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# Similar effects of ozone on four cultivars of lettuce in open top chambers during winter

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## Abstract

Ozone is the major phytotoxic air pollutant that reduces the yield of several agricultural crops in the Spanish Mediterranean area. We studied four lettuce cultivars (*Lactuca sativa* L.) for the effects of different $O_3$ concentrations during the winter on chlorophyll (Chl) *a* fluorescence, lipid peroxidation, and root length in outdoor open-top chambers. Under $O_3$ the photosynthetic quantum conversion declined while heat emissions increased in all cultivars; these results provide more evidence of non-filtered air with additional ozone (NFA+$O_3$) treatment compared with non-filtered air (NFA) and charcoal filtered ozone-free air (CFA). Changes in the Chl *a* fluorescence may be associated with an increase in membrane lipid peroxidation as well as with observed reduction of root length under $O_3$ stress.

*Additional key words*: chlorophyll fluorescence; *Lactuca sativa*; lipid peroxidation; malondialdehyde; root length.

## Introduction

Ozone is three-atom allotrope oxygen, known as a toxic air pollutant in the troposphere. There is evidence that many Mediterranean areas suffer a critical photochemical oxidant problem (Gimeno *et al.* 1999, Calatayud and Barreno 2000, 2001) especially during the spring and summer but little is known of the sensitivity to $O_3$ during the winter. These studies are interesting because we have detected a high $O_3$ concentration in Valencia (Spain) during the winter which may modify crop response.

The effect of $O_3$ on biological organisms is attributed to its ability to spontaneously dismutate or react with cellular constituents to generate excess active oxygen species (AOS) (Rao *et al.* 2000). In the plant world $O_3$ is considered to be one of the most important air pollutants that causes significant yield losses on sensitive crop species (Heck *et al.* 1988), even though the action mechanism of $O_3$ has not been fully elucidated (Tingey and Andersen 1991). Many studies have stated that $O_3$ impairs membrane functions, as it diminishes photosynthesis, impacts on root and shoot tissues, and induces premature senescence (Lefohn 1992).

Measurement of Chl *a* fluorescence provides information on light reaction of photosynthesis and serves as a non-invasive indicator of the status of photosynthetic reaction centres of plants.

The photosynthetic apparatus is a crucial contact point between the plant and the environment and is particularly sensitive to $O_3$. Its flexibility on the one hand and stability on the other are instrumental to plant survival (Mattos *et al.* 1999). When plants are exposed to $O_3$ or other stresses, the sunlight may become too excessive for photosynthesis and can lead to a decrease in the efficiency of

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*Abbreviations*: AOS, active oxygen species; CFA, charcoal filtered ozone-free air; Chl, chlorophyll; $F_m$, maximum Chl fluorescence yield obtained with dark-adapted sample; $F_m'$, maximum Chl fluorescence yield in irradiated sample; $F_0$, minimum Chl fluorescence yield in the dark-adapted state; $F_0'$, Chl fluorescence yield during a brief interruption of actinic irradiation in the presence of far-red irradiation; $F_s$, Chl fluorescence yield during actinic irradiation; $F_v$, variable Chl fluorescence of the dark-adapted leaf; MDA, malondialdehyde; NFA, non-filtered air; NFA+$O_3$, non-filtered air with additional ozone; NPQ, non-photochemical quenching calculated from Stern-Volmer equation; OTC, open top chamber; PS2, photosystem 2; $q_P$, photochemical quenching; Rfd, potential photochemical quantum conversion; RL, root length; %D, fraction of photons absorbed that is dissipated in the PS2 antennae; %P, fraction of photons absorbed that is utilised in PS2 photochemistry; %X, fraction of photons absorbed by PS2 that is neither used in photochemistry nor dissipated in the PS2 antennae; $\Phi_{exc}$, intrinsic efficiency of open PS2 reaction centres; $\Phi_{PS2}$, quantum efficiency of PS2.

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photosystem 2 (PS2). Plants may dissipate the excess of energy by non-photochemical quenching as an adaptive protection mechanism to avoid PS2 damage. If the limits are exceeded and adaptive capacity is overworked, permanent damage or even death may result (Demmig-Adams and Adams 1992, Foyer *et al.* 1994, Krause 1994, Ciompi *et al.* 1997, Calatayud and Barreno 2001). Photosynthetic apparatus is not the only thing affected by $O_3$, membranes are another vulnerable point on the cells that results in modifications to membrane structure producing functional changes in the ionic and solute relationships of cellular components, which may then alter photosynthesis (Heath 1988).

We studied the effect of $O_3$ fumigation compared with ambient air and charcoal filtered air on four lettuce cultivars grown during winter in open top chambers (OTCs). Chl *a* fluorescence, lipid peroxidation, and root length (RL) were investigated to evaluate ozone damage on the leaves.

## Materials and methods

Experimental site was the "Centro de Capacitación Agraria (Generalitat Valenciana)" in Carcaixent (39°7′N, 0°27′W, and 22.1 m above sea level) in a rural environment. The site is about 40 km south of Valencia on the Spanish Eastern Mediterranean Coast.

**Plants**: Lettuce seeds (*Lactuca sativa* L.) of cultivars Valladolid, Del Prat, Odra, and Maravilla verde were germinated in vermiculite in a greenhouse at the Experimental Station. Environmental conditions in the greenhouse during plant growth were as follows: 10-15/6-12 °C (day/night); relative humidity 50 to 95 %; photosynthetically active radiation of day-time maximum at plant height in the glasshouse of 500-700 $\mu$mol $m^{-2}$ $s^{-1}$. Four-week-old seedlings were transplanted into large pots containing a commercial soil mixture (*Terraplant*, *BASF*, Uchte, Germany) and transferred into OTC's where they remained for 30 d (from the middle of December to the middle of January). During the growth period plants were watered daily. Each OTC contained ten plants of each cultivar.

**Open top chamber treatment**: Potted plants were grown in 9 OTC's located at the Carcaixent Experimental Station. OTC's were based on the original design employed in the NCLAN programme (see Heagle *et al.* 1973). During the growing season, three OTC's were ventilated continuously (24 h $d^{-1}$) by passing air through activated charcoal and dust filters of type *SF/Q* class *EU-3* and *EU-4*, *SF/CG*, and *RBAA2* (CFA, ozone-free air), three OTC's were ventilated with non-filtered air (NFA), and three received additional ozone (NFA+$O_3$). Ozone was generated electrically (ozone generator *S-3003*, *G.O.A.C.*, *DASIBI*) using pure compressed oxygen which was added to ambient air. A flow controller regulated the flow of ozone-enriched air to the OTC's. The $O_3$ additions were carried out from 10 h to 15 h GMT five days a week. Concentration of gaseous pollutants ($O_3$, NO, $NO_2$, $SO_2$), wind speed, wind direction, air temperature, irradiance, and relative humidity were continuously monitored. The concentrations of $O_3$, $NO_x$, and $SO_2$ were monitored using EPA-approved analysers, specific to each pollutant (*DASIBI* models *1008*, *2108*, and *4108*, respectively). The ozone analyser was calibrated on a twice-monthly basis with a *DASIBI* model *5008* (*Dasibi Environmental Corp.*, Glendale, CA, USA).

**Chl *a* fluorescence**: At the end of the growth period, Chl *a* fluorescence was measured at ambient temperature *in situ* in the OTC's, using a portable fluorometer (*PAM-2000*, *Walz*, Effeltrich, Germany). Leaves without visible injury symptoms were darkened for 30 min prior to measurement. The minimum (dark) fluorescence $F_0$ was obtained upon excitation of leaves with a weak beam from a light-emitting diode. The maximum fluorescence ($F_m$) was determined following a 600 ms pulse of saturating "white light". The yield of variable fluorescence ($F_v$) was calculated as $F_m - F_0$. Following 2 min of dark re-adaptation, actinic "white light" [430 $\mu$mol(photon) $m^{-2}$ $s^{-1}$] was switched on and a saturating pulse [14 000 $\mu$mol(photon) $m^{-2}$ $s^{-1}$] was applied at 60 s intervals for 15 min to determine: (*1*) maximum fluorescence yield during actinic irradiation ($F_m'$), (*2*) the level of modulated fluorescence during a brief interruption of actinic irradiation in the presence of far-red radiation ($F_0'$), and (*3*) the Chl fluorescence yield during actinic irradiation ($F_s$). Quenching due to non-photochemical dissipation of absorbed radiant energy (NPQ) was calculated at each saturating pulse, using the equation $NPQ = (F_m - F_m')/F_m'$ (Bilger and Björkman 1991). The coefficient for photochemical quenching, $q_P$, which represents the fraction of open PS2 reaction centres was calculated as $(F_m' - F_s)/(F_m' - F_0')$ (Schreiber *et al.* 1986). The quantum efficiency of PS2 photochemistry, $\Phi_{PS2}$, closely associated with the quantum yield of non-cyclic electron transport, was estimated from $(F_m' - F_s)/F_m'$ and the intrinsic PS2 efficiency was calculated as $F_v'/F_m'$, $\Phi_{exc}$ (Genty *et al.* 1989). The ratio $(1 - q_P)/NPQ$ was used as an estimate of photon excess and, therefore, of the susceptibility of PS2 to high irradiance (Park *et al.* 1995). The fractions of photons absorbed dissipated in the PS2 antennae (%D) and utilised in PS2 photochemistry (%P) were estimated from $1 - (F_v'/F_m') \times 100$ and $(F_v'/F_m')\, q_P \times 100$, respectively (Demmig-Adams *et al.* 1996). The fraction of photons absorbed by PS2 that is not used in photochemistry nor dissipated in the PS2 antenna (%X) was estimated from $(F_v'/F_m')(1 - q_P) \times 100$ (Demmig-Adams *et al.* 1996). The ratio of fluorescence decrease (from $F_m$

to $F_s$) to the steady-state fluorescence $F_s$ (Rfd = $F_d/F_s$) is an indicator of the potential photosynthetic quantum conversion (Lichtenthaler and Buschmann 1987).

**Lipid peroxidation**: The extent of lipid peroxidation was estimated using malondialdehyde (MDA) as an indicator, according to the protocol adapted for leaves by Heath and Parker (1968), employing the modifications suggested by Dhindsa *et al.* (1981). Leaves used did not display visible symptoms at the end of the growth period.

**Root length**: At the end of growing season the plants were harvested and the major root was measured.

## Results

**Air quality**: Plants exposed to elevated $O_3$ (NFA+$O_3$) showed visible injury symptoms in the form of blackish, necrotic bi-facial lesions, mostly located in the interveinal and marginal area of the leaf. During the growth period $SO_2$ and $NO_x$ concentrations were low (less than 2 $mm^3$ $m^{-3}$ for $SO_2$ and 8 $mm^3$ $m^{-3}$ for $NO_x$). The $O_3$ concentrations and environmental conditions are summarised in Table 1. The 12-h period (daylight hours) monthly mean concentrations of $O_3$ inside the elevated $O_3$ chambers (NFA+$O_3$) (mean for 30 d was 62.7 $mm^3$ $m^{-3}$) were about twice those of ambient $O_3$ concentrations (NFA mean for 30 d was 30.1 $mm^3$ $m^{-3}$). The mean concentration of $O_3$ in CFA chambers was below 10 $mm^3$ $m^{-3}$. The environmental conditions were similar in all treatments.

Table 1. Summary of ozone concentrations and environmental conditions in open top chambers during the winter growing season of lettuce plants. Mean concentrations of $O_3$ for 12 h during 30 d [$mm^3$ $m^{-3}$], $T_{mean}$, mean 24 h air temperature [°C], $T_{min}$, minimum air temperature [°C], $T_{max}$, maximum air temperature [°C], and RH, air relative humidity [%].

| Treatment | Mean [$O_3$] | $T_{mean}$ | $T_{min}$ | $T_{max}$ | RH |
|---|---|---|---|---|---|
| CFA | 10.2 | 11.5 | 7.80 | 15.71 | 74.3 |
| NFA | 30.1 | 11.3 | 7.55 | 15.85 | 77.5 |
| NFA+$O_3$ | 62.7 | 12.2 | 8.60 | 15.60 | 70.1 |

Table 2. Maximum quantum yield of PS2, $F_v/F_m$, in lettuce leaves at the end of growing season (30 d in the field) in CFA, NFA, and NFA+$O_3$ treatments. Means of ten samples. For comparison of means, variance analysis (ANOVA) followed by the least significance difference (LSD) test, calculated at 95 % confidence level, were performed. Significant differences did not show up.

| Treatment | Valladolid | Del Prat | Odra | Maravilla verde |
|---|---|---|---|---|
| CFA | 0.824 | 0.831 | 0.822 | 0.815 |
| NFA | 0.813 | 0.815 | 0.814 | 0.802 |
| NFA+$O_3$ | 0.817 | 0.800 | 0.802 | 0.801 |

**Chl *a* fluorescence parameters**: Table 2 shows the Chl *a* fluorescence dark-adapted parameters for the leaves subjected to CFA, NFA, and NFA+$O_3$ treatments at the end of the growing period (30 d). The maximum quantum yield of PS2 photochemistry, $F_v/F_m$, did not change significantly with air pollution. This indicates that leaves in NFA and NFA+$O_3$ showed no signs of photoinhibition. The slight decline in this ratio from CFA to NFA+$O_3$ was the result of a decrease in the fluorescence parameters $F_m$ and $F_v$, since the minimum level of fluorescence $F_0$ was constant (values not shown) in all treatments.

The quenching of Chl *a* fluorescence under steady-state kinetics was measured in four lettuce cultivars (Table 3). Ozone fumigation (NFA+$O_3$) led to a decrease in the actual PS2 efficiency ($\Phi_{PS2}$) and in the fraction of open PS2 centres ($q_P$) in all cultivars of lettuce. NPQ is a good estimate of heat dissipation by PS2: an increase in

Table 3. Changes in chlorophyll *a* parameters in lettuce cultivars after 30 d in open top chambers. Means of 8 samples. For comparison of means, variance analysis (ANOVA) followed by the least significance difference (LSD) test, calculated at 95 % confi-dence level, was performed. Values followed by the same letter indicate no significant differences.

| Parameter | Valladolid | | | Del Prat | | | Odra | | | Maravilla verde | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CFA | NFA | NFA+$O_3$ | CFA | NFA | NFA+$O_3$ | CFA | NFA | NFA+$O_3$ | CFA | NFA | NFA+$O_3$ |
| $\Phi_{PS2}$ | 0.605a | 0.485b | 0.447c | 0.573a | 0.543a | 0.445b | 0.540a | 0.524a | 0.429b | 0.608a | 0.544b | 0.493c |
| $q_P$ | 0.810a | 0.793a | 0.720b | 0.847a | 0.809a | 0.715b | 0.851a | 0.845a | 0.803b | 0.855a | 0.819b | 0.792b |
| NPQ | 1.072a | 1.469b | 1.899c | 0.875a | 1.013b | 1.661c | 1.290a | 1.326a | 1.548b | 0.629a | 0.850b | 1.028c |
| 1 − $q_P$/NPQ | 0.17a | 0.15a | 0.11b | 0.17a | 0.19a | 0.17a | 0.11a | 0.12a | 0.13a | 0.23a | 0.22a | 0.21a |
| Rfd | 3.48a | 3.40a | 3.25a | 3.56a | 3.08b | 2.54c | 3.42a | 3.47a | 3.22a | 2.83a | 2.70a | 2.45b |
| $\Phi_{exc}$ | 0.747a | 0.611b | 0.620b | 0.676a | 0.671a | 0.622b | 0.634a | 0.620a | 0.534b | 0.711a | 0.664b | 0.622b |
| %P | 61a | 49b | 44b | 58a | 54a | 44b | 54a | 52a | 43b | 60a | 54b | 49c |
| %D | 25a | 38b | 39b | 32a | 33a | 39b | 37a | 38a | 47b | 30a | 34a | 38a |
| %X | 14a | 13a | 17a | 10a | 13a | 17b | 9a | 10a | 10a | 10a | 12a | 13a |

NFA and more so in NFA+$O_3$ treatments was observed in all cultivars. The ratio between excitation pressure ($1 - q_P$) and NPQ is an estimate of the photon excess. No changes in this ratio occurred in NFA and NFA+$O_3$ leaves with respect to the control, except in the Valladolid cultivar. The intrinsic PS2 efficiency of excitation trapping by PS2, $\Phi_{exc}$ decreased in NFA and NFA+$O_3$ leaves in all cultivars, indicating that the PS2 centres which were open, showed a lowered efficiency of excitation trapping. The ratio Rfd of Chl *a* fluorescence decreased for ozone fumigation in all cultivars. The Chl *a* fluorescence quenching parameters enable to estimate the different fractions of absorbed photons (Table 3). The fraction of photons absorbed by the PS2 antenna used in photochemistry (%P) decreased from CFA to NFA+$O_3$ treatment in all cultivars and the fraction thermally dissipated in the antenna (%D) and the fraction neither used in photochemistry nor dissipated in the antenna (%X) increased in all cultivars.

**Lipid peroxidation**: Table 4 shows the results of MDA assay in lettuce plants subjected to different air quality. Lipid peroxidation in leaves increased significantly after exposure to ambient ozone and fumigation. As compared to CFA, this increase was greater under NFA+$O_3$ treatment than under NFA.

Table 4. Malondialdehyde (MDA) content [μmol(MDA) $kg^{-1}$(f.m.)] in Valladolid, Del Prat, Ordra, and Maravilla verde cultivars after 30 d in the open top chambers. Means of five replications. In each column, numbers flanked by the same letters are not different for $p < 0.05$.

| Treatment | Valladolid | Del Prat | Ordra | Maravilla verde |
|---|---|---|---|---|
| CFA | 16.88a | 24.27a | 24.25a | 16.45a |
| NFA | 22.69b | 32.19b | 27.48a | 22.83b |
| NFA+$O_3$ | 32.74c | 37.63b | 36.90b | 37.84c |

**Root length** [cm] decreased at the end of the growing season (Table 5) in all cultivars in NFA and NFA+$O_3$ with respect to the control (CFA). There were no significant differences in shoot length.

Table 5. Major root length, RL [cm] in Valladolid, Del Prat, Ordra, and Maravilla verde cultivars measured at the end of the growing season. Means of 30 replications. In each column, numbers flanked by the same letters are not different for $p < 0.05$.

| Treatment | Valladolid | Del Prat | Ordra | Maravilla verde |
|---|---|---|---|---|
| CFA | 17.38a | 18.41a | 17.03a | 17.13a |
| NFA | 15.93ab | 15.04b | 15.30ab | 17.00a |
| NFA+$O_3$ | 13.90b | 13.08b | 13.21b | 13.50b |

## Discussion

The exposure of lettuce plants to winter ozone concentration (NFA) and ozone fumigation (NFA+$O_3$) modified Chl *a* parameters, variable Chl *a* fluorescence ($F_v$) declined together with decreases in non-cyclic electron transport and photochemical quenching, and thus heat dissipation and lipid peroxidation rate increased while RL decreased in the four cultivars grown in OTC's.

Leaves from NFA and NFA+$O_3$ treatments did not show any sign of photoinhibition, as indicated by the non-significant differences in the $F_v/F_m$ ratio compared to the control. Under steady-state Chl *a* fluorescence kinet-ics, NFA and NFA+$O_3$ leaves showed a lower actual PS2 efficiency ($\Phi_{PS2}$) and lower fraction of open PS2 centres ($q_P$) compared to the control indicating that $O_3$ decreased the quantum efficiency of non-cyclic electron transport and the capacity for re-oxidising $Q_A$ during actinic irradiation. When plants grow under stressful environment, the activation of the quenching process allows the plants to modulate their primary photochemical reactions. As a consequence $\Phi_{PS2}$ can be limited and the over-reduction of photosynthetic electron carriers prevented (Foyer *et al.* 1994), and an increase in non-radiative dissipation of excitation energy as heat occurs and may be a photoprotective strategy (Osmond 1994, Horton *et al.* 1996). Parallel to reduced $q_P$, an increase in NPQ was observed in NFA and NFA+$O_3$ leaves. The increased NPQ in both treatments with respect to control (CFA) corresponds with a higher fraction of the reduced $Q_A$, favouring non-photochemical quenching at the expense of photochemical utilisation of excitation energy (lower $\Phi_{exc}$ and $\Phi_{PS2}$). In our study, excitation energy reaching the PS2 centres decreased, as indicated by the decrease in $\Phi_{exc}$ relation with the efficiency of excitation capture by open PS2 reaction centres. The ratio $(1 - q_P)$/NPQ relates the values between the excitation pressure and non-photochemical quenching and is an estimate of photon excess. The values of this ratio were similar for all treatments (CFA, NFA, and NFA+$O_3$) in all cultivars. This indicated that although the excitation pressure values ($1 - q_P$) increased with respect to control, the NPQ values were high enough to maintain this ratio and prevented greater damage in PS2 by dissipating the excitation energy as heat. Our results indicate that the decrease in quantum yield of PS2 electron transport and in the efficiency of excitation capture by open PS2 reaction centres, the increase in the proportion of the closed PS2 reaction centres, and the increase of NPQ may be a down-regulation mechanism in the photosynthetic process. The Chl *a* fluorescence-decrease ratio, Rfd, decreased during $O_3$ fumigation indicating a minor potential photosynthetic quantum conversion (Lichtenthaler and Buschmann 1987).

The energy distribution in leaves exposed to $O_3$ was different with respect to control in all cultivars. The $O_3$ ambient and $O_3$ fumigation limit photosynthetic activity, as indicated by a decrease in %P and an increase in thermal energy dissipation and alternative ways. The distribution of energy is correlated with the quenching fluorescence parameters and is a useful tool to understand the routes of energy absorbed by PS2.

Changes in Chl *a* fluorescence yield in NFA and NFA+$O_3$ leaves are associated with changes in the thylakoid membrane. This may suggest that $O_3$ alters the membrane properties (Guidi *et al.* 2000, Calatayud and Barreno 2001, Calatayud *et al.* 2001). $O_3$ or its AOS may react with unsaturated fatty acids forming hydroperoxides (Knox and Dodge 1985). Membrane damage due to lipid peroxidation was found in all treatments and cultivars. MDA analysis seemed to confirm higher lipid peroxidation of the membrane in NFA and NFA+$O_3$ treatment compared to CFA leaves. The MDA content has often been correlated with the degree of $O_3$ exposure in higher plants (Prince *et al.* 1990, Yoshida *et al.* 1994, Ranieri *et al.* 1996, Calatayud and Barreno 2001).

Increase in $O_3$ concentration led to a reduction of RL in all cultivars after 30 d of exposure. The reduction in root length may be associated with decreased root growth (Ollerenshaw *et al.* 1999). Many studies indicate that $O_3$ causes lesser root than shoot growth (Cooley and Manning 1987, Kasana 1991). This response has been discussed in terms of an $O_3$-induced alteration in sink-source relationships (Cooley and Manning 1987, Miller 1988, Renaud *et al.* 1997). Reduced carbon availability for export toward roots could therefore account for the decrease in root growth as opposed to shoot. This hypothesis is in agreement with the obtained Chl *a* fluorescence parameters.

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