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Early Inhibition of Photosynthesis during Development of Mn Toxicity in Tobacco[1]

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ABSTRACT

Early physiological effects of developing Mn toxicity in young leaves of burley tobacco (*Nicotiana tabacum* L. cv KY 14) were examined in glasshouse/water cultured plants grown at high (summer) and low (winter) photon flux. Following transfer of plants to solutions containing 1 millimolar Mn^{2+}, sequential samplings were made at various times for the following 9 days, during which Mn accumulation by leaves increased rapidly from ~70 on day 0 to ~1700 and ~5000 microgram per gram dry matter after 1 and 9 days, respectively. In plants grown at high photon flux, net photosynthesis declined by ~20 and ~60% after 1 and 9 days, respectively, and the onset of this decline preceded appearance (after 3 to 4 days) of visible foliar symptoms of Mn toxicity. Intercellular CO_2 concentrations and rates of transpiration were not significantly affected; moreover, the activity of the Hill and photosystem I and II partial reactions of chloroplasts remained constant despite ultimate development of severe necrosis. Though the activity of latent or activated polyphenol oxidase increased in parallel with Mn accumulation, neither leaf respiration nor the activity of catalase [EC 1.11.1.6] and peroxidase [EC 1.10.1.7] were greatly affected. These effects from Mn toxicity could not be explained by any changes in protein or chlorophyll abundance. Additionally, they were not a consequence of Mn induced Fe deficiency. Therefore, inhibition of net photosynthesis and enhancement of polyphenol oxidase activity are early indicators of excess Mn accumulation in tobacco leaves. These changes, as well as leaf visual symptoms of Mn toxicity, were less severe in plants cultured and treated at low photon flux even though the rates of leaf Mn accumulation at high and low photon flux were essentially equivalent.

Excess Mn accumulation by plants is generally associated with the development of visual symptoms (leaf chlorosis/necrosis, altered leaf morphology, and/or a discoloration of the roots) and a decrease in yield (see Refs. 7 and 8 for reviews), although yield responses have been recorded without symptom development (23). The extent of injury from Mn toxicity is approximately proportionate to the concentration of excess Mn accumulated; however, considerable inter- and intraspecific variation exists in tolerance to excess Mn (7, 8). Climatic factors such as photon flux and temperature have also been reported to modulate the severity of expression of injury from Mn toxicity (7, 8, 29). Some of the differences in the susceptibility between cultivars and the modulation by temperature have been attributed to nonhomogeneous accumulations in various leaf cells (12) and to differences in the extent of Mn compartmentation within the vacuole (29).

Two general hypotheses have been proposed to explain the physiological disorders caused by Mn toxicity. First, several workers have postulated that excess Mn accumulation results in increased peroxidative destruction of IAA and increased synthesis of ethylene (6, 24, 25, 31). Second, numerous workers have postulated that the symptoms of Mn toxicity reflect a Mn/Fe interaction thereby leading to physiological disorders as a consequence of limitation of uptake/utilization of Fe (7, 8).

The effects of Mn toxicity on various enzymic activities of extracts from leaves of cotton (*Gossypium hirsutum* Linn.) have shown: (a) peroxidase activity is increased (6, 24, 25, 31) while other Fe-containing enzymes/enzyme complexes such as catalase and Cyt *c* oxidase are diminished (31); and (b) the activity of the Cu-containing polyphenol oxidase is increased early during excess Mn accumulation, but the activity of another Cu-enzyme, ascorbic acid oxidase, is diminished, particularly after extended (80 d) exposure to Mn toxicity (31). The increase of polyphenol oxidase activity may lend some support to the hypothesis of Morgan *et al.* (6, 24, 25), but the decreased activity of catalase and Cyt *c* (31) oxidase can be interpreted to support the hypothesis invoking interference of Fe uptake/utilization by Mn toxicity. No cogent explanation is apparent for the reported increase of polyphenol oxidase activity (31) which is associated with chloroplasts (9, 13, 30, 32, 33).

Most of the past work dealing with Mn toxicity has been done with tissue after prolonged exposure to excess Mn and usually after the appearance of visual symptoms; thus, primary and secondary effects may not have been distinguished. Here we report studies on the early events in the development of Mn toxicity in leaves of tobacco, a plant which frequently exhibits this disorder during cultivation (11, 23, 29). These studies are focused primarily on toxicity effects on photosynthesis, a process which becomes inhibited following long-term excess accumulation of leaf Mn (27).

MATERIALS AND METHODS

Plant Growth and Harvests. Seeds of burley tobacco (*Nicotiana tabacum* L. cv KY 14) were germinated on agar containing basal nutrients (half concentration of the nutrient solution described previously [26], except for FeEDTA which was retained at 30 μM), and cultured in the laboratory for 21 d under metal halide lamps (300–400 μmol/s·m^2 of PAR; 27°C). Plants of similar size were then transferred to water culture (basal nutrients) in a temperature controlled glasshouse (28°C day/14°C night). Initially, plants were cultured for 14 d (45/10 L container) then transferred to 20 L containers (5 plants/container) for the remainder of an experiment. The pH of nutrient solutions was maintained at pH 6.0 ± 0.5 with KOH. After 14 d in 20 L containers, nutrient solutions were renewed and either 0.5 μM $MnSO_4$ (control) or 1000 μM $MnSO_4$ (Mn-treated) was added; equivalent results were obtained with $MnCl_2$. The effect of Fe

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supply on the development of Mn toxicity was studied by providing plants with either 30 or 100 μM FeEDTA on transfer to nutrient solutions. All treatments were imposed in triplicate.

During the summer, the maximum PAR was approximately 1400 μmol/s·m^2 (14 h photoperiod). During the winter, plants were grown with supplementary light from high pressure Na lamps (1500 μmol/s·m^2 of PAR, 15 h photoperiod) or only natural light with maximum PAR of approximately 900 μmol/s·m^2 (10 h photoperiod).

In all experiments, at least one plant was harvested from each container at the time Mn treatments were imposed (designated d 0) and on each day up to 9. Measurements were made on the leaf which was the third youngest on d 0 and which, independent of Mn treatment, increased in length from 15 to 25 cm and was the fourth youngest leaf on d 9. All the results reported represent the mean of two experiments (each with three replicates).

Gas Exchange Measurements. Net photosynthesis and transpiration were determined on attached leaves using an open gas exchange system which employed: (a) a Plexiglas clamp-on chamber (21) that enclosed a 16 cm^2 section of a leaf (midway along on the leaf and avoiding the midvein), (b) an Analytical Development Company model 225 differential IR gas analyzer, (c) thermistors in the gas stream entering and leaving the chamber, and (d) two EG and G hygrometers. Compressed air containing 370 μl CO_2/L was humidifed (35%) by passage (0.8 L/min) through a saturated $CaCl_2$ solution, passed over both sides of the leaf section and through a hygrometer, an ice bath, and anhydrous $Mg(ClO_4)_2$ before IR gas analysis. The reference gas circuit (no leaf chamber) was similar.

Unless otherwise noted, plants were kept in light for 2 or 3 h prior to the measurements which were made at 27°C (measured in the chamber) at a photon flux (1100 μmol/s·m^2 of PAR) which saturated net photosynthesis. Light from a 150 W flood lamp was filtered through 5 cm of 0.1% (w/v) $CuSO_4$ and a Schott 116 heat filter then focused onto the leaf chamber.

Following gas exchange measurements, the leaf section within the chamber was excised and FW,[3] DM, Mn, and Fe determined. For Chl determinations, a 16 cm^2 leaf section was excised from a comparable position on the opposite side of the leaf midvein. Intercellular CO_2 concentrations were calculated from the equations of Farquhar and Sharkey (5).

Dark respiration was measured polarographically at 27°C using a 1.3 cm diameter preweighed leaf disc in a 1.5 cm diameter thermostatted vessel (0.5 ml internal volume), essentially as described by Delieu and Walker (4). Plants previously used for net photosynthesis measurements were transferred to darkness for 2 h, then four separate measurements were made on individual leaf discs excised from the opposite region of the leaf used in gas exchange measurements.

Chloroplast Isolation and Assay. Chloroplasts were isolated from the same leaf used in gas exchange measurements. The leaf was excised, chilled in ice water for 30 min, then a blotted leaf section (15 g without midvein) was homogenized for 60 s in 60 ml of 0.4 M sucrose/20 mM Hepes-NaOH, pH 7.5/5 mM $MgCl_2$/20 mM Na-isoascorbate in a blender at full voltage. After filtration through six layers of cheesecloth, centrifugation at 200g/5 min, and resuspension in homogenization buffer (Na-isoascorbate omitted), Hill activity, and PSI and PSII partial reactions were assayed as described elsewhere (1).

Enzyme Assays. Assays were made on various fractions of the homogenate from the chloroplast isolation procedure (see individual Figs./Tables). Catalase [EC 1.11.1.6] was assayed polarographically (1 ml reaction vessel) using 50 mM Hepes-NaOH (pH 7.5) containing 50 mM H_2O_2. Polyphenol oxidase [EC 1.10.3.1] was assayed essentially as described in Goldbeck and Cammarata (9) with the exception that 0.1% (w/v) LDS was used for activation where indicated. Peroxidase [EC 1.10.1.7] activity was determined spectrophotometrically (15). Appropriate aliquots of the various centrifugal fractions were used such that the initial rates were linear for >10 s. The high concentrations of Mn^{2+} in homogenates of Mn-treated leaves caused high rates of chemical oxidation of the substrates employed in the polyphenol oxidase and peroxidase assays. Since 5 mM EDTA eliminated the chemical oxidation but had no effects on the polyphenol oxidase or peroxidase activity in extracts from control leaves, 5 mM EDTA was routinely added to all assays of polyphenol oxidase and peroxidase.

Other Methods. Chl was determined on chloroplasts/homogenate fractions and leaf sections using 80% (v/v) acetone and *N,N*-dimethylformamide, respectively (14). Protein was determined by the procedure of Lowry *et al.* (20) using 0.1% (w/v) Na-deoxycholate with BSA as the standard. Mn and Fe determinations were made by flame atomic absorption and on dried leaf samples subjected to total digestion (26).

RESULTS

Effects of Mn Treatment on Leaf Mn/Fe Concentration and Visible Symptoms. The effect of Mn treatment (plus Mn) of tobacco plants is presented for leaf Mn concentrations (Fig. 1a) and leaf Fe concentrations (Fig. 1b). Very similar data were obtained irrespective of the photon flux (summer *versus* winter *versus* winter plus supplemental lighting) used during culture and Mn treatment of plants; thus the observed differences in effect of Mn toxicity observed at high versus low photon flux on leaf chlorosis/necrosis, polyphenol oxidase activity, and net photosynthesis shown later are not a consequence of differing total leaf Mn concentrations. Mn treatment caused approximately a 25- and 70-fold increase in leaf Mn after 1 and 6 to 9 d, respectively (Fig. 1a). Total leaf Fe concentration declined by about 25% relative to controls after 5 to 9 d Mn treatment; however, the maximum extent of decline was to a value which was still 2-fold greater than the Fe critical value (70 μg Fe/g DM) believed necessary for maximal growth of young tobacco leaves (34). A similar small lack of an effect of Mn treatment on Fe concentration in tops of tobacco has been reported (11). Moreover, and in contrast to data in Hiatt and Ragland (11), increasing the leaf Fe concentration by 30% by increasing the FeEDTA in the nutrient solution 3-fold, did not diminish the visual symptoms of

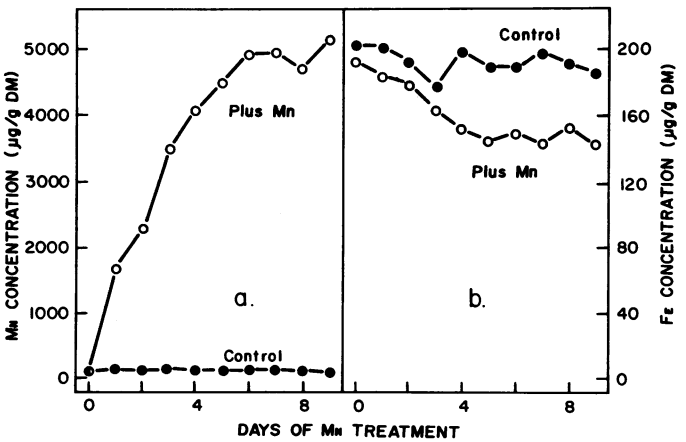

FIG. 1. Effects of Mn treatment (plus Mn) of tobacco plants on the concentration of Mn (a) and Fe (b) in leaves of summer-cultured plants.

[3] Abbreviations: FW, fresh weight; DM, dry matter; LDS, lithium dodecyl sulfate; DOPA, DL-β-3,4-dihydroxyphenylalanine; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; FeCN, potassium ferricyanide; MV, methyl viologen; $DCIPH_2$, reduced 2,6-dichlorophenolindophenol.

Mn toxicity.

Visible symptoms observed in these studies with Mn-treated plants at high photon flux included: (a) a discoloration of root tips within 24 h which subsequently intensified and spread to the entire root system by d 9; and (b) a development in leaves of slight paleness (d 3), minor interveinal chlorosis/necrosis (d 7), and pronounced interveinal chlorosis/necrosis (d 9) even though leaf Mn/Fe concentrations did not change significantly between 7 to 9 d (Fig. 1). By contrast, leaves of Mn-treated plants cultured at low photon flux (winter) showed no visible symptoms until d 5 (slight paleness) and then only mild interveinal chlorosis with few necrotic lesions even after 9 d treatment. Nevertheless, within the short 9-d Mn-treatment period described here, no decreases in leaf growth/expansion relative to controls was detected (FW or DM/leaf area) at either high or low photon flux.

Effects of Mn Toxicity on Catalase, Peroxidase, and Respiration of Tobacco Leaves. Although results described above suggested no gross Mn/Fe interaction resulting in Fe deficiency, analyses of total leaf Fe concentrations (Fig. 1b) do not provide critical information regarding the availability/utilization of Fe by various Fe-enzymes in different cellular compartments (28). Moreover, the previously reported increase in peroxidase activity (6, 24, 25, 31) but decreased activity of catalase and Cyt *c* oxidase following development of Mn toxicity in cotton leaves (31) might reflect differential effects of Mn toxicity on these Fe-enzymes thereby lending some credence to the hypothesis that Mn toxicity is a consequence of Mn/Fe interactions (7, 8) at the cellular level.

Table I summarizes the effects of developing Mn toxicity in tobacco leaves on the specific activity of catalase and polyphenol oxidase in crude homogenates (200*g*/2 min supernatants) of control *versus* Mn-treated plants. As shown, the total activities are expressed on both a DM and a protein basis. (The short-term Mn-treatment of summer or winter cultured plants caused no decrease in DM, but resulted in a 20% decrease, after 9 d, of the initial total leaf protein value of 475 mg protein/g DM.) We observed no major change in the activity of either catalase or peroxidase even though by d 8 plants showed severe visual symptoms of Mn toxicity. Any small decrease in activities when expressed per g DM was not observed on a per mg protein. These results contrast with those previously reported (31) where apparently no chemical oxidation of substrate in peroxidase assays was observed by high Mn^{2+} present in homogenates from Mn-treated plants. The data of Table I lend no support to the hypothesis that Mn toxicity results from Mn/Fe interactions leading to interference of Fe utilization. Analyses of leaf respiration during development of Mn toxicity also do not support this hypothesis. Leaf respiration of Mn-treated plants remained equivalent to control plants (260 μmol O_2/g DM·h) to d 4 then declined by only 10% by d 9 when expressed per mg protein. If increased ethylene synthesis is involved in the development of Mn toxicity and is dependent on an increase of peroxidative activity (6, 17, 24, 25, 31), then the data of Table I also lend no support to the idea that Mn toxicity symptoms are a consequence of hormonal imbalance induced by high Mn^{2+} concentrations.

Effects of Mn Treatment and Photon Flux on Polyphenol Oxidase Activity. Polyphenol oxidase exists primarily in a latent form on thylakoid membranes of healthy green tissue (9, 13, 18, 22, 30, 32, 33). In such tissue the function(s) of this enzyme is not clear, its phenol oxidase activity limited either by latency or by the compartmentation of substrate(s) within the vacuole (30, 33). However, phenol oxidase activity is generally expressed during senescence (15) and/or following injury, conditions leading to activation of the latent enzyme and mixing of vacuole and plastid contents (32, 33).

The effect(s) of Mn treatment of summer cultivated tobacco plants on the LDS-activated polyphenol oxidase activity in leaf homogenates is shown in Figure 2. Since EDTA was included in assays to eliminate any chemical oxidation of substrate by high Mn^{2+} concentrations present in homogenates of leaves from Mn-treated plants ("Materials and Methods"), the rates shown are catalyzed specifically by polyphenol oxidase. As shown, the polyphenol oxidase activity in leaf homogenates of control plants remained rather invariant whether expressed per g DM or per mg protein. In contrast, the polyphenol oxidase activity from Mn-treated plants increased approximately 1.5-fold after d 1 then continued to increase and ultimately reach a 2.5-fold increase by d 9. A comparison of the time-courses of Mn accumulation in leaves (Fig. 1a) and the increase in polyphenol oxidase activity (Fig. 2) *versus* the sequence of development of visual foliar symptoms of Mn toxicity in summer cultured tobacco suggested that these processes might be interrelated.

The total LDS-activated polyphenol oxidase activities found in both control and Mn-treated plants cultured without supplementary light during the winter were markedly less compared to winter plants receiving supplementary light (Fig. 3) which had polyphenol oxidase activities similar to those from plants cultured during summer (Fig. 2). This effect of photon flux as a determinant of activated polyphenol oxidase activity has been reported previously (9, 30, 32). As indicated in a previous section, the rate of Mn accumulation in leaves was the same under high *versus* low photon flux; thus, any differences in rate and extent of increase of LDS-activated polyphenol oxidase activity shown in Figure 3 cannot be attributed to differences in accumulation of total leaf Mn.

Nevertheless, despite the approximately 8-fold difference in total LDS-activated polyphenol oxidase activity of control plants

Table I. *Catalase and Peroxidase Activities of Leaf Homogenates from Control and Mn-Treated Tobacco Leaves*

Homogenates were prepared from 15 g leaf tissue ("Materials and Methods") from summer-cultured/Mn-treated plants. The 200 g/2 min supernatant was used in the assays and the activities shown represent the total activities present in the homogenate. EDTA (5 mM) was included in the peroxidase assays to eliminate chemical oxidation of substrate by the high Mn^{2+} concentrations present in homogenates from Mn-treated plants.

Treatment Duration	Catalase Activity				Peroxidase Activity			
	Control	Plus Mn	Control	Plus Mn	Control	Plus Mn	Control	Plus Mn
d	*μmol O_2/g DM·h*		*mmol O_2/mg protein·h*		*Δ425 nm/mg DM·h*		*Δ425 nm/mg protein·h*	
0	1120	1080	2.40	2.52	588	602	1.25	1.25
1	1152	1184	2.40	2.48	560	588	1.24	1.28
2	1096	1120	2.32	2.40	576	600	1.20	1.30
3	1104	1064	2.40	2.52	540	552	1.12	1.18
4	1064	1144	2.52	2.28	552	592	1.20	1.31
6		1080	2.40	2.52	576	588	1.20	1.14
7	1128							
8	1088	1000	2.40	2.60	568	504	1.20	1.16

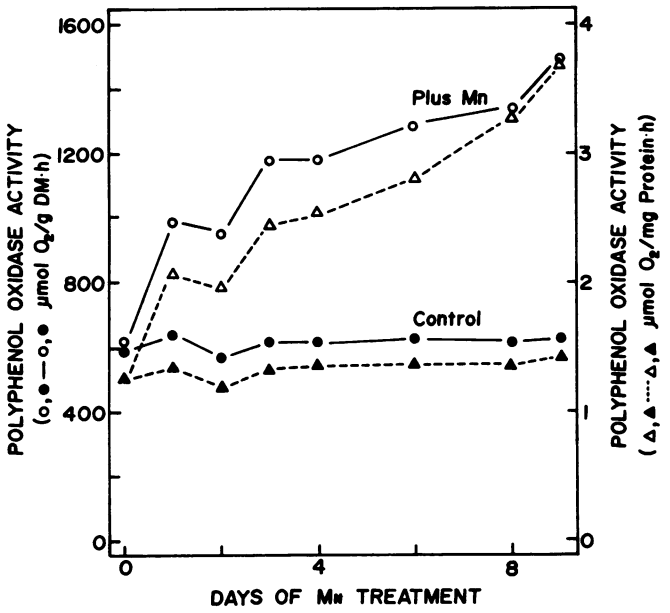

FIG. 2. Effects of Mn treatment (plus Mn) of tobacco plants on the total LDS-activated polyphenol oxidase activity in homogenates of leaves from summer-cultured plants.

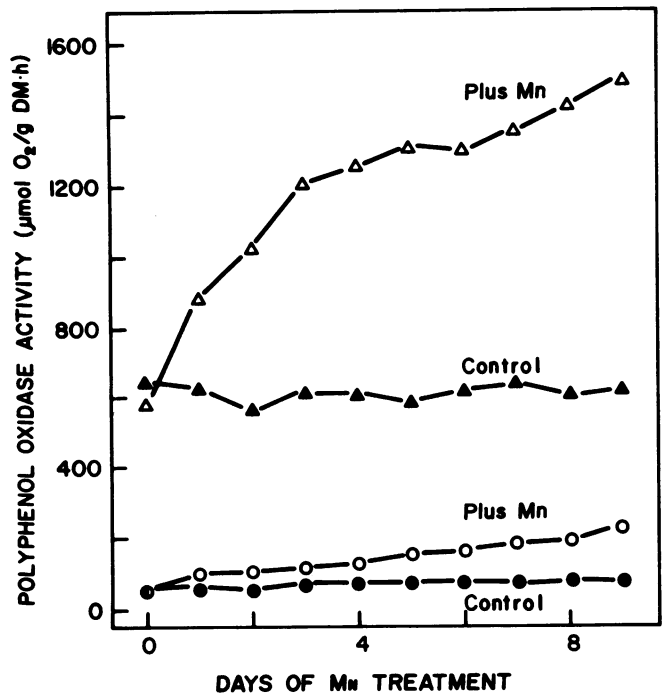

FIG. 3. Effects of Mn treatment (plus Mn) and photon flux on total LDS-activated polyphenol oxidase activity in homogenates of leaves from leaves of winter-cultured tobacco plants. Plants were cultured with (▲, △) or without (●, ○) supplementary light.

cultured at high (Fig. 2) *versus* low (Fig. 3) photon flux, Mn treatment at either high or low photon flux yielded maximally a 2.5-fold increase in LDS-activated polyphenol oxidase activity by d 9. However, at the winter photon flux, the increase of LDS-activated polyphenol oxidase activity from Mn treatment showed a conspicuously slower and different time-course than the increase in summer (Fig. 2) or winter cultured plants receiving supplementary light (Fig. 3). Though quantification of visual foliar symptoms was not possible, the appearance of these symptoms seems to be generally correlated with the rate of increase of LDS-activated polyphenol oxidase activity; namely, at low photon flux, the appearance of visual symptoms of Mn toxicity was delayed as well as less severe at 9 d Mn treatment.

Effects of Mn Treatment on Polyphenol Oxidase Activation State. *In vitro* studies have shown that thylakoid bound latent polyphenol oxdase is activated by chemically diverse compounds (9, 13, 33), light (32), and homogenization procedures leading to its solubilization from thylakoids (9, 30, 33). The experiments summarized in Table II represent attempts to determine if Mn-treatment led to *in vivo* activation of latent polyphenol oxidase. They also were made to gain some insights into the underlying basis for the ≤2.5-fold greater LDS-activated polyphenol oxidase activity in homogenates of leaves from Mn-treated plants than from control tobacco plants (Figs. 2 and 3).

Most of the total polyphenol oxidase activity in homogenates of leaves from control and Mn-treated plants was in 30,000g/20 min supernatant regardless of treatment duration. The activity in this fraction showed no latency. Apparently, our homogenizaton procedure resulted in activation/solubilization of a major fraction of the plastidic polyphenol oxidase. Significantly, the specific activity (μmol O_2/mg protein·h) in the soluble supernatant from Mn-treated plants increased by 2.2- and 3.9-fold relative to controls after 3 and 9 d treatment, respectively. This increased specific activity is reflected in an increase of total polyphenol oxidase activity and a decrease in the amount of protein in this fraction, particularly at 9 d treatment.

The polyphenol oxidase activity in the broken chloroplast fraction from leaves of Mn-treated plants also showed an increase of total and specific activity with or without LDS-activation. The data of Table II therefore tend to suggest that excess Mn accumulation leads to partial *in vivo* activation of the plastidic latent enzyme as well as ≥2-fold increase in activated enzyme following only 1 to 3 d of Mn treatment. No explicit explanation can be offered for the observed increase of total activated polyphenol oxidase activity (chloroplast plus 30,000g/20 min supernatant) in leaf homogenates prepared from Mn-treated plants.

Effects of Mn Treatment and Photon Flux on Photosynthesis. Oxidation products from phenolic compounds are very reactive and capable of causing nonspecific inhibition of enzyme systems (10, 19, 33). If Mn-treatment were to cause mixing of polyphenol oxidase substrates located in the vacuole (33) with the polyphenol oxidase activated by Mn treatment, then inhibition of chloroplast processes could possibly result.

Table III records the activities of the Hill and PSII/PSI partial reactions of chloroplasts from control and Mn-treated plants exposed to excess Mn for various durations at a summer photon flux. By d 9, leaves of Mn-treated plants showed severe chlorosis/necrosis which was reflected by diminished Chl abundance and chloroplast yield (data not shown). However, no decrease in the activities measured occurred despite the development of such symptoms and an increase of polyphenol oxidase activity (Figs. 2 and 3; Table I) in Mn-treated leaves.

In contrast, net photosynthesis of leaves was inhibited by Mn treatment. The development of inhibition in summer cultured Mn-treated plants is shown in Figure 4. After only 1 d of Mn treatment, rates of photosynthesis were diminished by 20% whether expressed on a DM or Chl basis. When expressed on a DM basis, the rates declined progressively reaching 60% inhibition by d 9. The maximum extent (33%) and the course of development of inhibition was different when rates were expressed on a Chl basis (Fig. 4, inset), the difference reflecting the onset of Chl loss at d 3 to 4 in Mn-treated plants cultured during summer.

The extent of inhibition of net photosynthesis shown in Figure 4 cannot be explained by diminished stomatal conductance in leaves of summer-cultured Mn-treated tobacco plants. A com-

Table II. *Latent and LDS Activated Polyphenol Oxidase Activity in Homogenates of Control and Mn-Treated Tobacco Leaves*
Fractions were prepared from 15 g of leaf tissue as described in "Materials and Methods" from plants cultured in summer.

Mn Treatment	Treatment Duration	Total Polyphenol Oxidase Activity			Protein		Specific Polyphenol Oxidase Activity		
		Chloroplast		30,000 g/20 min supernatant[a]	Chloroplast	30,000 g/20 min supernatant[a]	Chloroplast		30,000 g/20 min supernatant[a]
		−LDS	+LDS				−LDS	+LDS	
	d	*mol O_2/h*			*mg*		*μmol O_2/mg protein·h*		
Control	0	33	93	660	108	636	0.31	0.86	1.04
	3	32	98	693	123	651	0.26	0.80	1.07
	9	40	109	639	109	702	0.37	1.00	0.91
Mn Treated	0	41	103	630	109	663	0.38	0.94	0.95
	3	74	192	1481	115	624	0.64	1.67	2.37
	9	84	243	1830	92	516	0.91	2.64	3.56

[a] Values were equivalent whether assayed in the presence or absence of LDS (see "Materials and Methods") and the activities in the 2700 g/5 min supernatant and the 30,000 g/20 min supernatant were equivalent.

Table III. *Hill Activity and PSII and PSI Donor Photooxidation Activity of Broken Chloroplasts from Control and Mn-Treated Tobacco Leaves*
Chloroplasts were prepared from summer-cultured plants. See "Materials and Methods" for details of the preparation and the assays.

Mn Treatment	Treatment Duration	Hill Activity ($H_2O \rightarrow$ FeCN)	PSII → PSI ($NH_2OH \rightarrow$ MV)	PSI ($DCIPH_2 \rightarrow$ MV)
	d	*μequivalents/mg Chl·h*		
Control	0	999	158	1124
	3	1153	169	1051
	9	1196	162	1058
Mn treated	0	1059	170	979
	3	1090	158	1093
	9	1159	168	1111

parison of the effects of Mn treatment on transpiration (Fig. 5) in relation to net photosynthesis reveals: (a) no significant change in transpiration of Mn-treated plants (relative to controls) occurred through d 4 at which time net photosynthesis had declined by 41% (Fig. 4, main figure); and (b) at a time (d 9) when rates of net photosynthesis (per mg Chl) were diminished 33% by Mn treatment (Fig. 4, inset), the same leaves exhibited a 39% increase of transpiration (per mg Chl) relative to controls (Fig. 5, inset). Moreover, calculations of intercellular CO_2 concentrations for leaves of both control and Mn-treated plants cultured during summer showed they remained essentially invariant (330 μl CO_2/L) throughout the course of the 9 d experiment (5); thus, the observed inhibition of net photosynthesis from excess Mn accumulation in tobacco leaves is not a consequence of diminished leaf CO_2 conductance. Inhibition of net photosynthesis with no inhibition of transpiration has been previously reported with wheat plants subjected to excess Mn for a 12 d period (27).

The effects of photon flux on the development of visible leaf symptoms and polyphenol oxidase activity of leaves of Mn-treated plants (Figs. 2 and 3) prompted an examination of the possible relationship between Mn treatment, photon flux, and net photosynthesis. Accordingly, plants were cultured and subjected to Mn treatment at low (winter) or high (winter plus supplementary light) photon flux for durations shown on the abscissa then net photosynthesis was measured. Results from these experiments are summarized in Figure 6. At the winter photon flux (Fig. 6), upper panel, the development of inhibition of net photosynthesis was slower than observed with winter plants receiving supplementary light (Fig. 6), lower panel. As a consequence, on d 9 the inhibition of net photosynthesis in winter cultured and Mn-treated plants was approximately 50% less than found with winter cultured and Mn-treated plants receiving supplementary light or plants cultured at summer photon flux (Fig. 4), irrespective of whether net photosynthesis was expressed on a DM or a Chl basis. Clearly, photon flux modulates the inhibition of net photosynthesis by Mn toxicity even though the rate and extent of leaf Mn accumulation in tobacco leaves is independent of photon flux.

DISCUSSION

Occurrence of Mn toxicity in plants is rather common particularly among plants cultivated in acid soils (7, 8, 11, 23, 29). The effects of excess Mn accumulation in leaves are most frequently associated with leaf chlorosis/necrosis and diminished productivity (7, 8); however, decreased productivity without appearance of leaf visual symptoms sometimes occurs (23). Two hypotheses have been offered to explain the physiological disorders induced by Mn toxicity: (a) an imbalance of auxin is created which leads to increased concentration of ethylene (6, 24, 25, 31) and subsequent acceleration of senescence processes; and (b) the excess Mn^{2+} concentration leads to interference(s) of cellular utilization of Fe and possibly other cations (7, 8) thereby causing decreased activity of enzyme systems requiring these elements. Generally, these hypotheses are based on data from experiments focused on effects following long-term Mn treatment. The present experiments were undertaken to examine early physiological responses in attempts to detect some of the primary physiological consequences of Mn toxicity in tobacco. The duration of the Mn treatment used here was sufficiently long, however, to permit comparisons of results from previous studies.

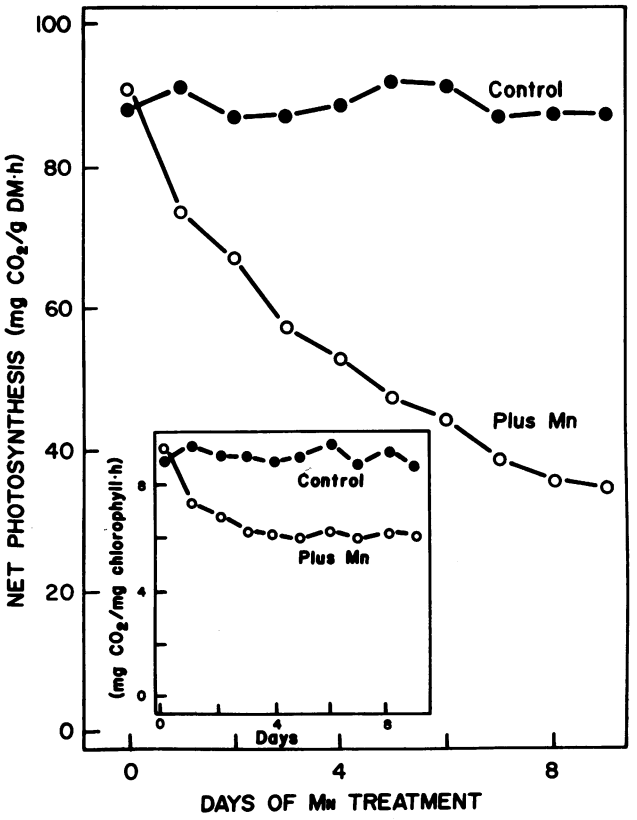

FIG. 4. Effects of Mn treatment (plus Mn) on net photosynthesis of leaves of summer-cultured tobacco plants. Rates are expressed on a DM (main Fig.) or a Chl (inset) basis. Relative to controls, the Chl abundance of leaves of Mn-treated plants showed no decrease through d 3; thereafter, decreases of 11.5, 24.3, and 44.3% were measured at d 4, 6, and 9, respectively.

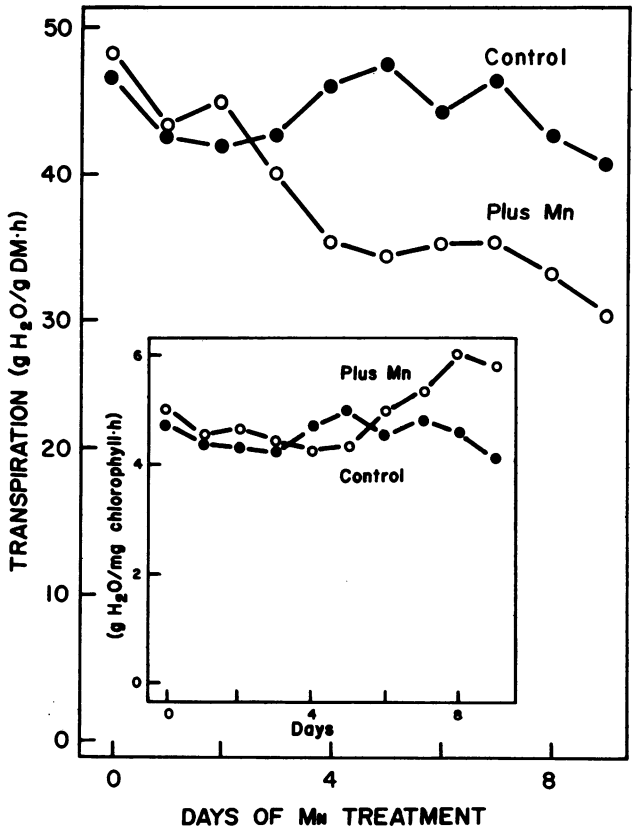

FIG. 5. Effects of Mn treatment (plus Mn) on transpiration rates of leaves of summer-cultured tobacco plants. Rates are expressed on a DM (main Fig.) or a Chl (inset) basis.

Our data lend no support to the idea that Mn toxicity directly or indirectly results from an impairment of Fe utilization. First, the activities of Fe-requiring enzymes/complexes of different organelles (28) such as catalase, peroxidase, the respiratory complex, and the photosynthetic electron transport complex were not affected significantly throughout the development of Mn toxicity. Second, the accumulation of leaf Mn to approximately a 10 mM concentration (FW basis) diminished leaf Fe concentrations by only 25%, a value well above the Fe concentration necessary for normal growth of young leaves (34); moreover, an increase of this diminished leaf Fe concentration by 30% (by increasing Fe supply) did not delay or diminish the severity of chlorosis/necrosis resulting from Mn toxicity (*cf.* Ref. 11). The reported inhibition of Fe-requiring steps in Chl synthesis by high Mn^{2+} concentrations (2, 3) was therefore not detected in our analyses. Even if we accept arguments for the inhibition of Chl synthesis by Mn toxicity (2, 3), the data here indicate that an increase of polyphenol oxidase activity and a decrease of net photosynthesis occur at least 2 to 3 d prior to inhibition of Chl synthesis, at least as indicated by a decrease of Chl abundance of leaves after ≥3 to 4 d Mn treatment.

The rapid increase of polyphenol oxidase activity (Figs. 2 and 3; also Ref. 3) and decrease of net photosynthesis (Figs. 4 and 6), seen with tobacco plants subjected to excess Mn particularly at high but also at low photon flux, indicate that these chloroplast associated processes are early indicators of excess leaf Mn accumulation. At high photon flux, changes in these activities were observed within 24 h at which time total leaf Mn concentration had increased about 12-fold relative to controls and to about 32% of the maximum Mn accumulated at d 9. Moreover, over the course of the 9 d Mn treatment at high photon flux, the changes in activites of polyphenol oxidase and net photosynthesis paralleled leaf Mn accumulation when activities were expressed on either a DM or Chl basis, if corrections were made for the loss of Chl beginning after 3 d of Mn treatment. Such analyses suggest that inhibition of net photosynthesis, without inhibition of Hill activity, and increase of polyphenol oxidase activity are closely correlated with the development of the Mn toxicity syndrome at high photon flux conditions. The inhibition of photosynthesis by leaf Mn accumulations producing no visual symptoms of Mn toxicity reported here yields a plausible explanation for the reported decrease of yield of field-grown tobacco by long-term low, but excess leaf Mn concentrations producing no visual foliar symptoms (23).

Generally, this apparent correlation between inhibition of net photosynthesis and increase of polyphenol oxidase activity *versus* appearance of visual symptoms of Mn toxicity also is observed but less clearly at low photon flux. Though low photon flux did not affect the rate or final extent of leaf Mn accumulation, it did diminish the rate and final extent of inhibition of net photosynthesis (Fig. 6), the rate of increase of polyphenol oxidase activity (Fig. 3), and the loss of Chl accompanying the appearance of visual foliar symptoms of Mn toxicity. If we postulate that the effects on polyphenol oxidase and photosynthesis activities reflect excess Mn accumulation by chloroplasts, then we suggest that photon flux modulates the distribution/solubility of Mn^{2+} within leaf cell compartments as well as the activation state of polyphenol oxidase (Figs. 2 and 3; Refs. 9, 13, 18, 22, 30, 32, 33). If we further postulate that the inhibition of photosynthesis is causally related to the increased polyphenol oxidase activity,

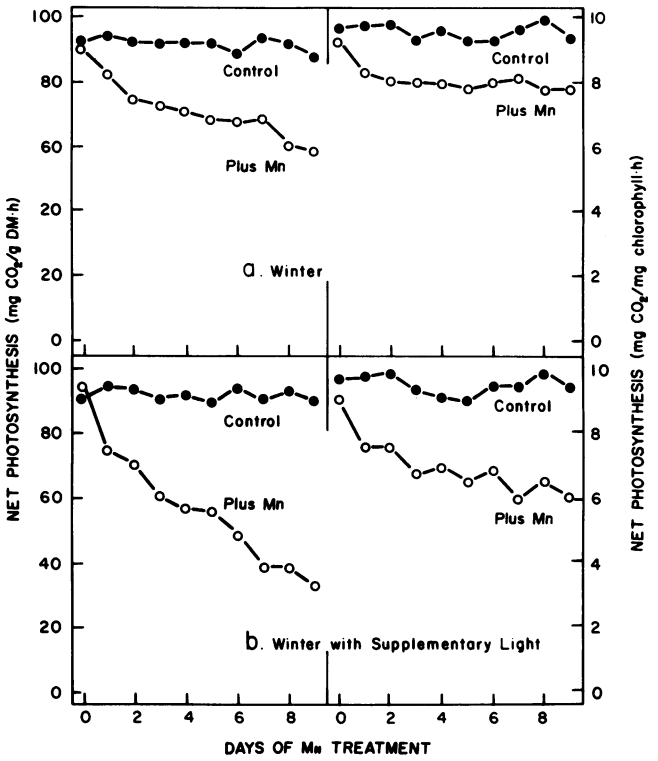

FIG. 6. Effects of Mn treatment (plus Mn) and photon flux on net photosynthesis of leaves of winter-cultured tobacco plants. Plants were cultured without (a) or with (b) supplementary light. Rates are expressed on a DM (left panels) or a Chl (right panels) basis.

then we must assume: (a) excess Mn^{2+} causes a destabilization of the tonoplast (16) which results in leakage of vacuolar compartmentalized reduced polyphenol oxidase substrates (33) into chloroplasts containing activated polyphenol oxidase; and (b) the resulting oxidation products of phenolics inhibit photosynthesis by binding to enzymes of the reductive photosynthetic carbon cycle enzymes (19).

This hypothesis as well as some others which can be advanced to explain the basis of inhibition of photosynthesis by Mn toxicity are explored in the accompanying companion publication.

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