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Seasonal Variation in the Antioxidant System of Eastern White Pine Needles[1]

Evidence for Thermal Dependence

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ABSTRACT

Antioxidant metabolites in eastern white pine (*Pinus strobus* L.) needles increased two- to fourfold from the summer to the winter season. Antioxidant enzymes in needle tissue increased between 2- and 122-fold during this same period. These seasonal changes were determined by monitoring ascorbate and glutathione concentrations and the activity of ascorbate peroxidase, glutathione reductase (GR), and superoxide dismutase. Levels of antioxidant metabolites and enzymes were observed always to be lowest during the summer, or active growing season, and highest during the winter, or dormant season. These data correlated well with the thermal kinetic window for purified GR obtained from summer needles. The minimum, apparent $K_{m,NADPH}$ for two isoforms of GR (GR_A and GR_B) occurred at 5 and 10°C, respectively. The upper limit of the thermal kinetic window (200% of the minimum K_m) for GR_A and GR_B was 20 and 25°C, respectively, indicating that needle temperatures exceeding 25°C may result in impairment of antioxidant metabolism. The needle content and kinetic properties of GR, the increased activities of other enzymes, and the high substrate concentrations observed during the winter are consistent with the protective function this pathway may provide against photooxidative, winter injury.

Eastern white pine (*Pinus strobus* L.) is a temperate climate, coniferous species that is indigenous to the northeastern United States. Populations extend westward to Minnesota throughout the Great Lakes region, and southward to Tennessee along the slopes of the Appalachian mountains. Throughout its growing range, this evergreen species is exposed to subfreezing temperatures that range from −10 to −30°C during the winter and high temperatures exceeding 30°C during the summer. These extremes are generally indicative of conditions leading to photooxidative stress, especially during periods of high irradiance and reduced CO_2 fixation, when the primary sink for the removal of electrons from PSI is limited.

At subfreezing temperatures, net photosynthesis in white pine approaches zero (8, 17), and photodynamic damage to the chloroplast can occur if energy from absorbed photons is not dissipated (16, 27). Generally, cold-acclimated conifer species, such as *Pinus sylvestris* and *Picea abies*, undergo a decrease in the efficiency of PSII (photoinhibition), which is assumed to provide a mechanism for the thermal and radiative dissipation of excess excitation energy of the photosynthetic apparatus (24). However, some cold-hardened species demonstrate a resistance to photoinhibition that may result from increased metabolism of active oxygen scavengers (16). Cold acclimation has been associated with increased levels of ascorbate (8, 11, 24), ascorbate peroxidase (11, 21, 24), dehydroascorbate reductase (11, 21), ascorbate free radical reductase (21, 24), glutathione (8–10, 15), glutathione reductase (9, 10, 14, 23), and superoxide dismutase (3, 24). These metabolites and enzymes are all components of the chloroplast antioxidant system that functions in the scavenging of oxygen free radicals and H_2O_2 (1). The antioxidant system also may function to protect drought-stressed plants against oxidative damage during CO_2-limiting carbon fixation, possibly by lowering the NADPH/$NADP^+$ ratio (12).

GR,[3] the enzyme responsible for maintaining a high ratio of GSH/GSSG in plant cells, is thought to prevent the oxidation of proteins and membranes during high and low temperature stress (1, 25). This enzyme has been used to demonstrate correlations between the TKW and the optimum growing temperature of warm and cold-tolerant crop plants that differ in their response to thermal stress (7, 19, 20). The TKW is defined by Mahan *et al.* (19, 20) as the temperature range over which the apparent K_m for an enzyme remains within 200% of the minimum K_m. The TKW concept is a modification from Teeri (26), who suggested that a change of less than twofold in the value of the apparent K_m would not significantly alter enzyme function. He based this hypothesis on the observation that apparent K_m values for plants grown within their native environment vary less than twofold over

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[3] Abbreviations: GR, glutathione reductase; GSSG/GSH, oxidized glutathione/reduced glutathione; TKW, thermal kinetic window; SOD, superoxide dismutase; AP, ascorbate peroxidase.

the range of temperatures likely to occur during the lifetime of the plant. Native plants generally do not show altered TKW characteristics (acclimation) when grown outside their normal temperature regimes (7). As a consequence, crop plants grown in nonnative environments have the potential for reduced dry matter accumulation as a direct result of the time during which foliage temperatures exceed the TKW (7).

Enzyme K_m values in excess of 200% of the minimum K_m may indicate a loss in catalytic and regulatory capacity (26). The K_m is a function of enzyme:ligand interactions and, because these interactions are thermally dependent, the TKW for GR may be a useful indicator for determining the temperature range over which a species experiences optimum metabolism or, conversely, when it experiences thermal stress (7, 19, 20).

Although research has been directed toward understanding the relationship between the TKW and temperature stress in crop plants (7, 19, 20, 26), this concept has not been applied to conifers. Existing studies on the thermal response of GR in conifers generally have been conducted *in vitro* at 25°C and may have little, if any, relationship to protection against low or high temperature stress *in vivo*. In the present paper, we report seasonal variations of antioxidant enzyme activity and metabolite concentrations in eastern white pine needles over a 3-year period, and the relationships between antioxidant components and the TKW for two isoforms of GR present in whole needles. We discuss the use of the TKW as a means for determining the temperature range over which eastern white pine might show a transition from normal to abnormal metabolism (thermal stress) and how this might affect yearly growth and ecological distribution.

MATERIALS AND METHODS

Plant Material

Three eastern white pine (*Pinus strobis* L.) trees, 8 to 10 years old, growing within a 15 m radius in a nursery plantation in Montgomery County, VA, were selected as study specimens. Needles were collected from June 1988 through January 1991. All needles samples were taken from the trees between 9 and 11 AM eastern standard time. Each year's needle flush was collected from emergence in June through senescence and shed 14 to 16 months later.

Acid Soluble Metabolite Extraction

Three sets of whole needles, 500 mg each, were collected randomly from each of the trees at approximately breast height. The needle samples were cut into 2 to 4 mm sections immediately prior to freezing in liquid N_2. Each sample was then homogenized in 3 mL of ice-cold 6% *meta*-phosphoric acid (pH 2.8), containing 1 mM EDTA, using a Polytron (Brinkmann Instrument Co., Westbury, NY) at top speed for 30 s. Homogenates were centrifuged at 20,000*g* for 15 min (4°C) using a fixed angle rotor (Beckman JA-20), and the resulting supernatants were filtered through a 0.45 μm ultrafilter. Samples were stored at −20°C prior to analysis of ascorbic acid and glutathione without loss or change in ratios of oxidized or reduced forms of these metabolites.

Ascorbic Acid Analysis

Prior to analysis, 1 mL of the acid-soluble metabolite extract was incubated for 24 h at 25°C in the presence of 30 mM DTT to reduce dehydroascorbate to ascorbic acid. Ascorbic acid was measured using a modified procedure of Lee *et al.* (18). Reduced ascorbic acid content was measured at 245 nm (maximum absorbance of the nonionized form) using HPLC. Separation was accomplished using a 250 × 4.6 mm PartiSphere C_{18} octadectyl reverse-phase column (Whatman, Clifton, NJ) protected with a prepacked PartiSphere anion exchange guard cartridge (Whatman) and a 250 × 4.6 mm Solvecon precolumn (Whatman). The eluent was aqueous 2% $NH_4H_2PO_4$ (pH 2.8) at a flow rate of 1 mL min^{-1} and a column pressure of 1800 p.s.i. Peak areas were calculated using a Spectra Physics System I integrator. A standard curve was based on L-ascorbate (Fisher Scientific, 99.2% pure) from 50 to 800 μg mL^{-1} in *meta*-phosphoric acid. Dehydroascorbic acid values were determined by comparing the difference between total reduced ascorbic acid in extracts with or without 30 mM DTT.

Glutathione Analysis

Glutathione concentrations in the acid-soluble extracts were analyzed according to the procedure of Brehe and Burch (6) with the following modifications. Total glutathione was measured in a 1.2 mL reaction mixture containing 400 μL reagent 1 (110 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 15 mM EDTA, 0.3 mM 5,5′-dithiobis-(2-nitrobenzoic acid), and 0.04% BSA), 320 μL reagent 2 (1 mM EDTA, 50 mM imidazole solution, and 0.02% BSA), which contained an equivalent of 1.5 units GR activity (bakers' yeast, type III, Sigma Chemical Company) mL^{-1}, and 400 μL of a 1:50 dilution of acid extract in 5% Na_2HPO_4 (pH 7.5). The dilution of acid extract was done immediately prior to starting the reaction by the addition of 80 μL of 9.0 mM NADPH. Change in absorbance of the reaction mixture was measured at 412 nm.

Oxidized glutathione was measured by first incubating 1 mL of the 1:50 diluted acid extract with 40 μL of 2-vinylpyridine for 1 h at 25°C. The samples incubated in 2-vinylpyridine were then assayed to estimate GSSG content. Reduced glutathione was determined as the difference between total glutathione and GSSG. All values are reported as GSH equivalents.

Protein Extraction

Three whole needle samples, 1 g each, were collected from each of the trees and frozen in liquid N_2 as described above. The needles were homogenized with a Polytron at top speed for 30 s in 5 mL of 50 mM Pipes buffer (pH 6.8), 6 mM L-cysteine hydrochloride, 10 mM D-isoascorbate, 1 mM EDTA, 0.3% Triton X-100, 1% (w/v) PVP (mol wt 10,000), 1% polyclar-AT, and 1 drop of antifoam A emulsion. After centrifugation at 20,000*g* for 15 min (4°C), the supernatants were saved and the remaining insoluble material was reextracted with an additional 2 mL of extraction buffer using a Polytron at top speed for 10 to 15 s. The homogenates were again centrifuged and the resulting supernatants were pooled

together with the corresponding supernatants of the first extraction.

A 2 mL fraction of each extract was desalted on a G-25 desalting column (Pharmacia Fine Chemicals) using the quick centrifugation method of Neal and Florini (22). Each column was preequilibrated with 50 mM Tris-HCl (pH 7.5), containing 1 mM EDTA, prior to desalting of the extract.

Protein Determination

Protein was determined by the method of Bradford (5) using lyophilized BSA (Bio-Rad) as a standard.

Ascorbate Peroxidase Assay

Ascorbate peroxidase activity in desalted protein extracts was measured spectrophotometrically by monitoring the ascorbic acid-dependent reduction of H_2O_2 at 265 nm. Assays were performed at 25°C in a 1 mL reaction mixture containing 500 μL of 166 mM Hepes-KOH (pH 7.0), 100 μL each of 1.0 mM EDTA, 1.5 mM Na ascorbate, and 1.0 mM H_2O_2.

Purification and Analysis of Glutathione Reductase (EC 1.6.4.2)

The assay for, and the purification of, GR was performed as previously described by Anderson *et al.* (2). The substrate-binding affinity of GR for NADPH and GSSG was determined at 10, 20, 30, and 40°C. A 5 × 5 concentration matrix of NADPH and GSSG was used to determine the K_m value at each temperature. The NADPH and GSSG concentrations ranged from 1 to 100 μM and 5 to 200 μM, respectively. The K_m values also were obtained for GR at 5, 10, 15, 20, 30, and 40°C using the procedure described by Mahan *et al.* (20), which consisted of a 1 × 5 matrix of GSSG and NADPH, respectively. Initial velocities were calculated over the first 30 s of the assay using 0.04 units of GR. The apparent K_m values were estimated using the Pennzyme program for analyzing kinetic data.

Superoxide Dismutase Analysis

SOD activity was assayed on 7.5% native PAGE using the nitroblue tetrazolium activity staining procedure of Beauchamp and Fridovich (4). Standard SOD from *Escherichia coli* was loaded at 0.5, 1, and 2 μg protein for comparison with tissue samples. Gels were scanned using a Shimadzu model 3 densitometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) at 560 nm and peak areas were determined.

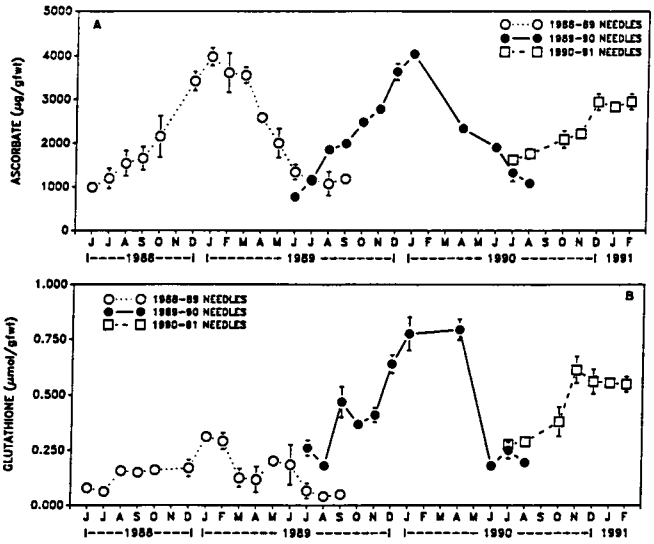

Figure 1. Seasonal variation in ascorbate (A) and glutathione (B) content of eastern white pine needles. Error bars represent 95% confidence limits and are contained within the symbols when not shown ($n = 9$).

Environmental Measurements

Temperature was monitored at the Environmental Protection Agency's Dry Deposition Station located at the Horton Research Center, Giles County, VA.

RESULTS

Ascorbate

The concentration of ascorbate in field-grown eastern white pine needles changed in a consistent annual pattern. In young tissue, ascorbate was low and ranged between 1000 and 1500 μg g fresh weight^{-1} (Fig. 1A). The ascorbate content increased throughout the summer and fall and reached maximum concentrations during December through February. The increase in ascorbate between summer and winter was approximately

Table I. *Summary of the Average Minimum and Maximum Values of Metabolites, Enzyme Activities, and Total Extractable Protein in Eastern White Pine Needles over a 3-Year Period*

Variable	1988–1989 Needles			1989–90 Needles			1990–91 Needles		
	Min	Max	FI[a]	Min	Max	FI	Min	Max	FI
ASC[b]	989	3974	4.0	1158	4036	3.5	1619	2948	1.8
GSH[c]	0.12	0.33	2.8	0.23	0.80	3.4	0.28	0.67	2.4
AP[d]	NA[e]	NA	NA	0.03	1.82	65	0.06	2.28	40
GR[f]	NA	NA	NA	0.14	4.55	32	0.04	5.25	122
SOD[g]	NA	NA	NA	45.2	215	4.7	81.2	117	1.44
Protein[h]	NA	NA	NA	1.25	8.85	7.1	1.07	12.6	11.7

[a] Fold increase. [b] μg ascorbate g fresh wt^{-1}. [c] μmol total glutathione g fresh wt^{-1}. [d] Units ascorbate peroxidase g fresh wt^{-1}. [e] Data not available. [f] Units glutathione reductase g fresh wt^{-1}. [g] Units superoxide dismutase g fresh wt^{-1}. [h] mg protein g fresh wt^{-1}.

Table II. *Seasonal Variation in Oxidized and Reduced Glutathione and Ascorbic Acid Content in Eastern White Pine Needles*

Month	1988–89 Needles			1989–90 Needles		
	GSSG[a]	GSH	GSSG/GSH	GSSG	GSH	GSSG/GSH
July	65[b]	63	1.01	30	259	0.12
October	29	160	0.18	13	366	0.04
January	19	310	0.06	24	775	0.03
April	6	117	0.05	4	793	0.01
July[c]	25	66	0.38	16	233	0.07
	dAsc[d]	Asc	dAsc/asc	dAsc	Asc	dAsc/Asc
July	117	1190	0.10	112	1153	0.10
October	141	2398	0.06	215	2476	0.09
January	18	3974	0.004	18	4036	0.004
April	152	2585	0.06	411	2328	0.19
July[c]	153	1150	0.13	171	1321	0.13

[a] Oxidized (GSSG) and reduced (GSH) glutathione in nmol/g fresh wt. [b] Values are mean needle content (n = 9). [c] Needles in second year. [d] Oxidized (dAsc) and reduced (Asc) ascorbate in μg/g fresh wt.

three- to fourfold in the 1988–89 and 1989–90 year needles (Table I), but was only twofold in the 1990–91 year needles. Ascorbate content began to decrease in March and reached minimum levels in the August–September period, just prior to needle shed. The ratio of oxidized to reduced ascorbate was always lowest during the winter months (Table II), when total ascorbate was at peak concentrations.

Glutathione

Glutathione content in needles of eastern white pine varied in a seasonal pattern similar to that for ascorbate, with the lowest levels in the summer and the highest concentrations during mid-winter (Fig. 1B). However, the year-to-year variability in glutathione was considerably greater compared with that in ascorbate. Minimum concentrations of glutathione were observed in the summer in both young and mature tissue and ranged between 75 and 100 μmol g fresh weight^{-1} (1988–89 year needles) and 200 and 275 μmol g fresh weight^{-1} (1989–90 and 1990–91 year needles). Maximum levels of glutathione ranged between 300 μmol g fresh weight^{-1} (1988–89 year needles) and 650 to 800 μmol g fresh weight^{-1} (1989–90 and 1990–91 year needles). Although the seasonal glutathione content was variable from year to year, the difference between minimum and maximum levels remained consistent at approximately 2.4- to 3.4-fold (Table I). As with ascorbate, the ratio of oxidized to reduced glutathione was lowest during the winter and highest during the summer months (Table II).

Ascorbate Peroxidase and Glutathione Reductase

Enzyme activities were measured only in the 1989–90 and 1990–91 year needles (Fig. 2A and B) and varied annually in the same general pattern as ascorbate and glutathione. The lowest activities were present in the summer and the highest activities in the winter. Some variability was observed when comparing the timing at which maximum levels of these two enzymes occurred in the 1989–90 and 1990–91 year needles. In the 1989–90 year needles, AP maximum activity was evident in September and October, whereas in the 1990–91 year needles, maximum activity occurred in December and February (Fig. 2A).

Glutathione reductase activity was maximum in December and January in the 1989–90 year needles. In the 1990–91 year needles, GR levels peaked earlier than in the previous year, September and October, and then declined during December and January before attaining maximum levels in February (Fig. 2B). The decrease in the enzyme activity of AP and GR during December and January in the 1990–91 year needles paralleled the pattern seen for total extractable protein in the same year needles (Fig. 3A). Seasonal variation in total enzyme activity was attributed mainly to changes in protein content, because needle dry weight to fresh weight ratios remained essentially constant throughout the year (Fig. 3B).

The specific activity of AP and GR followed a similar seasonal pattern as total activity (Table III), increasing during the winter and decreasing the following spring. In second year tissue, just prior to the initiation needle shed, enzyme activity was greater than in first year tissue.

The magnitude of change in activity for these two enzymes between summer and winter was substantially greater than the seasonal differences in the enzymes' substrates. Ascorbate

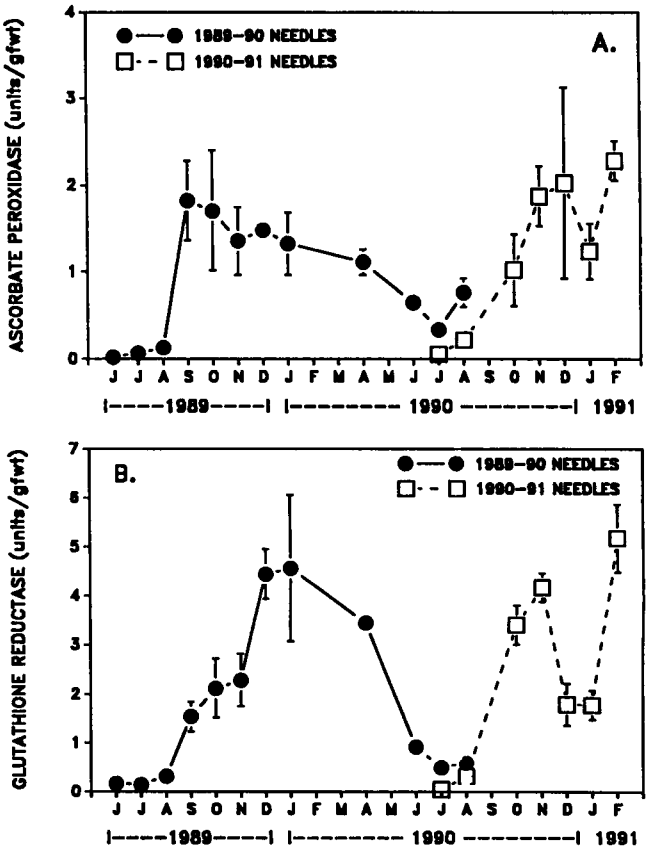

Figure 2. Seasonal changes in (A) ascorbate peroxidase activity (g fresh wt^{-1}) and (B) glutathione reductase activity (g fresh wt^{-1}) in Eastern white pine needles. Error bars represent 95% confidence limits and are contained within the symbols when not shown (n = 9).

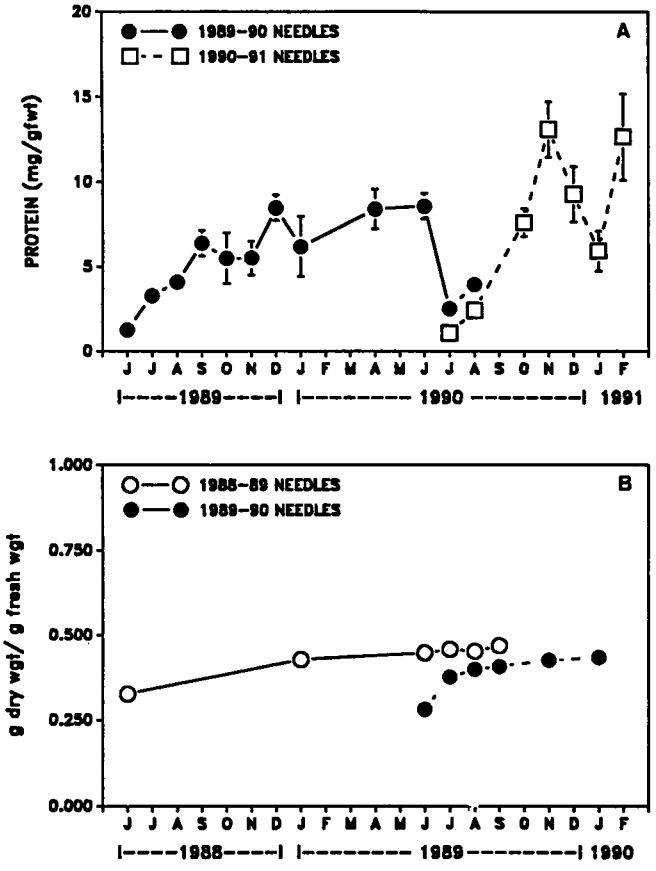

Figure 3. Seasonal variation in (A) extractable protein (g fresh wt^{-1}) and (B) fresh weight to dry weight ratios in eastern white pine needle tissue. Error bars represent 95% confidence limits and are contained within the symbols when not shown; $n = 9$ (A), $n = 3$ (B).

peroxidase showed a 65- (1989–90 year needles) and 40-fold (1990–91 year needles) increase in units of activity g fresh $weight^{-1}$ between summer and winter (Table I) and GR activity g fresh $weight^{-1}$ increased 32- (1989–90 year needles) and 122-fold (1990–91 year needles) during this period (Table I). The 90-fold greater increase in minimum *versus* maximum GR activity in the 1990–91 year needles compared with the 1989–90 year needles may be related, in part, to the greater maximum concentration of extractable protein present in the 1990–91 year needles (Fig. 3A).

Superoxide Dismutase

Seasonal variation in SOD activity was similar to the pattern observed for AP and GR (Fig. 4A and B). Total SOD activity g fresh $weight^{-1}$ was greater in the winter than in the summer in both the 1989–90 and 1990–91 year needles (Fig. 4B). However, the magnitude of increase from summer to winter was 4.7-fold in the 1989–90 year needles compared with only 1.4-fold in the 1990–91 year needles (Table I). Specific activity of SOD (Fig. 4A), from summer to winter, decreased 3.3-fold in the 1990–91 year needles, but increased 3.7-fold in the 1989–90 year needles. Despite greater extractable protein from the 1990–91 year needles (Fig. 3A), the total SOD activity g fresh $weight^{-1}$ was less compared with 1989–90 year needles (Fig. 4B).

Temperature *Versus* K_m for Glutathione Reductase

Two isoforms of GR, GR_A and GR_B, were purified from eastern white pine during early summer as described by Anderson *et al.* (2) and were used to determine the apparent K_m values between 5 and 40°C. The apparent minimum $K_{m,GSSG}$ for GR_A and GR_B was 11.1 and 29.1 μM, respectively, and occurred at 10°C (Fig. 5A). The $K_{m,GSSG}$ for GR_A increased in a linear fashion and showed a maximum value of 75.2 μM at 40°C. The maximum apparent $K_{m,GSSG}$ value of 71 μM for GR_B occurred at 30°C. The $K_{m,GSSG}$, especially for GR_A, at 25°C interpolated from the temperature curves is higher than previously reported by Anderson *et al.* (2). This difference may be a consequence of sampling needles at different seasons (early summer *versus* October [2]) and/or at different tissue age (overwintered-early summer *versus* young-October [2]).

The apparent minimum $K_{m,NADPH}$ for GR_A and GR_B of 4.05 and 2.9 μM occurred at 10 and 20°C, respectively (Fig. 5B). The $K_{m,NADPH}$ values for GR_A and GR_B varied only slightly between 10 and 20°C, but increased considerably between 20 and 30°C. Both GR_A and GR_B had maximum apparent $K_{m,NADPH}$ values of 11 and 15.6 μM, respectively, at 40°C. A temperature-dependent range of $K_{m,NADPH}$ values for the two isoforms of purified GR from eastern white pine also was estimated as described by Mahan *et al.* (20). Under these conditions, the apparent minimum $K_{m,NADPH}$ for GR_A and GR_B were 4.1 and 2.6 μM and occurred at 5 and 10°C, respectively (Fig. 6). The $K_{m,NADPH}$ for GR_A increased gradually from 5°C and showed a maximum value of 21 μM at 40°C. The $K_{m,NADPH}$ values for GR_B showed little difference between 5 and 15°C, but increased gradually from 15°C, yielding a maximum value of 11.7 μM at 40°C.

Using 200% of the minimum K_m values as the criteria for the TKW, the thermal response data indicated that the upper limit of the TKW for GR_A was 18°C for GSSG and 26°C for NADPH. For GR_B, the upper limit of the TKW was 23°C for GSSG and 25°C for NADPH.

Annual Temperature Variation (1988–1991)

In the summer of 1988, the total number of degree hours above 25°C was approximately 650 (the majority occurring

Table III. *Seasonal Variation in the Specific Activity (units/mg protein) of Glutathione Reductase and Ascorbate Peroxidase in Eastern White Pine Needles*

Month	Glutathione Reductase		Ascorbate Peroxidase	
	1989–90	1990–91	1989–90	1990–91
July	0.047[a]	0.043	0.041	0.054
October	0.401	0.449	0.273	0.140
January	0.720	0.314	0.218	0.210
April	0.425	NA[b]	0.136	NA
July[c]	0.500	NA	0.340	NA

[a] Values are mean specific activity ($n = 9$). [b] Data not available. [c] Activity of needles in second year.

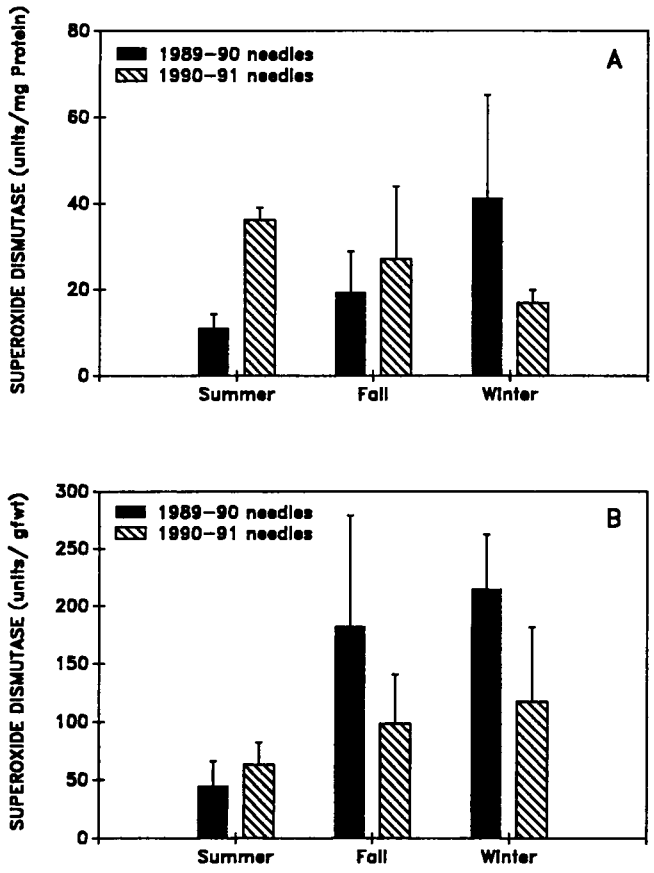

Figure 4. Seasonal changes in superoxide dismutase (A) specific activity (units mg^{-1} protein) and (B) activity (g fresh wt^{-1}) in eastern white pine needles. Error bars represent 95% confidence limits ($n = 9$).

in July and August), whereas in 1989 and 1990, the total number of degree hours above 25°C was approximately 80 and 130, respectively. The total number of degree hours below −5°C also varied from year to year. The winter of 1989–90 had approximately 2650 degree hours below −5°C (primarily in December). In comparison, the milder winters of 1988–89 and 1990–91 had 1500 and 550 degree hours, respectively, below −5°C.

DISCUSSION

The variation in glutathione and GR activity through a summer-winter period has been reported for *P. abies* (10) and was similar to the changes observed in eastern white pine in this study. A seasonal fluctuation of GR has been documented in 21 other evergreen species (10) and in poplar twigs (23). Cold-hardened *P. abies* and poplar twigs additionally had greater AP activity and higher ascorbate levels compared with nonhardened specimens (11, 21). However, the 40- to 65-fold and 40- to 122-fold increase in AP and GR activity g fresh $weight^{-1}$, respectively, from summer to winter needles of eastern white pine was considerably larger than the fivefold increase in AP in poplar twigs (21) and the fourfold increase in GR in *P. abies* needles (10).

The relationship between low temperature stress and increased levels of total glutathione, GR, and the ratio of GSSG/GSH is not understood (25). Guy *et al.* (15) observed increases in all of the above metabolic components in twigs of citrus acclimated at 10°C. Artificially increasing the concentration of glutathione in the twigs, however, did not enhance tolerance to freezing temperatures. de Kok and Oosterhuis (9) reported that increases in glutathione and GR made spinach more tolerant to low temperatures. Wise and Naylor (27) observed a decrease in GSH and an increase in GSSG in chilling-sensitive cucumber under low temperatures, whereas chilling-resistant peas showed only a slight reduction in GSH and no accumulation of GSSG. In this study, we observed decreased ratios of GSSG/GSH during the winter, which were generally 0.05:1 (1988–89 year needles) and 0.03:1 (1989–90 and 1990–91 year needles), compared with summer ratios, which typically exceeded 0.10:1. Furthermore, the levels of GSSG during the winter were lower than during the summer months, which may be a direct reflection of the seasonal changes in GR activity in eastern white pine. Similar variations, from summer to winter, in the ratio of oxidized to reduced ascorbate and in the activity of AP in needle tissue occurred also, suggesting a general increase in antioxidant metabolism in response to cold temperatures.

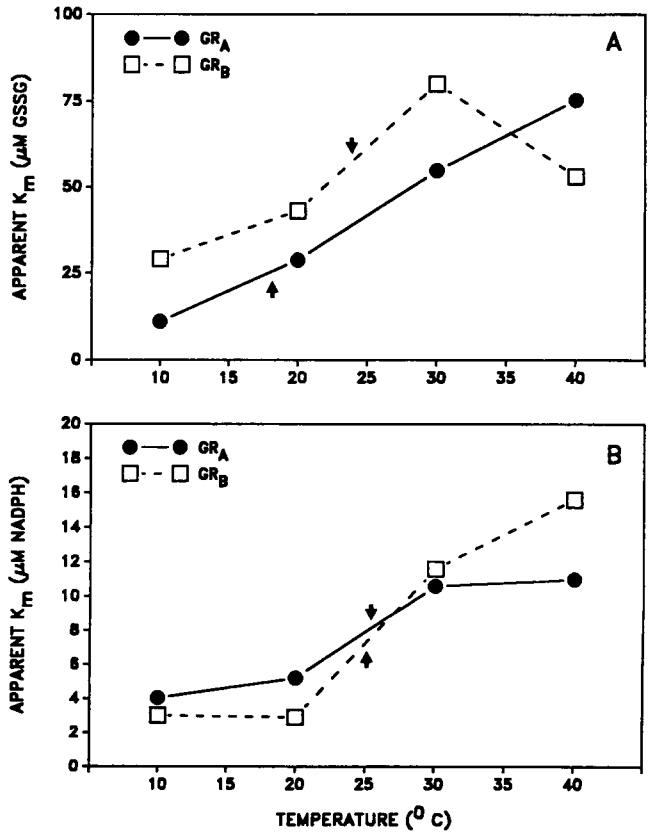

Figure 5. Thermal response of apparent K_m values for (A) GSSG and (B) NADPH of two purified isoforms of glutathione reductase (GR_A and GR_B) from eastern white pine needles sampled in early summer from overwintered tissue. Each data point represents the apparent K_m estimated from a 5 × 5 concentration matrix of GSSG and NADPH. Arrows designate the upper limits of the TKW for each isoform.

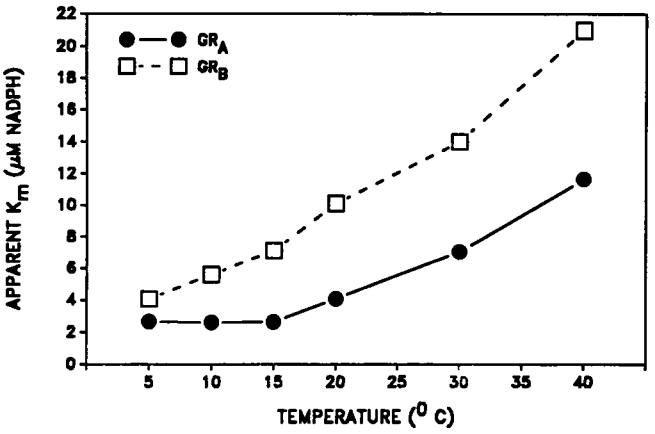

Figure 6. Thermal response of apparent K_m values for NADPH of two purified isoforms of glutathione reductase as in Figure 5. Each data point represents the apparent K_m estimated from a 1 × 5 concentration matrix of GSSG and NADPH, respectively (20).

Guy and Carter (14) suggested that increases in GR activity in hardened spinach may, in part, be due to the synthesis of new isoforms of the enzyme that have different kinetic properties. They observed a 66% increase in the activity of total GR, along with new isoforms, in cold-acclimated spinach. The GR extracted from the hardened spinach had a lower $K_{m,NADPH}$ value than nonhardened tissue (14). More recently the $K_{m,NADPH}$ for GR has been shown to vary more than sixfold between 5 and 45°C (7, 19, 20). These data indicate that increases in GR activity and decreases in the ratio of GSSG/GSH in plants during low temperature may be due to new isoforms of GR that have different affinities for GSSG and NADPH. However, Smith *et al.* (25) have emphasized the need for caution when interpreting changes in the total activity and apparent K_m value of GR in extracts from plants under thermal stress, especially if the plant expresses multiple isoforms of the enzyme that have different affinities for the substrates.

In our study, the two isoforms of GR (GR_A and GR_B) from white pine needles were isolated and purified as previously described by Anderson *et al.* (2). Using chromatography and electrophoresis, we observed no new isoforms of GR when comparing summer and winter tissue (data not shown), and both isoforms showed a proportional increase in activity from summer to winter. Hence, the GR activity in both summer and winter needles can be attributed to approximately 20% GR_A and 80% GR_B (2).

The thermal responses of the K_m for GR_A and GR_B presented in this paper are consistent with the normal growing range of eastern white pine, which is restricted to the northeastern/north central region of the United States and southward along the slopes of the Appalachian Mountains. Because the upper limit of the TKW ranged from 18 to 26°C for GR_A and from 23 to 25°C for GR_B, eastern white pine GR may show thermal inhibition when needle temperatures exceed 25°C. That GR_A and GR_B had a lower affinity for GSSG and NADPH above 15°C may be indicative of the observed higher concentrations of oxidized glutathione in summer compared with winter needles. Also, because GR levels are at their lowest during the summer months, the enzyme provides less antioxidant protection during periods of high temperature stress than low temperature stress. Reports of increased GSSG levels during exposure to low temperatures, specifically in soybean (25), citrus (15), and cucumber (27), were made using "warm weather plants." Such plants have TKWs for GR that generally range between 22 and 40°C (20) and, therefore, enzyme affinity for GSSG is lower at the temperatures conducive to cold stress.

The overall accumulation and magnitude of change in antioxidant components in eastern white pine foliage appear to correlate with the annual temperature patterns recorded during the 3-year period the trees were evaluated. The sixfold greater number of hours above 25°C in the summer of 1988 may have influenced the accumulation of total glutathione observed in the 1988–89 year needles (Fig. 1B). This hypothesis is consistent with altered metabolic function above 25°C that would occur in a cool weather plant. Because glutathione is synthesized enzymatically, rather than being produced from direct translation of mRNA (1), the pathway responsible for glutathione synthesis may be altered at higher temperatures. Our data suggest that glutathione metabolism is more sensitive to thermal stress than ascorbate, because seasonal concentrations of the latter metabolite were less variable in successive years than were glutathione levels.

The greater number of degree hours below −5°C in the winter of 1989–90 compared with 1990–91 appeared to correlate with the magnitude of change between summer and winter levels of ascorbate and glutathione; colder temperatures were associated with higher metabolite concentrations. Conversely, warm winter temperatures were associated with lower levels of antioxidant enzyme activity during December and January of 1990–91 (Fig. 2).

Photooxidation, resulting from cold temperatures and high light intensities, has been associated with damage due to $O_2^{\cdot -}$ (27). The higher levels of SOD observed in white pine needles during the winter would coincide with a mechanism for the scavenging of $O_2^{\cdot -}$ and the production of H_2O_2. In eastern white pine needles, H_2O_2 concentrations have been observed to be 10 to 20 μmol g fresh weight^{-1} during the winter months (J.L. Hess, personal communication). The higher levels of antioxidants observed during the winter in hardened needles would provide a mechanism for the removal of $O_2^{\cdot -}$ and H_2O_2. However, this metabolic pathway would require a source of reductant to remove oxidized ascorbate and glutathione. Normally, antioxidants in the chloroplast are maintained in the reduced state by drawing reducing potential from the photosynthetic apparatus via NADPH. Because approximately 70 to 80% of the AP and GR are contained in the chloroplast (13), the increase in components of the antioxidant system observed in hardened eastern white pine needles should provide a mechanism for the dissipation of captured light energy when carbon fixation is minimal. Cold-acclimated white pine needles may express increased antioxidant metabolism as a means for resistance to putative photoinhibition and for protection against oxidative damage to the cell during low temperature stress at high light intensities.

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LITERATURE CITED

1. **Alscher RG** (1989) Biosynthesis and antioxidant function of glutathione in plants. Physiol Plant **77:** 457–464
2. **Anderson JV, Hess JL, Chevone BI** (1990) Purification, characterization, and immunological properties for two isoforms of glutathione reductase from eastern white pine. Plant Physiol **94:** 1402–1409
3. **Asada K, Takahasi M** (1987) Production and scavenging of active oxygen in photosynthesis. *In* DJ Kyle, CB Osmond, CD Arntzen, eds, Topics In Photosynthesis. "Photoinhibition," Vol 9. Elsevier, Amsterdam, pp 227–287
4. **Beauchamp C, Fridovich I** (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem **44:** 276–287
5. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
6. **Brehe JE, Burch HB** (1976) Enzymatic assay for glutathione. Anal Biochem **74:** 189–197
7. **Burke JJ** (1990) High temperature stress and adaptations in crops. *In* RG Alscher, JR Cumming, eds, Stress Responses in Plants: Adaptations and Acclimation Mechanisms, Plant Biology Series, Vol 12. Wiley-Liss, New York, pp 295–310
8. **Chevone BI, Lee WS, Anderson JV, Hess JL** (1989) Gas exchange rates and needle ascorbate content of eastern white pine exposed to ambient air pollution. Proceedings Air and Waste Management Association, Paper 89.89.2, Pittsburgh, pp 1–15
9. **de Kok LJ, Oosterhuis FA** (1983) Effects of frost-hardening and salinity on glutathione and sulfhydryl levels and on glutathione reductase activity in spinach leaves. Physiol Plant **58:** 47–51
10. **Esterbauer H, Grill D** (1978) Seasonal variation of glutathione and glutathione reductase in needles of *Picea abies*. Plant Physiol **61:** 119–121
11. **Esterbauer H, Grill D, Welt R** (1980) Der jahreszeitliche rhythmus des ascorben-sauresytens in nadein von *Picea abies*. Z Pflanzenphysiol **98:** 393–402
12. **Gamble PE, Burke JJ** (1984) Effects of water stress on the chloroplast antioxidant system: alterations in glutathione reductase activity. Plant Physiol **76:** 615–621
13. **Gillham DJ, Dodge AD** (1986) Hydrogen-peroxide-scavenging systems within pea chloroplasts. A quantitative study. Planta **167:** 246–251
14. **Guy CL, Carter JV** (1984) Characterization of partially purified glutathione reductase from cold-hardened and non-hardened spinach leaf tissue. Cryobiology **21:** 454–464
15. **Guy CL, Carter JV, Yelenosky G, Guy CT** (1984) Changes in glutathione content during cold acclimation in *Cornus sericea* and *Citrus sinensis*. Cryobiology **21:** 443–454
16. **Hallgren J-E, Oquist G** (1990) Adaptations to low temperature. *In* RG Alscher, JR Cumming, eds, Stress Responses in Plants: Adaptations and Acclimation Mechanisms, Plant Biology Series, Vol 12. Wiley-Liss, New York, pp 265–294
17. **Jurik JW, Briggs GM, Gates DM** (1988) Springtime recovery of photosynthesis activity of white pine in Michigan. Can J Bot **66:** 138–141
18. **Lee EH, Jersey JA, Gifford C, Bennett JH** (1984) Differential ozone tolerance in soybean and snapbeans: analysis of ascorbic acid in O_3-susceptible and O_3-resistant cultivars by high performance liquid chromatography. Environ Exp Biol **24:** 331–341
19. **Mahan JR, Burke JJ, Orzech KA** (1987) The thermal kinetic window as an indicator of optimum plant temperature (abstract No. 521). Plant Physiol **83:** S-87
20. **Mahan JR, Burke JJ, Orzech KA** (1990) Thermal dependence of the apparent K_m of glutathione reductases from three plant species. Plant Physiol **93:** 822–824
21. **Nakagawara S, Sagisaka S** (1984) Increases in enzyme activities related to ascorbate metabolism during cold acclimation in poplar twigs. Plant Cell Physiol **25:** 899–906
22. **Neal MW, Florini JR** (1973) A rapid method for desalting small volumes of solution. Anal Biochem **55:** 328–330
23. **Sagisaka S** (1985) Injuries of cold acclimated poplar twigs resulting from enzyme inactivation and substrate depression during frozen storage at ambient temperatures for a long period. Plant Cell Physiol **26:** 1135–1145
24. **Schoner S, Foyer C, Lelandais M, Krouse GH** (1989) Increase in activities of scavengers for active oxygen in spinach related to cold acclimation in excess light: VIII. International Congress on Photosynthesis, Stockholm. Physiol Plant **76:** A99
25. **Smith IK, Polle A, Rennenberg H** (1990) Glutathione. *In* RG Alscher, JR Cumming, eds, Stress Responses in Plants: Adaptation and Acclimation Mechanisms, Plant Biology Series, Vol 12. Wiley-Liss, New York, pp 201–215
26. **Teeri JA** (1980) Adaptation of enzymes to temperature variability. *In* NC Turner, PJ Kramer, eds, Adaptation of Plants to Water and High Temperature Stress. John Wiley and Sons, New York, pp 251–260
27. **Wise RR, Naylor AW** (1987) Chilling-enhanced photooxidation. Evidence for the role of singlet oxygen and superoxide in the breakdown of pigments and endogenous antioxidants. Plant Physiol **83:** 278–282