



Research article

Response of water balance and nitrogen assimilation in cucumber seedlings to CO₂ enrichment and salt stress



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ABSTRACT

The effects of CO₂ enrichment on water balance and nitrogen (N) assimilation in cucumber (*Cucumis sativus* L. cv. Jinyou No.35) seedlings under salt stress were investigated. Two-way randomized block design was used: the main treatment consisted of two [CO₂] levels, ambient and enriched (400 and 800 ± 40 μmol mol⁻¹, respectively), and the minor treatment consisted on two salinity treatment levels, 0 and 80 mmol L⁻¹ NaCl. The results showed that, under the experimental conditions, enriched [CO₂] and salt stress significantly inhibited the N assimilation process in cucumber leaves; however, enriched [CO₂] had no effect on the nitrate (NO₃⁻) reduction or ammonium (NH₄⁺) assimilation of leaves under salt stress, inhibiting only the transamination. Moreover, enriched [CO₂] increased the plasma membrane H⁺-ATPase activity, vacuolar membrane H⁺-ATPase activity and root hydraulic conductivity under salt stress, thereby increasing the ion selective absorption and water absorption capacity. To a certain extent, enriched [CO₂] promoted the accumulation of K⁺ in plants, which significantly reduced the Na⁺/K⁺ ratio; moreover, the enrichment ultimately improved the water state conditions and helped to maintain the ion balance in plants under stress, ensuring normal enzymatic activity.

1. Introduction

Plants are frequently challenged by abiotic stresses, including drought, salinity, alkalinity and extreme temperature. Among abiotic stresses, soil salinization is an important growth-limiting factor for most plants. Salinity causes many plant physiological changes, including ion imbalance and/or ion toxicity (Wang et al., 2006), reduced photosynthetic rates (Piñero et al., 2016), and altered nitrogen (N) metabolism (Pérez-López et al., 2013). Accordingly, plants can avoid the adverse effects of salt stress via endogenous mechanisms such as accumulating osmoregulatory substances and activating Na⁺/K⁺ antiporters (Flowers, 2004). N is considered an essential element and is a constituent of many important compounds in plants. Nitrate (NO₃⁻) absorbed by plants from the soil can be converted into amino acids through a series of assimilation processes, which are often affected by salt stress (Gouia et al., 1994). The change in key enzyme activities during NO₃⁻ assimilation under salt stress depends on the plant species

and their sensitivity to salt stress (Mansour, 2000). Moreover, salt can also lower the extracellular water potential, affect the expression and activity of aquaporins, increase the resistance of root water absorption (Qian et al., 2015), alter the cellular state of water, and cause dehydration, which in turn affects plant growth and yield. In contrast, enriched [CO₂] can increase plant water use efficiency, and under conditions of salinity, this phenomenon may constitute an advantage for plant tolerance (Zaghdoud et al., 2013). In addition, enriched [CO₂] allows plants to better counteract the water stress caused by saline conditions by increasing photosynthesis (Pérez-López et al., 2012) and improving plant water relations (Pérez-López et al., 2009, 2010; Zaghdoud et al., 2016), and it also reduces the accumulation of Na⁺ in plants and reduces ion toxicity (Zhu et al., 2016). Pérez-López et al. (2013) reported that enriched [CO₂] improves plant water status and increases carbohydrate levels; however, when salt stress is severe, nitrate reductase (NR) activity is similar under both ambient and enriched [CO₂] conditions, indicating a possible direct toxic effect of ions

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on NR activity. Moreover, the effect of CO₂ concentration on nitrogen assimilation is related to changes in photosynthesis process. Enriched [CO₂] enhances photosynthesis and provides more NAD(P)H and ATP for plants, thereby promoting carbohydrate synthesis and increasing carbon skeleton for nitrogen assimilation. On the other hand, carbon assimilation and nitrogen assimilation are a pair of competing relationships. The assimilation process requires the NAD(P)H and the participation of ATP. Therefore, enriched [CO₂] tends to inhibit nitrogen assimilation while promoting carbon assimilation (Li et al., 2017). At the same time, some researchers have suggested that enriched [CO₂] promotes the nitrogen uptake rate (NUR) and nitrogen translocation rate (NTR) in barley plant (Pérez-López et al., 2013, 2014), thereby promoting nitrogen assimilation-related enzyme activities (Robredo et al., 2011).

Although studies have enhanced our understanding of how enriched [CO₂] improves the ionic state of plants and alters N assimilation, less is known about the effects of enriched [CO₂] on the water balance and N assimilation in cucumber seedlings exposed to salt stress. Cucumber (*Cucumis sativus* L.) is one of the most extensively cultivated crops worldwide, and its yield increases with increasing CO₂ levels; however, salt stress reduces cucumber productivity. In this experiment, we used cucumber seedlings as test materials to study N assimilation and water balance under enriched [CO₂] and salt stress to clarify the physiological mechanism of enriched [CO₂] in alleviating the salt stress-induced adverse effects on cucumber growth.

2. Materials and methods

2.1. Plant materials and treatments

This experiment was conducted in a greenhouse situated at the experimental station of Shandong Agricultural University, China. Cucumber (*Cucumis sativus* L. cv. Jinyou No.35, from the Tianjin Kerun Research Institution, China) seeds were rinsed in distilled water, imbibed in water for 6–8 h, sown on moistened filter paper and germinated at 28 °C for 18–20 h in darkness. The seeds were then sown in plastic trays (54 × 28 × 5 cm) containing a mixture of peat, perlite and vermiculite (3/1/1, v/v/v), which were subsequently placed in a greenhouse. Representative seedlings with one pair of true leaves were transplanted and cultured hydroponically in darkened plastic containers (length: 37.5 cm, width: 29 cm and height: 12 cm; six plants per container) supplied with full-strength Yamazaki cucumber nutrient solution [1.00 mmol L⁻¹ NH₄H₂PO₄, 3.50 mmol L⁻¹ Ca(NO₃)₂·4H₂O, 6.00 mmol L⁻¹ KNO₃, 1.96 mmol L⁻¹ MgSO₄·7H₂O and a full suite of trace elements; the NO₃⁻/ammonium (NH₄⁺) ratio was 13:1]. Additionally, the solutions were aerated every 3 min by air pumps and replaced every 4 d.

The experiment was arranged in a randomized complete block. The main treatments were [CO₂] treatments, which consisted of an ambient [CO₂] and an enriched [CO₂] of approximately 400 and 800 ± 40 μmol mol⁻¹, respectively, while the minor treatments were salt treatments, which consisted of control conditions [nutrient solution + 0 mmol L⁻¹ NaCl, electrical conductivity (EC) = 2.97 ± 0.03 mS cm⁻¹] and salt stress conditions (nutrient solution + 80 mmol L⁻¹ NaCl, EC = 11.87 ± 0.56 mS cm⁻¹). Each CO₂ treatment was replicated in two open-top greenhouses (length: 6 m, width: 6 m and ridge height: 2.6 m) equipped with an environmental control system (Auto 2000; Beijing, China) to supply CO₂ from a compressed CO₂ gas cylinder controlled by a solenoid valve. The CO₂ was automatically injected into each greenhouse to maintain the target concentration. The other two open-top greenhouses were maintained at the ambient [CO₂]. Light condition was sunlight, and the average maximum light intensity during the test was 3342 μmol m⁻² s⁻¹. Cucumber seedlings were treated when the second leaves were fully expanded, and each treatment consisted of five containers of seedlings. Upon CO₂ enrichment, NaCl was dissolved in the nutrient solution (one-

time addition) to simulate rhizosphere salt stress in accordance with the experimental design. After 7 d of treatment, the third fully developed leaf (from nadir) and roots were sampled from random plants, immediately frozen in liquid N, and then stored at -70 °C for subsequent analyses. Each of the measured physiological parameters was repeated at least three times.

2.2. Measurement of Na⁺ and K⁺ contents

The shoots and roots were dried in an oven at 105 °C for 15 min and then at 80 °C to a constant weight. After the plant samples were pulverized, they were passed through a 30-mesh sieve and accurately weighed. Approximately 0.5 g (g) was pulverized and digested in a concentrated HNO₃/HClO₄ (2/1, v/v) solution. The extract was used to determine Na⁺ and K⁺ contents by flame spectrophotometry (F-100, Metash, Shanghai, China) (Hunt, 1982).

2.3. Root activity

Root activity was determined by the triphenyltetrazolium chloride (TTC) reduction method (Comas et al., 2010). A total amount of 0.5 g of root tissue was added to 10 mL of 0.5 mmol L⁻¹ phosphate buffered solution (PBS) (pH 7.0) that contained 0.4% (w/v) TTC at 37 °C for 1 h, after which the reaction was stopped with 2 mL of 1 mol L⁻¹ H₂SO₄. The product of the reaction (triphenyl formazan) was washed using acetic ether, and the root activity was analyzed by the reduction in TTC at 485 nm.

2.4. Root hydraulic conductance

The root hydraulic conductance (*L_p*) was measured as described by Liu et al. (2001). The root surface area was determined using a root scanner (LA-S, Wseen, Hangzhou, China), and the root hydraulic conductance was calculated as $L_{p_r} = Q_v / (S \times P) = J_v / P$, where Q_v (m³·min⁻¹) is the water flux density (i.e., exuded sap was collected in Eppendorf tubes 1 min periods for each stabilized pressure, and weighed; set the density of the juice to 1 g cm⁻³), S (m²) is the root surface area, P (MPa) is the pressure on the root, and J_v (m·min⁻¹) is the flow rate.

2.5. Measurement of total N, free amino acid, soluble protein, NO₃⁻ and NH₄⁺ contents

A 0.2 g sample of dry leaf tissue was digested in a solution of H₂SO₄-H₂O₂, and the extract was used to determine the total N content via the Kjeldahl method (Bao, 2000).

The free amino acid content was measured using the ninhydrin method (Aurisano et al., 1995).

The soluble protein was extracted from leaves in a medium that contained 50 mmol L⁻¹ PBS (pH 7.8), after which the extract was centrifuged at 12000 × g for 20 min at 4 °C. The supernatant was used to determine the content of soluble protein according to the Bradford method, with bovine serum albumin (BSA) used as a standard (Bradford, 1976).

The NO₃⁻ and NH₄⁺ contents were measured using the salicylic acid method (Cataldo et al., 1975) and the phenol-hypochlorite method (Solorzano, 1969), respectively.

2.6. Enzyme assays

NR and NiR activities were measured using the sulfanilic acid method (Datta and Sharma, 1999), and the activities were assayed based on the absorbance at 540 nm and the micrograms of NO₂⁻ consumed per hour per gram.

Glutamine synthetase (GS) activity was expressed as micromoles of γ-glutamyl-hydroxamate (γ-GHM) produced per hour per gram of

sample based on the absorbance at 540 nm (Lin and Kao, 1996).

Glutamate synthase (GOGAT) activity in the supernatant was determined by measuring the decrease in absorption at 340 nm caused by the enzymatic oxidation of NADH (Wang et al., 2005). The reaction mixtures contained 100 mmol L⁻¹ phosphate buffer (pH 7.6), 20 mmol L⁻¹ L-glutamine, 20 mmol L⁻¹ α-ketoglutaric acid (α-KG), 10 mmol L⁻¹ KCl, 3 mmol L⁻¹ NADH and 0.2 mL of crude enzyme.

Glutamate dehydrogenase (GDH) activity in the supernatant was determined by measuring the decrease in absorption at 340 nm caused by the enzymatic oxidation of NADH (Singh and Srivastava, 2010). The reaction mixtures contained 25 mmol L⁻¹ phosphate buffer (pH 7.6), 150 mmol L⁻¹ NH₄Cl, 0.5 mmol L⁻¹ CaCl₂, 20 mmol L⁻¹ α-KG, 3 mmol L⁻¹ NADH and 0.3 mL of crude enzyme.

Glutaminoxaloacetic transaminase (Asp-AT) and glutamic-pyruvic transaminase (Ala-AT) were extracted in a chilled mortar in 50 mmol L⁻¹ Tris-HCl (pH 7.8) containing of 1 mmol L⁻¹ EDTA, 15% (v/v) glycerol, 14 mmol L⁻¹ 2-mercaptoethanol and 0.1% (v/v) Triton X-100. The homogenate was centrifuged at 10000 × g for 10 min at 4 °C and the supernatant was used for Asp-AT and Ala-AT activities assay as described by Tang (1999).

Iso citrate dehydrogenase (IDH) activity was determined by monitoring the reduction of NADP⁺ at 340 nm (Zhong et al., 2016).

A total amount of 1 g of root tissue was added to 5 mL grinding buffer that contained [25 mmol L⁻¹ Hepes-Tris (pH 7.6), 250 mmol L⁻¹ mannitol, 5 mmol L⁻¹ EGTA, 5 mmol L⁻¹ EDTA, 10 mmol L⁻¹ KCl, 2 mmol L⁻¹ PMSF, 1.5% PVP, 0.5% BSA, 5 μg L⁻¹ BHT, 5 mmol L⁻¹ K₂S₂O₈, 1 mmol L⁻¹ DTT] rapidly homogenized. The homogenate was filtered through four layers of cold-water-soaked gauze. The suspension was centrifuged at 13000 × g for 30 min to remove coarse matter and then at 60000 × g for 30 min at 4 °C. The supernatant was carefully discarded, and the pellet was suspended in 1 mL resuspension buffer that contained [2.5 mmol L⁻¹ Hepes-Tris (pH 7.6), 250 mmol L⁻¹ mannitol, 1 mmol L⁻¹ EDTA, 1 mmol L⁻¹ DTT] and placed in a discontinuous sucrose gradient [containing 45%, 36%, and 22% (W/V)] and then centrifuged at 70000 × g for 2 h. Finally, take 22% and 36% of the solution as the vacuolar microcapsules, take 36% and 45% of the solution as plasma membrane microcapsules (Wang et al., 2005).

The activities of the plasma membrane H⁺-ATPase (P-H⁺-ATPase), vacuolar membrane H⁺-ATPase (V-H⁺-ATPase), and vacuolar membrane H⁺-PPase (V-H⁺-PPase) were determined by measuring the release of inorganic phosphate (Pi). V-H⁺-ATPase activity: The reaction mixtures contained 30 mmol L⁻¹ Hepes-Tris (pH 7.8), 50 mmol L⁻¹ KCl, 30 mmol L⁻¹ MgSO₄, 0.1 mmol L⁻¹ H₈MoN₂O₄, 0.1 mmol L⁻¹ Na₃VO₄, 3 mmol L⁻¹ ATPN₂, and 0.1 mL of crude enzyme. Preincubated 30 min at 37 °C, then stop reaction by adding 50 μL of 55% TCA. Incubate for 15 min at room temperature, add Pi protectant (contained 16 mmol L⁻¹ EDTA-Na₂, 4% H₈MoN₂O₄, 1 mmol L⁻¹ PVP, 0.172 mol L⁻¹ H₈N₂O₆S, 87.5 mmol L⁻¹ H₂SO₄) and 6.47 mmol L⁻¹ NaOH. For the P-H⁺-ATPase activity, the buffer is 30 mmol L⁻¹ Hepes-Tris (pH 6.5), substitute KCl with 50 mmol/L KNO₃, with 0.1 mmol L⁻¹ NaN₃ inhibited mitochondrial activity, added 0.2% TritonX-100, dissolved the membrane lipid to bind the substrate and the enzyme. The remaining steps were the same as the determination of the V-H⁺-ATPase activity. For the V-H⁺-PPase activity, the buffer is 30 mmol L⁻¹ Hepes-Tris (pH 8.0), substitute ATPN₂ with 3 mmol L⁻¹ Na₄PPi.

2.7. RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from 0.2 g root samples using an RN09-EASYspin Plant RNA Rapid Extraction Kit (Aidlab, Beijing, China). The RNA was reverse transcribed to generate cDNA using a 5X All-In-One RT MasterMix Kit (with an AccuRT Genomic DNA Removal Kit) (Abm, Vancouver, Canada). qRT-PCR was performed using a LightCycler[®] 96 real-time PCR system (Roche, Basel, Switzerland). The relative expression level of the genes was calculated using the 2^{-ΔΔCT} method. The gene-specific primers used are shown in Table 1.

Table 1

Primer sequences of qRT-PCR.

| Genes | Accession No. | Primer sequences (forward/reverse) |
|-----------------|---------------|--|
| <i>Tua</i> | XM011654602 | F 5'-CTCTCAACCCATTCTCTCTTGG-3' R 5'-CGGTTGAGGTTCGAGTAGTTAG-3' |
| <i>CsPIP1-2</i> | KF641170 | F 5'-TTGGGAGCGGCCATTATTTA-3' R 5'-TGAAAGGGATGGCTCTGATTAC-3' |
| <i>CsPIP2-4</i> | KF641175 | F 5'-CCCAGAGAGCTACCAAAT-3' R 5'-CTTCCGTGGGAGTAACCAATAA-3' |
| <i>CsPMA</i> | AF289025 | F 5'-CACGAGCAGGCATAAGAGAA-3' R 5'-GCTTGTCTCGATGCCAATTAC-3' |
| <i>CsVHA-A</i> | AY580162 | F 5'-CATGAAGCGTACCACTCTTGTA-3' R 5'-CTCTGAAGTACTCGGCCAAAG-3' |
| <i>CsVHA-c</i> | EF373537 | F 5'-GCTTATGGAACGGCGAAGA-3' R 5'-CAGCCATAACCACTGGAACA-3' |
| <i>CsVP</i> | EF373538 | F 5'-GGTGCTATGCTGCCATACT-3' R 5'-GACCTGGGATTGTGTTGAATTG-3' |

2.8. Statistical analyses

The statistical analyses were carried out using DPS software 17.10 (Zhejiang University, Hangzhou, China). Two-way analysis of variance was applied to evaluate the salt and CO₂ interaction. All the data were tested for significant treatment differences using Duncan's multiple range test ($\alpha = 0.05$).

3. Results

3.1. Root activity, hydraulic conductivity and PIP gene expression

As shown in Table 2, salt stress significantly inhibited the root activity and hydraulic conductivity of cucumber seedlings. At ambient [CO₂], compared with the control treatment, the 80 mmol L⁻¹ NaCl treatment reduced the root activity and hydraulic conductivity by 47.5% and 74.0%, respectively. Enriched [CO₂] alleviated the inhibition of root stress caused by salt stress. However, the expression of the plasma membrane aquaporin gene is relatively complex. The expression of the *CsPIP1-2* and *CsPIP2-4* genes was markedly upregulated under salt stress. In contrast, enriched [CO₂] downregulated the expression of the *CsPIP2-4* gene, but the *CsPIP1-2* transcript level was unaffected.

3.2. Ion balance

As shown in Table 3, at the ambient [CO₂], the 80 mmol L⁻¹ NaCl treatment significantly increased the Na⁺ content in the shoots and roots of the plants and significantly reduced the K⁺ content, resulting in a significant increase in the Na⁺/K⁺ ratio. Enriched [CO₂] reduced the Na⁺ content to some extent in the shoots and increased the K⁺ content, thus significantly reducing the Na⁺/K⁺ ratio. The contents of Na⁺ and K⁺ and the Na⁺/K⁺ ratio in the roots under salt stress subjected to enriched [CO₂] are similar to those in the shoots.

As shown in Table 4, at ambient [CO₂], compared with the control treatment, the 80 mmol L⁻¹ NaCl treatment significantly increased the activities of P-H⁺-ATPase, V-H⁺-ATPase and V-H⁺-PPase in the cucumber seedling roots by 60.8%, 20.6% and 33.9%, respectively. Enriched [CO₂] further increased the P-H⁺-ATPase and V-H⁺-ATPase activities but reduced the V-H⁺-PPase activity. In terms of the gene transcript level, the effect of salt stress on the transcript level was consistent with that of the enzyme activity; however, enriched [CO₂] significantly increased the expression of only the *CsVHA-c* gene in the roots under salt stress and significantly decreased the expression of the *CsVP* gene; the expression of the *CsPMA* and *CsVHA-A* genes was unaffected.

Table 2

Effects of CO₂ enrichment on root activity, root hydraulic conductance and the transcript levels of the *CsPIP1-2* and *CsPIP2-4* genes in cucumber seedlings under salt stress.

| CO ₂ concentration (μmol·mol ⁻¹) | NaCl concentration (mmol·L ⁻¹) | Root activity (μg TTF·h ⁻¹ ·g ⁻¹ FW) | Root hydraulic conductance (10 ⁻⁶ m ³ ·m ⁻² ·min ⁻¹ ·MPa ⁻¹) | <i>CsPIP1-2</i> | <i>CsPIP2-4</i> |
|---|--|--|--|-----------------|-----------------|
| 400 | 0 | 146.42 ± 21.79b | 1.54 ± 0.10b | 1.00 ± 0.03b | 1.00 ± 0.03c |
| | 80 | 76.89 ± 23.78d | 0.40 ± 0.11d | 4.67 ± 0.10a | 3.13 ± 0.18a |
| 800 | 0 | 186.45 ± 23.08a | 2.22 ± 0.24a | 1.03 ± 0.13b | 0.44 ± 0.03d |
| | 80 | 112.23 ± 9.78c | 0.62 ± 0.07c | 4.30 ± 0.36a | 2.48 ± 0.11b |

Different lowercase letters in the same column indicate significant differences at the 0.05 level among treatments. Values are the means ± SD (n = 3). The same is true below.

3.3. Total N, free amino acid and soluble protein contents

The salt stress significantly increased the soluble protein content in the leaves of cucumber seedlings but significantly reduced the total N content (Table 5). Enriched [CO₂] not only reduced the free amino acid content in cucumber seedling leaves under salt stress but also reduced the total N content, but the soluble protein content was not affected.

3.4. Leaf NO₃⁻ concentration and its reduction

Fig. 1 shows that salt stress significantly reduced the NO₃⁻ content (Fig. 1A) and NR activity (Fig. 1B) but increased the NiR activity (Fig. 1C) in the leaves of cucumber seedlings. The change trend of NiR activity in response to enriched [CO₂] was consistent with that of salt stress but had no effect on the NO₃⁻ content or NR activity.

3.5. Leaf NH₄⁺ concentration and assimilation

At ambient [CO₂], compared with the control treatment, the 80 mmol L⁻¹ NaCl treatment inhibited the activity GS (Fig. 2B), GOGAT (Fig. 2C), Asp-AT (Fig. 2E) and IDH (Fig. 3) by 36.8%, 60.4%, 61.0%, and 27.4%, respectively. Moreover, enriched [CO₂] further reduced the activities of GS, GOGAT, Asp-AT and IDH under salt stress and reduced the activity of Ala-AT (Fig. 2F), while the activity of GDH (Fig. 2D) was significantly increased; however, enriched [CO₂] and salt treatment had no significant effect on the NH₄⁺ content (Fig. 2A).

4. Discussion

The Na⁺ content and Na⁺/K⁺ ratio are usually used to measure ion toxicity (Shabala and Cuin, 2007). In the present study, salt stress induced the accumulation of Na⁺ in cucumber plants, which was accompanied by a sharp decrease in K⁺ content (Table 3). This inverse relationship could be due to the competition between Na⁺ and K⁺ uptake in the roots. Because their ionic radii are similarly hydrated, Na⁺ and K⁺ are difficult to discriminate, resulting in the basic mechanism of Na⁺ toxicity (Blumwald, 2000); moreover, Na⁺ has no activated cations in plants, and excessive accumulation can inactivate related metabolic enzymes. Enriched [CO₂] can reduce Na⁺ and promote K⁺ accumulation in plants, thereby significantly reducing the Na⁺/K⁺ ratio, maintaining the ion balance in plants under stress conditions, and ensuring normal enzymatic activity. In addition, plants

can also provide energy for the plasma membrane Na⁺/H⁺ antiporters by the P-H⁺-ATPase to discharge Na⁺ from cells (Ashraf and Akhtar, 2004) or by the V-H⁺-ATPase and V-H⁺-PPase to hydrolyze ATP and PPI, respectively, to generate proton kinetic potential to provide a driving force for tonoplast Na⁺/H⁺ antiporters, whose activity pumps Na⁺ from within the cytoplasm into the vacuole (Blumwald, 2000) to increase salt tolerance. Therefore, we determined the transcript levels of both genes involved in root proton pump activity and related genes and found that the change in the activity of P-H⁺-ATPase in response to enriched [CO₂] was not dependent on changes in the expression of the *CsPMA* genes (Table 4). These results indicate that alterations of P-H⁺-ATPase activity in cucumber roots are due primarily to posttranslational alterations induced by enriched [CO₂]; however, activation or inhibition of V-H⁺-ATPase and V-H⁺-PPase activities involves mRNA levels. Salt stress increases mainly the activity of P-H⁺-ATPase and V-H⁺-PPase in plants to adapt to salt damage, and enriched [CO₂] regulates proton pumps by activating P-H⁺-ATPase and V-H⁺-ATPase activity. Salt stress can decrease stomata opening [through osmotic shock, hydraulic signal and ABA (Christmann et al., 2013; Zhang et al., 2006)] and, suppresses CO₂ influx, thereby, suppress the Calvin cycle and increase the cyclic electron flow (Sukhov et al., 2015), cause photosynthetic phosphorylation to be blocked. At the same time, oxidative phosphorylation may be inhibited under stress, resulting in a sharp decrease in ATP (Zhong et al., 2016). Compared with ATP contents, PPI contents remain relatively constant under adverse conditions (Hong et al., 2010), so plants adapt to stress mainly by increasing their V-H⁺-PPase activity; increase of CO₂ can eliminate limitation of CO₂ influx, stimulate the Calvin cycle and decrease cyclic electron flow around PSI and, enriched [CO₂] can improve the synthesis of ATP by increasing both the number of mitochondria and the carbohydrate content (Li et al., 2013) and by increasing the amount of substrates for P-H⁺-ATPase and V-H⁺-ATPase, thus ensuring the function of the proton pumps.

Maintaining a good water state under stress conditions and diluting salt within a whole plant to reduce ion toxicity are very important for improving the salt tolerance of plants (Romero-Aranda et al., 2006). Our previous research showed that enriched [CO₂] alleviated the adverse effects of high NaCl by increasing stomatal closure and reducing transpiration rates (Li et al., 2019), and in this study, enriched [CO₂] increased root hydraulic conductance and increased root activity, thereby enhancing root water absorption (Table 2), which may be one of the means for plants to maintain water balance under salt stress.

Table 3

Effects of CO₂ enrichment on the Na⁺ and K⁺ contents and the Na⁺/K⁺ ratio in cucumber seedlings under salt stress.

| CO ₂ concentration (μmol·mol ⁻¹) | NaCl concentration (mmol·L ⁻¹) | Na ⁺ (mmol·g ⁻¹ DW) | | K ⁺ (mmol·g ⁻¹ DW) | | Na ⁺ /K ⁺ | |
|---|--|---|----------------|--|----------------|---------------------------------|----------------|
| | | Shoot | Root | Shoot | Root | Shoot | Root |
| 400 | 0 | 0.036 ± 0.010b | 0.062 ± 0.008b | 1.482 ± 0.364a | 1.994 ± 0.317a | 0.025 ± 0.007c | 0.032 ± 0.010c |
| | 80 | 0.769 ± 0.153a | 0.512 ± 0.145a | 0.776 ± 0.207b | 1.196 ± 0.318b | 1.003 ± 0.089a | 0.430 ± 0.059a |
| 800 | 0 | 0.040 ± 0.012b | 0.056 ± 0.014b | 0.986 ± 0.161ab | 1.938 ± 0.305a | 0.040 ± 0.006c | 0.030 ± 0.008c |
| | 80 | 0.659 ± 0.100a | 0.428 ± 0.034a | 0.842 ± 0.187ab | 2.297 ± 0.438a | 0.792 ± 0.069b | 0.193 ± 0.052b |

Table 4

Effects of CO₂ enrichment on the activities of P-H⁺-ATPase, V-H⁺-ATPase, and V-H⁺-PPase and the transcript levels of the *CsPMA*, *CsVHA-A*, *CsVHA-c* and *CsVP* genes in cucumber seedlings under salt stress.

| CO ₂ concentration (μmol·mol ⁻¹) | NaCl concentration (mmol·L ⁻¹) | P-H ⁺ -ATPase activity (μg·g ⁻¹ FW) | V-H ⁺ -ATPase activity (μg·g ⁻¹ FW) | V-H ⁺ -PPase activity (μg·g ⁻¹ FW) | <i>CsPMA</i> | <i>CsVHA-A</i> | <i>CsVHA-c</i> | <i>CsVP</i> |
|---|--|---|---|--|---------------|----------------|----------------|--------------|
| 400 | 0 | 4.77 ± 0.69d | 5.88 ± 1.10c | 6.05 ± 0.36c | 1.00 ± 0.14b | 1.00 ± 0.12b | 1.00 ± 0.10c | 1.00 ± 0.08c |
| | 80 | 7.67 ± 1.49b | 7.09 ± 0.54b | 8.10 ± 0.16a | 18.79 ± 2.56a | 1.18 ± 0.04a | 1.42 ± 0.05b | 4.23 ± 0.10a |
| 800 | 0 | 5.86 ± 0.21c | 6.49 ± 0.53bc | 5.96 ± 0.32c | 0.89 ± 0.22b | 0.98 ± 0.03b | 0.99 ± 0.15c | 0.92 ± 0.10c |
| | 80 | 10.21 ± 0.35a | 9.24 ± 0.44a | 7.23 ± 0.41b | 16.99 ± 1.36a | 1.24 ± 0.13a | 1.80 ± 0.28a | 1.22 ± 0.14b |

Table 5

Effects of CO₂ enrichment on the total N, free amino acids and soluble protein contents in the leaves of cucumber seedlings under salt stress.

| CO ₂ concentration (μmol·mol ⁻¹) | NaCl concentration (mmol·L ⁻¹) | Total nitrogen (mg·g ⁻¹ DW) | Free amino acids (μg·g ⁻¹ FW) | Soluble protein (mg·g ⁻¹ FW) |
|---|--|--|--|---|
| 400 | 0 | 34.4 ± 2.3a | 309.24 ± 14.02 ab | 12.21 ± 0.29b |
| | 80 | 28.7 ± 1.2b | 317.88 ± 6.32a | 12.91 ± 0.32a |
| 800 | 0 | 34.9 ± 3.5a | 273.64 ± 18.85c | 12.45 ± 0.16b |
| | 80 | 24.5 ± 0.9c | 284.03 ± 7.18bc | 13.02 ± 0.06a |

According to reports, 70–90% of the water flowing through the roots is transported through plasma membrane aquaporins, and the expression of the plasma membrane aquaporin genes *CsPIP1-2* and *CsPIP2-4* accounts for approximately 80% of the protein expression of all the water channels in the cucumber root system (Qian et al., 2015). Therefore, in this experiment, we measured mainly the expression levels of *CsPIP1-2* and *CsPIP2-4* and found that expression of the root plasma membrane aquaporin genes was stimulated under salt stress (Table 2), indicating that salt promoted the transcription level of the aquaporin gene, but the strength of the root hydraulic conductance is decision by the ability of root aquaporin to transport water (i.e., the number and function of the aquaporin). The mRNA produced by transcription needs to undergo two stages of mRNA translation and post-translational modification to synthesize functional proteins. Therefore, although salt stress stimulated the expression of the root plasma membrane aquaporin gene, the root hydraulic conductance was still inhibited. Enriched [CO₂] significantly reduced the expression of *CsPIP2-4* and but no effect on *CsPIP1-2*. Moreover, Zaghudud et al. (2013) reported that enriched [CO₂] significantly increased the hydraulic conductance of broccoli

roots under 90 mmol L⁻¹ NaCl stress but significantly reduced the abundance of PIP1 and PIP2 proteins in root tissues; thus, the authors proposed that the effect of CO₂ on aquaporin functionality must be considered.

Roots are not only the main part of plants that absorb water but also the place where mineral elements are absorbed. Salt stress inhibited the root activity of cucumber seedlings (Table 2), and due to the toxic effects of salt ions and a lack ATP (Pérez-López et al., 2013; Parida and Das, 2004), the NO₃⁻ transport rate was reduced, which weakened the absorption capacity of NO₃⁻ by the roots, thereby reducing the leaf NO₃⁻ content (Fig. 1A). NR acts as an inducible enzyme whose activity is activated by NO₃⁻; salt stress reduced the NO₃⁻ content in the leaves, resulting in inhibition of NR activity (Fig. 1B). NR is the rate-limiting enzyme of NO₃⁻ reduction; however, under salt stress, the NiR activity increased (Fig. 1C), but the inhibited NO₃⁻ reduction was still unaffected. Enriched [CO₂] reversed the inhibition of root activity caused by salt stress (Table 2) and enhanced the net photosynthetic rate (Li et al., 2019). A higher net photosynthetic rate may increase the proportion of total biomass allocated to underground tissues, especially

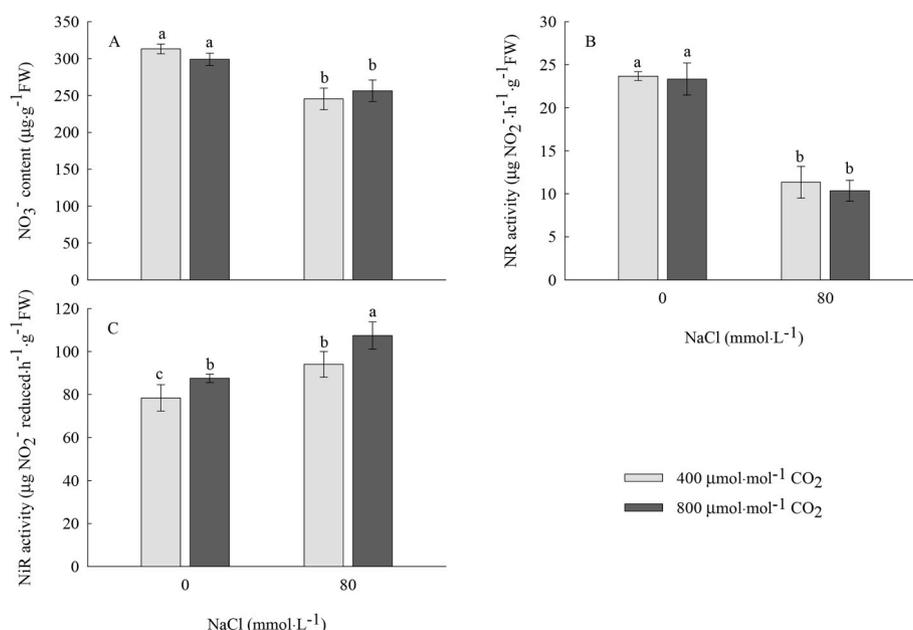


Fig. 1. Effects of CO₂ enrichment on the NO₃⁻ content, NR activity and NiR activity in the leaves of cucumber seedlings under salt stress. Different lowercase letters in the same column mean significant differences at the 0.05 level among treatments. Values are the means ± SD (n = 3). The same is true below.

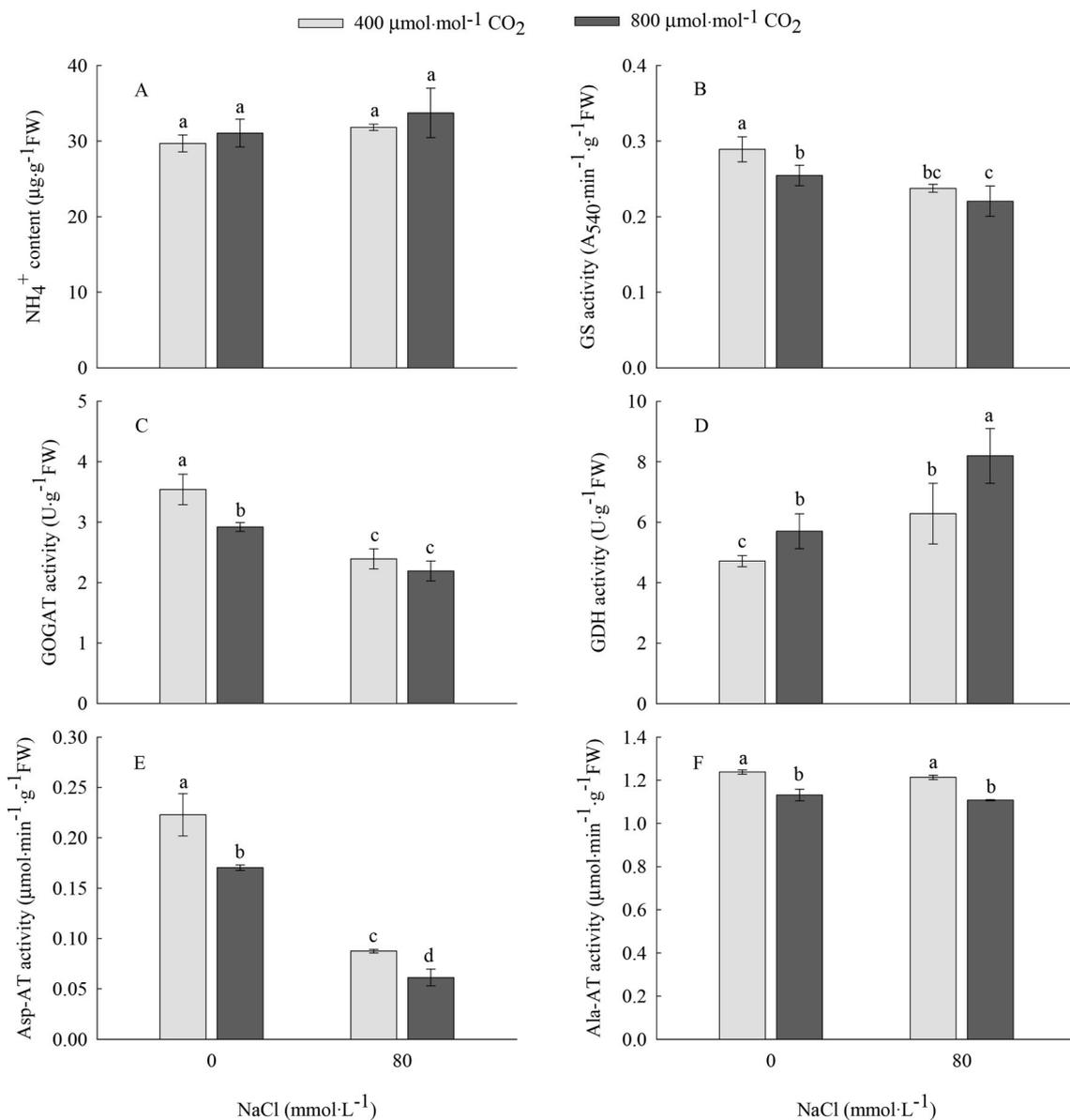


Fig. 2. Effects of CO₂ enrichment on the NH₄⁺ content and its enzymatic assimilation activity in the leaves of cucumber seedlings under salt stress.

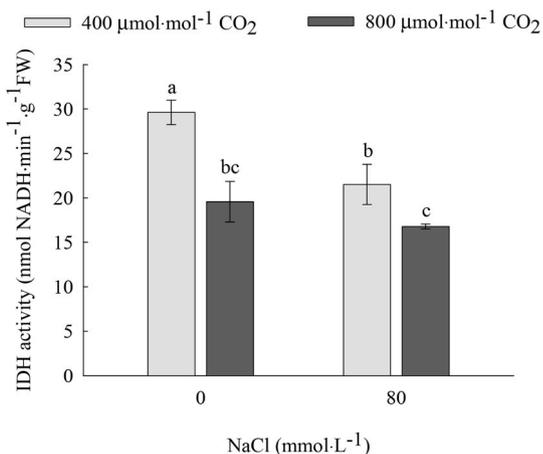


Fig. 3. Effects of CO₂ enrichment on IDH activity in leaves of cucumber seedlings under salt stress.

fine roots. This scenario should allow plants to exploit a larger soil volume and ultimately provide increased N uptake even under saline conditions (Bassirirad et al., 1996). However, our results indicate that enriched [CO₂] has no significant effect on leaf NO₃⁻ content or NR activity under salt stress (Fig. 1); these findings may be because at high [CO₂], the relatively low transpiration rates may reduce the driving force for NO₃⁻ movement from the soil to the leaves (Fangmeier et al., 2002), decreasing the content of NO₃⁻ in the leaves and reducing the NR activity. Agüera et al. (2006) reported no differences in NR activity between cucumber plants grown under ambient and enriched [CO₂] conditions.

On the other hand, the increase in NiR activity in response to enriched [CO₂] promotes the conversion of NO₂⁻ to NH₄⁺ under salt stress; free NH₄⁺ is usually toxic, so NH₄⁺ absorbed by plants or NH₄⁺ produced by reduction must be assimilated immediately. NH₄⁺ assimilation in plants occurs mainly via the GS-GOGAT pathway. Our results show that salt stress and enriched [CO₂] significantly inhibited the activity of both GS and GOGAT (Fig. 2B and C); GOGAT catalyzes the conversion of glutamine (Gln) to glutamic acid (Glu), which requires the formation of a carbon skeleton by α-KG catalyzed by IDH (Du

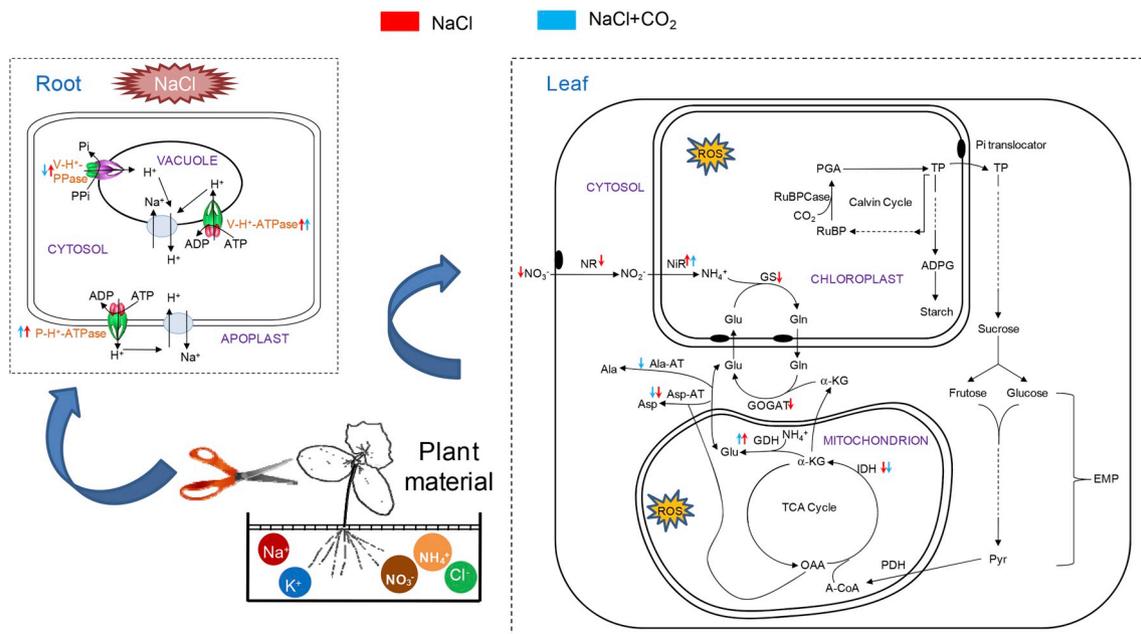


Fig. 4. Simplified metabolic map in response to NaCl and enriched $[\text{CO}_2]$ + NaCl based on the relevant metabolic pathways in cucumber after 8 d. The NaCl treatment is shown in red, and the enriched $[\text{CO}_2]$ + NaCl treatment is shown in blue. A downward arrow indicates inhibition, and an upward arrow indicates promotion. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2017), and salt stress and enriched $[\text{CO}_2]$ significantly inhibited IDH activity (Fig. 3), resulting in a decrease in the production of $\alpha\text{-KG}$. In addition, Li et al. (2018) used ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) technology to perform a metabolomics analysis on cucumber leaves and found that enriched $[\text{CO}_2]$ significantly reduced the relative content of $\alpha\text{-KG}$, thus affecting GOGAT activity and inhibiting the assimilation of NH_4^+ . Interestingly, even though enriched $[\text{CO}_2]$ inhibited the activity of IDH under salt stress, a change in GOGAT activity was not observed under the experimental conditions; thus, the GOGAT mechanism of influence needs to be further studied. Although GDH has a relatively high K_m value for NH_4^+ , it is also an important pathway in rapid NH_4^+ assimilation, especially when the GS-GOGAT pathway in plant cells is inhibited considerably. Under the action of enriched $[\text{CO}_2]$ and salt stress, increased GDH activity (Fig. 2D) effectively promoted the conversion of NH_4^+ to Glu, thereby reducing the toxicity of NH_4^+ to cucumber seedlings. Glu produced by both the GS-GOGAT pathway and GDH transformation is the major amino donor for the synthesis of other amino acids and can be converted to aspartic acid (Asp) and alanine (Ala) by transamination of Asp-AT and Ala-AT (Hodges, 2002). Therefore, in this experiment, the inhibition of transaminase by salt and enriched $[\text{CO}_2]$ (Fig. 2E and F) is related mainly to the weakened activity of both GS and GOGAT.

Metabolic disorders induced by salinity can result in great changes in the metabolism of organic solutes essential to cellular function (Ferreira-Silva et al., 2010). In this study, salinity induced alterations in the leaf contents of free amino acids and soluble protein, which were also influenced by CO_2 ; salt stress increased the free amino acid content in the studied plants, regardless of the CO_2 concentration (Table 5). Salt-induced changes in the contents of organic solutes such as amino acids (especially proline) and proteins may represent metabolic alterations that are associated with resistance and/or sensitivity to salinity (Ashraf and Harris, 2004). However, enriched $[\text{CO}_2]$ reduced the free amino acid and total N concentrations in the leaves under salt stress (Table 5). Stitt and Krapp, (1999) stressed that the decrease in N content in response to enriched $[\text{CO}_2]$ is partly a consequence of the increase in nonstructural carbohydrates and indicates that NO_3^- uptake and assimilation often fail to keep pace with photosynthesis and

growth under enriched $[\text{CO}_2]$. In this experiment, although enriched $[\text{CO}_2]$ significantly reduced the activity of IDH, Asp-AT and Ala-AT under salt stress, it had no significant effect on the activity of NR, GS or GOGAT, indicating that the decrease in free amino acid and total N concentrations was not completely due to the inhibition of NO_3^- assimilation, which may also be related to the dilution effect caused by the rapid growth of leaves under enriched $[\text{CO}_2]$. On the other hand, Robredo et al. (2011) reported that enriched $[\text{CO}_2]$ resulted in higher NR activity in barley leaves (supplied with $20 \text{ mmol L}^{-1} \text{ NO}_3^- \text{-N}$), but when the nutrient solution supplied with $14 \text{ mmol L}^{-1} \text{ NO}_3^- \text{-N}$, this phenomenon was not observed in the same barley cultivar (Pérez-López et al., 2013). Thus, N supply has a significant effect on N assimilation in plants grown in a high- CO_2 environment. So in order to improve the rate of N assimilation and ensure the coordination of carbon assimilation and N assimilation in a high- CO_2 environment in the future, we intend to investigate the response of N assimilation to enriched $[\text{CO}_2]$ and salt stress in cucumber seedlings further under different N supply levels and/or N forms.

5. Conclusion

In summary, enriched $[\text{CO}_2]$ can activate P-H + -ATPase and V-H + -ATPase activity in the roots of plants under salt stress to ensure the function of proton pumps, which improves root activity and root hydraulic conductivity and ameliorates root growth status and water absorption (Fig. 4). Moreover, enriched $[\text{CO}_2]$ promotes both the efflux/regionalization and the dilution of Na^+ within plants under the action of membrane proton pumps and water, thereby reducing ion toxicity. On the other hand, although enriched $[\text{CO}_2]$ improves the water status of plants under salt stress, it does not promote N assimilation, which may be related to N availability (N supply level and/or N form). Additional studies on this phenomenon are needed.

Conflicts of interest

The authors declare that they have no conflict of interest.

Author contributions

Shuhao Li performed the main experiments, analyzed the data and wrote the manuscript. Yiman Li and Xinrui He performed some of the experiments. Qingming Li and Binbin Liu designed the experiments. Xizhen Ai and Dalong Zhang analyzed the data.

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