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Quantification of Ozone Decay and Requirement for Ascorbate in *Phaseolus vulgaris* L. Mesophyll Cell Walls

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Summary

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Enhancement of ascorbate concentration from 0.6 mM to 1.0 mM and significant activation of peroxidase activity in cell walls of bean leaves was observed during acute exposure to ozone concentrations 0.45-0.65 μL L^{-1} for 3.5 h. Ozone flux to the mesophyll cell walls, its decay in the direct reaction with ascorbate and in the cyclic chain reaction were quantified by measuring stomatal conductance, cell wall ozone exposed area and cell wall thickness. At the start and at the end of exposure, respectively, 61% and 43% of ozone impinging on mesophyll cell surface was detoxified in cell wall (thickness 0.3 μm) nonenzymatically, mainly due to the direct reaction with ascorbate. Use rates of ascorbate in peroxidase catalyzed H_2O_2 scavenging reactions were calculated to be comparable to those in the direct reaction only at cell wall H_2O_2 concentration exceeding 1 μM and concentration of phenolics 1mM. It is concluded that ascorbate induction in the thin mesophyll cell wall by ozone quarantees no full detoxification of the pollutant in the nonenzymatic reactions.

Introduction

Increasing emission of precursor pollutants of ozone in human activities has caused levels of this powerful oxidant in the lower troposphere to be higher than many plant species are able to tolerate. The results are perturbations in

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membrane functioning, injury to cells and, ultimately, loss of productivity. Much research has been devoted to the biochemistry of ozone toxicity in leaves, and a rather complex picture has been emerged. It is being believed that the antioxidant ascorbate (AA) in mesophyll cell walls (CW) is of paramount importance in protecting plasmalemma and cell interior against ozone. AA reacts rapidly with ozone in weakly acidic CW solution (KANOFSKY & SIMA 1995). AA is able to react also with active oxygen species generated in CW during ozone degradation in cyclic reaction. CW contains also nonspecific peroxidases catalyzing enzymatic scavenging of H_2O_2 by AA and phenolics. In the latter case AA is used for the reduction of phenoxy-radicals produced (TAKAHAMA 1993).

There are still few attempts to quantify the relative roles of various components of ozone degradation in the CW region. It was shown by model calculations that in the case of relatively thick CW (above 1 µm) most of ozone, entering liquid phase after its diffusion through stomata, could be degraded in direct nonenzymatic reaction with AA present in CW up to millimolar concentrations (CHAMEIDES 1989).

However, in most of the mesophytes CW thickness is only a few tenths of a micrometer; in these cases the direct scavenging of ozone by CW AA appears to be much less effective and significant amount of the toxicant is anticipated to reach the vulnerable plasma membrane. This raises the need for quantification of other processes of ozone-related chemistry in CW. In this study the induction of AA and of the antioxidant peroxidase system in mesophyll CW of an ozone-tolerant French bean variety under acute ozone exposure is analyzed with the aim to quantify the relative capacities of the direct and enzymatic systems to detoxify ozone and its oxidative intermediates in CW region.

Material and Methods

Plants of a dwarf *Phaseolus vulgaris* L. var. "Vaia" were individually grown in a growth box as described in (MOLDAU & al. 1996).

Two weeks after seedling emergence the primary leaves were removed to have only nearly full-grown first trifoliate on shoot. Three hours after the start of the next light period the above-ground part of an intact plant was sealed into an air- and temperature-controlled through-flow fumigation chamber (vol. 9 L) where it was exposed to 0.45-0.65 µL L^{-1} ozone for 3.5 h. Other conditions in the chamber were maintained close to those in the growth box. Control plants were treated similarly but without ozone in the chamber. During exposure transpiration rate of the above-ground parts and ozone concentration in the chamber were continuously monitored. After exposure the trifoliate was immediately detached and used for analyses of CW AA content and peroxidase activity.

CW solute extraction, determination of AA and enzyme activities, and plasmalemma integrity analyses were performed as described in PADU & al. 1996 and MOLDAU & al. 1996.

To get ozone flux to the mesophyll, stomatal conductances of leaves for ozone (g_s) were calculated after correcting for transpiration rates of stem, petioles and leaf cuticle and using the ratio of 0.61 for the diffusion coefficients of ozone versus water vapor. Ozone exposed mesophyll area and CW thickness were measured from leaf sections using light and electron microscopes, respectively. Ozone decay rate in CW due to the direct reaction with AA was calculated according

to CHAMEIDES 1989, using physical constants therein and the bimolecular rate constant of $6x10^7$ M^{-1} s^{-1} for the reaction between ozone and AA.

Decomposition rate of ozone in CW due to the cyclic chain reaction involving hydroxyl ions, superoxide, ozonide and hydroxyl radicals was calculated after STAEHELIN & HOIGNE 1985. Induction of the OH-radical by phenolics and scavenging of this radical by AA, as well promotion of the cycle were estimated using data given in Table 1.

Table 1. Rate constants k_I, k_S, k_P (in M^{-1} s^{-1}, symbols correspond to those in sceme II in STAEHELIN & HOIGNE 1985) used to estimate influence of promotor and scavenger processes on the cyclic chain reaction. Concentrations 1mM for all three organic solutes were used.

Reaction	Rate constant	Notes
OH-radical induction by phenolics	$k_I=3x10^4$	on the base of Fig.1 & 2 from (GRIMES & al. 1983).
OH-radical scavenging by ascorbate	$k_S=7x10^9$	from (BUCKLAND & al.1991).
promotion of the cyclic reaction	$k_P=7x10^9$	assumed equal to k_S. Solute(s) not identified.

The oxidation rate of AA in CW peroxidase-catalyzed reaction with H_2O_2 was estimated on basis of substrate dependent APX and GPX activities. It was assumed that GPX activity represents activity of peroxidases acting upon native phenolics in CW.

Ozonation experiments were performed in triplicate. Two measurements of CW [AA] per experiment were made. PX activities are means over 2-4 measurements. In model calculations the mean values were used.

Results

There was no visual injury in ozone-treated leaves. CW extracts were apparently not contaminated with intracellular proteins as revealed by the absence of cytosolic marker enzymes in the extracted fluids.

During the first 1.2 h of ozonation stomatal conductance for ozone (g_s), CW ascorbate concentration ([AA]) and peroxidase activities remained similar to corresponding values in control plant. Thereafter g_s under ozone started to decline reaching 40% of the initial value at the end of the exposure (Table 2).

Table 2. Ozone concentrations over leaves z (μL L^{-1}), stomatal conductances for ozone g_s (cm s^{-1}), ascorbate concentrations [AA] (mM), and guaiacol and ascorbate peroxidase activities GPX, APX (nmol cm^{-3} s^{-1}) in cell walls during initial and final phases of a 3.5 h exposure of bean leaves to 0.45-0.65 μL L^{-1}ozone . Values in parentheses: SD, n = 2 -4 . Differences in g_s, [AA] and GPX were significant ($P<0.05$, Student's test).

Oz. time (h)	z	g_s	[AA]	GPX	APX
0-1.2	0.45	0.20(0.02)	0.60(0.05)	380(10)	10(1)
3.5	0.65	0.08(0.03)	1.00(0.10)	830(270)	13(4)
Ratio	1.44	0.40	1.67	2.16	1.3

During the same time [AA] in CW increased significantly (by 1.67 times), GPX and APX activities increased by 2.16 and 1.30 times, respectively.

In Table 3 the calculated components of ozone flux to and through the mesophyll CW are presented for the first 1.2 h of exposure and for the end of the exposure. Stomatal closure decreased ozone flux rate to CW, F_i, to 61 % of the initial value. At the same time the amount of ozone, scavenged in CW in the direct reaction, F_{sd}, decreased only by 11% due to increase of [AA] in CW. The amount of ozone decomposed in cyclic reactions, F_{dc}, was negligible. To the end of the experiment ozone flux to the plasmalemma, F_p, decreased to 43 % of the initial value.

Table 3. Calculated ozone fluxes: impinging on mesophyll cell surface F_i, scavenged by ascorbate in cell wall in the direct reaction F_{sd}, decomposed in cell wall in cyclic chain reaction F_{dc}, and reaching plasmalemma F_p (all fluxes are in fmol O_3 cm^{-2} s^{-1}) during initial and final phases of a 3.5 h exposure of bean leaves to 0.45-0.65 μL L^{-1} ozone. The measured anatomical parameters used in calculations, were: mesophyll ozone-exposed area 16.6 cm^2 CW cm^{-2} leaf projective area, CW thickness 0.3 μm.

Oz. time (h)	F_i	F_{sd}	F_{dc}	F_p	F_p/F_i
0-1.2	230	90	0.05	140	0.61
3.5	140	80	0.04	60	0.43
Ratio	0.61	0.89	0.8	0.43	0.70

In Table 4 the calculated rates of AA used in direct and enzymatic reactions at the initial and final phases of ozonation are given, for concentrations of H_2O_2 and phenolics found in literature (see Discussion). As scavenging of active oxygen species by AA was low due to low degradation of ozone in the chain reaction (Table 3), use rates of AA in nonenzymatic reactions, U_{sn}, practically reflect ozone use rates in the direct reaction with AA. At 1 μM [H_2O_2] U_{sn} consisted 90% and 81% of the total need for AA at the start and the end of the exposure, respectively. Only at the value of [H_2O_2]≈10 μM use of AA in enzymatic reactions was comparable to that used in the direct reactions.

Table 4. Calculated use rates of ascorbate in mesophyll cell walls during initial and final phases of 3.5 h exposure of bean leaves to 0.45-0.65 μL L^{-1} ozone. [H_2O_2] - assumed concentration of hydrogen peroxide in cell wall (μM). U_{sn} - use rate of ascorbate in nonenzymatic reactions, U_{AA}, U_{Ph} - use rate of ascorbate in peroxidase-catalysed reactions using ascorbate or phenolic acids, respectively (all rates in fmol ascorbate cm^{-2} cell wall s^{-1}). Total = U_{sn} + U_{AA} + U_{Ph}. A concentration of 1 mM phenolic acids was assumed throughout.

Oz. time (h)	[H_2O_2]	U_{sn}	U_{AA}	U_{Ph}	Total	U_{sn}/Total
0-1.2	1	90	5	5	100	0.90
	10	90	40	40	170	0.53
3.5	1	80	9	10	99	0.81
	10	80	80	70	230	0.35

Discussion

The above results indicate that the stomata of an ozone tolerant variety of *Phaseolus vulgaris* L. are able for more than one hour to tolerate ozone concentrations exceeding the natural background concentrations up to 10-20 times. During this time 61% of the ozone flux impinging on the mesophyll cell surface (Table 3) was reaching the plasmalemma. During the following ozone-induced stomatal closure an increase in cell wall ascorbate concentration (Table 2) decreased the penetration of ozone flux to the plasmalemma to 43% of that reaching the cell surface. Evidently, the direct reaction with ascorbate in cell wall with thickness of 0.3 μm consisted no perfect barrier to ozone, allowing 140 - 60 fmol ozone to reach 1 cm^2 of the plasma membrane per second. Upon 3.5 h of exposure there was no leakage of intracellular marker proteins to cell wall and no visual injury on leaves. This indicates that either: 1) the plasmalemma has a high resistance to ozone, or 2) we have underestimated the ozone detoxification capacity of cell walls. As the fate of ozone in the plasma membrane could not be treated quantitatively on the base of scanty knowledge on the mechanisms of plasmalemma ozone tolerance, we will discuss only the version 2).

The measured ascorbate concentrations in cell walls up to 1mM (Table 2) are probably not underestimated as they enter the range observed for other species. However, cell wall thickness is a critical parameter for ozone absorption (CHAMEIDES 1989). Our measured value of 0.3 μm seems to be appropriate for young leaves of mesophytes. Still, circuitous trajectory around the microfibrils may increase the effective pathlength of molecules. This effect should be estimated in further calculations.

Ozone decomposition in the chain reaction was estimated to be at least three orders of magnitude lower than in the direct reaction with ascorbate (Table 3), although the induction of OH-radical production by phenolic acids was considered and half of OH-radicals produced in the chain reaction were assumed to be recycled to superoxide radicals ($k_P = k_S$, Table1). Consequently, formation of H_2O_2 as the termination product of the chain reaction was low. However, this relatively long-living toxicant could be formed in cell wall also in complex reactions (OBINGER & al. 1996). As H_2O_2 concentration values for cell walls under ozone were not found in literature and they were also not measured in our experiments, the potential rate of H_2O_2 scavenging in cell wall was estimated, using H_2O_2 concentrations up to 10 μM, considered to be toxic for the cells (FOYER & al. 1994). As follows from Table 4, only at these concentrations the flow of ascorbate through the enzymatic reaction is comparable with the flow through the nonenzymatic reaction with ozone. The same is true for enzymatic reaction with phenolics if their concentration approaches 1 mM, a value reported for cell walls in tobacco (LANGEBARTELS & al. 1991). Apparently, native levels of hydrogen peroxide and of phenolic compounds, as well as their induction in cell wall by ozone remain to be quantified, to promote in quantifying protective capacity of this compartment to ozone.

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