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Factors that affect leaf extracellular ascorbic acid content and redox status

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Leaf ascorbic acid content and redox status were compared in ozone-tolerant (Provider) and ozone-sensitive (S156) genotypes of snap bean (*Phaseolus vulgaris* L.). Plants were grown in pots for 24 days under charcoal-filtered air (CF) conditions in open-top field chambers and then maintained as CF controls (29 nmol mol^{-1} ozone) or exposed to elevated ozone (71 nmol mol^{-1} ozone). Following a 10-day treatment, mature leaves of the same age were harvested early in the morning (06:00–08:00 h) or in the afternoon (13:00–15:00 h) for analysis of ascorbic acid (AA) and dehydroascorbic acid (DHA). Vacuum infiltration methods were used to separate leaf AA into apoplast and symplast fractions. The total ascorbate content [AA + DHA] of leaf tissue averaged 28% higher in Provider relative to S156, and Provider exhibited a greater capacity to maintain [AA + DHA] content under ozone stress. Apoplast [AA + DHA] content was 2-fold higher in tolerant Provider (360 nmol g^{-1} FW maximum) relative to sensitive S156 (160 nmol g^{-1} FW maximum) regardless of sampling period or treatment, supporting the hypothesis that extracellular AA is a factor in ozone tolerance. Apoplast [AA + DHA] levels were significantly higher in the afternoon than early morning for both genotypes, evidence for short-term regulation of extracellular ascorbate content. Total leaf ascorbate was primarily reduced with AA/[AA + DHA] ratios of 0.81–0.90. In contrast, apoplast AA/[AA + DHA] ratios were 0.01–0.60 and depended on genotype and ozone treatment. Provider exhibited a greater capacity to maintain extracellular AA/[AA + DHA] ratios under ozone stress, suggesting that ozone tolerance is associated with apoplast ascorbate redox status.

Introduction

Tropospheric ozone is a major air pollutant that has adverse effects on the growth of agricultural crops and natural vegetation (Heagle 1989, Krupa et al. 2001). Ozone enters the plant through open leaf stomata and then rapidly decomposes (Laisk et al. 1989) to form reactive oxygen intermediates (ROI) within the cell wall (Grimes et al. 1983, Mehlhorn et al. 1990, Kanofsky and Sima 1995). Once formed, ROI are either neutralized in the cell wall or react with cellular components to initiate injury responses (Pell et al. 1997, Sandermann et al. 1998). Therefore, extracellular reactions that neutralize ozone or ROI may play a role in plant defense against ozone injury.

Ascorbic acid (AA) is synthesized inside cells (Smirnoff et al. 2001) and then transported into the leaf apoplast where it plays a role in many cell wall processes (Smirnoff 1996). One potential function of extracellular AA is to protect the plasma membrane from oxidative damage by participating in reactions that neutralize ozone and ROI (Moldau 1998). Although AA has the potential to react directly with ozone (Runeckles and Chevone 1992), experimental evidence suggests that direct reaction is not a major ozone detoxification pathway in the apoplast (Jakob and Heber 1998). Alternatively, apoplast AA can serve as a substrate in enzymatic reactions that deactivate the ROI formed during ozone de-

Abbreviations –, AA, ascorbic acid; DHA, dehydroascorbic acid; ASC_T, AA + DHA; AA/ASC_T, redox ratio of reduced/total ascorbate; AM, morning sampling period from 06: 00 to 08: 00; CF, charcoal filtered; Chl, chlorophyll; DAP, days after planting; G6P, glucose 6-phosphate; GuPOD, guaiacol peroxidase; IWF, intercellular wash fluid; PAR, photosynthetically active radiation; PM, afternoon sampling period 1300–15: 00; ROI, reactive oxygen intermediates.

composition (Polle 1998). Thus, apoplast AA dynamics may be a critical factor in plant response to ozone stress. This concept is featured in a model developed by Chameides (1989) and expanded by others (e.g. Moldau 1998, Plöchl et al. 2000) for ozone detoxification in the leaf apoplast.

Genotypes of snap bean have been identified that exhibit a range of ozone sensitivity and express differences in apoplast ascorbate content (Burkey and Eason 2002). This study compared apoplast AA dynamics in ozone-sensitive and tolerant snap beans to identify differences that may contribute to the observed differences in ozone response. The specific objective was to determine whether changes occur in apoplast ascorbate content and redox status during the day and assess the impact of ozone on these processes.

Material and methods

Plant growth and treatment

Seeds of ozone-sensitive (S156) and ozone-tolerant (Provider) genotypes of snap bean (*Phaseolus vulgaris* L.) were planted in cups of Metro Mix-200 (Scotts-Sierra Horticultural Products, Marysville, OH, USA) in a charcoal-filtered air (CF) greenhouse. Twelve days after planting (DAP) seedlings were transplanted into 10-l pots of Metro Mix-200 and placed in open-top field chambers (Heagle et al. 1973) under CF conditions. Pot numbers were limited to 14 per chamber to eliminate shading by neighbouring plants. Plants were watered daily, fertilized weekly with Peter's 20-10-20 water-soluble nutrient solution (Scotts-Sierra Horticultural Products), and fertilized once at 22 DAP with Peter's standard trace element mixture (Scotts-Sierra Horticultural Products). The third main-stem trifoliolate leaf completed expansion by 24 DAP and was tagged for later identification.

Beginning at 24 DAP, plants were either maintained under CF conditions or exposed to elevated ozone by adding ozone to CF air for 12 h each day (08:00–20:00 h eastern standard time). An ozone generator (Griffin Technics Corporation, Lodi, NJ, USA) was used to produce ozone by electrostatic discharge in dry oxygen. Ozone was monitored using a UV photometric ozone analyser (Thermo Environmental Instruments, Franklin, MA, USA). Ozone was dispensed in proportion to a daily profile that followed the average hourly concentration of ambient ozone measured at the experimental site from 1993 to 1996 during the months of June, July and August (F. Booker, unpublished data). The targeted 12 h mean was 75 nmol mol^{-1} ozone in the ozone treatment. The final experimental design consisted of 3 blocks, each containing one elevated ozone chamber and one CF control chamber. The study included two independent experiments with initial planting dates of May 12 and May 27, 2000.

Throughout the experiments ambient weather data, including photosynthetically active radiation (PAR), temperature and relative humidity, were recorded by a computer-based data acquisition system. Data were averaged over 2 min intervals and the averages stored to the system disk from 01:00 to 23:00 h each day. The daytime atmospheric water vapour saturation deficit was calculated from the temperature and relative humidity measurements, averaged across the daylight hours and expressed as kPa. The daily PAR data was integrated across each day and expressed as mol m^{-2}, while the air temperature (°C) was averaged over the entire day.

Tissue sampling

Following 10 days of ozone treatment, tissue harvests were conducted by block in the early morning between 06:00 and 08:00 h (AM) and again in the afternoon between 13:00 and 15:00 h (PM). Harvests were performed for the first experiment on June 14 (AM and PM) and June 15 (AM) and for the second experiment on June 28 (AM and PM). Each sample consisted of a third main stem trifoliolate leaf from a single plant. The leaf was excised and transported to an adjacent laboratory where processing of the tissue was begun within 5 min of excision. The centre leaflet was used to prepare intercellular wash fluid (IWF) and determine leaf ascorbate. Side leaflets were weighed and frozen with liquid nitrogen for analysis of chlorophyll (Chl) content and guaiacol peroxidase (GuPOD). Frozen tissue was stored at −80°C prior to analysis.

Isolation and assessment of IWF

The mid-vein was removed from the centre leaflet of the selected leaf and the initial FW determined. IWF was isolated using the vacuum infiltration procedure of Luwe and Heber (1995) adapted according to Burkey (1999). After IWF recovery, the leaf tissue was re-weighed, frozen with liquid nitrogen, and stored at −80°C for analysis of AA content.

The presence of glucose 6-phosphate (G6P) was used as a marker for cytoplasm contamination (Burkey 1999). An aliquot from each IWF sample was frozen with liquid nitrogen and stored at −80°C. G6P was detected using commercial glucose 6-phosphate dehydrogenase (EC 1.1.1.49) as described previously (Burkey 1999). If a G6P signal was observed, the individual IWF sample was not included in the data set.

AA extraction and assay

Frozen leaf tissue was ground in liquid nitrogen using a mortar and pestle and then extracted with cold extraction buffer [2% (w/v) meta-phosphoric acid, 2 m*M* EDTA]. The extraction buffer was prepared fresh each day and used in a ratio of 5 ml g FW^{-1}. The homogenate was subjected to centrifugation at 10 500 *g* for 10 min at 4°C. Extract supernatants were assayed for AA and DHA.

The ascorbic acid (AA) and dehydroascorbic acid

(DHA) present in IWF and total leaf extracts were determined independently by monitoring changes in A_{265nm} induced by commercially available ascorbate oxidase (EC 1.10.3.3, Calbiochem-Novabiochem, La Jolla, CA, USA) and dithiothreitol, respectively (Luwe and Heber 1995). ASC_T was used to express the total of AA + DHA in a sample. Ascorbate redox status was expressed as the AA/ASC_T ratio.

For calculation of apoplast ASC_T content, measurements of leaf weight before and after infiltration with 100 m*M* KCl and again following the IWF centrifugation step were used to calculate the recovery of the infiltrated solution (typically 70–80%). The recovery percentage for each sample was used in the calculation so that the reported values are normalized to reflect 100% recovery.

Chlorophyll and GuPOD

Chl was extracted from leaf tissue in dimethylformamide in the dark for 48 h at 4°C. The extract was then assayed for Chl using the spectrophotometric procedure of Moran (1982). GuPOD (EC 1.11.1.7) was extracted from frozen leaf tissue as described previously (Burkey et al. 2000). GuPOD activity was measured at 436 nm in an assay containing 100 m*M* KP_i pH 6.3, 10 m*M* H_2O_2, and 40 m*M* guaiacol using an extinction coefficient of 25 mM^{-1} cm^{-1} (Polle et al. 1990).

Statistics

The treatment design for these experiments was a 2×2 factorial with two ozone treatments and two snap bean lines. The experimental design was a split plot design with the open-top chambers serving as the main plots and arranged in a randomized complete block design having three replicates. The bean lines were the subplot treatments and were arranged such that two pots of a bean line would not be adjacent to one another within each chamber. For the variables measured in both the AM and PM, the treatment design was a $2 \times 2 \times 2$ factorial and the experimental design a split-split plot with open-top chambers serving as main plots, bean lines being the sub plot treatment, and the subsub plot treatment being the time of day the data were collected.

Leaf ASC_T, apoplast ASC_T, and ascorbate redox status in the symplast and apoplast were measured in the morning (AM) and afternoon (PM). Leaf Chl and GuPOD were measured only in the afternoon (PM). Because of expected genotype and treatment differences, several ratio variables were also analysed. Data for each dependent variable from both experiments were analysed separately using general linear models, and the residual plots were examined for heterogeneity of variances and non-normal data. In those cases where residual plots indicated the need for transformation, log transformation was chosen based on Box-Cox Tests (Rawlings 1988). Because AM ascorbate content and redox status were measured twice during the first experiment (June 14 and 15), contrast statements were used to show that the two AM measurements on June 14 and June 15 were not different and therefore could be averaged for all further analyses. Data from the two experiments were combined for analyses with experiment as a fixed factor. All fixed effects were considered significantly different at $P \leq 0.05$ and trends toward significance noted for $P \leq 0.20$.

Results

Ozone injury

Loss of leaf Chl and the induction of GuPOD activity were used to assess the ozone response based on previous reports that identified these parameters as indicators of chronic ozone stress in snap bean (Burkey et al. 2000, Burkey and Eason 2002). Significant genotype differences in the ozone response were observed for both GuPOD ($P = 0.04$) and Chl ($P = 0.03$). Ozone-tolerant Provider exhibited ozone/CF ratios of approximately 1.0 for GuPOD and leaf Chl, indicating that the ozone treatments imposed here did not induce a significant injury response (Table 1). In contrast, GuPOD activity increased 2- to 5-fold and leaf Chl was reduced by 20–40% in ozone-sensitive S156 as the result of ozone treatment. These results confirm the genotype differences in ozone sensitivity of Provider and S156.

Based on GuPOD stimulation and leaf Chl reduction, ozone injury in S156 was greater in the second experiment, although elevated ozone treatments were similar in the two experiments (Table 2). Differences in plant damage between experiments may have been due to environmental variables. Mean daily temperatures between experiments differed by less than 6% and the difference was not significant (Table 2). Overall, PAR was 22% greater in experiment 1 than in experiment 2, with the difference being statistically marginal ($P = 0.078$), but there was a statistically significant 55% difference in the atmospheric water saturation deficit between experiments 1 and 2. Weather conditions during experiment 1 may generally be characterized as brighter and drier than experiment 2. The dry air and high light would tend to increase transpiration demand with the consequence that mild water stresses may have developed during experiment 1, the consequences of which would have been a moderate stomatal closure and restriction of

Table 1. Ozone injury assessed as stimulation of GuPOD activity and loss of leaf Chl. Values are the average ± SE of three replications.

Experiment	Genotype	GuPOD Ozone/CF ratio	Leaf Chl Ozone/CF ratio
1	Provider	0.9 ± 0.1	0.98 ± 0.11
	S156	2.5 ± 0.7	0.80 ± 0.07
2	Provider	1.4 ± 0.2	0.97 ± 0.09
	S156	5.3 ± 1.9	0.63 ± 0.06

Table 2. Comparison of environmental conditions during the 10-day ozone treatment period for experiment 1 (June 5–14, 2000) and experiment 2 (June 19–28, 2000).

Experiment	$[O_3]$ nmol mol^{-1}		Temperature (°C)	PAR (mol m^{-2})	Water Saturation Deficit (kPa)
	CF	Ozone			
1	33	73	25.7	50.9	1.999
2	25	69	27.1	41.9	1.291
P-value	<0.001	0.781	0.272	0.078	0.017

Table 3. Total leaf tissue ascorbate content and redox status. Reported values of leaf ascorbate content represent the summation of ASC_T from the apoplast and symplast fractions. Values are the average ± SE of three replications for each experiment.

Experiment	Genotype	Harvest Time	Leaf ascorbate content (μmol ASC_T $g^{-1}FW$)			Leaf ascorbate redox status (AA/ASC_T)	
			CF	Ozone	Ozone/CF ratio	CF	Ozone
1	Provider	AM	5.5 ± 0.3	6.2 ± 0.3	1.15 ± 0.08	0.89 ± 0.02	0.89 ± 0.02
	Provider	PM	6.1 ± 0.2	6.6 ± 0.3	1.08 ± 0.08	0.89 ± 0.01	0.88 ± 0.01
	S156	AM	5.1 ± 0.4	4.3 ± 0.4	0.86 ± 0.10	0.91 ± 0.01	0.86 ± 0.01
	S156	PM	4.9 ± 0.4	3.8 ± 0.5	0.80 ± 0.17	0.89 ± 0.01	0.86 ± 0.02
2	Provider	AM	6.0 ± 0.2	6.0 ± 0.5	1.01 ± 0.11	0.90 ± 0.01	0.91 ± 0.01
	Provider	PM	5.5 ± 0.5	6.0 ± 0.4	1.13 ± 0.16	0.89 ± 0.01	0.91 ± 0.01
	S156	AM	3.8 ± 0.3	3.6 ± 0.2	0.94 ± 0.03	0.85 ± 0.03	0.81 ± 0.03
	S156	PM	4.4 ± 0.3	3.0 ± 0.5	0.67 ± 0.08	0.85 ± 0.01	0.83 ± 0.02

ozone entry into the leaves. In contrast, conditions during experiment 2, with substantially higher humidity and lower light, were conducive to maximum stomatal opening, which may have resulted in a significantly higher effective ozone exposure. Although the injury patterns were consistent with this speculation, actual measurements of transpiration or leaf conductance were not made so confirmation of this hypothesis was not possible.

Leaf tissue ascorbate content and redox status

Significant ($P = 0.001$) genotype differences in leaf ASC_T content were observed. For the two experiments, ozone-tolerant Provider averaged 28% more leaf ASC_T than sensitive S156 under CF conditions and exhibited greater capacity to maintain ASC_T content under ozone stress than did S156 (Table 3). A comparison of leaves in the early morning versus afternoon showed that leaf ASC_T content did not change significantly during the day (Table 3).

For all genotype × treatment combinations, AA/ASC_T ratios were 0.81–0.9 for leaf tissue (Table 3), indicating the presence of sufficient ascorbate-glutathione cycle capacity to maintain a majority of the ascorbate in the reduced AA form. In experiment 1, Provider and S156 had similar AA/ASC_T ratios under CF and elevated ozone conditions. In experiment 2, where ozone injury was greatest, a small but significant ($P = 0.02$) genotype difference was observed with S156 having lower AA/ASC_T ratios than Provider and the difference being more pronounced under elevated ozone conditions.

Extracellular ascorbate content and redox status

Leaf extracellular ASC_T levels were 2-fold higher in tolerant Provider compared with sensitive S156 under both CF and elevated ozone conditions (Fig. 1). There was a trend toward higher extracellular AA in ozone treated plants, but the ozone effect was not significant ($P = 0.17$).

Leaf apoplast ASC_T increased significantly ($P = 0.0001$) from morning to afternoon by approximately 2-

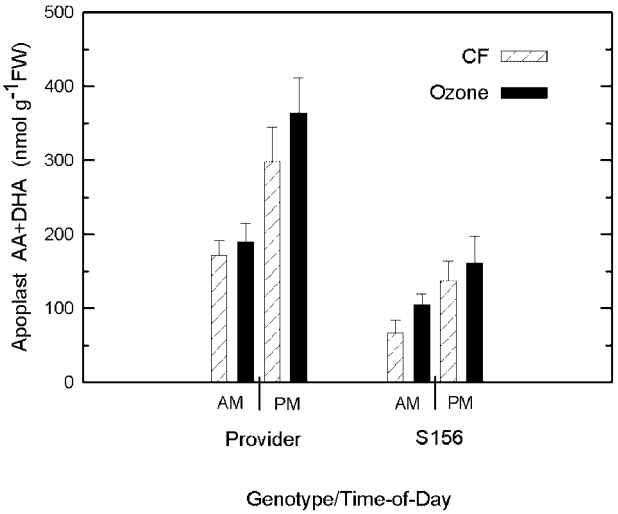

Fig. 1. Apoplast ascorbate (AA + DHA) in control (hatch bars) and ozone treated (solid bars) plants. Data from the two experiments were combined. Values represent the average ± SE (n = 5–6).

fold (Fig. 1). There was no genotype-time interaction ($P = 0.77$) indicating that the induction of apoplast ASC_T was a common response in both Provider and S156. The AM to PM increase in apoplast ASC_T was observed in both the CF and ozone treatments.

Significant differences ($P = 0.04$) in apoplast AA/ASC_T ratio response were observed between the two experiments of this study (Table 4), most likely reflecting the greater ozone injury in the second experiment. Both genotype ($P = 0.0001$) and ozone treatment ($P = 0.05$) differences in apoplast AA/ASC_T ratio were observed, but time of day was not a factor ($P = 0.58$). In the first experiment, AA/ASC_T ratios of approximately 0.5 were observed for both Provider and S156 under CF conditions. Ozone stress had no effect on Provider but caused a 4-fold decrease in the AA/ASC_T ratios of S156. In the second experiment, Provider exhibited greater AA/ASC_T ratios than S156 under both CF and ozone conditions. Ozone did not affect the apoplast ascorbate redox status in Provider, but essentially all of the apoplast ascorbate was oxidized in ozone treated S156.

Discussion

Several factors were identified that affected apoplast ascorbate dynamics. First, genetic differences were found in both leaf tissue ascorbate content and the capacity to accumulate ascorbate in the leaf apoplast. Under CF conditions, the ozone-tolerant genotype Provider contained 28% more total leaf ASC_T (Table 3) and 2-fold greater apoplast ASC_T (Fig. 1) relative to the ozone-sensitive genotype S156. The relationship between elevated apoplast ascorbate and ozone tolerance supports the hypothesis that AA in the cell wall plays a role in the detoxification of ozone and related ROI. These results confirmed the findings of an earlier controlled environment study (Burkey and Eason 2002) where apoplast ascorbate content was correlated with ozone tolerance in snap bean.

Models for ozone detoxification in the leaf extracellular space have identified apoplast AA concentration as one of several important parameters that affect plant response to ozone stress (Chameides 1989, Moldau 1998, Plochl et al. 2000). Measurements of leaf apoplast ascorbate content have now been made for a number of species (summarized in Table 5). Apoplast ascorbate levels in a majority of plant species are less then 200 nmol g FW^{-1} (i.e. less then 2 m*M* assuming the apoplast volume is 10% of FW). The exceptions include certain tree species (e.g. beech) where extracellular ASC_T levels are significantly higher. The maximum apoplast ASC_T level reported here for ozone-sensitive S156 (160 nmol g FW^{-1} or 1.6 m*M*) was in the range of values reported for a most plants. In contrast, maximum apoplast ASC_T levels for ozone-tolerant Provider (360 nmol g FW^{-1} or 3.6 m*M*) was higher than for other herbaceous plants studied to date. Although preliminary and highly speculative, the results suggest that elevation of extracellular ascorbate levels into the 3–4 m*M* range protected snap bean from ozone injury under the open-top chamber conditions employed in this study. Studies are underway to compare the two genotypes for differences in other critical parameters associated with ozone tolerance (e.g. stomatal conductance).

Time-of-day was a second factor that affected apoplast ascorbate dynamics. Both genotypes exhibited an increase of approximately 2-fold in apoplast ASC_T content between early morning and afternoon, and the increase occurred in both the elevated ozone and CF air conditions (Fig. 1). The daily pattern observed for snap bean was distinct from the pattern reported for barley and wheat (Kollist et al. 2000) where extracellular ascorbate levels were similar in the morning and early afternoon but declined during late afternoon. The different results may reflect species differences in extracellular ascorbate dynamics or differences in the timing of morning harvests (06:00 versus 09:00). Additional studies are needed to determine whether the time-of-day effect was the result of light activation or a biological clock. A light activation mechanism is supported by a previous snap bean study where extracellular AA levels increased in ozone treated plants following re-illumination at moderate light levels (Moldau et al. 1998). If light activation is involved, then partially shaded leaves within a plant canopy may have less extracellular ascorbate available for ozone detoxification. High plant density and the associated canopy shading may explain why genotype differences in extracellular ascorbate content were not consistently observed in previous open-top chamber studies (Burkey 1999, Burkey and Eason 2002).

Ozone treatment was a third factor that affected apoplast ascorbate dynamics. In this study, there was a trend toward higher apoplast ASC_T content in the ozone treated plants (Fig. 1), but the effect was not significant. An increase in apoplast ASC_T content following ozone

Table 4. Ascorbate redox status in the leaf apoplast. Values are the average ± SE of three replications. Only two replications were included for the Provider PM ozone value in experiment 1 because one IWF sample was excluded from the data set due to G6P contamination.

		Experiment 1 AA/ASC_T		Experiment 2 AA/ASC_T	
Genotype	Harvest Time	CF	Ozone	CF	Ozone
Provider	AM	0.54 ± 0.05	0.68 ± 0.04	0.48 ± 0.02	0.51 ± 0.09
Provider	PM	0.52 ± 0.04	0.43 ± 0.18	0.60 ± 0.09	0.61 ± 0.10
S156	AM	0.46 ± 0.08	0.12 ± 0.05	0.25 ± 0.03	0.06 ± 0.03
S156	PM	0.48 ± 0.06	0.14 ± 0.07	0.14 ± 0.07	0.01 ± 0.00

Table 5. Leaf apoplast ascorbate content (ASC_T) reported for various plant species. Values originally reported in units of concentration (m*M*) were converted using the assumption that apoplast volume was 10% of FW (Speer and Kaiser 1991).

Species	Apoplast ASC_T Content (nmol g^{-1} FW)		Reference
	Control	+ ozone	
Barley	200–300	Not applicable	Vanacker et al. (1998)
(*Hordeum vulgare* L.)	60–120	70–130	Kollist et al. (2000)
Beech	200–300	200–300	Luwe and Heber (1995)
(*Fagus sylvatica* L.)	100–600	Not applicable	Luwe (1996)
Broad bean	75	75	Luwe and Heber (1995)
(*Vicia faba* L)	60–90	40–90	Turcsanyi et al. (2000)
Plantago major L.	25–75	20–60	Zheng et al. (2000)
Pumpkin	40	160	Ranieri et al. (1996)
(*Cucurbita pepo* L.)			
Radish	50–100	175	Maddison et al. (2002)
(*Raphanus sativus* L.)			
Sedum album L.	60	60–125	Castillo and Greppin (1988)
Snap bean	100	70–200	Moldau et al. (1998)
(*Phaseolus vulgaris* L.)	40–150	50–150	Burkey (1999)
	25–75	25–150	Bichele et al. (2000)
Spinach	50–60	Not applicable	Takahama and Oniki (1992)
(*Spinacia oleracea* L.)	50	15	Luwe et al. (1993)
	80	190	Luwe and Heber (1995)
Spruce	26	Not applicable	Polle et al. (1990)
(*Picea abies* L)	80–150	250–350	Polle et al. (1995)
Wheat	70–140	80–150	Kollist et al. (2000)
(*Triticum aestivum* L.)			

exposure has been reported in a majority of studies (Table 5), although there are examples where ozone treatment had no effect (Luwe and Heber 1995) or resulted in a decline (Luwe et al. 1993, Turcsanyi et al. 2000). This inconsistency may arise from limitations in utilizing steady-state measurements to describe a dynamic system. Measured values of extracellular ASC_T content are a steady-state reflection of two processes, apoplast AA utilization and AA transport from the cytoplasm, that probably vary with species and ozone treatment (e.g. acute versus chronic ozone exposure). Thus, scenarios can be developed where a range of ozone effects is possible by combining different rates of AA supply and utilization.

The mechanism for the movement of AA and DHA across the plasma membrane is not well understood. Hypotheses have been proposed that are based on either facilitated transport by specific membrane carriers (Horemans et al. 2000) or diffusion (Bichele et al. 2000). An important feature of the diffusion mechanism is a linear relationship between the AA content of leaf tissue and the amount of AA present in the apoplast. In this study, no consistent relationship was observed between the leaf ASC_T content and the ASC_T content of the corresponding IWF. Large genotype and time-of-day effects on apoplast ASC_T content (Fig. 1) were associated with small changes or no change in the leaf tissue ASC_T content (Table 3). Therefore, the results are more compatible with the facilitated transport hypothesis where the number and/or specific activity of plasma membrane carriers could be regulated to change the apoplast ASC_T content under conditions where the tissue ascorbate content does not vary.

AA/ASC_T ratios in the leaf apoplast (Table 4) were low compared with whole leaf tissue (Table 3), indicating a more oxidized redox status in the cell wall. This observation presumably reflects a limitation in the transport of DHA from the apoplast to cytoplasm where DHA is reduced to AA by the ascorbate-glutathione cycle (Noctor and Foyer 1998). Apoplast AA/ASC_T ratios were higher in ozone-tolerant Provider than in ozone-sensitive S156, particularly in ozone-treated plants. Elevated extracellular DHA in S156 under ozone stress may reflect higher rates of AA conversion to DHA by extracellular antioxidant reactions (Polle 1998) or differences in the plasma membrane DHA carriers described by Horemans et al. (1998a, 1998b) that transport DHA from the apoplast into the cytoplasm.

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