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ORIGINAL PAPER

Effects of nickel nutrition in the mineral form and complexed with histidine in the nitrogen metabolism of onion bulb

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Abstract Excess accumulation of nitrate in edible plants particularly onion is a great concern for human health. In this experiment, we investigated the possibility of reducing nitrate accumulation in onion bulb by application of Ni. Two onion cultivars (*Allium cepa* L. cvs. Dorrcheh and Cebolla Valenciana) were supplied with three levels of Ni (0, 25, and 50 μM) in the form of synthetic Ni–histidine complex [$Ni(His)_2$] and $NiCl_2$. Addition of Ni significantly increased fresh bulb mass of onion cultivars. In 'Valenciana', increasing Ni concentration from 25 to 50 μM reduced fresh bulb mass while in 'Dorrcheh', no significant difference was found between 25 and 50 μM Ni treatments. Supplement with Ni resulted in lower accumulation of nitrate in onion bulb. This reduction was associated with higher activity of nitrate reductase (NR) and glutamine synthetase (GS). Onion plants supplied with Ni had higher concentration of ammonium and total N in their bulb compared with those unsupplied with Ni. The [$Ni(His)_2$] complex was more effective than $NiCl_2$ in improving yield, stimulating activity of NR and GS, and reducing nitrate concentration in onion. According to the results obtained, application of 25 μM Ni particularly in the form of [$Ni(His)_2$] can be effective in reducing nitrate accumulation and enhancing health quality of onion.

Keywords *Allium cepa* L. · Nickel · Histidine · Nitrate synthetase · Glutamine synthetase

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Introduction

Nitrate is the most important source of nitrogen for plant species which have no N_2-fixing symbioses, but its excess accumulation in edible parts of food plants is a serious concern for human health (Zhu 2002). Excess nitrate concentration in the human diet causes several disorders such as cancers and blue baby syndrome (Dich et al. 1996; Zhu 2002). Vegetables are the main source of nitrate in the human body and consist about 80 % of daily intake of nitrate (Shen et al. 1982). Therefore, many governments and international organizations have set maximum nitrate levels for vegetables (Gunes et al. 1996; Santamaria and Elia 1997).

The onion (*Allium cepa*) that is known as the bulb onion or common onion is used as a vegetable and is the most widely cultivated species of the genus *Allium*. It is usually served cooked, as a vegetable or part of a prepared spicy dish, but can also be eaten raw or used to make pickles or chutneys. The total area under cultivation of onion in Iran is about 62,000 ha with average production of 2.15 million ton per year (Khodadadi and Hassanpanah 2010). There are several reports indicating excess accumulation of nitrate in onion produced in Iran (Rostamforoudi et al. 1999). Therefore, applying appropriate strategies to reduce accumulation of nitrate in onion is necessary. Improving nitrate metabolism and stimulating activity of enzymes involved in nitrate reduction particularly nitrate reductase (NR) can significantly reduce nitrate accumulation in edible plants.

Nickel (Ni) is a micronutrient element for plants and plays role in nitrogen metabolism (Brown et al. 1987). Although the necessity for activity of urease enzyme is well distinguished (Dixon et al. 1975), there is limited information on the effect of Ni on activity of NR. There are some reports indicating higher activity of NR in plants

supplied with Ni. For example, Tabatabai (2009) found that addition of Ni enhanced activity of NR and reduced nitrate accumulation in cucumber. Gad et al. (2007) also found that addition of 30 mg kg^{-1} Ni reduced concentration of nitrate and ammonium in tomato. Brown et al. (1990) reported higher concentrations of nitrate in shoots of barley plants at the Ni-deficient conditions than the Ni-sufficient conditions.

Activity of NR is a limiting step in uptake and assimilation of nitrate in plant (Matraszek 2008). After uptake by plant roots, nitrate is converted to ammonium by NR and nitrite reductase enzymes. Ammonium is converted to glutamine by glutamine synthetase (GS) that is the most important enzyme involved in assimilation of ammonium. Activity of GS is strongly dependent on the ammonium concentration as enzyme substrate (Bashan et al. 2008).

We hypothesized that Ni nutrition can reduce accumulation of nitrate in onion via improving assimilation of nitrate. To test this hypothesis, we investigated the effect of Ni on activity of NR and GS and concentration of nitrate in hydroponically-grown onion. The efficacy of Ni in the form of mineral salt and $[Ni(His)_2]$ was compared. The metal-amino acids chelates have recently been synthesized and characterized as novel fertilizer sources with high efficiency in supplying metal micronutrients for plants (Ghasemi et al. 2011, 2013a). Histidine has the ability to bind ion metals via amine and carboxyl groups and thereby enhances uptake and translocation of metals in plants (Ghasemi et al. 2013b). The effective role of histidine on uptake and translocation of Ni in plants has been reported (Richau et al. 2009; Singer et al. 2007).

Materials and methods

Plant culture

Seeds of two onion cultivars (*A. cepa* L., cvs. Dorrcheh and Cebolla Valenciana), most commonly grown in Iran, were surface-sterilized in a 1 % aqueous solution of Na-hypochlorite for 10 min, rinsed with distilled water and germinated on moist filter paper in an incubator at 28 °C. Four days later, uniformly-sized seedlings were transferred to 2 kg polyethylene pots, which contained sterilized quartz sand. One plant was planted in each pot and continuously irrigated with Johnson nutrient solution. The nutrient solution (electrical resistivity = 18 MΩ cm^{-1}) contained: 1.5 mM NH_4NO_3, 1.0 mM $CaCl_2$, 1.0 mM KH_2PO_4, 1.0 mM $MgSO_4$, 100 μM Fe–EDTA, 50 μM KCl, 25 μM H_3BO_3, 2.0 μM $MnSO_4$, 2.0 μM $ZnSO_4$, 0.5 μM $CuSO_4$, and 0.5 μM $H_2Mo_7O_4$ adjusted to pH 6 with NaOH or HCl as a buffer.

Experiment was performed in a greenhouse under controlled conditions with an 8 h light period at intensity of 390 μmol m^{-2} s^{-1}, 25/20 °C day/night temperature, and 65–75 % relative humidity. Soluble nickel was supplied to the plants by amending the nutrient solution with 0, 25, and 50 μM Ni in the form of $NiCl_2$ or $[Ni(His)_2]$ complex. Each treatment was run with six replicates under the same conditions.

Plants were harvested approximately 16 weeks after seeding, when 80 % of the plant tops had collapsed. Upon harvest, the roots and leaves were removed from the bulbs. Bulb fresh matter yields were determined for each pot.

Ni concentration in onion bulb

The plant materials were dried for 48 h in a forced-air oven at 70 °C and ground to a fine powder in a Wiley mill to pass through a 20-mesh sieve. About 0.500 g of samples digested in APCU-40 75 mL TFM Teflon vessel of microwave (Milestone Srl, START D, Sorisole, Italy) using 5 mL HNO_3 and 3 mL H_2O_2, and then filtered through Whatman no. 42 filters, transferred to 50-mL volumetric flasks, and diluted with deionized, distilled water. Concentrations of Ni in the bulbs were determined in solutions of acid digested samples by atomic absorption spectrophotometer (Perkin-Elmer 3030, PerkinElmer, Wellesley, MA). All plant tissue concentrations were expressed on a dry weight basis.

Ammonium, nitrate, and total nitrogen concentration

Concentration of NH_4-N and NO_3-N in the plant extracts was measured by steam distillation (Keeney and Nelson 1982(. The total N concentration of onion bulb was measured using Autotech (Model 300) according to Kjeldahl method (Bremner and Mulvaney 1982).

Total amino acid (AA) concentration

Total amino acid (AA) concentration was measured using the method of Rosen (1957): The plant samples were extracted with 2 M KCl (1:10 plant to solution ratio) for 1 h by shaking at 200 rpm and the extract was filtered through Whatman no. 42 filter paper. Aliquots of 1 mL of extracts were mixed with 0.5 mL cyanide–acetate buffer and 0.5 mL 3 % ninhydrin solution in Methyl Cellosolve and heated for 15 min in a 100 °C water bath. Concentrations of AA were determined by comparing the optical absorbance (570 nm) of the samples relative to a standard curve prepared with leucine.

Nitrate reductase (NR)

Frozen plant material was homogenized in chilled mortar and pestle with 100 mM potassium phosphate buffer (pH 7.5) containing 5 mM cysteine, 2 mM EDTA and 0.5 % (w/v) poly venil pyrolidon. The homogenate was

centrifuged at 20,000*g* for 20 min at 4 °C. NR activity was determined according to the method as described by Debouba et al. (2006). The extract (0.1 mL) was incubated in a reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.5), 0.14 mM NADH, and 7 M KNO_3 at 27 °C for 30 min. NR was incubated with excess of 5 mM EDTA (for maximum NR determination). The reaction was stopped by 0.5 mM zinc acetate. Nitrite ions were assayed after diazotization with 1 % (w/v) sulfanilamide, 0.01 % (w/v) *N*-naphthylethylenediamine-dichloride. After 20 min of incubation at room temperature, the absorbance was measured at 540 nm and amount of nitrite was calculated using standard calibration curve prepared for $NaNO_2$.

Glutamine synthetase (GS)

Fresh bulb tissue was homogenized (1:5, w/v) in the ice cold mortar using 50 mM TRIS–HCl buffer (pH 7.6) containing 1 mM EDTA, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dithiothreitol, and 0.5 % (w/v) polyvinylpyrrolidone (PVP). After centrifugation (20,000*g*) for 20 min, the supernatant was used for the enzymatic assay.

Activity GS (EC 6.3.1.2) in the onion bulbs was determined according to Agbaria et al. (1998). The GS assay mixture, with a total volume of 2 mL, contained 50 mM TRIS–HCl buffer (pH 7.2), 1 mM ADP, 50 mM glutamine, 20 mM $MgCl_2$, 20 mM sodium arsenate, enzyme extract and 13 mM hydroxylamine. The reaction was initiated by the addition of hydroxylamine. After 30 min incubation at 30 °C, the reaction was terminated by addition of 3 mL the mixture consisted of 0.5 M HCl, 0.2 M $FeCl_3$ and 0.24 M trichloroacetic acid. After centrifugation (3,000*g*, 10 min), the absorbance was measured at 540 nm. The enzyme activity was expressed in units, each representing the amount of enzyme catalyzing the formation of 1 nmole glutamylhydroxamate min^{-1}.

Statistical analysis

The experiments were set up in a completely randomized design; each treatment contained six replicates. A total of 72 pots were used in this experiment. One plant was planted in each pot. Treatments effects were analyzed by analysis of variance using general linear models (GLMs). Means were compared using least significant differences (LSDs) at $P < 0.05$ (SAS Institute 2000).

Results

Fresh bulb mass

The use of Ni promoted the increase of bulb fresh weight, but the increase of Ni concentration from 25 to 50 µM reduced fresh bulb mass (Fig. 1). It seems that 25 µM is the optimum concentration to promote a substantial increase of fresh bulb mass and at the higher levels of Ni, an inhibition of mass accumulation is observed. The effectiveness of $[Ni(His)_2]$ on fresh bulb mass of onion was dependent on the plant cultivar. In 'Dorrcheh', $[Ni(His)_2]$ was more effective than $NiCl_2$ while in 'Valenciana' the effect of both Ni sources on the fresh mass of onion bulb was similar.

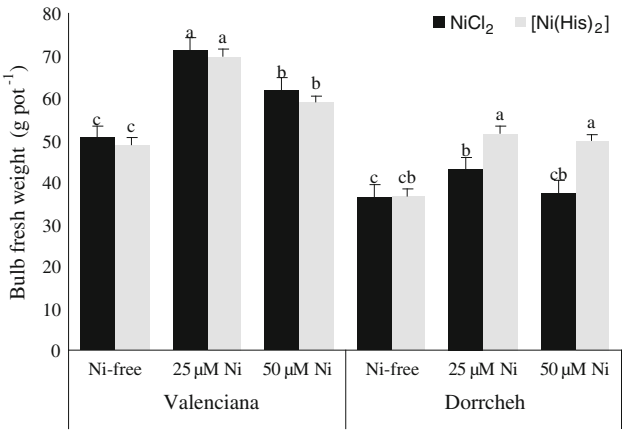

Fig. 1 The effect of Ni in the form of $NiCl_2$ and $[Ni(His)_2]$ complex on bulb fresh weight of two onion cultivars. *Bars* with the *same letter* are not significantly different at $P \leq 0.05$ by LSD multiple range test

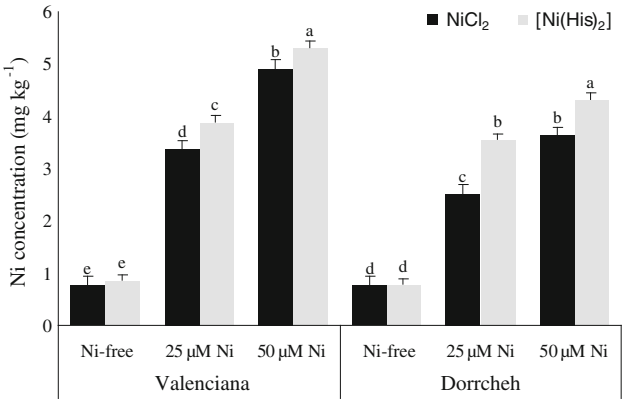

Fig. 2 The effect of Ni in the form of $NiCl_2$ and $[Ni(His)_2]$ complex on Ni concentration in bulbs of two onion cultivars. *Bars* with the *same letter* are not significantly different at $P \leq 0.05$ by LSD multiple range test

Nickel concentration

In both onion cultivars, addition of Ni, proportional to the added dose, significantly increased concentration of Ni in onion bulb (Fig. 2). In spite of onion cultivar, greater Ni was accumulated in plants supplied with $[Ni(His)_2]$ in comparison with those supplied with $NiCl_2$.

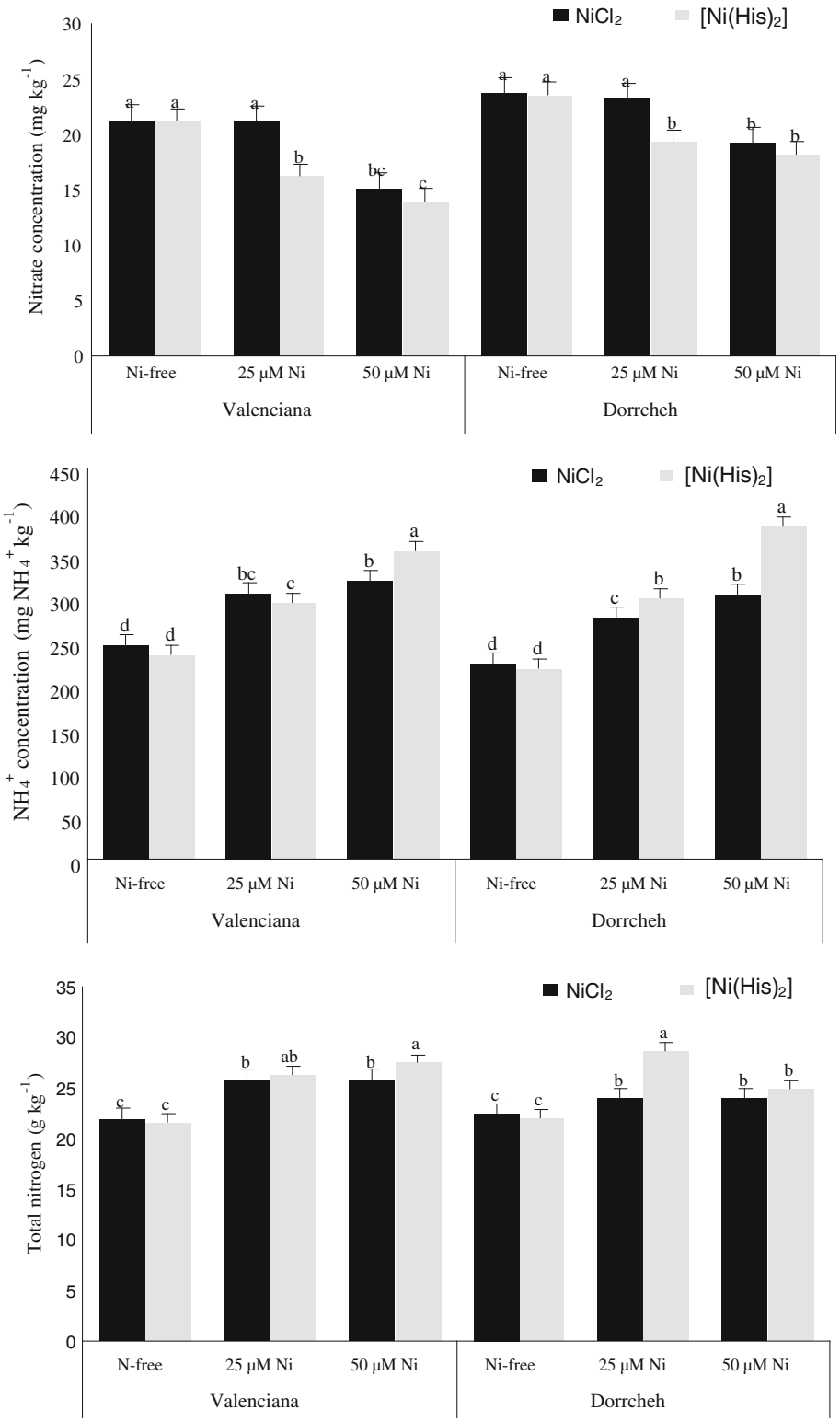

Fig. 3 The effect of Ni in the form of $NiCl_2$ and $[Ni(His)_2]$ complex on nitrate (*above*), ammonium (*middle*) and total N (*below*) concentration in bulbs of two onion cultivars. *Bars* with the *same letter* are not significantly different at $P \leq 0.05$ by LSD multiple range test

Nitrate concentration

The effect of Ni on concentration of nitrate in onion bulbs varied dependent on the cultivar and Ni source (Fig. 3). The $[Ni(His)_2]$ in both levels promoted reduction of nitrate concentration in both cultivars while $NiCl_2$ only at the 50 µM was effective in promoting reduction of nitrate concentration. Addition of 25 µM $NiCl_2$ had no effect on

nitrate concentration. In 'Valenciana' only 50 μM $NiCl_2$ was effective in reducing concentration of nitrate.

Addition of Ni reduced accumulation of nitrate by 23–31 % in 'Valenciana' and by 10–19 % in 'Dorrcheh'. In spite of onion cultivar, at the 25 μM Ni treatment, lower nitrate was accumulated in plants supplied with $[Ni(His)_2]$ in comparison with those supplied with $NiCl_2$. At the 50 μM Ni level, both Ni sources had similar effects on bulb nitrate concentration.

Ammonium concentration

Onion plants supplemented with Ni accumulated higher amounts of ammonium in their bulbs compared with those grown at the Ni-free treatment (Fig. 3). In 'Dorrcheh', higher ammonium was accumulated in plants supplied with $[Ni(His)_2]$ in comparison with those supplied with $NiCl_2$. In 'Valenciana', at the 50 μM Ni level, $[Ni(His)_2]$ was more effective than $NiCl_2$ while at the 25 μM level, both Ni sources had similar effects on bulb ammonium concentration.

Total N concentration

Application of Ni significantly increased total N concentration of onion bulb (Fig. 3); although no significant difference was found between 25 and 50 μM Ni treatments. The effect of Ni source on the total N concentration was dependent on the onion cultivar and applied metal dose. In 'Dorrcheh', at the 25 μM Ni level, $[Ni(His)_2]$ was more effective than $NiCl_2$ in enhancing the total N content of onion bulb. In 'Valenciana', at the 50 μM Ni level, $[Ni(His)_2]$ was more effective than $NiCl_2$ while at the 25 μM treatment, both Ni sources had similar effects on the total N concentration.

Activity of nitrate reductase (NR)

Application of Ni resulted in significant increase of NR activity (Fig. 4). In 'Valenciana', the increase in activity of NR was proportional to the applied level of Ni and greater NR activity was found for plants supplied with 50 μM than those supplied with 25 μM Ni. In 'Dorrcheh', increasing concentration of Ni from 25 to 50 μM in the form of $[Ni(His)_2]$ enhanced activity of NR while a reduction in activity of NR was found when the concentration of $NiCl_2$ increased from 25 to 50 μM.

The effect of Ni source on the total N concentration was dependent on the onion cultivar. In 'Dorrcheh', at both Ni levels, $[Ni(His)_2]$ was more effective than $NiCl_2$ in enhancing activity of NR while in 'Valenciana', at the 50 μM Ni level, $NiCl_2$ was more effective than $[Ni(His)_2]$.

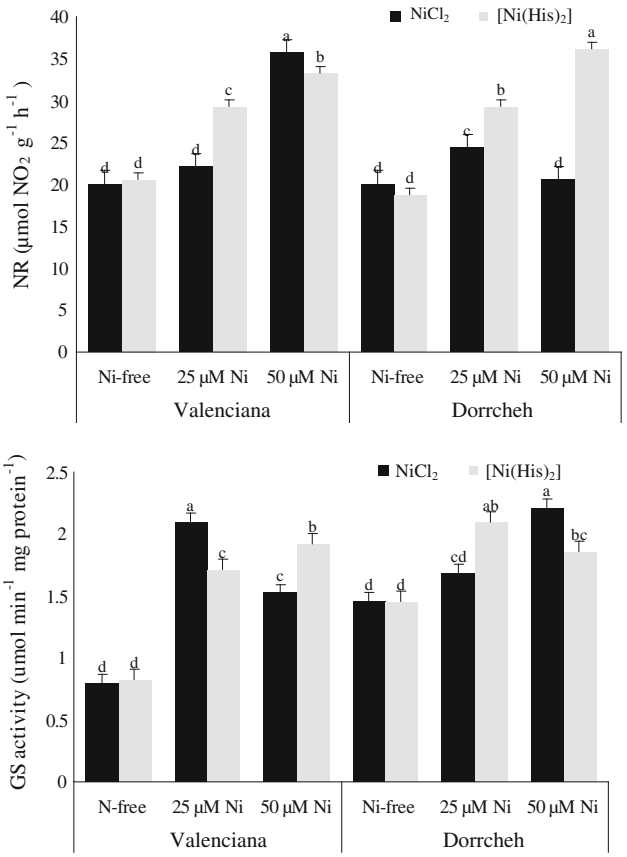

Fig. 4 The effect of Ni in the form of $NiCl_2$ and $[Ni(His)_2]$ complex on activity of nitrate reductase (*above*) and glutamine synthetase (*below*) in two onion cultivars. *Bars* with the *same letter* are not significantly different at $P \leq 0.05$ by LSD multiple range test

Activity of glutamine synthetase (GS)

Addition of Ni enhanced GS activity and the magnitude of this increase was dependent on the plant cultivar and Ni source (Fig. 4). In 'Valenciana', GS activity increased proportionally to the increase of $[Ni(His)_2]$ concentration (from 25 to 50 μM). With supplementation of 25 μM, the GS activity promoted by $NiCl_2$ was greater than the effect promoted by $[Ni(His)_2]$.

In 'Dorrcheh', activity of GS in plants supplied with 50 μM Ni in the form of $[Ni(His)_2]$ was greater than those supplied with the same concentration of Ni in the form of $NiCl_2$.

Total amino acid concentration

Application of Ni significantly increased total AA concentration of onion bulb (Fig. 5). The effect of Ni source on the total AA concentration was dependent on the onion cultivar and applied metal dose. In 'Dorrcheh', at both 25 and 50 μM Ni levels, $[Ni(His)_2]$ was more effective than

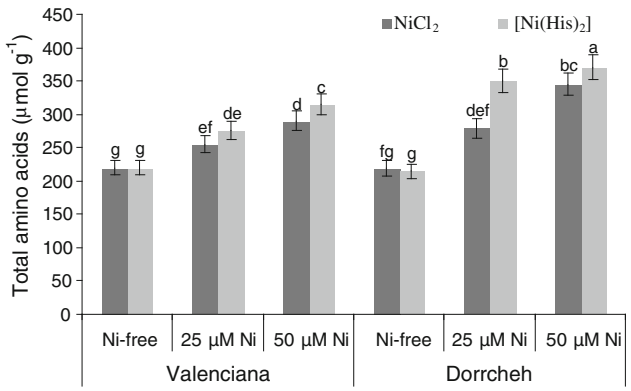

Fig. 5 The effect of Ni in the form of $NiCl_2$ and $[Ni(His)_2]$ complex on concentration of total amino acids in two onion cultivars. *Bars* with the *same letter* are not significantly different at $P \leq 0.05$ by LSD multiple range test

$NiCl_2$ in enhancing the total AA content of onion bulb. In 'Valenciana', at the 50 µM Ni level, $[Ni(His)_2]$ was more effective than $NiCl_2$ while at the 25 µM treatment, both Ni sources had similar effects on the total AA concentration.

Discussion

Excess accumulation of nitrate in edible plants particularly vegetables is a serious concern for human health (Atta-Aly 1999; Gad et al. 2007). In this study we investigated the role of Ni supply on nitrate accumulation in hydroponically-grown onion plants.

The study showed that supplement with 25 µM Ni in the form of $[Ni(His)_2]$ was effective in reducing concentrations of nitrate in onion (Fig. 3). Addition of 50 µM Ni regardless the form used ($NiCl_2$ or $[Ni(His)_2]$) was effective in reducing concentrations of nitrate in onion. This reduction in nitrate accumulation of Ni-supplied plants was accompanied with higher activity of NR and GS (Watanabe and Shimada 1990). The stimulating effect of Ni nutrition on activity of enzymes involving in nitrogen metabolism has been reported in several studies (Matraszek 2008; Atta-Aly 1999; Brown et al. 1990). A possible reason for increasing activity of GS by Ni is elevated concentration of ammonium that is the main substrate for activity of this enzyme (Arkoun et al. 2013). In the present study, addition of Ni at both applied levels (25 and 50 µM) enhanced ammonium concentration in the studied onion cultivars. Similar with our result, Gajewska et al. (2009) reported that addition of 50 µM Ni resulted in significant increase of ammonium concentration in wheat roots. The positive effect of Ni on activity of GS in leaves and roots of different plant species has been reported by Gerendás and Sattelmacher (1998). Tan et al. (2000) also found that addition of 0.1 mg L^{-1} Ni increased concentration of total N, NH_4-N, and chlorophyll content in urea-fed plants.

Higher concentration of ammonium in Ni-fed plants compared with those unfed with Ni might also be due to the positive effect of Ni on urease activity, an enzyme that is involved in hydrolysis of endogenous urea. Endogenous urea is originated from ureides and arginine products (Polacco and Holland 1993) Ammonium is converted to glutamine by GS and then to glutamate by glutamate synthetase (Bhushan Jha and Dubey 2004; Arkoun et al. 2013). Glutamate, as the end-product of ammonium assimilation plays role in biosynthesis of nitrogen-containing compounds i.e., chlorophylls, nucleotides, polyamines, alkaloids, and other AAs (Bhushan Jha and Dubey 2004; Gajewska et al. 2009). It has been reported that higher activity of GS results in stimulation of AA synthesis (Gerendás and Sattelmacher 1997, 1998). Accordingly, in the present study, enhanced activity of GS in the Ni-supplied onion plants was accompanied with higher bulb concentration of AAs (Fig. 4).

Onion plants accumulated higher Ni in their bulbs at the $[Ni(His)_2]$ treatment than at the $NiCl_2$ treatment. Histidine can form stable complex with Ni and enhance its uptake and root to shoot translocation in plant (Richau et al. 2009; Singer et al. 2007). For example, Kerkeb and Krämer (2003) showed that histidine in the nutrient solution formed complexes with Ni and thereby enhanced apoplastic pathway and release of Ni into the xylem. Persans et al. (1999) found that histidine formed complexes with Ni and increased loading to the xylem sap and translocation to aerial parts of Ni in hyperaccumulator Thlaspi. Krämer et al. (1996) found a positive correlation between concentration of Ni and histidine in xylem sap of different hyperaccumulator plants.

In general, the $[Ni(His)_2]$ was more effective in reduce accumulation of nitrate comparatively with $NiCl_2$. For example, at the 25 µM level, $[Ni(His)_2]$ was more effective than 25 µM $NiCl_2$ to Valenciana in accumulation of nitrate, but $NiCl_2$ was more effective than $[Ni(His)_2]$ at 25 and 50 µM levels to Dorrcheh. Direct inhibiting effect of certain AAs i.e., histidine on nitrate uptake by plant roots from the surrounding nutrient solution is another possible reason for lower accumulation of nitrate in plant tissues. AAs are end-products of nitrate assimilation in plant cells and their concentrations regulate nitrate assimilation. Sufficient concentrations of AAs inhibit induction of nitrate uptake mechanism. For example, Wang et al. (2007) showed that glutamine and arginine significantly reduced concentration of nitrate in cabbage. Gunes et al. (1996) found that replacing a part of nitrate in the nutrient solution by glycine or a mixture of certain AAs resulted in reduction of nitrate concentration in onion bulb. AAs can also inhibit transcription of HvNRT2 gene in the roots. Therefore,

synthesis of nitrate-transporter coding mRNA that is directly involved in nitrate uptake is inhibited. Aslam et al. (2001) reported that certain AAs are very effective in reducing nitrate accumulation in barley roots by inhibiting activity of nitrate uptake system.

Conclusion

The results showed that application of 25 μM Ni not only increased onion yield, but it was effective in reducing nitrate accumulation in edible bulbs. This was associated with stimulating activity of NR and GS in the presence of Ni. In general, the plants supplied with $[Ni(His)_2]$ had higher yield and accumulated lower nitrate in their bulbs compared with those supplied with $NiCl_2$. According to the results obtained, supplement with 25 μM Ni particularly in the form of $[Ni(His)_2]$ can be considered as a useful approach to improve yield and health quality of onion.

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