Response of spinach leaves (*Spinacia oleracea* L.) to ozone measured by gas exchange, chlorophyll *a* fluorescence, antioxidant systems, and lipid peroxidation

A. CALATAYUD[*,***], D.J. IGLESIAS[**], M. TALÓN[**], and E. BARRENO[*]

Universidad de Valencia, Facultad de Ciencias Biológicas, Departamento de Biología Vegetal, C/Dr. Moliner, 50, 46100-Burjasot, Valencia, Spain[*]
Departamento de Citricultura, Instituto Valenciano de Investigaciones Agrarias I.V.I.A., Conselleria de Agricultura, Pesca y Alimentación, Generalitat Valenciana, Ctra. Moncada-Naquera km. 4.5, 46113-Moncada, Valencia, Spain[**]

Abstract

Spinach (*Spinacia oleracea* L. cv. Clermont) leaves grown in open-top chambers and exposed to three different concentrations of ozone were measured for gas exchange, chlorophyll *a* fluorescence, antioxidant systems, and lipid peroxidation at the end of growing season. High O_3 concentration reduced F_v/F_m, indicating that the efficiency in the energy conversion of photosystem 2 (PS2) was altered. The rate of non-cyclic electron transport rate and the capacity to reduce the quinone pool were also affected. The development of non-photochemical quenching was not high enough to decrease the photon excess in the PS2. The limitation of photosynthetic activity was probably correlated with stomata closure and with an increase in intercellular CO_2 concentration. Under oxidative stress, superoxide dismutase (SOD) activity was stimulated in parallel with lipid peroxidation. We did not find any differences in the ascorbate (AsA) pool and ascorbate peroxidase (APX) or glutathione reductase (GR) activities between air qualities. Small, but similar responses were observed in spinach leaves exposed to ambient ozone concentration.

Additional key words: antioxidant enzymes; ascorbate; chlorophyll fluorescence; intracellular CO_2 concentration; malondialdehyde; photosynthetic rate; quantum efficiency of photosystem 2; quenching; stomatal conductance.

Introduction

Ozone is the main phytotoxic air pollutant in the Mediterranean area (Lorenzini *et al*. 1994, Velissariou *et al*. 1996, Gimeno *et al*. 1999, Calatayud *et al*. 2002a). The influence of O_3 on vegetation is dependent on doses, genetic backgrounds, and the development phase of the plants (Heath 1994). The phytotoxicity of O_3 is due to its high oxidative capacity (redox potential +2.07 V) through the induction of reactive oxygen species (ROS) in exposed plants, such as hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), superoxide (O_2^{-}), and singlet oxygen ($^1O_2$). These chemical species can initiate multiple oxidation events in plant cells. ROS may react with unsaturated double bonds of fatty acids (Mudd 1982) or through oxidation of SH-groups and residues producing carbonyl groups of proteins (Eckardt and Pell 1995). In the chloroplast, these reactions could directly or indirectly impair the light and dark reactions of photosynthesis. Effects of O_3 on the changes in photosynthetic capacities can be assessed non-destructively by gas exchange measurements and chlorophyll (Chl) *a* fluorescence analysis. These techniques are complementary and have been widely used to search for the primary site of photosynthetic limitations.

Received 10 September 2003, *accepted* 10 March 2004.
[***]Corresponding author; fax: +34-6-3544372, e-mail: angeles.calatayud@uv.es
Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; C_i, intercellular CO_2 concentration; CFA, charcoal filtered ozone-free air; Chl, chlorophyll; DM, dry mass; F_m, maximum Chl fluorescence yield obtained a 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 radiation; F_s, Chl fluorescence yield during actinic irradiation; F_v, variable Chl fluorescence of the dark-adapted leaf; g_s, stomatal conductance to water vapour; GR, glutathione reductase; MDA, malondialdehyde; NFA, non-filtered air; NFA+O_3, non-filtered air with additional ozone; NPQ, non-photochemical quenching calculated from the Stern-Volmer equation; OTC, open top chamber; P_{max}, photosynthetic rate at photon saturation; PFD, photon flux density; PS2, photosystem 2; PVP, polyvinylpyrrolidone; q_P, photochemical quenching; ROS, reactive oxygen species; SOD, superoxide dismutase; Φ_{PS2}, quantum efficiency of PS2.
Acknowledgements: The authors thank Miquel Juan Delhom and personnel of the Carcaixent Experimental Station for their technical assistance. A.C. is beneficiary of a project of the Conselleria d`Agricultura, Pesca i Alimentació (GV-683.2/3897).

The need to alleviate the negative effects of ROS on plants prompted the search for chemical compounds such as antioxidant enzymes and antioxidant substances that are effective in counteracting O_3-induced phytotoxicity. Antioxidant enzymes such as SOD, catalase, peroxidases, enzymes of the ascorbate (AsA)-glutathione cycle, and contents of AsA, glutathione, or α-tocopherol provide endogenous defence against the accumulation of harmful concentrations of ROS (Lee *et al*. 1984, Castillo and Greppin 1988, Lyons *et al*. 1999, Calatayud and Barreno 2000, 2001). Upon long-term exposure, plant perturbation by O_3 can depend on the ability to reduce O_3 through its capacity to detoxify ROS by antioxidant systems. However, there are many conflicting reports about the effects of O_3 on antioxidant systems (Bowler *et al*. 1992, Lyons *et al*. 1999, Calatayud and Barreno 2001).

The aim of this work was to characterize the effects of ozone on the photosynthetic processes of spinach leaves (through the measurement of gas exchange parameters and Chl *a* fluorescence analysis) and on the antioxidative capacity as one of the main tolerance mechanisms to O_3 (through the analysis of the levels of antioxidant systems). As a probe of oxidative stress by O_3 we determined the levels of lipid peroxidation. Spinach cv. Clermont is a cultivar that is commonly cultivated during winter at the Spanish Eastern Mediterranean Coast. The plants grown in OTCs in the field were fumigated with O_3, ambient air, and charcoal filtered air.

Materials and methods

Plants: The experimental site was established in the Centro de Capacitación Agraria (Conselleria de Agricultura, Pesca y Alimentación, Generalitat Valenciana) at Carcaixent (see Calatayud *et al*. 2002b for more details).

Spinach seeds (*Spinacia oleracea* L.) cv. Clermont (*Rijk Zwaan*, De Lier, The Netherlands), were germinated in vermiculite and maintained in a greenhouse at the Carcaixent Experimental Station (Valencia, Spain). Environmental conditions in the greenhouse during plant growth were as follows: 15–20 ºC (day), 10–15 ºC (night); relative humidity 50–95 %; photosynthetically active radiation of day-time maximum values at plant height in the greenhouse 500–700 μmol 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 OTCs where they remained for 60 d (from the beginning of February to the end of March). At the end of this period the spinach plants were suitable for commercialization. During the growth period plants were watered daily. Each OTC contained three spinach plants. All measurements (gas exchange, Chl *a* fluorescence, AsA content, antioxidant enzymes, and lipid peroxidation) were performed on mature and external leaves (20–25 cm length by 10–15 cm wide) in asymptomatic areas on the upper zone of the leaves.

At least twenty leaves of the same physiological characteristics described for antioxidant systems and lipid peroxidation measurements were collected from each treatment. Each leaf was weighed and dried in an oven at about 70 ºC. Dry mass (DM) was computed as mean values obtained after weighing twenty leaves per treatment. Antioxidant systems' activities and lipid peroxidation were expressed on a DM basis due to the effect of O_3 on decreasing leaf turgor.

OTC treatments: Potted plants were grown in 9 OTCs located at the Carcaixent Experimental Station. OTCs were based on the original design employed in the NCLAN programme (see Heagle *et al*. 1973). Over the course of growing season, three OTCs were ventilated continuously (24 h d^{-1}) by passing air through activated charcoal and dust filters (CFA, ozone-free air), three OTCs were ventilated with non-filtered air (NFA), and three received additional ozone (NFA+O_3) (for details see Calatayud *et al*. 2002b).

Gas exchange measurements were performed at the end of the growing season using an IRGA (*LICOR-6400, LI-COR,* Lincoln, NE, USA) (Calatayud *et al*. 2002b). Within the leaf chamber, environment did not show statistical differences between measurements during the study. Environmental parameters were maintained stable during the measurements: mean temperature 18.0±1.5 ºC; relative humidity 60.6±5.0 %, and leaf-to-air vapour pressure deficit (VPD) 1.0±0.1 kPa. Photosynthetic rates were taken into account only when the coefficient of variation for each measurement was lower than 1 %. Determinations were initially performed by increasing photon flux densities (PFD) to estimate saturating values for external mature leaves. All determinations were performed at 1 000 μmol(photon) m^{-2} s^{-1}, the minimum saturating PFD determined in preliminary experiments. The gas exchange parameters determined at saturation irradiance were: maximum photosynthetic rate, P_{max} [μmol(CO_2) m^{-2} s^{-1}], stomatal conductance to water vapour, g_s [mol(H_2O) m^{-2} s^{-1}], and the intercellular CO_2 concentration, C_i [μmol(CO_2) mol^{-1}(air)]. Gas exchange determinations were performed between 10:00 and 11:00 ($n \geq 3$ leaves per plant). Measurements were repeated on the same or similar leaves 30 and 60 d after the beginning of treatments. Since the highest differences among treatments were obtained in the last determinations, only those values have been presented (60 d).

Chl *a* fluorescence measurements: During the growth period, measurements of Chl *a* fluorescence were taken 60 d after the treatment at ambient temperature in the OTCs, using a portable fluorimeter (*PAM-2000, Walz*, Effeltrich, Germany). Measurements performed previously did not show differences in the studied parameters.

The environmental conditions during fluorescence determinations were similar to those reported for gas exchange (see above). Leaves were kept in the dark for 30 min prior to measurements (see Calatayud *et al*. 2002b). Calculation of quenching due to non-photochemical dissipation of absorbed light energy (NPQ), was determined at each saturating pulse, according to 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 $(F_m` - F_s)/(F_m` - F_o`)$ (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 $(F_m` - F_s)/F_m`$ (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).

AsA determination: The petiole and veins were removed from excised mature and external leaves at the end of growing season. Each sample included three leaves and determinations were repeated independently, at least three times per sample. We did not find significant differences between values obtained for samples of the same treatment. Samples were also used for determinations of the activity of antioxidant enzymes and of lipid peroxidation. Subsequently, 1 g was weighed, pulverised in liquid nitrogen, and homogenised with 5 cm^3 of 2 % metaphosphoric acid. To pellet all debris, the homogenate was centrifuged (4 360×*g*, 4 ºC, 10 min) and the supernatant was filtered (*Millipore, Mitex* 0.5 μm). AsA was measured spectrophotometrically as described by Takahama and Oniki (1992) with a spectrophotometer (*8452 A Hewlett-Packard*, Palo Alto, USA). AsA amount was determined by monitoring the absorbance decrease at 265 nm induced by the oxidation of AsA to dehydroascorbate by ascorbate oxidase (EC 1.10.3.3, from *Cucurbita* sp.).

Determination of antioxidant enzymes: Mature and external leaves (2 g) exhibiting no visible injury symptoms and without the main midrib were homogenised in 10 cm^3 of 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA and 2 % (m/v) soluble polyvinyl-pyrrolidone (PVP-10) and 5 mM AsA for measurement of APX activity. The slurry was centrifuged at 15 000×*g* for 20 min. The supernatant was filtered (*Millipore, Mitex* 0.5 μm) and utilized for enzyme analysis. All operations were carried out at 3–5 ºC until analysis. The enzymatic assays were performed on leaves removed from plants after 60 d of growth under OTCs conditions.

APX (EC 1.11.1.11) was determined by monitoring the decrease in A_{290} for 4 min in 3 cm^3 of reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM ascorbate, 0.4 mM H_2O_2, and the enzyme aliquot (Nakano and Asada 1981). Corrections were made for the oxidation of AsA in the absence of H_2O_2.

GR (EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm as described by Rao (1992) and Rao *et al*. (1996). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.8), 0.2 mM NADPH, 0.5 mM GSSG, and the leaf extract. The assays were initiated by the addition of NADPH at room temperature. Corrections were made for NADPH oxidation in the absence of the addition of GSSG to the reaction mixture.

SOD (EC 1.15.1.1) activity was measured as described by Beyer and Fridovich (1987). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM methionine, 57 μM nitroblue tetrazolium (NBT), 0.9 μM riboflavin, 0.025 % (m/v) *Triton X-100*, and the appropriate amount of leaf extract. A_{560} was recorded after a 7-min irradiation period. In this assay, 1 unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50 %.

Lipid peroxidation: The extent of lipid peroxidation was estimated at the end of the growth period using malondialdehyde (MDA) as an indicator, according to the protocol adapted for leaves by Heath and Packer (1968), employing the modifications suggested by Dhindsa *et al*. (1981).

Statistical analysis: Variance analysis (ANOVA) was performed on experimental data, statistical significance ($p \leq 0.05$), and judged by the least significant difference (LSD) method. Statistical analyses were performed using the statistical package SPSS (*SPSS Inc.*, Chicago, IL, USA).

Results

Air quality: Ozone is the most important pollutant in the study area. SO_2 and NO_x concentrations were kept low (less than 2 $mm^3\ m^{-3}$ and 5 $mm^3\ m^{-3}$). The data for O_3 concentration and environmental conditions during the growth period are summarized in Table 1. The twelve (daylight)-hours' mean monthly O_3 concentration inside the chambers with the highest O_3 concentration ($NFA+O_3$), including days and hours without O_3 additions, was about 2.5-fold that of ambient concentrations of O_3 (NFA). The mean of high-peak in $NFA+O_3$ chambers every day during daylight hours was approximately 3-fold higher than the high-peak mean in NFA conditions. The mean O_3 concentration in CFA chambers was lower than 10 $mm^3\ m^{-3}$. The environmental conditions were similar in all treatments during the measurements.

Gas exchange measurements: At the end of the growing season, P_{max} (Table 2) was considerably depressed in $NFA+O_3$ plants in comparison to control plants (CFA) (by *ca*. 61 %). The decrease was minor in NFA plants, by

Table 1. Summary of ozone concentrations and environmental conditions in open top chambers during the growing season of spinach plants. CFA, charcoal filtered ozone-free air; NFA, non-filtered air; NFA+O_3, non-filtered air with additional ozone; $[O_3]_{mean}$, mean O_3 concentration [$mm^3\ m^{-3}$]; $[O_3]_{min}$ and $[O_3]_{max}$, means of the minimum and maximum concentrations during 60 d in cycles of 12 h [$mm^3\ m^{-3}$], respectively; T_{mean}, mean 24-h air temperature [ºC]; T_{min} and T_{max}, minimum and maximum air temperatures [ºC], 0 ; and RH [%], air relative humidity.

Treatment	$[O_3]_{mean}$	$[O_3]_{min}$	$[O_3]_{max}$	T_{mean}	T_{min}	T_{max}	RH
CFA	10.2	–	–	13.00	8.02	17.60	72.22
NFA	30.1	17	40	13.10	8.22	18.00	72.32
NFA+O_3	73.0	35	110	13.00	8.10	17.80	72.26

about 25 %. g_s was also affected: in NFA+O_3 spinach plants it decreased in comparison to CFA plants by about 42 %, whereas in NFA plants it decreased only slightly, by about 6 %. Changes in P_{max} and g_s in NFA+O_3 plants were associated with a statistically significant increase in C_i, by about 22 %.

Table 2. Gas exchange and chlorophyll *a* fluorescence parameters in mature spinach leaves (cv. Clermont) without injury symptoms measured at the end of the growing period (60 d in the field) in CFA, NFA, and NFA+O_3 treatments. Gas exchange was measured at ambient CO_2 and O_2 concentrations (345 $cm^3\ m^{-3}$ and 21 %, respectively) and at saturating irradiance (1 000 µmol $m^{-2}\ s^{-1}$). P_{max}, photosynthetic activity at irradiance saturation [µmol(CO_2) $m^{-2}\ s^{-1}$], g_s, stomatal conductance to water vapour [mol(H_2O) $m^{-2}\ s^{-1}$], and C_i, intercellular CO_2 concentration [µmol(CO_2) mol^{-1}]. Values are means (n = 10). Variance analysis (ANOVA) followed by the least significance difference (LSD) test, calculated at 95 % confidence level, was performed. Values followed by the same letter indicate no significant differences.

Treatment	P_{max}	g_s	C_i	F_0	F_m	F_v/F_m	Φ_{PS2}	q_P	NPQ	$1 - q_P/NPQ$
CFA	23.0a	0.458a	246a	0.239a	1.470a	0.838a	0.627a	0.901a	0.583a	0.171a
NFA	17.27a	0.432a	279b	0.240a	1.472a	0.836a	0.617a	0.880a	0.590a	0.205b
NFA+O_3	8.95b	0.267b	301c	0.249a	1.300b	0.807b	0.509b	0.702b	0.640b	0.465c

Table 3. Ascorbate (AsA) content [g kg^{-1}(DM)] and activities of antioxidant enzymes: APX, ascorbate peroxidase [mmol kg^{-1}(DM) s^{-1}]; GR, glutathione reductase [mmol kg^{-1}(DM) s^{-1}]; SOD, superoxide dismutase [U g^{-1}(DM)], and leaf malondialdehyde (MDA) content [µmol(MDA) kg^{-1}(DM)] at the end of growing period in spinach leaves cv. Clermont. Means (n = 8). For comparison of means, variance analysis (ANOVA) followed by the least significance difference (LSD) test was calculated at 95 % confidence level. Values followed by the same letters indicate no significant differences.

Treatment	AsA	APX	GR	SOD	MDA
CFA	3.46a	5.175a	1.055a	6430a	242a
NFA	3.65a	6.286a	1.105a	6200a	246a
NFA+O_3	3.20a	5.784a	1.035a	6950b	350b

Chl *a* fluorescence parameters at dark-adaptation for the mature spinach leaves subjected to CFA, NFA, and NFA+O_3 treatments were changed at the end of the growing period (60 d) (Table 2). The maximum quantum yield of PS2 photochemistry, F_v/F_m, was affected by O_3 exposure (NFA+O_3), which was mainly due to the decrease in F_m. The minimum level of fluorescence, F_0, was not affected by O_3 fumigation.

Also modulated Chl *a* fluorescence at the end of actinic irradiation changed (Table 2). The actual quantum yield (Φ_{PS2}) and photochemical quenching coefficient (q_P) at steady-state kinetics significantly decreased in leaves treated with O_3 (NFA+O_3). Non-photochemical quenching (NPQ) was similar in control and NFA leaves, although a significant increase was observed in NFA+O_3 leaves (by about 10 %). The ratio $(1 - q_P)/NPQ$, a value proportional to the photon excess, was 2.7-fold increased from CFA to NFA+O_3.

AsA pool and activities of antioxidant enzymes (Table 3): AsA pool was similar in all treatments, although a slight insignificant decrease was observed in NFA+O_3 leaves. The APX and GR activities were similar in all treatments. The SOD activity increased in a statistically significant manner in the presence of high O_3 concentration (by about 8 %).

Lipid peroxidation (Table 3) was measured as MDA content. This parameter increased significantly at the end of the growing season in leaves of NFA+O_3 plants, showing a large increase in relation to CFA plants (by about 45 %).

Discussion

O_3 affected the photosynthetic processes in spinach leaves of cv. Clermont, inducing changes in gas exchange and Chl *a* fluorescence and increasing lipid peroxidation. On the other hand, only SOD activity increased in response to O_3. APX and GR activities and AsA content were similar in the three applied air qualities.

The analyses of gas exchange parameters showed a decrease in CO_2 fixation in the NFA+O_3 treatment, which was associated with a strong reduction in g_s and an increase in C_i. These results seem to indicate that O_3 not only induced stomatal closure but also modified the mesophyll CO_2 fixation, as indicated by the increase in C_i contributing to the decrease in P_{max}. The changes in gas exchange parameters were accompanied by variations in Chl *a* fluorescence parameters. The efficiency of primary photochemistry of PS2 can be expressed by the ratio of variable to maximum fluorescence (F_v/F_m) of dark-adapted leaves exposed to saturating radiation pulse. This ratio was affected in response to excess O_3 exposure (Farage 1996, Meinander *et al*. 1996, Calatayud *et al*. 2002a,b). In our case, F_v/F_m decreased in spinach leaves, which can be a result of an increase in protective non-radiative energy dissipation, photodamage of PS2 centres, or both (Krause 1988). The minimum fluorescence yield in the dark-adapted state of the leaf (F_0) remained unchanged in all treatments. This indicated that O_3 did not induce modifications in the antenna pigments, did not decrease the efficiency of excitation trapping at the active centres of PS2, and did not induce photon damage to PS2. The changes in F_v/F_m were due to a decrease in the maximum fluorescence yield in the dark-adapted leaves (F_m), suggesting that the photochemistry of PS2 and its ability to reduce the primary acceptor, Q_A, was affected by O_3. Changes in F_v/F_m can be attributed to an increase in protective non-radiative energy, as shown by NPQ increase in NFA+O_3 plants. Our results are similar to those obtained by Ciompi *et al*. (1997), Guidi *et al*. (1997), and Calatayud and Barreno (2001).

The fraction of closed PS2 reaction centres can be estimated by Chl *a* fluorescence as q_P. This parameter decreased significantly in plants treated with NFA+O_3, indicating an increase in the reduction state of Q_A, thus leading to an increased fraction of closed PS2 centres during actinic irradiation (Calatayud *et al*. 2002a). Closed PS2 centres have fully reduced Q_A, thus the possibility of electron transport to photosystem 1 and beyond is inhibited (Seaton and Walker 1990). According to this suggestion, Φ_{PS2} closely correlates with the quantum yield of non-cyclic electron transport (Genty *et al*. 1989), being reduced by high O_3 concentration and therefore indicating alterations of the electron flow around PS2. Considering the adverse effects of O_3 on the electron transport rate, the inhibition of photosynthesis could be a result, at least to some extent, of a decreased availability of ATP and reducing power. However, the possibility of O_3 damage on Calvin cycle enzymes also must be considered (Baker *et al*. 1994, Miller *et al*. 1999, Guidi *et al*. 2001). A greater fraction of the reduced Q_A favoured the development of NPQ at the expense of photochemical quenching (Calatayud and Barreno 2001, Guidi *et al*. 2001), as found in our experiment. NPQ increased in both treatments (NFA and NFA+O_3) with respect to control plants (CFA), although differences were only significant upon high O_3 fumigation. A combined indicator of irradiance stress is given by the ratio $1 - q_P/NPQ$, which tends to increase as PS2 reaction centres close (q_P lower or $1 - q_P$ higher), but decreases when photoprotective NPQ is enhanced (Park *et al*. 1995). In spinach leaves this ratio increased to CFA from NFA+O_3 with statistically significant differences, indicating a greater photoinactivation of PS2 in plants grown in NFA+O_3 atmosphere. This fact is confirmed by the minor values of F_v/F_m found in those leaves.

The ability of O_3 and/or its products to interact with lipids may have a destructive effect on cell membranes (Guidi *et al*. 1999, Plażek *et al*. 2000, Calatayud *et al*. 2002a,b). Lipid peroxidation measured by MDA content is considered a suitable injury index in plants grown under polluted atmosphere (Ranieri *et al*. 1993) and thus has been correlated with the degree of O_3 exposure to plants (Prince *et al*. 1990, Ranieri *et al*. 1996, Calatayud *et al*. 2002a,b). In spinach plants, lipid peroxidation increased in NFA+O_3 treatment. ROS have been proposed as lipid peroxidation initiators (Elstner *et al*. 1988, Winston 1990). Biological systems have evolved protective scavenging or buffering mechanisms for ROS (Tingey and Taylor 1982, Lyons *et al*. 1999). SOD activity is a primary scavenger for $O_2^{\cdot-}$ radicals and plays a central role in the defence against toxic ROS accumulation (Bowler *et al*. 1992). In our experiments, SOD activity increased in NFA+O_3 plants, although there were non-significant differences in the activities of APX and GR between treatments. The unchanged GR and APX activities in plants grown under O_3 could indicate that the constitutive activities in spinach plants might be sufficient to allow a normal functioning of the AsA-glutathione cycle. The invariability of GR activity is consistent with reports of Cakmak and Marschner (1992) and Calatayud and Barreno (2001). AsA content may be an important factor in the tolerance mechanism. A close relation between AsA and O_3 tolerance has been reported by many researchers (Lyons *et al*. 1999, Pasqualini *et al*. 2001, Calatayud *et al*. 2002b). However, the exposure to O_3 did not produce an effect on AsA content. No significant variations in the intracellular AsA content after O_3 fumigation have been reported in other spinach cultivars (Luwe *et al*. 1993), pumpkin (Ranieri *et al*. 1996), or tobacco (Pasqualini *et al*. 2001). The contents and activities of antioxidant systems depend on a number of factors including the stage of leaf and plant development (Bowler

et al. 1992, Bender *et al*. 1994), growth conditions (Menser 1964), and nutritional status (Polle and Rennerberg 1994, Matyssek *et al*. 1997). Therefore, it is not surprising that extensive surveys of the literature reveal a variable pattern in the response of antioxidative defences to specific pollutants (Polle 1998, Barnes *et al*. 1999).

In summary, the photosynthetic CO_2 fixation in the chloroplast was inhibited by O_3 absorbed into the leaves. This inhibition induced an increase in C_i and stomatal closure, resulting in the reduction of g_s. The limitation of photosynthesis appears to be correlated with a reduction of the efficiency in the energy conversion of PS2, decreasing the rate of non-cyclic electron flow and the capacity to reduce the quinone pool. The oxidative stress was also manifested through an increase in membrane lipid peroxidation. The antioxidant systems showed a minor response to ozone, being only observed as an enhancement of SOD activity, probably because this enzyme acts as a primary scavenger for $O_2^{\cdot -}$ radicals generated both from normal physiological activities and from exposure to stress factors, such as O_3. AsA content was not modified by O_3 and can be associated to unchanged APX and GR activities.

References

Baker, N.R., Nie, G., Tamasevic, M.: Responses of photosynthetic light-use efficiency and chloroplast development on exposure of leaves to ozone. – In: Alscher, R.G., Wellburn, A.R. (ed.): Plant Responses to the Gaseous Environment: Molecular, Metabolic and Physiological Aspects. Pp. 219-238. Chapman and Hall, London 1994.

Barnes, J., Davison, A., Balaguer, L., Manrique-Reol, E.: Resistance to air pollutants: from cell to community. – In: Pugnaire, F.I., Valladares, F. (ed.): Handbook of Functional Plant Ecology. Pp. 735-770. Marcel Dekker, New York – Basel 1999.

Bender, J., Weigel, H.J., Wegner, U., Jager, H.J.: Response of cellular antioxidants to ozone in wheat flag leaves at different stages of plant development. – Environ. Pollut. **84**: 15-21, 1994.

Beyer, W.F., Fridovich, I.: Assaying of superoxide dismutase activity: some large consequences of minor changes in conditions. – Anal. Biochem. **161**: 559-566, 1987.

Bilger, W., Björkman, O.: Temperature dependence of violaxanthin de-epoxidation and non-photochemical fluorescence quenching in intact leaves of *Gossypium hirsutum* L. and *Malva parviflora* L. – Planta **184**: 226-234, 1991.

Bowler, C., van Montagu, M., Inzé, D.: Superoxide dismutase and stress tolerance. – Annu. Rev. Plant Physiol. Plant mol. Biol. **43**: 83-116, 1992.

Cakmak, I., Marschner, H.: Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. – Plant Physiol. **98**: 1222-1227, 1992.

Calatayud, A., Alvarado, J.W., Barreno, E.: Differences in ozone sensitivity in three varieties of cabbage (*Brassica oleracea* L.) in the rural Mediterranean area. – J. Plant Physiol. **159**: 863-868, 2002a.

Calatayud, A., Barreno, E.: Foliar spraying with zineb increases fruit productivity and alleviates oxidative stress in two tomato cultivars. – Photosynthetica **38**: 149-154, 2000.

Calatayud, A., Barreno, E.: Chlorophyll *a* fluorescence, antioxidant enzymes and lipid peroxidation in tomato in response to ozone and benomyl. – Environ. Pollut. **115**: 283-289, 2001.

Calatayud, A., Ramirez, J.W., Iglesias, D.J., Barreno, E.: Effects of ozone on photosynthetic CO_2 exchange, chlorophyll *a* fluorescence and antioxidant systems in lettuce leaves. – Physiol. Plant. **116**: 308-316, 2002b.

Castillo, F.J., Greppin, H.: Extracellular ascorbic acid and enzyme activities related to ascorbic acid metabolism in *Sedum album* L. leaves after ozone exposure. – Environ. exp. Bot. **28**: 231-238, 1988.

Ciompi, S., Castagna, A., Ranieri, A., Nali, C., Lorenzini, G., Soldatini, G.F.: CO_2 assimilation, xanthophyll cycle pigments and PSII efficiency in pumpkin plants as affected by ozone fumigation. – Physiol. Plant. **101**: 881-889, 1997.

Dhindsa, R.S., Plumb-Dhindsa, P., Thorpe, T.A.: Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. – J. exp. Bot. **32**: 93-101, 1981.

Eckardt, N.A., Pell, E.J.: Oxidative modification of Rubisco from potato foliage in response to ozone. – Plant Physiol. Biochem. **33**: 273-282, 1995.

Elstner, E.F., Wagner, G.A., Schutz, W.: Activated oxygen in green plants in relation to stress situations. – In: Randall, D.D., Blevins, D.G., Campbell, W.H. (ed.): Current Topics Plant Biochemistry and Physiology. Vol. 7. Pp. 159-187. University of Missouri, Columbia 1988.

Farage, P.K.: The effect of ozone fumigation over one season on photosynthetic process of *Quercus robur* seedling. – New Phytol. **134**: 279-285, 1996.

Genty, B., Briantais, J.-M., Baker, N.R.: The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – Biochim. biophys. Acta **990**: 87-92, 1989.

Gimeno, B.S., Bermejo, V., Reinert, R.A., Zheng, Y., Barnes, J.D.: Adverse effects of ambient ozone on watermelon yield and physiology at a rural site in Eastern Spain. – New Phytol. **144**: 245-260, 1999.

Guidi, L., Bongi, G., Ciompi, S., Soldatini, G.F.: In *Vicia faba* leaves photoinhibition from ozone fumigation in light precedes a decrease in quantum yield of functional PSII centres. – J. Plant Physiol. **154**: 167-172, 1999.

Guidi, L., Nali, C., Ciompi, S., Lorenzini, G., Soldatini, G.F.: The use of chlorophyll fluorescence and leaf gas exchange as methods for studying the different responses to ozone of two bean cultivars. – J. exp. Bot. **48**: 173-179, 1997.

Guidi, L., Nali, C., Lorenzini, G., Filippi, F., Soldatini, G.F.: Effects of chronic ozone fumigation on the photosynthetic process of poplar clones showing different sensitivity. – Environ. Pollut. **113**: 245-254, 2001.

Heagle, A.S., Body, D.E., Heck, W.W.: An open-top chamber to asses the impact of air pollution on plants. – J. environ. Quality **2**: 365-368, 1973.

Heath, R.L.: Possible mechanisms for the inhibition of photosynthesis by ozone. – Photosynth. Res. **39**: 439-451, 1994.

Heath, R.L., Parker, L.: Photoperoxidation in isolated chloroplasts I. Kinetics and stoichiometry of fatty acid peroxidation. – Arch. Biochem. Biophys. **125**: 189-198, 1968.

Krause, G.H.: Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. – Physiol. Plant. **74**: 566-574, 1988.

Lee, E.H., Jersey, J.A., Gifford, C., Bennett, J.H.: Differential ozone tolerance in soybean and snap beans: analysis of ascorbic acid in O_3-susceptible and O_3-resistant cultivars by high performance lipid chromatography. – Environ. exp. Bot. **24**: 331-341, 1984.

Lorenzini, G., Nali, C., Panicucci, A.: Surface ozone in Pisa (Italy): a six-year study. – Atmos. Environ. **28**: 3155-3164, 1994.

Luwe, M.W.F., Takahama, U., Heber, U.: Role of ascorbate in detoxifying ozone in the apoplast of spinach (*Spinacia oleracea* L.) leaves. – Plant Physiol. **101**: 969-976, 1993.

Lyons, T., Plöchl, M., Turcsányi, E., Barnes, J.D.: Extracellular antioxidants: a protective screen against ozone? – In: Agrawal, M., Krizek, D.T. (ed.): Environmental Pollution and Plant Responses. Pp. 183-201. CRC Press/Lewis Publisher, Boca Raton 1999.

Matyssek, R., Maurer, S., Gunthardt-Goerg, M., Landolt, W., Saurer, M., Polle, A.: Nutrition determines the "strategy" of *Betula pendula* for coping with ozone stress. – Phyton **37**: 157-168, 1997.

Meinander, O., Somersalo, S., Holopainen, T., Strasser, R.J.: Scots pines after exposure to elevated ozone and carbon dioxide probed by reflectance spectra and chlorophyll *a* fluorescence transients. – J. Plant Physiol. **148**: 229-236, 1996.

Menser, H.A.: Response of plant to air pollution. III. A relation between ascorbic acid levels and ozone susceptibility in light-preconditioned tobacco plants. – Plant Physiol. **39**: 564-567, 1964.

Miller, J.D., Arteca, R.N., Pell, E.J.: Senescence-associated gene expression during ozone-induced leaf senescence in *Arabidopsis*. – Plant Physiol. **120**: 1015-1023, 1999.

Mudd, J.B.: Effects of oxidants on metabolic function. – In: Unsworth, M.H., Ormrod, D.P. (ed.): Effects of Gaseous Air Pollution in Agriculture and Horticulture. Pp. 189-203. Butterworth Scientific, London – Boston – Sydney – Wellington – Durban – Toronto 1982.

Nakano, Y., Asada, K.: Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. – Plant Cell Physiol. **22**: 867-880, 1981.

Park, Y.-I., Chow, W.S., Anderson, J.M.: The quantum yield of photoinactivation of photosystem II in pea leaves is greater at low than at high photon exposure. – Plant Cell Physiol. **36**: 1163-1167, 1995.

Pasqualini, S., Batini, P., Ederli, L., Porceddu, A., Piccioni, C., Marchis, F., Antonielli, M.: Effects of short-term ozone fumigation on tobacco plants: response of the scavenging systems and expression of the glutathione reductase. – Plant Cell Environ. **24**: 245-252, 2001.

Plażek, A., Rapacz, M., Skoczowski, A.: Effects of ozone fumigation on photosynthesis and membrane permeability in leaves of spring barley, meadow fescue, and winter rape. – Photosynthetica **38**: 409-413, 2000.

Polle, A.: Photochemical oxidants: Uptake and detoxidication mechanisms. – In: de Kok, L.J., Stulen, I. (ed.): Responses of Plant Metabolism to Air Pollution and Global Change. Pp. 95-116. Backhuys Publ., Leiden 1998.

Polle, A., Rennenberg, H.: Photo-oxidative stress in trees. – In: Foyer, C., Mullineaux, P. (ed.): Causes of Photo-oxidative Stress and Amelioration by Defence Systems in Plants. Pp. 119-218. CRC Press, Boca Raton 1994.

Prince, A., Lucas, P.W., Lea, P.J.: Age dependent damage and glutathione metabolism in ozone fumigated barley: a leaf section approach. – J. exp. Bot. **41**: 1309-1317, 1990.

Ranieri, A., Lencioni, L., Schenone, G., Soldatini, G.F.: Glutathione-ascorbic acid cycle in pumpkin plants grown under polluted air in open-top chambers. – J. Plant Physiol. **142**: 286-290, 1993.

Ranieri, A., D`Urso, G., Nali, C., Lorenzini, G., Soldatini, G.F.: Ozone stimulates apoplastic antioxidant systems in pumpkin leaves. – Physiol. Plant. **97**: 381-387, 1996.

Rao, M.V.: Cellular detoxifying mechanisms determine the age dependent injury in tropical trees exposed to SO_2. – J. Plant Physiol. **140**: 733-740, 1992.

Rao, M.V., Gopinadhan, P., Ormrod, D.P.: Ultraviolet-B and ozone induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. – Planta **110**: 126-136, 1996.

Schreiber, U., Schliwa, U., Bilger, W.: Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorimeter. – Photosynth. Res. **10**: 51-62, 1986.

Seaton, G.G.R., Walker, D.A.: Chlorophyll fluorescence as a measure of photosynthetic carbon assimilation. – Proc. roy. Soc. London B **242**: 29-35, 1990.

Takahama, U., Oniki, T.: Regulation of peroxidase-dependent oxidation of phenolics in the apoplast of spinach leaves by ascorbate. – Plant Cell Physiol. **33**: 379-387, 1992.

Tingey, D.T., Taylor, G.E., Jr.: Variation in plant responses to ozone: a conceptual model of physiological events. – In: Unsworth, M.H., Ormrod, D.P. (ed.): Effects of Gaseous Air Pollution in Agriculture and Horticulture. Pp. 113-138. Butterworth Scientific, London – Boston – Sydney – Wellington – Durban – Toronto 1982.

Velissariou, D., Gimeno, B.S., Badiani, M., Fumagalli, I., Davison, A.W.: Records of O_3 visible injury in the ECE Mediterranean region. – In: Kärelampi, L., Skärby, L. (ed.): Critical Levels for Ozone in Europe: Testing and Finalising the Concept. Pp. 343-350. UN-ECE Workshop Report, University of Kuopio, Kuopio 1996.

Winston, G.W.: Physicochemical basis for free radical formation in cells: production and defense. – In: Alscher, R.G., Cumming, J.R. (ed.): Stress Response in Plants: Adaptation and Acclimation Mechanisms. Pp. 58-86. Wiley-Liss, New York 1990.