Extracellular Ascorbate: A Potential First-Line of Defence against Ozone

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Abstract

The mechanisms underlying the differential sensitivity of plants to the ubiquitous air pollutant ozone (O_3) are far from fully understood. There is, however, a growing realisation that, following the uptake of the pollutant into the leaf interior, the first reactions take place in the aqueous matrix associated with the leaf cell walls (i.e. the leaf apoplast). This compartment forms the primary boundary between atmosphere and biosphere. The leaf apoplast is known to contain several antioxidants that react readily with O_3 (and/or it's primary dissolution products) to yield ostensibly harmless compounds. There is therefore the possibility that significant amounts of O_3 are scavenged (i.e. detoxified) prior to reaching the primary target - the plasmalemma. If this is the case, antioxidants situated in the leaf apoplast may afford an important first-line of defence against O_3. Herein, we focus on the role played by one of these compounds, ascorbate (vitamin C), in screening the plasmalemma from O_3-induced oxidative insult.

***Key Words:** Antioxidants, Apoplast, Ascorbate, Cell wall, Detoxification, Modelling, ozone*

Introduction

The past century has witnessed a steady rise in tropospheric ozone (O_3) concentrations in the Northern hemisphere (Volz and Kley, 1988; Marenco *et al.*, 1994), plus a sharp increase in the frequency and duration of potentially damaging photochemical episodes (Stockwell *et al.*, 1997). Indeed, there is unequivocal evidence that current ground-level concentrations of the O_3 are high enough to depress crop yields (Heck *et al.*, 1983; Fuhrer *et al.*, 1997), affect the composition and diversity of unmanaged ecosystems (Davison and Barnes, 1998) and contribute to localised declines in tree vitality in parts of Europe, North and Central America and the Far East (Chappelka and Chevone, 1992; Sandermann *et al.*, 1997; Izuta, 1998).

The phytotoxicity of O_3 arises primarily as a result of the oxidative damage it causes to plasmalemma constituents (Heath, 1980, 1987, 1988). The pollutant is taken-up into the leaf interior, *via* the stomates (Kersteins and Lendzian, 1989), where it reacts with constituents of

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the aqueous matrix associated with the cell wall (the apoplast) to yield a suite of reactive oxygen species which, in addition to O_3, result in the oxidation of sensitive components of the plasmalemma, and subsequently the cytosol (Heath, 1980; Chameides, 1989; Moldau, 1998). In this sense, the oxidative stress induced by O_3 shares similarities with the initial events associated with other plant pathologies and the downstream consequences of O_3-induced oxidative damage are well-documented i.e. visible leaf injury (i.e. localised cell death), stimulated rates of respiration, the suppression of photosynthesis, enhanced emissions of ethylene, premature leaf senescence and shifts in resource partitioning (Harris and Bailey-Serres, 1994; Kangasjärvi *et al.*, 1994; Schraudner *et al.*, 1997). These responses are manifested in decreased plant growth, depressed crop yields and reduced quality of harvested components (Runeckles and Chevone, 1992; Davison and Barnes, 1998; Barnes *et al.*, 1999a, b; Turcsányi *et al.*, 2000a). Since the plasmalemma is the principle site of attack, the interception and detoxification of O_3 (and/or its reactive products) by constituents of the leaf apoplast may play a crucial role in averting cellular damage (Heath, 1988; Kelly *et al.*, 1995; Dietz, 1997; Cross *et al.*, 1998; Lyons *et al.*, 1999a). In this contribution, we focus on evidence supporting a potentially important role for extracellular ascorbate (ASC) in mediating the tolerance of plants to O_3.

Ozone resistance

Herein, the term 'O_3 resistance' is defined as the "ability to maintain growth and remain free from injury in a polluted environment" (*sensu* Roose *et al.*, 1982). Resistance need not be complete and, based on the conceptual model proposed by Tingey and Andersen (1991), is envisaged to be mediated through effects on pollutant uptake (avoidance through changes in stomatal conductance) and/or changes in the tolerance of plant tissues following uptake (through effects on metabolism resulting in an increased capacity for the detoxification of the pollutant and its potentially damaging reaction products).

There is considerable variation in O_3 resistance within, as well as between, species (see Figure 1 which shows data for plantain [*Plantago major*]). This variation, which has been shown in many cases to be heritable, has been capitalised upon in several plant breeding programmes - directed selection resulting in the improved O_3 resistance of strains of alfalfa (*Medicago sativa* L.), sweet corn (*Zea mays* L.), snap bean (*Phaseolus vulgaris* L.), eastern white pine (*Pinus strobus* L.) and aspen (*Populus tremuloides* Michx.) (Howell *et al.*, 1971; Cameron, 1975; Mebrahtu *et al.*, 1990; Karnosky, 1991; Karnosky *et al.*, 1992).

It is generally concluded from such breeding experiments that O_3 resistance is a quantitative trait, governed by the additive effects of several genes (Sand, 1960; Howell *et al.*, 1971; DeVos *et al.*, 1982; Roose, 1991). However, Macnair (1991) has questioned this view, providing evidence that in many cases it is inevitable that air pollution resistance has been identified as a polygenic trait. Hence, it remains possible that O_3 resistance is controlled by a relatively small number of major genes and several modifiers. There is incontrovertible evidence that at least one of these genes is connected with the biosynthesis of L-ascorbate (vitamin C).

Evidence supporting a role for ascorbate in mediating ozone resistance

Ascorbate is an abundant and powerful antioxidant which serves essential metabolic functions in both plants and animals (Loewus 1980, 1988; Smirnoff, 1996; Smirnoff and

Pallanca, 1996; Noctor and Foyer, 1998). The central position occupied by ASC in the metabolic control of reactive oxygen species is illustrated in Figure 2.

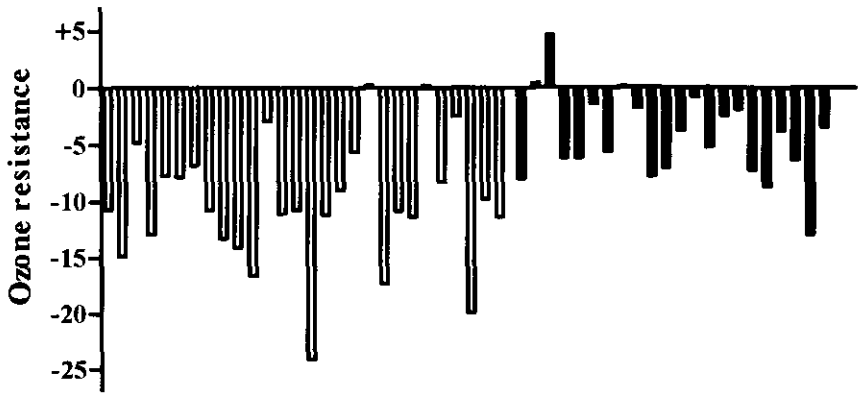

Fig. 1 Ozone resistance in a range of geographically-distinct populations of *Plantago major*. Ozone resistance assessed in terms of the % change in relative growth rate induced by a two-week exposure to 70 ppb ozone for 7 h d^{-1}. Data from Reiling and Davison (1992) (open bars) and Lyons *et al.* (1997) (closed bars).

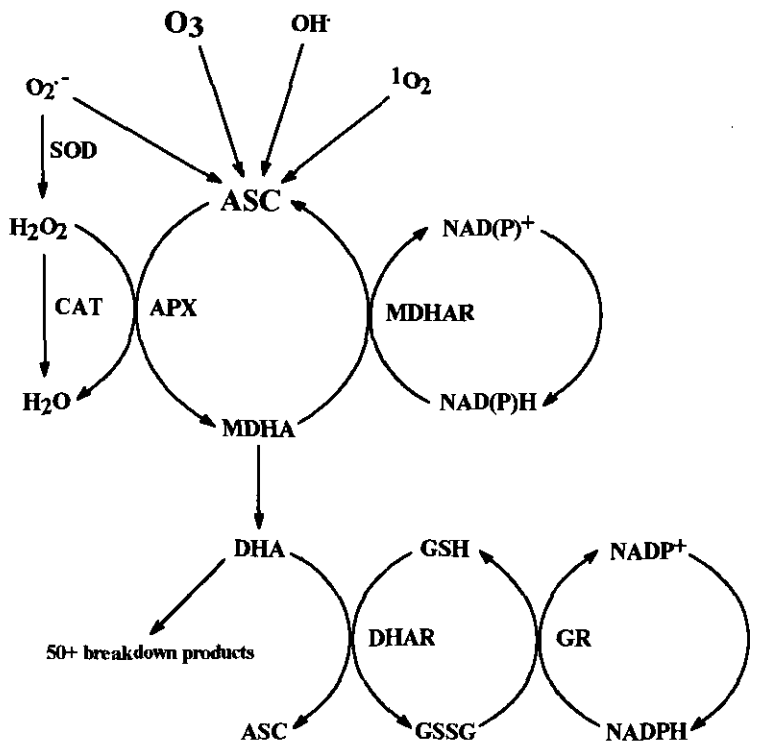

Fig. 2 Schematic representation of the central position occupied by reduced ascorbate (ASC), and its oxidised forms, monodehydroascorbate radical (MDHA) and dehydroascorbate (DHA), in the metabolism of reactive oxygen species: ozone (O_3); superoxide radical ($O_2^{\cdot-}$); hydroxyl radical ($OH^{\cdot}$); singlet oxygen ($^1O_2$); Hydrogen peroxide (H_2O_2). Enzyme and other metabolites involved are: superoxide dismutase (SOD); catalase (CAT); ascorbate peroxidase (APX); monodehydroascorbate radical reductase (MDHAR); dehydroascorbe reductase (DHAR); glutathione reductase (GR); reduced glutathione (GSH); oxidised glutathione (GSSG). Redrawn from Barnes *et al.* (1999b).

There are several strong lines of evidence linking leaf ASC content with O_3 resistance: (i) research with *Arabidopsis* mutants. Work performed by Conklin and co-workers (1996, 1997, 1999) has shown that O_3-resistance co-segregates with the capacity to synthesize ASC in a range of *Arabidopsis thaliana* L. mutants derived from *vtc1* (formerly *soz1*); an ultrasensitive genotype that accumulates only 25% of the wild type leaf ASC concentration due to a deficiency in a key enzyme of the ASC biosynthetic pathway, GDP-mannose pyrophosphorylase. (ii) Correlative evidence. Endogenous levels of ASC correspond with variations in O_3 resistance in some cases (Lee *et al.*, 1984; Bilodeau and Chevrier, 1998), but not others (Menser, 1964; Ranieri *et al.*, 1999). (iii) Manipulation of leaf ASC content. Treatments employed to enhance leaf ASC content e.g. feeding ASC to roots (Mächler *et al.*, 1995) or spraying foliage (Freebairn, 1960; Freebairn and Taylor, 1960) have been shown to afford additional protection against O_3. This effect is illustrated in Figure 3 using some of the authors' data to show that the extra protection observed in ASC-sprayed leaves cannot be explained through reduced rates of O_3 uptake (i.e. protection occurred in ASC-sprayed leaves of common plantain (*P. major* L.) in the absence of shifts in stomatal conductance).

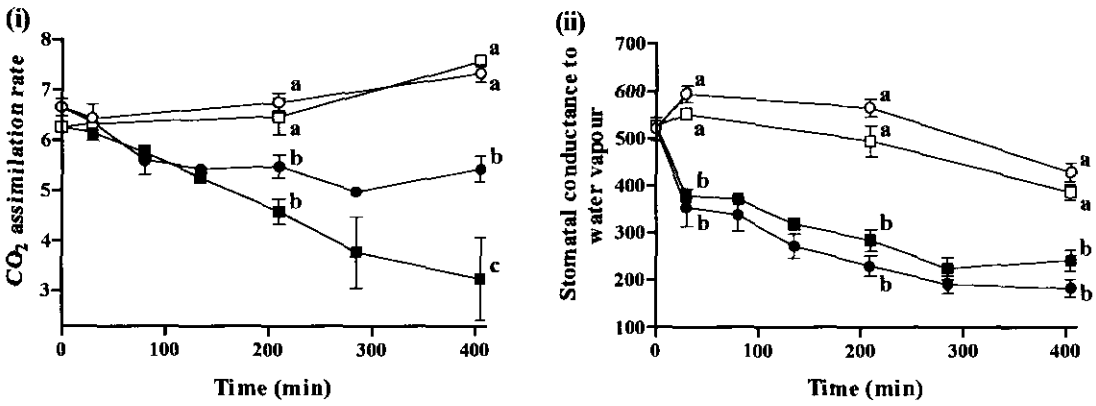

Fig. 3 The effect of foliar ascorbate application on the response of (i) CO_2 assimilation rate (μmol m^{-2} s^{-1}) and (ii) stomatal conductance to water vapour (mmol m^{-2} s^{-1}) in leaves of *Plantago major* exposed to charcoal/Purafil®-filtered air (CFA, open symbols) or CFA plus 400 ppb ozone (closed symbols). Squares represent control plants sprayed with 10 mM $NaHCO_3$, circles represent plants sprayed with 10 mM Na-ascorbate. Data represent means (n = 4) ± SE. Different letters denote significant differences (P< 0.05). Redrawn from Zheng *et al.* (2000).

In contrast, treatments which reduce leaf ASC content have been shown to enhance sensitivity to O_3 (Menser, 1964; Moldau *et al.*, 1998). More recently, some researchers have employed the recently-identified biosynthetic precursor of ASC, L-galactono-1, 4-lactone (L-GL), to enhance the ASC content of plant tissue. Bilodean and Chevrier (1998), utilising an O_3 sensitive 'colourless-mutant' of *Euglena gracilis*, demonstrated that the addition of L-GL to the growth medium resulted in elevated levels of ASC in the cells, and increased their tolerance (in terms of cell viability) to O_3 exposure. In parallel experiments conducted by some of the authors (J. Maddison, T. Lyons & J. Barnes, unpublished), leaf ASC content was doubled in radish (*Raphanus sativus* L.) fed 50mM L-GL without effects on stomatal conductance. The treatment was found to afford complete protection against the negative effects of O_3 (in terms of visible injury and effects on plant relative growth rate).

Importance of the sub-cellular localisation of ascorbate in the interception of ozone

The first reactions of ozone occur at the air/liquid interface (i.e. the leaf apoplast). Around 1-2% of the ASC found in leaves is located at this boundary. Hence, the ASC concentration in leaf cell walls ranges from 10 to 4000 μM (Castillo and Greppin, 1988; Takahama and Oniki, 1992; Polle *et al.*, 1990, 1995; Luwe and Heber, 1995; Vanacker *et al.*, 1998a; Lyons *et al.*, 1999b; Ranieri *et al.*, 1999; Turcsányi *et al.*, 2000b). This raises the question of how much of the incoming O_3 can be scavenged by this pool of ASC, and what the background rate of ASC consumption is (since ASC also participates as a co-factor in several enzyme-based reactions). *In vitro* studies on pure solutions have revealed that the ASC/O_3 reaction proceeds rapidly (second-order reaction rate constant is between 4.8×10^7 and 6.0×10^7 M^{-1} s^{-1} at physiologically-relevant pHs [Giamalva *et al.*, 1985; Kanofsky and Sima, 1995a]). Experiments involving the ozonation of isolated biological fluids, e.g. apoplastic washing fluid (Kanofsky and Sima, 1995b), respiratory tract lining fluid (Kelly *et al.*, 1995; Cross *et al.*, 1998) and blood plasma (Cross *et al.*, 1992; van der Vliet *et al.*, 1995) reveal that ASC is consumed at a rate dependent upon the concentration and the duration of exposure to the pollutant. Moreover, these studies have demonstrated that ASC is oxidised prior to reaction with lipid and protein constituents. These findings substantiate reports from *in vivo* studies that O_3 exposure results in the depletion of extracellular ASC (Castillo and Greppin, 1988; Luwe *et al.*, 1993; Polle *et al.*, 1995; Luwe and Heber, 1995; Mudway *et al.*, 1999; Turcsányi *et al.*, 2000b). However, some researchers - Jakob and Heber (1998), in particular - are not yet convinced that *in vitro* findings are reciprocated *in vivo*. These authors have found that ASC does not prevent changes in the fluorescence of oxidation-sensitive dyes vacuum-infiltrated in to the apoplast of spinach (*Spinacia oleracea* L.) leaves, in contrast to experiments carried out in the test tube.

The product of ASC oxidation, dehydroascorbic acid (DHA) is reduced to regenerate ASC, or is rapidly and irreversibly hydrolysed to yield 2, 3-diketogulonic acid and an array of degradation products (Loewus, 1980, 1988; Smirnoff 1996; Deutsch, 1998a). Since DHA cannot be reduced efficiently in the apoplast, it is believed to be returned to the cytosol for recycling (Castillo and Greppin, 1984; Polle *et al.*, 1990; Luwe *et al.*, 1993). This view is supported by the presence of a carrier-mediated system on the plasma membrane for the transport of ASC/DHA (Rautenkranz *et al.*, 1994; Foyer and Lelandais, 1996; Horemans *et al.*, 1996). This system displays a higher affinity for DHA than for ASC (Horemans *et al.*, 1997, 1998). Recent reports by Vanacker and co-workers (Vanacker *et al.*, 1998a, b, 1999) suggest that there is the possibility, at least in some species, that DHA may be enzymatically reduced back to ASC in the apoplast - this finding awaits further investigation. It is also possible that monodehydroascorbate radical is formed as an intermediate during the reaction of ASC and O_3. If this is the case, plasmalemma-bound monodehydroascorbate radical educates may facilitate the rapid regeneration of apoplastic ASC *in situ* (Navas *et al.*, 1994; Asard *et al.*, 1995; Bérczi and Møller, 1998).

Estimation of ozone detoxification in the leaf apoplast

Semi-quantitative estimates of the degree of protection afforded by apoplastic ASC were first provided by Chameides (1989). This author's insightful approach attempted to describe the uptake of O_3 from the atmosphere to the mesophyll cell wall. The study indicated that the ASC/O_3 reaction in the leaf apoplast could provide a major sink for the pollutant, based on a

first-order loss co-efficient for the reaction of between 3,000-60,000 s^{-1}; governed by the rate of the reaction and the concentration of apoplastic ASC (taken to be 50-1000 μM). More recently, a model (SODA; Simulating Ozone Detoxification in the Leaf Apoplast) which extends the one-dimensional approach adopted by Chameides (1989) to encompass the re-supply of ASC into the leaf apoplast under O_3 exposure has been developed (Plöchl *et al.*, 2000). SODA estimates O_3 uptake through the usual corollary with a series of resistances to diffusion first described by Gaastra (1959). Sub-cellular distribution of ASC is estimated from the diffusional movement of the neutral form, ascorbic acid (AA), and the 'trapping' of this compound in compartments of differing pH (*sensu* Slovik *et al.*, 1992). The detoxification of O_3 in the leaf apoplast through direct reaction with ASC is modelled using a biomolecular reaction rate of 4.8×10^7 M^{-1} s^{-1} (Kanofsky and Sima, 1995a). A schematic representation of the modelled resistance-reaction network is presented in Figure 4.

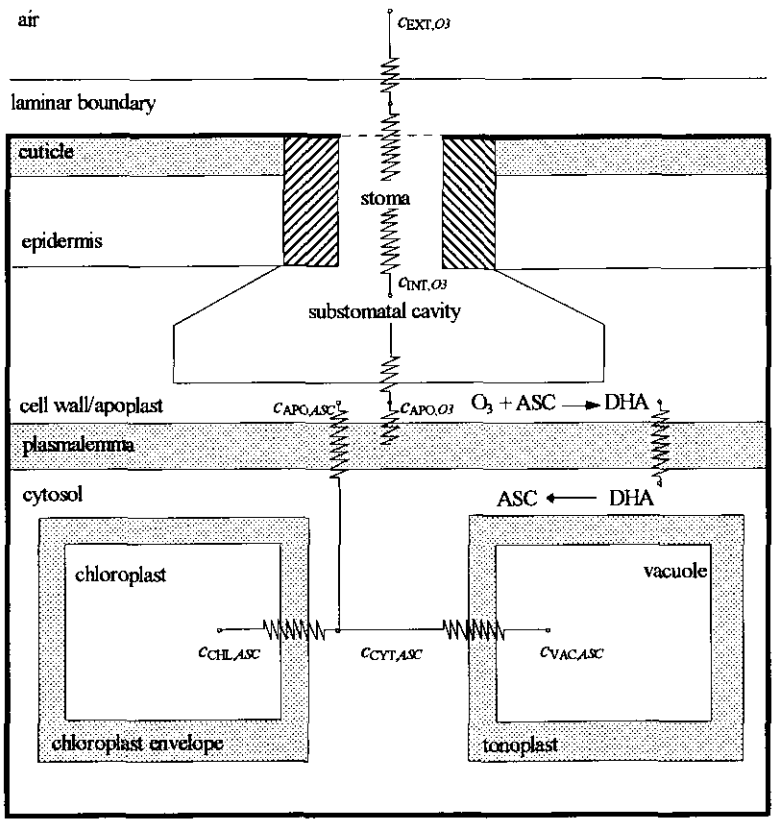

Fig. 4 Schematic representation of the diffusion-reaction network modelled in SODA; considering the uptake of ozone (O_3) from the air ($_{EXT}$) through the laminar boundary, stoma, intercellular air space ($_{INT}$), and its dissolution and reaction with ascorbate (ASC) in the aqueous phase of the cell wall (the apoplast, $_{APO}$), the distribution of ASC between cell compartments, as well as the transfer of dehydroascorbic acid (DHA) from apoplast to cytosol and its subsequent regeneration. Other abbreviations: c = concentration; $_{PL}$ = plasmalemma; $_{CHL}$ = chloroplast; $_{VAC}$ = vacuole. Redrawn from Plöchl *et al.* (2000).

The full complement of parameters necessary to rigorously test the theoretical prediction of mesophyll cell wall ASC concentrations, and O_3 interception, have not yet been determined for an archetypal plant under standardised conditions. However, the best available dataset at this time, collected by Turcsányi *et al.* (2000b) for broad bean (*Vicia faba* L.) demonstrates a

reasonable agreement between modelled and measured ASC data (Figure 5). It is also interesting to note that recent work reveals (i) a positive correlation between the ASC content of the leaf apoplast and O_3 resistance (expressed in terms of reductions in growth rate induced by the pollutant) across a range of genotypes of *P. major* and *R. sativus* (Figure 6), and (ii) that pre-exposure of *P. vulgaris* plants to continuous darkness decreases the ASC content of the leaf apoplast by 80%, and results in an increase in injury to the plasmalemma upon exposure to acute O_3 (Moldau *et al.*, 1998).

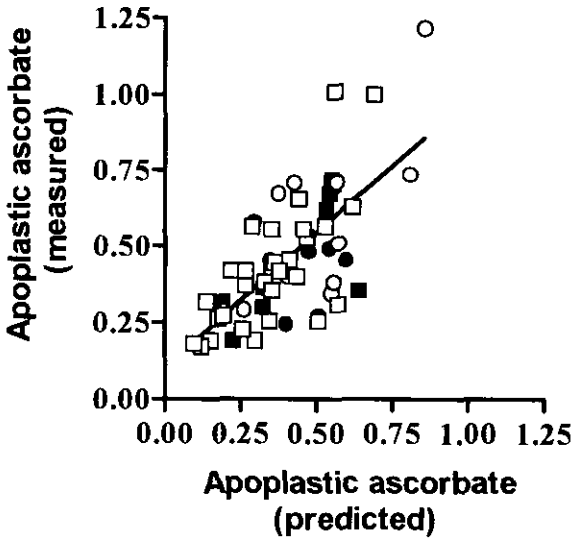

Fig. 5 Relationship between measured and modelled concentration of ascorbate (mM) in the apoplast of *Vicia faba* leaves raised in CFA (charcoal/Purafil®-filtered air; squares) or ozone (O_3) (CFA plus 75 nmol mol^{-1} O_3 for 7 h d^{-1}; circles) and exposed to CFA (open symbols) or 150 nmol mol^{-1} O_3 (closed symbols). Line represents the least square regression fit; $r^2 = 0.48$, $P < 0.0001$, $y = 0.885\ x + 0.099$ (replotted from Turcsányi *et al.*, 2000b).

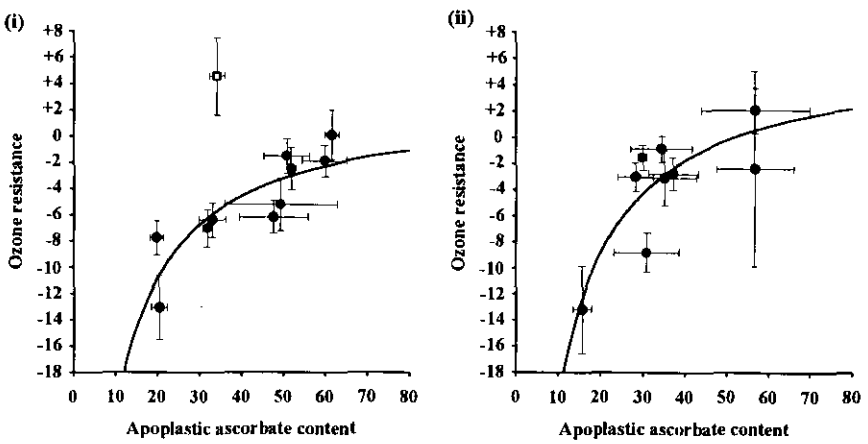

Fig. 6 Relationship between ascorbate content of the leaf apoplast (nmol g^{-1} FW) and ozone resistance in (i) populations of *Plantago major* ($r^2 = 0.731$, $P = 0.002$) and (ii) cultivars of *Raphanus sativus* ($r^2 = 0.714$, $P = 0.004$). Ozone resistance was assessed in terms of the % change in relative growth rate induced by a two-week exposure to 70 ppb ozone for 7 h d^{-1}. Data represent means (n = 10-15) ± SE (replotted from Barnes *et al.*, 1999b).

Model simulations emphasize the importance of pollutant concentrations in the atmosphere, stomatal conductance and the concentration of ASC in the leaf apoplast, in governing the flux of O_3 that impinges on the plasmalemma (see Plöchl *et al.*, 2000). This point is emphasised in Figure 7 which shows (i) the effect of apoplastic ascorbate concentration on the flux of impinging on the plasmalemma. It is worthy of note that, at a concentration of ascorbate in the leaf apoplast of 500 μM, ~60 % of the incoming O_3 could be detoxified before it reaches the plasmalemma; (ii) the strong effect exerted by cell wall thickness on the detoxification of O_3, because of the way in which it modifies the length of the aqueous diffusion pathway to the plasmalemma (Nobel, 1991) and therefore the residence time of the pollutant in the cell wall. The thicker the wall, the greater the interception of the pollutant - reinforcing the conclusion of Chameides (1989); and (iii) the way in which the sub-cellular distribution of ASC is potentially affected by apoplast pH. The distribution of ASC between cell compartments is considered to be driven by a combination of pH and carrier-mediated transport systems, so shifts in the pH of the apoplast would be predicted to result in considerable changes in the concentration of ASC in the cell wall, and hence, O_3 flux to the plasmalemma. This infers that environmental factors which alter apoplast pH e.g. water deficit (Hartung *et al.*, 1988), light (Mühling *et al.*, 1995), nitrogen supply (Hoffmann *et al.*, 1992) and gaseous pollutants including O_3 itself (Heath, 1988; Wellburn, 1990; Pfanz and Oppmann, 1991; Moldau, 1998) may affect resistance to the pollutant in a predictable manner. This hypothesis is currently under investigation in the principle authors' laboratory.

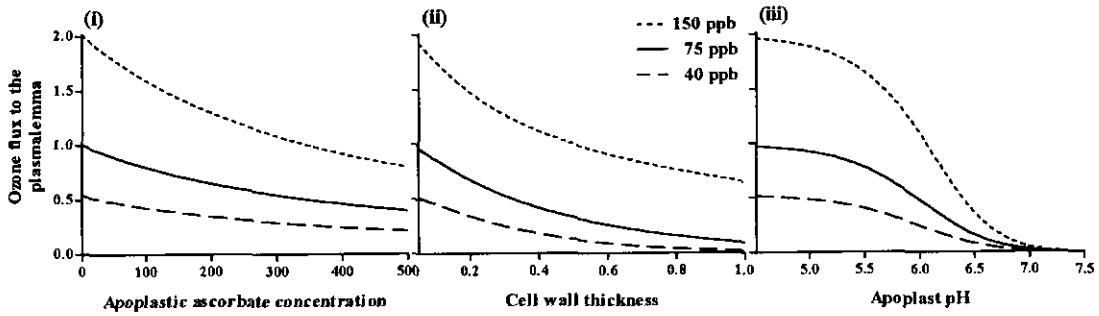

Fig. 7 The influence of (i) apoplastic ascorbate concentration (μM), (ii) cell wall thickness (μm) and (iii) apoplast pH on the simulated flux of ozone to the plasmalemma over a range of external ozone concentrations. Model inputs (unless otherwise shown) were: leaf ascorbate concentration = 6 mM; stomatal conductance to water vapour = 200 mmol m^{-2} s^{-1}; cell wall thickness = 0.2 μm; apoplast pH = 5.7; ascorbate/ozone reaction rate constant = 4.8×10^7 M^{-1} s^{-1}.

Present model formulations consider that O_3 detoxification occurs solely the direct reaction of the pollutant with apoplastic ASC. There are numerous other apoplastic constituents that can scavenge O_3 and its reactive products e.g. metabolites, such as polyamines and phenolic compounds (Bors *et al.*, 1989; Langebartels *et al.*, 1991; Eckey-Kaltenbach *et al.*, 1993, 1994), and enzymes, such as peroxidases and superoxide dismutase (Castillo *et al.*, 1984; Castillo and Greppin, 1988; Polle *et al.*, 1990; Streller and Wingsle, 1994; Ogawa *et al.*, 1996; Ranieri *et al.*, 1996; Vanacker *et al.*, 1998a, b, 1999; Lyons *et al.*, 1999b). Furthermore, the work of Deutsch (1998b) suggests that several of the oxidation products of ASC (including DHA) may act as strong antioxidants. There are also numerous reactions leading to the consumption of apoplastic ASC including the oxidative scission of cell wall polysaccharides *via* the formation of hydroxyl radicals (Fry, 1998), reaction with phenoxy

radicals formed by cell wall peroxidases (Takahama and Oniki, 1992), regeneration of α-tocopherol in the plasmalemma (Smirnoff, 1996) and the provision of substrate for ASC-dependant peroxidases (Castillo and Greppin, 1988) and oxidases (Takahama and Oniki, 1994). Our ultimate objective is to extend SODA to account for these reactions. However, a better understanding of the complex biochemistry of the apoplast is needed before this goal can be realized.

Conclusions

Model simulations, allied to the available experimental data, indicate that extracellular ASC may play a significant role in the detoxification of O_3 under environmentally-relevant conditions. Modulation of ASC content through various treatments leads to predictable shifts in O_3 resistance; suggesting that it maybe possible to manipulate resistance *via* transgenic technology, particularly given recent advances in the characterisation of the biosynthetic pathway of ASC in plants. However, the data presented suggest that the protection against O_3 afforded by apoplastic ASC is unlikely to complete. It appears likely that additional mechanisms may be important in capturing O_3 in the leaf apoplast. Future work should be directed towards elucidating the nature of these extracellular defences and to the manipulation of these defences through present-day gene technology.

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