1 **Interactive effects of nitrate-ammonium ratios and temperatures on**
2 **growth, photosynthesis, and nitrogen metabolism of tomato seedlings**

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8 Running title: Interaction of the nitrate-ammonium ratio and temperature

9
10 ABSTRACT: Growth of many species of plants is optimal when the two major forms
11 of N that are assimilated by plants are supplied at a particular ratio. This ratio is
12 affected by both species and the environment. We assessed the effects of different
13 ratios of nitrate to ammonium (N-A ratio) supplied to hydroponically grown chilling
14 stressed and non-stressed tomato seedlings on several parameters. When the plants
15 were grown in normal temperature (14-30°C), growth parameters, photosynthetic rate,
16 chlorophyll concentration, soluble protein in roots, and leaf nitrates were greatest
17 when the N-A ratio was 75:25. The activities of glutamine synthetase (GS) and
18 NADH-dependent glutamate synthetase (NADH-GOGAT) in leaves were maximal
19 when the N-A ratio was 50:50, while NADH-GOGAT in roots was maximal when the
20 ratio was 25:75. Soluble protein in leaves and NO₃⁻ content in roots and nitrate

Abbreviations: CT, chilling temperature; NR, nitrate reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; Pn, net photosynthetic rate; NO₃⁻, nitrate; NH₄⁺, ammonium; SP, soluble protein; FAA, free amino acids

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reductase activity were positively correlated with N-A ratio, while free amino acids, total N, and NH_4^+ content were negatively with this ratio. Under chilling temperature (5-18°C), growth parameters, photosynthetic rate, soluble protein in roots, leaf nitrate, and GS activity in roots also had an optimal N-A ratio of 50:50, while GS activity in leaves and NADH-GOGAT activity were the highest when the N-A ratio was 25:75. Increased NH_4^+ , resulting in an N-A ratio of 75:25, improved NR activity, and NO_3^- in roots in CT. The leaf chlorophyll content was not affected by the N-A ratio. Plant growth parameters, N content in roots, NO_3^- and NH_4^+ concentrations, photosynthetic rate, leaf chlorophyll content and key enzymes activities of nitrogen metabolism were influenced by the N-A ratio, while the concentrations of soluble protein, free amino acids, and total leaf N did not appear to be influenced by the interaction of temperature and N supply. The optimal N-A ratio of tomato seedling appears to be 75:25 for unstressed plants and 50:50 for plants grown in chilling temperatures.

Keywords: Nitrate-ammonium ratio; Nutrient solution; Chilling temperature; Tomato

1. Introduction

Nitrogen is an essential nutrient for plants and, when limiting, can reduce growth and crop productivity (Glass, 2005; Piwpuan et al., 2013). Plants assimilate both ammonium (NH_4^+) and nitrate (NO_3^-) from the soil solution and these two inorganic forms are their most important nitrogen sources (Miller and Cramer, 2005; Luo et al., 2013). Uptake and assimilation of ammonium by plants requires less energy than uptake and assimilation of nitrate, leading to the proposal that, of the two, ammonium may be the more efficient and advantageous nitrogen source. However, it has been shown that the growth and development of crop plants cultured without soil may be more dependent on nitrate than ammonium. It is also clear that the relative availability of the two nitrogen forms differs between environments (Britto et al., 2001), making the nitrogen source an important consideration in determining optimal growth conditions. In higher plants, uptake of NH_4^+ follows its electrochemical gradient, utilizing a uniport carrier system across the plasma membrane, while NO_3^- uptake is active, via a proton-anion carrier employing co-transport of two protons (Tischner, 2006; Lambers et al., 2008). Nitrate must be reduced to ammonium in cells by two consecutive reductions. First, nitrate is reduced to nitrite by nitrate reductase (NR), followed by reduction of the nitrite to ammonium by nitrite reductase. These reactions employ the reducing power of NADH or NADPH from respiration or photosynthesis (Piwpuan et al., 2013). The ammonium can be incorporated into carbon skeletons to produce a variety of amino acids (Miller and Gramer, 2005). These complex processes may account for the higher energy costs associated with nitrate as a nitrogen source compared to ammonium (Britto and Kronzucker, 2002; Guo et al., 2007). In support of this suggestion, the addition of NH_4^+ to the nutrient solution increased the growth of several plant species (Sonneveld, 2002).

Despite the apparent preference of plants for one of the two nitrogen sources, it would appear that most plants perform best at a particular nitrate-ammonium ratio (N-A ratio). This ratio appears to regulate distribution of assimilated N between shoots and roots. It may also differ between species (Britto et al., 2001; Britto et al., 2002; Sonneveld, 2002), as Chinese cabbage exhibited maximal growth when the N-A ratio was 75:25 (Chen et al., 2005), while spinach grew maximally when it was 50:50 (Mills et al., 1976). The optimal ratio may also depend on environmental conditions such as pH (Borgognone et al., 2013), light intensity (Tabatabaei et al., 2008), and the temperature of the root zone (Sattelmacher et al., 1993). Of particular interest to us is the relationship between species' optimal N-A ratio and temperature. Little is known about this issue, although it has been shown that nitrate uptake is more temperature sensitive than ammonium uptake: nitrate uptake predominates at warm temperatures while ammonium predominates at cool temperatures (Warren, 2009).

Cold temperatures can severely restrict plant growth, development, and result in physiological disorders (Theocharis et al., 2012) such as decreased chlorophyll biosynthesis, net photosynthetic rate (Pn), carbohydrate metabolism, and crop yield and quality (Tewari and Tripathy, 1998; Beck et al., 2004). Chilling can also damage membrane lipids, proteins, nucleic acids, and disrupt cellular homeostasis (Kang and Saltveit, 2002; Feng et al., 2003; Theocharis et al., 2012). In agriculture areas subjected to chilling temperatures (CT), including high latitude regions such as northern China (Qin et al., 2011; Liu et al., 2013), production of chilling sensitive plants may be compromised during cold seasons, even when they are grown in a greenhouse (Miao et al., 2009). Tomato (*Lycopersicon esculentum* Mill.), of tropical origin, is sensitive to temperatures less than 10°C (Lurie and Klein, 1991). This is an important agricultural issue, as tomatoes are often grown in unheated greenhouses in

the winter, where they experience low temperatures. Night temperatures of 6 or 9°C led to an irreversible reduction in Pn, stomatal limitation of CO₂ supply, and decreased activity of ribulose-bis-phosphate carboxylase in tomato (Liu et al., 2012).

Uptake and assimilation of nitrogen may be affected by chilling temperatures and, specifically, the optimal N-A ratio in a particular species may be altered by CT. This experiment was undertaken to assess the effect on various parameters of different N-A ratios on tomato plants subjected to CT. Specifically, we examined growth, Pn, and nitrogen metabolism characteristics in tomato seedlings.

2. Materials and methods

2.1 Plant material and growth conditions

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Xi Nong 2011) were sown in hole trays in a seedling substrate (pH 5.5–6.5) with an organic mass fraction $\geq 50\%$ and a humic acid mass fraction $\geq 20\%$. When the third leaves were fully expanded, seedlings were transplanted into rectangular hydroponic containers and supplied with half-strength modified ShanQi's nutrient solution containing 0.75 mM Ca(NO₃)₂, 2 mM KNO₃, 0.5 mM (NH₄)₂HPO₄, 0.5 mM MgSO₄·7H₂O, 25 μ M EDTA-Fe, 1.25 μ M H₃BO₃, 1.0 μ M MnSO₄·4H₂O, 1.0 μ M ZnSO₄·7H₂O, 0.25 μ M CuSO₄·5H₂O, and 0.25 μ M (NH₄)₆Mo₇O₂₄·4H₂O. The containers were supplied with air bubblers to ensure adequate oxygen supply. The plants were placed in a greenhouse with an average temperature of 25°C during the day and 15°C during the night. Plants received 16 h of light (800 mmol·photons m⁻²·s⁻¹) and 8 h of dark. The relative humidity was 60%–90%.

2.2 Chilling temperature and NO₃⁻-N/NH₄⁺-N ratio treatments

Five days after transplanting to the hydroponic containers, tomato seedlings were

divided into two groups and randomly placed in two identical environmentally controlled multi-span greenhouses. Temperature was maintained by regulating the radiator heating system, a wet-pad cooling system, and opening and closing a skylight. The CTs ranged from 5°C to 18°C and the normal temperatures ranged from 14°C to 30°C. The greenhouse temperatures were monitored and recorded with automated sensors. The highest and lowest temperatures are presented in Figure 1.

Five N-A ratios were selected and supplied in Modified ShanQi's solution. These ratios were: 100:0, 75:25, 50:50, 25:75, and 0:100. The details are shown in Table 1. The final concentrations of the macrolelements were 8 mM N, 4 mM K, 2.5 mM Ca, 1 mM P, and 1 mM Mg. The final concentration of the microelements were 2.5 µM B, 2.0 µM Mn, 2.0 µM Zn, 0.5 µM Cu, and 0.5 µM Mo. Iron was supplied as EDTA-Fe for a final concentration of 50 µM and 7 µM dicyandiamide was added in all nutrient solutions to prevent the conversion of ammonium into nitrate. The nutrient solution was replaced every 5 days. The changes in N-A ratios were balanced by varying the Cl^- concentration to maintain constant N, P, K, Ca, and Mg concentrations in all treatments. The electrical conductivity was 2.5-2.6 dS m⁻¹. The pH was adjusted daily within the range of 6.0–6.1.

2.3. Plant growth analysis

At the start of the experiment and 20 days later, five seedlings from each treatment were selected randomly to measure the fresh and dry weights of roots and shoots. Plant material was weighed and then dried in an air-forced oven at 105°C for 20 min and then at 80°C 48 h, at which time the material had reached constant weight. The data were used to calculate relative growth rate (RGR). The RGR in d⁻¹ = $(\ln W_2 - \ln W_1) / (t_2 - t_1)$, where W1 and W2 were the initial and final biomass and t1 and t2

were the initial and final time in days, respectively (Kingsbury et al., 1984).

2.4 Photosynthetic rate and chlorophyll analysis

Net photosynthesis and the chlorophyll content were measured in the third youngest fully expanded leaf of five plants. Photosynthesis was measured over a 2-h period early in the photoperiod with a portable photosynthetic system, Li-6400 (LI-COR, USA). Chlorophyll was analyzed according to the method described by Harmuutk (1987).

2.5 Analysis of activities of key enzymes nitrogen metabolism

The activities of key enzymes of N metabolism in the plant tissues were analyzed 20 days after the start of treatment. After harvesting, the fresh tissues were frozen immediately in liquid nitrogen and stored at -80°C until analysis. For analysis, tissues were ground to powder in a mortar that had been pre-cooled with liquid nitrogen and then homogenized in the appropriate extraction buffer.

Nitrate reductase (NR) activity was estimated according to Scheible et al. (1997) and Piwpuan et al. (2013). Five-hundred milligrams of ground tissue were homogenized in 3 mL ice-cold extraction buffer consisting of 100 mM HEPES-KOH (pH 7.5), 1% (w/v) polyvinylpyrrolidone, 1 mM EDTA, 5 mM $\text{Mg}(\text{CH}_3\text{COOH})_2$, 5 μM Na_2MoO_4 , 10% (v/v) glycerol, 5 mM dithiothreitol (DTT), 0.1% Triton X-100, 1% (w/v) BSA, 20 μM flavin adenine dinucleotide, 25 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, the latter in 99% ethanol. One-hundred microliters of the extraction mixture were transferred to an eppendorf tube placed on ice, then added to 0.5 mL of assay medium containing 100 mM HEPES-KOH (pH 7.5), 5 mM EDTA, 5 mM KNO_3 , and 0.25 mM NADH at 25°C. The mixture was incubated at 25 °C for 30 min and the reaction was stopped by adding a stop reagent containing 75 μL 0.15

mM phenazine methosulphate and 25 μ L 0.6 M $\text{Zn}(\text{CH}_3\text{COOH})_2$. For the control, the stop reagent was added before the assay mixture. Samples were incubated at room temperature for 15 min and 300 μ L of a color reagent, 0.02% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 3 M HCl, was added. The reagent will react with the nitrite ion and permit quantification of NR activity by spectrophotometry. The reaction proceeded for 30 min at room temperature, after which it was centrifuged at $14,000\times g$ (Biofuge Stratos, Osterode, Germany; used throughout) for 2 min. The absorbance of the supernatants was measured at 540 nm and NR activity was expressed as $\mu\text{g NO}_2^- \cdot \text{g fresh weight}^{-1} \cdot \text{h}^{-1}$.

Glutamine synthetase (GS) activity in roots and leaves was measured following the method described by Wang et al. (2008) and Luo et al. (2013). The ground samples of five-hundred milligrams were homogenized in 50 mM Tris-HCl (pH 8.0), 2 mM MgCl_2 , 0.4 M sucrose, and 2 mM DTT and centrifuged at $15,000\times g$ for 20 min, all at 4°C. The supernatants were incubated at 37°C for 30 min in reaction medium contained 0.8 mL of 0.1 M Tris-HCl buffer (pH 7.4), 0.35 mL of 40 mM ATP, 80 mM NH_2OH , 2 mM EGTA, 20 mM Na-glutamate, 80 mM MgSO_4 , and 20 mM cysteine, after which the reaction was stopped with ferric chloride reagent (0.6 M HCl, 0.2 M trichloroacetic acid, 0.37 M FeCl_3). The reaction mixtures were centrifuged at $5000\times g$ for 15 min at 4°C and the absorbance of the resultant reaction mixture was measured at 540 nm. One unit of GS activity was defined by the appearance of 1 μmol of γ -glutamyl hydroxamate per hour.

Activity of the NADH-dependent glutamate synthetase (NADH-GOGAT) was measured by colorimetric assay as reported by Lin and Kao (1996). Ground tissues of five-hundred milligrams were homogenized in 10 mM Tris-HCl buffer (pH 7.6) with

1 mM β -mercaptoethanol, 1 mM EDTA, and 1 mM MgCl_2 on ice and centrifuged at 15,000 $\times g$ for 30 min at 4 °C. The supernatant was used for determination of NADH-GOGAT activity in a reaction mixture containing 0.875 mL 25 mM Tris-HCl buffer (pH 7.6), 0.1 mL 3 mM NADH, 0.2 mL 20 mM l-glutamine, 50 μL 10 mM KCl, 25 μL 0.1 M 2-oxoglutarate, and 0.25 mL of enzyme extract. The decreases in absorbance were read at 340 nm for 5 min. Enzyme activity was calculated from a standard curve prepared with NADH. One unit of enzyme activity is defined as 1 μmol NADH oxidized $\cdot \text{g}$ fresh weight⁻¹·h⁻¹.

2.6 The analysis of NO_3^- -N, NH_4^+ -N and N contents

Tissue samples were dried in an air-forced oven at 80°C to constant weight. Two-hundred milligrams of the dried tissue were ground to a powder and extracted in 10 mL of distilled water for 2.5 h. The nitrate content of the solution was spectrophotometrically after mixing 0.2 mL of solution with 10% (w/v) salicylic acid in 96% sulfuric acid. The values were quantified after generating a standard curve (Cataldo et al., 1975). The ammonium content was determined with the colorimetric assay described by Krom (1980). Total N in the plant tissues was measured by the Kjeldahl method.

2.7 Analysis of soluble protein and free amino acid content

Five-hundred milligrams frozen root and leaf were extracted in a 50 mM cold potassium-phosphate buffer at pH 7.0, and centrifuged at 4°C for 15 min at 12,000 $\times g$ in a refrigerated high-speed centrifuge (Biofuge Stratos, Osterode, Germany). The resulting supernatant was used to quantify of soluble protein (SP) and free amino acids (FAA). The SP content was estimated by the Coomassie blue staining method with BSA as standard (Bradford, 1976). The FAA content was estimated with the

ninhydrin method described by Yemm et al. (1955).

2.8 Statistic analysis

All the measurements were measured from 5 replicates. Data were statistically analyzed with SAS software (Version 8.1; SAS Institute, Cary, NC, USA) and were tested for homogeneity of variance by means of LSD's multiple range test at the level of 0.05. Graphs were drawn with Excel 2013 software.

3. Results

3.1 Plant growth

Biomass and RGR of tomato seedlings were affected by temperature, as is apparent in Table 2. There was a significant reduction in both fresh and dry weight in CT treatments compared to the control (grown at normal temperatures; $p < 0.05$). The relative growth rate of plants in CT was only 65.5% of the control. Biomass accumulation and RGR were also affected by the N-A ratio. As the ratio decreased, starting with 100% nitrate, the growth parameters increased, but then decreased, indicating to us that there was an optimal ratio, and that growth was optimized when both N forms were supplied. This ratio, however, differed in the two temperature regimes: it was 75:25 for the control and 50:50 for CT. As the contribution of nitrate decreased beyond these ratios and ammonium predominated, biomass accumulation decreased. We conclude that growth was negatively affected by a high concentration of ammonium in the solution.

3.2 Photosynthetic rate and chlorophyll content

Chilling stress dramatically reduced Pn, while slightly reduced chlorophyll content in the leaves of the tomato seedlings (Fig. 2) compared to the control ($p <$

0.05). The Pn and chlorophyll content in CT plants were 74.25% and 92.87%, respectively, of these values in the control plants.

When the N supply was 100% ammonium (an N-A ratio of 0:100), Pn and the chlorophyll content of leaves were reduced compared to other N-A ratios, in both temperatures (Fig. 2). As with the growth parameters, there appeared to be an optimal N-A ratio for Pn, but this ratio differed with respect to temperature. Net photosynthesis was greatest when the N-A ratio was 75:25 for the control, unchilled plants and 50:50 for plants grown at CTs. These optimal ratios were the same for both photosynthesis and growth (Fig. 2, Table 2). The amount of chlorophyll was affected by the N-A ratio in the control plants, but not in the chilled plants.

3.3 Key enzymes of nitrogen metabolism

Nitrate reductase activity: The activity of NR was measured in both the roots and shoots of the tomato seedlings (Fig. 3). Activity was greater in the shoots than in the roots in all treatments. The results presented in Figure 3 also show that NR activity was dependent on temperature. Chilling temperatures reduced NR activity in both leaves and roots, by 31.25% and 39.55%, respectively, of the control values.

Nitrate reductase activity was positively correlated with the nitrate contribution to the N source in the control plants. When plants were grown under CTs, however, the NR activity increased when the ammonium contribution was increased from 0 to 25%. It was lower when the N-A ratio was 50:50, but the highest NR activity was observed at a ratio of 75:25. In general, when the ammonium ion was greater than 25% of the N supplied to the seedling, NR activity was markedly reduced regardless of temperature.

Glutamine synthetase activity: Glutamine synthetase activity was also measured

in the tomato seedlings. The results are presented in Figure 4. The activity of this enzyme was higher in the leaves than in the roots, regardless of temperature treatment.

The activity of GS was reduced by CTs in both leaves and roots of tomato seedlings compared to the unchilled control, by 40.11% and 43.53%, respectively. In general, GS activity of both leaves and roots of the control plants increased when the ammonium contribution increased and the highest activity was observed when the N-A ratio was 50:50. For chilled seedlings, the highest activity was observed at an N-A ratio of 25:75 in leaves and 50:50 in roots.

Activity of NADH-dependent glutamate synthetase. The activity of NADH-GOGAT in tomato seedling is presented in Figure 5. Chilling stress reduced the enzyme's activity both leaves and roots, by 44.45% and 29.45%, respectively, as compared to the control at normal temperature. In the control, as the N-A ratio was decreased from 100:0, the activity of NADH-GOGAT was first increased by the increased ammonium, but was then reduced as the ammonium concentration increased. The highest enzyme activity was observed when the N-A ratio was 50:50 in leaves and at 25:75 in roots. In seedlings grown at CTs, the highest NADH-GOGAT activity in leaves was observed at an N-A ratio of 25:75.

3.4 The concentrations of soluble protein and free amino acids

The concentrations of SP and FAA of tomato seedling leaves and roots are presented in Table 3. In general, the SP concentration was positively correlated with the N-A ratio and the FAA was negatively correlated, in both leaves and roots and regardless of temperature treatment. No difference in SP content with respect to temperature regime was apparent in roots, while the SP content in leaves was reduced

in leaves of plants exposed to CT compared to the control. Chilling stress also notably increased FAA content of tomato seedling leaves and roots compared to plants grown in normal temperatures, by 38.95% and 18.87%, respectively, as compared to NT treatment. There was no obvious interaction between the N-A ratio and temperature on SP and FAA content.

3.5 The concentrations of N, NO₃⁻, and NH₄⁺ in seedlings

The concentrations of N, NO₃⁻, and NH₄⁺, and various other forms of N in plant tissues are given in Table 4. The results indicate that the nitrate and ammonium contents in the leaves were significantly influenced by the N-A ratio in the solution and by temperature, while these factors had little influence on the N forms present in roots ($p < 0.01$). The concentrations of N were higher and the NO₃⁻ and NH₄⁺ concentrations were lower in leaves than in roots. Chilling resulted in a decrease in the concentrations of NO₃⁻ and NH₄⁺ and an increase in N concentration, in both leaves and roots.

There was a strong positive correlation between N and NH₄⁺ concentrations and the ammonium concentration in the nutrient solution in both leaves and roots. Control plants grown on an N-A ratio of 75:25 contained more NO₃⁻ than plants exposed to the other N-A ratios. The highest levels of NO₃⁻ in chilled plants was when the N-A ratio was 50:50 in leaves and 75:25 in roots.

4. Discussion

Chilling stress suppresses growth and disrupts physiological processes in many species, including tomato. Such species are described as chilling sensitive (Brüggemann and Linger, 1994; Ahn et al., 1999; Rosa et al., 2009; Ruelland and Zachowski, 2010). Another factor in plant growth is the form of N present in the soil

303 solution. Exclusive supply of either ammonium or nitrate can impair growth
304 (Claussen, 2002; Guo et al., 2007), as demonstrated by Tabatabaei et al. (2008), who
305 observed that increased concentration of NO_3^- reduced strawberry plant growth.
306 Indeed, a proper combination NH_4^+ and NO_3^- has been recognized to be important in
307 growth of most plant species (Britto and Kronzucker, 2002).

308 The ideal N-A ratio is known to be affected by environmental factors and, among
309 others factors, it depends on root zone temperature (Sattelmacher et al., 1993). In this
310 study, we chose to examine the relationship between this ratio and temperature in
311 chilling sensitive plants. Tomato plants, which are chilling sensitive and are grown in
312 areas and situation in which they are exposed to cold temperatures, were selected.
313 This study should be of value to agriculture as the relative availability of NH_4^+ and
314 NO_3^- differs between environments (Britto et al., 2001; Haghghi et al., 2014) and
315 may affect crop yields.

316 First, we confirmed the negative effects of both cold and an unbalanced N supply
317 in tomato seedlings. Biomass accumulation and RGR were negatively affected by
318 both CTs and when N was supplied as solely nitrate or ammonium.

319 Our results showed that the tomato seedlings grew best at a particular N-A ratio,
320 but this optimal ratio differed with respect to temperature. Under CTs, the optimal
321 N-A ratio was less than it was for plants grown under normal temperatures, that is, the
322 stressed plants seemed to benefit from a supply that was enriched in ammonium. A
323 possible explanation for this is that the higher concentration of ammonium reduces
324 energy required for assimilation of inorganic nitrogen. However, a high concentration
325 of NH_4^+ appeared to be toxic to the seedlings, which could not tolerate more than 2
326 mM NH_4^+ in normal temperature or more than 4 mM NH_4^+ in CT.

It is often reported that low temperatures decrease Pn, presumably due to accumulation of phosphorylated intermediates and decreased ATP:ADP, which result when sucrose synthesis is inhibited (Hurry et al., 2000; Catoni and Gratani, 2014; Sales et al., 2013). In our study, CT severely reduced plant Pn of tomato seedlings.

There are numerous studies on the negative effects of high NH_4^+ concentrations on Pn, which we confirmed here. We observed decreased Pn when the N-A ratio was 0:100 compared to the other N supply regimes, that is, when the seedlings received only ammonium. This result is in agreement with the findings of Borgognone et al. (2013), who reported reduction of Pn in NH_4^+ fed plants. The unfavorable effect of ammonium on Pn has been attributed to NH_4^+ accumulation in plant leaves, which could cause uncoupling of the electron transport from photophosphorylation in chloroplasts (Guo et al., 2006). Moreover, the assimilation of high NH_4^+ should increase the demand for carbon skeletons to detoxify the excess NH_4^+ (Schortemeyer et al., 1997).

In contrast to the numerous studies of the effects high NH_4^+ on Pn, there are few reports on the effects of high nitrate on Pn. We observed that the optimal N-A ratio for Pn was 75:25 in unstressed plants and 50:50 for chilled plants. In other words, 100% nitrate was inhibitory to both, compared to the optimal ratio. Since this finding is novel, it would be of interest to explore this inhibitory effect of NO_3^- on plant Pn.

It is also apparent that, under chilling conditions, Pn varied with the N-A ratio. It may be that some of the negative effects of low temperature are mitigated by the N supply, particularly by an adequate level of ammonium. Chlorophyll may be a key player here. Chlorophyll levels are indicators of stress on the photosynthetic system, and tropical plants often show symptoms of chlorosis in low temperature, perhaps due

to inhibition of chlorophyll precursors or accelerated photodestruction of chlorophyll relative to its synthesis (Martino and Ort, 1992; Haghhigh et al., 2014). Here, chilling reduced chlorophyll in the seedlings, perhaps accounting for decreased Pn. Chlorophyll is an indicator of nitrogen nutrition in the rhizosphere and N stress negatively affects chlorophyll synthesis. In our experiments, high NH_4^+ levels and as well as 100% NO_3^- both reduced the chlorophyll content in unstressed seedlings, although the N-A ratio had no obvious influence on the chlorophyll content of the chilling stressed seedlings. This result is discrepant and may be due to unknown effects on absorption of the two forms of N.

Enzymes of nitrogen metabolism catalyze nitrogen reduction and assimilation, an important adaptation of plants to certain environmental conditions (Debouba et al., 2006). Nitrate reductase is the first enzyme involved in nitrate assimilation and catalyzes the reduction of nitrate to nitrite, using NADH, in the cytoplasm of higher plant cells (Hong et al., 2011). Nitrate reductase is regulated at the transcriptional as well as the post-translational level by various external and endogenous signals. However, rapid posttranslational modifications of the enzyme are thought to dominate the regulation of NR activity. (Jones et al., 1998; Lea et al., 2006). Low temperature reduced the diurnal activation of NR by delaying the circadian pattern of critical protein phosphatases (Jones et al., 1998). We observed reduced NR activity in chilled tomato seedling plants, consistent with previous studies (Finch-Savage et al., 2007). Generally, NR is induced by nitrate (Cedergreen and Madsen, 2003). In our study, it was clearly affected by the composition of the N supplied in the culture solutions. It has been frequently reported that NR activity is positively associated with the NO_3^- concentration in leaf tissues (Jampeetong and Brix, 2009; Munzarova et al., 2006) and has been reported to decrease dramatically or even completely when plants are

376 supplied with ammonium rather than nitrate (Konnerup and Brix, 2010). When
377 tomato seedlings were supplied solely with ammonium, they had lower NR activity
378 than control plants supplied with nitrate alone or chilling stressed plants supplied with
379 an N-A ratio of 75:25. The finding that an appropriate level of ammonium is
380 necessary for NR activity in seedling grown in CTs suggests that the additional
381 ammonium contributes to the improved Pn under stress.

382 For growth and development, plants must assimilate ammonium. This critical
383 biochemical process is catalyzed by the combined activity of GS and GOGAT. The
384 GS-GOGAT cycle is the main pathway by which plants incorporate ammonium into
385 carbon skeletons (Fernandez and Galvan, 2007; Martínez-Andújar et al., 2013). The
386 activities of both enzymes were reduced by chilling in this study and they may have
387 been negatively affected by reactive oxygen species that are produced during cold
388 stress. Due to amino acids in proteins and enzymes are a target for oxidative damage
389 and direct oxidation of the side chains leads to the formation of carbonyl groups
390 (Burton and Jauniaux, 2011). It is reported that when NH_4^+ instead of NO_3^- is used as
391 a nitrogen source, GS activity increases (Arnozis et al., 1988; Mihaljević et al., 2011),
392 although Sagi et al. (1998) did not observe an effect of the N form on GS activity in
393 ryegrass. The different responses, of course, may be due to comparisons between
394 genetically different species and cultivars, or environmental effects. We conclude,
395 though, that in this study, temperature and the N-A ratio interacted such that increased
396 NH_4^+ increased GS and GOGAT activity in chilling stressed plants.

397 Excessive ammonium ions are toxic in plants and, consequently, NH_4^+ uptake
398 and metabolism must be tightly regulated (Pérez-Tienda et al., 2014). Ammonium
399 assimilated by the roots is transported as amino acids, proteins, and other compounds
400 (Peoples and Gifford, 1997). Protein may constitute a major form of nitrogen storage

401 for many species and protein synthesis in plants can be regulated by temperature. In
402 addition, most SPs are metabolic enzymes and the SP content has been considered to
403 be an important index of overall metabolic activity in plants (Doganlar et al., 2010).
404 Although gene and protein expression in herbaceous species is altered by cold
405 temperatures, studies of the effect of cold on protein and gene expression are
406 inconsistent. Soluble proteins serve a protective function and accumulate in many
407 plants subjected to chilling stress (Corbel et al., 1999). This accumulation may be due
408 to either enhanced protein synthesis or reduced protein degradation (Zhang et al.,
409 2015). It is important to note that there are also studies that report no changes in
410 protein concentrations or protein patterning, for example in scots pine needles
411 exposed to low temperatures (Kontunen-Soppela et al., 2002). In this study, SP
412 decreased in leaves but not in roots of the stressed seedlings. Again, inconsistencies in
413 the literature may be due to the highly complex genetic background of the plant
414 varieties and the complicated natural environment. The relationship between soluble
415 protein content and cold stress is expected to be complex, and different tomato
416 varieties and species might have different mechanisms by which protein synthesis
417 responds to chilling temperature (Zhang et al., 2015). Amino acids in plants are
418 derived from ammonia assimilation, which is catalyzed by GS and GOGAT. Free
419 amino acids have a special role in cold acclimation, since their accumulation may
420 prevent the water loss from cells that occurs at low temperatures. In our study, CT
421 resulted in a large increase in total FAA of the tomato seedlings, which is in
422 agreement with Kovács et al., (2011), who found that cold acclimation increased total
423 FAA content in all the wheat genotypes studied. The amount of FAAs was positively
424 correlated with the N-A ratio in the two temperatures, leading us to suggest that the
425 ideal contribution of ammonium to the N supply increases total FAA, which would

increase tolerance of plants to low temperature.

It has been suggested that, when plants recover from low-temperature stress, additional N is required to support the increased net assimilation rate and the development of new tissues that accompanies recovery (Atkinson et al., 2015), a suggestion that was supported by a greater N concentration in cold-grown plants than in the unstressed control. These workers suggested that photosynthesis and respiration rates correlate with the N concentration, and the increases in N content would up-regulate carbon metabolism. In our study, the concentration of total N was greater in CTs than in the control, consistent with the proposal that the plants require more N to counterbalance the depressed biochemical efficiency caused by low temperature (Calatayud et al., 2008; Körner, 2003). The ability of the seedlings to assimilate NH_4^+ and NO_3^- may have been reduced under CT stress, resulting in less NH_4^+ and NO_3^- in the stressed plants than in the unstressed control. Nevertheless, many previous studies suggest that uptake of NO_3^- from nutrient solutions is more temperature sensitive than uptake of NH_4^+ . This may provide an important link between the form of N that is absorbed and temperature, with nitrate uptake being more inhibited than ammonium, so that the relative absorption rate of NH_4^+ was greater in CT. The total N concentration in the tomato seedling was affected by the N-A ratio; as this ratio increased, the total N concentration in tomato seedling plants generally decreased, in both temperatures. This is consistent with the findings of Bar-Tal et al. (2001) and Konnerup and Brix (2010), who both concluded that a decreased N-A ratio resulted in increased N concentration in the stem, leaves, and fruits of pepper. In our study, the concentration of nitrate and ammonium in tomato roots correlated with the N-A ratio in the nutrient solution, as was observed by others (Cruz et al., 2006; Horchani et al., 2010). Also, we noted a positive correlation between NH_4^+ concentration in the

nutrient solution and NH_4^+ concentration in tomato seedling plants. High concentrations of NH_4^+ in the nutrient solution may lead to NH_4^+ toxicity, as excess NH_4^+ can induce nutrient deficiency due to acidification of the root zone, impaired uptake of ions, alterations in the osmotic balance, modification of phytohormones, and impairment of N enzyme metabolism (Claussen and Lenz, 1999; Tabatabaei et al., 2008). As reported by Cruz et al. (2006) plant responses to NH_4^+ nutrition in the nutrient solution depend on the tissue concentration. When the N-A ratio of the nutrient solution was lower than 50:50, the concentration of NH_4^+ in the seedlings was great enough to disrupt plant growth, regardless of temperature.

5. Conclusions

We conclude that temperature affects not only growth but also nitrogen metabolism of tomato seedlings. The effects of chilling may also, in turn, be modulated by the form of N supplied in the soil solution. When the N-A ratio was 75:25 in unstressed plants, all parameters assessed—the seedling biomass; Pn; chlorophyll level—were greater than any of the other nutrient regimes. The activity of the key N metabolism enzymes; and total N, SP, and FAA also reaching higher level. Sustained higher activities of NR under this regime should enhance the absorption of nitrate and improve plant growth and quality. High GS and GOGAT activity should also promote growth, while the high content of SP and FAA should increase the resistance to low temperature.

The optimal N-A ratio for seedlings grown in CTs was 50:50. Apparently, the chilling stressed plant benefitted from more ammonium than was optimal for the unstressed plant, which may be due to the fact that less energy is consumed when ammonium is assimilated, leaving more resources for adaptation and coping with CTs.

It is possible that manipulating the N-A ratio may improve cold tolerance of chilling sensitive species. Determining and implementing the optimal N supply regime may also enhance yields of tomato and other plants.

Acknowledgments

This work was supported by National Science Foundation of China (No. 31471916), National Science and Technology Support Plan Project (No. 2014BAD14B06) and China Agriculture Research System.

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Fig. 1. The highest and lowest temperatures at two greenhouse during the entire period of experimentation

Fig. 2. Effect of nitrate-ammonium ratios and temperatures on net photosynthetic rate and chlorophyll content of tomato seedlings. The symbols in the figures are the means of five replicates and the error bars in the figures represent standard error. Different letters indicate significant differences between treatments according to LSD's test at $P < 0.05$.

■ 100/0 ■ 75/25 ■ 50/50 ■ 25/75 □ 0/100

Fig. 3. Effect of nitrate-ammonium ratios and temperatures on nitrate reductase activity in the leaves (left) and roots (right) of tomato seedlings. The symbols in the figures are the means of five replicates and the error bars in the figures represent standard error. Different letters indicate significant differences between treatments according to LSD's test at $P < 0.05$.

■ 100/0 ■ 75/25 ■ 50/50 ■ 25/75 □ 0/100

Fig. 4. Effect of nitrate-ammonium ratios and temperatures on glutamine synthetase activity in the leaves (left) and roots (right) of tomato seedlings. The symbols in the figures are the means of five replicates and the error bars in the figures represent standard error. Different letters indicate significant differences between treatments according to LSD's test at $P < 0.05$.

■ 100/0 ■ 75/25 ■ 50/50 ■ 25/75 □ 0/100

Fig. 5. Effect of nitrate-ammonium ratios and temperatures on GOGAT activity in the leaves (left) and roots (right) of tomato seedlings. The symbols in the figures are the means of five replicates and the error bars in the figures represent standard error. Different letters indicate significant differences between treatments according to LSD's test at $P < 0.05$.

Table 1. Nutrient solution composition (mmol/L)

Nutrient source	Nitrate-ammonium ratios in the nutrient solutions				
	100/0	75/25	50/50	25/75	0/100
KNO ₃	3	1			
Ca(NO ₃) ₂	2.5	2.5	2	1	
KH ₂ PO ₄	1	1	1	1	1
KCl		2	3	3	3
MgSO ₄	1	1	1	1	1
CaCl ₂			0.5	1.5	2.5
NH ₄ Cl		2	4	6	8

Table 2. Effect of nitrate-ammonium ratios and temperatures on growth of tomato seedlings.

Temperature	NO ₃ ⁻ -N/NH ₄ ⁺ -N	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight	RGR
	ratio	(g • plant ⁻¹)	(g • plant ⁻¹)	(g • plant ⁻¹)	(g • plant ⁻¹)	(d ⁻¹)
Normal temperature (14-30℃)	100/0	19.554±2.229 ^a	1.663±0.097 ^b	4.120±0.564 ^a	0.265±0.020 ^b	0.108±0.006 ^{ab}
	75/25	22.041±2.585 ^a	1.935±0.126 ^a	4.356±0.549 ^a	0.290±0.020 ^a	0.113±0.006 ^a
	50/50	16.510±1.836 ^b	1.540±0.087 ^b	2.876±0.311 ^b	0.220±0.014 ^c	0.098±0.005 ^b
	25/75	6.502±1.180 ^{de}	0.699±0.050 ^{ef}	1.348±0.174 ^{def}	0.119±0.008 ^{fg}	0.052±0.008 ^{de}
	0/100	4.155±0.565 ^{ef}	0.510±0.053 ^{gh}	1.024±0.128 ^{ef}	0.097±0.008 ^{gh}	0.032±0.007 ^f
Chilling temperature (5-18℃)	100/0	7.431±1.683 ^d	0.807±0.095 ^{de}	1.577±0.343 ^{cd}	0.145±0.020 ^e	0.059±0.011 ^d
	75/25	8.106±0.997 ^d	0.863±0.065 ^d	1.442±0.202 ^{de}	0.134±0.012 ^{ef}	0.062±0.006 ^d
	50/50	11.645±1.969 ^c	1.152±0.093 ^c	2.086±0.211 ^c	0.184±0.010 ^d	0.080±0.008 ^c
	25/75	5.395±0.699 ^{def}	0.611±0.034 ^{fg}	1.295±0.1361 ^{def}	0.1247±0.007 ^{ef}	0.044±0.006 ^e
	0/100	3.106±0.363 ^f	0.378±0.024 ^h	0.828±0.055 ^f	0.088±0.003 ^h	0.018±0.005 ^g
Normal temperature (14-30℃)		13.735±7.540 ^a	1.269±0.585 ^a	2.745±1.458 ^a	0.198±0.081 ^a	0.080±0.034 ^a
Chilling temperature (5-18℃)		7.137±3.146 ^b	0.762±0.274 ^b	1.446±0.459 ^b	0.135±0.034 ^b	0.053±0.023 ^b
Temperature		0.001	0.001	0.001	0.001	0.001

nitrate-ammonium	0.001	0.001	0.001	0.001	0.001
Temperature×nitrate-ammonium	0.001	0.001	0.001	0.001	0.001

Each value is the mean $\pm$ SE of 5 independent experiments. Values within a column followed by different letters indicate significant differences among treatments at the $P < 0.05$ level. (ns) non significance; (0.05) significance at 0.05 probability level; (0.01) significance at 0.01 probability level, and (0.001) significance at 0.001 probability level.

Table 3. Effect of nitrate-ammonium ratios and temperatures on the concentration of soluble protein and free amino acids of tomato seedlings.

Temperature	NO ₃ ⁻ -N/NH ₄ ⁺ -N	Soluble protein content in leaves	Soluble protein content in roots	Free amino acids content in leaves	Free amino acids content in roots
	ratio	(mg·g ⁻¹)	(mg·g ⁻¹)	(mg·g ⁻¹)	(mg·g ⁻¹)
Normal temperature (14-30℃)	100/0	23.225±3.232 ^a	6.785±0.891 ^{abc}	0.597±0.097 ^e	0.327±0.047 ^e
	75/25	21.011±1.849 ^{ab}	7.251±0.809 ^{ab}	0.657±0.139 ^e	0.640±0.114 ^d
	50/50	19.560±2.196 ^b	5.650±0.881 ^{cde}	0.747±0.129 ^{de}	0.663±0.080 ^{cd}
	25/75	16.037±1.712 ^c	5.388±0.454 ^{de}	0.963±0.120 ^{cd}	0.757±0.114 ^{bcd}
	0/100	13.956±1.024 ^{cd}	4.807±0.426 ^{ef}	1.400±0.189 ^b	0.897±0.102 ^b
Chilling temperature (5-18℃)	100/0	21.680±2.932 ^{ab}	6.244±0.919 ^{bcd}	0.780±0.140 ^{de}	0.470±0.076 ^e
	75/25	19.834±1.885 ^b	7.782±0.650 ^a	0.967±0.115 ^{cd}	0.670±0.092 ^{cd}
	50/50	15.406±1.344 ^c	5.551±1.147 ^{cde}	1.090±0.196 ^c	0.777±0.095 ^{bcd}
	25/75	13.073±1.366 ^{cd}	4.696±0.583 ^{ef}	1.470±0.183 ^b	0.837±0.075 ^{bc}
	0/100	10.901±0.992 ^d	3.520±0.674 ^f	1.750±0.212 ^a	1.150±0.111 ^a
Normal temperature (14-30℃)		18.758±3.908 ^a	5.976±1.119 ^a	0.873±0.325 ^b	0.657±0.211 ^b
Chilling temperature (5-18℃)		16.179±4.462 ^b	5.567±1.652 ^a	1.213±0.393 ^a	0.781±0.243 ^a

Temperature	0.010	ns	0.001	0.001
nitrate-ammonium	0.001	0.001	0.001	0.001
Temperature× nitrate-ammonium	ns	ns	ns	ns

Each value is the mean $\pm$ SE of 5 independent experiments. Values within a column followed by different letters indicate significant differences among treatments at the $P < 0.05$ level. (ns) non significance; (0.05) significance at 0.05 probability level; (0.01) significance at 0.01 probability level, and (0.001) significance at 0.001 probability level.

Table 4. Effect of nitrate-ammonium ratios and temperatures on the concentration of N, NO_3^- and NH_4^+ of tomato seedlings.

Temperature	NO_3^- -N/ NH_4^+ -N	N content in leaves	N content in roots	NO_3^- content in leaves	NO_3^- content in roots	NH_4^+ content in leaves	NH_4^+ content in roots
	ratio	(mg $\cdot$ g $^{-1}$ DW)	(mg $\cdot$ g $^{-1}$ DW)	(mg $\cdot$ g $^{-1}$ DW)	(mg $\cdot$ g $^{-1}$ DW)	(mg $\cdot$ g $^{-1}$ DW)	(mg $\cdot$ g $^{-1}$ DW)
Normal temperature (14-30°C)	100/0	39.883 $\pm$ 2.162 ^f	30.37 $\pm$ 0.445 ^f	29.397 $\pm$ 2.049 ^b	41.557 $\pm$ 2.204 ^a	0.397 $\pm$ 0.086 ^f	0.500 $\pm$ 0.099 ^f
	75/25	41.283 $\pm$ 1.570 ^{ef}	32.173 $\pm$ 0.973 ^e	32.503 $\pm$ 2.268 ^a	36.9833 $\pm$ 1.3878 ^b	1.057 $\pm$ 0.180 ^e	2.023 $\pm$ 0.270 ^f
	50/50	45.563 $\pm$ 1.273 ^{cd}	35.613 $\pm$ 0.909 ^d	26.120 $\pm$ 1.230 ^c	30.310 $\pm$ 1.022 ^d	2.207 $\pm$ 0.225 ^c	8.090 $\pm$ 1.096 ^d
	25/75	47.540 $\pm$ 1.860 ^{bc}	38.643 $\pm$ 1.312 ^c	19.583 $\pm$ 1.007 ^d	23.830 $\pm$ 2.132 ^e	3.283 $\pm$ 0.225 ^b	15.480 $\pm$ 0.832 ^b
	0/100	48.993 $\pm$ 1.406 ^b	39.530 $\pm$ 0.859 ^c	4.353 $\pm$ 0.965 ^f	3.567 $\pm$ 0.897 ^g	4.197 $\pm$ 0.323 ^a	18.440 $\pm$ 2.063 ^a
Chilling temperature(5-18°C)	100/0	43.367 $\pm$ 1.083 ^{de}	32.463 $\pm$ 0.920 ^e	20.210 $\pm$ 2.118 ^d	30.533 $\pm$ 2.378 ^{cd}	0.270 $\pm$ 0.046 ^f	0.310 $\pm$ 0.089 ^f

	75/25	47.223±0.591 ^{bc}	33.337±0.859 ^e	23.967±1.238 ^c	33.087±1.796 ^c	0.917±0.1498 ^e	1.660±0.210 ^f
	50/50	48.877±1.119 ^b	38.097±1.162 ^c	24.753±1.373 ^c	28.510±0.908 ^d	1.750±0.111 ^d	5.430±0.282 ^e
	25/75	52.770±2.327 ^a	41.623±0.836 ^b	14.573±1.183 ^e	17.860±1.284 ^f	2.467±0.258 ^c	12.577±1.754 ^c
	0/100	53.493±1.237 ^a	44.550±1.340 ^a	2.103±0.419 ^f	1.220±0.345 ^g	3.123±0.230 ^b	12.957±1.271 ^c
Normal temperature (14-30° C)		44.653±3.916 ^b	35.266±3.766 ^b	22.391±10.423 ^a	27.249±13.811 ^a	2.228±1.455 ^a	8.907±7.421 ^a
Chilling temperature (5-18°		49.146±4.027 ^a	38.014±4.900 ^a	17.121±8.697 ^b	22.242±12.199 ^b	1.705±1.074 ^b	6.587±5.568 ^b
Temperature		0.001	0.001	0.001	0.001	0.001	0.001
nitrate-ammonium		0.001	0.001	0.001	0.001	0.001	0.001
Temperature × nitrate-ammonium		ns	0.05	0.001	0.001	0.01	0.01

Each value is the mean ± SE of 5 independent experiments. Values within a column followed by different letters indicate significant differences among treatments at the P<0.05 level. (ns) non significance; (0.05) significance at 0.05 probability level; (0.01) significance at 0.01 probability level, and (0.001) significance at 0.001 probability level.