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Nickel toxicity in *Brassica rapa* seedlings: Impact on sulfur metabolism and mineral nutrient content

Nickeltoxizität bei *Brassica rapa* Sämlingen: Einfluss auf den Schwefelstoffwechsel und den Mineralstoffgehalt

Abstract

Throughout the world anthropogenic activity has resulted in enhanced soil nickel (Ni^{2+}) levels, which may negatively affect plant productivity. The physiological background of Ni^{2+} phytotoxicity is still largely unclear. Ten-day exposures of *Brassica rapa* seedlings to 1, 2 and 5 μM $NiCl_2$ resulted in strongly enhanced tissue Ni levels, a decreased biomass production and leaf chlorosis at ≥ 2 μM Ni^{2+}. At 5 μM Ni^{2+} plant growth was completely halted. Ni toxicity occurred when the content of the shoot exceeded 1.0 μmol g^{-1} dry weight and that of the root, 23 μmol g^{-1} dry weight. Ni^{2+} exposure at ≤ 2 μM only slightly affected the mineral nutrient content of both shoot and root. Hence, Ni^{2+} exposure hardly affected the sulfur metabolite content of the plant. At ≥ 1 μM Ni^{2+} the total sulfur content of the root was only slightly lowered, which could fully be ascribed to a decreased sulfate content. Moreover, the water-soluble non-protein thiol content of both shoot and root was only enhanced at 5 μM Ni^{2+}. From these results it was clear that sulfur metabolism was unlikely to be directly involved in the Ni^{2+} tolerance mechanisms of *B. rapa*.

Key words: toxic metals; heavy metals; nickel; sulfur; thiols; glutathione; mineral composition

Zusammenfassung

Weltweit haben anthropogene Aktivitäten zu erhöhten Nickelgehalten im Boden (Ni^{2+}) geführt, was sich negativ auf die Pflanzenproduktivität auswirken kann. Der physiologische Hintergrund der Ni^{2+} Phytotoxizität ist noch weitgehend unklar. Eine zehntägige Exposition von *Brassica rapa* Sämlingen mit 1, 2 und 5 μM $NiCl_2$ führte zu stark erhöhten Ni Gehalten im Gewebe, einer verringerten Biomasseproduktion und zu Blattchlorosen bei Konzentrationen von ≥ 2 μM Ni^{2+}. Bei einer Konzentration von 5 μM Ni^{2+} war kein Pflanzenwachstum mehr zu beobachten. Eine Ni Toxizität trat auf, wenn der Ni Gehalt im Sprosses 1,0 μmol g^{-1} Trockengewicht und der in der Wurzel 23 μmol g^{-1} Trockengewicht überschritt. Eine Ni^{2+} Exposition von 2 μM beeinflusste den Mineralstoffgehalt in Spross und Wurzel nur geringfügig. Daher beeinflusste eine Ni^{2+} Exposition die Gehalte an Schwefelmetaboliten in der Pflanze kaum. Bei ≥ 1 μM Ni^{2+} war der Gesamtschwefelgehalt der Wurzel nur geringfügig erniedrigt, was vollständig auf einen verminderten Sulfatgehalt zurückzuführen war. Darüber hinaus war der Gehalt an wasserlöslichen Nicht-Protein-Thiolen sowohl im Spross als auch in der Wurzel nur bei 5 μM Ni^{2+} erhöht. Aus diesen Ergebnissen geht hervor, dass der Schwefelstoffwechsel wahrscheinlich nicht direkt an den Ni^{2+} Toleranzmechanismen von *B. rapa* beteiligt ist.

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Introduction

Nickel (Ni) is considered as an important micronutrient for the physiological functioning of plants (Wood et al., 2004; Brown, 2006; Polacco et al., 2013; Shahzad et al., 2018). Plants acquire Ni in the divalent form (Ni^{2+}), which is both passively and actively taken up by the root (Cataldo et al., 1988). After uptake, Ni^{2+} is complexed with organic acids and amino acids, which may subsequently be transported from the root to the shoot or stored in the vacuole (Cataldo et al., 1988).

Ni is required for the activation of ureases, which catalyze the conversion of urea into ammonium (Polacco, 1977; Polacco et al., 2013). Consequently, if plants are grown with urea as sole nitrogen source, Ni deprivation may retard growth by inducing nitrogen deficiency (Polacco, 1977; Polacco et al., 2013). By contrast, if plants are supplied with other nitrogen sources, Ni-deprivation may retard growth by strongly enhancing tissue urea levels (Gerendas et al., 1999; Brown, 2006; Shahzad et al., 2018). Apart from activating ureases, Ni may also be involved in the activation of other enzymes. For instance, it may activate glyoxalase I, which functions in the degradation of methylglyoxal, a toxic molecule produced during cellular processes (Fabiano et al., 2015). Ni may also be transported from plant cells to plant-associated microbes (Brown, 2006). Nitrogen-fixing symbionts and leaf commensals contain Ni-dependent hydrogenases, whose activity is inhibited upon colonization of Ni-deficient plants (Holland & Polacco, 1992).

Despite its significance in plant functioning, elevated Ni levels in the root environment may cause growth retardations and leaf chlorosis (Freeman et al., 2004; Brown, 2006; Shahzad et al., 2018). Elevated soil Ni levels may be the consequence of anthropogenic activities, which include mining, smelting, waste disposal and industrial undertakings where Ni is used as catalyst (e.g., the production of electrical batteries; Brown, 2006; Shahzad et al., 2018). In polluted regions soil nickel levels have increased up to 20 to 30-fold (up to 26 g kg^{-1}) compared to unpolluted regions, which is now seriously threatening agricultural productivity (Brown, 2006; Shahzad et al., 2018).

The primary cause of Ni phytotoxicity remains poorly understood. Analogous to other heavy metals, Ni might react with thiol moieties present in enzymes and other proteins (Pilon et al., 2009; Yadav, 2010). Additionally, exposure to excessive Ni could possibly hamper the root uptake of other essential mineral nutrients (Pilon et al., 2009; Yadav, 2010). Consequently, it may disturb metabolism, which may lead to the production of reactive oxygen species and subsequently lipid peroxidation, protein denaturation and DNA mutation reactions (Pilon et al., 2009; Yadav, 2010).

To tolerate elevated Ni levels, plants could sequester Ni in the vacuole, synthesize Ni-chelator complexes in the cytosol or increase antioxidant levels (Brown, 2006; Shahzad et al., 2018). Sulfur metabolism may have significance in these Ni tolerance strategies. The thiol groups of cysteine and phytochelatins have the chemical properties to complex with heavy metals (Freeman et al., 2004; Sirko & Gotor, 2007; Cuypers et al., 2009). Furthermore, glutathione has the capability to bind reactive oxygen species, which presence might be induced in plants upon exposure to heavy metal stress (Freeman et al., 2004; Sirko & Gotor, 2007; Cuypers et al., 2009). Nevertheless, the physiological significance of these non-protein thiols for Ni tolerance remains elusive. Therefore, the current research describes Ni toxicity and its impact on S metabolism and mineral nutrient content in *Brassica rapa* seedlings.

Materials and methods

Seeds of *Brassica rapa* cv. Komatsuna (Nickerson-Zwaan, Made, The Netherlands) were germinated in vermiculite in a climate-controlled room. After 10 days, seedlings were transferred to an aerated 25% Hoagland nutrient solution (see Koralewska et al., 2007 for composition) containing 0, 1, 2 or 5 µM $NiCl_2 \cdot 4\,H_2O$ in 30 l plastic containers (10 sets of plants per container, 3 plants per set). Day and night temperatures were 21 and 18°C (± 1°C), respectively, relative humidity was 70–80% and the photoperiod was 14 h at a photon fluence rate of 400 ± 30 µmol m^{-2} s^{-1} (within the 400–700 nm range) at plant height, supplied by Philips GreenPower LED (red/white 120) production modules. After 10 days of exposure, plants were harvested 3 h after the onset of the light period. The roots were rinsed in ice-cold de-mineralized water (3 × 20 s). Subsequently, shoots and roots were separated and weighted. Plant biomass production was calculated by subtracting the initial, pre-exposure, weight from that at harvest. For the determination of dry matter content, plant material was dried at 80°C for 24 h. Four independent experiments were performed for the analyses of the different parameters.

Chlorophyll was extracted from shoots, which were stored at –80°C after harvest, by homogenization in 96% ethanol using an Ultra Turrax (10 ml g^{-1} fresh weight). After centrifugation at 800 *g* for 20 min, the chlorophyll content was determined according to Lichtenthaler (1987). Chlorophyll *a* fluorescence was measured on the adaxial side of fully-expanded leaves as described by Shahbaz et al. (2010a) using a modulated fluorometer (PAM 2000, Walz GmbH, Effeltrich, Germany). For the analyses of Ni, S and other mineral nutrients dried whole shoots and roots were pulverized with a Retsch MixerMill (type MM2; Haan, Germany), digested with nitric acid/perchloric acid and analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) as described by Reich et al. (2017). Sulfate was extracted from frozen plant material and determined refractrometrically after

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HPLC separation (SHAHBAZ et al., 2010a). Water-soluble non-protein thiols were extracted from fresh shoots and roots (SHAHBAZ et al., 2010a) and determined colorimetrically after reaction with 5,5'-dithiobis(2-nitrobenzoic acid), according to DE KOK et al. (1988).

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). To compare treatment means a one-way ANOVA with a Tukey's HSD test as *post-hoc* test at the $P \leq 0.05$ level was performed.

Results and discussion

Seedlings of *B. rapa* were highly susceptible to elevated Ni^{2+} levels in the root environment, since already at 2 µM Ni^{2+} the plant biomass production was reduced by 35% (Fig. 1). At 2 µM Ni^{2+} shoot biomass production was more affected than root biomass production, since the shoot/root ratio was reduced by 21% (Fig. 1). 5 µM Ni^{2+} completely halted plant growth and it reduced biomass production by 100% (Fig. 1). Ni^{2+} exposure hardly affected the dry matter content of the seedlings. The dry matter content of the shoot was only slightly enhanced at 5 µM Ni^{2+}, whereas that of the root was not affected by Ni^{2+} exposure (Fig. 1). A similarly high Ni^{2+} susceptibility was observed in e.g., soybean (*Glycine max*; REIS et al., 2017). Moreover, the susceptibility of *B. rapa* to Ni^{2+} was comparable with the susceptibility of *Brassica* species to elevated Zn^{2+} and Cu^{2+} levels in the root environment, which also substantially reduced plant growth at concentrations ≥ 2 µM (SHAHBAZ et al., 2010a; STUIVER et al., 2014). Nevertheless, the Ni^{2+} susceptibility of *Brassica* was much higher than that for Mn^{2+} and MoO_4^{2-}, which only reduced plant growth at concentrations ≥ 20 and

100 µM, respectively (NEVES et al., 2017; ZUIDERSMA et al., 2020). Exposure of *B. rapa* to Ni^{2+} inhibited the shoot biomass production more than that of the root (Fig. 1). Exposure of *Brassica* species to toxic Zn^{2+} and Mn^{2+} levels also mainly inhibited shoot growth, whereas exposure to Cu^{2+} and MoO_4^{2-} mainly inhibited root growth (SHAHBAZ et al., 2010a; STUIVER et al., 2014; ZUIDERSMA et al., 2020).

Upon Ni^{2+} exposure of *B. rapa* and Cu^{2+}, MoO_4^{2-}, Ni^{2+} and Zn^{2+} exposure of other *Brassica* species, the decrease in biomass production was accompanied or even preceded by a decrease in the chlorophyll content (Table 1; SHAHBAZ et al., 2010a; STUIVER et al., 2014; NEVES et al., 2017; ZUIDERSMA et al., 2020). At 5 µM Ni^{2+} the chlorophyll content was lowered by 53% (Table 1). However, the chlorophyll a/b ratio and chlorophyll *a* fluorescence (F_v/F_m) were not substantially affected by exposure of *Brassica* to Ni^{2+} and other heavy metals (Table 1; SHAHBAZ et al., 2010a; STUIVER et al., 2014; NEVES et al., 2017; ZUIDERSMA et al., 2020). The chlorophyll *a* fluorescence was only slightly decreased upon exposure to 5 µM Ni^{2+} (Table 1). Apparently, heavy metal exposure of *Brassica* hampers the development of new chloroplasts, but not the functioning of the already existing chloroplasts (SHAHBAZ et al., 2010a; STUIVER et al., 2014; NEVES et al., 2017; ZUIDERSMA et al., 2020). Thus, the photosystems of the remaining chloroplasts are not or only minimally suffering from photo-inhibition.

Exposure of *B. rapa* to Ni^{2+} resulted in a strongly enhanced Ni content of the shoot and root (Table 2). However, Ni contents increased more in the root than in the shoot and at toxic Ni^{2+} levels (2 µM) its content in the shoot increased 34-fold (to 1.04 µmol g^{-1} dry weight) and in the root 450-fold (to 22.5 µmol g^{-1} dry weight; Table 2). It has been suggested that excessive Ni^{2+} and other heavy metal levels in the root environment might
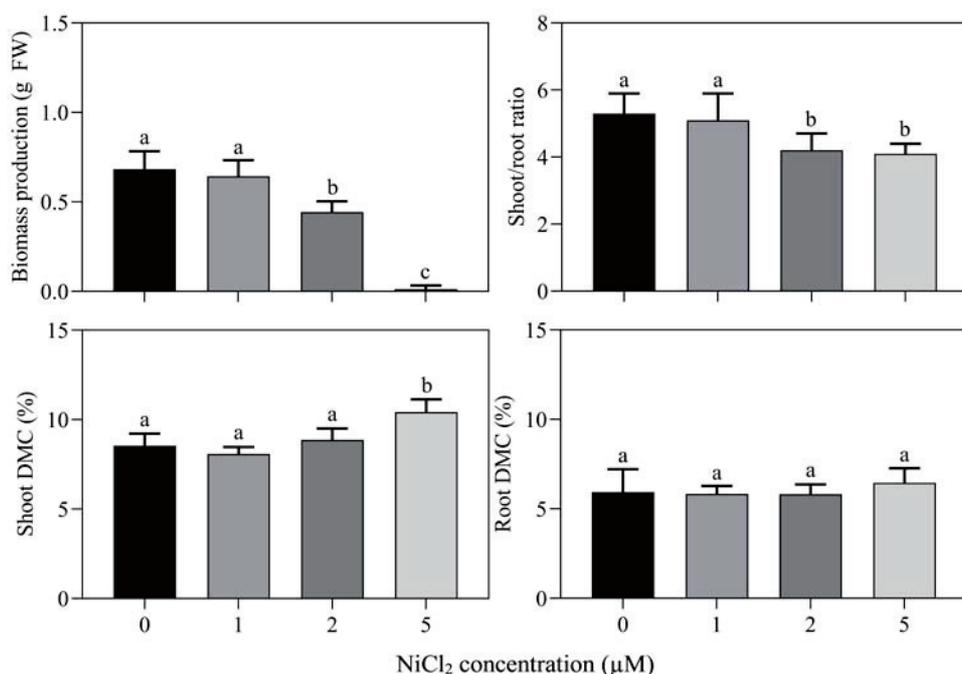
Fig. 1. Impact of Ni^{2+} exposure on the growth of *Brassica rapa*. 10-day old seedlings were grown on 25% Hoagland nutrient solutions containing supplemental $NiCl_2$ levels of 0, 1, 2 or 5 µM for 10 days. The initial plant weight was 0.178 ± 0.007 g. Data on biomass production (g FW) and shoot/root ratio represent the mean of four independent experiments with nine to ten measurements with three plants in each (± SD). Data on dry matter content (DMC; %) represent the mean of three independent experiments with three measurements with three plants in each (± SD). Different letters indicate significant differences between treatments ($P \leq 0.05$, one-way ANOVA; Tukey's HSD test as a *post-hoc* test).

Table 1. Impact of Ni²⁺ exposure on the chlorophyll content and fluorescence of *Brasica rapa*. 10-day old seedlings were grown on 25% Hoagland nutrient solutions containing supplemental NiCl₂ levels of 0, 1, 2 or 5 μM for 10 days. Data on chlorophyll content (mg g⁻¹ FW) and ratio represent the mean of two independent experiments with three measurements with three plants in each (± SD). Data on chlorophyll *a* fluorescence (F_v/F_m) represent the mean of ten measurements on different plants (± SD). Different letters indicate significant differences between treatments ($P \leq 0.05$, one-way ANOVA; Tukey's HSD test as a *post-hoc* test).

	NiCl₂ concentration (μM)			
	0	1	2	5
Shoot				
Chlorophyll	0.70 ± 0.12a	0.70 ± 0.06a	0.64 ± 0.04a	0.33 ± 0.06b
Chlorophyll a/b	3.0 ± 0.3a	3.0 ± 0.2a	2.7 ± 0.3a	3.5 ± 1.1a
F_v/F_m	0.84 ± 0.02a	0.83 ± 0.01ab	0.80 ± 0.01b	0.74 ± 0.05c

Table 2. Impact of Ni²⁺ exposure on the mineral content of *Brassica rapa*. 10-day old seedlings were grown on 25% Hoagland nutrient solutions containing supplemental NiCl₂ levels of 0, 1, 2 or 5 μM for 10 days. Data on mineral content (μmol g⁻¹ DW) represent the mean of three measurements with three plants in each (± SD). Different letters indicate significant differences between treatments ($P \leq 0.05$, one-way ANOVA; Tukey's HSD test as a *post-hoc* test).

Mineral content (μmol g⁻¹ DW)	NiCl₂ concentration (μM)			
	0	1	2	5
Shoot				
Calcium	766 ± 14a	761 ± 51a	781 ± 15a	815 ± 15a
Copper	0.08 ± 0.01a	0.06 ± 0.01a	0.06 ± 0.01a	0.14 ± 0.02b
Iron	1.43 ± 0.32a	1.33 ± 0.06a	1.01 ± 0.14a	1.28 ± 0.20a
Magnesium	184 ± 2a	191 ± 10a	201 ± 1a	252 ± 8b
Manganese	3.1 ± 0.2a	3.2 ± 0.3a	2.8 ± 0.1a	1.9 ± 0.1b
Nickel	0.03 ± 0.03a	0.53 ± 0.03b	1.04 ± 0.03c	2.12 ± 0.02d
Phosphorus	235 ± 10a	225 ± 18a	210 ± 5a	213 ± 4a
Sulfur	249 ± 14a	257 ± 10a	258 ± 12a	241 ± 5a
Potassium	1720 ± 42a	1350 ± 52b	1375 ± 20b	1089 ± 22c
Zinc	0.54 ± 0.05a	0.59 ± 0.02a	0.55 ± 0.01a	0.70 ± 0.12a
Root				
Calcium	182 ± 22a	182 ± 15a	170 ± 3a	189 ± 5a
Copper	0.29 ± 0.02a	0.23 ± 0.01b	0.28 ± 0.02ab	0.73 ± 0.03c
Iron	23.7 ± 0.8a	34.3 ± 1.9b	34.9 ± 1.1b	45.5 ± 3.7c
Magnesium	191 ± 12a	198 ± 9a	207 ± 22a	237 ± 26a
Manganese	44 ± 3a	52 ± 2ab	54 ± 3b	50 ± 4ab
Nickel	0.05 ± 0.01a	10.9 ± 0.4b	22.5 ± 1.3c	33.7 ± 1.9d
Phosphorus	322 ± 9a	321 ± 6a	316 ± 11a	305 ± 10a
Sulfur	358 ± 3a	295 ± 7b	307 ± 9bc	274 ± 16c
Potassium	1556 ± 11a	1595 ± 67a	1615 ± 86a	1319 ± 59b
Zinc	0.86 ± 0.16a	0.74 ± 0.03a	1.01 ± 0.07a	2.17 ± 0.40b

be phytotoxic, because their uptake might potentially hamper the uptake of other essential metal ions (Pilon et al., 2009; Yadav, 2010). However, it is doubtful if an altered essential metal ion uptake is the direct cause of Ni²⁺ toxicity in *B. rapa*. Exposure of plants to toxic Ni²⁺ levels (2 μM) only slightly affected the content of other essential mineral nutrients in the shoot and root (Table 2). There was a 30% decrease in the K content of the shoot and a 20 and 50% increase in the Mn and Fe content of roots, respectively, though this also occurred upon exposure to 1 μM Ni²⁺ (*viz*. a non-toxic Ni²⁺ concentration). At 5 μM Ni²⁺, where biomass production was complete halted, there was a much more pronounced impact on the plant's mineral nutrient content, with alter-

ations in the root and shoot Cu, Fe, K, Mg, Mn, S and Zn contents (Table 2). In accordance with these results, exposure of *Brassica* to excessive Zn^{2+}, Cu^{2+}, Mn^{2+} as well as MoO_4^{2-} also hardly affected the tissue content of mineral nutrients, suggesting that in *Brassica* an altered mineral nutrient uptake is unlikely to be the direct cause of toxicity of these metals (Shahbaz et al., 2010a,b; Stuiver et al., 2014; Neves et al., 2017; Zuidersma et al., 2020).

Exposure to elevated Ni^{2+} concentrations only slightly affected the S status of *B. rapa*. There was a 20% decrease in the total S content of the root at $\geq 1\,\mu M\ Ni^{2+}$, which could be fully attributed to a decrease in sulfate content (Table 3). Exposure to Ni^{2+} hardly affected the total sulfur and sulfate content of the shoot (Table 3). In *Brassica* the uptake of sulfate by the roots is regulated by the expression and activity of the Group 1 sulfate transporters, by *viz.* Sultr1;2 at sulfate-sufficient and Sultr1;1 and Sultr1;2 at sulfate-deprived conditions (Koralewska et al., 2007). Exposure of *Brassica* to excessive Zn^{2+} and Cu^{2+} resulted in an increased activity of the sulfate transporters and substantially enhanced sulfate content of the shoot (Shahbaz et al., 2010a; Stuiver et al., 2014). Evidently exposure to these toxic metals resulted in a deregulation of the uptake and metabolism of sulfur (Shahbaz et al., 2010a; Stuiver et al., 2014). By contrast, exposure to excessive Mn^{2+} and MoO_4^{2-} hardly affected tissue sulfate contents and the sulfate uptake capacity (expressed on a whole-plant fresh weight basis; Neves et al., 2017; Zuidersma et al., 2020). Thus, different heavy metals interfere to different extents with the signal cascade that regulates the sulfate transporters (Shahbaz et al., 2010a; Stuiver et al., 2014; Neves et al., 2017; Zuidersma et al., 2020). Interestingly, the concentration of hydrogen sulfide (H_2S) inside cells may regulate the transcription of sulfate transporters (De Kok et al., 2011). Heavy metals may react with H_2S, which may reduce cellular H_2S levels (Rumble, 2009). However, metals differ in their H_2S-reactivity (Rumble, 2009), which may potentially underlie variation in metal impact on the regulation of the sulfate transporters.

The S-containing compounds cysteine, glutathione and phytochelatins may be important for plant Ni^{2+} tolerance due to their molecular characteristics (Sirko & Gotor, 2007; Cuypers et al., 2009). However, although exposure to $2\,\mu M\ Ni^{2+}$ strongly inhibited growth of *B. rapa*, it did not affect the size of the water-soluble non-protein thiol pool (Table 3). Only at $5\,\mu M\ Ni^{2+}$, where the biomass production completely halted, there was a 2.3 and 1.6-fold increased water-soluble non-protein thiol content of the root and shoot, respectively (Table 3). Despite the increase in the content of water-soluble non-protein thiol compounds in the root of *B. rapa* at $5\,\mu M\ Ni^{2+}$, the absence of any impact of $2\,\mu M\ Ni^{2+}$ on the thiol pool suggests that cysteine, glutathione and phytochelatins are not directly important for Ni^{2+} tolerance of *B. rapa*. Notably, this may seem in contrast with earlier experiments, showing that transgenic *Arabidopsis thaliana* overproducing glutathione had a higher growth rate upon exposure to $100\,\mu M\ Ni^{2+}$ than wildtype plants (Freeman et al., 2004). However, genetic manipulation of the glutathione synthesis pathway may not only alter tissue glutathione content, but also the contents of other metabolites, which may affect Ni^{2+} tolerance. Accordingly, exposure of *Brassica* to excessive Mn^{2+} and MoO_4^{2-} similarly did not affect the tissue levels of water-soluble non-protein thiols (Neves et al., 2017; Zuidersma et al., 2020). Furthermore, although exposure of *Brassica* to excessive Zn^{2+} and Cu^{2+} enhanced the size of this pool, it was unlikely that this increase had any significance for heavy metal detoxification: experimental manipulation of the size and composition of the thiol pool did not alter the Cu^{2+} tolerance of Chinese cabbage (Shahbaz et al.,

Table 3. Impact of Ni²⁺ exposure on sulfate, water-soluble non-protein and total sulfur content of *Brassica rapa*. 10-day old seedlings were grown on 25% Hoagland nutrient solutions containing supplemental $NiCl_2$ levels of 0, 1, 2 or 5 μM for 10 days. Data on sulfate and water-soluble non-protein thiol content (μmol g^{-1} FW) represent the mean of two independent experiments with four to six (sulfate) or five to six (thiols) measurements with three plants in each (± SD). Data on total sulfur content (μmol g^{-1} FW) were calculated from the data in Table 2 by multiplying for each treatment the contents per g DW with the average dry matter content. Total sulfur data represent the mean of three measurements with three plants in each (± SD). Different letters indicate significant differences between treatments ($P \leq 0.05$, one-way ANOVA; Tukey's HSD test as a *post-hoc* test).

	NiCl₂ concentration (μM)			
	0	1	2	5
Shoot				
Sulfate	11.2 ± 2.2a	11.4 ± 1.3a	11.5 ± 1.0a	12.1 ± 1.2a
Thiols	0.33 ± 0.03a	0.36 ± 0.10a	0.37 ± 0.04a	0.54 ± 0.08b
Total sulfur	21.3 ± 1.2a	20.8 ± 0.8a	22.9 ± 1.1ab	25.1 ± 0.5b
Root				
Sulfate	12.5 ± 1.0a	8.9 ± 2.5b	8.6 ± 1.1b	7.0 ± 1.3b
Thiols	0.27 ± 0.07a	0.29 ± 0.04a	0.33 ± 0.04a	0.61 ± 0.11b
Total sulfur	21.3 ± 0.2a	17.2 ± 0.4b	17.9 ± 0.5b	17.7 ± 1.0b

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2010a; 2014). Thus, in conclusion, sulfur metabolism is unlikely to have direct relevance for Ni^{2+} tolerance mechanisms.

Acknowledgments

Conflicts of Interest

The authors declare no conflicts of interest.

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