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Ozone and Water Stress: Effects on the Behaviour of Two White Clover Biotypes

Massimo Fagnano*, Gerardo Merola

Dipartimento Ingegneria Agraria e Agronomia del Territorio, Università di Napoli Federico II
Via Università 100, 80055 Portici (NA), Italy

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Abstract

Ozone is a strong oxidizing pollutant which derives by alteration of the photolytic NOx cycle and it accumulates in the troposphere spreading in rural areas and therefore determining injuries on natural vegetation and crops. Since its penetration occurs mainly through stomata, all factors which alter plant-atmosphere relations could be able to modify plant response to ozone. Interaction between ozone and water stress in Mediterranean environment was studied on ozone resistant and sensitive biotypes of white clover, which were grown in charcoal filtered and not-filtered Open Top Chambers in factorial combination with different levels of water supply. Measurements of biomass, leaf area and stomatal conductance were made during the growth period. Ozone injuries were estimated as not-filtered/filtered OTC yield ratio; the stomatal flux of ozone was estimated multiplying stomata conductance x diffusivity ratio between ozone and water vapour (0.613) x ozone hourly concentrations. The hourly values of ozone uptake were cumulated throughout the cropping periods of the two years. In the sensitive biotype, water stress reduced yield losses due to ozone from 38% to 22%, as well as yield losses due to water stress were reduced by the presence of ozone from 43% to 29%, while no interaction between ozone and water stress was observed in the resistant biotype. Biomass yield losses of the sensitive biotype were strictly correlated to cumulated ozone uptake (R^2 = 0.99), while biomass yield losses of the resistant biotype were not affected by the ozone fluxes variations created by the treatments. Flux based models could better estimate yield losses due to ozone in Mediterranean environments in which other stresses could be contemporary present; therefore, the new European directives might replace the actual thresholds based on ozone concentration with others based on ozone flux models.

Key-words: ozone pollution, water stress, stomata conductance, ozone uptake, clover, OTC.

1. Introduction

Ozone is a strong oxidizing pollutant of the troposphere which is formed by the alteration of the photolytic NOx cycle due to the presence of reactive hydrocarbons. These pollutant spreads and accumulates mainly in rural areas, because of lower levels of NO which contribute to ozone degradation. Therefore, tropospheric ozone is nowadays considered the most dangerous pollutant for natural vegetation and crops, and it is considered responsible of 90% yield losses due to atmospheric pollution (Heck et al., 1982).

Ozone injuries to vegetation are known since long time (Heggestad and Heck, 1971) and also in Italy yield losses are well known in many crops (Postiglione and Fagnano, 1993; 1995; Postiglione et al., 2000; Fumagalli et al., 2001). Nevertheless, these trials were made without water limitation, while the typical environmental conditions of the Mediterranean area are characterized by summer water deficit.

Since ozone penetration into leaves occurs mainly through stomata, all the factors which alter plant-atmosphere relations could be able to modify plant response to ozone (Guderian, 1985). In particular, pedoclimatic factors which reduce stomatal conductance, could also reduce ozone uptake by leaves and the consequent damages to crops (Darrall, 1989; Iqbal et al., 1996; Mansfield and Pearson, 1996).

In Mediterranean conditions, water stress is one of the most important modifying factors of ozone sensitivity because it reduces stomatal

* Corresponding Author: Tel.: +39 081 2539129; Fax: +39 081 7755129. E-mail address: fagnano@unina.it

conductance and therefore also reduces ozone uptake by plants and its effects on crops (Guderian, 1985; Dixon et al., 1998; Emberson et al., 2000).

This aspect is of a particular importance in defining the thresholds of damage to the vegetation in Mediterranean environment, since it is characterized by the frequent occurrence of water and ozone stress during the summer season.

The Italian (16/5/96 DM) and European (directive 2002/3/CE) legislations uses a concentration-based critical level for calculating the thresholds for the protection of vegetation from ozone damages. However, previous studies made in Italy (Ferretti et al., 2006) reported that ozone concentrations were poorly correlated with yield losses, even though they were very often higher than the recommended threshold. Therefore it is necessary to review the European legislation by adopting flux oriented models, able to estimate the reduction of plant stomatal conductance and ozone uptake caused by water stress.

In the last years several researches were made about a flux-based critical level, with the aim to modelling stomatal flux of ozone, taking in account the effects of the environmental factors in modifying the response of plant to ozone (Emberson et al., 2000; Grünhage et al., 2001; Danielsson et al., 2003).

The aim of this trial was to study the effects of the interaction between ozone and water stress on two biotypes of white clover that are used in international biomonitoring networks. We tested the hypothesis that the reduction of stomatal conductance caused by water limitation can reduce the yield losses due to ozone.

2. Materials and methods

2.1 Location, plant cultivation and exposure

The experiment was carried out during summer of 2001 and 2002 in “Parco Gussone”, of the Agriculture Faculty of Portici (20 m s.l.m.), near Naples City, in an area characterized by high levels of air pollution caused by car traffic.

The experiment was made with two white clover (*Trifolium repens* L. cv. “Regal”) biotypes selected in North Caroline (Heagle et al., 1991), one resistant (R) and one sensitive (S) to ozone. These biotypes were used in North-American biomonitoring nets (Heagle et al., 1995) and now they are considered the most useful system for estimating risks due to ozone pollution, using the ratio between biomass yield of the sensitive and resistant biotypes: Yield Losses (%) = (1-S/R)*100.

The plants were grown in Open Top Chambers (OTC), that were suggested as an exposure system to investigate the effects of ozone on plants in a more ecologically realistic way (Heagle et al., 1973). OTC are cylindrical structures (2.7 m high with a diameter of 3 m) covered with plastic films, in which it is possible to inlet filtered, not-filtered or ozone enriched air. They represent a good compromise between the plants exposed to ambient air, and those grown in fully controlled environments (i.e. growth chambers), which are too artificial and different from natural conditions.

Despite the advantages (e.g. low cost for both structure and maintenance), many authors have underlined that OTC could alter the microclimate (Unsworth, 1986; Norris and Bailey, 1996), determining an increase in temperature (Fuhrer et al., 1992) and VPD (Rana and Mastrorilli, personal communication) and a decrease in boundary layer resistance (Nussbaum and Fuhrer, 2000). However, Norris and Bailey (1996), showed that cover films with a light transmission coefficient near to 85% and a fan system which allows at least 3 changes of air per minute, can reduce the differences between the ambient air and the OTC. Indeed, Fagnano et al. (2004) reported that yield losses estimated in OTCs built following these indications were similar to those calculated with other methods.

In this experiment 8 OTC were used: 4 chambers were equipped with charcoal filters (CF-OTC) with a filtering area of 8.5 m^2 (SCF1/2-FPP/AFP Luwa Filters Shelter Technology) and an efficiency of ozone exclusion of up to 85%. Other 4 chambers received non-filtered ambient air (NF-OTC).

Clover plants were grown in 15 L pots with a water reservoir. Four fibreglass wicks per pot connecting the soil with the water reservoir provided a continuous supply of water. Plants were subjected to 2 water treatments: well watered (100%) and water stress (50%). Water reservoirs in 100% treatment were refilled each two days, while half of the amount used for the 100% treatment was used to irrigate the 50% one.

Eight pots per biotype (4 well watered and 4 water stressed) were placed in each OTC, so obtaining 4 pots per replication.

Stolons cuts of clover, provided by the co-ordination centre of UN/ECE ICP-Vegetation (United Nation Economic Commission for Europe, International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops), were grown according to the common protocol (UN/ECE, 1996). Cuttings were placed in a rooting substrate (H2 from BAAT®) in 14 cm pots, located in a greenhouse. After rooting, plants were transplanted in 15 L pots filled with a commercial substrate (90% peat and 10% perlite) enriched with slow release fertilizer Osmocote® (NPK = 14:14:14).

2.2 Measurements

In all the ambients, temperature, relative humidity and total radiation were recorded with Vaisala sensors connected to a data logger. Ozone concentrations were detected at the top of the canopy with a spectrophotometric UV device Dasibi™ 1108. Ozone data were recorded as hourly means which were used to calculate the following ozone pollution indices:

O_3max (daily maximum values);

$O_3$24 (average of 24 hour means);

$O_3$7 (average of 7 hour means: 10-16);

AOT40 (accumulated values exceeding the threshold of 40 ppb):

$$AOT_{40} = \sum_{I=1}^{n} ([O_3]_i - 40) \text{ if } [O_3]_i > 40 \text{ ppb}$$

where n is the number of hours of the period, $[O_3]_i$ is the ozone concentration of the hour i.

The first growth period, which ended on 26 and 27 June in 2001 and 2002 respectively, was not taken into account for the data analyses because it was considered as an acclimatization period.

The following 4 harvests were made every 28 days. At each harvest, biomass yield and leaf area were measured. The ratio leaf weight/leaf area was used to calculate leaf thickness, here expressed as Specific Leaf Weight (Hunt, 1978). One week before the harvests, gas exchange measures were made in the morning (8-9 am) at noon (1-2 pm) and in the afternoon (4-5 pm) using a LiCor 6200 device. Each two days, the whole-plant water use was measured by weighting the water reservoir before and after re-filling.

Yield losses due to ozone were estimated as the ratio between the yield in NF and CF OTC and they were expressed as

$$YL\ (\%) = (1 - NF/CF) \times 100$$

Yield losses due to water stress were estimated as the ratio between the yield in 50% and 100% water treatments and they were expressed as

$$YL\ (\%) = (1 - 50\%/100\%) \times 100$$

Stomatal fluxes of ozone (nmol m^{-2} s^{-1}) were calculated multiplying hourly values of stomatal conductance (mol m^{-2} s^{-1}) x diffusivity ratio between ozone and water vapour (0.613) x hourly ozone concentrations (nmol mol^{-1}), as suggested by Emberson et al. (2000). The daily fluxes from 7.00 am to 6.00 pm, were cumulated throughout the clover cropping periods of the 2 years.

All the data were subjected to analysis of variance, and the separation of mean was made with the LSD test with $P \leq 0.05$.

Stomatal conductance data were analyzed separately for the two years, using a split plot design: Ambients (NF-OTC vs. CF-OTC), as main plots, x Date (July, August, September, October), as sub-plots, x Hours (9-10, 13-14, 17-18), as sub-sub-plots, x Water treatments (100% vs. 50%), as sub-sub-sub-plots, x Biotypes (R vs. S), as sub-sub-sub-sub-plots.

Plant growth and water use data were analyzed according to the following split-plot design: Years (2001 vs. 2002), as main plots, x Ambients (NF-OTC vs. CF-OTC), as sub-plots, x Water treatments (100% vs. 50%), as sub-sub-plots, x Biotypes (R vs. S), as sub-sub-sub-plots.

Yield losses due to ozone were analyzed using the following split-plot design: Years, as main plots, x Water treatments, as sub-plots, x Biotypes, as sub-sub-plots.

Yield losses due to water stress were analyzed using the following split-plot design: Years, as main plots, x Ambients, as sub-plots, x Biotypes as sub-sub-plots.

2.3 Meteorological conditions

The lowest temperatures, the highest total amount of rainfall and the best distribution of rainfalls were recorded during 2002 (Fig. 1). The maximum temperatures in OTC, were 5° C higher than in ambient air, while minimum temperatures were often lower in OTC. On the av-

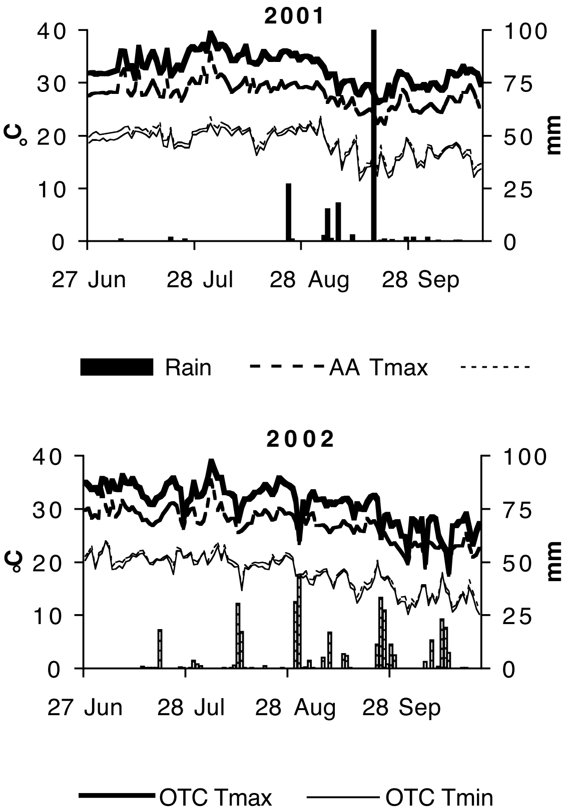

Figure 1. Daily temperatures and rainfalls in Ambient Air (AA) and in Open Top Chambers (OTC) during the two years.

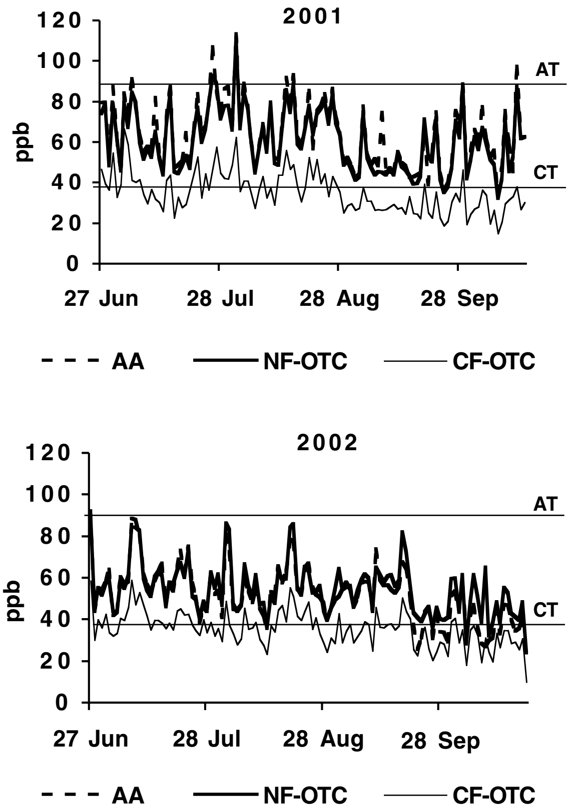

Figure 2. Daily maximum values of ozone in ambient air (AA), non filtered OTC (NF-OTC) and charcoal filtered OTC (CF-OTC) during the two years.
AT = attention threshold for human health (90 ppb); CT = critical threshold for ozone pollution (40 ppb).

erage of the whole cropping period, temperatures in ambient air were 23.5 and 22.6° C in 2001 and 2002, respectively, whereas in OTC mean temperatures were 25.5 and 24.4 in the 2 years; rainfalls were 178 mm in 19 days during 2001 and 351 mm in 47 days during 2002. 56%

Table 1. Ozone pollution indices in the 3 ambients during the cropping periods of the two years.

Ambient	Index	Unit	2001	2002
Ambient air	O_3 max	ppb	64.2	51.8
	O_3 24h	ppb	32.2	28.8
	O3 7h	ppb	52.7	41.8
	AOT40	ppb h	14789	7401
NF-OTC	O_3 max	ppb	61.1	54.4
	O_3 24h	ppb	30.9	42
	O3 7h	ppb	50.2	29
	AOT40	ppb h	12443	7901
CF-OTC	O_3 max	ppb	35.2	35.5
	O_3 24h	ppb	16.5	16.4
	O3 7h	ppb	27.2	26.9
	AOT40	ppb h	491	416

of rainfalls in 2001 fell in only one day (100 mm on 15 September).

2.4 Ozone trend

Ozone concentration levels were lower in 2002 (Table 1) during which higher rainfalls were recorded, confirming that ozone pollution is negatively correlated to rainfalls and particularly to the number of rainy days (Fagnano et al., 2004). Ozone concentrations were very similar in both ambient air (AA) and NF-OTC, whereas the lowest values were recorded in CF-OTC. AOT40 values, cumulated in 112 and 116 days during the years 2001 and 2002 respectively, were very higher than the thresholds stated in the UE Directive (2002/3/CE) for the protection of vegetation from ozone injuries (9000 ppb h in 2010 and 3000 in 2020).

The analysis of maximum daily values suggests (Fig. 2) that the ozone concentration of 90 ppb, which is reported in the Italian low DM – 16/5/96 as the warning threshold for human health protection, was exceeded in 7 days in 2001 and in only one day in 2002. On the other hand, the value of 40 ppb, which is considered the critical threshold of ozone pollution (Kärenlampi and Skärby, 1996) was exceeded in 96% of the monitored days in 2001 and 82% of the days in 2002.

In both years the hourly trend of ozone, calculated as the average of the whole cropping period (Figure 3), was that typical of plain areas (Manes et al., 2002), showing a quick increase from early morning (7-8 am) to the central hours of the day (1-2 pm), and a decrease during the afternoon, reaching the minimum value very late at night: the threshold of 40 ppb was exceeded from 10 am to 7 pm (10 hours) in 2001 and from 12 am to 6 pm (7 hours) during 2002; in CF-OTC ozone values exceeded the threshold of 40 ppb only in few days: therefore AOT_{40} was negligible.

3. Results and discussion

3.1 Stomatal conductance

Stomatal conductance (Table 2) showed similar values in the two years (0.35 mol m^{-2} s^{-1} in 2001 and 0.37 in 2002, on the average). From the analysis of variance, the interactions Date x Water treatment, Hour x Water treatment and Ambient x Biotype were significant in both the years.

The interaction Date x Water treatment showed that water stress significantly reduced stomatal conductance in July in both the years and in August only in the first year that was characterized by lower rainfalls and higher temperatures (Fig. 4).

The interactions Hour x Water treatment showed that the water stress reduced stomatal conductance in the central hours of the day (13-14) in both the years and in the morning (9-10) in the first year only (Fig. 5).

From these data, it clearly rises that the more frequent rainfalls and the lower temperature of the second year reduced the effect of the water treatments.

The interaction Ambient x Biotype confirmed that ozone injuries can reduce stomatal conductance in the sensitive genotypes (Danielsson et al., 2003): the stomatal conductance of the resistant biotype was not modified by the

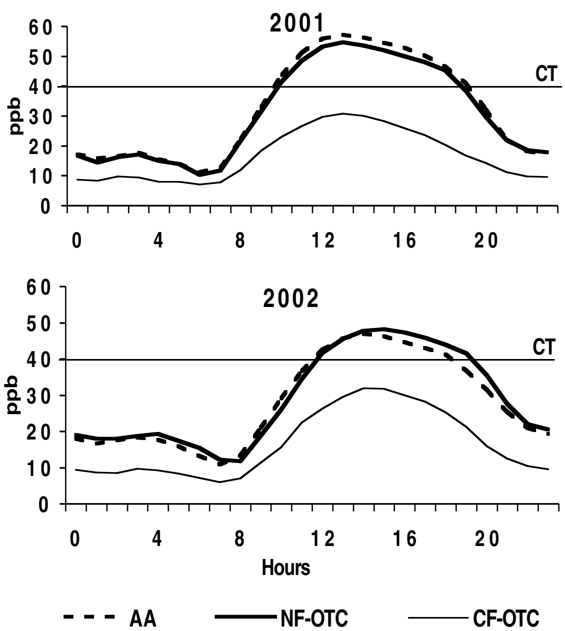

Figure 3. Daily trend of ozone in Ambient Air (AA), Non Filtered OTC (NF-OTC) and charcoal Filtered OTC (CF-OTC): hourly value (average of the crop period).
CT = critical threshold for ozone pollution (40 ppb).

Table 2. Stomatal conductance in the two years: average values of main factors.

	2001	2002
Ambient		
NF-OTC	0.32	0.35
CF-OTC	0.38	0.39
Significance (P)	*n.s.*	*n.s.*
Date		
July	0.43	0.33
August	0.32	0.32
September	0.27	0.37
October	0.39	0.46
Significance (P)	***	***
Hour		
9-10	0.36	0.36
13-14	0.42	0.39
17-18	0.28	0.36
Significance (P)	***	*n.s.*
Water Supply		
100%	0.39	0.39
50%	0.31	0.34
Significance (P)	***	**
Biotype		
Resistant	0.32	0.36
Sensitive	0.38	0.38
Significance (P)	***	*n.s.*

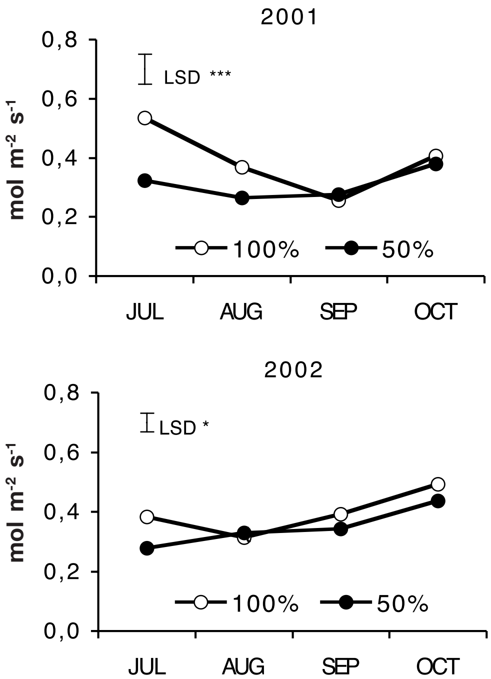

Figure 4. Stomatal conductance in the two years: interaction Date x Water treatment.

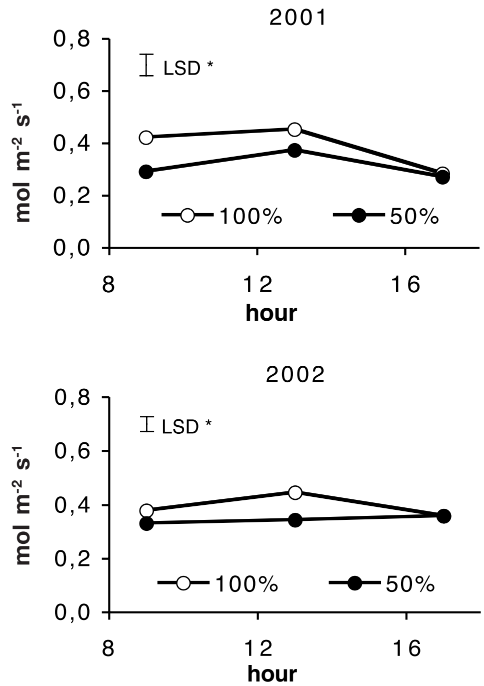

Figure 5. Stomatal conductance in the two years: interaction Hour x Water treatment.

presence of ozone, while that of the sensitive biotype was significantly reduced in presence of ozone (TaB. 3).

These data also shows that the sensitive biotype has a higher stomatal conductance when grown in clean air. Therefore the ozone uptake of this genotype is potentially higher, and this could be one of the factors of its sensitivity to ozone.

3.2 Plant growth and water use

The effect of the years on plant behaviour was not very marked (Table 4). Little differences were observed only in the average area per leaf, total leaf area per plant and water use that were lower in the first year, and in weight/area ratio of leaves (SLW) that was higher. These differences could be due to the more severe drought conditions recorded in 2001.

Table 3. Stomatal conductance (mol m^{-2} s^{-1}): Interaction Ambient x Biotype in the 2 years.

	2001		2002	
	Resistant	Sensitive	Resistant	Sensitive
NF-OTC	0.304	0.336	0.352	0.349
CF-OTC	0.341	0.419	0.367	0.410
LSD	*0.046*		*0.041*	

Dry Weight (Fig. 6) and Leaf Area (Fig. 7) showed similar behaviours. In both the cases, the interaction Ambient x Water treatment x Biotype was significant.

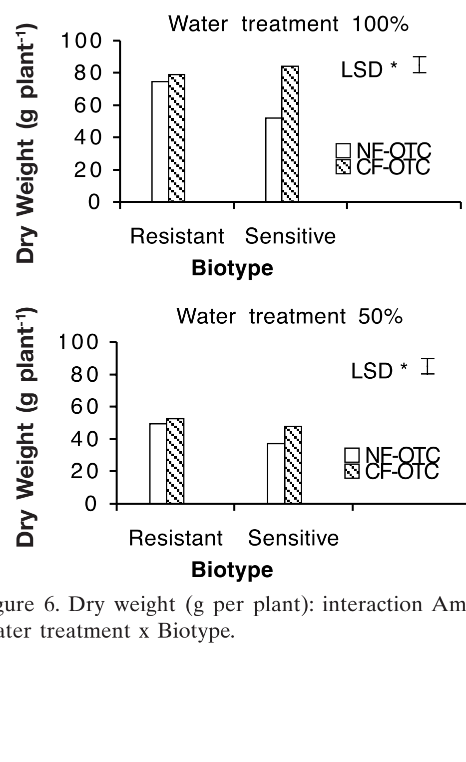

Figure 6. Dry weight (g per plant): interaction Ambient x Water treatment x Biotype.

Table 4. Plant growth and water use: average values of main factors.

Main factors	DW g $plant^{-1}$	Leaves Nr. $plant^{-1}$	Leaf area cm^2 $leaf^{-1}$	Leaf area cm^2 $plant^{-1}$	SLW g m^{-2}	WU L pt^{-1} d^{-1}
Year						
2001	60.1	780	12.0	0.96	44.7	1.52
2002	58.9	847	12.6	1.10	39.1	2.06
Significance (P)	*n.s.*	*n.s.*	*	*	***	***
Ambient						
NF-OTC	53.2	726	12.2	0.91	41.9	1.68
CF-OTC	65.8	901	12.4	1.15	41.9	1.90
Significance (P)	***	***	*n.s.*	***	*n.s.*	***
Water Supply						
100%	72.4 A	945	13.7	1.30	40.7	2.24
50%	46.7 B	683	11.0	0.76	43.1	1.34
Significance (P)	***	***	***	***	*	***
Biotype						
R	63.8	888	11.6	1.06	44.1	1.77
S	55.2	739	13.0	1.00	39.7	1.80
Significance (P)	***	***	***	*n.s.*	***	*n.s.*

Note: DW = Dry Weight, SLW = Specific Leaf Weight, WU = Water use per plant.

The sensitive biotype showed a lower growth than the resistant one in presence of ozone, while no difference between the biotypes was observed in filtered air, confirming the results obtained in previous trials (Fagnano et al., 2004). These differences were more marked in well watered conditions.

Number of leaves per plant and the average area of leaves were both reduced by water stress, while SLW was increased (Ta. 4). The average area of leaves and SLW were not influenced by the presence of ozone, but the former was higher in the sensitive biotype, while SLW was lower as reported in previous experiments carried out in the same environment (Postiglione et al., 2000).

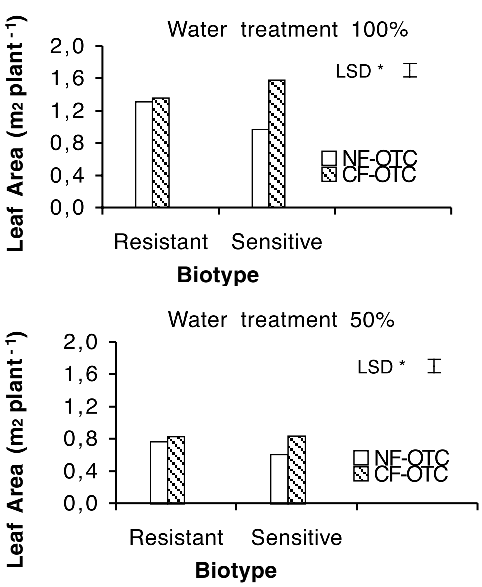

Figure 7. Leaf Area (m^2 per plant): interaction Ambient x Water treatment x Biotype.

The lower SLW of this biotype could be another factor causing ozone sensitivity, since the lower SLW can be associated to an increased apoplastic space that may facilitate the ozone diffusion among the target cells (Bennett et al., 1992; Evans et al., 1996).

As regards the number of leaves per plant, the interaction between Ambient and Biotype was significant (Fig. 8a): the sensitive biotype showed a decrease in the number of leaves in presence of ozone, while no difference was observed in the resistant one.

The Water Use per plant of the two biotypes was differently influenced by the presence/absence of ozone (Fig. 8b). The sensitive biotype showed the highest water use in filtered air and a severe reduction in presence of ozone, while no variation was observed in the water use of the resistant biotype in relation to the presence/absence of ozone.

The lowering of water used in the sensitive biotype in not filtered air can be related to the already mentioned effects of ozone on leaf area and on stomatal conductance.

3.3 Yield losses

The effect of the year did not influenced yield losses due to ozone, and that due to water stress.

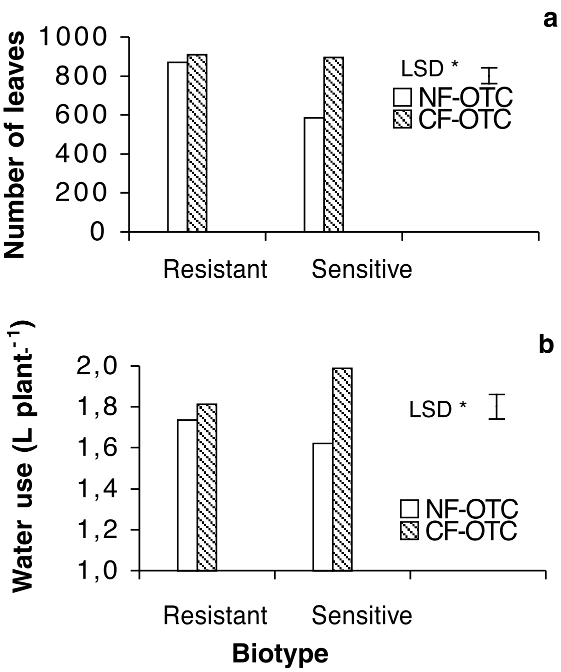

Figure 8. Number of leaves per plant (a) and water use (b): Interactions Ambient x Biotype

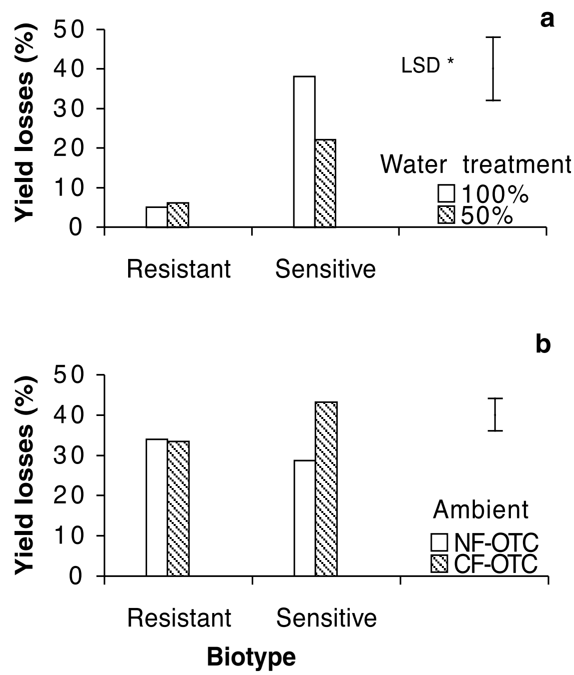

Figure 9. Yield losses due to ozone (a) and to water stress (b).

The interactions Water treatment x Biotype in the first case, and Ambient x Biotype in the second one, were significant.

Water stress reduced yield losses due to ozone in the sensitive biotype from 38% to 22%, while no difference was observed in the resistant one (Fig. 9a). This can be related to the decrease in stomata conductance caused by water stress which reduced ozone uptake by plants.

Water stress effect (Fig. 9b) caused severe yield losses in both biotypes, but the presence of ozone in NF-OTC reduced its effect on the sensitive biotype from 43% to 29%. Also in this case the presence/absence of ozone did not modify the response of the resistant biotype.

The effect of water stress in reducing ozone damages, because of the reduction of stomatal conductance and of ozone uptake by plants, is very clear from the regression analysis between yield losses and ozone uptake (Fig. 10), here calculated on a hourly basis by the stomatal conductance measured during the day and throughout the cropping periods of the two years and by the respective hourly values of ozone. The hourly values of ozone uptake were cumulated throughout the cropping periods of the two years.

Yield losses of the sensitive biotype were strictly correlated ($R^2 = 0.99$) with the cumulative uptake of ozone, while yield losses of the resistant biotype were not related to the ozone flux variations created by the treatments.

4. Conclusions

In a sub-urban environment of Southern Italy, ozone values during summer were very high and exceeded the thresholds for vegetation and human health protection. Ozone values constant-

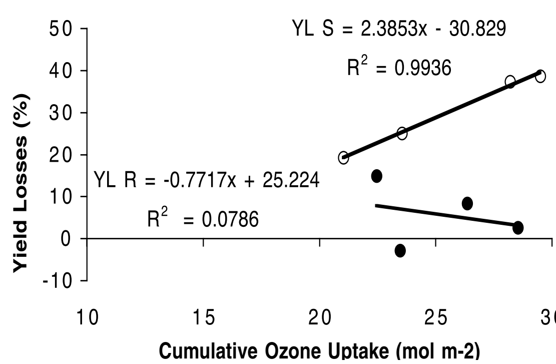

Figure 10. Relations between Yield Losses of Sensitive (open circles) and Resistant biotypes (filled circles) and cumulative ozone uptake.

ly resulted higher than the objective thresholds reported in recent European directives, even during years with unfavourable conditions for ozone accumulation.

In Mediterranean conditions, the white clover showed severe yield losses as highlighted by the comparison between sensitive vs. resistant biotype. Some factors associated to ozone sensitivity could be the higher stomatal conductance (higher ozone uptake by plant) and the lower specific leaf weight (higher internal air space volume) found in the sensitive biotype.

Interaction between water and ozone stresses, as typical conditions of the Mediterranean area, seemed to be antagonistic: water stress caused a partial protection for the sensitive types reducing stomatal conductance and ozone uptake by plants.

Yield losses due to ozone was positively correlated to ozone flux in the sensitive biotype of clover.

Models based on ozone stomatal flux represent better the negative effects of this pollutant on crops, especially in Mediterranean area characterized by frequent conditions in which many stresses (i.e. water, thermal, salt) contemporarily damage the natural vegetation and crops. The results of the present study suggest that the new European directives should modify the actual thresholds based on ozone concentrations, by adopting specific models for ozone uptake estimation.

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