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Research Report

Growth and Physiological Response to Manganese Toxicity in Chinese Cabbage (*Brassica rapa* L. ssp. *campestris*)

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Abstract. This study was conducted to assess the effect of manganese (Mn) treatment on the growth and physiological characters of Chinese cabbage (*Brassica rapa* L. ssp. *campestris*). Seedlings were transplanted to plastic pots filled with soil and then grown in a plastic house. Four treatments with Mn (control, 15 μM, 300 μM, and 1.5 mM) were applied to plants along with half-strength Hoagland solution. The plant growth characters leaf length, leaf size, chlorophyll content, and fresh and dry weight of shoots and roots decreased significantly with high (1.5 mM) Mn treatment. As the concentration of Mn increased, K, Ca, Mg, Fe, Zn, and Cu content in outer leaves decreased, but the concentration of total N, P, and Mn increased significantly. Chlorophyll a decreased significantly with increasing Mn concentration. Maximum photochemical efficiency (F_v/F_m) was highest (0.893) in the control followed by 15 μM and 300 μM Mn-treated plants, whereas CO_2 assimilation decreased with increasing Mn. Total free amino acids also significantly decreased with an increasing Mn. Hence, growth and physiological characters of Chinese cabbage demonstrated tolerance upto 300 μM Mn with nutrient solution in pot culture.

***Additional key words*:** chlorophyll content, CO_2 assimilation rate, Mn, maximum photochemical efficiency, tolerance, total free amino acid

Introduction

Manganese (Mn) is considered a limiting factor for crop production on acid soils but can also be a problem in neutral or alkaline soils with poor aeration (Foy et al., 1988). Manganese toxicity leads to brown spots on older leaf surfaces, chlorosis and necrosis, and browning of roots (Horst and Marschner, 1978a). In *Brassica* crops, Mn toxicity is primarily seen on older leaves as interveinal chlorosis, and brown and necrotic spots (Foy et al., 1978). Excess Mn interferes with the absorption, translocation, and utilization of other mineral elements such as Ca, Mg, Fe, and P (Clark, 1982). Generally, Mn toxicity involves a decline in photosynthetic efficiency, a reduction of respiration, and decreased photosynthetic pigment content as physiological effects (Macfie and Taylor, 1992). The Mn-induced brown leaf spots contain oxidized Mn and oxidized phenolic compounds, especially in epidermal cell walls (Horiguchi, 1987). It has been proposed that oxidation of Mn^{2+} and phenol is mediated by apoplastic peroxidases (PODs) as the key reaction leading to Mn toxicity symptoms (Fecht-Christoffers et al., 2006).

The level of Mn leading to toxicity varies widely among the plant species (El-Jaoual and Cox, 1998), depending on the biochemical pathway used, genotype, and environmental and nutritional factors, such as temperature and Si, Ca, Mg, or Fe status (Alam et al., 2001; Le Bot et al., 1990). *Brassica* species are very sensitive to high concentrations of Mn in the soil, although some genotypes such as rapeseed (*B. napus* and *B. rapa* L.) have been identified as resistant to high Mn (Moroni et al., 2003).

Chinese cabbage (*Brassica pekinensis* Rupr.) is generally grown on farmlands, near highways, and in mining areas, and can now even be grown in soils contaminated by heavy metals (Xiong et al., 2006). Kim et al. (2010) studied the physiological mechanisms of Chinese cabbage in response to NaCl stress; however studies of the effects of Mn toxicity on growth and physiological characters have not yet been undertaken in Chinese cabbage. Therefore, the aim of this study was to investigate the growth and physiological response to Mn in Chinese cabbage.

Materials and Methods

Plant Material

Chinese cabbage (*Brassica rapa* L. *spp. campestris*) seeds 'Ilpoom' were sown in plastic plug trays in May 2010. Seedlings were transplanted to plastic pots (25 cm × 25 cm × 25 cm) filled with horticultural soil and grown for two months in a plastic house under the natural photoperiod of 14-16 h with ambient temperature (day 30-35℃ night, 17-20℃). For acclimation of the plants, pots were placed at outdoor for 2 weeks and watered before treatment. Four treatments consisting of three manganese (Mn) concentrations (15 μM, 300 μM, and 1.5 mM) and the control were laid out in randomized complete block design with three replications and each replication consisted in three plants. $MnSO_4$ was used for Mn supplement along with a modified half strength Hoagland solution (Hoagland and Arnon, 1950). Solutions were applied at 2-days interval for 2 weeks and, then 4-days interval following 2 weeks. Total 200 mL of each treatment solution were applied during 4 weeks period. Physicochemical properties of soil before the treatment applications were analyzed and are presented in Table 1.

Growth Parameters and Nutrient Analysis

After 28 days of treatment application, number of leaves, leaf height and width (cm) were measured by scale, leaf area (cm^2) estimated by leaf area meter (Area meter, Delta-T, UK), and chlorophyll contents was determined by CCM-200 apparatus (CCM-200, Opti-Science, USA). Fresh weight and dry weight of shoot and roots were taken after harvesting. Dry weight of shoots and roots were taken through oven-dry method at 60℃ for 72 h until constant weight achieved.

For nutrient analysis, samples were collected from inner and outer leaves at 21 days after treatment. Samples were washed twice in tap water followed by rinsing with distilled water. Then, samples were dried in oven at 60℃ for 76 h until constant weight. Then, 0.2 g samples were dissolved in HNO_3 and H_2SO_4 (2:1 v/v) following AOAC procedures (Association of Official Analytical Chemists, USA). Calcium, Mg, K, Na and heavy metals (Mn, Fe, Zn, and Cu) were determined by Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES, OPTIMA 7300DV, Perkin Elma, USA) and total nitrogen (T-N) was estimated by spectrophotometrical method (RDA, 2008) and phosphorus was estimated by the molybdate vanadate method (Abadia et al., 1985; Igartua et al., 2000).

Chlorophyll Content Estimation and Fluorescence Measurement

For chlorophyll estimation, three leaves were taken from each plant. Fresh leaf samples were taken from the youngest fully expanded leaf after 21 days of Mn treatment and extracted with 90% acetone. Readings were observed at 470, 663, 645, and 750 nm wavelength using a UV-vis spectrophotometer (Neosys-2000, Scinco, Japan). Absorbance at 750 nm was subtracted from absorbance at 663 and 645 nm to eliminate the turbidity in solution. Chlorophyll concentrations in leaves were calculated according to formulas by Strain and Svec (1966).

The photosynthetic activity of Mn was estimated by chlorophyll fluorescence and subsequently determined the photosystem II efficiency. The measurements were taken in 15 to 18th leaves from apex, using a photosynthesis yield analyzer (MINI-PAM, Heinz Walz GmbH, Germany). Prior to the measurements, plants were illuminated with 68.39 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon for 30 min. F_o value was obtained during excitation of leaf surface with switched light (0.1 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon). F_m was measured at pulse of 6,000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon intensity halogen-lamp light for 1 sec. Maximum PSII efficiency was calculated using F_v/F_m, where F_v value is F_m-F_o (Krause, 1991). It was performed at ambient CO_2 concentration, fixed temperature; 25℃ and a relative humidity of 15 % inside the growth chamber.

CO_2 Exchange Rate Measurement

After 21 days of treatment application, 15 to 18th leaves from apex were exposed to saturating 68.39 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon at 25℃ with 15% relative humidity for the CO_2 exchange measurement (S151, Qubit system, USA). The measurement was taken in growth chamber for 30 min with ambient CO_2 concentration and results were calculated by Jarvis's formula (Jarvis et al., 1971).

Analysis of Total Free Amino Acid

After 21 days of Mn treatment, fresh young leaves (0.5 g) were taken and homogenized in 10 mL of 10% ethanol with a pestle and mortar and diluted to 100 mL with distilled water, and then the extract was filtered through a filter paper

Table 1. Physicochemical properties of soil before planting Chinese cabbage.

Sample	pH	EC (mS/cm)	CEC ($cmol \cdot kg^{-1}$)	OM (%)	NO_3-N	P_2O_5	K	Mg	Ca	SiO_2	Mn
								$mg \cdot kg^{-1}$			
Soil	6.05	0.4	6.33	20.1	151.9	30.1	329.5	49.7	47.6	113.1	2.1

EC: electrical conductivity; CEC: cation exchange capacity; OM: organic matter.

(Whatman No. 4). Total free amino acid contents were determined according to modified ninhydrin reaction method (Rosen, 1957). To make ninhydrin reaction, 1.0 mL filtered solution was added in vial, to which included 1.0 ml of acetic acid-sodium acetate buffer solution (0.1 M, pH 4.6) 0.1 ml of 3% ascorbic acid, and 3 ml of ninhydrin solution (1% w/v resolved in n-BuOH). The vial was placed in a water bath at 100℃ for 15 min and was further kept at room temperature. The solution was filled up to 20 mL with 60% ethanol. Then, the absorbance of the solution was measured at 570 nm using a UV-vis spectrophotometer (S-1000, Scinco, Japan).

All data were statistically analyzed using SAS (v9.1, SAS institute Inc, USA) program and treatment means were separated by least significant difference (LSD) test.

Results

Growth Parameters

After 21 days of exposure to 1.5 mM Mn, necrosis and brown spots were observed in the outer leaf tissues of plants (Fig. 1). Plant growth parameter responses to different concentrations of Mn are given in Table 2. Leaf width and leaf area significantly increased with 15 μM and 300 μM Mn; however, leaf length at both of these concentrations was unchanged (Table 2). But the number of leaves, leaf width and leaf area were not statistically different with control. Chlorophyll content was significantly higher (9.2) in 15 μM Mn-treated plants, but decreased in plants treated with 300 μM to 1.5 mM Mn.

The control had the highest (207.8 g) shoot fresh weight (Table 2). The shoot fresh decreased with increased level of Mn but the dry weight of shoots from 15 μM to 300 μM Mn was not statistically different with control. The root fresh and dry weight at 15 μM and 300 μM Mn was statistically similar with control. Plants given 1.5 mM Mn significantly decreased leaf length, chlorophyll content, and fresh and dry weight of shoots and roots.

Effect of Mn on Nutrient Content in Leaves

Total nitrogen and phosphorus in inner leaf tissues increased significantly with increasing Mn concentration but potassium content remained statistically similar in all Mn treatments. Calcium and magnesium levels significantly decreased in leaf tissues of plants grown with 300 μM and 1.5 mM Mn (Table 3). In the outer leaf tissues, except phosphorus, total nitrogen, calcium, magnesium and potassium content at 15 mM, 300 mM and 1.5 mM Mn was statistically similar with control.

Compared to the control, Mn concentration in inner leaf tissues significantly increased in plants grown with 15 μM to 300 μM Mn, and was highest (229.04 $\mu g \cdot g^{-1}$) in 1.5 mM Mn-treated plants (Table 4). Similarly, Mn concentration in outer leaf tissues increased significantly with increasing Mn in the growth medium. The highest (2062.3 $\mu g \cdot g^{-1}$) Mn content was observed in plants grown with 1.5 mM Mn. The levels of Fe and Zn decreased in inner leaf tissues when Mn increased from 15 μM to 1.5 mM but Cu levels significantly increased above control levels in plants grown with 1.5 mM

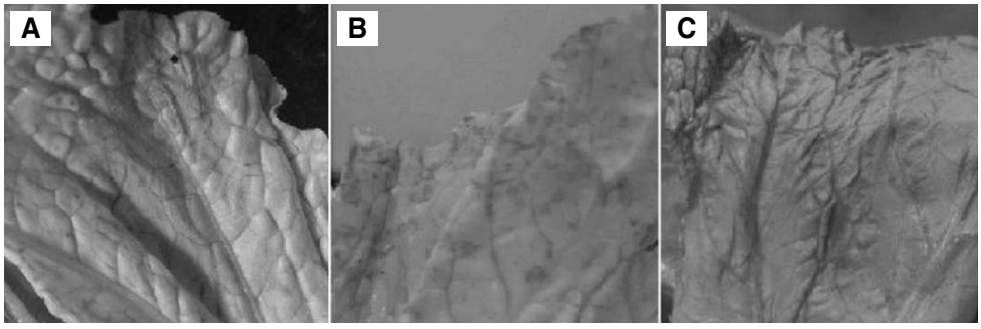

Fig. 1. Visible symptoms in outer leaves of plants grown with 1.5 mM Mn for (A) 21, (B) 24, and (C) 28 days.

Table 2. Effects of different concentrations of Mn on growth parameters of Chinese cabbage.

Mn concentration	No. of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Chlorophyll content[z]	Shoot weight (g)		Root weight (g)	
						Fresh	Dry	Fresh	Dry
Control	33.7 a[y]	14.7 b	8.9 a	95.6 a	8.8 ab	207.8 a	26.4 a	22.3 a	3.4 a
15 μM	30.3 a	16.6 a	10.0 a	100.1 a	9.2 a	195.8 b	27.5 a	22.4 a	3.8 a
300 μM	32.7 a	16.4 a	10.4 a	103.8 a	8.2 b	198.3 b	27.9 a	20.7 a	3.7 a
1.5 mM	32.3 a	15.2 b	9.3 a	95.0 a	6.3 c	175.6 c	23.6 b	16.6 b	2.5 b

[z]Values obtained with a chlorophyll meter (model CCM-200, Opti-Science, USA).
[y]Means within the same column followed by the same letter are not significantly different by LSD ($P \leq 0.05$).

Table 3. Effect of manganese (Mn) treatments on macronutrient contents in leaves of Chinese cabbage.

Parts of leaves	Mn concentration	T-N	P	K	Ca	Mg
Inner leaf	Control	6.24 b[z]	0.74 d	1.34 a	0.24 a	0.10 a
	15 μM	6.24 b	0.83 c	1.36 a	0.23 ab	0.11 a
	300 μM	6.26 b	0.87 b	1.38 a	0.18 bc	0.09 b
	1.5 mM	7.09 a	1.05 a	1.38 a	0.14 d	0.07 c
Outer leaf	Control	4.27 a	0.65 c	2.22 b	1.52 c	0.37 c
	15 μM	4.12 a	0.76 b	2.85 a	2.29 a	0.63 a
	300 μM	3.42 b	0.76 b	2.66 a	1.90 b	0.51 b
	1.5 mM	3.92 a	0.84 a	2.14 b	1.38 c	0.31 c

[z]Macronutrient content expressed as percentage (%) dry weight. Means within a column followed by the same letter are not significantly different by LSD ($P \leq 0.05$).

Table 4. Micronutrient concentrations in leaves of Chinese cabbage grown at different manganese (Mn) concentrations.

Parts of leaves	Mn concentration	Mn	Fe	Zn	Cu
Inner leaf	Control	18.06 d[z]	81.53 c	39.88 d	2.43 b
	15 μM	21.39 c	114.84 a	55.74 a	2.61 ab
	300 μM	50.02 b	89.88 b	47.07 b	2.59 ab
	1.5 mM	229.04 a	68.35 d	43.57 c	2.88 a
Outer leaf	Control	105.64 d	165.68 a	46.77 bc	4.34 a
	15 μM	211.08 c	194.09 a	81.42 a	6.52 a
	300 μM	466.48 b	149.27 ab	49.60 b	8.10 a
	1.5 mM	2062.3 a	109.51 b	38.29 c	7.92 a

[z]Micronutrient contents are expressed as $\mu g \cdot g^{-1}$ dry weight. Means within the column followed by the same letters are not significantly different by LSD ($P \leq 0.05$).

Table 5. Effect of manganese (Mn) toxicity on photosynthetic pigments of leaves in Chinese cabbage.

Mn concentration	Chl a	Chl b	Total Chl	Carotenoids	Chl a/b
	$mg \cdot g^{-1}$ freshweight				
Control	4.63 a[z]	1.77 a	6.40 a	1.56 b	2.62
15 μM	4.55 a	1.33 c	5.88 bc	1.81 a	3.42
300 μM	4.58 a	1.57 b	6.15 ab	1.83 a	2.93
1.5 mM	4.12 b	1.58 b	5.70 c	1.78 a	2.62

[z]Means within the column followed by the same letters are not significantly different by LSD ($P \leq 0.05$).

Mn. The Fe and Zn concentrations in outer leaf tissues decreased in plants grown at 1.5 mM Mn, but Cu levels in outer leaves of plants grown at high (1.5 mM) Mn were not significantly different from those in other Mn treatments.

Effect of Mn on Photosynthetic Pigments

The effect of Mn on the content of photosynthetic pigments in leaves was statistically significant ($P \leq 0.05$) (Table 5). The Chl *a* and total chlorophyll content decreased after 1.5 mM Mn treatment, while the Chl *a/b* ratio was highest (3.42) in plants grown with 15 μM Mn compared to control (2.62) values.

Chlorophyll Fluorescence and CO_2 Assimilation Parameters

Treatment effects of Mn on chlorophyll fluorescence were significant ($P \leq 0.05$) (Fig. 2). Control plants, followed by plants grown with 15 μM Mn, exhibited maximum photochemical efficiency of PSII (F_v/F_m); however, the PSII (F_v/F_m) value obtained in plants grown with 300 μM Mn was statistically similar to those of control and 15 μM Mn-treated plants. The F_v/F_m value decreased in plants treated with high

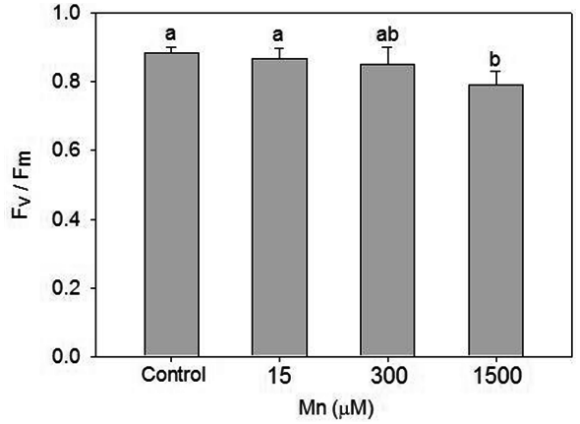

Fig. 2. Effect of Mn on photochemical efficiency of PSII (F_v/F_m) in Chinese cabbage leaves. Means separation at each treatment by LSD ($P \leq 0.05$). Vertical bars indicate standard deviation.

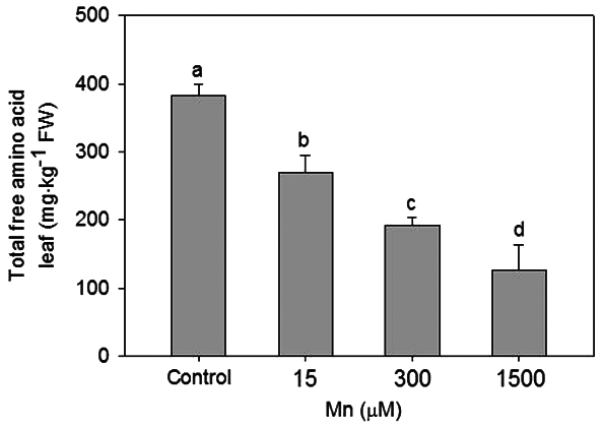

Fig. 4. Effect of Mn on total amino-N (expressed as total free amino acids) of Chinese cabbage. Mean separation at each treatments by LSD ($P \leq 0.05$). Vertical bars indicate standard deviation.

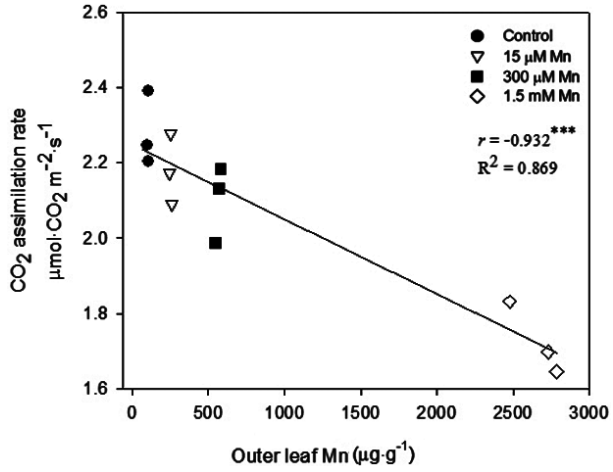

Fig. 3. Relationship between CO_2 assimilation rate and Mn concentration in outer leaves of Chinese cabbage grown under different Mn concentrations.

1.5 mM Mn concentrations.

Likewise, CO_2 assimilation rate and Mn^{2+} concentration in outer leaves (Fig. 3) decreased with increasing Mn growth concentration, and there was a linear relationship between CO_2 assimilation and Mn concentration in outer leaf tissues.

Total Free Amino Acid Content

The amino acid content of leaves after 21 days varied significantly with level of Mn treatment ($P \leq 0.05$). Total free amino acid content (free amino-N) in leaves significantly decreased with increasing Mn concentration (Fig. 4). At 1.5 mM Mn, amino-N levels were three-fold lower than in the control.

Discussion

Excess Mn leads to the development of visible symptoms such as brown spots and necrosis in older leaves of Chinese cabbage (Foy et al., 1978); the same symptoms have been reported in lettuce, celery, and cabbage (*Brassica oleracea* Capitata group; Osawa and Ikeda, 1976). Excess Mn increases the accumulation of reactive oxygen species, which mediate oxidative stress (Gonzalez et al., 1998) and are associated with oxidation of phenolics (Horiguchi, 1987), which in turn induces visible toxicity symptoms of Mn in plants. Plants grown with 15 μM and 300 μM Mn had no significant difference in leaf width and area, but chlorophyll content was affected at higher Mn concentrations. Supplementary low Mn can promote the growth of many plant species (Balamey et al., 1986; Mehdi et al., 2008; Xianghua et al., 2007).

Macronutrient content of the leaves was also affected in plants grown at excess Mn concentrations. Total nitrogen (T-N), calcium (Ca), and magnesium (Mg) concentrations were low in plants grown with high concentrations of Mn. Clark (1982) reported that excess Mn interferes with the absorption, translocation, and utilization of mineral elements such as Ca, Mg, and Fe. Similarly, Galvez et al. (1989) reported that high levels of Mn in the nutrient solution decreased shoot concentrations of Si, K, Ca, Mg, Zn, and Cu but increased shoot concentrations of Mn and P in sorghum. In this study, increasing levels of Mn from 15 μM to 1.5 mM in nutrient solution decreased leaf concentrations of K, Mg, Fe, Zn, and Cu, but increased leaf concentrations of Mn and P, in agreement with Galvez et al. (1989). However, N and Ca concentrations were not significantly different with the control. Some researchers (Heenan and Campbell, 1981; Horst and Marschner, 1978b; Kazda and Znacek, 1989) have reported Ca and Mg deficiency in plants caused by competition between Mg and Mn for binding sites in roots during mineral absorption. Mn inhibits Mg absorption and competes more effectively than Mg. Similarly, uptake of Ca might be affected by high levels of Mn, because of a decrease in cation exchange capacity of the leaf tissue.

Chinese cabbage grown with 15 and 300 μM Mn had an increased Chl *a/b* ratio compared to the control. Hajiboland and Hasani (2007) reported that photosynthetic pigments increased in rice and sunflower grown at 50-100 μM Mn, and Xianthua et al. (2007) reported the same result in *Phytolacca acinosa* grown with 500 μM Mn. In contrast, chlorophyll content decreased even at low Mn concentrations in tomato (Moshe et al., 2004), barley (Alam et al., 2006), and kidney bean (Gonzalez and Lynch, 1998). This might be due to inhibition of chlorophyll synthesis (Clairmont et al., 1986), or leaf necrosis induced by Mn toxicity.

Chlorophyll *a* fluorescence and maximum PSII efficiency were also affected by Mn concentration. Plants grown at high Mn concentrations showed decreased maximum photochemical efficiency of PSII (F_v/F_m). Likewise, CO_2 assimilation rate suppressed linearly with increasing concentrations of Mn. Some researchers (Mitsutoshi et al., 1997; Subrahmanyam and Rathore, 2000) have reported that CO_2 assimilation decreased simultaneously with maximal quantum yield of PSII (F_v/F_m) at increasing Mn concentrations in the outer leaf tissues of plants, e.g., in wheat (Ohki, 1985) and tobacco (Nable et al., 1988). As the concentration of Mn increased, total free amino acid content in leaves also decreased. The reduction of total free amino acids in shoots after Mn exposure may be related to a decrease in nitrate uptake by roots and feedback repression of downstream metabolites such as amino acids or other factors that regulate this process (Nazoa et al., 2003; Xiong et al., 2006).

In conclusion, plants grown at high (1.5 mM) Mn concentrations reduced the chlorophyll content, fresh and dry weight of shoots and roots, photosynthetic pigment (Chl a and Chl b), photochemical efficiency, CO_2 assimilation, as well as total free amino acid content. Plants grown with 15 μM Mn and 300 μM exhibited the highest Chl *a/b* ratio, maximum photochemical efficiency of PSII and highest CO_2 assimilation rate; hence, Chinese cabbage can tolerate up to 300 μM Mn in nutrient solution under the pot culture.

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