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# Changes in chlorophyll *a* fluorescence, lipid peroxidation, and detoxificant system in potato plants grown under filtered and non-filtered air in open-top chambers

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## Abstract

Its high oxidant capacity and ability to generate reactive oxygen species cause ozone toxicity. We studied the effect of ambient ozone on chlorophyll (Chl) *a* fluorescence, antioxidant enzymes, ascorbate contents, and lipid peroxidation in potatoes grown in open-top chambers in the field. In plants grown in non-filtered air (NFA), the development of non-photochemical quenching brought about a decrease in photosystem 2 (PS2) photochemical efficiency. Also the ability of PS2 to reduce the primary acceptor $Q_A$ was lower than in charcoal-filtered, ozone-free air (CFA). Changes in Chl fluorescence yield were associated with changes in the thylakoid membrane. Ozone altered chloroplast membrane properties, as indicated by an increase in membrane lipid peroxidation in NFA-leaves compared to CFA plants. The ascorbate pool and activities of antioxidant enzymes were used for an indication of the detoxification system state in NFA and CFA leaves, whereby ozone affects the ascorbate concentration and decreases the antioxidant enzymes activities. The capacity of both detoxifying systems together was not high enough to protect potato plants against ambient ozone concentrations which reduced the photosynthetic yield in this potato cultivar.

*Additional key words*: antioxidant enzymes; ascorbate; lipid peroxidation; non-photochemical quenching; ozone; photochemical quenching; *Solanum tuberosum*.

## Introduction

Tropospheric ozone is one of the most phytotoxic air pollutants. It is formed by the interaction of nitrogen oxides, hydrocarbons, and UV radiation. Ozone or its free radical impairs membrane functions, and leads to a decline in photosynthesis, foliar injury, reduction in shoot and root growth, and premature senescence (Lefohn 1992). Photosynthesis is particularly sensitive to ozone. PS2 activity regulates the response of photosynthesis to environmental perturbations (Baker *et al.* 1994). The photochemistry of PS2 has been extensively studied using Chl fluorescence emission. Chl *a* fluorescence is a sensitive indicator of photosynthetic energy conversion (Papageorgiou 1975). At physiologically optimal conditions, the major part of the photons absorbed by plants is converted into photochemistry whereas de-excitation by heat emission and Chl fluorescence is fairly low. Under stress the photosynthetic quantum conversion declines and heat emission and Chl fluorescence increase considerably (Lichtenthaler 1996, McMichael *et al.* 1989). At high irradiance, the increase in the amount of excess radiant energy absorbed by leaves can lead to a depression in the efficiency of PS2 by photoinhibition (Powles 1984, Lichtenthaler *et al.* 1992, Krause 1994).

Negative effects on the photosynthetic apparatus

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*Abbreviations*: APX, ascorbate peroxidase; AsA, ascorbate; CFA, charcoal-filtered ozone-free air; Chl, chlorophyll; DHA, dehydroascorbate; $F_m$, maximum Chl fluorescence yield obtained a with dark-adapted sample; $F'_m$, maximum Chl fluorescence yield in irradiated samples; $F_0$, 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; $F_s$, chlorophyll fluorescence yield during actinic irradiation; $F_v = (F_m - F_0)$ variable Chl fluorescence in the dark-adapted leaf; MDA, malondialdehyde; NFA, non-filtered air; NPQ, non-photochemical quenching calculated from Stern-Volmer equation; OTC, open top chamber; PS, photosystem; $q_p$, photochemical fluorescence quenching; SOD, superoxide dismutase; TAA, total ascorbate; $\Phi_{PS2}$, quantum efficiency of PS2.
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caused by ozone or its reactive oxygen species (AOS) are considered to be of secondary order in the chain reaction of oxidative degradation in plants even in cases without visible leaf injury (Heath 1980). The plants metabolize AOS by invoking the antioxidative defense system, which consists of low molecular mass antioxidants such as ascorbate, glutathione, α-tocopherol, and carotenoids (see Alscher and Hess 1993), as well as several enzymes such as superoxide dismutase, catalase, peroxidase, glutathione reductase, and ascorbate peroxidase (see Bowler *et al.* 1994, Creissen *et al.* 1994). Under non-stressed conditions, the antioxidants adequately protect against these AOS (Asada and Takahashi 1987). If the concentration of ozone is high, the anti-oxidative capacity increases but it may be insufficient to protect the plant completely (Foyer *et al.* 1994). These AOS can also cause the disruption of plasma membrane integrity due to the oxidation of protein sulfhydryl groups and/or lipids (Beckerson and Hofstra 1980, Guidi *et al.* 1999). The ability to deal with ozone, once it has entered the cell, might account for differences in the sensitivity of the species to a particular pollutant. In this context, non-enzymatic antioxidants and antioxidant enzymes must play a crucial role in the detoxification of AOS generated by ozone (Foyer and Mullineaux 1994).

Monitoring ozone data (Ribas *et al.* 1998, Sanz and Millán 1998) shows that crops in few areas of Europe are at greater risk from $O_3$ than on the East Coast of Spain (mainly in Catalunya, Valencia, Murcia, and Almeria). This area is the major centre for fruit and vegetable production. Potatoes are one of the most widely cultivated crops in the Valencia area. Their sensitivity or tolerance to ozone depends on the cultivar (De Vos *et al.* 1983). In order to characterise the $O_3$ sensitivity in potato plants var. Obelix, we have measured Chl *a* fluorescence, the activities of antioxidant enzymes, an oxidative strain in plants cultivated in charcoal-filtered, ozone-free air (CFA) *versus* non-filtered air (NFA) open top chambers.

## Materials and methods

**Plants**: Half of tuber cores (*ca.* 10×6 cm size) of potato (*Solanum tuberosum* L. var. Obelix) were germinated in vermiculite and maintained in a glasshouse at the Carcaixent Experimental Station (Valencia, Spain). Environmental conditions in the glasshouse during plant growth were as follows: 15 to 20 °C (day), 8 to 12 °C (night); relative humidity 60 to 95 %; maximum photosynthetically active radiation at plant height 700-1 000 μmol $m^{-2}$ $s^{-1}$. After 4 weeks, plants (14-20 leaves) were transplanted into large pots containing a commercial soil mixture (*Terraplant, BASF*, Uchte, Germany) and transferred into open top chambers (OTCs) where they were exposed to CFA and NFA for 75 d from the beginning of February to the middle of April. Each chamber contained 4 pots. During the growth period all plants were watered daily with the same amount of water (drip-feed system).

**Open top chambers**: Potted plants were grown in 8 OTCs, 3 m in diameter and 3 m in height, located at the Carcaixent Experimental Station. OTCs were based on the original design employed in the US National Crop Loss Assessment Network (NCLAN) (see Heagle *et al.* 1973). During the growing season, four OTCs were ventilated continuously with a pump, air passed through activated charcoal and dust filters (type *SF/Q* class *EU-3* and *EU-4*, *SF/CG* and *RBAA2*) (CFA) and the remainder ones were ventilated with non-filtered air (NFA). The concentration of gaseous pollutants [$O_3$, oxides of nitrogen (NO and $NO_2$), and sulphur dioxide ($SO_2$)], 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 for 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 Env. Corp.*, Glendale, CA, USA).

**Air quality**: The concentrations of $SO_2$ and $NO_x$ during the growth period were low, less than 2 $mm^3$ $m^{-3}$ for $SO_2$ and 5 $mm^3$ $m^{-3}$ for $NO_x$ in the Carcaixent Experimental Station. Ozone concentration was high during the growing season: the accumulated hourly ozone exposure when the concentration was over 40 $mm^3$ $m^{-3}$ (AOT40) between sunrise and sunset was 4 028 $mm^3$ $m^{-3}$ $s^{-1}$. This exceeded the UN-ECE critical level for the protection of crop yield (set at an AOT40 out of 3 000 $mm^3$ $m^{-3}$ $s^{-1}$, based on AOT40 during daylight hours for the consecutive 3-month period). Maximum $O_3$ concentration reached 70 $mm^3$ $m^{-3}$ during the growing season, and the total number of hours exceeding the 40 $mm^3$ $m^{-3}$ threshold over the exposure period was 436. Ozone concentrations in CFA chambers never attained the UN-ECF threshold of 40 $mm^3$ $m^{-3}$. Mean temperatures inside the OTCs reached a maximum during April (24±6 °C), minimum temperatures occurred during February (10±5 °C). The daily mean relative humidity was approx. 83±10 % during the growing season.

**Chl *a* fluorescence**: At the end of the growth period, Chl fluorescence was measured at ambient temperature *in situ* in the OTCs, using a portable fluorometer (*PAM-2000, Walz*, Effeltrich, Germany). Mature leaves without visible injury symptoms were darkened for 30 min prior to the measurement. The minimum (dark) Chl fluorescence, $F_0$, was obtained on exciting leaves with a weak beam from a light-emitting diode. The maximum Chl fluorescence ($F_m$) was determined following a 600 ms pulse of saturating "white light". The yield of variable Chl fluores-

cence ($F_v$) was calculated as $F_m - F_0$. Following 2 min of dark re-adaptation, actinic "white light" was switched on and different irradiances were applied for each Chl fluorescence induction kinetic (430, 940, 1 460, and 2 230 μmol $m^{-2}$ $s^{-1}$) (Calatayud and Barreno 2001). 600 ms saturating pulses (8 000 μmol $m^{-2}$ $s^{-1}$) were applied at 5 min intervals for 15 min to determine the maximum Chl fluorescence yield during actinic irradiation ($F'_m$). The constant Chl fluorescence yield during a brief interruption of actinic irradiation in the presence of far-red radiation ($F'_0$), and the Chl fluorescence yield during actinic irradiation ($F_s$) were also measured. The calculation of non-photochemical quenching (NPQ) was determined using the equation $NPQ = (F_m - F'_m)/F'_m$) (Bilger and Björkman 1991). The coefficient for photochemical quenching, $q_P$, 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 $(F'_m - F_s)/F'_m$) (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).

**Analyses of antioxidant enzymes** were performed at the end of the growth period. Mature leaves (2 g) exhibiting no visible injury symptoms, 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 ascorbate for measurement of ascorbate peroxidase activity. The slurry was centrifuged at 15 000×*g* for 20 min. The supernatant was filtered (*Millipore, Mitex* 0.5 μm) and utilised for enzyme analysis. All operations (until analysis) were carried out at 3 to 5 °C.

Ascorbate peroxidase (APX) was determined by monitoring the decrease in $A_{290}$ for 4 min in 3 $cm^3$ of a 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 ascorbate in the absence of $H_2O_2$.

NADPH-dependent glutathione reductase (GR) activity was determined as the oxidation of NADPH (Rao 1992, 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 GSSG in the reaction mixture.

Superoxide dismutase (SOD) activity was measured as described by Beyer and Fridovich (1987). The reaction mixture was composed of 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. The $A_{560}$ was recorded after a 7-min irradiation. 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 using malondialdehyde (MDA) as an indicator, following the protocol adapted for leaves by Heath and Parker (1968), employing the modifications made by Dhindsa *et al.* (1981). Mature leaves without visible symptoms were used at the end of the growth period.

**Ascorbate determination**: The petiole and veins were removed from the excised mature leaves. Subsequently, 1 g was weighed and 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 °C, 10 min) and the supernatant filtered (*Millipore, Mitex* 0.5 μm). Ascorbate (AsA) and dehydroascorbate (DHA) were measured as described by Takahama and Oniki (1992) using the *8452 A Hewlett-Packard* spectrophotometer (Palo Alto, USA). Ascorbate concentration was determined by monitoring the absorbance decrease at 265 nm induced by the oxidation of AsA to DHA by ascorbate oxidase (EC 1.10.3.3, from *Cucurbita* sp.). In the second assay, to determine total ascorbate (TAA), DHA was reduced to AsA by adding dithiothreitol to a final concentration of 0.5 mM. The DHA concentration was calculated as the difference between TAA and AsA.

**Statistical analysis**: Variance analysis (ANOVA) was performed on experimental data, statistical significance ($p < 0.05$) was judged by the least significant differences (LSD) method. The statistical analysis was performed using the statistical program SPSS® (*SPSS*, Chicago, IL, USA).

## Results

**Chl *a* fluorescence analysis**: The maximal photochemical efficiency after a 30-min dark adaptation, estimated by the ratio $F_v/F_m$, did not change significantly with air pollution. Leaves in CFA had $F_v/F_m$ ratios of 0.81±0.02, whereas for leaves in NFA it was 0.80±0.04. This indicates that leaves in NFA had no signs of photoinhibition. The fluorescence parameters after 15 min of actinic irradiation are shown in Fig. 1. The actual photochemical efficiency ($\Phi_{PS2}$) (Fig. 1*A*) and the fraction of open PS2 centres ($q_P$) (Fig. 1*B*) decreased as actinic irradiance

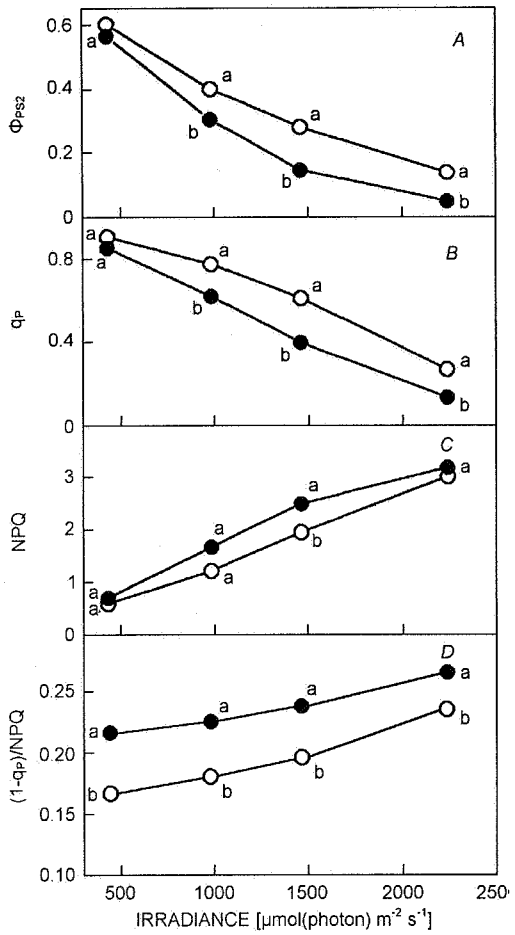


Fig. 1. Changes in chlorophyll fluorescence parameters at different actinic irradiance, $\Phi_{PS2}$ (*A*), $q_P$ (*B*), NPQ (*C*), and $(1 - q_P)/NPQ$ (*D*) in potato leaves. Plants in charcoal-filtered chambers (○), plants in non-filtered air (●). Means of 8 determinations. Symbols in the graph followed by different letters indicate significant differences between the two growth conditions at $p < 0.05$ (LSD-test).

increased. These parameters showed a significant reduction in NFA leaves as compared with control treatments. NPQ (Fig. 1*C*) is a good estimate of heat dissipation by PS2. The values of NPQ at the end of kinetic fluorescence induction were similar for all irradiances and showed no significant differences in either treatment, except for an irradiance of 1 460 μmol $m^{-2}$ $s^{-1}$ where the values between NFA and CFA plants showed a significant difference. The ratio between excitation pressure and NPQ $[(1 - q_P)/NPQ]$ is an estimate of the excess of photons during photosynthesis induction. The changes in this ratio (Fig. 1*D*) indicate that a large excess of photons occurred in the leaves under NFA treatment at all irradiances with significant differences between either treatment.

**Changes in antioxidant enzymes activities**: Activities of APX (Table 1) and SOD measured at the end of the growing season were significantly lower in NFA potato leaves than CFA leaves, with a decrease of 25 and 36 %, respectively. The GR activity was not significantly different between NFA and CFA leaves.

**Lipid peroxidation** was significantly higher in NFA leaves [195.52±8.40 μmol(MDA) $kg^{-1}$(f.m.)] than in CFA plants [96.92±7.00 μmol(MDA) $kg^{-1}$(f.w.)]. The degree of peroxidation induced air quality in NFA plants increased by 102 % compared to CFA leaves.

**AsA and DHA concentrations**: The $O_3$ in the air decreased the contents of AsA in NFA leaves. CFA plants (Table 1) had nearly an 8-fold increase over NFA leaves in AsA content. DHA concentration was higher in NFA than CFA leaves. The AsA/TAA ratio was higher in CFA leaves than NFA plants with significant differences. However, TAA content did not differ significantly in CFA and NFA leaves.

Table 1. Activities of antioxidant enzymes, APX = ascorbate peroxidase [mmol $kg^{-1}$(f.m.) $s^{-1}$], GR = [mmol $kg^{-1}$(f.m.) $s^{-1}$], SOD = suoperoxide dismutase [U $g^{-1}$(f.m.)], and ascorbate (AsA), dehydroascorbate (DHA), and total ascorbate (TAA = AsA + DHA) contents [g $kg^{-1}$(f.m.)] and the AsA/TAA ratio in potato leaves at different treatments. CFA plants in charcoal-filtered air and NFA plants in non-filtered air. Means±SD, $n = 4$. To compare means, variance analysis (ANOVA) followed by the least significance difference (LSD) test, calculated at $p < 0.05$ were carried out. Values followed by the same letter are not significantly different.

| Treatment | APX | GR | SOD | AsA | DHA | TAA | AsA/TAA | AsA/DHA |
|---|---|---|---|---|---|---|---|---|
| CFA | 0.546±0.020a | 0.102±0.003a | 1854.2±125.0a | 0.059±0.010a | 0.131±0.020a | 0.190±0.050a | 0.312±0.080a | 0.451±0.100a |
| NFA | 0.416±0.040b | 0.098±0.005a | 1194.3±160.0b | 0.007±0.002b | 0.188±0.020b | 0.195±0.060a | 0.036±0.010b | 0.037±0.010b |

## Discussion

Environmental stresses affect plant photosynthetic processes and decrease the photosynthetic quantum conversion, and thus increase Chl fluorescence and heat dissipation (Lichtenthaler 1996). In the present paper we studied the effect of ambient ozone concentrations on the photosynthetic process in potato leaves using Chl fluorescence techniques and other events, such as enzyme antioxidant activities, ascorbate concentration, and lipid peroxidation,

to gain a greater understanding of the effects of ozone on plants. Leaves from ozone NFA treatments did not show signs of photoinhibition, as indicated by the unchanged $F_v/F_m$ ratios after 30 min of dark adaptation. But after 15 min irradiation, the NFA leaves showed a lower PS2 photochemical efficiency ($\Phi_{PS2}$). Moreover, the lower $q_P$ values indicate that NFA plants experienced a higher excitation pressure on PS2 and more reaction centres were closed in NFA treatment compared to the control (CFA) plants. When the plants grow under stressful environmental situations, 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). The ratio $(1 - q_P)/NPQ$ relates the values between photo-chemical and non-photochemical quenching associated with an excess of photons during the photosynthetic process. In NFA leaves this ratio was higher than CFA indicating that a great excess of photons occurred and the excitation pressure values $(1 - q_P)$ were not low enough in NFA plants or NPQ values were not high enough to decrease this ratio. NPQ values can be affected by ascorbate concentration. This compound is required specifically to synthesise zeaxanthin in the xanthophyll cycle for harmless and radiationless dissipation of excess excitation energy from the Chl pigment bed (Foyer *et al.* 1991, Pfündel and Bilger 1994, Demmig-Adams *et al.* 1995, Calatayud *et al.* 1999). In addition, the ascorbate is important in the antioxidant cell system. Under our experimental conditions, the ozone concentration affected the AsA endogenous concentration in NFA leaves from CFA plants: this decrease in the AsA content might be a con-sequence of sustained oxidative stress induced by ambient ozone. A decline in the AsA pool in the course of pollutant stress has been documented by Castillo and Greppin (1988), Luwe (1996), Wellburn *et al.* (1996), Calatayud *et al.* (1999), Herrero-Martinez *et al.* (2000), and Turcsányi *et al.* (2000). Moreover, DHA contents increased at the expense of its reduced form, however, the TAA were not modified. The AsA/DHA ratio in the cell depends mainly on two events, AsA biosynthesis and AsA oxidoreduction (Arrigoni 1994). This ratio severely decreases in NFA leaves, indicating that the AOS concentration in the cell presumably exceeds the regeneration rate of AsA. This event, which could explain the lower APX activity in NFA plants, is highly sensitive to the presence of AsA (Groden and Beck 1979). Ozone degrades into superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen as a result of reaction with biological molecule (Foyer *et al.* 1994). This $H_2O_2$ is eliminated by APX, but if its turnover is low, it can accumulate in the cell, causing the inactivation of endogenous SOD, together with a reversible inhibition of Calvin cycle enzymes (Tanaka *et al.* 1985). This could explain the lower activity of SOD found in NFA leaves from CFA plants. In our experiments we observed that GR activity did not change under NFA treatments: the constitutive GR activity in potato plant leaves may be sufficient for it to function normally.

The ozone or its AOS alter membrane properties, modify the Chl fluorescence yield, activities of anti-oxidant enzymes, or other metabolic processes in the cell (Prince *et al.* 1990, Ranieri *et al.* 1996, Calatayud *et al.* 2000, Calatayud and Barreno 2000). MDA analysis seems to confirm higher lipid peroxidation of the membrane in NFA compared to CFA leaves. The MDA content has been correlated with the degree of $O_3$ exposure in higher plants (Prince *et al.* 1990, Yoshida *et al.* 1994, Ranieri *et al.* 1996).

In conclusion, ambient ozone induces alterations in the light reactions of photosynthesis, the CFA leaves present a higher PS2-mediated electron flow and higher fraction of open PS2 centres than NFA leaves, whereas through heat dissipation they are similar in CFA and NFA potato leaves. Ozone decreases the AsA pool and the antioxidant enzyme activities that then can not act in the neutralisation of toxic oxygen species as shown by the increased lipid peroxidation in NFA leaves.

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