Seasonal profiles of leaf ascorbic acid content and redox state in ozone-sensitive wildflowers

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Wildflower species exhibit differences in ascorbic acid content and redox status that affect ozone sensitivity.

Abstract

Cutleaf coneflower (*Rudbeckia laciniata* L.), crown-beard (*Verbesina occidentalis* Walt.), and tall milkweed (*Asclepias exaltata* L.) are wildflower species native to Great Smoky Mountains National Park (U.S.A.). Natural populations of each species were analyzed for leaf ascorbic acid (AA) and dehydroascorbic acid (DHA) to assess the role of ascorbate in protecting the plants from ozone stress. Tall milkweed contained greater quantities of AA ($7-10$ µmol g^{-1} fresh weight) than crown-beard ($2-4$ µmol g^{-1} fresh weight) or cutleaf coneflower ($0.5-2$ µmol g^{-1} fresh weight). DHA was elevated in crown-beard and cutleaf coneflower relative to tall milkweed suggesting a diminished capacity for converting DHA into AA. Tall milkweed accumulated AA in the leaf apoplast ($30-100$ nmol g^{-1} fresh weight) with individuals expressing ozone foliar injury symptoms late in the season having less apoplast AA. In contrast, AA was not present in the leaf apoplast of either crown-beard or cutleaf coneflower. Unidentified antioxidant compounds were present in the leaf apoplast of all three species. Overall, distinct differences in antioxidant metabolism were found in the wildflower species that corresponded with differences in ozone sensitivity.
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Keywords: Antioxidant capacity; Ascorbic acid; Foliar injury; Great Smoky Mountains National Park; Native plants; Ozone

1. Introduction

Ascorbic acid (AA) is a key metabolite of antioxidant systems that protect plants from reactive oxygen species (ROS) formed as part of normal metabolism in the chloroplast and mitochondria or during periods of environmental stress associated with ozone exposure (Runeckles and Chevone, 1992; Smirnoff, 1996; Conklin and Barth, 2004). Key elements of the ascorbate mechanism include the synthesis of sufficient quantities of AA (Smirnoff et al., 2001), and adequate ascorbate–glutathione cycle capacity (Noctor and Foyer, 1998) which converts the dehydroascorbic acid (DHA) formed by antioxidant reactions into AA that can be re-utilized as an antioxidant metabolite.

While synthesized inside leaf cells, AA is present in the leaf extracellular space in many plant species (summarized in Burkey et al., 2003). The localization of AA in the leaf apoplast is thought to involve specific plasma membrane carriers that transport AA and DHA between the cytoplasm and leaf extracellular space (Horemans et al., 2000). In the case of ozone stress, AA in the leaf apoplast represents a third element of oxidative stress response (Chameides, 1989; Plöchl et al.,

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2000; Turcsanyi et al., 2000) with the potential to scavenge ROS formed as products of ozone breakdown, protecting plasma membrane components and preventing the initiation of responses (Rao and Davis, 2001) that lead to ozone injury. Formation of ROS in the leaf cell wall following ozone exposure is a complex process. Initially, ozone decomposes in the leaf apoplast and ROS accumulate. In ozone-sensitive plants, initial ROS formation from ozone breakdown is followed by a plant-derived secondary oxidative burst in the form of hydrogen peroxide or superoxide depending on the species (Pellinen et al., 1999; Wohlgemuth et al., 2002). The secondary oxidative burst is initially localized in the leaf apoplast, and later expands into the cytoplasm and sub-cellular compartments leading to the formation of visible lesions (Pellinen et al., 1999). Potentially, ascorbic acid could serve as either a direct chemical scavenger of ozone in the apoplast, although this mechanism has been questioned (Jakob and Heber, 1998), or as a substrate for extracellular enzymes (e.g. ascorbate peroxidase) that attenuate ROS levels and thus affect the propagation of the initial ozone signal. Changes in leaf extracellular ascorbate content and redox status have been observed in ozone-treated plants (Castillo and Greppin, 1988; Luwe and Heber, 1995; Burkey, 1999), evidence that extracellular ascorbic acid is involved in detoxification of ozone and related ROS. Recent studies have found higher levels of AA in the leaf apoplast of ozone-tolerant genotypes of *Phaseolus vulgaris* (Burkey et al., 2003) and *Plantago major* (Zheng et al., 2000) compared with sensitive lines, suggesting that localization of AA in the cell wall is important.

A number of studies have examined the relationship between ozone sensitivity and the ascorbate content and redox status in leaves. The most convincing evidence that ascorbate plays a critical role in plant response to ozone stress is found in the *vtc1* mutant of Arabidopsis (Conklin et al., 1996) where a decrease in AA content is associated with increased ozone sensitivity. While it is clear that minimum levels of AA are required, the concentration of this antioxidant metabolite that naturally occurs in leaf tissue is not always well correlated with ozone tolerance (Guzy and Heath, 1993; Wellburn and Wellburn, 1996; Burkey et al., 2000). In general, these studies have focused on cultivated plants and selected tree species with less attention given to natural vegetation.

Great Smoky Mountains National Park (GRSM) encompasses an area greater than 206,000 ha in the states of Tennessee and North Carolina, and contains a wide diversity of plant and animal species characteristic of a large portion of the eastern U.S.A. There are more wildflower species in GRSM than in any other National Park (Shaver et al., 1994). Over one-half of the old-growth forest in the eastern U.S.A. is found in the Park, and greater than three-fourths of the spruce–fir ecosystem in the southern Appalachians is located in GRSM. Therefore, any detrimental effects to the vegetation are of major concern for the entire region (Shaver et al., 1994). Studies in GRSM have identified ambient ozone as an important stress factor. Ozone-sensitive, native wildflower species have been identified in the Park and species differences and seasonal trends in ozone-induced foliar injury have been documented

(Neufeld et al., 1992; Chappelka et al., 1997, 2003). Given the episodic nature and unpredictability of ambient ozone episodes, plant response to ozone stress may depend on the capacity to maintain leaf antioxidant systems throughout the season. The objective of this study was to assess seasonal patterns of ascorbate pool size and redox status in leaves from natural populations of three wildflower species to determine possible relationships with ozone-induced foliar injury.

2. Materials and methods

2.1. Plant material and injury assessment

The wildflowers utilized in this study were randomly selected from natural stands in Great Smoky Mountains National Park. Species and locations included tall milkweed (*Asclepias exaltata*) at Mt. Sterling Gap, NC [Lat: 35°42′01″N, Long: 83°05′52″W, elevation 1525 m]; cutleaf coneflower (*Rudbeckia laciniata* L. var. *laciniata*) at Clingmans Dome, TN [Lat: 35°33′46″N, Long: 83°30′04″W, elevation 2015 m]; and crown-beard (*Verbesina occidentalis* Walt.) near (<2 km) the Twin Creeks Natural Resource Center in Gatlinburg, TN [Lat: 35°41′17″N, Long: 83°29′53″W, elevation 572 m]. Plants were assessed on three dates during the summer of 2001: June 12–14, July 10–12, and August 7–9.

Plants were selected and tagged during the June sampling and the same individuals assessed again in July and August. For tall milkweed, nine ozone-insensitive and 11 ozone-sensitive individuals identified the previous year (Souza et al., in press) were tagged in June when plants consisted of four to six leaf pairs. One leaf from leaf pair #2 above the base of the plant was analyzed in June and again in July with a leaf from leaf pair #3 analyzed during the August harvest. For cutleaf coneflower, 10 individuals were tagged in June within each of two populations at Clingmans Dome. The two populations were physically located either on or off the paved trail (Chappelka et al., 2003) and have been shown previously to be genetically distinct (Davison et al., 2003). The cutleaf coneflower study was initiated by analyzing the fourth leaf below the apical bud in June with leaves located one and two nodes above the original leaf analyzed during July and August, respectively. For crown-beard, six individuals were tagged and the fourth leaf pair below the apical bud was analyzed in June with leaves located one and two nodes above the original leaf analyzed during July and August, respectively.

A six point scale was used to quantify relative severity of symptoms on injured leaves (classes = 0%, 1–6%, 7–25%, 26–50%, 51–75%, and 76–100%). The mid-point of each class was used to calculate average leaf area injured.

2.2. Ambient ozone measurements

Average ozone concentrations were calculated on a biweekly basis using passive ozone samplers (Ogawa & Co., Inc., Pompano Beach, FL, U.S.A.). These passive samplers collect ozone onto a filter coated with the absorbent sodium nitrite (Krupa et al., 2001). Sampling began in May and continued through early October. Ozone was sampled at 0.5 m above the soil surface. Samplers were retrieved and mailed to the Research Triangle Institute (Research Triangle Park, NC) for analysis at the end of each sampling period.

2.3. Extracellular ascorbic acid isolation and leaf tissue harvest

Isolation of intercellular wash fluid (IWF) was conducted adjacent to remote experimental plots using an automobile battery and a DC/AC converter to provide electrical power for operating the analytical balance and clinical centrifuge required by the procedure. Leaves were excised, placed in plastic bags, and transported to the remote laboratory so that the IWF isolation began within 5 min of harvest. The mid-vein was removed from the selected leaf and the initial fresh weight determined. The leaf tissue was vacuum infiltrated with 100 mM KCl using a 60-mL polyethylene syringe as described previously

(Burkey, 1999). Excess KCl solution was removed from the infiltrated leaf surfaces by blotting with paper towels and the tissue re-weighed. The IWF containing extracellular ascorbic acid and other antioxidant compounds was recovered from the infiltrated leaf tissue by centrifugation at 556 *g* under ambient temperature into an aliquot of 2% (w/v) meta-phosphoric acid, 2 mM EDTA used to stabilize AA. Following IWF recovery, the leaf tissue was re-weighed, frozen with liquid nitrogen, transported to the lab under dry ice, and stored at −80 °C prior to analysis of AA content.

IWF was initially stored on wet ice for up to 12 h. For each species, AA in the IWF was shown to be stable based on addition of commercial AA into aliquots of IWF. Extracellular protein precipitated in the presence of 2% (w/v) meta-phosphoric acid, 2 mM EDTA was removed by micro-centrifugation at $11,000 \times g$ for 2 min. Recovered IWF was frozen under dry ice, transported to the lab, and stored at −80 °C prior to analysis of AA content. The presence of glucose 6-phosphate (G6P) was used as a marker for cytoplasm contamination (Burkey, 1999). If a G6P signal was observed, the individual IWF sample was not included in the data set.

2.4. Tissue extraction protocol

Frozen leaf tissue was ground in liquid nitrogen using a mortar and pestle and then extracted with cold 6% (w/v) meta-phosphoric acid, 0.2 mM diethylenetriaminepentaacetic acid. The extraction buffer was prepared fresh each day and used in a ratio of 10 mL g FW^{-1}. The homogenate was subjected to centrifugation at $10,500 \times g$ for 10 min at 4 °C. Extract supernatants were assayed for AA and DHA. Recovery experiments based on the addition of commercial AA to leaf extracts showed that this protocol efficiently extracted AA without changes in redox state. An extraction buffer containing 6% (w/v) meta-phosphoric acid was critical because preliminary studies showed that AA was not stable in coneflower tissue when extracted in solutions containing 2% (w/v) meta-phosphoric acid or 0.1 M HCl.

2.5. Assay of AA and DHA

The AA and DHA present in IWF and leaf tissue extracts were determined independently by monitoring changes in $A_{265 \, nm}$ induced by commercially available ascorbate oxidase and dithiothreitol, respectively (Luwe and Heber, 1995). For calculation of apoplast ascorbate content, measurements of leaf weight before and after infiltration with 100 mM KCl and again following the IWF centrifugation step were used to calculate the recovery of the infiltrated solution. A range of IWF recovery was observed for coneflower (35−63%, average 46%), crown-beard (22−46%, average 34%) and tall milkweed (43−78%, average 61%). The recovery percentage for each leaf was used in

the calculation so that the reported values are normalized to reflect 100% recovery.

2.6. Assay of total antioxidant capacity

Total antioxidant capacity of leaf IWF was determined using the "FRAP" method of Benzie and Strain (1996) that has been successfully applied to plant samples for the analysis of fruit juices (Deighton et al., 2000). Briefly, an aliquot (typically 30 μL) of IWF was combined with 900 μL "FRAP" working solution, and distilled de-ionized water in a total volume of 1 mL. Reactions were conducted in individual micro-centrifuge tubes, incubated at 37 °C for 5 min, and then centrifuged at $12,000 \times g$ for 2 min to remove a precipitate formed by the presence of KCl in the infiltration solution used to isolate the IWF. The $A_{593 \, nm}$ of the supernatant was determined and compared with a standard curve (100−1000 μM ferrous ion). Preliminary tests showed that precipitate formation did not interfere with color formation in the assay, but for protocol consistency KCl equivalent to the unknowns was included in the ferrous sulfate reactions used to construct the standard curve. The IWF recovery percentage for each leaf was used to calculate antioxidant capacity in ferrous ion equivalents so that the reported values are normalized to reflect 100% recovery.

2.7. Statistics

For tall milkweed, ozone-sensitive and insensitive individuals were compared as separate populations. For cutleaf coneflower, plants located on and off the paved trail were compared as separate populations. Crown-beard plants were treated as a single population. For each species, dependent variables were subjected to analysis of variance using general linear models with population and month as independent variables. Inspection of residual plots revealed that all dependent variables could be analyzed without transformation. Means were compared using a Tukey analysis.

3. Results

3.1. Tall milkweed

Ozone-sensitive and insensitive tall milkweed plants were compared as separate populations throughout the season. Foliar injury was relatively low in 2001. Negligible injury was observed on ozone-insensitive plants (Fig. 1A). Low
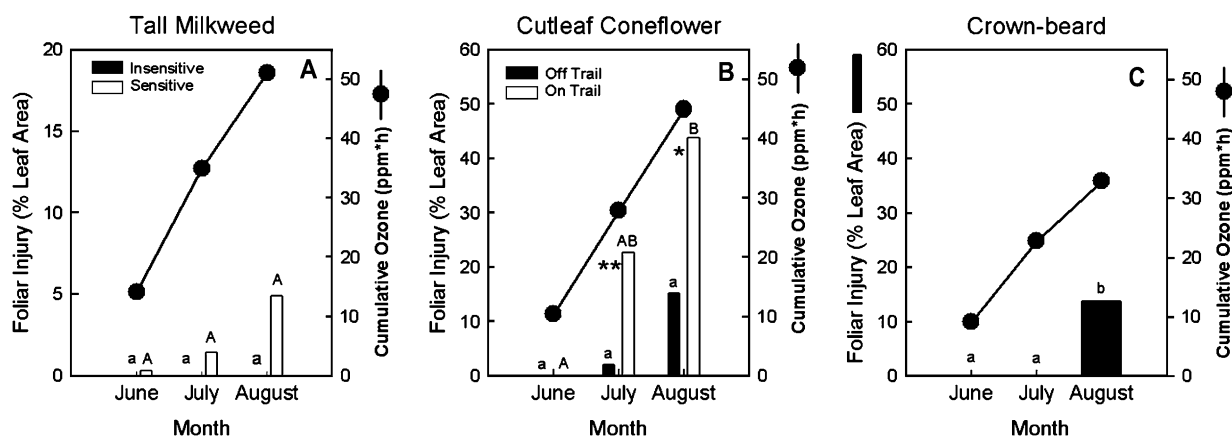

Fig. 1. Seasonal profiles of foliar injury (bars) and cumulative ozone exposure (filled circles) for tall milkweed (panel A), cutleaf coneflower (panel B), and crown-beard (panel C). Foliar injury values are means for each harvest date. Note the foliar injury scale is expanded for tall milkweed (panel A) to show the relatively low levels of injury for this species. For a given harvest date, significant differences in foliar injury between tall milkweed populations or cutleaf coneflower populations from analysis of variance are noted at the 0.05 (**) and 0.1 (*) confidence level. Within each population, monthly foliar injury values across the season followed by the same letter were not significantly different using a Tukey means analysis.

levels of foliar injury were observed on leaves of ozone-sensitive individuals and injury tended to increase throughout the season as cumulative ambient ozone levels reached 50 ppm h (Fig. 1A).

Leaf AA content was approximately 10 μmol g FW^{-1} in both populations during June and decreased throughout the season, although the decline was only significant in the sensitive population (Fig. 2A). Leaf DHA levels were relatively low ($\leq$0.5 μmol g FW^{-1}) throughout the season in both populations (Fig. 2B) so that 93–96% of the total leaf ascorbate was present in the reduced form throughout the study.

Leaf extracellular AA content in both populations was highest in June with values in the range of 80–100 nmol g FW^{-1} (Fig. 3A). Extracellular AA levels declined later in the season for the ozone-sensitive population and were significantly lower than the insensitive population at the July and August harvest dates (Fig. 3A). Extracellular DHA content was in the range of 60–80 nmol g FW^{-1} in both populations throughout the season (Fig. 3B).

The total antioxidant capacity of the IWF isolated from tall milkweed leaves was approximately 300 nmol g FW^{-1} for both populations at the June harvest (Fig. 4A). By the end of season, total extracellular antioxidant capacity declined to approximately 200 nmol g FW^{-1} in the ozone-sensitive population and to a lesser extent in the insensitive population.

3.2. Cutleaf coneflower

Populations of cutleaf coneflower located either on or off the paved trail at Clingmans Dome were compared. Foliar injury was not observed in June at the leaf position selected for this study for either population, but developed in both populations later in the season as cumulative ambient ozone levels reached 45 ppm h (Fig. 1B). During the July and August harvests, plants in the on-trail population exhibited significantly greater foliar injury than the off-trail population (Fig. 1B).

Leaf AA content was approximately 2 μmol g FW^{-1} in both populations during June and decreased to values <1 μmol g FW^{-1} in July and August (Fig. 2C). Leaf DHA levels were approximately 1.5 μmol g FW^{-1} in both populations during June and July, and decreased to approximately 1 μmol g FW^{-1} in August (Fig. 2D) so that approximately 60% of the total leaf ascorbate was present in the reduced form in June but only 20–30% later in the season.

Leaf extracellular AA levels were negligible in both populations of cutleaf coneflower throughout the season (Fig. 3C).
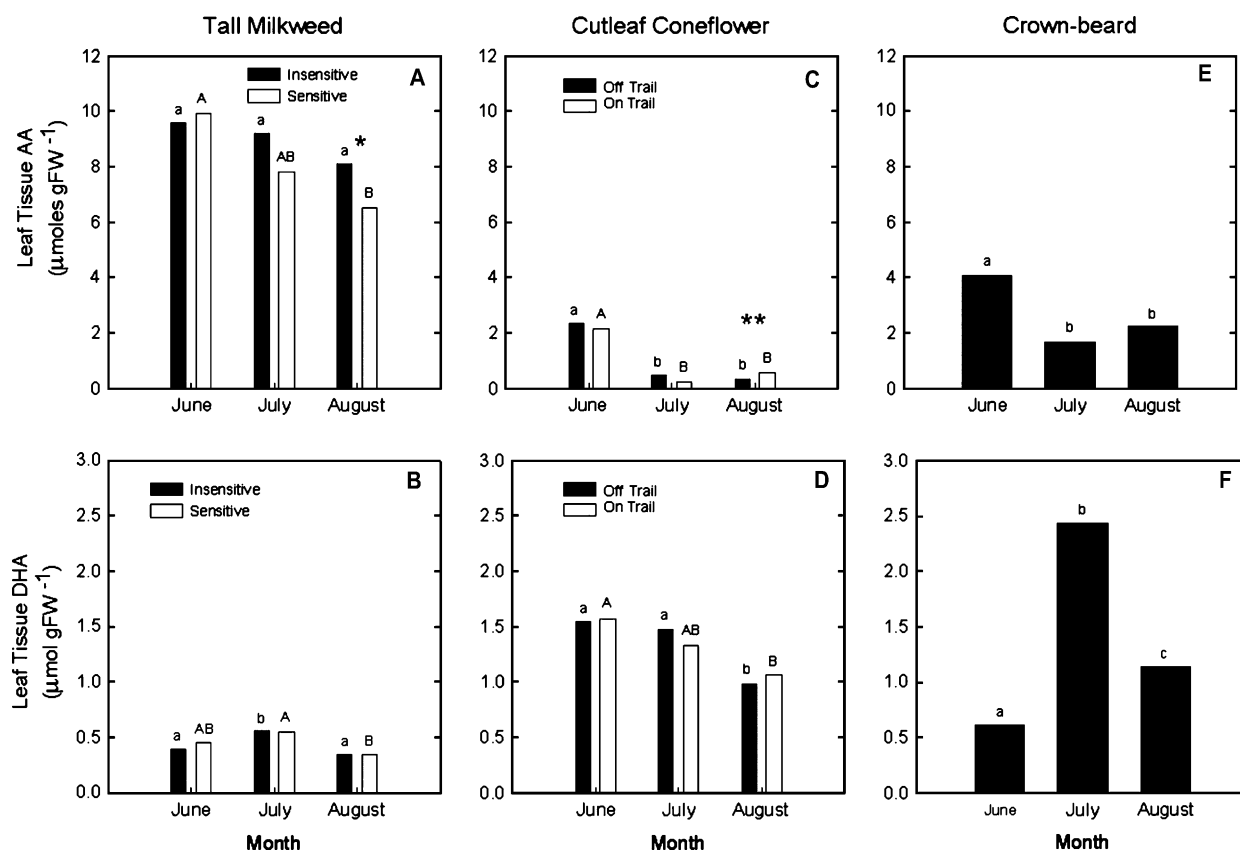

Fig. 2. Seasonal profiles of total leaf AA and DHA contents, respectively, for tall milkweed (panels A and B), cutleaf coneflower (panels C and D), and crown-beard (panels E and F). Values are means for each harvest date. For a given harvest date, significant differences from analysis of variance between tall milkweed populations or cutleaf coneflower populations are noted at the 0.05 (**) and 0.1 (*) confidence level. Within each population, monthly values across the season followed by the same letter were not significantly different using a Tukey means analysis.
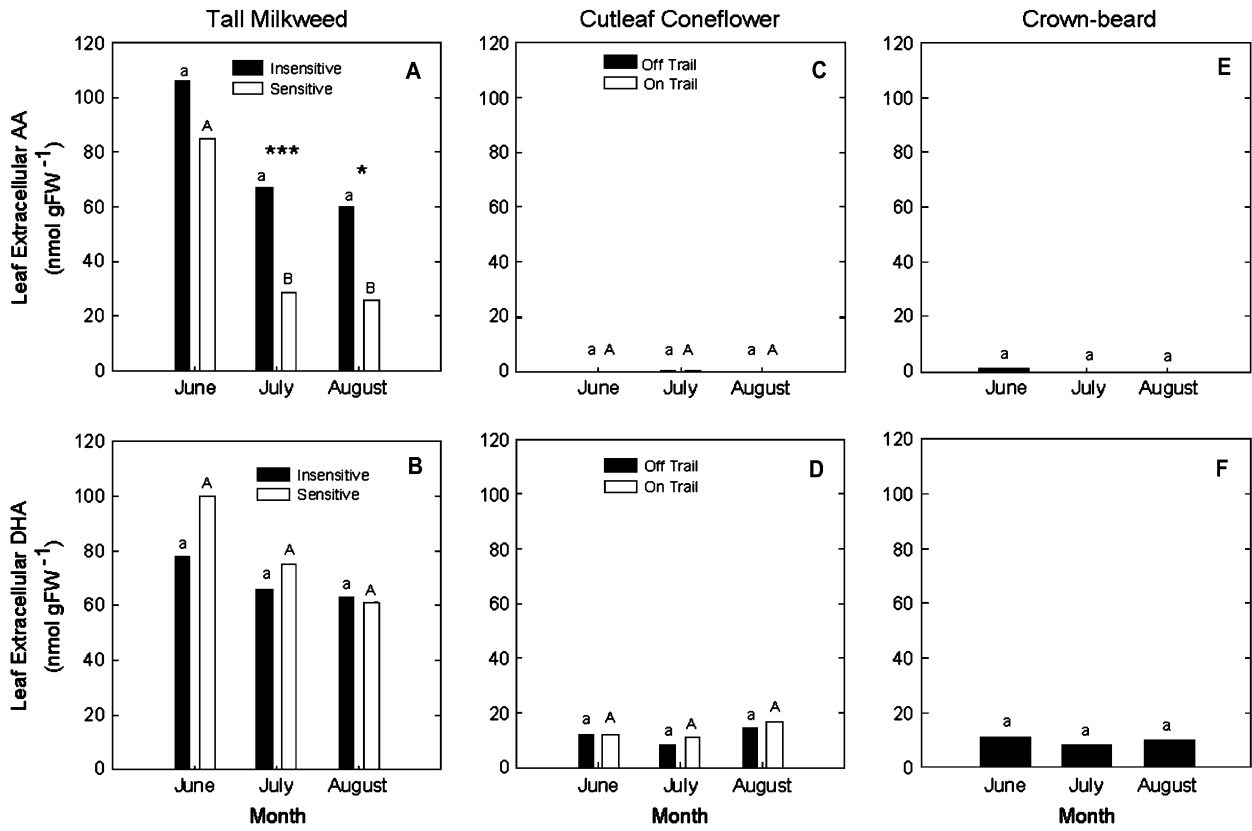
Fig. 3. Seasonal profiles of leaf extracellular AA and DHA contents, respectively, for tall milkweed (panels A and B), cutleaf coneflower (panels C and D), and crown-beard (panels E and F). Values are means for each harvest date. For a given harvest date, significant differences from analysis of variance between tall milkweed populations or cutleaf coneflower populations are noted at the 0.001 (***) and 0.1 (*) confidence level. Within each population, monthly values across the season followed by the same letter were not significantly different using a Tukey means analysis.

Extracellular DHA levels were <20 nmol g FW^{-1} in both populations at all harvest dates (Fig. 3D).

The total antioxidant capacity of the IWF isolated from cutleaf coneflower leaves was <200 nmol g FW^{-1} for both populations at the June harvest and increased dramatically throughout the season (Fig. 4B). At each harvest, total leaf extracellular antioxidant capacity was significantly greater in the on-trail population than the off-trail group with the difference between populations increasing as the season progressed.

3.3. Crown-beard

For crown-beard, foliar injury on the selected upper canopy leaves was not observed in either June or July, but became
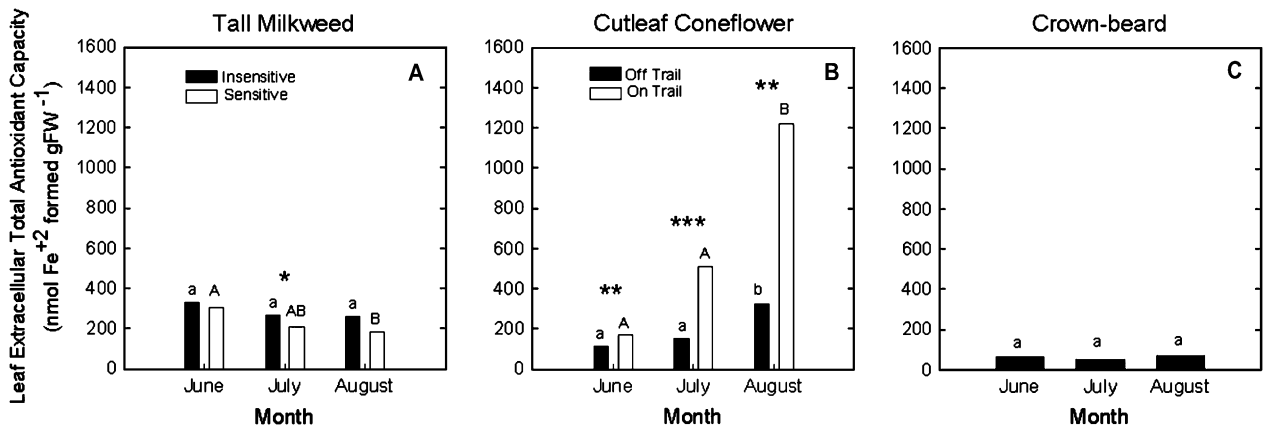

Fig. 4. Seasonal profiles of leaf extracellular antioxidant capacity for tall milkweed (panel A), cutleaf coneflower (panel B), and crown-beard (panel C). Values are means for each harvest date. For a given harvest date, significant differences from analysis of variance between tall milkweed populations or cutleaf coneflower populations are noted at the 0.001 (***), 0.05 (**) and 0.1 (*) confidence level. Within each population, monthly values across the season followed by the same letter were not significantly different using a Tukey means analysis.

significant at the August harvest as cumulative ambient ozone levels reached 33 ppm h (Fig. 1C).

Leaf AA levels were approximately 4 µmol g FW^{-1} in crown-beard at the June harvest and decreased to approximately 2 µmol g FW^{-1} in July and August (Fig. 2E). Leaf DHA levels were approximately 0.5 µmol g FW^{-1} in June and significantly higher in July and August (Fig. 2F) so that 87% of the total leaf ascorbate was present in the reduced form in June but only 40–60% later in the season.

Leaf extracellular AA levels were negligible in crown-beard throughout the season (Fig. 3E). Extracellular DHA levels were <20 nmol g FW^{-1} at all harvest dates (Fig. 3F). The total antioxidant capacity of the IWF isolated from crown-beard leaves was <100 nmol g FW^{-1} throughout the season with no significant changes over time (Fig. 4C).

4. Discussion

Ascorbate metabolism was assessed in three wildflower species based on the leaf content of AA and the oxidized form DHA. Levels of AA reflect the capacity of ascorbate to participate in antioxidant reactions, and thus the potential to deal with the oxidative stress associated with ozone exposure. DHA is the product of AA oxidation and in this chemical form is no longer an effective antioxidant compound. However, since the ascorbate synthesis pathway (Smirnoff et al., 2001) is the original source for each AA and DHA molecule, the total ascorbate [AA + DHA] can be used as a measure of the leaf capacity to synthesize ascorbate. Finally, the ascorbate redox status assessed as the AA percent relative to the total [AA + DHA] can be used as a measure of leaf capacity to convert DHA to AA by the ascorbate–glutathione cycle (Noctor and Foyer, 1998).

Using the criteria outlined above, distinct differences were found in the ascorbate metabolism of the three species. In tall milkweed, leaf AA content was relatively high throughout the season compared with the other species (Fig. 2). Tall milkweed leaf AA levels of 6–10 µmol g FW^{-1} were similar to values reported for soybean (Robinson and Britz, 2000) and somewhat higher than observed in snap bean (Burkey et al., 2000) and *Plantago* (Zheng et al., 2000). More than 90% of the tall milkweed ascorbate was present in the tissue as AA, typical of plants with sufficient ascorbate–glutathione cycle capacity. In contrast, cutleaf coneflower leaf AA levels were extremely low, particularly later in the season when values were less than 1 µmol g FW^{-1} (Fig. 2C), and thus similar to AA levels found in the *vtc1* ascorbate deficient Arabidopsis mutant described by Conklin et al. (1996). By August, total ascorbate [AA + DHA] levels in cutleaf coneflower had declined to less than 2 µmol g FW^{-1}, suggesting a diminished synthesis capacity. In July and August, cutleaf coneflower leaves contained only 20–30% of the leaf ascorbate in the reduced AA form, implying diminished ascorbate–glutathione cycle capacity as well. Crown-beard presented a somewhat different scenario with leaf AA levels of approximately 4 µmol g FW^{-1} in June that declined to approximately 2 µmol g FW^{-1} in July and August (Fig. 2E). However, total ascorbate [AA + DHA] levels were consistently 3–4 µmol g FW^{-1} throughout the season. Thus, ascorbate synthesis in crown-beard was sufficient to maintain [AA + DHA] levels throughout the season, but the capacity to convert DHA to AA declined as the season progressed.

Tall milkweed was the only species where leaf apoplast AA appeared to be a factor in scavenging extracellular ROS. Apoplast AA was found in both populations of tall milkweed with a tendency to decline over time (Fig. 3A). Apoplast AA was twofold lower (calculated from Fig. 3A) and total leaf AA was 30% lower (calculated from Fig. 2A) in ozone-sensitive plants relative to insensitive plants later in the season. These declines in extracellular and intracellular AA occurred as foliar injury increased in sensitive individuals. This result supports the hypothesis that extracellular AA plays a role in ozone tolerance (Burkey et al., 2003; Zheng et al., 2000). However, in both cutleaf coneflower and crown-beard, extracellular ascorbate levels were extremely low and essentially all of the ascorbate was present as DHA (Fig. 3). Thus, AA does not appear to be an effective scavenger of extracellular ROS in either of these species.

Extracellular AA was found to be only a fraction of the total antioxidant capacity of the leaf apoplast based on a comparison of AA content (Fig. 3A, C, and E) and ferrous reducing power (Fig. 4A–C) in isolated IWF. For reference, 1 mol of ascorbic acid will reduce 2 mol of Fe^{+3} to Fe^{+2} in the FRAP assay. In tall milkweed, AA represented approximately 60% of the total extracellular antioxidant capacity in both populations at the June harvest, and later in the season represented 50% of total capacity in the insensitive population and 30% in the sensitive population. In cutleaf coneflower and crown-beard, apoplast AA levels were essentially zero, and thus AA did not contribute to the total antioxidant capacity reported in Fig. 4B and C. This suggests that substantial quantities of antioxidant compounds other than AA are localized in the leaf apoplast. A significant topic for future research will be the identification of these compounds and whether they are associated with chemical or enzymatic reactions that scavenge extracellular ROS. In cutleaf coneflower, these extracellular compounds increased dramatically (Fig. 4B) as injury appeared (Fig. 1B), suggesting that synthesis and accumulation were related to an injury response. In contrast, total extracellular antioxidant capacity in tall milkweed and crown-beard was relatively constant across the season, suggesting the compounds play a constitutive role that may include a ROS scavenging function.

The inherent complexities of working with natural plant populations allowed only general inferences to be made regarding the relationship between ozone sensitivity and leaf ascorbate. The wildflowers were in different locations resulting in slightly different cumulative ozone levels for tall milkweed (50 ppm h), cutleaf coneflower (45 ppm h), and crown-beard (33 ppm h) during the experimental period (Fig. 1). Ozone uptake leading to foliar injury can vary between sites depending on how local environmental conditions affect stomatal conductance, although in general, the three species exhibit similar stomatal conductance patterns

(A. Davison, L. Souza, unpublished data). Harvesting tissue from the same individual plants across the season required the use of leaves from two or three adjacent nodes, but leaf age differences were considered to be minimal because all leaves destined to be harvested during the two-month study period were present on the plants in June. While these factors do impose limitations, species differences in the foliar injury were found to be associated with certain aspects of leaf AA content and oxidation state. Ozone-induced foliar injury was much greater in cutleaf coneflower than in crown-beard, confirming an earlier report (Chappelka et al., 2003). Cutleaf coneflower contained the lowest quantities of total leaf [AA + DHA], exhibited the lowest capacity to reduce DHA to AA, and did not accumulate AA in the leaf apoplast. Crown-beard contained sufficient quantities of total leaf [AA + DHA], but exhibited diminished capacity to reduce DHA to AA during July and August and did not accumulate AA in the leaf apoplast. The diminished capacity for converting DHA to AA was associated with the appearance of foliar injury in crown-beard late in the season, suggesting that the redox state of the ascorbate pool plays a role in plant response to ozone stress. In contrast, tall milkweed contained the greatest leaf AA content and capacity to reduce DHA to AA, accumulated AA in the leaf apoplast for extracellular ROS scavenging, and exhibited the lowest foliar injury of the three wildflower species.

5. Conclusions

To our knowledge, this study is the first to characterize leaf ascorbate in natural populations of wildflower species under field conditions. Techniques were developed to assess leaf extracellular AA in remote locations. Cutleaf coneflower, crown-beard, and tall milkweed exhibited differences in leaf AA content and oxidation state that corresponded with species differences in ozone-induced foliar injury. The study identified three aspects of ascorbate metabolism that contributed to plant defense against ozone stress: synthesis of sufficient quantities of AA, maintenance of a reduced ascorbate pool, and the capacity to accumulate reduced ascorbate in the leaf apoplast.

Acknowledgements

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