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# Effect of selenium on $CO_2$ and $NO_3^-$ assimilation under low and adequate nitrogen supply in wheat (*Triticum aestivum* L.)

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## Abstract

In order to study the mechanisms of Se-mediated growth improvement as related to carbon (C) and nitrogen (N) metabolism, wheat plants were cultivated hydroponically with adequate (4 mM, $N_a$) or low (1 mM, $N_d$) N supply and treated with 10 and 50 μM $Na_2SeO_4$ for six weeks. The Se supplementation enhanced plant biomass; it was significant for shoots of $N_a$ plants at 50 μM Se. Chlorophyll fluorescence parameters were significantly lowered under $N_d$ conditions but restored completely by Se addition reaching values of those in $N_a$ plants. Net $CO_2$ assimilation rate ($P_N$) decreased only slightly by limited N availability, but it enhanced significantly in both $N_d$ and $N_a$ plants equally by 10 and 50 μM Se. Effect of Se on $P_N$ in the $N_a$ plants occurred mainly due to the stomata opening, while it was related to both stomatal and nonstomatal mechanisms in the $N_d$ plants. The Se treatment resulted in enhancement of nitrate reductase (NR) activity in both $N_a$ and $N_d$ plants with an optimal response at 10 μM Se. Negative correlations between nitrate concentration and NR activity indicated a partial nitrate depletion in the roots following by elevated NR activity in $N_d$ plants. In contrast, nitrite concentrations were higher in the Se treated plants. Higher amino acids and protein concentrations in the Se-treated plants might be an indication of a general upregulation of N metabolism. However, in $N_a$ plants, the stimulation of N metabolism was not observed at 50 μM Se which could not be attributed to lesser availability of C skeletons because of maintaning higher $CO_2$ fixation under these conditions. It implies the function of some regulatory mechanisms that are responsible for coordination of C and N metabolism in whole plant.

*Additional key words*: chlorophyll fluorescence; net assimilation rate; nitrate reductase; nutrients.

## Introduction

Se is not an essential element for higher plants, but its beneficial effects on plant stress tolerance have been frequently reported (Terry *et al.* 2000, Germ *et al.* 2007). Effects of Se have been mainly attributed to the activation of antioxidative defense, thus enhancement of plant ability for counteracting latent stress factors (Pilon-Smits *et al.* 2009). The Se beneficial effects were found under various stress conditions, such as UV radiation (Pennanen *et al.* 2002, Heijari *et al.* 2006), drought (Yao *et al.* 2009, Hasanuzzaman and Fujita 2011, Habibi 2013), and salinity (Hawrylak-Nowak 2009).

However, Se acts also independently and improves plant performance under optimal growth conditions as observed in various *Brassica* species (Hajiboland and Amjad 2007). Se is also effective in stimulation of flowering in canola (Hajiboland and Keivanfar 2012) and delaying senescence in soybean plants (Djanaguiraman *et al.* 2004).

The stimulation of plant growth and performance under unstressful conditions may be primarily attributed to an improved C and N metabolism (Nunes-Nesi *et al.* 2010). Enhancement of photosynthesis provides extra energy and C skeletons for other anabolic pathways requiring ATP and reducing equivalents including nutrient assimilation.

In addition, an elevated protein synthesis is required for achievement and maintenance of the higher growth rate

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*Abbreviations:* Car – carotenoids; Chl *a*(*b*) – chlorophyll *a*(*b*); DM – dry mass; ETR – electron transport rate; $F_v'/F_m'$ – excitation capture efficiency of open PSII; $F_0$ – initial fluorescence of dark-adapted leaf; FM – fresh mass; *E* – transpiration rate; $F_m$ – maximum fluorescence of dark-adapted leaf; $F_m'$ – maximum fluorescence of light-adapted leaf; $F_s$ – steady-state fluorescence of light-adapted leaf; $F_v$ – variable fluorescence of dark-adapted leaf; $F_v/F_m$ – maximum quantum yield of PSII; $g_s$ – stomatal conductance; $N_a$ – N-adequate; $N_d$ – N-deficient; NiR – nitrite reductase; NPQ – nonphotochemical quenching; NR – nitrate reductase; $P_N$ – net assimilation rate; $q_P$ – photochemical quenching; SD – standard deviation; $\Phi_{PSII}$ – effective quantum yield of PSII.
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that, in turn, is dependent on the assimilation rate of $NO_3^-$ in plants. Higher $P_N$ of Se-supplemented plants (Owusu-Sekyere *et al.* 2013) and elevated shoot protein contents (Hajiboland and Keivanfar 2012) were reported in plants grown under optimal growth conditions.

Nitrate is taken up by roots and assimilated by NR in roots or shoots, with their relative contribution depending on the species and conditions (Hawkesford *et al.* 2012). Since assimilation of $NO_3^-$ is an energy-requiring process, photosynthesis plays, directly or indirectly, a key role in $NO_3^-$ reduction and assimilation. The production of reduced C in photosynthesis and its reoxidation in respiration and photorespiration are necessary to produce both the energy and C skeletons required for the incorporation of $NO_3^-$ into amino acids (Lawlor 2002, Stitt *et al.* 2002, Foyer *et al.* 2003, Nunes-Nesi *et al.* 2010). Conversely, N assimilation is required to sustain the output of organic C and N. NR together with phospho*enol*pyruvate carboxylase, are important control points for regulation and coordination of primary N and C assimilation (Nunes-Nesi *et al.* 2010). Activity of NR is regulated at both transcriptional and post-translational levels and light, $NO_3^-$, glutamine, and sugars are important factors in this regulation (Stitt *et al.* 2002, Foyer *et al.* 2003). There are reports on the enhancement of NR activity by some nutrients, such as K fertilizers (Ruiz and Romero 2002). It has been recently reported that Se at low concentrations [8–12 mg $kg^{-1}$(soil)] increased NR activity in tobacco plants (Xu *et al.* 2013).

In a preliminary experiment with wheat plants grown under field conditions, we observed substantially higher dry mass (DM) and grain yield after application of 5–15 g(Se) $ha^{-1}$ (unpublished results). We hypothesized that, in addition to the increase of $P_N$, N metabolism might be also upregulated by Se application in wheat plants.

The aim of this work was to study the effect of Se supplementation on C and N metabolism in plants with different N nutritional status. Our working hypothesis was that Se supplementation would impact positively the $P_N$ and $NO_3^-$ reduction in both $N_d$ and $N_a$ plants.

## Materials and methods

**Plant culture and treatments**: Seeds of wheat (*Triticum aestivum* L. cv. Homa) provided by Agricultural Research Organization (Tabriz, Iran) were surface-sterilized and germinated in dark. Five-day-old, young seedlings were transferred to 50% wheat nutrient solution, pH 6.0 (Hajiboland *et al.* 2003), with the following composition [mM]: $Ca(NO_3)_2$ 2.5, $Mg(SO_4)$ 1.0, $K_2SO_4$ 0.9, $KH_2PO_4$ 0.25, KCl 0.1 and (μM): $H_3BO_3$ 2.0, $MnSO_4$ 0.4, $ZnSO_4$ 1.0, $CuSO_4$ 0.4, $(NH_4)_6Mo_7O_{24}$ 0.04, Fe–EDTA 0.1. Twelve seedlings were cultured in each 2 L pot. Plants were precultured for 5 d with $N_a$ adequate [4 mM, as 2 mM $Ca(NO_3)_2$] or low $N_d$ [1 mM, as 0.5 mM $Ca(NO_3)_2$] N supply. In order to equilibrate $Ca^{2+}$ concentration between both N treatments, 1.5 mM $CaCl_2$ was added to the $N_d$ nutrient solution. Thereafter, Se (as $Na_2SeO_4$) was applied at 0, 10, and 50 μM. In order to equilibrate $Ca^{2+}$ concentration between both N treatments, 1.5 mM $CaCl_2$ was added to the $N_d$ nutrient solution. Nutrient solutions were replaced every 4 d and plants were grown under controlled environmental conditions with a temperature regime of 25/18°C day/night, 14/10 h light/dark period, a relative humidity of 70/80%, and at a PPFD of about 400 μmol $m^{-2}$ $s^{-1}$. The plants were harvested six weeks after starting the Se treatments. After a separation of shoots and roots, fresh mass (FM) and dry mass (DM) of plants (after drying at 70°C for 2 d) were determined. Before harvest, leaf fluorescence and gas-exchange parameters were determined in the attached leaves.

**Chlorophyll (Chl) fluorescence and gas-exchange parameters**: Chl fluorescence parameters were recorded using a fluorometer (*OSF1, ADC Bioscientific Ltd.*, Great Amwell, UK). Initial ($F_0$), maximum ($F_m$), variable ($F_v = F_m - F_0$) fluorescence, and maximum quantum yield of PSII ($F_v/F_m$) were recorded in the dark-adapted leaves, steady-state ($F_s$), and maximum ($F_m'$) fluorescence were measured in the light-adapted leaves. Calculations were made for: $F_0' = F_0/[(F_v/F_m) + (F_0/F_m')]$,
$F_v'/F_m' = [(F_m' - F_0')/F_m']$,
$q_P = [(F_m' - F_s)/(F_m' - F_0')]$, and
$NPQ = [1 - (F_m' - F_0')/(F_m - F_0)]$, where $q_P$ is photochemical quenching and NPQ is nonphotochemical quenching (Genty *et al.* 1989). Gas-exchange parameters including $P_N$, transpiration rate ($E$), and stomatal conductance ($g_s$) were recorded using a calibrated portable gas-exchange system (*LCA-4, ADC Bioscientific Ltd.*, Great Amwell, UK) during the light period between 9:00 and 13:00 h under a PPFD of about 400 μmol $m^{-2}$ $s^{-1}$. For Chl fluorescence and gas-exchange analyses, an average of four records from different parts of each individual leaf was considered for each replicate.

**Leaf pigments, soluble carbohydrates, and starch**: Chl *a*, Chl *b*, and carotenoids (Car) were determined according to Lichtenthaller and Wellburn (1983). Leaves were homogenized in 80% cold acetone in the dark at 4ºC. After 24 h, the absorption of samples was determined at 663 (Chl *a*), 646 (Chl *b*), and 470 nm (Car) using spectrophotometer (*Specord 200, Analytik Jena,* Jena, Germany).

For determination of nonstructural carbohydrates, samples were homogenized in 100 mM phosphate buffer (pH 7.5) at 4ºC. After centrifugation at 12,000 × *g* for 15 min, the supernatant was used for determination of total soluble sugars whereas the pellets were kept for starch analysis (Magné *et al.* 2006). An aliquot of the supernatant was mixed with anthrone-sulfuric acid reagent and incubated for 10 min at 100ºC. After cooling, the absorbance was determined at 625 nm. Standard curve was

created using glucose (*Merck*, Darmstadt, Germany). For determination of starch, the pellet was resuspended in a 4:1 (v/v) mixture of 8 N HCl/dimethylsulfoxide (*Merck*). Starch was dissolved for 30 min at 60ºC under agitation. After centrifugation, the supernatant was mixed with iodine-HCl solution and after 15 min at room temperature the absorbance was determined at 600 nm. Starch (*Merck*) was used for preparation of a standard curve.

**NR activity and nitrogenous metabolites**: *In vivo* NR (E.C. 1.6.6.1) activity was determined using the method described by Jaworski (1971). Leaf blades and root samples were cut into 5-mm sections and placed in incubation buffer (100 mg tissue for 10 mL of buffer) containing 25 mM potassium phosphate buffer (pH 7.2), 25 mM $KNO_3$, and 1% Triton X-100 (*Sigma*, St. Louis, USA). The samples were infiltrated using vacuum (80 kPa). After 1 h (roots) or 5 h (leaves), the vacuum was released and the samples were incubated at 30ºC in darkness for 1 h, then placed in a boiling water bath to stop the NR activity. The resulting nitrite was determined spectrophotometrically at 540 nm in a reaction mixture containing sulfanilamide and naphthylethylenediamine dihydrochloride (*N-NEDA, Sigma*, St. Louis, USA). Activity of NR was calculated from a standard curve established with $NaNO_2$ (*Merck*) and expressed in produced $\mu mol(NO_2^-)\ g^{-1}(FM)\ s^{-1}$ or $\mu mol(NO_2^-)\ mg^{-1}(protein)\ s^{-1}$.

Nitrate was determined according to the procedure developed by Cataldo *et al.* (1975). The standard curve was created by using $KNO_3$ (*Merck*) in the range of 0–10 mM. Nitrite was extracted from fresh materials using 25 mM potassium phosphate buffer (pH 7.5). After centrifugation and proper dilution, the nitrite concentration was assayed after the formation of a red-violet colored, water-soluble azo dye as used for determination of nitrite described above.

Soluble proteins were determined using a commercial reagent (*Bradford reagent, Sigma*, St. Louis, USA) and bovine serum albumin (*Merck*, Darmstadt, Germany) as standard. Content of total free α-amino acids was assayed using a ninhydrin colorimetric method (Hwang and Ederer 1975) with glycine (*Merck*) as a standard.

Experiment was undertaken in complete randomized block design with four independent replications. Statistical analyses were carried out using *Sigma Stat 3.5* software (*Systat Software Inc.*, San José, California) with *Tukey*'s test ($p<0.05$).

## Results

Plant fresh (FM) and dry mass (DM) was influenced slightly or significantly by both N supply concentration and Se supplementation. The shoot (but not root) FM and DM decreased under $N_d$ supply. The root DM was even higher in $N_d$ compared with $N_a$ plants in the absence and presence of 10 μM Se. Supplementation with Se increased plant biomass that was significant for shoots of the $N_a$ plants at 50 μM Se. In addition, Se increased slightly shoot growth in $N_d$ plants and root growth in $N_a$ plants (Table 1).

Different N supply did not affect concentration of leaf pigments significantly. Added Se caused a slight, but consistent increase in the concentrations of leaf Chl *a* and Car under both N supply levels (Table 2).

Some Chl fluorescence parameters were affected by N supply concentration as well as by Se supplementation. $F_v/F_m$ was not changed by both treatments, while $q_P$ slightly increased by Se treatment, particularly in $N_d$ plants. For other parameters, including excitation capture efficiency of open PSII ($F_v'/F_m'$), quantum yield of PSII ($\Phi_{PSII}$), and electron transport rate (ETR), a significant reduction under $N_d$ conditions was observed in the absence of Se. In the presence of Se, however, such a reduction was not observed; these parameters were similar to $N_a$ plants. The NPQ showed the same trend but, as expected, in an opposite direction. Enhanced NPQ in $N_d$ plants returned to its basal values upon the Se supplementation (Table 3).

Table 1. Fresh mass (FM) and dry mass (DM) of shoots and roots in wheat plants grown under adequate (4 mM) or low (1 mM) N supply in the hydroponic medium and supplemented with different concentrations of Se ($Na_2SeO_4$). Data are means ± SD ($n = 4$). Difference among data of each column followed by *the same letter* was not statistically significant ($p<0.05$).

| N treatment | $Na_2SeO_4$ [μM] | Shoots | | Roots | |
|---|---|---|---|---|---|
| | | FM [mg pot$^{-1}$] | DM [mg pot$^{-1}$] | FM [mg pot$^{-1}$] | DM [mg pot$^{-1}$] |
| Adequate | 0 | 23.9 ± 2.3$^{b}$ | 3.23 ± 0.26$^{b}$ | 6.67 ± 1.18$^{a}$ | 0.59 ± 0.08$^{b}$ |
| | 10 | 23.9 ± 0.7$^{b}$ | 3.29 ± 0.47$^{b}$ | 6.84 ± 0.47$^{a}$ | 0.60 ± 0.04$^{b}$ |
| | 50 | 31.7 ± 1.9$^{a}$ | 3.98 ± 0.22$^{a}$ | 8.04 ± 0.04$^{a}$ | 0.73 ± 0.03$^{ab}$ |
| Low | 0 | 11.7 ± 0.8$^{c}$ | 2.08 ± 0.08$^{c}$ | 8.00 ± 0.55$^{a}$ | 0.75 ± 0.03$^{a}$ |
| | 10 | 12.8 ± 1.1$^{c}$ | 2.20 ± 0.12$^{c}$ | 9.23 ± 1.93$^{a}$ | 0.77 ± 0.08$^{a}$ |
| | 50 | 13.3 ± 1.7$^{c}$ | 2.16 ± 0.16$^{c}$ | 8.22 ± 2.10$^{a}$ | 0.72 ± 0.09$^{ab}$ |

Table 2. Leaf chlorophyll (Chl) *a*, Chl *b*, and carotenoid (Car) concentrations in wheat plants grown under adequate (4 mM) or low (1 mM) N supply in the hydroponic medium and supplemented with different concentrations of Se ($Na_2SeO_4$). Data are means ± SD ($n$ = 4). Difference among data of each column followed by *the same letter* was not statistically significant ($p$<0.05).

| N treatment | $Na_2SeO_4$ [μM] | Chl *a* [mg $g^{-1}$(FM)] | Chl *b* [mg $g^{-1}$(FM)] | Car [mg $g^{-1}$(FM)] |
|---|---|---|---|---|
| Adequate | 0 | 0.92 ± 0.16$^a$ | 0.23 ± 0.04$^a$ | 97 ± 14$^{ab}$ |
| | 10 | 1.00 ± 0.14$^a$ | 0.24 ± 0.02$^a$ | 100 ± 8$^{ab}$ |
| | 50 | 1.13 ± 0.23$^a$ | 0.20 ± 0.03$^a$ | 106 ± 12$^{ab}$ |
| Low | 0 | 0.91 ± 0.08$^a$ | 0.22 ± 0.04$^a$ | 86 ± 13$^b$ |
| | 10 | 1.07 ± 0.13$^a$ | 0.27 ± 0.04$^a$ | 115 ± 17$^a$ |
| | 50 | 1.14 ± 0.10$^a$ | 0.26 ± 0.04$^a$ | 119 ± 11$^a$ |

Table 3. Chlorophyll fluorescence parameters including the maximum photochemical efficiency of PSII ($F_v/F_m$), excitation capture efficiency of open PSII ($F_v'/F_m'$), photochemical quenching ($q_P$), nonphotochemical quenching (NPQ), quantum yield of PSII ($\Phi_{PSII}$), and electron transport rate (ETR) in wheat plants grown under adequate (4 mM) or low (1 mM) N supply in the hydroponic medium and supplemented with different concentrations of Se ($Na_2SeO_4$). Data are means ± SD ($n$ = 4). Difference among data of each column followed by *the same letter* was not statistically significant ($p$<0.05).

| N treatment | $Na_2SeO_4$ [μM] | $F_v/F_m$ | $F_v'/F_m'$ | $q_P$ | NPQ | $\Phi_{PSII}$ | ETR |
|---|---|---|---|---|---|---|---|
| Adequate | 0 | 0.86 ± 0.01$^a$ | 0.78 ± 0.00$^a$ | 0.95 ± 0.01$^a$ | 0.11 ± 0.08$^b$ | 0.75 ± 0.01$^a$ | 125 ± 1.2$^a$ |
| | 10 | 0.88 ± 0.01$^a$ | 0.74 ± 0.02$^{ab}$ | 0.97 ± 0.05$^a$ | 0.09 ± 0.07$^b$ | 0.72 ± 0.02$^{ab}$ | 123 ± 2.6$^a$ |
| | 50 | 0.88 ± 0.01$^a$ | 0.79 ± 0.02$^a$ | 0.94 ± 0.03$^a$ | 0.11 ± 0.03$^b$ | 0.74 ± 0.02$^a$ | 124 ± 2.6$^a$ |
| Low | 0 | 0.86 ± 0.04$^a$ | 0.69 ± 0.02$^b$ | 0.92 ± 0.05$^a$ | 0.29 ± 0.07$^a$ | 0.64 ± 0.04$^b$ | 106 ± 6.4$^b$ |
| | 10 | 0.88 ± 0.01$^a$ | 0.75 ± 0.03$^{ab}$ | 0.98 ± 0.04$^a$ | 0.14 ± 0.07$^{ab}$ | 0.73 ± 0.04$^a$ | 123 ± 2.5$^a$ |
| | 50 | 0.88 ± 0.01$^a$ | 0.77 ± 0.05$^a$ | 0.98 ± 0.07$^a$ | 0.10 ± 0.07$^b$ | 0.75 ± 0.07$^a$ | 125 ± 0.7$^a$ |

In the absence of Se, $P_N$ decreased only slightly due to low N supply. The Se supplementation, however, increased it significantly at both $N_d$ and $N_a$ concentrations. The stimulatory effect was not dependent on the Se concentration. The similar trend was observed in $E$ and $g_s$ (Fig. 1).

In general, the effect of N and Se was similar for both soluble sugars and starch. The concentration of soluble sugars in the leaves and roots was slightly or significantly higher in $N_d$ plants compared with $N_a$ ones. After the Se treatment, the concentration of soluble carbohydrates slightly increased in the leaves and roots of $N_a$ plants. In $N_d$ plants, however, the Se treatment lowered the contents of soluble sugars in the leaves (significantly) and in the roots (slightly). Similarly, the leaf (but not root) starch content was higher in $N_d$ plants in the absence of Se. Under the Se treatment, the starch content of the $N_a$ plants slightly increased while it decreased in the $N_d$ ones. The root starch content was not influenced by any of the applied treatments (Table 4).

As expected, the activity of NR (expressed on both FM and protein content basis) was lower in $N_d$ plants in both leaves and roots compared with the plants supplied with $N_a$. The Se treatment, in general, resulted in significant or slight enhancement of NR activity in both leaves and roots of both $N_a$ and $N_d$ plants. However, the effect of Se on the root NR activity was significant only in the $N_d$ plants. On the other hand, the effect of 10 μM Se on the leaf NR activity was observed only when expressed on FM basis. Higher (50 μM) Se concentration reduced leaf NR activity in the $N_a$ (but not in $N_d$) plants (Fig. 2).

As expected, leaf and root concentrations of nitrate were lower in the $N_d$ compared with $N_a$ plants. The Se treatment, however, did not influence nitrate concentration of the $N_a$ plants either in the leaves or roots. In $N_d$ plants, in contrast, the Se treatment resulted in a reduction of the nitrate concentration; this effect was significant in the roots (Table 5). Similarly to nitrate, nitrite concentration was lower in the $N_d$ plants in both leaves and roots. The Se treatment, however, influenced the nitrite concentration differently. A significant rise of the nitrite concentration in the $N_a$ plants was observed in the leaves and roots. In the $N_d$ ones, however, such effect was observed only in the roots (Table 5).

As expected and similarly with nitrate and nitrite, $N_d$ conditions caused significant reduction of total free amino acids in both leaves and roots in the absence of Se. Under supplementation with Se, the leaf and root amino acid contents declined in the $N_a$ plants while they increased in the $N_d$ ones (Table 5). Soluble protein contents were slightly lower in the leaves (but not in the roots) of $N_d$ plants while the increase due to the Se treatment was found in the leaves and roots (Table 5).

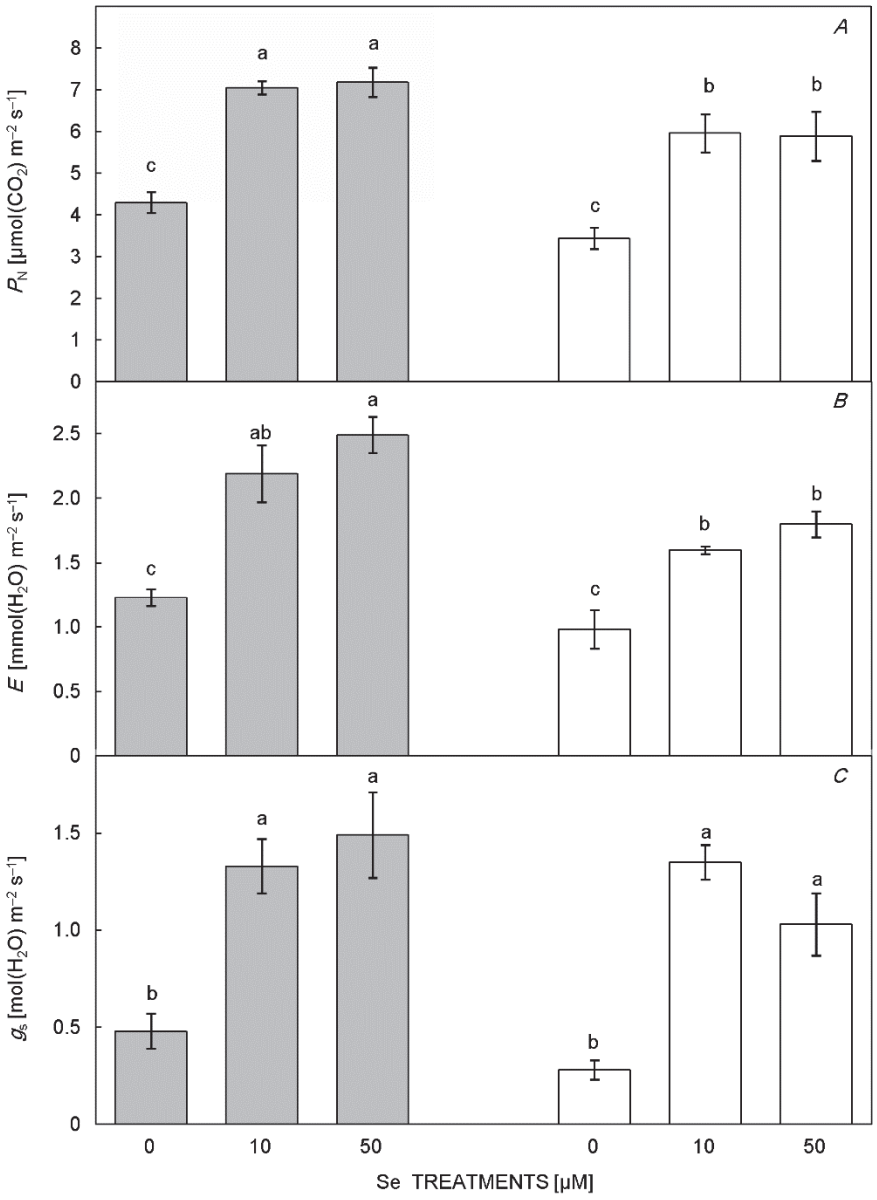


Fig. 1. Gas-exchange parameters including *A*: net photosynthetic rate ($P_N$), *B*: transpiration rate ($E$), and *C*: stomatal conductance to water vapor ($g_s$) in wheat plants grown under adequate (4 mM, *dark columns*) or low (1 mM, *open columns*) N supply in the hydroponic medium and supplemented with different concentrations of Se ($Na_2SeO_4$). Data are means ± SD ($n$ = 4). Difference among columns indicated by *the same letter* was not statistically significant ($p$<0.05).

Table 4. Effect of Se supplementation on the concentrations of soluble sugars and starch in the leaves and roots of wheat plants grown under adequate (4 mM) or low (1 mM) N supply in the hydroponic medium and supplemented with different concentrations of Se ($Na_2SeO_4$). Data are means ± SD ($n$ = 4). Difference among data of each column followed by *the same letter* was not statistically significant ($p$<0.05).

| N treatment | $Na_2SeO_4$ [µM] | Leaves | | Roots | |
|---|---|---|---|---|---|
| | | Soluble sugars [mg(eq. glucose) $g^{-1}$(FM)] | Starch [mg $g^{-1}$(FM)] | Soluble sugars [mg(eq. glucose) $g^{-1}$(FM)] | Starch [mg $g^{-1}$(FM)] |
| Adequate | 0 | $4.44 \pm 0.57^{c}$ | $0.46 \pm 0.04^{b}$ | $0.77 \pm 0.12^{b}$ | $0.10 \pm 0.02^{a}$ |
| | 10 | $6.12 \pm 0.97^{bc}$ | $0.48 \pm 0.04^{b}$ | $0.93 \pm 0.19^{b}$ | $0.09 \pm 0.01^{a}$ |
| | 50 | $5.42 \pm 0.95^{bc}$ | $0.59 \pm 0.11^{ab}$ | $1.96 \pm 0.27^{ab}$ | $0.10 \pm 0.02^{a}$ |
| Low | 0 | $10.12 \pm 0.71^{a}$ | $0.70 \pm 0.06^{a}$ | $2.99 \pm 0.72^{a}$ | $0.09 \pm 0.01^{a}$ |
| | 10 | $6.46 \pm 0.78^{b}$ | $0.59 \pm 0.07^{ab}$ | $2.41 \pm 0.67^{a}$ | $0.06 \pm 0.01^{a}$ |
| | 50 | $9.92 \pm 0.57^{a}$ | $0.46 \pm 0.08^{b}$ | $2.27 \pm 0.98^{a}$ | $0.09 \pm 0.03^{a}$ |

## Discussion

**Effect of N deficiency on plant growth and parameters of C and N metabolism**: $N_d$ plants exhibited the lower shoot biomass while the root DM was significantly higher than that in $N_a$ plants. Greater root biomass and larger root surface area in response to limiting N availability has been reported for several plant species and could be regarded as

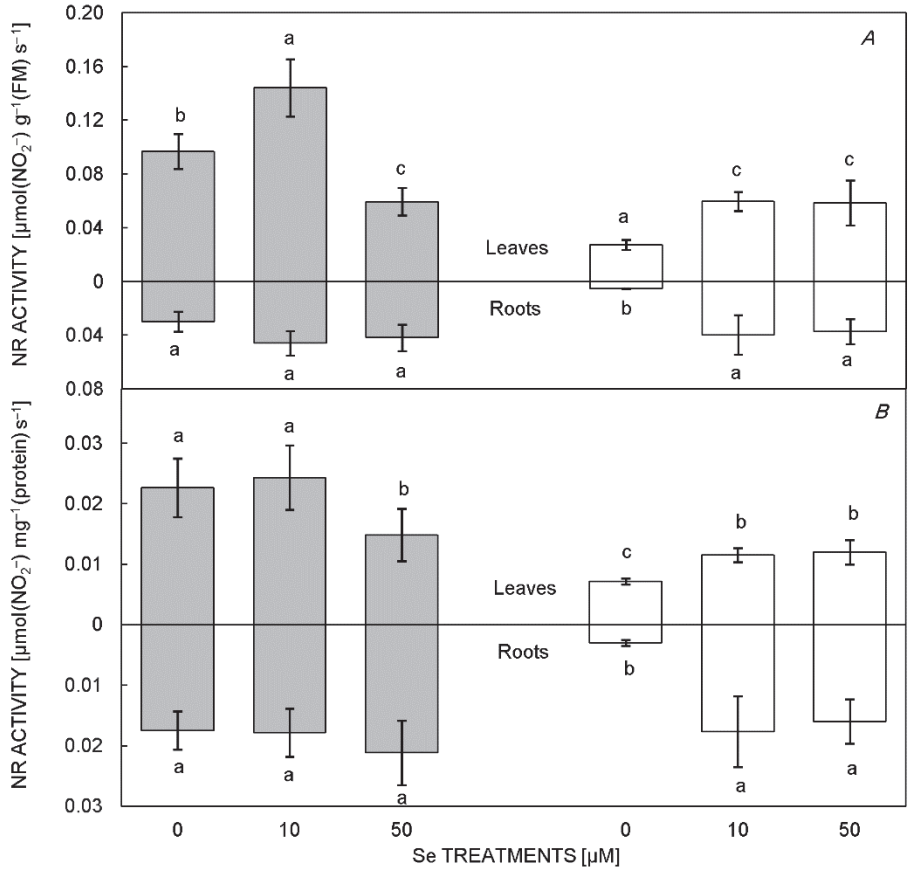


Fig. 2. Activity of nitrate reductase (NR) on the basis of fresh mass (*A*) and protein content (*B*) in the leaves and roots of wheat grown under adequate (4 mM, *dark columns*) or low (1 mM, *open columns*) N supply in the hydroponic medium and supplemented with different concentrations of Se ($Na_2SeO_4$). Data are means ± SD ($n$ = 4). Difference among columns within each plant organ indicated by *the same letter* was not statistically significant ($p$<0.05).

Table 5. Effect of Se supplementation on the concentrations of nitrate, nitrite, total free α-amino acids, and proteins in the leaves and roots of wheat plants grown under low (1 mM) or adequate (4 mM) N supply in the hydroponic medium and supplemented with different concentrations of Se ($Na_2SeO_4$). Data are means ± SD ($n$ = 4). Difference among data of each column within each plant organ indicated by *the same letter* was not statistically significant ($p$<0.05).

| N treatment | $Na_2SeO_4$ [µM] | Nitrate [mmol $g^{-1}$(FM)] | Nitrite [µmol $g^{-1}$(FM)] | Amino acids [µmol $g^{-1}$(FM)] | Proteins [mg $g^{-1}$(FM)] |
|---|---|---|---|---|---|
| | | Leaves | | | |
| Adequate | 0 | $3.30 \pm 0.83^{ab}$ | $7.75 \pm 1.50^{b}$ | $79 \pm 20^{a}$ | $4.39 \pm 0.89^{ab}$ |
| | 10 | $3.51 \pm 0.46^{a}$ | $11.8 \pm 0.87^{a}$ | $42 \pm 11^{b}$ | $6.02 \pm 0.61^{a}$ |
| | 50 | $3.21 \pm 0.43^{ab}$ | $11.6 \pm 2.59^{a}$ | $56 \pm 10^{ab}$ | $4.14 \pm 0.53^{b}$ |
| Low | 0 | $2.33 \pm 0.97^{ab}$ | $2.88 \pm 1.71^{c}$ | $44 \pm 12^{b}$ | $3.89 \pm 0.77^{b}$ |
| | 10 | $2.88 \pm 0.20^{ab}$ | $3.88 \pm 1.75^{c}$ | $72 \pm 15^{a}$ | $5.14 \pm 1.06^{ab}$ |
| | 50 | $2.04 \pm 0.45^{b}$ | $2.75 \pm 0.65^{c}$ | $78 \pm 11^{a}$ | $5.20 \pm 0.21^{ab}$ |
| | | Roots | | | |
| Adequate | 0 | $6.70 \pm 0.44^{a}$ | $2.38 \pm 0.85^{b}$ | $17 \pm 5.5^{a}$ | $1.46 \pm 0.47^{b}$ |
| | 10 | $5.99 \pm 0.16^{a}$ | $3.00 \pm 0.82^{ab}$ | $9 \pm 4.1^{ab}$ | $2.69 \pm 0.45^{a}$ |
| | 50 | $5.11 \pm 0.62^{a}$ | $4.12 \pm 0.48^{a}$ | $15 \pm 6.5^{a}$ | $2.11 \pm 0.54^{ab}$ |
| Low | 0 | $5.16 \pm 1.31^{a}$ | $1.75 \pm 0.29^{b}$ | $4 \pm 0.8^{b}$ | $1.73 \pm 0.32^{b}$ |
| | 10 | $3.10 \pm 1.25^{b}$ | $3.50 \pm 0.41^{a}$ | $15 \pm 3.9^{a}$ | $2.14 \pm 0.24^{ab}$ |
| | 50 | $2.56 \pm 0.45^{b}$ | $3.00 \pm 0.58^{ab}$ | $17 \pm 5.5^{a}$ | $2.35 \pm 0.40^{ab}$ |

a critical response to acclimation of plants to low N supply (Shen *et al.* 2013). N acquisition depends on root characteristics and morphology and plants with higher root surface area may better exploit nutrient resources in rhizosphere (Kraiser *et al.* 2011).

N deficiency influenced significantly various Chl fluorescence parameters while it only slightly reduced gas exchange parameters and did not modify the concentration of Chl in the absence of Se. Several studies demonstrated that N deficiency negatively influences photosynthetic capacity of plants. Reduction of Rubisco activity (Huang *et al.* 2004, Antal *et al.* 2010), stomata closure (Shrestha *et al.* 2012), and depression of photochemical processes (Lima *et al.* 1999, Lu and Zhang 2000, Antal *et al.* 2010) have been reported as the main mechanisms for reduction of photosynthetic capacity in $N_d$ plants. However, the extent to which each of these parameters is affected by limiting N availability seems to be dependent on the plant species and/or genotype as well as the severity and duration of N deficiency. A significant reduction of $F_v'/F_m'$, $\Phi_{PSII}$, and ETR under $N_d$ conditions in this work suggested likely the occurrence of photoinhibition. Photoinhibition is defined as photochemical inactivation mainly of PSII and is the consequence of stresses such as low and high temperature, drought, and low $CO_2$ availability (stomatal closing), particularly in a combination with high light conditions (Niyogi 1999). Although $P_N$ was only slightly lowered by low N supply in this work, lack of any decline in the Chl concentration and relatively higher light intensities [400 μmol(photon) $m^{-2}$ $s^{-1}$] in growth chamber might lead to an overreduction of the photosynthetic electron transport chain and therefore to photoinhibition. Photoinhibition, however, is not necessarily associated with damages to photosystems (Niyogi 1999) as it could be confirmed by stable values of $F_v/F_m$ in this work. The stable ratio of $F_v/F_m$ was reached likely due to an efficient nonradiative dissipation of excess energy in $N_d$ plants reflected in the elevated NPQ, also an indication of heat dissipation *via* xanthophyll cycle. This protective mechanism converts excess excitation energy into heat and prevents formation of reactive oxygen species in the chloroplasts (Müller *et al.* 2001).

Leaf and root contents of soluble sugars and leaf concentration of starch were higher under N starvation concomitant with lower amino acids concentrations in both leaves and roots. These alterations in the concentration of C and N metabolites indicate first the lower availability of reduced N for synthesis of N compounds and/or reduced demand of C skeletons for N compounds (Luo *et al.* 2013). In addition, it could be the consequence of photosynthesis exceeding the demands of respiration and growth under $N_d$ conditions (Lawlor 2002). Accumulation of soluble sugars in the roots of $N_d$ plants suggested likely that C export from source leaves and/or phloem transport was not influenced by low N supply (Luo *et al.* 2013). Insignificant reduction of leaf protein content in the shoots might be partly the result of simultaneously lowered FM in $N_d$ plants. Despite increase in FM, the protein content in roots (similar to that of shoot) was not influenced by N supply level.

The NR activity was higher in the leaves than in the roots but it was similarly influenced by low N supply. Lower activity of NR in the absence of adequate, external $NO_3^-$ supply has been well documented (Masclaux-Daubresse *et al.* 2010). Nitrate concentration in $N_d$ plants was only slightly lower than that in $N_a$ ones, while nitrite concentration declined more under limiting N supply, particularly in the leaves. Because of down-regulation of NR concomitant with reduction of nitrate influx (Masclaux-Daubresse *et al.* 2010), $NO_3^-$ is likely less depleted than nitrite in $N_d$ plants. Reduction of NR activity and $NO_3^-$ uptake is a part of a mechanism for the slowing down N metabolism under low N availability. Transcriptional regulation of genes involved in $NO_3^-$ influx, assimilation, and further metabolism (Hirai *et al.* 2004) plays a fundamental role in response to N starvation in plants.

**Effect of Se on plant growth and photosynthesis at low and adequate N supply**: Se improved plant growth under both N supply concentrations, but this effect was greater in $N_a$ than that in the $N_d$ plants in both shoots and roots. Leaf Car concentrations increased after the Se treatments being significant in the $N_d$ plants. Car exhibit important protective role *via* the xanthophyll cycle in heat dissipation, *e.g.*, nonphotochemical quenching of excess excitation energy. Although increase of Car concentrations in $N_d$ plants by Se was not accompanied by higher NPQ in the leaves, it could be effective under conditions of higher excess excitation energy, *i.e.*, severer N deficiency and/or higher light intensities. It has been reported that severe N deficiency conditions, particularly at high light, was linked to qualitative changes in the Car composition, upregulation of the xanthophyll cycle, and increased thermal dissipation (Pompelli *et al.* 2010). Various Chl fluorescence parameters, including $F_v'/F_m'$, $\Phi_{PSII}$, and ETR, which decreased under limiting N availability, recovered completely by the Se treatment and reached the level of those in $N_a$ plants. Acceleration of electron flow and enhancement of excitation capture of PSII reaction centers might be attributed to depletion of ATP and reducing equivalents resulting from higher $CO_2$ assimilation in Se-supplemented plants. Enhancement of efficient excitation energy quenching was reflected well in lower NPQ values in $N_d$ plants in the presence of Se compared with the plants without Se.

In the $N_a$ plants, the Se treatment elevated significantly $g_s$ up to the level of the $N_a$ plants. Similarly, $P_N$ increased significantly becoming even higher than that in the $N_a$ plants without Se. Enhancement of $g_s$ by Se and higher $CO_2$ assimilation was observed in our previous works (Hajiboland and Keivanfar 2012, Owusu-Sekyere *et al.* 2013). The mechanism of Se-induced increase in $g_s$ remained obscure and it is likely attributable to activated proton pumping with an unknown mechanism that

promotes $K^+$ inward currents. Higher $P_N$ in $N_a$ plants could be attributed mainly to the stomata opening. In $N_d$ plants, however, both stomatal and nonstomatal mechanisms, *e.g.*, leaf photochemistry and likely the activity of enzymes involved in photosynthetic C metabolism, contributed to higher $CO_2$ assimilation rate. Increase in the activity of fructose-1,6-bisphosphatase was reported in alfalfa plants upon Se treatment and it was attributed to Se-evoked changes in the ferrodoxin/thioredoxin system (Owusu-Sekyere *et al.* 2013).

Despite the significant stimulation of photosynthesis, soluble carbohydrates and starch concentrations rather declined under Se treatment in the $N_d$ plants. Nonetheless, carbohydrate concentration might not be a good measure of C metabolism in our experiment as it could be depleted more rapidly because of enhanced utilization as C and ATP sources for amino acids and protein synthesis in the Se-treated plants. In accordance with this, lower carbohydrate concentration in the plants treated with 10 μM Se was associated with the increase of amino acids and proteins in the leaves. Thus, a shift from C to N metabolism might be responsible for lower carbohydrates in the Se-treated, $N_d$ plants.

**Effect of Se on plant N metabolism at low and adequate N supply**: Beside enhancement of leaf photosynthesis, Se supplementation increased NR activity of both $N_d$ and $N_a$ plants. The optimum Se concentration was 10 μM for $N_a$ plants, while the effect was similar at both Se concentrations in the $N_d$ plants. Although NR activity in $N_d$ plants, supplemented with Se, was higher than that in the plants without Se, it was still significantly lower than that in $N_a$ plants. It indicated likely that Se did not substitute the effect of $NO_3^-$ as the important regulatory signal for NR activity. NR activity is an important control point in N assimilation pathway. Maximal NR activity is governed by multiple factors, the most important of which are light, nitrate, glutamine, and sugars (Campbell 1999, Stitt *et al.* 2002). NR is subjected to both transcriptional and post-translational regulation and carbohydrate supply affects several control points of NR activity. Exogenous sugars influence the transcripts abundance and are also involved in post-translational regulation of NR, *i.e.*, enhanced stability and increase in activation state (Kaiser and Huber 1994, Morcuende *et al.* 1998). Elevation of NR activity by Se has also been reported in soybean (Djanaguiraman *et al.* 2004) and tobacco (Xu *et al.* 2013). There are several potential mechanisms for the effect of Se on NR activity. Minor amino acids, such as histidine, asparagine, and cystein, as regulators of NR activity (Stitt *et al.* 2002) are subjected to changes by Se (Lee *et al.* 2005). Plant hormones are involved in regulation of NR activity and in coordination of C and N metabolism (Zhang *et al.* 2011, Reguera *et al.* 2013) and there is evidence for the Se-induced expression of hormone-responsive genes (Tamaoki *et al.* 2008). Regarding relationships between Se assimilation and glutathione synthesis pathways (Bañuelos *et al*. 2005) and a central role of glutathione in nitrate assimilation and N metabolism (Kopriva and Rennenberg 2004), it could be also speculated that Se might influence NR activity by changes in the glutathione redox state. Detailed studies are needed for elucidation of mechanisms how Se affects NR activity.

Roots of $N_a$ plants did not respond to Se in the same way because of limited capacity for $NO_3^-$ reduction in the roots as compared with the leaves (Hawkesford *et al.* 2012). In the roots of the Se-treated plants, nitrate concentrations were negatively correlated with the activity of NR. In $N_d$ plants, lower nitrate concentration was accompanied by higher NR activity ($r^2 = 0.91$, $p<0.05$); in the $N_a$ plants, stable amounts of nitrate was associated with stable NR activity ($r^2 = 0.86$, $p<0.05$). It might indicate that Se did not affect nitrate uptake under lower external supply, thus, partial nitrate depletion in the roots could be attributed to elevated reduction. Such a negative correlation, however, was not observed in the leaves.

Nevertheless, nitrite concentrations were slightly or significantly higher in the Se treated plants. Nitrite is toxic to the cell because it forms diazo compounds with amino groups of nucleobases and it leads to mutations. Under normal metabolic conditions, very efficient reduction of nitrite by nitrite reductase (NiR) prevents nitrite from accumulating in the cell (Heldt 2005). Here, inadequate coupling of NR and NiR activities, likely because of limitations in the reducing equivalents that are required for NiR activity, was the probable cause of nitrite accumulation in Se-treated plants. However, considering positive effect of Se on plants growth, nitrite concentration was not obviously at toxic levels in this work.

In the $N_d$ plants, concentrations of free amino acids and soluble proteins were significantly higher due to Se without any difference between both Se concentrations. Higher amino acids (Djanaguiraman *et al.* 2004, Lee *et al.* 2005, Hajiboland and Keivanfar 2012) and protein synthesis in the Se-treated plants (Hajiboland and Keivanfar 2012) have been reported previously and it may be an indication of a general upregulation of N metabolism. This upregulation was associated with concomitantly higher NR activity, leaf photochemistry, and gas exchange parameters at two Se concentrations.

In the $N_a$ plants, however, the concentration of free α-amino acids showed distinctly different pattern: a reduction by the lower Se concentration (10 μM), but the increase by the higher (50 μM) Se concentration. Protein contents were negatively correlated with the amino acids concentrations in the leaves ($r^2 = 0.98$, $p<0.05$) and roots ($r^2 = 0.91$, $p<0.05$). Similar pattern of changes was observed for NR activity (Fig. 2*A*). Lower NR activity and protein synthesis at 50 μM Se could not be attributed to reduced provision of energy substrate and C skeletons because higher $CO_2$ fixation was maintained under the higher Se concentration (Fig. 1*A*). Mechanisms involved in the whole-plant N and C economy (Lawlor 2002), particularly, those linked with plant genetic capacity, may

play a fundamental role in this feed-back regulation. In contrast to the parameters of N metabolism, the highest biomass production was observed at 50 μM Se.

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