Chapter 2.

Ozone phytotoxicity in Catalonia: Bioindication studies

2.1. Preliminary studies on bioindication in the study area: different methodological approaches

2.1.1. Evaluation of tobacco cultivars as bioindicators and biomonitors of ozone phytotoxical levels

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Summary

A field study was conducted from May to October 1995 to assess ozone (O_3) phytotoxicity in Catalonia (NE Spain) by determining a percentage value of leaf area injured by ozone on three tobacco cultivars, Bel-W3, Bel-C and Bel-B as bioindicators. Colorimetric parameters were also determined in an effort to have an objective assessment of ozone injury. The study was conducted simultaneously on eight sites where ozone levels and several meteorological parameters were continuously monitored. Two sets of plants were used at each site. The first one was composed of six plants of each cultivar which were changed every two weeks while the second one involved two plants of each cultivar which were kept in the plots throughout the whole experimental period. Open Top Chambers were also used to test the response of the three cultivars to ozone under controlled conditions. The ozone levels correlated well with ozone injury on the Bel-W3 cultivar but not as well on the other two cultivars. The ozone levels did not fully account for all the observed injury. The response of tobacco plants to ozone concentrations and therefore its biomonitoring capacity depended also on different environmental conditions linked to stomatal behaviour such as temperature, humidity, wind or altitude. These environmental conditions had some effects on the intensity of Ozone injury. Ozone concentrations accumulated over a threshold of 60 ppb_v (AOT60) when VPD was below 1 kPa. Correlated well with the ozone injury and best explained the intensity tobacco injury symptoms. For large plants growing throughout the whole period of study, Bel-C was the best indicator cultivar for AOT60 over the 3 days prior to the last ozone injury assessment. The colorimetric parameters were indicators of seasonal changes but they were not good ozone damage indicators. It is concluded that tobacco cultivars were good bioindicators but meteorological and other environmental factors need to be considered in there use as biomonitors.

Key words: Ozone, Tobacco cultivars, Bel-W3, Bel-C, Bel-B, phytotoxicity, colorimetry, Catalonia.

Introduction

The coincidence of high solar radiation with industrialization and the high density of population involving vehicular traffic favour ozone formation on the Spanish Mediterranean coast (Gimeno et al., 1993b; Peñuelas et al., 1995a). In areas like Catalonia, the orographic situation determines the existence of local concentration differences. For example, coastal mountains facilitate the existence of local high ozone concentrations since the recirculation of polluted air masses due to air flow regimes associated to the coupling of mountain-valley and sea-land breezes determines the aging of ozone precursors through photochemical reactions (Martin et al., 1991; Millán et al., 1996). These high ozone levels may become toxic to plants and animals (Peñuelas, 1993).

The exposure of plants, to concentrations of ozone ranging from 40 to $70ppb_v$, quite common in many Mediterranean coastal sites, can produce growth reduction, and other physiological and ecological effects (Peñuelas et al., 1994, 1995 a; Elvira et al., 1995). Deleterious effects of ozone on Mediterranean crops, fruit trees and natural species have been reported under experimental or field conditions (Reinert et al., 1992; Gimeno et al., 1993a, 1994, 1995a; Peñuelas et al., 1995 a; Elvira et al., 1995; Paolacci et al., 1995; Velissariou et al., 1996).

Ozone enters the plant leaves via open stomata through gas exchange between the plant and its immediate environment. Leaves that are still expanding and are just approaching full expansion are the most susceptible to ozone injury. For this reason, ozone injury is usually found on older leaves. Palisade parenchyma cells are injured first and mesophyll cells later, if injury is severe or exposure prolonged. Thus, injury is often more severe on upper leaf surfaces, but may occur on both surfaces (Manning and Feder, 1980).

Ozone causes a wide array of symptoms on many plants. A common symptom of ozone injury on plants is "flecking". It is usually an indication of acute injury. Small necrotic spots or flecks occur due to death of palisade cells. These flecks are metallic or brown and often bleach to tan or white with age. Flecks may coalesce to form blotches which can result in chlorosis and leaf fall (Manning and Feder, 1980).

These characteristic symptoms on plants can be used to assess ozone phytotoxicity. Many plants have been described as ozone sensitive, such as bean, cucumber, potato, or tobacco plants (Manning and Feder, 1980). There are three tobacco (*Nicotiana tabacum*) cultivars (Bel-B, Bel-C and Bel-W3) differing in their ozone sensitivity that have been often used in combination as bioindicators (Heggestad, 1991). However, most bioindicator programs conducted all over the world have involved the use of only tobacco cv. Bel-W3 to assess ozone phytotoxicity (Mignanego et al., 1992; Bytnerowicz et al., 1993; Lorenzini, 1994).

Previous to the appearance of injury, plants develop mechanisms of defense in response to ozone stress. For example, quinone by-products are produced (Howell and Kremer, 1973) or there is activation of the defense-related proteins in tobacco leaves (Langebartels et al., 1990; Rosemann et al.,1991; Schraudner et al.,1992; Kangasjärvi et al., 1994). The measurement of these changes in physiological or biochemical parameters can be used as an ozone damage index.

The high ozone concentrations and phytotoxicity in crops and natural vegetation is an important problem in eastern Spain (Salleras et al., 1989; Reinert et al., 1992; Gimeno et al., 1995a). Preliminary studies to assess ozone phytotoxicity involving the use of tobacco cultivars Bel-W3, Bel-C and Bel-B have been carried out in Catalonia (NE Spain) (Gimeno et al., 1995b; Peñuelas et al., 1995b). We conducted the present study to assess the sensitivity and efficiency of the three cultivars of tobacco as ozone bioindicators, to obtain a ozone damage map of Catalonia and to study the possibilities of colorimetric indices for the assessment of ozone damage and early detection. Furthermore, we attempted to relate plant ozone damage with other environmental parameters such as temperature, wind and humidity (vapor pressure deficit, VPD) in order to study the possible use of tobacco cultivars for biomonitoring ozone levels.

Material and methods

Measurement sites

The study was performed in 1995 at Catalonia (N.E. Spain). The bioindicator plants were distributed in eight rural sites of Catalonia covering a variety of environmental conditions ranging from sea level to 1040 meters high and from sea-shore to 250 km in-land (Figure 1, Table 2). The plants were placed in the monitoring stations of the Rural Network of the Environmental Department of the Generalitat de Catalunya containing ozone monitors and meteorological sensors.

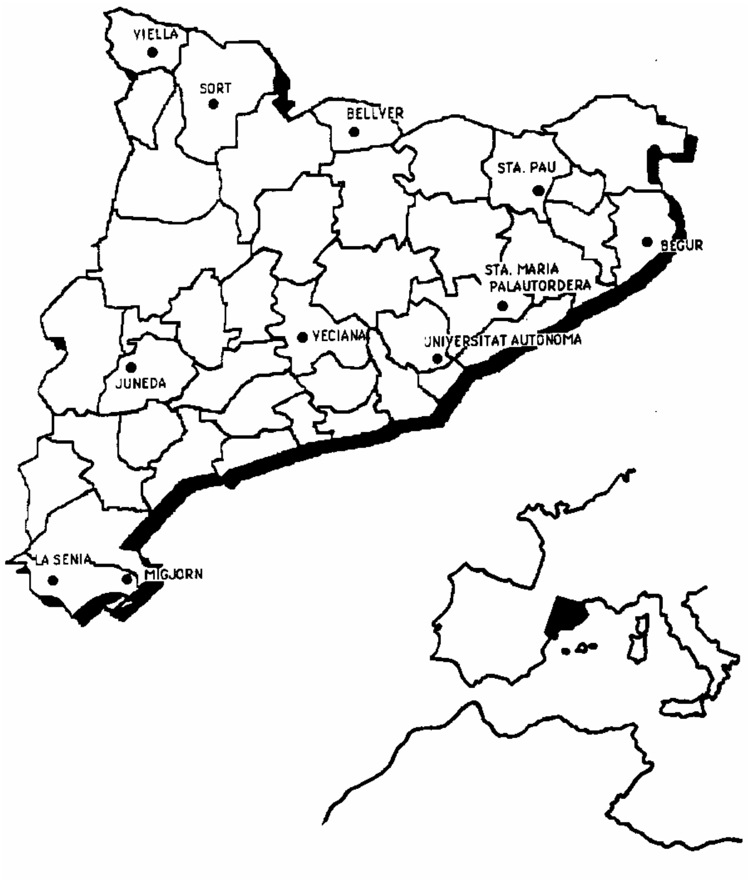


Figure 1. Map of the studied stations in Catalonia (N.E.Spain).

Plant bioindicators

Six tobacco (*Nicotinana tabacum*) plants of each cultivar Bel-B, Bel-C and Bel-W3 were used as ozone bioindicators in each site. The Bel-W3 cultivar is considered as sensitive, the Bel-C cultivar is considered as moderately sensitive, and Bel-B cultivar is considered as resistant. Tobacco seeds were germinated in open top, charcoal filtered air, free of ozone to obtain homogeneous plants and to avoid possible early contamination of ozone. When seedlings were at the 4th leaf stage, they were transplanted to 8 L pots filled with 25 % peat, 25 % vermiculite, 25 % perlite and 25 % sand. Soil pH of these pots was adjusted to 6.0 by adding $CaCO_3$. A NPK 15:11:13 slow-release fertilizer (Osmocote plus) was also added. A self-watering system was used in each pot by placing them above individual self-watering reservoirs communicated by two wicks. Ozone-induced visible injury was assessed after two weeks for the presence or absence of injury on the three cultivars and by measuring colorimetric parameters. New plants (small plants, SP) were replaced every two weeks from May to October.

Ozone phytotoxicity was characterized as low (level 1) when ozone injury was only recorded on Bel-W3, as medium (level 2) when it was recorded on Bel-W3 and Bel-C, and as high (level 3) when the three cultivars, including the most resistant cv Bel-B, were injured. When no visual symptoms were found in any of the three cultivars, the level of phytotoxicity was considered 0.

As a complementary assessment, two plants (large plants, LP) of each cultivar were grown for the whole period of study (May through October) in 16 L pots in each station. Damage and color evolution was measured in their leaves every two weeks.

Visual damage parameters

Percentage of damaged leaf area, number of damaged leaves per plant, and number of damaged plants per cultivar were visually recorded. Percentage classes of damaged leaf area and damaged leaf number were estimated in 5 % intervals and only those measures higher than 5 % were considered. Numbers of damaged plants lower than 20 % were not considered. The average percentage of damaged leaf area was calculated in the four first leaves, except in large plants, where the average percentage of damaged total leaf area was calculated considering only the damaged leaves.

Colorimetric parameters

A Croma Meter Cr-200 (Minolta Osaka, Japan) was used to measure the colorimetric parameters of tobacco plants. Color as perceived has three dimensions: hue, chroma and lightness. Hue and chroma, specified by two chromaticity coordinates, a (red—>green) and b (yellow—>blue). Since these two coordinates cannot describe a color completely, a lightness factor (L) must also be included to identify a color precisely. The first whole three leaves of each one of the plants were measured (beginning with the leaf closer to root neck).

Exposure of tobacco cultivars in OTC

Parallel experiments were conducted in the same period to check the response of the three cultivars to controlled ozone concentrations. Bel-W3, Bel-C and Bel-B plants were exposed in the Open Top Chambers (OTC) to either Charcoal Filtered Air (FI), Non Filtered Air (NF) or Non Filtered Air plus 40 ppb_v ozone (NF+). Eight plants per chamber were used. Two experiments were performed at the OTC experimental field in the Ebro Delta (managed by CIEMAT). The first experiment was run from the 16th to the 31st of August, while the second one took place from the 1st to the 15th of September. The damage assessment was conducted by measuring the percentage of affected leaves and the percentage of affected plants.

Ozone and meteorological measurements

AOT40 and AOT60 (Accumulated exposure Over a Threshold of 40 ppb_v and 60 ppb_v respectively) calculations were performed to make ozone values biologically meaningful. The AOT40, the sum of the differences between the hourly concentrations in ppb_v and 40 ppb_v for each hour when the concentration exceeds 40 ppb_v (Fuhrer and Acherman, 1994), was calculated for those diurnal hours with solar radiation above 50 W m^{-2} following the definitions stated at the UN/ECE workshops held at Bern in 1993 and Kuopio in 1996. AOT40 values were calculated for every three days and for the fortnightly assessment periods. We also calculated AOT40 without considering a cut-off of diurnal radiation and AOT40 considering different cut-offs of vapor pressure deficit from 0 to 1.5 kPa. The AOT60 calculations were conducted the same way but taking a cut-off of 60 ppb_v.

To characterize weather conditions, meteorological data (pressure (HPa), solar radiation (W m^{-2}), rain (mm), wind speed (m s^{-1}), wind direction (^{O}), vapour pressure deficit (kPa), relative humidity (%) and temperature (^{O}C)) were also recorded. We also calculated accumulated humidities over cut-offs of 50 % and 70 % (AHOT50 and AHOT70 respectively) in a similar way to the above mentioned AOT40 and AOT60 for ozone. Wind speeds below 2 m s^{-1} were not considered.

Statistical Analyses

For wind direction statistics (means and variances) a circular statistic programme package (Oriana, Kovach Computing Services, Anglesey, Wales, UK) was used. For all the other statistical analyses and variables Statwiew 4.5 (Abaccus Concepts Inc., Berkeley, LA, USA) was used. Data were transformed in several ways: logarithmic, $\sin^{-1} x^{1/2}$ or $x^{1/2}$ with no improvement of correlations between biological damage parameters and environmental parameters. Besides from correlation analyses, stepwise multiple regression was used because of its ability to determine the order of importance of the meteorological variables in best explaining variation in leaf damage and of biological variables in best explaining variation in ozone concentrations.

Results

Open Top Chamber experiments

No visible injury was recorded in any tobacco cultivar when plants were exposed to the FI treatment open top chambers (OTC) in Ebro Delta (Figure 2). Different amounts of ozone injury were recorded in the different cultivars depending on their sensitivity following exposure to ozone. Thus, ozone injury was most severe in Bel-W3 plants, followed by Bel-C and Bel-B plants in NF and NF+ treatments. Similarly, the intensity of injury was the highest in the NF+ treatment for any given cultivar. The percentage of affected leaves appeared to be a better indicator of ozone exposure than the percentage of plants affected, which reached 100 % earlier (Fig.2).

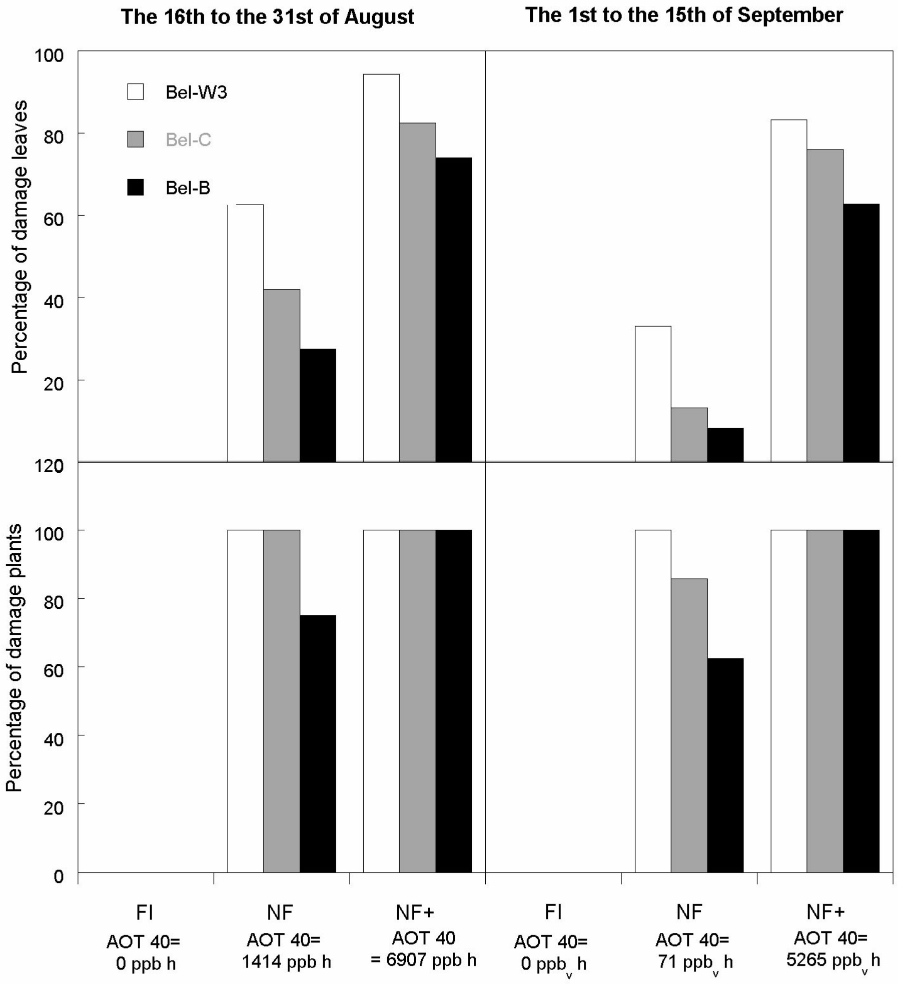


Figure 2. Percentage of the damaged leaves and damaged plants in the three treatments (FI Filtered air, NF Non filtered air, and NF+ Non filtered air + 40 ppb$_v$ O_3) of Open Top Chamber controlled measurements.

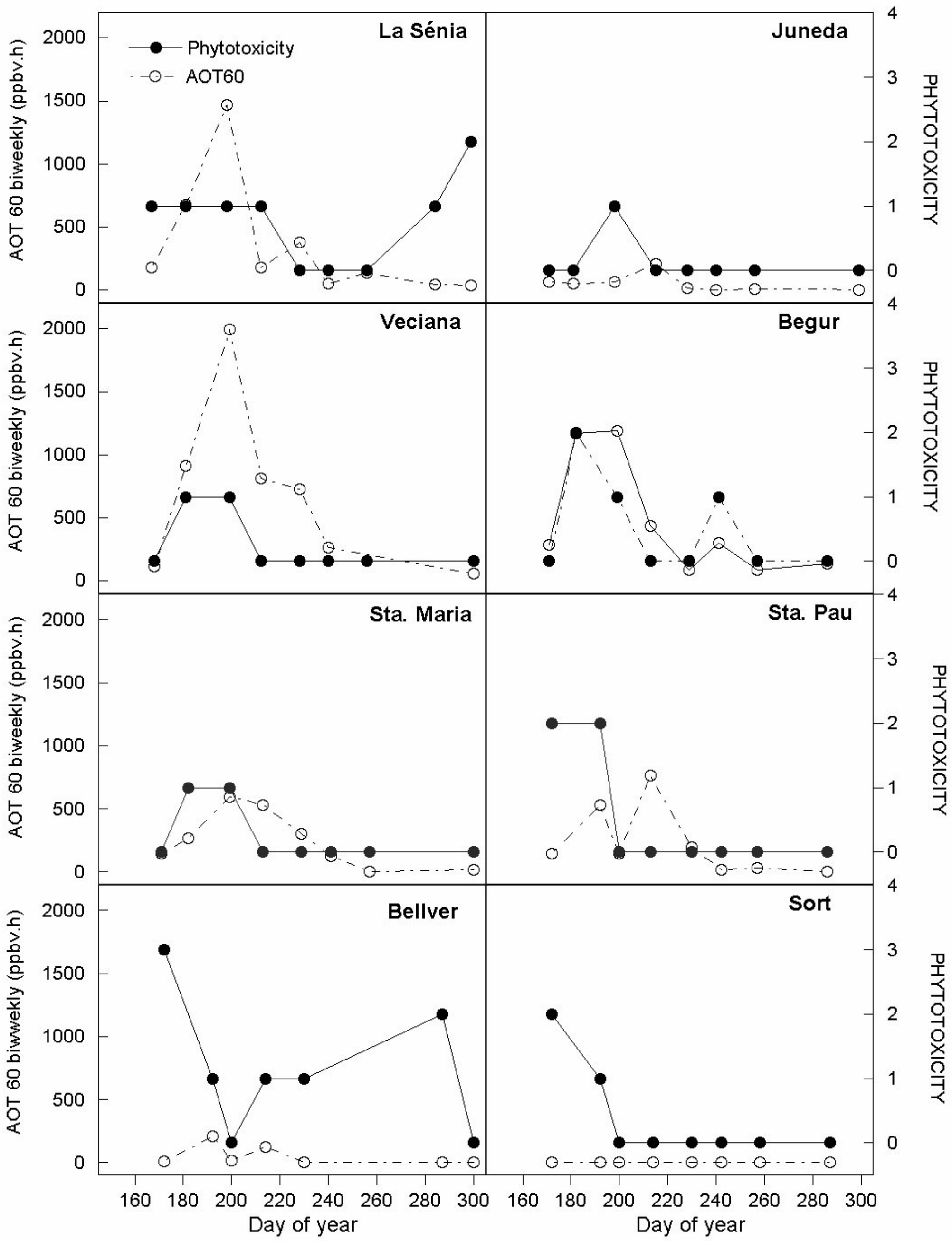


Figure 3. Phytotoxicity (defined in text depending on the presence of symptoms in the three different cultivars Bel-W3, Bel-C, and Bel-B) and AOT60 (Accumulated over a threshold of 60 ppb$_v$) evolution, for each station, throughout experimental period.

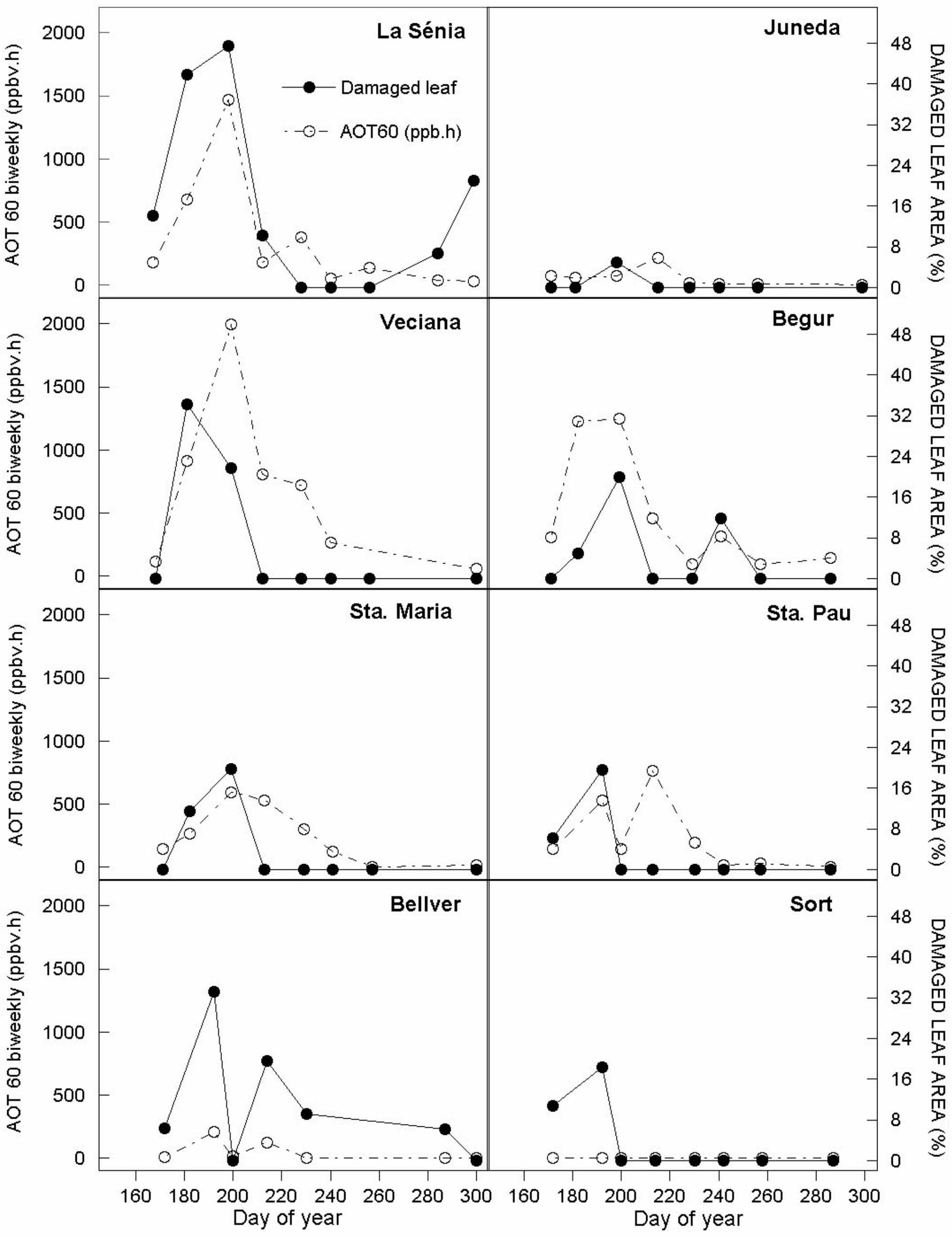
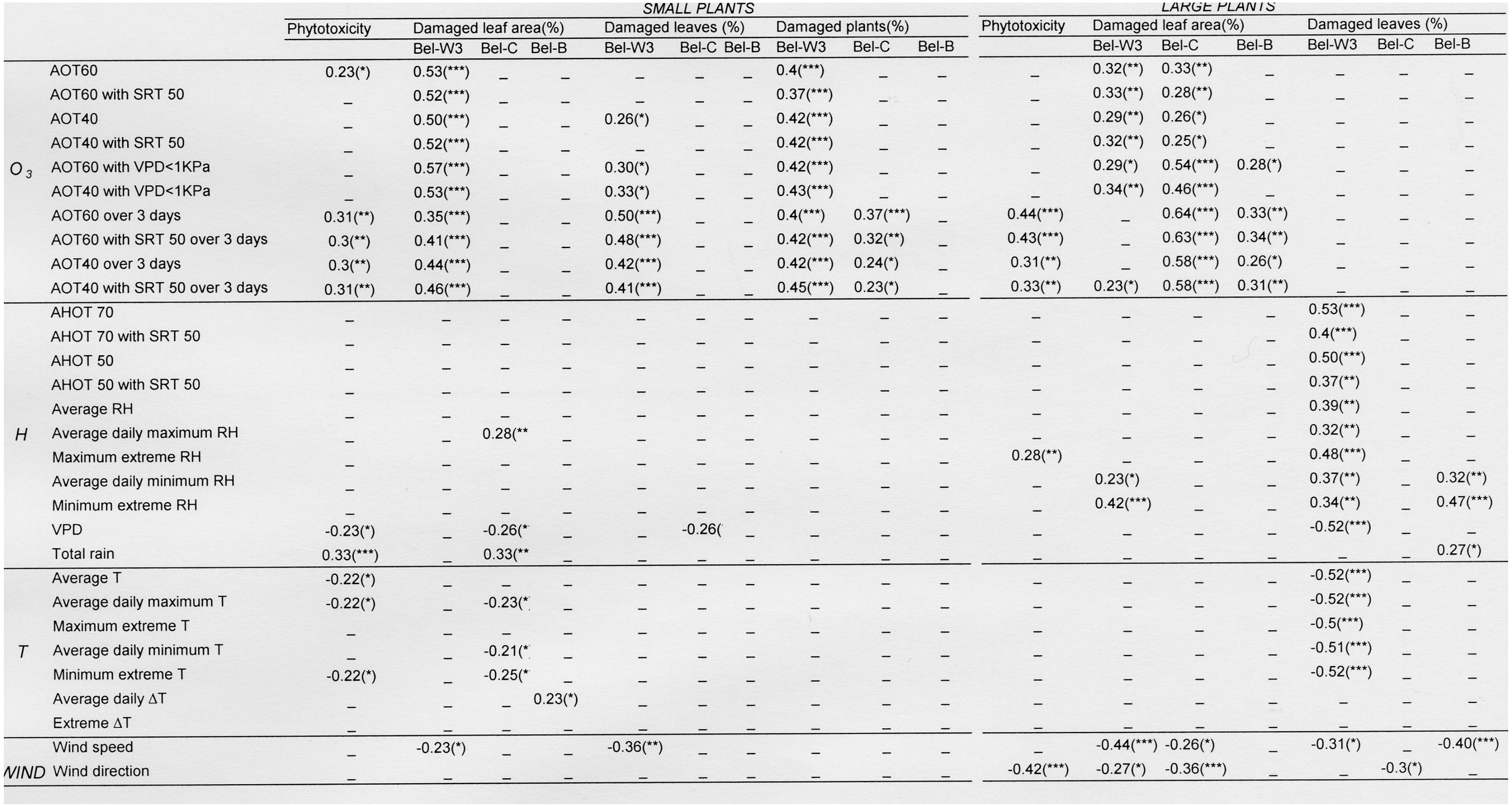


Figure 4. Damaged leaf area (%) in Bel-W3 cultivar and AOT60 evolution, for each station, throughout experimental period.



		SMALL PLANTS										LARGE PLANTS						
		Phytotoxicity	Damaged leaf area(%)			Damaged leaves (%)			Damaged plants(%)			Phytotoxicity	Damaged leaf area(%)			Damaged leaves (%)		
			Bel-W3	Bel-C	Bel-B	Bel-W3	Bel-C	Bel-B	Bel-W3	Bel-C	Bel-B		Bel-W3	Bel-C	Bel-B	Bel-W3	Bel-C	Bel-B
O_3	AOT60	0.23(*)	0.53(***)	_	_	_	_	_	0.4(***)	_	_	_	0.32(**)	0.33(**)	_	_	_	_
	AOT60 with SRT 50	_	0.52(***)	_	_	_	_	_	0.37(***)	_	_	_	0.33(**)	0.28(**)	_	_	_	_
	AOT40	_	0.50(***)	_	_	0.26(*)	_	_	0.42(***)	_	_	_	0.29(**)	0.26(*)	_	_	_	_
	AOT40 with SRT 50	_	0.52(***)	_	_	_	_	_	0.42(***)	_	_	_	0.32(**)	0.25(*)	_	_	_	_
	AOT60 with VPD<1KPa	_	0.57(***)	_	_	0.30(*)	_	_	0.42(***)	_	_	_	0.29(*)	0.54(***)	0.28(*)	_	_	_
	AOT40 with VPD<1KPa	_	0.53(***)	_	_	0.33(*)	_	_	0.43(***)	_	_	_	0.34(**)	0.46(***)	_	_	_	_
	AOT60 over 3 days	0.31(**)	0.35(***)	_	_	0.50(***)	_	_	0.4(***)	0.37(***)	_	0.44(***)	_	0.64(***)	0.33(**)	_	_	_
	AOT60 with SRT 50 over 3 days	0.3(**)	0.41(***)	_	_	0.48(***)	_	_	0.42(***)	0.32(**)	_	0.43(***)	_	0.63(***)	0.34(**)	_	_	_
	AOT40 over 3 days	0.3(**)	0.44(***)	_	_	0.42(***)	_	_	0.42(***)	0.24(*)	_	0.31(**)	_	0.58(***)	0.26(*)	_	_	_
	AOT40 with SRT 50 over 3 days	0.31(**)	0.46(***)	_	_	0.41(***)	_	_	0.45(***)	0.23(*)	_	0.33(**)	0.23(*)	0.58(***)	0.31(**)	_	_	_
H	AHOT 70	_	_	_	_	_	_	_	_	_	_	_	_	_	_	0.53(***)	_	_
	AHOT 70 with SRT 50	_	_	_	_	_	_	_	_	_	_	_	_	_	_	0.4(***)	_	_
	AHOT 50	_	_	_	_	_	_	_	_	_	_	_	_	_	_	0.50(***)	_	_
	AHOT 50 with SRT 50	_	_	_	_	_	_	_	_	_	_	_	_	_	_	0.37(**)	_	_
	Average RH	_	_	_	_	_	_	_	_	_	_	_	_	_	_	0.39(**)	_	_
	Average daily maximum RH	_	_	0.28(**	_	_	_	_	_	_	_	_	_	_	_	0.32(**)	_	_
	Maximum extreme RH	_	_	_	_	_	_	_	_	_	_	0.28(**)	_	_	_	0.48(***)	_	_
	Average daily minimum RH	_	_	_	_	_	_	_	_	_	_	_	0.23(*)	_	_	0.37(**)	_	0.32(**)
	Minimum extreme RH	_	_	_	_	_	_	_	_	_	_	_	0.42(***)	_	_	0.34(**)	_	0.47(***)
	VPD	-0.23(*)	_	-0.26(*	_	_	-0.26(	_	_	_	_	_	_	_	_	-0.52(***)	_	_
	Total rain	0.33(***)	_	0.33(**	_	_	_	_	_	_	_	_	_	_	_	_	_	0.27(*)
T	Average T	-0.22(*)	_	_	_	_	_	_	_	_	_	_	_	_	_	-0.52(***)	_	_
	Average daily maximum T	-0.22(*)	_	-0.23(*	_	_	_	_	_	_	_	_	_	_	_	-0.52(***)	_	_
	Maximum extreme T	_	_	_	_	_	_	_	_	_	_	_	_	_	_	-0.5(***)	_	_
	Average daily minimum T	_	_	-0.21(*	_	_	_	_	_	_	_	_	_	_	_	-0.51(***)	_	_
	Minimum extreme T	-0.22(*)	_	-0.25(*	_	_	_	_	_	_	_	_	_	_	_	-0.52(***)	_	_
	Average daily ΔT	_	_	_	0.23(*)	_	_	_	_	_	_	_	_	_	_	_	_	_
	Extreme ΔT	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
WIND	Wind speed	_	-0.23(*)	_	_	-0.36(**)	_	_	_	_	_	_	-0.44(***)	-0.26(*)	_	-0.31(*)	_	-0.40(***)
	Wind direction	_	_	_	_	_	_	_	_	_	_	-0.42(***)	-0.27(*)	-0.36(***)	_	_	-0.3(*)	_

Table 1. Correlation coefficients between damage parameters in bioindicators, O_3 and environmental parameters. (LP= Large Plants, remaining in the stations the whole period of study, SP= Small Plants, changed every two weeks, AOT40= Accumulated over a threshold of 40 ppb_v, AOT60= Accumulated over a threshold of 60 ppb_v, AHOT50= Accumulated Humidity over a threshold of 50 %, AHOT70= Accumulated Humidity over a threshold of 70 %, and SRT 50= Solar radiation Threshold > 50 Wm^{-2}). Phytotoxicity, defined in methods section depending on the presence of symptoms in the different tobacco cultivars. Significance levels (*)= p<0.1, (**)= p<0.05 and (***)=p<0.01.

Field measurements

The highest ambient ozone levels and greatest plant damage were found mainly in the coastal areas (La Sénia, Begur) (Figures 3 and 4). These high ozone values were evident at the end of spring and the beginning of summer. Later, at the end of summer and beginning of autumn, ozone and damage levels decreased. This was the general pattern in all the sampling stations. The spatial distribution and the seasonality are in agreement with the maps of ozone in Catalonia developed by Gimeno et al (1996). Although there was not always a perfect parallel between ozone phytotoxicity rating and ozone levels (Figures 3), in general the ozone phytotoxicity level 0 corresponded to biweekly AOT60 values ranging between 0 and 500 ppb_v, while phytotoxicity levels 1, 2 and 3 were related to biweekly AOT60 values ranging from 500-1200 $ppb_v \cdot h$, 1200-1700 $ppb_v \cdot h$ and higher than 1700 $ppb_v \cdot h$ respectively.

The percentage of damaged leaf area of Bel-W3 correlated better with the ozone level than the phytotoxicity parameter defined, depending on the presence of symptoms in the three different cultivars (Figures 3 and 4). The correlation coefficient (r) between AOT60 and phytotoxicity in SP was 0.23 ($p=0.07$ and $n=63$) while the r between AOT60 and damaged leaf area of Bel-W3 was 0.53 ($p< 0.0001$, $n=63$). Correlations were not higher for LP but for a slight increase of r values when AOT60 levels in the last three days of period exposure were considered (Table 1).

These relations improved only slightly when using a cut-off depending on average VPD (Vapour pressure deficit) (Table 1). The correlation coeficient between AOT60 and damaged leaf area of Bel-W3 was 0.57 ($p< 0.0001$, $n=49$) when VPD< 1kPa, and 0.53 ($p< 0.0001$, $n=60$) with a VPD< 1.5 kPa.

In order to get a better knowledge of the relationships between ozone injury and environmental parameters, we obtained a matrix of their correlations (Table 1). Some of the damage variables presented a significant relationship with ozone and meteorological data, but the highest significance corresponded to Bel-W3 cultivar wich was the most ozone-sensitive cultivar.

The most significant correlation was found between percentage of damaged leaf area in Bel-W3 small plants (changed every two weeks) and AOT60. There was not much difference whether considering the solar radiation cut-off or not in the calculation of AOT60 (Table 1). Damaged leaf area also showed some significant relationships with wind speed for Bel-W3, with humidity and temperature parameters in Bel-C, and showed only a significant relation with average daily thermal oscilation in Bel-B. The number of damaged leaves presented significant relations with ozone parameters, especially those calculated for the last three days of exposure, and with wind speed for Bel-W3. The percentage of damaged plants presented a significative correlation with ozone parameters in the Bel-W3 cultivar and only when considering the last three days in the Bel-C cultivar.

In the large plants, the most significant relations were also found between the damaged leaf area of the three cultivars and ozone parameters, especially those calculated for the last three days of exposure. Humidity and wind were also significant in the Bel-W3 and Bel-C cultivars. Damaged leaf number was not correlated with ozone parameters (Table 1). In these large plants, Bel-C was a more useful indicator of ozone levels during the last three days of exposure than Bel-W3.

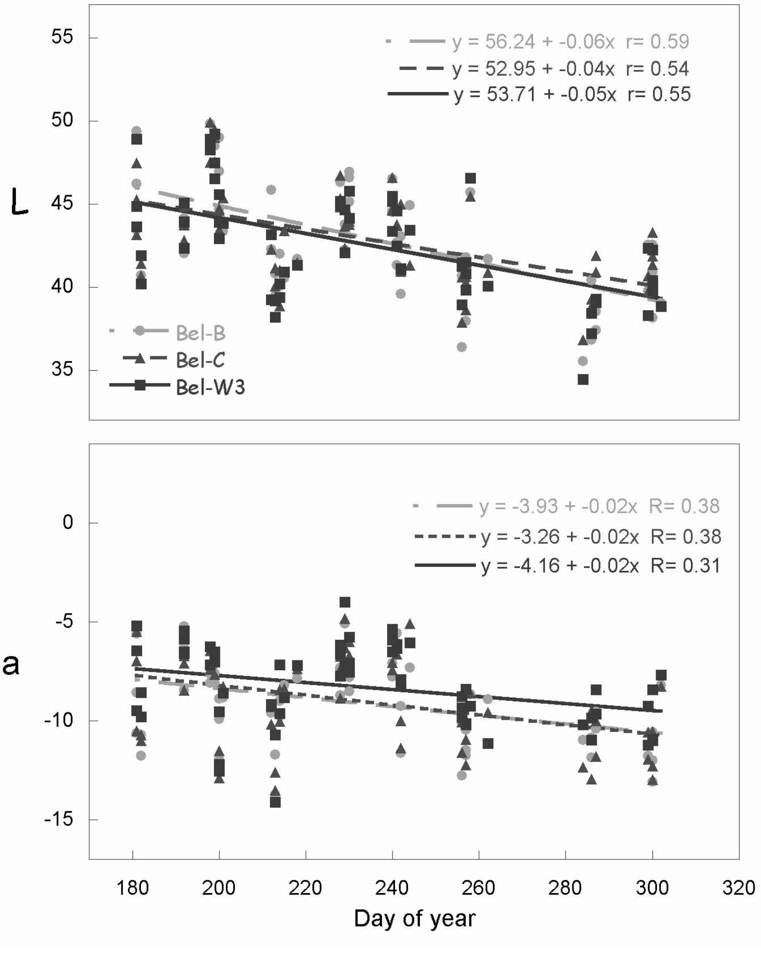


Figure 5. Evolution of the three tobacco cultivars of the colorimetric parameters, L (lightness) and a (hue) throughout the analysis period.

General leaf color did not follow leaf damage. No significant correlation was found between the color parameters L (lightness), a (hue), b (chroma) and the damage parameters. But there were some significant relationships between colorimetric variables (especially L) and ozone levels and day of year (Figure 5). However, this was probably due to the correlation of day of year and ozone levels ($r=-0.34$, $p< 0.01$ and $n=63$). Leaves were increasingly darker throughout the year because of phenological changes and this coincided with decreasing ozone levels from May and June to October.

Station	Altitude	Longitude	Latitude	Intercept	AOT60 Coefficients	RMSR	r	P-Value
Bellver	1040	1° 45'	42° 20'	3.59	0.14	4.2	0.95	0.001
La Sénia	370	0° 15'	40° 35'	4.9	0,031	11.38	0.8	0.009
Begur	190	3° 15'	41° 55'	-0,47	0.011	6,013	0.67	0.069
Veciana	725	1° 30'	41° 40'	-1.28	0.013	12,000	0.63	0.13
Sta. Maria	210	2° 30'	41° 42'	-1.12	0.021	6.52	0.6	0.11
Sta. Pau	540	3° 00'	42° 10'	0.91	0.01	6.88	0.4	0.32
Juneda	275	0° 50'	41° 35'	0.53	0.002	1.9	0.07	0.86
Sort	700	1° 00'	42° 30'	_	_	_	_	_

Table 2. Simple regression equations between percentages of damaged leaf area in Bel-W3 and AOT60 in the eight stations. AOT coefficients are considered as damage response rates to ozone. There is no data for Sort because AOT60 was always 0.

Discussion

The different cultivars showed the expected sensitivity to ozone, in the OTC experiments. Bel-W3 was the most sensitive cultivar, followed by Bel-C and Bel-B. Thus, at each location it was expected that Bel-W3 should have a higher percentage of affected leaves than in any other cultivar. In fact this is what was observed. However, ozone phytotoxicity did appear to be not only the result of ozone exposure but also by other environmental factors.

The study of colorimetric L (lightness) and a (hue) variables for ozone damage monitoring in the tobacco plants did not show any correlation between injury from ozone in tobacco and changes in the colorimetric values at least in the range of damage found in this study. But there was a significant correlation of colorimetric variables with ozone levels and also day of year. That indicates parallel phenological changes in leaf color and annual ozone concentrations. Throughout the summer and autumn growing period the tobacco leaves tended to become darker (L decreased) and greener (a decreased) whole air ozone concentrations decreased progressively.

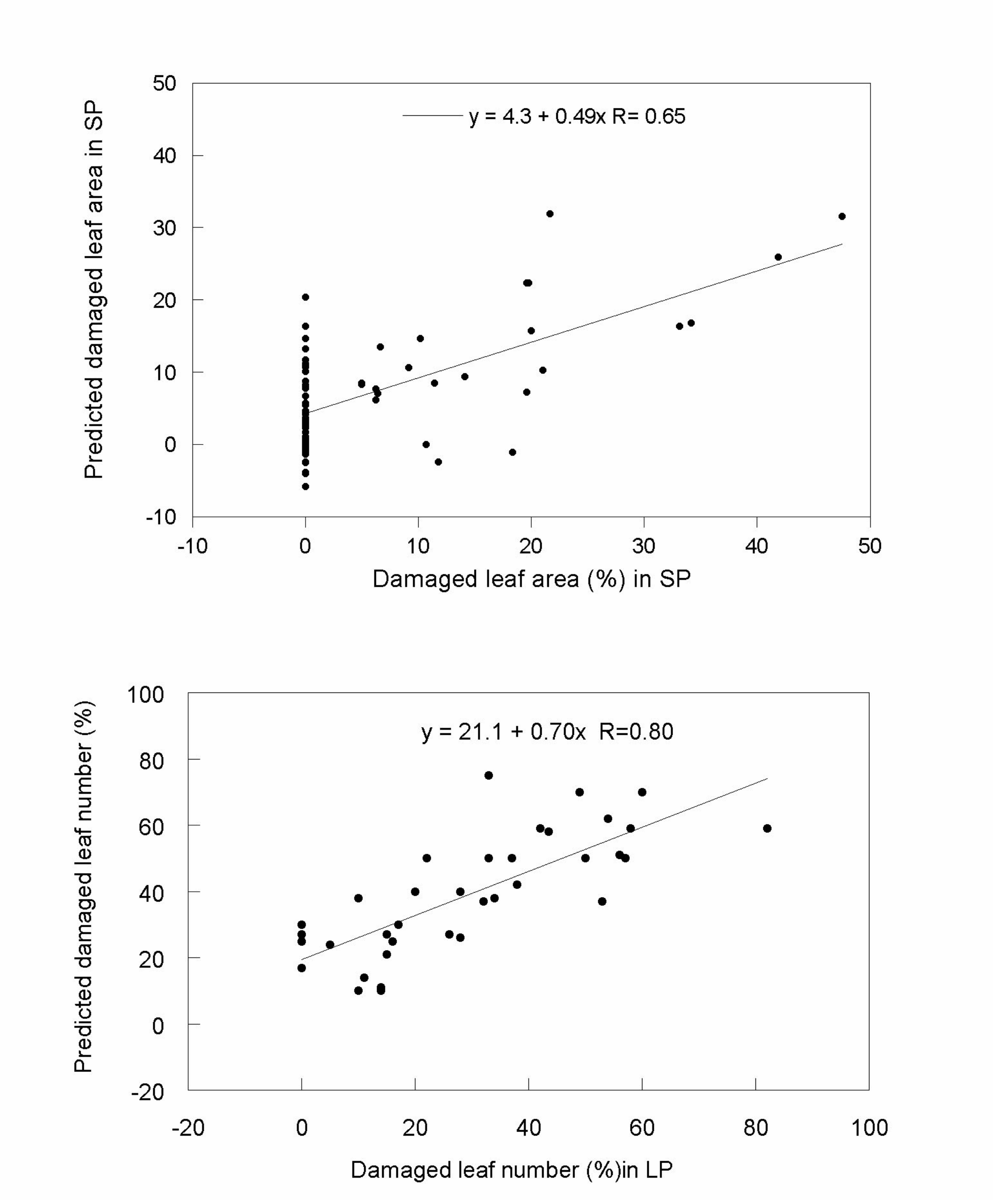


Figure 6. Actual versus predicted values by regression models a) Damaged leaf area percentage in SP=13.28+0.02(AOT60)-3.3(Wind speed) (r=0.61, n=62, F-value= 17.51, p<0.0001, RMSR=8.93), and b) Damaged leaf number percentage in LP= 86.85-2.14(average daily maximum T)+0.66(minimum extreme RH)-0.01(AOT40)-0.09(wind direction)+0.07(AOT60 with RST 50Wm^{-2} in the last 3 days) (r=0.81, n=38, F-value=12.46, p<0.0001, RMSR=12.92). (SP, small plants changed every two weeks; LP, large plants remaining in the stations the whole period study).

These studies suggested the possibility of estimating ozone levels through the visible injury in each tobacco cultivar. To determine the importance of tobacco as a biomonitor, a stepwise multiple regression model was developed with the AOT60 parameter as dependent variable and damaged leaf area in Bel-W3, damaged leaf area in Bel-C and damaged leaf area in Bel-B as three independent variables. The model only selected the Bel-W3 cultivar: (F-to-include=23.60) AOT60= 137.71 + 19.04*Damaged leaf area in Bel-W3 ($R=0.53$, $n=63$, $p < 0.0001$). Thus, the percentage of damaged leaf area in the Bel-W3 cultivar was chosen as the most representative indicator of ozone levels.

Phytotoxic levels defined according to the presence of damage on the three tobacco cultivars, Bel-W3, Bel-C and Bel-B did not correlate very well with the ozone levels. The correlation improved when they were calculated for the last three days of period exposure. In fact the presence of visual injury in Bel-C and Bel-B cultivars did not present significant relationships with ozone levels except only in the percentage of damaged plants in Bel-C and in the damaged leaf area of LP in Bel-C and Bel-B. Thus, Bel-W3 damage development indicated ozone levels better than the other two cultivars in SP, but Bel-C was better indicator in LP.

Cultivar behaviour seemed to overestimate ozone levels, especially in the Pyrinees stations (Bellver, Sort and Santa Pau) in the first sampled period (spring) (Figure 3). In these stations low ozone values were accompanied by high phytotoxic levels when defined depending on the three cultivars. Thus, the use of a phytotoxicity variable including the injury in the Bel-B and Bel-C cultivars was not as useful for biomonitoring in this biweekly study as in others (Mignanego et al., 1992; Bytnerowicz et al., 1993; Lorenzini, 1994; Wäber et al., 1994). In this study, phytotoxicity was more correlated with the ozone exposure during the previous three days than the previous fifteen days. Although there was not a strong relationship between ozone exposure and the ozone phytotoxicity index derived from the use of the three tobacco cultivars, it was still significant (Table 1). We still think that it is valid to use the set of the three cultivars because it helps to make a diagnosis of the ozone injury on tobacco leaves since a gradation on the extension of injury should be observed at any site following the sensitivity of the cultivars to ozone as it was shown in the controlled OTC experiments. However, in the field, much higher AOT60 values were necessary to produce similar symptoms than in the controlled OTC experiments (Fig. 2 and 3). Although the cultural conditions of the bioindicator plants were standardized as much as possible, it might have happened that variations in pot soil moisture were induced by site-specific environmental conditions.

Thus, the effect of ozone may be modified by environmental factors, and by co-occurring pollutants (Sanders et al., 1995; Benton et al., 1996). In our case very diverse climatological conditions could have caused some distortion of the linkage between ozone and damage when this damage is used as ozone biomonitor. Multiple linear regressions were developed relating the damage variables in Bel-W3 cultivar and the ozone and meteorological variables. In small plants a multiple stepwise regression model with damaged leaf area as the dependent variable selected AOT60 (F-to-include= 30.77) and wind speed (F-to-include= 8.62) as independent variables ($r=0.61$, $n=62$, $p <0.0001$ and RMSR=8.93) (Figure 6). In large plants the model selected average daily maximum temperature (F-to-include= 16.81), minimum extreme relative humidity (F-to-include= 11.33), AOT40 (F-to-include= 9.91), wind direction (F-to-include= 9.97) and AOT60 in the last 3 days of exposure (F-to-include= 4.79) as independent variables ($r=0.81$, $n=38$, $p<0.0001$ and RMSR=12.92). The predictive ability of these regression equations for bioindicator symptoms was assessed using a cross-validation method with 50 % randomly selected samples, and the resulting correlation coefficients were similar to those of regressions developed with all sampling data. It is interesting that both a variable of chronical ozone exposition (AOT40) and a variable of punctual exposition to high ozone concentration (AOT60 in the last three days of exposure) entered in the regression model as selected variables. Apparently, ozone injury on small plants was slightly more related to acute exposures while ozone injury on LP was slightly more affected by chronic exposures. The lack of very high ozone levels in most of the sites would explain their low correlation with damage on Bel-C and Bel-B cultivars; therefore, only Bel-W3 had enough sensitivity to track the variations in ozone exposure. The still small correlation coefficients indicated that these regression models were not wholly explanatory of the damaged symptoms found in the tobacco bioindicators. Other environmental factors difficult to define and control may be involved in the expression of the observed injury.

Figure 4 and Table 2 show a clear difference in the response of Bel-W3 to ozone levels according to the site. In some stations such as Bellver or La Sénia there was a strong relationship between damage and ozone variables. But in other stations, such as Juneda and Sort, this relation did not exist partly because the ozone concentration were not high enough (AOT60 was always 0 in Sort) (Figure 4). The response of bioindicators was likely affected by other environmental parameters of the stations, such as temperature parameters or the altitude. In fact, there was also a significant relation between the station damage response rate to ozone (AOT60 coefficients of Table 2) and the station average temperature (R=-0.72, p=0.04, n=8) and altitude (R=-0.66, p=0.07, n=8).

However, when overall station data were considered, AOT 60 was the main variable explaining damaged leaf area, especially when accumulation was only computed for VPD below 1kPa values. Thus, the appearance of injury in the bioindicator leaves was mainly driven by ozone concentrations. Humidity and wind were other variables to be considered in the expression of damage (Table 1). Higher humidity and lower wind speed would have increased stomatal conductance and thus ozone diffusion into leaves (Gimeno et al., 1995b; Peñuelas et al., 1995 b; Benton et al., 1995). Other environmental variables linked to ozone effects such as temperature or direction of wind were also partly explanatory of the injury in these bioindicators. Therefore, in the determination of critical ozone levels and on biomonitoring it is important to characterize the meteorological conditions, mainly those linked to stomatal behaviour such as humidity and wind speed and also to conduct a good control of growth conditions such as irrigation.

It is concluded that tobacco cultivars behaved as good bioindicators of ozone phytotoxicity but meteorological and environmental factors need to be considered when using tobacco cultivars for biomonitoring ozone levels. Further studies of relations between bioindicators symptomatology, ozone levels and environmental conditions are warranted. Special emphasis should be awarded to the extraordinarily variable environmental field conditions.

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