Tree Physiology 23, 923–929

Variation in morphological and biochemical O_3 injury attributes of mature Jeffrey pine within canopies and between microsites

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Received November 22, 2002; accepted February 15, 2003; published online August 1, 2003

Summary Crown morphology and leaf tissue chemical and biochemical attributes associated with ozone (O_3) injury were assessed in the lower, mid- and upper canopy of Jeffrey pine (*Pinus jeffreyi* Grev. & Balf.) growing in mesic and xeric microsites in Sequoia National Park, California. Microsites were designated mesic or xeric based on topography and bole growth in response to years of above-average precipitation. In mesic microsites, canopy response to O_3 was characterized by thinner branches, earlier needle fall, less chlorotic leaf mottling, and lower foliar antioxidant capacity, especially of the aqueous fraction. In xeric microsites, canopy response to O_3 was characterized by higher chlorotic leaf mottling, shorter needles, lower needle chlorophyll concentration, and greater foliar antioxidant capacity. Increased leaf chlorotic mottle in xeric microsites was related to drought stress and increased concurrent internal production of highly reactive oxygen species, and not necessarily to stomatal O_3 uptake. Within-canopy position also influenced the expression of O_3 injury in Jeffrey pine.

Keywords: antioxidants, drought stress.

Introduction

Drought-induced stomatal closure has been shown to reduce ecosystem ozone (O_3) uptake in a pine plantation (Panek and Goldstein 2001) and could protect trees from O_3 injury. However, drought itself can generate oxidative stress in foliage (Smirnoff 1993), making a deleterious interaction of drought and O_3 exposure conceivable (Grulke et al. 2002). In wheat, antioxidative defense systems responded more to drought than elevated O_3 exposure (Herbinger et al. 2002). In this paper, we describe responses of antioxidative defense systems to microsite water regime in a stand of mature Jeffrey pine (*Pinus jeffreyi* Grev. & Balf.) in Sequoia National Park, California.

To assess O_3 injury in pine in the western USA, a collection of morphological symptoms is commonly considered (Miller et al. 1996). In yellow pine (i.e., Jeffrey and ponderosa (*Pinus ponderosa* Dougl. ex P. Laws & C. Laws.) pine), a characteristic chlorotic leaf mottling develops in response to moderate O_3 exposure (Miller et al. 1963, 1983, Temple et al. 1992). In the montane forests of Sequoia National Park, most yellow pine exhibits some O_3 injury to older needles. Needle chlorophyll and nitrogen concentrations, the number of needle age classes retained, within-whorl needle retention, and branch and bole growth are all correlated to the extent of chlorotic leaf mottling in yellow pine (Grulke and Lee 1997).

In a previous study of Jeffrey pine at the same site (Grulke et al. 2003), both leaf- and canopy-level gas exchange measurements indicated that trees in xeric microsites had lowest stomatal conductances during the latter half of the summer. Lower conductance translated to 20% lower cumulative O_3 uptake over the growing season relative to trees in mesic microsites. Based on the commonly used Ozone Injury Index (Miller et al. 1996), trees in mesic microsites had slightly higher injury (primarily due to lower needle retention) but showed less chlorotic leaf mottling than trees in xeric microsites. In this paper we tested the effects of canopy level (lower, mid- and upper), drought stress, and O_3 uptake on the expression of symptoms of O_3 injury. We further tested whether patterns in morphological attributes and tissue chemistry were correlated with biochemical attributes.

Materials and methods

Research site

Ozone produced in San Francisco and the San Joaquin Valley is transported east to the Sierra Nevada (Van Ooy and Carroll 1995). Daily maximum 1-h average O_3 concentrations in Sequoia National Park exceeded California state standards (95 ppb) an average of 32 times per summer over the last 16 years (National Park Service, Air Resources Division). A Jeffrey pine stand on a south-facing slope (36.6° N, 118.73° W) at 2170 m was selected for study in Sequoia National Park, approximately 1 to 4 km west of the village of Lodgepole, Cal-

ifornia. The stand was dominated by Jeffrey pine, white fir (*Abies concolor* (Gord.) Lindl. ex Hildebr.), lodgepole pine (*Pinus contorta* Dougl. ex Loud.), incense cedar (*Libocedrus decurrens* Torr.) and sugar pine (*Pinus lambertiana* Dougl.). A few red fir (*Abies magnifica* A. Murr.) and ponderosa pine trees also occurred in the stand.

Sampled trees

Mature Jeffrey pine trees were sampled in 30 mesic microsites and 30 xeric microsites. Microsites of trees were classified mesic or xeric based on topography. Riparian microsites and seeps were mesic, and rocky outcrops xeric. Trees on rocky outcrops and seeps were open-grown and intermingled across the slope. Riparian trees were dominant or codominant, but not sampled if less than 2.8 m from another tree > 10 cm in diameter (see Grulke and Lee 1997 for effects of inferred competition on canopy response to O_3 exposure).

Microsite classification was confirmed by comparing annual ring growth of trees in mesic and xeric microsites. Radial growth was measured (Peterson et al. 1992), and annual increase in basal area increment (BAI) calculated and linearized when necessary. Long-term mean precipitation was estimated from records for Lodgepole (1969 to 2000), 1 km east of the site, and Giant Forest (1932 to 1968), 6 km to the south at a similar elevation (California Department of Water Resources, California Data Exchange Center). Percent BAI in years of average or above-average (> 120% of long-term mean) precipitation was compared to that in years of below-average (< 80% of long-term mean) precipitation. A year was compared only if it was preceeded by a year of similar precipitation because groundwater may take more than one year to recharge after a drought, and trees may take more than one year to recover from a drought. Bole wood production in mesic and xeric microsites was compared in 11 years of average or above-average precipitation and 13 years of below-average precipitation by an analysis of variance. Several trees in mesic microsites had limited ring growth in dry years (e.g., 1987 to 1992) but exhibited higher growth in subsequent wet years (1995 to 1998) (California Department of Water Resources, California Data Exchange Center). Percent BAI in mesic microsites was 15% greater in years of above-average precipitation than in years of below-average precipitation ($P = 0.001$), and it was significantly greater than percent BAI in xeric microsites in any year. Bole growth in xeric microsites increased in years of above-average precipitation (+7% BAI), but the increase was not statistically significant (Grulke et al. 2003).

Morphological and tissue chemistry attributes associated with O_3 injury

We evaluated crown morphology and leaf chemistry attributes in the lower, mid- and upper canopy of trees. The attributes tested were the ones recommended for ponderosa pine (second whorl leaf chlorotic mottling, number of needle age classes retained, proportion of the branchlet foliated (length of branchlet foliated divided by the total branchlet length), branchlet diameter, percent foliar nitrogen content, distance to nearest conspecific neighbor) (Grulke and Lee 1997) and two others (percent foliar carbon content and foliar C:N ratio). Lower, mid- and upper canopy thirds were determined by eye based on height of the live crown. Branches were cut, and a representative subset of foliage frozen in liquid nitrogen within 4 min for chemical and biochemical analysis. Morphological measurements were made on the remaining branch tissue. Branchlet length and diameter, needle length and retention, and chlorotic mottling were measured on previous-year foliage on secondary branches on the southern aspect of trees, between 0830 and 1400 h for full sun exposure, important for the antioxidant development, over a two-week period in mid-August 2000.

Foliar chemical and biochemical attributes associated with O_3 injury

Foliage was transported in liquid nitrogen and stored at –80 °C until lyophilization. For chemical analysis, tissue was ground in a Wiley mill and analyzed on a Carlo-Erba C-N-S analyzer. For biochemical analysis, tissue was ground in a dismembrator and stored frozen in humidity-proof plastic vials prior to analysis. Pigments were extracted from dry needle powder with acetone and analyzed by the HPLC gradient method of Pfeifhofer (1989). Tocopherols were measured according to Wildi and Lütz (1996). Ascorbate and dehydroascorbate were determined with an isocratic HPLC method (Tausz et al. 1996) and glutathione in its oxidized and reduced state was measured according to Kranner and Grill (1993). The concentrations of photoprotective carotenoids were expressed on a unit chlorophyll basis to relate their defense capacity to light absorbance.

Statistical analyses

Comparisons between canopy positions and microsites were evaluated by a two-way analysis of variance with canopy position as the replicate factor and microsite as the independent factor. Homogeneity of variances was tested with Levene's test, and if variances were not homogeneous, data were log-transformed. We tested to ensure that means and variances were not correlated, and that data did not significantly deviate from normality (using the Shapiro-Wilkis test). We applied a canonical correlation analysis (McCune and Mefford 1999) to test whether morphological and tissue chemistry attributes were correlated with biochemical defense attributes. In this study, input variables were chosen according to previous papers on canopy attributes (Grulke and Lee 1997) and on biochemical defense in ponderosa pine (Tausz et al. 2001). Only input variables that did not significantly deviate from the normal distribution were used. On this basis, one morphological attribute (number of whorls) was rejected. In the test, 180 cases (three canopy positions × 60 trees) were processed.

Results

Morphological and tissue chemistry attributes

Crown morphology and leaf tissue chemistry attributes are summarized in Table 1. Chlorotic leaf mottling was higher in

Table 1. Summary of statistics for morphology and tissue chemistry attributes in upper, mid- and lower canopy of 30 Jeffrey pine trees in each of mesic and xeric microsites. Means (1 SE) are presented for previous-year tissue only. A two-way nested analysis of variance was used to test for significance between canopy position, microsite and their interaction (ns indicates $P > 0.05$).

Attribute	Canopy position	Mesic	Xeric	*P*-value		
				Canopy position	Microsite	Interaction
Numbers of whorls	Upper	5.0 (0.2)	4.7 (0.2)	0.05	ns	ns
	Mid-	4.6 (0.2)	4.9 (0.2)			
	Lower	4.5 (0.2)	4.7 (0.1)			
Chlorotic mottle (%)	Upper	3.1 (0.4)	4.2 (0.4)	ns	0.01	ns
	Mid-	2.8 (0.3)	4.5 (1.2)			
	Lower	3.2 (0.3)	4.8 (0.7)			
Needle length (cm)	Upper	15.6 (0.4)	15.5 (0.4)	ns	0.01	ns
	Mid-	15.7 (0.4)	14.8 (0.3)			
	Lower	15.7 (0.6)	14.0 (0.3)			
Branchlet length (mm)	Upper	65 (5)	49 (3)	< 0.01	< 0.01	ns
	Mid-	41 (3)	35 (2)			
	Lower	34 (2)	27 (2)			
Percent foliation	Upper	75 (2)	73 (2)	< 0.01	0.01	0.02
	Mid-	68 (1)	73 (2)			
	Lower	64 (2)	71 (2)			
Branch diameter (mm)	Upper	14.1 (0.5)	14.4 (0.5)	< 0.01	0.06	ns
	Mid-	11.6 (0.3)	12.4 (0.4)			
	Lower	10.4 (0.3)	11.2 (0.3)			
Foliar carbon (%)	Upper	48.3 (0.3)	48.4 (0.3)	ns	0.03	ns
	Mid-	48.4 (0.2)	47.7 (0.4)			
	Lower	49.0 (0.6)	47.5 (0.4)			
Foliar nitrogen (%)	Upper	1.13 (0.03)	1.14 (0.02)	0.03	ns	ns
	Mid-	1.08 (0.02)	1.13 (0.02)			
	Lower	1.08 (0.02)	1.06 (0.02)			
Foliar C:N ratio	Upper	43.6 (1.0)	42.9 (0.7)	0.04	0.05	ns
	Mid-	45.5 (0.9)	42.6 (0.8)			
	Lower	45.9 (0.8)	45.0 (0.8)			

foliage of trees in xeric microsites at each canopy position ($P = 0.007$). Foliar N was highest at the top of the tree in both microsites ($P = 0.032$). Foliar C was highest in the lower canopy in mesic microsites, but was highest in the upper canopy in xeric microsites. Foliar C was greater in mesic than xeric microsites in lower and mid-canopy positions ($P = 0.032$). Foliar C:N ratio was greater in lower than higher canopy positions ($P = 0.045$), and greater in mesic than xeric microsites ($P = 0.050$). Measures of elongation growth (needle and branchlet length) were greater in mesic than xeric microsites ($P = 0.012$ and $P = 0.0003$, respectively), and branchlet elongation growth also differed significantly between canopy positions ($P = 0.0001$). Branchlet enlargement growth differed significantly between microsites ($P = 0.057$) and canopy positions ($P = 0.0001$). Needle retention within a whorl was greatest in the upper canopy ($P = 0.001$) and was greater in xeric than mesic microsites ($P = 0.014$). The number of needle age classes retained (number of whorls) was significantly greater in the upper canopy of trees in both microsites ($P = 0.050$).

Biochemical antioxidant defense attributes

Biochemical defense attributes are summarized in Table 2 and Figures 1 and 2. Total concentrations of the water-soluble antioxidants ascorbic acid (AA) and glutathione and the de-epoxidation state (DEPS) of the xanthophyll cycle (Figure 1) were significantly affected by canopy position. Antioxidant concentrations were highest in the upper canopy, whereas the xanthophylls were more de-epoxidized in the lower canopy. The glutathione pool was more oxidized in the lower canopy. Canopy position had no significant effect on chlorophyll and carotenoid concentrations or on the lipophilic antioxidant tocopherol.

Foliage of trees in xeric microsites had 20% greater total ascorbate concentrations and a higher dehydroascorbate (DHA) to AA ratio, and contained a larger pool of the xanthophyll cycle pigments in a more de-epoxidized state (Figure 2). The glutathione system, chlorophyll concentrations and α-carotene, β-carotene, lutein, neoxanthin and α-tocopherol contents were unaffected by microsite or canopy position.

Table 2. Summary of statistics for antioxidant and photoprotective defense systems in upper, mid- and lower canopy of 30 Jeffrey pine trees in each of mesic and xeric microsites. Means (1 S.E.) is presented for previous-year tissue only. A two-way nested analysis of variance was used to test for significance between canopy position, microsite and their interaction. All relationships were insignificant ($P > 0.05$) except the effect of canopy position on total glutathione ($P = 0.01$) at mesic microsites. Abbreviation: chl = chlorophyll.

Attribute	Microsite	Upper canopy	Mid-canopy	Lower canopy
Total glutathione (nmol g_{DM}^{-1})	Mesic	720 (54)	594 (41)	528 (42)
	Xeric	712 (68)	674 (61)	600 (74)
Glutathione disulfide (%)	Mesic	12 (1)	13 (1)	14 (1)
	Xeric	13 (2)	12 (2)	14 (2)
α-Tocopherol (nmol g_{DM}^{-1})	Mesic	162 (4)	164 (4)	159 (4)
	Xeric	167 (6)	168 (5)	157 (6)
Total chlorophyll (μmol g_{DM}^{-1})	Mesic	1.68 (0.09)	1.80 (0.09)	1.60 (0.08)
	Xeric	1.61 (0.12)	1.70 (0.11)	1.57 (0.09)
Neoxanthin (nmol μmol^{-1} chl)	Mesic	72 (4)	67 (3)	73 (4)
	Xeric	68 (5)	67 (4)	69 (5)
Lutein (nmol μmol^{-1} chl)	Mesic	181 (9)	171 (7)	189 (9)
	Xeric	179 (13)	173 (10)	185 (12)
α-Carotene (nmol μmol^{-1} chl)	Mesic	34 (3)	32 (2)	33 (2)
	Xeric	33 (4)	31 (3)	32 (3)
β-Carotene (nmol μmol^{-1} chl)	Mesic	96 (6)	87 (5)	98 (6)
	Xeric	96 (7)	88 (7)	90 (7)

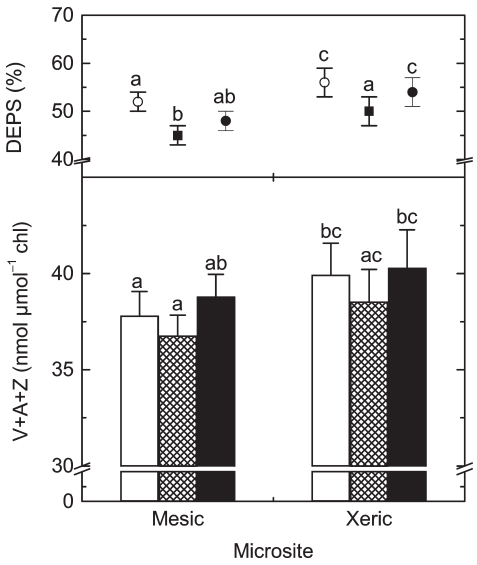

Figure 1. Concentrations of xanthophyll cycle pigments and the de-epoxidation state (DEPS). Abbreviations: V = violaxanthin; A = antheraxanthin; and Z = zeaxanthin. Lower canopy is represented by white bars and open symbols, mid-canopy by shaded bars and black squares, and upper canopy by black bars and circles. Bars with different letters are statistically significant at the $P \leq 0.05$ level.

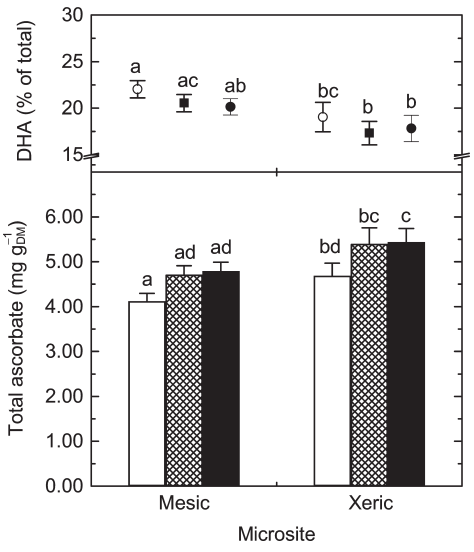

Figure 2. Concentrations of ascorbate and the redox state of ascorbate (dehydroascorbate, DHA). Lower canopy is represented by white bars and open symbols, mid-canopy by shaded bars and black squares, and upper canopy by black bars and circles. Bars with different letters are statistically significant at the $P \leq 0.05$ level.

Relationship between biochemical attributes and morphological and tissue chemistry attributes

Two significant roots representing correlated, accumulated variables from each of the two sets of attributes were extracted by canonical correlation analysis. Variables that did not contribute to an increase in the correlation coefficient were removed and the resulting model is shown in Table 3. For crown morphology and tissue chemistry attributes, both extracted ca-

nonical roots were related to chlorotic leaf mottling, foliar N and C:N ratio. Within-whorl needle retention (percent foliated branch) and branch diameters contributed to Root 1, whereas needle length contributed strongly to Root 2. Lower scores on Root 1 were correlated (see canonical loadings in Table 3) to higher leaf chlorotic mottling, longer needle retention, thicker branchlets, more foliar N and a lower C:N ratio. Lower scores on Root 2 also corresponded to higher chlorotic leaf mottling, but in combination with shorter needles, less foliar N and C and a higher C:N ratio. For antioxidant biochemistry, Root 1 consisted mainly of contributions of the water-soluble antioxidants ascorbate and glutathione. Lower scores on this root were related to higher ascorbate contents, a lower DHA:AA ratio, a lower percentage of oxidized glutathione and more total glutathione. Root 2 was constructed mainly from chlorophyll content, tocopherol, and the DEPS of the xanthophyll cycle. Lower scores on Root 2 were correlated mainly with lower total chlorophyll contents, a higher DEPS of the xanthophylls, more oxidized glutathione, but less total glutathione, and more tocopherol.

Discussion

Trees in xeric microsites had lower cumulative O_3 uptake than trees in mesic microsites (Grulke et al. 2003), so they should have suffered less O_3 injury. Yet foliage of trees in xeric microsites exhibited greater chlorotic leaf mottling than foliage of trees in mesic microsites. Foliar chlorotic mottle is considered a definitive symptom of O_3 injury in yellow pine (Miller et al. 1996). However, greater chlorotic leaf mottling may be indicative of greater stress in general (O_3 plus drought stress), not just O_3 injury. When O_3 is taken up, reactive oxygen species (ROS) formed in the substomatal cavity directly injure cell membranes and instigate a series of biochemical stress responses (Polle 1998, De Kok and Tausz 2001). Reactive oxygen species produced in the chloroplast when drought induces photooxidation (Smirnoff 1993) may elicit the same visible symptoms. Alternatively, O_3 uptake and drought may have combined to elicit greater oxidant foliar injury as a generalized stress response, or a greater sensitivity to O_3 in trees in xeric microsites.

Plants have antioxidant and photoprotective defense systems to deal with oxidative stress. Changes in the investigated variables (e.g., antioxidant contents and pigment composition) are widely used as markers for stress responses (reviewed in Tausz et al. 2001). For example, xanthophylls counteract excess light energy absorption (a higher DEPS of xanthophylls indicates enhanced photoprotection), and tocopherol in the lipid phase of membranes, glutathione in the aqueous phase within the symplast, and ascorbate in the symplast and in the cell walls all neutralize stress-related ROS. In the present study, trees in xeric microsites had higher contents of xanthophylls and ascorbate, suggesting a higher O_3 protection capacity. It is possible that the higher protection capacity was associated with long-term drought, which is known to specifically increase antioxidant defense systems (Kronfuß et al. 1998, Wonisch et al. 1999, Tausz et al. 2002*a*, 2002*b*).

Canopy position significantly affected the degree of expression of morphological, chemical and biochemical attributes associated with O_3 injury in Jeffrey pine. For conifers with pronounced light-shade crown architecture, such differences are well-known (Givnish 1988, Sprugel et al. 1996, Weiser et al. 2002). Shade (or lower canopy) foliage commonly contains lower concentrations of antioxidants and protective caroten-

Table 3. Canonical analysis model and results for $n = 180$ samples of 60 trees. Canonical R: first roots, 0.51 ($P < 0.001$) and second roots, 0.45 ($P = 0.004$). Redundancy: morphological and tissue chemistry variables, 11% and biochemical variables, 9%. Canonical weights (for standardized original variables) and loadings (correlations of the original variables with the accumulated canonical roots) are given. Only loadings significantly different from 0 ($P < 0.05$) are shown. Abbreviation: DEPS = de-epoxidation state of the xanthophyll cycle.

Original variable	Canonical weights		Canonical loadings	
	Root 1	Root 2	Root 1	Root 2
Morphological and tissue chemistry variables				
Chlorotic mottle	–0.49	–0.34	–0.41	–0.46
Needle length	0.02	0.83		0.90
Percent foliation	–0.23	0.01	–0.61	
Branch diameter	–0.69	–0.04	–0.80	
% C	–0.26	–0.10		0.17
% N	–0.05	0.83	–0.36	0.35
C:N ratio	0.25	0.70	0.33	–0.25
Biochemical variables				
Total ascorbate	–0.56	–0.20	–0.74	
% Dehydroascorbate	0.18	0.35	0.50	0.23
Total chlorophyll	0.20	1.28		0.54
DEPS	0.32	–0.46		–0.66
% Glutathione disulfide	–0.45	–0.45	0.35	–0.40
Total glutathione	–0.90	–0.05	–0.72	0.26
α-Tocopherol	0.28	1.36		–0.20

oids, and higher concentrations of chlorophylls (Weiser et al. 2002). In our study, chlorophyll concentrations and most carotenoids did not differ with canopy position, suggesting a fairly consistent light environment across canopy positions. Antioxidant and xanthophyll differences were small, but still significant. These data suggest that lower-canopy positions may have the highest risk of O_3 injury because they have the least biochemical protection. The upper canopy had higher potential antioxidant protection, as suggested by total glutathione concentrations, and may have the lowest risk of O_3 injury. More than half the foliar biomass resides in mid-canopy (Grulke and Balduman 1999).

The canonical correlation analysis suggested two response complexes. One complex (Root 1) consisted of shorter needle retention, smaller branch diameters, lower foliar nitrogen contents, lower foliar chlorotic mottling, and lower ascorbate and glutathione contents with a higher proportion of these antioxidants in an oxidized state. This pattern is consistent with responses expected from stomatal uptake of oxidants. According to previous studies, O_3 exposure promotes foliar senescence in yellow pines, and the water-soluble antioxidants (mainly ascorbate) are the first defense systems evoked in O_3 resistance (Heath 1980, Tausz 2001). Lower foliar N and lower branch diameters also suggest higher stress. This pattern was related to lower aqueous-phase foliar antioxidants in a more oxidized state: the higher stress was related to a weaker defense capacity. Trees in mesic microsites (which take up more O_3, Grulke et al. 2003) had larger components of this response complex than trees in xeric microsites (Figure 3), suggesting that this pattern is a response to O_3 uptake.

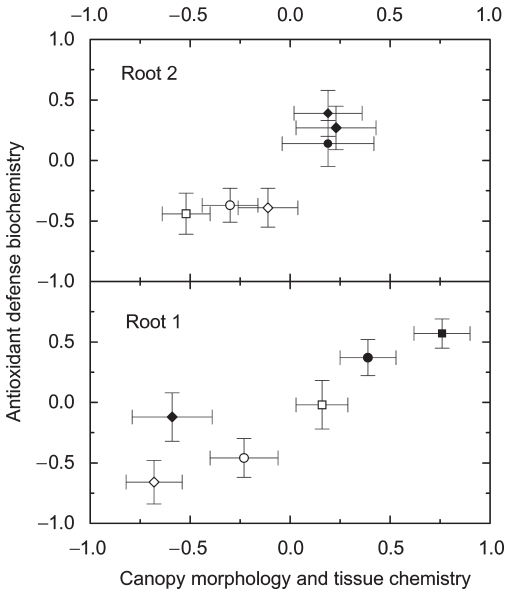

Figure 3. Canonical correlation analysis results. Root scores of canopy morphology and tissue chemistry variables are plotted against scores of antioxidant defense biochemistry variables classified by microsite and canopy level. Numbers are normalized canonical scores on Roots 1 and 2; means ± 1 standard error; $n = 30$ in each point. Open symbols = xeric microsites and solid symbols = mesic microsites. The upper canopy is represented by circles, mid-canopy by diamonds and lower canopy by squares.

A second complex of responses (Root 2) comprised higher foliar chlorotic mottling, shorter needles, lower foliar N content and C:N ratio, lower chlorophyll and higher α-tocopherol concentrations, and a high DEPS of xanthophylls. Lower scores on the second canonical root corresponded to higher stress according to established criteria. Trees in xeric microsites displayed more pronounced symptoms of stress than trees in mesic microsites, irrespective of canopy position (Figure 3). The observed biochemical responses suggested photooxidation stress in the chloroplast (Smirnoff 1993, Demmig-Adams and Adams 1994, Tausz 2001), because chloroplast membrane systems (chlorophyll, xanthophylls, tocopherol) were more clearly related to this response. If the response had been due to stomatal uptake of O_3, then a response from ascorbate, the main water-soluble antioxidant in the cell wall, would have been evoked. In general, more protective pigments and higher DEPS suggest photooxidative stress, rather than stomatal uptake of O_3 in trees in xeric microsites.

Acknowledgments

This research was supported by PRIMENET, a program jointly funded by the U.S. EPA and NPS. This presentation has not been subjected to the EPA's internal review, and does not necessarily reflect their view. The field work was supported by Liz Ballenger, Tony Gomez, Janet Kirsch, Tony Davila, Veronica Pile, Crystal Dickard and Terrence Kastner. Many thanks to Susan Schilling for technical support. M. Tausz gratefully acknowledges a grant of the Fulbright Commission that provided for his stay in California. N. Grulke acknowledges support by a Guest Professorship at the University of Graz and by the Dr. Heinrich-Jörg Foundation, which granted a short term visit, and expedited this cooperative work with M. Tausz.

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