Manganese Toxicity Hardly Affects Sulfur Metabolism in *Brassica rapa*

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Abstract Manganese (Mn) is an essential plant nutrient, though at elevated levels in plant tissues it may become toxic. The physiological basis for phytotoxicity is largely unclear. Exposure of *Brassica rapa* to elevated levels of Mn^{2+} in the nutrient solution resulted in decreased biomass production at $\geq$20 μM and chlorosis. The Mn content in the shoot increased with the Mn^{2+} concentration in the nutrient solution and became toxic when it exceeded a four-fold concentration of the control. In contrast to observations with Cu and Zn, elevated and toxic Mn^{2+} levels did not affect the water-soluble non-protein thiols in both root and shoot and the expression the sulfate transporters, Sultr1;1 and Sultr1;2, in the root.

Mn is an essential plant nutrient and its availability in soil strongly affects plant growth and development (Kováčik et al. 2014; Sadana et al. 2003). Mn functions in several physiological processes, viz. in photosynthesis, where it is associated with the water-oxidizing complex of photosystem II, which catalyzes the photosynthetic O_2 evolution (Mukhopadhyay and Sharma 1991; Millaleo et al. 2013). Moreover, Mn is an important cofactor of several enzymes, e.g., manganese-dependent superoxide dismutase (MnSOD), catalases, glycotransferases, pyruvate carboxylase, nitrate reductase and is involved in amino acid and lignin synthesis (Marschner 1995; Pedas et al. 2005; Humphries et al. 2006; Pittman 2008). Mn is taken up by the plant root as Mn^{2+}, which availability is strongly affected by the pH of the soil (Humphries et al. 2006; Socha and Guerinot 2014). In alkaline soils (high pH) Mn availability to plants may be low and deficiency may occur, whereas in in acidic soils (low pH) excessive availability may result in toxicity (Humphries et al. 2006;

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Mundus et al. 2012). Little is known about manganese transporters in plants, though the iron (Fe^{2+}), zinc (Zn^{2+}) and calcium (Ca^{2+}) family transporters seem to be the most probable candidates (Socha and Guerinot 2014). In the xylem, Mn is transported as Mn^{2+} ion or as complex with citrate or malate; in the shoot high levels of Mn may accumulate in the vacuole (Pittman 2005). The physiological basis for Mn toxicity is largely unclear. Differential tolerance of plants to manganese cannot solely be explained by a restricted Mn^{2+} uptake and transport to the shoot but additionally by intrinsic strategies that enhance cellular accumulation capacity (Foy et al. 1978). Sequestration into the vacuole, activity of antioxidant enzymes and formation of chelation complexes in the cytosol are some of the strategies proposed to promote toxic metal tolerance (Pittman 2005). Sulfur metabolites play a role in the detoxification of potential toxic metals (Yadav 2010). Complexation of sulfur compounds (e.g., cysteine, phytochelatins, metallothioneins) with toxic metal ions as a mechanism to overcome their toxicity is widely described for different elements and plant species (Ernst et al. 2008; Yadav 2010; Leitenmaier and Küpper 2013). Other sulfur metabolites, such as glutathione, are also crucial for antioxidant protection against reactive oxygen species, of which levels might be induced upon toxic metal stress (Na and Salt 2011). Some species capable of high manganese accumulation, such as *Phytolacca americana*, show a positive relationship between sulfur and manganese (Peng et al. 2008; Yadav 2010). Moreover, it has been observed that toxic metals, e.g., Cu, Zn may directly induce changes in sulfur uptake by affecting the activity of the sulfate transporters and affect the regulation of enzymes involved in S assimilation and activity of sulfate transporters (Nocito et al. 2002; Sun et al. 2007; Schiavon et al. 2008; Shahbaz et al. 2010, 2013; Na and Salt 2011; Stuiver et al. 2014). All changes induced in sulfur status of the plant could be linked a toxic metal-induced change in activity of the sulfate transporters (Yoshimoto et al. 2002; Sun et al. 2007; Stuiver et al. 2014). *Brassica* species have high sulfur requirements for growth (Ernst 2000) and are generally considered to be susceptible to Mn toxicity (Foy et al. 1978; Humphries et al. 2006; Lee et al. 2011). In the current paper the interaction between Mn and sulfur metabolism was studied in the *Brassica rapa*.

Brassica. rapa var. perviridis (Komatsuna) seeds were germinated in vermiculite and were subsequently transferred to an aerated 25% Hoagland nutrient solution, containing supplemental concentrations of 0, 10, 20, 50 and 100 μM $MnCl_2$ (pH 5.9) in 30 l plastic containers (20 sets of plants per container, three plants per set) in a climate-controlled room for 10 days. Day and night temperatures were 21 and 18 °C (±1 °C), respectively, relative humidity was 70–80%. The photoperiod was 14 h at a photon fluence rate of 300 ± 20 μmol m^{-2} s^{-1} (400–700 nm) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules. After 10 days of Mn^{2+} exposure, plants were harvested 3 h after the start of the light period and shoots and roots separated and weighed. Shoot and root biomass production was calculated by subtracting pre-exposure weight from that after Mn^{2+} exposure. Shoot/root ratio was calculated from the shoot and root fresh weight after the exposure. For the determination of pigments and anions, plant material was frozen in liquid N_2 immediately after harvest and stored at −80 °C. For analysis of water-soluble non-protein thiols freshly harvested material was

used. Chlorophyll a + b content, chlorophyll a fluorescence and the content of sulfate, nitrate, water-soluble non-protein thiol content, free amino acid and the Mn and S mineral nutrient content were determined as described by Shahbaz et al. (2010) and Stuiver et al. (2014). Total RNA was isolated from shoots and roots of *B. rapa* plants as described by Aghajanzadeh et al. (2014). The full length sequences of sulfur transporter genes are found under the following accession numbers: Sulfur transporter 1.1 (Sultr1;1 XM009128953), Sulfur transporter 1.2 (Sultr1;1 XM009108197, XM009108195 and XM009108196). Transcription was determined by quantitative real-time polymerase chain reaction (qRT-PCR; see Reich et al. 2017). Statistical analysis of the results was performed using unpaired Student's t-test. Different letters indicate significant differences at $P < 0.01$ between different treatments.

Exposure of *B. rapa* to elevated levels of Mn^{2+} in the nutrient solution resulted in decreased biomass production at ≥20 μM (Table 1). Shoot growth was relatively slightly more affected upon Mn^{2+} exposure than root growth resulting in a decrease in shoot to root ratio. Mn^{2+} exposure also resulted in a substantial increase in dry

Table 1 Impact of Mn^{2+} exposure on biomass production, dry matter content, chlorophyll a fluorescence and contents of pigments, nitrate and sulfate, amino acids and water-soluble non-protein thiols of *Brassica rapa*

	Mn^{2+} concentration (μM)				
	0	10	20	50	100
Shoot					
Biomass production (g FW)	0.97 ± 0.18c	0.85 ± 0.11c	0.73 ± 0.19bc	0.59 ± 0.14b	0.32 ± 0.06a
Dry matter content (%)	7.7 ± 0.3a	7.9 ± 0.3a	8.3 ± 0.3ab	8.9 ± 0.7bc	9.4 ± 0.4c
Chl a + b (mg g^{-1} FW)	0.69 ± 0.04b	0.61 ± 0.05b	0.54 ± 0.13ab	0.52 ± 0.09a	0.47 ± 0.05a
Chl a/b	2.4 ± 0.2a	2.6 ± 0.1a	2.5 ± 0.3a	2.2 ± 0.4a	2.7 ± 0.3a
Chl a + b/Car	3.0 ± 0.0a	2.9 ± 0.1ab	2.9 ± 0.2ab	2.9 ± 0.2ab	2.8 ± 0.1b
F_v/F_m	0.81 ± 0.03a	0.81 ± 0.05a	0.82 ± 0.04a	0.80 ± 0.05a	0.82 ± 0.05a
Manganese (μmol g^{-1} DW)	3.3 ± 0.2a	9.8 ± 0.6b	16.4 ± 1.1c	30.5 ± 0.4d	53.6 ± 4.7e
Sulfur (μmol g^{-1} DW)	241 ± 21a	277 ± 7a	265 ± 7a	289 ± 25ab	300 ± 10b
Sulfate (μmol g^{-1} FW)	14 ± 2a	13 ± 1a	15 ± 4a	26 ± 3b	34 ± 4b
Thiols (μmol g^{-1} FW)	0.56 ± 0.01a	0.57 ± 0.09a	0.56 ± 0.05a	0.59 ± 0.04a	0.60 ± 0.06a
Nitrate (μmol g^{-1} FW)	84 ± 7a	105 ± 1b	102 ± 4b	85 ± 3a	85 ± 0a
Amino acids (μmol g^{-1} FW)	14 ± 1a	14 ± 2a	15 ± 1a	15 ± 2a	16 ± 2a

(continued)

Table 1 (continued)

	Mn^{2+} concentration (μM)				
	0	10	20	50	100
Root					
Biomass production (g FW)	0.17 ± 0.03c	0.18 ± 0.03c	0.16 ± 0.05bc	0.12 ± 0.04b	0.07 ± 0.02a
Dry matter content (%)	6.5 ± 0.3a	7.1 ± 0.4a	7.0 ± 0.5a	7.9 ± 1.1ab	8.6 ± 0.5b
Manganese (μmol g^{-1} DW)	49 ± 5a	162 ± 6b	168 ± 75b	180 ± 53b	154 ± 19b
Sulfur (μmol g^{-1} DW)	241 ± 21a	277 ± 7a	265 ± 7a	289 ± 25ab	300 ± 10b
Sulfate (μmol g^{-1} FW)	28 ± 2a	23 ± 2a	27 ± 2a	25 ± 4a	22 ± 2a
Thiols (μmol g^{-1} FW)	0.42 ± 0.03a	0.41 ± 0.08a	0.44 ± 0.02a	0.45 ± 0.06a	0.53 ± 0.06a
Nitrate (μmol g^{-1} FW)	44 ± 5a	39 ± 2a	48 ± 3b	43 ± 4ab	42 ± 3a
Amino acids (μmol g^{-1} FW)	15 ± 1a	15 ± 2ab	18 ± 2ab	18 ± 1b	17 ± 2ab
Plant					
Shoot/root ratio	5.6 ± 0.4a	4.7 ± 0.3b	4.5 ± 0.7b	4.9 ± 0.9b	4.4 ± 0.8b

Ten day-old seedlings were grown on a 25% Hoagland nutrient solution containing supplemental concentrations of 0, 10, 20, 50 and 100 μM $MnCl_2$. The initial shoot and root fresh weights were 0.100 ± 0.01 g and 0.040 ± 0.01 g, respectively. Data on biomass production, dry matter, pigment and amino acid content represent the mean of two independent experiments, with a total of 12, 6, 6, and 6 measurements with 3 plants in each, respectively (± SD). Data on chlorophyll a fluorescence represents the mean of 10 measurements (± SD). Data on nitrate, sulfate and water-soluble non-protein thiol content represent the mean of 3 measurements with 3 plants in each (± SD). Different letters indicate significant differences at $P < 0.01$ between different treatments

matter content of both root and shoot at 100 μM. Mn^{2+} exposure resulted in interveinal chlorosis (especially of the younger leaves) at ≥50 μM, however the chlorophyll a/b and the chlorophyll/carotenoid ratios were hardly affected (Table 1). Chlorophyll a fluorescence (measured as the quantum yield of the photosynthetic system II photochemistry, F_v/F_m ratio) was not affected upon Mn^{2+} exposure, even at toxic levels (Table 1). The Mn content in the shoot increased with the Mn^{2+} concentration in the nutrient solution (Table 1). However, in the root there was a strong increase in the Mn content at 10 μM Mn^{2+}, which hardly increased further at higher Mn^{2+} concentrations. This increase was markedly higher in the shoot than in the root (Table 1). Evidently, Mn became toxic and reduced the biomass production when the content in the shoot was ≥16 μmol g^{-1} dry weight and exceeded four-fold of that of the control. Exposure of plants to toxic Mn^{2+} levels hardly affected the content of other essential mineral nutrients in both root and shoot (data not presented); there was only a 30% and 19% decrease at 100 μM in the K content of the root and shoot, respectively. Moreover, the Zn content increased

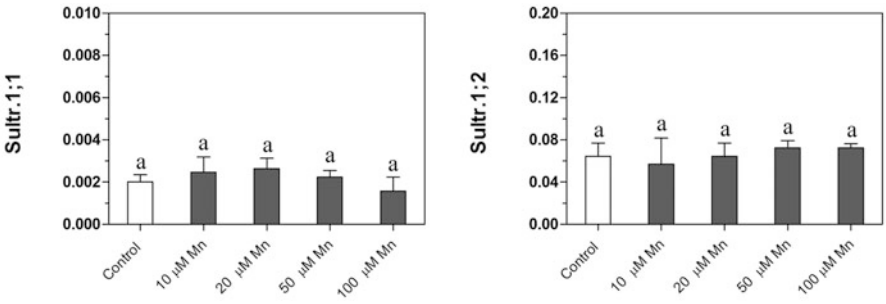

Fig. 1 Impact of Mn^{2+} exposure on the transcript levels of Sultr1;1 and Sultr1;2 in the root of *B. rapa*. For experimental details, see legends of Table 1. Relative gene expression of these genes was determined by qRT-PCR and the mRNA levels were compared to actin. Data on relative expression represent the mean of 3 measurements with 3 plants in each (±SD). *Different letters* indicate significant differences at $P < 0.01\%$ between different treatments

two-fold in both root and shoot; a similar increase in the Cu content was observed in the root. Mn^{2+} hardly affected the nitrate and free amino acid content of the plants (Table 1). The total sulfur and sulfur metabolite contents of *B. rapa* were only slightly affected at toxic Mn^{2+} levels. There was only a 1.25-fold increase in sulfur content in the shoot at 100 µM Mn^{2+}, which could be attributed to an increase of the sulfate content. Mn^{2+} exposure did not affect the total sulfur and sulfate content of the root and the water-soluble non-protein thiols in both root and shoot (Table 1). The sulfate transporters Sultr1;1 and Sultr1;2 are involved in the primary uptake of sulfate by the roots, though the transcript level of Sultr1;2 in the roots of *B. rapa* was 30-fold higher than that of Sultr1;1 (Fig. 1). The expression of these sulfate transporters were not affected upon Mn^{2+} exposure.

Similar to other essential potentially toxic metals, viz. Cu and Zn, exposure of *B. rapa* to elevated Mn^{2+} levels in the nutrient solution resulted in a strong accumulation of the metal in both root and shoot, resulting in decreased plant biomass production and chlororis of the shoot. *B. rapa* was much less susceptible to Mn than *B. pekinenis* to Cu and Zn toxicity: Mn^{2+} became toxic at $\geq$20 µM, whereas Cu^{2+} and Zn^{2+} already affected plant biomass production at $\geq$2 µM (Shahbaz et al. 2010, 2013, 2014; Stuiver et al. 2014). The decrease in biomass production due to toxic metal exposure was accompanied or even preceded by a decrease in pigment content (Foy et al. 1978, Shahbaz et al. 2010, 2013; Stuiver et al. 2014), although chlorophyll a fluorescence upon Cu^{2+} (Shahbaz et al. 2010) and Mn^{2+} exposure remained unaffected, which indicated that development rather than chloroplast functioning was negatively affected. High Mn levels also reduced the pigment content in tobacco (Clairmont et al. 1986), mungbean (Sinha et al. 2002), Chinese cabbage (Lee et al. 2011), spearmint (Asrar et al. 2005), tomato (Shenker et al. 2004) by affecting the chlorophyll, carotenoid and flavonoid biosynthesis (Clairmont et al. 1986; González and López 2013).

Exposure of *B. pekinensis* to elevated levels of Cu^{2+} and Zn^{2+} in the nutrient solution substantially affected the uptake, distribution and metabolism of sulfur (Shahbaz et al. 2010, 2013, 2014; Stuiver et al. 2014). Cu^{2+} and Zn^{2+} exposure resulted in an up-regulation of the activity of sulfate transporters and expression of the Group 1 sulfate transporters, viz. Sultr1;2, which are involved in the uptake of sulfate by the root in *Brassica* species (Shahbaz et al. 2010, 2013, 2014; Stuiver et al. 2014). The up-regulation of the sulfate transporters was most likely not due to a higher plant sulfur requirement upon Cu^{2+} and Zn^{2+} exposure, since it was accompanied by a substantial increase in the sulfate content of the shoot (Shahbaz et al. 2010, 2013, 2014; Stuiver et al. 2014). It was presumed that the up-regulation of the sulfate transporters was the consequence of a direct interference of these metal ions with the signal transduction pathway resulting in a disturbed regulation of the transporters (Shabaz et al. 2014; Stuiver et al. 2014). However, exposure of *B. rapa* to elevated Mn^{2+} levels did not affect the transcript levels of the sulfate transporters Sultr1;1 and Sultr1;2 in the root, despite a slight increase in the sulfate content in the shoot. The impact of elevated Mn^{2+} levels was also measured in *B. juncea* and all results on growth, pigment content and metabolite content were quite similar to that in *B. rapa*, with the exception that Mn toxicity was not accompanied with higher sulfur and sulfate contents in the shoot, and that it was even slightly decreased (data not presented). Apparently, in contrast to Cu^{2+} and Zn^{2+}, Mn^{2+} exposure did not interfere with the signaling of the regulation of the sulfate transporters. In general under normal conditions glutathione is the major water-soluble non-protein thiol compound present in plant tissues. Exposure of *B. pekinensis* to Cu^{+} (Shahbaz et al. 2010, 2013, 2014) and Zn^{2+} (Stuiver et al. 2014) resulted a strong increase in the water-soluble non-protein thiol content of the root and to a lesser extent in the shoot. This increase could partially be ascribed to an increase in phytochelatins (Shahbaz et al. 2010) and cysteine (Stuiver et al. 2014). An increase in water-soluble non-protein thiols (e.g., cysteine and glutathione) is expected as a defense mechanism against heavy metal toxicity (Leitenmaier and Küpper 2013). Moreover, cysteine and glutathione are the precursors for the synthesis of phytochelatins, which may complex with metals and increase toxic metal tolerance (Ernst et al. 2008). However, Mn^{2+} exposure did not affect the water-soluble non-protein content of both root and shoot of *B. rapa* (and *B. juncea*, data not presented). Apparently, an exposure of *B. rapa* to elevated and toxic Mn^{2+} levels did not trigger the synthesis of thiols (e.g. cysteine, glutathione and/or phytochelatins).

In conclusion, in contrast to Cu^{2+} and Zn^{2+}, elevated and toxic Mn^{2+} levels in the root environment hardly affected the uptake and metabolism of sulfate in *Brassica*.

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