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Antioxidant metabolite levels in ozone-sensitive and tolerant genotypes of snap bean

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Ozone-sensitive and tolerant genotypes of snap bean (*Phaseolus vulgaris* L.) were compared for differences in leaf ascorbic acid (vitamin C), glutathione and α-tocopherol (vitamin E) content to determine whether antioxidant levels were related to ozone tolerance. Seven genotypes were grown in pots under field conditions during the months of June and July. Open top chambers were used to establish either a charcoal filtered (CF) air control (36 nmol mol^{-1} ozone) or a treatment where CF air was supplemented with ozone from 8:00 to 20:00 h with a daily 12 h mean of 77 nmol mol^{-1}. Fully expanded leaves were analyzed for ascorbic acid, chlorophyll, glutathione, guaiacol peroxidase (EC 1.11.1.7) and α-tocopherol. Leaf ascorbic acid was the only variable identified as a potential factor in ozone tolerance. Tolerant genotypes contained more ascorbic acid than sensitive lines, but the differences were not always statistically significant. Genetic differences in glutathione and α-tocopherol were also observed, but no relationship with ozone tolerance was found. Guaiacol peroxidase activity and leaf α-tocopherol content increased in all genotypes following a one week ozone exposure, indicative of a general ozone stress response. Ozone had little effect on the other variables tested. Overall, ozone sensitive and tolerant plants were not clearly distinguished by differences in leaf antioxidant content. The evidence suggests that screening for ozone tolerance based on antioxidant content is not a reliable approach.

Introduction

Plants are sensitive to tropospheric ozone (Heagle 1989, Runeckles and Chevone 1992, Sandermann 1996), but the ozone response can be quite variable depending on the species and environmental factors such as soil moisture (Heagle 1989). Dicots appear to be more sensitive than monocots (Heagle 1989), and within a given species both sensitive and tolerant genotypes can be identified (Lee et al. 1984, Guzy and Heath 1993, Wellburn and Wellburn 1996). The physiological basis for the genetic variation in ozone sensitivity is not known, but is postulated to involve stomatal limitation of ozone uptake and/or metabolic differences in ozone detoxification and injury repair.

Plant antioxidant systems are thought to play a role in ozone detoxification mechanisms (Runeckles and Chevone 1992). Central to the proposed mechanisms are antioxidant metabolites that include ascorbic acid, glutathione and α-tocopherol (vitamin E). Ascorbic acid is a critical metabolite in the ascorbate-glutathione and xanthophyll cycles that protect chloroplasts from oxidative stress during photosynthesis (Noctor and Foyer 1998, Niyogi 1999) and is postulated to play a role in the removal of activated oxygen species within the cell wall (Smirnoff 1996). Glutathione is also critical for the operation of the ascorbate-glutathione cycle (Noctor and Foyer 1998) and in the removal of toxic products from ozone-induced lipid peroxidation (Price et al. 1990). α-Tocopherol protects membranes from photo-oxidative damage by scavenging activated oxygen species and polyunsaturated fatty acid radicals (Fryer 1992). While all

Abbreviations – AA, reduced form of ascorbic acid; CF, charcoal-filtered; DAP, days after planting; DHA, dehydroascorbic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; GuPOD, guaiacol peroxidase.

plants contain these antioxidant metabolites, it is not clear whether sufficient quantities are present to maintain metabolic functions and participate in ozone detoxification reactions. Genetic differences in the antioxidant content of leaf tissue could account for differences in ozone sensitivity. The hypothesis tested in this study was that ozone tolerant plants contain elevated levels of antioxidant metabolites. The first objective was to compare the concentrations of ascorbic acid, glutathione and α-tocopherol in leaves of snap bean genotypes that differ in ozone sensitivity. A second objective was to determine the effect of ozone treatment on the concentration of these antioxidants in an attempt to identify response patterns that distinguish ozone sensitive and tolerant genotypes.

Materials and methods

Plant material and treatments

Five cultivars (Oregon-91, Provider, Strike, Tenderette and Wade) and two experimental lines (R337 and S248, R. Reinert, unpublished data) of snap bean (*Phaseolus vulgaris* L.) were planted in cups of Metro Mix-220 (Scotts-Sierra Horticultural Products, Marysville, OH, USA) in a charcoal-filtered (CF) greenhouse on June 2, 1997. Fourteen days after planting (DAP), uniform plants of each genotype were selected and transplanted into 15-l pots of MetroMix-220 in each of six open-top field chambers (Heagle et al. 1973) under CF conditions. Plants were watered daily, fertilized weekly with Peter's 20-10-20 (Scotts-Sierra Horticultural Products) water soluble nutrient solution, and fertilized once at 28 DAP with Peter's standard trace element mixture (Scotts-Sierra Horticultural Products). The fourth main stem trifoliate leaf was tagged at 30 DAP and expansion of the tagged leaf was completed by 35 DAP.

Beginning at 36 DAP, plants were either maintained under CF conditions or exposed to elevated ozone by adding ozone to CF air for 12 h each day (8:00 to 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 analyzer (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 1993–1996 during the months of June, July and August (F. Booker, unpublished data). The targeted 12 h mean was 80 nmol mol^{-1} ozone in the CF + ozone treatment. The final experimental design consisted of 3 blocks, each containing one elevated ozone and one CF chamber.

Tissue sampling and plant harvests

Leaf tissue was analyzed for antioxidant metabolites, chlorophyll and guaiacol peroxidase (GuPOD). To facilitate multiple tasks, leaf tissue for GuPOD, ascorbic acid and glutathione analyses was harvested following a 7-day ozone exposure while leaf tissue for α-tocopherol analysis was harvested after an additional day of treatment. Analysis of leaf chlorophyll content was conducted on both days. Sampling was conducted by block between 12:00 and 17:00 each day using leaflets from the fourth main stem trifoliate. Leaves were excised and transported to an adjacent laboratory where processing of the tissue was complete within 10 min of excision. For each sample, four 3.5 cm^2 disks were removed, weighed and frozen with liquid nitrogen for chlorophyll analysis. The remainder of the tissue was divided into samples, weighed and frozen with liquid nitrogen for analysis of ascorbic acid, glutathione and α-tocopherol contents and GuPOD activity. Frozen tissue was stored at -80°C prior to analysis.

Total plant biomass was analyzed at 71 DAP following 36 days of ozone treatment. Two plants per genotype from each chamber were harvested, soil removed from the roots, and dried at 50°C to a constant weight.

Seed yield per plant was determined for two plants per genotype from each chamber. Dried pods were removed on a weekly basis beginning at 91 DAP and the various harvests combined for each plant. Seeds were separated from dried pod tissue and weighed.

Ascorbic acid and glutathione 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^{-1} fresh weight. The homogenate was clarified by centrifugation at 10 500 *g* for 10 min at 4°C.

For ascorbic acid analysis, TCA was added to an aliquot of clarified leaf extract to a final concentration of 5% (w/v). After a 15 min incubation on ice, the TCA precipitated proteins were removed by centrifugation in a micro-centrifuge. The TCA treated extracts were assayed for reduced ascorbic acid (AA) using a colorimetric method based on the reduction of ferric ions by AA and the formation of a ferrous-dipyridyl complex (Okamura 1980). TCA treated extracts were assayed for total ascorbic acid (AA + DHA) by the same method after DTT reduction of dehydroascorbic acid (DHA) to AA and removal of excess DTT with N-ethylmaleimide (Okamura 1980).

Total glutathione in the clarified leaf extract was assayed according to Tietze (1969). Oxidized glutathione (GSSG) was measured by the same procedure after removal of reduced glutathione (GSH) by treatment with N-ethylmaleimide (Sacchetta et al. 1986) and alkaline hydrolysis of excess N-ethylmaleimide (Redegeld et al. 1988).

Tocopherol extraction and assay

In screw-capped test tubes, 125 mg of lyophilized snap bean leaf tissue was mixed with 100 μg of ε-tocopherol (internal quantitative standard) and extracted overnight at room temperature with chloroform:methanol (2:1 v/v). Following extraction, the mixture was filtered and the solvent evaporated with nitrogen. The tocopherols were separated from the crude extract by column chromatography using florisil

(50–100 mesh) as the stationary phase. The solvent system consisted of hexane followed by hexane:ether (9:1 v/v) with the tocopherols eluting in the hexane/ether fraction. Purified tocopherols were converted to trimethylsilyl ether derivatives with pyridine:bis(trimethylsilyl)-trifluoroacetamide (1:1 v/v) under nitrogen at 65°C for 15 min. Derivatized samples were analyzed on a Varian 3500 capillary gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a flame ionization detector (FID) and a Varian 8100 auto sampler. A 30 m × 0.32 mm SPB-1 capillary column (Supelco Inc., Bellfonte, PA, USA) with 0.32 μm film thickness was used under the following operating conditions: oven 230°C isothermal, injector and detector 300°C. The carrier gas was helium with an average linear velocity of 76.5 cm s^{-1}, column head pressure 230 kPa at a split ratio of 20:1. Known amounts of ε-tocopherol were injected into the GC to generate the response factors used by the computer for quantification of α-tocopherol in the unknowns. The α-tocopherol content of unknowns was corrected for losses during extraction, based on the recovery (typically 75–80%) of the added ε-tocopherol internal standard.

Chlorophyll extraction and assay

Chlorophyll was extracted from frozen leaf tissue in dimethylformamide in the dark for 48 h at 4°C. The extract was then assayed for chlorophyll using the spectrophotometric procedure of Moran (1982).

Guaiacol peroxidase (EC 1.11.1.7) extraction and assay

A liquid nitrogen powder was prepared by grinding frozen leaf tissue in a mortar with 100 mg insoluble polyvinylpolypyrrolidone g^{-1} fresh weight. The powder was extracted in cold 100 m*M* Tris-HCl pH 7.5, 1 m*M* EDTA (5 ml g^{-1} fresh weight) followed by centrifugation at 38 000 *g* for 10 min at 4°C. The supernatant was diluted with extraction buffer as required. 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).

Statistical analysis

The treatment design was a 2 × 7 factorial with two ozone levels (CF or elevated ozone) and seven snap bean genotypes. The experimental design was a split plot with open-top chambers serving as main plots and arranged in a randomized complete block design with three blocks. The seven snap bean genotypes were the subplot treatments with four plants per genotype randomly placed within each chamber. Biomass and seed yield variables were the mean of two individual plants from the same chamber. A single measurement was made for antioxidant metabolites, GuPOD and chlorophyll from each chamber. The chlorophyll data from the two harvest dates were averaged for further analyses, because no differences were found in the genotype response or the ozone response for the two dates.

Data for each dependent variable were analyzed separately using general linear models. Residual plots for each variable were examined to identify variables that required transformation. Box-Cox Tests (Rawlings 1988) were used to select the most appropriate transformation. Four variables (biomass, GuPOD, total leaf ascorbic acid (AA + DHA), and α-tocopherol) were transformed using $\log_e$. Ozone, genotype and genotype × ozone interactions were tested for significance at $P \leq 0.05$. Least significant differences ($LSD_{0.05}$) were used to make all possible pair-wise comparisons among genotype means within ozone levels. The magnitude of change in a variable caused by ozone exposure was examined by calculating the ozone/CF ratio for each genotype within each block. Genotype ratio means were separated with $LSD_{0.05}$. Pearson product moment correlations were computed between all of the antioxidant metabolites, chlorophyll, GuPOD, biomass and yield to explore relationships among the responses of variables under the experimental regime.

Results

Genotype differences in ozone sensitivity

Significant genotype differences were observed for plant size and seed yield in the CF controls (Table 1), showing that the seven genotypes exhibited a range of yield potential. Thus,

Table 1. Assessment of ozone tolerance. Total plant biomass was analyzed at 71 DAP following 36 days of ozone treatment. Seed yield was measured at the end of the season. The 12 h mean for the ozone treatment was 70 nmol mol^{-1} ozone for the biomass study and 72 nmol mol^{-1} ozone for the seed yield study. The CF control ozone level was 30 nmol mol^{-1} ozone in both cases. Each value is the mean ± SE for the three experimental blocks. Values within each column followed by the same letter were not significantly different using a $LSD_{0.05}$ means analysis. Biomass data were log-transformed prior to analysis, and the transformed genotype means were separated within ozone levels. Untransformed means are presented here.

Genotype	Biomass		Seed yield	
Tolerant ↓ Sensitive	CF control (g $plant^{-1}$)	Ozone/CF ratio	CF control (g $plant^{-1}$)	Ozone/CF ratio
Provider	94 ± 19ab	1.00 ± 0.30a	52 ± 7b	0.64 ± 0.14a
Tenderette	114 ± 20a	0.82 ± 0.22ab	74 ± 4a	0.45 ± 0.10ab
Strike	99 ± 6ab	0.70 ± 0.09ab	49 ± 2bc	0.56 ± 0.04a
Oregon-91	70 ± 8abc	0.68 ± 0.16ab	34 ± 2d	0.49 ± 0.08a
Wade	122 ± 21a	0.59 ± 0.14ab	70 ± 6a	0.37 ± 0.03ab
R337	87 ± 10ab	0.39 ± 0.03b	39 ± 7cd	0.38 ± 0.12ab
S248	48 ± 5c	0.36 ± 0.09b	21 ± 2e	0.19 ± 0.04b

Table 2. Genotype comparison of leaf components in CF controls. The fourth main stem trifoliate leaf from CF control plants was analyzed at 42 or 43 DAP for the biochemical markers and antioxidant metabolites shown. The 12 h mean ozone concentration during the week before harvest was 36 nmol mol^{-1}. Each value is the mean ± SE for the three experimental blocks. Values within each column followed by the same letter were not significantly different using a $LSD_{0.05}$ means analysis. GuPOD, α-tocopherol and total ascorbate were log-transformed prior to analysis, and the transformed genotype means were separated using LSD. Untransformed means are presented here.

Genotype Tolerant ↓ Sensitive	Chlorophyll (mg g^{-1} FW)	GuPOD activity (μmol mg^{-1} protein min^{-1})	α-tocopherol (μg g^{-1} DW)	Total ascorbate (AA+DHA) (μmol g^{-1} FW)	Ascorbate redox status AA/(AA+DHA)	Total glutathione (nmol g^{-1} FW)	Glutathione redox status GSSG/(GSH+GSSG)
Provider	1.45 ± 0.14b	1.47 ± 0.33ab	374 ± 23de	3.35 ± 0.39abc	1.06 ± 0.05a	111 ± 5c	0.040 ± 0.016a
Tenderette	1.47 ± 0.11b	0.68 ± 0.12bc	486 ± 29bcd	4.07 ± 0.60ab	0.97 ± 0.03ab	220 ± 33a	0.052 ± 0.001a
Strike	1.57 ± 0.07b	0.89 ± 0.21abc	388 ± 46cde	4.38 ± 0.94a	1.02 ± 0.03a	189 ± 38abc	0.039 ± 0.001a
Oregon-91	1.89 ± 0.08a	0.80 ± 0.21abc	527 ± 86bc	3.13 ± 0.30abc	0.95 ± 0.01ab	196 ± 34ab	0.019 ± 0.012a
Wade	1.53 ± 0.11b	0.59 ± 0.01c	317 ± 19e	3.29 ± 0.14abc	1.03 ± 0.05a	183 ± 33abc	0.032 ± 0.012a
R337	1.42 ± 0.06b	0.93 ± 0.14abc	611 ± 57ab	2.63 ± 0.28bc	0.95 ± 0.06ab	137 ± 23bc	0.051 ± 0.002a
S248	1.62 ± 0.08ab	1.58 ± 0.17a	816 ± 82a	2.30 ± 0.33c	0.86 ± 0.07b	200 ± 42ab	0.050 ± 0.018a

direct comparisons of yield data under ozone stress could not be used to rank the genotypes for ozone sensitivity. Instead, ratios between ozone treated plants and CF controls were determined for biomass at 71 DAP (36 day ozone treatment) and dry seed yield of mature plants. Ozone/CF ratios were calculated for each experimental block and the mean values compared. In general, ozone had a greater impact on seed yield (36 to 81% loss) than on biomass (0 to 64% loss). Ozone had an effect on all seven genotypes tested, but genotype differences were observed and used to rank ozone sensitivity. Table 1 is organized so that the genotypes are ranked from the most ozone-tolerant (Provider) to the most ozone-sensitive (S248) in descending order.

Genotype differences in leaf chlorophyll and antioxidants

Biochemical markers and antioxidant metabolites were measured in CF control plants to determine if an increased capacity to accumulate a particular component was related to ozone tolerance. The results are presented in Table 2 with the genotypes listed in descending order of ozone tolerance. Significant genotype differences were found for several parameters (leaf chlorophyll, GuPOD and glutathione) that showed no apparent relationship to ozone sensitivity. For example, genetic differences in leaf glutathione content were observed for two tolerant lines Provider and Tenderette, yet glutathione levels were similar in tolerant Tenderette and sensitive S248.

Plants contain several isomeric forms of tocopherol that differ in the number of methyl groups attached to the ring system of the molecule. The most common are γ-, δ- and α-tocopherol. In general, α-tocopherol is the predominate form in leaves. For snap bean leaf tissue in this study, α-tocopherol represented ≥ 98% of the total tocopherol. No endogenous ε-tocopherol was observed in snap bean leaf tissue. Leaf α-tocopherol varied from 300 to 800 μg g^{-1} dry weight and significant genotype differences were observed (Table 2). The most sensitive genotypes R337 and S248 contained higher levels of α-tocopherol than all of the other genotypes.

Genetic differences were also observed in total leaf ascorbic acid content (AA + DHA) with values in the range of 2.3 to 4.4 μmol g^{-1} fresh weight (Table 2). Tolerant genotypes typically had higher levels of ascorbic acid than sensitive lines, but in most cases the differences were not significant. However, the most sensitive genotypes S248 and R337 contained the lowest levels of total leaf ascorbate.

Measurements of the reduced and oxidized forms of the ascorbic acid and glutathione showed that greater than 90% of each metabolite was present in the reduced form (Table 2). Small but statistically significant genetic differences were observed in the AA/(AA + DHA) ratio, but the differences were not related to ozone sensitivity. No genetic variation was found in the GSSG/(GSH + GSSG) ratio.

Ozone effects

Ozone effects on biochemical markers and antioxidant metabolites were evaluated as an alternative way to assess genetic differences. Ozone/CF ratios were again used for this purpose because CF control plants exhibited genetic differences in several biochemical components (see Table 2). Plants exposed to elevated ozone (77 nmol mol^{-1} ozone, 12 h mean) for one week were compared with control plants maintained under the CF conditions (36 nmol mol^{-1} ozone). CF data for the calculation of ozone/CF ratios are given in Table 2.

An ozone/CF ratio less than 1.00 would indicate an ozone-induced loss of leaf chlorophyll. Only the most sensitive genotype S248 exhibited a large decrease in leaf chlorophyll following a one week ozone exposure (Table 3), and the decrease was not statistically significant from the other genotypes. Therefore, ozone-induced loss of leaf chlorophyll was not an effective early indicator of ozone injury for plants exposed to the moderate ozone stress utilized in this study.

In contrast, GuPOD activity increased in all genotypes in response to a brief ozone treatment (Table 3). The two most ozone-sensitive genotypes R337 and S248 exhibited the largest increase in GuPOD, but smaller increases in GuPOD were also observed in tolerant lines such as Provider and Tenderette. As a result, a negative correlation was observed between GuPOD activity early in ozone treatment and both biomass ($R = -0.58$, $P = 0.0001$) and seed yield ($R = -0.57$, $P = 0.0001$) later in the season. In general, GuPOD activity was a good early indicator of ozone stress, but was not a good marker to distinguish sensitive and tolerant genotypes.

Leaf α-tocopherol content increased up to 2-fold following ozone treatment (Table 3). Ozone stimulation of α-tocopherol content was observed in both sensitive and tolerant genotypes, suggesting that the effect was a general response to ozone stress and not a factor in ozone tolerance. Because ozone treatment was associated with increased α-tocopherol content, a negative correlation was also observed between leaf α-tocopherol early in ozone treatment and both biomass ($R = -0.71$, $P = 0.0001$) and seed yield ($R = -0.68$, $P = 0.0001$) later in the season.

Effects of ozone treatment on leaf ascorbic acid and glutathione content and redox status were minimal. No significant changes were found in ascorbic acid content in any genotype (Table 3). For six of seven genotypes, leaf glutathione was either unaffected or showed an insignificant decline following ozone treatment (Table 3). The exception was the tolerant line Provider where ozone treatment caused an increase in glutathione content. However, this apparent ozone stimulation reflected the low glutathione content of Provider CF controls (Table 2), not the accumulation of glutathione in Provider to levels that were significantly higher than other genotypes. Ozone treatment did not have a significant effect on AA/(AA + DHA) or GSSG/(GSH + GSSG) ratios (data not shown), so both antioxidant metabolites were found primarily in the reduced form in both CF controls (Table 2) and in ozone treated plants.

Discussion

Significant genetic variation was found in the ascorbic acid, glutathione and α-tocopherol contents of leaf tissue. However, elevated antioxidant content did not generally correlate with increased ozone tolerance. High levels of glutathione and α-tocopherol were found in both sensitive and tolerant genotypes (Table 2). Leaf ascorbic acid was weakly related to ozone tolerance, primarily because the two most ozone sensitive genotypes R337 and S248 contained low levels of ascorbate (≤ 2.6 μmol g^{-1} fresh weight) compared to the other genotypes. Moderately sensitive genotypes such as Oregon-91 and Wade contained levels of ascorbate similar to tolerant Provider, showing that leaf ascorbic acid content alone did not explain the observed differences in ozone sensitivity. Overall, the antioxidant content of leaf tissue was not a reliable marker for distinguishing ozone-sensitive and tolerant genotypes. This does not exclude the possibility that other metabolites with antioxidant activity (e.g. polyamines, flavonoids) might prove to be more reliable indicators of ozone tolerance.

Ascorbic acid is clearly involved in plant defense against ozone injury. Previously, low leaf ascorbic acid content has been associated with ozone sensitivity in cultivars of snap bean and soybean (Lee et al. 1984). In addition, an *Arabidopsis* mutant with reduced levels of leaf ascorbic acid was found to be extremely ozone sensitive compared to wild type plants (Conklin et al. 1996). Thus, a minimum level of ascorbic acid is required to protect against ozone stress. The optimal level of ascorbate for maximum protection is not known and may depend on plant species, environmental conditions and leaf age. Overall, the evidence suggests that ozone tolerance might be improved by manipulation of ascorbic acid content to ensure that leaf ascorbate levels are above a critical threshold. Additional studies are needed to clearly define this threshold.

Cellular localization of ascorbic acid may be an important factor in ozone tolerance. Ascorbic acid in the leaf extracellular space has been proposed to play a role in the protection of plasma membranes via reactions that detoxify ozone and associated reactive oxygen species (Smirnoff 1996). In a study conducted in parallel with the present study, apoplast ascorbic acid levels were higher in leaves of ozone-tolerant Tenderette than in the ozone-sensitive genotypes S248 or Oregon-91 when grown in open top chambers under CF conditions (Burkey 1999). This preliminary evidence suggests that ozone tolerance in snap bean is associated with extracellular ascorbic acid content. Manipulation of ascorbic acid exchange between the cytoplasm and the apoplastic space may be another approach to improve ozone tolerance.

In this study, chronic ozone exposure did not have a significant effect on the glutathione content of leaves (Table 3) or glutathione redox status. Glutathione was found primarily (95–98%) in the reduced form in both CF and ozone treated plants. Similar results were found in a study of spruce seedlings where chronic ozone exposure up to twice ambient ozone concentration did not alter glutathione levels, and GSSG/(GSH + GSSG) ratios were in the range of

Table 3. Ozone effect on leaf components (ozone/CF ratios). The fourth main stem trifoliate leaf was analyzed at 42 or 43 DAP following a 7 or 8-day ozone treatment. The 12 h mean ozone concentration during the week before harvest was 36 nmol mol^{-1} and 77 nmol mol^{-1} for the CF control and ozone treatments, respectively. Each value is the mean ± SE for the three experimental blocks. Values within each column followed by the same letter were not significantly different using a $LSD_{0.05}$ means analysis. GuPOD, α-tocopherol and total ascorbate were log-transformed prior to analysis, and the transformed genotype means were separated using LSD. Untransformed means are presented here.

Genotype Tolerant ↓ Sensitive	Chlorophyll	GuPOD activity	α-tocopherol	Total ascorbate	Total glutathione
Provider	0.95 ± 0.21a	1.74 ± 0.23bc	1.53 ± 0.03ab	1.24 ± 0.35a	1.52 ± 0.27a
Tenderette	0.91 ± 0.10a	1.48 ± 0.19bcd	1.14 ± 0.22b	0.69 ± 0.16a	0.75 ± 0.12b
Strike	0.89 ± 0.05a	1.18 ± 0.23d	1.60 ± 0.50ab	1.04 ± 0.42a	0.96 ± 0.29ab
Oregon-91	0.87 ± 0.04a	1.95 ± 0.53b	1.15 ± 0.21b	1.08 ± 0.09a	1.02 ± 0.03ab
Wade	0.87 ± 0.10a	1.29 ± 0.13cd	2.25 ± 0.28a	1.29 ± 0.45a	0.86 ± 0.33ab
R337	0.86 ± 0.07a	2.60 ± 0.56a	1.19 ± 0.27ab	1.01 ± 0.25a	0.85 ± 0.38ab
S248	0.65 ± 0.07a	2.65 ± 0.52a	1.39 ± 0.28ab	1.10 ± 0.16a	0.60 ± 0.04b

0.01–0.03 (Hausladen et al. 1990). The absence of a chronic exposure effect on glutathione is in sharp contrast to the effects of acute ozone exposure. Glutathione levels doubled and high levels of oxidized glutathione (GSSG) were observed in poplar leaves following a 3 h exposure to 180 nmol mol^{-1} ozone (Sen Gupta et al. 1991). Glutathione levels declined in barley exposed for 5 days to 200 nmol mol^{-1} ozone (Price et al. 1990). Clearly, the exposure dynamics are a critical factor in determining the antioxidant response.

Tocopherol was the only metabolite measured in this study that responded to ozone. In general, α-tocopherol content increased in response to ozone treatment for all genotypes (Table 3). An increase in α-tocopherol content following ozone treatment agreed with previous observations for conifers (Melhorn et al. 1986), but not for oak trees where ozone had no effect (Kurz et al. 1998). Ozone stimulation of α-tocopherol content may have been underestimated in the most sensitive snap bean genotypes R337 and S248. CF plants of R337 and S248 contained high levels of α-tocopherol (Table 2), suggesting that a stress response may have occurred in these genotypes at control levels of ozone as low as 36 nmol mol^{-1}. If α-tocopherol content was partially induced in the R337 and S248 controls, then further increases in α-tocopherol content may have been limited in the high ozone treatment. Presumably, the increase in α-tocopherol content is part of a response mechanism to stabilize biomembranes against lipid oxidation under ozone stress and protect new membranes synthesized in conjunction with repair processes. It would be of interest to determine if the additional α-tocopherol is preferentially distributed to the plasma membrane or internal membranes of leaf cells.

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