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Research article

Effects of nitrogen combined with zinc application on glutamate, glutamine, aspartate and asparagine accumulation in two winter wheat cultivars

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ABSTRACT

Zinc (Zn) deficiency remarkably depresses the protein concentration in the grain of winter wheat. Cultivar 'Pingan 8' showed lower Zn concentrations in the grain than did cultivar 'Yangao 006' after nitrogen (N) combined with Zn application. However, little is known about how amino acids are influenced by Zn combined with N application or about the differences in amino acid accumulation between the two winter wheat cultivars. A pot experiment was conducted to characterize amino acid accumulation in the low Zn-accumulating cultivar 'Pingan 8' and the high Zn-accumulating cultivar 'Yangao 006' at various growth stages (seedling, jointing, grain filling and maturity) as influenced by N and Zn supply. The N ($N_{0.2}$) combined with Zn (Zn_{10}) application significantly increased grain yields and the concentrations of N, Zn and crude protein in the grain of both wheat cultivars. N combined with Zn application significantly increased the concentrations of glutamate (Glu) and asparagine (Asn) but decreased the concentrations of glutamine (Gln) and aspartate (Asp) in cultivar 'Yangao 006'; the N combined with Zn application decreased the concentrations of Glu and Gln but increased the concentrations of Asp and Asn in cultivar 'Pingan 8' at the jointing, grain filling and mature stages. Correlation analysis results showed that there were significant relationships between grain yields, spike number, grain number and Zn, N, crude protein, Glu, Gln, Asp and Asn concentrations in the shoots and grain of winter wheat at different growth stages. These results demonstrate that N combined with Zn application enhanced protein synthesis by altering amino acid accumulation in both winter wheat cultivars. Cultivar 'Pingan 8' had lower Gln, Asp and Asn concentrations and higher Glu concentrations than did cultivar 'Yangao 006' after the $N_{0.05}$ treatment but had higher Glu, Gln, Asp, and Asn concentrations and lower Glu concentrations than did cultivar 'Yangao 006' after the $N_{0.2}$ treatment. These results revealed that the difference in amino acid concentrations between the two cultivars was related to the N application level.

1. Introduction

In many countries, wheat is the main component of the diet and the most important source of both calories and protein (Cakmak, 2008). In China, the Northern Winter Wheat Region contributes approximately 70% of the national wheat production (Zhuang, 2003). Wheat flour is widely used in the production of breads, pasta, noodles and cookies. Undoubtedly, proteins play an important role in wheat flour functionality (Veraverbeke and Delcour, 2002). Zinc (Zn) exerts a positive effect on protein formation and metabolism in plants, acting as a functional, structural or regulatory cofactor for a large number of enzymes. More than 70 metalloenzymes containing Zn have been identified, and these enzymes occur in all of six classes of enzymes, including oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (Alloway and Alloway, 2008). Zn is necessary for the activity of the enzyme RNA polymerase, which is involved in protein synthesis. In general, low activity of RNA polymerase is a typical feature of Zn-deficient plants. As a consequence, the causal effect of Zn deficiency is a sharp decrease in the amount of protein (Brown et al., 1993). Moreover, the most fundamental effect of Zn on protein metabolism occurs through its involvement in the stability and function of the genetic material. However, the specific mechanisms by which protein levels are decreased by Zn deficiency are not clear.

Protein synthesis has a close relationship with nitrogen (N) metabolism in plants. After uptake by the roots, nitrate is reduced to nitrite by nitrate reductase (NR) using NADH as a reductant. Nitrite reductase

Abbreviations: asparagine, Asn; asparagine synthetase, AS; aspartate, Asp; aspartate aminotransferase, AST; glutamate, Glu; glutamate synthase, GOGAT; glutamine, Gln; glutamine synthetase, GS; nitrate reductase, NR; nitrite reductase, NiR; glutamate dehydrogenase, GDH

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(NiR) catalyzes the ferredoxin-dependent, six-electron reduction of nitrite to ammonia (Lea et al., 1999). The resulting ammonia is converted into the amide nitrogen of glutamine (Gln) via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle, which is a recognized route of N assimilation in higher plants (Lea and Miflin, 2003). First, the reaction catalyzed by GS involves the ATP-dependent amination of glutamate (Glu) to yield Gln; then, the transfer of the amide group from Gln to α-ketoglutarate is catalyzed by GOGAT to yield two molecules of Glu. One molecule of Glu can be used to replenish the pool of Glu for subsequent GS catalysis, and the other molecule of Glu donates its amino group to form other nitrogen-containing compounds (Temple et al., 1998). For example, aspartate (Asp) is produced from Glu in a reaction catalyzed by aspartate aminotransferase (AST). Asparagine synthetase (AS) requires the energy-producing hydrolysis of ATP to catalyze asparagine (Asn) synthesis via the amidation of Asp. Glu and Asp can be used to form various amino acids that are the substrates for protein synthesis (Vitale et al., 1993). Therefore, the accumulation of Glu, Gln, Asp and Asn directly influences protein synthesis in plants. In addition, amino acids play important roles in the synthesis of endogenous hormones, nutrient uptake, resistance to stress and disease resistance in plants. Moreira and Moraes (2017) showed that the foliar application of low molecular weight amino acids increased nutrient uptake and consequently increased productivity in common bean. Moreira et al. (2014) reported that applying amino acids increased shoot dry weight yields and chlorophyll concentrations but decreased phosphorus, potassium, magnesium and boron concentrations in alfalfa in the presence of a Zn supply.

There are few studies of how Zn application affects N metabolism. Harper and Paulsen (1969) showed that the effect of Zn application on N metabolism did not result in a higher concentration of protein in wheat seedlings because of the activities of glutamate dehydrogenase (GDH) and GS, and protein content was not affected by Zn application, although nitrate accumulated in Zn-efficient plants. Nevertheless, Zn application increased the activity of NR and GS in wheat, rice and millet (Liu et al., 2015; Seethambaram and Das, 1986) and enhanced NH_4^+ assimilation, thus increasing amino acid and protein concentrations in millet (Seethambaram and Das, 1986). Kitagishi and Obata (1986) also reported that Zn deficiency resulted in disorders of N metabolism, severely depressed protein production, and induced the accumulation of free amino acids and amides in meristematic tissues of rice. Therefore, whether the enhanced protein synthesis observed after Zn application is related to the amino acid accumulation involved in N metabolism in plants requires further study.

As a component of proteins, N plays an important role in amino acid accumulation in plants. Eppendorfer (1968) reported that changes in the concentrations of protein amino acids were closely correlated with corresponding changes in the total N content in barley. Mulder and Bakema (1956) also suggested that N nutrition had a pronounced effect on the protein content and amino-acid composition of proteins in potato. Zhang et al., (2017a) conducted a field experiment and demonstrated that increased N application significantly increased the protein and total, essential and nonessential amino acid contents in the grain of winter wheat. However, little is known about how amino acid accumulation is influenced by N combined with Zn application in plants.

Our previous studies reported that Zn application increased protein levels in the grain and altered the flour protein composition in winter wheat (Liu et al., 2015). Two cultivars (cultivar 'Pingan 8', with low grain Zn concentrations, and cultivar 'Yangao 006', with high grain Zn concentrations) were screened from 38 winter wheat varieties growing in the area surrounding the Huang-Huai-Hai Plain. Cultivar 'Yangao 006' showed higher yields, Zn concentrations and Zn accumulation in the grain than did cultivar 'Pingan 8' (data unpublished). The aim of this study was to i) re-examine the effects of N combined with Zn application on protein concentrations; ii) investigate the accumulation of Glu, Gln, Asp and Asn in response to N combined with Zn application; and iii) investigate the difference in amino acid accumulation between two winter wheat cultivars that differ in their capacity to accumulate Zn in the grain. These results will improve our understanding of the interaction of N and Zn in plants and our ability to more effectively regulate the nutritional quality of winter wheat grain via the application of N and Zn fertilizers in agricultural practice.

2. Materials and methods

2.1. Plant growth description

Two cultivars of winter wheat (*Triticum aestivum*), the low Zn-accumulating cultivar 'Pingan 8' and the high Zn-accumulating cultivar 'Yangao 006', were grown in ceramic pots filled with 8 kg of Zn-deficient calcareous soil. The physical and chemical properties of the soil were as follows: pH 7.3 (soil:water ratio of 1:2.5), organic matter ($C_{org.}$) 6.96 g kg^{-1}, alkaline hydrolysis N 78 mg kg^{-1}, Olsen P 11 mg kg^{-1}, available K 164 mg kg^{-1}, and DTPA-extractable Zn 1.0 mg kg^{-1}.

Two rates of Zn, 0 and 10 mg kg^{-1} soil, were applied by using $ZnSO_4{\cdot}7H_2O$, according to Liu et al. (2015). Two N levels, 0.05 (low) and 0.2 (optimal) g kg^{-1} soil, were applied by using urea. The Zn and N treatments were applied in a fully factorial arrangement: $N_{0.05}Zn_0$, $N_{0.2}Zn_0$, $N_{0.05}Zn_{10}$ and $N_{0.2}Zn_{10}$. To meet the P and K requirements, KH_2PO_4 and KCl were applied in each pot at rates of 39.3 mg P kg^{-1} soil and 62.2 mg K kg^{-1} soil, respectively. All fertilizers were mixed throughout the soil profile before sowing, except the N fertilizer (0.2 g N kg^{-1} soil), which was split into three applications. Half of the N fertilizer (0.1 g) was supplied before sowing, and the remaining N fertilizer was split into two equal applications in the soil at the jointing and booting stages. Each treatment was replicated three times. All chemical regents used were analytical grade, and deionized water was used during the experimental period.

The seeds were disinfected in a solution of 0.5% v/v NaClO, and fifteen seeds were then sown in each pot; 10 seedlings were retained in each pot after thinning. The leaves of both winter wheat cultivars at the seedling (Zadoks stage: 19), jointing (Zadoks stage: 36) and grain filling stages (Zadoks stage: 77) and the grain of both cultivars at maturity (Zadoks stage: 99) were sampled for chemical analyses. The samples were washed with running water and then deionized water at least three times. The leaves and grains of seven plants were oven-dried at 65 °C and analyzed for elemental concentrations. The other three plants were immediately frozen in liquid nitrogen and stored at −20 °C for further biochemical analyses.

2.2. Glu, Gln, Asp and Asn analyses

According to the method of Kim et al. (2007), Glu, Gln, Asp and Asn in the leaves and grain were measured by enzyme-linked immunosorbent assays (ELISA). One gram of fresh leaf or grain sample was homogenized in 4 mL of ice-cold 0.01 mol L^{-1} phosphate-buffered solution (PBS, pH 7.2–7.4, containing 2.0 mmol L^{-1} KH_2PO_4, 4.0 mmol L^{-1} $Na_2HPO_4{\cdot}12H_2O$, 137 mmol L^{-1} NaCl and 2.7 mol L^{-1} KCl). The homogenate was centrifuged at 1500 × *g* for 15 min. The Glu, Gln, Asp and Asn concentrations of the supernatant were then determined with plant Glu, Gln, Asp and Asn ELISA kits, respectively, according to the manufacturer's instructions. Briefly, 96-well microplates were coated with standard diluent overnight at 4 °C and then washed and blocked with assay diluent (Glu, Gln, Asp and Asn, Pharmingen) for 1 h at 37 °C. Supernatants (containing Glu, Gln, Asp and Asn) were then added to the plates to form antibody-antigen-antibody complexes, and working detector (avidin-HRP) was added to the wells and incubated for 30 h at 37 °C. The plates were washed, and the tetramethylbenzidine (TMB) substrate reagent was added to the wells for 15 min at 37 °C in the dark. At the end of the incubation, stop solution (1 M H_3PO_4) was added, and the absorbance was read at 450 nm using a microplate spectrophotometer 352 (Labsystems Multiskan MS, Finland). The measurements were carried out at Enzyme-linked