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Research Article

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Effect of nitrate-to-ammonium ratio on changes in pakchoi folate constituents and contents in nutrient solution under hydroponic conditions

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Abstract: Vegetables are important dietary sources of folate for human nutrition. The influence of different nitrogen doses and forms on changes in primary nitrogen metabolism, such as amino acid and protein synthesis, in plants is well established. However, the impacts of nitrate-N (NO_3^-)-to-ammonium-N (NH_4^+) ratio on folate synthesis and accumulation in vegetables are unclear. This study used a hydroponic experiment with six different NO_3^-/ NH_4^+ ratio treatments to investigate the effects of the integrated application of NO_3^- and NH_4^+ on the folate constituents and contents of pakchoi (*Brassica rapa subsp. Chinensis*). The results indicated that an appropriate NO_3^-/ NH_4^+ ratio in nutrient solution could promote pakchoi growth and increase folate contents by increasing polyglutamylated 5-formyl-tetrahydrofolate (5-CHO-THF) and polyglutamylated 5-methyl-THF (5-CH_3-THF). The activities of enzymes related to folate biosynthesis (except folylpolyglutamate synthase (FPGS)) were lower with an NH_4^+-N supply at the same nitrogen concentration. The statistical results revealed a significant negative correlation between folate contents and 14 detected metabolites (including fructose and sucrose, glutamine (Gln) in the nitrogen assimilation, shikimate in the shikimate pathway, citrate, succinate, malate, α-oxoglutarate in the tricarboxylic acid cycle, and *p*-aminobenzoate (*p*ABA) and 6-hydroxymethyldihydropterin pyrophosphate (HMDH-P_2) in the folate biosynthesis pathway), implying that the enhancement of folates biosynthesis with NH_4^+-N supply increased the consumption of the folate precursors and intermediate metabolites. Additionally, NH_4^+-N supply could improve folate stability by increasing polyglutamylated folates and reducing γ-glutamyl hydrolase (GGH) activity; the latter could weaken folate deglutamylation. As the best growth and highest total folate content were obtained at the appropriate NO_3^-/ NH_4^+ ratio, strategic selection of the NO_3^-/ NH_4^+ ratio should be considered for the hydroponic cultivation of foliar vegetable crops.

Key words: Pakchoi (*Brassica Rapa Subsp. Chinensis*); Nitrate; Ammonium; Folate; Biosynthesis

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1 Introduction

Folate (vitamin B9) is the general term for tetrahydrofolate (THF) and its derivatives (folates). Folates play a crucial role in one-carbon (1C) metabolism in living organisms, acting as an enzyme cofactor capable of receiving and donating 1C units. Folates are essential micronutrients for humans that must be obtained through diet due to our lack of ability to synthesize them (de la Garza et al., 2019). Folate deficiency in the periconceptional period tends to increase the risk of neural tube deformities (NTDs) in infants, and the increased

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risk of several chronic disorders in humans has also been associated with folates deficiency, including cancer, cardiovascular disease, and neurological conditions (Bailey et al., 2015; Force et al., 2017). Adequate dietary intake of folates is crucial for human health. The recommended dietary allowance (RDA) for adults is 400 μg/d. For pregnant women, it is 600 μg/d, but not exceeding 1000 μg/d (Baily, 1998). Synthetic folic acid (FA) has lower bioavailability than natural folate, and excessive intake may have negative health effects (Blancquaert et al., 2014; Scaglione and Panzavolta, 2014). Optimizing folate supply from natural foods is therefore essential.

The folate molecule comprises three main components: a *p*-aminobenzoate (*p*ABA) moiety bound to a 2-amino-4-hydroxypteridine (pterin) ring via a carbon (C)-nitrogen (N) bond, linked to a glutamate (Glu) tail via a peptide bond (Fig. 1). THF contains numerous derivatives with different properties, determined by the substituents on N_5 and N_{10}, as well as the length of the Glu tail (Gorelova et al., 2017; de la Garza et al., 2019). Folates are stabilized through polyglutamylation and accumulate in vacuoles as polyglutamates (Akhtar et al., 2010; Tyagi et al., 2023). Fig. 1 illustrates the metabolic pathway for folate biosynthesis in plants, which can be divided into four parts: precursor synthesis, folate assembly, polyglutamylation and deglutamylation, and transport and distribution (Gorelova et al., 2017; Hanson and Gregory, 2011). Carbohydrate metabolism is the source of upstream substrates for the folate precursors. These substrates provide chorismate (CHI) for *p*ABA synthesis and supply guanosine triphosphate (GTP) for the conversion to pterin (Fig. 1) (Kołton et al., 2022; Yokoyama et al., 2022).

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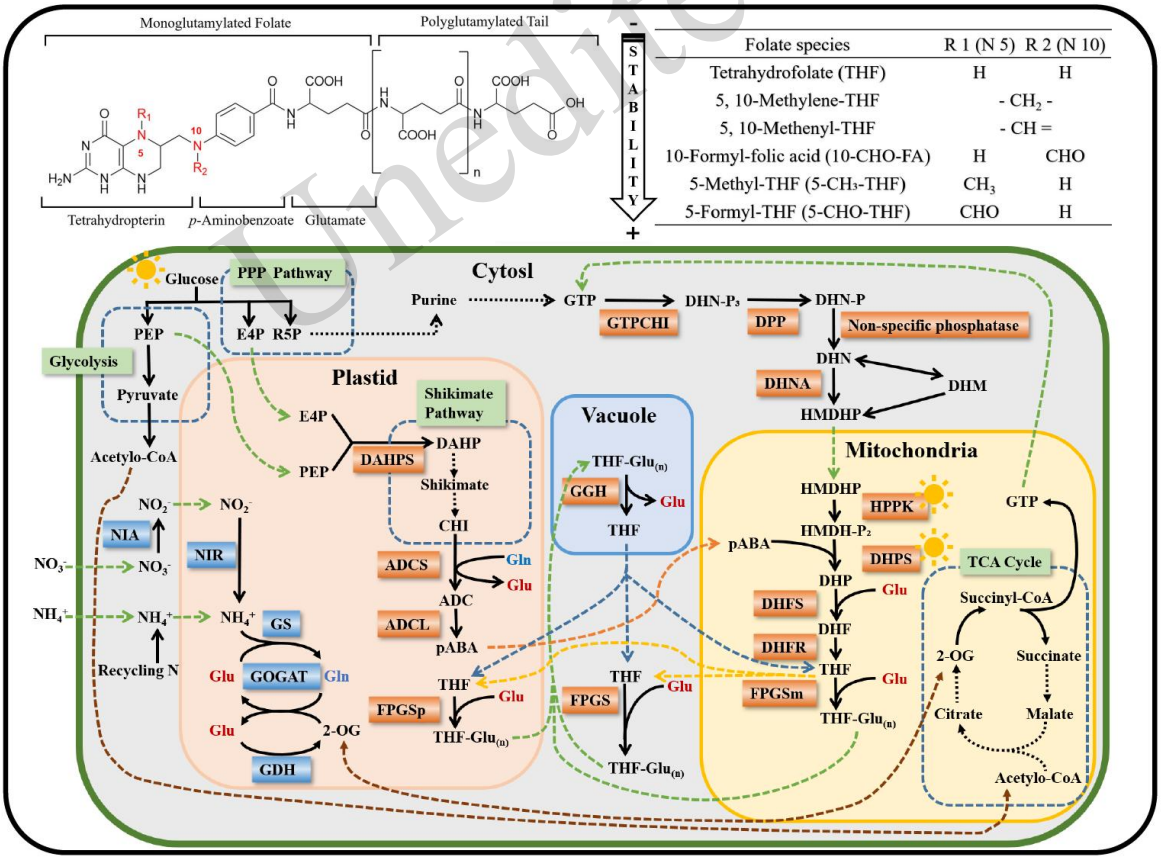

Fig. 1 Folate constituents and their biosynthetic pathway in plants. The two red Ns indicate the positions of one-carbon groups. R1 and R2 represent various one-carbon substituents. Black arrows indicate biosynthetic steps: the solid line indicates the direct step, and the dotted line indicates the indirect step. Non-black dotted arrows indicate transmembrane transport of different substances. PPP Pathway, pentose phosphate pathway. TCA Cycle, tricarboxylic acid cycle. Chemical substances abbreviations: PEP, phos-phoenolpyruvate; E4P, erythrose 4-phosphate; R5P, ribsose-5-phospate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; CHI, chorismate; ADC, aminodeoxychorismate; *p*ABA,

p-aminobenzoate; GTP, guanosine triphosphate; DHN-P_3, dihydroneopterin (DHN) triphosphate; DHN-P, DHN monophosphate; DHM, dihydromonapterin; HMDHP, 6-hydroxymethyldihydropterin; HMDH-P_2, 6-hydroxymethyldihydropterin pyrophosphate; DHP, dihydropteroate; DHF, dihydrofolate; THF-Glu(n), THF-polyglutamate; Glu, glutamate; Gln, glutamine; 2-OG, α-oxoglutarate. Enzymes abbreviations: DAHPS, DAHP synthase; ADCS, ADC synthase; ADCL, ADC lyase; GTPCHI, GTP cyclohydrolase I; DPP, DHN triphosphate diphosphatase; DHNA, DHN aldolase; HPPK-DHPS, HMDHP pyrophosphokinase-DHP synthase; DHFS, DHF synthase; DHFR, DHF reductase; FPGS (m or p), folylpolyglutamate synthase (mitochondrial or plastidial isoform); GGH, γ-glutamyl hydrolase; NIA, nitrite reductase; NR, nitrate reductase; GS, Gln synthetase; GOGAT, Gln 2-OG aminotransferase; GDH, Glu dehydrogenase.

Currently, crops folate biofortification can be strategically achieved by promoting folate over-synthesis, enhancing its stability and bioavailability via: (1) breeding, (2) metabolic engineering, (3) environmental regulation (light or temperature), (4) chemical elicitation (phytohormones or folates synthesis precursors), and (5) post-harvest processing (Delchier et al., 2016; Gorelova et al., 2017; Kołton et al., 2022; Saini et al., 2016). However, these approaches may meet obstacles such as long development cycles, regulation or restriction of transgenic food, additional production costs, and the risk of potential yield reductions. For example, adding Glu and phenylalanine significantly increased the folate content of hydroponically cultivated spinach but reduced its biomass (Watanabe et al., 2017). It is therefore worth exploring universal, inexpensive, and safe means of crop folates biofortification.

N is essential for plant growth and development and is a crucial component of macromolecules such as proteins, nucleic acids, hormones, and vitamins. Plants absorb N in two forms: inorganic N (nitrate-N (NO_3^-) and ammonium-N (NH_4^+)) and organic N (urea, amino acids, and peptides). Inorganic N is the primary form absorbed by plants and the dominant input to agriculture (Boschiero et al., 2019; Krapp, 2015; Liu and von Wiren, 2017). NH_4^+ whether absorbed directly from the outside or converted from NO_3^- reduction is assimilated into glutamine (Gln) and further converted into Glu (Fig. 1) (Xu et al., 2012). The products Gln and Glu affect folate synthesis and accumulation. Gln acts as a N donor for purine biosynthesis, which is used to produce pterin (Lee et al., 2023). In pABA synthesis, under the catalysis of aminodeoxychorismate (ADC) synthase (ADCS), Gln is hydrolyzed to Glu and NH_4^+, and CHI is converted to ADC using the produced NH_4^+ (Nakamichi et al., 2023; de la Garza et al., 2019). Glu, the direct synthetic precursor of folate, serves as a tail, the length (the extent of polyglutamylation) of which determines folate stability (Akhtar et al., 2010; Tyagi et al., 2023).

The evidence suggests that NH_4^+ inhibits net NO_3^- uptake, and NO_3^- accelerates net NH_4^+ uptake. Since the coexistence of NO_3^- and NH_4^+ at a specific ratio can overcome the inhibition, plants acquire more N under specific NO_3^-/NH_4^+ ratios, as compared to NO_3^- or NH_4^+ alone (Hachiya and Sakakibara, 2017). Although absorbing NH_4^+ consumes less reducing equivalents, excess NH_4^+ can cause toxic action in plants by interfering with energy metabolism and ion uptake and/or disrupting pH balance (Esteban et al., 2016; Krapp, 2015). N assimilation is energetically costly, requiring reducing equivalents and adenosine triphosphate (ATP) and C skeletons, which are initially derived from photosynthate. Specifically, these C skeletons are derived from α-oxoglutarate in the tricarboxylic acid (TCA) cycle (Wang et al., 2014). Exogenous NH_4^+ stimulates cellular NH_4^+ assimilation by bypassing NO_3^- reduction as the rate-limiting step of N assimilation. Compared to sole NO_3^-, more C skeletons are required by the coexistence of NO_3^- and NH_4^+ in response to the increased N assimilation, thus enhancing C input to the TCA cycle (Hachiya and Sakakibara, 2017).

N supply is essential for folate synthesis, including its availability and forms. N sufficiency is critical for folate synthesis; lower folate content is exhibited under N deficiency (Wang et al., 2023). Plants' N acquisition and N assimilation change under different NO_3^-/NH_4^+ ratios, thus altering Glu and Gln contents; however, the effect of these changes on folate content has not yet been explored. The substrates of folate biosynthesis precursors and the C skeletons for N assimilation are both derived from the C metabolism of glycolysis (Kołton et al., 2022; Wang et al., 2014). One study indicated that folate accumulation can be controlled by pyruvate metabolism in glycolysis (Lian et al., 2022). Whether folate biosynthesis is regulated by changes in C and N metabolism induced by different NO_3^-/NH_4^+ ratios remains poorly understood. Additionally, vegetables in

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soilless culture rarely use NH_4^+-N as a source of N. Thus, we chose a folate-rich, leafy vegetable, pakchoi (*Brassica rapa subsp. Chinensis*), as the experimental material, and through hydroponic experiments, we studied the effect of N with different NO_3^-/NH_4^+ ratios on folate biosynthesis and accumulation. The results of this study can inform folate enhancement strategies with reasonable nitrogen fertilization.

2 Materials and methods

2.1 Hydroponic experiment and plant sampling.

Pakchoi (*Brassica rapa subsp. Chinensis*) seeds II2B8210 were used for this study. Seeds were surface sterilized by soaking in 2% H_2O_2 for 10 min and fully rinsed with deionized water. Subsequently, the seeds were soaked in deionized water for 24 h in the dark and then germinated in a moist sand substrate. After 10 days, healthy seedlings of a similar size were selected and cultured in pots containing 4 L of nutrient solutions (Table 1) in an aerated medium. We prepared six kinds of nutrient solutions with different NO_3^-/NH_4^+ ratios (100/0, 90/10, 80/20, 70/30, 60/40, and 50/50) in a 15 mmol/L N concentration. After transplanting, all plants were cultured over three periods: for the first 6 d in the 100/0-ratio nutrient solution with 25% ionic strength, then up to 50% ionic strength for 6 d, and finally in the complete (100% ionic strength) nutrient solution for 6 d. Next, all plants were randomly divided into six groups, each cultured in one of the six different complete nutrient solutions for 12 d. The experiment was performed in controlled environmental conditions: temperature ((25±5) °C), photoperiod (light/dark cycle, 12 h/12 h), relative humidity ((55±5)%), pH of all nutrient solutions (adjusted to 5.8 before application), renewal of all nutrient solutions (every 3 d throughout the experiment). Pots were randomly placed with three replicates per treatment, and eight plants were hydroponically grown in each pot.

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Table 1 Nitrogen, phosphorus and potassium concentrations of the nutrient solution with different NO_3^-/NH_4^+ ratios

NO_3^-/NH_4^+	Nutrient concentration (mmol/L)				
ratio	NO_3^--N	NH_4^+-N	Total-N	P	K
100/0	15.0	0.0	15.0	1.0	6.0
90/10	13.5	1.5	15.0	1.0	6.0
80/20	12.0	3.0	15.0	1.0	6.0
70/30	10.5	4.5	15.0	1.0	6.0
60/40	9.0	6.0	15.0	1.0	6.0
50/50	7.5	7.5	15.0	1.0	6.0

Micronutrients were supplied at the following concentrations: 5 mmol/L Ca, 2 mmol/L Mg, 0.1 mmol/L Fe, 0.01 mmol/L Mn, 0.001 mmol/L Cu, 0.001 mmol/L Zn, 0.033 mmol/L B, 0.002 mmol/L Mo.

After culturing for 30 d to reach commercial maturity, the plants were harvested, and the fresh weights were measured. Subsequently, the fresh edible tissues were washed with deionized water and then dried with absorbent paper. The first part was immediately placed into liquid nitrogen and then cryogenically homogenized into fine powder and stored at -80 °C before undergoing metabolite and enzyme activity analysis. Another part was oven-dried at 105 °C for 30 min, then at 60°C to a constant weight, before being ground into a fine powder for element determination.

2.2 Folate extraction and quantification

Folates were extracted using tri-enzyme (α-amylase, protease, and γ-glutamyl hydrolase [GGH]) treatment without light, as described in a previous study with some modifications (He et al., 2022). Briefly, 0.2 g

cryogenically stored sample was added to a 2.0 mL brown capped tube with 1 mL extraction buffer (50 mmol/L phosphate buffer containing 1% (volume fraction) L-ascorbic acid and 0.1% (volume fraction) 2-mercaptoethanol, pH7.2; freshly prepared). After mixing, the tube was placed at 100 °C for 5 min and flash-cooled on ice at room temperature, preventing the conversion metabolism of folate derivatives. To capture potential folates in carbohydrates and proteins, the extract was treated with α-amylase and papain. After being mixed with 50 μL of α-amylase (20 mg/mL), the extract was incubated at 37 ℃ for 30 min under vibration. Subsequently, 100 μL of papain (a protease) (20 mg/mL) was added, and then the sample was incubated at 37 ℃ for 1 h under vibration. The sample was placed at 100 °C for 5 min to denature the enzyme, then flash-cooled on ice at room temperature. After centrifuging at 12 000*g*, 4 °C for 20 min, the supernatant was collected. The most common sources of GGHs for folate analysis are rat plasma, hog kidney, and chicken pancreas extracts (Ramos-Parra, 2013). Next, 500 μL supernatant was mixed with 100 μL rat serum and incubated at 37 ℃ for 4 h under vibration to deconjugate polyglutamylated folates. To eliminate the effect of endogenous folates on the quantification, the rat serum was mixed with activated charcoal (1:10, m/v), centrifuged after an ice bath for 1 h, and then filtered before folate deconjugation. After repeating the enzyme denaturation and cooling steps, the sample was centrifuged at 12000*g*, 4 °C for 20 min, and then filtered through a 0.45 μm nylon membrane filter into a 2 mL brown Agilent vial for quantification.

To quantify monoglutamylated folates (MG) and polyglutamylation (PG), the extraction followed the same steps as the folates, except the step of rat serum treatment was omitted. MG was the quantified value without GGH treatment. PG was calculated by subtracting the MG from the value quantified in the deglutamylated sample, indicated by the equation below (Ramos-Parra, 2013).

$$(MG_{GGH\text{-}treated}) - (MG_{non\text{-}treated}) = PG.$$

The sample was introduced into the ultra-high performance liquid chromotography (UHPLC) system (Acquity UHPLC M-Class system, Waters, USA)-triple quadruple mass spectrometry (MS) (Xevo TQ-S, Waters, USA) for mass analyses and quantification according to the previous liquid chromatography-tandem mass spectrometry (LC-MS/MS) settings with some modifications (Wan et al., 2019). The sample was separated using an Acquity UPLC BEH-C18 column (Waters, 2.1 × 100 mm, 1.7 μm) at a flow rate of 0.35 mL/min. The injection volume was 1.2 μL at 4 °C, and the column temperature was set at 30 ℃. The mobile phases were 0.1% (volume fraction) formic acid in water (phase A) and 0.1% (volume fraction) formic acid in acetonitrile (phase B). The gradient program lasted a total of 6 min: initiated with 10% B with a 0.5 min hold, increased to 50% over 0.7 min, and then increased to 95% over 1.8 min. After holding at 95% for 1 min, it immediately adjusted to its initial composition over 0.2 min, followed by an equilibration time of 1.8 min. The mass spectrometer was operated in positive ion mode. The parameters were optimized for folates analysis with a capillary voltage of 3160 V, desolvation temperature of 350 °C, desolvation gas flow of 800 L/h, cone gas flow of 152 L/h, collision gas flow of 0.12 mL/min, and nebulizer pressure of 0.7 MPa. Data were acquired in the optimized multiple reaction monitoring (MRM) mode (Table 2). Using external folate standards, the folate concentrations were calculated from the calibration curve. The commercial folates standards (FA HPLC ≥ 98%, THF HPLC = 65%, 10-formyl-FA (10-CHO-FA) HPLC ≥ 95%, 5-methyl-THF (5-CH_3-THF) HPLC ≥ 98%, 5-formyl-THF (5-CHO-THF) HPLC ≥ 98%) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd, China.

Table 2 MS/MS-MRM parameters and retention time of five folates species

Folate analytes	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone energy (V)	Collision energy (V)
FA	442.1	295.1	30	14
THF	446.1	299.1	30	20
10-CHO-FA	470.3	294.9	40	24
5-CH_3-THF	460.2	313.1	20	18

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5-CHO-THF	474.2	327.1	29	18

FA, folic acid; THF, tetrahydrofolate; 10-CHO-FA, 10-formyl-FA; 5-CH_3-THF, 5-methyl-THF; 5-CHO-THF, 5-formyl-THF.

2.3 Metabolites extraction and determination

The metabolite extraction was performed using a method similar to that described by Gao et al. (2023). The cryogenically stored sample was extracted using the same extraction buffer as used for folate extraction, then centrifuged at 1000*g*, 4 ℃ for 20 min, and the supernatant was collected. To detect the metabolites (CHI, ADC, *p*ABA, GTP, dihydroneopterin triphosphate (DHN-P_3), 6-hydroxymethyldihydropterin (HMDHP), 6-hydroxymethyldihydropterin pyrophosphate (HMDH-P_2), dihydropteroate (DHP), dihydrofolate (DHF), Glu, Gln, folate-binding protein (FBP)), the supernatant was treated with the plant-specific enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China) according to the manufacturer's instructions. The content of each metabolite was calculated as the fresh weight (FW).

For sugar components (glucose, fructose, and sucrose) analysis, 0.3 g of cryogenically stored sample and 2 mL of ultrapure water (autoclave before use) were mixed, then extracted at 100 ℃ for 20 min. After cooling and centrifugation, the supernatant was collected and then filtered for injection into the HPLC (Waters 2695, USA) coupled with a refractive index (RI) detector. The sample was separated using an Amino (NH_2) Column (Waters, 3.9 mm× 300 mm, 10 μm). The injection volume was 10 μL, and the column temperature was set at 80℃. The mobile phase was the ultrapure water at a flow rate of 0.6 mL/min.

For organic acid components (pyruvate, shikimate, citrate, succinate, malate, α-oxoglutarate) analysis, 0.3 g of cryogenically stored sample and 2 mL of 2 g/L metaphosphate buffer solution were mixed, then extracted with an ice bath for 10 min. After centrifugation at 4 ℃, the supernatant was collected and then filtered for injection into the HPLC (Waters 2695, USA) coupled with an Ultraviolet-Visible (UV/Vis) detector. The sample was separated using an XSelect HSS T3 Column (Waters, 4.6 mm× 250 mm, 3.5 μm). The injection volume was 10 μL, and the column temperature was set at 35 ℃. The mobile phase was the 0.2% metaphosphate buffer (filtered through a 0.45 μm filter) at a flow rate of 1 mL/min.

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2.4 Enzymes extraction and activity determination

The enzyme extraction was performed using the same method as that used for metabolite extraction. To detect enzyme activities (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), ADCS, ADC lyase (ADCL), GTP cyclohydrolase I (GTPCHI), HMDHP pyrophosphokinase-DHP synthase (HPPK-DHPS), DHF synthase (DHFS), DHF reductase (DHFR), folylpolyglutamate synthase (FPGS), GGH), the supernatant was treated with the plant-specific ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The units of enzyme activity per g (FW) were abbreviated as U/g FW, according to the kit instructions.

2.5 Statistical analysis

Three independent biological replicates were used for all tests. All the data were collated and analyzed for basic statistics using Microsoft Excel. Analysis of variance (ANOVA), followed by multiple comparisons with Duncan's ($P < 0.05$), was also performed using SAS 9.2. Correlation analysis was performed using IBM SPSS Statistics 20, and heat maps of the correlation analyses were generated using ChiPlot (https://www.chiplot.online/).

3 Results

3.1 Fresh weights of different NO_3^-/NH_4^+ ratio treatments

Root and whole plant fresh weights of the treatments with NH_4^+-N (90/10 to 50/50 NO_3^-/NH_4^+ ratio) were significantly higher than those of the control (100/0 NO_3^-/NH_4^+ ratio), as were the shoot fresh weights (Table 3). The treatment with 70/30 NO_3^-/NH_4^+ ratio was the best. NH_4^+ did not inhibit the growth of pakchoi (a NO_3^--preferred vegetable crop) at the same N concentration, even with a 50/50 NO_3^-/NH_4^+ ratio.

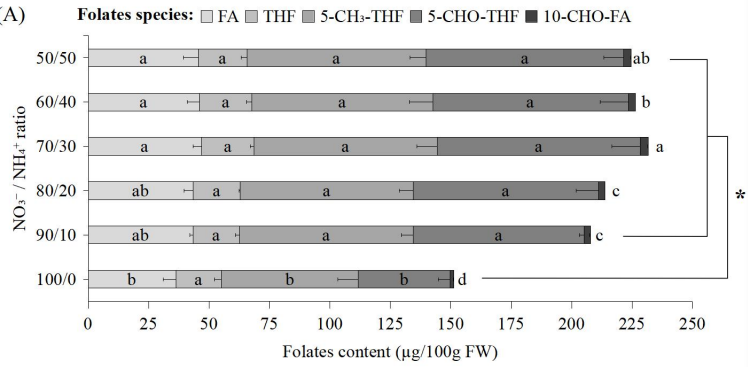
Table 3 Pakchoi fresh weights of different NO_3^-/NH_4^+ ratio treatments

NO_3^-/NH_4^+ ratio	Fresh weight (g/plant)		
	Shoot	Root	Whole plant
100/0	8.98±0.52^a	0.59±0.02^d	9.58±0.51^c
90/10	9.14±0.83^a	0.73±0.04^c	9.87±0.87^b
80/20	9.33±0.42^a	0.91±0.03^b	10.24±0.42^{ab}
70/30	9.98±0.10^a	1.00±0.02^a	10.98±0.12^a
60/40	9.23±0.26^a	0.89±0.04^b	10.12±0.24^{ab}
50/50	9.21±0.25^a	0.73±0.02^c	9.94±0.24^b

The biomass of the whole plant is equal to the sum of root (underground) and shoot (aboveground) biomass**.** Data are the mean values per plant and are expressed as mean±standard deviation (SD), n=3. Different letters indicate significant difference among treatments according to the Duncan test (P<0.05).

3.2 Folate constituents and contents of different NO_3^-/NH_4^+ ratio treatments

As shown in Fig. 2a, the total folate contents in pakchoi shoots with NH_4^+-N treatments were significantly higher, approximately 1.3-fold to 1.5-fold, than those of the 100/0 NO_3^-/NH_4^+ ratio treatment ((151.2±17.6) μg per 100 g FW). The treatment with 70/30 NO_3^-/NH_4^+ ratio was the best for increasing total folate contents ((231.7±15.0) μg per 100 g FW). Among five detected folate vitamers, the FA contents of 70/30, 60/40, and 50/50 NO_3^-/NH_4^+ ratio treatments were significantly higher than those of the 100/0 NO_3^-/NH_4^+ ratio treatment. The 5-CH_3-THF, 5-CHO-THF, and 10-CHO-FA contents of all five treatments with NH_4^+-N were significantly higher than those of the 100/0 NO_3^-/NH_4^+ ratio treatment, whereas the differences in THF contents among all six treatments were not significant. The treatment with the 70/30 NO_3^-/NH_4^+ ratio was the best for increasing the contents of FA, 5-CH_3-THF, 5-CHO-THF, and 10-CHO-FA (for specific folate contents, see the Supplementary Materials Table S1).



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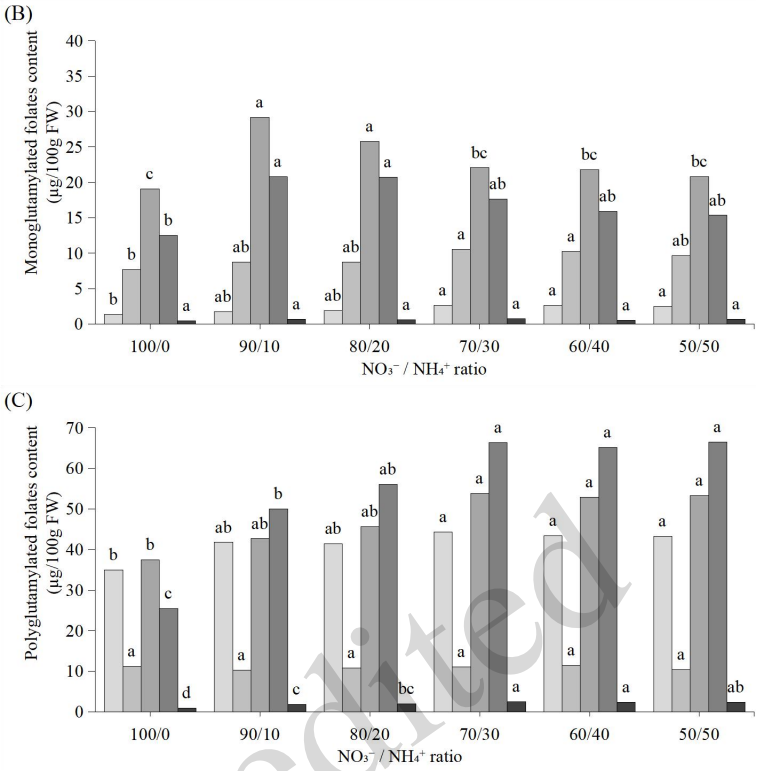

Fig. 2. Folates constituents and contents in pachoi fresh shoots hydroponically cultivated at different NO_3^-/NH_4^+ ratios. (**A**) Total folates and five folate vitamers, viz. FA (folic acid), THF (tetrahydrofolate), 5-CH_3-THF (5-methyl-THF), 5-CHO-THF (5-formyl-THF) and 10-CHO-FA (10-formyl-FA), were analyzed from the fresh shoots of pakchoi. (**B**) **M**ono**g**lutamylated (MG) folate contents. (**C**) **P**oly**g**lutamylated (PG) folate contents. In all figures, FW, fresh weight. Data are expressed as mean±standard deviation (SD), n=3. The different letters indicate significant differences among treatments according to the Duncan test ($P<0.05$). In (**A**), an asterisk (∗) shows a significant difference in total folate contents ($P<0.05$) between the treatment groups.

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As shown in Fig. 2 B, the monoglutamylated (MG) FA contents of the treatments with 70/30, 60/40, and 50/50 NO_3^-/NH_4^+ ratios were significantly higher than those of the 100/0 NO_3^-/NH_4^+ ratio treatment, as were the treatments with 70/30 and 60/40 NO_3^-/NH_4^+ ratios with respect to MG THF contents. As shown in Fig. 2 C, the polyglutamylated (PG) FA, PG 5-CH_3-THF, and PG 5-CHO-THF contents of the treatments with 70/30, 60/40, and 50/50 NO_3^-/NH_4^+ ratios were significantly higher than those of the 100/0 NO_3^-/NH_4^+ ratio treatment. The PG 10-CHO-FA contents of all five treatments with NH_4^+-N were significantly higher than those of the 100/0 NO_3^-/NH_4^+ ratio treatment.

It was also found that the higher 5-CHO-THF (36%), 5-CH_3-THF (32%), and FA (20%) mainly contributed to the highest total folate contents of the treatment with a 70/30 NO_3^-/NH_4^+ ratio. Increases in 5-CHO-THF and 5-CH_3-THF were exhibited with the higher PG 5-CHO-THF and PG 5-CH_3-THF, respectively. The FA contents increased with the higher MG FA and PG FA contents. In general, total folate contents increased with both the higher PG 5-CHO-THF and PG 5-CH_3-THF.

3.3 Activities of enzymes affected by different NO_3^-/NH_4^+ ratio treatments

The activities of nine enzymes related to folate biosynthesis, such as GTPCHI, DAHPS, ADCS, ADCL, HPPK-DHPS, DHFS, DHFR, FPGS, and GGH, were analyzed. As shown in Fig. 3, the activities of GTPCHI, DHFS, DHFR, and GGH in pakchoi shoots after the five treatments with NH_4^+-N were significantly lower than those after the 100/0 NO_3^-/NH_4^+ ratio treatment, and the ADCS activities of the treatments with 90/10, 80/20, and 50/50 NO_3^-/NH_4^+ ratios were also significantly lower than those of the 100/0 NO_3^-/NH_4^+ ratio treatment.

The activities of DAHPS, ACDL, and HPPK-DHPS of all six treatments were similar, except for the 50/50 NO_3^-/NH_4^+ ratio treatment for DAHPS, ACDL activities, and the 70/30 NO_3^-/NH_4^+ ratio treatment for HPPK-DHPS activity. The differences in FPGS activities among all six treatments were not significant (for specific activities of the enzymes, see the Supplementary Materials Table S1).

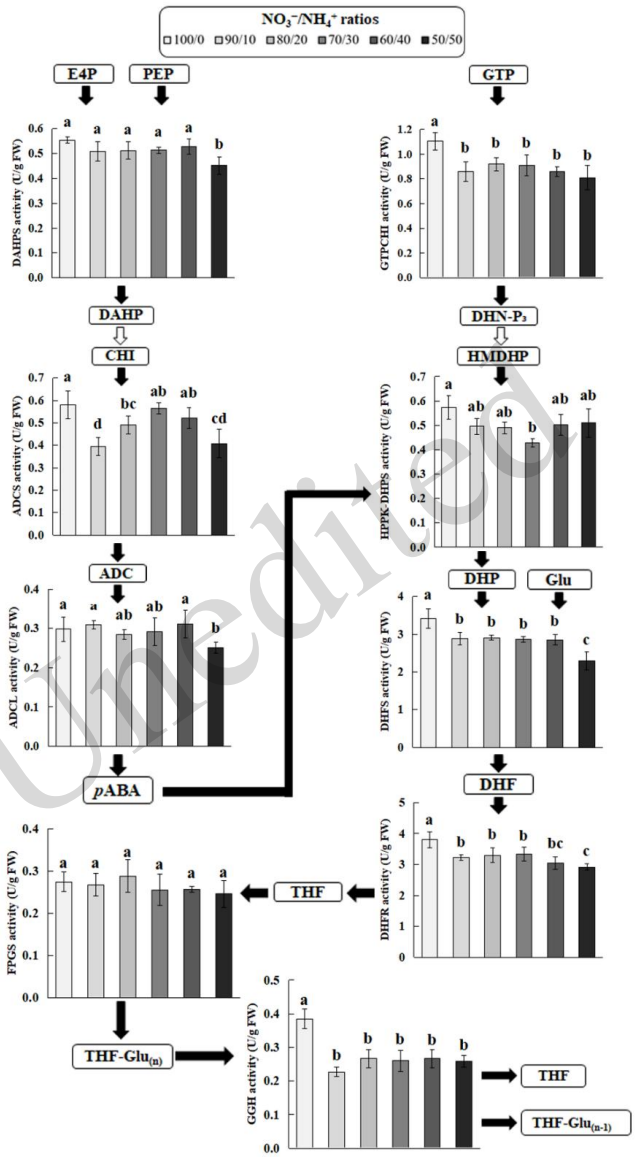

Fig. 3. Changes in the activities of enzymes related to folate biosynthesis in pakchoi shoots among different NO_3^-/NH_4^+ ratio treatments. FW, fresh weight. U/g FW, the units of enzyme activity per g (fresh weight). The text in the black boxes represents metabolites: E4P, erythrose 4-phosphate; PEP, phos-phoenolpyruvate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; CHI, chorismite; ADC, aminodeoxychorismate; *p*ABA, *p*-aminobenzoate; GTP, guanosine triphosphate; DHN-P_3, dihydroneopterin triphosphate; HMDHP, 6-hydroxymethyldihydropterin; DHP, dihydropteroate; DHF, dihydrofolate; THF, tetrahydrofolate; THF-Glu(n), THF-polyglutamate; Glu, glutamate. The histograms are activities of enzymes and enzymes abbreviations: DAHPS, DAHP synthase; ADCS, ADC synthase; ADCL, ADC lyase; GTPCHI, GTP cyclohydrolase I; HPPK-DHPS, HMDHP pyrophosphokinase-DHP synthase; DHFS, DHF synthase; DHFR, DHF reductase; FPGS, folylpolyglutamate synthase; GGH, γ-glutamyl hydrolase. Data are expressed as mean±standard deviation (SD), n=3. The different letters indicate significant differences among treatments according to the Duncan test (P<0.05).

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3.4 Changes of metabolites in folate biosynthesis with different NO_3^-/NH_4^+ ratio treatments

The biosynthesis of folates in plants is based on substances derived from carbon metabolism. Thus, the primary metabolites of carbon metabolism, such as sugars and organic acids in pakchoi shoots, were analyzed. As shown in Fig. 4, the glucose contents of the five treatments with NH_4^+-N were significantly higher than those of the 100/0 NO_3^-/NH_4^+ treatment, as the sucrose and fructose contents decreased significantly, except for sucrose content after the 90/10 NO_3^-/NH_4^+ ratio treatment. Pyruvate and shikimate contents of the five treatments with NH_4^+-N were significantly lower than those of the 100/0 NO_3^-/NH_4^+ treatment, except for the 90/10 NO_3^-/NH_4^+ ratio treatment. Citrate, malate, α-oxoglutarate, and succinate contents of the five treatments with NH_4^+-N were also significantly lower than those of the 100/0 NO_3^-/NH_4^+ treatment, except for the 90/10 NO_3^-/NH_4^+ ratio treatment for malate and succinate contents (for specific metabolites contents, see the Supplementary Materials Table S1).

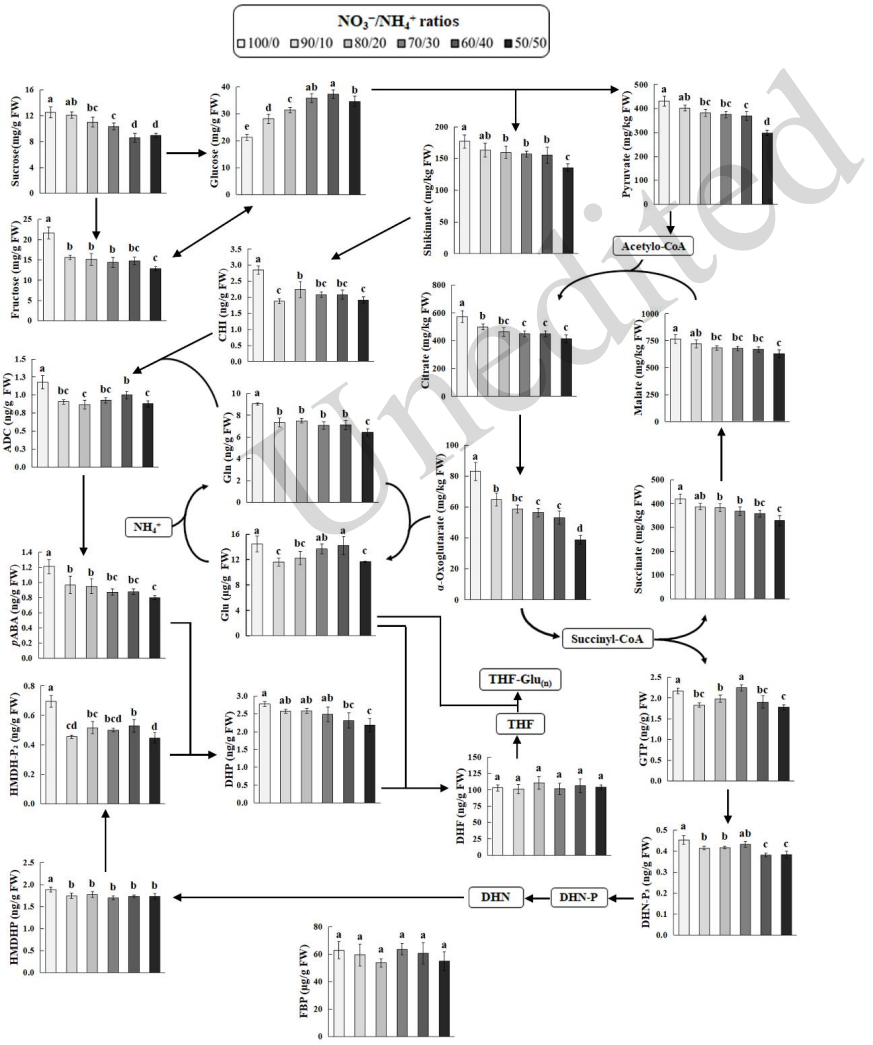

Fig. 4. Changes in the metabolic profiles in the pakchoi shoots among different NO_3^-/NH_4^+ ratio treatments. The text in the histogram and black boxes represents metabolites: FW, fresh weight; CHI, chorismite; ADC, aminodeoxychorismate; *p*ABA, *p*-aminobenzoate; GTP, guanosine triphosphate; DHN-P_3, dihydroneopterin (DHN) triphosphate; DHN-P, DHN monophosphate; HMDHP, 6-hydroxymethyldihydropterin; HMDH-P_2, 6-hydroxymethyldihydropterin pyrophosphate; DHP, dihydropteroate; DHF, dihydrofolate; THF, tetrahydrofolate; THF-Glu(n), THF-polyglutamate; Glu, glutamate; Gln, glutamine; FBP, folate-binding protein. Data are expressed as mean±standard deviation (SD), n=3. Different letters indicate significant differences among treatments according to the Duncan test (P<0.05).

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Glu and Gln, the products of N assimilation involved in folate synthesis in pakchoi shoots, were analyzed (Fig. 4). The Gln contents of all five treatments with NH_4^+-N were significantly lower than those of the 100/0 NO_3^-/NH_4^+ treatment, and the Glu contents of the treatments with 90/10, 80/20 and 50/50 NO_3^-/NH_4^+ ratios were also significantly lower than those of the 100/0 NO_3^-/NH_4^+ treatment.

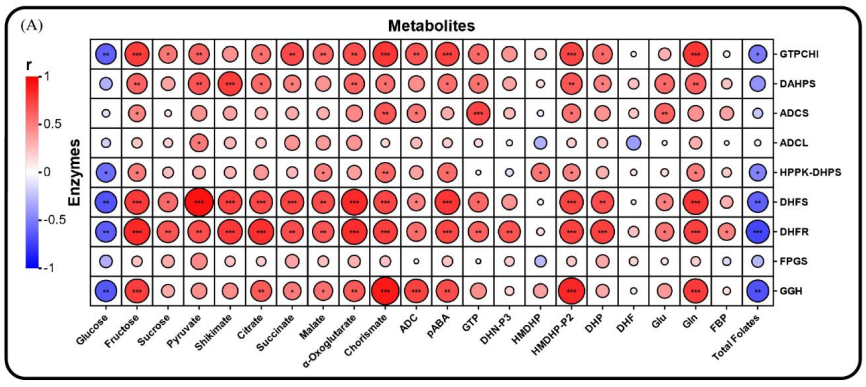
To examine the impacts of NO_3^--N and NH_4^+-N supply on folate accumulation in pakchoi shoots, the intermediate metabolites such as CHI, ADC, *p*ABA, GTP, DHN-P_3, HMDHP, HMDH-P_2, DHP, and DHF in the folate synthesis pathway were analyzed. As shown in Fig. 4, in the *p*ABA synthesis section, the contents of CHI, ADC, and *p*ABA of all five treatments with NH_4^+-N were significantly lower than those of the 100/0 NO_3^-/NH_4^+ treatment. In the HMDH-P_2 synthesis section, the contents of GTP, DHN-P_3, HMDHP, and HMDH-P_2 of five treatments with NH_4^+-N were significantly lower than those of the 100/0 NO_3^-/NH_4^+ treatment except for the 70/30 NO_3^-/NH_4^+ ratio treatment for GTP and DHN-P_3 contents. In the folate assembly section, only the 50/50 NO_3^-/NH_4^+ ratio treatment had significantly lower DHP contents than the 100/0 NO_3^-/NH_4^+ treatment, and the differences in DHF contents among all six treatments were not significant.

FBP, a protein with folate binding ability, can improve folate stability and contribute to folate accumulation (Blancquaert et al., 2015). FBP was analyzed to examine the impacts of NO_3^--N and NH_4^+-N supply on FBP in pakchoi shoots. As shown in Fig. 4, FBP contents did not vary significantly among all six treatments.

3.5 Correlations of enzyme activities and folates with metabolites

The correlations of enzyme activities with corresponding metabolites are presented in Fig. 5 A. The GTPCHI, ADCS, HPPK-DHPS, and DHFS activities were significantly positively correlated with their corresponding substrate as (1) GTP, (2) chorismite, (3) *p*ABA, HMDHP, and HMDH-P_2, and (4) DHP and Glu. The consequential products, ADC, Glu, and DHP, were significantly positively correlated with their corresponding enzyme activity. Total folate contents were significantly negatively correlated with both DHFR and GGH activities.

The correlations of folates with 21 metabolites are shown in Fig. 5 B. FA, THF, 5-CH_3-THF, 5-CHO-THF, 10-CHO-FA, and total folates were significantly positively correlated with glucose (the initial metabolite). Negative correlations were demonstrated among total folates or between each folate form and 20 other metabolites, even significant associations, such as FA with 10 metabolites (fructose, sucrose, shikimate, citrate, succinate, malate, α-oxoglutarate, *p*ABA, HMDH-P_2, and Gln); THF with one metabolite (*p*ABA); 5-CH_3-THF with 12 metabolites (fructose, sucrose, shikimate, citrate, succinate, malate, α-oxoglutarate, chorismite, ADC, *p*ABA, HMDHP-P_2, and Gln); and 5-CHO-THF, 10-CHO-THF, and total folates each with 14 metabolites (fructose, sucrose, pyruvate, shikimate, citrate, succinate, malate, α-oxoglutarate, chorismite, ADC, *p*ABA, HMDH-P_2, DHP, and Gln).



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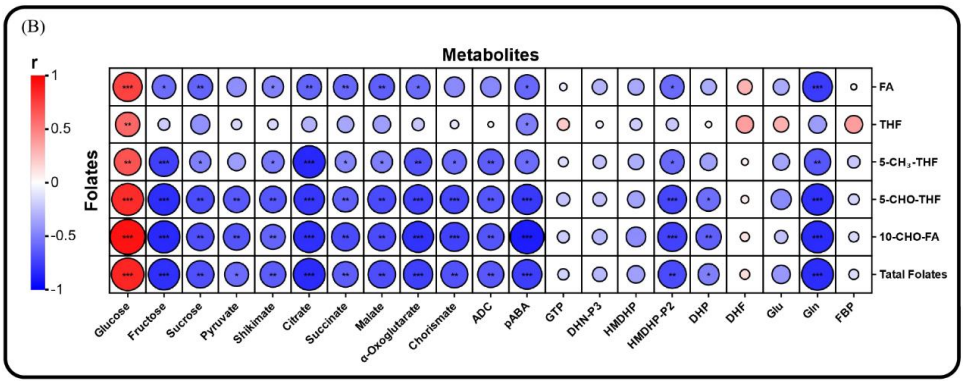

Fig. 5. Map of correlations between enzyme activities, folates, and metabolites. (A) Correlations between enzyme activities and metabolites. (B) Correlations between folates and metabolites. Correlation analyses of all metabolite contents and enzyme activities were based on fresh weight. Correlations were calculated using Pearson's coefficients (r). The size of the circle indicates the degree of the correlation. Positive correlations are red, and negative correlations are blue. (∗), (∗∗) and (∗∗∗) indicate the significance level at 0.05, 0.01, and 0.001. Abbreviations: FA, folic acid; THF, tetrahydrofolate; 5-CH_3-THF, 5-methyl-THF; 5-CHO-THF, 5-formyl-THF; 10-CHO-FA, 10-formyl-FA; ADC, aminodeoxychorismate; *p*ABA, *p*-aminobenzoate; GTP, guanosine triphosphate; DHN-P_3, dihydroneopterin (DHN) triphosphate; HMDHP, 6-hydroxymethyldihydropterin; HMDH-P_2, 6-hydroxymethyldihydropterin pyrophosphate; DHP, dihydropteroate; DHF, dihydrofolate; THF, tetrahydrofolate; Glu, glutamate; Gln, glutamine; FBP, folate-binding protein; DAHPS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; ADCS, ADC synthase; ADCL, ADC lyase; GTPCHI, GTP cyclohydrolase I; HPPK-DHPS, HMDHP pyrophosphokinase-DHP synthase; DHFS, DHF synthase; DHFR, DHF reductase; FPGS, folylpolyglutamate synthase; GGH, γ-glutamyl hydrolase.

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4 Discussion

Our research revealed that simply optimizing NO_3^-/ NH_4^+ ratio leads to a 1.5-fold increase in folate level (231.7 μg/100g FW) in fresh shoots of hydroponically cultivated pakchoi, with a 70/30 NO_3^-/NH_4^+ ratio treatment producing the best growth compared to no NH_4^+-N treatment. The higher folate level with the 70/30 NO_3^-/NH_4^+ ratio may reflect a higher rate of folate biosynthesis or a reduction in folate degradation. We speculate that the appropriate (70/30) NO_3^-/NH_4^+ ratio induces folate enrichment for the following reasons.

First, an appropriate NO_3^-/NH_4^+ ratio could stimulate C metabolism to provide additional material support for folate biosynthesis. GTP and chorismate, both required for folate biosynthesis, are derived from C metabolism. Carbohydrates can be converted into GTP via glycolysis through the TCA cycle or into chorismate via glycolysis through the shikimate pathway (Kołton et al., 2022). Additionally, the C skeletons that are required for the biosynthesis of Glu and Gln, which are essential for folate synthesis, are derived from *α*-oxoglutarate in the TCA cycle (Wang et al., 2014). C input to the TCA cycle via the glycolysis through pyruvate kinase was enhanced when both NO_3^--N and NH_4^+-N supply were present compared with NO_3^--N supply alone (Hachiya and Sakakibara, 2017). In our study, the low levels of pyruvate in the glycolysis, and the low levels of citrate, succinate, malate, and α-oxoglutarate in the TCA cycle of the treatments with NH_4^+-N may be responsible for the enhanced C fluxes, providing more C skeletons for N assimilation. Fructose requires fewer catalytic steps than glucose to enter glycolysis (Patrick et al., 2013). It is possible that the low fructose levels reflect more efficient utilization for enhancing C metabolism with NH_4^+-N application. Since sucrose is formed by glucose and fructose, the decreased sucrose level may result from accelerated fructose consumption. It seems reasonable that with the addition of NH_4^+-N, a low GTP level was observed due to the enhanced GTP consumption for folate biosynthesis; as the NO_3^-/NH_4^+ ratio declined, the GTP level increased due to the increased output from the TCA cycle.

However, excessive NH_4^+-N may weaken photosynthesis, thereby reducing C metabolism input (Esteban

et al., 2016). With an excessively low NO_3^-/NH_4^+ ratio (e.g., 50/50 NO_3^-/NH_4^+), the GTP level decreased due to the lower C input to the TCA cycle and the lower α-oxoglutarate level. As with GTP, low chorismite levels in the treatments with NH_4^+-N may result from enhanced chorismite consumption due to increased folate biosynthesis. DAHPS catalyzes the first step of enzymatic reactions in the shikimate pathway (Yokoyama et al., 2022). In our study, low DAHPS activity at the 50/50 NO_3^-/NH_4^+ ratio may limit the C flux into the shikimate pathway. Excessive NH_4^+ can also enhance the anaplerotic routes for greater C input to the TCA cycle through pyruvate kinase (Esteban et al., 2016). Low chorismate levels at the 50/50 NO_3^-/NH_4^+ ratio may also be a response to the decreased shikimate caused by the reduced C flux input and/or the additional C redirected to the TCA cycle. Overall, this evidence suggests that an appropriate NO_3^-/NH_4^+ ratio enhanced C metabolism to promote fluxes for folate synthesis.

Second, an appropriate NO_3^-/NH_4^+ ratio could stimulate the conversion of GTP to HMDHP via a series of steps. The GTPCHI catalyzes a net expansion of the GTP to form a pteridine derivative, DHN-P_3, which represents a rate-limiting step controlling metabolic flux into the folate biosynthesis pathway (Gorelova et al., 2017; Grawert et al., 2013). In our study, folate content increased with the addition of NH_4^+-N, despite the reduced GTPCHI activity. This reduction in GTPCHI activity is more likely to be attributable to negative feedback regulation by the enhanced metabolism. In pakchoi, folate levels were elevated by controlled illumination, which is attributed to decreased GTP and HMDHP levels (Gao et al., 2023). Similarly, with the addition of NH_4^+-N, low GTP, DHN-P_3, and HMDHP levels may be the result of their greater consumption for folate biosynthesis.

Third, an appropriate NO_3^-/NH_4^+ ratio could also stimulate the conversion of chorismate to *p*ABA. *p*ABA biosynthesis starts with chorismate by catalyzing two reactions: NH_4^+ release from Gln and hydroxyl group replacement with the amino group yielding ADC (de la Garza et al., 2019). In our study, the levels of chorismate, ADC, *p*ABA, and Gln decreased with the addition of NH_4^+-N due to the enhanced folate biosynthesis consuming more of them. Folate levels in the pakchoi were elevated by controlled illumination, which is attributed to the decreased chorismate and ADC levels (Gao et al., 2023). This evidence supports that the decline of *p*ABA resulted from the enhanced folate biosynthesis in our study.

Additionally, an appropriate NO_3^-/NH_4^+ ratio could promote folate assembly. Folate is produced in mitochondria, where HMDHP is phosphorylated by HPPK to form HMDH-P_2, and this moiety is condensed with *p*ABA to form DHP by DHPS. DHP is then glutamylated by DHFS to form DHF, which is further reduced to THF by DHFR (Gorelova et al., 2017). In our study, the low HMDH-P_2 and DHP levels associated with the addition of NH_4^+-N may be the result of the promotion of folate biosynthesis. Notably, high folate levels at 70/30 and 60/40 NO_3^-/NH_4^+ ratios may be attributed to high Glu levels because Glu acts as a donor for glutamylation. With the NH_4^+-N supply, HPPK-DHPS, DHFS, and DHFR activities were reduced by varying degrees, subject to a series of complex feedback regulation processes by folate or its synthesis intermediates, for instance, negative feedback of DHP and DHF on HPPK-DHPS, and 5-CHO-THF negative feedback on DHFR (Gorelova et al., 2017; Li et al., 2021).

The final reason for folate enhancement is the increased stability of folates. The extent of folate polyglutamylation, which is defined by the polyglutamylated tail length, can affect cellular folate breakdown to alter folate levels. The extent is determined by FPGS adding a glutamate tail and GGH removing it. In tomatoes, the overexpression of GGH caused extensive deglutamylation of polyglutamylated folates and resulted in a total folate content reduction of approximately 40%. In contrast, lower GGH activity in tomatoes was associated with an increased folate level (Akhtar et al., 2010; Tyagi et al., 2023). With NH_4^+-N application, GGH activity was significantly lower than with NO_3^--N supply alone. Correspondingly, the higher polyglutamylated folate level with NH_4^+-N supply seems to be the result of reduced GGH activity, weakening the deglutamylation process. In addition to folate polyglutamylation, the folate form can affect folate breakdown. 5-CHO-THF is the most stable form among folates, followed by 5-CH_3-THF (de la Garza et al., 2019). The increase in total folate level with NH_4^+-N application was mainly due to the increase of polyglutamylated 5-CHO-THF, followed by polyglutamylated 5-CH_3-THF, of which 5-CHO-THF was dominant. Thus, the folates of the treatments with

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NH_4^+-N are more stable and less susceptible to degradation. 5-CHO-THF is the only derivative that does not function as a one-carbon donor while modulating C and N metabolism (Li et al., 2021). It is very likely that the increased 5-CHO-THF with the NH_4^+-N treatments regulated the change in C and N metabolism.

Enhanced folate accumulation means that more substances are consumed to support folate synthesis. In our study, most of the metabolites of C and N metabolism or folate synthesis showed a significant negative correlation with folate contents, which can be explained by the enhancement of folate biosynthesis by NH_4^+-N supply increasing the consumption of these metabolites. Additionally, the significantly positive correlations between GTPCHI, ADCS, HPPK-DHPS, and DHFS activities and their corresponding substrates and consequential products suggest that they may be the main control points for increased metabolite consumption. Given that low GGH activity is conducive to enhanced folate synthesis, the significant negative correlation between GGH activity and folate contents indicates that low GGH activity is a key factor in the enhancement of folate biosynthesis with NH_4^+-N supply.

In conclusion, an appropriate NO_3^-/NH_4^+ ratio in nutrient solution could promote pakchoi growth and enhance the metabolic flux of C and N metabolism, especially with respect to glucose accumulation in shoots and folate biosynthesis. This therefore increases folate contents in pakchoi shoots, mainly in the form of polyglutamylated 5-CHO-THF and polyglutamylated 5-CH_3-THF. The regulation of the rate of folate biosynthesis could be controlled by GTPCHI, ADCS, HPPK-DHPS, and DHFS activities. At the same N concentration, NH_4^+-N supply could increase polyglutamylated folates by reducing folate deglutamylation via lower GGH activity, which is conducive to folate stability. With good growth responses and folate biofortification, the appropriate 70/30 NO_3^-/NH_4^+ ratio in nutrient solution should be recommended for the hydroponic cultivation of pakchoi. For the optimal growth and efficient folate accumulation of other foliar vegetable crops under hydroponic conditions, the appropriate ratio of NH_4^+-N to NO_3^--N needs further exploration.

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Data availability statement

All datasets analyzed in this study are available from the corresponding author upon request.

Acknowledgments

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Author contributions

Yongcong ZHU performed the experimental research and data analysis, and wrote and edited the manuscript. Wei CHENG performed the experimental research and data analysis. Yuemin NI contributed to the study design. Wuzhong NI contributed to the study design, writing and editing of the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yongcong ZHU, Wei CHENG, Yuemin NI and Wuzhong NI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Table S1

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