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EFFECT OF SILICON SUPPLY ON GROWTH AND MALATE-METABOLIZING ENZYMES IN Mn-TREATED PEA PLANTS

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Summary: The effects of exogenously applied silicon (Si) on plant growth and the isoform patterns of NAD-dependent malate dehydrogenase (NAD-MDH, EC 1.1.1.37) and NADP-dependent malic enzyme (NADP-ME, EC 1.1.1.40) were studied in two pea genotypes (cultivar Auralia and mutant line 1/220) subjected to differential Mn treatments (50, 200 and 500 μM Mn) under control conditions. The excess Mn inhibited plant growth more strongly in the mutant line than in the cultivar Auralia referred to as Mn sensitive and Mn tolerant, respectively. The difference in Mn sensitivity of pea plants was related to the higher activity of the isoforms of NADP-ME and NAD-MDH in the Mn-tolerant cultivar Auralia. Mn treatment increased the activity of two isoforms of NADP-ME and one isoform of NAD-MDH in the leaves of both pea genotypes. The addition of Si improved plant growth in Mn-treated plants and its protective influence was more effective in the Mn-sensitive mutant line than in the Mn-tolerant cultivar Auralia. The alleviation of Mn toxicity by Si was related to decreased activity of the isoforms of NAD-MDH and NADP-ME, thus pointing to normalization of the redox status in Mn-exposed pea plants. This response was stronger in the tolerant cultivar. Noticeably, when applied to control plants Si had no effect on all parameters measured; it exerted its protective effect only in Mn-treated plants. Our data suggest a role of malate-metabolizing enzymes in Si-induced defense responses to excess Mn and in the difference in sensitivity of the two pea genotypes to Mn.

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Keywords*:* NAD-dependent malate dehydrogenase; NADP-dependent malic enzyme; manganese toxicity; pea plants.

INTRODUCTION

Manganese (Mn) is a micronutrient essential for all stages of plant development. It is involved in photosynthesis, respiration, and lignin and amino acid biosynthesis, in addition to performing a key function in the activation of several enzymes, including decarboxylating malate dehydrogenase, malic enzyme, isocitrate dehydrogenase, and nitrate reductase (Mukhopadhay and Sharma, 1991). Mn depending on its content in the soil, pH redox potential, soil moisture, microbial activity, extreme climatic conditions (water logging, dry, hot conditions) can achieve levels that

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are toxic for plants. Under conditions of increased Mn availability, high Mn concentrations in plant tissues induce the appearance of Mn toxicity symptoms and affect plant growth (Doncheva et al., 2009). Mn toxicity is one of the most limiting factors for crop production in acid soils (Horst, 1988).

Malate plays an important role in plant responses to stress conditions, as a component of the root exudates and a regulatory osmolyte affecting stomatal functioning (Fernie and Martinoia, 2009). Enzymes directly involved in malate metabolism are NAD-dependent malate dehydrogenase (NAD-MDH) (E.C. 1.1.1.37) and malic enzyme (NADP-ME) (E.C. 1.1.1.40).

Malate dehydrogenase catalyzes a reversible NAD-dependent dehydrogenase reaction involved in central metabolism and redox homeostasis between organelle compartments. MDH is a ubiquitous enzyme, for which several isoforms have been identified, differing in their subcellular localization and their coenzyme specifity. The enzyme participates in the Krebs cycle, photorespiration, metabolite shuttling and other catabolic and anabolic pathways (Musrati et al., 1998). The isoforms localized in subcellular organelles like peroxisomes, mitochondria, and cytosol are NAD-dependent, whereas the chloroplastic one is NADP-dependent (Gietl, 1992). Isoforms operate in mitochondria, chloroplasts, peroxisomes and the cytosol, but due to the ready transport and utilization of malate and oxaloacetate and the availability of NAD, this reaction can cooperate across compartments and is the basis for malate/oxaloacetate shuttling of reducing equivalents in many different metabolic schemes of plant cellular function (Krömer, 1995). Differential expression of MDH isoforms and changes in their activity has been reported in many plant species under abiotic stresses (Kumar et al., 2000).

Malic enzyme catalyzes the conversion between malate and pyruvate and is potentially involved in both malate synthesis and degradation, depending on the isoforms present, cellular conditions and the availability of substrates, and functions in both photosynthetic and non-photosynthetic pathways in plants (Drincovich et al., 2001; Maier et al., 2011). In C_3 plants such as *Arabidopsis thaliana* and rice (*Oryza sativa*) with known genome, the sequences of three cytosolic (NADP-ME_1 to NADP-ME_3) and one chloroplastic isoforms were found (Chi et al., 2004). In addition to participating in photosynthesis, plastidic and cytosolic NADP-ME isoforms may also be involved in plant defense reactions and environmental stress responses (Casati et al., 1999a; Pinto et al., 1999). Recently, it has been shown that over-expression of NADP-ME in transgenic *Arabidopsis* plants enhances tolerance of plants to salt and osmotic stress. Two different *NADP-ME* transcripts *TaNADP-ME1* [NCBI: EU170134] and *TaNADP-ME2* [NCBI: EU082065] were identified in plastidic and cytosolic counterpart, respectively, with a role in response of wheat plants to abscisic acid and salicylic acid as well as low temperature, salt, dark and drought stresses (Fu et al., 2009). Enhanced activity of NADP-ME was found in plants under various types of abiotic stress, such as drought, high salt concentration, ozone, the absence of phosphate and iron or the presence of heavy metals in

the soil (Doubnerova et al., 2010). An increase in the level of NADP-ME could provide building blocks and energy for biosynthesis of defense compounds (Casati at al., 1999b). Recently, it has been suggested that different rates of exudation of carboxylates may play a role in differential genotypic tolerance to Mn deficiency in lucerne (Gherardi and Rengel, 2004). Silicon is known to effectively mitigate various abiotic stresses such as Mn, Al and heavy metal toxicities, as well as salinity, drought, chilling and freezing stresses (Liang et al., 2007; Doncheva et al., 2009)

The aim of this study was to investigate the effects of exogenous Si on growth and isoform patterns of malate-metabolizing enzymes (NAD-MDH and NADP-ME) in two Mn-treated pea genotypes in order to elucidate some physiological and biochemical mechanisms underlying the protective effect of Si in plants experiencing Mn toxicity.

MATERIALS AND METHODS

Plant material and growth conditions

Pea (*Pisum sativum* L.) plants – cv. Auralia and mutant line 1/220 - were used as plant material. The mutant line 1/220 was obtained by gamma-ray-induced mutation in the parent cultivar Auralia and characterized by more wax on the upper leaf surface (named *more waxbloom*) (Naidenova and Vassilevska-Ivanova, 2004; Vassilevska-Ivanova and Naidenova, 2006). Seeds were germinated on wet filter paper. Five-day-old seedlings were transferred to 1200 ml-pots containing aerated nutrient solution in a greenhouse under natural light. The basic nutrient solution contained (μM): 200 $Ca(NO_3)_2.4H_2O$, 100 $MgSO_4.7H_2O$, 400 KNO_3, 300 NH_4NO_3, 10 Fe-EDTA, 5 $NaH_2PO_4.H_2O$, 8 H_3BO_3, 5 $MnSO_4.H_2O$, 0.16 $CuSO_4.5H_2O$, 0.38 $ZnSO_4.7H_2O$, 0.06 $(NH_4)_6Mo_7O_{24}.H_2O$ (pH 4.3) (Doncheva et al, 2005). After 5 days half of the plants received this nutrient solution (–Si), while the rest was exposed to a solution supplemented with 1 mM Si (+Si). Silicon was supplied at a concentration of 1 mM as a silicic acid got by passing sodium silicate through a H^+ loaded Dowex 50 W x 8 cation exchange resin (Marschner et al., 1990). After 72 h growth in solutions in the presence or absence of Si, the plants were transferred to a new solution with the same composition as above without Si, but supplemented with 50, 200, or 500 μM Mn as $MnSO_4$. Control plants received the basic nutrient solution containing 5 μM Mn throughout the experiment. The nutrient solution was changed two times a week. The plants were harvested for analyses 5 days after Mn treatment.

Plant growth analysis

Growth was determined by the relative root weight (RRW) and relative shoot weight (RSW) measured as the ratio of root or shoot dry weight under excess Mn to root or shoot dry weight at a basic Mn concentration (control).

Native PAGE analysis and detection of NAD-MDH and NADP-ME isozymes

Soluble proteins were extracted from finely ground leaf material with 0.1 M K-phosphate buffer (pH 7.8) and 0.05 M Tris-HCI buffer (pH 7.2) containing 6 mM cystein hydrochloride, 6 mM ascorbic acid and 0.5 M sucrose. The crude extracts were centrifuged at 15 000 x *g* for 20 min at 4°C and the supernatants

were used for electrophoretic analyses. Isoforms of NAD-MDH and NADP-ME were separated by native polyacrylamide gel electrophoresis (PAGE) using 7.5% polyacrylamide gel according to Davis (1964). Equal amounts of protein (50 μg) were loaded on each lane. Bands corresponding to NADP-ME were detected after incubation of gels in a solution of 100 mM Tris–HCl (pH 7.5) containing 1 M L-malate, 1 M $MgCl_2$, 0.2 M NADP, 0.15 mg/ml nitroblue tetrazolium and 0.05 mg/ml phenazine methosulfate at 30°C in the dark. Bands corresponding to NAD-MDH activity were detected by incubation of gels in a solution of 100 mM Tris-HCl (pH 7.5) containing 0.2 M NAD, 0.15 mg/ml thiazolyl blue tetrazolium bromide and 0.05 mg/ml phenazine methosulphate at 30°C in the dark (Adams, Joly, 1980).

The activity of bands of the isoforms obtained on a polyacrylamide gel were quantified by converting the stained area and intensity into relative units by gel scanning using ImageJ software (http://rsb.info.nih.gov/ij/).

Statistical analysis

Results are means of three independent experiments. Comparison of means was performed by the Fisher LSD test ($P \leq 0.05$) after performing multifactor ANOVA analysis.

RESULTS AND DISCUSSION

RSW and RRW were used to evaluate pea sensitivity to Mn. Both pea genotypes differed in their response to excess Mn in the nutrient solution (Table 1). RSW and RRW were much more affected by Mn treatment in the mutant genotype 1/220 than in cv. Auralia. RSW and RRW of cv. Auralia declined by 18% and 25% at the highest Mn concentration (500 μM) in the nutrient solution, respectively. Treatment of the mutant genotype with 500 μM Mn reduced RSW and RRW by 43% and 48%, respectively. According to Moroni et al. (2003) the genotypes sensitive to high Mn level have a RSW about 60% or below of the control plants, and thus were defined as Mn-sensitive, while the plants tolerant to Mn have a RSW >70% of the control and thus were confirmed as Mn-tolerant. Based on these parameters, the mutant genotype 1/220 could be defined as sensitive to Mn toxicity while its parent cv. Auralia as tolerant. When applied to control plants Si didn't influence plant growth. However, it had a clear amelioration effect on plants exposed to Mn toxicity. The growth of Mn-treated pea plants was significantly improved in the presence of Si. This was much more pronounced in the Mn-sensitive pea mutant line 1/220 than in the Mn-tolerant cv. Auralia (Table 1). The differences in the growth-improving effect of Si in the two Mn-treated genotypes allowed us to suggest that Mn-tolerance of cv. Auralia was genetically-based in contrast to the Si-induced Mn-tolerance of the mutant line.

Three NADP-ME isoforms were visualized on polyacylriamide gels in the leaves of both pea genotypes (Fig. 1). Data obtained for plants exposed to 500 μM Mn were selected as representative. The activities of all isoforms were higher in control plants of cv. Auralia compared to the mutant line 1/220 (Fig. 1), thus suggesting a relation of constitutive levels of NADP-ME activity with expression of Mn-tolerance. Treatment with 500 μM Mn did not change NADP-ME isoform profiles

Table 1. Dry weight of shoots and roots, relative shoot weight (RSW) and relative root weight (RRW) of pea plants of cv. Auralia and mutant line 1/220 exposed to increasing Mn concentrations (50, 200, 500 μM) in the presence or absence of 1 mM Si. Different letters indicate significant differences assessed by Fisher LSD test ($P<0.05$) after performing ANOVA multifactor analysis.

Genotypes	Mn [μM]	Shoots [g plant^{-1}]	Roots [(g plant^{-1}]	RSW [%]	RRW [%]
cv. Auralia	5 (control)	0.158 ± 0.0017^{e}	0.112 ± 0.0035^{d}	100	100
	5 + Si	0.158 ± 0.0023^{d}	0.108 ± 0.0012^{d}	100	96
	50	0.144 ± 0.0035^{bc}	0.089 ± 0.0029^{bc}	91	80
	50 + Si	0.151 ± 0.0026^{cd}	0.094 ± 0.0052^{c}	96	84
	200	0.137 ± 0.0029^{ab}	0.079 ± 0.0015^{ab}	87	81
	200 + Si	0.147 ± 0.0035^{c}	0.083 ± 0.012^{abc}	93	84
	500	0.130 ± 0.0046^{a}	0.084 ± 0.004^{a}	82	75
	500 + Si	0.134 ± 0.0012^{ab}	0.087 ± 0.0058^{ab}	85	78
	LSD ($P\leq0.05$)	0.0089	0.0170		
Mutant line 1/220	5 (control)	0.199 ± 0.0014^{d}	0.081 ± 0.0016^{d}	100	100
	5 + Si	0.195 ± 0.0062^{d}	0.084 ± 0.0008^{d}	98	103
	50	0.161 ± 0.0020^{b}	0.066 ± 0.0023^{c}	81	81
	50 + Si	0.179 ± 0.0006^{c}	0.072 ± 0.0013^{c}	90	88
	200	0.121 ± 0.0044^{a}	0.055 ± 0.0015^{b}	61	68
	200 + Si	0.158 ± 0.0026^{b}	0.065 ± 0.0028^{c}	79	80
	500	0.113 ± 0.0017^{a}	0.042 ± 0.0035^{a}	57	52
	500 + Si	0.151 ± 0.0068^{b}	0.056 ± 0.0040^{b}	76	69
	LSD ($P\leq0.05$)	0.0118	0.0074		

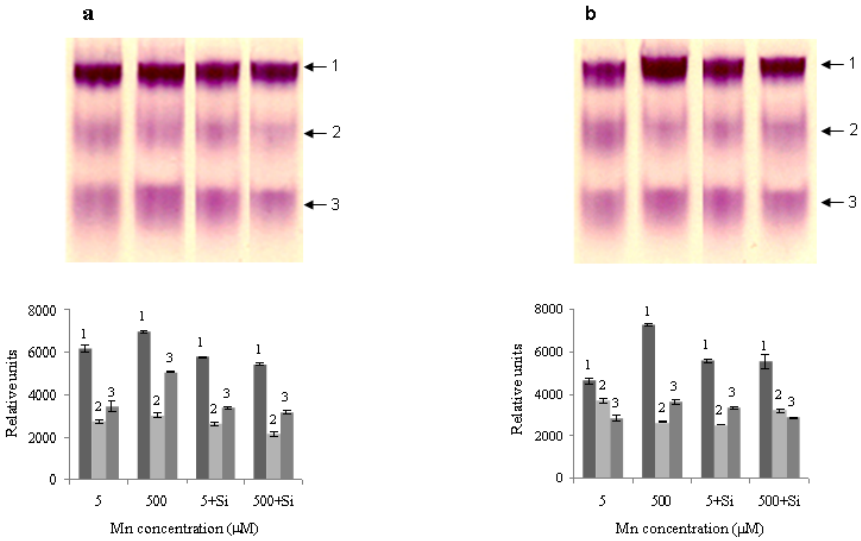

Figure 1. Identification of NADP-ME isoenzymes in leaves of cv. Auralia (a) and the mutant line 1/220 (b) after treatment with 500 μM Mn in the presence or absence of 1 mM Si. Control – 5μM Mn ± Si. Isoenzymes of NADP-ME were separated by native PAGE and identified by gel activity staining. The activities of NADP-ME isoforms were quantified by converting the stained area and intensity into relative units using gel scanning ImageJ software (http://rsb.info.nih.gov/ij/). 1-3: isoenzymes detected.

in both genotypes. However, leaves of Mn-treated plants exhibited increased activity of isoforms 1 and 3 in both genotypes indicating that pea NADP-ME responded to Mn stress. Si supply to control plants had no definite effect on the activity and pattern of NADP-ME. Contrarily, in Mn-exposed plants Si led to a decrease in the isozyme activity in both genotypes to levels comparable to control in the tolerant cv. Auralia while remaining still higher than control in the sensitive mutant line.

NADP-ME may facilitate defensive responses in plants to biotic or abiotic stresses (Pinto, 1999; Casati 1999; Maurino et al., 2001) by providing NADP(H) for the biosynthesis of lignin and flavonoids (Drincovich et al., 2001). It was reported that the four rice NADP-ME genes responded to stress conditions (Chi et al., 2004). Mn induced oxidation stress by generation of reactive oxygen species (ROS) including superoxide radical ($O_2^{\cdot-}$) (Li et al., 2010). The production of NADP(H) is involved in the metabolism for balancing ROS as the early-activated antioxidative defense mechanism (Smeets et al., 2005; Liu et al., 2007).

Probably, the production of NADP(H) by reactions catalyzed by NADP-ME could be considered as a defense mechanism for establishment of redox balance. The lowering of NADP-ME activity in Si-supplied Mn-treated plants suggested that Si had effectively contributed to the normalization of their redox status, this being better expressed in the Mn-tolerant cultivar.

Two NAD-dependent malate dehydrogenase bands were detected on polyacriamide gels in both genotypes (Fig. 2). The slowest band (1) is of mitochondrial origin while the fast migrating one (2) is considered as a cytosolic isoform of MDH

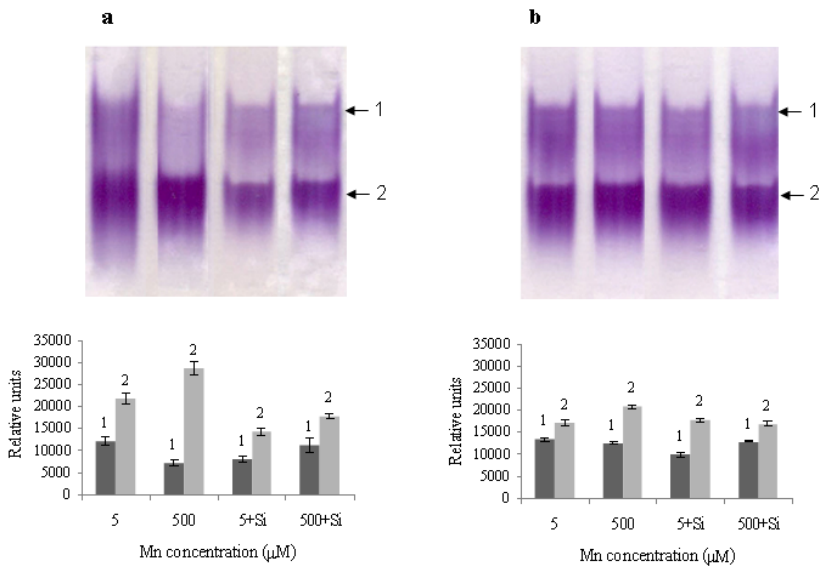

Figure 2. Identification of NAD-MDH isoenzymes in leaves of cv. Auralia (a) and the mutant line 1/220 (b) after treatment with 500 μM Mn in the presence or absence of 1 mM Si. Control – 5μM Mn ± Si. Isoenzymes of NADP-ME were separated by native PAGE and identified by gel activity staining. The activities of NADP-ME isoforms were quantified by converting the stained area and intensity into relative units using gel scanning ImageJ software (http://rsb.info.nih.gov/ij/). 1, 2: isoenzymes detected.

(Musrati et al., 1998).

The activity of the cytosolic isoform was higher in cv. Auralia control plants as compared with the mutant genotype (Fig. 2). The two MDH isoforms responded in a different manner to Mn excess. Exposure to 500 μM Mn increased cytosolic MDH activity and decreased mitochondrial MDH in the leaves of both genotypes, this being more expressed in cv. Auralia. The response of cytosolic and mitochondrial isoforms to Mn treatment was altered by Si supply which led to a decrease of cytosolic MDH activity and an increase of mitochondrial MDH in both genotypes. Similarly to NADP-ME, MDH was not influenced by the application of Si to control plants.

It has been hypothesized that enhanced release of organic acids, such as citrate, malate, and oxalate in response to toxic metals leads to metal complexation in the root apoplast and/or rhizosphere, thus avoiding its interaction with root cellular components and its entry in the root symplast (Ma, 2000; Mariano et. al., 2005).

Because malate is a crucial component of plant nutrient acquisition and adaptation to environmental stress, it is hypothesized that improving malate synthesis might be an effective strategy for improving plant nutrition. Increased synthesis of citrate and malate is intimately related with plant tolerance to Al stress (Delhaize and Ryan, 1995; Kochian, 1995).

CONCLUSION

At toxic levels Mn negatively influenced the growth of pea plants, the effect being much more pronounced in the genotype referred to as Mn-sensitive as compared to the genotype referred to as Mn-tolerant. Moreover, Mn induced a rise in the activity of isozymes of NADP-ME and NAD-MDH probably as a defensive tool to counteract the oxidative stress known to occur during Mn exposure. The higher constitutive levels of these isozymes observed in the tolerant cultivar can be related to the expression of Mn tolerance. The addition of Si to control plants didn't lead to changes in growth and activity of malate-metabolizing enzymes. Its protective action was expressed only under Mn toxicity leading to oxidative stress, and it was evidenced by improvement of plant growth and normalization of the redox status as judged by the decreased activity of malate-metabolizing enzymes. Our data reveal physiological and biochemical mechanisms underlying the protective effect of Si in plants experiencing Mn toxicity.

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