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Effects of wheat intercropping on the nitrogen metabolism during senescence of cucumber leaves

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ABSTRACT

In Greenhouse experiment, we studied the effects of wheat (*Triticum aestivum* L.) intercropping on the nitrogen metabolism during the senescence of cucumber (*Cucumis sativus* L.) leaves. The results showed that compared with cucumber monoculture, intercropping with wheat increased the content of soluble protein, nitrate nitrogen, free amino acid and proline and enhanced the activity of nitrate reductase (NR), glutamine synthase (GS), glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) of cucumber leaves. The qRT-PCR analysis revealed that compared with cucumber monoculture, the expression of *N1*, *N2* and *N3*, which belong to *NRT* (Nitrate Transporter Protein) family and *A1*, which belong to *AMT* (Ammonium Transporter Protein) family and *GS1* (cytosolic glutamine synthase gene) were up-regulated in intercropped cucumber. In short, the cultivation mode of intercropping with wheat increased the content of nitrogen compounds and enzyme activity and promoted the expression of genes related to nitrogen metabolism in cucumber leaves, which delayed the senescence of cucumber leaves.

Key words: Cucumber, *Cucumis sativus* L., enzymes, intercropping, leaf senescence, nitrogen metabolism, qRT-PCR, *Triticum aestivum* L., wheat.

INTRODUCTION

Continuous monocropping is major limiting factor in vegetable cultivation (69), which not only increases the plant diseases and insect pests, reduces yield and quality of vegetables, but also causes premature aging of plants (31). Senescence is necessary stage during the growth and development of crops (49) and has become major problem to increase crop yield and quality. Senescence is a complex process of a series of physiological and biochemical changes, which are induces by growth and development stages of plants and environmental stress (40). Nitrogen metabolism is one of the important metabolic activities of crops, which affects the crop growth and development. The nitrogen metabolism is decreased during the senescence of crops and this leads to premature aging and yield reduction (16,21). Nitrogen inefficient genotypes are prone to premature senescence. Accordingly, nitrogen metabolism is closely related to leaf senescence of crops and the nitrogen metabolism is also decreased during the leaf senescence (72).

The advantages of intercropping system can improve the ecological adaptability of plants to environmental stress. The intercropping of potatoes is more beneficial to farmers

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than respective sole cropping (62). The peanut/maize intercropping leads to variation in the proteins related to carbon and nitrogen metabolism (54). Most of the legume species are involved in phosphorus, nitrogen and allelochemicals metabolism through proteomics analyses in faba bean/maize intercropping system (59). In intercropping systems, legumes are commonly combined with other crops (often cereals) to reduce fertilizer requirements, specifically nitrogen (6). Compare to wheat or barley monocultures systems, N uptake by wheat and barley in wheat-maize or barley-maize intercropping systems were significantly higher (66). This shows that intercropping plays important role in nutrients absorption and nutrients metabolism of crops.

Cucumber (*Cucumis sativus* L.) is one of the largest protected vegetables in China. Its continuous monocropping has resulted in premature aging of leaves, further decreasing the yield and fruit quality of cucumber (14). Wheat is major crop globally with allelopathic properties. Intercropping of wheat with cucumber significantly improves the flora composition of soil microorganisms, reduces the number of soil-borne pathogens and alleviates the problems of soil sickness in continuous monocropping (48). This promotes the growth of cucumber, increases fruit yield, reduces the incidence and disease index of angular leaf spot of cucumber and improves the diversity of soil microbial community (20,44,51). Intercropping of wheat with cucumber also reduces the degradation rate of chlorophyll and protein in cucumber, increases the antioxidant enzyme activities of cucumber and delays the senescence of cucumber leaves (12). However, there is no report about the change in nitrogen metabolism during the senescence of cucumber leaf, in intercropping with wheat. Therefore, this greenhouse experiment aimed to determine the content of nitrogen compounds, the activity of enzymes related to nitrogen metabolism and the expression of related genes in cucumber leaves. Besides, we studied the effects of intercropping with wheat on nitrogen metabolism during the cucumber leaf senescence.

MATERIALS AND METHODS

The study was conducted from March to July 2017, at Northeast Agricultural University, Harbin, China [(45°41′N, 126°37′E). Altitude: 165 m; Annual rainfall: 529 nm; Maximum temperature: 32.6℃; Minimum temperature: 22.1℃]. Cucumber cultivar ‘Jinzao-9’ was obtained from the Academy of Agricultural Sciences, Tianjin, China. Wheat cultivar ‘Pinzi II-5’ was obtained from the Laboratory of Vegetables, Physiological Ecology, Northeast Agricultural University, Harbin, China. Test soil for the greenhouse studies was taken from the field under continuous monocropping of cucumber. The chemical characteristics of soil were determined by the methods of Bao (4) and the chemical composition was: ammonium nitrogen: 47.65 $mg \cdot kg^{-1}$, nitrate nitrogen: 149.66 $mg \cdot kg^{-1}$, total nitrogen: 1.41 $g \cdot kg^{-1}$, available P: 319.27 $mg \cdot kg^{-1}$, total P: 1.43 $g \cdot kg^{-1}$, available K: 316.00 $mg \cdot kg^{-1}$, organic matter: 68.38 $mg \cdot kg^{-1}$, EC (1:2.5, w/v): 1.03 $mS \cdot cm^{-1}$ and pH 7.72 (1:2.5, w/v).

Greenhouse experiments

Cucumber variety ‘Jinzao-9’ were sown on March 28, 2017 and harvested on July

10, 2017. Wheat variety ‘Pinzi II-5’ was sown on April 24, 2017. This experiment does not aim at wheat harvesting, because wheat plants were sown too near to cucumber plants and hence, will affect the normal growth of cucumber. The experiment included two treatments: (i) Cucumber monoculture, (ii) Cucumber + wheat intercropping. Cucumber seeds were washed several times with distilled water and then soaked at 55℃ or 30 min, stirred for 10 minutes. After standing at room temperature for 6 h, the seeds was evenly spread in a tray

Photograph 1. The plot 1 showing intercropping of cucumber + wheat in greenhouse

Photograph 2. The plot 2 showing intercropping of cucumber + wheat in greenhouse

(30 cm×17 cm×6 cm) filled with moist gauze, and its surface was also covered with moist gauze. The germination was done in incubator at 28℃. Two days later, the germinated cucumber seeds were sown into the trays (40 cms ×30 cms×10 cms). One week later, cucumber seedlings with two cotyledons were transplanted in pots (10 × 10 cms). After 20 d, cucumber seedlings with 2-true-leaves were transplanted into greenhouses in one row on April 24, 2017. Same day wheat seeds were sown after transplanting of cucumber seedlings. Harbin [125°42′-130°10′E and 44°04′-46°40′N, mean Annual Temp: 5.6℃] has mid-temperate continental monsoon climate, long winter (November to March) and short summer (July to August). Thus when the southern China farmers starts crops sowing, Harbin land is still in frozen state until late April. Hence the Harbin farmers, first raise the crops seedlings in greenhouse for transplanting in the field. The distance between cucumber seedlings was 30 cm and the distance between the ridges was 60 cm. Single factor randomized block design was used in cucumber cultivation. Each treatment was repeated thrice and each repetition was a plot (5 m × 0.6 m), so there were 6 plots in this experiment. Protected lines were kept on both sides of each plot. The cucumber seedlings were planted first on top of ridges (30 cms Height and 30 cms ridge to ridge spacing) on April 24, 2017, thereafter, wheat seeds were sown by dibbling method on the side of ridge by bunch planting (Photographs). The distance between the wheat and the cucumber plants was only 5 cms. The numbers of wheat companion crop seedlings with one cucumber seedlings were 40, for this 40 wheat seeds were sown near (5 cms away) each cucumber plant..

Sampling

When the wheat plants were 30 cms tall (10- days old), these plants were harvested at 10 cms height i.e. left about 10 cms stubble, which do not affect the cucumber plants growth. The 2-true-leaves emerged on April 21, 2017, the fourth leaf emerged on April 28, 2017 and the fifth leaves on May 17, 2017. The random samples were taken 5-times at 10 days intervals i.e. for 50 days. A part of leaves was used to determine the content of soluble protein, nitrate nitrogen, free amino acids and proline. While the other part of the leaves was stored at -80 ℃, to determine the enzymes activity [Nitrate reductase (NR), glutamine synthase (GS), glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT)] and to extract the leaf RNA.

NITROGENOUS COMPOUNDS CONTENT

These were determined by the Li *et al*. (26) method given below:

(i). Soluble protein content: Fresh leaf samples (0.5 g) were homogenized using distilled water (2 ml) and combined with residues flushed by distilled water (6 ml) in 10 ml tube. The homogenate was centrifuged for 20 min at 4 000 r/min after incubating for 30 min at room temperature. The supernatant was transferred to a 10 ml volumetric flask and filled with distilled water. Colour reaction: Leaf protein extract (1 ml) was homogenized with Coomassie brilliant blue G-250 (5 ml) and allowed to set for 2 min, after which the absorbance was measured at 595 nm.

(ii). Free amino acids: Fresh leaf samples (0.5 g) were homogenized with 10 % acetic acid (5 ml), combined with distilled water in a tube (10 ml) and filtered in to conical flask. Filtrate was transferred into graduated test tubes containing nitrogen (per tube containing 0, 1, 2, 3, 4, 5 µg) with distilled water (1 ml), ninhydrin (3 ml) and 0.1% ascorbic acid (0.1 ml). The tubes had a seal, boiled for 15 min and quickly cooled, these were shaken resulting in blue purple instead of red that was gradually oxidized. Then made up the volume of tubes to 20 ml with 60% ethanol and the absorbance was measured at 570 nm.

(iii). Proline content: Fresh leaf samples (0.5 g) were put into tubes with 3% Sulfonyl salicylic acid solution (5 ml). Then the tubes with seal were incubated for 10 min in boiling water and homogenized. Take another clean tube, pipetted filtrate (2 ml), added glacial acetic acid (2 ml) and acid ninhydrin solution (2 ml). The solution turned red after mixing and kept in boiling in a boiling water for 30 min until turned red. When the mixture was at room temperature, added toluene (4 ml), shaked for 30 s, and let stand for a while. The supernatant was gently added into a 96-well plate, followed by measuring the absorbance at 520 nm.

(iv). Nitrate nitrogen content: Fresh leaves (0.5 g) were grinded and put into a tube (10 ml) with ultrapure water (8 ml) then heated for 30 min in boiling water until homogenized. The homogenate was centrifuged for 10 min at 5000 r/min after cooled at room temperature and transferred to a new tube. One treatment of supernatant (0.1 ml) was combined with the solution of 5% salicylic acid-sulfuric acid (0.4 ml), another was treated with distilled water and combined with similar solution as control. The treatments were kept still for 20 min, added 8％NaOH (9.5 ml) before homogenized and cooled to room temperature and the absorbance was measured at 410 nm.

ENZYME ACTIVITY

(i). Nitrate reductase (NR) (26) activity: In brief, enzyme extract (0.4 ml) and 1.2 mL of 0.1 mmol/L KNO_3 phosphate buffer containing 0.4 ml NaOH were mixed at room temperature for 30 min; then the mixture was mixed with 1ml sulfanilamide and 1-Naphthylamine. The colour of solution changed to pink. After 15 min the colour developed ande the absorbance was measured at 540 nm.

(ii). Glutamine synthetase (GS) activity (65): To prepare the reaction mixture in deionized water, we added HCl of (pH 7.4), Tris (1.2236g), $MgSO_4 \cdot 7H_2O$ (1.9918 g), glutamic acid sodium salt (0.3451 g), cysteine (0.2422 g) EDTA-Na_2 (0.0744 g) and hydroxylamine hydrochloride (0.5560 g) This solution without hydroxylamine hydrochloride was used as blank. However in all treatments, above reaction mixture was further mixed with enzyme extract solution (0.5 ml) containing 40 mmol/L ATP (0.5 ml) in a centrifuge tube of 10 ml for 30 min at 37℃. The mixture was finally treated with TCA (1 ml) and kept still for 5 min, then it was centrifuged for 10 min at 3500 r. The supernatant was compared with blank. and absorbance was measured at 540 nm.

(iii). Glutamate dehydrogenase (GDH) Activity (30):The reaction mixture (3 ml) containing 115.4 mmol/L Tris-HCl (pH8.0), 23.1 mmol/L α-ketoglutarate, 231 mmol/L NH_4Cl, 30 mmol/L $CaCl_2$ and ddH_20. NADH (6 mmol/L) was prepared fresh and added (0.1 ml) in the treatments. The enzyme extract was not added to control, while 0.1 ml enzyme extract was mixed in rest of treatments.

The absorbance was measured at 340 nm after kept on water bath (30℃) for few minutes. When the first absorbance was recorded, the treatment was kept on water bath for about 30 min and second absorbance was measured again at the same condition to the first. Calculate the difference twice for the activity. The active unit of GDH was expressed as the amount of 1μmol NADH catalyzed per minute at 30℃.

(vi). Glutamate Synthase (GOGAT) Activity(39): The reaction mixture consisted of 20 mmol/L L- glutamine, 100 mmol/L α-ketoglutarate (0.05 ml), 10 mmol/L KCl (1.95 ml), 25 mmol/L Tris-HCl (pH7.6) (2.45 ml) and 0.2 ml of freshly prepared NADH (3 mmol/L). However in Control treatment 0.4 ml L- glutamine (20 mmol/L) was added while in all other treatments were supplemented with 0.3 ml enzymolysis liquid.

All treatments were kept for few minutes after water bath (30℃) and then absorbance was measured at 340 nm . When the first absorbance was recorded, the treatment was kept on the water bath for about 30 min and second absorbance was measured again at the same condition to the first. Calculate the difference twice for the activity. The active unit of NADH-GOGAT was expressed as the amount of 1μmol NADH catalyzed per minute at 30℃.

RT-PCR analysis

After searching all the N transporter genes in plant from http://www.tcdb.org/ and obtaining the amino-acid sequence of nitrogen transport proteins, these sequences were compared to tblastn software from the Cucurbita Genome Database (http://www.icugi.org/) and the EST sequences of cucumber nitrogen transport protein wase searched. According to the obtained EST sequences, we compared the sequences with other plants on NCBI and selected four high-similarity sequences as the nitrogen transport protein gene sequences of cucumber, named as *N1*, *N2*, *N3* and *A1*, respectively. The glutamine synthase gene of cytoplasmic type of cucumber (*GS1*) (10). The PCR cycle profile consisted of 30 s at 95℃, 30 s at 55℃ and 45 s at 72℃. The following primer pairs were used:

Actin-F:5’-CGCTCTTCTTGCTTTCACCCTT-3’;
Actin-R:5’-TACCTTGCCTTGGAGTATTTG G-3’; *N1*-F:
5’-GCTCCGACGGTGTTTTG-3’; *N1*-R: 5’-CCCGTTCACAAGCCCTATTA-3’;
N2-F:5’-AACGGCTCTCAGTGGCTAAA-3’; *N2*-R:
5’-GCTCTTCACAACTGCAACCA-3’;
N3-F:5’-GTATCGGGTCGTTGTTTGCT-3’; *N3*-R:
5’-TCACTGTGAGAGGGCTTCCT-3’;
A1-F:5’-GTGTCCCATTGGTTCTGGTC-3’;
A1-R:5’-GCCAATTCGTGGACCTTCTA-3’;

GS1-F:5'-CTGCCCAAGTGGAACTATGATG-3';
GS1-R:5'-CACGCCTGCTGGTGTGTATG-3'.

The obtained data was used to calculate the relative expression level of the gene by the method of Ct ($2^{-\Delta\Delta Ct}$) (8).

Statistical analysis

The raw data was organized using Qrigin8.5 software, data processing using SAS 9.2 software and ANOVA using Turkey SAS ($P < 0.05$).

RESULTS AND DISCUSSION

Nitrogen compounds content

The soluble protein content of cucumber leaves decreased in all sampling dates. It was higher in intercropping than in monoculture system and the difference was significant at 20, 40 and 50 d ($P < 0.05$) (Fig 1, a).

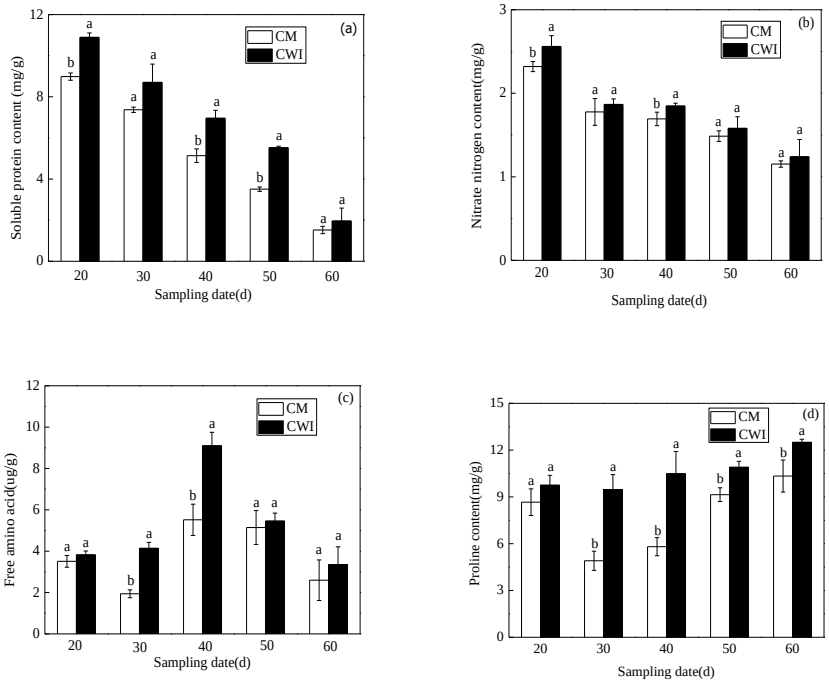

Figure 1. Effects of wheat intercropping on nitrogen compounds content in cucumber leaves.
Note: Cucumber monoculture (CM) and cucumber/wheat intercropping (CWI) were two treatments in the experiment. Soluble protein (a), nitrate nitrogen (b), free amino acid (c) and proline (d) were determined when the 4th cucumber leaf age was 20, 30, 40, 50 and 60 days, respectively. Different letters a, b indicate significant difference at the 0.05 level by Tukey's HSD test.

The nitrate nitrogen content of cucumber leaves decreased in the whole sampling period. It was higher in intercropping system than in monoculture system at all sampling dates. The nitrate nitrogen content in cucumber leaves increased by 10.07 % and 9.21 % at 20 and 40 d, respectively, which significantly differed between the intercropping and monoculture system ($P<0.05$) (Fig 1,b).

The free amino acid content of cucumber leaves was higher in intercropping system than in monoculture system at all sampling dates, which significantly differed in monoculture system at 30 and 40 d ($P< 0.05$). The free amino acid content of cucumber leaves were increased by 113.30 % and 64.85 %, respectively. The content was highest (9.10 μg/g) in intercropping at 40 d (Fig 1,c).

The proline content of cucumber leaves was higher in intercropping system than in monoculture system at all sampling dates. The proline content of cucumber leaves other sampling dates were significantly higher than that in monoculture system except for 20 d (Fig 1,d).

Most soluble proteins in plants are enzymes involved in various metabolisms and their content is important indicator of total plant metabolism (44). Liu and Li (28) found that maize/soybean intercropping effectively increased the content of soluble protein in maize leaves and delayed the senescence of maize Zhao *et al*. (67) found that the appropriate rotation slowed down the degradation of soluble protein in buckwheat leaves and delayed the senescence of leaves. Our experimental results showed that the intercropping system promoted the ability of metabolites to accumulate in cucumber leaves and delayed the degradation of soluble protein in leaves, which provide nitrogen to young leaves and seeds (34,35) and delayed the senescence of cucumber leaves.

Amino acids are basic components of biological macromolecular proteins and important nitrogen metabolites in plants, play important role in nitrogen metabolism of plants (57). Higher level of free amino acids delays the senescence of cucumber leaf (13) and peanut leaves (64). The intercropping system to some extent increased the free amino acid content of cucumber leaves, which may regulate intracellular pH, ion transport and stomatal conductance and thus maintain the integrity of proteins and membrane (25,41). The change of free amino acids content directly or indirectly responds to biotic or abiotic stress in environment (2) and regulate the physiological process of nitrogen metabolism in plants (63), which delays the senescence of cucumber leaves.

The main forms of active nitrogen in senescing leaves are amino acids, small peptides, urea and ammonium nitrate. A large amount of nitrate accumulation in plant vacuole for activation, which is not the same event that nitrate limits plant growth (33). Gao *et al*. (14) found that higher nitrate content delayed the senescence of cucumber leaves. Our results stated that compared with the monoculture system, the intercropping system increased the ammonium nitrogen content of cucumber leaves. Because the intercropping system induces the generation of nitrate from leaves to remobilize the inorganic nitrogen in senescent tissue (7), further improving the level of nitrogen metabolism of cucumber, thus delayed cucumber leaves senescence.

Proline is an intracellular osmotic regulator, whose accumulation maintains cell shape and membrane permeability and stabilize protein structure and enzyme activity. Therefore, the accumulation of proline is a physiological response to delaying plant senescence (61). Wu *et al*. (53) found that leaf senescence was delayed when the proline content of soybean leaves increased. Xue *et al*. (55) showed that wheat cultivars with higher proline content had slower senescence rates during wheat growth and after anthesis. Liu *et al*. (29) found that the increase of proline content in pear leaves delayed their senescence. In the current research, intercropping system increased the proline content in leaves. The accumulation of proline in the senescing leaves provides protection against oxidative stress, which delays the senescence of leaves (63).

Many plants roots secrete allelochemicals [lily (56), rice (21), wheat (50), soybean (32), chili pepper (23), cucumber (43), onion (58), garlic (73) and eggplant (46)]. These allelochemicals in root exudates can produce allelopathic effects on other plants around, which influence the physiological and biochemical process of plants and this promote or inhibit the growth of surrounding plants (60).

Wheat is typical allelopathic crop and its root exudates contain variety of allelochemicals components (50). The intercropping with wheat, increased the content of soluble protein, free amino acid and proline in cucumber leaves. The reason may be that allelochemicals in wheat root exudates could inhibit the protein degradation, induce the synthesis of new protein or improve the synthesis of the original protein (1). The cucumber irrigation with fresh root extract of celery influenced the content of nitrogen metabolism substances in cucumber leaf and increased the content of soluble protein, serine, arginine, proline and total amino acids in cucumber leaf were than the control (5). The treatment of Alopecurus with water extracts from allelopathic wheat root tissues increases its content of soluble protein significantly (36). Our experiment results agree with previous results.

Enzyme activity

The NR activity in cucumber leaves showed decreasing trend during the whole sampling period. At 20, 30, 40 and 60 d, the intercropping system significantly increased the NR activity of cucumber leaves by 42.78%, 46.73%, 66.73% and 27.15%, respectively, than in monoculture system ($P < 0.05$), (Fig 2, a).

The GS activity in cucumber leaves also declined during the whole sampling period. At 20, 30, 50 and 60 d, the intercropping system significantly increased the GS activity of cucumber leaves, by 21.14%, 46.12%, 47.99% and 52.92%, respectively, compare to monoculture system ($P <0.05$) (Fig 2, b).

The lowest GDH activity in cucumber leaves was recorded at 20 d. At 30 and 40 d, the GDH activity of cucumber leaves increased but activity of intercropping was 14% i.e. higher than monoculture (12%) ($P <0.05$). However, at 50 and 60 d a gradual decline in activity was observed but it was still higher than that of 20 d (Fig 2, c).

The GOGAT activity in cucumber leaves also showed downward trend during the whole sampling period. At 20, 30 and 40 d, the GOGAT activity of cucumber leaves in intercropping was higher by 39.51%, 23.04% and 67.50%, respectively, than monoculture ($P<0.05$), (Fig 2, d).

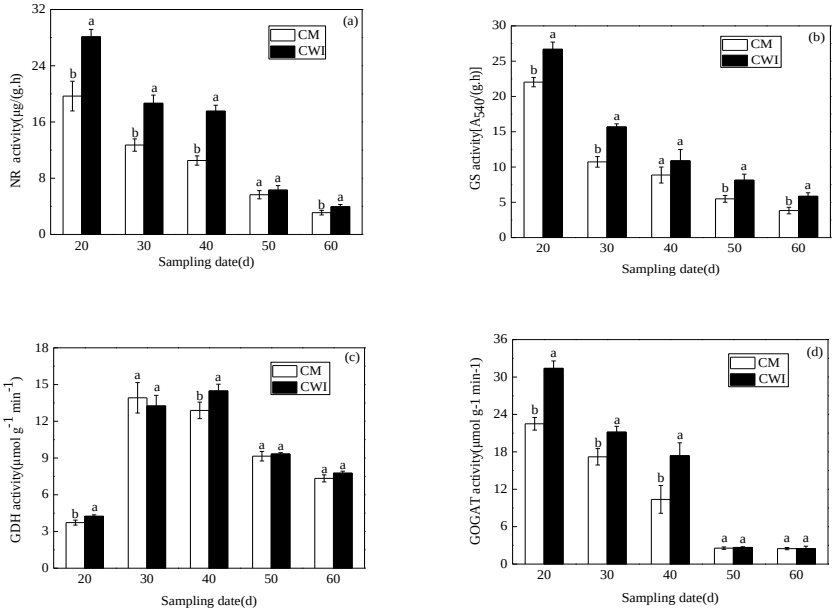

Figure 2. Effects of wheat intercropping on the activity of enzymes related to nitrogen metabolism in cucumber leaves.
Note: Cucumber monoculture (CM) and cucumber/wheat intercropping (CWI) were two treatments in the experiment. NR (a), GS (b), GDH (c) and GOGAT (d) were determined when the 4th cucumber leaf age was 20, 30, 40, 50 and 60 days, respectively. Different letters a, b indicate significant difference at the 0.05 level by Tukey's HSD test.

Some studies found that the senescence was delayed with higher NR, GS and GDH activity in the leaves of peanut (66) and muskmelon (68). Gao *et al*. (15) showed that enhanced activity of NR, GS and GOGAT delayed the cucumber leaf senescence; Chu *et al*. (9) believed that higher NR, GS and GDH activity in peanut leaf helped nitrogen assimilation and protein synthesis during the later developmental of peanut and thereby delayed the leaf senescence. The addition of decomposed peanut or corn straw increases the NR, GS and GOGAT activity in cucumber leaves and maintains higher level of nitrogen metabolism (14). This study showed that, on the whole, compared with the monoculture system, the intercropping system increased the activity of NR, GS and GOGAT in cucumber leaves, which indicated that intercropping promoted the nitrogen assimilation. So we can see that these enzymes in intercropping system mainly have strong activity in the prometa phase of leaf senescence, their activity becomes weaker in the later stages of senescence. Furthermore, the main form of ammonium assimilation is GS/GOGAT cycle in cucumber leaves. Because GS and GOGAT can be oxidized (3), so one possible reason for the increase of enzyme activity is intercropping with wheat enhanced the antioxidant capacity of cucumber (37), which delays the senescence of cucumber leaves.

Plant allelochemicals can alter many enzymes activity like NR, GS, GDH and indoleacetic acid synthase and influences the related metabolic pathways (56). The coptis root extracts increased nitrate reductase activity of faba bean and pea seedlings (27). In this study, intercropping with wheat, to some extent improved the activity of NR, GS, GDH and GOGAT in cucumber leaves, which may produce some kinds of substances in wheat root exudates, which effects the activity of related enzyme of nitrogen metabolism in cucumber leaves and cucumber leaves maintain high nitrogen metabolism level, thus delayed their senescence (14).

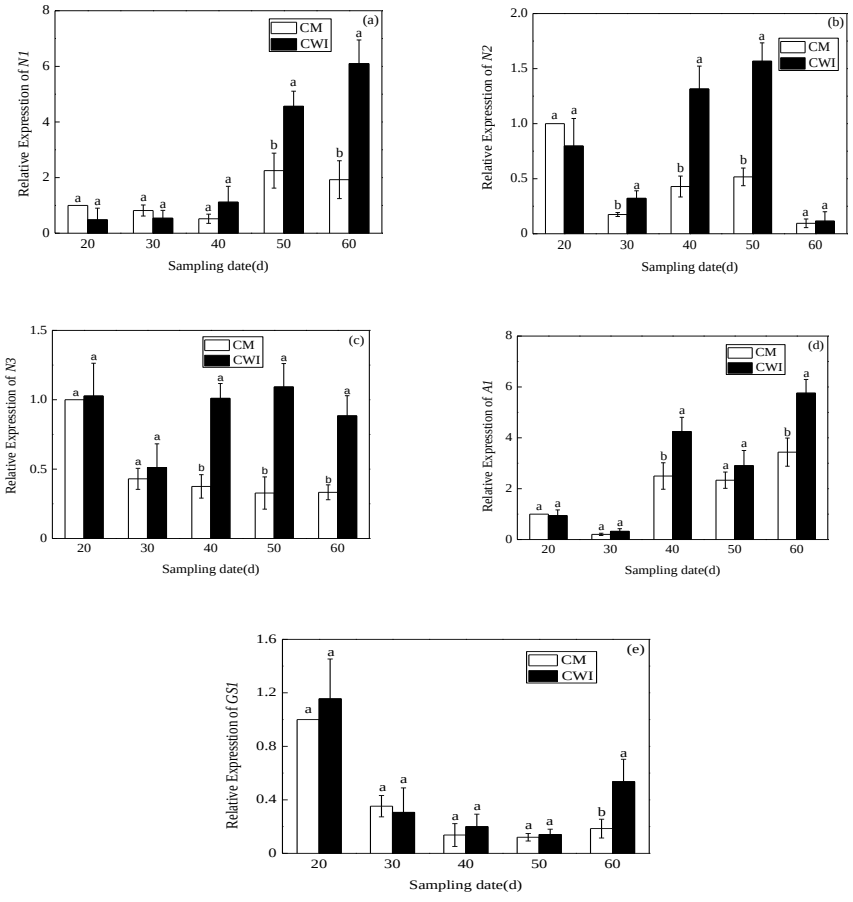

Figure 3. Effects of wheat intercropping on nitrogen metabolism related genes expression in cucumber leaves

Note: Cucumber monoculture (CM) and cucumber/wheat intercropping (CWI) were two treatments in the experiment. The relative expression of *N1* (a), *N2* (b), *N3* (c), *A1* (d) and *GS1* (e) were determined when the 4th cucumber leaf age was 20, 30, 40, 50 and 60 days, respectively. Different letters a, b indicate significant difference at the 0.05 level by Tukey's HSD test.

The root exudates of plants contains many compounds vanillin, pyruvic acid, cinnamic acid, ferulic acid, malic acid and p-hydroxybenzoic acid) with allelopathic potential (70). However, the question is which kind of compounds in wheat root exudates affects the nitrogen metabolism during the senescence of cucumber leaves still need further research, including dynamic content and types of allelopathic substances, to better explain the effects of wheat intercropping on nitrogen metabolism during the senescence of cucumber leaves.

Real-time quantitative PCR

Compared with monoculture system, the expression of *N1* in intercropping system was significantly up-regulated in 50 and 60 d ($P < 0.05$) (Fig 3, a). At 30, 40 and 50 d, the expression of *N2* in intercropping system was significantly up-regulated compared to monoculture system ($P < 0.05$) (Fig 3, b). At 40, 50 and 60 d, the expression of *N3* in intercropping system was significantly up-regulated ($P < 0.05$) (Fig 3, c).

Also, the expression of *A1* in intercropping system was significantly up-regulated in 40 and 60 d compared to monocluture system ($P < 0.05$), but there was no significant difference in other sampling dates (Fig 3, d). At 60 d, the expression of *GS1* in intercropping system was significantly up-regulated ($P < 0.05$), but, there was no significant difference in other sampling dates (Fig 3, e).

Senescence is an extremely complex process involving the expression of thousands of genes (41). NRT (nitrate transporter gene) family and AMT (ammonium transporter gene) family are the major gene families related to nitrogen absorption and translocation (18). Nitrate transporters are responsible for transferring exogenous NO^{3-} into cells, whereas ammonium transporters can make cells absorb NH^{4+} from the environment (17). He *et al*. (22) found that nitrogen transport genes are involved in the process of nitrogen metabolism and the coordination of nitrogen metabolism with other metabolism as a regulator. In this study, *N1*, *N2* and *N3* from NRT family, *A1* from AMT family and *GS1* (cytoplasmic glutamine synthase gene) were identified, respectively. We found that these genes were up-regulated to certain extent in intercropping system. However, gene specificity and limitations of primer design may limit detection of the expression of genes related to nitrogen metabolism, so further studies are needed.

The premature senescence under low nitrogen conditions can be alleviated by nitrogen supply (19,39). However, it is not known how the intercropping with wheat can supply nitrogen to the leaves. Perhaps the wheat root exudates (i). directly or indirectly changes the soil microbial community structure and diversity (51,58,71). (ii). improves the nutrient (especially nitrogen) use efficiency of crops, which may delay the leaf senescence (24) and thereby promotes the cucumber growth (47,50,52). All these changes may directly or indirectly affect the expression of related genes、the content of nitrogen compounds and the activity of enzymes of nitrogen metabolism in cucumber leaves, thereby regulating the activity of nitrogen metabolism in leaves and delaying leaf senescence. The mechanism remains to be further studied and the result is a preliminary exploration of effects on nitrogen metabolism of intercropping with wheat during senescence of cucumber leaves. In fact, nitrogen is the main control factor of senescence.

Although it is important for plant growth, there is no knowledge how N signals effect the network of leaf senescence in the cultivation model of intercropping system. Accordingly, we probably not just care about a simple metabolic chain, there is need for further research.

CONCLUSIONS

Intercropping with wheat increased the activity of nitrogen metabolism in cucumber leaves. Intercropping also increased the content of nitrogen compounds, the activities of enzymes related to nitrogen metabolism and the expression of nitrogen metabolism related genes in cucumber leaves. These delayed the senescence of cucumber leaves. Intercropping with wheat may increase the cucumber yields by improving the activity of nitrogen metabolism.

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