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Photosynthetic and antioxidant enzyme responses of sugar maple and red maple seedlings to excess manganese in contrasting light environments

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Abstract. Manganese (Mn) toxicity may be a significant constraint to forest health on acidic, non-glaciated soils. We hypothesised that sugar maple (*Acer saccharum* Marsh.) and red maple (*Acer rubrum* L.) seedlings differ in their tolerance to excess Mn, and that photosynthetic sensitivity to excess Mn is exacerbated at higher light intensities through photo-oxidative stress. To test these hypotheses, we assessed photosynthesis and antioxidant enzyme responses of sugar maple and red maple seedlings at variable Mn and light levels in a greenhouse study. In both species, high Mn treatments impaired photosynthetic function, particularly in high light conditions. Responses to Mn and light depended on the developmental stage of the leaves. All sugar maple leaves were sensitive to Mn toxicity except shaded young leaves. For red maple, only mature leaves exposed to high light were prone to Mn toxicity. Antioxidant enzyme and $\Phi_{PSII} / \Phi_{CO_2}$ data suggested that photo-oxidative stress did not explain the observed photosynthetic responses to treatment variables. Our results indicate that in natural forest environments, sugar maple and red maple foliage exposed to high light intensity (outer canopy, canopy gaps) may be more prone to Mn toxicity.

Keywords: chlorophyll, metals, nutrition, photo-oxidation, photosynthesis, stress.

Introduction

The eastern forests of North America, which include both the mixed deciduous forest and transitional forest, are a principal terrestrial biome covering about 65 million hectares. Much of this forest is in the northeast USA where anthropogenic influences are significant. Over the last few decades, symptoms of declining health in sensitive tree species have been observed (Tomlinson and Tomlinson 1990). The decline of sugar maple has been particularly well documented (Bernier and Brazeau 1988*b*; Sharpe and Drohan 1999; Horsley *et al.* 2000; Drohan *et al.* 2002). Symptoms include a reduction in growth, canopy deterioration, leaf chlorosis, tree mortality and low rates of seedling regeneration (Bernier and Brazeau 1988*a*; Tomlinson and Tomlinson 1990; McWilliams *et al.* 1996; Horsley *et al.* 2000; Duchesne *et al.* 2002).

In contrast with sugar maple, the range and dominance of red maple has expanded significantly in North American forests over the last 100 years (Abrams 1998). Starting in the early 1990s, significant decline symptoms were observed in trees of the Allegheny National Forest (McWilliams *et al.* 1996). Surveys revealed that mortality and health decline symptoms were three times greater in sugar maple than red maple (McWilliams *et al.* 1996). Soil acidification and the disruption of nutrient cycles appears to be a central factor that weakens sugar maple resistance to both abiotic and biotic stresses and predisposes it to health decline symptoms (Horsley *et al.* 2000; Driscoll *et al.* 2001; Duchesne *et al.* 2002; Tomlinson 2003). In contrast, it has been proposed that tolerance of edaphic stresses is one factor that has contributed to the expansion of red maple populations in eastern forests of North America (Abrams 1998). Interspecific variation in tolerance to edaphic stresses may be an important factor influencing the differential success of these two species.

The majority of research examining the influence of nutrient stress on forest health has focused on aluminum (Al) toxicity and foliar deficiencies in calcium (Ca) and

Abbreviations used: A / C_i, assimilation of CO_2 / intercellular CO_2; APX, ascorbate peroxidase; CER, CO_2 exchange rate; ETR, electron transport rate; F'_m, maximal fluorescence in the light adapted state; F_s, steady state fluorescence; GR, glutathione reductase; ROS, reactive oxygen species; TSP, total soluble protein; Φ_{CO_2}, quantum yield of CO_2 fixation; Φ_{PSII}, quantum yield of PSII.

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magnesium (Mg) (Cronan and Grigal 1995; Huttl and Schaaf 1997; Kobe *et al.* 2002). There is evidence that metal toxicity of leaves also contributes to decline symptoms (Gawel *et al.* 1996). Manganese is an essential plant micronutrient that can become phytotoxic at high foliar concentrations (El-Jaoual and Cox 1998). The bioavailability of Mn increases as soils acidify below pH 5.5 (Lucas and Davis 1961). Health decline symptoms on acidic, non-glaciated forest soils of the Allegheny Plateau have been correlated with high foliar concentrations of Mn (Horsley *et al.* 2000). Microscopic examination of sugar maple leaves exposed to excess Mn has shown unusual cellular responses in palisade and spongy mesophyll cells (McQuattie and Schier 2000) such as irregularities in cell shape and the presence of electron-dense regions in the thylakoid membranes of the chloroplast. Chloroplasts accumulate Mn (McCain and Markley 1989; Gonzalez and Lynch 1999) that, at higher concentrations, impairs photosynthetic function in several plant species (Gonzalez and Lynch 1997; Kitao *et al.* 1997, 1998; Nable *et al.* 1988). Manganese toxicity varies among plant species and genotypes (Foy *et al.* 1988; Gonzalez *et al.* 1998). Tolerance to Mn toxicity could be one trait conferring differential success among tree species growing on acidic forest soils, where the hyperaccumulation of foliar Mn is common.

High light intensity can exacerbate nutrient stress in plants (Marschner and Cakmak 1989; Cakmak and Marschner 1992). This relationship may be particularly important in forest ecosystems where suboptimal nutrient conditions and high light gradients overlap. In crop plants the detrimental effects of excess Mn on net photosynthesis and leaf chlorophyll concentration are increased with higher light through photo-oxidative mechanisms (Gonzalez *et al.* 1998; Lidon and Teixeira 2000).

The objective of this study was to compare the photosynthetic and antioxidant responses of sugar maple and red maple seedlings to excess Mn under variable light regimes, in order to test the following hypotheses: that

1. sugar maple and red maple seedlings differ in their tolerance of excess Mn; and
2. photosynthetic sensitivity to excess Mn is exacerbated at higher light intensities through photo-oxidative stress.

Materials and methods

Plant material and growth conditions

Experiments were undertaken with 2-year-old bare-rooted seedlings of *Acer saccharum* Marsh. (Penn Nursery, Spring Mills, PA) and *Acer rubrum* L. (Musser Forest Nursery, Indiana, PA) grown from seed. Seedling roots were thoroughly rinsed with deionised water and seedlings then planted in 11.37-L plastic tree pots (15 cm wide × 41 cm deep; Steuwe and Sons, Corvallis, OR) filled with a mixture of (1 : 1 v / v) 20 mesh silica sand (US Silica Co., Ottawa, IL) and medium grade (2–4 mm particles) vermiculite (Schundler Co., Metuchen, NJ). Seedlings were irrigated three times a day with an automated drip system, which delivered approximately 500 mL of nutrient solution to each pot during each irrigation period. Delivery of this volume resulted in the loss of approximately 200 mL of solution from the bottom of each pot, thereby preventing nutrient accumulation in the pots. The nutrient solution contained (in $\mu mol\ L^{-1}$): 3214 N (3 : 1 $NO_3 : NH_4$); 212 P (PO_3); 1200 K; 1400 Ca; 400 Mg; 241 S (SO_4); 10 Fe (Fe EDTA); 168 Cl; 4.2 B; 5.0 Mn; 1.8 Zn; 0.3 Cu; 0.24 Mo. Solution pH was maintained at 5.0 with HCl.

Two greenhouse experiments examining the influence of Mn availability and light intensity on photosynthesis of red maple and sugar maple seedlings were conducted. In the first experiment, seedlings were planted on 13 May 2002. On 30 May 2002, treatment conditions were imposed. The experiment was a completely randomised 3 × 2 × 2 factorial split-plot design replicated five times. The factors were three Mn levels (5, 250 and 500 $\mu mol\ L^{-1}$, in the form of $MnSO_4$), two leaf-level light intensities [1500 $\mu mol\ m^{-2}\ s^{-1}$ and 500 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density (PPFD)] imposed by placing seedlings under in full sunlight or underneath shade cloth, and two species (sugar maple and red maple). The high Mn treatment was increased incrementally to 750 µM on day 46 and to a final concentration of 1 mM on day 57.

The second experiment included a total of 30 seedlings (20 sugar maple and 10 red maple). Sugar maple seedlings were planted 29 May 2002 and treatment conditions were imposed 12 June 2002. Red maple seedlings from unshaded blocks in experiment one served as treatment units in experiment two. The factors for experiment two were two Mn levels (5 and 500 $\mu mol\ L^{-1}$), and two light levels (1500 $\mu mol\ m^{-2}\ s^{-1}$ and 300 $\mu mol\ m^{-2}\ s^{-1}$ PPFD). In this experiment, shading was imposed on individual leaves of fully-exposed seedlings (as opposed to shading entire seedlings) by two layers of 125 mm, #1 Whatman filter paper (Whatman Co., Maidstone, Kent, UK). The filter paper was fixed to the petiole by a small piece of clear tape. The high Mn treatment was increased incrementally to 750 µM on day 33 and then to a final concentration of 1 mM on day 44.

Red maple seedlings in both experiments exhibited continuous apical growth throughout the course of the study. Sugar maple seedlings in the two experiments differed in their growth habit. In experiment one, sugar maple seedlings produced a single determinate flush of leaves, and in experiment two, they produced an initial flush followed by a second flush, resulting in continuous apical growth. Based on these growth patterns, measurements in experiment one were focused exclusively on the physiological responses of mature leaves. The continuous growth observed in experiment two allowed us to examine how leaves at different developmental stages (i.e. young *v.* mature leaves) responded to Mn and light treatments. In both species, mature leaves were defined as the third emerging leaf pair from the initial flush. Young leaves in both species were defined as the youngest leaf pair that had reached full expansion at the August measurement date, which in both species was usually the third leaf pair below the apical meristem. The development of these young leaves began 10–15 d after treatment conditions were imposed.

Both experiments were conducted in the same climate-controlled greenhouse at Penn State University (40°49′N, 77°49′W). Temperature and relative humidity in the shaded and full sunlight blocks were monitored with HOBO data loggers (MicroDAQ, Warner, NH). Mean day time temperature was 28 ± 5°C in unshaded blocks and 25 ± 4°C in shaded blocks. Night time temperature in both blocks averaged 23 ± 2°C. Relative humidity averaged 62 ± 6% regardless of the diurnal period or shade treatment. Leaf-level PPFD varied throughout the day, with a maximum of 1500 $\mu mol\ m^{-2}\ s^{-1}$.

Leaf gas exchange and chlorophyll fluorescence

Leaf CO_2 exchange rate (CER), and electron transport rate (ETR), were measured with a Li-Cor 6400 gas exchange system with a 6400-40 leaf chamber fluorometer (Li-Cor, Lincoln, NE). Gas exchange and fluorescence were measured at targeted leaf chamber irradiances of 1500 $\mu mol\ m^{-2}\ s^{-1}$ PPFD for the unshaded treatments

and 500 μmol m^{-2} s^{-1} PPFD (experiment one) or 300 μmol m^{-2} s^{-1} PPFD (experiment two) for the shaded treatments. Measurements were taken at ambient temperature and humidity. Measurements were initiated by sealing the leaf chamber onto each leaf proximally to the major leaf sinus avoiding the primary veins on either side. When dF/dt (the rate of change in the fluorescence signal) approached zero and CO_2 concentrations in the leaf chamber reached a steady state (60–90 seconds), F_s (steady state fluorescence) was measured followed by a 0.8 s saturating light flash to induce maximal fluorescence in the light adapted state (F'_m). These two coefficients were used to calculate $\Phi_{PSII} = F'_m - F'_s) / F'_m$ according to Genty *et al.* (1989). ETR was calculated according to the equation, ETR = $\Phi_{PSII} \times$ PFDa $\times$ 0.5 (Maxwell and Johnson 2000) where PFDa is assumed to be approximately 0.85. Gas exchange data was logged concurrently with fluorescence data and exchange rates were calculated according to von Caemmerer and Farquhar (1981). Photosynthetic measurements were taken between 1000 and 1500 h as diurnal gas-exchange measurements taken on red maple and sugar maple seedlings indicated that CER values were stable during these hours. The measurement sequence was randomised so that treatment effects were not biased by the time of day when measurements were taken.

In experiment one, gas exchange and chlorophyll fluorescence of the third emerging leaf pair from the flush were measured 28, 50 and 72 d after initiating treatment conditions. In experiment two, gas exchange and fluorescence measurements of mature leaves were taken on the third leaf pair in the original flush and measurements of young leaves were taken on the third leaf pair of the second expanding flush. Measurements were taken on 10 August 2002, which corresponded to day 72 for red maples and day 60 for sugar maples.

Assimilation of CO_2 / intercellular CO_2 (A / C_i) response curves were developed from experiment two. The curves were generated from measurements taken on four young leaves of unshaded seedlings within each Mn and species category. Leaf chamber CO_2 concentrations were modulated in the following sequence to create the A / C_i curves: 400, 300, 200, 100, 50, 400, 600, 800 μmol mol^{-1}. Light intensity was held constant at 1500 μmol m^{-2} s^{-1}. Light response curves were taken on 11 and 12 August 2002. Light response curves were also developed, generated from measurements taken on four young sugar maple leaves and four mature red maple leaves of unshaded seedlings within each Mn treatment category. Leaf chamber light levels were modulated in the following sequence to create the light curves: 2000, 1400, 800, 200, 50 and 0 μmol m^{-2} s^{-1} PAR. Each light level was imposed for a minimum of 60 s and a maximum of 120 s.

There is generally a strong linear relationship between the quantum yield of PSII (Φ_{PSII}) and the quantum yield of CO_2 fixation (Φ_{CO_2}) (Genty *et al.* 1989). PSII / CO_2 above the linear range of healthy leaves indicates an electron flow in excess of that being quenched through CO_2 fixation, which suggests that reactions generating a reactive oxygen species (ROS) may be operating (Fryer *et al.* 1998). Simultaneous measurements of Φ_{PSII} and Φ_{CO_2} were taken in this study to calculate $\Phi_{PSII} / \Phi_{CO_2}$. These measurements were made under non-photorespiring conditions so that energy dissipation through photorespiration and ROS generation could be distinguished (Fryer *et al.* 1998). Non-photorespiring conditions were achieved by connecting a gas cylinder with a 99 : 1 nitrogen : oxygen gas mixture to the air intake valve on the Li-Cor 6400.

Biomass, chlorophyll and elemental analysis

Following the gas-exchange and fluorescence measurements in August, the leaves upon which physiological measurements had been made were harvested for element, chlorophyll and antioxidant enzyme analysis. The leaves were stored in freezer bags purged with 100% nitrogen, and stored at −80°C until analysis was performed. Chlorophyll *a* and *b* were extracted by placing 28-mm^2 leaf disks from the tip and side of each leaf in 1 mL of *N*,*N*-dimethylformamide overnight at 4°C. Aliquots (0.2 mL) were diluted with 0.8 mL of *N*,*N*-dimethylformamide and measured at A_{664} and A_{647} with a UV 160 spectrophotometer (Shimadzu America, Columbia, MD). Chlorophyll *a* and *b* content were calculated according to the methods of Wellburn (1994). The remaining leaf tissue was divided into two subsamples for elemental and biochemical analyses. One subsample was dried for 72 h at 65°C, ashed at 500°C for 14 h, dissolved in 0.1 N HCl and analysed for Mn, Ca, Mg, K, Fe and Zn by atomic absorption spectrophotometry (AAnalyst 100, Perkin Elmer Co., Shelton, CT) according to Gonzalez *et al.* (1998). Seedling tissue from the second experiment was dried at 65°C for 1 week and weighed to determine total seedling biomass.

Antioxidant enzymes

Total soluble protein (TSP) extraction for determination of ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2) activity was performed according to Pell *et al.* (1999). The extraction buffer (pH 7.8) contained 90 mM potassium phosphate, 1 mM EDTA, 4% polyvinylpolypyrrolidone (PVPP), 1.5% PVP-40T, 5 mM ascorbate, and 8% glycerol. Leaf samples were ground in liquid nitrogen with a mortar and pestle. The ground samples (0.2 g) were transferred to a 5-mL plastic vial on ice. Ice-cold extraction buffer (2 mL) was added and the sample was homogenised on ice for 30 s with a hand-held homogeniser (Tissue-tearor, Biospec Products, Bartlesville, OK) on medium speed. The homogenate was then centrifuged at 15 850 *g* for 15 min at 4°C. Sample aliquots were immediately frozen and stored at −80°C.

Antioxidant enzyme activity was determined on young sugar maple leaves and mature red maple leaves from experiment two because of their significant photosynthetic responses to Mn and light treatment variables. APX activity was determined by following the linear decrease (for 60 s) in absorbance at 290 nm as APX catalyses the oxidation of ascorbate in the presence of H_2O_2 (Nakano and Asada 1981). The 1-mL reaction was initiated by combining in a cuvette the following reagents: potassium phosphate (50 mM, pH 7.8), ascorbate (0.5 mM), 10 μL of sample extract and H_2O_2 (0.1 mM). Final APX activity was calculated by subtracting for the ascorbate oxidase and non-enzymatic breakdown of ascorbate. GR activity was determined by following the linear increase (for 120 s) in absorbance at 412 nm as GR catalyses the reaction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and oxidised glutathione to form TNB (Smith *et al.* 1988). The 1-mL reaction was initiated by combining in a cuvette the following reagents: potassium phosphate (50 mM, pH 7.8), 50 μL of sample extract, DTNB (0.5 mM), NADPH (0.2 mM) and GSSG (0.2 mM). TSP was quantified by Bradford reagent (Bio-Rad Laboratories, Hercules, CA) with BSA standards so that antioxidant enzyme analysis could be calculated per mg of protein. Assays for GR, APX, and TSP were conducted by spectrophotometry (Lambda 25, Perkin Elmer Co., Shelton, CT).

Statistical analysis

The influence of Mn and light variables on dependent variables was tested with ANOVA, with significance defined as $\alpha \leq 0.05$. The light variable was omitted from the ANOVA model when examining the influences of Mn on foliar element concentrations because it was not significant. For experiment one, repeated measures of CER and ETR on days 28, 50 and 72 were examined for significant responses to treatment effects over time, with time as the ‘within’ factor (Gumpertz and Brownie 1993). Statistical analysis was performed by Statview statistical software (SAS Institute, Cary, NC).

Results

Elemental analysis

Experiment 1

In both experiments, foliar Mn accumulations were 1.5–2 times higher than the mean foliar Mn concentrations in sugar

maple and red maple seedlings collected from non-glaciated soils of Pennsylvania's Allegheny Plateau (St.Clair 2004). In experiment one, both maple species had similar patterns of foliar Mn accumulation, which increased relative to the Mn concentrations provided in the nutrient solution (Table 1). Excess Mn significantly decreased foliar concentrations of K and Fe in sugar maple seedlings and Mg in red maple seedlings (Table 1). Foliar K and Fe concentrations in sugar maple were more sensitive to Mn treatments than red maple as indicated by the significant Mn × species interaction (Table 1).

Experiment 2

Foliar Mn concentrations generally followed the patterns observed in experiment one, with the exception that some sugar maple foliage had lower Mn concentrations (Table 2). Excess Mn significantly decreased foliar K and Fe concentrations in mature sugar maple leaves and K, Fe, and Ca in young sugar maple leaves (Table 2). In mature red maple foliage, excess Mn significantly decreased foliar concentrations of Mg, Ca, and Fe. In young red maple leaves, decreases in Mg, Ca, and Fe in response to excess Mn were marginally significant (Table 2), but Zn was significantly increased.

Photosynthesis

Experiment 1

Repeated measures analysis indicated that excess Mn significantly decreased CER ($P<0.001$) and ETR ($P<0.001$) in sugar maple under both light treatments across all three

Table 1. Effect of Mn on foliar nutrition

Foliar concentrations (μg g^{-1} DW) of five elements in sugar maple and red maple seedlings in response to Mn treatments (5, 250, 1000 μM) from experiment one. Means ± 1 SE ($n = 5$) and P-values for foliar element responses to Mn treatments and the Mn × species interaction are presented

Mn (μM)	Mn (μg g^{-1} DW)	Mg (μg g^{-1} DW)	Ca (μg g^{-1} DW)	K (μg g^{-1} DW)	Fe (μg g^{-1} DW)
Sugar maple					
5	1623 ± 74	3857 ± 462	12 694 ± 1335	6653 ± 615	391 ± 68
250	6555 ± 541	2831 ± 232	11 702 ± 818	4187 ± 222	79 ± 17
1000	8462 ± 747	3281 ± 305	10 538 ± 1145	3720 ± 179	41 ± 10
P-level	< 0.001	0.131	0.408	< 0.001	< 0.001
Red maple					
5	1242 ± 73	2493 ± 144	11 523 ± 1107	9663 ± 579	39 ± 10
250	6519 ± 442	2251 ± 193	12 003 ± 1423	10 559 ± 970	31 ± 11
1000	8835 ± 402	1847 ± 151	9750 ± 650	8868 ± 746	31 ± 11
P-level	< 0.001	0.027	0.316	0.321	0.841
Mn × species	0.83	0.24	0.79	0.02	< 0.001

Table 2. Effect of Mn on nutrient status at different stages of leaf development

Foliar concentrations (μg g^{-1} DW) of six elements in young and mature foliage of sugar maple and red maple seedlings in response to Mn treatments (5, 1000 μM) from experiment two. Means ± 1 SE ($n = 10$ sugar maple, $n = 5$ red maple) and P-values for foliar element responses to Mn treatments are presented

	Mn (μM)	Mn (μg g^{-1} DW)	Mg (μg g^{-1} DW)	Ca (μg g^{-1} DW)	K (μg g^{-1} DW)	Fe (μg g^{-1} DW)	Zn (μg g^{-1} DW)
Sugar maple							
Young leaves	5	416 ± 36	1582 ± 99	8488 ± 557	9812 ± 316	237 ± 22	46 ± 1
	1000	8675 ± 514	1344 ± 102	6287 ± 422	7136 ± 275	114 ± 15	49 ± 1
	P-level	< 0.001	0.109	0.003	< 0.001	< 0.001	0.126
Mature leaves	5	776 ± 40	2885 ± 193	9708 ± 386	5177 ± 230	200 ± 14	48 ± 2
	1000	6473 ± 323	2869 ± 200	8917 ± 210	3971 ± 135	101 ± 11	52 ± 3
	P-level	< 0.001	0.955	0.067	< 0.001	< 0.001	0.362
Red maple							
Young leaves	5	1065 ± 98	2280 ± 174	11 835 ± 1228	11 653 ± 1117	75 ± 4	55 ± 2
	1000	9472 ± 850	1812 ± 159	8658 ± 970	12 029 ± 1255	63 ± 5	66 ± 2
	P-level	< 0.001	0.064	0.058	0.825	0.087	0.004
Mature leaves	5	1172 ± 77	2582 ± 200	13 534 ± 842	11 033 ± 1321	70 ± 4	52 ± 2
	1000	8835 ± 402	1991 ± 153	10 774 ± 343	8994 ± 901	52 ± 5	49 ± 5
	P-level	< 0.001	0.031	0.007	0.219	0.013	0.564

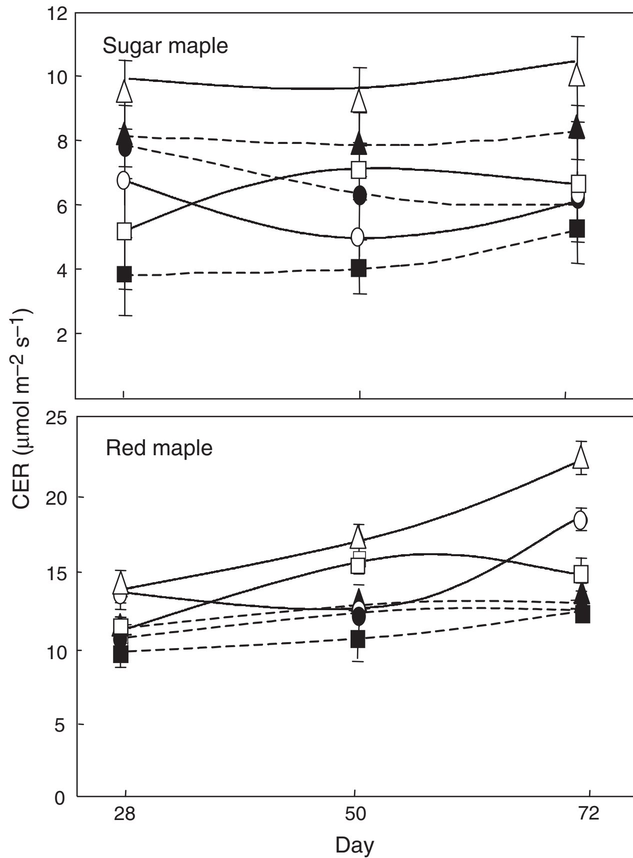

Fig. 1. Time-course analysis of experiment one examining CO_2 exchange rates (CER) of maple seedlings in response to three Mn concentrations (△, 5 µM; ○, 250 µM; □, 1000 µM) and two light levels (500 µmol m^{-2} s^{-1}, 1500 µmol m^{-2} s^{-1}). Dashed lines with solid symbols are shaded seedlings (500 µmol m^{-2} s^{-1} light), solid lines with open symbols are unshaded seedlings (1500 µmol m^{-2} s^{-1} light). Error bars represent ± 1 SE.

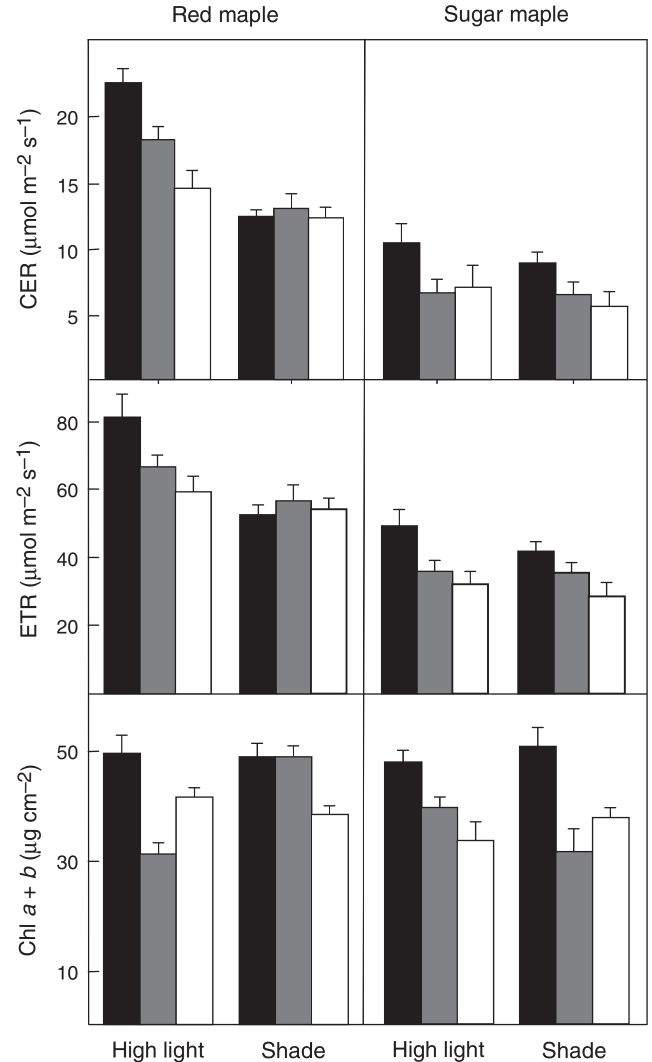

Fig. 2. The influence of Mn availability (black, 5 µM; grey, 250 µM; white, 1000 µM) and light intensity (1500 and 500 µmol m^{-2} s^{-1}) on the light and dark reactions of photosynthesis (CER, ETR) and chlorophyll $a + b$ in red and sugar maple leaves of experiment one. Error bars represent ± 1 SE.

measurement dates (Fig. 1). In contrast, high Mn significantly decreased CER in red maple seedlings only under high light conditions, as indicated by a significant Mn × light interaction ($P<0.01$). This response increased over the course of the experiment, as indicated by a significant Mn × light date interaction for red maple seedlings ($P<0.05$).

Excess Mn significantly reduced CER, ETR, stomatal conductance and chlorophyll $a + b$ in sugar maple on day 72 under both light treatments (Fig. 2) as indicated by the significant Mn variable in the ANOVA models ($P<0.05$). In red maple seedlings, excess Mn significantly decreased CER, stomatal conductance and chlorophyll $a + b$, and the Mn effect on ETR was marginally significant ($P=0.06$). Red maple was characterised by its differential sensitivity to excess Mn depending on light intensity, as Mn decreased CER and ETR only under high light conditions (Fig. 2) as indicated by significant Mn × light interactions for these two variables ($P<0.05$). In red maple chlorophyll $a + b$ content and stomatal conductance significantly decreased in response to excess Mn under both light conditions (Fig. 2).

Experiment 2

In young sugar maple leaves, excess Mn reduced carboxylation efficiency by 60% (Fig. 3), and significantly decreased CER, ETR, stomatal conductance and chlorophyll $a + b$ (Fig. 4; Table 3). High light increased the sensitivity of CER and ETR of young sugar maple leaves to excess Mn as indicated by the significant interactions between Mn and light (Fig. 4; Table 3) and light response curves (Fig. 5). In mature sugar maple leaves, reductions in chlorophyll $a + b$ under excess Mn was the only statistically significant response to treatment conditions (Fig. 4;

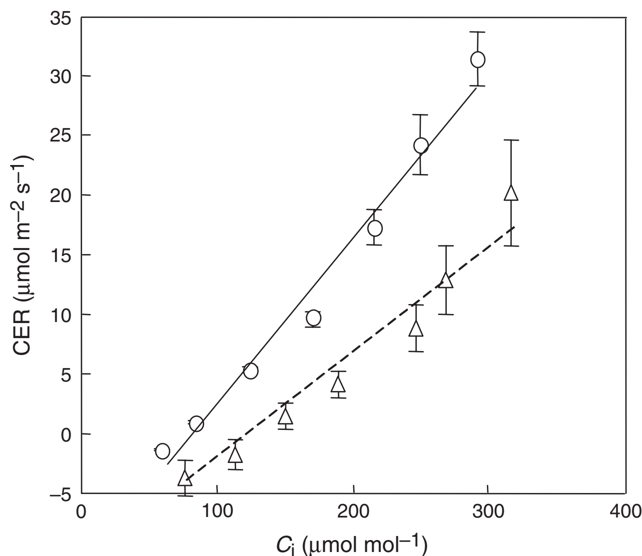

Fig. 3. The influence of Mn availability (○, 5 µM; △, 1000 µM) on CO_2 exchange rates (CER) with increasing intercellular CO_2 concentrations (C_i) in young sugar maple leaves. Light intensity was constant during all measurements (1500 µmol m^{-2} s^{-1}). Error bars represent ± 1 SE (n = 4).

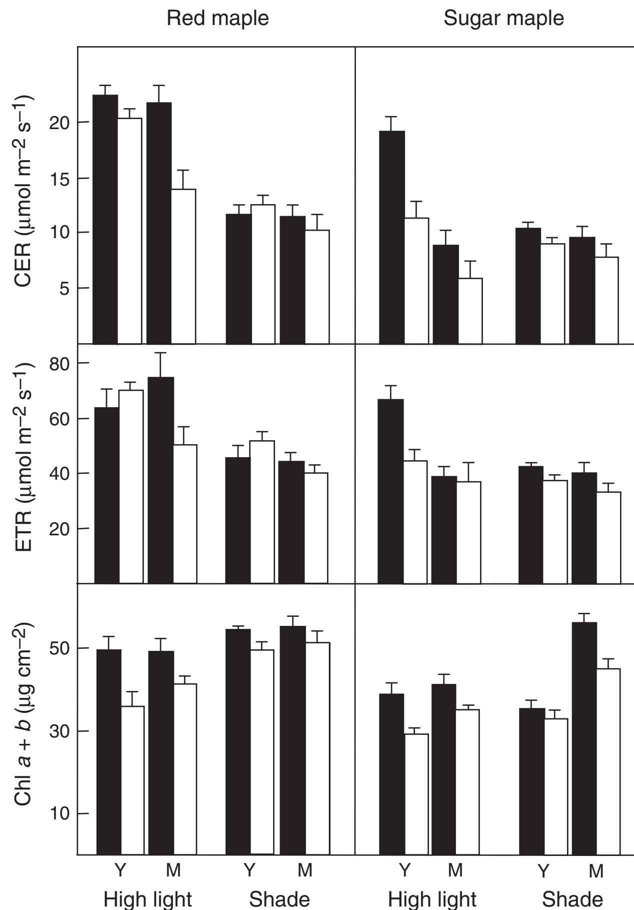

Fig. 4. The influence of Mn availability (black, 5 µM; white, 1000, µM), light intensity (1500 and 300 µmol m^{-2} s^{-1}) and leaf age (Y, young leaves; M, mature leaves) on the light and dark reactions of photosynthesis (CER, ETR) and chlorophyll $a+b$ in red maple and sugar maple seedlings in experiment two. Error bars represent ± 1 SE.

Table 3), although CER was marginally significant (P=0.07).

As observed in experiment one, CER, ETR and chlorophyll $a+b$ of red maple leaves were not responsive to excess Mn under low light conditions (Fig. 4). Under high light conditions, photosynthesis of young red maple leaves was more tolerant of excess Mn than mature leaves (Fig. 4). In young leaves, chlorophyll $a+b$ was the only parameter significantly reduced by excess Mn under high light conditions (Fig. 4; Table 3). In mature leaves under high light, CER, ETR and stomatal conductance

Table 3. Effects of Mn and light on photosynthesis at different stages of leaf development

F-values from ANOVA of experiment two, examining photosynthesis-related responses of sugar maple and red maple seedlings to Mn (5, 1000 µM) and Mn × light (responses under different combinations of Mn and light intensities, 1500 and 300 µmol m^{-2} s^{-1}). *, P<0.05; **, P<0.01; ***, P<0.001

	Source of variation	CER	ETR	Stomatal conductance	Chlorophyll $a+b$
Sugar maple					
Young leaves	Mn	14.99***	10.95**	17.34***	7.27**
	Mn × light	7.46**	4.38*	0.23	3.01
Mature leaves	Mn	3.45	0.73	1.09	15.14***
	Mn × light	0.29	0.31	<0.01	1.65
Red maple					
Young leaves	Mn	0.45	1.85	1.71	9.91**
	Mn × light	2.64	<0.01	0.69	2.43
Mature leaves	Mn	9.12**	5.94*	5.94*	3.98
	Mn × light	5.13*	2.71	<0.01	0.40

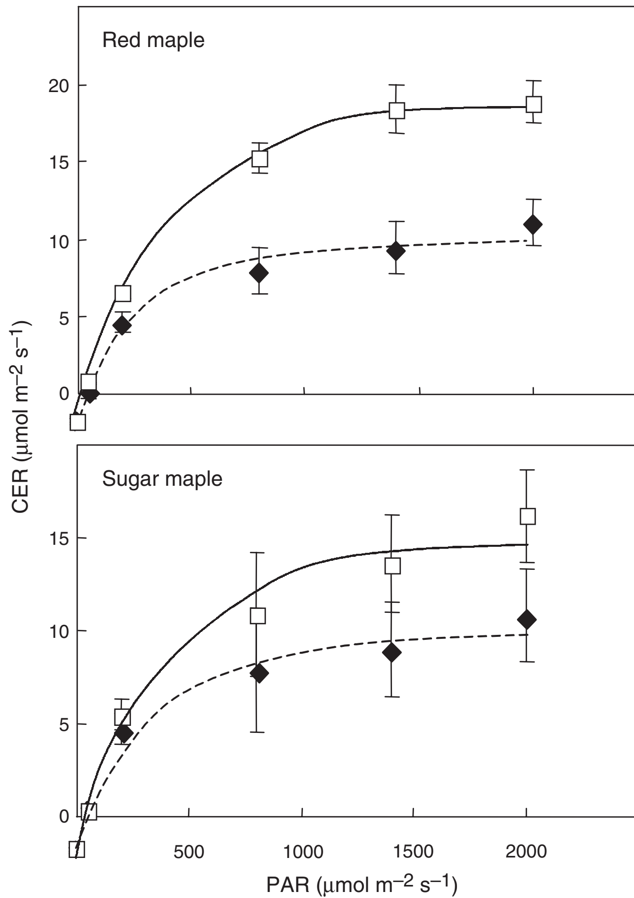

Fig. 5. The influence of Mn availability (□, 5 μM; ◆, 1000 μM) on CO_2 exchange rate with increasing light intensity (0, 50, 200, 800, 1400 and 2000 μmol m^{-2} s^{-1}) on mature red maple and young sugar maple leaves from experiment 2 (n = 4). Error bars represent ± 1 SE.

were significantly reduced by high Mn, and reductions in chlorophyll $a + b$ were marginally significant (P=0.06, Fig. 4; Table 3). The Mn-induced decreases in the CER of mature leaves were potentiated by high light (Fig. 4) as indicated by the significant Mn × light interaction (Table 3) and light response curves (Fig. 5). The relationships between CER and stomatal conductance and ETR in both species across all treatment conditions were strongly linear (Fig. 6).

Biomass (experiment 2)

In experiment two, excess Mn reduced biomass by 31% in sugar maple (F = 7.3, P=0.015) and 37% (F = 10.6, P=0.013) in red maple seedlings.

Antioxidant enzymes and $\Phi_{PSII} / \Phi_{CO_2}$ *(experiment 2)*

In young sugar maple leaves, none of the treatment variables significantly influenced the activity of either antioxidant enzyme (Fig. 7). In mature red maple leaves, GR activity was significantly (P<0.05) up-regulated in response to excess Mn under both light conditions (Fig. 7).

The relationship between Φ_{PSII} and Φ_{CO_2} was linear (Fig. 8*A*). The $\Phi_{PSII} / \Phi_{CO_2}$ ratio did not differ significantly among any of the treatment conditions. The $\Phi_{PSII} / \Phi_{CO_2}$ ratio of mature sugar maple leaves was significantly greater than the linear slope, but there was substantial variation as indicated by the error bars (Fig. 8*B*).

Discussion

This study examined the photosynthetic responses of sugar maple and red maple seedlings to excess Mn at variable light intensities to test the following hypotheses: (1) sugar maple and red maple seedlings differ in their tolerance to excess Mn;

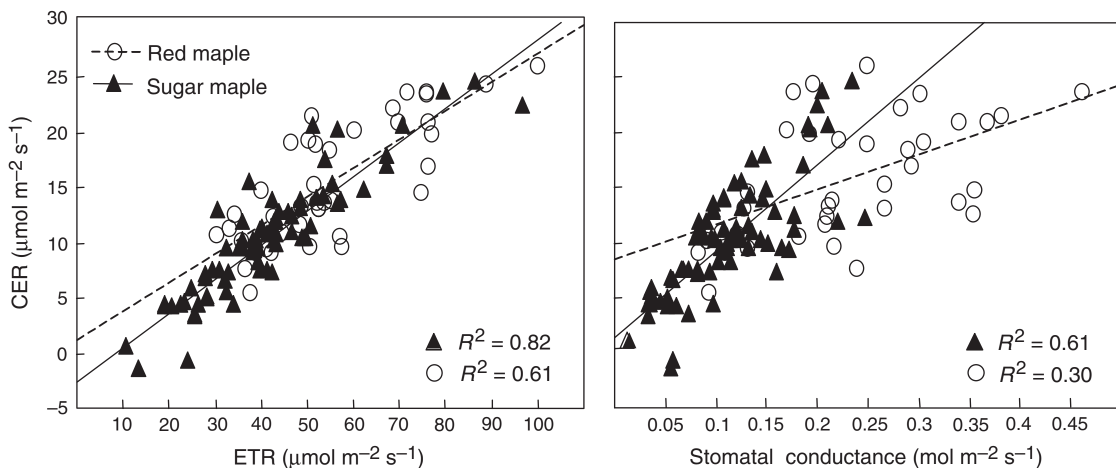

Fig. 6. Relationships between CER and ETR or stomatal conductance in sugar maple and red maple seedlings in experiment 2 across all treatment conditions. All coefficients of determination are significant at P<0.001.

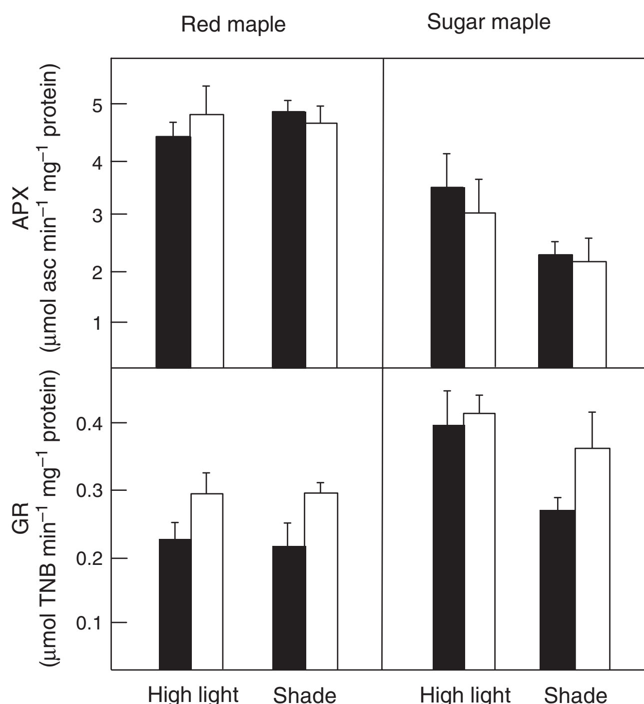

Fig. 7. GR and APX activity in young sugar maple leaves and mature red maple leaves exposed to two Mn levels (black, 5 µM; white, 1000 µM) and two light levels (1500 and 300 µmol m^{-2} s^{-1}) in experiment 2. Error bars represent ± 1 SE.

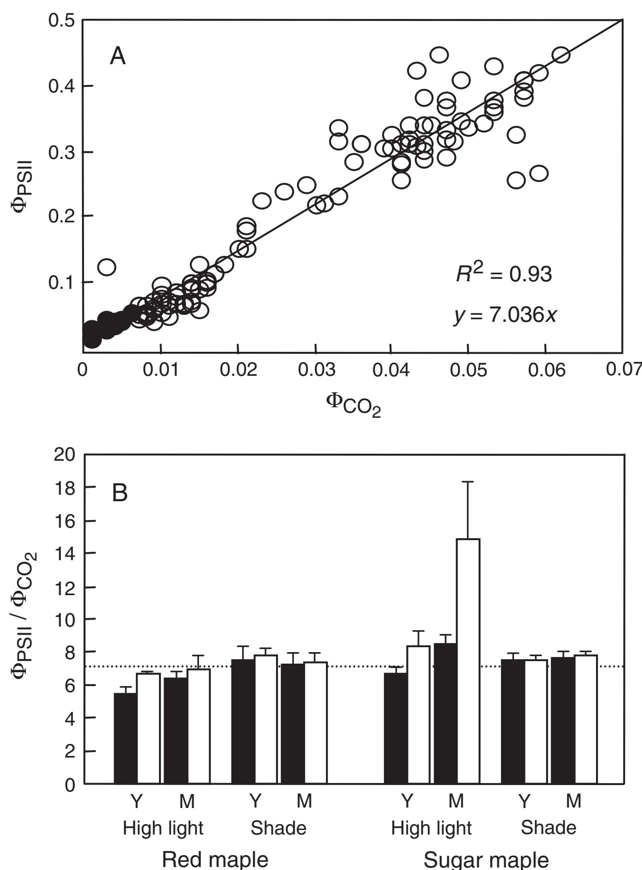

Fig. 8. Regression analysis examining relationship of Φ_{PSII} and Φ_{CO_2} of red maple and sugar maple leaves under all treatment conditions in experiment 2 (*A*), and histogram indicating treatment effects on $\Phi_{PSII} / \Phi_{CO_2}$ (*B*). The dotted line (*A*) represents the linear slope $y = 7.036x$. Black bars, 5 µM Mn; white bars, 1 mM Mn. Y, young leaves; M, mature leaves. Error bars represent ± 1 SE.

(2) photosynthetic sensitivity to excess Mn is exacerbated at higher light intensities through photo-oxidative mechanims. Our results demonstrate that excess Mn decreased the photosynthetic capacity and growth of sugar maple and red maple seedlings. Differences in species tolerance to Mn outlined in the first hypothesis varied depending on the developmental stage of the leaf, as observed in other studies examining metal toxicity in plants (Maksymiec and Baszynski 1996; Gonzalez and Lynch 1997; Krupa and Moniak 1998) and light environment. In summary, all sugar maple leaves, except shaded young leaves, were sensitive to excess Mn. For red maple only mature leaves exposed to high light were prone to Mn toxicity. Our results support the hypothesis that high light potentiates Mn toxicity of the photosynthetic apparatus in these two maple species but antioxidant enzyme and PSII / CO_2 data does not suggest that this response was the result of photo-oxidative stress.

The deleterious effects of metals on the photosynthetic apparatus are often the result of competitive interference with essential metal cofactors (Krupa and Baszynski 1995). In crop plants, Mn interferes with the uptake and function of Fe, Mg, Ca and K (El-Jaoual and Cox 1998). Results from the present study, and that of McQuattie and Schier (2000), indicate that Mg, Ca, K and Fe were the elements most often decreased by excess Mn in maple seedlings (Tables 1, 2). In this study, Mn interference of foliar Fe accumulation was drastic, which may be important because of the role of Fe in electron transport and the strong relationship between ETR and CER (Fig. 6). Sugar maple and red maple differed markedly in their patterns of foliar Fe accumulation. At low Mn, red maple seedlings accumulated only a small amount of the foliar Fe found in sugar maple seedlings in this study and others (McQuattie and Schier 2000). We have observed this difference in Fe accumulation among sugar maple and red maple seedlings growing on natural forest soils (St. Clair 2004). The strong photosynthetic and growth responses in control seedlings of both species indicate that these two species function well at vastly different Fe concentrations.

Excess Mn decreased stomatal conductance in sugar maple, which was strongly correlated with CER (Fig. 6). The influence of Mn on stomatal conductance could have occurred indirectly through interference with K, which is an important regulator of stomatal function. Further studies would be required to confirm this hypothesis.

Differences in carboxylation efficiency suggest that the direct effect of excess Mn on carbon fixation were important. These results may indicate that Rubisco is a target of Mn toxicity as in crop plants (Houtz *et al.* 1988). However, differences in the A / C_i slopes presented in Fig. 3 are also likely to be influenced by observed differences in photochemical capacity and chlorophyll content. These A / C_i measurements were made on young sugar maple leaves, which accumulated the lowest foliar Mg concentrations in this study (Tables 1, 2), resulting in low foliar Mg / Mn ratios in seedlings exposed to the high Mn treatment. Low Mg / Mn in leaves could decrease carboxylation efficiency, as Mn^{2+} competes with Mg^{2+} as Rubisco's cofactor, but has only a fraction of its activating potential (Wildner and Henkel 1978; Christeller and Laing 1979). This is relevant in the eastern forests of North America as Mg / Mn in sugar maple foliage is often very low, as Mg deficiency overlaps with Mn hyperaccumulation in trees growing on non-glaciated acidic soils (Horsley *et al.* 2000).

Sugar maple seedlings show strong photosynthesis and growth responses to Ca concentrations in the range of values found in this study (Kobe *et al.* 2002; St.Clair 2004). Even the moderate interference of Ca uptake by excess Mn observed in this study may have had important effects on photosynthesis. However, connections between excess Mn and leaf function through interference of Fe, K, Mg and Ca are only suggestive, as the critical nutrient thresholds for sugar maple seedlings are not precisely known, and photosynthetic sensitivity may not entirely be a function of foliar nutrient concentrations.

Light intensity was an important variable affecting photochemical responses to excess Mn in this study, as indicated by Mn × light interactions and the light response curves. Stresses that impair the photosynthetic apparatus can be exacerbated by high light through photo-oxidation (Cakmak and Marschner 1992; Smirnoff 1993; Massacci *et al.* 1995). Antioxidant systems that are up-regulated in response to photo-oxidative stress increase in the leaves of crop plants exposed to excess Mn and high light (Gonzalez *et al.* 1998; Bueno and Piqueras 2002). However, antioxidant enzyme data did not suggest that photo-oxidative stress explained the observed physiological responses to the Mn and light variables in this study. Although GR was significantly up-regulated in response to excess Mn in mature red maple leaves, this response was independent of light intensity. Leaf tissue (young sugar maple leaves and mature red maple leaves) that was photosynthetically sensitive to the interactive effects of Mn and high light did not have $\Phi_{PSII} / \Phi_{CO_2}$ ratios greater than control leaves. Therefore, these leaves appear to effectively down-regulate electron transport relative to carbon fixation rates, a response that would limit ROS formation through the Mehler reaction (Fryer *et al.* 1998).

Our results support those of Nable *et al.* (1988) and Gonzalez *et al.* (1998) in showing that high light intensity increases the deleterious effects of excess Mn on the photosynthetic apparatus. This indicates that in natural forest ecosystems, sugar maple and red maple foliage exposed to high light intensity (outer canopy, canopy gaps) could be more prone to Mn toxicity. In summary, these results suggest that excess Mn in acid soils may be an important factor influencing the health decline of sugar maple seedlings, as observed in overstorey sugar maple (Horsley *et al.* 2000), but do not necessarily demonstrate that responses of red maple to excess Mn contribute to its tolerance of acid, nutrient-poor soils (Abrams 1998). As processes affecting foliar Mn hyperaccumulation and light intensity (soil acidification, global climate change) are not static in the biosphere, the influence of Mn phytotoxicity on maple species may be increasingly important in the future.

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