

Seasonal Variation in Glutathione Reductase Activity in Coastal and Montane Populations of Lodgepole Pine (*Pinus contorta*)

Abstract

Increased activity of antioxidant defense enzymes, such as glutathione reductase (GR), is one mechanism employed by plants to prevent oxidative damage during low-temperature stress. Lodgepole pine (*Pinus contorta* Dougl.) was chosen to test the hypotheses that 1) enhanced GR activity would accompany cold acclimation and 2) montane populations (*P. contorta* var. *latifolia*) would have a higher level of GR activity than coastal populations (*P. contorta* var. *contorta*). Seasonal changes in specific activity of GR in needles of *P. contorta* were monitored in montane and coastal populations. Greenhouse and growth chamber experiments were performed to investigate the effect of temperature on GR activity and total glutathione content. Glutathione content and specific activity of GR were consistently higher in the montane site than in the coastal site during the winter. GR activity peaked at the onset of freezing low temperatures in the montane population but soon decreased again despite the persistence of freezing temperatures. Seedlings exposed to low temperatures in a growth chamber had higher levels of GR activity than did comparable seedlings maintained under higher temperatures, however, total glutathione was similar for both treatments. These data suggest that GR and glutathione may play a central role in cold acclimation especially during early phases and that these factors account in part for the considerable cold tolerance of lodgepole pine.

Introduction

Antioxidant defense systems are an important component of cold acclimation in plants (Guy 1990; Kuroda et al. 1991). As temperatures decrease with the onset of winter, the rate of the temperature-dependent enzymatic reactions of metabolism, including the carbon-fixing Calvin cycle of photosynthesis, slows. However, the temperature-independent light-harvesting Hill reactions continue to supply high-energy electrons to the metabolic pathways of the cell at a constant and relatively high rate. The reduced energy demands of the plant's slowed metabolism can no longer provide a sufficient acceptor pool for these electrons, which may instead reduce oxygen, leading to the formation of oxygen radicals (Scandalios 1990). Thus, colder temperatures promote an abundance of oxyradicals in the tissues of the plant, and the activity of the antioxidant defense system is necessary to degrade these potentially damaging oxygen species.

The initial oxygen free radical and the source of other oxyradical species in plants is the superoxide radical ($O_2^{\cdot-}$). In the first step of the ascorbate peroxidase-glutathione reductase antioxidant defense pathway (Dalton 1995), superoxide disproportionates to hydrogen peroxide in a reaction catalyzed by superoxide dismutase. Hydrogen peroxide is then reduced to water by ascor-

bate peroxidase in a reaction coupled to the oxidation of ascorbate to form monodehydroascorbate. Monodehydroascorbate is further oxidized to dehydroascorbate, which is then reduced back to ascorbate by dehydroascorbate reductase in a reaction coupled with the oxidation of glutathione (GSH) to its oxidized form (GSSG). In the final step of the pathway, GSSG is recycled back to GSH by NADPH-dependent catalysis with glutathione reductase (GR; EC 1.6.4.2). Thus, this pathway provides a mechanism for detoxification of oxygen radicals, including those produced during low-temperature stress. Those species best able to neutralize oxyradicals produced during photooxidative stress associated with cold acclimation are at a competitive advantage in low-temperature environments.

Photooxidative stress in cold acclimation is of particular importance for evergreen coniferous species, which retain their photosynthetic apparatus throughout the winter. Several studies have confirmed the role of antioxidant defense in cold acclimation in species of conifers recognized as cold-tolerant. Anderson et al. (1992) reported increases in activity and substrates of the enzymes of the ascorbate-glutathione pathway during the winter months in needles of eastern white pine (*Pinus strobus*), with the peaks disappearing again during the summer. A similar response has been

well-documented in spruce (*Picea rubens* and *P. abies*, Hausladen and Alscher 1994; Doulis et al. 1993; Madaminchi et al. 1990). The primary role of GR in preventing photooxidative stress is indicated by the observation that approximately 80 % of all GR in photosynthetic cells is located in the chloroplasts (Anderson et al. 1990).

Lodgepole pine (*Pinus contorta*) is an interesting study species for the investigation of the role of antioxidant defense in cold acclimation. This species is one of the most diverse both in terms of geographical distribution (northern Canada to Baja California, Pacific coast to South Dakota) and environmental tolerance (Critchfield 1980). Various characters are characterized as drought-tolerant (Sorenson 1992), flood-tolerant (Youngberg and Dyrness 1959), and cold-tolerant (Sorenson and Miles 1974), *P. contorta* thrives in a variety of harsh environments. Four varieties (or subspecies) have been recognized on the basis of morphology, and more recent molecular studies have supported this taxonomic arrangement (Wheeler and Guries 1982). Of these varieties, var. *contorta* (shore pine) occupies the coastal areas (Alaska to California) of the species range. Variety *murrayana* predominates in the Cascade Mountains of Oregon (Wheeler and Guries 1982), but var. *latifolia* extends into the northern Oregon Cascades and some authorities (Hitchcock and Cronquist 1974) place all non-coastal *P. contorta* within this latter variety. This article uses the *latifolia* nomenclature. The varieties of *P. contorta* display marked morphological differences, which are accompanied by physiological distinctions in lignification (Hagner 1970), monoterpene composition (Forrest 1980), and photosynthetic rate (Lopushinsky 1975). Since the maritime habitat of var. *contorta* experiences milder winter temperatures than the higher-elevation range of var. *latifolia* and few tree species can match this range of environmental tolerance, *P. contorta* is ideal for the study of the effect of temperature on the antioxidant defense systems of evergreen conifers.

This study examined the role of GR, as the terminal enzyme in the ascorbate-glutathione antioxidant defense pathway, in the freezing tolerance of *P. contorta*. Seasonal activity of GR was compared in geographically distinct populations of var. *contorta* (coastal provenance) and *latifolia* (montane provenance) to test the hypothesis that the latter, being exposed to colder win-

ter temperatures, would exhibit consistently higher activity of GR and higher levels of glutathione. The hypothesis that lower temperatures result in increased levels of antioxidant activity was tested both by regression analysis of the relationship between temperature and GR activity in field experiments and by temperature-controlled experiments on seedlings of var. *contorta* maintained in a growth chamber.

Materials and Methods

Study sites and field collection

Coast site. The study population of shore pine (*Pinus contorta* var. *contorta*) was located on the Oregon coast at the Sand Lake recreational area of the Hebo Ranger District in the northern portion of the Siuslaw National Forest (45° 16' 30" N 123° 57' 00" W). The site was located within 10 m of sea level approximately 800 m inland from the shore zone. Two samples were taken from each of 3 trees within a 25-m radius of each other. Each tree had a height of 6-7 m and a dbh of 70-100 cm.

Montane site. The study population of lodgepole pine (*Pinus contorta* var. *latifolia*) was located in the White River drainage on the southern side of Mount Hood (45° 22' 00" N 121° 42' 30" W), at an elevation of approximately 1070 m. The site was approximately 200 m south of the junction of U. S. Highway 26 and Oregon State Route 35. Two samples were taken from each of 3 trees within a 25-m radius of each other. Each tree had a height of 10-11 m and a dbh of 70-100 cm.

Sample collection. Each site was visited every two weeks in the fall, beginning in the second week of October 1995. No needles were collected between the second week of December 1995 and the third week of January 1996. Beginning in the third week of January 1996, collections were made at monthly intervals. Needles were collected from each of the three study trees on each visit. Collection was consistently carried out during the noon hour. Needles were removed from the distal branch ends at breast height in a circuit around the entire tree, frozen in liquid nitrogen, and stored on dry ice for transport back to the lab. Specific activity of GR was monitored throughout the season in the field study, while glutathione content was determined only for the final collection date in each site.

Meteorological data. Values for daily high and low temperatures were obtained from the USFS "Cedar" weather station on the south side of Mount Hebo for the coast site and from the Government Camp weather station for the Cascade site. Temperature values used in regression analyses were means of daily high or low temperatures for the two weeks preceding the collection date.

Glutathione reductase assay. One-gram samples of needles were weighed, cut into 1-2 mm segments with a razor blade, and frozen in liquid nitrogen. Frozen needles were crushed with mortar and pestle and then macerated in 5 ml extraction buffer (50 mM Pipes, 6 mM L-cysteine, 10 mM D-isoascorbate, 1 mM EDTA, 0.3% Triton X-100, 1% PVP-10 and 1% Polyclar-AT), pH 6.8 (Anderson et al. 1990, 1992). This mixture was poured off and the remnants were rinsed from the mortar with another 5 mL of extraction buffer. Extracts were centrifuged at 20,000 g for 15 minutes at 4° C. The resultant supernatant was assayed spectrophotometrically for GR activity by monitoring the decrease in A_{340} due to GSSG-dependent oxidation of NADPH. Assays were performed at 25° C in a 1 mL-reaction mixture consisting of 0.25 μ mol GSSG and 0.125 μ mol NADPH in a buffer of 50 mM Tris-HCl (pH 7.8) plus 0.2 mM EDTA (Dalton et al. 1986), to which was added 50 μ L of crude extract. Controls consisted of a non-enzymatic treatment (no enzyme added) and an assay for NADPH oxidase activity (no GSSG added). Activity units were defined as nmol NADPH oxidized per min. Activity was expressed as units \cdot mg protein⁻¹ or units \cdot g FW⁻¹.

Protein determination. Protein content of crude extracts was determined by the method of Bradford (1976) using BSA as the standard.

Glutathione assay. Needle samples weighing 0.175 g were cut into 1-2 mm segments and placed in a 2.0 mL flat-bottomed Eppendorf tube with 1 mL of 5 % sulfosalicylic acid. The samples were thoroughly macerated with a Tissumizer (Tekmar TR-5T). Tubes were then microfuged at full speed for 5 min. The supernatant was removed and analyzed for total glutathione equivalents (GSH and GSSG) with a spectrophotometric assay based on the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) in the presence of excess glutathione reductase (Griffith 1980). Assays were performed at 25° C in a reaction mixture consisting of 300 μ L 143 mM potassium phosphate buffer with 6.3 mM Na-EDTA (pH 7.5), 350 μ L NADPH (0.496 mg \cdot mL⁻¹), 100 μ L distilled H₂O, 100 μ L 6 mM 5,5'-

dithio-bis-2-nitrobenzoic acid (DTNB), 50 μ L 5% sulfosalicylic acid, and 50 μ L extract. The above reagents were added to the cuvette in a 32° C water bath, and the reaction was initiated with the addition of 50 μ L yeast GR (Sigma) at a concentration of 10 units/mL. GSSG content was determined after derivitization of 250 μ L of the above extract with 5 μ L of 2-vinyl pyridine and 20 μ L of 50% (v/v) triethanolamine. For field extraction, a 2.0 μ L flat-bottomed Eppendorf tube was filled to the 0.5 mL mark with segmented needles, and 1.0 mL 5% sulfosalicylic acid was added. The tubes were then placed on dry ice for transport back to the lab, where their contents were thawed and extracted as above.

Greenhouse and growth chamber experiments. One hundred 2-year old seedlings of shore pine (*P. contorta* var. *contorta*) were obtained from D. Wells Farms (Aurora, OR). Seedlings were grown from seed stock originating from the central coast of Oregon. The seedlings were removed from the field and transported to Reed College between 14 Feb. and 21 Feb. Seedlings were individually potted in vermiculite in 6-inch pots and watered daily. Nutrients were supplied via irrigation with modified Hoagland's solution once per week (Jiang et al. 1989). Photoperiod was 16 h, with a thermoperiod of 32° C days/19° C night. Halide lights provided a photon flux density at seedling height of 300-400 μ E \cdot m⁻² \cdot s⁻¹. After two weeks in the greenhouse, 20 seedlings were chosen at random and transported from the greenhouse to a Percival Model MB60-B Plant Growth Chamber to induce cold acclimation response. Photoperiod in the growth chamber was 11 h, with a thermoperiod of 13° C day/ minus 4° C night. The photon flux density was 35 μ E \cdot m⁻² \cdot s⁻¹. Needles were collected from seedlings in the greenhouse (GH) and the growth chamber (GC) at weekly intervals beginning one week after potting. Specific activity of GR and glutathione content were determined at each collection. The new flush of needles began appearing on the GH seedlings approximately three weeks after arrival, and by the sixth week were abundant enough for collection and analysis as a third treatment.

Results

Seasonal Trends in GR Activity

GR activity in extracts from needles from the montane site (var. *latifolia*) showed a 2.3 -fold

increase between the collection dates of 27 Oct. and 10 Nov. (Figure 1). Residual plots showed no evidence of non-normalcy or non-constant variance and thus met the assumptions for use of ANOVA. A one-way ANOVA for the entire sampling period showed a significant effect ($F=9.187$, $p < 0.0001$) of collection date. GR remained elevated through the 27 Nov. sampling, but then returned to previous levels for samples collected from 8 Dec. to 27 March. The overall mean of GR activity for all dates was 210 units · mg protein⁻¹ or 214 units · g needle FW⁻¹. This seasonal pattern was essentially identical regardless of whether activity was expressed on a protein basis or a FW basis.

In contrast, GR activity in extracts from needles from the coastal site (*var. contorta*) remained relatively unchanged throughout the sampling period (Figure 1, 3 Nov. - 1 Apr.). A one-way ANOVA for the entire sampling period showed no significant effect ($F = 1.428$, $p = 0.2327$) of collection date. The overall mean of GR activity for all dates was 112 units · mg protein⁻¹ or 116 units · g FW⁻¹. The activity of coastal extracts was consistently below activity of montane extracts, however these differences were significant only for samples collected in November, where GR activity in montane samples was 3.0-fold higher than activity in coastal samples. One-way ANOVA analysis of GR activity (all dates)

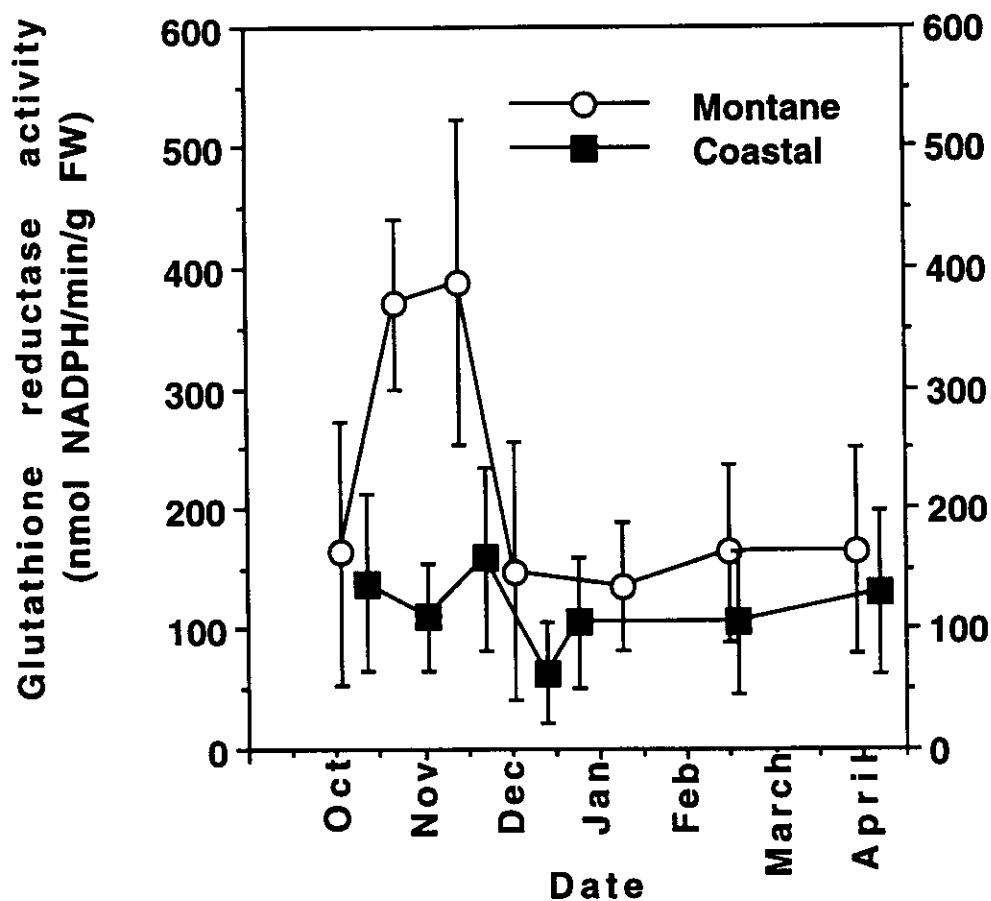


Figure 1. Glutathione reductase activity in extracts from needles of *P. contorta* var. *latifolia* (montane) and *P. contorta* var. *contorta* (coastal) over the course of the collection period (27 October 1995 - 1 April 1996). Each value is the mean of 6 trials (2 replicates from each of 3 trees); error bars show 95% confidence intervals.

indicated a high level of significance between GR activity at the 2 sites ($p < 0.0001$).

Correlation of GR activity with temperature in field sites. At the montane site, the increase in GR activity from the first collection date to the second was accompanied by the first drop below freezing low temperatures. Regression analysis for all sampling dates at the montane site revealed a significant relationship between GR activity and mean low temperatures in the 2-week period preceding sampling ($p = 0.0175$, $r^2 = 0.116$). No significant relationship was found between temperature and GR activity at the coastal site.

Glutathione content. Glutathione content was assayed only on the final collection date at each site. Levels of oxidized glutathione were too low to be detected, but it was possible to estimate the total glutathione content (both oxidized and reduced forms) in the needles. Total glutathione content was significantly greater (unpaired t-test: $p = 0.0031$) in needles of trees at the montane site ($24.2 \mu\text{mol} \cdot \text{g FW}^{-1}$) than in those at the coast ($15.0 \mu\text{mol} \cdot \text{g FW}^{-1}$).

Greenhouse and grown chamber experiment. Specific activity of GR in extracts from needles of GH seedlings displayed a steady decline from the time the seedlings were placed in the greenhouse until the end of the experiment, evident as a significant effect of collection date on GR activity in the GH treatment (one-way ANOVA: $F = 10.177$, $p = 0.0005$). There was no similar significant effect of date on GR activity in GC seedlings (one-way ANOVA: $F = 0.476$, $p = 0.7075$). GH and GC needles from any one date (8 Mar. through 29 Mar.) did not have significant differences. However, if values for all above dates were combined GR activity was significantly higher (unpaired t-test: $df = 22$, $t = 2.907$, $p = 0.0082$) in extracts from needles of seedlings from the GC treatment than in those in the GH (Figure 2).

New needles became abundant enough for collection from GH seedlings by the 29 Mar. collection date. GR activity in extracts from new needles of the GH seedlings was significantly higher (unpaired t-test, $p = 0.0114$) than in those from their older counterparts (Figure 2).

Glutathione content was assayed in old needles of both GC and GH seedlings beginning on the 8 Mar. collection date, when seedlings were first placed in the GC. There was no significant dif-

ference (unpaired t-test, $p = 0.8473$) between extracts from the GH and GC in total glutathione content over the entire course of this experiment (Figure 3). On the final collection date, glutathione content was also assayed in the new needles of the GH treatment, which displayed significantly higher (Mann-Whitney U-test: $U = 9$, $Z = 1.964$, $p = 0.0495$) total glutathione content than the older needles of the same treatment (Figure 3).

Discussion

The finding that GR activity over the winter was significantly higher in the montane population (var. *latifolia*) than in the coast population (var. *contorta*) is in keeping with the initial hypothesis that those individuals in a location subjected to longer intervals of freezing would have higher levels of GR activity to counteract the harmful effects of photooxidative stress associated with chilling. It is unlikely that cold temperatures are the only factor influencing levels of GR activity in the needles of *P. contorta*. Though these experiments provided no platform for assaying genetic differences between varieties, it is certainly possible that different isoforms of GR with different temperature optima might exist between or within varieties. GR isoforms specific to cold-hardened and nonhardened needles have been identified in red spruce (Doullis et al. 1993; Hausladen and Alscher 1994).

The lack of variation in GR activity in needles of var. *contorta* at the coast site over the course of the winter suggests either that the environmental cues which would trigger an increase in activity levels were absent at the site, or that such a response is not present in that variety. As no prolonged periods of nightly freezing occurred at the coast site, it is more reasonable to adopt the former as the more conservative explanation. Anderson et al. (1992) offer a similar rationale for *Pinus strobus*, in terms of fewer degree hours below 5°C , to account for reduced activity levels of GR in a study site which had exhibited high levels in the previous year.

The pronounced peak in GR activity at the montane site confirms the expectation that GR activity increases in response to low-temperature stress. However, in contrast to the report of Anderson et al. (1992) on *P. strobus*, the peak occurred early in the winter, well in advance of the prolonged interval of freezing low temperatures. One

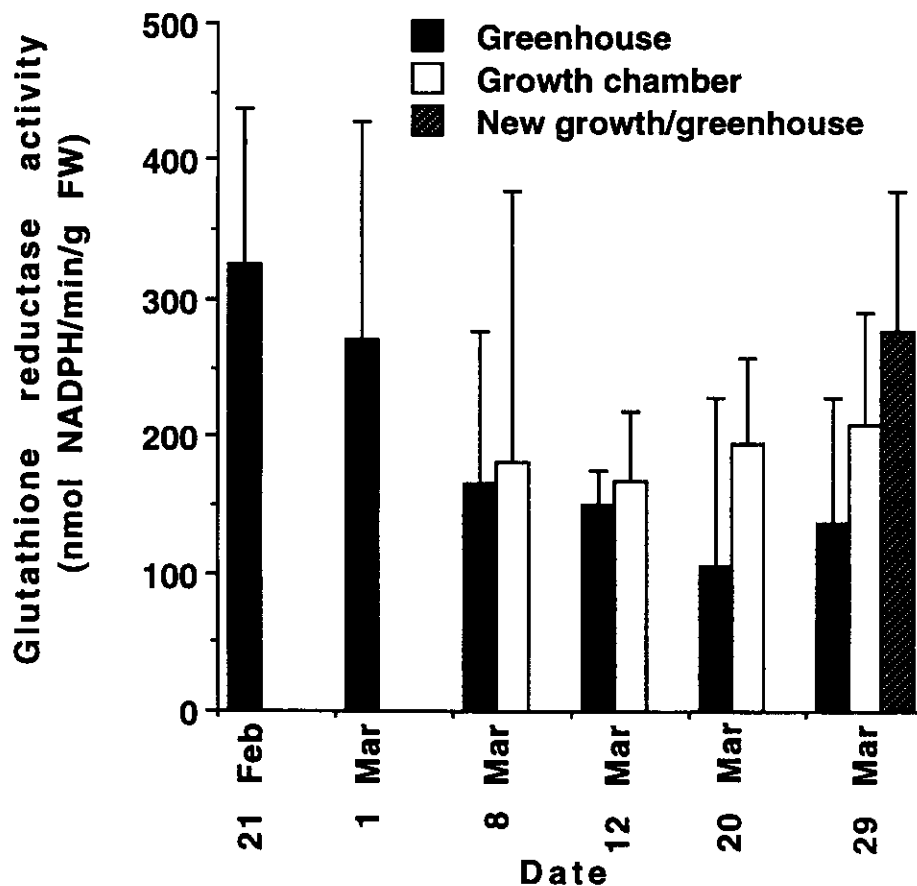


Figure 2. Glutathione reductase activity in extracts from needles of *P. contorta* var. *contorta* seedlings over the course of the temperature-controlled experiments (21 Feb. 1996 - 29 March 1996). The greenhouse thermoperiod (day/night) was 32°/19° C and the growth chamber thermoperiod was 13°/-4° C. Each value is the means of 6 trials (2 replicates from each of 3 trees); error bars show 95% confidence intervals.

possible explanation for our observations between GR activity levels, time of year, and temperature would involve an initial increase of GR activity during cold acclimation followed by a decrease with the onset of winter dormancy. Havranek and Tranquillini (1995) review a number of cellular alterations leading to reduced light-harvesting as a result of cold acclimation in conifers, including reduced cytochrome and chlorophyll content of the thylakoid membranes, clumping and migration of chloroplasts to light-protected regions of the cell, and increased opacity of the cytoplasm due to vacuolar fragmentation. The carbon fixation pathways of photosynthesis stop abruptly upon initial formation of ice in plant tissues (Larcher 1995), but the physical rearrangement of cellular components probably occurs over a longer time

frame, necessitating an intervening protective mechanism. Assuming that similar cytological rearrangements occur during cold acclimation of *P. contorta*, a scenario can be envisioned in which GR activity increases early in acclimation to protect the thylakoid membranes from photooxidative damage, but declines as the light-harvesting apparatus is sequestered.

As var. *contorta* exhibited no response to low temperature in field conditions, the opportunity to subject seedlings of that variety to freezing low temperatures was of interest. The higher levels of GR activity in needles of the GC treatment support the hypothesis that low-temperature stress triggers an increase in the activity of antioxidant defense enzymes even in var. *contorta* which would not normally be exposed to conditions of low temperature.

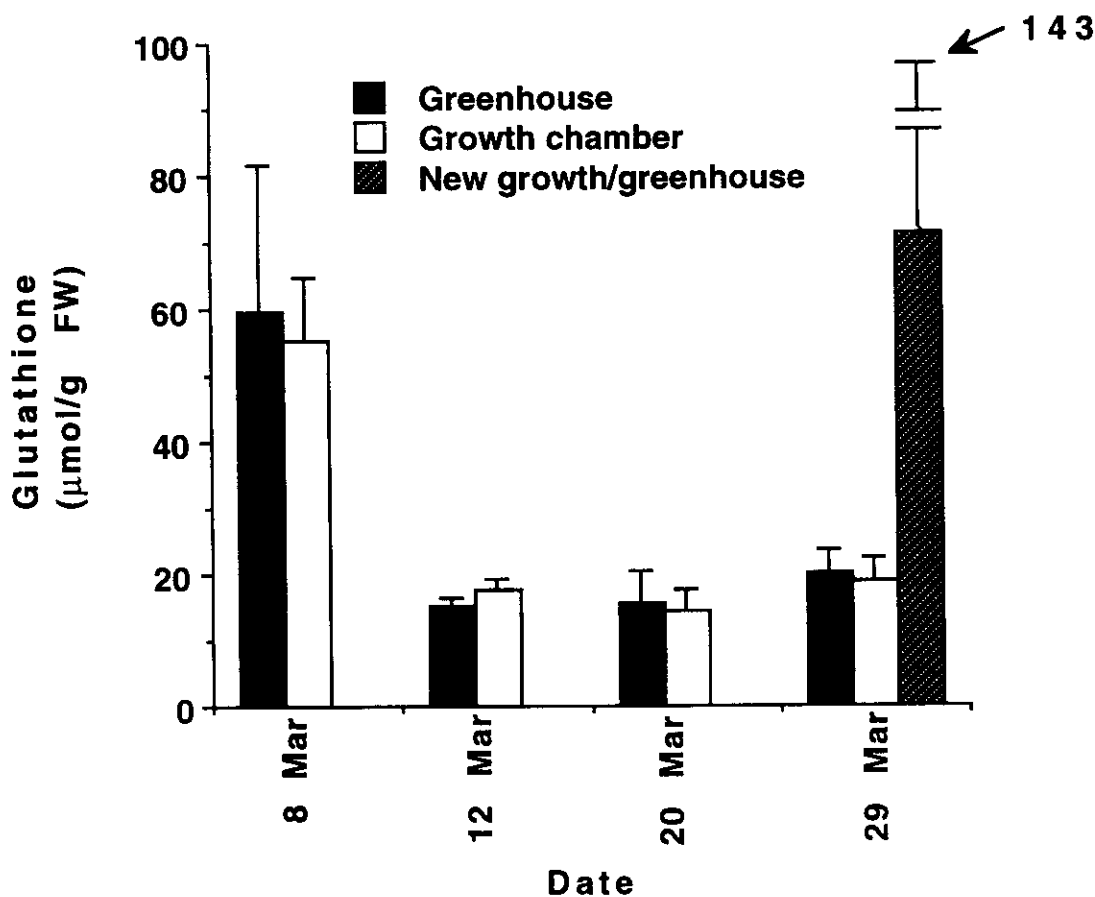


Figure 3. Total glutathione (oxidized + reduced) content in extracts from needles of *P. contorta* var. *contorta* seedlings over the course of the temperature-controlled experiments. Thermoperiod was as described in Fig. 2. Each value is the mean of 6 trials (2 replicates from each of 3 trees); error bars show 95% confidence intervals.

The greater mean total glutathione content in needles of trees at the montane site agrees with reports of higher glutathione content in red spruce in response to winter conditions (Madaminchi et al. 1990). A more informative measure would have been the ratio of reduced to oxidized glutathione, however, levels of oxidized glutathione were too low to detect despite the high sensitivity (low nmolar range) of the assay. Thus, it appears that GR was operating with high efficiency in all cases.

Pinus contorta is one of the most ecologically tolerant of conifer species. This species is found at elevations from sea level to over 3,300 m and in habitats with annual rainfall from 27 to over 150 cm. It displays a remarkable tolerance to extreme edaphic situations, including wet, droughty,

or infertile soils (reviewed by Lotan and Perry 1983) although the physiological mechanisms responsible for this environmental resiliency are not clear. Ability to withstand extreme cold can be attributed in part to a number of factors to reduce light-harvesting (reviewed by Havranek and Tranquillini 1995). These mechanisms for reduction in light harvesting are advantageous in times of stress because the plants' diminished capacity for CO₂ fixation results in a lowered photon-utilizing capacity and a tendency for reduced intermediates of the light reactions of photosynthesis to produce harmful activated forms of oxygen such as superoxide, H₂O₂, and hydroxyl radicals (reviewed by Asada 1996). Antioxidant defenses are extremely critical under such situations. Our studies suggest that such defenses are partly

responsible for the extreme cold tolerance of *Pinus contorta*. According to Sakai and Weiser (1973), *P. contorta* from Montana can withstand temperatures as low as -80°C . No other conifers in these authors' Pacific coast grouping is capable of surviving these extremes of cold. Furthermore, many Pacific Northwest conifers conduct a substantial part of their total annual photosynthesis during the winter (Waring and Franklin 1979). Enhanced antioxidant defenses may be an essential feature of these conifers that allows for this ability.

In conclusion, cold acclimation in *Pinus contorta* var. *latifolia* growing in a harsh montane climate involves elevated levels of glutathione and an initial increase in GR activity. This increase is not evident in individuals of *P. contorta* var. *contorta* growing in a more moderate coastal climate. Because numerous types of environmental

stress (e.g., chilling, drought, heat, strong light, and xenobiotics) promote oxidative damage, it may be that the antioxidant defenses are a primary mechanism that explains the environmental tolerance of *Pinus contorta* as well as many other conifer species. Further studies are required to examine these possibilities.

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Literature Cited

- Anderson, J. V., J. L. Hess, and B. I. Chevone. 1990. Purification, characterization, and immunological properties for two isoforms of glutathione reductase from eastern white pine needles. *Plant Physiol.* 94: 1402-1409.
- Anderson, J. V., B. I. Chevone, and J. L. Hess. 1992. Seasonal variation in the antioxidant system of eastern white pine needles. *Plant Physiol.* 98: 501-508.
- Asada, K. 1996. Radical production and scavenging in the chloroplasts. In N. R. Baker (ed.), *Photosynthesis and the Environment*. Kluwer Acad., Dordrecht. Pp. 123-150.
- Bradford, M. 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.
- Critchfield, W. B. 1980. Genetics of lodgepole pine. USDA Forest Service Res. Paper WO-37.
- Dalton, D. A. 1995. Antioxidant defenses of plants and fungi. In S. Ahmad (ed.) *Oxidative Stress and Antioxidant Defenses in Biology*. Chapman & Hall, New York. Pp. 298-355.
- Dalton, D. A., S. A. Russell, F. J. Hanus, G. A. Pascoe, and H. J. Evans. 1986. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *Proc. Nat. Acad. Sci.* 83: 3811-3815.
- Doullis, A. G., A. Hausladen, B. Mondy, R. G. Alscher, B. I. Chevone, J. L. Hess, and R. L. Weiser. 1993. Antioxidant response and winter hardiness in red spruce (*Picea rubens* Sarg.). *New Phytol.* 123: 365-374.
- Forrest, G. I. 1980. Geographical variation in the monoterpenes of *Pinus contorta* oleoresin. *Biochem. System. Ecol.* 8: 343-359.
- Griffith, O. W. 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106: 207-212.
- Guy, C. L. 1990. Cold acclimation and freezing stress tolerance: Role of protein metabolism. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 41: 187-223.
- Hagner, M. 1970. A genecological investigation of the annual rhythm of *Pinus contorta* Dougl. and a comparison with *Pinus sylvestris* L. *Studia Forestalia Suecica* 81: 1-26.
- Hausladen, A. and R. G. Alscher. 1994. Purification and characterization of glutathione reductase isozymes specific for the state of cold hardiness of red spruce. *Plant Physiol.* 105: 205-213.
- Havranek, W. M. and W. Tranquillini. 1995. Physiological processes during winter dormancy and their ecological significance. In W. K. Smith and T. M. Hinckley (eds.) *Ecophysiology of Coniferous Forests*, Academic Press, San Diego. Pp. 95-124.
- Hitchcock, C. L. and A. Cronquist. 1974. *Flora of the Pacific Northwest*. Univ. of Washington Press, Seattle.
- Jiang, I. B.-J., A. Jonsson, and G. Eriksson. 1989. Within- and between-population variation in growth of *Pinus contorta latifolia*: a combined study of growth-chamber and field-trial experiments. *Silvae Genetica* 38: 201-211.
- Kuroda, H., S. Sagisaka, M. Asada, and K. Chiba. 1991. Peroxide scavenging systems during cold acclimation of apple callus in culture. *Plant Cell Physiol.* 32: 635-641.
- Larcher, W. 1995. *Physiological Plant Ecology*. Springer, Berlin.
- Lopoushinsky, W. 1975. Water relations of lodgepole pine. In D. Baumgartner (ed.), *Management of Lodgepole Pine Ecosystems*. Symposium Proceedings. Washington State University Cooperative Extension Service, Pullman, WA. Pp. 135-153.

- Lotan, J. E. and D. A. Perry. 1983. Ecology and regeneration of lodgepole pine. U. S. Dept. of Agriculture, Forest Service handbook no. 606.
- Madaminchi, N. R., A. Hausladen, R. G. Alscher, R. G. Amundson, and S. Fellows. 1990. Seasonal changes in antioxidants in red spruce (*Picea rubens* Sarg.) from three field sites in the northeastern United States. *New Phytol.* 118: 331-338.
- Sakai, A. and C. J. Weiser. 1973. Freezing resistance of trees in North America with reference to tree regions. *Ecol.* 54:118-126.
- Scandalios, J. G. 1990. Response of plant antioxidant defense genes to environmental stress. *Adv. Gen.* 28: 1-41.
- Sorenson, F. C. 1992. Genetic variation and seed transfer guidelines for lodgepole pine in central Oregon. USDA Forest Service Res. Pap. PNW-RP-453.
- Sorenson, F. C. and R. S. Miles. 1974. Differential frost tolerance of ponderosa and lodgepole pine megasporangiate strobili. *For. Sci.* 20: 377-378.
- Waring, R. H. and J. F. Franklin. 1979. Evergreen coniferous forests of the Pacific Northwest. *Sci.* 204:1380-1386.
- Wheeler, N. C. and R. P. Guries. 1982. Population structure, genetic diversity, and morphological variation in *Pinus contorta*. *Can. J. For. Res.* 12: 595-606.
- Youngberg, C. T. and C. T. Dyrness. 1959. The influence of soils and topography on the occurrence of lodgepole pine in central Oregon. *Northwest Sci.* 33: 111-120.

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