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Acclimation of Foliar Antioxidant Systems to Growth Irradiance in Three Broad-Leaved Evergreen Species[1]

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The protective role of leaf antioxidant systems in the mechanism of plant acclimation to growth irradiance was studied in *Vinca major*, *Schefflera arboricola*, and *Mahonia repens*, which were grown for several months at 20, 100, and 1200 μmol photons m^{-2} s^{-1}. As growth irradiance increased, several constituents of the "Mehler-peroxidase" pathway also increased: superoxide dismutase, ascorbate peroxidase, glutathione reductase, ascorbate, and glutathione. This occurred concomitantly with increases in the xanthophyll cycle pool size and in the rate of nonphotochemical energy dissipation under steady-state conditions. There was no evidence for photosystem II overreduction in plants grown at high irradiance, although the reduction state of the stromal NADP pool, estimated from measurements of NADP-malate dehydrogenase activity, was greater than 60% in *V. major* and *S. arboricola*. Ascorbate, which removes reactive O_2 species generated by O_2 photoreduction in the chloroplast and serves as a reductant for the conversion of the xanthophyll cycle pigments to the de-epoxidized forms A plus Z, generally exhibited the most dramatic increases in response to growth irradiance. We conclude from these results that O_2 photoreduction occurs at higher rates in leaves acclimated to high irradiance, despite increases in xanthophyll cycle-dependent energy dissipation, and that increases in leaf antioxidants protect against this potential oxidative stress.

Plants acclimated to high irradiance use various mechanisms to protect the photosynthetic apparatus against the deleterious effects of excess light absorption. Much attention has been focused on elucidating the role of the xanthophyll cycle in the dissipation of excess excitation energy in the light-harvesting antennae (Demmig-Adams et al., 1996). The associated increases in the xanthophyll cycle pool size, V plus A plus Z, and the capacity for nonphotochemical energy dissipation, NPQ, is a well-characterized and apparently fundamental acclimatory response to high PFDs (Björkman and Demmig-Adams, 1994; Demmig-Adams and Adams, 1994). Xanthophyll cycle-dependent energy dissipation lowers the photon efficiency of PSII, thus providing a mechanism to balance the synthesis of ATP and NADPH with the rate at which these metabolites can be utilized in photosynthesis (Foyer, 1993). It is not known, however, whether this dynamic, regulatory process compensates fully for the fraction of absorbed photons that are not utilized in assimilatory electron flow. A key question is whether exposure to high PFDs causes an increase in the rate of pseudocyclic electron flow to molecular O_2 via the Mehler reaction in fully acclimated leaves. Such nonassimilatory flow may act to drain excess electrons from the transport chain (Osmond and Grace, 1995) or serve a regulatory role by augmenting the transthylakoid proton gradient (Schreiber and Neubauer, 1990). Although the magnitude of O_2-dependent flux in vivo remains controversial, direct measurements using MS suggest that in C_3 plants up to 25% of the total noncyclic electron transport is consumed by this process at light saturation (Canvin et al., 1980; Badger, 1985; Osmond and Grace, 1995).

Protection against the possible toxicity of ROS is afforded by an integrated system of enzymatic and nonenzymatic antioxidants that are concentrated in the chloroplast (Asada, 1994). The superoxide anion, the initial product of photosynthetic O_2 reduction, is either dismuted by SOD or reduced by ascorbate to H_2O_2, which is then removed in the Mehler-peroxidase pathway of electron transport (Schreiber and Neubauer, 1990). Ascorbate and glutathione, which serve as intermediary redox metabolites in this pathway, are maintained in the reduced state by MDAR (Hossain et al., 1984) and GR (Foyer and Halliwell, 1976) using photosynthetically generated reductants. Direct photoreduction of the ascorbate radical by the electron transport chain also occurs at high rates (Miyake and Asada, 1994; Grace et al., 1995). Catalase is concentrated in the peroxisome, where it scavenges H_2O_2 that is generated in the photorespiratory cycle. Cytosolic and mitochondrial isoforms of SOD, APX, GR, and catalase also exist, where they may function to scavenge ROS that is generated by mitochondrial electron transport (Cadenas, 1995), autoxidation of sugars (Fridovich, 1995), or plasma membrane-linked redox reactions (Rubinstein and Luster, 1993).

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Abbreviations: APX, ascorbate peroxidase; DHA, dehydroascorbate; F_V'/F_M', efficiency of PSII in the light ($F_M' - F_O'/F_M'$); GR, glutathione reductase; MDAR, monodehydroascorbate reductase; MDH, malate dehydrogenase; NPQ, Stern-Volmer-type nonphotochemical quenching ($F_M/F_M' - 1$); q_P, coefficient of photochemical quenching ($F_M' - F/F_M' - F_O'$); ROS, reactive O_2 species; SOD, superoxide dismutase; V plus A plus Z, xanthophyll cycle components, violaxanthin, antheraxanthin, and zeaxanthin.

Few studies have directly addressed whether growth at high irradiance induces an increase in cellular antioxidant systems due to higher rates of O_2 photoreduction. Although short-term exposure of plants (minutes to hours) to high PFDs at normal temperatures (18–25°C) appears to have little effect on foliar antioxidant levels (Foyer et al., 1989; Mishra et al., 1993), longer-term acclimation to high PFDs appears to have a positive effect on these systems. Gillham and Dodge (1987) reported that greenhouse-grown peas had the highest antioxidant content in the summer, coinciding with maximal daily integrated PFDs. Logan et al. (1996) observed that, with increasing levels of solar irradiance, there were concomitant increases in the ascorbate and xanthophyll cycle pools in several Australian rainforest species. Plants growing in the field typically have the highest antioxidant levels in the winter, when even low PFDs can be excessive for photosynthesis (Anderson et al., 1992). This is consistent with the well-known synergistic effect of high-light and low-temperature stress on leaf antioxidant systems (Schöner and Krause, 1990; Mishra et al., 1993).

To understand the role of antioxidation in the process of long-term light acclimation, we have investigated changes in the leaf antioxidant system in three herbaceous evergreen species, *Vinca major*, *Schefflera arboricola*, and *Mahonia repens*, that were grown for several months at three different PFDs under controlled environmental conditions. Chlorophyll fluorescence data were used to derive estimates of PSII efficiency (F_V'/F_M'), photochemical quenching (q_P), and rates of energy dissipation (NPQ) during steady-state photosynthesis. The pool size and conversion state of the xanthophyll cycle carotenoids were analyzed by HPLC. In addition, the reduction state of the chloroplast NADP pool was estimated from measurements of NADP-dependent MDH activity, with the aim of elucidating the relationship between the xanthophyll cycle-associated energy dissipation in the antennae, the redox state of the stroma, and the rates of O_2 photoreduction in vivo.

MATERIALS AND METHODS

Growth of Plants

Vinca major L., *Schefflera arboricola* (Hayata) Merrill, and *Mahonia repens* (Lindley) Don were obtained from local greenhouses and grown in chambers under controlled environmental conditions at 20, 100, and 1200 μmol photons m^{-2} s^{-1}. These treatments will be referred to as very low, low, and high PFDs, respectively. Individual plants were transferred into soil in 10-gallon pots and allowed to acclimate to their respective PFDs for approximately 4 months prior to sampling. Plants were grown under a 12-h photoperiod at a day/night temperature of 22/18°C and 75% RH. Daytime air temperature in the high-PFD chamber was maintained at 18°C to avoid excessive leaf temperatures. Tissue samples were harvested from mature leaves approximately 4 h into the diurnal light period and either extracted and analyzed immediately or frozen and stored in liquid nitrogen until analysis.

Photosynthesis Measurements

O_2 evolution as a function of PFD was measured in air containing 5% CO_2 in a leaf-disc O_2 electrode (Hansatech, King's Lynn, UK), as described by Delieu and Walker (1981). Leaf discs (3.2 cm^2) were excised from fully expanded leaves 2 h into the light period for photosynthesis measurements. White actinic illumination was varied at the leaf surface using neutral density filters.

Fluorescence Measurements

Room temperature chlorophyll fluorescence was measured on intact leaves using a field-portable pulse modulation fluorescence system (PAM-2000, Walz, Effeltrich, Germany). NPQ of maximal fluorescence was calculated according to the method of Bilger and Björkman (1990). Other fluorescence parameters were calculated according to the method of Schreiber et al. (1986) and are expressed using the nomenclature of van Kooten and Snel (1990).

Pigment Determination

Carotenoids and chlorophylls were analyzed by HPLC according to the method of Gilmore and Yamamoto (1991). Leaf tissue (0.25 cm^2) was extracted according to the method of Adams and Demmig-Adams (1992).

Antioxidant Metabolite Determination

Ascorbate

Ascorbate content was measured according to the method of Foyer et al. (1989). Leaf tissue (1 cm^2) was ground in a prechilled mortar with acid-washed sand in 1 mL of 6% perchloric acid. The crude extract was centrifuged at 2°C for 10 min at 10,000*g* and the supernatant was collected for analysis. The pH of the extract was increased to approximately 1 by stepwise addition of saturated Na_2CO_3. Ascorbate was assayed spectrophotometrically at 265 nm in 0.2 M sodium acetate buffer, pH 5.6, before and after a 15-min incubation with 5 units of ascorbate oxidase (from *Curcurbita*, Sigma) at room temperature. Ascorbate levels were determined from a standard curve.

Glutathione

Glutathione was extracted as above, with the exception that 7% sulfosalicylic acid was used in place of $HClO_4$. Total glutathione was determined spectrophotometrically at 412 nm by the cycling method described by Griffith (1980). A portion of the extract was neutralized by a 30-fold dilution in 0.5 M KH_2PO_4, 6.3 mM EDTA, pH 7.6, and subsequently assayed in the presence of 1.2 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 0.2 mM NADPH. The reaction was started by the addition of 0.2 unit of GR (from yeast, Boehringer Mannheim) in a total volume of 2 mL. All values are expressed as GSH equivalents, determined from a standard curve.

α-Tocopherol

Approximately 0.15 g of leaf tissue was ground in a prechilled mortar with acid-washed sand in 3 mL of 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT, pH 7.4. The extract was sonicated on ice for 30 s. The sample was then thoroughly mixed with an equal volume of cold 3:2 (v/v) hexane:2-propanol containing 0.025% butylated hydroxytoluene and centrifuged at 1000*g* for 2 min. The organic layer was collected and dried in vacuo. Samples were resuspended in 0.2 mL of ethanol, filtered through a 0.45-μm microfilter, bubbled with nitrogen, and stored on ice until analysis. α-Tocopherol was determined by HPLC on a reverse-phase column (Lichrosorb RP-8, 5 μm, Alltech Deerfield, IL) using an isocratic solvent method (9:1, v/v, methanol:H_2O) run at 0.9 mL min^{-1}. Peak areas were calibrated against α-tocopherol standards of known concentrations.

Antioxidant Enzyme Analysis

For determination of antioxidant enzyme activities, 1 cm^2 of leaf tissue was homogenized as above in 1.5 mL of extraction buffer containing 50 mM KH_2PO_4, 0.1 mM EDTA, 0.3% (w/v) Triton X-100, and 4% (w/v) soluble PVP-10. This extraction buffer yields excellent recovery of SOD and other antioxidant enzymes (Tandy et al., 1989). For GR determination, 1.5 cm^2 of leaf tissue was used and Triton X-100 was omitted from the extraction buffer. Samples were mixed and allowed to incubate on ice for approximately 10 min and then centrifuged for 10 min at 10,000*g* at 2°C, and the supernatant was collected for analysis. Desalting the crude extract on a Sephadex G-25 column was found to have no effect on enzyme activity and so this step was omitted. All enzyme assays were carried out at 25°C in a total volume of 2 mL.

SOD activity was measured spectrophotometrically at 550 nm by the Cyt *c* method described by McCord and Fridovich (1969). The assay medium contained 50 mM KH_2PO_4, 0.1 mM EDTA, 0.01 mM Cyt *c*, and 0.05 mM xanthine, pH 7.6. The reaction was started by the addition of sufficient xanthine oxidase (approximately 0.03 unit) to achieve a control rate of Cyt *c* reduction (in the absence of sample) of 0.025 absorbance units min^{-1}. One unit of SOD activity is defined as the amount necessary to produce a 50% inhibition of the Cyt *c* reduction rate (McCord and Fridovch, 1969). The volume of the sample in the assay was adjusted so that the sample contained approximately 1 unit of activity.

APX activity was measured spectrophotometrically at 290 nm according to the method of Nakano and Asada (1981). The assay medium contained 50 mM Hepes-KOH, 0.1 mM EDTA, and 0.5 mM ascorbate, pH 7.6. The reaction was started by the addition of 0.2 mM H_2O_2. Activities were corrected for the uncatalyzed rate of ascorbate oxidation by H_2O_2 in the absence of the sample. A molar extinction coefficient of 2800 cm^{-1} was used to calculate activity.

GR activity was measured spectrophotometrically at 340 nm in 100 mM Tris-HCl, 1 mM EDTA, and 0.5 mM GSSG, pH 8.0. The reaction was started by the addition of 0.05 mM NADPH. The rate of nonspecific NADPH oxidase activity (in the absence of GSSG) was subtracted. A molar extinction coefficient of 6200 cm^{-1} was used to calculate activity.

MDAR activity was measured spectrophotometrically at 340 nm according to the method of Foyer et al. (1989). The assay medium contained 50 mM Hepes-KOH, 0.1 mM EDTA, 2.5 mM ascorbate, and 0.1 mM NADH, pH 7.6. The reaction was started by the addition of 4 units of ascorbate oxidase to generate the monodehydroascorbate radical. The rate of nonspecific NADH oxidase activity (in the absence of sample) was subtracted.

Catalase activity was measured polarographically in a liquid-phase O_2 electrode (DW2/2, Hansatech) in 50 mM KH_2PO_4, 0.1 mM EDTA, and 10 mM H_2O_2, pH 7.0. The reaction was started by the addition of 10 to 50 μL of sample and activity was monitored as O_2 evolution. Inhibition by 1 mM NaN_3 confirmed that all of the activity was due to the catalase that was present in the sample. One unit of activity is defined as the amount necessary to convert 1 μmol H_2O_2 min^{-1}.

NADP-MDH Activity Measurements

Frozen leaf tissue (1.5 cm^2) was homogenized in a mortar and pestle in ice-cold extraction buffer consisting of 50 mM sodium acetate, pH 6.0, previously bubbled with nitrogen gas and containing 0.1% Triton X-100, 0.1% BSA, and 4 mM DTT. The extract was centrifuged at 10,000*g* for 10 min and the supernatant was collected for analysis. NADP-specific MDH activity was assayed immediately after extraction according to the method of Scheibe et al. (1986). The assay medium consisted of 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM oxaloacetate, 0.2 mM NADPH, and 50 to 100 μL of extract, and the decline in the A_{340} was monitored. To obtain the activity of the fully reduced enzyme, a portion of the extract was incubated in 250 mM Tris-HCl and 125 mM DTT, pH 9.0, under anaerobic conditions according to the method of Scheibe and Stitt (1988). Activities were corrected for nonspecific NADPH oxidase activity (in the absence of sample) and for the side activity of NAD-dependent MDH, which was assumed to be 0.2% of the total (Scheibe and Stitt, 1988). To ensure that the activation state of the enzyme was not being overestimated, a number of controls were performed. Time-course experiments confirmed that the low concentrations of DTT that were used in the extraction medium (4 mM) did not cause any detectable activation of the enzyme. This conclusion is supported by the fact that activation states near zero could be obtained from dark-adapted plants or in plants growing in low light.

RESULTS

Pigment Acclimation

Table I shows that there was a progressive increase in the chlorophyll *a*/*b* ratio with increasing growth PFD in two of the species studied, *V. major* and *S. arboricola*. Previous investigations of light acclimation in higher plants showed that higher chlorophyll *a*/*b* ratios were correlated with higher rates of electron transport and photophosphoryla-

Table I. *Changes in chlorophyll a plus b content and a/b ratio and xanthophyll cycle pool size, expressed per unit of chlorophyll and as a fraction of total carotenoids, in leaves grown under 20, 100, and 1200 μmol photons m^{-2} s^{-1}*

Values represent the means ± SD of three to six leaves.

Species/Growth PFD	Chlorophyll a + b	Chlorophyll *a/b* Ratio	V + A + Z	V + A + Z
μmol photons m^{-2} s^{-1}	*μmol (g fresh wt.)$^{-1}$*		*mmol (mol chlorophyll a + b)$^{-1}$*	*% total carotenoids*
V. major				
20	2.56 ± 0.24	2.87 ± 0.10	28.6 ± 2.2	11.6 ± 0.7
100	1.80 ± 0.41	3.41 ± 0.14	34.2 ± 1.6	12.9 ± 0.5
1200	1.41 ± 0.15	3.85 ± 0.14	89.6 ± 10	25.5 ± 1.4
S. arboricola				
20	1.35 ± 0.08	2.82 ± 0.09	28.1 ± 2.9	10.4 ± 1.0
100	1.30 ± 0.13	3.14 ± 0.09	48.2 ± 7.8	17.8 ± 3.2
1200	1.37 ± 0.14	3.46 ± 0.23	81.2 ± 7.0	22.9 ± 0.9
M. repens				
20	3.91 ± 0.10	3.23 ± 0.06	29.4 ± 0.9	12.4 ± 0.3
100	2.95 ± 0.29	3.24 ± 0.19	28.2 ± 1.8	11.7 ± 0.7
1200-Green	2.32 ± 0.37	3.88 ± 0.10	74.1 ± 6.1	22.3 ± 1.0
1200-Red	1.23 ± 0.41	3.30 ± 0.27	108.2 ± 20	26.0 ± 4.6

tion per unit of total chlorophyll (reviewed by Anderson and Osmond, 1987). Light-response curves for photosynthetic O_2 evolution showed a corresponding increase in photosynthetic capacity with increasing growth PFD in all species (Fig. 1). The chlorophyll *a/b* ratio did not differ between the leaves of *M. repens* that were acclimated to very low PFD (20 μmol photons m^{-2} s^{-1}) and low PFD (100 μmol m^{-2} s^{-1}), although mature leaves of plants acclimated to high PFD (1200 μmol m^{-2} s^{-1}) did have a predictably higher *a/b* ratio (Table I). It is important to note that, in contrast to the other PFD treatments, emergent and developing leaves of *M. repens* that were grown in the high-PFD chamber were red due to a high anthocyanin content in the epidermis and gradually became dark green during the course of several weeks. Red leaves of *M. repens* had a lower chlorophyll *a* plus *b* content and a lower chlorophyll *a/b* ratio than did fully developed green leaves, which were associated with a lower maximal photosynthetic rate (data not shown).

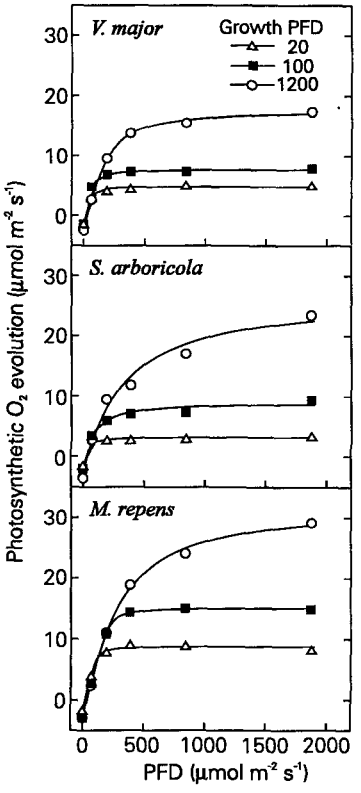

Figure 1. Photosynthetic light-response curves for leaves of *V. major* (top), *S. arboricola* (middle), and *M. repens* (bottom) acclimated to 20 (△), 100 (■), and 1200 (○) μmol photons m^{-2} s^{-1}. Rates of O_2 evolution were measured under saturating CO_2. Data for high-PFD-grown *M. repens* were taken from mature green leaves.

As expected, there was an increase in the pool size of the xanthophyll cycle carotenoids with increasing growth PFD, both per unit of chlorophyll and as a fraction of the total carotenoid pool (Table I). It is interesting to note that unlike the chlorophyll *a* plus *b* content, which typically showed the largest differences between plants grown at 20 and 100 μmol photons m^{-2} s^{-1}, the largest difference in the pool size of the xanthophyll cycle occurred between plants grown at 100 and 1200 μmol photons m^{-2} s^{-1}. Red leaves of high-PFD-grown *M. repens* had the largest xanthophyll cycle pool size overall, which is typical for developing leaves (Krause et al., 1995). These changes in the xanthophyll cycle pool size are consistent with a greater requirement for photoprotective energy dissipation in leaves acclimated to high PFD (Demmig-Adams et al., 1996).

PSII Efficiency and Conversion State of the Xanthophyll Cycle

The photon efficiency of open PSII centers, given by the fluorescence parameter F_V'/F_M' (Demmig-Adams, 1990), was compared with the de-epoxidation state of the xanthophyll cycle pigments under steady-state conditions in the light. Plants growing at 20 and 100 μmol photons m^{-2} s^{-1} maintained near maximal PSII efficiencies in the light and retained nearly all of the xanthophyll cycle pool as V (Table II). In contrast, in high-PFD-grown plants there was an approximately 35% decline in F_V'/F_M', coinciding with

Table II. *PSII efficiency (F_V'/F_M') and the conversion state of the xanthophyll cycle pigments in leaves grown under 20, 100, and 1200 μmol photons m^{-2} s^{-1}*

Fluorescence measurements were taken on intact leaves 6 h into the diurnal light period, and pigment analysis was carried out on the same leaves. Values represent the means ± SD of three leaves.

Species/Growth PFD	PSII Efficiency	Conversion State (A+Z)/(V+A+Z)
μmol photons m^{-2} s^{-1}	F_V'/F_M'	
V. major		
20	0.80 ± 0.006	0
100	0.78 ± 0.002	0.07 ± 0.02
1200	0.67 ± 0.024	0.58 ± 0.05
S. arboricola		
20	0.79 ± 0.002	0
100	0.76 ± 0.006	0.11 ± 0.01
1200	0.62 ± 0.047	0.55 ± 0.08
M. repens		
20	0.78 ± 0.004	0
100	0.76 ± 0.003	0
1200-Green	0.53 ± 0.025	0.53 ± 0.08
1200-Red	0.59 ± 0.058	0.75 ± 0.06

conversion of more than 50% of the xanthophyll cycle carotenoids to the de-epoxidized forms A plus Z. These results are generally consistent with the idea that losses in PSII efficiency are associated with xanthophyll cycle-dependent energy dissipation in the antennae (Björkman and Demmig-Adams, 1994).

Photochemical Quenching, Energy Dissipation, and Stromal Reduction State

The q_P provides an estimate of the fraction of oxidized PSII quinone acceptor or "open" PSII centers (Schreiber et al., 1986). In plants grown at 20 and 100 μmol photons m^{-2} s^{-1}, q_P was greater than 97% among all species during steady-state illumination (Table III). In part because of decreases in PSII efficiency (Table II), q_P also remained high in high-PFD-grown plants, ranging from 80 to 85%.

Under steady-state conditions, NPQ of F_M increased with increasing growth PFD in all species (Table III), with the highest values observed in high-PFD-grown *M. repens*. Even higher values of NPQ are typically observed during peak irradiance in sun-exposed populations of these species in the field. However, we do not believe our results are inconsistent with reported field measurements, since the highest growth PFD used in these experiments (1200 μmol photons m^{-2} s^{-1}) was still well below the maximum solar irradiance at midday. Higher values of NPQ were observed in chamber-grown plants at 2000 μmol photons m^{-2} s^{-1} (data not shown).

The activation state of the chloroplast-localized NADP-dependent MDH was used as a metabolic indicator of the reduction state of the stromal NADP pool (Harbinson et al., 1990). Among all species, plants acclimated to low PFDs exhibited a consistently low activation state (<0.15), indicating a largely oxidized NADP pool under steady-state conditions (Table III). The total activity (i.e. in the fully reduced extract) in plants that were grown under very low PFDs was quite variable and typically very low; therefore, data from these experiments were not used. The MDH activation state was unexpectedly high, approximately 0.65, in high-PFD-grown *V. major* and *S. arboricola*, indicating a much higher stromal reduction state in comparison with the low-PFD treatment. In contrast, the NADP-MDH activation state was only 0.15 in high-PFD-grown *M. repens*. It is also important to note that the total MDH activity of high-PFD-grown *M. repens* was more than twice that of the low-PFD treatments and considerably higher than the high-PFD treatment in the other two species (data not shown).

Antioxidant Metabolites

Levels of the major soluble antioxidant metabolites ascorbate and glutathione exhibited a consistent increase in response to higher growth PFDs in both *V. major* and *S. arboricola* (Fig. 2). The most dramatic differences were observed between plants grown at 100 and 1200 μmol photons m^{-2} s^{-1}. The increase in the ascorbate content was less dramatic in high-PFD-grown *M. repens*, with similar levels observed in both green and red leaves. However, it is noteworthy that the ascorbate content of *M. repens* that were grown at 20 μmol photons m^{-2} s^{-1} was approximately 10-fold higher than in the other two species.

α-Tocopherol contents did not exhibit any consistent trend in response to growth PFD (Fig. 2). Red leaves of high-PFD-grown *M. repens* had a very low α-tocopherol content, which is typical for developing leaves (Hess, 1993).

Table III. *q_P, NPQ, and the activation state of NADP-dependent MDH in leaves grown under 20, 100, and 1200 μmol photons m^{-2} s^{-1}*

The activation state is defined as the ratio of NADP-MDH activity obtained immediately following extraction to the activity of the fully reduced enzyme. Fluorescence measurements were taken on intact leaves 6 h into the diurnal light period. Tissue for enzyme analysis was harvested on a separate day. The time between taking a leaf punch and freezing in liquid nitrogen was approximately 20 s. NPQ values were corrected based on a maximal dark-adapted F_V/F_M of 0.84 (Demmig-Adams, 1990). Values represent the means ± SD of three leaves or two leaves where no SD is shown.

Species/Growth PFD	q_P	NPQ	NADP-MDH Activation State
μmol photons m^{-2} s^{-1}			
V. major			
20	0.98 ± 0.002	0.08 ± 0.039	–[a]
100	0.97 ± 0.005	0.28 ± 0.051	0.13 ± 0.05
1200	0.80 ± 0.014	0.84 ± 0.080	0.66
S. arboricola			
20	0.98 ± 0.001	0.19 ± 0.024	–
100	0.97 ± 0.009	0.42 ± 0.029	0.12 ± 0.08
1200	0.79 ± 0.043	0.97 ± 0.105	0.65
M. repens			
20	0.99 ± 0.003	0.27 ± 0.022	0.093 ± 0.08
100	0.97 ± 0.006	0.42 ± 0.016	0
1200-Green	0.85 ± 0.066	1.81 ± 0.343	0.15 ± 0.10
1200-Red	0.87 ± 0.011	1.62 ± 0.490	–

[a] –, Data not available.

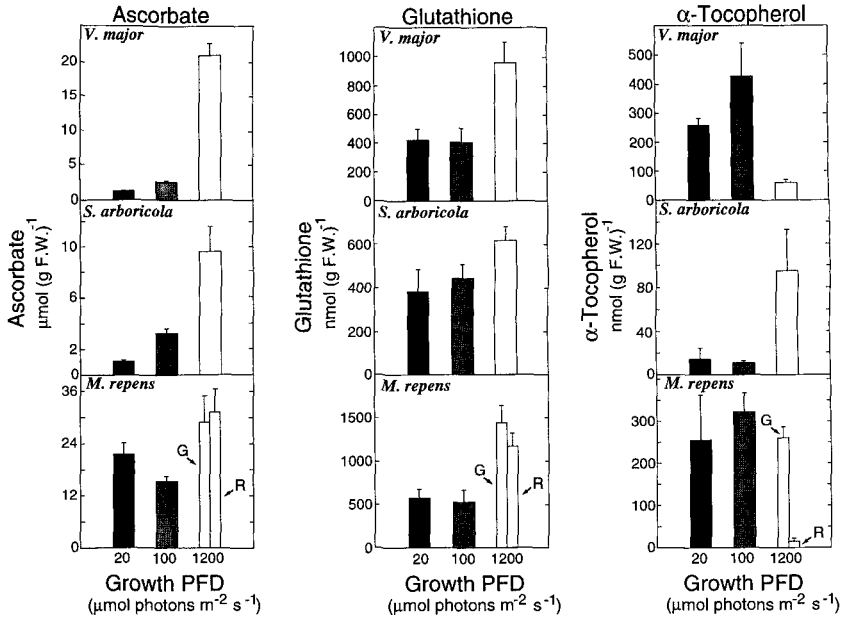

Figure 2. Foliar ascorbate, glutathione, and α-tocopherol content in leaves grown under 20, 100, and 1200 μmol photons m^{-2} s^{-1}. Values represent the means ± SD of three leaves. F.W., Fresh weight; G, Green leaves; R, red leaves.

Antioxidant Enzymes

The three primary enzymes of the Mehler-peroxidase pathway (SOD, APX, and GR) exhibited a general trend toward higher activity with increasing growth PFD in all species (Fig. 3). A notable exception was the developing red leaves of high-PFD-grown *M. repens*, which exhibited approximately 3-fold less APX and GR activity relative to mature green leaves acclimated to the same PFD. However, a similar reduction in soluble protein levels suggests that low enzyme activities were a consequence of the developmental state of the leaves (data not shown). An enigmatic opposing trend was observed with respect to SOD, with approximately 30% more activity found in red leaf than in green leaf extracts. We suggest that a large fraction of this apparent SOD activity was due to the radical-scavenging properties of anthocyanins, which were particularly abundant in these samples. Anthocyanins, derived from the flavonoid biosynthetic pathway, have been shown to react with a variety of free radical substrates (Rice-Evans et al., 1995; van Acker et al., 1996). We are currently investigating whether anthocyanins react directly with the superoxide radical.

As was the case with ascorbate and glutathione, leaves of *M. repens* had notably higher antioxidant enzyme activities in comparison with the other two species. The high "total antioxidant capacity" was correlated with a lower leaf water content and a 3-fold higher soluble protein content per unit fresh weight (not shown). To our knowledge, the values for ascorbate content and antioxidant enzyme activity of *M. repens* are among the highest reported for any species.

Catalase activities in *V. major* and *S. arboricola* did not exhibit strong trends in relation to growth PFD (Table IV). However, in *M. repens* catalase activity increased approximately 2-fold in the high-PFD treatment in both red and green leaves. Contrary to our expectation, MDAR activities were highest in plants grown at 100 μmol photons m^{-2} s^{-1} for all species (Table IV).

DISCUSSION

It has been suggested that leaves acclimated to high PFDs compensate fully for the fraction of absorbed light that is not utilized in photosynthesis by dissipating the excess energy nonphotochemically in the light-harvesting system (Foyer et al., 1990; Demmig-Adams et al., 1996). However, our investigations of the acclimation of leaf antioxidant systems suggest that the rate of O_2 photoreduction increases with increasing growth irradiance, despite concomitant increases in xanthophyll cycle-dependent energy dissipation.

The antioxidant enzyme system comprises multiple isoforms that are located in different subcellular compartments. In leaves the activities of each of the major antioxidant enzymes, SOD, APX, GR, and MDAR, are concentrated within the chloroplast (Foyer and Halliwell, 1976; Jackson et al., 1978; Foster and Edwards, 1980; Hossain et al., 1984; Gillham and Dodge, 1986, Edwards et al., 1990). These enzymes act in concert both to ensure the efficient removal of ROS generated during photosynthesis and to allow photon utilization in electron transport (Osmond and Grace, 1995). We propose that changes in their activity reflect changes in the rate of photosynthetic O_2 reduction in vivo.

Most of the total catalase activity in leaves is concentrated within the peroxisome (Volk and Feierabend, 1989), where it is involved in removing H_2O_2 that is generated by glycolate oxidation during photorespiration. Photorespiration increases during water stress as intracellular CO_2 becomes limiting for photosynthesis (Cornic and Briantais, 1991), leading to increased leaf catalase activity (Mittler and Zilinskas, 1994). However, there is no evidence that photorespiration constitutes an energy-dissipating process

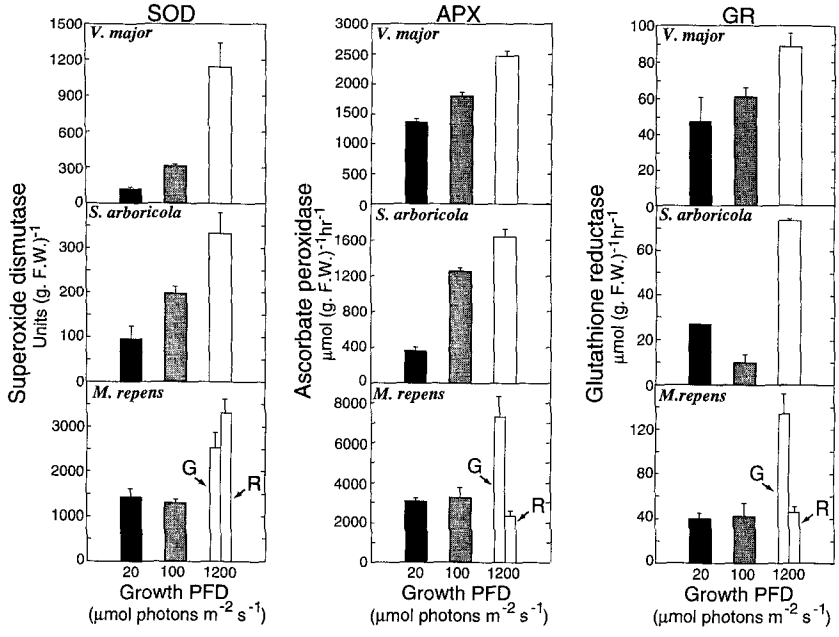

Figure 3. Activities of SOD, APX, and GR in leaves grown under 20, 100, and 1200 µmol photons m^{-2} s^{-1}. Samples were collected 4 h into the diurnal light period. Values represent the means ± SE of three leaves. F.W., Fresh weight; G, Green leaves; R, red leaves.

under normal conditions, since the rate of photorespiration, relative to photosynthesis, remains nearly constant over a range of light intensities (Canvin et al., 1980; Ogren, 1984). An increase in the absolute flux through the pathway, concomitant with an increased rate of photosynthesis, would nevertheless be expected to result in higher leaf catalase activities. Growth at high PFD was associated with an increase in total catalase activity in *M. repens*, although no such relationship was observed in the other two species. High-PFD-grown *V. major*, in fact, exhibited a slight decline in leaf catalase activity. There have been reports of catalase inactivation in leaves exposed to high light, especially under chilling conditions (Volk and Feierabend, 1989; Mishra et al., 1993). Catalase-deficient leaves show no increase in H_2O_2 levels relative to control plants (Volk and Feierabend, 1989), consistent with the view that enzymatic removal of H_2O_2 by the ascorbate/APX system is the dominant pathway in leaves.

Table IV. *Activities of catalase and MDAR in leaves grown under 20, 100, and 1200 µmol photons m^{-2} s^{-1}*

Values represent the means ± SD of three leaves.

Species/Growth PFD	Catalase	MDAR
µmol photons m^{-2} s^{-1}	*units (g fresh wt.)$^{-1}$*	*µmol (g fresh wt.)$^{-1}$ h^{-1}*
V. major		
20	1543 ± 71	312 ± 34
100	1543 ± 138	632 ± 31
1200	1255 ± 72	120 ± 25
S. arboricola		
20	742 ± 191	72 ± 19
100	1147 ± 99	232 ± 24
1200	991 ± 128	51 ± 4
M. repens		
20	407 ± 54	113 ± 14
100	527 ± 61	497 ± 29
1200-Green	1015 ± 69	241 ± 67
1200-Red	935 ± 240	–[a]

[a] –, Data not available.

Ascorbate exhibited the most dramatic response to growth PFD among the antioxidants studied. This may be due to its many roles in photoprotection of the chloroplast. Ascorbate is the reductive substrate of V de-epoxidase, the enzyme that catalyzes the conversion of V to A and Z (Yamamoto, 1979). It is also the reductant for APX, the enzyme that reduces photosynthetically generated H_2O_2 (Nakano and Asada, 1981). Thus, it plays a critical role in both upstream xanthophyll cycle-dependent energy dissipation and downstream detoxification of ROS via the Mehler-peroxidase pathway. Another function of ascorbate involves the electron transfer-mediated recovery of vitamin E, thereby affording protection against the deleterious effects of lipid peroxidation (Packer et al., 1979). The direct radical-scavenging activity of both ascorbate and glutathione in the soluble phase may also provide an important antioxidant function outside the chloroplast (Cadenas, 1995).

To function as an antioxidant, ascorbate must be maintained in the reduced state. In leaves the ascorbate radical is reduced either enzymatically by MDAR, using NADH and to a lesser extent NADPH as electron donors, or by direct photoreduction in the chloroplast using electrons derived from Fd (Miyake and Asada, 1994; Grace et al., 1995). The latter reaction demonstrates a high capacity and, given an adequate rate of electron transport, appears to take precedence over the enzymatic pathway for ascorbate regeneration inside the chloroplast (Grace and Osmond, 1995).

We found, somewhat unexpectedly, the highest MDAR activities in plants acclimated to 100 µmol photons m^{-2} s^{-1}. The low activities observed in leaves acclimated to 20 µmol photons m^{-2} s^{-1} are consistent with a low rate of ascorbate oxidation under these conditions. However, the

approximate 4-fold reduction in MDAR activity that was observed in the high-PFD treatment, relative to the low-PFD treatment, seems inconsistent with the high rates of ascorbate utilization in the Mehler-peroxidase reaction. This result can be explained by an increase in the relative importance of the direct photoreduction of ascorbate at high PFD. We have consistently observed higher ascorbate reduction states in sun- versus shade-acclimated leaves in the field, suggesting that direct photoreduction is a more efficient pathway for ascorbate regeneration (B. Logan and S. Grace, unpublished observations).

The two-electron oxidation product of ascorbate, DHA, is recycled back to ascorbate using glutathione as an electron donor. This reaction occurs readily in the absence of catalysis at pH values greater than 7.5, which are likely to prevail in the chloroplast stroma during illumination (Foyer and Halliwell, 1976; Winkler et al., 1994). The oxidized product of this reaction, GSSH, is reduced by GR using NADPH as an electron donor. Photosynthetic electron transport provides a source of NADPH in the light, whereas the dark-activated enzyme Glc-6-P dehydrogenase provides a source in darkness (Foyer and Halliwell, 1976). The up-regulation of both glutathione and GR in response to growth PFD supports a role for this system in scavenging the products of photosynthetic O_2 reduction.

NADP-MDH is one of many thiol-modulated enzymes in the chloroplast, which, in addition, is allosterically regulated by NADP (Scheibe, 1987). The combination of reductive activation and metabolite regulation allows NADP-MDH to be used as a "sensor" of the reduction state of the stromal NADP pool (Harbinson et al., 1990). It has been suggested that measurements of NADP-MDH activity provide a more accurate reflection of the stromal redox state due to uncertainties in direct metabolite measurements in leaves (Foyer, 1993). NADP-MDH allows reducing power in the form of malate to be efficiently exported from the chloroplast via the dicarboxylic acid translocator. Since the enzyme is inhibited by a high NADP/NADPH ratio, export by this pathway will occur only when the photosythetic rate is saturated (Scheibe, 1987).

Measurements of NADP-MDH activity revealed an unexpectedly high stromal reduction state in high-PFD-grown *V. major* and *S. arboricola*. Previous studies using plants with high rates of photosynthesis (spinach and pea) have shown that NADP-MDH activity in vivo seldom exceeds 30% of the maximum, even in saturating light (Scheibe and Stitt, 1988; Harbinson et al., 1990). This supports the hypothesis that photoprotective "down-regulation" of PSII prevents overreduction of the chloroplast NADP pool (Foyer, 1993). However, when photosynthesis is constrained, e.g. by low temperature (Scheibe and Stitt, 1988) or low [CO_2] (Harbinson et al., 1990), NADP-MDH activity dramatically increases, indicating that regulation of PSII activity is not always fully effective in preventing overreduction of the stroma. The low photosynthetic capacities of *V. major* and *S. arboricola* suggest that similar limitations on photon utilization may exist in these evergreen species. However, unlike the redox state of the stroma, there was no evidence of PSII overreduction (Table III), suggesting that electron flow to alternative acceptors such as O_2 may indeed be occurring.

High-PFD-grown *M. repens* was unique in many respects. This species had the highest rates of photosynthesis (Fig. 1) as well as a substantially lower MDH activation state (Table III). Total NADP-MDH activity was also considerably higher in this species (data not shown), presumably reflecting an increased capacity to export reducing power in the form of malate out of the chloroplast. An important energy-consuming process that occurs in the cytosol of this species is flavonoid biosynthesis, known to be a light-activated process (Beggs and Wellman, 1994). Although a functional role for these compounds in screening potentially harmful UV radiation has been demonstrated (Lois and Buchanan, 1994; Burger and Edwards, 1996), there is growing evidence that certain types of flavonoids also possess significant antioxidant properties (Rice-Evans et al., 1995; van Acker et al., 1996). We are currently investigating whether induction of flavonoid biosynthesis is part of the mechanism of high light acclimation in *M. repens*. It is interesting to note that, even though high-PFD-grown *M. repens* had a more highly oxidized stroma than did *V. major* or *S. arboricola*, the overall response of its antioxidant systems was similar, suggesting that electron flow to O_2 is not strictly dependent on the stromal redox state.

Although the role of O_2 as an alternative electron acceptor during the induction phase of photosynthesis has been established (Radmer and Kok, 1976; Marsho et al., 1979), the magnitude of the O_2-dependent flow during steady-state photosynthesis remains controversial. Studies of in vivo chlorophyll fluorescence show little evidence for direct electron flow to O_2 (Cornic and Briantais, 1991), although this has recently been questioned (Lovelock and Winter, 1996). Direct measurements of O_2 uptake using MS have demonstrated that even under nonphotorespiratory conditions there is evidence for a substantial fraction of electron flow to O_2 in C_3 plants at light saturation (Canvin et al., 1980; Badger, 1985). Studies with isolated spinach chloroplasts revealed that O_2 photoreduction could account for 16 to 33% of the total electron flow, depending on the availability of NADP (Robinson and Gibbs, 1982). In intact protoplasts of *Xanthium strumarium* under nonphotorespiratory conditions, up to 19% of the total electron flow was accounted for by O_2 reduction in the chloroplast (Furbank et al., 1982). These studies suggest that, even in the presence of NADP, O_2 can act as a competitive electron acceptor.

Our results do not distinguish whether electron flow to O_2 via the Mehler-peroxidase pathway acts to potentiate nonphotochemical energy dissipation by modulating the proton gradient (Neubauer and Yamamoto, 1992) or serves as a dissipative process itself (Osmond and Grace, 1995). This study, however, provides indirect evidence that upstream, xanthophyll cycle-dependent energy dissipation, even in a plant acclimated to its growth PFD, does not completely eliminate the photoreduction of molecular O_2. The antioxidant system, therefore, appears to be a fundamental part of the acclimatory response of plants to high PFDs.

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