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Ammonium Toxicity Alleviation by Silicon is Dependent on Cytokinins in Tomato cv. Micro-Tom

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Abstract

The objectives were to verify the effects of the lack of cytokinins (CKs), comparing tomato cv. Micro-Tom (MT, wild type) to *MT CKX2* (transgenic with less CKs) fed with nitrate (NO_3^-) and ammonium (NH_4^+), in the presence and absence of silicon (Si); verify if the attenuation of NH_4^+ toxicity by Si depends on the increase of CKs in MT; and verify if 6-benzyladenine (6-BA) attenuates NH_4^+ toxicity in MT. Three experiments were performed with treatments via nutrient solution. First, MT and *MT CKX2* were grown with NO_3^- or NH_4^+ (5.9 mmol L^{-1}), in the absence and presence of Si (1.28 mmol L^{-1}). Second, MT was grown with NO_3^- or NH_4^+ (5.9 mmol L^{-1}), in the absence and presence of Si (1.28 mmol L^{-1}). Third, MT was grown with NO_3^- or NH_4^+ (5.9 mmol L^{-1}) and 6-BA (from $1e^{-10}$ to $1e^{-6}$ mol L^{-1}) associated with NH_4^+. The MT and *MT CKX2* had a decrease of 18% and 48% in the shoot dry weight, respectively, when fed with NH_4^+, compared to NO_3^-. Si attenuated NH_4^+ toxicity in MT, but not in *MT CKX2*. This attenuation in MT was accompanied by a decrease in *trans*-zeatin (*t*Z) content in the roots and increase in the shoots. 6-BA did not improve the shoot growth of MT fed with NH_4^+. In conclusion, the alleviation of NH_4^+ toxicity by Si was dependent on the increase in *t*Z content in shoots. In CK-deficient plants, Si did not alleviate NH_4^+ toxicity, and 6-BA did not alleviate NH_4^+ toxicity in MT shoots.

Keywords *Solanum lycopersicum* · Cytokinins deficiency · *CKX2* · Beneficial element · 6- benzyladenine · Ammonium nutrition

Introduction

Agriculture requires the intensive use of nitrogen fertilizers, which are predominantly up taken by plants in nitrate (NO_3^-) and ammonium (NH_4^+) forms; however, the latter can be toxic, particularly in ecosystems with low temperatures, acidic soils and hypoxic conditions where NH_4^+ concentrations can be up to threefold greater than those of NO_3^- (Miller et al. 2007). Typical growth inhibition due to NH_4^+ toxicity is caused by alteration of the pH gradient required for electron transport in photosynthesis and respiration, the high energy cost to maintain low NH_4^+ levels in cells, oxidative stress, degradation of photosynthetic pigments, decreased photosynthesis (Britto and Kronzucker 2002), reduced accumulation of cations, such as K, Ca and Mg (Barreto et al. 2018) and decreased levels of cytokinins (CKs) (Walch-Liu et al. 2000).

In this context, silicon (Si) is a beneficial element that relieves stress, such as NH_4^+ toxicity, because it increases nutrient use efficiency, antioxidant system activity,

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photosynthetic pigment content and photosynthesis (Gao et al. 2014; Barreto et al. 2017). However, some similar effects between Si and CKs are noteworthy given that this hormone also induced tolerance to NH_4^+ toxicity. For example, exogenous CK administered via nutrient solution in its *trans*-zeatin (*t*Z) form activated leaf growth and prevented a reduction in chlorophyll content in *Arabidopsis thaliana* fed NH_4^+ (Shtratnikova et al. 2015), and the effect of the cheaper synthetic CK 6-benzyladenine (6-BA) on NH_4^+ toxicity has not been studied to date. In addition, *A. thaliana* and *Sorghum bicolor* with Si accumulation exhibited delayed leaf senescence. As the response was similar in both species, the authors suggested a general role of Si in stress relief because it increased expression of the *IPT7* and *ARR5* genes, which act on CK biosynthesis and signal transduction, respectively (Markovich et al. 2017).

In addition to the application of CKs and the use of substances that may stimulate CK biosynthesis, such as Si, plants that overexpress the enzyme cytokinin oxidase and consequently exhibit reduced levels of CKs are important models used to generate information on the processes regulated by CKs (Werner et al. 2001). In this context, the tomato-dwarf cultivar Micro-Tom (MT) was genetically transformed to overexpress the enzyme *CYTOKININ OXIDASE 2* (*MT CKX2*) (Pino-Nunes 2009). Thus, we hypothesized that (i) Si alleviates NH_4^+ toxicity in MT but not in *MT CKX2*, (ii) the attenuation of NH_4^+ toxicity by Si is dependent on increased levels of CKs, and (iii) 6-BA alleviates NH_4^+ toxicity in tomato MT. Thus, the objectives were to verify the effects of the lack of CKs, comparing tomato cv. MT (wild type) to *MT CKX2* (transgenic with less CKs) fed with NO_3^- and ammonium NH_4^+, in the presence and absence of Si; verify if the attenuation of NH_4^+ toxicity by Si depends on the increase of CKs in MT; and verify if 6-BA attenuates NH_4^+ toxicity in MT.

Material and Methods

Plant Material and Growth Conditions

The experiments were performed in a growth chamber with a 12-h photoperiod, and light was supplied at 70 µmol m^{-2} s^{-1} using fluorescent tubes. Air temperatures at sites of plant growth were 26/22 ± 3 °C (day/night), and the relative air humidity was between 35 and 45%.

Tomato (*Solanum lycopersicum* L.) seeds of MT and *MT CKX2* were seeded in polystyrene trays containing vermiculite and Bioplant® substrate (1:1, v:v). The trays were suspended with 5-cm supports to avoid root contact with the cultivation bench and root damage during plant removal for transplantation. Substrate moisture was maintained with potable water using a hand irrigator once daily, and irrigation was stopped at the first signs of drainage.

When plants had two nonexpanded leaves (12 d after sowing (DAS) for MT and 22 DAS for *MT CKX2*), they were removed from the trays. The substrate was removed from the roots with running water. The plants were placed in polypropylene vases 11 cm high and 14 cm wide containing 2 L of nutrient solution (Hoagland and Arnon 1950) diluted to 25%. The sources of Fe, Cu, Zn and Mn micronutrients were modified to chelated forms (EDDHA for Fe and EDTA for Cu, Mn and Zn).

Si supplementation was initially provided during transplanting and maintained throughout the experimental period. The nutrient solution was prepared in the following sequence: Si was added to water, pH was adjusted to 5.7 ± 0.3, nutrients were added, and pH was adjusted to 5.7 ± 0.3. The nutrient solution was prepared with deionized water. The pH value was monitored daily and adjusted when necessary to 5.7 ± 0.3 with NaOH or HCl (10%) solutions. The solution was aerated using an air compressor during the photoperiod. The source of Si was potassium silicate (128 g L^{-1} Si, 126 g L^{-1} K_2O). The same amount of K in all treatments was obtained with potassium chloride (KCl). When the plants had two expanded leaves, the nutrient solution was modified according to each treatment and experiment. When MT had 6 expanded leaves and *MT CKX2* had 4 expanded leaves (between 37 and 39 DAS), the evaluations were performed.

Experiment 1: MT and Transgenic *MT CKX2* Overexpressing a Cytokinin Oxidase

The experiment was designed using a 2 × 2 factorial scheme corresponding to two N forms, i.e., NO_3^- or NH_4^+ (5.9 mmol L^{-1}), in the absence and presence of Si (1.28 mmol L^{-1}). These factors were combined, resulting in 4 treatments (NO_3^-; NO_3^- + Si; NH_4^+ and NH_4^+ + Si) in a randomized block design (RBD) with at least 5 replicates. The composition of the nutrient solution was presented in Table 1.

A previous experiment with MT (data not shown) was performed to calculate the critical level of NH_4^+ toxicity corresponding to the nutrient concentration in the plant that reduces growth. Davis et al. (1978) indicated that this value corresponds to a 10% decrease in yield. Thus, the plants were grown at NH_4^+ concentrations between 0.25 and 7.5 mmol L^{-1}. The critical toxicity level was obtained with 5.9 mmol L^{-1} NH_4^+. In addition, 5.9 mmol L^{-1} NO_3^- served as the control.

To verify whether NH_4^+ toxicity was alleviated by Si in MT tomato, a previous experiment (data not shown) with Si concentrations between 1 and 2.5 mmol L^{-1} obtained from potassium silicate was performed. Here, 1.28 mmol L^{-1} Si

Table 1 Composition of nutrient solution with NO_3^- or NH_4^+ in the absence or presence of Si in which the experiment was conducted

Stock nutrient solution (mol L^{-1})	NO_3^- mL L^{-1}	NO_3^- + Si	NH_4^+	NH_4^+ + Si
KH_2PO_4	0.50	0.50	0.50	0.50
$MgSO_4$ $7H_2O$	1.00	1.00	1.00	1.00
KCl	2.50	1.75	2.50	1.75
NH_4Cl	–	–	5.90	5.90
Ca $(NO_3)_2$ $4H_2O$	2.95	2.95	–	–
$CaCl_2$ $2H_2O$	–	–	2.95	2.95
SiK[a]	–	0.28	–	0.28
Micronutrients[b]	0.50	0.50	0.50	0.50
Fe-EDDHA[c]	1.00	1.00	1.00	1.00

[a]Potassium silicate: 128 g L^{-1} Si, 126 g L^{-1} K_2O

[b]For 1 L: 2.86 g H_3BO_3; 0.02 g $H_2MoO_4H_2O$; 3.87 g Mn-EDTA (12%); 0.343 g Zn-EDTA (14%), 0.126 g Cu-EDTA (15%)

[c]83.3 g L^{-1} Fe-EDDHA (6%)

alleviated NH_4^+ toxicity. In addition, 1.28 mmol L^{-1} Si combined with 5.9 mmol L^{-1} NO_3^- served as the control.

Plant Images

The plants were anchored in tubes containing 100 mL water. A black cotton cloth was used for the background. The images were recorded with an 8-megapixel camera attached to a smartphone, and the background was completely black.

Plant Height and Leaf Area

Using a ruler, the height of the plants was obtained from the base of the phenolic foam to the insertion of the last leaf. The leaf area without the petiole was measured using a leaf area meter (L-3100, Li-Cor).

Chlorophylls and Carotenoids

Briefly, 25–30 mg fresh mass from the apex to the base of the fourth leaf in MT and the second leaf in *MT CKX2* was collected and transferred to an Eppendorf tube protected from light that contained 1.5 mL 80% acetone. Samples were stored at 4 °C for 48 h. The extracts were measured at 663 nm (chlorophyll A), 647 nm (chlorophyll B) and 470 nm (carotenoids), and the concentration of each pigment was obtained using the corresponding equations (Lichtenthaler 1987).

Malondialdehyde (MDA) and Hydrogen Peroxide (H_2O_2)

Approximately 250 mg of plant material (one root after excess water is removed using a paper towel and the fourth and fifth leaves from top to bottom) from MT and approximately 180 mg of fresh mass (one root with excess after water is removed with paper towel and all leaves) from *MT CKX2* were used to obtain fresh mass data and were immediately frozen in liquid nitrogen. Subsequently, the plant material was stored at -80° C for quantification of MDA and H_2O_2 content.

MDA content was determined using the 2-thiobarbituric acid test (TBA) as described by Heath and Packer (1968). Reactive metabolites of cell membranes, especially MDA, react with TBA and can be quantified using spectrophotometry. Fresh samples were macerated with 1 mL 0.1% trichloroacetic acid (TCA) and 20% polyvinylpolypyrrolidone in an ice bath. After homogenization, samples were transferred to tubes and centrifuged at 10,000×*g* for 5 min. Then, 250 µL supernatant was withdrawn and mixed with 1 mL 20% TCA and 0.5% TBA. The procedures were performed in triplicate. The mixture was placed in a dry bath for 30 min at 95 °C and then cooled on ice. Samples were centrifuged for 10 min at 10,000×*g*. The samples were assessed on a spectrophotometer at 535 and 600 nm.

The H_2O_2 content was determined based on the reaction with potassium iodide (KI) according to Alexieva et al. (2001). Fresh samples were macerated with 1 mL 0.1% TCA and 20% polyvinylpolypyrrolidone in an ice bath. After homogenization, samples were transferred to tubes and centrifuged at 10,000×*g* for 15 min at 4 °C. Then, 200 µL of the supernatant was obtained. Next, 200 µL 100 mM potassium phosphate buffer (pH 7.5) and 800 µL 1 M KI solution were added. Reactions were performed in triplicate. The blank sample contained the same blend described above but 200 µl 0.1% TCA was used instead of the supernatant. The reaction tubes were placed on ice and remained in the dark for 1 h. Samples were assessed using a spectrophotometer at 390 nm.

Dry Weight of Shoot and Root

Shoots and roots from plants were separated. Shoots were washed in running water, neutral detergent (0.1%), hydrochloric acid (0.3%) and deionized water. The plant material was dried in a forced air circulation unit at 65 ± 5 °C until a constant mass was obtained. After drying, the dry weights of shoots and roots were obtained using an analytical balance.

Nitrogen Content and Accumulation in Shoots

After obtaining the dry weight, shoots were milled in a Willey mill, and the N content was chemically analyzed (Bataglia et al. 1983). The level of N accumulation was obtained by multiplying the content with the shoot dry weight.

Table 2 Composition of nutrient solution with NO_3^- or NH_4^+ and 6-BA concentrations associated with NH_4^+ in which the experiment was conducted

Stock nutrientsolution (mol L^{-1})	NO_3^-	NH_4^+					
	6-benzyladenine (BA, mol L^{-1})						
	0	0	$1e^{-10}$	$1e^{-9}$	$1e^{-8}$	$1e^{-7}$	
KH_2PO_4	0.50	0.50	0.50	0.50	0.50	0.50	
$MgSO_4$ $7H_2O$	1.00	1.00	1.00	1.00	1.00	1.00	
KCl	2.50	2.50	2.50	2.50	2.50	2.50	
NH_4Cl	–	5.90	5.90	5.90	5.90	5.90	
$Ca(NO_3)_2$ $4H_2O$	2.90	–	–	–	–	–	
$CaCl_2$ $2H_2O$	–	2.90	2.90	2.90	2.90	2.90	
BA [a] (µmol L^{-1})	–	–	0.10	1.00	10.00	100.00	
Micronutrients [b]	0.50	0.50	0.50	0.50	0.50	0.50	
Fe-EDDHA [c]	1.00	1.00	1.00	1.00	1.00	1.00	

[a] $C_{12}H_{11}N_5$

[b] For 1 L: 2.86 g H_3BO_3; 0.02 g $H_2MoO_4H_2O$; 3.87 g Mn-EDTA (12%); 0.343 g Zn-EDTA (14%), 0.126 g Cu-EDTA (15%)

[c] 83.3 g L^{-1} Fe-EDDHA (6%)

Experiment 2: CK Content in MT Shoots and Roots

MT plants grown under the same treatments described in Sect. Experiment 1: MT and Transgenic *MT CKX2* Overexpressing a Cytokinin Oxidase were used to quantify CKs. The roots were rinsed in deionized water, separated from the shoot and transferred to 15 mL Falcon tubes. The shoot was transferred to 50 mL Falcon tubes. The tubes were immediately frozen in liquid nitrogen and placed in a lyophilizer (Savant Super Modulyo) for 72 h. After lyophilization, the samples were stored in a plastic bag to avoid contact with air.

Lyophilized shoot and root tissues were used for the quantification of CKs. Approximately 50 mg of root and shoot samples were macerated in liquid nitrogen with the addition of 1.8 mL extraction solution (80% methanol, 1% acetic acid and 19% distilled water) in 2 mL tubes. Subsequently, the internal standards of the respective hormone to be quantified were added. This mixture was stirred for 1 h at 4 °C and then centrifuged at 10,000×*g* for 4 min at 4 °C. The supernatant was removed and transferred to a fresh 2 mL tube. The sample was stored at −20 °C for 24 h for protein precipitation. Then, the samples were centrifuged at 10,000×*g* for 4 min at 4 °C, and the supernatant was transferred to a 5 mL glass tube. Samples were concentrated in a rotovapor for 3 h. The concentrated samples were quenched to 1 mL with 1% acetic acid. After rapid stirring, the mixture was filtered on Oasis HLB® columns (reversed-phase). The hormones were recovered by applying 1 mL 95% methanol. Samples were rotovapor dried (Thermos Scientific®) and subsequently dissolved in 150 µl acetylnitrile (5%) + acetic acid (1%). Finally, readings were performed using a high-resolution mass spectrometer coupled to an Ultra-high Performance Liquid Chromatography (UHPLC) system (Thermo Scientific®) (Seo et al. 2011).

Experiment 3: Exogenous Application of 6-BA in MT Plants

This experiment was performed to determine whether 6-BA application attenuates NH_4^+ toxicity. On 19 DAS, the nutrient solution was modified to provide 5.9 mmol L^{-1} of N in the forms of NO_3^- (control) and NH_4^+ and 6-BA concentrations (0; $1e^{-10}$; $1e^{-9}$; $1e^{-8}$; $1e^{-7}$ e $1e^{-6}$ mol L^{-1}) associated with NH_4^+ (Table 2). The treatments were arranged in a RBD with 4 replicates. 6-BA concentrations were the same as those described by Shtratnikova et al. (2015); however, these experiments were performed in *A. thaliana* using the source *t*Z.

Root Area and Diameter

1 g fresh weight of roots was collected and stored in 20% alcohol solution at 4 °C. The roots were subject to methylene

blue staining for 5 min, washed in running water to remove excess staining, and assessed using a Hewlett Packard scanner. The area and mean root diameter were determined using Delta-T Scan Root Analysis System software.

The assessment of plant height and leaf area was described in Sect. Plant Height and Leaf Area. The assessment of root and shoot dry weight was described in Sect. Dry Weight of Shoot and Root. The assessment of plant images was described in Sect. Plant Images.

Statistical Analysis

In experiment 1, we used a factorial analysis of variance (ANOVA two-way) to test the effects of genotypes and treatments via nutrient solution, as well as their interaction. In experiments 2 and 3, we used an analysis of variance (ANOVA one-way) to test the effects of treatments via nutrient solution. Means were compared using a Tukey test ($p \leq 0.05$ and $p \leq 0.01$). Data were analyzed using SISVAR software (Ferreira 2011). Excel software was used to draw the figures.

Results

Experiment 1: MT and Transgenic *MT CKX2* Overexpressing a Cytokinin Oxidase

In MT, we observed that plants treated with NO_3^- and $NO_3^- + Si$ were similar. Plants treated with NH_4^+ exhibited reduced growth compared with NO_3^--treated plants; $NH_4^+ + Si$ yielded increased growth compared with NH_4^+ treatment alone (Fig. 1a). However, the addition of Si had no effect on NO_3^-- or NH_4^+-treated *MT CKX2* plants. In addition, *MT CKX2* plants treated with NH_4^+ were smaller than plants treated with NO_3^- (Fig. 1b).

MT and *MT CKX2* plants grown in NO_3^- in the presence and absence of Si exhibited similar shoot N levels (Fig. 2a). However, NH_4^+ treatment in the presence of Si increased N content in MT. Furthermore, the increase in N content in NH_4^+-treated *MT CKX2* plants occurred independently of Si and was approximately 50%. These results suggest that NH_4^+ toxicity may be more severe in CK-deficient transgenic plants due to increased shoot N concentrations upon treatment with NH_4^+.

In MT, N accumulation was lower only when grown in NH_4^+ in the absence of Si. However, no difference in N accumulation was observed among different nutrient solution treatments. In addition, *MT CKX2* exhibited lower N accumulation than MT (Fig. 2b).

MT plants exhibited increased total chlorophyll content when grown in NH_4^+ in the presence of Si. However, in *MT CKX2*, total chlorophyll was not altered among the different nutrient solution treatments. In addition, *MT CKX2* exhibited lower total chlorophyll than MT (Fig. 2c). Carotenoid levels increased in MT grown in $NH_4^+ + Si$. Moreover, in *MT CKX2*, no difference in carotenoid levels was noted among the different treatments (Fig. 2d).

In leaves, different responses were noted for stress indicators. In MT, MDA levels were increased in plants fed NH_4^+ compared with NO_3^- independent of Si supply. In *MT CKX2*, the opposite was noted; specifically, plants grown in NH_4^+ exhibited reduced MDA levels (Fig. 3a). In MT roots, the lowest MDA content was observed in NO_3^--treated plants. There was no difference in MDA content in *MT CKX2* roots among the different treatments (Fig. 3b). Regarding H_2O_2, Si supplementation reduced MDA levels in leaves of MT plants treated with NH_4^+ compared with NO_3^- in the presence of Si. However, H_2O_2 levels were reduced in the leaves of *MT CKX2* plants grown in NH_4^+ regardless of Si supply (Fig. 3c). In roots of MT plants grown in NH_4^+, H_2O_2 levels decreased upon supplementation with Si; this effect was not observed in *MT CKX2* roots (Fig. 3d).

MT and *MT CKX2* plants exhibited reduced height when grown in NH_4^+ regardless of Si application (Fig. 4a). However, different leaf area results were noted between MT and

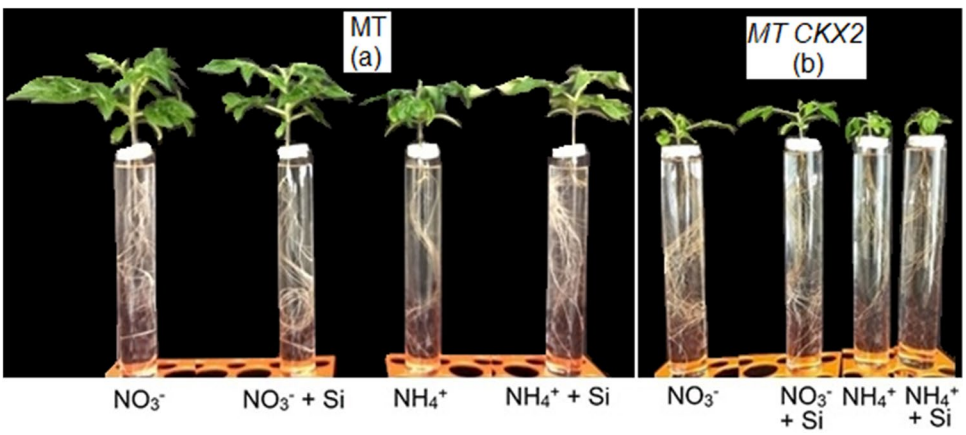

Fig. 1 Tomato cv MT (**a**) and *MT CKX2* (**b**) grown in nutrient solution with NO_3^- or NH_4^+ in presence and absence of Si

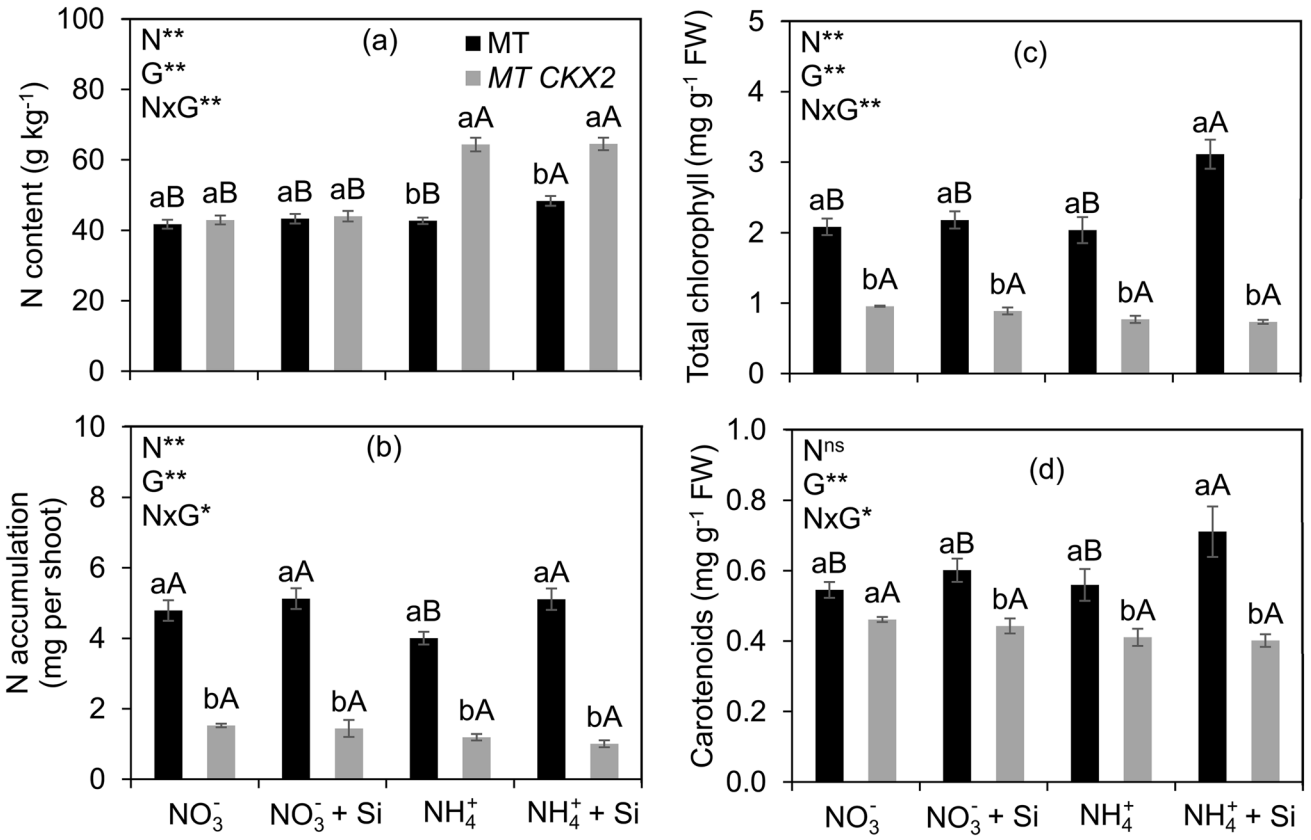

Fig. 2 N content (**a**), N accumulation (**b**), total chlorophyll (**c**) and carotenoids (**d**) in tomato cv MT and *MT CKX2* grown in nutrient solution with NO_3^- and NH_4^+ in the presence and absence of Si ^{ns}, * and **: not significant, significant at 5 and 1%, respectively N: nutrient solution; G: genotype; NxG: interaction between nutrient solution and genotype. Same lowercase letters indicate no difference between the genotypes for the same treatment in nutrient solution. Same uppercase letters indicate no difference in the same tomato genotype among the different nutrient solution treatments

MT CKX2. MT cultivated in the NH_4^+ and Si supply exhibited increased leaf area, and this effect was not observed in *MT CKX2* (Fig. 4b). In this context, the results indicated that Si mitigated NH_4^+ toxicity exclusively in MT plants.

In the absence of Si, MT plants cultivated in NH_4^+ exhibited a reduction in shoot and root dry mass compared with NO_3^- plants. However, in the presence of Si, the shoot and root dry mass of plants grown in NH_4^+ did not differ from those in NO_3^-. Thus, Si alleviated NH_4^+ toxicity in MT but not *MT CKX2* tomato plants because the shoot and root dry mass was reduced in plants treated with NH_4^+ regardless of the Si in the nutrient solution (Fig. 5a and b).

Experiment 2: CK Content in MT Shoots and Roots

MT plants grown in NO_3^-, NO_3^- + Si and NH_4^+ exhibited reduced shoot *tZ* content compared with NH_4^+ + Si plants (Fig. 6a). However, opposite results were observed in the roots, i.e., plants grown in NO_3^- and NO_3^- + Si exhibited increased root *tZ* content compared with NH_4^+ and NH_4^+ + Si plants (Fig. 6b).

The iP content in shoots and roots was reduced in plants supplemented with NO_3^- + Si, NH_4^+ and NH_4^+ + Si, compared with plants cultivated in NO_3^-. Plants cultivated in NH_4^+ exhibited reduced iP levels in roots (Fig. 6c and d).

Experiment 3: Exogenous 6-BA in MT Plants Subject to NH_4^+ Toxicity

NH_4^+ inhibited shoot and root growth compared with NO_3^-. Increased root growth was observed in plants treated with NH_4^+ combined with $1e^{-9}$ and $1e^{-8}$ mol L^{-1} of 6-BA. In addition, increasing the 6-BA concentration to $1e^{-7}$ mol L^{-1} inhibited shoot and root growth (Fig. 7).

The plants cultivated in NO_3^- exhibited increased height, leaf area and shoot dry mass compared with plants grown in NH_4^+. No significant differences in these parameters were observed when 6-BA was provided in the nutrient solution with the exception of increased leaf area and height upon treatment with $1e^{-8}$ mol L^{-1} 6-BA. However, upon treatment with $1e^{-7}$ mol L^{-1} 6-BA, plant height, leaf area and shoot dry mass decreased. Thus, the high 6-BA concentration was

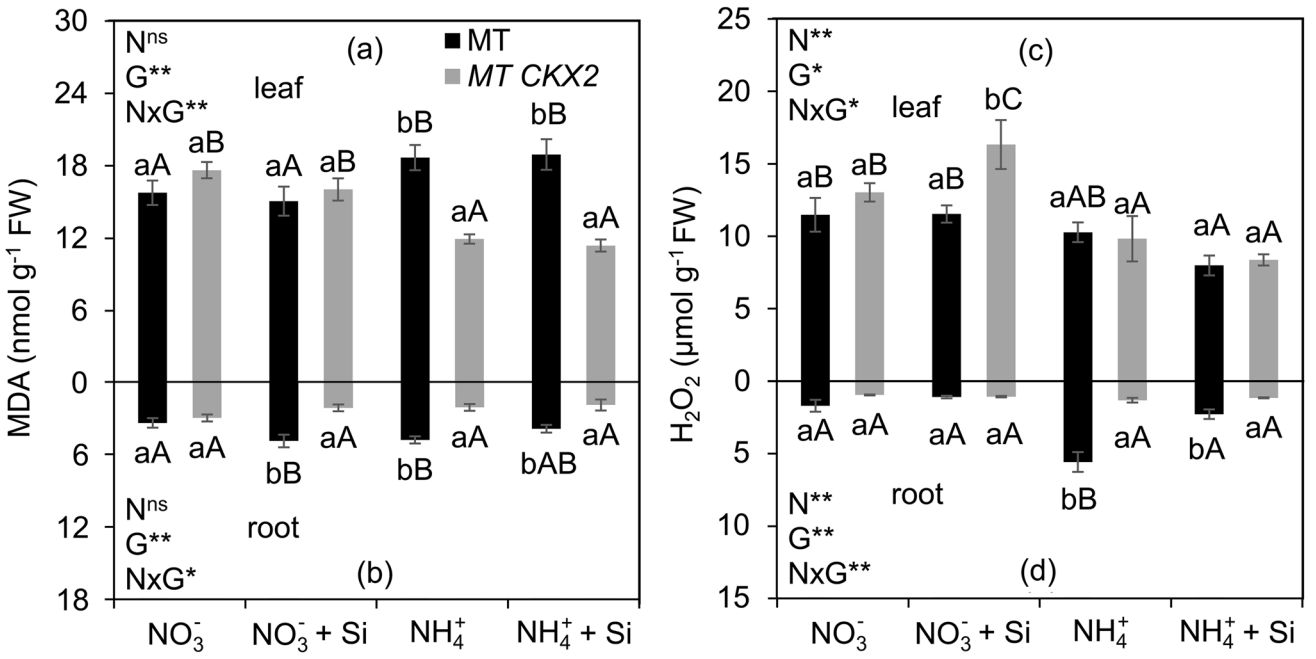

Fig. 3 Malondialdehyde (MDA) levels in leaves (**a**) and roots (**b**) as well as hydrogen peroxide (H_2O_2) levels in leaves (**c**) and roots (**d**) of tomato cv MT and *MT CKX2* grown in nutrient solution with NO_3^- and NH_4^+ in the presence and absence of Si ^{ns}, * and **: not significant, significant at 5 and 1%, respectively N: nutrient solution; G: genotype; NxG: interaction between nutrient solution and genotype. Same lowercase letters indicate no difference between the genotypes for the same treatment in nutrient solution. Same uppercase letters indicate no difference in the same tomato genotype among the different nutrient solution treatments

toxic. However, root area and root dry mass increased with BA concentrations between $1e^{-10}$ and $1e^{-8}$ mol L^{-1}, suggesting that the beneficial concentration of this growth regulator in roots is not the same as that noted for shoots or 6-BA does not benefit shoots (Fig. 8).

Discussion

To assess our hypotheses that (i) Si alleviates NH_4^+ toxicity in MT but not *MT CKX2*, (ii) the attenuation of NH_4^+ toxicity by Si is dependent on increased levels of CKs, and (iii) 6-BA alleviates NH_4^+ toxicity in tomato MT, we performed the following experiments. First, we subjected MT and *MT CKX2* plants to NH_4^+ toxicity. Second, we quantified endogenous CKs (*tZ* and iP) in MT plants under conditions of NH_4^+ toxicity. Third, we supplied an exogenous cytokinin (6-BA) to MT plants under conditions of NH_4^+ toxicity. In all experiments, NO_3^- served as a control. Our findings are discussed below.

Silicon Alleviates NH_4^+ Toxicity in MT but not in *MTCKX2* Plants

Silicon was beneficial for MT plants primarily through increasing chlorophyll and carotenoid levels and decreasing H_2O_2 levels in leaves and roots. The role of Si in alleviating NH_4^+ toxicity was demonstrated in tomato (Barreto et al. 2016) and other species, such as cucumber (Gao et al. 2014), broccoli and cauliflower (Barreto et al. 2017). It has been reported that abiotic stresses disrupt the structure of chloroplasts by dilating the thylakoid membranes; thus, Si-mediated increases in antioxidant capacity prevent damage to the chloroplast structure (Zhu and Gong 2014). Given that the role of Si is most evident under stress conditions, this finding serves as a possible explanation for the lack of an effect of Si in plants grown in NO_3^-, i.e., control conditions; however, there are reports of Si benefits in nonstressed plants (Savvas and Ntatsi 2015).

The late development stage, reduced height, and narrow leaf phenotype of *MT CKX2* plants grown with NO_3^-, i.e., control conditions, occurred because the lack of CKs induce reduced apical meristem cell division in the shoot, reduced leaf cell division rates, and early termination of cell division (Werner et al. 2003). *MT CKX2* plants exhibit an even greater reduction in growth when fed NH_4^+, and Si does not alleviate NH_4^+ toxicity in *MT CKX2* plants in contrast to its effects in MT plants. In addition, the MT and *MT CKX2* had a decrease of 18% and 48% in the shoot dry weight, respectively, when fed with NH_4^+, compared to NO_3^-. In other words, *MT CKX2* was more sensitive to NH_4^+. In this context, when comparing lower and higher stress conditions

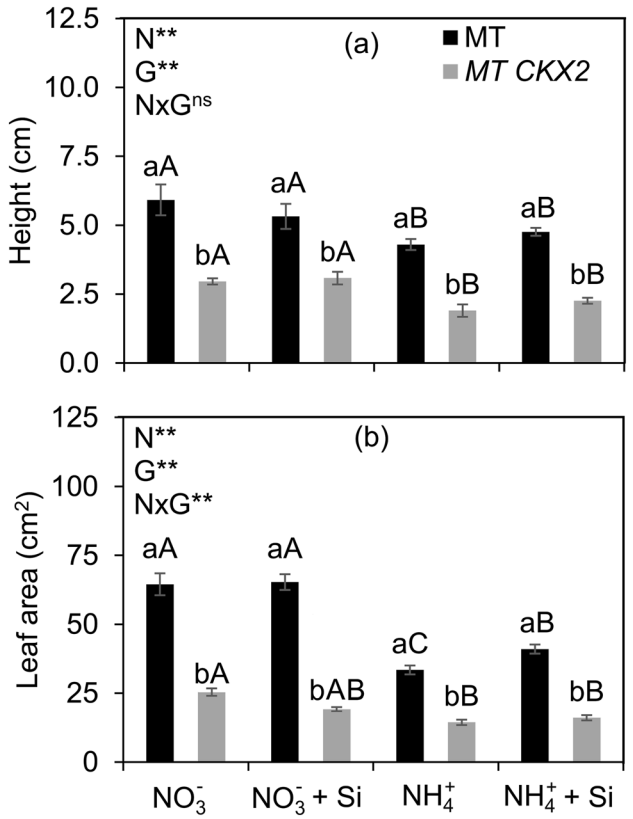

Fig. 4 Height (**a**) and leaf area (**b**) of tomato cv MT and *MT CKX2* grown in nutrient solution with NO_3^- and NH_4^+ in the presence and absence of Si ^{ns} and **: not significant and significant at 1%, respectively N: nutrient solution; G: genotype; NxG: interaction between nutrient solution and genotype. Same lowercase letters indicate no difference between the genotypes for the same treatment in nutrient solution. Same uppercase letters indicate no difference in the same tomato genotype among the different nutrient solution treatments

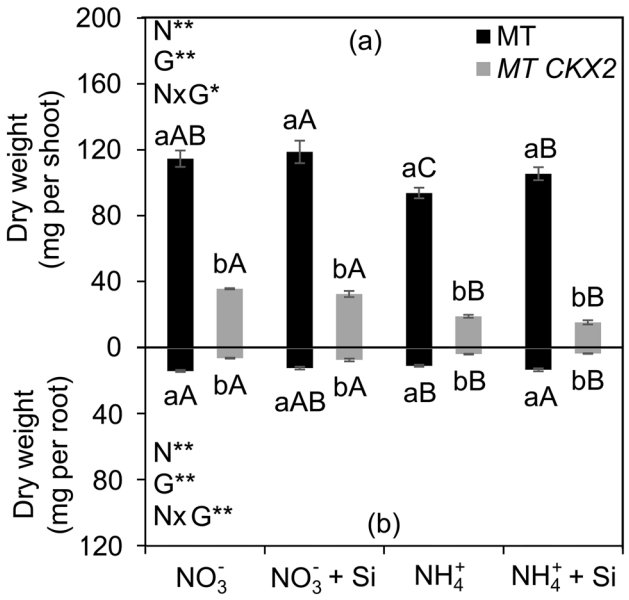

Fig. 5 Dry weight of shoots (**a**) and roots (**b**) of tomato cv MT and *MT CKX2* grown in nutrient solution with NO_3^- and NH_4^+ in the presence and absence of Si * and **: significant at 5% and 1%, respectively N: nutrient solution; G: genotype; NxG: interaction between the nutrient solution and genotype. Same lowercase letters indicate no difference between the genotypes for the same treatment in nutrient solution. Same uppercase letters indicate no difference in the same tomato genotype among the different nutrient solution treatments

associated with Si, the element has been more efficient in lower stress conditions (Kleiber et al. 2015; Barreto et al. 2016). We provide three possible reasons why Si does not alleviate NH_4^+ toxicity in *MT CKX2* plants: (i) *MT CKX2* plants do not uptake Si; (ii) *MT CKX2* plants uptake Si, but Si has no effect on CK biosynthesis; and (iii) *MT CKX2* plants uptake Si, and Si increases *tZ,* but *tZ* is degraded by the enzyme *CYTOKININ OXIDASE 2*.

MT CKX2 exhibited reduced growth compared MT and similar N levels in shoots when cultivated in NO_3^-, and the increase in N levels with NH_4^+ treatment occurs independently of Si. These results indicate that N is more concentrated in shoots in transgenic plants. In this regard, it has been proposed that CKs negatively regulate N uptake; for example, CKs act as a N satiety signal to inhibit N uptake by roots (Kiba et al. 2011). Thus, the lack of CKs may result in more concentrated N in plant tissues as noted here. In addition, it is possible that transgenic plants exhibited lower chlorophyll and carotenoid levels because CKs stimulate chloroplast formation and chlorophyll biosynthesis. Thus, CK-deficient tobacco plants exhibited reduced chlorophyll levels and disorganized, dilated chloroplasts and are cheaper to maintain compared with wild-type plants (Werner et al. 2008).

Since CKs combat reactive oxygen species, such as H_2O_2 (Zavaleta-Mancera et al. 2007), we expected *MT CKX2* plants to exhibit higher H_2O_2 contents, but this was not observed possibly because decreasing CKs and increasing H_2O_2 is exclusively observed in the context of stressors that cause chlorosis in tomato leaves (Cueno et al. 2012). Although NH_4^+ toxicity causes chlorosis in tomato leaves (Barreto et al. 2018), we did not study this level of toxicity here because Si is beneficial in the context of less severe forms of toxicity compared with the more severe forms that cause leaf chlorosis (Kleiber et al. 2015; Barreto et al. 2016).

Our reduced MDA data in *MT CKX2* fed with NH_4^+ compared to NO_3^- (Fig. 3a) indicates that *MT CKX2* is less stressed in NH_4^+. In fact, MDA content is widely used as an indicator of damage in plant membranes. This holds true if MDA levels remain high, irreparably modifying proteins and nucleic acids. However, MDA increases may represent acclimation processes rather than damage, since MDA can exert a positive role by activating regulatory genes involved

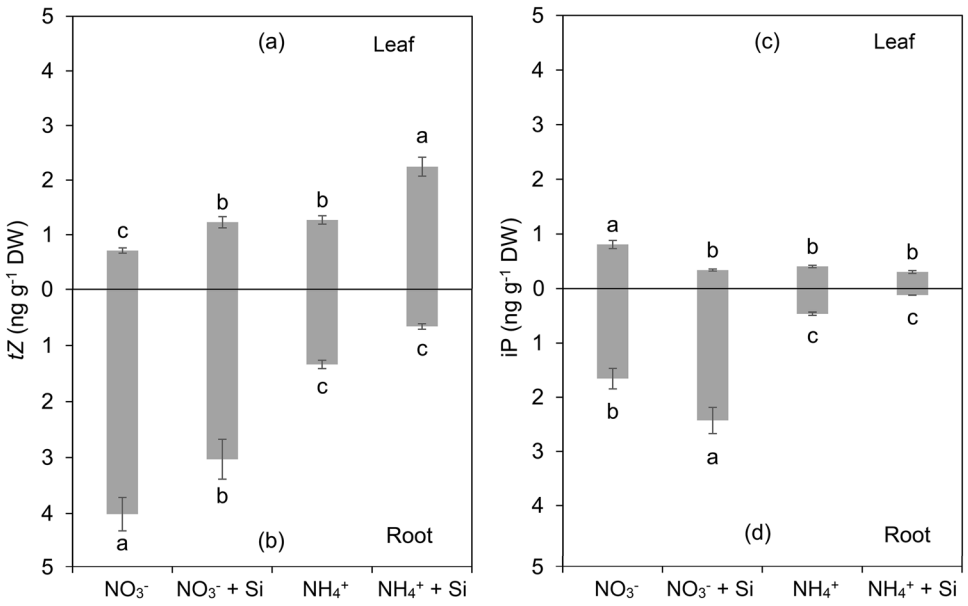

Fig. 6 *trans*-Zeatin (*tZ*) content in shoots (**a**) and roots (**b**) as well as isopentenyladenine (iP) content in shoots (**c**) and roots (**d**) of tomato cv MT grown in nutrient solution with NO_3^- and NH_4^+ in the presence and absence of Si ^{ns}, * and **: not significant, significant at 5 and 1%, respectively. Same lowercase letters indicate no difference between N forms for the same Si condition. Same uppercase letters indicate no difference in the same N form between the Si conditions

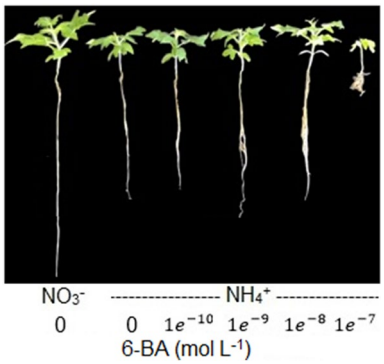

Fig. 7 Features of tomato cv MT grown in nutrient solution with NO_3^- or NH_4^+ and 6-BA concentrations associated with NH_4^+

in plant defense and development. In this respect, it has been suggested that MDA may act as a protection mechanism rather than being an indicator of damage (Morales and Munné-Bosch 2019). Therefore, we presume that *MT CKX2* was not less stressed in NH_4^+ but more protected in NO_3^-. Finally, the plant's growth measures reflect its adaptation to a particular growth environment, such as the chemical form of N (Ariz et al. 2013). Therefore, the reduced dry weight of *MT CKX2* fed with NH_4^+ (Fig. 5) indicates that this chemical form of N was stressful.

The Alleviation of NH_4^+ Toxicity by Si is Dependent on Increased *tZ* Levels in MT Shoots

To date, the known mechanisms by which Si alleviates NH_4^+ toxicity involve increases in antioxidant enzyme activity to combat reactive oxygen species, membrane physical integrity, chlorophyll and carotenoid levels, photosynthetic rates and water use efficiency (Gao et al. 2014; Barreto et al. 2017). In this study, Si increased *tZ* levels in shoots. These observations are consistent with the studies of Markovich et al. (2017), which demonstrated that Si alters the expression of biosynthesis genes including CKs, and Shtratnikova et al. (2015), which demonstrated that the supplementation of *tZ* to the culture medium resulted in the alleviation of NH_4^+ toxicity in *A. thaliana* plants, indicating that naturally occurring CK forms in plants may be more efficient in alleviating NH_4^+ toxicity. However, the decrease in *tZ* content in the roots and an increase in the shoots observed as a function of Si in MT plants suggests that Si may be involved in the transport of *tZ*. In this respect, changes in CK concentrations in plants were mainly attributed to higher or lower synthesis of CKs in roots (Rahayu et al. 2005), cytokine oxidase activity (Brugiere et al. 2003) and the control mechanism of CK loading in the xylem, which involves the transmembrane transport of CKs by root cells that is mediated by groups of purine permease transporters (PUPs) and equilibrium nucleoside transporters (ENTs) (Kang et al. 2017). To the best of our knowledge, the role of Si in these carriers is unknown. Therefore, we recommend that the role of Si in CK transporters should be studied in the future.

Exogenous 6-BA Does not Alleviate NH_4^+ Toxicity in MT

The search for natural CKs from plants led to the identification of *tZ*, iP and dihydrozeatine (Ha et al. 2012). However, the first CK discovered (kinetin) is not derived from plants but rather from degraded herring sperm DNA; this CK was identified in the search for substances that promote cell

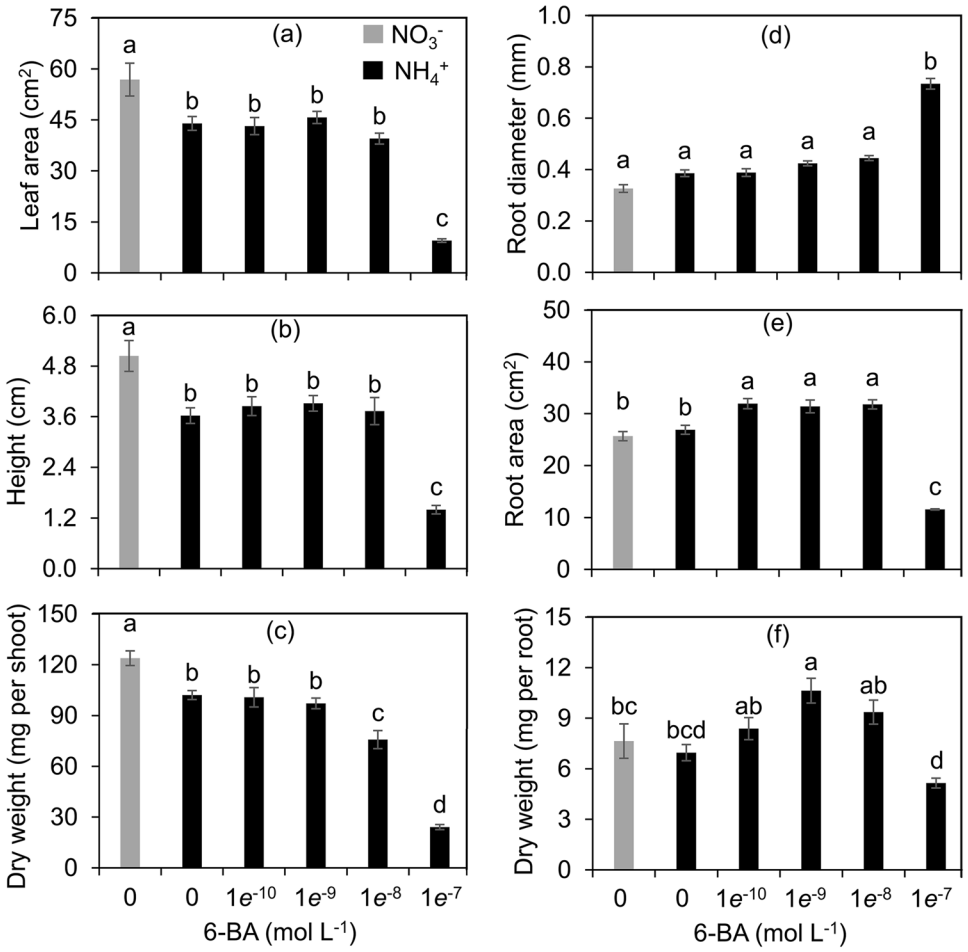

Fig. 8 Leaf area (**a**), height (**b**) shoot dry weight (**c**), root mean diameter (**d**), root area (**e**) and root dry weight (**f**) of tomato cv MT grown in nutrient solution with NO_3^- or NH_4^+ and BA concentrations associated with NH_4^+. Same letters indicate no difference

division in tissue culture (Miller et al. 1956). Based on the discovery of kinetin, several studies have aimed to develop synthetic CKs by chemical modification of the kinetin molecule (Jiang and Asami 2018). Briefly, 6-BA is a synthetic CK used in agriculture primarily to increase fruit resistance to cracking (Ginzberg and Stern 2016).

In addition, *tZ* and 6-BA have been used in hydroponic experiments and provided via nutrient solution to relieve stresses that result in decreased CK content in plants, such as NH_4^+ toxicity and salt stress. Moreover, *tZ* has the advantage of being the most abundant and active CK found in plants, and 6-BA is cheaper than *tZ* (Wu et al. 2014; Shtratnikova et al. 2015).

The study by Wu et al. (2014) of eggplant genotypes supplemented with 6-BA via nutrient solution through the roots revealed the greatest shoot growth in plants subjected to saline stress. However, the majority of studies showed that the benefits of 6-BA are restricted to the local area of application. For example, spraying 6-BA on fruits stimulates epidermal cell division and decreases cracking as shown in the review by Ginzberg and Stern (2016). Our results also provide evidence that the benefits of 6-BA may be limited to the application site because there was an increase in plant root dry mass but no effect on shoots. Thus, 6-BA supplementation via nutrient solution does not alleviate NH_4^+ toxicity in tomato MT. Future research on 6-BA application via leaf spray may elucidate new plant responses under conditions of NH_4^+ toxicity.

In conclusion, the alleviation of NH_4^+ toxicity by Si was dependent on the increase in *tZ* content in shoots but not iP. In CK-deficient transgenic plants, Si did not alleviate NH_4^+ toxicity, and 6-BA did not alleviate NH_4^+ toxicity in MT shoots. Thus, we provide strong evidence that the alleviation of NH_4^+ toxicity by Si is dependent on the increase in CK levels, specifically *tZ*, in MT shoots.

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Author Contributions RFB: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Roles/Writing—original draft; Editing. RMP: Conceptualization; Funding acquisition; Project administration; Supervision; Methodology; Writing—review. JCBL: Formal analysis; Investigation; Methodology. ILD:

Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Supervision. EC: Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Supervision. RFC: Conceptualization; Funding acquisition; Project administration; Methodology; Supervision; Writing—review.

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Data Availability All data were presented in the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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