COMPARATIVE STUDY OF AN ANTIOXIDANT DEFENSE MECHANISM IN GENOTYPES OF EASTERN WHITE PINE WHICH SHOW DIFFERENTIAL FOLIAR CHARACTERISTICS

by

James Valentine Anderson

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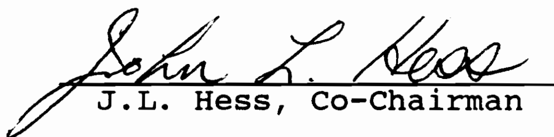
APPROVED:

B.I. Chevone, Co-Chairman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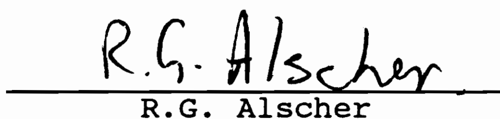
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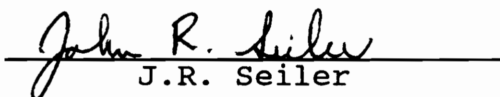
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(ABSTRACT)

Approximately 10-15% of field-grown eastern white pine (<u>Pinus strobus</u> L.) within a nursery plantation expressed foliar characteristics similar to that induced by oxidant pollution. Sensitive genotypes (based on foliar characteristics), had a 50% reduction in needle growth, severe needle tip burn, mottling, and early needle shed during a high O_3, drought-type growing season (1988) compared to a low O_3, non-drought growing season (1989). Tolerant genotypes showed little difference in needle growth or visible injury during the two growing season.

Seasonal needle ascorbate concentrations were similar during the two years however, needle glutathione (GSH) content has not. Total GSH content was two-to three-fold greater in both genotypes during the summer of 1989 compared to 1988. Cloned, tolerant trees also had 23% more total GSH when exposed to forced ambient air compared to charcoal-filtered air in open-top chambers. Cloned sensitive trees had similar GSH concentrations when exposed to

different chamber treatments.

One-year-old needles always had lower ratios of ascorbate/dehydroascorbate, ascorbate/α-tocopherol and GSH/GSSG than current year needles. One-year-old needles from the tolerant tree also maintained a higher glutathione reductase (GR) activity than the sensitive tree during the late summer.

Needles of eastern white pine had two isoforms of GR (GR_A and GR_B). GR_A and GR_B accounted for 17% and 83% of the GR recovered, respectively. GR_A and GR_B had different physical and kinetic properties. Antibody produced from GR_B was reactive with both native and denatured GR_B, but was cross-reactive with only native GR_A.

Tolerant and sensitive clones exposed to control (< 0.025 ppm) or high (4.5 ppm•hr total dose) O_3 for 0 to 72 hr, showed no increase in GR activity. Only in the high-O_3-treated trees did the amount of GR protein increase. Needles from the sensitive clone contained 14, 62, and 464 ng GR mgP^{-1} and needles from the tolerant clone contained 21, 138, and 2800 ng GR mgP^{-1} after 0, 24 and 72 hr O_3 exposure, respectively.

The results of this dissertation indicate that differential foliar characteristics in eastern white pine may be correlated with GSH turnover and its regulation by GR during periods of high oxidant stress.

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Finally, the author dedicates this dissertation in memory of his late mother, Dorthy May Anderson.

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CHAPTER 1

LITERATURE REVIEW

OXYGEN AS A HILL REDUCTANT IN CHLOROPLASTS

Oxygen (O_2) is a very perplexing molecule, which through time, has evolved as a "Jekell and Hyde molecule". Oxygen constitutes 21% of our atmosphere and is essential for all aerobic life. In the presence of strong reductants it can act as a strong oxidant (E'_o for a half cell reaction of $O_2 \text{---}> H_2O_2$ = +0.30 V) and has the unique ability to undergo univalent and bivalent reduction to produce superoxide radical ($O_2^{\bullet -}$)and hydrogen peroxide (H_2O_2), respectively (Elstner, 1982; Robinson, 1988). Photosynthetic organisms not only absorb O_2 by natural processes, they also posses reaction centers to oxidize water to yield electrons, protons and O_2 (O_2 evolution). Due to this unique ability to evolve O_2, plant chloroplasts under illumination are the most aerobic organelles in nature (Robinson, 1988). Under optimal conditions O_2 concentration in plant chloroplasts have been experimentally estimated to be 250 - 300 μM (Steiger et al., 1977). Elevated O_2 conditions (equal to or greater than atmospheric levels) can inhibit chloroplast development, decrease seed viability and root growth, damage membranes, stimulate leaf abscission, and increase growth abnormalities (for review

see Halliwell, 1984). Thus, aerobic organisms must have evolved parallel mechanisms to overcome the toxic effects of elevated O_2.

Chloroplasts ultimately use electrons derived from the splitting of H_2O (light reaction of photosynthesis) to reduce CO_2 (dark reaction of photosynthesis). In this process, $NADP^+$ serves as a Hill reductant to accept electrons from photosystem I (PSI) producing NADPH. ATP, derived from photophosphorylation, and NADPH are the energy and reductant sources, respectively, for CO_2 fixation. However, in 1951 Mehler was the first to report that photosynthetic electrons could be used to reduce O_2 to H_2O_2 (Mehler reaction). Since that time it has been established that O_2 can serve as a Hill reductant in the chloroplast (Marsho et al., 1979; Robinson and Gibbs, 1982). Radmer and Ollinger (1979) used wild type Scenedesmus (containing PSI) and mutants (lacking PSI) and clearly showed that, in contrast to the wild type, mutants were not capable of light-driven O_2 uptake. These data confirmed that O_2 uptake occurred on the reducing side of PSI and was similar to the non-cyclic reduction of $NADP^+$ in the chloroplast.

Competition for $NADP^+$ and O_2 as Hill reductants in the chloroplast is a reflection of the plastid CO_2 level (Robinson, 1988). Non-cyclic electron transport is not affected by different CO_2 concentrations in the chloroplast since under both high and low CO_2, O_2 evolution was similar

(Marsho et al., 1979; Robinson and Gibbs, 1982). However, O_2 was shown to be the principal Hill reductant when CO_2 fixation was not optimal. At high CO_2 concentrations, $NADP^+$ was the major Hill reductant, as demonstrated by low O_2 uptake and unchanged O_2 evolution. But, at low CO_2 levels, O_2 was the major Hill reductant as determined by the high O_2 uptake rates. Explanations for this competition for different Hill reductants have been that O_2 reduction prevents the over-reduction of components of the photosynthetic electron transport chain (Heber et al., 1978) while allowing the proper stoichiometric production of NADPH and ATP for efficient photosynthetic CO_2 fixation (Heber 1976). Similar conclusions were suggested by Furbank et al. (1983) who reported that C_4 mesophyll cells, which lack ribulose bisphosphate carboxylase/oxygenase (Rubisco), also showed O_2 photoreduction linked to ATP production that was strongly regulated by $NADP^+$ levels. The previous data was significant in showing that photorespiratory O_2 uptake, by the oxygenase of Rubisco, could not account for the O_2 photoreduction seen in C_4 mesophyll cells. However, there still remains questions as to the true function of O_2 photoreduction in chloroplasts (for reviews see Badger, 1985; Robinson, 1988).

Univalent photoreduction of O_2 produces superoxide radicals ($O_2^{\bullet -}$), (Badger, 1985; Elstner, 1982; Halliwell, 1984; Salin, 1988). Superoxide radicals can spontaneously

dismutate to H_2O_2 at a rate constant of 10^5 $M^{-1}s^{-1}$ (Elstner, 1982) or the reaction can be catalyzed by superoxide dismutase (SOD), (McCord and Fridovich, 1969) at a rate constant of 2×10^9 $M^{-1}s^{-1}$ (Elstner, 1982). At a concentration of 10^{-5} M, H_2O_2 can inhibit CO_2 fixation by 50% (Kaiser, 1976) due to its toxic effect on the light activated enzymes of the Calvin cycle (Kaiser 1979). Assuming that the stromal volume of a chloroplast is 30 $\mu l \cdot mg$ chl^{-1} (Heldt et al., 1973) and based on Robinson and Gibbs (1982) suggestion that H_2O_2 formation in CO_2-fixing chloroplasts does not exceed 1 $\mu mol \cdot mg$ chl^{-1} h^{-1}, a concentration of 10^{-5} M H_2O_2 could be achieved in 1 s (Robinson, 1988). In the presence of transition metals, H_2O_2 and O_2 can also react non-enzymatically to form $OH^{\bullet}$ (hydroxyl radical) which is extremely reactive (lifetime of 1 μs), thereby making its site of reaction close to the site of formation (Elstner, 1982). The production of $OH^{\bullet}$ adjacent to membranes can bring about lipid peroxidation (Pryor, 1976). The radical by-products of lipid peroxidation, as well as $OH^{\bullet}$, can bring about damage to proteins, DNA and pigments. To overcome the toxic effects of O_2 and H_2O_2 in the chloroplast, plants must have evolved a system to scavenge $O_2^{\bullet -}$, $OH^{\bullet}$ and H_2O_2.

H_2O_2 SCAVENGING BY THE ASCORBATE-GLUTATHIONE CYCLE

The chloroplast ascorbate-glutathione cycle, linked to

NADPH produced from PSI ferredoxin, was suggested by Foyer and Halliwell (1976) and Nakano and Asada (1980) as a mechanism for the scavenging of H_2O_2. Law et al. (1983) reported that isolated spinach chloroplasts have an ascorbate/dehydroascorbate ratio of 12.9:2.8 mM and a GSH/GSSG ratio of 4.2:0.3 mM under both light and dark conditions. Addition of 100 μM H_2O_2 to these chloroplasts lowered the GSH/GSSG ratio to 1.4:2.82 mM after 2 hrs in the dark. These results could be reversed by illumination for 2 min to yield a GSH/GSSG ratio of 3.83:0.32 mM. Similar shifts in the ratio of GSH/GSSG were not observed if H_2O_2 was added to the chloroplasts during illumination.

Ruptured chloroplasts have a low photoscavenging activity for H_2O_2 compared to intact chloroplasts (Nakano and Asada, 1981). However, addition of 4mM dehydroascorbate and 1.5 mM GSH to ruptured chloroplasts under illumination resulted in enhanced photoscavenging of added H_2O_2 along with the evolution of O_2. After O_2 evolution ceased, addition of more H_2O_2 resulted in additional O_2-evolution and the disappearance of H_2O_2. These data suggest that evolution of O_2, after the disappearance of H_2O_2, was due to the photoreduction of dehydroascorbate produced during the reduction of H_2O_2. Further evidence for this reaction results from the decomposition of H_2O_2 in the dark, without O_2 evolution, after chloroplasts have been in the presence of dehydroascorbate and GSH (Nakano and Asada, 1981). This

observation indicates that, in the dark, H_2O_2 may be reduced by ascorbate formed from dehydroascorbate during illumination.

Ruptured chloroplasts under illumination, in the presence of 0.2 mM NADPH and 1.5 mM GSSG, also showed a GSSG-dependent evolution of O_2 (Nakano and Asada, 1981). This O_2 evolution only occurred with ruptured chloroplasts, indicating that the chloroplast envelope is impermeable to GSSG. From these results, the authors concluded that glutathione reductase was localized in the chloroplast stroma.

Addition of paraquat to illuminated chloroplasts was reported to cause rapid oxidation of ascorbate and GSH (Law et al., 1983). However, paraquat caused a much slower oxidation in the dark. Since paraquat involves the formation of $O_2^{\cdot -}$ and H_2O_2, inhibits NADPH formation by reducing electron flow to PSI, and oxidizes ascorbate and GSH, these results further suggest that H_2O_2 reduction is linked to NADPH as an electron donor.

Generally, 30-40% of the cellular ascorbate (Foyer et al.,1983; Gillham and Dodge, 1986) and 10%-50% of the glutathione (Gillham and Dodge, 1986; Smith et al., 1985) are contained in the chloroplast. Ruptured chloroplasts can photoreduce dehydroascorbate to ascorbate with the evolution of O_2 in the presence of GSH (Nakano and Asada, 1981) with a stoichiometry of one mole O_2 evolved for the photoreduction of two moles of dehydroascorbate. These

data, and those previously discussed, are all conclusive evidence that dehydroascorbate is reduced by GSH, with the evolution of O_2, and that GSH is regenerated by NADPH produced in the thylakoids of chloroplasts. Since glutathione is impermeable to the chloroplast membrane (Jablonski and Anderson, 1982; Nakano and Asada, 1981) and the rate of ascorbate uptake by the chloroplast is too slow to account for the rate of H_2O_2 reduction (Anderson et al., 1983), the enzymes which catalyze the turnover of the antioxidant metabolites, ascorbate and glutathione, are considered to play an important role in the ascorbate-glutathione cycle for the scavenging of H_2O_2. This catalysis is especially required in the case of ascorbate, since dehydroascorbate will rapidly decompose to diketogulonic acid (Pachla et al., 1983) and oxalic acid (Foyer et al., 1983) if not taken to the reduced state.

Gillham and Dodge (1986) reported that 85% intact pea chloroplasts contain 79% of the ascorbate peroxidase, 77% of the glutathione reductase, and 65% of the dehydroascorbate reductase contained in whole pea leaf tissue. The enzymes ascorbate peroxidase (Nakano and Asada, 1981, 1988), dehydroascorbate reductase (Hossain et al., 1984; Nakano and Asada, 1981), and ascorbate free radical reductase (Hossain et al., 1984) also have been associated with spinach chloroplasts. Thus, it is currently believed that the ascorbate-glutathione cycle scavenges H_2O_2 in the

chloroplast starting with the ascorbate peroxidation of H_2O_2, catalyzed by ascorbate peroxidase, to yield 2 H_2O. The resulting ascorbate free radical can spontaneously dismutate to form dehydroascorbate and ascorbate. Ascorbate free radical can also be reduced to ascorbate via ascorbate free radical reductase which uses NADPH as a reductant. Dehydroascorbate is reduced to ascorbate by GSH, in a dehydroascorbate reductase catalyzed reaction, with the resulting GSSG being recycled back to GSH via glutathione reductase and photogenerated NADPH (See Figure 1). For a more extensive review of the ascorbate-glutathione cycle see Alscher and Amthor (1988), Halliwell (1984), Robinson (1988), Thompson et al. (1987), Salin (1988).

Catalase is not endogenous to the chloroplast and in the peroxisomes it has a relatively low affinity for H_2O_2 (Halliwell, 1974). Thus, it is unlikely that catalase is involved in H_2O_2 detoxification in chloroplasts. However, since H_2O_2 is permeable to the chloroplast membrane (Nakano and Asada, 1981), the chloroplast may serve as a sink for the photoreduction of H_2O_2 formed in the cytosol as an alternative to catalase reduction of H_2O_2 in the peroxisome. However, the argument could also be made that since 50%-90% of the glutathione in a plant is not chloroplastic (Gillham and Dodge, 1986; Smith et al., 1985), and since 90-95% of the glutathione content of plant tissue is in the form of GSSG when plants are exposed to methyl viologen and

ANTIOXIDANT REGENERATION AND FREE RADICAL SCAVENGING

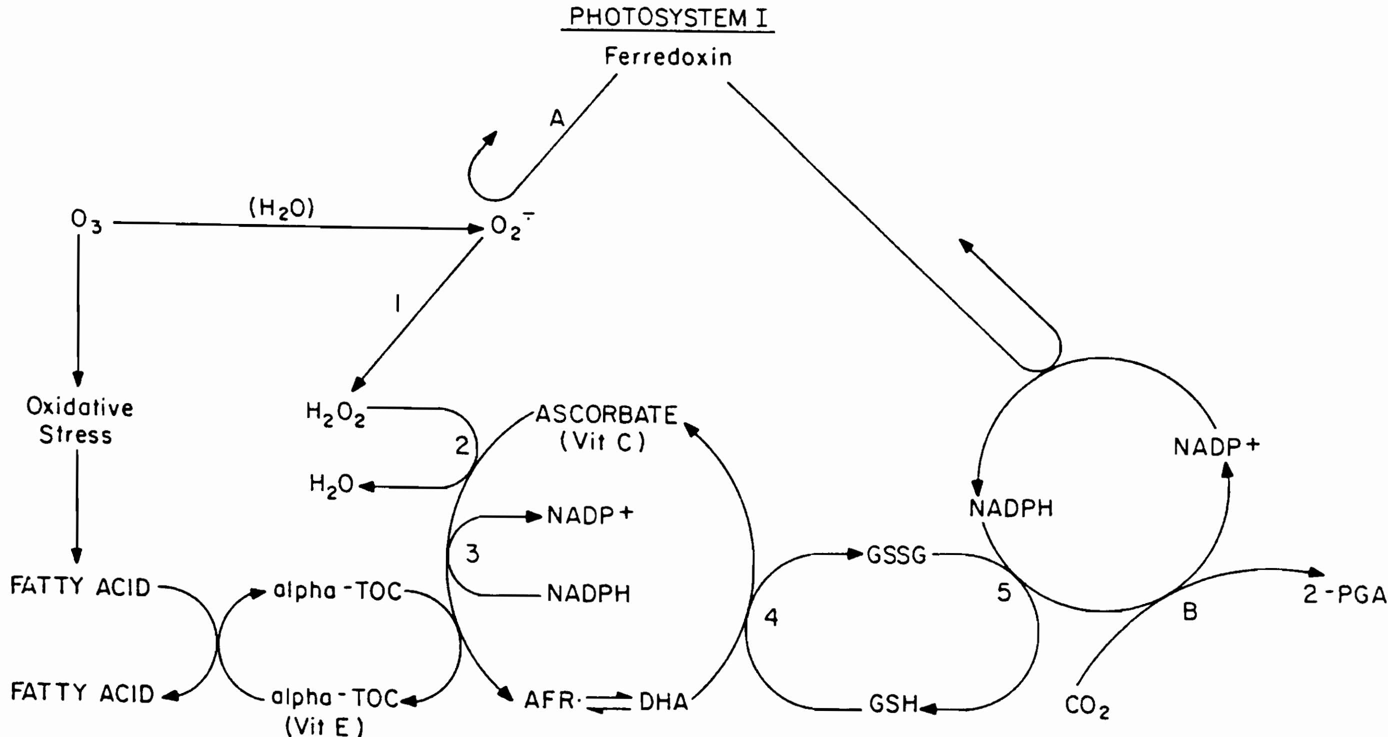

Figure 1.1 Antioxidant mechanism for the scavenging of oxy-free radicals in plant chloroplasts. A = Mehler reaction. B = CO_2 fixation. 1 = Superoxide dismutase. 2 = Ascorbate peroxidase. 3 = Ascorbate free radical reductase. 4 = Dehydroascorbate reductase. 5 = Glutathione reductase. AFR = Ascorbate free radical. DHA = Dehydroascorbate.

light (Law et al., 1983), H_2O_2 produced in the chloroplast may be detoxified in the cytosol. The ascorbate-glutathione cycle has been reported to also function in the cytosol (Castillo and Greppin, 1988; Klapheck et al., 1987). However, since only 21% of the ascorbate peroxidase and 23% of the glutathione reductase are present in the cytosol, and based on unpublished data (Anderson et al., 1990) which indicates that a putative cytosolic form of glutathione reductase has a lower V_{max} and greater affinity for GSSG and NADPH, the previous argument appears weak.

Alpha-TOCOPHEROL AS A LIPID SOLUBLE ANTIOXIDANT

Alpha-tocopherol (Vitamin E), a lipid soluble antioxidant, exists in the cellular membranes of both animals (Burton et al., 1983) and plants (Grumbach, 1983). It's primary function is to protect membranes against free radical-dependent, chain reaction lipid peroxidation by reacting with lipid peroxy radicals to form α-tocopherol semiquinone. Since nearly all of the α-tocopherol in plants is located in the thylakoid membranes of the chloroplast (Grumbach, 1983), it may be an important antioxidant against lipid peroxidation of thylakoid membranes.

Alpha-tocopherol semiquinone can be reduced by GSH either directly or via a cycling reaction with ascorbate (McCay, 1985). Schmidt and Kunert (1986) used acifluorfen to induce lipid peroxidation in beans and reported a cor-

responding increase in ascorbate and glutathione production, along with increased glutathione reductase activity. Lipid peroxidation in bilayer membranes was not affected by glutathione, but instead was controlled by the ratio of ascorbate to α-tocopherol (Liebler et al., 1986). Finckh and Kunert (1985) also suggested that the specific ratio of ascorbate to α-tocopherol plays a key role in protection against lipid peroxidation in plants which are under oxidative stress due to herbicide interaction. These results suggest that increased production of antioxidants, which can interact with α-tocopherol in the ascorbate-glutathione cycle, may be an important mechanism for reducing lipid peroxidation in plants which are under oxidative stress. Ascorbate and α-tocopherol interactions have been associated with protection against lipid peroxidation during chilling-enhanced photooxidation (Wise and Naylor, 1987), herbicide interaction (Finckh and Kunert, 1985), O_3-exposure (Giamalva et al., 1985; Mehlhorn et al., 1986), and senescence (Kunert and Ederer, 1985) all of which have been associated with the evolution of activated oxygen species.

ENVIRONMENTAL CONDITIONS AFFECT GLUTATHIONE TURNOVER

Although the ratio of ascorbate to α-tocopherol may be a signal of oxidative stress, other markers in plants which are under oxidative stress are glutathione and the enzyme

which keeps the ratio of GSH/GSSG high, glutathione reductase. Increased levels of glutathione have been reported in plants which are under oxidative stress due to air pollutants (Chiment et al., 1986; Grill et al., 1979; Mehlhorn et al., 1986), low temperature (de Kok and Oosterhuis, 1983; Esterbauer and Grill, 1978; Guy et al., 1984a; Wise and Naylor, 1987), drought (Burke et al., 1985), heat shock (Nieto-Sotelo and Ho, 1986), and excess radiation (Smith et al., 1990). The increase in glutathione reductase activity during exposure of plants to 75% O_2 (Foster and Hess, 1980 and 1982), air pollutants (Mehlhorn et al., 1986, Tanaka et al., 1988), drought (Burke et al., 1985; Smirnoff and Colombe, 1988), low temperature (Esterbauer and Grill, 1978; Guy et al., 1984b) and excess radiation (Gillham and Dodge, 1987) is consistent with required protection depending on glutathione turnover during photosynthesis.

In chilling-sensitive cucumber, increased oxidative damage due to low temperatures was correlated with an overall decrease in GSH but, and an increase in the pool of GSSG (Wise and Naylor, 1987). In comparison, a chilling-resistant cultivar of pea showed only a slight decrease in total glutathione and no accumulation of GSSG. Similar increases in GSSG content have been reported for chilling-sensitive soybeans when grown at 5^oC (Smith et al., 1990). However, the author also reported an overall increase in the total pool of glutathione. These data suggest that the

ratio of GSH/GSSG, which is normally maintained at 10:1 in the chloroplast (Halliwell, 1984), plays an important role in protecting plants against oxidative stress. Glutathione levels also are thought to have a negative feedback on the pathway for glutathione biosynthesis (Alscher, 1989), suggesting that shifts in the ratio of GSH/GSSG and the total pool may have a bearing on the increased levels of glutathione seen in plants which show resistance to oxidative stress.

For the above reasons it is likely that glutathione reductase may also be regulated directly, or indirectly, by environmental variables which enhance oxidative conditions within plant cells. Tanaka et al. (1988) showed that increased glutathione reductase activity in spinach exposed to O_3 was due to induction of the protein. Guy and Carter (1984b) reported that the differences seen between cold-hardened and non cold-hardened spinach for glutathione reductase K_m values was due to the synthesis of different isozymes. These two examples suggest that increases in glutathione reductase activity, due to oxidative stress, can be due to induction of one or several isozymes of glutathione reductase.

The differences seen in glutathione reductase K_m values from different species can vary as much as 6-fold between 10-45°C. Burke et al. (1987) suggested the "thermal kinetic window theory" which is the temperature range where

the apparent K_m is minimal. This is considered to be the optimal thermal range of glutathione reductase from a specific organism. Plants grown in cool environments do have glutathione reductase with lower thermal kinetic windows than plants grown in high temperature environments (Mahan et al., 1987). These observations suggest that oxidative stress, due to high and low temperature, can bring about the induction of different isozymes of glutathione reductase which could influence a particular organism's thermal kinetic window for the turnover of glutathione.

O_3 AND SO_2 GENERATE OXY-RADICALS AND PEROXIDES IN PLANTS

Ozone decomposes in aqueous solutions through chain reactions to yield $OH^{\bullet}$ (Grimes et al., 1983), $O_2^{\bullet -}$ and peroxy radicals (Hoigne and Bader, 1975; Peleg, 1976; Pryor, 1986). Whether decomposition occurs directly or through reactions with cellular constituents in plants is uncertain. However, Pryor et al. (1983) have suggested that radical formation, resulting from O_3, occurs via an alkyl hydrotrioxide intermediate (ROOOH). Application of the experimental chemical N-[2-(2-oxo-1-imidazolidinyl)-ethyl]-N'-phenylurea (EDU) to snap beans increased their tolerance against O_3 exposures (Lee and Bennet, 1982). The increased O_3 tolerance seen in snapbean was always paralleled by an increased SOD activity. Immature tissue of snap bean always had higher levels of SOD activity than did

mature tissue which corresponded to the younger tissues showing less visible damage during O_3 exposures. These data implicate SOD in the detoxification of O_3 (implicating $O_2^{\bullet -}$ as a substantial intermediate of O_3 interaction within the plant cell). However, SOD does not appear to be responsible for differential tolerance among plants, since both O_3-sensitive and O_3-tolerant cultivars of bean had increased SOD activity due to either 0.2 ppm O_3 for 24 hrs (Mckersie et al., 1982) or due to cumulative chronic or acute O_3 exposure (Chanaway and Runeckles, 1983).

In the case of sulfur dioxide (SO_2), Asada et al. (1974) observed that $O_2^{\bullet -}$ was formed on thylakoid membranes under illumination and could initiate the aerobic oxidation of sulfite to yield $O_2^{\bullet -}$, singlet oxygen (O_2), H_2O_2, and $OH^{\bullet}$. Superoxide dismutase also inhibited the oxidation of sulfite by illuminated chloroplasts which had been damaged by SO_2 (Asada, 1980). The photooxidative damage to the chloroplast system seen during O_3 exposure appears to have similarities with that of leaf damage due to (SO_2) exposure.

EFFECTS OF AIR POLLUTANTS ON PLANT PHYSIOLOGY

Ozone (O_3) is generally considered to cause the most damage to vegetation of any air pollutant (Heck et al. 1977). Ozone flux into leaf tissue, via the stomata, is directly proportional to the change in O_3 concentration

between the ambient air and the leaf interior, if the stomatal conductance remains constant (Adams et al., 1986). Since plants generally have their stomata open during daylight hours, when photogenerated O_3 levels are maximum in the atmosphere, gas exchange would allow for a significant uptake of O_3. Plants exposed to O_3 have been correlated with varying degrees of visible and invisible injury, such as, water logging, necrosis, chlorosis, decreased photosynthetic activity, altered metabolic pools, changes in enzyme activities, altered membrane permeability, and overall reduction in plant biomass and quality (for reviews see, Adams et al., 1986; Heath, 1980; Heck et al., 1977; Hostede et al., 1987; Reich and Amundson, 1985; Tingey and Taylor, 1982). Differential tolerance to O_3 exposure has been reported for many cultivars of crop and tree species (Adams et al., 1986). However, the mechanism(s) for this inherent tolerance are still in question.

The effects of air pollutant stress on the net photosynthetic assimilation (P_n) of CO_2 should be directly related to effects on growth and yield and could be the primary way by which air pollutants reduce plant growth (Reich and Amundson, 1985). Stomatal closure (Engle and Gabelman, 1966; Reich, 1987; Rich and Turner, 1972) and reduced stomatal density (Butler and Tibbitts, 1979) have been implicated as playing key regulatory roles in gas exchange for those cultivars of plants which exhibit toler-

ance against O_3 exposure. However, other reports indicate that stomatal closure and density do not account for the induced alterations in gas exchange characteristics between cultivars during O_3 exposure. Instead, differential tolerance is proposed to be an expression of biochemical sensitivity (Boyer et al., 1986; Chanway and Runeckles, 1983; Olszyk and Tingey, 1984a; Reich and Amundson, 1985; Tingey and Taylor, 1982; Yang et al., 1983ab).

Reich and Amundson (1985) used 4 tree species and 3 crop species to test the effect of O_3 exposure on the P_n, leaf diffusive conductance and water use efficiency. In all seven species, long-term exposure to O_3 resulted in a linear reduction in P_n which was concentration dependent. The reductions in P_n were also related to reductions in growth. They reported that the reduction in P_n in white pine, red oak and sugar maple was directly due to O_3 since declining leaf conductance was not observed. In species which did show decreased leaf conductance, which also resulted in decreased P_n, the lower conductance was not directly attributed to O_3 effects on stomates, but instead, was a result of increase internal leaf CO_2 concentrations.

The results of Reich and Amundson (1985) agree well with Yang et al. (1983a, b), who reported a greater inhibition of P_n as opposed to light-dependent transpiration in response to O_3 exposure in eastern white pine clones which show differential sensitivity to O_3. The results of Yang

et al., (1983a, b) suggested that the difference in clonal sensitivity to O_3 occurred at the biochemical level in foliar tissue. Short-term O_3 exposure (< 0.12 $\mu l \cdot L^{-1}$) can inhibit Rubisco activity in rice plants (Nakamura and Sada, 1978). Short-and long-term O_3 exposure has also been reported to reduce the content of Rubisco in alfalfa (Pell and Pearson, 1983) and potato foliage (Dann and Pell, 1989). These results suggest a mechanism for the reduction of P_n and increased CO_2 concentrations in plants exposed to O_3 and may explain an indirect mechanism for those plants which show stomatal closure during O_3 exposure.

Results correlating exposure to O_3 with decreased Rubisco activity and quantity in plants (see previous discussion) also suggest the partial inhibition of electron flow to carbon which could cause enhanced pseudocyclic electron flow through O_2. Such a system could explain the 30% or greater increase in foliar necrosis in pea following exposure to O_3 during illumination, as opposed to O_3 exposure in the dark, when the stomata of tomato were stimulated to stay open using fusicoccin, or when mutants (stomata stay open in dark) were used (Olszyk and Tingey, 1984a, b). Also, increased fluorescence has been reported in bean up to 20 hours before necrosis was observed when exposed to O_3 and illumination (Schreiber et al., 1978). Such observations indicate that O_3 causes photooxidative damage to the chloroplast system and that the process is

light-enhanced.

Shimazki et al. (1980) observed the breakdown of chlorophyll a in spinach leaves which had been fumigated with 2 ppm SO_2 for 2 hours but, observed no breakdown in chlorophyll b over an 8 hour period. No chlorophyll destruction was observed in SO_2-fumigated and illuminated leaf disks, when incubated in the dark. Pre-fumigated leaf disks were also used to test the inhibition of chlorophyll a damage with scavengers of $O_2^{\bullet -}$, $^1O_2$, and $OH^{\bullet}$. Hydroquinone, trion, and ascorbate ($O_2^{\bullet -}$ scavengers) showed inhibition of chlorophyll a destruction, while 1,4-diazabicyclo-[2,2,2]-octane, methionine and histidine ($^1O_2$ scavengers) and benzoate and formate ($OH^{\bullet}$ scavengers) showed no inhibition of chlorophyll a destruction. These data also indicate that O_3 and SO_2 show similar photo-oxidative damage to the chloroplast which appears to be reduced in the presence of $O_2^{\bullet -}$ scavengers.

THE CHLOROPLAST ANTIOXIDANT SCAVENGING MECHANISM

The majority of SOD activity in plants has been observed in the chloroplast (Foster and Edwards, 1980; Jackson et al., 1978). Thus, superoxide radicals within the chloroplast, which result either from pseudocyclic electron flow or from air pollutant interactions, are reduced to H_2O_2 and O_2. However, since H_2O_2 is toxic to the SH-containing enzymes of the Calvin cycle (Kaiser,

1979; Nakano and Asada, 1981; Tanaka et al., 1982), it is likely that H_2O_2 is scavenged by the ascorbate-glutathione cycle discussed earlier. Increased ascorbate content has been associated with less foliar injury in O_3-tolerant cultivars of snapbean (Lee et al., 1984) and pine (Barnes, 1972). Ascorbate can also directly interact with $OH^{\bullet}$ (Halliwell and Gutteridge, 1985) and quench singlet oxygen (Bodannes and Chan, 1979). Sakaki et al. (1985) observed that the application of ascorbate to spinach leaves decreased the extent to which O_3 caused foliar pigment and lipid breakdown. Increased activity of ascorbate peroxidase (Mehlhorn et al., 1987; Tanaka et al., 1985) and glutathione reductase (Mehlhorn et al., 1987; Tanaka et al., 1988) in plants which have been exposed to O_3 would be consistent with the previously discussed data.

Alpha-TOCOPHEROL AND LIPID PEROXIDATION

Substantial evidence indicates that O_3 can affect membrane permeability in plants (Dominy and Heath, 1985; McKersie et al., 1981; Perchorowicz and Ting, 1974; Swanson et al., 1973; Sutton and Ting, 1977). These changes have generally been attributed to fatty acid peroxidation (Sakaki et al., 1985; Fong and Heath, 1981) and free radical cleavage of the ester linkages between the glycerol moiety and the fatty acids of phospholipids (MacKay et al., 1987). Alpha-tocopherol, a lipid soluble antioxidant, can

protect membranes against chain reaction lipid peroxidation by reacting with lipid peroxy radicals to form α-tocopherol semiquinone (see previous discussion). MacKay et al. (1987) demonstrated increased α-tocopherol concentration in wheat plants using triazole S-3307 and showed a correlative decrease in both visible injury and cleavage of phospholipids due to O_3 exposure. Since α-tocopherol semiquinone can be reductively regenerated by GSH either directly or via a cyclic reaction with ascorbate (McCay, 1985), and since acifluorfen induced lipid peroxidation results in increased ascorbate, glutathione, and glutathione reductase (Schmidt and Kunert, 1986), it appears that the interaction of the SOD/ascorbate/glutathione cycle and α-tocopherol may play an important role in those cultivars of plants which show differential tolerance to O_3.

In animals, ascorbate is found in high concentrations in lung surfactant (Snyder et al., 1984) and is thought to regenerate α-tocopherol in the pulmonary membranes which become oxidized during the repair of peroxidized polyunsaturated fatty acids resulting from O_3-exposure. At pH 7.0-7.5, O_3 shows a reaction rate of 6×10^7 M s^{-1} with ascorbate which is greater than that of the reaction rates for α-tocopherol or uric acid (Giamalva et al., 1985). Although glutathione shows a reaction rate constant with O_3 greater than that for ascorbate, it is not found in the lung lavage (Ames et al., 1981). Glutathione is found in

the whole blood where it reduces lipid hydroperoxides and ozonides. These results suggest that depletion of ascorbate, through ozonation, could lower the levels of other antioxidants in the membranes and whole blood, leading to a weakening of the body's defenses against O_3 and other free radicals and oxidative damage (Giamalva et al., 1985).

O_3 GENERATED FORMATION OF $O_2^{\cdot -}$ AND H_2O_2 IN THE APOPLAST

More recent evidence indicates that the ascorbate-glutathione mechanism for the scavenging of free radicals produced by O_3 may extend into the cytosol and extracellular spaces of plants. Laisk et al. (1989) used sunflower leaves, which have stomata on both sides of the leaf, and observed that fumigation with 1.5 ppm O_3 resulted in the uptake of O_3 through the stomata on one side of the leaf but, no flux of O_3 could be detected on the opposite side of the leaf. These results were obtained using a chamber which employs the leaf as a membrane separating two compartments. Uptake of CO_2 and stomatal resistance were measured prior to and during the experiment to monitor leaf physiology. Ozone was introduced into one compartment, and then measured in the opposite compartment. Thus, if O_3 is taken up through the stomata on one side of an amphistomatous leaf, then, unless the intercellular concentration of O_3 is zero, some of the O_3 molecules should diffuse out from the leaf through the stomata of the opposite side.

These same procedures have been used in the past to measure the intercellular concentration of CO_2 (Oja, 1972, 1983). Since no detectable O_3 diffused through the sunflower leaf, it was concluded that O_3 rapidly decomposes, or is absorbed, in the cell wall or plasmalemma region.

Castillo et al. (1987) observed a significant increase in SOD activity in the extracellular fluid of one-year-old Norway spruce after 2 and 30 days when grown in charcoal-filtered, open-top chambers supplemented with 300 $\mu l \cdot M^{-3}$ O_3. They did not see a significant difference in SOD levels of current year needles after 2 days in either charcoal-filtered or charcoal-filtered and O_3 supplemented chambers, but did see a significant increase in SOD in the extracellular fluid of current year needles after 30 days in the charcoal-filtered, O_3 supplemented chamber. These results suggest that O_3 produces $O_2^{\cdot -}$ in the extracellular spaces and is most likely converted to H_2O_2 by SOD.

APOPLASTIC ASCORBATE AND ASCORBATE PEROXIDASE

The previous data support the report by Castillo and Greppin (1988) who observed an increase in dehydroascorbic acid in the apoplastic spaces of Sedum album 2 hours after exposure to either 0.2, 0.4 or 0.6 $\mu l \cdot L^{-1}$ O_3. Although the amount of reduced ascorbate decreased in the apoplast, the overall concentration of ascorbate (ascorbate + dehydroascorbate) increased in the apoplast. The authors also

reported that the overall levels of ascorbate and glutathione decreased in the whole leaf during the fumigation, but rapidly recovered to control levels after the fumigation ended. Their results showed no dehydroascorbate reductase or glutathione reductase in the apoplastic spaces. However, dehydroascorbate reductase activity did increase in the whole leaf while the activity of glutathione reductase stayed approximately the same.

Castillo and Greppin (1986) also reported that an ascorbate specific peroxidase was stimulated in the extracellular space of Sedum album during a 3 hour fumigation with 0.4 $\mu l \cdot L^{-1}$ O_3. Increased ascorbate specific peroxidase activity also has been reported to increase in the extracellular spaces of pinto beans (Peters et al., 1989) and Picea abies (Castillo et al., 1987) when exposed to O_3. Peters et al. (1989) observed no change in the ascorbate peroxidase activity in the extracellular spaces when pinto bean was fumigated with SO_2. These observations may explain differences that are seen in response to O_3 and SO_2 singly or in combination (Boyer et al., 1986; Olszyk and Tingey, 1984a, b). If SO_2 does not decompose in the apoplast, then it does have a greater potential for reacting with the chloroplast thylakoids to produce $O_2^{\bullet -}$ as discussed previously. This compartmentation also could explain why tomato plants show 65-80% less foliar necrosis in the light vs the dark when fumigated with SO_2 as opposed

to plants fumigated with O_3 which show 30% more foliar necrosis in the light then in the dark (Olszyk and Tingey, 1984a, b).

REGULATION OF ASCORBATE PEROXIDASE SECRETION BY Ca^{++} DURING EXPOSURE TO O_3

Based on the work of Sticher et al. (1981) who showed that Ca^{++} was required for the secretion of peroxidases by plant cell suspensions, Castillo et al. (1984) tested the effect of Ca^{++} on the secretion of peroxidases from leaves of <u>Sedum</u> <u>album</u>. They observed that plants fumigated with 0.4 $\mu l \cdot L^{-1}$ O_3 secreted more peroxidase when the leaves were incubated in solution containing Ca^{++}. Addition of Ca^{++} to the roots prior to fumigations also increased the level of peroxidase in the extracellular fluid after fumigation. Addition of EGTA, a Ca^{++} chelator, to the roots in the presence of Ca^{++} inhibited the increase in extracellular peroxidase activity after fumigations. From these data it does appear that Ca^{++} plays a regulatory role over the activity of peroxidases found in the extracellular spaces during O_3 fumigations.

Inhibitors of protein synthesis do not inhibit the early increases seen in ascorbate specific peroxidase (Gaspar et al., 1982), nor are radioactive labels incorporated into the extracellular ascorbate specific peroxidases (Castillo et al., 1984) during short-term exposures to O_3.

These data indicate that increased extracellular ascorbate specific peroxidase was not due to de novo protein synthesis but suggested that enzyme activity is activated by Ca^{++}.

The influx of Ca^{++} into cells during O_3 exposure may be associated with oxidation of proteins in the plasma membrane which may be important for the controlled regulation of intercellular Ca^{++} concentrations. Dominy and Heath (1985) reported that fumigation of a sensitive variety of pinto bean with 0.2 to 0.5 $\mu l \cdot L^{-1}$ O_3 resulted in the inhibition of $(K^{+}+Mg^{++})$-ATPase of the plasma membrane. This inhibition was not observed in O_3-resistant pinto beans. Addition of dithiolerythritol, a sulfhydryl reagent, to the damaged membranes reversed the inhibition and suggested that the inhibition of the ATPase was due to oxidation of sulfhydryl moieties. Oxidation of sulfhydryl groups of proteins associated with the plasma membrane could explain the increased permeability of membranes during exposures to O_3. Such increased permeability could allow for the increase influx of Ca^{++} into the cell and as a result stimulate the secretion of peroxidase into the extracellular spaces.

CYTOSOLIC ASCORBATE-GLUTATHIONE CYCLE MECHANISM FOR THE SCAVENGING OF $O_2^{\bullet -}$ and H_2O_2

It seems quite clear that the ascorbate-glutathione

cycle exists in the cytoplasm. From the data presented in this chapter, it would appear that ascorbate and ascorbate peroxidase can be transported across the plasma membrane to scavenge toxic by-products of O_3 breakdown in the apoplast. Beck et al. (1983) have shown that ascorbate can be transported across the chloroplast envelope by a specific carrier system and a similar carrier system might exist in the plasma membrane. Since no dehydroascorbate peroxidase was observed in the apoplast, dehydroascorbate must be shuttled into the cytosol to be reduced to ascorbate. In the dark, NADH generated during glycolysis could act as a reductant for the regeneration of ascorbate in the cytosol (Beck et al., 1983). However, from the data presented in this chapter, it would appear that the majority of ascorbate is probably regenerated by GSH and dehydroascorbate reductase. The resulting GSSG could then be reduced to GSH by glutathione reductase in the cytosol. The reductant for the production of GSH from GSSG could come from the chloroplast via the dicarboxylic acid transport system (Edwards and Walker, 1983). Alternatively, an increase in the $NADP^+$/NADPH ratio could stimulate the pentose-phosphate shunt (Yoshida, 1973). Glucose 6-phosphate dehydrogenase, the first regulated enzyme of the pentose phosphate shunt, can be stimulated by O_3 exposure (Castillo et al., 1984; Tingey et al., 1975). Thus, increased production of NADPH by glucose 6-phosphate dehydrogenase, in the cytosol, could

provide additional reductant for keeping the GSH/GSSG ratio at a level adequate to maintain a reduced ascorbate pool in the cytoplasm.

In summary, the SOD/ascorbate/glutathione mechanism for the reduction of toxic by-products of O_3 and SO_2 breakdown, whether occurring in the cytosol or in the chloroplast, appears to show protection against oxidative conditions but, at the expense of drawing reductant (NADPH) away from the assimilatory pathway for CO_2 fixation. The extra metabolism and reductant required for radical scavenging and repair mechanisms probably contributes to the reduced accumulation of dry matter and overall vigor of plants which experience oxidative stress.

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CHAPTER 2

COMPARATIVE STUDY OF FOLIAR CHARACTERISTICS AND ANTIOXIDANTS IN NEEDLES OF FIELD-GROWN EASTERN WHITE PINE DURING DIFFERENT GROWING SEASONS

INTRODUCTION

Over 60 major urban areas annually violate the National Ambient Air Quality Standard for ozone (O_3) which is 120 ppb (Abelson et al., 1988). Prevailing weather patterns can cause urban air pollutants to drift over agricultural and heavily forested regions of the United States. In the Appalachian and Blue Ridge Mountains of the eastern United States, O_3 episodes also can occur as a result of slow moving, or stagnant high pressure systems which are conducive to the photochemical production of O_3 (Duchelle et al., 1983). During the spring and early summer, when forest trees are in a rapid growth phase, mean, hourly, ambient O_3 concentrations can range between 45 and 65 ppb (Duchelle et al., 1982) and can exceed 90 ppb for several hours daily during O_3 episodes (Duchelle et al., 1983).

In the eastern United States, O_3 alone has been shown to cause significant reductions in forest tree growth, even in the absence of foliar symptoms (Benoit et al., 1982; Duchelle et al., 1982, 1983). Ozone has also been impli-

cated in the forest decline seen in eastern Europe (Blank, 1985).

Eastern white pine is an important commercial and ornamental species that may be injured to a greater extent by air pollutants than any other eastern United States timber species (Gerhold, 1977). The phytotoxic effects of O_3 on eastern white pine have been well documented (Barnes, 1972; Boyer et al., 1986; Gerhold, 1977; Yang et al., 1983a, b) and generally include shortened needle length, reduced needle retention, chlorosis, mottling, banding and needle tip necrosis. Heterogenic variation in native eastern white pine populations is expressed in plants which show differential sensitivity when exposed to O_3 (Anderson et al., 1988; Benoit et al., 1982; Houston and Stairs, 1973; Mann et al., 1980; Yang et al., 1983ab).

Growth reduction in eastern white pines exposed to O_3 has consistently been correlated with an inhibition of the net photosynthetic (P_n) rate (Barnes, 1972; Botkin et al., 1972; Boyer et al., 1986; Reich and Amundson, 1985; Yang et al., 1983a, b). Previous research at the Air Pollution Laboratory at Virginia Tech has demonstrated a positive correlation between the severity of P_n inhibition, in grafted scions (branches), with the sensitivity of eastern white pine parent trees to oxidant stress (based on visible foliar symptomatology). The greater P_n inhibition as opposed to conductance in response to O_3 concentration, in

all clones, suggested that the difference in clonal sensitivity of eastern white pine to O_3 occurred at the biochemical level in foliar tissue (Yang et al., 1983a, b). Reich and Amundson (1985) reported a similar reduction in P_n with no change in conductance during exposure of eastern white pine to O_3. However, these data are not consistent with Reich (1987) who concluded that primary O_3 tolerance among plant species is related to inherent stomatal conductance and the associated control over pollutant flux into the leaf interior. In plants which show a correlation between lower P_n and partial stomatal closure, it is still not clear whether O_3 directly stimulates stomatal closure or if stomatal closure is an indirect response to increased intracellular CO_2 concentration resulting from O_3 altered cell physiology. Since plants exposed to O_3 show a reduction in the activity of ribulose bisphosphate carboxylase/oxygenase (Nakamura and Sada, 1978; Pell and Pearson, 1983; Dann and Pell, 1989), the resulting increase in internal CO_2 has been suggested as a stomatal signal for those species of plants which do show partial stomatal closure during O_3 exposure (Reich and Amundson, 1985).

Ozone decomposes in water to generate superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and H_2O_2 (Peleg, 1976). Ascorbate and glutathione have the greatest capacity to interact with O_3 and the toxic oxygen species generated from O_3 (Pryor et al., 1984; Giamalva et al., 1983).

Barnes (1972) showed increased ascorbate concentrations in several genotypes of pine after exposure to O_3. Since the ascorbate/glutathione (GSH) cycle has been reported to exist in the chloroplast (Foyer and Halliwell, 1976; Nakono and Asada, 1980) and in the cytosol (Castillo and Greppin, 1988; Klapheck et al., 1989) it may play an important role in those genotypes of eastern white pine which show tolerance to air pollutants.

Superoxide dismutase (SOD) is also a major plant defense protein which catalyzes the formation of H_2O_2 and O_2 from 2 H^+ and 2 $O_2^{\bullet -}$ (Rabinowitch et al., 1983). Since SOD activity increased in both the apoplast (Castillo et al., 1987) and the cytosol (Lee and Bennett, 1982) in association with O_3 exposure, it appears that the toxic oxy-radicals produced during O_3 exposure can be reduced to H_2O in a mechanism which involves SOD, the ascorbate/ glutathione cycle, and photosynthetically derived NADPH. However, α-tocopherol may also play an important role as a lipid soluble antioxidant which protects against, or repairs, damage due to lipid peroxidation since it is a radical chain breaking substance (Burton et al., 1983) of chloroplast membranes (Grumbach, 1983). Alpha-tocopherol semiquinone can be reduced by GSH either directly or through the ascorbate/ glutathione cycle (McCay, 1985).

The objectives of this research were to determine the effects of ambient air pollutants and climate on the levels

of antioxidants (α-tocopherol, ascorbate, and glutathione) and enzymes (ascorbate peroxidase, and glutathione reductase) from needles of eastern white pine trees which show differential foliar characteristics under field conditions. This information will further serve as a reference point for comparing data obtained from needles of grafted scions made from the same field-grown trees and then exposed to O_3 in either open-top field chambers or laboratory fumigation chamber (CSTRs).

MATERIALS AND METHODS

Plant Material

Three eastern white pine trees, 8 to 10 years old, growing within 15 m of each other in a nursery plantation were selected based on differences in foliar characteristics. The "sensitive" tree showed reduced needle growth, chlorotic mottling and needle tip necrosis which are characteristics similar to that induced by oxidant pollution. Approximately 10-15% of the eastern white pine in the nursery plantation displayed foliar symptoms similar in appearance to the sensitive tree. Both the "tolerant" and the "very tolerant" tree displayed uniform green needles and long needle growth, however; the very tolerant tree displayed needles which showed a deeper green pigmentation.

Needles were collected from June 1988 through January

1990. The needles from this time period fell into two different categories of tissue. Those needles which emerged from the new candles in the spring of 1988, and shed from the branches in the fall of 1989, were termed 1988-89 year needles. Those needles which emerged from the new candles in the spring of 1989, and shed from the branches in the fall of 1990, were termed 1989-90 year needles.

Needle fresh weight/dry weight ratios were measured by drying two, 0.5 g (fresh weight) needle samples for 48 hr at 100^{0}C and then allowed to cool to room temp in a desiccator prior to weighing. Fresh/dry weight ratios, for one tree of each genotype, were determined by dividing the fresh weight of needles by the dry weight of the same needles. Comparison of needle length was determined by measuring the cm growth of 10 current year needles from one tree of each genotype after the first active growing season.

Acid Soluble Metabolite Extraction

Three replicate samples, 500 mg each, of needles were collected monthly from each of the three trees, at approximately breast height, between 9:30 AM to 10:30 AM EST. The needle samples were cut into 2-4 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 sec.

Homogenates were centrifuged at 20,000 x g for 15 min ($4^{o}C$) using a fixed angle rotor (Beckman JA-20) and the resulting supernatants were filtered through a 0.45 μ ultrafilter. Samples were stored at $-20^{o}C$ 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 hr at room temperature ($25^{o}C$) in the presence of 30 mM dithiothreitol (DTT) to reduce dehydroascorbate to ascorbic acid. Ascorbic acid was measured using a modified procedure of Lee et al. (1984). Reduced ascorbic acid content was measured at 245 nm (maximum absorbance of the non-ionized form) using a high performance liquid chromatography (Spectra Physics model 770) equipped with a variable wavelength UV/visible detector. Separation was accomplished using a 250 x 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 x 4.6 mm Solvecon pre-column (Whatman). The eluant was aqueous 2% $NH_4H_2PO_4$ (pH 3.0) at a flow rate of 1 ml min^{-1} and a column pressure of 1800 psi. Peak areas were calculated using a Spectra Physics System I integrator. A standard curve was based on L-ascorbate (Fischer Scientific, 99.2% pure) from 50 to 800 $\mu g \cdot ml^{-1}$ in meta-phosphoric acid. Dehydroascorbic

acid values were determined by comparing the difference between total reduced ascorbic acid in acid extracts with or without 30 mM DTT added.

Glutathione Analysis

Total and reduced glutathione concentrations in the acid soluble extracts were analyzed according to the procedure of Brehe and Burch (1976) with the following modifications. Total glutathione (GSH and GSSG) 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% bovine serium albumin (BSA)), 320 μl reagent 2 (1 mM EDTA, 50 mM imidazole solution, and 0.02% BSA) which contained an equivalent of 1.5 units glutathione reductase 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 with the addition of 80 μl of 9.0 mM NADPH. Change in absorbance of the reaction mixture was measured at 412 nm using a LKB Ultraspec II (model 4050 UV/visible) spectrophotometer. The glutathione content was calculated from standard. The reaction rates were proportional to glutathione concentrations between 0.1 and 0.8 nmol per assay.

Oxidized glutathione (GSSG) was measured by first incubating 1 ml of the 1:50 diluted acid extract with 40 μl of

2-vinylpyridine for 1 hr at room temperature. The samples incubated in 2-vinylpyridine were then assayed as previously described to determine the change in the total glutathione value for an individual sample. The difference in total glutathione values (obtained using samples with and without 2-vinylpyridine) were used to determine GSSG content in each sample. Standard curves were established in the presence of vinylpyridine for each set of samples.

Lipid Soluble Metabolite Extraction

Three replicate samples, 500 mg each, were collected from each of the three trees as previously discussed. The needles were cut into 2-4 mm segments and frozen in liquid N_2. The needles were then homogenized in 3 ml of ice cold 80% ETOH using a Polytron at top speed for 30 sec. After centrifugation at 20,000 x g for 15 min (4^oC), each sample was filtered through a 0.45 μ ultrafilter. Exactly 2 ml of supernatant was then transferred to a test tube on crushed ice under subdued lighting conditions. The supernatants were partitioned twice using 500 μl of ice cold HPLC-grade hexane. The hexane fractions were pooled together and stored at -20^oC prior to analysis.

Alpha-tocopherol Analysis

Detection of α-tocopherol was accomplished using HPLC-fluorography as described by Finckh and Kunert (1985). A Perkin-Elmer series 400 liquid chromatograph, with a model LC-15B UV detector and a model LS-3 fluorescence detector,

was used to monitor the concentration of α-tocopherol in each sample at an excitation wavelength of 294 nm and an emission wavelength of 325 nm. Hexane fractions were separated on a Nucleosil 50, 5 μ particle size, column (Machery-Nagel, Düren), at a constant temp of 40°C, pro-tected with a 40 x 4.6 mm column of HC Pellosil (Whatman) and a 30 x 4.6 mm precolumn of Spheri-5 RP-18 (Brownlee Labs, Santa Clara, CA). The eluant was 0.6% dioxane in 100% hexane (V/V) at a flow rate of 1.5 $ml \cdot min^{-1}$. A standard curve was established from 12.5 to 250 $\mu g\ ml^{-1}$ of DL α-tocopherol standard (ICN Pharmaceuticals, Inc., Cleveland, OH) using the fluorescence peak area.

Protein Extraction

Three replicate samples, 1 g each, were collected from each of the three trees as previously discussed. Needles were then cut into 2-4 mm segments and frozen in liquid N_2. The needles were homogenized with a Polytron at top speed for 30 sec 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 polyvinylpyrrolidone (mol wt 10,000; PVP-10), 1% polyclar-AT, and 1 drop of antifoam A emulsion. After centrifugation at 20,000 x g for 15 min (4°C), the supernatants were saved and the remaining insoluble material was re-extracted with an additional 2 ml of extraction buffer using a polytron at top speed for 10-15 sec. The homogenates were again centrifuged and the

resulting supernatants were pooled together with the corresponding supernatants of the first extraction.

A fraction of each extract was desalted on a prepacked G-25 desalting column (Pharmacia, Uppsala, Sweden) using the quick centrifugation method of Neal and Florini (1973). Each column was pre-equilibrated with 50 mM Tris-HCL (pH 7.5), containing 1 mM EDTA, prior to desalting of the extract.

Total Protein Analysis

Protein determinations were done using the Commassie blue dye binding assay (Bradford, 1976). Protein values for crude desalted extracts were obtained from a standard protein curve which was generated based on the dye binding capacity for Bovine Serum Albumin-Standard II (Bio-Rad, Richmond, CA) vs the change in absorbance at 595 nm.

Ascorbate Peroxidase Analysis

Ascorbate peroxidase activity in the desalted extracts was measured by the method of Peters et al. (1988). This assay measures the decrease in absorbance$\cdot$min^{-1} at 265 nm due to the oxidation of ascorbic acid in the ascorbate peroxidase catalyzed reduction of H_2O_2. A 1 ml reaction mixture contained 500 μl of 166 mM Hepes-KOH (pH 7.0), 100 μl each of 1.0 mM EDTA, 1.5 mM NaAscorbate, and 1.0 mM H_2O_2. The reaction was started with the addition of the desalted extract (sample + buffer to = 100 μl). The extinction coefficient for ascorbic acid = 13.4 mM^{-1} cm^{-1}.

Glutathione Reductase Analysis

Glutathione reductase activity was assayed according to the procedure of Schaedle and Bassham (1977). This assay measures the rate of oxidation of NADPH during a glutathione reductase catalyzed reduction of GSSG by monitoring the decrease in absorbance$\cdot min^{-1}$ at 340 nm. A 1 ml reaction mixture contained 700 μl of 50 mM Tris-HCL (pH 7.5) - X μl of desalted extract, and 100 μl each of 30 mM $MgCl_2$, 5 mM GSSG, and 1.5 mM NADPH. The reaction was started by adding the desalted extract. One unit of glutathione reductase activity is = to 1 μmole NADPH oxidized$\cdot min^{-1}$. The extinction coefficient for NADPH = 6.22 μM^{-1} cm^{-1}.

Environmental and Climatic Measurements

Temperature, precipitation, and hourly O_3 concentrations were all monitored by the EPA Dry Deposition Station located at the Horton Research Center, Giles County, VA. (elevation = 3450 feet). Monitoring protocol followed guidelines detailed in the EPA document (EPA-450/4-87-007) "Ambient Monitoring Guidelines for Prevention of Significant Deterioration (PSD)", (1987).

RESULTS AND DISCUSSION

ATMOSPHERIC AND CLIMATIC CONDITIONS VS NEEDLE GROWTH

The mean length of the 1988-89 year tolerant needles (n=10 fascicles) in October, 1988 was 9.88 ± 0.47 cm,

whereas, the mean length of the 1988-89 year sensitive needles (n=10) was 5.39 ± 0.09 cm (Chevone et al., 1989). The 45% reduction observed in the growth of the sensitive needles also was paralleled by severe needle tip burn and mottling (Figure 2.1, A). Needles from the tolerant tree showed very little tip burn or mottling. In the 1989-90 year needles, only a 30% reduction in length was observed in the sensitive needles compared to the tolerant needles in October, 1989 (Figure 2.1, B). The mean length of the 1989-90 year sensitive needles (n=10 fascicles) was 7.01 ± 0.59 cm, whereas, the mean length of the 1989-90 year tolerant needles (n=10) was 10.15 ± 0.90 cm (Personal communication, W.S. Lee). furthermore, very little difference in visible injury was observed between the 1989-90 year sensitive and tolerant needles. The difference seen between needle length and visible injury for 1988-89 vs 1989-90 may be a reflection of the difference in the atmospheric and climatic conditions between 1988 and 1989.

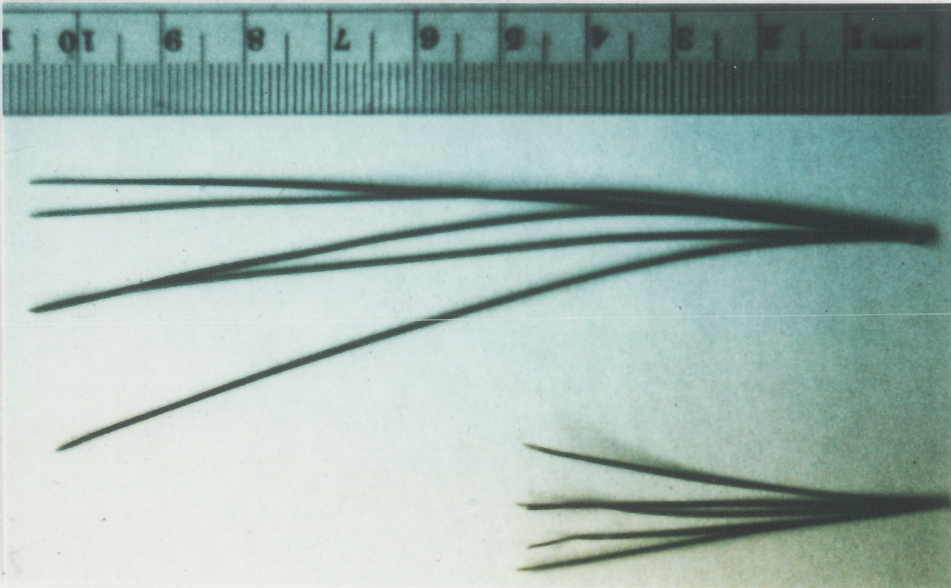
During the 1988 growing season, there were 23 days when the maximum daily O_3 concentration at the Horton Research Center, Giles County, Virginia was greater than 100 ppb compared to 1989 when this concentration was exceeded on only 1 day (Figure 2.2, A). There were also 8 days in the 1988 growing season when the maximum daily O_3 concentration was greater than 120 ppb in comparison to 1989 when there were no O_3 episodes greater than 120 ppb. In addition, at

Figure 2.1 Foliar characteristics of needles taken from Field-grown eastern white pine during different growing seasons. (A) 1988-89 year needles emerged in May, 1988. (B) 1989-90 year needles emerged in May, 1989. SY1 = Sensitive, TY1 = Tolerant, VTY1 = Very-tolerant (classified based on visible foliar characteristics.

B. 1989-90 NEEDLES

SY1 TY1 VTY1

A. 1988-89 NEEDLES



SY1 TY1

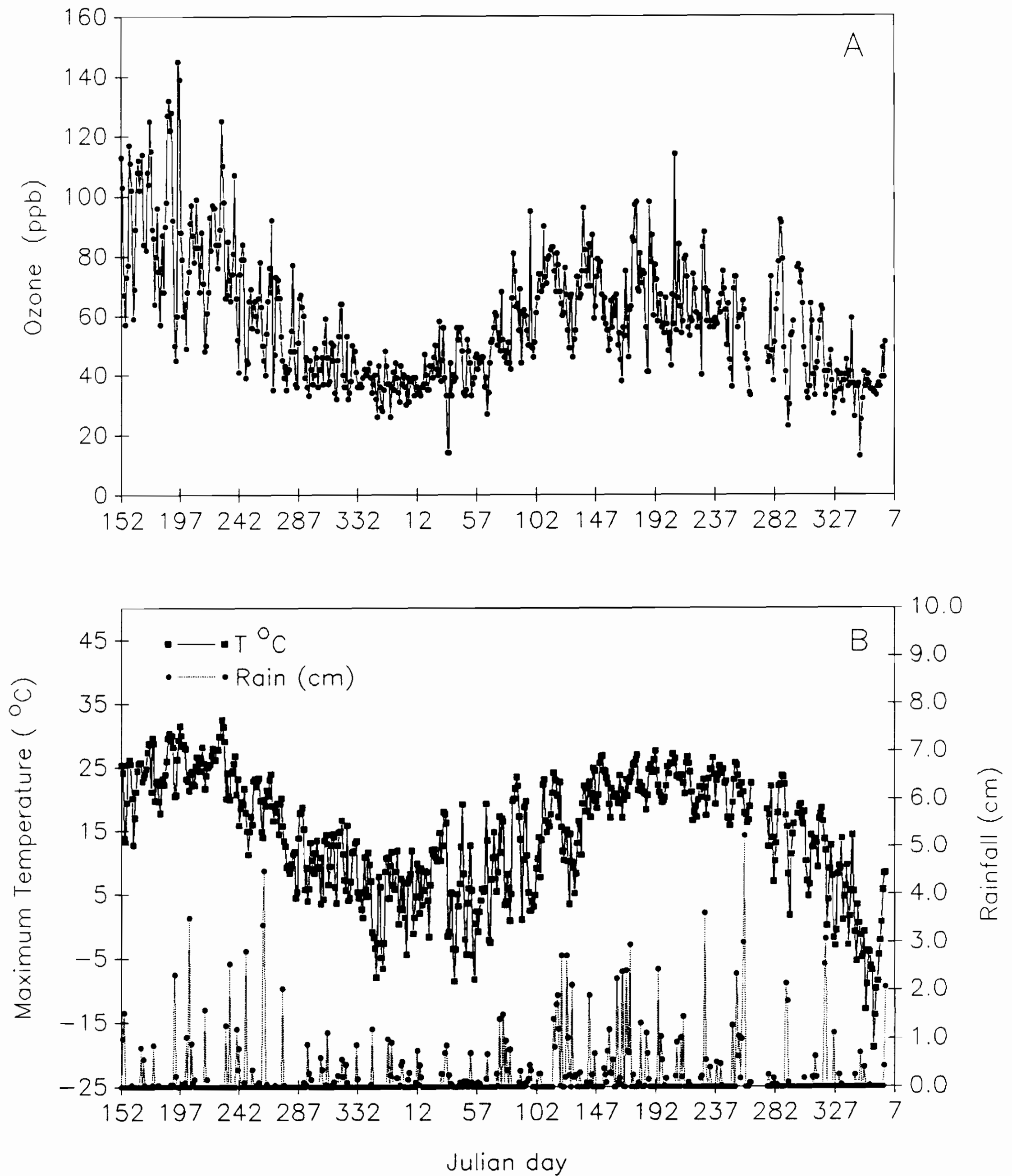


Figure 2.2 Seasonal change in the maximum daily ozone concentration during June 1, 1988 through December 31, 1989 (A). Seasonal change in the maximum daily temperature and rainfall during June 1, 1988 through December 31, 1989 at the Horton Research Center, Giles County, Va.

the Horton Research Center, during the 1988 growing season, daily temperatures were higher and rainfall was lower compared to the 1989 growing season (Figure 2.2, B). The maximum monthly mean temperatures at a low elevation site (Turf Center, VPI, Blacksburg, Va., elevation = 2000 feet) was also greater during 1988 compared to 1989 (Table 2.1). During July and August of 1988 the maximum monthly mean temperature was greater than the 30 year average (1941-1970), whereas, the maximum monthly mean temperature during May through October of 1989 was less than the 30 year average. At the low elevation site, the monthly rainfall in May, June, September and October of 1988 was less than compared to 1989 (Table 2.1). The rainfall in May, June and October of 1988 was also less than the 30 year monthly average, whereas, the monthly rainfall in May through October of 1989 was greater than the 30 year monthly average. The 15% greater reduction in needle length and the greater visible injury observed on needles collected during a high O_3, drought-type growing season (1988) compared to a low O_3, non-drought growing season (1989) may suggest that the needles of the tolerant-type pine trees express a greater capacity to withstand environmental conditions which can enhance oxidative stress.

NEEDLE TISSUE WEIGHT

The fresh weight to dry weight ratio of needles among the three trees never varied more than 10% during any

Table 2.1 Maximum monthly mean temperature and monthly rainfall for 1988, 1989 and 30 year average. Data collected from the Turf Center, VPI, Blacksburg, Va.

	MAXIMUM MEAN TEMP			MONTHLY RAINFALL		
MONTH	**1988**	**1989**	**30 YEAR**	**1988**	**1989**	**30 YEAR****
MAY	72.6	67.1	73.8	1.68	4.40	3.46
JUNE	78.1	76.5	80.1	3.21	7.04	3.75
JULY	85.3	81.4	83.1	5.39	4.81	4.00
AUG	84.6	79.2	82.0	5.57	4.53	3.51
SEPT	73.2	73.2	76.3	4.14	8.09	3.16
OCT	59.6	70.4	66.9	1.56	3.17	2.49

** 30 year average monthly rainfall during 1941-1970.

sampling period (Table 2.2). However, the fresh weight to dry weight ratio decreased between summer to winter in both the 1988-89 and 1989-90 year needles. These data indicate that the percentage dry weight of needles increases during the active expansion period and prior to winter. The data presented here indicate that the three genotypes of pine have similar fresh/dry weight ratios during a low O_3, non-drought type growing season (1989) and during a high O_3, drought type growing season (1988).

NEEDLE ASCORBIC ACID CONCENTRATIONS

For the 1988-89 year needles, only the sensitive and the tolerant trees were sampled. No samples were taken from the very-tolerant tree until the 1989-90 year needles emerged. Ascorbate concentrations in the 1988-89 year needles in June, 1988 were approximately 850 $\mu g\ gfwt^{-1}$ in the sensitive needles and approximately 1100 $\mu g\ gfwt^{-1}$ for the tolerant tree (Figure 2.3, A). The concentration increased through the summer and fall months and showed a maximum peak in January 1989 of approximately 3800 and 4150 $\mu g\ gfwt^{-1}$ in the sensitive and tolerant needles, respectively. Ascorbate remained high through March of 1989 and then showed a steady decline before leveling off in July of 1989. The concentration in the 1988-89 year needles then remained constant at approximately 1000 to 1200 $\mu g\ gfwt^{-1}$ from July of 1989 through September 1989 after which the majority of the 1988-89 needles were shed from the trees.

Table 2.2 Fresh weight to dry weight ratio of needles taken from field-grown eastern white pine. Genotypes of pine are classified based on visible foliar characteristics. 1988-89 needles emerged in May, 1988 and 1989-90 year needles emerged in May, 1989.

		GENOTYPIC CLASSIFICATION		
NEEDLES	**DATE**	**SENSITIVE**	**TOLERANT**	**VERY-TOLERANT**
		(fresh/dry weight ratio)*		
1988-89	6-28-88	3.11	3.03	----
1988-89	1-08-89	2.35	2.33	----
1988-89	6-14-89	2.23	2.34	2.15
1988-89	7-14-89	2.11	2.13	2.33
1988-89	8-22-89	2.12	2.22	2.32
1988-89	9-14-89	2.10	2.17	2.13
1989-90	6-14-89	3.58	3.57	3.54
1989-90	7-14-89	2.63	2.62	2.72
1989-90	8-22-89	2.59	2.50	2.46
1989-90	9-14-89	2.41	2.51	2.46
1989-90	11-29-89	2.32	2.36	2.38
1989-90	1-18-90	2.25	2.32	2.32

*mean (n=2) fresh/dry weight ratio of needles taken from one tree of each genotype.

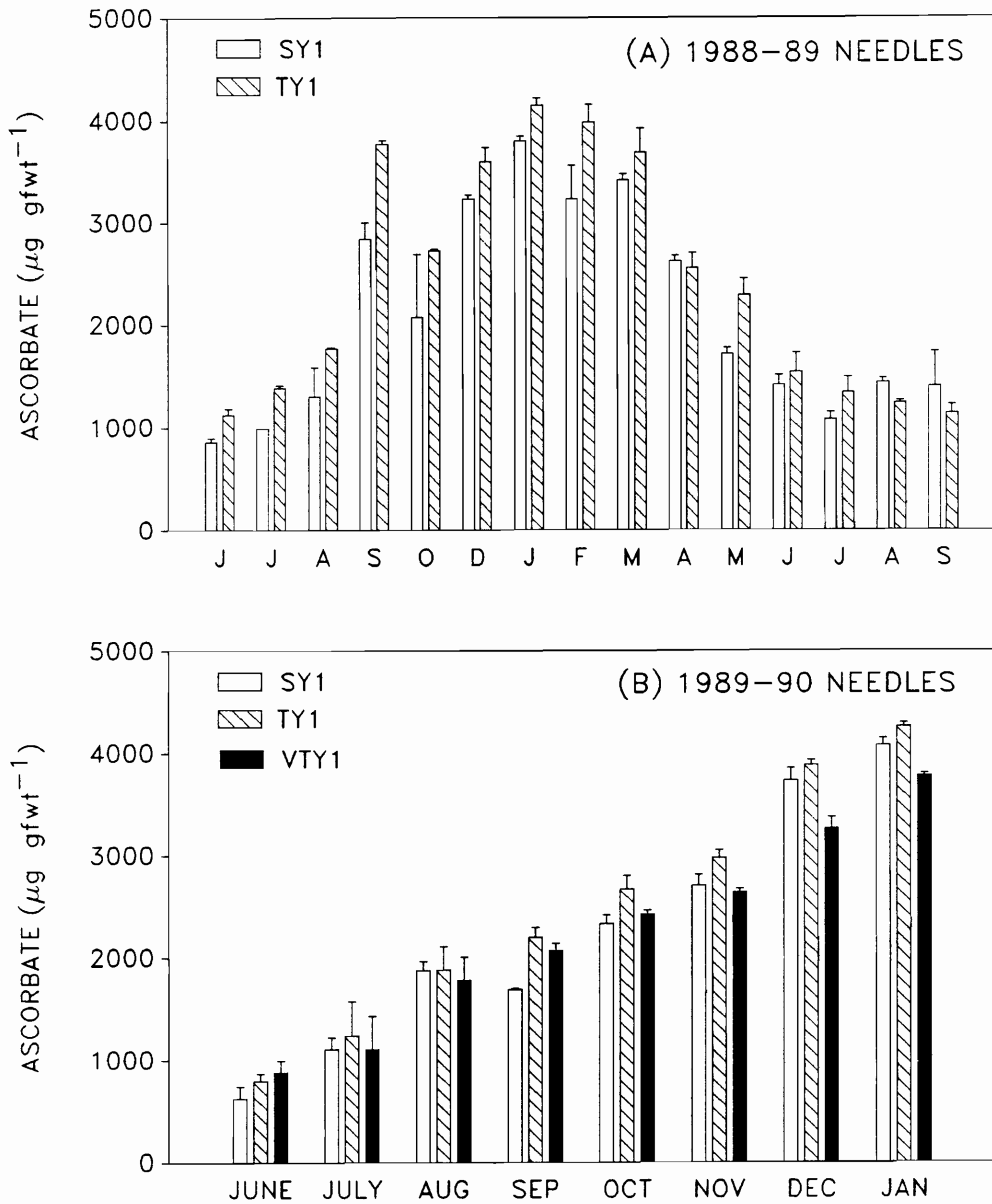


Figure 2.3 Monthly needle mean ascorbate concentrations in 1988-89 year needles (A) and 1989-90 year needles (B) of sensitive (SY1), tolerant (TY1) and very-tolerant (VTY1) field-grown eastern white pine. Error bars represent 95% confidence limits (n=2 from June, 1988 through May, 1989; n=3 from June, 1989 through January, 1990). Genotypes of pine are classified based on visible foliar characteristics.

The ascorbate content in the 1988-89 year needles was always greater in the tolerant needles than in the sensitive needles, with the exception of April, August and September, 1989 (Figure 2.3, A). This observation is consistent with the positive correlation between ascorbate content and increased protection against oxidative damage (Lee and Bennett, 1982). However, this hypothesis was not supported by the ascorbate data collected from the 1989-90 year needles. When a third genotype of eastern white pine (very-tolerant) was included in the sampling, the very-tolerant tree's needles had less ascorbate content $gfwt^{-1}$ than either the sensitive or the resistant needles (Figure 2.3, B). Although the difference in the ascorbate content was not as noticeable between the needles of the sensitive and tolerant trees in the 1989-90 year needles, the overall ascorbate content and seasonal concentration patterns were similar during the two years (Figure 2.3, A and B). From the results reported here, it was not possible to associate the degree of visible needle injury with the concentration of ascorbate.

Wise and Naylor (1987) reported that exposure of chilling-sensitive cucumber to cold temperature and high light for 12 hrs resulted in the oxidation of up to 50% of the total ascorbate. However, chilling-resistant pea showed no such oxidation of ascorbate under the same conditions. Our data showed that ascorbic acid content increased by as much

as 4-fold in the needles from all three trees during the winter months. This data may indicate that ascorbate plays a role in the overall cold hardiness of needle tissue. Since photooxidation, resulting from cold temperature and high light intensities, have been associated with damage due to $O_2^{\bullet -}$ (Wise and Naylor, 1986), and results in the oxidation of ascorbate in chilling-sensitive plants, it became apparent that eastern white pine needles under similar conditions might result in the oxidation of ascorbate. Since our HPLC assay only detected the total reduced ascorbic acid, and not the dehydroascorbic acid, it was not possible to determine the affect ascorbate concentration had on needle physiology and visible injury until both oxidized and reduced ascorbate were quantified.

Dehydroascorbate content of needles was determined by incubating acid extracts with 30 mM DTT at room temperature for 24 hr. Addition of 30 mM DTT to a dehydroascorbic acid standard resulted in a 70% conversion to ascorbic acid (Figure 2.4, A). Addition of dehydroascorbic acid to an acid extract from needles of eastern white pine, in the presence of 30 mM DTT, resulted in 78.5% recovery as ascorbic acid (Figure 2.4, B). With this method it was possible to determine the ratio of oxidized to reduced (or the total) ascorbate concentration in needles.

Incubation of 1988-89 year needle acid extracts in 30 mM DTT showed that very little of the ascorbate pool in

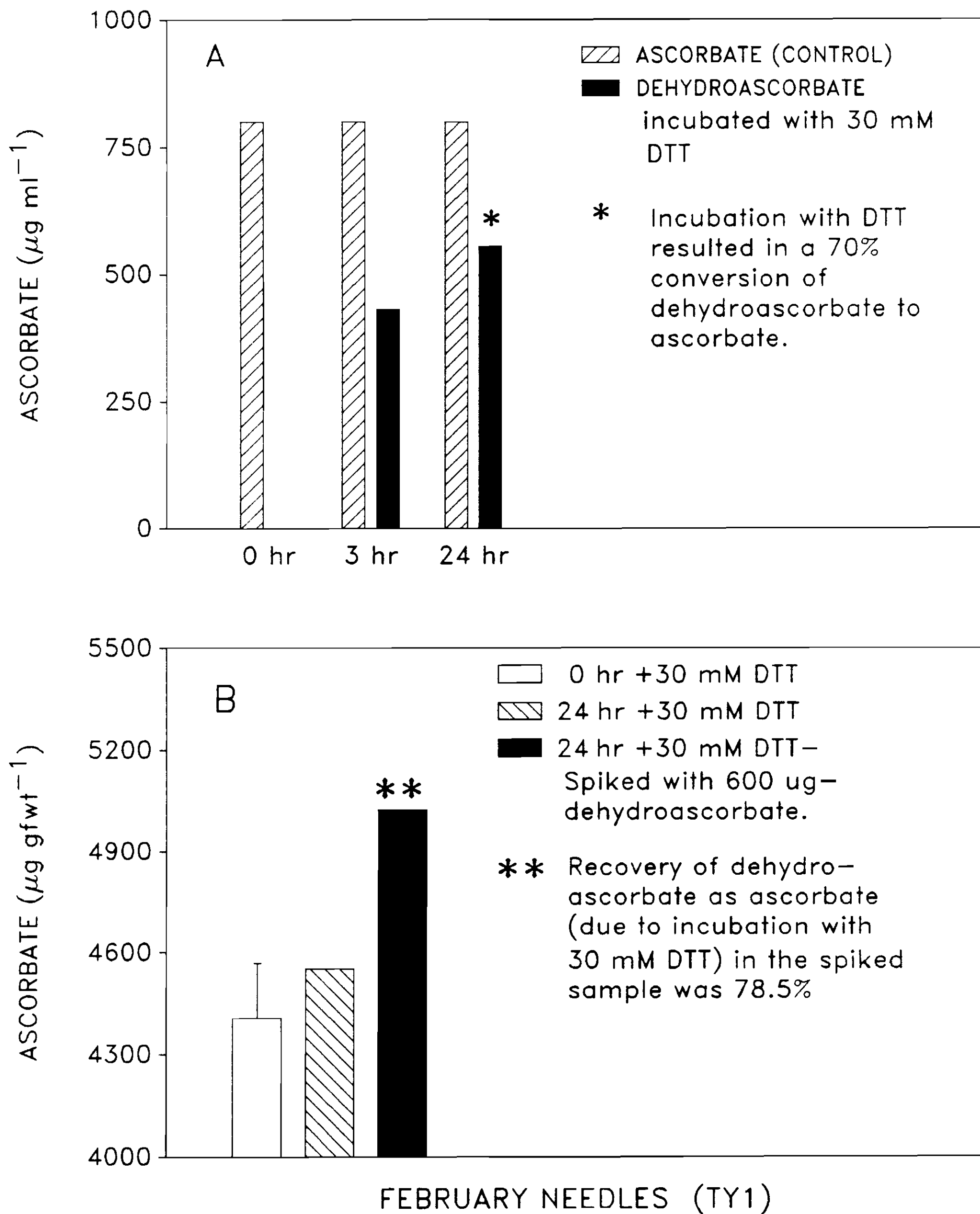


Figure 2.4 Conversion of dehydroascorbic acid standard to ascorbic acid during incubation with DTT at pH 2.8 (A). Recovery of dehydroascorbic acid as ascorbic acid in acid extracts of eastern white pine spiked with dehydroascorbic acid standard (B).

needles was in the form of dehydroascorbate. Prior to the winter of 1988-89, dehydroascorbate constituted less than 10% of the total ascorbate in the needles (Figure 2.5, A). During the winter months, dehydroascorbate concentrations were less, being never greater than 5% of the total pool of ascorbate in the needles. The greatest percentage of dehydroascorbate occurred as the temperature began to increase in the spring of 1989 (Figure 2.5, A), and was highest in June and July when the average of 3 replicate samples ranged between 10 to 15%.

The percentage of dehydroascorbate in the 1989-90 year needles paralleled that seen for the 1988-89 year needles with the exception of June, 1989 (Figure 2.5, B). In June, dehydroascorbate in the needles (average of 3 replicates) was 45.8, 34.1 and 31.2% for the sensitive, tolerant and very-tolerant needles, respectively.

Based on these data, differences in visible injury and growth of the 1988-89 and 1989-90 year needles did not appear to be due to differences in the percentage of dehydroascorbate content. However, since one-year-old needles have a greater percentage of the total ascorbate pool as dehydroascorbate, it appears that they may be under a greater oxidative stress than current year needles.

NEEDLES ASCORBATE PEROXIDASE ACTIVITY

Development of an extraction procedure for reproducible recovery of total protein from eastern white pine needles

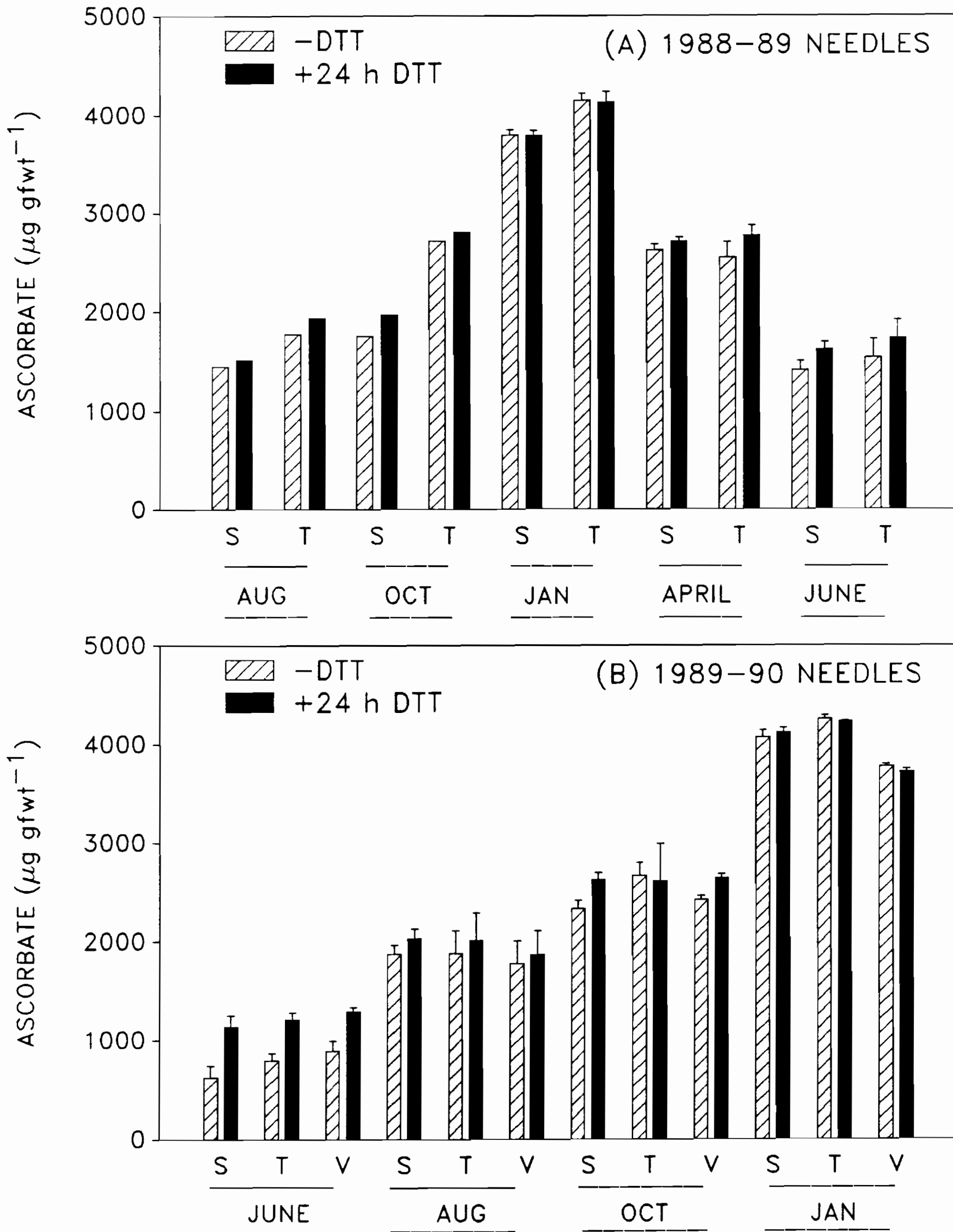


Figure 2.5 Comparison of the mean reduced (-DTT) vs total ascorbate (+24 h DTT) concentration of 1988-89 year needles (A) and 1989-90 year needles (B) from field-grown eastern white pine trees. Sensitive (S), tolerant (T), and very-tolerant (V) are genotypically classified based on visible foliar characteristics. Error bars represent 95% confidence limits (n=2, Aug through April, 1988; n=3, June, 1988 through Jan, 1989).

permitted comparison of enzyme activities in the three genotypes beginning in 1989. Both the total activity and the specific activity of ascorbate peroxidase was very low during the early growth (June-August) of the 1989-90 year needles (Figures 2.6, A and B). However, in September, the total activity of ascorbate peroxidase increased by a factor of 14.2, 16.5 and 21.4 and the specific activity increased by a factor of 6.0, 5.5 and 3.45 for the sensitive, tolerant and very-tolerant needles, respectively, compared to August values. The activity of ascorbate peroxidase remained high through the fall and winter months of 1989-90. The ascorbate peroxidase activity was always greater in the sensitive and very-tolerant 1989-90 year needles during September and October based on both total and specific activity.

The increase in ascorbate peroxidase activity observed between August and September in the 1989-90 year needles did not appear to be entirely due to a general increase in protein, since the soluble protein only increased by a factor of two-fold among the three varieties of needles sampled (Figure 2.7). However, if the increase in the specific activity of ascorbate peroxidase were related to cold hardening, and only specific proteins were increased, then the two-fold increase in total protein could account for the observed increase in total units. A decrease in the daily temperature was observed between August and

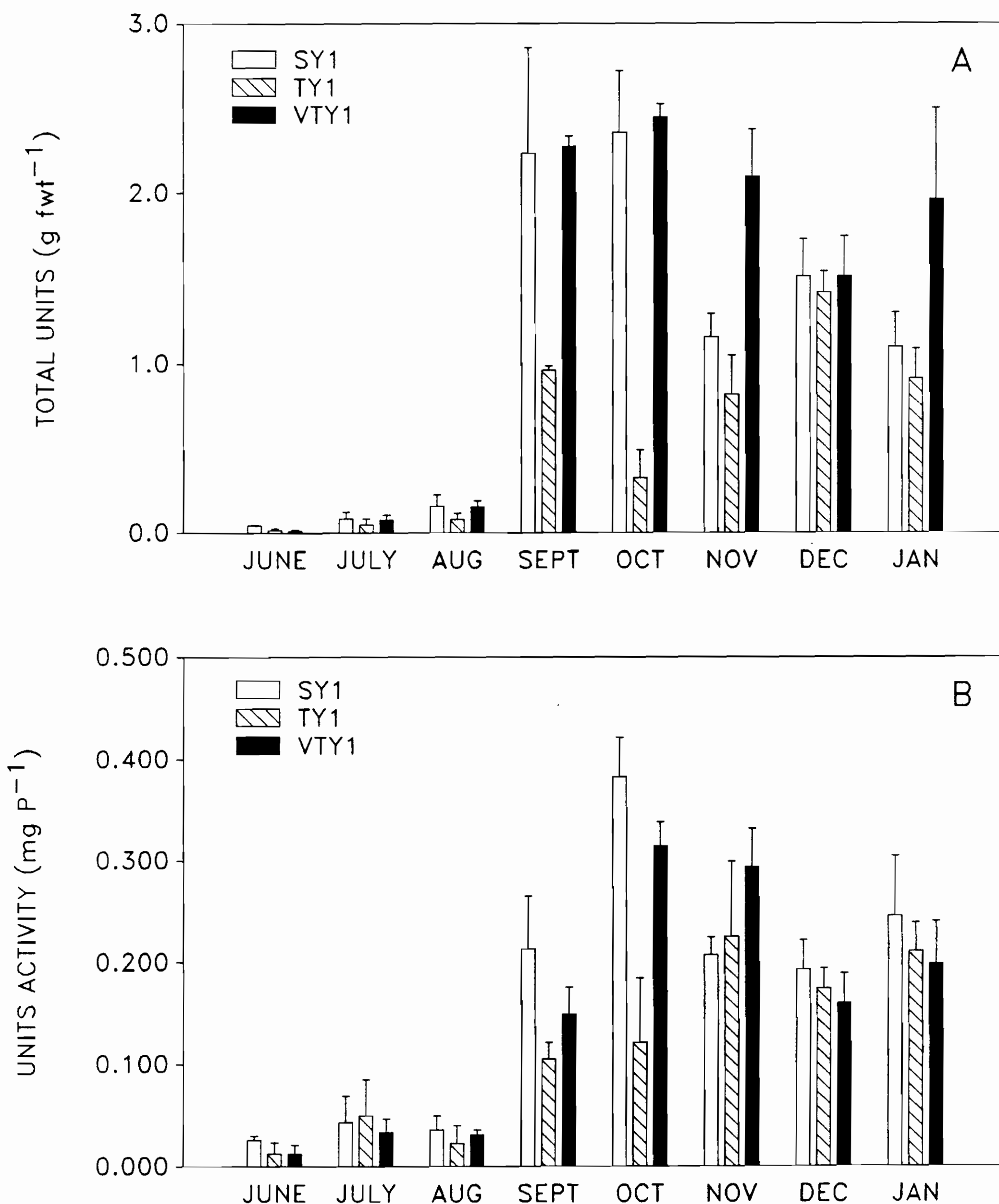


Figure 2.6 Monthly mean ascorbate peroxidase activity in the 1989-90 year needles of sensitive (SY1), tolerant (TY1) and very-tolerant (VTY1) genotypes of field-grown eastern white pine based on both total units activity (A) and specific activity (B). Error bars represent 95% confidence limits (n=3). Genotypes of pine are classified based on visible foliar characteristics.

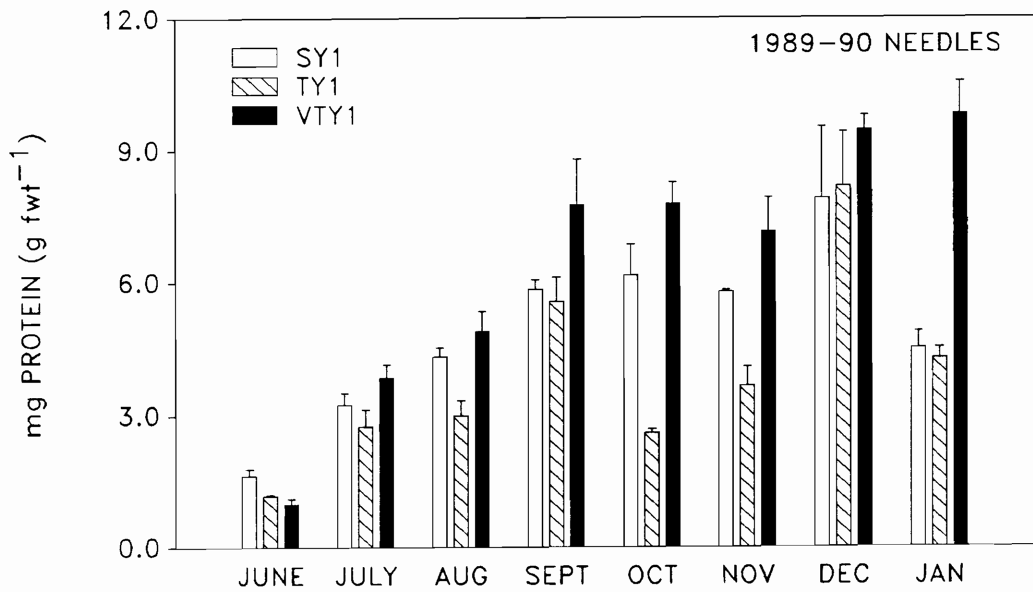


Figure 2.7 Mean soluble protein concentrations in 1989-90 year needles of sensitive (SY1), tolerant (TY1) and very-tolerant (VTY1) field-grown eastern white pine. Error bars represent 95% confidence limits (n=3). Genotypes of pine are classified based on visible foliar characteristics.

September, 1989 (Figure 2.2, B) and could account for a cold hardening response. The increase seen in specific activity between August and September probably indicates the synthesis of new isozymes of ascorbate peroxidase.

An alternative explanation for the observed increase in ascorbate peroxidase activity in September may be that a major shift occurred in needle physiology and function away from the one-year-old needles (1988-89 year needles) and favoring the current year needles (1989-90 year needles). Further justification for such an explanation is that a majority of the one-year-old needles were shed from the three pine trees during October. Such a shift in physiology may cause the synthesis of new isozymes of ascorbate peroxidase which could explain the increase in specific activity observed in September.

An increase of four-fold was observed for total ascorbate peroxidase activity in the 1988-89 year needles between the months of June and September 1989 (Figure 2.8, A). However, no significant changes were seen in the specific activity of ascorbate peroxidase during the same time period (Figure 2.8, B). The four-fold increase in total ascorbate peroxidase activity in the 1988-89 year needles between June and September was not due to an increase in total protein. These data suggest that the pine needles may experience increased oxidative metabolism prior to needle shed which stimulates the production of ascorbate

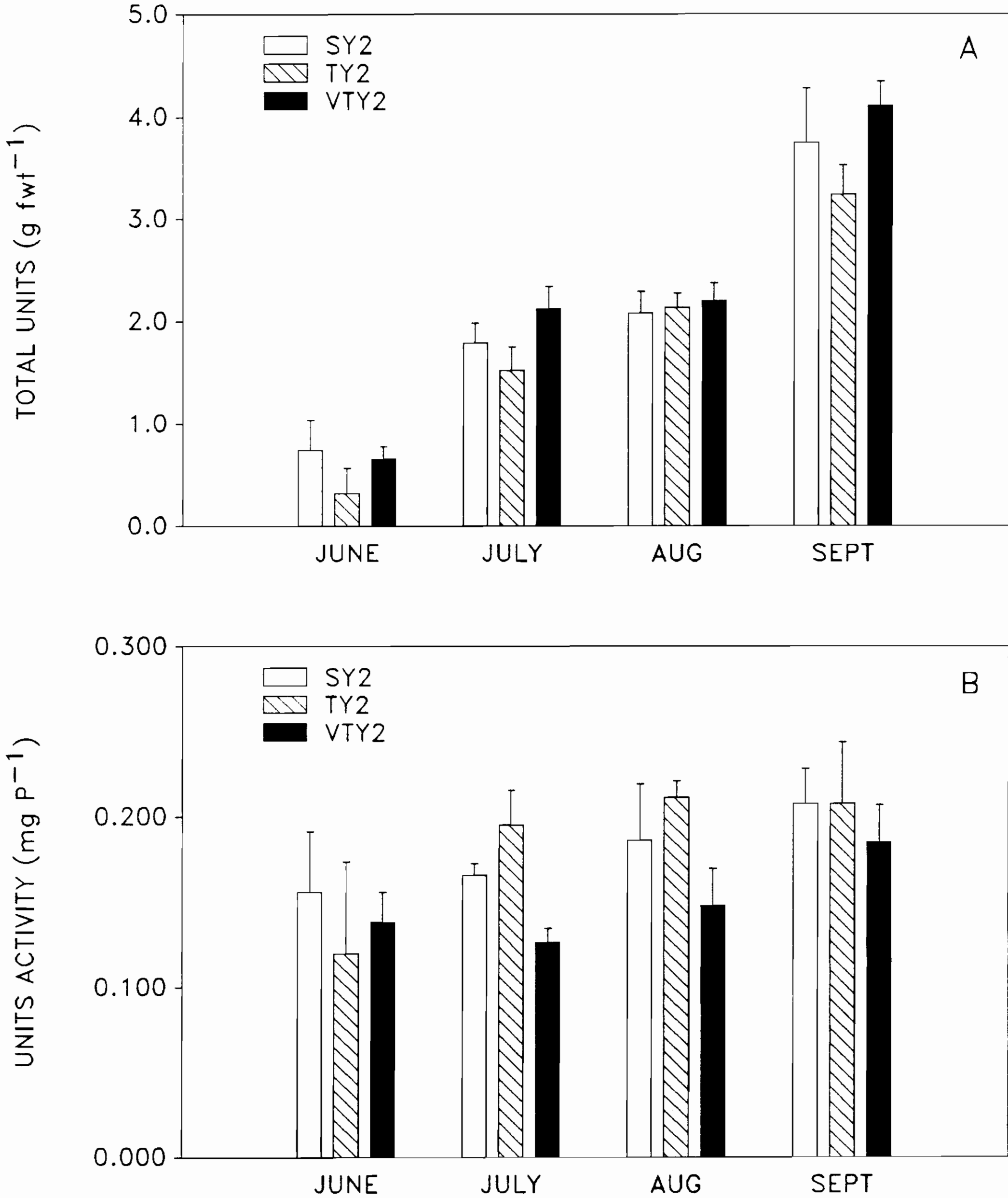


Figure 2.8 Mean ascorbate peroxidase activity in 1988-89, one-year-old needles of sensitive (SY2), tolerant (TY2) and very-tolerant (VTY2) field-grown eastern white pine based on both total units activity (A) and specific activity (B). Error bars represent 95% confidence limits (n=3). Genotypes of pine are classified based on visible foliar characteristics.

peroxidase. Furthermore, Thompson et al., (1987) have indicated that peroxidase activity does increase in senescing tissue.

NEEDLE GLUTATHIONE CONTENT

Total glutathione, reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in both 1988-89 and 1989-90 year needles. In the 1988-89 year needles, total glutathione content in the young needles (June and July 1988) ranged between 0.1 and 0.15 μmol gfwt^{-1} and progressively increased through the summer and fall months until reaching a maximum concentration in January, 1989 between 0.3 and 0.35 μmol gfwt^{-1} (Figure 2.9, A and B). Needles from the sensitive tree showed a steady decrease in total glutathione content during the spring months of 1989. However, in the needles from the tolerant tree, total glutathione levels decreased only between February and March of 1989 and then increased again to approximately 0.25 μmol gfwt^{-1} in June. Between the months of June and September, 1989, total glutathione content ranged between 0.025 to 0.075 μmol gfwt^{-1} in the sensitive needles compared to a range of 0.10 to 0.25 μmol gfwt^{-1} in the tolerant needles.

Generally the reduced/oxidized glutathione content in photosynthetic tissue is high (10:1), Halliwell (1984). However, in June and July, 1988 current year needles of both sensitive and tolerant genotypes had a GSH/GSSG ratio of approximately 1:1. As the needles matured during the

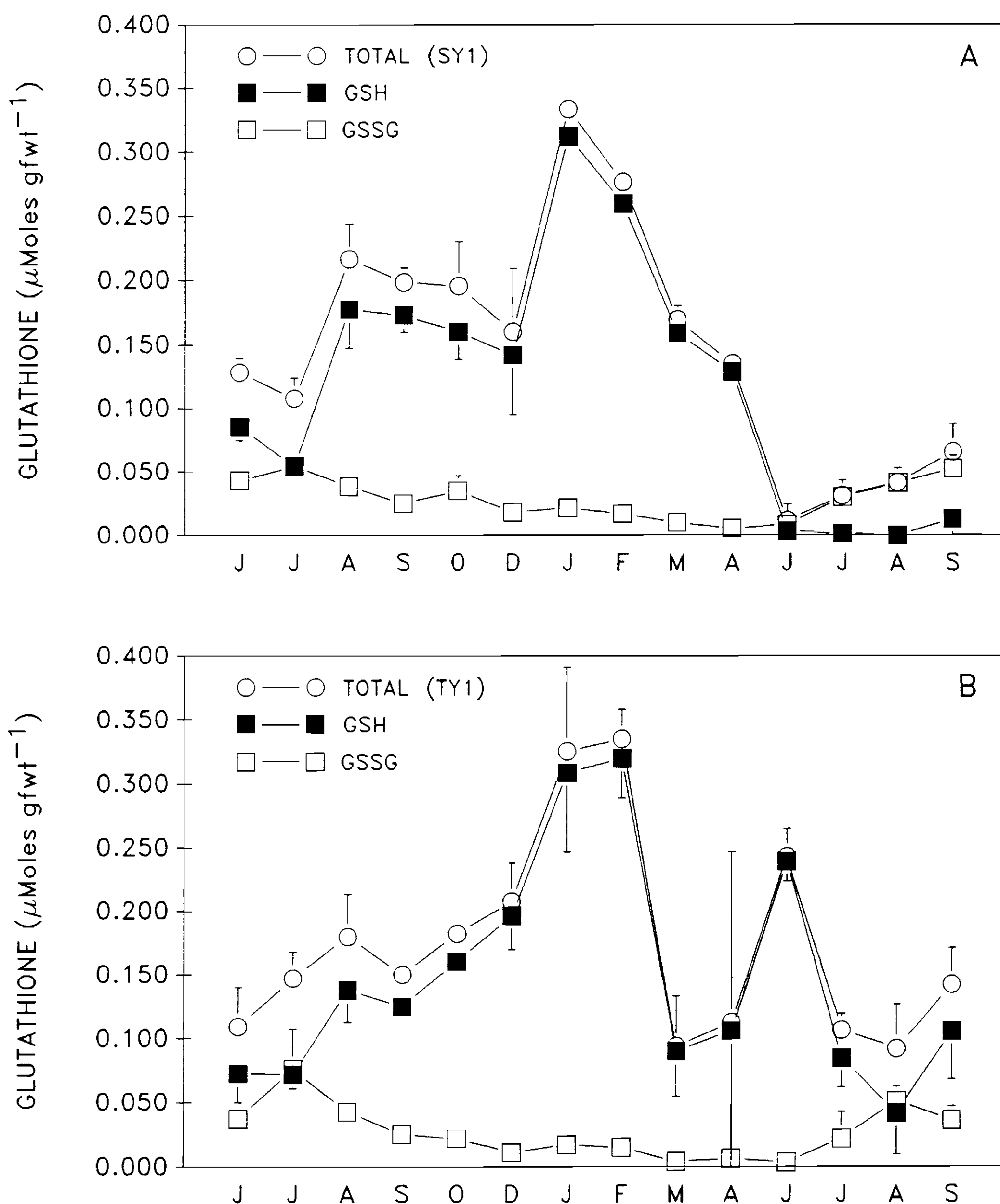


Figure 2.9 Monthly needle mean concentrations of total, oxidized (GSSG) and reduced (GSH) glutathione in 1988-89 year needles of sensitive (A) and tolerant (B) field-grown eastern white pine trees. Error bars represent 95% confidence limits (n=2, for June through May, 1988-89; n=3, for June-September, 1989). Genotypes of pine are classified based on visible foliar characteristics.

late summer and fall of 1988, the GSH/GSSG ratio increased. In January, 1989, the GSH/GSSG ratio approached 10:1 in both sensitive and tolerant needles (Figure 2.9, A and B). During the months of June through September, 1989, the tolerant one-year-old needles always had a GSH/GSSG ratio greater than that of the sensitive one-year-old needles. These data indicate that the sensitive and tolerant one-year-old needles have different physiological characteristics during their second growing season (June through September, 1989).

The total glutathione content in the 1989-90 year needles was different from that seen in the 1988-89 year needles. In June, 1989, the sensitive, tolerant and very-tolerant 1989-90 year needles had a total glutathione content which ranged between 0.55 and 0.65 μmol $gfwt^{-1}$ (Figure 2.10, A through D). These values were five- to six-fold greater than those seen in June, 1988 for the 1988-89 year needles (Figure 2.11, A and B). The total glutathione content decreased in the 1989-90 year needles through July and August, 1989. Only in August, 1989, did the total glutathione content in the 1989-90 year needles compare to the 1988-89 year needles (Figure 2.11, A and B). The total glutathione content in the 1989-90 year needles increased from August, 1989 through January, 1990 and showed a maximum content of between 0.7 and 1.0 μmol $gfwt^{-1}$ in January, 1990. In contrast to the 1988-89 year needles, at no time

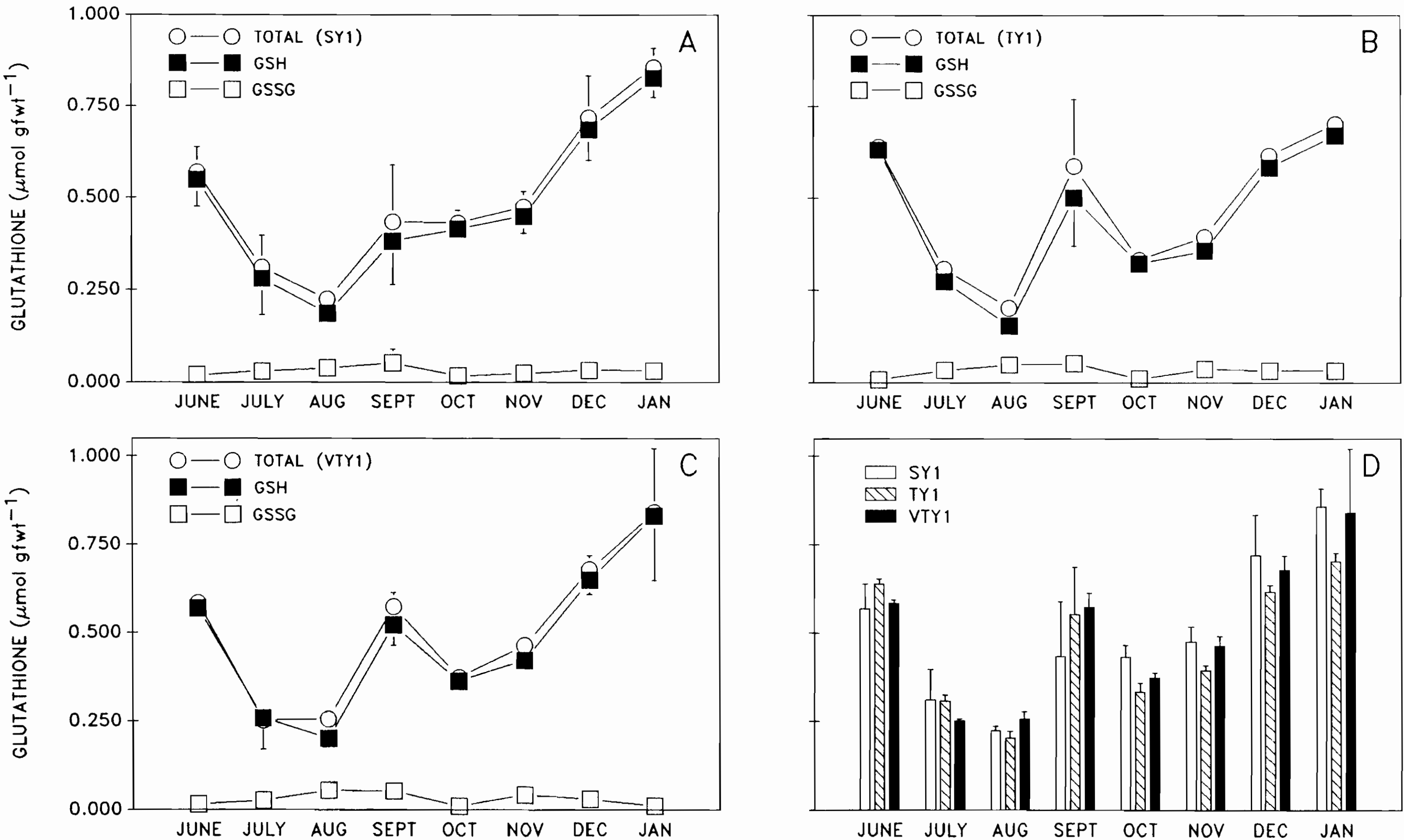


Figure 2.10 Monthly needle mean concentration of total, oxidized (GSSG) and reduced (GSH) glutathione in 1989-90 year needles of sensitive (A), tolerant (B), and very-tolerant (C) field-grown eastern white pine trees. (D) Comparison of monthly total GSH in all three genotypes. Error bars represent 95% confidence limits (n=3). Genotypes of pine are classified based on visible foliar characteristics.

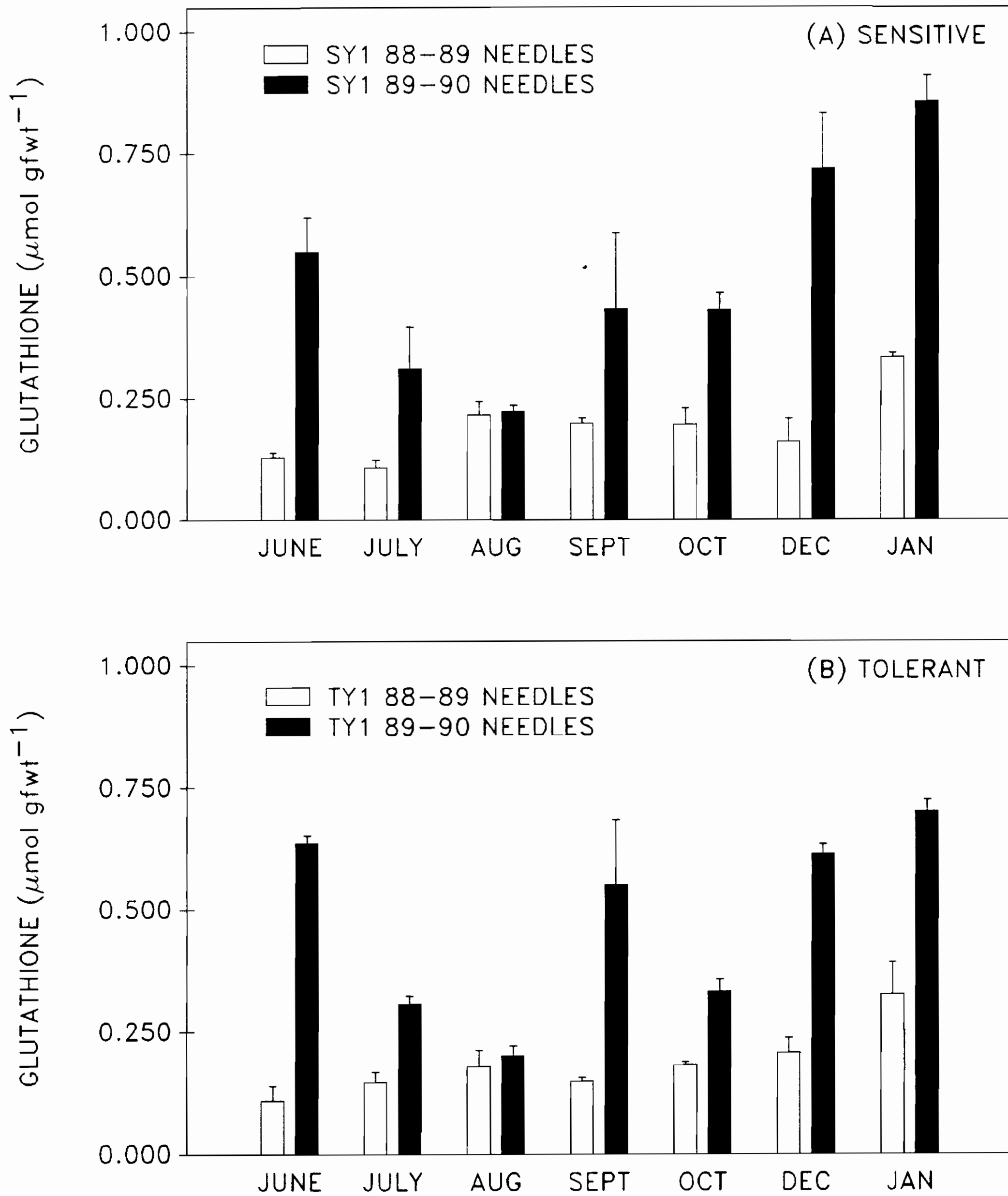


Figure 2.11 Comparison between the monthly mean total glutathione concentrations in 1988-89 year needles vs 1989-90 year needles of sensitive (A) and tolerant (B) field-grown eastern white pine trees. Error bars represent 95% confidence limits (n=2 for 1988-89 needles; n=3 for 1989-90 needles). Genotypes of pine are classified based on visible foliar characteristics.

during the 1989 growing season did the GSH/GSSG ratio fall below 3:1 in the 1989-90 year needles.

The seasonal pattern seen for glutathione content in pine needles was very similar to that reported by Esterbauer and Grill (1978) who observed an increased glutathione content in field-grown red spruce between August and February of 70 to 700 nmol $gfwt^{-1}$. Guy et al., (1984) also observed an increase in total glutathione and the GSH/GSSG ratio in the tissue of both dogwood stem and orange tree stem during cold acclimation. These results, and those reported in this study, suggest that increased glutathione, and in particular increased GSH/GSSG ratios, may function to protect against low temperature injury. However, orange seedlings which were treated with herbicide, to increase glutathione content, did not have increased frost tolerance (Guy et al., 1984) and would suggest that increased glutathione may not protect plants against low temperature injury. Nevertheless, one should not overlook the numerous reports which show that plants have increased glutathione content during the winter months. Furthermore, the use of orange (a sub-tropical plant species) as a tissue source for drawing conclusions on frost tolerance may be unrealistic. The available data suggest that Levitt's cold tolerance hypothesis (1962) may be valid in that increased glutathione protects against protein denaturation by inhibiting the formation of intermolecular disulfide bonds result-

ing from cell dehydration.

The data presented for atmospheric and climatic conditions, which show a higher incidence of O_3 and lower moisture availability during the 1988 growing season, might explain the difference seen among seasonal needle glutathione content when comparing 1988-89 and 1989-90 year needles. These data would suggest that a high incidence of air pollutants, high temperature, and low rainfall during the 1988 growing season may have caused an overall reduction in needle glutathione content in field-grown white pine trees. A decrease in glutathione content has been observed in Sedum during short term O_3 exposure (Castillo et al., 1988) and in bean leaves which have been exposed to O_3 (Guri, 1983). However, under field conditions, Osswald et al. (1987) observed an increase in the glutathione content of needles from spruce grown in Bavaria which were presumably injured by air pollutants. It was not clear whether the increase in glutathione was due to an O_3 or an SO_2 response, since, in Bavaria, SO_2 is a problem. Alscher et al. (1987) reported that SO_2-insensitive pea showed an increase in reduced glutathione content when exposed to 0.8 ppm SO_2 under a controlled environment, whereas, an SO_2-sensitive cultivar of pea did not show an increased glutathione response when exposed to SO_2. These data, along with the data presented in this study, indicate that air pollutant and/or drought conditions may effect the

overall glutathione content.

When comparing the two growing seasons (1988 vs 1989), (Figure 2.2, A and B) and the different needle growth patterns (1988-89 vs 1989-90), (figures 2.1, A and B), there were noticeable differences among needle glutathione content and needle length. From these data it appears that the mechanism for the turnover of glutathione and maintenance of a high GSH/GSSG ratio may be important in the protection of pine needles against oxidative stress.

NEEDLE GLUTATHIONE REDUCTASE ACTIVITY

In the 1989-90 year needles, the total units of glutathione reductase activity $gfwt^{-1}$ remained low during June through August (Figure 2.12, A). The specific activity also was lowest in the months June through September (Figure 2.12, B). An increase in the total glutathione reductase activity in the 1989-90 year needles was observed between August and September, 1989. However, the increase was not as great as was observed for ascorbate peroxidase during the same time period. Total glutathione reductase increased by a factor of approximately five-fold for all needle varieties between August and September. The increase in specific activity may again suggest the synthesis of different isozymes of glutathione reductase in response of either cold hardening or needle abscission as previously discussed.

In contrast to ascorbate peroxidase specific activity,

an increase in the specific activity of glutathione reductase in the 1989-90 year needles was not observed until October, 1989. Glutathione reductase activity increased throughout the Fall and Winter of 1989. Starting in September, the very-tolerant needles always contained the greatest total units of glutathione reductase activity. However, the total extractable protein was also greatest in the very-tolerant needles during this same time period (Figure 2.7). Only in November and December of 1989 was the total glutathione reductase activity of the tolerant needles equal to or greater than that of the sensitive needles. The specific activity of glutathione reductase in the tolerant 1989-90 year needles was always equal to or greater than that of either the sensitive or very-tolerant needles with the exception of January, 1990.

The increase seen in glutathione reductase activity in pine needles between summer and winter, reported here, was similar to that observed in red spruce needles by Esterbauer and Grill (1978) who reported a four-fold increase in glutathione reductase during the winter. Since the concentration of total glutathione and GSH/GSSG was also observed to be the greatest during the winter months, it would not be unreasonable to assume that increased glutathione reductase activity would be required to keep a proper GSH/GSSG ratio. de Kok and Oosterhuis (1983) reported that plants with both increased levels of glutathione and glutathione

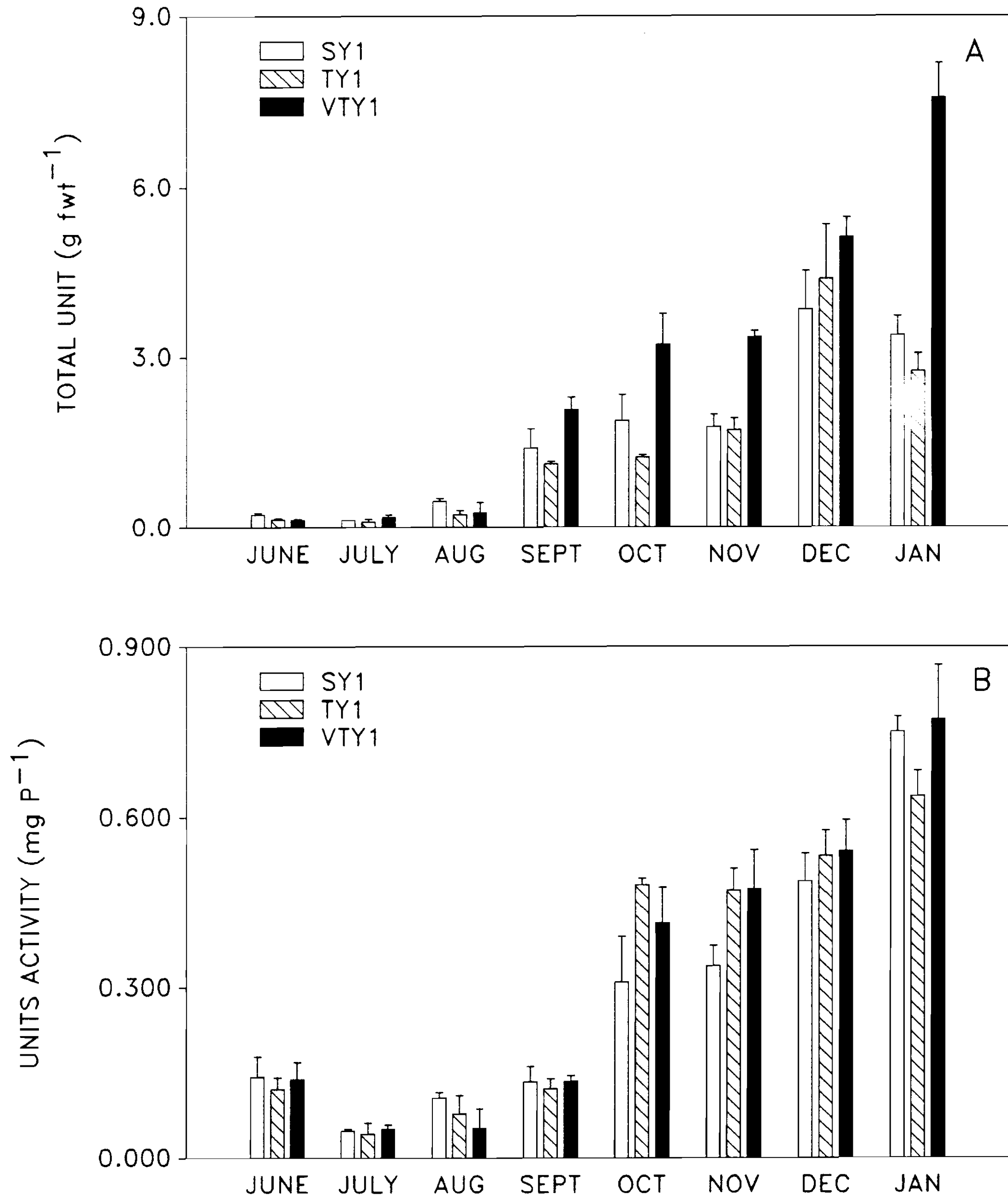


Figure 2.12 Monthly mean glutathione reductase activity in 1989-90 year needles of sensitive (SY1), tolerant (TY1) and very-tolerant (VTY1) field-grown eastern white pine trees based both on total units activity (A) and specific activity (B). Error bars represent 95% confidence limits (n=3). Genotypes of pine are classified based on visible foliar characteristics.

reductase are more resistant to low temperatures. Also, Wise and Naylor (1987) observed a decrease in total GSH, and an increase in GSSG, in chilling-sensitive cucumber under low temperature, whereas, chilling-resistant peas showed only a slight reduction in GSH and no accumulation of GSSG. Since electron transport occurs during the winter, whereas, CO_2 fixation is at it's lowest during the winter, the mechanism for the turnover of glutathione may be important for the scavenging of $O_2^{\bullet -}$ and might also partially limit it's formation by reducing the flow of electrons to O_2. Such a mechanism might help to explain the greater glutathione content and glutathione reductase activity observed in field-grown eastern white pine during the winter months.

Guy and Carter (1984) reported that glutathione reductase from cold acclimated spinach had lower K_ms due to the synthesis of new isozymes of glutathione reductase. Mahan et al. (1987) also reported that the K_ms for glutathione reductase can vary as much as six-fold between 15 and 45^{0}C. These data indicate that increases in glutathione reductase, and in the GSH/GSSG ratio, during low temperatures may be due to new isozymes of glutathione reductase which have a different affinity for GSSG and NADPH. Thus, the increases observed for glutathione reductase in white pine (this study) and other plant species, between the summer and winter, may reflect changes due to the production of

isozymes of glutathione reductase which have different K_ms and specific activity and may also explain the greater GSH/GSSG ratios observed in white pine during the winter.

Increased glutathione reductase has been reported in plants which experience drought stress (Burke et al., 1985; Smirnoff and Colombe, 1988) and O_3 exposure (Mehlhorn et al., 1986; Tanaka et al., 1988). Guri (1983) also reported that the activity of glutathione reductase was also higher in bean leaves which were relatively O_3 resistant. These reports also suggest that glutathione reductase can be induced by more than one type of oxidative stress. Shifts in glutathione reductase activity during oxidative stress implicate the importance in maintaining glutathione turnover in the cell.

The low GSH/GSSG ratio observed in the 1988-89 year needles during June through September, 1989 (Figure 2.9, A and B) can in part be explained by the level of glutathione reductase activity during this same time period. Glutathione reductase activity declined in the 1988-89 year needles during June through September, 1989 based both on total units and specific activity (Figure 2.13, A and B). In September, 1989, the sensitive needles had no detectable glutathione reductase activity. The greater loss of glutathione reductase activity in the sensitive, one-year-old needles may also help to explain the lower GSH/GSSG ratios which occurred in the sensitive compared to the tolerant,

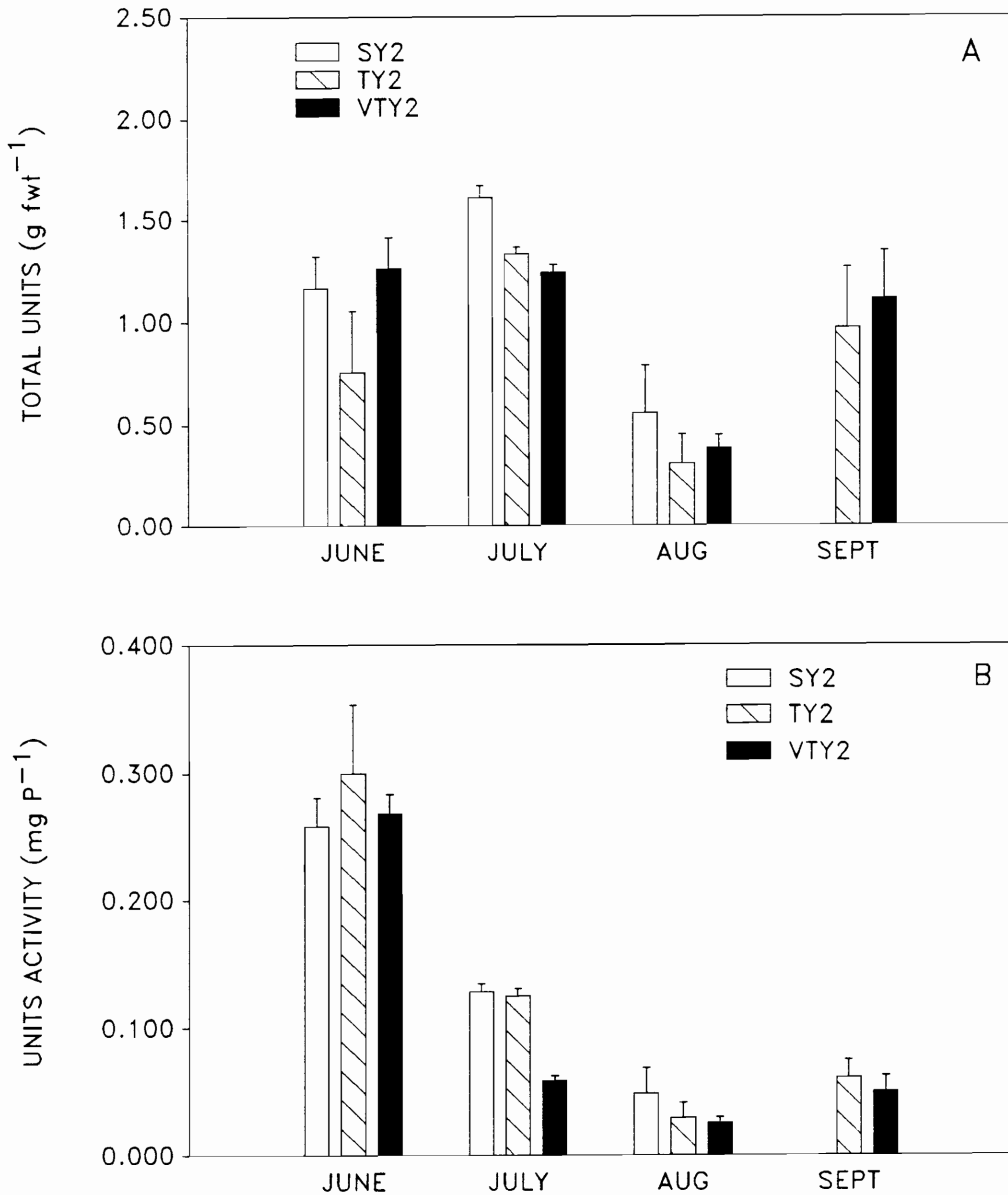


Figure 2.13 Monthly mean glutathione reductase activity in 1988-89, one-year-old needles of sensitive (SY2), tolerant (TY2) and very-tolerant (VTY2) field-grown eastern white pine trees based on both total units activity (A) and specific activity (B). Error bars represent 95% confidence limits (n=3). Genotypes of pine are classified based on visible foliar characteristics.

one-year-old needles.

NEEDLE α-TOCOPHEROL CONTENT

Measurements of α-tocopherol content began in February of 1989 using the 1988-89 year needles. The level of α-tocopherol ranged between 75 to 110 μg $gfwt^{-1}$ from February through April of 1989 in the 1988-89 year needles (Figure 2.14, A). Alpha-tocopherol content then increased, reaching a maximum concentration ranging between 150 to 250 μg $gfwt^{-1}$ in May and June of 1989. The level of α-tocopherol then leveled off between 100 to 150 μg $gfwt^{-1}$ and remained constant until the 1988-89 needles were shed in October. In the 1989-90 year needles the α-tocopherol content was less than 50 μg $gfwt^{-1}$ in June of 1989 and then increased gradually through the summer and fall before reaching a concentration of between 70 to 100 μg $gfwt^{-1}$ (Figure 14, B). These results for α-tocopherol content do not appear to contribute to any differences which might occur in oxidant induced visible needle injury under field conditions. However, these measurements were only taken during periods which were not conducive to oxidative stress.

The concentration of α-tocopherol throughout the year in pine needles was similar to that reported by Kunert and Ederer (1985) in fir needles. However, they also reported that a ratio between 10:1 and 15:1 for ascorbic acid/α-tocopherol provided the best antioxidant protection against peroxidative conditions and that a ratio of 1:1 or

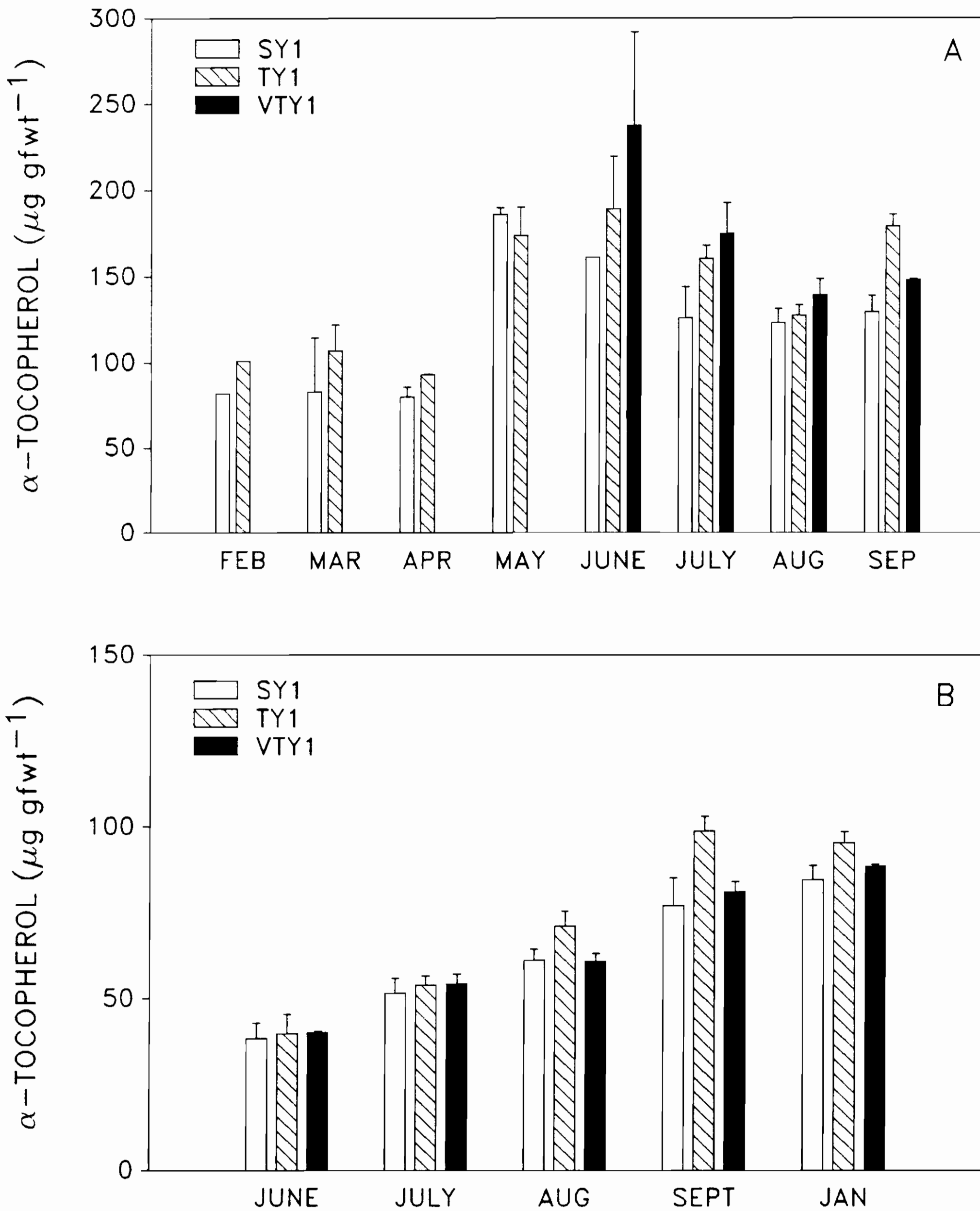


Figure 2.14 Monthly mean α-tocopherol concentration in 1988-89 year needles (A) and 1989-90 year needles (B) of sensitive (SY1), tolerant (TY1) and very-tolerant (VTY1) field-grown eastern white pine. Error bars represent 95% confidence limits (n=2, February through May; n=3 June through January). Genotypes of pine are classified based on visible foliar characteristics.

lower allowed for peroxidative damage. The ratio of ascorbic acid/α-tocopherol in pine needles was generally greater than 20:1 during the summer, and greater than 30:1 during the fall and winter months in current year needles. The ascorbic acid/α-tocopherol ratio was the least in one-year-old needles which had a ratio of approximately 10:1 from May through needle shed. These data may indicate that one-year-old needles have a lower capacity to protect against oxidative damage.

Mclaughlin et al. (1982) reported that young needles of white pine, in the expansion stage, draw on the carbon reserves and photosynthetically fixed carbon from the previous year needles. Davis and Wood (1972) also reported that susceptible conifers were most sensitive to O_3 four through thirteen weeks after bud break. Based on the data presented in this study, the sensitive, one-year-old needles may retain a lower capacity for providing a supply of carbon to expanding tissues when grown under conditions which could enhance oxidative stress. This hypothesis may be justified by the 15% greater reduction in growth which occurred in the sensitive 1988-89 year needles during a high O_3, drought-type growing season compared a low O_3, non-drought growing season. Further evidence for this conclusion was based on Chevone et al. (1989) who reported that the P_n of one-year-old, sensitive needles from eastern white pine was less than that of the tolerant, one-year-old

needles during the high O_3, drought-type growing season (1988). However, during a low O_3, non-drought growing season (1989), the sensitive, one-year-old needles had a greater P_n than did the tolerant, one-year-old needles.

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CHAPTER 3

COMPARATIVE STUDY OF AN ANTIOXIDANT DEFENSE MECHANISM IN CLONES OF EASTERN WHITE PINE WHICH SHOW DIFFERENTIAL FOLIAR CHARACTERISTICS: A PRELIMINARY ESTIMATE OF DIFFERENCES DUE TO AMBIENT VS CHARCOAL-FILTERED AIR EXPOSURES

INTRODUCTION

Interpretations of cause and effect relationships between environmental stress factors and changes in plant physiology and biochemistry, under natural field conditions, are generally complicated by the absence of appropriate controls. Differences in total needle GSH content and needle length were observed in field-grown Eastern White pine when comparing needles which matured under two distinctly different growing environments (Chapter 2). However, since no trees grew under conditions of controlled levels of O_3, temperature, and rainfall, it was not possible to determine which factor or factors affected total needle GSH and needle length. For these reasons research methods have been developed for studying plants grown under field conditions which allow for some control of environmental variables.

The open-top chamber system (Heagle et al, 1973; Mandl et al, 1973) is one of the most popular field-exposure sys-

tems presently used to monitor plant responses to air pollutants. The system consists of an upright cylinder with a clear plastic covering around the sides. This system uses a high-volume flow of air to reduce ambient pollutant influx through the open tops. The chamber design also eliminates ambient air pollutants from the high-volume air flow by first drawing the ambient air through a charcoal filtering system. Heagle et al. (1979) and Reich and Amundson (1984) have also reported that open-top chambers do not significantly affect either plant response or sensitivity to air pollutants.

Another limitation in field studies can be the availability of material which is genetically uniform. To reduce genetic variability, sampling error, and bias it is often desirable to increase the size of a sampled population. The Eastern White pine sample population was increased, while at the same time genetic diversity was reduced, by grafting scions to common root stock (Yang et al, 1983a, b). This method allowed for an established sample population, from a single parent tree, which shows specific genetic characteristics when exposed to environmental variables. This method also provided a uniform population of plants which can be transplanted into open-top chamber systems.

The objective of this study was to compare the levels of total ascorbic acid (ascorbic acid + dehydroascorbic

acid), total glutathione (GSH + GSSG), ascorbate peroxidase, and glutathione reductase activity in the needles of sensitive and tolerant clones of eastern white pine which have been grown in open-top chambers supplied with either charcoal-filtered, non-filtered or ambient air. Tissue collected in the summer and winter were also compared to determine if summertime air pollutant exposure might affect the antioxidant system during low temperature stress. The results obtained from the clones were compared to those from parent, field-grown, sensitive and tolerant pine trees.

MATERIALS AND METHODS

Plant Material

Clones of tolerant and sensitive (Chapter 2) eastern white pine (based on foliar characteristics) were used in this study to minimize variation in biochemical responses to O_3 exposure. Clones were produced by grafting scions, from field specimens at the Laurel Creek Nursery plantation, Montgomery County, Virginia (Chapter 2), to two-year old root stock (open pollinated seed source obtained from Virginia Division of Forestry, Waynesburo, Va) after the procedure of Yang et al (1983a, b). The clones were maintained in 10 cm plastic pots which contained soil obtained from the Reynolds' Agricultural Experiment Station (Critz,

Virginia). The clones were graphed in late March, 1988 and grown in a glasshouse for 3 months supplied with charcoal-filtered air to reduce ambient O_3 to below 0.025 ppm. The clones were then transferred to a lathhouse until transplanted into the open-top chambers.

Open-Top Chambers

Cloned-Eastern white pine were transplanted into native soil either in charcoal-filtered, non-filtered, or ambient open-top chambers located at the Horton Research Station (Salt Pond Mt., Giles County, Virginia) in the Fall of 1988 or spring of 1989. Soil pH in the chambers ranged from 5.5 to 6.3. At least three grafted plants/clone were placed in each of two replicate chambers/treatment. The plants were hand watered as needed with well water. The open-top chambers were only operated during the active growing season (May-October). During the winter and early spring, when O_3 concentrations are generally < 0.035 ppm, all clonal material was exposed to ambient air conditions. Ozone was monitored on a time shared basis (EPA Dry Deposition Station located at Horton Research Center) within one replicate chamber of ambient forced air and charcoal-filtered air treatments. The concentration of O_3 in the monitored, charcoal-filtered chamber ranged between 30-50 ppb (Figure 3.1). The concentration of O_3 in the monitored, ambient forced air chamber during July 18-25, 1989 was approximately 20-30 ppb greater than in the charcoal-

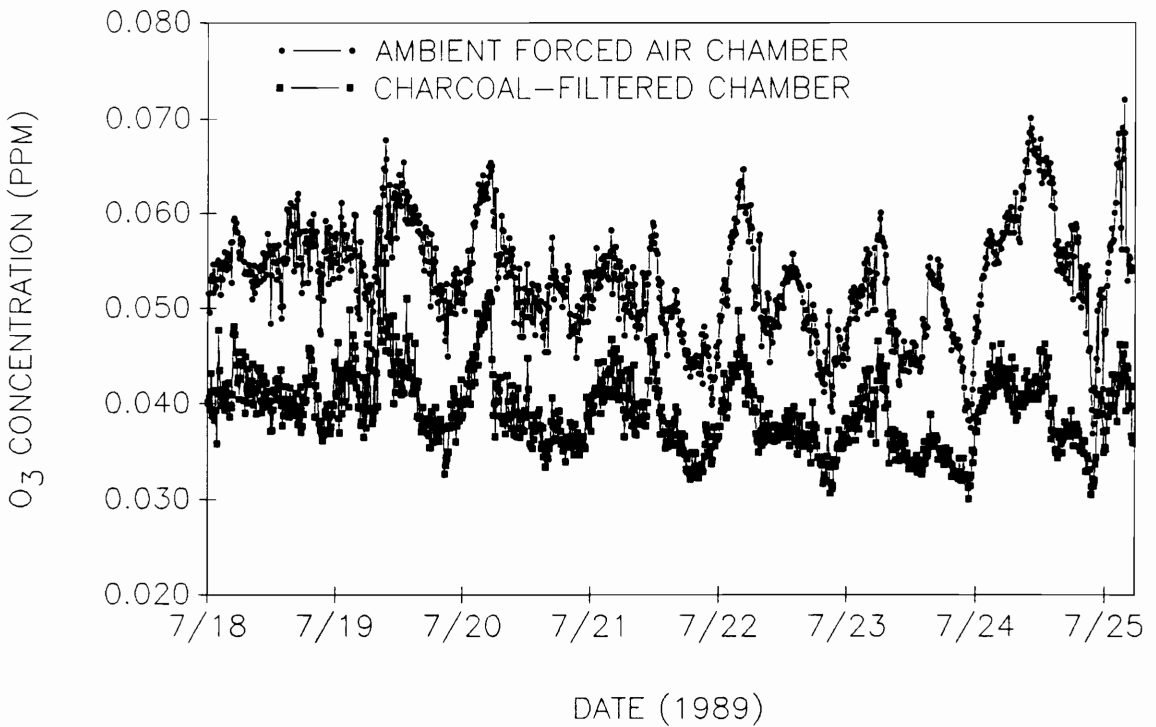


Figure 3.1 Comparison of O_3 concentration (PPM) in ambient and charcoal-filtered, open-top chambers located at Horton Research Center, Salt Pond Mountain, Giles County, Va.

filtered chamber.

Biochemical Measurements

Measurements of needle biochemistry were done on the new growth of the 1989-90 year needles. Measurements were taken in September, 1989 and January, 1990. Due to the limited amount of needle tissue available from the current year growth (1989-90 year needles) sampling size was reduced to approximately 250 mg. Preparation of tissue extracts, and their analysis for ascorbic acid, glutathione, protein, ascorbate peroxidase, and glutathione reductase, were done as described in Chapter 2.

RESULTS AND DISCUSSION

NEEDLE ASCORBIC ACID CONTENT

All data presented here are based on the mean of three replicate samples/clone/chamber (one chamber = one treatment) unless otherwise stated. Data presented in range values represent the mean (n=3) from each of two replicate chambers. The content of reduced ascorbate in sensitive 1989-90 year needles from September, 1989 ranged between 1567-1940 µg, 1774-2311 µg and 1761-2463 µg $gfwt^{-1}$ for clones grown under ambient, non-filtered and charcoal-filtered air, respectively (Figure 3.2, A). The total ascorbate content of the sensitive needle in September, 1989 ranged between 1942-2134 µg, 1944-2501 µg and

1926-2602 μg $gfwt^{-1}$, respectively, for samples taken from ambient, non-filtered and charcoal-filtered chambers. These data show that 10-17.5% of the total ascorbate content of sensitive needles taken from the ambient chambers was dehydroascorbate (Table 3.1). Whereas, 7.5-12% and 5.5-8.5% of the total ascorbate in sensitive needles taken from the non-filtered and charcoal-filtered chambers, respectively, was dehydroascorbate.

The content of reduced ascorbate in September, 1989 from the tolerant needles of cloned material grown under ambient, non-filtered and charcoal-filtered air ranged between 1662-1707 μg, 1978-2045 μg and 1972-1996 μg $gfwt^{-1}$, respectively (Figure 3.2, A). Total ascorbate content during the same time period ranged between 1942-2169 μg, 2140-2218 μg and 2132-2196 μg $gfwt^{-1}$ in tolerant needles taken from ambient, non-filtered, and charcoal-filtered chambers, respectively. In September 12-23% of the total ascorbate content in the tolerant needles taken from ambient chambers was dehydroascorbate. Whereas, 7.5% and 7.5-9.0% of the total ascorbate content of tolerant needles taken from the non-filtered, and charcoal-filtered chambers, respectively, was dehydroascorbate (Table 3.1).

Castillo et al. (1987) concluded that ascorbic acid may be an important indicator of ozone injury in plants. They observed a two-fold decrease in the concentration of ascorbate in the intercellular fluid of Norway spruce which were

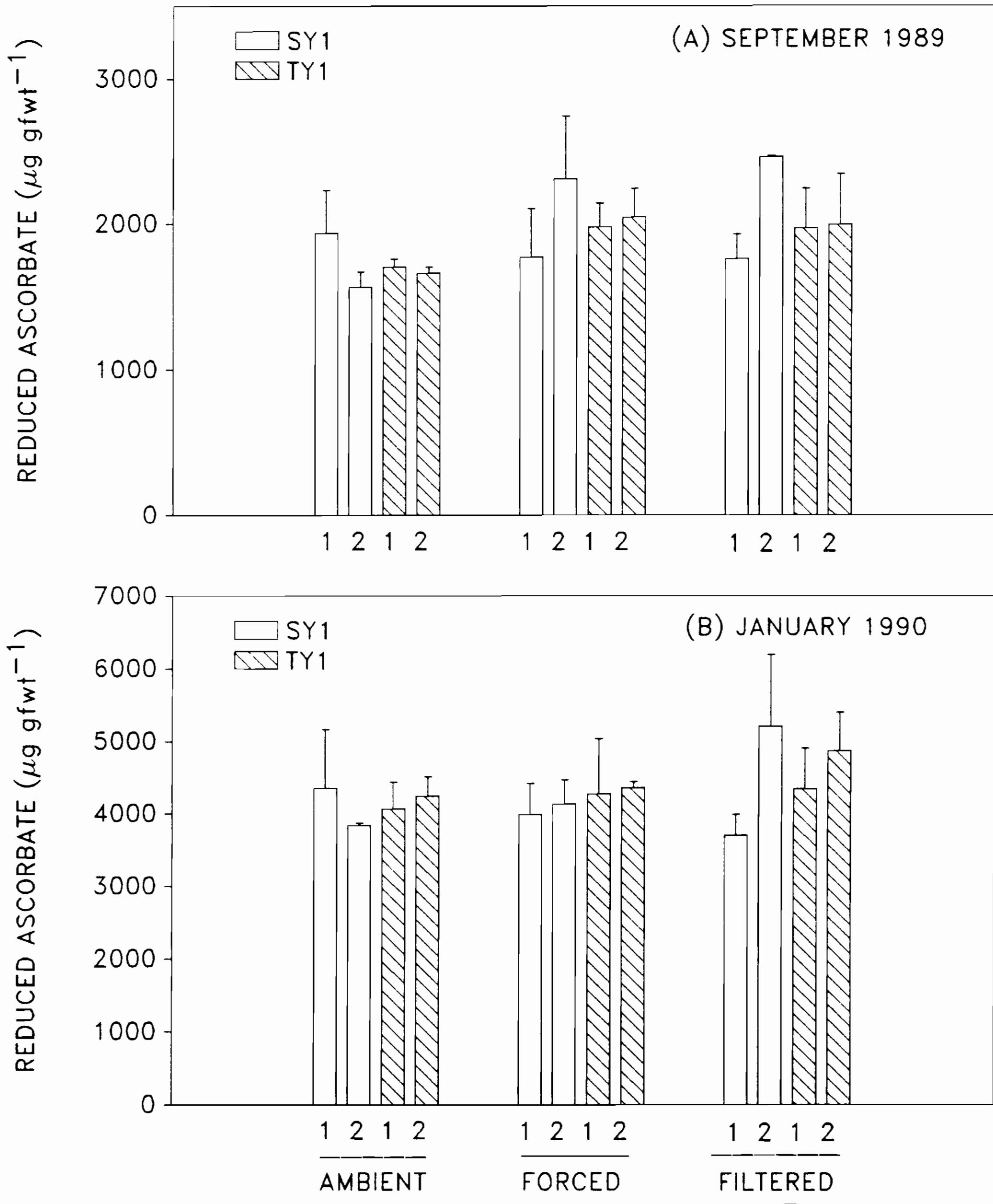


Figure 3.2 Comparison of the ascorbate concentration in needles of sensitive (SY1) and tolerant (TY1) clones of eastern white pine grown in either ambient, non-filtered (forced), and charcoal-filtered (filtered) open-top chambers. Samples were taken in September, 1989 (A) and January, 1990 (B). Error bars represent 95% confidence limits (n=3) for values in each treatment replicate chamber (1 vs 2). Genotypes of pine are classified based on visible foliar characteristics.

Table 3.1 Comparison of the mean (n=6) ascorbate (reduced and oxidized) concentrations in needles from eastern white pine clones grown either in ambient (AA), non-filtered (FA), or charcoal-filtered (CFA) open-top chambers. Genotypes classified based on visible foliar characteristics.

		SENSITIVE		TOLERANT	
DATE	**TRT**	**REDUCED**	**OXIDIZED**	**REDUCED**	**OXIDIZED**
			($\mu g\ gfwt^{-1}$)		
9-89	AA	1791 ± 275**	292 ± 159	1689 ± 45	344 ± 167
9-89	FA	2096 ± 417	209 ± 59	2018 ± 142	168 ± 48
9-89	CFA	2042 ± 398	154 ± 18	1984 ± 247	180 ± 43
1-90	AA	4096 ± 534	ND	4134 ± 270	ND
1-90	FA	3871 ± 512	ND	4329 ± 284	ND
1-90	CFA	4306 ± 913	ND	4606 ± 522	ND

** Mean value ± standard deviation.
ND = Not detectable.
TRT = Chamber treatment.

either grown under ambient air or in charcoal-filtered chambers supplemented with 300 $\mu g\ m^{-3}$ O_3 compared to seedlings grown in charcoal-filtered chambers alone. The authors also reported a slight decrease in the concentration of cellular ascorbate from needles grown under ambient and O_3 supplemented chambers. The data from this study showed no apparent significant differences in total ascorbate content when comparing needles of sensitive or tolerant clones exposed to either ambient or charcoal-filtered air (Table 3.1). The contrast between Castillo et al. (1987) and our ascorbate data may be a reflection of the lower O_3 concentration which eastern white pine clones were exposed to during 1989.

The ascorbate content in sensitive 1989-90 year needles taken from ambient, non-filtered and charcoal-filtered plots, in January, 1990 ranged between 3837-4354 μg, 3989-4139 μg and 3706-5207 $\mu g\ gfwt^{-1}$, respectively (Figure 3.2, B). No appreciable concentration of dehydroascorbate could be detected in any of the sensitive or tolerant needles in January. The ascorbate content in tolerant 1989-90 year needles taken from ambient, non-filtered and charcoal-filtered plots, in January, 1990 ranged between 4064-4240 μg, 4277-4363 μg and 4343-4870 $\mu g\ gfwt^{-1}$ (Figure 3.2, B).

The mean (n=6) ascorbate content in sensitive and tolerant needles taken from charcoal-filtered plots were

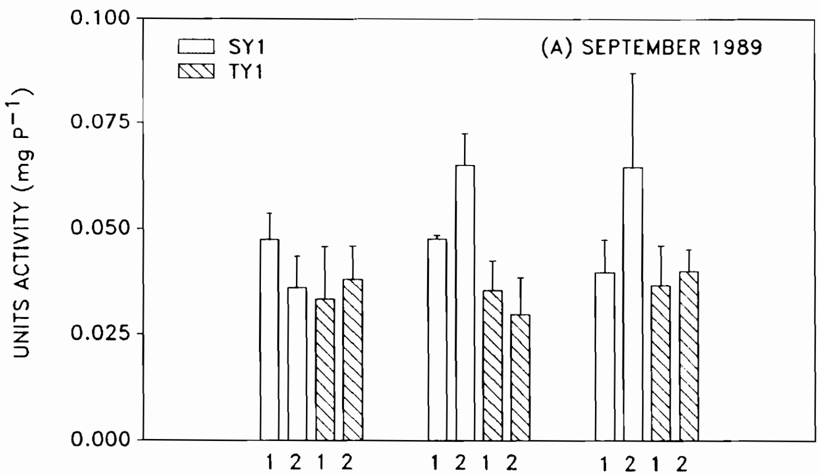
slightly greater than that seen in needles taken from either the ambient (5 and 10%, respectively) or non-filtered plots (10 and 6%, respectively), Table 3.1. These data indicate that exposure to air pollutants during the summer growing season may reduce winter ascorbate content in needles.

The ascorbate values observed in the needles of sensitive and tolerant clones taken from ambient chambers in September and January were similar to those observed in needles taken from the parent trees during the same time period. These data indicate that needle metabolite concentrations in the cloned material is parallel to that of the parent plants.

NEEDLE ASCORBATE PEROXIDASE ACTIVITY

Differences in ascorbate peroxidase activity were observed in the September, 1989-90 sensitive year needles when comparing effects due to chamber treatments. Needles taken from the sensitive clones in the ambient chamber during September had less ascorbate peroxidase activity than did needles taken from similar plants in the non-filtered and charcoal-filtered chambers (Figure 3.3, A). However, needles from the clones of the tolerant tree, sampled from all chambers in September, 1989, showed no apparent differences in ascorbate peroxidase specific activity (Figure 3.3, A). Differences were observed in ascorbate peroxidase specific activity when comparing sensitive and tolerant

Figure 3.3 Ascorbate peroxidase specific activity (A and B) and total activity $gfwt^{-1}$ (C and D) in needles of sensitive (SY1) and tolerant (TY1) clones of eastern white pine grown in either ambient, non-filtered (forced) and charcoal-filtered (filtered) open-top chambers in September, 1989 (A and C) and January, 1990 (B and D). Error bars represent 95% confidence limits (n=3) for values in each treatment replicate (1 vs 2). Genotypes of pine are classified based on visible foliar characteristics.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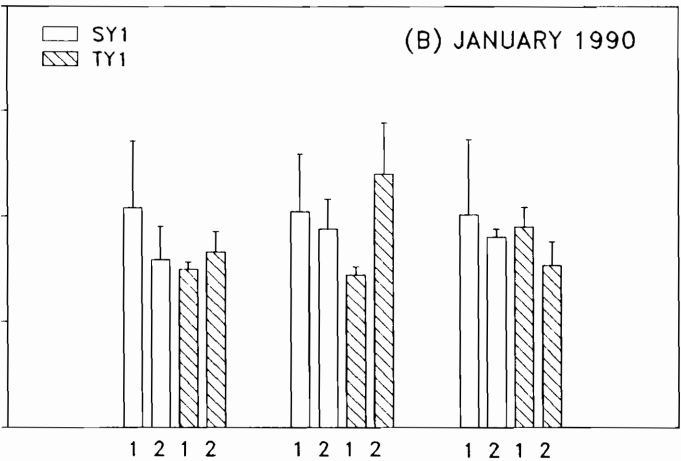
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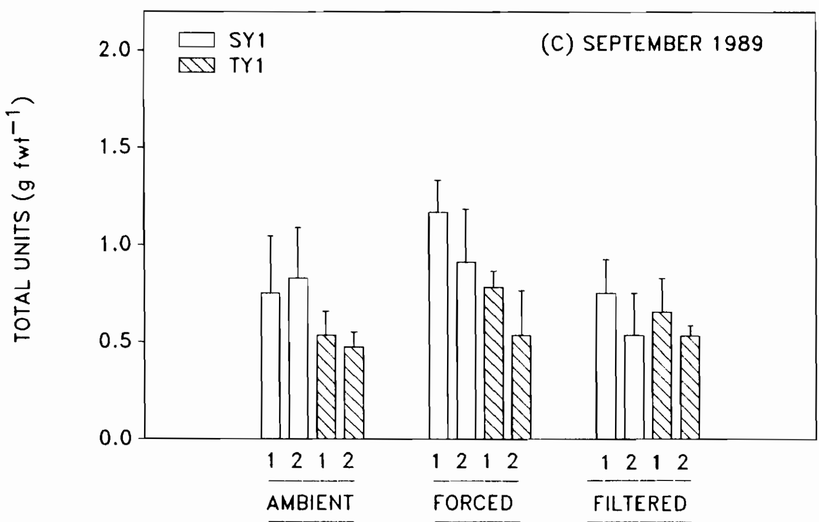
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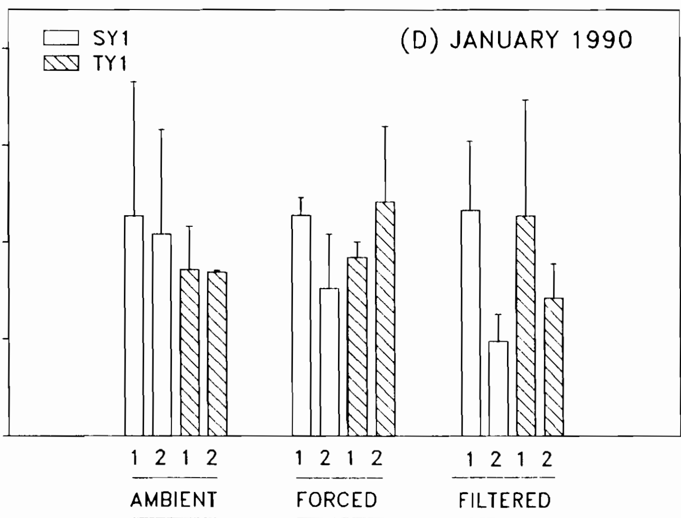
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needles taken from ambient chambers. The range of ascorbate peroxidase specific activity was greater in needles taken from the sensitive clones in the non-filtered and charcoal-filtered chambers compared to tolerant needles taken from the same chambers (Figure 3.3, A). Based on the total units of ascorbate peroxidase activity $gfwt^{-1}$, no differences were observed when comparing sensitive (0.534-0.751) and tolerant (0.530-0.654) needles taken from charcoal-filtered chambers (Figure 3.3, C). Total activity however, was greater in the sensitive needles taken from the ambient (0.748-0.823) and non-filtered chambers (0.909-1.16) compared to tolerant needles taken from the same chambers (0.471-0.534 and 0.534-0.780, respectively, Figure 3.3, C). This observation was significant in that needles from the sensitive parent tree grown under field conditions had two-fold more total units of ascorbate peroxidase activity $gfwt^{-1}$ than did needles from the tolerant parent tree in September, 1989. The fact that sensitive needles had more total enzyme activity only when grown in the ambient and non-filtered chambers (Table 3.2) indicates that the response could be due to increased oxidative stress caused by ambient air pollutants. Only in the sensitive clones was total ascorbate peroxidase activity increased. This data agrees well with that reported by Castillo et al. (1987) who observed a two- to three-fold increase in the peroxidase activity of Norway spruce during

Table 3.2 Comparison of the mean values (n=6) of ascorbate peroxidase activity based both on specific activity and on total units $gfwt^{-1}$ in needles of sensitive and tolerant clones of eastern white pine taken from either ambient (AA), non-filtered (FA), or charcoal-filtered (CFA) open-top chambers. Genotypes of pine are classified based on visible foliar characteristics.

		SENSITIVE		TOLERANT	
DATE	TRT	SPECIFIC ACTIVITY	TOTAL UNITS	SPECIFIC ACTIVITY	TOTAL UNITS
			($x\ 10^{-2}$)		
9-89	AA	4.1 ± 0.8**	78 ± 22	3.5 ± 0.8	51 ± 9
9-89	FA	5.8 ± 1.0	101 ± 23	3.2 ± 0.6	63 ± 19
9-89	CFA	4.9 ± 2.1	66 ± 17	3.8 ± 0.6	59 ± 21
1-90	AA	4.5 ± 1.2	108 ± 49	3.9 ± 0.3	85 ± 14
1-90	FA	4.8 ± 0.6	91 ± 27	5.0 ± 1.5	109 ± 29
1-90	CFA	4.8 ± 1.1	89 ± 43	4.2 ± 0.6	92 ± 41

** Mean value ± standard deviation.
TRT = Chamber treatment.

long-term exposure of Norway spruce to 300 $\mu g\ m^{-3}\ O_3$ in charcoal-filtered open-top chambers. The authors reported that the increase in peroxidase activity occurred in the intercellular and cellular fluid of both current and one-year-old needles. They also reported a significant increase in the intercellular and cellular peroxidase activity in Norway spruce exposed to only ambient air, although the increase was less than that observed in the needles of O_3 supplemented chambers.

No appreciable differences in specific or total ascorbate peroxidase activity were observed between sensitive and tolerant needles in any of the chambers during January, 1990. (Figure 3.3, B and D). One exception was that the total activity in the sensitive needles taken from charcoal-filtered plot 2 were greatly reduced (Figure 3.3, D). The reduction seen in ascorbate peroxidase activity in the sensitive needles of charcoal-filtered plot 2 were attributed to an overall decline in the health of the cloned material.

The seasonal change in needle ascorbate peroxidase activity between September, 1989 and January, 1990, in cloned sensitive and tolerant plants taken from ambient chamber plots, were similar to those described in the needles of the sensitive and tolerant parent trees (Chapter 2) with the exception of total units of activity, which increased between summer and winter in the cloned material.

Also, the overall ascorbate peroxidase values reported in the cloned needles are between two- to three-fold less than those reported in the parent field needles.

NEEDLE GLUTATHIONE CONTENT

In September 1989, the 1989-90 year needles from the sensitive clone, in all chamber treatment combinations, showed no appreciable differences in total glutathione content ranging between 0.22 to 0.26 μmol gfwt^{-1} (Figure 3.4, A). The level of total glutathione in needles of the sensitive clone were approximately two-fold less than observed in the parent sensitive needles during the same time period. McLaughlin (1985) has suggested that physiological balances and the sensitivity of tree seedlings in chambers may differ from those of mature forest trees. The difference in total glutathione content between the cloned and parent sensitive needles in September was not due to diurnal fluctuations Rennenberg (1982), since the needles (parent and cloned) were always collected and extracted between 9:00 and 11:00 A.M. EST.

The needles from the tolerant clones did show differences in total glutathione content. In September 1989, the average total glutathione content in the tolerant needles, taken from the ambient and non-filtered chambers, was always greater than that seen in the sensitive needles taken from the same chambers (Figure 3.4, A). The tolerant needles taken from the non-filtered chamber showed 42% more

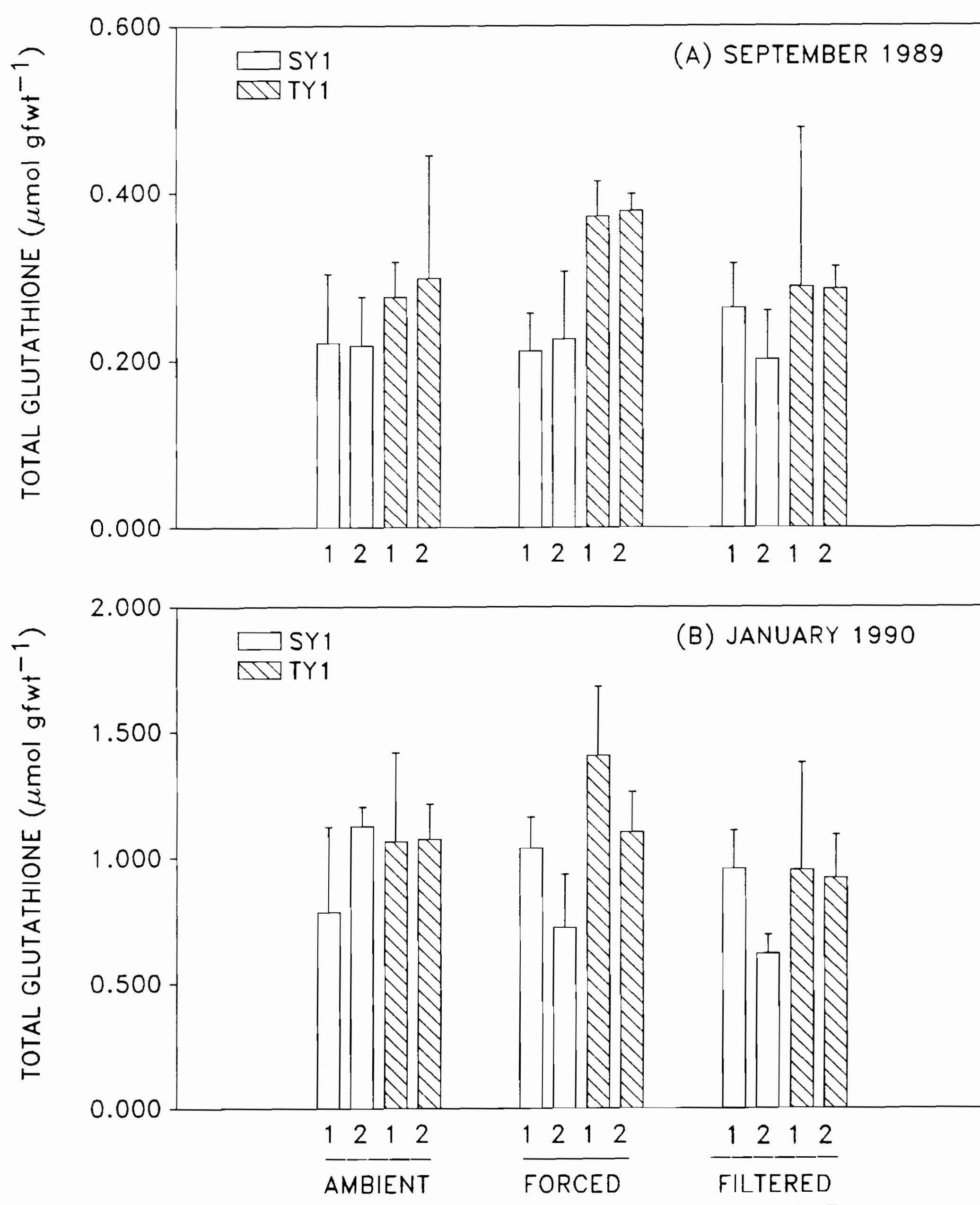


Figure 3.4 Comparison of GSH concentration in needles of sensitive (SY1) and tolerant (TY1) clones of eastern white pine grown in either ambient, non-filtered (forced) and charcoal-filtered (filtered) open-top chambers. Samples were taken in September, 1989 (A) and January, 1990 (B). Error bars represent 95% confidence limits (n=3) for values in each treatment replicate (1 vs 2). Genotypes of pine are classified based on visible foliar characteristics.

total glutathione content compared to sensitive needles taken from the same chambers. The tolerant needles taken from the ambient chamber showed only 23% more total glutathione compared to the sensitive needles and was similar to that observed when comparing total glutathione content from the parent needles.

The level of GSSG in needles of both the sensitive and tolerant clones grown in open-top chambers was not detectable with the amount of needle tissue used (generally less than 250 mg). Sample size was limited by the amount of current year needle growth in 1989. For this reason it was not possible to compare differences in GSH/GSSG ratios in the cloned material.

Since needles of the tolerant clone, taken from the ambient and non-filtered chambers, had greater total mean (n=6) glutathione content than needles of the sensitive clone, and no difference was observed in the mean glutathione content of needles taken from the charcoal-filtered chambers (Table 3.3), tolerant needles may have the capacity to accumulate increased glutathione levels when exposed to air pollutants. This data is in contrast to Mehlhorn et al. (1986) who observed a decrease in glutathione in the current year needles of spruce when exposed to 0.037 $\mu l/L$ O_3 for 12 hours daily in charcoal-filtered open-top chambers for 2 years. However, the authors did report an increase in the glutathione content of one-year-old needles

Table 3.3 Comparison of the mean (n=6) GSH concentration in needles of sensitive and tolerant clones of eastern white pine grown in either ambient (AA), non-filtered (FA), or charcoal-filtered (CFA) open-top chambers. Genotypes of pine are classified based on visible foliar characteristics.

		TOTAL GSH	
DATE	**TREATMENT**	**SENSITIVE**	**TOLERANT**
		(μmol gfwt^{-1})	
9-89	AA	0.219 ± 0.055**	0.285 ± 0.060
9-89	FA	0.218 ± 0.052	0.375 ± 0.019
9-89	CFA	0.234 ± 0.051	0.287 ± 0.106
1-90	AA	0.955 ± 0.208	1.070 ± 0.226
1-90	FA	0.881 ± 0.222	1.220 ± 0.215
1-90	CFA	0.822 ± 0.208	0.938 ± 0.255

** Mean value ± standard deviation.

exposed to the same conditions.

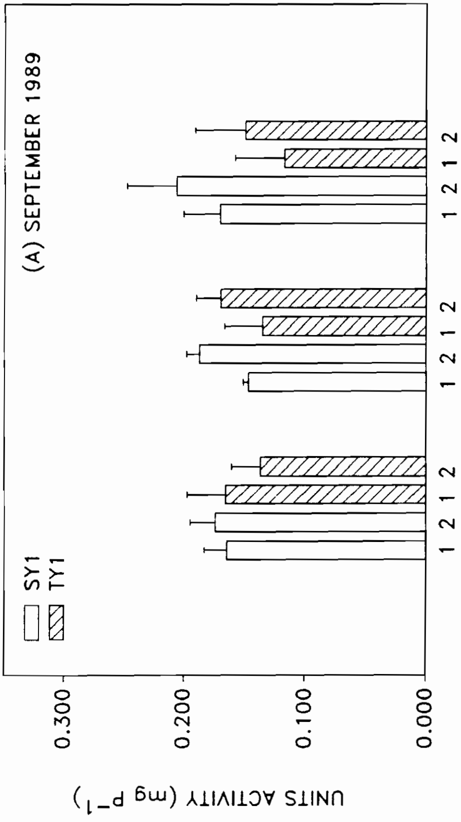
Total glutathione content in needles taken from ambient plots in January, 1990 ranged between 0.79 to 1.12 μmol gfwt^{-1} (Figure 3.4, B) and was similar to the values observed in the sensitive and tolerant parent needles in January. Again the total glutathione content was slightly greater in needles taken from clones in the non-filtered plots compared to the sensitive needles taken from the same chamber plots (1.10-1.40 μmol gfwt^{-1} vs 0.72-1.04 μmol gfwt^{-1}, respectively).

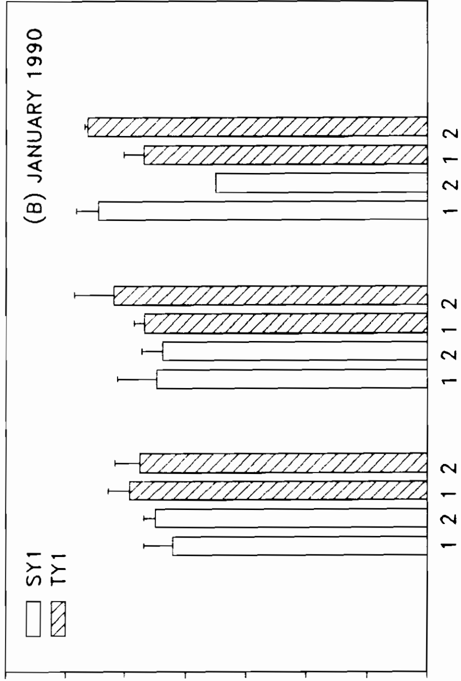
NEEDLE GLUTATHIONE REDUCTASE ACTIVITY

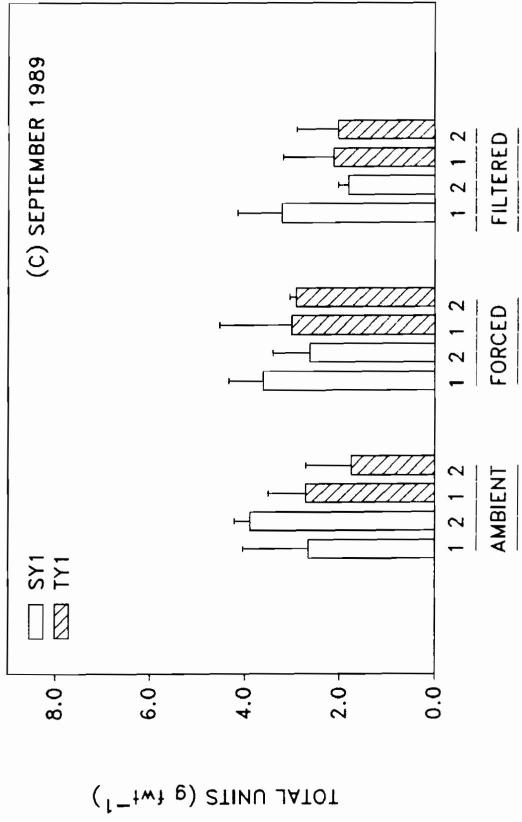
In September, 1989, the specific activity of glutathione reductase in needles taken from the sensitive clones in the forced ambient and charcoal-filtered chambers was slightly greater than that of the tolerant clones grown in the same chambers (Figure 3.5, A). However, based on the mean value (n=6), both the specific activity and the total units of glutathione reductase was greater in needles of the sensitive clones compared to needles of the tolerant clones in all chamber treatments in September, 1989 (Table 3.4). The specific activity of glutathione reductase in needles of cloned trees taken from the ambient chambers in September, 1989 was very similar to the specific activity observed in the needles of the parent trees during the same time period.

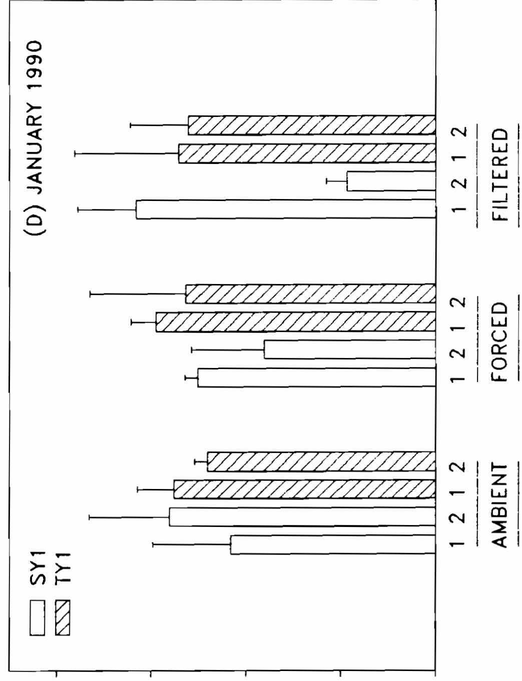
In January, 1990, needles of the sensitive and tolerant

Figure 3.5 Glutathione reductase specific activity (A and B) and total activity $gfwt^{-1}$ (C and D) in needles of sensitive (SY1) and tolerant (TY1) clones of eastern white pine grown in either ambient, non-filtered (forced) and charcoal-filtered (filtered) open-top chambers. Samples were taken in September, 1989 (A and C) and January, 1990 (B and D). Error bars represent 95% confidence limits (n=3) for values in each treatment replicate (1 vs 2). Genotypes of pine are classified based on visible foliar characteristics.


(A) SEPTEMBER 1989
SY1
TY1
1 2 1 2
1 2 1 2
1 2 1 2
0.300
0.200
0.100
0.000
UNITS ACTIVITY (mg P-1)


(B) JANUARY 1990
SY1
TY1
1 2 1 2
1 2 1 2
1 2 1 2


(C) SEPTEMBER 1989
SY1
TY1
1 2 1 2
AMBIENT
1 2 1 2
FORCED
1 2 1 2
FILTERED
8.0
6.0
4.0
2.0
0.0
TOTAL UNITS (g fwt-1)


(D) JANUARY 1990
SY1
TY1
1 2 1 2
AMBIENT
1 2 1 2
FORCED
1 2 1 2
FILTERED

clones taken from the open-top chamber plots showed no appreciable differences in glutathione reductase activity (Figure 3.5, B and D). One exception was that the total glutathione reductase activity in the sensitive needles taken from charcoal-filtered plot 2 was 70% less than that in the sensitive needles taken from charcoal-filtered plot 1. This reduction in activity was attributed to an overall reduction in the health of the cloned trees. Based on the mean value (n=6) both the specific and total units of glutathione reductase was greater in the needles of the tolerant clones compared to needles of the sensitive clones in all chamber plots during January, 1990 (Table 3.4).

In September, 1989, the specific activity of glutathione reductase was slightly greater (based on mean values) in the needles of the sensitive clones (Table 3.4). In contrast in January, 1990, the specific activity of glutathione reductase was slightly greater in needles of the tolerant than in the needles of the sensitive clone. This data may indicate that the synthesis of new isozymes of glutathione reductase occurs during the winter which have different affinity (K_ms) for their substrates. This difference would be consistent with the synthesis of new isozymes of glutathione reductase, which have different K_m values, observed by Guy and Carter (1984) in spinach leaves in response to cold acclimation.

The specific activity of glutathione reductase in the

Table 3.4 Comparison of the mean values (n=6) of glutathione reductase activity based both on specific activity and on total units $gfwt^{-1}$ in needles of sensitive and tolerant clones of eastern white pine taken from either ambient (AA), non-filtered (FA), or charcoal-filtered (CFA) open-top chambers. Genotypes of pine are classified based on visible foliar characteristics.

		SENSITIVE		TOLERANT	
DATE	TRT	SPECIFIC ACTIVITY	TOTAL UNITS	SPECIFIC ACTIVITY	TOTAL UNITS
			($x\ 10^{-2}$)		
9-89	AA	16.9 ± 1.6**	327 ± 103	15.4 ± 2.6	233 ± 79
9-89	FA	17.1 ± 2.3	302 ± 77	15.5 ± 2.5	293 ± 56
9-89	CFA	18.4 ± 3.0	265 ± 96	13.3 ± 3.6	207 ± 77
1-90	AA	21.7 ± 1.6	495 ± 150	24.2 ± 1.4	522 ± 62
1-90	FA	22.0 ± 1.5	416 ± 122	24.8 ± 2.5	551 ± 133
1-90	CFA	23.3 ± 5.4	453 ± 255	25.8 ± 2.8	530 ± 141

** Mean value ± standard deviation.
TRT = Chamber treatment.

cloned trees in January, 1990 was approximately two-to three-fold less than that observed in needles of the parent trees during the same time period. However, the total units of glutathione reductase activity gfwt^{-1} in the cloned trees in January, 1990 was approximately two-fold greater than that of the parent trees. This data indicates a greater extractable protein content from the needles of the cloned material in January, 1990. Although needles of the cloned trees showed more extractable protein gfwt^{-1}, the increase in total glutathione reductase gfwt^{-1} between September, 1989 and January, 1990 was approximately two-fold in needles from both cloned and parent trees, and indicates that the glutathione reductase response to low temperature was similar in both parent and cloned material.

VISIBLE NEEDLE CHARACTERISTICS

It was observed that needle tip burn in eastern white pine may be due to factors other than O_3 injury. The cloned tolerant trees grown in the charcoal-filtered chambers often showed the greatest degree of needle tip burn, whereas, the cloned sensitive trees showed no needle tip burn. This data is in contrast to that previously reported (see chapter 2, introduction). No apparent differences in needle growth or injury were observed due to the different treatment combinations. These data again may be a reflection of the low O_3, non-drought growing season of 1989.

OTHER FACTORS AND COMMENTS

The data presented in this chapter are only preliminary observations of the first year's needle growth. These data also were collected during a low O_3, non-drought season (Chapter 2, Figure 2.2, A and B). The limited sampling periods and the low O_3 season may explain the difficulty in discerning appreciable differences among treatment chambers. Each successive year of growth will allow for more frequent samplings during the active growing season in both current and one-year-old needles.

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CHAPTER 4

IMMUNOLOGICAL PROPERTIES FOR TWO PURIFIED ISOFORMS OF GLUTATHIONE REDUCTASE AND THE INDUCTIVE RESPONSE OF THIS ENZYME TO O_3 IN DIFFERENT GENOTYPES OF EASTERN WHITE PINE

INTRODUCTION

Plants have demonstrated altered antioxidant levels when exposed to air pollutants, low temperature, drought, or xenobiotics such as herbicides (for review see Alscher, 1989). These plant-stress interactions, at one time or another, have been associated with increased cellular concentrations of superoxide anion radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Superoxide radicals and H_2O_2 can directly attack lipid membranes and certain sulfhydryl-containing enzymes of the reductive pentose cycle (Nakano and Asada, 1981), or they can further interact to generate hydroxyl radicals (OH•) which also can react with membrane lipids and proteins (Alscher and Amthor, 1988; Smith et al, 1990). Such alterations to cell metabolism and membrane biochemistry would limit normal growth and vigor. Mechanisms for the scavenging of toxic free radicals have previously been reviewed in the plant literature (Alscher, 1989; Alscher and Amthor, 1988; Badger, 1985; Halliwell, 1984; Smith et al, 1990).

The univalent reduction of oxygen to $O_2^{\bullet -}$ via the pseudocyclic flow of electrons (Mehler reaction) naturally occurs in photosynthetically active, illuminated chloroplasts. Consequently, when the ratio of $NADPH/NADP^+$ is high, or when carbon fixation is not optimal, O_2, in the presence of thylakoids, can compete with $NADP^+$ as a Hill reductant (Badger, 1985; Halliwell, 1984). CO_2 fixation can be limited by factors such as short-term exposure to O_3 which results in the inactivation and decrease of ribulose bisphosphate carboxylase oxygenase in potato foliage (Dann and Pell, 1989). Also, during water deficits, when stomata close to limit water loss, the reduction in internal CO_2 leads to CO_2-limited carbon fixation. Such conditions could decrease the availability of $NADP^+$ as an electron acceptor for PS I and thus enhance O_2 uptake. Therefore, endogenous antioxidants, and their metabolism, could both reduce the toxic effects of activated oxygen species, and maintain a turnover of NADPH, thereby providing inherent tolerance to oxidative stress.

Glutathione, a ubiquitous free thiol-containing tripeptide in plants, possesses antioxidant properties which can protect plant membranes and protein thiols against oxidation by $O_2^{\bullet -}$ and H_2O_2 (Alscher, 1989; Smith et al, 1990). Glutathione reductase (EC 1.6.4.2) catalyzes the reduction of oxidized (GSSG) to reduced (2 GSH) glutathione in an NADPH-dependent reaction which can maintain GSH/GSSG ratios

of 10:1 in the chloroplast (Halliwell and Foyer, 1978). Glutathione reductase is also involved in the ascorbate-glutathione cycle for the scavenging of H_2O_2 (Halliwell and Foyer, 1978; Nakano and Asada, 1981). This pathway functions in the chloroplast (Halliwell, 1984) and may also occur in the cytosol (Bielawski and Joy, 1986; Castillo and Greppin, 1988; Klapheck et al, 1987). Increased cellular glutathione and glutathione reductase has been reported in plants which show tolerance to air pollutants (Mehlhorn et al, 1987; Tanaka et al, 1988; Yang et al, 1983), cold stress (de Kok and Oosterhuis, 1983; Esterbauer and Grill, 1978; Guy and Carter, 1984; Guy et al, 1984), drought (Gamble and Burke, 1984), and O_2-enriched air (Foster and Hess, 1982).

Glutathione reductase has been isolated from plant chloroplasts (Bielawski and Joy, 1986; Connell and Mullet, 1986; Kalt-Torres et al, 1984; Mahan and Burke, 1987; Schaedle and Bassham, 1977), roots (Bielawski and Joy, 1986), whole leaf (Halliwell and Foyer, 1978; Wingsle, 1989), and seeds (Ida and Morita, 1971; Mapson and Isherwood, 1963). Purified or partially purified glutathione reductase generally shows a K_m for NADPH between 1-13 μM and a K_m for GSSG of between 10-200 μM at 25^oC. These reports also have indicated large variations among glutathione reductase native molecular weights (106-190 kD), subunit molecular weight (41-72 kD) and pH optimum (7.6-9.0).

In only a few reports are different isozymes of glutathione reductase described. Guy and Carter (1984) reported that cold-hardened spinach showed increase glutathione reductase activity and had different kinetic and molecular forms of glutathione reductase than did non-hardened tissue. Also, plastidic and cytoplasmic isoforms of glutathione reductase have been separated from mustard cotyledons. These isoforms show different characteristics when exposed to light and photooxidative conditions (Drumm-Herrel et al, 1989). These data suggest that plants contain different isozymes of glutathione reductase which may be stimulated by different environmental signals. Tanaka et al (1988) have reported that increased glutathione reductase activity in spinach, exposed to O_3, was due to biosynthesis of the enzyme but not due to synthesis of new isoforms.

The objectives of this study were to isolate, purify and characterize glutathione reductase from eastern white pine needles. Another objective of this study was to use antibody, produced from purified pine glutathione reductase, to determine if O_3 exposure of sensitive and tolerant clones (based on foliar symptomatology) caused the induction of the enzyme.

METHODS AND MATERIALS

Plant Material

For the purification of glutathione reductase current year needles from a 30-year-old Eastern white pine, growing on the campus of Virginia Polytechnic Institute and State University, were collected between August and October, 1989.

Clones of sensitive and tolerant eastern white pine were used for CSTR O_3 fumigations. All clones were obtained as described in Chapter 3 Methods and Materials. The cloned material was transferred from a lathhouse to a greenhouse in early January, 1990 which allowed for bud to occur. The greenhouse was supplied with charcoal-filtered air to remove any ambient air pollutants. Clones were used for O_3 fumigation in late April, 1990 after full expansion of the new needles had occurred.

Ozone-Fumigation

Fumigations in CSTR-chambers were conducted at the Air Pollution Laboratory at Virginia Polytechnic Institute and State University. Cloned material was equilibrated in the CSTR-chambers for 16 hr prior to starting the fumigations. Ozone fumigations occurred for 16 hrs day^{-1} for 3 consecutive days. Cloned material was either exposed to <0.025 ppm O_3 (controls) or to a step function O_3 profile that peaked at 0.120 ppm for 2 hrs each day (total O_3 dose

of 4.48 ppm•hr during light period). Fumigations started at 500 EST and continued until 2000 EST. Two replicate seedlings/clone were exposed to each O_3 concentration. Temperature during the fumigations was maintained at 25°C ± 1.5°C, with a RH of 55% ± 10% (vapor pressure deficit of 1.5 KPa), and a PPFD of 575 ± 25 μmol $m^{-2}s^{-1}$. Ozone was generated from O_2 by UV irradiation (Welsbach Laboratory Ozonator Model T-408, Welsbach Ozone Systems Corporation, Philadelphia, Pa) and monitored with a Teco UV O_3 Analyzer (Model 49s, Thermo Electron Corporation, Environmental Instruments Division, Hopkinton, Mass) calibrated with a Photocal 3000 Automated O_3 calibrator (Columbia Scientific Industries, Austin, Tex).

Enzyme Purification

All steps in the purification were carried out at 4°C except for chromatography by fast protein liquid chromatography (FPLC) and high performance liquid chromatography (HPLC) which were performed at 20 to 22°C.

Needles (300 g fresh weight) were crushed in liquid N_2 using a chilled mortar and pestle. The needles were homogenized with a Polytron homogenizer (Brinkmann Instrument Co., Westbury, NY) at top speed for 3 min in 1 l 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 polyvinylpyrrolidone mol wt 10,000 (PVP-10), and 1% insoluble polyclar-AT. The homogenate was filtered through 8 layers

of cheesecloth. The remaining soluble material in the cheesecloth was reextracted with an additional 1 L of extraction buffer. The filtrates were pooled and remaining insoluble material was removed by centrifugation for 30 min at 22,000 x g in a fixed angle rotor (Beckman JA-14).

The supernatant was brought to 35% saturation with solid ammonium sulfate and stirred for 30 min. After centrifugation at 22,000 x g for 30 min the resulting supernatant was brought to 75% saturation with additional solid ammonium sulfate and stirred for 1 hr. Following centrifugation at 22,000 x g for 35 min the residue was resuspended in 150 ml of buffer A: 50 mM Tris-HCL (pH 7.5), 1 mM EDTA, and 0.1 mM DTT. The resuspended residue was reprecipitated by making it 1 M with solid ammonium sulfate and stirring for 15 min. Centrifugation at 22,000 x g for 20 min removed the dark green pigmentation. The resulting supernatant was brought to 2 M saturation with additional solid ammonium sulfate which produced a pale yellow precipitate. The precipitate was removed from solution by centrifugation at 22,000 x g for 20 min and resuspended in 30 ml of buffer A.

After desalting on Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden), samples containing glutathione reductase activity were applied to a 1.5 x 8 cm column of 2′,5′-ADP-Sepharose 4B (Pharmacia Fine Chemicals) equilibrated with buffer A. After washing the column with 30 ml

of buffer A, followed by an additional 30 ml wash with 25 mM NaCl in buffer A, the $NADP^+$-dependent enzymes were eluted with a 5 ml pulse of 10 mM $NADP^+$. The column was washed with an additional 20 ml of buffer A followed by 20 ml of 500 mM NaCl in buffer A.

Fractions containing glutathione reductase activity were pooled, concentrated (Amicon model 52, having a cut-off filter of 10 kD), and dialyzed overnight against 4 l of buffer B: 20 mM Tris-HCl (pH 7.5), containing 1 mM EDTA. The sample was filtered through a 22 micron Acrodisk (Gel-man Sciences Inc., Ann Arbor, Michigan) and chromatographed on anion-exchange chromatography. Individual peaks of glu-tathione reductase activity (2 peaks, GR_A and GR_B), eluted from anion-exchange chromatography, were made 1 M by adding solid ammonium sulfate. The samples were individually applied to a hydrophobic interaction column (Phenyl-Superose HR 5/5, Pharmacia Fine Chemicals) equilibrated with 1 M ammonium sulfate in buffer B (buffer C).

GR_A and GR_B were individually concentrated to 500 μl and separately applied to a gel filtration column (Super-ose-12, Pharmacia Fine Chemicals) equilibrated with buffer D: 50 mM Tris-HCL (pH 7.5), and 10 mM NaCl. After gel fil-tration both isoforms were reapplied individually to an anion-exchange column (Mono Q HR 5/5) equilibrated with 20 mM Tris-HCL (pH 9.0), containing 1 mM EDTA (buffer E). Factions containing glutathione reductase activity were

pooled and stored at -4°C in buffer E.

Protein Extraction of Fumigated Needles

Needles collected during the O_3 fumigation were extracted as described in chapter 2 Methods and Materials. Approximately 500 mg fresh tissue were collected for analysis of glutathione reductase activity prior to fumigation and at 24 and 72 hr during the exposure period.

Protein Determination

Protein was determined by the method of Bradford (4) using lyophilized BSA (BioRad, Richmond, CA) as standard.

Glutathione Reductase (EC 1.6.4.2) Assay

Glutathione reductase was assayed spectrophotometrically by monitoring the GSSG-dependent NADPH oxidation at 340 nm. Assays were performed at 25°C in a 1 ml reaction mixture of 50 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 1.5 mM NADPH, and 0.5 mM GSSG unless otherwise specified.

Preparation of Antisera

Antibody to purified GR_B (215 μgP) was prepared by Cocalico Biologicals, Reamstown, PA. The rabbit was given booster injections at 14 and 21 days and serum collected at 6 and 10 wks after the initial injection of antigen, contained sufficient levels of antibody.

Electrophoresis

SDS-PAGE (10% gels) and native-PAGE (7.5 and 10% gels) were done as described by Smith (1989). Visualization of proteins on gels was accomplished by silver staining as

described by Sasse (1989).

Glutathione reductase activity was detected on 7.5% native-PAGE gels by incubating them in 100 ml solution containing 10 mg MTT, 10 mg 2,6-dichlorophenolindophenol, 34 mM GSSG, 0.4 mM NADPH and 50 mM Tris (pH 7.5). Duplicate gels were assayed for glutathione reductase activity: one with and one without GSSG.

Western Blotting

Proteins were transferred from polyacrylamide gels to nitrocellulose using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (BioRad) at 20 volts for 15-30 min. Transfers were done using 48 mM Tris, 39 mM glycine, 0.130 mM SDS, and 20% MeOH (pH 9.2) as a transfer buffer. Transblots were probed with antisera produced from purified GR_B (anti-GR_B) and glutathione reductase was visualized on the membranes using a 1:100 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG (BioRad). Color development was done using 2.8 mM 4-chloro-1-naphthol (BioRad) and 0.03% H_2O_2.

Immunological Detection of Antigen

Antibody sensitivity and cross-reaction of native glutathione reductase with anti-GR_B were done using both ELISA and Slot-blot analysis. Serial dilutions of GR_B were visualized on ELISA plates using anti-GR_B and a 1:100 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG. Color development was done using 0.18 mM 2,2'-azino-di(3-

ethylbenzothiazoline sulfonate) and 0.09% H_2O_2. Serial dilutions of GR_A and GR_B bound to nitrocellulose by slot-blot application were visualized as previously described for Western transblot analysis.

Molecular Weight Determination

The subunit mol wt of glutathione reductase was determined by SDS-PAGE using prestained Electrophoresis mol wt standards (Diversified Biotech, Newton Centre, MA).

The native mol wt of the enzyme was determined by gel filtration chromatography using a 1.5 x 30 cm Superose 6 FPLC column (Pharmacia Fine Chemicals) and a 7.5 x 300 mm Bio-Sil TSK-250 (BioRad) HPLC Gel filtration column equilibrated with 0.1 M Na_2SO_4 and 0.02 M NaH_2PO_4 (pH 6.8). The columns were precalibrated with 50 μl of gel filtration mol wt standards (BioRad).

RESULTS AND DISCUSSION

PROTEIN PURIFICATION

The protein purification procedure used resulted in the isolation of two forms of glutathione reductase from eastern white pine needles. These two forms were labeled GR_A and GR_B. Purified GR_A had a specific activity of 1.81 μkat mgP^{-1} and represented a 170-fold purification (Table 4.1). Purified GR_B had a specific activity of 6.08 μKat mgP^{-1} which represented a 570-fold purification (Table 4.1).

TABLE 4.1: Purification of glutathione reductase from eastern white pine.

PURIFICATION STEP	SPECIFIC ACTIVITY μ Kat (mg P^{-1})	TOTAL ACTIVITY UNITS	PURIFICATION FACTOR	RECOVERY PERCENT
G-25 Sephadex	0.01	246	1	
2',5'-ADP Sepharose	0.41	253	38.1	103%
Mono Q (GR_A), pH 7.5	0.12	9.2	10.7	
Mono Q (GR_B), pH 7.5	1.05	116	98.7	A+B = 51%
Phenyl Superose (GR_A)	0.31	4.6	29.1	
Phenyl Superose (GR_B)	1.76	82.2	165	A+B = 35%
Superose-12 (GR_A)	0.45	3.2	41.2	
Superose-12 (GR_B)	4.8	76	443	A+B = 32%
Mono Q (GR_A), pH 9.0	1.81	2	109	
Mono Q (GR_B), pH 9.0	6.08	82	570	A+B = 28% [a]

[a] A total of 12 units of GR_A were applied; a total of 167 units of GR_B were applied.

The specific activity for GR_A is more representative of previously reported values for glutathione reductase isolated from plants which range from 0.43 to 4.1 μkat (Connell and Mullet, 1986; Halliwell and Foyer, 1978; Kalt-Torres et al, 1984; Mahan and Burke, 1987 Mapson and Isherwood, 1963); however, GR_B has a greater specific activity than that previously reported in the plant literature.

Affinity chromatography with 2',5'-ADP-Sepharose 4B resulted in a 38.1 fold purification step for the isolation of glutathione reductase (Table 4.1). Approximately 80-90% of the protein containing glutathione reductase activity was eluted with a 5 ml pulse of 10 mM $NADP^+$ (Figure 4.1). The remaining glutathione reductase activity was eluted from the affinity column with 500 mM NaCl. Wingsle (1989) reported that the majority of glutathione reductase from scots pine eluted from 2',5'-ADP-Sepharose 4B only after 500 mM NaCl was applied, a result in contrast to our results and previous reports (Guy and Carter, 1984; Halliwell and Foyer, 1978; Kalt-Torres et al, 1984) which indicated that the majority of glutathione reductase activity eluted with 10 mM $NADP^+$. The inability of scots pine glutathione reductase to elute from the affinity column with 10 mM $NADP^+$ may have been a result of using PVP-360 in the extraction medium (Wingsle, 1989). When we replaced PVP-10 with PVP-360 in our extraction buffer we also were unable to elute glutathione reductase from the affinity column

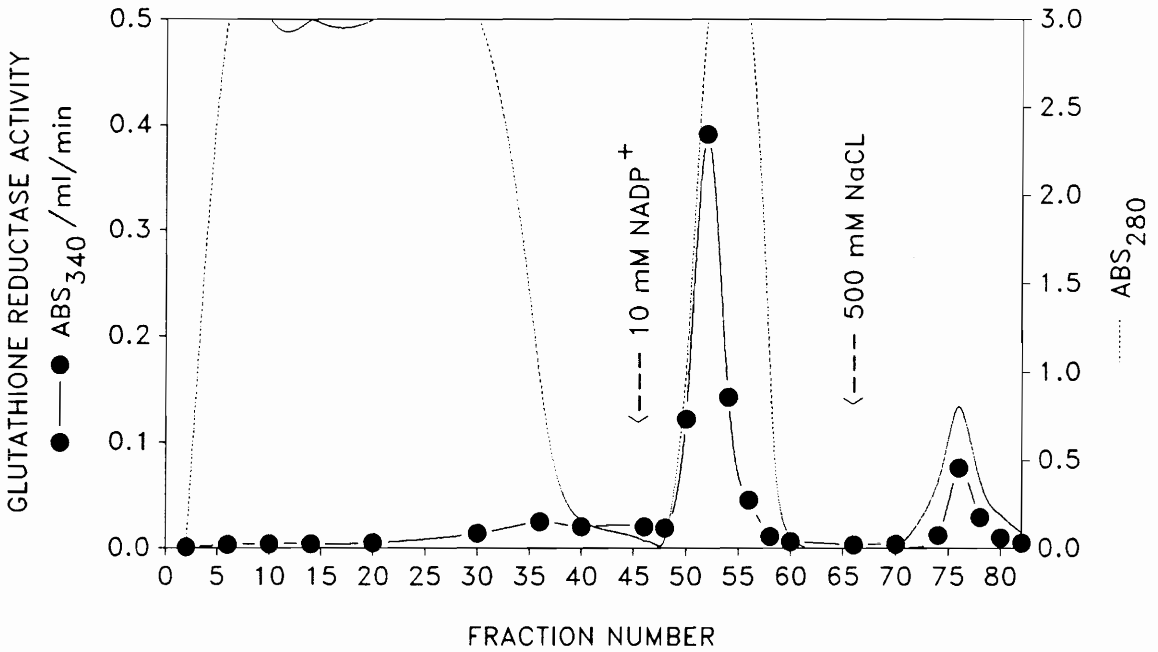

Figure 1.1 Chromatography profile of glutathione reductase from eastern white pine on 2'5'-ADP-Sepharose 4B. Glutathione reductase activity was first eluted with a 5 ml pulse of 10 mM DTT and 25 mM $NADP^+$. The remaining glutathione reductase activity was eluted with 20 ml of 500 mM NaCl. Each fraction represents 2 ml of eluant. The flow rate was 1 ml min^{-1}. Fractions 49-58 contained 253 total units of activity and fractions 74-81 contained 15.

10 mM $NADP^+$ and found that the enzyme only eluted after addition of 500 mM NaCl. We also observed a 4-fold decrease in the recovery of glutathione reductase from crude extracts when we replaced PVP-10 with PVP-360.

Anion-exchange with a Mono Q HR 5/5 column (FPLC) provided a simple and rapid method for the separation of different isoforms of glutathione reductase. Approximately 8% of the total glutathione reductase recovered from anion-exchange was GR_A and 83% was GR_B (Figure 4.2). The remaining 9-10% of glutathione reductase was recovered in the unbound fraction of activity (Figure 4.2). This unbound fraction of glutathione reductase activity was determined to be the same GR_A isoform which eluted from the anion-exchange column with 150 mM NaCl. This conclusion was obtained by comparing the migration patterns of the unbound fraction, GR_A and GR_B on native and SDS-PAGE gels. GR_A and the unbound fraction of glutathione reductase always showed the same migration patterns on native gels which were stained for either activity (Figure 4.3, A) or protein (Figure 4.3, B). Similar migration patterns were also seen for GR_A and the unbound fraction on SDS-PAGE gels (Figure 4.4, A). The specific activity of GR_A and the unbound fraction of glutathione reductase were also similar throughout all steps in the purification procedure.

Drumm-Herrel et al (1989) also used FPLC anion-exchange chromatography to isolate two peaks of glutathione

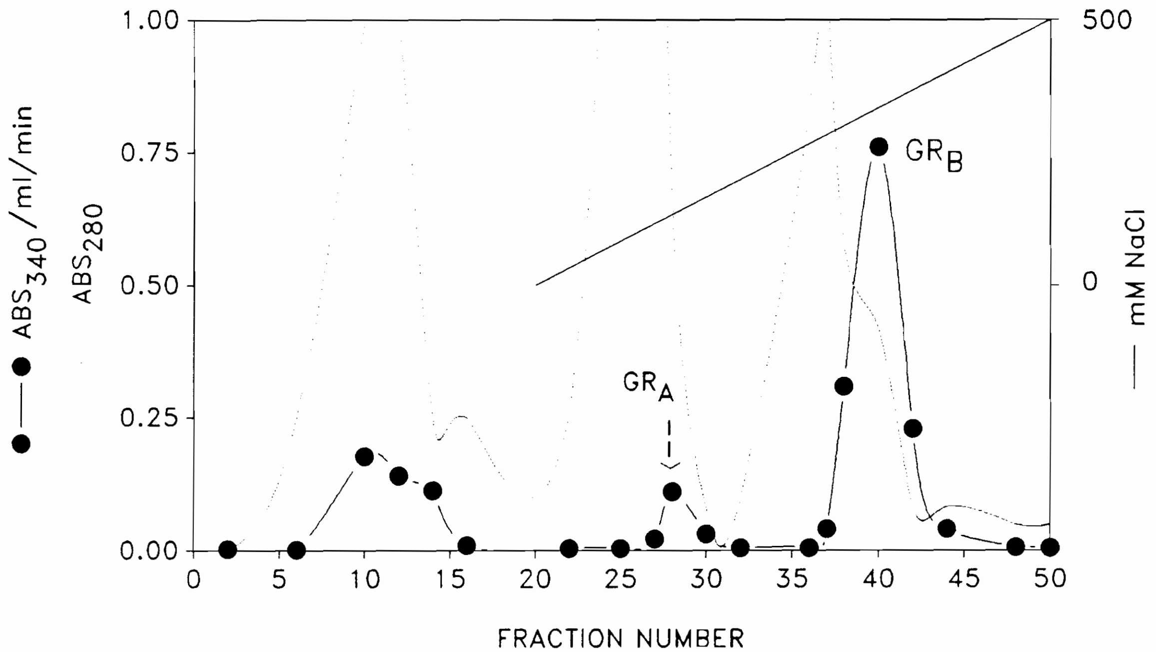

Figure 4.2 Chromatography profile of affinity purified glutathione reductase for eastern white pine on Mono Q HR 5/5 (FPLC) anion-exchange. A 4 ml prepared sample was applied to the column. The sample was eluted with 15 ml of a linear gradient of 0 to 500 mM NaCl. Solvents were delivered at a flow rate of 0.5 ml min^{-1}. Each fraction represents 500 μl of eluant. Fractions 9-15 contain 14.3 total units of activity, fractions 27-30 contain 9.1 total units and fractions 38-43 contain 115.5 total units of activity.

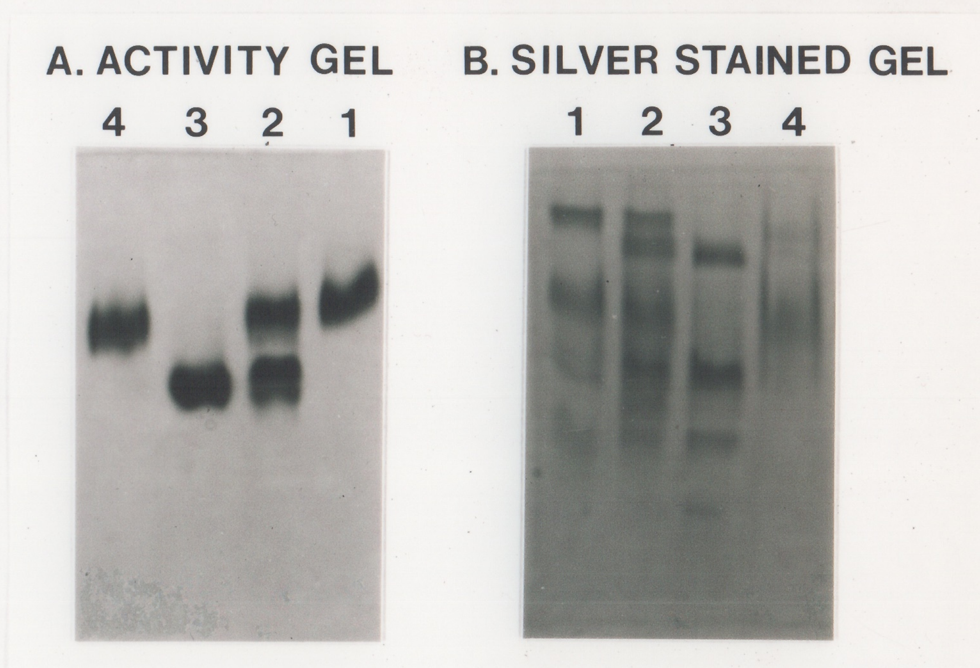

Figure 4.3 Electrophoretic separation of proteins from eastern white pine containing glutathione reductase activity on 7.5% native-PAGE gels. (A) Glutathione reductase activity stain. (B) Silver stain of gel. lane 1 = 1 μg protein of unbound fraction from anion exchange chromatography, lane 2 = 1 μg of protein recovered from 2'5'-ADP-Sepharose 4 B, lane 3 = 250 ng of protein (GR_B) recovered from anion-exchange chromatography, lane 4 = 250 ng of protein (GR_A) recovered from anion-exchange chromatography.

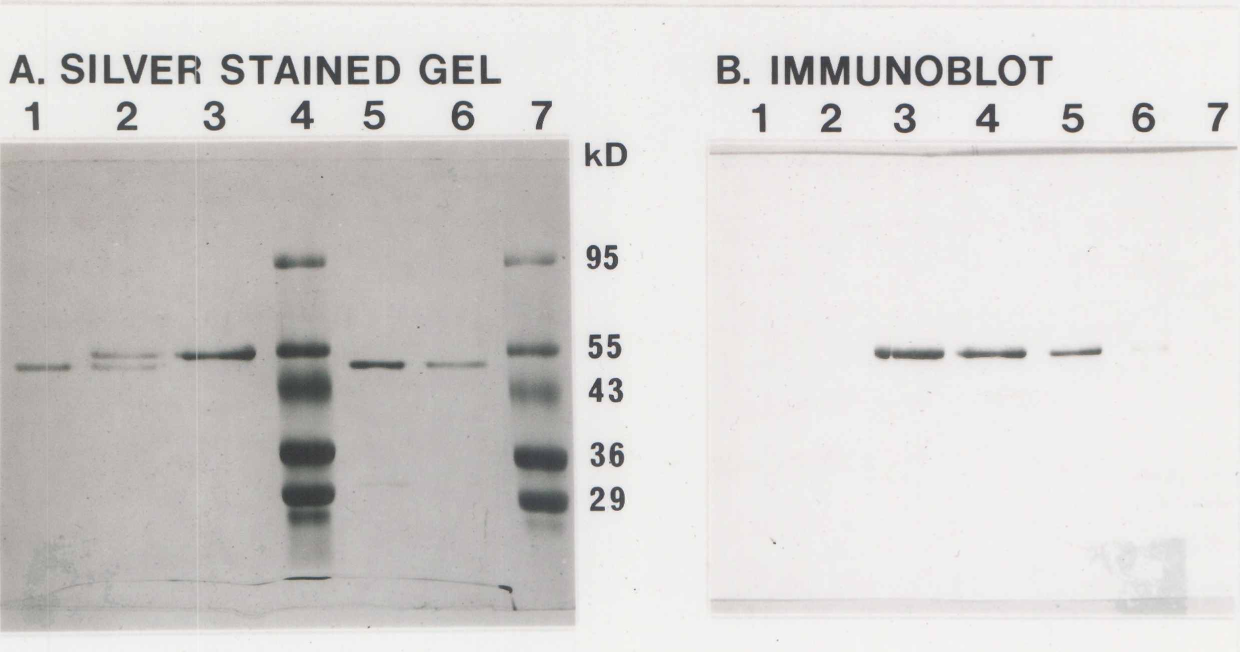

Figure 4.4. Electrophoretic separation of purified unbound, GR_A and GR_B glutathione reductase from eastern white pine on SDS-PAGE gels and Western transblot. (A) Silver stain of gel; lanes 1 and 6 = 150 ng GR_A, lane 2 = 100 ng each of GR_A and GR_B, lane 3 = 200 ng GR_B, lane 4 and 7 = 2 μl of standards, lane 5 = 200 ng purified unbound fraction from anion-exchange chromatography. (B) Western transblot probed with antibody produced against purified GR_B glutathione reductase from eastern white pine; lanes 1 and 2 = 150 ng purified unbound and GR_A glutathione reductase, respectively, lanes 3-7 = 150, 100, 50, 10 and 5 ng each of GR_A and GR_B, respectively.

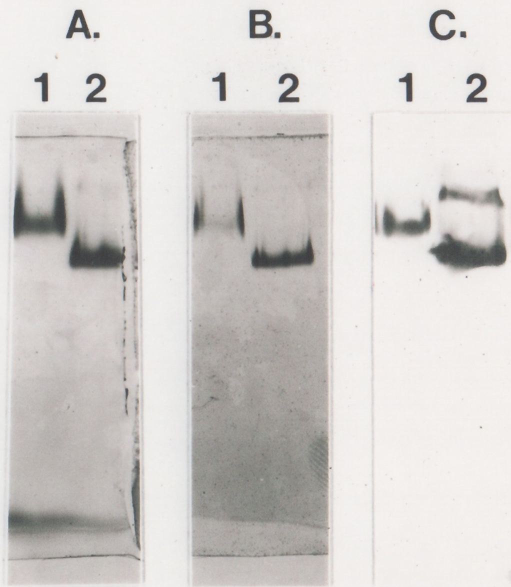

Figure 4.5 Electrophoretic separation of purified GR_A and GR_B glutathione reductase from eastern white pine on 10% native-PAGE gels and Western transblot of native-PAGE gel. (A) Glutathione reductase activity stain. (B) Silver stain of gel. (C) Western transblot probed with antibody produced to GR_B. lane 1 = 210 ng GR_A, lane 2 = 280 ng GR_B.

reductase activity from mustard cotyledons. In contrast to our results, they reported the first eluting peak, "GR_1", to be the major peak of activity and the second eluting peak, "GR_2", to be the minor peak of activity. They also termed GR_2 as a plastidic isoform based on its sensitivity to photooxidation, whereas GR_1 showed no sensitivity to photooxidation.

After further purification using Phenyl-Superose HR 5/5 and Superose-12 (FPLC) GR_A and GR_B were reapplied individually to a Mono Q HR 5/5 (FPLC) chromatography column equilibrated at pH 9.0. At this point both GR_A and GR_B were characterized by a single band of glutathione reductase activity (Figure 4.5, A) and a single band of protein on native polyacrylamide gels (Figure 4.5, B). SDS-PAGE gels also showed only a single band of protein for both GR_A and GR_B (Figure 4.4, A). It was concluded that both GR_A and GR_B were purified to homogeneity.

MOLECULAR WEIGHT DETERMINATION

The native mol wt determined by Superose-6 (FPLC) chromatography were 103 ± 1.5 kD for GR_A and 95 ± 2 kD for GR_B. Native mol wts were also determined using a TSK-250 gel filtration column (HPLC). In this case the native mol wt of GR_A was 104 ± 2 kD and 88 ± 2 kD for GR_B. The subunit mol wt as determined by SDS-PAGE were 53-54 kD for GR_A and 57 kD for GR_B (Figure 4.4, A). The subunit mol wt for glutathione reductase from eastern white pine are similar to

those reported for glutathione reductase from pea chloroplast (Connell and Mullet, 1986), scots pine (Wingsle, 1989), and rice kernel (Ida and Morita, 1971), Table 4.2. However, these subunit values are higher than the 41 and 42 kD subunits reported by Kalt-Torres et al (1984) for pea chloroplast and lower than the 72 kD subunits reported by Halliwell and Foyer (1978) for spinach leaves. Although Mahan and Burke (1987) reported subunit weights of 65, 63, 34 and 32 kD for glutathione reductase isolated from corn mesophyll chloroplast, most literature on glutathione reductase suggests that this protein is a dimer composed of two identical subunits (Connell and Mullet, 1986; Halliwell and Foyer, 1978; Ida and Morita, 1971; Wingsle, 1989). Our results for both GR_A and GR_B are also consistent with two dimeric structures each consisting of identical subunits.

The native mol wt of glutathione reductase from eastern white pine (GR_A and GR_B) are somewhat lower than reported for most plant glutathione reductases which range between 106-190 kD (Table 4.2). That the native mol wt of GR_A is greater than the mol wt of GR_B, yet shows smaller subunit mol wt than GR_B, is somewhat interesting. Possibly 1) GR_A may include a short signal sequence which dissociates from the polypeptide during the denaturing process, or 2) GR_A may bind more SDS under denatured conditions than does GR_B which allows it to migrate faster on SDS-PAGE. Similar explanations were suggested by Connell and Mullet (1986).

Table 4.2 Physical and kinetic properties of purified glutathione reductase from different sources of plant tissue.

	mol wt, kD		K_m, μM		pH	
Source	**Native**	**Subunit**	**GSSG**	**NADPH**	**Optimum**	**Ref**[1]
Chloroplast						
-Pea	-	60	62	3.0	-	6
-Pea	156	41/42	11	1.7	7.6	19
-Spinach	-	-	70	-	6.5-8.0	25
-Corn -mesophyll	190	32/34-63/65	14	1.7	8.0	20
Whole Leaf						
-Spinach	145	72	196	2.8	8.5-9.0	17
-hardened	#	#	52	6.0	8.0	14
-nonhardened	#	#	38	8.0	8.0	14
Roots						
-Pea	-	-	10	2.3	7.7	5
Seeds						
-Pea	120	-	17	4.7	-	21
-Rice	106	52	34	13.0	7.9	18
Pine Needles						
-Scots	-	59	28	1.0	7.7	29
-Estern white						
-GR_A	103-104	53-54	15.3±1.6[a]	3.7±0.2	7.2-7.8	**
-GR_B	88-95	57	39.8±1.7	8.8±0.8	7.25	**

**This study, #Multiple isoforms
[a]Mean of 4 replicates (GR_A) and 5 replicates (GR_B) ± SE
[1]For references see Anderson et al. (1990)

KINETIC PARAMETERS

For the substrate NADPH, GR_A had a K_m of 3.7 μM and GR_B had a K_m of 8.8 μM (Table 4.2). For GSSG, GR_A had a K_m of 15.3 μM whereas GR_B had a K_m of 39.8 μM. Although these two isoforms of glutathione reductase show different kinetic constants, the K_m values do agree well with those previously reported in plant tissues (Table 4.2).

pH OPTIMUM

The pH optimum of GR_A was 7.25-7.75 with 50% of the activity ranging between 7.0-8.0 (Figure 4.6). The pH optimum for GR_B was 7.25 with 50% of the activity ranging between 6.5 and 8.0. These values are lower than those previously reported for plant glutathione reductase which range between 7.7-9.0 for whole leaf (Guy and Carter, 1984; Halliwell and Foyer, 1978; Wingsle, 1989), root (Bielawski and Joy, 1986) and seeds (Ida and Morita, 1971), Table 4.2.

The data reported in this study has drawn us to the conclusion that GR_B may be a putative plastidic form of glutathione reductase. GR_B represented approximately 80% of the total glutathione reductase recovered from needles which is in close agreement with Gillham and Dodge (1986) who reported that 76.8% of the total glutathione reductase recovered from pea leaves was contained in the chloroplast. Further evidence (Drumm-Herrel et al, 1989) in which the second eluting peak of glutathione reductase from FPLC anion-exchange was reported to be a plastidic form, based

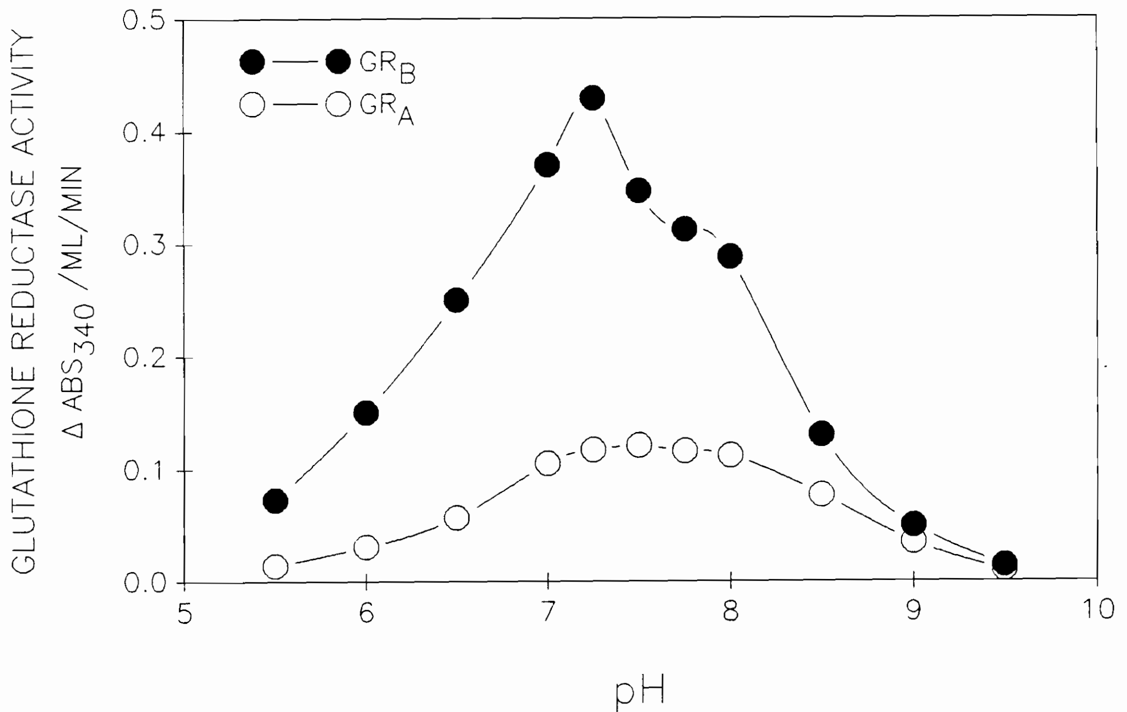

Figure 4.6 pH profile of purified glutathione reductase (GR_A and GR_B) from eastern white pine. Reactions at pH 5.5-7.0 were done using 50 mM MES buffer and reactions at pH 7.0-9.5 were done using 50 mM Tris buffer. Each reaction was done using 175 ng protein for purified GR_A and 25 ng for GR_B.

on its' sensitivity to photooxidation, may also suggest that our GR_B (also the second peak of activity to elute from FPLC anion-exchange, Figure 4.2) is a plastidic form. The lower pH optimum of 7.25 for GR_B, with a range between 6.5 and 8.0, also agrees well with Schaedle and Bassham (1977) who reported spinach chloroplast glutathione reductase to have a pH optimum of between 6.5 and 8.0. Such a pH range would allow a plastidic form of glutathione reductase to operate efficiently over stromal pH changes that occur during active photosynthesis and in the dark.

Identifying GR_B as a putative plastidic form is consistent with its' greater K_m for GSSG and NADPH and greater specific activity compared to GR_A. The concentration of NADPH in the chloroplasts of spinach is approximately 700 μM in the dark (Halliwell, 1984). Although only approximately 10-50% of the total glutathione of a leaf is chloroplastic (Gillham and Dodge, 1986; Smith et al., 1985), the chloroplast GSH concentrations generally range between 1-3.5 mM and GSSG between 100-200 μM (Halliwell, 1984). Such substrate concentrations would be more than sufficient to saturate eastern white pine glutathione reductase (GR_B). Since it has been established that glutathione and glutathione reductase are components of the ascorbate-glutathione cycle for the removal of H_2O_2 in the chloroplast (Halliwell and Foyer, 1978; Nakano and Asada, 1981), it should be expected that the chloroplast form of glutathione reductase

would have a greater K_m for GSSG and NADPH and a greater turnover rate to keep the GSH/GSSG at 10:1. Under illumination or limiting carbon reduction conditions, or when NADPH concentrations are high, the 3.3-fold greater specific activity (V_{max}, Table 4.1) for the putative chloroplastic isoform of glutathione reductase (GR_B) would also be consistent with the function of this enzyme in maintaining $NADP^+$ availability.

IMMUNOLOGICAL CHARACTERIZATION

Antiserum (anti-GR_B) from rabbit was tested for cross-reactivity with both purified GR_A and GR_B. Anti-GR_B binding was quantified with ELISA and Slot blot analysis. With ELISA the sensitivity of the reaction between GR_B and anti-GR_B could be detected at 0.150 ng total protein (Figure 4.7, A). Slot blot analysis of GR_B on nitrocellulose showed a reactivity with anti-GR_B which was sensitive at approximately 0.80 ng (Figure 4.7, B). Slot blot analysis of GR_A on nitrocellulose showed a cross-reactivity with anti-GR_B which was sensitive at approximately 10-20 ng (Figure 4.7, B).

Anti-GR_B probed Western transblots of SDS-PAGE gels resulted in no cross-reactivity with GR_A but did show primary-reactivity with GR_B (Figure 4, B). The data indicated that 5-10 ng of protein was the limit for reactivity between anti-GR_B and denatured GR_B. Western transblots of native gels were also probed with anti-GR_B. The

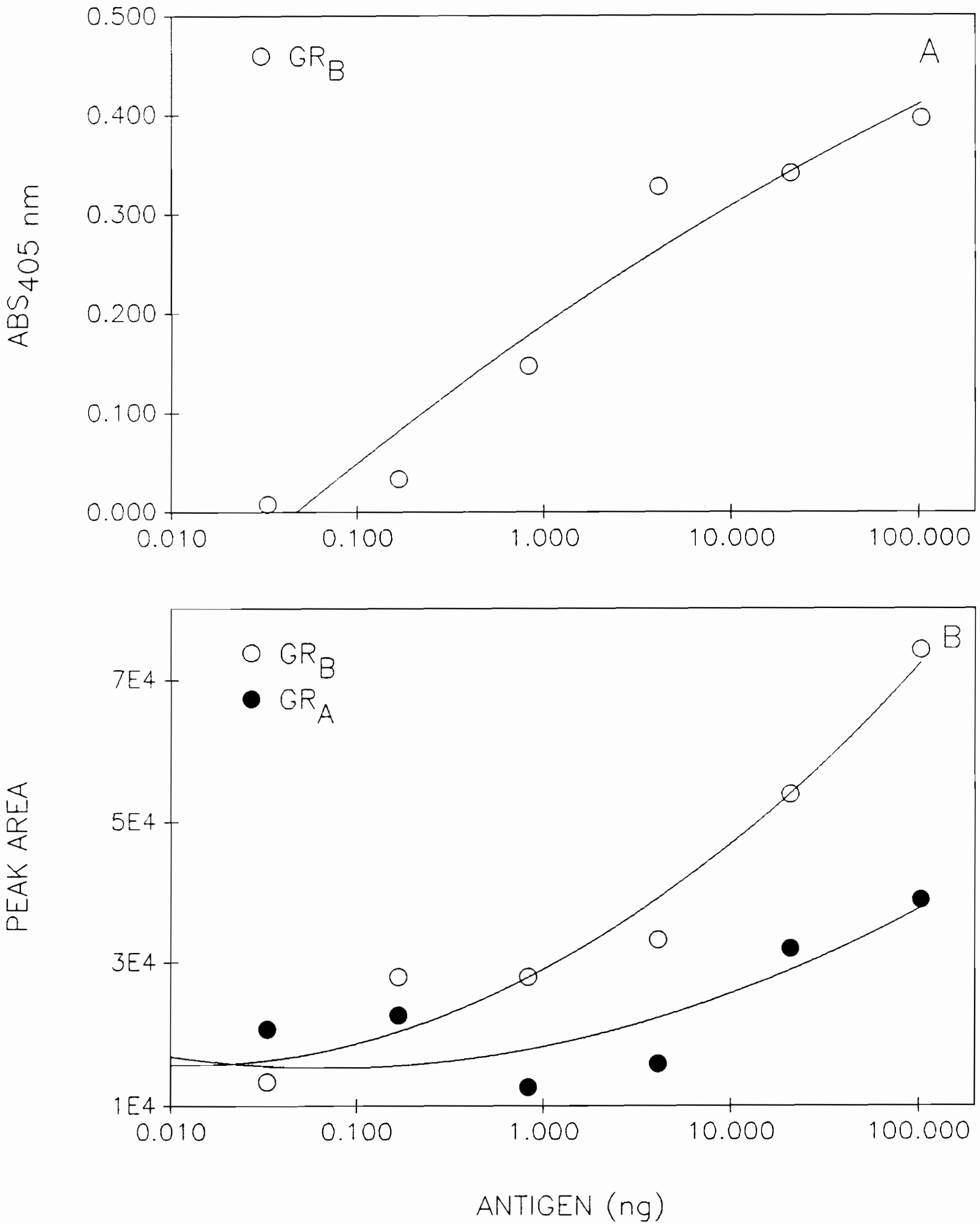

Figure 4.7 Sensitivity for antibody detection of glutathione reductase from eastern white pine. Antibody to GR_B was used in either an ELISA assay against GR_B with detection limit of > 0.1 ng (A), or slot blot analysis with detection limits for GR_B > 0.8 ng and for GR_A > 10 ng (B). The sensitivity for GR_B was greater than for GR_A. Data presented with a second order regression line.

native form of GR_A did show cross-reactivity with anti-GR_B (Figure 4.5, C). Thus, it appears that a determinant or conserved site for cross reactivity is lost or altered when GR_A is denatured.

GLUTATHIONE REDUCTASE ACTIVITY IN FUMIGATED PLANTS

Glutathione reductase activity in needles taken from plants in both the control and O_3-fumigated CSTR-chambers decreased after the first 24 hr of fumigation. In the sensitive clone, the decrease in glutathione reductase specific activity was 34% for both control and fumigated tissue (Figure 4.8, A). In the needles of the tolerant clone, taken from the control and fumigated chambers, the decrease in glutathione reductase specific activity was 10% and 31%, respectively (Figure 4.8, A). After 72 hr fumigation, glutathione reductase specific activity in the needles from sensitive and tolerant clones taken from control chambers was still less than that observed in control needles prior to fumigation (10% and 30%, respectively). However, in the needles of both the sensitive and tolerant clones taken from O_3-fumigated chambers after 72 hr, glutathione reductase specific activity was either equal to or greater than that observed prior to fumigation (100% and 105%, respectively).

Needles taken from both control and O_3-fumigated sensitive clones contained approximately 40-50% less total glutathione reductase activity $gfwt^{-1}$ after 24 and 72 hr

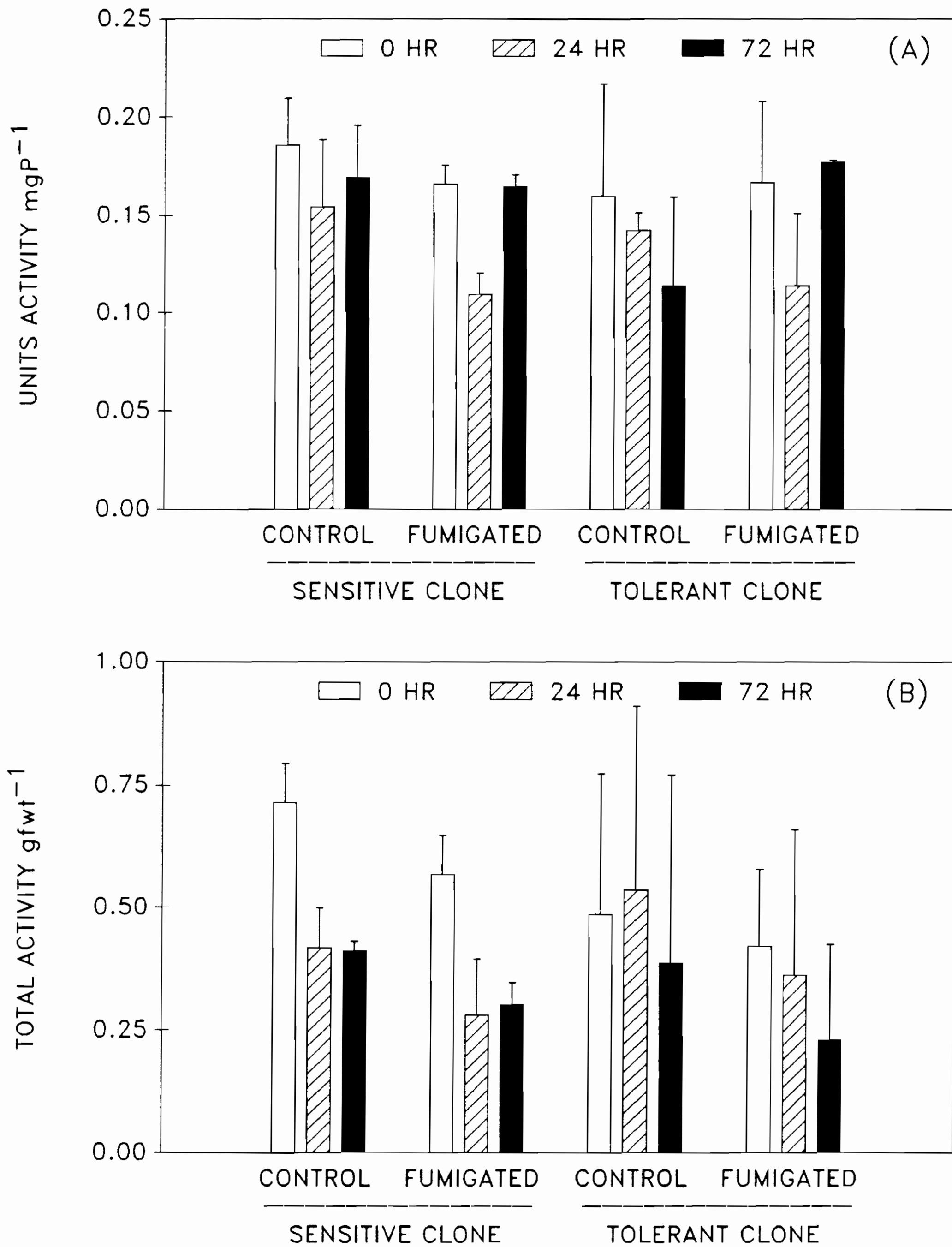

Figure 4.8 Glutathione reductase activity in needles of sensitive and tolerant clones of eastern white pine fumigated for 72 hr with either < 0.025 ppm O_3 (controls) or 4.48 ppm•hr total dose O_3 (Fumigated) during light period. Data is based on specific activity (A) and total units activity $gfwt^{-1}$ (B). Error bars represent 95% confidence limits. Genotypes of pine are classified based on visible foliar characteristics.

(Figure 4.8, B). The reduction in total units of glutathione reductase activity may in part be due to the 25-35% reduction in total extractable protein from the needles of the sensitive clones after 24 and 72 hr (Figure 4.9, A). Total units of glutathione reductase activity $gfwt^{-1}$ in needles of tolerant clones taken from the control chambers increased by approximately 10% after 24 hr and then decreased by 20% after 72 hr compared to prior to fumigation (Figure 4.8, A). Needles of the tolerant clones taken from the O_3-fumigated chamber contained 14 and 45% less total units of glutathione reductase activity $gfwt^{-1}$ after 24 and 72 hr, respectively. The 20% reduction in total units of activity in tolerant needles taken from the control chamber could not be attributed to a decrease in protein since the total extractable protein from these needles increased by 12.5% after 72 hr (Figure 4.9, A). However, the 45% reduction in total units of activity in the tolerant needles taken from the O_3-fumigated chamber was paralleled by a 47% reduction in total extractable protein after 72 hr (Figure 4.9, A).

Based on the peak areas obtained from Western transblots probed with anti-GR_B, no reduction in the quantity of glutathione reductase was observed after 24 hr fumigation. In fact, a 40% increase in glutathione reductase was observed in the needles of both the sensitive and tolerant clones taken from the O_3-fumigated chambers after 24 hr

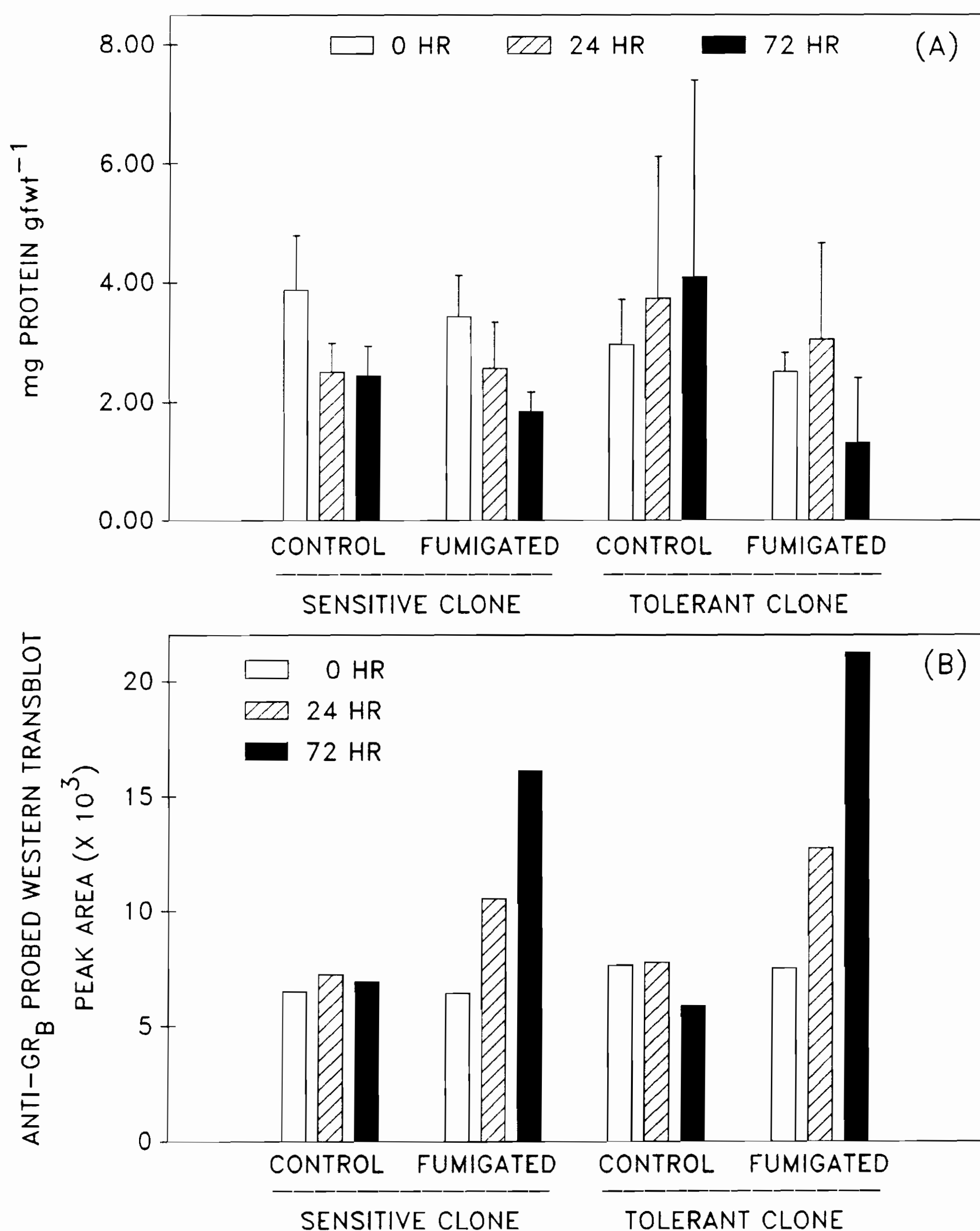

Figure 4.9 Comparison of soluble protein (A) and peak areas of Western transblots probed with antibody produced from purified glutathione reductase (GR_B) from eastern white pine (B). Data obtained from current year needles of sensitive and tolerant clones of eastern white pine fumigated for 72 hr with either < 0.025 ppm O_3 (controls) or 4.5 ppm·hr total dose O_3 (fumigated) during the light period. Error bars represent 95% confidence limits. Genotypes are classified based on visible foliar characteristics.

fumigation (Figure 4.9, B). No appreciable change in glutathione reductase quantity was observed in needles of either the sensitive or tolerant clones in the control chambers (Figure 4.9, B). No increase in the quantity of glutathione reductase was observed in needles of the sensitive clone taken from the control chamber after 72 hr fumigation (Figure 4.9, B). However, in the tolerant clone a 23% reduction in the quantity of glutathione reductase was observed on Western transblots (Figure 4.9, B) and this could contribute to the 30% reduction in glutathione reductase activity observed in needles of the tolerant clone taken from the control chamber after 72 hr. Needles from both the sensitive and tolerant clones showed a 60-65% increase in the quantity of glutathione reductase after 72 hr O_3-fumigation compared to needles taken prior to fumigation (based on peak area of transblots). This increase was also 20-25% greater than that observed at 24 hr O_3-fumigation.

Using the standard curve developed with purified glutathione reductase GR_B and Anti-GR_B (Figure 4.7, B) absolute values for the quantity of needle glutathione reductase were estimated using the peak areas obtained from Western transblots. Prior to O_3-fumigation, needles from both the sensitive and tolerant clones contained 14-21 ng glutathione reductase mgP^{-1} (Table 4.3). In the control chambers, these values remained consistent throughout the

Table 4.3 Quantity of glutathione reductase contained in needles of sensitive and tolerant clones of eastern white pine fumigated for 72 hr with either <0.025 ppm O_3 (control) or 4.48 ppm•hr total dose O_3 during light period. Genotypes of pine are classified based on visible foliar characteristics.

hrs-fumigated	SENSITIVE control	SENSITIVE $+O_3$	TOLERANT control	TOLERANT $+O_3$
	(ng glutathione reductase mgP^{-1})			
0	14	14	22	21
24	19	62	23	138
72	17	464	14	2800

fumigation. In needles of the sensitive clone taken from O_3-fumigated chambers at 24 and 72 hr, the quantity of glutathione reductase was 62 and 464 ng mgP^{-1}, respectively. In needles of the tolerant clone taken from the O_3-fumigated chambers at 24 and 72 hr, the quantity of glutathione reductase was 138 and 2800 ng mgP^{-1}, respectively. Based on these values, the quantity of glutathione reductase was two-fold greater after 24 hr O_3-fumigation and six-fold greater after 72 hr O_3-fumigation in needles of the tolerant clone compared to needles of the sensitive clone. Needles from the sensitive clone showed a 33-fold increase and needles from the tolerant clone showed a 133-fold increase in the quantity of glutathione reductase over a 72 hr O_3-fumigation period (based on ng glutathione reductase mgP^{-1}, Table 4.3).

The data presented in this study demonstrated that exposure of eastern white pine to the CSTR-chamber environment resulted in the reduction of glutathione reductase activity after 24 and 72 hr. The reduction in total units of glutathione reductase activity $gfwt^{-1}$ during the 72 hr period could generally be attributed to a reduction in total extractable needle protein. The fact that the glutathione reductase specific activity in the O_3-fumigated needles recovered to equal that observed in the needles prior to fumigation, whereas, the needles from the control chambers did not recover to the same degree, may indicate

the biosynthesis of new enzyme. The data observed from the antibody-probed Western transblots indicated that the quantity of glutathione reductase in O_3-fumigated tissue was 40-65% greater (based on peak area) after 24-72 hr fumigation compared with prior to fumigation. Also, based on absolute values for the quantity of glutathione reductase, it appears that O_3-tolerant clones have a greater capacity to maintain glutathione turnover during O_3 exposure since they contain greater quantities of the enzyme than do the O_3-sensitive clones which have been exposed to O_3. However, since no appreciable difference was observed in the specific activity at 0 and 72 hr O_3-fumigation, these data suggest that the O_3 induced enzyme was either still at an inactive stage or that the induced enzyme had a lower turnover rate (V_{max}). Since the anti-GR_B-probed transblots of native gels only showed one reaction band, with similar migration rates, it does not appear that the increase in the quantity of glutathione reductase was due to the synthesis of a different isoform of the enzyme.

Another explanation for the observed induction of glutathione reductase without an increase in specific activity, in eastern white pine needles, may be that O_3 results in the oxidation of sulfhydryl group which are important in it's catalytic function as an enzyme. Dominy and Heath (1985) reported that inhibition of (K^+ + Mg^{++})-ATPase during exposure of pinto bean to O_3 was due to the oxidation

of sulfhydryl moieties. Thus, the partial recovery of the specific activity of glutathione reductase in needles from the sensitive and tolerant clones between 24 and 72 hr O_3-fumigation may reflect a balance between oxidation and induction of the enzyme.

The data presented here does agree with Tanaka et al. (1988) who observed an increase in the biosynthesis of glutathione reductase during exposure of spinach to 0.07ppm O_3 for 4 days. However, in contrast to the 2.5-fold increase in glutathione reductase activity they observed after 2 to 4 days exposure, no such increases were observed in this study.

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SUMMARY AND CONCLUSIONS

After a high O_3, drought-type growing season (1988), current year needles taken from a sensitive genotype of eastern white pine had a 45% reduction in growth compared to current year needles taken from a tolerant tree. In contrast, after a low O_3, non-drought growing season (1989), current year needles taken from the sensitive tree had only a 30% reduction in growth compared to current year needles taken from the tolerant tree. The tolerant tree had similar needle growth during both growing seasons. Ascorbate concentration and the ratio of ascorbate/dehydroascorbate, were similar in the needles of both the sensitive and tolerant tree during the two different growing seasons. However, differences were observed in the concentration of glutathione when comparing needles from different growing seasons. Needles which expanded and matured during the 1989 growing season always had a greater concentration of glutathione than did needles which expanded and matured during the 1988 growing season. These data suggested that needles which matured during oxidative conditions expressed a limited capacity for the accumulation of total glutathione.

Although, needle glutathione content differed from one growing season to another, total glutathione in needles taken from the sensitive and tolerant trees were always

similar during a given sampling period. However, in one-year-old tissue, the GSH/GSSG ratio was always lower in the needles taken from the sensitive tree compared to needles taken from the tolerant tree. During the 1989 growing season the GSH/GSSG ratio in the one-year-old sensitive needles was always less than that in one-year-old tolerant needles. The inability of the one-year-old sensitive needles to maintain glutathione turnover was attributed to the greater loss of glutathione reductase activity prior to needle shed.

This study also indicated that the one-year-old needles experience a greater oxidative metabolism than did the current year needles. This hypothesis was further supported by the observed decrease in the ratio of ascorbate/ dehydroascorbate, GSH/GSSG and the ascorbate/α-tocopherol in the one-year-old needles, whereas, the current year needles always had increasing ratios of ascorbate/dehydroascorbate, GSH/GSSG and ascorbate/α-tocopherol.

The data presented in this study also indicated that the antioxidant mechanism may have a role in the cold tolerance of plants. All antioxidant parameters measured in eastern white pine increased during the fall and reached maximum levels during the winter months. However, the 1988-89 year needles had less total glutathione than did the 1989-90 year needles during the winter months. This data may suggest that lower accumulation of glutathione in

the current year needles during a high O_3, drought-type growing season (similar to the summer of 1988), may also have contributed to the lower accumulation of glutathione observed during the winter of 1988-89. These lower concentrations of glutathione may impair the cells ability to protect against winter injury.

Current year needles taken from sensitive and tolerant clones of eastern white pine grown in open-top chambers containing ambient or charcoal-filtered air displayed little difference in growth during the 1989 growing season. Needles taken from the sensitive clones in the ambient and non-filtered chambers had greater levels of ascorbate peroxidase activity than did sensitive needles taken from the charcoal-filtered chambers. No difference in ascorbate peroxidase was observed in the needles of tolerant clones due to different chamber treatments. The greater ascorbate peroxidase activity in the sensitive clones may indicate that the trees were experiencing greater oxidative metabolism when exposed to ambient air pollutants.

The mean concentration of GSH in the needles of tolerant clones was always greater than that observed in the sensitive clones in September, 1989. Different chamber treatments had no effect on GSH concentration in the sensitive needles. However, needles from the tolerant clone grown in the ambient forced air chambers had 23% more total GSH in September compared to tolerant needles taken from

either the ambient or charcoal-filtered plots. Needles from the tolerant clones exposed to ambient forced air in open-top chambers also had 43% more total GSH than needles of sensitive clones from the same chambers. The higher concentrations of needle glutathione in the tolerant clones of eastern white pine may indicate a greater capacity to respond to oxidative stress.

The difference in needle GSH content observed between the sensitive and tolerant clones in September, 1989 could not be explained due to differences in glutathione reductase activity. In fact the sensitive needles from all chamber treatments in September, 1989 generally had a slightly greater mean glutathione reductase activity based both on specific activity and total units of activity. However, in January, 1990, the glutathione reductase activity was always equal to or greater in the tolerant needles than in the sensitive needles based on both specific activity and total units of activity. Since the specific and total activity in both the sensitive and tolerant clones increase by at least two fold in January, 1990, it appears that the cold hardening period may signal the biosynthesis of new isoforms of glutathione reductase which have different kinetic properties than the major form of glutathione reductase extracted from needles in September, 1989.

The observation from the open-top chamber study which show increased ascorbate peroxidase activity in sensitive

needles exposed to non-filtered air, higher levels of GSH in tolerant needles during summer and winter, and the greater shift in glutathione reductase activity in tolerant needles from summer to winter, may suggest that the tolerant clones have a greater capacity to withstand oxidative stress. Although the data presented in this study were preliminary, it was significant in that the cloned material had physiological characteristic similar to those of the parent trees and that differences were observed between sensitive and tolerant clones exposed to different chamber treatments, even during a low O_3 growing season. This data suggests the potential for more pronounced response during a high O_3 growing season.

From the data collected in this study it was concluded that the maintenance of glutathione turnover in the plant cell is probably the most important mechanism of the antioxidant scavenging system and is the most sensitive marker of increased oxidative metabolism. Since differences were observed in the seasonal activity of glutathione reductase when comparing needles from sensitive and tolerant trees, it appeared that glutathione reductase also may be a key regulatory enzyme in plants which show tolerance to oxidative stress. To better understand the role glutathione reductase plays in the turnover of glutathione in eastern white pine this enzyme was isolated, purified and characterized.

The data presented in this study clearly demonstrated the presence of two isoforms of glutathione reductase (GR_A and GR_B) in eastern white pine needles. GR_A accounted for 17% of the total glutathione reductase recovered from anion-exchange separation and GR_B accounted for 83%. The native mol wt for GR_A was 103-104 kD and for GR_B was 88-95 kD. Both isoforms of glutathione reductase were dimers composed of identical subunit mol wts which were 53-54 kD for GR_A and 57 kD for GR_B. Purified GR_A had a specific activity of 1.81 μKat mgP^{-1} and GR_B had a specific activity of 6.08 μKats mgP^{-1}. At 25°C the K_m for GSSG was 15.3 and 39.8 μM for GR_A and GR_B, respectively. For NADPH, the K_m was 3.7 and 8.8 μM for GR_A and GR_B, respectively. The pH optimum for GR_A was 7.25 to 7.75 and for GR_B was 7.25. Antibody produced from purified GR_B was reactive with both native and denatured GR_B, but was cross-reactive with only native GR_A.

Based on the percent recovery, K_m for GSSG and NADPH, the greater V_{max}, and lower pH optimum for GR_B, compared to GR_A, GR_B was suggested to be a putative plastidic isoform of glutathione reductase. Also, GR_B had 50% maximum activity between pH 6.5 to 8.0, which would allow a plastidic form of glutathione reductase to operate efficiently over stromal pH changes that occur during active photosynthesis and in the dark. Based on the characteristics of GR_A, it was suggested to be either a cytosolic isoform of glutath-

ione reductase or a precursor form of the plastidic glutathione reductase.

Antibodies produced from GR_B were used as probes to determine if glutathione reductase was induced in cloned eastern white pine during O_3 exposures. Although increased glutathione reductase activity was not observed during the O_3-fumigation, an increase in the quantity of glutathione reductase protein did occur. The sensitive needles taken from the O_3-fumigated chamber contained 14, 62 and 464 ng glutathione reductase mgP^{-1} after 0, 24 and 72 hr, respectively in comparison to tolerant needles which contained 21, 138 and 2800 ng glutathione reductase after 0, 24 and 72 hr. These data demonstrated that the tolerant needles contained a two-fold and six-fold greater quantity of glutathione reductase after 24 and 72 hr O_3-fumigation, respectively.

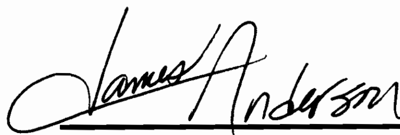
Immunoblot analysis indicated that the increased quantity of glutathione reductase without an increase in specific activity was not due to the inductive biosynthesis of an isoform with different kinetic characteristics. The hypothesis concluded from these data was that O_3 either inhibited glutathione reductase activity by oxidizing sulfhydryl groups such as those which are light activated or that the induced enzyme was still in an inactive state. However, the partial recovery of glutathione reductase specific activity between 24 and 72 hr O_3-fumigation may have

reflected a balance between oxidation and induction of the enzyme. These data also indicated that the tolerant clone would have a much greater capacity to maintain glutathione turnover after exposure to O_3, since it has a greater induction of glutathione reductase.

The data presented in these studies indicated that differential sensitivity to air pollutant stress in eastern white pine may be correlated to the turnover of glutathione and its regulation by glutathione reductase. It also appears that the reduction in current year needle growth in genotypes of eastern white pine which show visible foliar characteristic similar those caused by ambient air pollutants may be directly related to the capacity of one-year-old needles to maintain the mechanism for efficient glutathione turnover during the active growing season. It also appears that needles grown during conditions which enhance oxidative metabolism may reduce the accumulation of glutathione during the winter. Such a reduction may impair the trees ability to withstand winter injury.

VITA

James Valentine Anderson was born in Chippewa Falls, Wisconsin on January, 1, 1957 to Rolf and Dorthy Anderson. He graduated from Cadott Public High School, Cadott, Wisconsin in 1975. After serving in the U.S. Navy, he entered the University of Wisconsin at Eau Claire and received a B.S. degree in Biology in 1983. After attending graduate school at the University of Wisconsin at La Crosse for one semester, he transferred to South Dakota State University, Brookings, South Dakota and received an M.S. degree in Chemistry in 1987. He then enrolled in graduate school at Virginia Polytechnic Institute and State University to pursue a Ph. D. degree in Plant Physiology in 1987. Mr. Anderson married Jan Kistler on June 7, 1986. They have a daughter, Cassandra and a son, Nathan.



James Valentine Anderson