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ORIGINAL ARTICLE

Effect of NO_3^-:NH_4^+ ratios on growth, root morphology and leaf metabolism of oilseed rape (*Brassica napus* L.) seedlings

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Abstract The effect of NO_3^-:NH_4^+ ratio (14:1, 9:6, 7.5:7.5, 1:14, total 15 mmol/L N) in the nutrient solution on biomass, root morphology, and C and N metabolism parameter in hydroponically grown oilseed rape (*Brassica napus* L.) was evaluated. The dry weights of leaves and roots were significantly largest at the equal NO_3^-:NH_4^+ ratio (7.5:7.5) compared with those of high NO_3^-:NH_4^+ ratio (14:1) or low NO_3^-:NH_4^+ ratio (1:14). Additionally, low NO_3^-:NH_4^+ ratio (1:14) reduced total root length and root surface area compared with the equal NO_3^-:NH_4^+ ratio (7.5:7.5), while high NO_3^-:NH_4^+ ratio (14:1) did not show any significant effect on root morphology except average diameter. The maximum of chlorophyll *a*, chlorophyll *b* and carotenoid were obtained under 7.5:7.5 treatment, whereas the maximum of the leaf net photosynthetic (P_n), stomatal conductance (G_s) and transpiration rate (T_r) were increased with increase in NH_4^+ concentration in the nutrient solution. The activity of nitrate reductase (NR) showed a significant difference at different NO_3^-:NH_4^+ ratios and ranged 9:6 > 7.5:7.5 > 14:1 > 1:14, whereas the range of soluble sugar and soluble protein was 7.5:7.5 > 1:14 > 9:6 > 14:1. Our study reveals that oilseed rape growth is greater under 7.5:7.5 treatment than that under three other treatments. Oilseed rape growth at high or low NO_3^-:NH_4^+ ratios was inhibited by decreased pigments, NR activity, soluble sugar, and soluble protein, whereas subdued root growth should be apprehended considerate under high NH_4^+ condition.

Keywords Nutrient solution · NO_3^-:NH_4^+ ratio · Growth · Root morphology · Physiological metabolism

Introduction

Nitrogen (N) is an important nutrient for plant growth in agricultural ecosystems. It is taken up from soils mainly as nitrate (NO_3^-) and/or ammonium (NH_4^+) by roots of higher plants. In plants, NO_3^- reduction into nitrite (NO_2^-) is catalyzed in the cytosol by the enzyme nitrate reductase (NR) (Meyer and Stitt 2001). After nitrate reduction, nitrite is reduced to ammonium by the nitrite reductase (NiR) in the chloroplast (Meyer and Stitt 2001). Ammonia is mainly assimilated in the plastid/chloroplast by the GS/GOGAT cycle (Lea and Forde 1994; Perez-Garcia et al. 2011; von Wirén and Merrick 2004). Nitrate is generally the preferred source for plant growth, while ammonium can be deleterious to the growth of many plant species if absorbed as the sole N source or in high concentration (Bittsánszky et al. 2015). Furthermore, the form in which N is taken up by plants affects numerous physiological processes including N-assimilation, cation–anion balance, respiration, water relations, photosynthesis and secondary metabolism. For instance, plant supplied with NH_4^+ as the sole N source could have some visual symptoms, growth suppression and chlorosis (Cramer and Lewis 1993; Miller and Cramer 2004). Moreover, the cation (K^+, Mg^{2+}, Ca^{2+} etc.) uptake,

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pH homeostasis and carbon–nitrogen imbalance due to excessive consumption of soluble sugars for NH_4^+ assimilation in root tissues were affected by different NO_3^- and NH_4^+ ratios (Ali et al. 1998; Roosta and Schjoerring 2007; Cao et al. 2004; Finnemann and Schjoerring 1999). It is also noteworthy that disruption in hormonal homeostasis, damaged chloroplast ultrastructure, uncoupling of photophosphorylation and disturbance in photosynthesis were also associated with NH_4^+ stress in plants (Britto and Kronzucker 2002; Cao et al. 2004).

However, previous studies found that the sensitivity to NH_4^+ is strongly dependent on whether NH_4^+ is supplied together with NO_3^- (Bialczyk et al. 2005; Zhu et al. 2015). Li et al. (2011) found that more than tenfold higher concentrations of NH_4^+ are required to induce growth inhibition in the presence of NO_3^-. Moreover, nitrate addition alleviates ammonium toxicity without lessening ammonium accumulation, organic acid depletion and inorganic cation depletion in *Arabidopsis thaliana* shoots (Hachiya et al. 2012).

Oilseed rape is one of the most important oil crops in China, with a cultivated area of 7.5 million hectares and a production about 14.4 million tons of seeds in 2013 (Gu et al. 2016). It not only provides edible oil, but is also an important source of biodiesel (Kim et al. 1994). As a major nutrient, N is involved in various biochemical and physiological processes vital for oilseed rape production. However, there is limited or no information on the effects of different N sources on the growth of oilseed rape. Therefore, in the present work, the effects of NO_3^-:NH_4^+ ratios on plant growth, root traits, leaf photosynthetic parameter, nitrate reductase, carbohydrates and protein were investigated.

Materials and methods

Plant materials and growth conditions

The experiment was conducted at Huazhong Agricultural University, China. Oilseed rape (*Brasscia napus* L.) seeds of ZS11 were sterilized using 5% (v/v) NaClO for 30 min and then rinsed several times in sterile distilled water. The seeds were then germinated on a breed tray under dark conditions. One-week-old morphologically uniform seedlings were selected and transferred to black plastic tanks containing 10 L of aerated nutrient solution. The nutrient solution contained 4 mM $Ca(NO_3)_2{\cdot}4H_2O$, 6 mM KNO_3, 1 mM $NH_4H_2PO_4$, 2 mM $MgSO_4{\cdot}7H_2O$, 46.2 μM H_3BO_3, 9.1 μM $MnCl_2{\cdot}4H_2O$, 0.8 μM $ZnSO_4{\cdot}7H_2O$, 0.3 μM $CuSO_4{\cdot}5H_2O$, 100 μM EDTA-Fe and 1 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. The seedlings were precultured in 1/2 nutrient solution for 7 days and then the full nutrient solution for 13 days. After an initial growth period of 20 days, the following four NO_3^-:NH_4^+ ratios were applied to the seeding: 14:1, 9:6, 7.5:7.5 and 1:14 with total N concentration fixed at 15 mM using NH_4Cl; see Chen et al. (2010) and for details Table 1). The solutions were replaced completely every 4 days. The changes in NO_3^-:NH_4^+ ratios were balanced by varying the Cl^- concentration. The pH of the culture solutions was adjusted to 6.5 ± 0.2 with 1 mM NaOH.

Table 1 The concentrations of salts (mM) used to prepare nutrient solutions at NO_3^-:NH_4^+ ratios of 14:1, 9:6, 7.5:7.5 and 1:14

Salts	NO_3^-:NH_4^+ ratios in the nutrient solutions			
	14:1	9:6	7.5:7.5	1:14
$Ca(NO_3)_2{\cdot}2H_2O$	4	4	3	0.5
KNO_3	6	1	1.5	0
KCl	0	5	4.5	6
NH_4Cl	0	5	6.5	13
$CaCl_2$	0	0	1	3.5
$MgSO_4$	2	2	2	2
$NH_4H_2PO_4$	1	1	1	1

Sampling and measurements

15-Day-old plants were harvested after growth on solution cultures of different NO_3^-:NH_4^+ ratios. Roots and shoot were separated from half of the plant and oven-dried at 80 °C for 48 h. The sample dry mass was recorded immediately prior to grinding in a mortar. Half of the plant leaves were frozen in dry ice immediately after harvesting and stored at −80 °C. The rest of the plant roots were harvested for root morphology and root activity measurement.

Measurements of root morphology

Images of the root systems were captured using a flatbed scanner (WinRHIZO 2009; Canada) after harvesting. Images were loaded into ImageJ, and the total root length, surface area, average diameter and tips were measured (Shi et al. 2012).

Measurements of chlorophyll contents and photosynthesis of leaves

The contents of chlorophyll *a*, chlorophyll *b* and carotenoid in leaves were analyzed as described by Saunkaew et al. (2011). The frozen leaves (approximately, 0.25 g) were extracted with

25 mL 95% ethanol in the dark at room temperature. After 24 h, the absorbance of the extracts was measured at 665, 649 and 470 nm using a spectrophotometer. The chlorophyll contents were calculated as follows: $C_a = 13.95A_{665} - 6.88A_{649}$, $C_b = 24.96A_{649} - 7.32A_{665}$, $C_{x \cdot c} = (1000A_{470} - 2.05C_a - 114.8C_b)/245$. Photosynthesis measurement: photosynthetic rate (P_n), stomatal conductance (G_s), transpiration rate (T_r) and intercellular CO_2 concentration (C_i) were measured by a portable infrared gas analyzer (Li-6400P, Li-Cor, USA) from three replications per treatment from 9:30 to 10:30 am on a sunny day before harvest.

Measurements of nitrate reductase (NR)

The activity of nitrate reductase (NR) in the young leaves samples was measured according to Tang et al. (2013) with some modification. Approximately, 0.5 g fresh leaves were taken and homogenized in 4 mL of extraction buffer. The mixed solution was centrifuged for 15 min at 4 °C and 4000 r/min. Then, 1.2 mL 0.1 mol/L KNO_3 in phosphate buffer and 0.4 mL NADH were added to 0.4 mL extraction solution, and the mixture was kept at 25 °C for 30 min. Then, 1 mL 1% (w/v) of sulfanilamide and 1 mL 0.02% (w/v) *N*-(1-naphthyl)ethylene-diaminedihydrochloride were added and the mixture was kept at room temperature for 15 min. The absorbance of the color intensity was measured using a spectrophotometer at 540 nm. NR activity was calculated as μmol NO_3^- reduced per gram fresh weight and per hour.

Measurements of soluble sugars

Soluble sugars in leaf tissue were assayed as described by Yan et al. (2015) with modifications. About 0.2 g fresh leaf tissue was incubated in boiling distilled water for 30 min. The extraction process as described above was repeated, and the collected supernatants were combined with constant volume. Then, 0.5 mL of the extracted solution, 1.5 mL water, 0.5 mL anthrone ethyl acetate and 5 mL H_2SO_4 were added to a new colorimetric tube and the mixture was incubated in boiling water for 1 min. After cooling it to room temperature, the absorbance of the mixture at 630 nm was measured using a spectrophotometer. The standard curve for soluble sugar was obtained by preparing a series of diluted glucose solutions.

Measurements of soluble protein

About 0.5 g of leaf sample was homogenized in 2 mL of distilled water under chilled condition. After incubation at room temperature for 30–60 min, the crude extract was centrifuged at 4000 r/min for 20 min at 4 °C and the supernatant was used for the determination of total soluble protein. The total soluble protein content was determined using the method of Bradford (1976) and bovine serum albumin was used as a standard.

Statistical analysis

Data were statistically analyzed with SPSS 20.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with LSD (least significance difference) test was used for the analysis of differences in four NO_3^-:NH_4^+ ratios. The sample means and standard errors were calculated using Microsoft Excel 2010. Sigma Plot 12.0 was used for generation of graphs.

Results

Biomass

As shown in Fig. 1, there was no significant difference in root dry weight (DW). The largest values of shoot DW were observed under 7.5:7.5 treatment. For 14:1 and 1:14 treatments, the shoot DW was reduced by 16.1 and 19.6%, respectively, as compared to those with 7.5:7.5 treatment.

Root morphology

As a result of the inhibitory effect displayed by NO_3^-:NH_4^+ ratios on root morphology, it was found that the total root length and root surface area were minimum under 1:14 treatment, while both the parameters were higher at higher NO_3^-:NH_4^+ ratios (14:1 and 7.5:7.5) (Fig. 2). A higher average of root diameter under 7.5:7.5 treatment was shown than that under 14:1 and 1:14 treatments, whereas there was no significant difference between the 14:1 and 1:14 treatments. There was no significant in tips among the 14:1, 7.5:7.5, and 1:14 treatments.

Pigment and photosynthetic characteristics

The data of leaf pigment concentration in relation to NO_3^-:NH_4^+ ratios are presented in Fig. 3. Chlorophyll *a* and chlorophyll *b* were significantly higher at 9:6 and 7.5:7.5 treatments, and the maximum of carotenoid was also observed in the 7.5:7.5 treatment. However, the lowest chlorophyll *a*, chlorophyll *b* and carotenoid were observed in the 1:14 treatment.

The leaf net photosynthetic (P_n) value was lowest at 14:1 treatment (Fig. 4), but there was no significant difference among 9:6, 7.5:7.5 and 1:14 treatments. It was

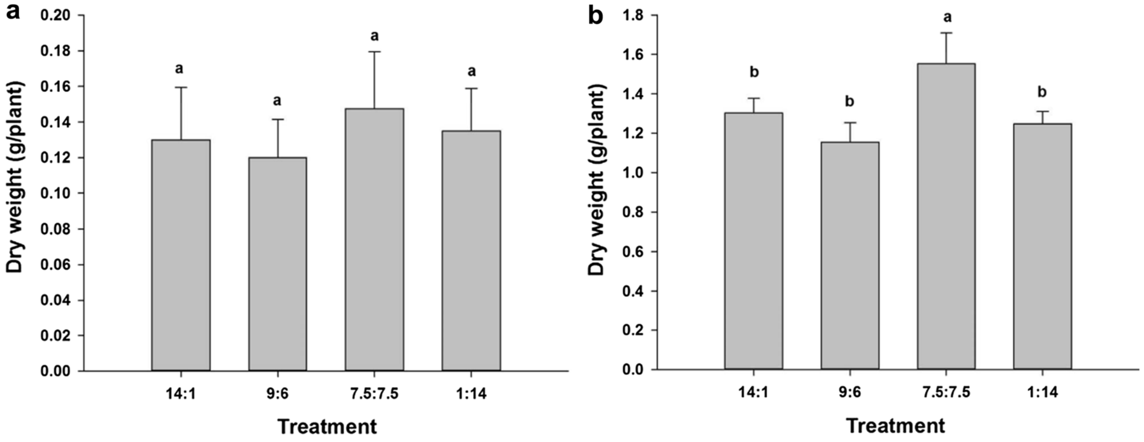

Fig. 1 The dry weight (DW) of roots (**a**) and shoot (**b**) of oilseed rape grown under different NO_3^-:NH_4^+ ratios. *Different letters* indicate significant difference at $P < 0.05$ by LSD test between means (±SE) of treatments ($n = 4$)

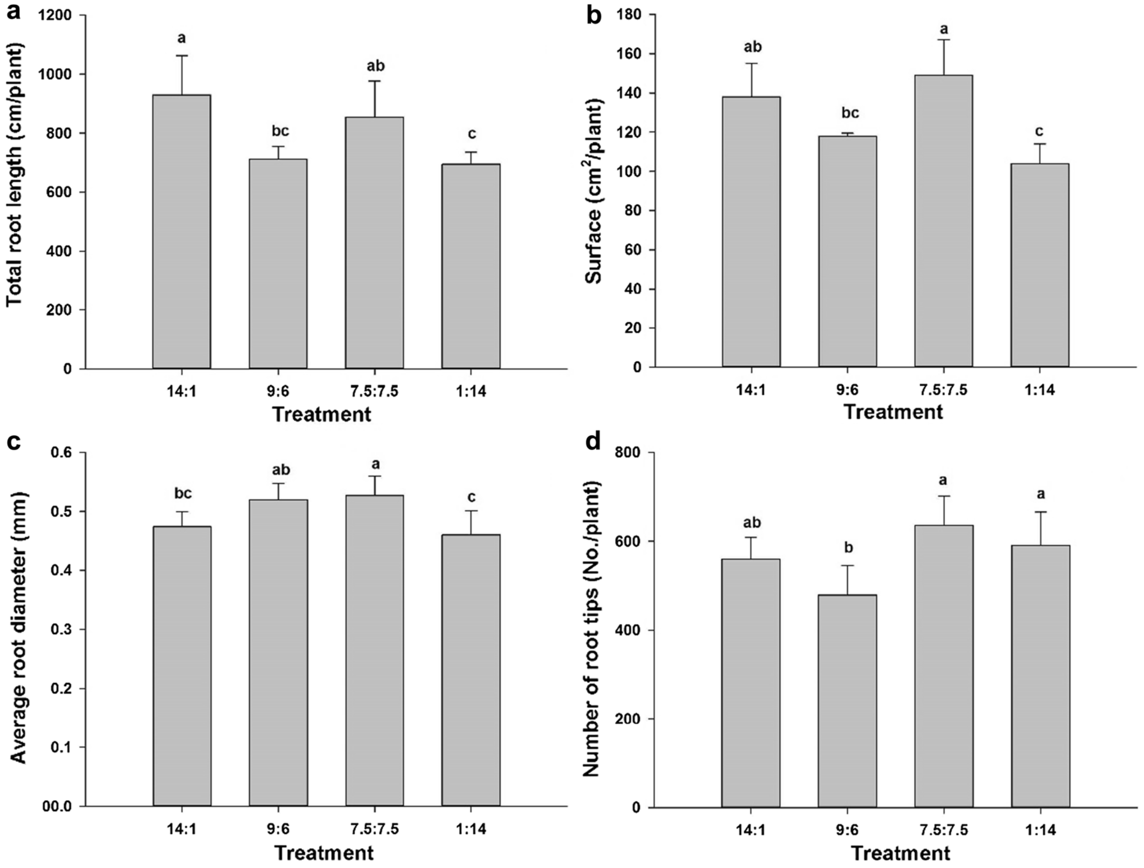

Fig. 2 The total root length (**a**), surface area (**b**), average diameter (**c**) and number of root tips (**d**) of oilseed rape under different NO_3^-:NH_4^+ ratios. *Different letters* indicate significant difference at $P < 0.05$ by LSD test between means (±SE) of treatments ($n = 4$)

observed that the highest stomatal conductance (G_s), intercellular CO_2 concentration (C_i) and transpiration rate (T_r) were observed in the 1:14 treatment, while no significant difference was seen in the 14:1, 9:6 and 7.5:7.5 treatments.

Nitrate reductase (NR), soluble sugar and soluble protein

Nitrate reductase catalyzes NAD(P)H reduction of nitrate to nitrite and is an enzyme highly regulated by nitrogen

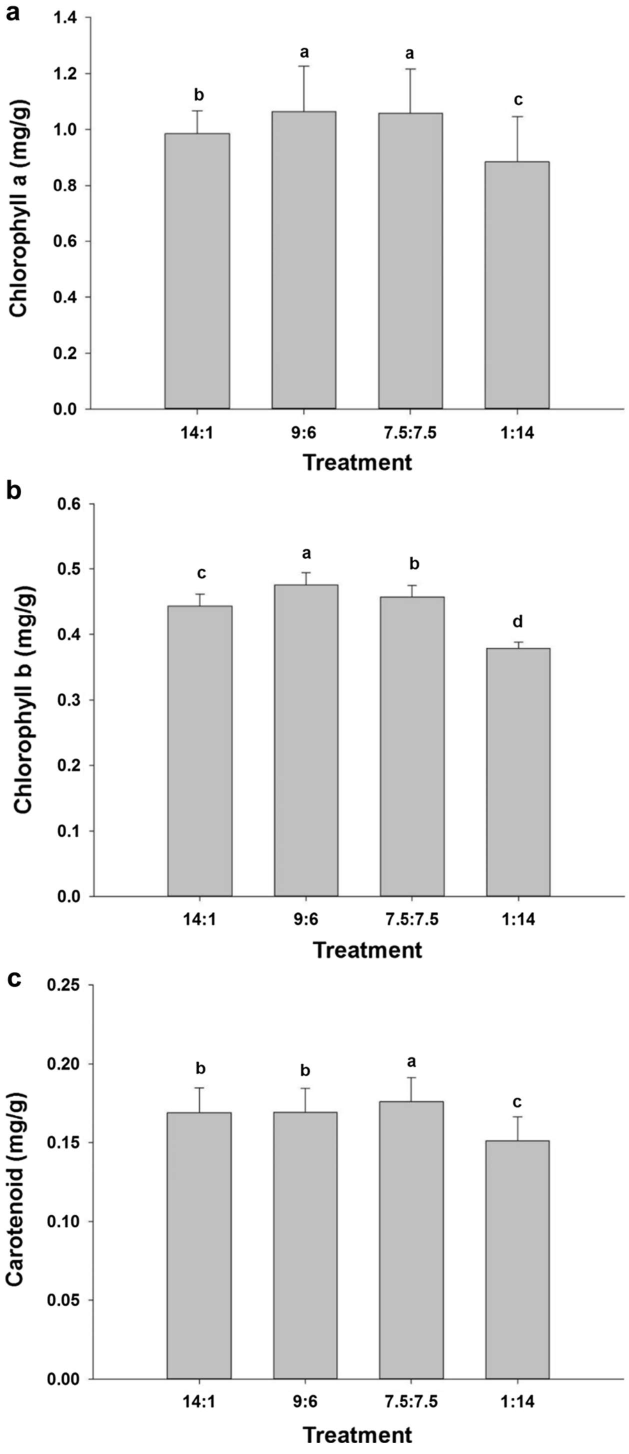

Fig. 3 The chlorophyll *a* (**a**), chlorophyll *b* (**b**) and carotenoid (**c**) contents of oilseed rapt under different NO_3^-:NH_4^+ ratios. *Different letters* indicate significant difference at $P < 0.05$ by LSD test between means (±SE) of treatments ($n = 4$)

availability. The maximum of NR activity observed was at 9:6 treatment, and the range of NR activity was 9:6 > 7.5:7.5 > 14:1 > 1:14 (Fig. 5). Compared with the 9:6 treatment, the NR activity of 14:1 and 1:14 was decreased to 57.0 and 66.1%, respectively.

The leaf soluble sugar and soluble protein were highest (2.75% and 83.9 mg/g) in the 7.5:7.5 treatment (Fig. 6). Compared with the 7.5:7.5 treatment, the leaf soluble sugar and soluble protein were decreased by 36.2 and 22.2% in the 14:1 treatment, and 17.5 and 14.4% in the 1:14 treatment.

Discussion

Domesticated plants are most sensitive to NH_4^+ toxicity including tomato, potato, barley, bean and pea (Britto and Kronzucker 2002). Further, growth inhibition by ammonium was initially observed in shoots more than in roots of plants, such as barley and sugar beets (Britto and Kronzucker 2002; Raab and Terry 1994). In our study, the oilseed rape leaf dry weight (DW) was affected by the ratio of NO_3^-:NH_4^+ in the nutrient solution. The maximum of DW was observed at 7.5:7.5, while there was no significant difference on root DW under NO_3^-:NH_4^+ ratios. Shoots are believed to be significantly more sensitive than roots when in direct contact with NH_4^+. Combining our results, it is likely that oilseed rape is also sensitive to high NO_3^-:NH_4^+ and low NO_3^-:NH_4^+ ratios, whereas high NO_3^-:NH_4^+ and low NO_3^-:NH_4^+ ratios inhibited plant growth at the shoot level first.

In addition, a stunted root system is the most visible phenotype when exposed to high NO_3^- or NH_4^+ in the growth medium, particularly for NH_4^+ (Balkos et al. 2010). Here, we showed that total root length, root surface area and average root diameter were inhibited by high concentration of NH_4^+ (1:14), while only average diameter was inhibited by a high concentration NO_3^- (14:1), indicating that NH_4^+ strongly damaged root development, but the NO_3^- signal appeared to have a localized stimulatory effect on lateral root development. Results also indicate that oilseed rape root growth is better in high NO_3^-:NH_4^+ than in low NO_3^-:NH_4^+ ratios. This result might be due to higher NH_4^+ accumulation in the roots than in leaves (Roosta and Schjoerring 2007), and evidences from previous studies indicated that the inhibition of root growth by NH_4^+ was mediated by the root tip and was linked to NH_4^+ cycling at the root plasma membrane and enzyme activities, such as GDP-mannose pyrophosphorylase (GMPase) (Qin et al. 2008; Barth et al. 2010; Li et al. 2010).

In hypersensitive plant, the chloroplasts and photosynthesis are the primary physiologic targets of enhanced NH_4^+ toxicity (Yang et al. 2015). What is more, photosynthetic pigments are the central part of the

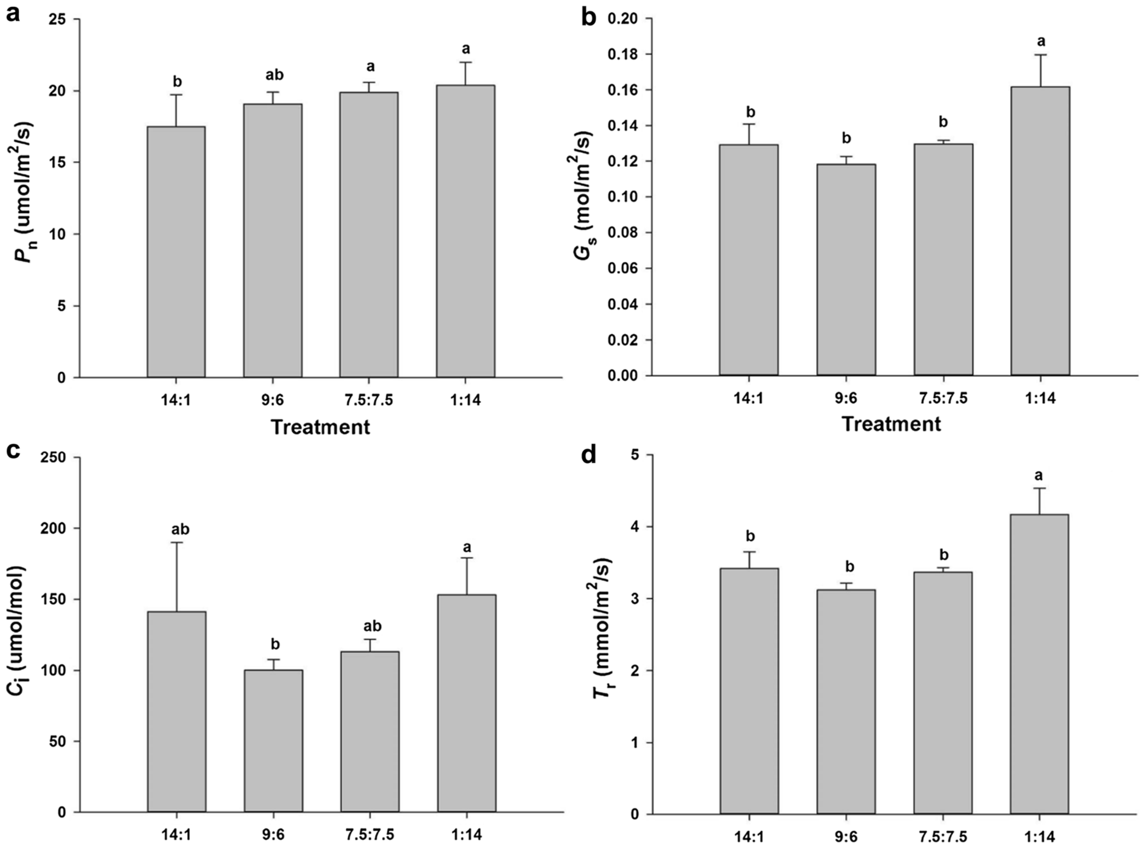

Fig. 4 The leaf net photosynthetic (**a**), stomatal conductance (**b**), intercellular CO_2 concentration (**c**) and transpiration rate (**d**) of oilseed rape at different NO_3^-:NH_4^+ ratios. *Different letters* indicate significant difference at $P < 0.05$ by LSD test between means (±SE) of treatments ($n = 4$)

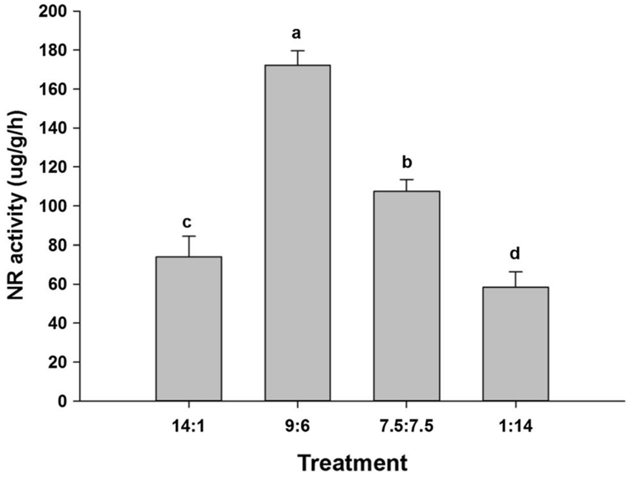

Fig. 5 The leaf nitrate reductase (NR) activities of oilseed rape under different NO_3^-:NH_4^+ ratios. *Different letters* indicate significant difference at $P < 0.05$ by LSD test between means (±SE) of treatments ($n = 4$)

photosynthetic system in green plants and any significant alteration in their levels is likely to cause a marked effect on the entire metabolism of the plant (Mishra and Agrawal 2006). In oilseed rape, the decreased chlorophyll *a*, chlorophyll *b* and carotenoid contents (Fig. 4) suggest that photosynthesis was inhibited. Similar research has also demonstrated that the photosynthetic rates were higher in combination with NO_3^- and NH_4^+ (Britto and Kronzucker 2002). In our study, however, the leaf net photosynthetic (P_n) values, stomatal conductance (G_s), intercellular CO_2 concentration (C_i) and transpiration rate (T_r) of oilseed rape were enhanced under a higher concentration of NH_4^+ (1:14), indicating that plants may excessively synthesize carbohydrate that was transported to root for NH_4^+ transport and metabolism to prevent NH_4^+ toxicity (Britto et al. 2001; Rare 1990). Additionally, the reduced photosynthetic rates and the need for a massive energy-consuming allocation of carbohydrates to the root for NH_4^+ assimilation in the roots have been associated with the negative effects of NH_4^+. It has been proved that the exogenous supply of organic or inorganic carbon could help the plant to tolerate NH_4^+ stress (Britto et al. 2001; Cao et al. 2009; Roosta and Schjoerring 2008).

To gain an insight into the physiological changes occurring during stress, we studied the changes in NR, soluble sugar and soluble protein content under different NO_3^-:NH_4^+ ratios. As it is known, nitrogen is required by plants in higher quantities to support the use of CO_2, water

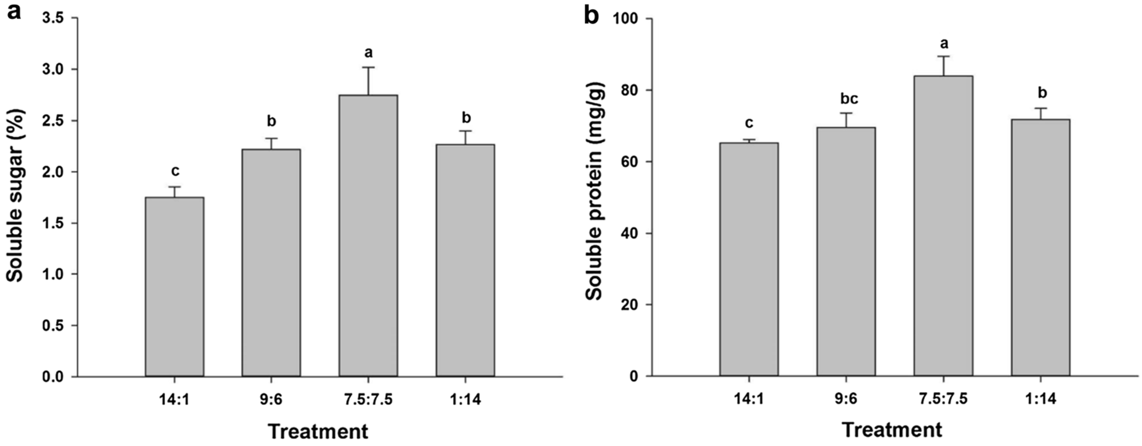

Fig. 6 The leaf soluble sugar (**a**) and soluble protein (**b**) of oilseed rape under different $NO_3^-:NH_4^+$ ratios. *Different letters* indicate significant difference at $P < 0.05$ by LSD test between means (±SE) of treatments ($n = 4$)

and inorganic nitrogen to produce sugar, organic acids and amino acid, the basic building blocks of biomass accumulation. The response to different $NO_3^-:NH_4^+$ ratios, the arrangement of nitrate reductase activity, soluble sugar and soluble protein in the leaves were $9:6 > 7.5:7.5 > 14:1 > 1:14$. Our results suggest that equal or near equal $NO_3^-:NH_4^+$ ratio had a highly favorable effect on photosynthetic pigments and leaf net photosynthetic values, which lead to enhanced photosynthesis and increase in the photosynthetic product, soluble sugar. In addition, NR involves the operation of nitrate and nitrite reductase to generate ammonium in leaves, which is then assimilated via the glutamine synthetase (GS)–glutamine-2-oxoglutarate aminotransferase (GOGAT) pathway. The operation of the GS/GOGAT pathway leads to the production of glutamate, which is the C and N source for the biosynthesis of most amino acids for proteins (Forde and Lea 2007; Nunes-Nesi et al. 2010). The maximum soluble protein sugar content was also observed at equal NO_3^-:-NH_4^+ ratio treatment, which revealed the highest N metabolism.

Conclusion

Results from the present study show that oilseed rape was more adaptive to growth at equal $NO_3^-:NH_4^+$ ratio than that at high or low $NO_3^-:NH_4^+$ ratio. The growth and physiological parameters, including biomass, pigment, NR activity, soluble sugar and soluble protein, are optimal at equal $NO_3^-:NH_4^+$ ratio. High or low $NO_3^-:NH_4^+$ ratio had a stronger inhibitory effect on the growth parameters. Overall, a combination of two forms of nitrogen in an equal ratio (7.5:7.5 $NO_3^-:NH_4^+$) appears to be beneficial to oilseed rape growth.

Author contribution statement Conceived and designed the experiments: XCS, XHZ and CXH. Performed the experiments: SYQ, JX and XW. Analyzed the data: QT. Wrote the paper: SYQ, XCS and QT. All authors have contributed significantly and all authors are in agreement with the content of the manuscript.

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