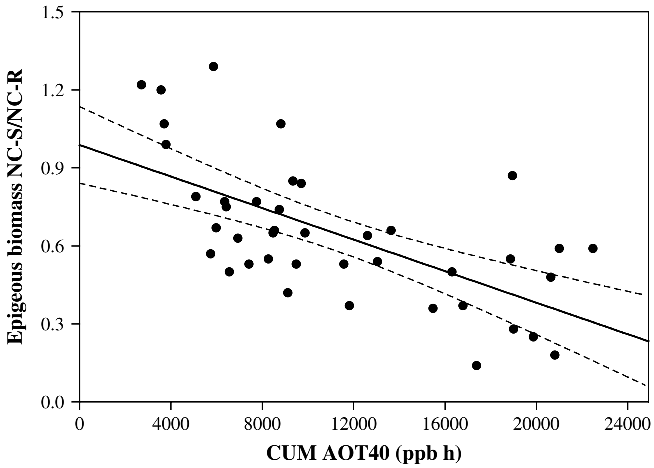

Fig. 2. Response of the NC-S/NC-R biomass ratio of white clover clones to AOT40 accumulated over the entire previous period between 1997 and 2007; broken lines represent the confidence interval ($P \leq 0.05$).

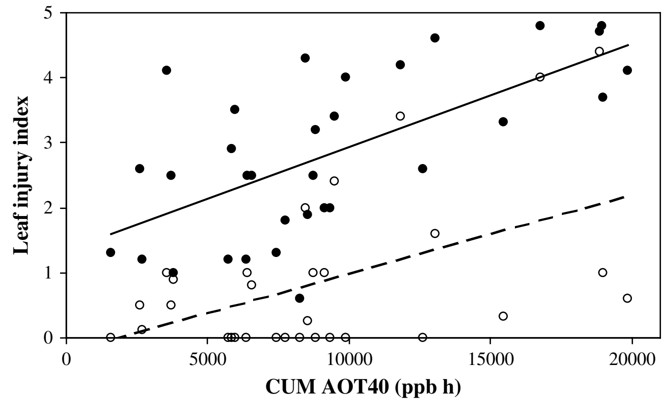

Fig. 4. Relationships between leaf injury index (0–5 scale) and O_3 concentration (CUM AOT40) for NC-S (closed circles and continuous line, $y = 1.3436 + 0.0002x$, $R^2 = 0.42$, $P < 0.001$) and NC-R (open circles and dotted line, $y = 0.2223 + 0.0001x$, $R^2 = 0.25$, $P < 0.01$) white clover clones exposed to ambient air during the growing seasons (1997–2007).

for population (M1 of 90 ppb) provided in the European law (*Directive 2002/3/EC, in Official Journal of European Communities, L 67, March 9, 2002*) is very frequently surpassed. The computation of the AOT40 values has systematically indicated high values (greater than 3 ppm h for three months, which is the long-term critical level for agricultural crops). Similar results have been reported from other Italian regions, such as Campania (Forlani et al., 2005) and in other sites of the Mediterranean area (Ribas and Peñuelas, 2003). The number of years of experimentation accounted for interannual variations of both ambient O_3 exposure and meteorological conditions: no temporal trend on a yearly basis was observed and the positive role of temperature and solar radiation in the photosmog complex was confirmed (Lorenzini et al., 1994). The lack also of the so-called "week-end effect" is in line with other studies still in the Mediterranean region (Nali et al., 2007). The analysis of the differences between weekday and week-end O_3 concentrations might help in identifying which pollutant reduction strategy [nitrogen oxides (NO_x) and/or volatile organic compound (VOC) control, according to the specific chemical regime, Sillman, 1999] would be more effective in reducing ambient O_3 concentration. Our results are in agreement with those recently reported by Viras (2002) in Athens during the 1984–1999 period: the reduction during week-ends of the mean values of O_3 precursors (NO_x and VOC) was estimated to be of the order of 10–20%, but the effect of this reduction has no consequence on the percentage of the violation of air quality limits for O_3. This is due to the fact that the reduction of O_3 depends not only on the absolute levels of NO_x and VOC, but also on their ratio. On the contrary, in California the presence of higher week-end O_3 concentrations suggests that the need for VOC control is greater than that for NO_x control (Altshuler et al., 1995).

Since 1991 to 2007, about 90 papers on white clover under O_3 exposure were published in ISI web of knowledge; among these, about 10 concerned the response of NC-R and NC-S biomass to this pollutant in open field. Although the contrasting behaviour of two clones in terms of biomass production and visible injury responses to O_3 is clearly admitted (Crous et al., 2006), there is generally disagreement on the association between O_3 indices and NC-S/NC-R biomass ratio or leaf injury index obtained from northern, central and south Europe. The same reality concerns Italian research groups who focused on the study of yield losses of white clover according to the ICP-Vegetation protocol. Studies conducted in Milan, Naples and Rome showed that yield losses of white clover clones were not significantly correlated with their respective AOT40 values (Manes et al., 2003). On the other hand, significant correlation was found in a survey carried out in Pisa (Nali et al., 2004) and nearby Florence (Nali et al., 2007). In our study, the extent of the biomass reduction was connected to the O_3 dose received over the entire previous period. This is in accordance with Fumagalli et al. (2003), who hypothesized a residual carry-over

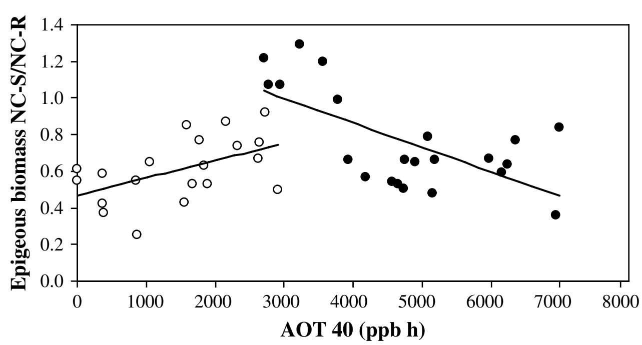

Fig. 3. Relationship between the NC-S/NC-R biomass ratio and AOT40 over the previous 28 days of each harvest. Continuous and dotted lines represent the best correlation between epigeous biomass ratio and AOT40 in the range of 0–3000 ppb h (open circles) and 3000–7000 ppb h (closed circles), respectively.

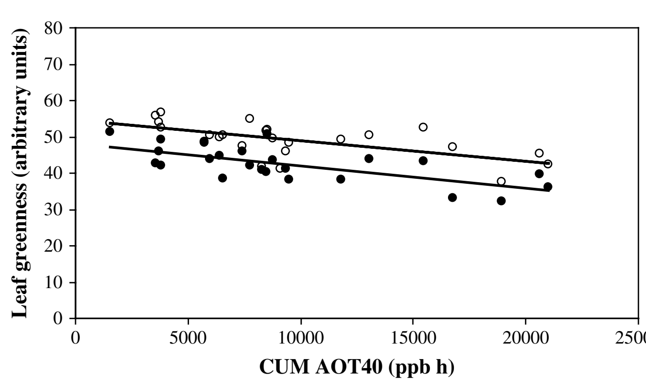

Fig. 5. Relationship between ozone dose (CUM AOT40) and leaf greenness for NC-S (closed circles and continuous line, $y = 47.965 - 0.0006x$, $R^2 = 0.46$, $P < 0.001$) and NC-R (open circles and dotted line, $y = 54{,}493 - 0.0006x$, $R^2 = 0.40$, $P < 0.001$) white clover clones exposed to ambient air during the growing seasons (1997–2007).

Table 4
Multiple regression analysis of the dependence of ozone foliar injury (in terms of leaf injury index, LII, scale 0–5) on mean temperature (T_{mean}, °C), total solar radiation (Sr, $W m^{-2}$) and rainfall (Rf, days). $LII = -2.8670 + 0.1367Rf - 0.0311Sr + 0.2162T_{mean}$. The asterisk indicates that differences are significant for $P \leq 0.05$.

Source	Sum of squares	Degree of freedom	Mean square	*F*-ratio	P
Total	36.06	21	1.72		
Regression	13.74	3	4.58	3.692	0.0313*
Rf	8.36	1	8.36	6.744	0.0182*
Sr	0.34	1	0.34	0.272	0.6085
T_{mean}	8.43	1	8.43	6.799	0.0178*
Error	36.07	18	1.24		

effect in the biomass responses of NC-S/NC-R between subsequent harvests. Moreover, when changes in climate occur from site to site this fluctuation in responses is not surprising. Bermejo et al. (2002) report that the response to O_3 of the clover biotype system is affected by environmental conditions: temperature and number of rainy days have strong influences on the system. That has been also found in our study.

A direct effect of O_3 on the flowering in the NC-S was also observed in our experiment, indicating that this pollutant influences the phenology of this clone. Although the ecological significance of O_3 effects on generative and vegetative reproduction has not been much accounted because most studies were finished before flowering of the plants, the potential impact of O_3 on the biomass and number of reproductive organs was shown for other species (Gimeno et al., 2004; Rämö et al., 2007).

Becker et al. (1989) found that white clover showed characteristic foliar necrotic lesions caused by ambient O_3 in Switzerland. This is also supported by Heagle et al. (1993). In our experiments, specific O_3 lesions are recorded on the leaves of both clones, but significant correlation was only found between cumulated AOT40 and score injury in NC-S. The determination coefficient indicates that performance of the O_3 index explained 41% of the variation in extent of visible injury. This reflects the conclusions by Karlsson et al. (2003): the cut-off concentration of 40 ppb left a rather large amount of visible injury unexplained, indicating that another cut-off concentration might be preferable. Emberson et al. (2000) also conclude that AOT40 explained the extent of leaf injury rather poorly.

Actually, there is strong evidence that other factors in addition to O_3 pollution influence the biomass ratios. Local parameters (the so-called 'level II factors'), such as phenological stage, nutritional state, co-occurrence with other pollutants and climatic elements should be considered when calculating site-specific critical levels of O_3 (Fuhrer and Achermann, 1999).

To improve O_3 dose/effect predictions, a more mechanistic approach, based on the flux of O_3 through stomata, has been recently developed (Emberson et al., 2000). On the other hand, Loreto and Fares (2007) report that O_3 uptake is not necessarily related to O_3 sensitivity and that probably O_3 scavenging molecules are involved. Because isoprenoids have an important antioxidant action, these authors conclude that a part of O_3 uptake may not be related to damage, at least in isoprenoid-emitting species, and may indicate the activation of defensive mechanisms scavenging reactive oxygen molecules and an overall reduced O_3 damage in leaves. Our data indicate that sensitive biotype has a higher Gw. Therefore O_3 uptake of this clone is potentially higher and this could be one of the factors for its sensitivity. Interestingly, Postiglione et al. (2000) have found that NC-S takes less O_3 than NC-R when measured at similar Gw, suggesting that O_3 uptake and O_3 damage are not necessarily directly correlated. White clover is not known as an isoprenoid-emitting species and no emission was detected by Loreto and Fares (2007). Thus, other compounds may be responsible for the large O_3 uptake of NC-R, such as ascorbate (Severino et al., 2007), although this finding is still controversial (D'Haese et al., 2005).

Regarding photosynthetic gas exchange parameters, it appears that limitations in mesophyllic CO_2 absorption should be regarded as a major responsible factor for reduced photosynthetic activity in NC-S. The extensive chlorosis observed in the sensitive clone suggests that severe damage to the photosynthetic system occurred. The extent of O_3-induced foliar injury and effects on photosynthetic activity also depend on environmental factors that affect Gw, as reported by Black et al. (2007) and as demonstrated by multiple regression between leaf injury index of NC-S and meteorological parameters in this study.

Results presented in this study indicate that: (a) the monitoring carried out with NC-S and NC-R white clover clones responds well to the concentrations of O_3 in our area, suggesting the good performance under Mediterranean conditions, also in the presence of interannual variations of both ambient O_3 exposure and meteorological conditions; (b) in this region, the variations in O_3 damage are linked with meteorological conditions; (c) the AOT40 based critical level approach is not a fully satisfactory predictor of O_3 risk on vegetation. Loreto and Fares (2007) have proved that O_3 flux into the leaves also may not necessarily be associated with damage. This finding calls for a redefinition of the stomatal O_3 uptake parameter to include a detoxification component. Further physiological and biochemical analyses are necessary to investigate if it is possible to find out a sensitive marker of the system, that may contribute to a better understanding of the mechanisms involved in plant response to pollutants.

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Table 5
Photosynthetic performance of white clover clones exposed to ambient air in 2000 and 2001. The values represent the average of 5 observations connected to harvests. A: photosynthetic activity; Ci: intercellular CO_2 concentration; Gw: stomatal conductance to water vapour; WUE: water use efficiency. Δ% is the percentage of reduction/increase of NC-S in comparison to NC-R. The asterisk indicates that differences are significant for $P \leq 0.05$.

Photosynhtetic parameters	2000			2001		
	NC-R	NC-S	Δ%	NC-R	NC-S	Δ%
A ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	17.9	15.6	−12.8*	21.7	18.1	−16.6*
Ci (ppm)	137	169	+23.3*	166	202	+21.7*
Gw ($mmol\ H_2O\ m^{-2}\ s^{-1}$)	205	215	+4.9*	266	329	+23.7*
WUE ($\mu mol\ CO_2\ mmol^{-1}\ H_2O$)	0.09	0.07	−22.2*	0.08	0.05	−37.1*

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