Does ascorbate in the mesophyll cell walls form the first line of defence against ozone? Testing the concept using broad bean (Vicia faba L.)

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Abstract

Broad bean (Vicia faba L.) plants were exposed, in duplicate controlled environment chambers, to charcoal/Purafil\textsuperscript{®}-filtered air (CFA-grown plants) or to 75 nmol mol\textsuperscript{-1} ozone (O\textsubscript{3}) for 7 h d\textsuperscript{-1} (O\textsubscript{3}-grown plants) for 28 d, and then exposed to 150 nmol mol\textsuperscript{-1} O\textsubscript{3} for 8 h. The concentration of ascorbate (ASC) was determined in leaf extracellular washing fluid (apoplast) and in the residual leaf tissue (symplast) after 0, 4 and 8 h acute fumigation, and after a 16 h ‘recovery’ period in CFA. Changes in stomatal conductance were measured \textit{in vivo} in order to model pollutant uptake, while the light-saturated rate of CO\textsubscript{2} assimilation (A\textsubscript{sat}) was recorded as an indicator of O\textsubscript{3}-induced intracellular damage. Measurements of A\textsubscript{sat} revealed enhanced tolerance to 150 nmol mol\textsuperscript{-1} O\textsubscript{3} in plants pre-exposed to the pollutant compared with equivalent plants grown in CFA, consistent with the observed reduction in pollutant uptake due to lower stomatal conductance. The concentration of ASC in the leaf apoplast (ASC\textsubscript{apo}) declined upon O\textsubscript{3}-treatment in both CFA- and O\textsubscript{3}-grown plants, consistent with the oxidation of ASC\textsubscript{apo} under O\textsubscript{3}-stress. Furthermore, the decline in ASC\textsubscript{apo} was reversible in O\textsubscript{3}-grown plants after a 16 h ‘recovery’ period, but not in plants grown in CFA. No significant change in the level and/or redox state of ASC in the symplast (ASC\textsubscript{sym}) was observed in plants exposed to 150 nmol mol\textsuperscript{-1} O\textsubscript{3}, and there was no difference in the constitutive level of ASC\textsubscript{sym} between CFA- and O\textsubscript{3}-grown plants. Model calculations indicated that the reaction of O\textsubscript{3} with ASC\textsubscript{apo} in the leaves of Vicia faba is potentially sufficient to intercept a substantial proportion (30–40%) of the O\textsubscript{3} entering the plant under environmentally-relevant conditions. The potential role of apoplastic ASC in mediating the tolerance of leaves to O\textsubscript{3} is discussed.

Key words: Apoplast, cell wall, ozone, detoxification, antioxidants, ascorbate.

Introduction

Tropospheric concentrations of ozone (O\textsubscript{3}) are known to pose a growing threat to the vitality of natural and managed ecosystems in many parts of the industrialized world (Runeckles and Chevone, 1992; Davison and Barnes, 1992, 1998; UNECE, 1996; Fuhrer \textit{et al.}, 1997). However, the mechanisms underlying the phytotoxicity of this ubiquitous air pollutant are just beginning to be unravelled (Harris and Bailey-Serres, 1993; Kangasjärvi \textit{et al.}, 1994; Mudd, 1996; Sharma and Davis, 1997). One area of growing interest has been the role played by ASC, and other potential antioxidants situated in the leaf apoplast, in mediating the tolerance of plant tissues to this powerful oxidant (Polle and Rennenberg, 1993; Lyons \textit{et al.}, 1999a).

The flux of O\textsubscript{3} to the leaf interior is controlled almost entirely by stomatal conductance (Kerstiens and Lendzian, 1989). Once inside the leaf, the pollutant dissolves into the aqueous matrix which overlays the...
surface of the cells lining the sub-stomatal cavity (the apoplast), resulting in an intercellular space O₃ concentration that is demonstrably close to zero (Laisk et al., 1989). Ozone is a strong oxidant (+2.07 V), and is considered to react first with oxidizable constituents of the apoplast, and, subsequently, if the pollutant and/or its reactive products escape interception, with components of the plasmalemma and cytosol (Heath, 1980, 1988). The dissolution chemistry of O₃ in the region of the cell wall is far from fully understood (Heath, 1980, 1988; Mudd, 1996; Moldau, 1998). However, the primary reactions of the pollutant have been suggested to yield several other potentially damaging reactive oxygen species (ROS), including the hydroxyl radical, superoxide, singlet oxygen, and hydrogen peroxide (Grimes et al., 1983; Kanofsky and Sima, 1995a, b; Mehlhorn et al., 1990; Pryor, 1994; Byvoet et al., 1995), and possibly organic compounds which can act as antioxidants, including Acute O₃ fl retention gas exchange was made on the fourth fully expanded leaf on 4-week-old plants.

Materials and methods

Plant culture and fumigation

Seed of broad bean (Vicia faba L. cv. Imperial White Windsor) was germinated in seed trays containing vermiculite, in duplicate (PPFD) of 200 μmol m⁻² s⁻¹ at plant height, supplied as a 14 h photoperiod (07.00 h–21.00 h). Relative humidity was maintained at 65±5%. All plants were transplanted into larger pots (3 dm³) containing a standard potting compost (John Innes No. 2) after 7 d fumigation. Further details of this controlled environment fumigation facility, and gas control/monitoring systems are provided elsewhere (Barnes et al., 1995; Zheng et al., 1998). Experiments were performed on the fourth fully expanded leaf on 4-week-old plants.

Acute O₃ exposures (150 nmol mol⁻¹, 8 h) were administered in a controlled environment chamber identical to those in which plants were grown, ventilated by the same air handling system. Plants were returned to their respective growth conditions following O₃-treatment.

Growth and dry matter partitioning

Between 10 and 12 plants (5–6 plants per chamber) grown in CFA and O₃ were harvested after 28 d. An initial harvest of 10 plants (5 plants per chamber) was made after 7 d—the root separated from the shoot, component plant parts dried (70 °C for 1 week) and then weighed. Mean plant relative growth rate (R), the relative growth rate of root (Rₘ) and shoot (Rₛ), and allometric root/shoot growth (K; Rₘ/Rₛ) were calculated as described previously (Hunt, 1990).

Leaf gas exchange measurements

In situ measurements of leaf gas exchange were made on the fourth (youngest fully expanded) leaf borne on six ‘control’ CFA- or O₃-grown plants and an equivalent number of plants transferred into 150 nmol mol⁻¹ O₃, at regular intervals over the course of the day. Additional measurements were made on the same leaves following 16 h recovery under pretreatment conditions. Experiments were repeated twice; the data presented representing the average of both experiments (i.e. n = 12). Rates
of CO₂/H₂O exchange were monitored using a portable infrared gas analysis system (CIRAS-1 portable IRGA system, PP Systems, Hitchin, Herts, UK). Measurements were made at a cuvette CO₂ concentration of 350 ± 1 μmol mol⁻¹ under chamber conditions (PPFD = 198 ± 4 μmol m⁻² s⁻¹ at the position occupied by the leaf in the cuvette; leaf temperature = 24 ± 0.5 °C) using a standard Parkinson leaf cuvette (model PLC-B, PP Systems), and under light-saturated conditions (PPFD = 1200 ± 2 μmol m⁻² s⁻¹ at the position occupied by the leaf; leaf temperature = 23 ± 0.5 °C) using a Parkinson leaf cuvette that incorporates automated light and temperature control (model auto-PLC-B, PP Systems). The light-saturated rate of CO₂ assimilation (A₅₅) and stomatal conductance to water vapour (gₛₛₛₒₒ; measured under chamber conditions) were calculated according to von Caemmerer and Farquhar (von Caemmerer and Farquhar, 1981).

### Preparation of extracellular washing fluid and residual leaf extracts

Extracellular washing fluid (EWF) and residual leaf extracts (RLEs) were prepared from independent ‘control’ (CFA- or O₃-grown) and ‘O₃-treated’ (CFA- or O₃-grown plants exposed to 150 nmol mol⁻¹ O₃) plants at regular intervals over the course of the fumigation. Additional measurements were performed on an equivalent number of plants allowed to recover for a 16 h-period under pretreatment conditions. Experiments were repeated twice; the data presented represent the average across the two experiments (n = 20–24 ‘control’; n = 6 acute O₃-treatment).

The fourth leaf (≈ 0.5 g FW) was detached, weighed, washed with double-distilled water, and then vacuum infiltrated (−70 kPa) with 100 ml of 66 mM K-phosphate buffer (pH 4.5) containing 100 mM KCl and 2.5 mM EDTA (two infiltration periods; each of 1 min duration). Immediately after infiltration, the leaf was blotted dry, re-weighed, rolled carefully and inserted into a syringe placed over a preweighed 1.5 ml Eppendorf tube containing 100 μl of cold 100 mM HCl. Extracellular washing fluid was collected under ‘soft’ centrifugation (5 min., 80 g) at 4 °C (3 K-18 centrifuge, Sigma-Aldrich, Poole, UK), and was kept on ice for only a short-time before the analysis of ASC/DHA. Eppendorf tubes were reweighed following centrifugation to determine the volume of recovered EWF. Approximately 500 μl of EWF was recovered per gram leaf fresh weight and there was no significant difference in the volume of EWF recovered (on the basis of leaf fresh weight) between plants grown in CFA and those exposed to O₃. The maximum time between leaf detachment to the analysis of EWF was 10 min.

Residual leaf extracts (RLEs) were prepared by homogenizing the leaf tissue remaining after the isolation of EWF; ≈ 0.1 g FW of leaf tissue was ground in 2 ml of ice-cold 100 mM HCl containing 2.5 mM EDTA. The homogenate was transferred to 50 ml tubes and centrifuged at 10000 g for 10 min at 4 °C. The supernatant was decanted and ASC/DHA content determined immediately.

Cytoplasmic contamination of EWF was checked by measuring the activity of glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) employing the method of Kornberg and Horecker (Kornberg and Horecker, 1955) using the technique described in detail elsewhere (Lyons et al., 1999b). The activity of G6PDH in RLEs was 6.95 ± 0.42 nkat g⁻¹ FW, but G6PDH activity was below the limits of detection in EWF (detection limit 0.5 nkat g⁻¹ FW), indicating that EWF was not contaminated with intracellular protein.

### Ascorbate determination

All assays were performed at 25°C using matched quartz cuvettes. Ascorbate/dehydroascorbate (ASC/DHA) content was determined using the spectrophotometric method described previously (Takahama and Oniki, 1992). For ASC, initial absorbance of a 50 μl aliquot of extract was measured at 265 nm in 100 mM K-phosphate buffer (pH 6.1), then remeasured following the addition of ascorbate oxidase (1 U ml⁻¹). Complete oxidation of ASC took no longer than 1 min. Dehydroascorbate content was determined in another 50 μl aliquot. Initial absorbance was recorded as for ASC, and then the sample was remeasured following the addition of 2 mM dithiothreitol (DTT). Complete reduction of DHA took no longer than 8 min. Measurements were corrected to account for the absorbance of DTT at 265 nm. An extinction coefficient of 14 mM⁻¹ cm⁻¹ for ASC at 265 nm was used in calculations (Nakano and Asada, 1981). Recovery of internal ASC standards was 93 ± 6% and 90 ± 7% for EWF and RLEs, respectively, and there was no evidence of significant oxidation during the extraction process. The redox state of ASC was calculated as

\[
\text{ASC/ASC} + \text{DHA}) \times 100
\]

### Modelling the extent of ozone protection afforded by apoplastic ascorbate

The fraction of O₃ detoxified through the direct reaction with ASC in the mesophyll cell wall was estimated using a computer-based model described elsewhere (Plochli et al., 2000). Measured input parameters are provided in Table 1. Cell wall thickness was determined from electron micrographs (according to Steer, 1981).

### Statistical analyses

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, USA). Data were first subjected to analysis of variance (ANOVA) investigating the influence of chamber, growth conditions, O₃-treatment, and time. No significant chamber effects were found within treatments, so data for individual plants were subjected to multivariate analysis of variance (MANOVA) to test the effects of growth conditions, O₃-treatment and time under the assumption that plants in replicate chambers were as likely to be as similar, or as different from plants within an individual chamber. Significant differences were determined using the least significant difference (LSD) calculated at the 5% level. Independent t-tests were used to compare individual means. Correlations were performed using Prism (GraphPad Software, San Diego, California), employing least square linear regression methods to test the goodness of fit.

### Results

#### Visible injury

Chronic O₃ exposure (28 d fumigation with CFA plus 75 nmol mol⁻¹ O₃ 7 h d⁻¹) resulted in no typical visible symptoms of injury on leaves at any stage of fumigation, nor were there any signs that O₃ exposure accelerated the rate of leaf senescence.

Acute O₃ exposure (150 nmol mol⁻¹ 8 h) resulted in the development of typical symptoms of visible damage (necrotic lesions developing on the adaxial leaf surface
within 8 h of exposure, affecting \(\approx 20\%\) of the leaf area) on the older leaves of plants transferred from CFA. Equivalent exposure resulted in no visible symptoms on the leaves of O\(_3\)-grown plants or on the leaf that was employed for all biochemical and physiological measurements (i.e. the youngest fully expanded leaf).

**Growth and dry matter partitioning**

Analysis of variance revealed no statistically significant effects of chronic O\(_3\) exposure on plant relative growth rate (\(R_{\text{CFA}} = 1.015 \pm 0.07\); \(R_{\text{O}} = 0.926 \pm 0.05\)) or allometric root:shoot growth (\(K_{\text{CFA}} = 0.765 \pm 0.06\); \(K_{\text{O}} = 0.717 \pm 0.04\)).

**Leaf gas exchange**

Figure 1 shows the effects of O\(_3\)-treatment on the light-saturated rate of CO\(_2\) assimilation (\(A_{\text{sat}}\)) and stomatal conductance (\(g_{\text{H}_{2}\text{O}}\)) for CFA- and O\(_3\)-grown plants. Analysis of variance revealed that acute O\(_3\) exposure resulted in a highly significant (\(P<0.001\)) decline in \(A_{\text{sat}}\) within 1 h in both CFA- and O\(_3\)-grown plants; O\(_3\)-treatment reduced \(A_{\text{sat}}\) by 22\% in CFA-grown plants and by 10\% in O\(_3\)-grown plants, the photosynthetic rate persisting at this reduced level for the remaining 8 h period of the treatment (Fig. 1A). Paired \(t\)-tests revealed a significant (\(P<0.05\)) depression in \(A_{\text{sat}}\) following acute O\(_3\) exposure in CFA-grown, but not in O\(_3\)-grown plants, while ANOVA revealed no statistically significant difference in the response of \(A_{\text{sat}}\) to acute O\(_3\) exposure in plants with contrasting O\(_3\) histories. Acute O\(_3\) exposure was found to result in a parallel decline in \(A_{\text{sat}}\) and \(g_{\text{H}_{2}\text{O}}\) (Fig. 1), but the average extent of the decline in \(g_{\text{H}_{2}\text{O}}\) with acute O\(_3\) exposure was significantly less in O\(_3\)-grown plants (\(-21\%) compared with their counterparts raised in CFA (\(-40\%). Consequently, a strong O\(_3\)-growth-concentration \(\times\) O\(_3\)-treatment interaction (\(P<0.001\)) was found. Differences in response were, at least partly, attributable to the fact that O\(_3\)-grown plants exhibited lower (44\%; \(P<0.001\)) \(g_{\text{H}_{2}\text{O}}\) at the start of the treatment compared with their counterparts raised in CFA.

**Effects of ozone on apoplastic ascorbate content**

Figure 2 shows changes in the concentration and redox status of ASC in EWF following acute O\(_3\) exposure. Despite the considerable replication employed in an effort to minimize the variation commonly associated with such measurements (Luwe and Heber, 1995), MANOVA revealed the impacts of the acute O\(_3\)-treatment on apoplastic ASC content (\(\text{ASC}_{\text{apo}}\)) and redox state to be, more often than not, on the borderlines of statistical significance. Acute fumigation resulted in a 30\% decline in \(\text{ASC}_{\text{apo}}\) in both CFA- and O\(_3\)-grown plants, within 4 h of exposure to the pollutant. Interestingly, no change in \(\text{ASC}_{\text{apo}}\) was observed in CFA-grown plants after 16 h of ‘recovery’. However, \(\text{ASC}_{\text{apo}}\) returned to pre-exposure levels within 16 h of transfer of O\(_3\)-grown plants to ‘control’ conditions (\(P<0.05\)).

After 4 h exposure to 150 nmol mol\(^{-1}\) O\(_3\), a 50\% decrease in the concentration of DHA in the apoplast (\(\text{DHA}_{\text{apo}}\)) of CFA-grown plants was observed (Fig. 2B). In contrast, \(\text{DHA}_{\text{apo}}\) in O\(_3\)-grown plants declined by only 15\% in response to the same treatment. Constitutive levels of \(\text{DHA}_{\text{apo}}\) in CFA- and O\(_3\)-grown plants were similar (Fig. 2B), and the redox state of ASC in the leaf apoplast of CFA- and O\(_3\)-grown plants remained unchanged during 8 h fumigation with 150 nmol mol\(^{-1}\) O\(_3\). However, a higher (\(P<0.05\)) redox state was found in EWF isolated from O\(_3\)-grown plants compared with their CFA-grown counterparts after 16 h ‘recovery’. The constitutive redox state of ASC in the leaf apoplast was similar in CFA-and O\(_3\)-grown plants; 67\% \(+\%\) in CFA-grown plants and 74\% \(+\%\) in O\(_3\)-grown plants (Fig. 2C).
Apoplastic ascorbate and ozone tolerance

Fig. 1. Impacts of O\textsubscript{3}-exposure on (A) the light-saturated rate of CO\textsubscript{2} assimilation (\(A_{\text{sat}}\)) and (B) stomatal conductance (\(g_{\text{H}_2\text{O}}\)) in the youngest fully expanded leaf of 28-d-old \textit{Vicia faba} plants. Plants were raised in CFA (charcoal/Purafiltr\textsuperscript{®}-filtered air; squares) or O\textsubscript{3} (CFA plus 75 nmol mol\textsuperscript{-1} O\textsubscript{3} for 7 h d\textsuperscript{-1}; circles) and exposed to CFA (open symbols) or 150 nmol mol\textsuperscript{-1} O\textsubscript{3} (closed symbols) for 8 h. Gas exchange measurements were made at PPFDs of 1200 and 200 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1} for \(A_{\text{sat}}\) and \(g_{\text{H}_2\text{O}}\), respectively, at an atmospheric CO\textsubscript{2} concentration of 350 \textmu mol mol\textsuperscript{-1}. Data represent the mean (±SE) of 12 measurements made on independent plants.

Effects of ozone on symplastic ascorbate content

Exposure of CFA- and O\textsubscript{3}-grown plants to acute O\textsubscript{3} resulted in no marked changes in ASC and DHA content in RLEs (Fig. 3). Symplastic ASC levels (ASC\textsubscript{symp}) declined by \(\approx\)25% within 4 h of O\textsubscript{3}-treatment in the CFA-grown plants, but this effect was not statistically significant. Ozone-treatment resulted in no shift in the redox state of symplastic ASC in CFA- or O\textsubscript{3}-grown plants (Fig. 3C) and constitutive ASC\textsubscript{symp} and DHA\textsubscript{symp} contents were similar.

A significant linear relationship (\(r^2=0.44, P<0.0001\)) was found between corresponding measurements of ASC\textsubscript{apo} and ASC\textsubscript{symp} for individual leaves (Fig. 4).

Modelling the extent of ozone protection afforded by apoplastic ascorbate

A linear relationship (\(r^2=0.48, P<0.0001\)) was found between measured and modelled apoplastic ASC concentrations (Fig. 5).

Modelled O\textsubscript{3} fluxes are presented in Table 2. Under chronic exposure (75 nmol mol\textsuperscript{-1} O\textsubscript{3}), the flux of O\textsubscript{3} to the leaf interior (\(J_{\text{INT}}\); the flux of O\textsubscript{3} determined purely by \(g_{\text{H}_2\text{O}}\)) and the flux of O\textsubscript{3} impinging on the plasmalemma (\(J_{\text{PLASMA}}\); the flux of O\textsubscript{3} determined by the combination of \(g_{\text{H}_2\text{O}}\) and the reaction of the pollutant with apoplasticASC) were decreased (\(P<0.05\)) in O\textsubscript{3}-grown plants compared with theoretical maximum O\textsubscript{3} fluxes at 75 nmol mol\textsuperscript{-1} (\(J_{\text{MAX}}\); the theoretical flux of O\textsubscript{3} based on
Fig. 3. Impacts of O₃-exposure on the content of ascorbate (ASCₚₒ) and dehydroascorbate (DHAₚₒ) plus redox state (ASC/ASC+DHAₚₒ) in residual leaf extracts of the youngest fully expanded leaf of 28-d-old *Vicia faba* plants. Plants were raised in CFA (charcoal/Purafil®-filtered air; squares) or O₃ (CFA plus 75 nmol mol⁻¹ O₃ for 7 h d⁻¹; circles) and exposed to CFA (open symbols) or 150 nmol mol⁻¹ O₃ (closed symbols) for 8 h under controlled conditions and then allowed to recover under pretreatment conditions for 16 h. Data represent the mean (±SE) of between 6 and 8 measurements made on independent plants.

Fig. 4. Relationship between the concentration of ASC in extracellular washing fluid (ASCₚₒ) and residual leaf extracts in the youngest fully expanded leaf of 28-d-old *Vicia faba* plants raised in CFA (charcoal/Purafil®-filtered air; squares) or O₃ (CFA plus 75 nmol mol⁻¹ O₃ for 7 h d⁻¹; circles) and exposed to CFA (open symbols) or 150 nmol mol⁻¹ O₃ (closed symbols). Line represents the least square regression fit; $r^2 = 0.44$, $P < 0.0001$, $y = 37.6x + 1920$.

Fig. 5. Relationship between measured and modelled concentration of ASC in the apoplast (ASCₚₒ) of the youngest fully expanded leaf of 28-d-old *Vicia faba* plants raised in CFA (charcoal/Purafil®-filtered air; squares) or O₃ (CFA plus 75 nmol mol⁻¹ O₃ for 7 h d⁻¹; circles) and exposed to CFA (open symbols) or 150 nmol mol⁻¹ O₃ (closed symbols). Line represents the least square regression fit; $r^2 = 0.48$, $P < 0.0001$, $y = 0.885x + 0.099$.

**Discussion**

The concentration of ASC in the apoplast of fully-expanded *Vicia faba* leaves was 0.50 ± 0.01 mM in CFA- and 0.59 ± 0.01 mM in O₃-grown plants, and 70 ± 4% of the apoplastic ASC pool was in the reduced state, irrespective of whether plants were raised in CFA or at environmentally-relevant O₃ concentrations. These findings agree well with data on ASCₚₒ for unstressed leaves of another cultivar of *Vicia faba* (Luwe and Heber, 1995), as well as with data for the leaves of other species (*Sedum album* L.: Castillo and Greppin, 1988; *Spinacia oleracea* L., Takahama and Oniki, 1992; Luwe et al., 1993; *Nicotiana tabacum* L.: Batini et al., 1995; *Cucurbita pepo* L.: Ranieri et al., 1996; *Kalancheïoa daigremontiana* Hamet et Perr.: Takahama, 1993; *Phaseolus vulgaris* L.: Moldau et al., 1997, 1998).
Exposure of CFA- and O₃-grown plants to 150 nmol mol⁻¹ O₃ was found to cause a decline in the ASC content of the leaf apoplast in vivo (Fig. 2). These findings suggest that ASC_apo is consumed upon exposure to O₃—a conclusion supported by a wealth of in vitro studies which have demonstrated O₃-driven oxidation of ASC in pure biochemical solutions (Giamalva et al., 1985; Mudway and Kelly, 1998), EWF (Kanofsky and Sima, 1995b), respiratory tract lining fluid (Kelly et al., 1995), and blood plasma (Cross et al., 1992; van der Vliet et al., 1995). Furthermore, the findings in the present study are consistent with O₃-induced changes in the composition of the leaf apoplast observed in several species (Castillo and Greppin, 1988; Luwe et al., 1993; Polle et al., 1995; Luwe and Heber, 1995), but are not consistent with the observations made by Jakob and Heber (Jakob and Heber, 1998). After ≈4 h exposure to 150 nmol mol⁻¹ O₃, the decline in ASC_apo was found to attain a steady state (Fig. 2). This finding suggests the attainment of an equilibrium between the rate of O₃-induced oxidation of ASC_apo and the replenishment of the ASC pool from the symplast. The concentration of ASC in the leaf apoplast is believed to be determined by a combination of free diffusion of the neutral species (ascorbic acid) (Plöchtl et al., 2000; Bichele et al., 2000) and the facilitated diffusion of ASC across the plasmalemma (Foyer and Lefebvre, 1986; Horemans et al., 1996), a view consistent with the linear relationship observed between corresponding measurements of ASC_apo and ASC_symp on individual leaves (Fig. 4). Following a period of ‘recovery’ in CFA, the O₃-induced decline in ASC_apo was found to be reversible in plants pre-exposed to the pollutant, but not in those grown in CFA. A possible explanation for this is that the oxidation of ASC in the apoplast continued in CFA-grown plants after the cessation of fumigation due to the elicitation of an unidentified source of oxidative stress (Schraudner et al., 1998; Rao and Davis, 1999).

The oxidized product of the ASC:O₃ reaction, DHA, cannot be reduced efficiently in the apoplast and must return to the cytosol for recycling (Castillo and Greppin, 1988; Polle et al., 1990; Luwe et al., 1993). The results of the present study indicated that ASC consumption in the apoplast was not mirrored by an increase in the concentration of DHA and as a consequence no shifts in the redox state of ASC_apo were observed (Fig. 2C). Similar findings were reported by Polle and co-workers (Polle et al., 1995) during their studies on the effects of O₃ on apoplastic/symplast antioxidant status in Picea abies [L.] Karst. These findings imply that there is rapid import of DHA into the cytosol under O₃ fumigation, a contention supported by the work of Horemans et al. which suggests that the carrier-mediated system for ASC/DHA on the plasmalemma has a strong preference for DHA, and/or DHA breaks down and is irreversibly lost from the apoplast (Horemans et al., 1997, 1998). Recent studies by Deutsch indicate that DHA itself may be a powerful antioxidant (Deutsch, 1998a, b). Hence, it is possible that the ‘loss’ of DHA from the apoplast may be the result of reaction with (and detoxification of) O₃. Further studies are required to confirm this.

In the present study, the increase in tolerance of O₃-grown plants in comparison with their CFA-grown counterparts appeared not to be mediated by differences in the potential detoxification of the pollutant by ASC_apo, but by the enhanced exclusion of O₃ through a decline in stomatal conductance in plants pre-exposed to the pollutant. However, model calculations revealed that a substantial fraction (30–40%) of the O₃ entering leaves (at environmentally relevant concentrations) may be intercepted by apoplastic ASC in Vicia faba plants per se. Similar findings have been reported for Phaseolus vulgaris L. (Moldau et al., 1997, 1998), where ~50% of the incoming O₃ was calculated to be detoxified by cell wall ASC. These findings suggest that the ASC pool located in the leaf apoplast may play a significant role as a first-line of defence against O₃ in some species, a view sup-

### Table 2. Predicted O₃ fluxes for the youngest fully expanded leaf of 28-d-old Vicia faba plants grown in CFA (charcoal/Parafilm®-filtered air) or O₃ (CFA plus 75 nmol mol⁻¹ O₃ for 7 h d⁻¹)

<table>
<thead>
<tr>
<th>Flux (nmol O₃ m⁻² s⁻¹)</th>
<th>Chronic exposure</th>
<th>Acute exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After 0 h O₃</td>
<td>After 4 h O₃</td>
</tr>
<tr>
<td>J_MAX</td>
<td>1.57 ± 0.22*</td>
<td>2.93 ± 0.31a</td>
</tr>
<tr>
<td></td>
<td>CFA-grown</td>
<td>O₃-grown</td>
</tr>
<tr>
<td>J_INT</td>
<td>0.91 ± 0.05</td>
<td>2.93 ± 0.31a</td>
</tr>
<tr>
<td>J_PLASMA</td>
<td>0.53 ± 0.02</td>
<td>2.13 ± 0.10</td>
</tr>
</tbody>
</table>

a 95% CI = 292 ± 35.
b 95% CI = 313 ± 50.
ported by several independent lines of evidence: (i) the concentration of ASC in the cell wall is relatively high, ranging from 0.01 to 4.00 mM (Castillo and Greppin, 1988; Luwe et al., 1993; Polle et al., 1995; Kollist et al., 1996; Luwe, 1996; Ranieri et al., 1996); (ii) the biomolecular rate constant for the reaction of O$_3$ with ASC (4.8 × 10$^{-1}$ M$^{-1}$ s$^{-1}$ at pH 6.0–7.0; Kanofsky and Sima, 1995a), is higher than that for other regular constituents of the apoplast or components of the plasma membrane (Giamalva et al., 1985; Heath, 1988; Chameides, 1989; Kanofsky and Sima, 1995a; Mudway and Kelly, 1998); (iii) extracellular ASC content has been shown to be inversely correlated with O$_3$ damage (Luwe et al. 1993; Barnes et al., 1999b; Kelly et al., 1995); (iv) manipulation of leaf ASC content (through feeding ASC, precursors or administering low/high light treatments) leads to predictable changes in O$_3$ tolerance—increased levels of ASC affording enhanced protection (Freebairn, 1960; Freebairn and Taylor, 1960; Menser, 1964; Mächler et al., 1995; Zheng et al., 2000; Maddison and Barnes, unpublished results) and decreased levels enhancing O$_3$ injury (Moldau et al., 1998); (v) O$_3$ resistance (assessed in terms of visible injury) co-segregates with the capacity to synthesize ASC in a range of mutants of Arabidopsis thaliana L. (Conklin et al., 1996).

In the present study, model estimates indicated that at 75 nmol mol$^{-1}$ O$_3$ a significant fraction of the O$_3$ flux (0.53 nmol O$_3$ m$^{-2}$ s$^{-1}$) escaped interception through direct reaction with cell wall ASC and might therefore be expected to impinge on the plasmalemma. Yet, leaves developed no visible symptoms of O$_3$ injury, and there were no significant changes in growth, root:shoot dry matter partitioning, intracellular ASC redox status or rates of CO$_2$ assimilation, following 28 d exposure to the pollutant. This is an interesting observation that is not consistent with model estimates of $J_{PLASMA}$ and suggests that factors in addition to ASC must probably play a role in intercepting O$_3$ in the leaf apoplast. This contention is supported by comparable data for hybrids of Populus deltoides (Ranieri et al., 1999), and for Triticum aestivum L. and Hordeum vulgare L. (Kollist et al., 1996).

In conclusion, the findings of the present work indicate that ASC has an important role to play in the interception of O$_3$ in the leaf cell walls of Vicia faba. However, the protection afforded is not complete, and the fate of the remaining O$_3$ is unclear. It appears likely that additional apoplastic constituents may be as important as ASC in the detoxification of O$_3$ in the leaf apoplast. The nature of these defences remain to be established.

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