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# Nitrogen fertilizer affects starch synthesis to define non-waxy and waxy proso millet quality

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ABSTRACT

Understanding the effect of nitrogen fertilization on the quality of proso millet is key to expanding the use of this crop to address water scarcity and food security. Therefore, this study determined the impact of nitrogen fertilization on the proso millet quality. Nitrogen fertilization significantly increased the NR and GS activities and decreased the GBSSase activity, resulting in an increase in protein content and reduction in amylose content and L*, which decreased the appearance quality. Nitrogen fertilization increased the proportion of short amylopectin chains, resulting in a more disordered carbohydrate structure, and decreased the proportion of hydrophilic functional groups, contributing to an increase in setback viscosity and decrease in pasting temperature in the waxy (w139) variety. In contrast, the non-waxy (n297) variety exhibited a larger proportion of long amylopectin chains, lower ordered structure and hydrophobic functional groups after nitrogen fertilization, which strengthened the inter- and intramolecular forces of starch colloids.

## 1. Introduction

Proso millet (*Panicum miliaceum L.*) is one of the oldest ancient grains. According to archaeological findings, China began to domesticate it about 10,000 years ago (Li et al., 2020). Proso millet (PM) possesses short growing season, high photosynthetic and water use efficiency. Studies have proved that PM can remediate saline soil and can be used as a cleaner production crop (Yuan et al., 2022). PM grain also is an excellent source of starch, protein, vitamins and minerals, with nutritional value equal to or better than common cereals. PM has about the same protein content (13 %) as wheat without being gluten-free, giving it the added advantage (Gulati et al., 2017). Therefore, PM has the potential to contribute to the sustainable diversification of agricultural production and address global issues such as increased food demand, water scarcity, and malnutrition.

At present, PM is the staple food in arid and semi-arid regions of China, and its quality traits are particularly important (Gong et al., 2021). PM quality is mainly related to appearance, nutritional, and cooking quality. Yellow pigmentation and grain size are important aspects of appearance quality. Protein and amino acid contents are indicators of nutritional quality. Cooking quality is mainly affected by viscosity properties, including breakdown viscosity (BV), setback viscosity (SV), and pasting temperature (PT). High BV and low SV result in relatively soft cooked grains, which may improve taste (Chen et al., 2021). PM can be categorized as waxy (low amylose content) or non-waxy (high amylose content), with significant differences in cooking quality. Starch, the primary factor influencing cooking and eating quality, composes of amylose and amylopectin (Wang, Yang, Ferdinand, et al., 2020; Yang et al., 2018). The accumulation of amylose and amylopectin strongly influences the structure and physicochemical properties of starch granules (Tang et al., 2019).

The internal structure and physicochemical properties of starch depend not only on genetics, but also on soil conditions, climate, and agricultural practices during crop growth (Yao et al., 2020). Nitrogen fertilization is an important agricultural practice that effectively improves crop yield and quality, especially during grain-filling (Gao et al., 2022). The effect of nitrogen fertilizer on grain quality is mainly due to the effects of carbohydrate biosynthetic enzyme activity (Gao et al., 2021). Nitrate reductase (NR) and glutamine synthase (GS) are key enzymes in nitrogen metabolism (Liang et al., 2011). Liang et al. (2011) reported that NR and GS promoted nitrogen metabolism and produced glutamate and aspartate, among other precursors to the synthesis of

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essential amino acids. Adenosine diphosphate (ADP)-glucose pyrophosphorylase (ADPGase) and granule-bound starch synthase (GBSSase) are primarily responsible for amylose biosynthesis. Amylopectin biosynthesis is driven by a series of coordinated enzymatic reactions, including ADPGase, soluble starch synthase (SSSase), starch-branching enzymes (SBE) and starch debranching enzymes (Bao et al., 2020). Gao et al. (2021), in a study on tartary buckwheat starch, found that four main enzymes involved in starch synthesis first increased and then decreased with increasing nitrogen fertilization. Zhou et al. (2020) found that moderate nitrogen application to rice crops resulted in a higher proportion of short amylopectin (DP 6–12), lower proportion of long amylopectin (DP $\geq$ 37), increased starch breakdown value, and decreased SV and PT. In general, nitrogen fertilization affects enzyme activity and substance accumulation, which further affect crop quality.

While previous studies examining the influence of nitrogen fertilizer on crop starch properties mainly focused on rice and wheat (Cao et al., 2018; Labuschagne et al., 2006), there are few such studies on PM. Furthermore, although our previous study showed that moderate nitrogen treatment can increase PM yield, whether it can also improve appearance, nutritional and cooking quality remains unknown (Gong et al., 2021). Therefore, this study aimed to determine the effect of nitrogen fertilization on the appearance, nutritional, and cooking quality of PM by comparing waxy and non-waxy varieties using four nitrogen fertilizer levels and examined the physiological mechanisms underpinning this effect by analyzing the activity of amylose, amylopectin biosynthesis, nitrogen metabolic enzymes, and the physicochemical properties of flours.

## 2. Materials and methods

### 2.1. Plant materials and experimental design

The proso millets varieties were provided by Northwest Agriculture and Forestry University. According to the differences in amylose content, the w139 variety (the lowest amylose content in waxy proso millets) and n297 variety (the highest amylose content in non-waxy proso millets) were selected for the study from 301 proso millets. Field experiment was conducted at the experimental site of the Northwest A & F University (109.7 E, 38.3 N, 1080 m altitude), Yulin, Shaanxi Province, China in 2021 using a split plot design. The main plots corresponded to four nitrogen fertilization rates: 0 (N0), 90 (N1), 180 (N2), and 270 (N3) kg/hm$^2$. Nitrogen was applied once as urea (nitrogen content >46 %) in a base fertilizer, and no topdressing was applied during proso millet growth. Subplots were assigned to two PM varieties: waxy (w139) and non-waxy (n297). All plots were treated with phosphorus (100 kg/hm$^2$) and potassium (75 kg/hm$^2$). The soil comprised sandy loam with a pH value of 8.69 and contained 3.01 g/kg of organic matter, 0.29 mg/g of total nitrogen, 21.29 mg/kg of available phosphate, and 116.75 mg/kg of available potassium. Sowing was performed on June 14, and harvesting was commenced on October 1. The experimental crops were managed in accordance with the technical regulations for local cultivation.

### 2.2. Sample preparation

In the PM flowering stage, spikes that blossomed on the same day and grew at the same rate were marked. Samples of the middle and top grains from 15 panicles were collected every 5 d up to 35 d after flowering, and were separated into two groups. The first group of grains was frozen using liquid nitrogen, stored at −80 °C, and used to measure the activity of key enzymes involved in nitrogen metabolism and starch synthesis. The second group of grains was greened at 105 °C for 30 min, then dried at 75 °C to constant weight, shelled, and ground to measure protein, amylose, and total starch content. The harvested grains had a moisture content of 12–14 % and were hulled with an experimental husker (SY88-TH, South Korea) to assess their appearance. Hulled grains were subsequently ground into flour, which was passed through a 100-mesh sieve to determine nutritional quality, viscosity, and structural properties.

### 2.3. Amylose, protein, and total starch content during grain-filling stage

10 mg of starch was weighed into a 15 mL centrifuge tube, then 100 μL of alcohol and 900 μL of sodium hydroxide were added. The mixture was subsequently vortexed and bathing in boiling water for 10 min. After the mixture had cooled, 0.1 mL of acetic acid and 0.2 mL of potassium iodide solution was added to 0.5 mL of the test supernatant, the volume of which was increased to 10 mL with distilled water. The amylose content of this mixture was determined by measuring its absorbance at 620 nm after pacing at room temperature (25 °C) for 10 min (Wang, Yang, Gao, et al., 2020). The protein content of the ground grain samples was measured via the Kjeldahl method, using a protein-nitrogen coefficient of 6.25 (Wang, Li, et al., 2021). The total starch content was measured using a starch content kit (No. ADS-W-DF001, FANKEL Industrial Co., Ltd., Shanghai, China) according to the manufacturer's instructions.

### 2.4. Activity of key starch synthesis and nitrogen metabolism enzymes

Fresh plant tissues were ground thoroughly in liquid nitrogen, the extract (9 times of the sample volume) was added, then centrifuged at 4 °C, 8000 rpm for 30 min, and the supernatant was stored in a 4 °C refrigerator for future analysis. The supernatant was used to measure the activities of key enzymes involved in starch synthesis and nitrogen metabolism. The activity of NR, GS, ADPGase, GBSS, SSSase, and SBE was measured using an enzyme-linked immunosorbent assay kit (Shanghai FANKEL Industrial Co., Ltd., No. F4992-A, F5047-A, F7904-A, F50055-A, F7906-A and F7907-A) according to the manufacturer's instructions. The detailed method is shown in the supplementary material.

### 2.5. Appearance quality of the grain

The color of the hulled grains was assessed using a chroma meter (Colorimeter Ci7600, Aisaili Color Technology Inc., Shanghai, China). The color was characterized using variables L*, a*, and b*, where L* represented a range from black (0) to white (100), a* represented a spectrum from red (+) to green (−), and b* represented a range from yellow (+) to blue (−) (Yang et al., 2018).

The hulled seeds were counted to 1000 seeds using multifunctional automatic seed counting instrument (DC-3, Yunhe Ltd., Jiangsu, China) and then weighed using an analytical balance. The length, width, and degree of circularity of the hulled grains were determined using an automatic seed-counting and -analyzing instrument (Model SC-G, Wanshen Ltd., Hangzhou, China).

### 2.6. Nutritional quality of the flour

The amylose content of the proso millet flour was determined by according to the method in Subsection 2.3. The protein content of the flour was determined via the Kjeldhal method, using a protein-nitrogen coefficient of 6.25. Albumin, globulin, prolamin and glutelin were extracted from the flour as described by Mao et al. (2014) and measured using the Kjeldahl method (Wang, Wang, et al., 2021). The detailed extraction methods were as follows:

1 g of flour was weighed in a 50 mL centrifuge tube and repeatedly extracted with 20 mL of deionized water. After 30 min of extraction at 50 °C and three rounds of subsequent centrifugation at 4000 r/min for 10 min, the liquid supernatant was used as the albumin extraction solution. After extracting the albumin, the precipitate was repeatedly extracted using 20 mL of 0.5 M sodium chloride solution. After 30 min of extraction at 50 °C and three rounds of subsequent centrifugation at 4000 r/min for 10 min, the supernatant was used as the globulin

extraction solution. After the extraction of globulin, the precipitate was repeatedly extracted with 20 mL of 75 % ethanol. After 30 min of extraction at 50 °C and three subsequent rounds of centrifugation at 4000 r/min for 10 min, the supernatant was used as the prolamin extraction solution. After extraction of glutelin, the precipitate was repeatedly extracted using 20 mL of 0.1 M sodium hydroxide solution. After 30 min of extraction at 50 °C and three rounds of subsequent centrifugation at 4000 r/min for 10 min, the supernatant was used as the glutelin extraction solution.

### 2.7. Viscosity properties of flour

A Rapid Visco Analyzer (RVA 4500, Perten, Sweden) was used to measure the pasting properties of the PM flour. Sample (3 g, 14 % moisture basis) was mixed with 25 mL of deionized water in a RVA sample can. The suspensions were left to rest at 50 °C for 1 min, heated from 50 to 95 °C at 12 °C/min, and maintained at 95 °C for 2 min. The suspension was subsequently cooled to 50 °C at 12 °C/min and left to rest for 1 min. Peak viscosity (PV), trough viscosity (TV), BV (PV − TV), final viscosity (FV), SV (FV − TV), and PT were then recorded (Zhang et al., 2022).

### 2.8. Structural properties

The starch isolation method was presented in the supplementary material. The chain length distribution (CLD) of amylopectin was analyzed using high-performance anion-exchange chromatography with the technical support provided by Sanshu Biotech. Co., Ltd. (Shanghai, China). The chromatographic conditions were shown as follows: flow rate, 0.4 mL/min; injection volume, 5 μL; solvent system, 0.2 M NaOH: (0.2 M NaOH, 0.2 M NaAc); gradient program, 90:10 V/V at 0 min, 90:10 V/V at 10 min, 40:60 V/V at 30 min, 40:60 V/V at 50 min, 90:10 V/V at 50.1 min, and 90:10 V/V at 60 min.

An X-ray diffractometer (D/Max2550VB+/PC, Rigaku Corporation, Tokyo, Japan) was used to determine the type of crystal and crystallinity of the PM flour. The samples were irradiated with an X-ray beam at 100 mA and 40 kV with a scan range of 5–50° 2θ and step size of 0.02° (Govindaraju et al., 2022).

The ordered structure of flour was determined using a Fourier transform infrared spectrometer (Nicolet iS50, Thermo Fisher Scientific, Massachusetts, USA). The flour was mixed with potassium bromide at a ratio of 1:100 and mounted on a pellet holder. The flour has a spectral range of 400–4000 $cm^{-1}$ with a resolution of 4 $cm^{-1}$, and a total of 32 spectral averages were obtained. The degree of order was determined using the ratio of 1045/1022 $cm^{-1}$, and amorphous and ordered carbohydrate structures were quantified using the ratio of 1022/995 $cm^{-1}$. The functional groups of the samples were identified and quantified based on the specific peaks displayed by the flour in the different wavelength bands (Chen et al., 2021).

### 2.9. Statistical analysis

The data in all the tables were average values of three repeated observations. Multiple comparisons were made using a one-way analysis of variance (ANOVA) and Duncan's test using SPSS 23.0. Statistical significance was set to $P \leq 0.05$. A correlation analysis using Spearman's correlation coefficient and correlation heatmap was generated using TBtools. Origin 2022 and Adobe Illustrator 2021 were used to plot the results of the analyses.

## 3. Results

### 3.1. Grain quality

The appearance, nutritional value, and cooking quality of PM are shown in Table 1 and Figs. 1 and 2. Good appearance quality was linked to higher grain mass and L* values and a positive b* value. As shown in Fig. 1, L*, a*, and b* values significantly decreased after the application of nitrogen fertilizer. Notably, b* increased in the following order: N0 > N2 > N3 > N1. Compared with N0 level, the length increased and circular degree decreased under nitrogen fertilizer level. For 1000 grain weight, N3 level was higher in w139 variety and N2 level was higher in n297 variety. The nutritional quality was related to proper protein content and amino acid balance. Amylose content was in the range of 1.45–2.55 % and 20.95–21.86 % in the w139 and n297 variety, respectively, and significantly decreased under the application of nitrogen fertilization. The protein content firstly decreased and then increased, while the protein components had higher value under N3 level. Good cooking quality was related to high BV, low SV, and low PT. For BV, the shear resistance of w139 variety was improved, while that of n297 variety was deteriorated by nitrogen fertilizer. While both varieties exhibited increasing SV with increasing nitrogen fertilization, the SV and PT of the w139 variety were lower than those of the n297 variety, indicating convenient storage.

**Table 1**
Amylose, protein, albumin, globulin, prolamin, glutelin content of flour and length, width, circular degree, 1000 grain weight of grain in w139 and n297 varieties under nitrogen fertilizer.[a]

| Treatment | Amylose (%) | Protein (%) | Albumin (%) | Globulin (%) | Prolamin (%) | Glutelin (%) | Length (mm) | Width (mm) | Circular degree | 1000 grain weight (g) |
|---|---|---|---|---|---|---|---|---|---|---|
| w139N0 | 1.99 ± 0.07e | 10.40 ± 0.13c | 1.03 ± 0.09cde | 0.48 ± 0.07c | 1.06 ± 0.02a | 1.88 ± 0.10 cd | 3.05 ± 0.01b | 2.75 ± 0.01 cd | 0.92 ± 0.02a | 6.37 ± 0.01e |
| w139N1 | 1.45 ± 0.18f | 9.88 ± 0.09d | 1.06 ± 0.03bcd | 0.79 ± 0.03ab | 0.89 ± 0.03b | 1.97 ± 0.08bc | 3.02 ± 0.02b | 2.73 ± 0.02 cd | 0.93 ± 0.02a | 6.33 ± 0.02e |
| w139N2 | 2.55 ± 0.23d | 11.03 ± 0.04b | 1.14 ± 0.06b | 0.81 ± 0.04ab | 0.81 ± 0.09bc | 2.00 ± 0.05b | 3.07 ± 0.04ab | 2.73 ± 0.02 cd | 0.91 ± 0.01ab | 6.39 ± 0.09e |
| w139N3 | 1.85 ± 0.09e | 11.50 ± 0.07a | 1.15 ± 0.06b | 0.82 ± 0.08a | 1.10 ± 0.06a | 2.13 ± 0.08a | 3.10 ± 0.01a | 2.71 ± 0.05d | 0.89 ± 0.01b | 6.51 ± 0.02d |
| Mean | 1.96 | 10.71 | 1.09 | 0.73 | 0.97 | 2.00 | 3.06 | 2.73 | 0.91 | 6.40 |
| n297N0 | 21.86 ± 0.09a | 7.93 ± 0.08f | 0.96 ± 0.02e | 0.72 ± 0.05b | 0.78 ± 0.03c | 1.45 ± 0.01e | 3.04 ± 0.03b | 2.78 ± 0.02abc | 0.93 ± 0.01a | 6.71 ± 0.02c |
| n297N1 | 20.95 ± 0.40c | 7.53 ± 0.06 g | 1.10 ± 0.06bc | 0.73 ± 0.04ab | 0.83 ± 0.06bc | 1.44 ± 0.03e | 3.06 ± 0.02ab | 2.80 ± 0.01ab | 0.93 ± 0.01a | 6.88 ± 0.04b |
| n297N2 | 21.52 ± 0.09ab | 8.19 ± 0.08e | 0.97 ± 0.04de | 0.56 ± 0.05c | 0.73 ± 0.10 cd | 1.35 ± 0.02e | 3.11 ± 0.03a | 2.83 ± 0.01a | 0.93 ± 0.01a | 7.06 ± 0.05a |
| n297N3 | 21.26 ± 0.18bc | 9.90 ± 0.19d | 1.25 ± 0.02a | 0.77 ± 0.02ab | 0.66 ± 0.03d | 1.82 ± 0.03d | 3.05 ± 0.03b | 2.77 ± 0.04bc | 0.93 ± 0.02a | 6.80 ± 0.04b |
| Mean | 21.40 | 8.39 | 1.07 | 0.69 | 0.75 | 1.52 | 3.06 | 2.79 | 0.93 | 6.86 |

[a] Datas are means ± standard deviation, n = 3. Values in the same column with different letters are significantly different ($P < 0.05$).

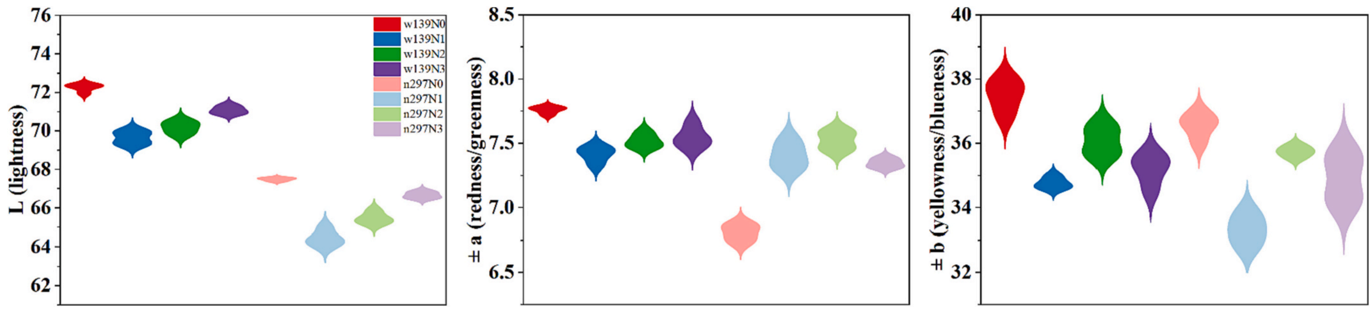

**Fig. 1.** L value, a value and b value of proso millet varieties under nitrogen treatment.

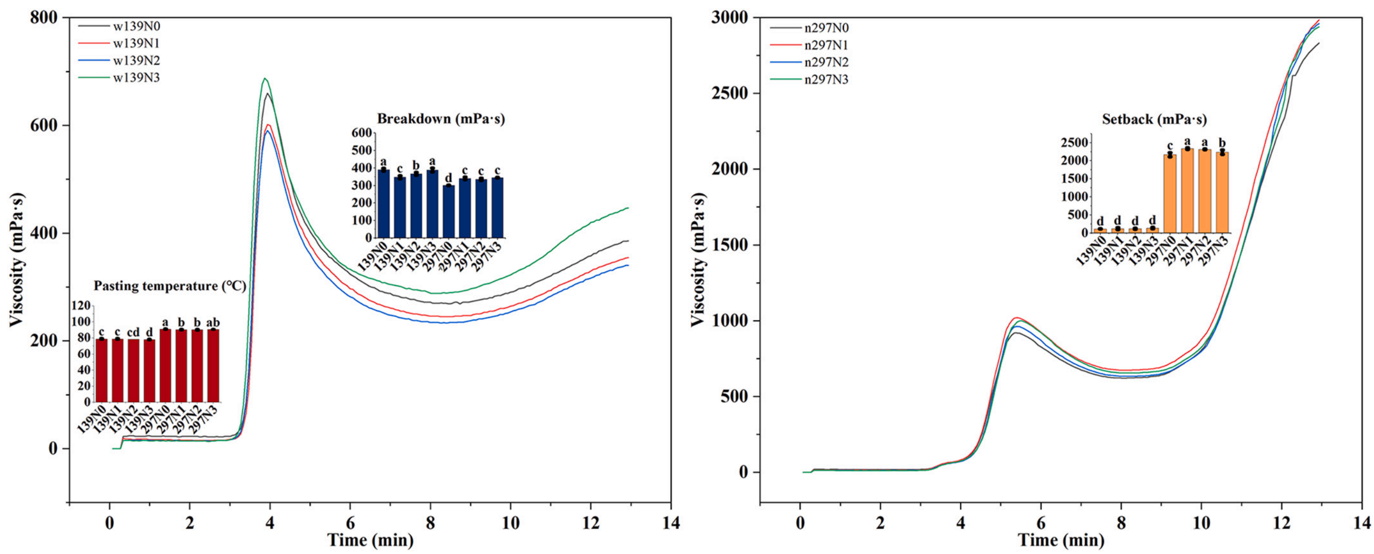

**Fig. 2.** Pasting temperature, breakdown viscosity and setback viscosity of proso millet varieties under nitrogen treatment.

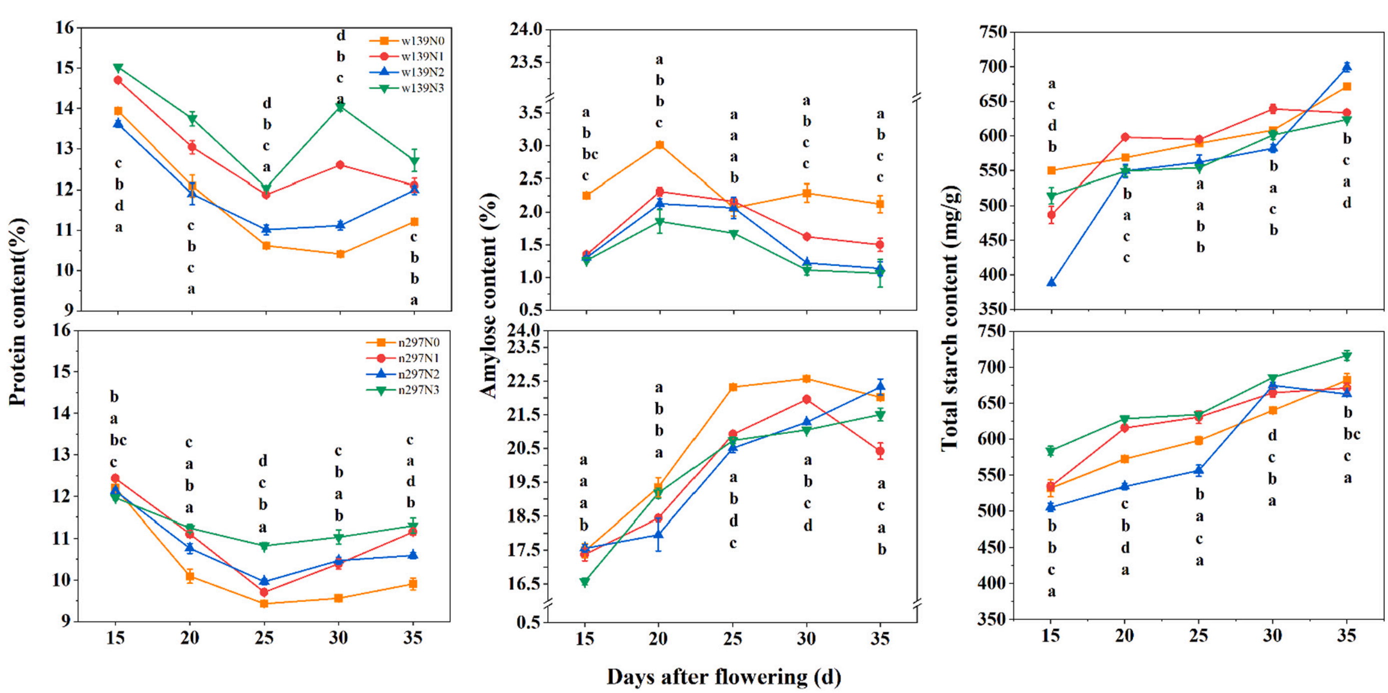

**Fig. 3.** Protein, amylose and starch content of proso millet varieties under nitrogen treatments at 15, 20, 25, 30 and 35 days after flowering. Different lowercase letters denote statistical differences between treatment at the 0.05 level in a column for (top to bottom) N0, N1, N2 and N3.

### 3.2. Accumulation of protein, amylose, total starch and their relationship with grain quality

Fig. 3 shows the accumulation of protein, amylose and total starch at different nitrogen levels. Overall, the protein content of both varieties increased with increasing nitrogen fertilization; it first decreased, with the lowest value recorded after 25 d, and then increased over time. The amylose content of the w139 variety first increased, peaking after 20 d, and then decreased over time, whereas the n297 variety increased consistently after 15 d – 35 d. Lastly, total starch content increased over time with extension time.

The relationship between PM quality and the accumulation of protein, amylose, and total starch content are illustrated in Fig. 4. Protein was negatively correlated with amylose content 15 d – 35 d after flowering ($P < 0.05$). As for the nutritional quality, Prolamin was negatively correlated ($P < 0.05$) with amylose content (15 d - 35 d). Glutelin had a negative correlation with the amylose and total starch content (15 d - 35 d). Glutelin was also positively correlated ($P < 0.05$) to BV and positively correlated ($P < 0.01$) to SV and PT. In terms of appearance, L* exhibited a positive correlation with protein content ($P < 0.05$). In terms of cooking quality, PT had a positive correlation ($P < 0.05$) with amylose content. In contrast, BV positively correlated ($P < 0.05$) to protein content (prolamin and glutelin) and L*. Lastly, SV exhibited positive correlation ($P < 0.01$) with amylose content and 1000 grain weight. These findings indicate that the accumulation of protein, total starch, and amylose after 15 d – 35 d of flowering significantly affected the appearance, nutritional value, and cooking quality of PM.

### 3.3. Activities of some key enzymes and their effects on grain quality

The activity of key enzymes involved in starch synthesis and nitrogen metabolism and their influence on grain quality are shown in Fig. 5 and Supplementary Fig. 1, respectively. The ADPGase activity of the w139 variety exhibited a unimodal distribution and peaked at 25 d, while the n297 variety showed a bimodal pattern and peaked at 20 and 30 d (Fig. 5). The GBSSase activity of the two varieties significantly decreased under nitrogen fertilization and peaked at 25 d (Fig. 5). This result suggest that nitrogen fertilization suppresses amylose synthesis, resulting in an increase in SV and decrease in PT. Nitrogen fertilizer application to the w139 variety increased SSSase activity and tended to produce more short-branched chains of amylopectin up to 20 d, which led to higher SV (Supplementary Fig. 1). Nitrogen fertilizer application of n297 variety maintained higher SBE activity to produce more long-branched chain of amylopectin during 10 d - 25 d and increased SSSase to accumulate short-branched chain of amylopectin after 20 d, which led to the increase in BV and SV of n297 variety after nitrogen fertilization (Supplementary Fig. 1). Compared with the w139 variety, the n297 variety exhibited higher NR and GS activity after 10 d – 35 d of flowering. Up to 20 d, NR activity of the w139 variety inhibited prolamin synthesis and promoted the synthesis of other proteins (albumin, prolamin, and glutelin), while the opposite trend was observed in the n297 variety (Supplementary Fig. 1). With increasing nitrogen level, two variety had trend to high NR activity with the increase of protein content, resulting in a decrease in PT. After 10 d, the accumulation of GS in w139 variety began to promote the synthesis of prolamin and glutenin, while the opposite was observed in the n297 variety, such that the prolamin and glutenin contents of the n297 variety was significantly lower than those of the w139 variety, resulting in higher PT (Fig. 4). The activity of NR and ADPGase reduced the 1000 grain weight of the w139 variety after 30 d–35 d and 15 d – 25 d of flowering, respectively, while increasing the 1000 grain weight of the n297 variety after 10 d – 25 d and 10 d – 30 d of flowering (Supplementary Fig. 1), respectively, such

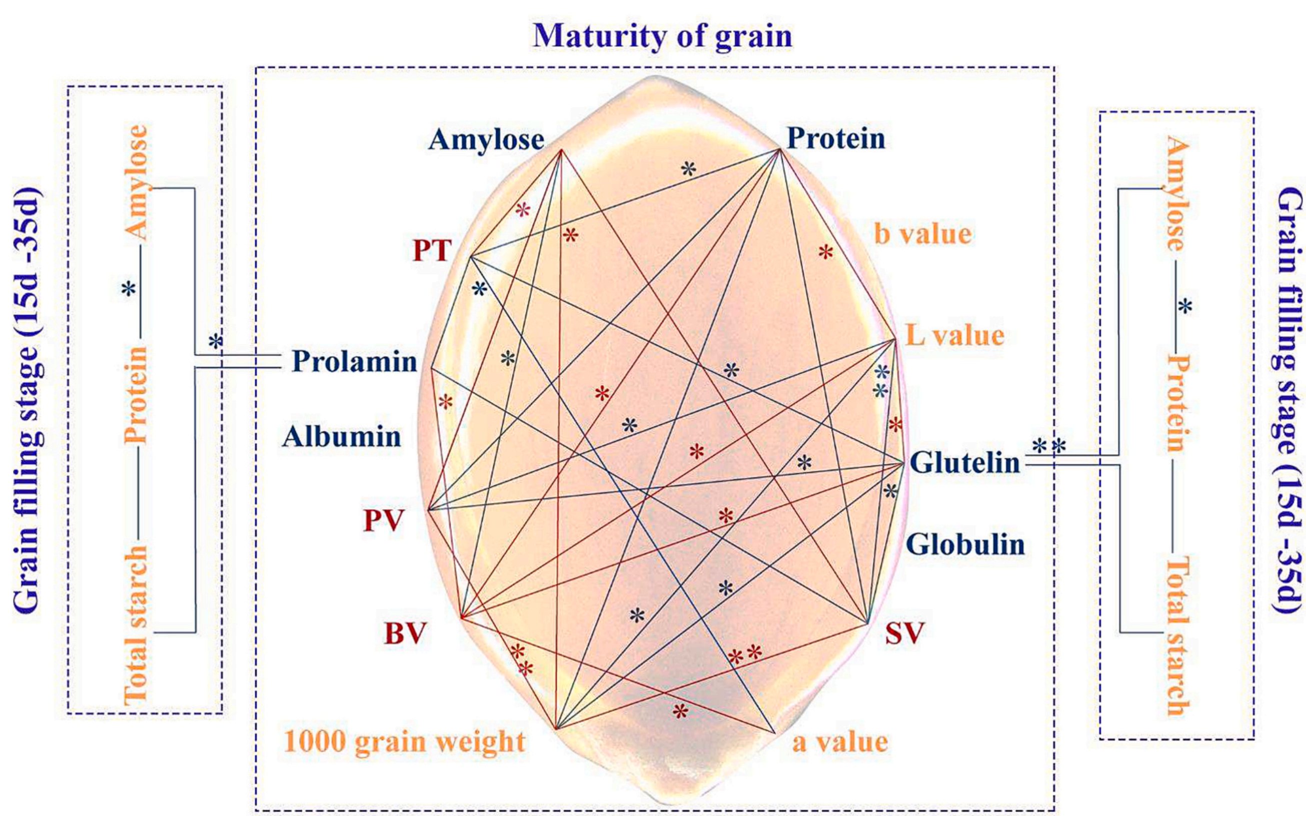


**Fig. 4.** Correlation analysis of protein, amylose and starch with L value, a value b value, 1000 grain weight, albumin content, globulin content, glutelin content, prolamine content, PT (pasting temperature), BV (breakdown viscosity), SV (setback viscosity) and PV (pasting viscosity) at 15, 20, 25, 30 and 35 days after flowering. * and ** indicate significant correlations at the 0.05 and 0.01 level, respectively. Red represents a positive correlation, while blue represents a negative correlation.

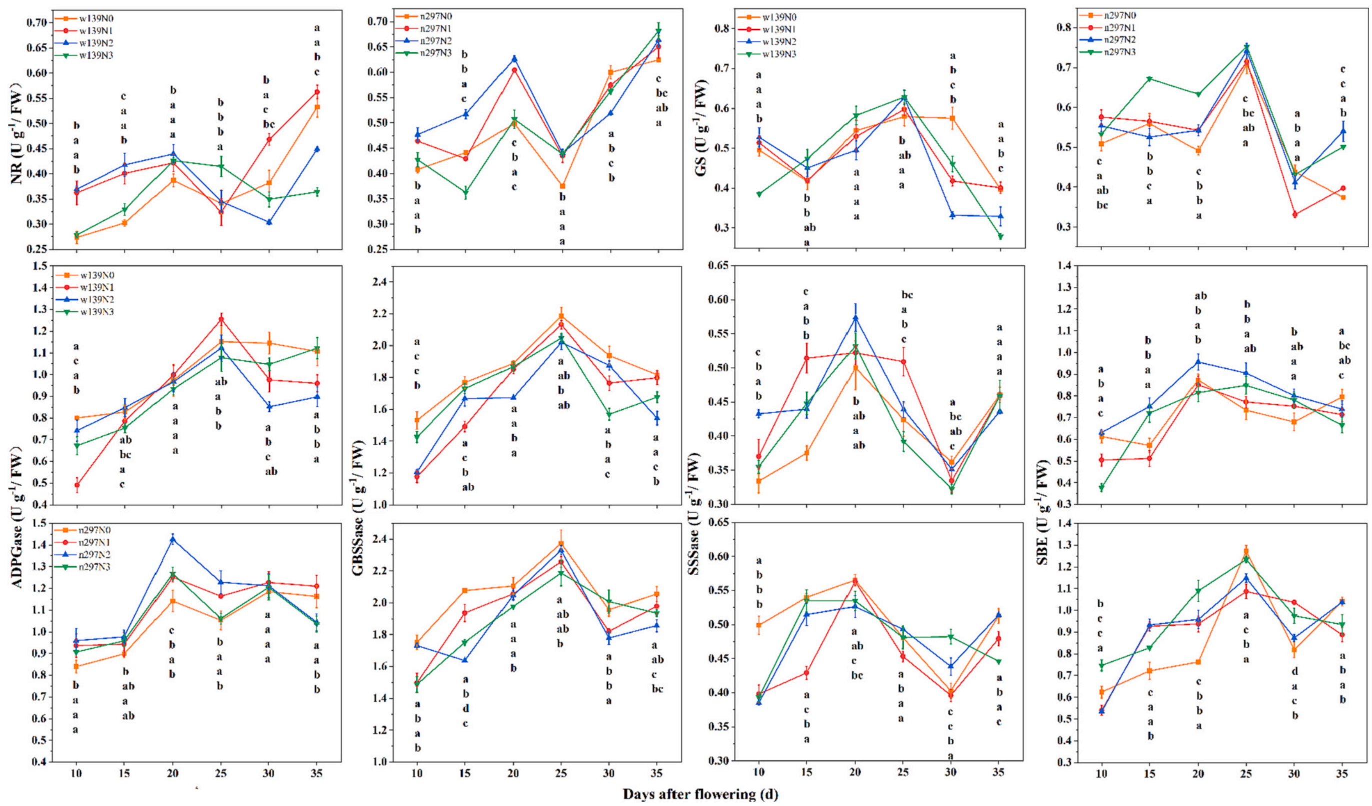


**Fig. 5.** Activities of nitrate reductase (NR), glutamine synthase (GS), ADP-glucose pyrophosphorylase (ADPGase), granule-bound starch synthase (GBSSase), soluble starch synthase (SSSase), starch branching enzyme (SBE) of proso millet varieties under nitrogen treatment at 10, 15, 20, 25, 30 and 35 days after flowering. FW: fresh weight. Different lowercase letters denote statistical differences between treatments at the 0.05 level in a column for (top to bottom) N0, N1, N2 and N3.

that the 1000 grain weight of the n297 variety was significantly higher than that of the w139 variety.

### 3.4. Structural properties and their relationship with grain quality

The fine structure of starch, particularly the CLD of amylopectin, is an important indicator for evaluating the quality properties of PM. Generally, the amylopectin chains can be divided into A (DP 6–12), B1 (DP 13–24), B2 (DP 25–36), and B3 (DP ≥ 37) types (Wang, Yang, Ferdinand, et al., 2020). As shown in Table 2, the A and B1 chains were the most abundant in the PM, which was consistent with the previous study (Yang et al., 2018). Compared with the control (N0), the increases in the percentage of short chains (A chain) with DP 6–12 and short-intermediate chains (B2 chain) with DP 25–36 were observed in w139 variety after applying nitrogen fertilizer, whereas the relative number of long branch chains (B3 chain) of amylopectin significantly increased and the percentage of short branch chains (A chain) of amylopectin decreased of n297 variety. The amounts of A and B2 chains in the starches of w139 variety were 24.67–25.38 % and 11.88–12.04 %, which were higher than those in the starches of n297 variety. By contrast, the n297 had higher amounts of B3 chain (11.35–12.82 %) than those of w139 variety (10.71.28–11.10 %). In the present study, the ACL of both varieties significantly increased with increasing nitrogen fertilization, ranging from 20.29 to 20.57 % for w139 variety and 20.33–20.50 % for n297 variety, respectively.

**Table 2**
Chain length distribution and average chain length (ACL) of amylopectin.[a]

| Treatment | Chain length distribution (%) | | | | ACL (%) |
|---|---|---|---|---|---|
| | DP 6–12 (A) | DP 13–24 (B1) | DP 25–36 (B2) | DP ≥ 37 (B3) | |
| w139N0 | 24.67 ± 0.03d | 52.35 ± 0.02b | 11.88 ± 0.01c | 11.10 ± 0.03e | 20.29 ± 0.02f |
| w139N1 | 24.79 ± 0.01c | 52.23 ± 0.10bc | 11.98 ± 0.06ab | 10.99 ± 0.05e | 20.36 ± 0.02de |
| w139N2 | 24.89 ± 0.02b | 52.33 ± 0.14b | 11.93 ± 0.06bc | 10.85 ± 0.05f | 20.31 ± 0.02f |
| w139N3 | 25.38 ± 0.01a | 51.87 ± 0.10e | 12.04 ± 0.03a | 10.71 ± 0.08 g | 20.57 ± 0.02a |
| Mean | 24.93 | 52.20 | 11.96 | 10.91 | 20.38 |
| n297N0 | 24.49 ± 0.03e | 52.50 ± 0.02a | 11.66 ± 0.02d | 11.35 ± 0.03d | 20.33 ± 0.02ef |
| n297N1 | 24.46 ± 0.04e | 52.17 ± 0.04 cd | 11.56 ± 0.04e | 11.81 ± 0.07c | 20.37 ± 0.01d |
| n297N2 | 23.67 ± 0.07f | 52.56 ± 0.10a | 11.57 ± 0.08de | 12.20 ± 0.06b | 20.41 ± 0.03c |
| n297N3 | 23.65 ± 0.05f | 52.08 ± 0.01d | 11.45 ± 0.07f | 12.82 ± 0.14a | 20.50 ± 0.01b |
| Mean | 24.07 | 52.33 | 11.56 | 12.05 | 20.40 |

[a] Datas are means ± standard deviation, n = 3. Values in the same column with different letters are significantly different ($p < 0.05$).

The X-ray diffraction patterns of PM flour at different nitrogen fertilizer levels were characterized as A-type, with strong reflection peaks at 15° and 23° at a 2θ diffraction angle and continuous peaks at 17° and 18° (Supplementary Fig. 2). Nitrogen fertilizer did not alter the crystal type, although variations in crystallinity were observed at different nitrogen fertilizer levels, with higher crystallinity of the w139 and n297 varieties recorded at N0 and N1, respectively (Fig. 6). The crystallinity of both varieties significantly decreased with increasing nitrogen levels in the order of N1, N2, and N3. The ordered structure and functional groups of the flour are shown in Fig. 6. Nitrogen fertilization significantly increased the 1045/1022 $m^{-1}$ ratio of the 139 variety and decreased the 1022/995 $m^{-1}$ ratio of the n297 variety. The w139 variety had a higher degree of order (1045/1022) and amorphous regions (1022/995) at N3 than other nitrogen levels. As nitrogen levels increased, all functional groups of the w139 variety decreased, whereas

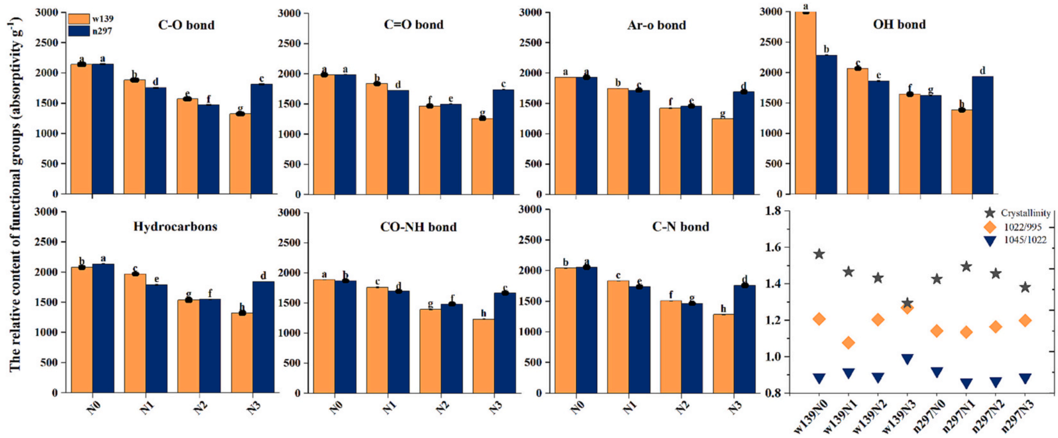


**Fig. 6.** Functional groups content, crystallinity, ratio of 1045/1022 and ratio of 1022/995 of proso millet varieties under nitrogen treatment.

hydrocarbons, C—O, C═O, C—N, and OH bonds in the n297 variety increased in the order of N0, N3, N1, and N2 and N0, N1, N3, and N2, as shown by Ar—O and CO—NH bonds, respectively. The w139 variety exhibited more functional groups than the n297 variety at N1, whereas the n297 variety had more functional groups than the w139 variety at N0 and N3 (except for Ar—O, CO-NH, and OH bonds).

The correlation analysis for functional groups and grain quality was shown in Fig. 7. These functional groups had different contributions to the varieties, especially the SV and PT of w139 and n297 varieties. The C—O, C═O, Ar—O, OH, hydrocarbons, CO—NH, C—N, order degree of carbohydrate structure had higher contributions to the BV in n297 variety. PV and BV of n297 variety was positively correlated with the amorphous regions, but negatively correlated with the order degree of carbohydrate structure and all functional groups. In the n297 variety, SV

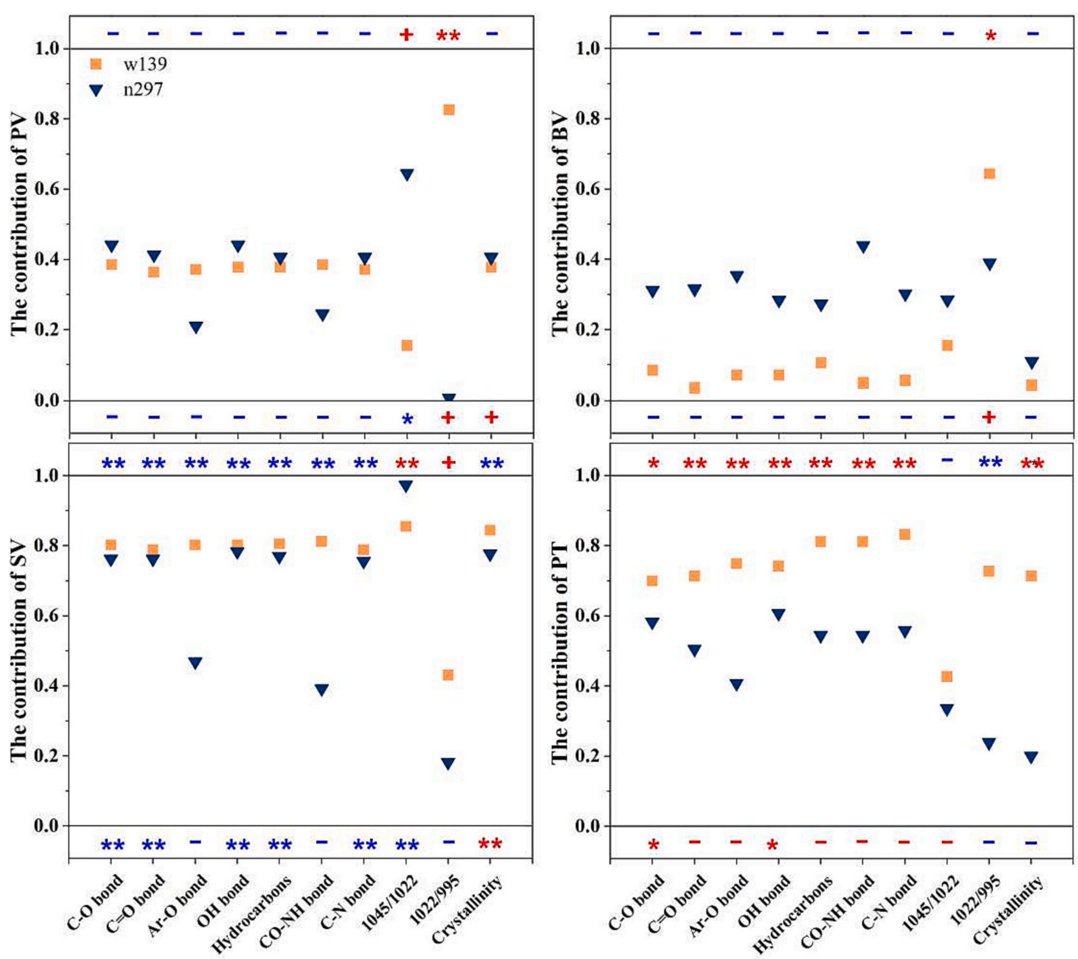


**Fig. 7.** Correlation analysis of the functional groups, crystallinity, ratio of 1045/1022 and ratio of 1022/995 with PT (pasting temperature), BV (breakdown viscosity), SV (setback viscosity) and PV (peak viscosity) of proso millet varieties. * and ** indicate significant correlations at the 0.05 and 0.01 level, respectively. Red represents a positive correlation, while blue represents a negative correlation. The top of the graph shows the significance of w139 varieties, while the bottom shows significance of n297 varieties.

positively correlated with crystallinity, but negatively correlated with carbohydrate structural and all functional groups. The SV of the w139 variety negatively correlated with crystallinity and all functional groups, but positively correlated with order degree of carbohydrate structural and amorphous regions. The crystallinity and all functional groups of w139 variety showed significant or extremely significant positive correlation with PT. These results indicate that the functional groups and structure of PM are closely related to grain quality.

## 4. Discussion

### *4.1. Accumulation of starch and protein affected the relationship among quality traits*

Mirroring the results of this study, Singh et al. (2011) reported that nitrogen treatment reduced the amylose content of different rice varieties. In grains, carbon and nitrogen metabolism utilize similar reducing power, ATP, and carbon skeletons. Competition for ATP and the carbon skeleton generally coincide with increasing rates of protein synthesis (Zhu et al., 2016). Therefore, increased protein synthesis results in lower starch content. Consistent with the results reported by Labuschagne et al. (2006), nitrogen fertilization coincided with higher albumin and glutelin contents, with the glutelin content of the w139 variety being significantly higher than that of the n297 variety. Albumin and glutelin have high nutritional value, although glutelin is difficult for the human body to digest. There was no significant difference in grain length and width between the two varieties at different nitrogen levels (Table 1), suggesting that nitrogen fertilization had no significant effect on these aspects. Observing a correlation between rice grain shape and quality, Thongbam et al. (2011) found that a slender caryopsis with low chalkiness and high transparency had a favorable appearance. Grain weight was determined using starch yield, which was consistent with our finding that total starch content during grain-filling positively correlated with 1000 grain weight (Supplementary Table 2). As the most important indicators of proso millet appearance quality, L* and b* values also had a significant impact on its nutritional value. The different shades of yellow in the grains were related to the composition and content of yellow pigment. Considering the competition between the metabolism of secondary yellow pigments and that of protein and fat, high levels of yellow pigment generally lead to protein and fat loss, which is consistent with our findings. Therefore, nitrogen treatment reduced L* and b* values, resulting in the relatively poor appearance quality of the grains.

The properties of PV, BV, and SV, are closely related to cooking quality (Fan et al., 2017; Sun et al., 2011). Peak viscosity reflects the degree to which starch can swell or water binding and is related to the quality of the final product, as the swelling and degradation of starch granules are related to the properties of the cooked starch. Breakdown viscosity measures the heat resistance and shear strength of the starch paste, reflecting the stability of the starch paste during cooking, and SV represents the retrograde characteristics of the starch paste (Kong et al., 2015). High BV and low SV are indicative of good cooking quality because the cooked product does not retrograde or become stiff upon cooling (Asante et al., 2013). Thus, the decrease in BV and increase in SV of the w139 variety after nitrogen fertilization are indicative of low cooking quality. Generally, the higher amylose content in starch corresponds to the higher PT and lower PV, which is consistent with the results of this study. The pasting properties of flour are also influenced by the protein content of the grains. Hydrophilic amino acids in protein bind to water, which limits water absorption, starch swelling, and the concentration of the dispersed and viscous forms of starch, thus impacting cooking quality (Martin & Fitzgerald, 2002; Wang, Li, et al., 2021). In this study, increasing nitrogen fertilization affected both protein and amylose content, which further influenced pasting properties. Both the two varieties under N2 level maintained better appearance, nutrition and cooking quality.

### *4.2. Regulation of enzyme activity of on grain quality (Fig. 8)*

The metabolism of carbon, nitrogen, and their products is an important determinant of grain quality (Tang et al., 2018). Studies have identified the major metabolic stages involved in starch and protein production in grains as well as the roles of key enzymes and regulators, such as NR and GS in nitrogen metabolism and GBSSase and SBE in starch synthesis. Increased GS and NR activity have been shown to increase nitrogen metabolism, protein synthesis, and amino acid transformation (Wang, Wang, et al., 2021). In this study, the increase in NR and GS activity with increasing nitrogen fertilization mainly occurred in the early stage of grain-filling (up to 20 d after flowering), which indicates that nitrogen was assimilated into organic substances, such as proteins and nucleic acids, and participated in the glutamine and glutamate synthase cycles to promote protein synthesis in both varieties. The inconsistent effects of NR and GS enzyme activity on the prolamin and glutelin contents of the two varieties were possibly due to different physiological pathways. Starch and protein contents are important components of 1000 grain weight. Therefore, because the n297 variety exhibited higher NR and ADPGase activity than the w139 variety, it can be inferred that the promotion of starch and protein synthesis was the reason for the higher 1000 grain weight of the n297 variety. The increased SSSase activity and proportion of short branch chain (A chain) of amylopectin in the w139 variety resulting from nitrogen treatment could not form double helices, which decreased the tightness and stability of starch granules and consequently reduced the L* value. However, the decrease in L* value was improved with increasing protein content. Additionally, nitrogen fertilization led to an increase in long branch chains of amylopectin after 20 d, which reduced the amylose content, further affecting the water absorption of starch granules, and decreased the PT of the w139 variety (Zhao et al., 2021). Nitrogen treatment of the n297 variety increased the synthesis of long branch chain (B3 chain) of amylopectin and maintained the formation of short amylopectin chains after 20 d. In starch granules, a high proportion of long amylopectin chains facilitates the formation of dense crystalline regions or concentration of amylopectin chains in amorphous lamellae of crystalline regions (Zhao et al., 2019). Short amylopectin branches fill the crystalline lamella, whereas long amylopectin branches increase the mobility of starch molecules, forming more intramolecular and intramolecular hydrogen bonds during degradation (Jeong et al., 2021). These factors contribute to low PT and high BV and SV of n297 variety. Greater amounts of short branched chains had lower PT than those having more long branched chains within the same botanical origin, whereas BV and SV negatively and positively correlate with the percentage of very long amylopectin chains, respectively (Zhang et al., 2022). Therefore, the different branched structures and amylose content of the n297 variety resulted in higher SV, PV, and PT values than those of the w139 variety.

### *4.3. PM cooking quality is affected by starch structure and functional groups (Fig. 8)*

The variation in the CLD of amylopectin influenced the stability of starch and the cooking quality of PM. The higher the content of short branch chains (A chain) of amylopectin, the lower the content of long branch chains (B3 chain), and the lower the stability and crystallinity of the starch (Wang, Yang, Gao, et al., 2020). In our research, the increase of A chain amylopectin for w139 variety could significantly decreased the crystallinity and increased the ratio of 1022/995 (amorphous regions), thereby decreasing the stability of the starch crystals. Simultaneously, pasting properties (cooking quality) were greatly influenced by CLD. PV and BV increased with the long amylopectin branch chain ratio (Bao et al., 2020). SV was generally taken as an indicator of cooking quality, which was positively correlated with the percentage of extremely short branch chain (A chain) of amylopectin (Bao et al., 2020). Therefore, compared with N0, nitrogen application could

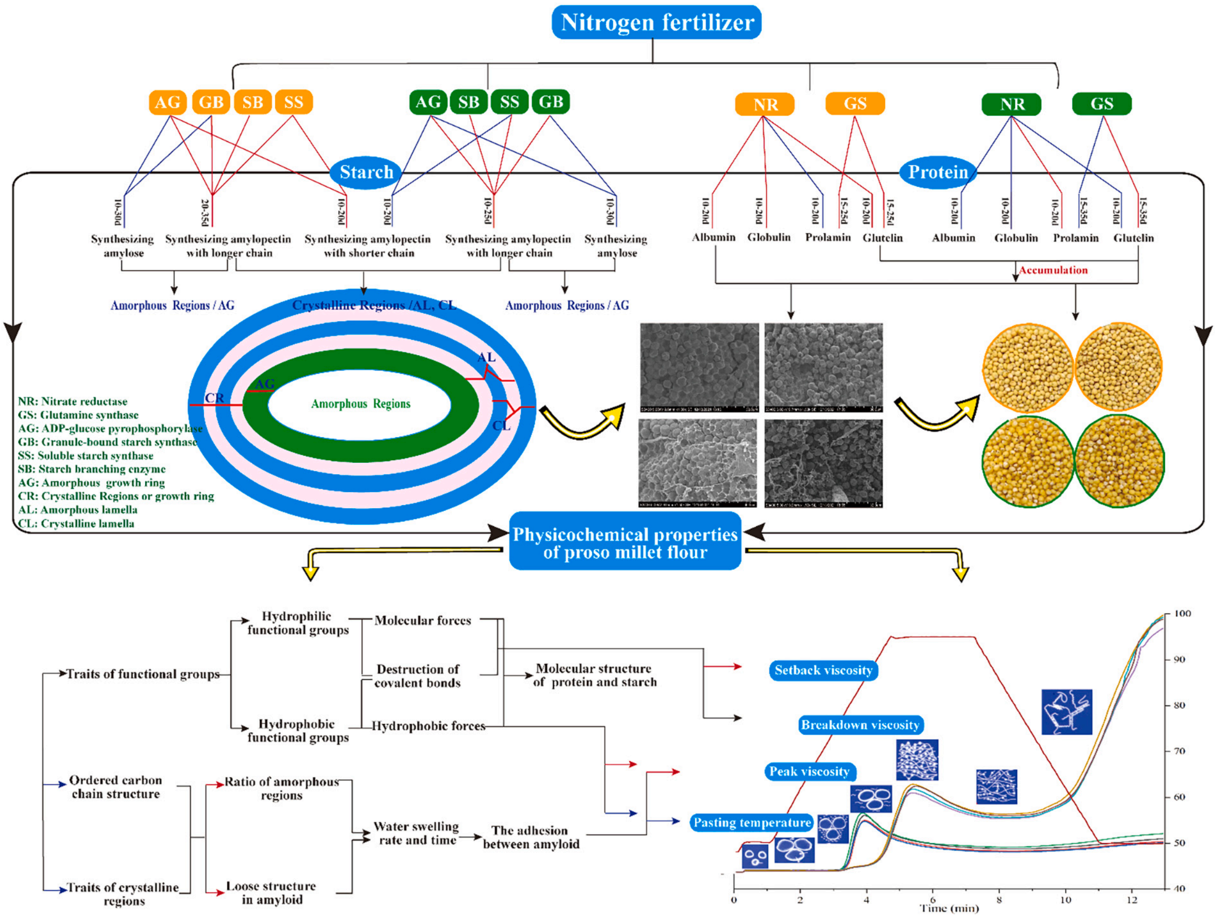


**Fig. 8.** Mechanisms by which assimilate accumulation forms, starch synthesis enzymes and starch granule physicochemical properties influence the appearance, nutritional and cooking quality of proso millet varieties under nitrogen treatment. Orange boxes represent w139 variety, green boxes represent n297 variety. Light red lines and arrows represents facilitation and light blue represents inhibition.

significantly increase the PV, BV and SV of n297 variety, and reduce the PV and BV of w139 variety. The changes in branch chain length distribution are due to changes in starch synthase activity including ADPGase, SSSase and SBE. Recent studies have indicated that enzymes SSSI are involved in the biosynthesis of short branch chain amylopectin, whereas SBEI play a critical role during the elongation of long chain amylopectin (Zhou et al., 2022). Our research indicated that nitrogen fertilizer significantly changed the activities of starch synthase and further regulated the short and long chains distribution of the two varieties, which ultimately affected the stability of starch and the cooking quality of PM.

The X-ray diffraction results of this study indicate that nitrogen treatment changed the crystallinity and influenced the stability of PM starch granules. Cao et al. (2018) found that late-stage nitrogen fertilization did not change the type of rice starch crystals, but reduced relative crystallinity, which may be due to differences in amylose content and amylopectin chain length distribution within starch granules (Chung et al., 2011). Native starch granules have a hierarchical structure, consisting of multiple concentric circles referred to as growth rings (Chen et al., 2021). Growth rings include both crystalline regions and amorphous lamellae, distributed in an alternating pattern (Sevenou et al., 2002). The crystalline layer comprises a lattice mainly formed by branched chains, whereas the amorphous layer contains amylopectin branch points and structurally disordered amylose and amylopectin molecules (Copeland et al., 2009; Yuryev et al., 2004). The differences in the proportion and composition of amylose and amylopectin in PM varieties at different nitrogen fertilizer levels affected the morphology of starch granules, resulting in different degrees of crystallinity. The degree of carbohydrate structural order in different PM varieties at different nitrogen fertilizer levels might be linked to the activity of key enzymes involved in starch synthesis (Guo et al., 2020). As the degree of ordered structure influences the resistance of starch to enzymes or acids, it can be used to determine the hydrolysis properties of starch and the structural changes that occur during starch processing (Wei et al., 2011).

The physicochemical properties of starch granules affect their viscosity properties. At N0, both varieties (especially w139) exhibited higher starch granule crystallinity and more hydrophobic (hydrocarbons) as well as hydrophilic (C—O, C═O, Ar—O, C—N, CO—NH, and OH bonds) functional groups than at higher nitrogen levels. At the N3, the w139 variety had a more ordered carbohydrate structure and lower crystallinity than at other levels, potentially resulting in a brittle granular structure and facilitating gelatinization at a lower temperature, thus decreasing PT (Wang, Yang, Ferdinand, et al., 2020). The n297 variety had more functional groups and a less ordered carbohydrate structure (ratio of 1045/1022) than the w139 variety. A disordered structure (ratio of 1022/995) promotes an increased release of amylose after water absorption by starch, thus increasing PV during gelatinization (Wang, Yang, Gao, et al., 2020).

At N0, the hydrophilic functional groups of the w139 variety maintained high levels and tended to form intermolecular hydrogen bonds with water molecules (Fumoto et al., 2020; Wang et al., 2019). After the pasting of starch granules, hydrophobic functional groups limit the water absorption capacity and increase the PT of starch. As temperature increases during gelatinization, the hydrophobic force decreases, and the molecular structure of the protein or starch becomes disordered, resulting in higher PV. The above phenomena lead to n297N0 with high hydrophobic functional groups and low protein content showed the high PT and low PV. Hydrogen bonds may be broken under continuous exposure to high temperature, whereas covalent bonds break more slowly (Wang et al., 2019). At N0, the n297 variety exhibited stronger covalent bonds, resulting in lower BV and higher shear resistance than at other nitrogen levels. Compared with the n297 variety, the w139 variety exhibited fewer functional groups, which can reduce the strength of intramolecular forces and allows chemical bonds to be more easily broken during gelatinization, thereby decreasing PT and PV and increasing BV. As the temperature decreased in the starch retrogradation stage, high hydrophilic functional groups and covalent bonds increased the difficulty of rearrangement of the dissolved loose molecules, resulting in the high SV observed in the n297 variety.

## 5. Conclusion

The synthesis of starch and protein were affected by nitrogen fertilizer and further affected the quality traits of PM, including protein component, grain color, intrinsic molecular structure, cooking and tasting quality. The results of this study showed that increasing nitrogen fertilization significantly decreased the appearance quality. After applying nitrogen fertilizer, the w139 variety exhibited an increase in the proportion of short amylopectin chains, disordered structure, resulting in an increase in SV and decrease in PT. In contrast, the n297 variety exhibited a larger proportion of long amylopectin chains and hydrophobic functional groups, strengthening the inter- and intramolecular forces of the starch colloids. These factors increased PV and reduced BV, thus improving the cooking quality of the n297 variety. Compared with the n297 variety, the w139 variety had a lower amylose content and number of functional groups, resulting in lower PT and PV and higher BV. Nitrogen fertilization has varying impacts on the quality traits of different PM. Notably, the amylose content of the w139 variety first increased, reaching a maximum after 20 d, and then decreased, while that of the n297 variety consistently increased. These findings provide new insight into starch synthesis pathways in waxy and non-waxy varieties of PM, forming the basis for future studies.

## CRediT authorship contribution statement

**Honglu Wang:** Conceptualization, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Dongmei Li:** Investigation, Data curation. **Qian Ma:** Software. **Enguo Wu:** Software. **Licheng Gao:** Writing – review & editing. **Pu Yang:** Formal analysis. **Jinfeng Gao:** Resources, Conceptualization, Funding acquisition, Supervision. **Baili Feng:** Resources, Conceptualization, Funding acquisition, Supervision.

## Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Data availability

Data will be made available on request.


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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.120423.

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