Changes in Growth and Photosynthetic Capacity of Cucumber Seedlings in Response to Nitrate Stress

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ABSTRACT

The effects of three nitrate levels - 14(CK), 56(T-1), and 140 mmol L^{-1} (T-2) - on growth and photosynthetic capacity of cucumber (*Cucumis sativus* L. cv. Xintaimici) seedlings grown in hydroponic culture were investigated. The results showed that at 12 d after treatment plant height, stem diameter, leaf area, and leaf number of cucumber seedlings were stimulated by 56 mmol L^{-1} nitrate, whereas were inhibited significantly by 140 mmol L^{-1} nitrate compared with CK. Short-term stimulation in photosynthetic rate occurred under T-1 treatment, and then recovered to the level of CK. Photosynthetic rate of T-2 seedlings significantly decreased over treatment course with respect to CK. Photosynthetic pigment content of T-1 and T-2 increased during the first 2 d, and gradually recovered to the level of CK thereafter. Chlorophyll a/b and carotenoids/chlorophyll of T-1 had no significant difference from CK during treatment period. During the first 4 d, there was no significant difference in chlorophyll a/b and carotenoids/chlorophyll between T-2 and CK. After 4 d, chlorophyll a/b of T-2 increased gradually, whereas carotenoids/chlorophyll decreased. Actual PSII efficiency (Φ_{PSII}) and photochemical quenching (qP) of T-1 had no significant difference from CK, and non-photochemical quenching (qN) was a little higher than CK after 2 d. During the first 2 d, there was little difference in Φ_{PSII} and qP between T-2 and CK. After 2 d, both Φ_{PSII} and qP of T-2 decreased to a great extend. A significant increase in qN of T-2 occurred over treatment course. With respect to CK, Hill reaction activity of T-1 slightly decreased, and T-2 treatment resulted in a significant decrease of Hill reaction activity. This evidence indicates that high-level nitrate stress may reduce photosynthesis through its effects not only on stomatal conductance but on the photosynthetic apparatus.

Key words: chlorophyll a fluorescence; Hill reaction; photosynthetic pigment content; photosynthetic rate

INTRODUCTION

Nitrogen, often a limiting resource for plant growth, is required by plants in great quantities than any other mineral element. Thus the availability of nitrogen is a significant determinant of crop yield (Foyer and Noctor, 2002). Complicating this for agriculture is the fact that often less than 50% of nitrogen fertilizer applied ultimately may be utilized by crops because nitrate ion is highly mobile and not absorbed by soil colloid (Allison, 1966). To satisfy the nitrogen demand, farmers often add nitrogen in large quantities to maintain adequate level in the rhizosphere (Zhu et al., 2005). This excessive use of nitrogen fertilizer has resulted in undesirable conditions such as the accumulation of nitrate in plant and soil. The large accumulation of nitrogen in the soil, on one hand, has contaminated the ground water (Barker and Mills, 1980),

on the other hand resulted in soil secondary salinization in protected farmland because of a lack of leaching by rainfall and strong evaporation of soil water (Kitamura et al., 2006).

China has the largest area of protected crops and is now the leading country in the world for protected agriculture, including multi-span greenhouse, solar lean-to greenhouse and plastic tunnels (Jiang and Du, 2000). However, soil secondary salinization has seriously limited sustainable development of agricultural production in protected farmland of China (Yu et al., 2005). According to the previous studies (Ju et al., 2007; Yu et al., 2005), accumulation of ions in protected farmland is greatly different from ordinary soil salinization. In protected farmland, the main cation and anion are Ca^{2+}, K^+ and NO_3^-, respectively, while Na^+ and Cl^- are the main forms of ions in ordinary soil salinization.

In the past several years, most of studies about salt stress to plant have been focused on NaCl (Debouba et al., 2007; Liu and Zhu, 1998; Stepien and Johnson, 2009; Zhu, 2002), however, there have been few investigations about nitrate stress in horticultural crops. Cucumber is one of the most important horticultural crops, and it has been reported that excessive accumulation of nitrate widely inhibits the growth and development of cucumber in protected farmland of China (Gao et al., 2008; Lü et al., 2007), while the underlying mechanisms are still not well understood. Photosynthesis is the fundamental metabolic process and plays a critical role in plant growth and development. This process is very sensitive to environmental stress. Drought (Lauteri et al., 1997), salt stress (Bongi and Loreto, 1989), and leaf aging (Loreto et al., 1994) all result in inhibition of photosynthesis because of the reduction of conductance to CO_2 diffusion in the leaf mesophyll (Delfine et al., 1999). But little information about changes of photosynthesis under nitrate stress exists. Therefore, we studied how excessive nitrate influenced the growth and photosynthetic capacity of cucumber seedlings. Plant growth, photosynthetic rate, photosynthetic pigment content, chlorophyll a fluorescence and Hill reaction activity were measured in nitrate-stressed cucumber seedlings under greenhouse conditions.

MATERIALS AND METHODS

Plants, growth conditions, and experimental design: Cucumber (*Cucumis sativus* L. cv. Xintaimici, mid-tolerant to salinity stress), was used in all experiments. The seeds were sterilized with a sodium hypochlorite solution (5% active Cl) for 5 min, washed five times, and soaked in deionized water for 12 h. The soaked seeds were raised in well-washed quartz sand and irrigated with tap water. The experiments were carried out in the greenhouse of Shandong Agricultural University from September to November in 2006. When plants had one fully expanded leaf, they were removed from the trays and their roots were washed with tap water to remove the substrate from the roots, and then transplanted to hydroponic boxes (40 cm×30 cm×12 cm, 8 plants per box) containing a complete cucumber nutrient solution (Table 1). The solution was continually aerated with an electric pump. The pH of nutrient solution was adjusted to 6.0 ± 0.5 by addition of 98% (W/W) H_2SO_4. The nutrient solutions in all the hydroponic boxes were completely renewed every four days. When the seedlings had developed three fully expanded leaves, nitrate was dissolved in nutrient solution directly. The excess nitrate test was carried out in a completely randomized design with a split plot arrangement of three replications, providing 8 plants per replication. Three treatments were applied (Table 2):

(CK) complete nutrient solution (control);

(T-1) complete nutrient solution + $Ca(NO_3)_2$ 10.5 mmol·L^{-1} + KNO_3 21 mmol·L^{-1};

(T-2) complete nutrient solution + $Ca(NO_3)_2$ 31.5 mmol·L^{-1} + KNO_3 63 mmol·L^{-1}.

At 0, 1, 2, 4, 6, 8, and 12 d of exposure to treatment, the second fully expanded leaves, counted from the top of seedlings, were sampled for the measurement of photosynthetic rate, chlorophyll content, chlorophyll a fluorescence and Hill reaction activity. At 12 d of exposure to treatment, 6 plants per treatment were collected for the determination of plant growth.

Table 1. Components of the complete cucumber nutrient solution (Guo, 2004)

Nutrient Chemicals	Concentration (mmol L^{-1})
$Ca(NO_3)_2$	3.5
KNO_3	7
KH_2PO_4	1
$MgSO_4$	2
H_3BO_3	0.05
$Na_2FeEDTA$	0.05
$MnSO_4$	0.01
$ZnSO_4$	0.0008
$CuSO_4$	0.0003
$(NH_4)Mo_7O_{24}$	0.02

Table 2. Nitrate concentration and osmotic potential of nutrient solution

Treatment	$Ca(NO_3)_2$ (mmol L^{-1})	KNO_3 (mmol L^{-1})	NO_3^- (mmol L^{-1})	Osmotic potential before treatment (MPa)	Osmotic potential after treatment for 3 d (MPa)
CK	3.5	7	14	-0.256	-0.218
T-1	14	28	56	-0.341	-0.315
T-2	35	70	140	-0.57	-0.567

Determination of plant growth: Plant height was measured from the border of the container to the top of the main plant stem with stainless steel ruler (500 mm, Kenta, Singapore). Stem diameter was measured at the border of the container with vernier caliper (530-101, Mitutoyo, Japan). Leaf area was calculated from leaf length and breadth according to the method of Robbins and Pharr (1987). The number of completely expended leaves was counted and recorded on each plant.

Determination of photosynthetic rate: Photosynthetic rate of individual leaf was measured with an open photosynthesis system (Ciras-II, PPsystems, UK) at 10:00 HR in the morning. The photosynthetic chamber provides leaf area of 2.5 cm^2, leaf temperature of 25 °C, relative humidity of 90%, leaf to air vapor pressure of 200 mbar, light intensity of 800 μmol $m^{-2} s^{-1}$, and CO_2 concentration of 380 μmol mol^{-1}.

Determination of photosynthetic pigment content: Chlorophyll and carotenoids were measured by extracting fresh leaf tissue in 80% acetone. The absorbance of the centrifuged extract was determined at 663, 645, and 440 nm. Chlorophyll and carotenoids content were calculated by the methods described before (Ikan, 1969; Strain and Svec, 1966).

Determination of chlorophyll a fluorescence: Chlorophyll a fluorescence was measured at room temperature with a portable fluorometer (FMS-2, Hansatech, UK). The experiment protocol described before (Genty et al., 1989) was basically followed. Fluorescence nomenclature was according to van Kooten and Snel (1990). All samples were dark-adapted for 30 min prior to fluorescence measurement.

Dark-adapted minimal fluorescence (Fo) with all PSII reaction centers open was measured by the measuring modulated light (< 0.1 μmol $m^{-2} s^{-1}$), which was sufficiently low not to induce any significant variable fluorescence. Dark-adapted maximal fluorescence (Fm) with all PSII reaction centers closed was determined by 0.8 s saturating pulse at 8000 μmol $m^{-2} s^{-1}$. The leaves were then continuously illuminated with white actinic at an intensity of 300 μmol $m^{-2} s^{-1}$. Fluorescence in steady state (Fs) was thereafter recorded and a second saturating pulse at 8000 μmol $m^{-2} s^{-1}$ was imposed to determine light-adapted maximal fluorescence (Fm′). The actinic light was removed and minimal light-adapted fluorescence level (Fo′) was determined by illuminating the leaf with 3 s far-red light. All measurements of Fo and Fo′ were performed with the measuring beam set to a frequency of 600 Hz, whereas all measurements of Fm and Fm′ were performed with the measuring beam automatically switching to 20 kHz during the saturating flash.

By using fluorescence parameters determined in both light- and dark-adapted leaves, calculations of actual PSII efficiency (Φ_{PSII}), photochemical quenching (qP), and non- photochemical quenching (qN) were made of: (1) Φ_{PSII}=(Fm′-Fs)/Fm′, (2) qP=(Fm′-Fs)/(Fm′-Fo′), (3) qN=1-(Fm′- Fo′)/(Fm-Fo).

**Isolation of chloroplasts and determination of Hill reaction activity:** Chloroplasts were isolated from the leaves of cucumber seedlings in 0.05 mol L^{-1} Tris-HCl buffer, pH7.6,

containing 0.4 mol sucrose and 0.01 mol NaCl as described by Gorham (1955) with some modifications. The chloroplasts were washed twice in the extraction buffer and finally suspended in the extraction buffer. The Hill reaction activity of the isolated chloroplasts was measured by the rate of photoreduction of $K_3Fe(SCN)_6$ as described by Vishniac (1957) with some modifications. The reaction mixture (final volume 3 ml) contained in mmol: Tris-HCl buffer, pH 7.6, 50; $MgCl_2$, 5; $K_3Fe(SCN)_6$, 2; chloroplast preparation containing about 20 μg of chlorophyll. The tubes were illuminated at 300 μmol m^{-2} s^{-1} of light intensity in the 4 °C water bath. The decrease in OD_{420} after 1 min was immediately measured with spectrometer (160 A, Shimadzu, Japan). Tube kept in complete darkness served as reagent blank. The Hill activity was calculated as μmol of reduced $K_3Fe(SCN)_6$ h^{-1} mg^{-1} chlorophyll. Chlorophyll content of the preparation was determined with the method above.

Statistical analysis: Date were analyzed with OriginPro8 (Version8E, OriginLab Corporation, Massachusetts, USA) and presented as means of three replicates ± standard errors.

RESULTS

Plant growth: Figure 1 shows the effects of nitrate stress on plant growth. At 12 d after nitrate stress imposition, plant growth at 56 mmol L^{-1} nitrate was stimulated in plant height, stem diameter, leaf area, and leaf number of, respectively, 9%, 4%, 9%, and 4% with respect to CK. By the same time, plant growth at 140 mmol L^{-1} nitrate suffered a severe reduction in plant height, stem diameter, leaf area, and leaf number of, respectively, 38%, 26%, 39%, and 38% with respect to CK.
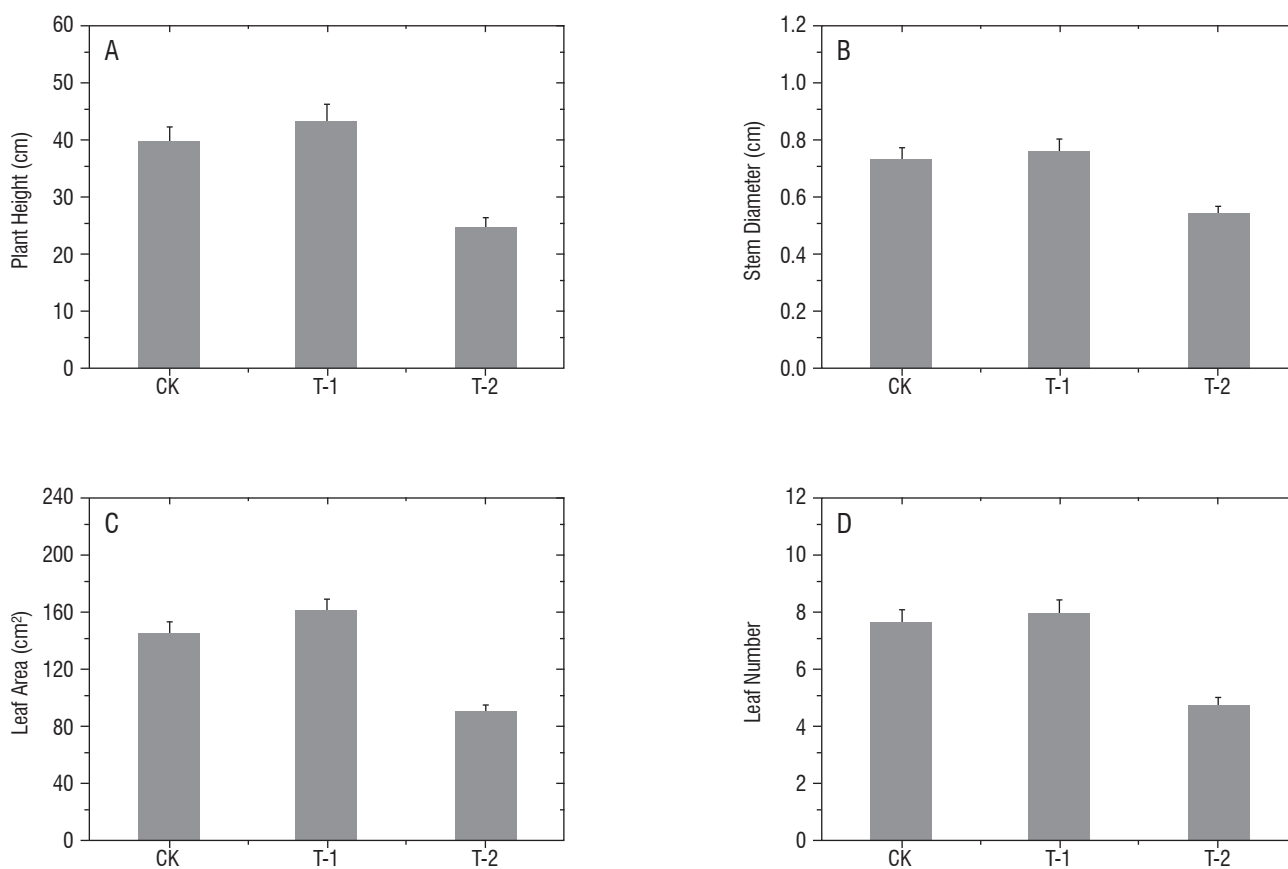

Figure 1. Changes in plant height (A), stem diameter (B), leaf area (C) and leaf number (D) of cucumber seedlings at 12 d after treatment. Plants were grown in nutrient solutions containing 14 (CK), 56 (T-1), and 140 mmol L^{-1} (T-2) nitrate, respectively. Vertical bars represent standard errors (n=3).

Photosynthetic rate: Figure 2 shows changes of photosynthetic rate in the leaves of cucumber seedlings under three nitrate levels. Photosynthetic rate of CK seedlings had few changes over treatment course. During the first 2 d, photosynthetic rate of T-1 seedlings increased by 27% with respect to CK. Thereafter, the rate recovered gradually to the level of CK. Compared with CK, photosynthetic rate of T-2 decreased significantly over treatment course. At 12 d, this decrease reached 40% with respect to CK.
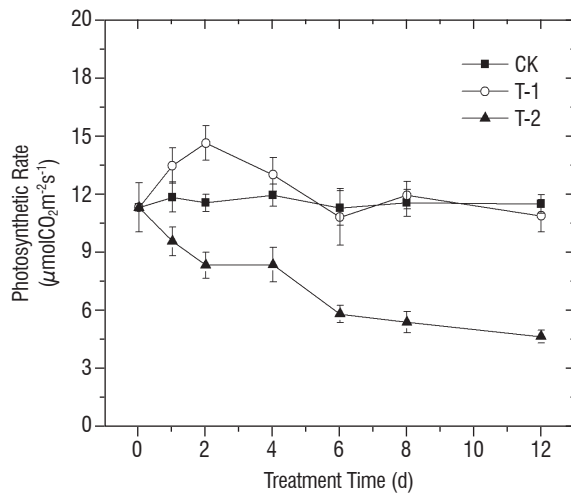
Figure 2. Effects of nitrate stress on photosynthetic rate in the leaves of cucumber seedlings. Plants were grown in nutrient solutions containing 14 (CK), 56 (T-1), and 140 mmol L^{-1} (T-2) nitrate, respectively, during 12 d. Vertical bars represent standard errors (n=3).

Photosynthetic pigment content: Figure 3 shows changes of total photosynthetic pigment content, chlorophyll a/b and carotenoids/chlorophyll in the leaves of cucumber seedlings under three nitrate levels. Over treatment course, photosynthetic pigment content, chlorophyll a/b and carotenoids/chlorophyll of CK seedlings had few changes. Photosynthetic pigment content of T-1 increased by 23% with respect to CK during the first 2 d. Thereafter, the content gradually recovered to the level of CK. Chlorophyll a/b and carotenoids/chlorophyll had no significant difference from CK during treatment period. Compared with CK, photosynthetic pigment content of T-2 increased by 25% during the first 2 d. Thereafter, the content gradually recovered to the level of CK. During the first 4 d, there was no significant difference in chlorophyll a/b and carotenoids/chlorophyll between T-2 and CK. After 4 d, chlorophyll a/b of T-2 increased gradually, whereas carotenoids/ chlorophyll decreased. At 12 d, the chlorophyll a/b was 23% higher than CK, and the carotenoids/chlorophyll was 15% lower than CK.
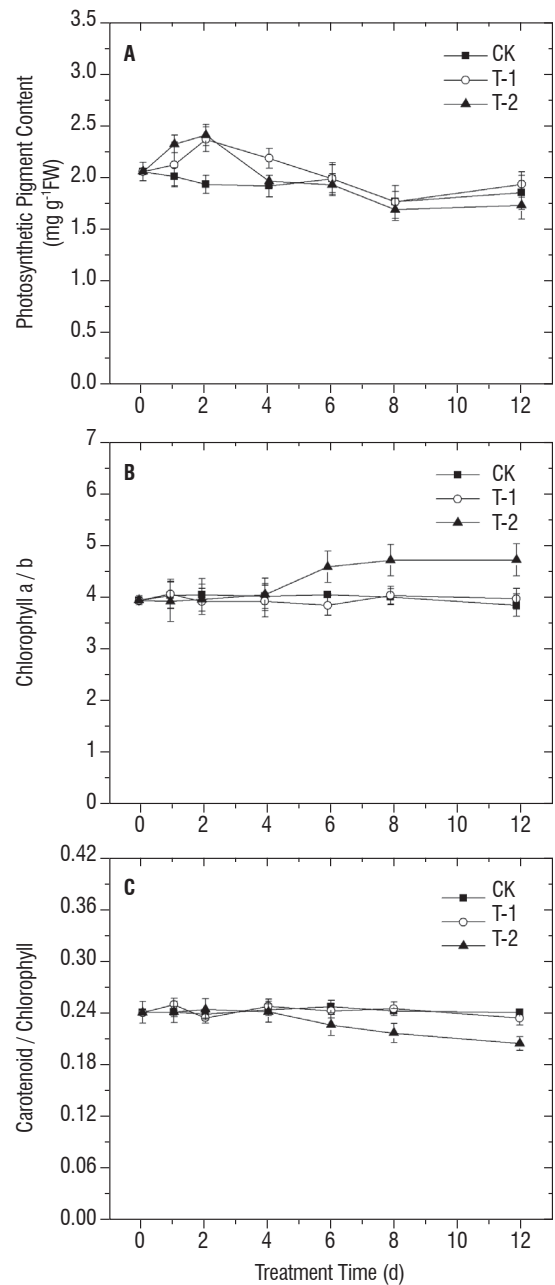
Figure 3. Effects of nitrate stress on photosynthetic pigment content (A), chlorophyll a/b (B), and carotenoids/chlorophyll (C) in the leaves of cucumber seedlings. Plants were grown in nutrient solutions containing 14 (CK), 56 (T-1), and 140 mmol L^{-1} (T-2) nitrate, respectively, during 12 d. Vertical bars represent standard errors (n=3).

Chlorophyll a fluorescence: Figure 4 shows changes of Φ_{PSII}, qP, and qN in the leaves of cucumber seedlings under three nitrate levels. Φ_{PSII}, qP, and qN of CK had few changes over treatment course. Φ_{PSII} and qP of T-1 had no significant difference from CK over treatment course, and qN was a little higher than CK after 2 d. During the first 2 d, there was no significant difference in Φ_{PSII} and qP between T-2 and CK. After 2 d, both Φ_{PSII} and qP

of T-2 decreased to a great extend. At 12 d, Φ_{PSII} and qP lowered by 44% and 48% with respect to CK, respectively. Over treatment course, a significant increase in qN of T-2 occurred. At 12 d, qN increased by 72% with respect to CK.
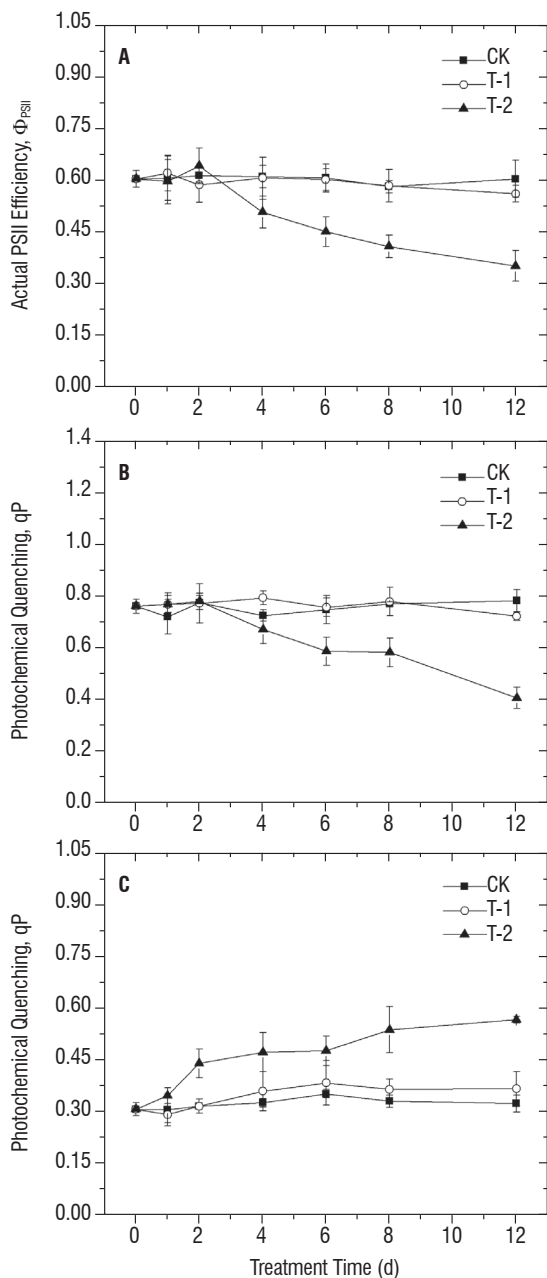
Figure 4. Effects of nitrate stress on Φ_{PSII} (A), qP (B), and qN (C) in the leaves of cucumber seedlings. Plants were grown in nutrient solutions containing 14 (CK), 56 (T-1), and 140 mmol L^{-1} (T-2) nitrate, respectively, during 12 d. Vertical bars represent standard errors (n=3).

Hill reaction activity: Figure 5 shows changes of Hill reaction activity in the leaves of cucumber seedlings under three nitrate levels. A little fluctuation in Hill reaction activity

of CK occurred over treatment course. With respect to CK, Hill reaction activity of T-1 slightly decreased. T-2 treatment resulted in a rapid decrease of Hill reaction activity of cucumber seedlings during treatment period compared with CK. At 12 d, the activity of T-2 was 70% lower than CK.
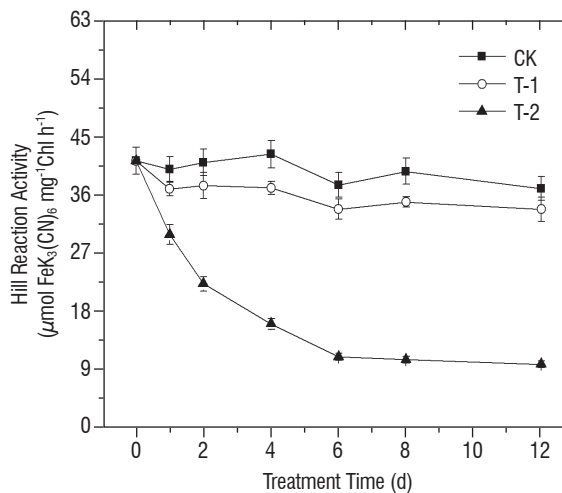
Figure 5. Effects of nitrate stress on Hill reaction activity in the leaves of cucumber seedlings. Plants were grown in nutrient solutions containing 14 (CK), 56 (T-1), and 140 mmol L^{-1} (T-2) nitrate, respectively, during 12 d. Vertical bars represent standard errors (n=3).

DISCUSSION

In the present study, a growth stimulation in cucumber seedlings was observed under 56 mmol L^{-1} nitrate (Figure 1), indicating that the additional input of nitrogen could promote plant growth (Burslem et al., 1996; Lawrence, 2003; Tanner et al., 1992). However, when nitrate concentration in nutrient solution increased to 140 mmol L^{-1}, a significant growth reduction in cucumber seedlings was observed (Figure 1). The inhibition of cucumber growth by high nitrate stress could be explained by osmotic effects, in which high nitrate concentration in the root medium results in low water potential and eventually may lead to water stress (Table 2), or by specific ion effects, in which toxicity or deficiency of one or more ions may cause growth reduction under stress. The maintenance of optimal C/N ratio is essential for plant growth and development. A high nitrate concentration in the root medium can result in entry of excessive NO$_3^-$, and cause disturbance of C/N radio. Ionic imbalance which results from high concentration of nitrate may cause slow plant growth. It

has been reported that root growth of many crops is severely inhibited by high saline concentration in the growing medium (Cramer et al., 1987). The reduction in root growth under salt stress reduces the volume of soil that can be explored by roots and therefore the quantity of nutritional ions, which moves by diffusion, is reduced from reaching plants. In addition, ions that transit the soil by mass flow (e.g. NO_3^-) may accumulate near the root surface and compete with nutritional ions for membrane uptake sites. Excessive nitrate environment may therefore induce suboptimal nutrient concentrations in plant tissues (Kakafi and Bernstein, 1996). For example, high concentration of NO_3^- has been shown to reduce Cl^- (Bar et al., 1987) and phosphate (Lamaze et al., 1987) uptake, and high concentration of K^+ and Ca^{2+} may reduce the uptake of Mg^{2+} (Barber, 1995).

Short-term stimulation in photosynthetic rate was detected under mid-level nitrate during the first 2 d (Figure 2), a trend which could be considered a short-term response to nitrate stress because of the increase of photosynthetic pigment content (Figure 3A), increases in the following samplings were hardly significant due to the recovery of photosynthetic pigment content (Figure 3A). High-level nitrate resulted in substantial decrease of photosynthetic rate over treatment course (Figure 2). High nitrate concentration can impose both ionic and osmotic stresses on cucumbers. Stomatal closure, which increases the diffusive resistance to the entry of CO_2 into the leaf (Sharp and Boyer, 1986), is often a rapid initial response to salt stress and can result in reduction of photosynthetic rate. In wheat, James et al. (2002) has shown that a stress-induced reduction in stomatal conductance was seen when the leaf emerged, but after some time there was a further decline, probably caused by chloroplast inhibition deriving from salt toxicity, which decreased the demand for CO_2. Yang (2008) reported that long-term nitrate stress resulted in large accumulation of NH_3 in cucumber seedlings, which was very toxic to plants (Cao et al., 2009) and could seriously damage photosynthetic electron flow (Izawa, 1977).

Photosynthetic pigment content plays a critical role in photosynthesis. Pigments can absorb solar energy and transfer it to chemical energy used for synthesis of ATP and glucose. A positive correlation between leaf nitrogen content and the photosynthetic pigment content is well documented for a number of plant species (Bojovic and Stojanovic, 2005; Fritshi and Ray, 2007; Houles et al., 2007; Sabo et al., 2002). In the

present study, not only mid- but also high-level nitrate resulted in the increase of photosynthetic pigment content during early treatment period (Figure 3A). But at the same time a significant decrease in photosynthetic rate occurred under high-level nitrate (Figure 2), revealing that the chlorophyll content was not certainly related to the photosynthesis rate, especially under stress conditions. Pulkrabek (1998) and Siddiqu et al.(2006) have reported similar changes in sugar beet. With the increase of treatment time, photosynthetic pigment content of T-1 and T-2 gradually recovered to the level of CK (Figure 3A). However, the underlying mechanisms are possibly different. In T-1 seedlings, dilution effect caused by the stimulation of plant growth (Figure 1) may play an important role in the recovery of photosynthetic pigment content. Inhibition of synthesis and stimulation of decomposition possibly cause the recovery under high nitrate stress (Reddy and Vora, 1986; Santos and Caldeira, 1999). Environmental stress severely affects not only photosynthetic pigment content but chlorophyll a/b and carotenoids/chlorophyll. In the present study, high nitrate stress resulted in the increase of chlorophyll a/b and the decrease of carotenoids/chlorophyll (Figure 3B, 3C). This is in accordance with the results reported by Ma et al.(1997). The increase of chlorophyll a/b is possibly due to less sensitivity of chlorophyll a to salt stress than chlorophyll b (Stoeva and Kaymakanova, 2008). The decrease of carotenoids/chlorophyll indicates that high nitrate stress may cause an increase in zeaxanthin and degradation of β-carotene, which are apparently involved in protection against photoinhibition (Sharma and Hall, 1991).

Photosystem II is considered to play an important role in the response of higher plants to environmental stress (Baker, 1991). The reduction of CO_2 assimilation by nitrate stress should therefore be reflected in the PSII behavior. Chlorophyll a fluorescence is a rapid and non-intrusive tool used to screen varieties for PSII under salt stress (Maxwell and Johnson, 2000). It has be reported that Φ_{PSII} and qP significantly decreased, but qN increased substantially under saline conditions (Corney et al., 2003; Netondo et al., 2004). In the present study, high nitrate stress resulted in significant decrease of Φ_{PSII} and qP, and increase of qN (Figure 4A, 4B, 4C). The decrease of Φ_{PSII} and qP reflects that less of the absorbed photon-energy captured by the light harvesting system is used in photochemical reaction. As a result, the amount of excessively absorbed photon flux is greater in nitrate-stressed leaves than in normal leaves, particularly under high photon flux. The excessively absorbed photo flux

can potentially lead to the production of $^1O_2$ and reduced reactive oxygen species, causing damage to photosynthetic apparatus (Han et al., 2009). The increase in qN may reflect a reduced demand for products of electron transport and, hence, increased heat dissipation. These results suggest that high-level nitrate stress may reduce photosynthesis through its direct effect on the photosynthetic apparatus.

With the increase of nitrate concentration, the rate of Hill reaction in cucumber seedlings was inhibited (Figure 5). This is similar to the results under salt stress reported by Tajdoost et al. (2007). The inhibition of Hill reaction possibly results from nitrate stress damage on photosynthetic systems and oxygen evolving complex (Allakhverdiev et al., 2000). Nitrate stress imposes a water stress because of low osmotic potential (Table 2) and may affect a wide variety of metabolic activities (Parida and Das, 2005). Krieger-Liszkay (2004) has proposed that the active oxygen forms could destroy the proteins, lipids, and important cell components, which could secondarily lead to damage in photosynthetic systems. In addition, NH_3 toxicity deriving from excessive accumulation of NH_3 under high nitrate stress may also lead to severe inhibition of Hill reaction activity (Izawa, 1977; Yang, 2008). The inhibition of Hill reaction activity in turn decreases the reduction of $NADP^+$ and phosphorylation of ADP, which will result in a strong inhibition of CO_2 assimilation.

In conclusion, high-level nitrate stress may reduce photosynthesis through its effects not only on stomatal conductance but on the photosynthetic apparatus.

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