Mechanism of Excessive Nitrogen Fertilization Aggravates the Damage of Cacopsylla Chinensis to Pear

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Abstract

Cacopsylla chinensis (Hemiptera: Psyllidae) is one of the most important pests on pear trees. Although nitrogen fertilization is known to often benefit the performance of many herbivores including psyllids, physiochemical and molecular mechanisms of how psyllids respond to excessive nitrogen application remain unclear. Study showed that nitrogen fertilizer concentrations significantly impacted phenolic acids compositions in pear leaves, there was an upward trend in ferulic acid concentration when increasing nitrogen fertilizers. The increased ferulic acid concentration accelerated the *C. chinensis* development. Moreover, high nitrogen fertilization also caused a significant increase in psyllid honeydew secretion and several amino acids concentrations in honeydew. The increased amino-acid content in pear leaves under high nitrogen fertilization improved the feed intakes of psyllid, however decreased more significantly the expression levels of several *C. chinensis* genes in amino-acid synthesis pathways. The mechanism of excessive nitrogen fertilization aggravating the damage of *C. chinensis* to pear trees was defined preliminarily.

Introduction

Pear psylla, *Cacopsylla chinensis* (Hemiptera: Psyllidae) is a main foliar pest on pear tree (Jiang et al.,2003; Dong et al.,2009; Inoue et al., 2012; Qiao et al.,2017). It is a monophagous insect feeding on phloem and xylem sap of pear trees (Gullan & Martin,2009), high density of psyllid populations can lead to premature leaves and fruit drop (Pfeiffer & Burts,1983). Honeydew deposited on leaves and fruits by the psyllids provides a substrate that fosters sooty molds' development, inhibits plant photosynthesis and reduces the quality of harvested products (Xu et al.,2000; Li et al.,2003). The growth of sooty mold on honeydew has caused severe economic losses in some agricultural crops (DuPont et al.,2021). The control of pear psylla relied mainly on chemical insecticides, because of the serious side-effects caused by chemical insecticides on the environment, the demand for alternative control methods, such as cultivation and administration technology is growing.

Nitrogen-based nutrients are necessary for insects, the deficiency of nitrogen can cause restrictions on their growth and reproduction (Mattson,1980; Huberty & Denno, 2006). Many of the insect herbivores increase feeding on host plants with higher nitrogen content. Nitrogen fertilizer addition has been linked with increased insect survival rates, feeding, larval development, fecundity and population growth (Wang et al.,2006; Zarghami et al.,2010). For those insects that feed exclusively on plant phloem sap like psyllids, they have to intake a large amount of plant sap to fulfill their nutritional need for amino acids due to the imbalanced diet nutrient composition in phloem sap. Therefore, nitrogen nutrition in host plants has long been considered as a limiting factor especially for herbivorous insects (Mattson,1980; Awmack & Leather,2002). It was reported that increases in the nitrogen application enhanced the feeding and development rates of the psylla species *Cacopsylla pyricola* on pear trees, leading to larger populations and increased fruit damage (Pfeiffer & Burts,1983; Pfeiffer & Burts,1984). The psylla population levels on pear trees reduced by using lower levels of nitrogen fertilization (Shaltiel-Harpaz et al.,2010; Gao et al. 2018). However, the mechanism of nitrogen fertilization regulating the performance,

population and damage of insect herbivores on host plants, and how nitrogen fertilization affects the amino acid processing in psyllids were much less explored.

Phenolic acids are the second most abundant secondary metabolite of plant, and play an important role in various plant defense responses to stresses such as herbivore and pathogen attack (Cheynier et al.,2013; Yahyaa et al.,2019).The phenolic acids from pear trees mainly include caffeic acid, chlorogenic acid, eugenic acid, ferulic acid, etc (Zheng et al.,2015; Zhang et al.,2017), these phenolic acids have certain effects on feeding pests. Ferulic acid is one of the natural phenolic compounds, this compound and its derivatives exhibit wide and important effects, including anticancer (Kim et al.,2011), antiviral activity (Huang et al.,2013), neuroprotective and antioxidant activity (Rukhsana et al.,2010). Nevertheless, little is known about the relationship between nitrogen fertilization and the presence of phenolic acids in pear tissue, and how the changes in phenolic acids of plant caused by nitrogen fertilizer further impact the development of insect herbivores .

Many of phloem-feeding hemipteran insects secrete honeydew during feeding which contains carbohydrates, amino acids, and other limiting nutrients. Honeydew production contributes to the regulation of osmotic stress during the feeding of herbivores (Wäckers, 2000). Many studies have measured the amount of honeydew secretion as a proxy for the food intakes of several species of Hemipteran insects, including psyllids. There is a positive correlation between food intakes of Hemipteran insects and their honeydew secretion, the excretion of honeydew can be used as a measurement index of the intake (Cameron et al.,2014). Researches have shown that the liquid honeydew might entrap small parasitoids or clot the mouthparts and sense organs of natural enemies, and attract ants' attendance to fight against natural enemies (Leroy et al. 2014). Research showed that honeydew of *C. chinensis* limited predator foraging with the potential to limit biological control, more generally, honeydew might form an important type of defense for stationary feeders like psyllids (Ge et al. 2020). Despite the importance of honeydew in the survival and control of psyllids, the effects of nitrogen fertilizers on the secretion and constitution of honeydew remains unclear.

Therefore, the mechanism of nitrogen fertilization regulating the performance of pear psylla *C. chinensis* on pear tree were tried to explored here from the following three aspects: 1) the relationships between phenolic secondary substances in the pear tree leaves and the amount of nitrogen fertilizer application, and the further impacts of ferulic acid on *C. chinensis*, 2) the impacts of nitrogen fertilizer on the honeydew secretion of *C. chinensis* and the amino acid contents in pear leaves and psyllid honeydew, 3) the impact of high concentration of nitrogen fertilizer on the expression levels of several important genes in serine, glycine, asparagine and glutamate synthesizing pathways of *C. chinensis*. Exposing the mechanism underlying how excessive nitrogen application aggravates the damage of *Cacopsylla chinensis* to pear trees will contribute to the rational nitrogen use in pear cultivation.

Materials And Methods

Pear seedlings and psyllid cultures

Pear (*Pyrus bretschneideri* Rehder,1915) leaves and seedlings were obtained from potted pear plants grown under controlled conditions (25 ± 2 ℃, RH 70 ± 5% and a 16:8 h L: D photoperiod). *Cacopsylla chinensis* colonies originated from field-collected individuals harvested from a pear orchard in Anhui Academy of Agricultural Sciences, China (31˚89’48"N, 117˚25’23"E). Insect colonies were maintained in growth chambers under the same conditions listed above. Psyllids were maintained on two-month-old pear seedlings. Discrimination of different instars of *C. chinensis* followed the description in Gai et al. 2000. All experiments were conducted in climate-controlled chambers (25 ± 2 °C; 50–70% RH). The nymphs with normal growth and physiological activity were selected for the experiment. Insects used in all assays were used only once.

Fertilization

Six-week old pear seedlings were treated with different concentrations (500mg/kg, 1000mg/kg and 1500mg/kg) of nitrogen fertilizer dissolved in distilled water, respectively. These three low, media, and high concentrations of nitrogen fertilizer were set according to the applied concentration in the field. A control group with the same volume of distilled water was set. After 9-11 days of fertilization, pear seedlings were used for the further experiments.

Analysis of phenolic acid compounds

The 3rd and 4th leaves from pear seedlings were picked off and put into 1.5 mL centrifuge tube and frozen with liquid nitrogen at once, and then grinded into freeze-dried powder by using ball mill (Mixer Mill MM 400, Verder Instruments and Equipment Co., Ltd., Shanghai, China). Then the samples were extracted with 1 mL 70% ethanol for 24 hours in an oscillator (220 r/min) so that it dissolves sufficiently. The extracted sample was centrifuged at 3500 r/min for 5 min (Eppendorf centrifuge 5424 R, Hamburg, Germany), the supernatant was filtered through an organic membrane filter with a 0.45 μm pore diameter (Tianjin Branch Billion Lung Experimental Equipment Co., Ltd., Tianjin, China), transferred into a vial and kept at 4℃ for UPLC analyses of phenolic compounds, the test was followed the method of Zheng et al. (2015). Identification and quantification of phenolic acids were performed by comparison with chromatographic retention times and areas of external standards. The standard products of phenolic acids were from Beijing Solarbio Technology Co. LTD, Beijing, China. The standard curve under experimental conditions was obtained, and the content of chlorogenic acid, ferulic acid and epicatechin of the samples were calculated according to the standard curve, the analysis was followed the methods of Li et al. (2015), Xie et al. (2013) and Borges & Pinto (1994) respectively.

Effect of ferulic acid on the developmental duration of *C. chinensis*

The ferulic acid was dissolved with acetone and then sprayed on pear seedlings leaves with concentrations of 0, 0.24, 0.36 ppm, respectively. The concentration was set according to the actual ferulic acid concentration in the pear leaves based on our UPLC measurement. The second instar pear psyllids were placed on pear leaves by using insect pins with 4 psyllids for every pear seedling and each leaf only one, No.2 ziplock bags (85mm×60mm) with pinholes for air permeability were covered on each

leaf in the same position. The pear seedlings were watered twice a week with a volume of 50 mL each time. The whole experiment was carried out under the conditions above described. The molting condition of *C. chinensis* was observed every 12 hours, the molting time was recorded, and the molt was removed, the developmental duration was got. The experiment consisted of three replicates with two pear seedlings in each replicate.

Effect of nitrogen fertilizer on honeydew secretion of *C. chinensis*

Two 3rd instar psyllids were put on the third and fourth leaves of one pear seedlings treated with different concentrations of N fertilizer, each leaf placed only one psyllid and was enclosed in ziplock bags with pinholes for ventilation, each treatment replicated 3 times with 30 nymphs of *C. chinensis* totally. The psyllids were removed 8 days after placing on pear leaves. We used two methods to determine the honeydew production of *C. chinensis* nymphs. Method one was direct volume determination, a 1cm×1cm square scale was placed aside the honeydew produced area, and then photos of the scale combined with psyllid produced honeydew were taken. The volume of honeydew was measured further by Image-j software(NIH, USA). Method two used water-sensitive paper (Syngenta, CH), we pressed water-sensitive paper in honeydew produced area lightly, and honeydew contacted area on water-sensitive paper would turn blue because of the bromocresol green in water-sensitive paper. The blue area on water-sensitive paper along with 1cm square scale were photographed subsequently and determinated by Image-j software.

Effect of nitrogen fertilizer on content of amino acids in pear leaves

The 3rd and 4th leaves of pear seedlings applied with different concentrations of nitrogen fertilizer were cut off and crushed with nitrogen by a mortar. Then put them into a 50 mL centrifuge tube, sealed with parafilm, poked holes with an insect needle for air permeability, and put them into a freeze dryer for freeze drying. Twenty milligram powders of each sample were weighed, dissolved in 1 mL water, and ultrasonically shaken for 30 min, centrifuged at 14000 rpm for 10 min, and then the supernatant was extracted for the further analysis. There are three replications for each nitrogen concentration treatment.

The sample of 10 μL were mixed with 50 μL borate buffer, added by 17 μL derivative (ACCQ® Fluor kit) , kept at room temperature for 1 min, then they were used water bath at 55℃ for 10 min, and cooled at room temperature for test. The samples above were analyzed by a Waters Acquity HPLC I-Class, equipped with an Agilent poreshell 120 BEH C18 (2.1* 100mm 1.7-Micron) column (Waters, Milford, USA). The mobile phase with a gradient of solvent A (20Mm NH_4Ac) and solvent B(80% ACN) was used at a flow rate of 0.3 mL min^{-1}. The gradient used for the amino acids analysis was as follows: from initial 97% A to 88% A (2 min), from 88% A to 66% A (7.5 min), from 66% A to 0 A (2.5 min), then from 0 A to 97% A for 0.5 min. The column temperature was 40℃, the injection volume was 1 μL.

Effect of nitrogen fertilizer on content of amino acids from honeydew of *C. chinensis*

The honeydew of 1 g collected from pear psylla raised on pear seedlings under different concentrations of nitrogen fertilizer was taken to determine the amino acids in honeydew, and 5 mL water was added to dilute it. After ultrasonically oscillating for 30 min, centrifuged at 14000 rpm for 10 min, 10 μL supernatant was taken for analysis. There are three replicates for the determination of amino acid contents in honeydew under each concentration of nitrogen fertilizer. The determination and analysis of the amino acid content in honeydew were same as the experimental procedures of the pear leave amino acids determination.

Effect of nitrogen fertilizer on the gene expression of amino acid synthesis of *C. chinensis*

RNA extraction

Twenty nymphs of the third instar of *C. chinensis* were inoculated to the leaves of the pear seedlings after nitrogen application (control, 1500 mg/kg), the 5th instar *C. chinensis* were collected after inoculation for 8 days. Each sample contained 20 *C. chinensis*, and there were three replicates for each treatment. Total RNA was extracted from each sample using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. The RNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Transcriptome sequencing, assembly, and annotation

Transcriptome sequencing was carried out by Beijing BioMics Technology Co., LTD. Transcriptome sequencing was performed following the steps of sample detection, library construction and quality control. The clean reads were subjected to assembly using software Trinity 2.8.3 (Haass et al.,2013). The sequencing data of each sample was compared with the assembled reference transcript (Ref) for statistical analysis, and RSEM soft was used for comparative analysis (Li and Colin, 2011), the bowtie2 parameter mismatch 2 was set as the default parameter (Haass et al., 2013). The Unigene sequence was searched against the NCBI NR(non-redundant protein) database by Blast2 software, then Unigene was compared with four commonly used protein databases: Swiss-Prot, GO, COG/KOG and KEGG. The results of Unigene in KEGG database were obtained by KOBAS2.0 software. According to the predicted amino acid sequence of Unigene and the Pfam database, the annotation information of Unigene was obtained.

Gene expression analysis

The differentially expressed genes (DEGs) of *C. chinensis* grown on pear leaves under different concentrations of nitrogen application (0, 1500mg/kg) were screened by DESeq2 software, and Fold Change ≥ 2 or Fold Change ≤ -2 and FDR < 0.05 were analyzed as standard. The Benjamini-Hochberg *p*-value correction method was used to avoid false positives. Four genes related to amino acid synthesis were selected from the DEGs analyzed by sequencing results for quantitative verification of transcriptome sequencing results. The primers used for the determination of DEGs expression were shown in the table 1. The cDNA was diluted in a 3-fold gradient, and the amplification efficiency of primers was measured. qPCR was conducted with a SYBR® Premix Ex TaqTM II RT-PCR Kit. The qPCR condition was as follows:

2 min of denaturation at 95°C, a two-step amplification cycle with 95°C for 5 s and 60°C for 30 s was cycled for 39 times95℃, denaturation 5 s, 60℃ extension 30 s. There are 3 to 4 technical replicates for each gene, and the mean value was taken as the Ct value of the biological replicate.

Table 1 The qPCR primers used to study the expression of amino acid synthesis pathway genes in *C. chinensis* affected by nitrogen fertilizer

E.C	Gene name	Sequences of qPCR primers（5’- 3’）
	Tubulin	F: GTCCTCGTGGTTAGTGCTTGTG
		R: CCACACTGACCAGCCTGTAC
	phosphoserine phosphatase	F:CGCAGAGTACATCAAGGACAAG
		R: CACATAGCGATCTCAGCGTC
2.6.1.1	*aspartate aminotransferase*	F: GTACTAGATGGAAGCAGCCG
		R: GGTGATTTGGACCGTGATGC
2.1.2.1	*Serine hydroxymethyltransferase*	F: CCGGTAACGAAGTGCTATACG
		R: GGTAGCCTGCTTTAGAGCGG
6.3.5.4	*asparagine synthase*	F: CAGCTTTCAGGCACACAGAC
		R: ACGACGTTACCGGACCAATC

Data Analysis

Ferulic acid, chlorogenic acid, and epicatechin contents in pear leaves and the developmental duration of *C. chinensis* in different nitrogen treatments were analyzed by one-way ANOVA. Least Significant Difference (LSD) test was used to determine the difference among different treatments described above ($P < 0.05$). The amount of honeydew secreted by *C. chinensis* and the contents of free amino acids in the honeydew of *C. chinensis* under different nitrogen fertilizer concentrations were compared by one-way ANOVA followed by Tukey HSD test. The amino acid content in honeydew and leaves of *C. chinensis* under the same nitrogen concentration treatment were analyzed by Mann-Whiteney test and student’s t test, all statistical analyses were performed by SPSS V21.0 software.

Results

Effects of nitrogen fertilizer on phenolic acids of leaves of pear tree

Nine phenolic acids were separated and identified from the leaves of pear seedlings, including chlorogenic acid, epicatechin, ferulic acid, vanillin, gallic, caffeic acid, syringic acid, p-coumaric acid, and

protocatechuic acid. chlorogenic acid, epicatechin, ferulic acid of them were the main phenolic acid from pear tree (Fig.S 1-4).

The content of ferulic acid from pear leaves treated with 500mg/kg and 1000mg/kg nitrogen fertilizers showed no significant different from that of the control, however the content of ferulic acid from pear leaves was significantly higher than that from the control pear leaves under the concentration of 1500mg/kg, ($F_{3,20}$= 4.649, P= 0.013) (Fig. 1A). The content of chlorogenic acid and epicatechin from pear leaves treated with nitrogen fertilizers showed no significant difference from that of the control (chlorogenic acid: $F_{3,20}$= 1.063, P= 0.387; Fig. 1B ; epicatechin: $F_{3,20}$= 0.104, P= 0.0957; Fig. 1C).

Effects of ferulic acid on developmental duration of nymphs of C. chinensis

The developmental duration of the 5th instar and the 3rd -5th instar of *C. chinensis* nymphs decreased significantly ($F_{2,57}$= 4.888, P= 0.011) in both two concentrations (0.24 ppm and 0.36 ppm) of ferulic acid treatment comparing with control treatment (Fig. 2). No significant differences were found among the developmental duration of the 3rd and 4th instar of *C. chinensis* in control and ferulic acid treatments (Fig. 2).

Effect of nitrogen fertilizer on honeydew secretion of C. chinensis

We found that nitrogen fertilizer could affect the secretion of *C. chinensis* honeydew, the secretion of honeydew gradually increased with the increase of nitrogen fertilizer. The secretion of honeydew from *C. chinensis* fed on pear leaves treated with nitrogen fertilizer was significantly higher than that of the control by testing with the direct measurement ($F_{3, 80}$=17.512, P<0.001) (Fig. 3A) or the water-sensitive paper method ($F_{3, 116}$=24.852, p< 0.001) (Fig. 3B).

Effect of nitrogen fertilizer on content of amino acids from pear leaves

A total of 19 amino acids were detected in the pear leaves treated with different concentrations of nitrogen fertilizer, including 9 non-essential amino acids and 10 essential amino acids for insects. The three main non-essential amino acids in leaves were glutamate, aspartate and asparagine, respectively. Arginine and threonine were the two most abundant essential amino acids in leaves. The result indicated that the content of amino acids in leaves showed an increase trend with the increase of nitrogen fertilizer. Most of the amino acids in leaves showed significantly difference under different concentrations of nitrogen fertilizer: (1) Non-essential amino acids: Aspartate $F_{3, 8}$=16.950, p = 0.001, asparagine $F_{3,}$

$_{8}$=12.585, p = 0.002, glutamine $F_{3,8}$=201.814, p < 0.001, alanine $F_{3,8}$=33.428, p <0.001, glycine $F_{3,8}$= 16.413, p = 0.001, serine $F_{3,8}$=11.261, p = 0.003, proline $F_{3,8}$=140.439, p < 0.001. (2) Essential amino acids: Arginine $F_{3,8}$=595.35, p < 0.001,valine $F_{3,8}$= 13.099, p = 0.002, phenylalanine $F_{3,8}$= 5.042, p = 0.03, threonine $F_{3,8}$= 8.856, p = 0.06, tryptophan $F_{3,8}$= 11.478, p = 0.03, histidine $F_{3,8}$= 14.307, p = 0.001 (Fig. 4).

Effect of nitrogen fertilizer on content of amino acids from honeydew of C. chinensis

Four essential amino acids (Fig. 5B) and eight non-essential amino acids (Fig. 5D) were identified in the honeydew secreted by *C. chinensis*. The results showed that the nitrogen fertilizer significantly affected the total content of amino acid (Fig. 5A & 5C) in the honeydew of *C. chinensis*, and the content of amino acids increased with the increase of nitrogen fertilizer, arginine ($F_{3,8}$=5.166, p = 0.028), threonine ($F_{3,8}$=52.898, p < 0.001), glutamine ($F_{3,8}$=11.896, p = 0.003), asparagine ($F_{3,8}$=4.534, p =0.039), glycine ($F_{3,8}$=4.306, p = 0.044), serine ($F_{3,8}$=9.455, p = 0.005) were significantly higher than that of the control respectively.

The amino acids contained in the pear leaves, such as tyrosine, leucine, isoleucine, methionine, phenylalanine, lysine, and tryptophan weren't detected from the honeydew of *C. chinensis*. The contents of amino acids in the honeydew of *C. chinensis* were significantly lower than that of pear leaves under non-treatment with nitrogen fertilizer, such as the non-essential amino acid glutamate (t_4=3.91. p = 0.017), essential amino acids arginine (t_4 = 7.74, p = 0.001), valine (t_4 = 8.89, p = 0.001), phenylalanine (t_4 = 12.03, p = 0.007), lysine (t_4 = 4.81, p = 0.009), threonine (t_4 =21.85, p< 0.001) (Fig. 5E). The content of amino acids in the honeydew of *C. chinensis* under the 1500 mg/kg of nitrogen fertilizer treatment, such as the non-essential amino acids alanine (t_4 = 8.42. p = 0.001), proline (t_4 = 4.28, p = 0.013), serine (t_4 = 5.78, p = 0.004), were also significantly lower than that in pear leaves (Fig. 5F).

Effect of nitrogen fertilizer on the gene expression of amino acid synthesis of C. chinensis

A total of 116 differentially expressed genes (DEGs) were obtained between *C. chinensis* fed on pear seedlings with and without nitrogen fertilization, of which 2 were up-regulated and 114 were down-regulated (Fig. 6). Two up-regulated genes failed to match the protein sequence after comparison with the database through Blastx. 107 out of the 116 differential genes were annotated.

The down-regulated differential genes were enriched in several pathways in the KEGG metabolic pathway analysis, mainly including amino acid anabolism, pentose phosphate pathway and peroxidase (Fig. 7). KEGG pathway analysis showed that several DEGs were related to amino acid metabolism pathways, and

all of them were down-regulated after treating with nitrogen fertilizer, including alanine, aspartic and glutamate metabolic pathways, tryptophan metabolic pathways, glycine, serine, and threonine metabolic pathways, etc. The results showed that the expression levels of the serine synthetic gene *phosphoserine phosphatase* (*PSP*), glycine synthetic gene *alanine-glyoxylate aminotransferase*, genes catalyzing the synthesis of glycine (*serine hydroxymethyltransferase*, *glyA*), asparagine (*asparagine synthase*) and glutamate (*aspartate aminotransferase*, *AAT*), peroxiredoxin and glutathione S-transferase, catalase (*CAT*), and sod-2 (*superoxide dismutase*) in *C. chinensis* were all down-regulated in the nitrogen-treated group. The expression level of the gene *cystathionine gama-lyase* that synthesized cysteine was not significantly changed after treatment with nitrogen fertilizer.

Verified by qPCR, the expression levels of genes *phosphoserine phosphatase* (t_2 = 11.02, p = 0.008) and *serine hydroxymethyltransferase* (t_2 = 10.068, p = 0.010) that synthesize serine and glycine were found significantly decreased in *C. chinensis* fed on pear seedlings treated with nitrogen fertilizer (Fig. 8). Meanwhile, the expression levels of asparagine synthesis gene *asparagine synthase* (t_2 = -9.19, p = 0.012) and glutamate synthesis gene *aspartate aminotransferase* (t_2 = -7.953. p = 0.015) were also significantly down-regulated in *C. chinensis* under nitrogen fertilizer treatment (Fig. 8).

Discussion

Nitrogen fertilizer can generally change or alter the morphological, physicochemical characteristics of host plants and the nutritional conditions of herbivores (Lu et al.,2007; Throop & Lerdau,2004). The changes of a nutritional level in plant tended to be accompanied by changes in the level of other nutrients, water and numerous allelochemicals (Mattson,1980; Lu et al.,2007). Studies have shown that, in pear leaves, the composition of phenolic compounds were influenced by pear cultivars, environmental conditions as well as ontogenetic leaf age (Andreotti et al.,2006; Dong et al.,2018). In this study, we found that the chlorogenic acid, epicatechin/catechin, ferulic acid, vanillic acid, and gallic acid are widespread in the pear leaves, the amounts of phenolic acids in pear leaves varied under different nitrogen fertilizer conditions. Our research indicated that chlorogenic acid, ferulic acid and epicatechin were the main phenolic acids in *P. bretschneideri* leaves, which is similar with *P. betulaefolia* (Zhang et al.,2015). Ferulic acid content in pear leaves increased with the increase of fertilization concentration, however nitrogen fertilizer had no significant effect on the contents of chlorogenic acid and epicatechin in the pear leaves. Several studies have shown that the synthesis of rosmarinic and caffeic acid increases under low nitrogen stress (Nguyen & Niemeyer,2008; Bénard et al.,2009), pear tree with high nitrogen treatment reduced the content of caffeic acid and p-coumaric acid, phloridzin, quercetin-glycosides in the young leaves of cultivars studied (Leser & Treutter,2005). Meanwhile, some studies have shown that the impact of lower nitrogen on secondary metabolites was less significant (Bénard et al.,2009). These showed that the effects of nitrogen fertilizer on different secondary metabolites was different. Our results showed that the content of ferulic acid increased under high nitrogen supply, and ferulic acid could improve the development of pear psylla. Similar findings have been reported on fruit fly that ferulic acid could accelerated the development of *Drosophila melanogaster* by modulating nutritional pathways for

development (Westfall et al., 2019). The accelerated developmental process of pear psylla induced by ferulic acid under high nitrogen supply we found in this study provided a new physiochemical mechanism of how the excessive nitrogen application aggravates the damage of psyllids.

Honeydew secretion plays important roles for pests themselves and other organisms, such as predators and ants. Even though honeydew serves as a supplementary nutritional source for beneficial insects, it could also attract ants' attendance and recruit ants to fight against natural enemies for the same source. The honeydew of the pear psylla was also found to disrupt the feeding, slowed the development, and reduced the longevity of its predator *Orius sauteri* (Ge et al. 2020). Therefore, the honeydew of *C. chinensis* could constitute an important component of the prey defense against natural enemies, was helpful for population increasing. Here we found that the secretion of honeydew from *C. chinensis* increased with the increase of nitrogen fertilizer concentration, which showed that *C. chinensis* increased feed intakes and aggravated the damage to pear trees under the high nitrogen fertilizer condition. The amino acids tyrosine, leucine, isoleucine, methionine, phenylalanine, lysine, and tryptophan contained in the leaves were not found in the honeydew. Furthermore, the concentration of amino acids glutamate, arginine, valine, phenylalanine, lysine, threonine, and serine contained in the honeydew of *C. chinensis* were significantly lower than those in pear leaves. These findings indicated that those above amino acids might be totally or partially assimilated or absorbed by *C. chinensis* while feeding and are therefore necessary for the development of *C. chinensis*. Our research proved that the content of amino acids from leaves of pear tree and honeydew of *C. chinensis* increased with the increase of nitrogen fertilizer. It was found that certain amino acids or combinations of several amino acids could induce feeding of aphids and scale insects (Calatayud et al., 2002). Aspartic acid, glutamate, glycine, arginine, asparagine have all been proved to play a certain role as phagostimulant for herbivores (Calatayud et al., 2002).The increase in feed intake of pear psylla related to the increase of amino acids of pear leaves demonstrated the mechanism of how excessive application of nitrogen fertilizer aggravating the damage of *C. chinensis* to pear trees from another aspect.

Amino acids are necessary for the development and growth of insects, many of the amino acids can be synthesized by the hemipterian themselves with the help of the endosymbionts, such as serine, glycine, asparagine and glutamine, etc. We found that the expression levels of *C. chinensis* genes *PSP*, *glyA*, *asparagine synthase*, *AAT* in the serine, glycine, asparagine and glutamate synthesis pathway decreased more significantly under high-concentration nitrogen treatment. This could be related to the insects adjust amino acid synthesis based on their dietary needs. Research showed that even with an excess of dietary glutamine, asparagine, glutamate or aspartic acid, the overall content of these amino acids in aphid bodies was mostly the product of catabolism and re-synthesis of dietary amino acids within aphids (Haribal et al., 2015). The decrease in amino acid synthesis level in *C. chinensis* under the high-concentration nitrogen treatment may be due to the increase in the concentration of essential and non-essential amino acids in diet, which provides more superfluous raw materials for the re-synthesis of amino acids in psyllid body and the redundant amino acids produced during the catabolism and *de novo* synthesis of amino acids in the psyllids were excreted with the honeydew. Therefore, high nitrogen

fertilization might increase the fitness of *C. chinensis* on plants by providing more nutrients and subsequently aggravated the damage caused by psyllids.

In conclusion, the nitrogen fertilizer increased the contents of ferulic acid and amino acids in pear leaves, which benefited the feeding and development of psyllid. The high nitrogen fertilization provided more nutrients in psyllid diet and decreased the expression levels of several psyllid genes in the serine, glycine, asparagine and glutamate synthesis pathway. We demonstrated the mechanism of how excessive application of nitrogen fertilizer aggravates the damage of *C. chinensis* to pear trees from physiochemical and molecular aspects. The results showed that rational application of nitrogen fertilization is very necessary to reduce the damage of *C. chinensis* and guarantee the yield and quality of pear.

Declarations

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Ethical approval. The article does not contain any studies with animals performed by any of the authors.

Author contribution statement.

W.P.S. and X.Z.H. conceived and supervised the study. Y.G., W.T.J. and Z.F.Q. designed and performed experiments. Y.G., W.T.J., L.Z., M.Y.F. and Z.F.Q. managed pear psylla, performed sample preparation, Y.G., W.T.J., L.Z. and Z.F.Q. performed data analysis and experiments. All authors contributed to editing and writing of the manuscript.

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Figures

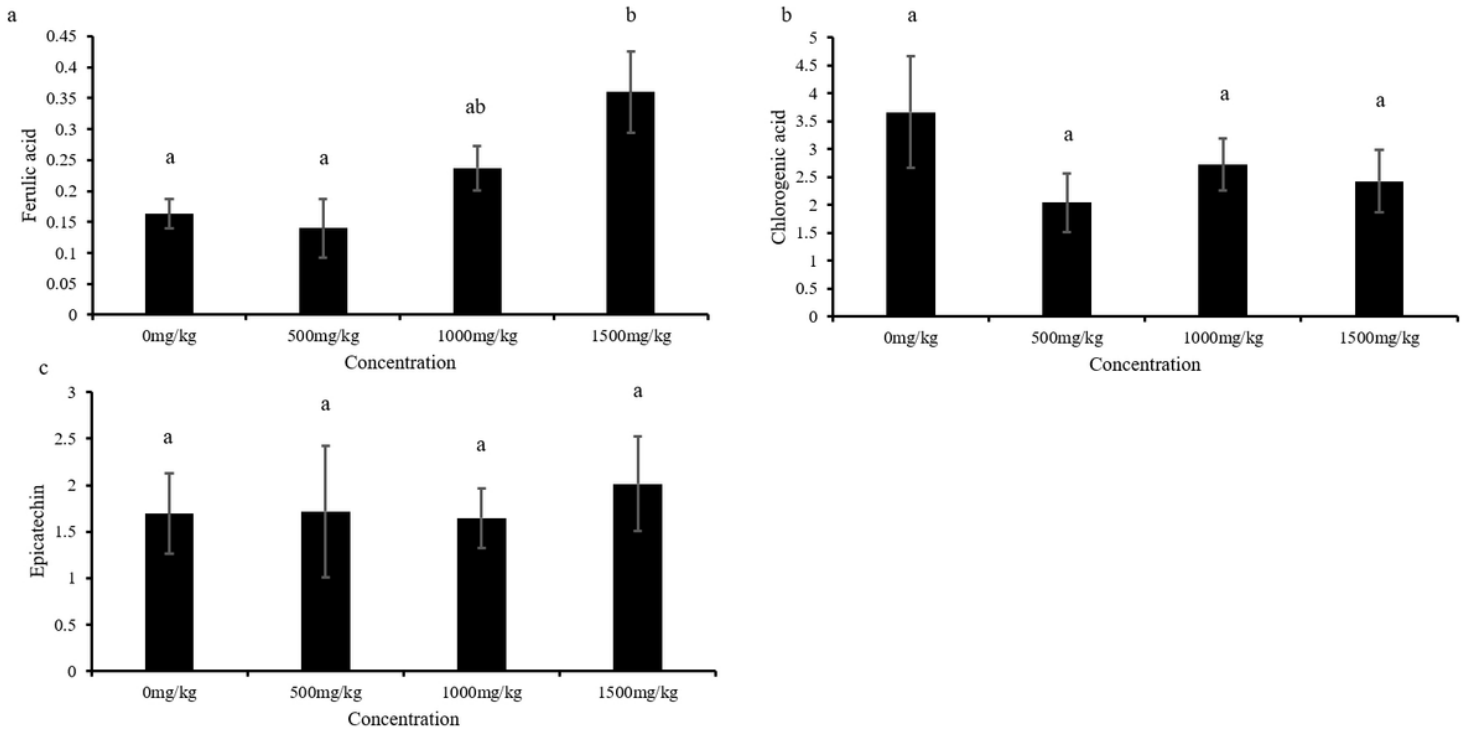

Figure 1

Effect of nitrogen fertilizer on ferulic acid (A),chlorogenic acid (B) , and epicatechin (C) concentration in pear leaves (n = 6; α = 0.05; ANOVA).Values in the bar chart are mean (±SE). The presence of the same

letter indicates that there is no significant difference between different treatments.

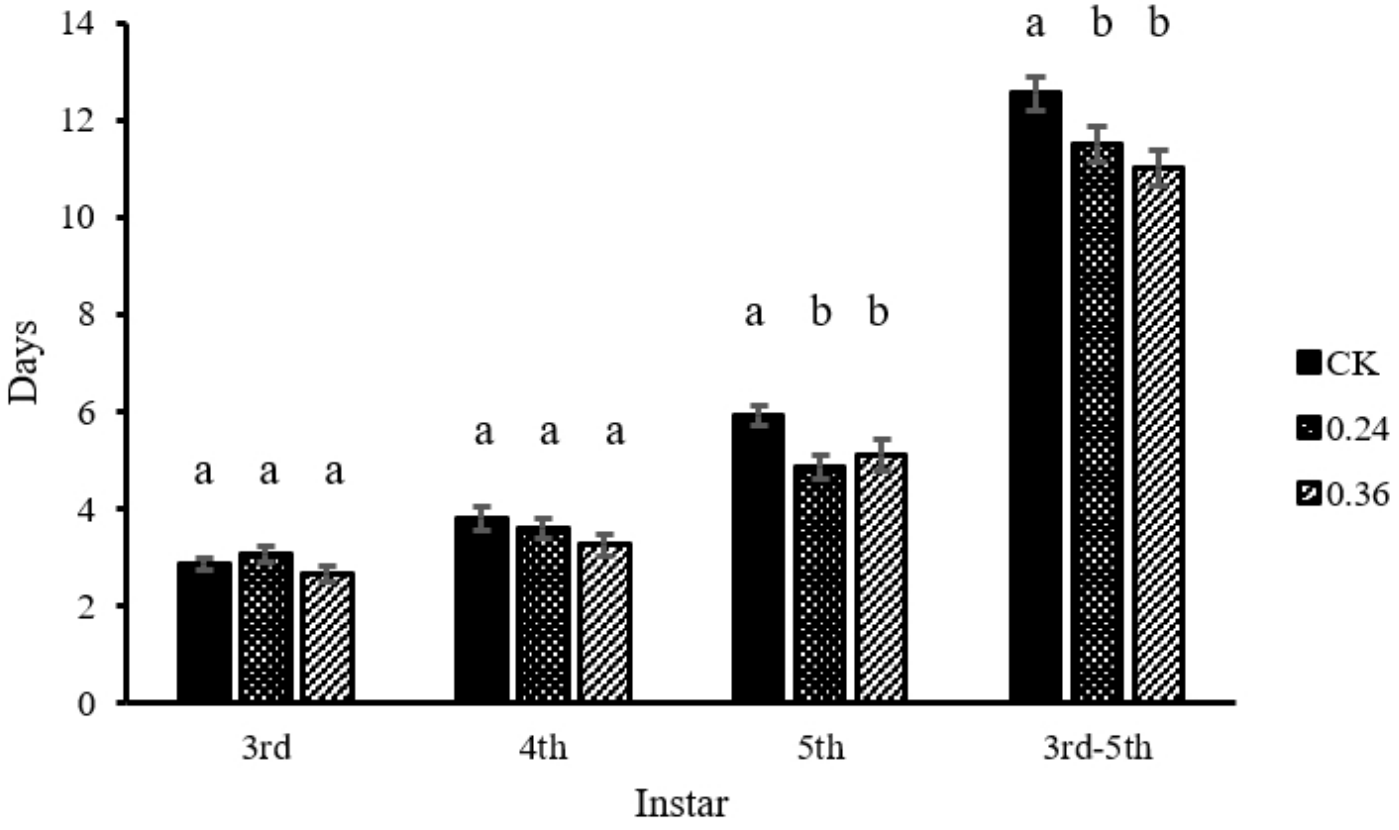

Figure 2

Effect of ferulic acid on developmental duration of the third to fifth instar nymphs of Cacopsylla chinensis (n = 20; α = 0.05; ANOVA).Values in the bar chart are (mean ±SE). The same letter indicates that there is no significant difference between different treatments.

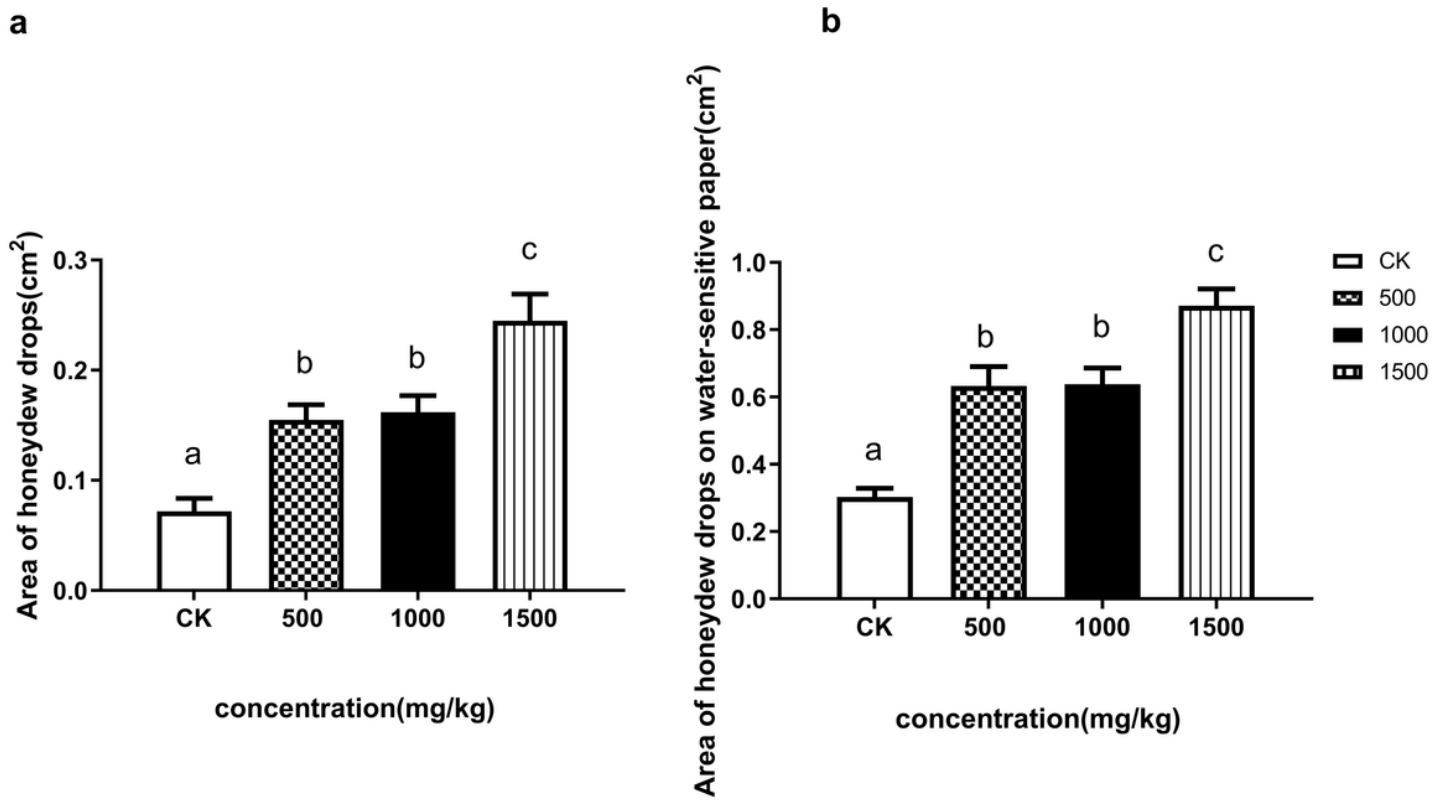

Figure 3

Effect of nitrogen fertilizer on honeydew production of Cacopsylla chinensis (A: direct determination; B: water sensitive paper). The values in the bar chart are (mean ±SE). The same letter indicates that there is no significant difference between different treatments (ANOVA).

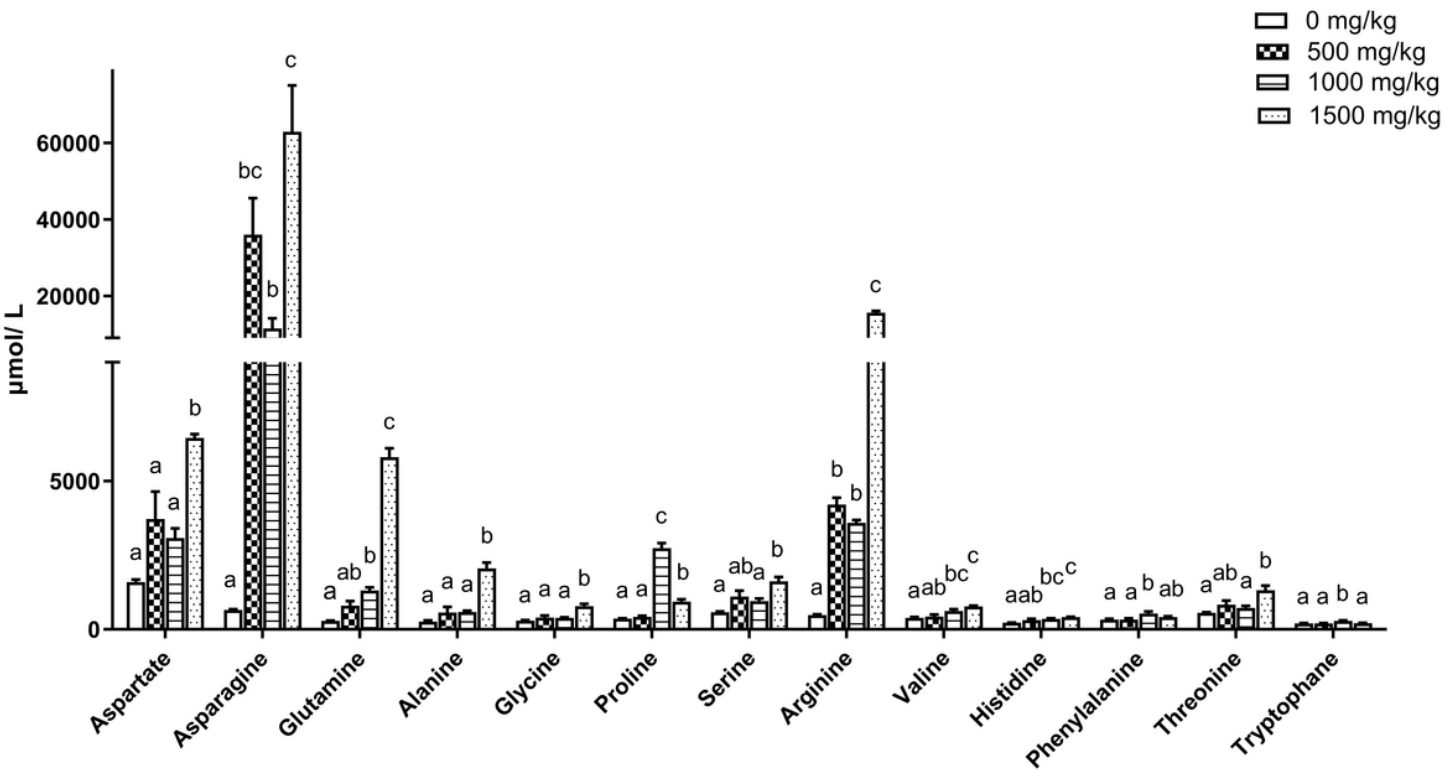

Figure 4

Effect of nitrogen fertilizer on content of amino acids in pear leaves. The values in the bar chart are mean (±SE). The presence of the same letter indicates that there is no significant difference between different treatments (ANOVA).

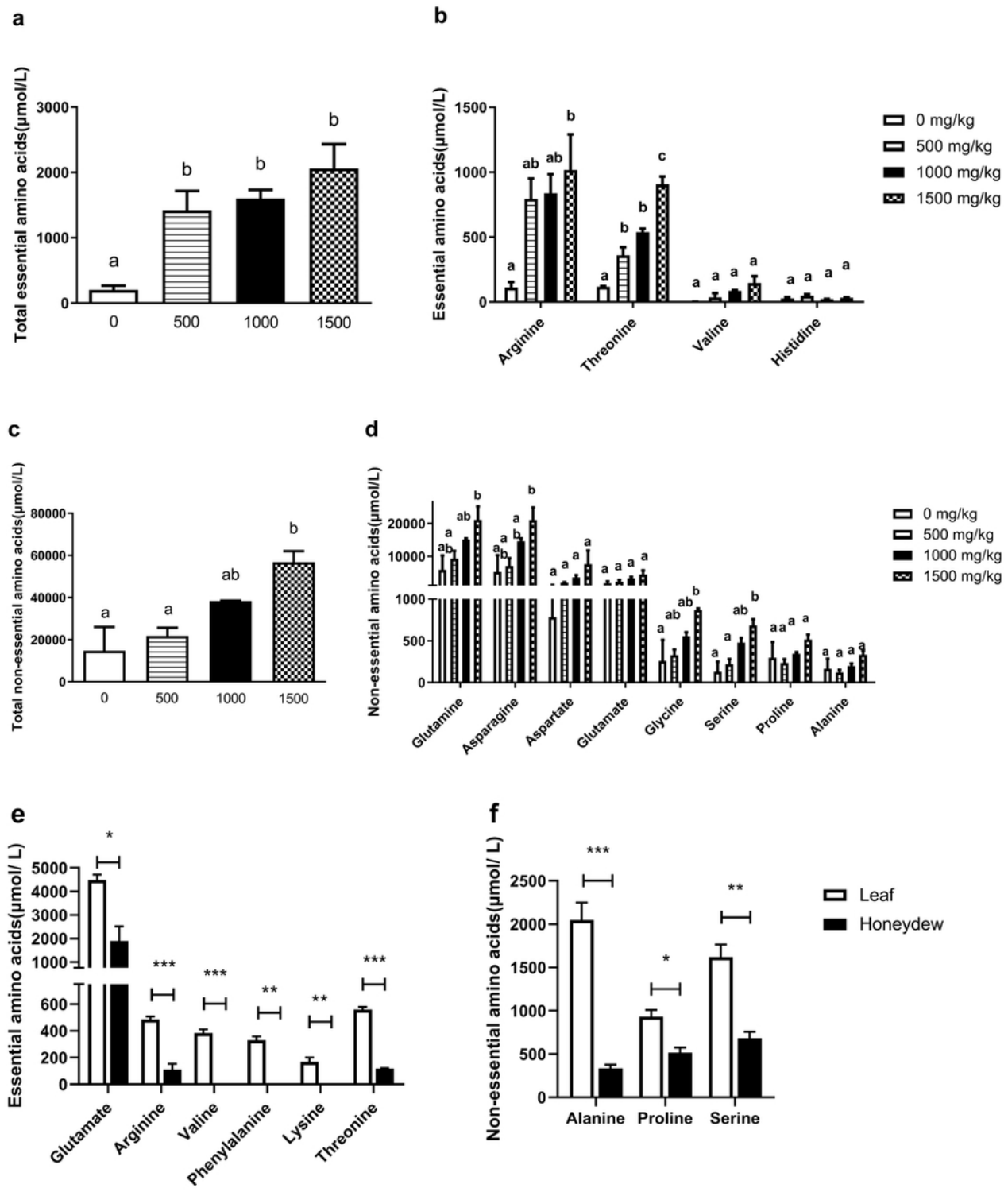

Figure 5

Effect of nitrogen fertilizer on content of amino acids from honeydew of C. chinensis. The values in the bar chart are mean (±SE). The presence of the same letter indicates that there is no significant difference between different treatments (ANOVA). A: Total content of essential amino acids; B: Content of different essential amino acids; C: Total content of non-essential amino acids; D: Content of different non-essential amino acids; E: Difference of contents of amino acids between the honeydew of C. chinensis and pear

leaves under non-treatment with nitrogen fertilizer; F: Difference of contents of amino acids between the honeydew of C. chinensis and pear leaves under the treatment with 1500 mg/kg of nitrogen fertilizer. * means there is a significant difference, $P<0.05$, student t-test; ** means there is a significant difference, $P \leq 0.01$, student t-test; *** means there is a significant difference, $P \leq 0.001$, student t-test.

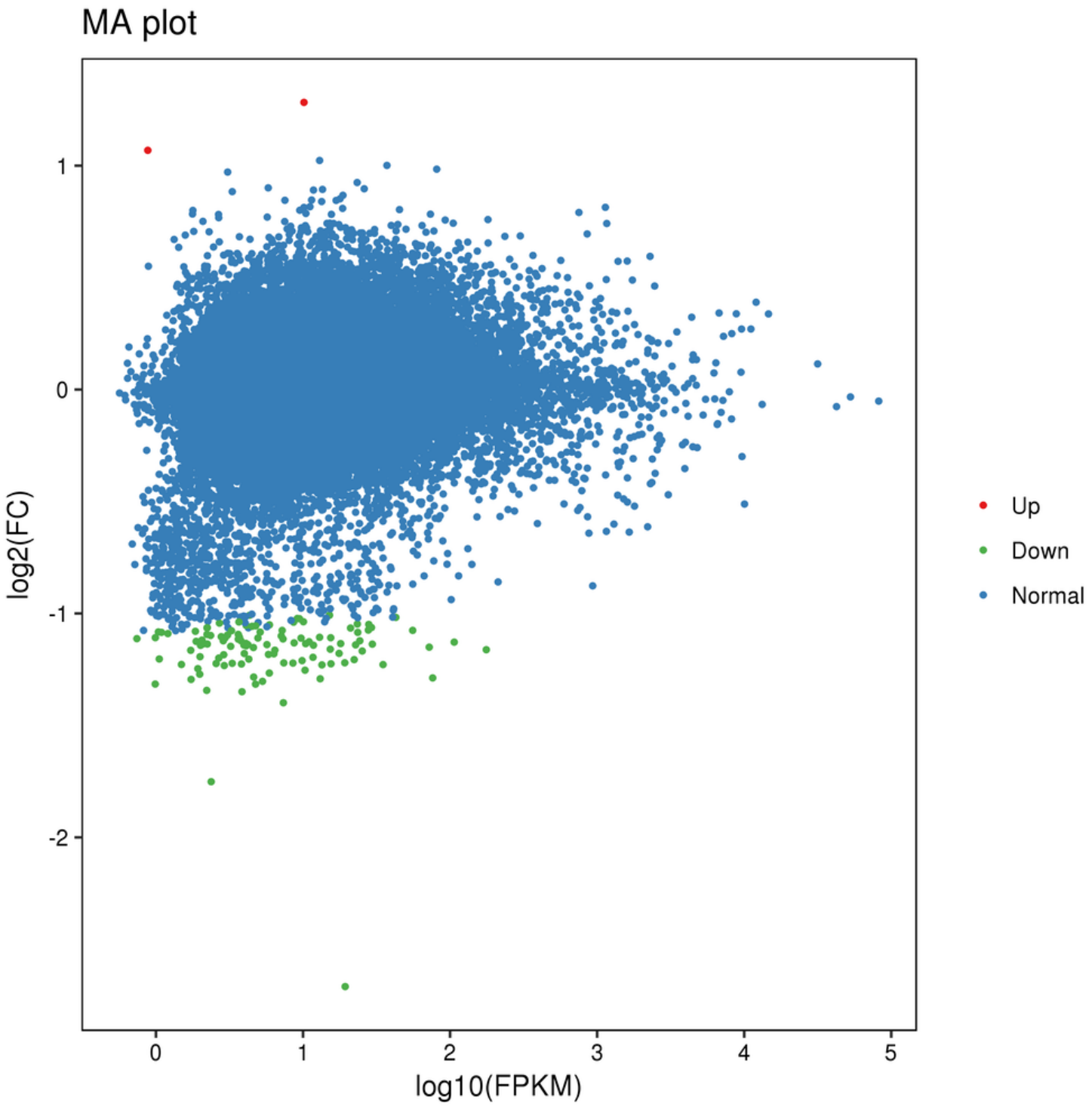

Figure 6

The level of differentially expressed genes between C. chinensis fed on pear seedlings without and with nitrogen fertilization. There were 2 up-regulated (red dot) and 114 down-regulated (green dot)

differentially expressed genes.

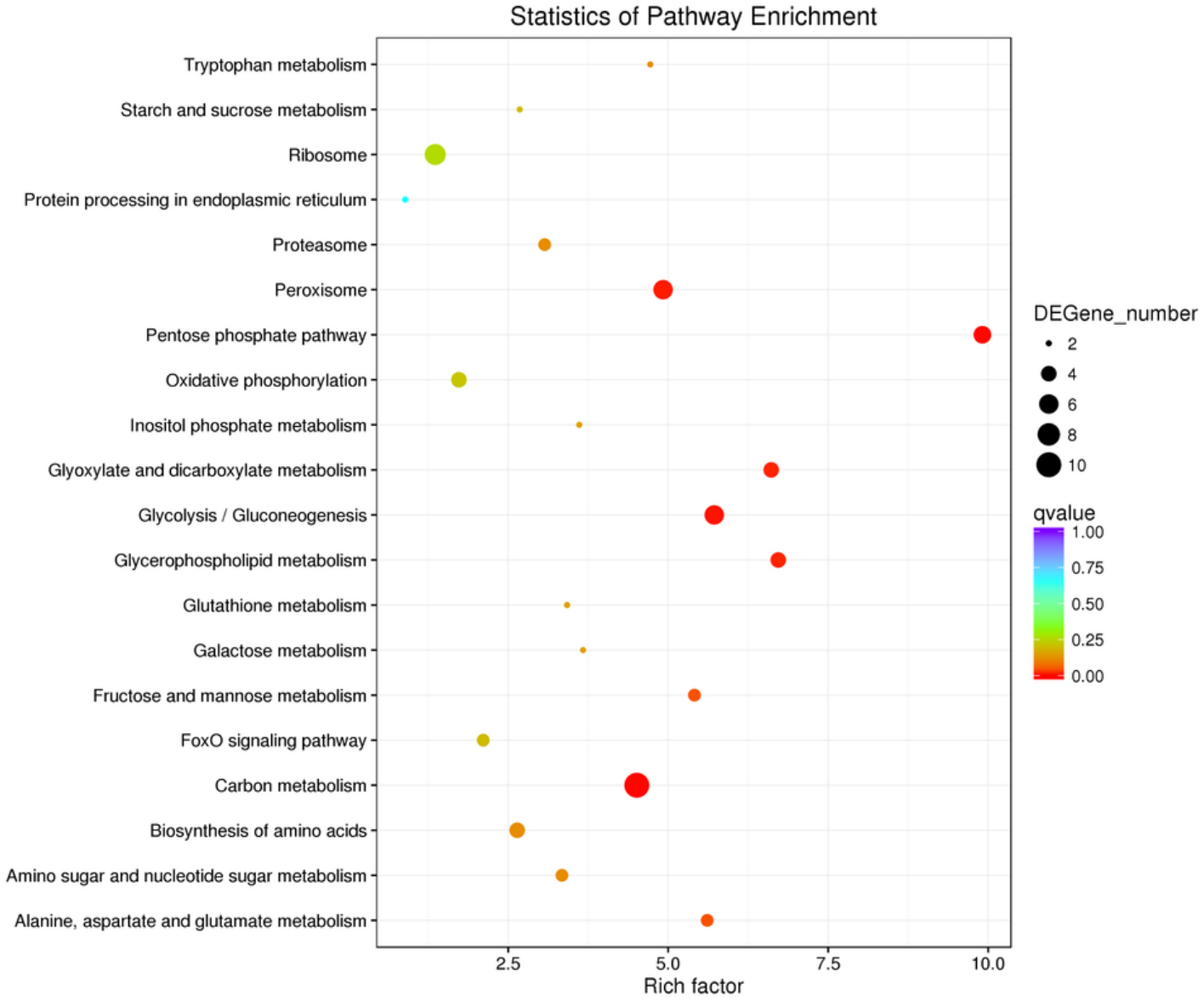

Figure 7

The KEGG metabolic enrichment pathway of differentially expressed genes. The vertical axis represents the names of 20 KEGG pathways with the most significant differentially enriched genes, and the horizontal axis refers to the number of differentially enriched genes in this pathway. The color of the dots corresponds to different ranges of q values. The smaller the q value, the more significant the enrichment.

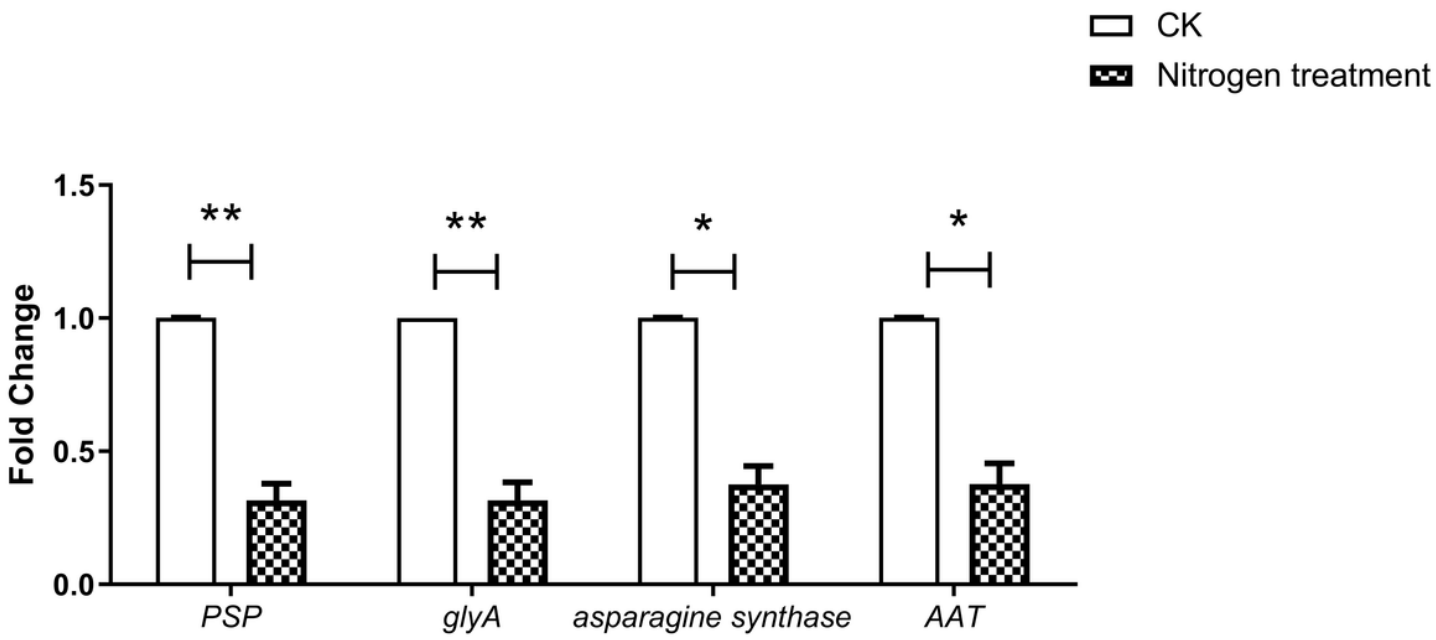

Figure 8

Gene expression of serine, glycine, asparagine and glutamate synthesis pathway. * means there is a significant difference between the nitrogen treatment group and the control treatment group, $P<0.05$, student t-test; ** means there is a significant difference between the nitrogen treatment group and the control treatment group, $P \leq 0.01$, student t-test.