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# High nitrogen application rate and planting density reduce wheat grain yield by reducing filling rate of inferior grain in middle spikelets

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Excessive use of nitrogen fertilizer and high planting density reduce grain weight in wheat. However, the effects of high nitrogen and planting density on the filling of grain located in different positions of the wheat spikelet are unknown. A two-year field experiment was conducted to investigate this question and the underlying mechanisms with respect to hormone and carbohydrate activity. Both high nitrogen application and planting density significantly increased spike density, while reducing kernel number per spike and 1000-kernel weight. However, the effects of high nitrogen and high plant density on kernel number per spike and 1000-kernel weight were different. The inhibitory effect of high nitrogen application and high planting density on kernel number per spike was achieved mainly by a reduction in kernel number per spikelet in the top and bottom spikelets. However, the decrease in 1000-kernel weight was contributed mainly by the reduced weight of grain in the middle spikelets. The grain-filling rate of inferior grain in the middle spikelets was significantly decreased under high nitrogen input and high planting density conditions, particularly during the early and middle grain-filling periods, leading to the suppression of grain filling and consequent decrease in grain weight. This effect resulted mainly from inhibited sucrose transport to and starch accumulation in inferior grain in the middle spikelets via reduction of the abscisic acid/ethylene ratio. This mechanism may explain how high nitrogen application and high planting density inhibit the grain filling of inferior wheat grain.



## 1. Introduction

Grain weight is one of the most important components of grain yield of wheat and is determined by grain filling. Accordingly, identifying the physiological mechanisms of grain filling is desirable for increasing wheat yield. Nitrogen (N) application rate and planting density strongly influence grain weight and yield [1,2]. In China, to maximize wheat yields, excessive amounts of N are often applied [3]. This practice results in large losses of N fertilizer [4,5] as well as

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inhibiting the grain filling of inferior kernels and reducing kernel weight [6].

Besides N, plant density also influences wheat grain weight [7]. To maintain annual crop yields under a winter wheat–summer maize double-cropping system, the practice of late harvest of summer maize and delayed sowing of winter wheat has been adopted in the Huang–Huai–Hai region [8]. In this system, winter wheat produces few tillers before winter owing to the shorter growing period caused by delayed sowing. In compensation for the reduction in spike numbers, the sowing density is increased. However, the high plant density inhibits grain filling and reduces kernel weight [9]. These studies suggest that high N fertilization and high plant density reduce wheat grain weight. An interaction between nitrogen fertilizer and plant density also influences grain filling and grain weight [10].

It has been hypothesized [1,3,11] that the main way in which high N fertilizer and high plant density reduce wheat kernel weight is by sharply increasing the number of tillers and spikes per unit area of wheat, intensifying competition among individual plants and leading to reduced plant uniformity and biomass accumulation. These effects in turn lead to increased lodging and disease and insect pest damage, reducing kernel number per spike and kernel weight. However, in a study of hybrid rice under increased nitrogen application, the number of spikes per unit area increased significantly, but the kernel number per spike was not affected [12]. Li et al. [13] reported that, at a planting density of 480 × $10^4$ wheat plants $ha^{-1}$, although the spike number per unit area increased significantly compared with 240 × $10^4$ plants $ha^{-1}$, both kernel weight and kernel number per spike remained unchanged. Liu et al. [14] suggested that using an advanced furrow-ridge rainwater harvesting farming technique, kernel number, kernel number per spike, and 1000-kernel weight of wheat could all be increased. These studies suggested that the increase in spike number per unit area induced by high N and high plant density may not the main reason for the decrease in kernel weight or kernel number per spike. Thus, the physiological mechanism underlying the regulatory effects of high nitrogen and high plant density on the inhibition of grain filling could not be explained from a population perspective.

Kernel filling in wheat is associated with the location of kernels on the spike [15]. Jiang et al. [16] classified wheat grain into superior and inferior grain based on the flowering period and the position of the kernels on the spike. Flowers of superior grain bloom early and are located at the bottom of the spikelet, while those of inferior grain bloom later and are located mainly at the top. Compared with inferior grain, superior grain generally has a greater filling rate and kernel weight [17]. Inferior grain shows greater spatial and temporal variation and is more sensitive to the environment than superior grain [18]. For some large-spike crop species, insufficient filling of inferior grain is the main factor limiting the full development of yield potential [19]. Thus, a key approach to increasing wheat grain weight is to increase the weight of inferior grain. However, it is unclear whether a grain position effect occurs even under high nitrogen and large-population conditions, and whether there are differences in the sensitivity of kernel filling of superior and inferior grain to high nitrogen fertilizer and high plant density.

Hormones regulate wheat grain filling. Auxin, cytokinin (CTK), abscisic acid (ABA), polyamines, ethylene (ETH), and other hormones are involved in this regulation [17,20–22]. Nitrogen fertilizer strongly influences the metabolism of multiple hormones [23–26], as does plant density [27–29]. Environmental factors such as moisture and temperature could modify grain filling of crop species by affecting hormone levels [15,30]. However, it is unknown whether the adverse effects of high nitrogen and high planting density on wheat grain filling involve hormones. The main component of the wheat kernel is starch, and the process of kernel filling involves the transport of carbohydrates to the kernel and the subsequent accumulation of starch [19]. Both nitrogen fertilizer and plant density influence carbohydrate metabolism and starch metabolism [31–33]. However, there is no consensus on whether the effects of high nitrogen and high planting density on the filling of wheat kernels at different positions on the spikelet involving carbohydrate transport and starch synthesis.

A two-year field study was conducted to investigate the responses of the grain filling performance of wheat grain located at different spikelet positions to N application rates and planting densities, and the underlying mechanisms with respect to activities of hormones and carbohydrates including ABA and ETH, soluble sugar, starch, nonstructural carbohydrate (NSC), and chlorophyll were investigated during the grain-filling period. The purpose of the present study was to (1) investigate whether the effects of high-N fertilizer and high plant density on the filling of kernels at different positions on the wheat spike were identical; (2) identify the kernel position most strongly affected by high N fertilization and high plant density with respect to final kernel weight, and (3) identify the physiological mechanisms underlying the effects of high N fertilization and high plant density on wheat kernel filling with respect to hormone and starch transport.

## 2. Materials and methods

### 2.1. Experimental design

The study was conducted at the Doukou Wheat and Maize Experimental Farm of Northwest A&F University, Shaanxi province, China (34°36′N, 108°52′E), during 2015–2017. The experimental site is at an elevation of 510 m above sea level. The annual mean temperature is 13.2 °C and the annual mean precipitation is 548.7 mm, 70% of which falls from June to September. The soil in the top 0.2 m is Eum-Orthrosols (Chinese soil taxonomy). The soil bulk density, organic matter content, available nitrogen, phosphorus, and potassium at a soil depth of 0–20 cm (before fertilization) were 1.03 g $cm^{-3}$, 15.03 g $kg^{-1}$, 97.33 mg $kg^{-1}$, 21.05 mg $kg^{-1}$, and 143.88 mg $kg^{-1}$, respectively.

A local widely adapted wheat cultivar, Xinong 979, was used in our study. The cultivar was sown on October 15, 2015 and October 18, 2016. Before the experiment arrangement, rotary tillage was applied and 120 kg $ha^{-1}$ $P_2O_5$ (ordinary superphosphate) were applied as a basal treatment. Two plant densities were tested: 3.00 × $10^6$ seedlings $ha^{-1}$ (DM, the plant density suggested for high-yield wheat) and 5.25 × $10^6$

seedlings $ha^{-1}$ (DH, the plant density used by local farmers). The row spacing was 0.25 m. For each plant density, four N treatments were applied: 180 kg N $ha^{-1}$ N fertilizer was applied as a basal application (NM1), 180 kg N $ha^{-1}$ N was applied half as basal application and the other half at jointing stage as topdressing (NM1), 270 kg N $ha^{-1}$ N was all applied as basal application (NH1) and 270 kg N $ha^{-1}$ N was applied half as basal application and half at jointing stage as topdressing (NH2). The 270 kg N $ha^{-1}$ rate was that used by local farmers [34] and the 180 kg N $ha^{-1}$ rate was that suggested for high-yield wheat. The experiment was laid out as a randomized complete block design. Each treatment had three replicates and each plot was of 20 $m^2$ (4 × 5 m).

### 2.2. Sampling and measurement

#### 2.2.1. Grain yield

For each plot, three 1-$m^2$ areas were harvested for measurement of grain yield. Spike number per unit area and grain yield (grain moisture content was measured and converted to 13.5%) were also measured. Two 1-m rows of plants were sampled and kernel number per spike and 1000-kernel weight were measured.

#### 2.2.2. Kernel number per spikelet and grain weight

In each plot, 100 spikes flowering on the same day were labeled at anthesis stage and sampled at maturity. Each spike was divided into three parts according to the kernel position [7]. Bottom spikelets were the basal to 6th spikelet (from down to up). Middle spikelets were the 7th to 15th, and top spikelets were the 16th to terminal spikelet. The kernels of each spikelet were sampled, and the kernel number per spikelet and weight of grain from the individual spikelet were measured.

#### 2.2.3. Grain filling

At anthesis, 400 spikes were collected on the same day and labeled. From anthesis to maturity, 30 spikes were sampled at 5-day time intervals. The grain of middle spikelets was divided into superior and inferior grain following Jiang et al. [16]. The first and second individual kernels from the bottom of the middle spikelet were considered as superior grain, and the remaining kernels as inferior grain. If the kernel number per spikelet was less than 3, only the superior kernels were analyzed. Half of the samples were flash frozen in liquid nitrogen and then stored at –40 °C for hormone measurement. The remaining samples were dried at 60 °C (after being heated for 15 min at 105 °C for deactivation of enzymes) to constant weight, weighed, and then used for sucrose and starch measurement. The grain-filling process was fitted by the Richards growth equation:

$$W = \frac{A}{(1 + Be^{-kt})^{\frac{1}{N}}} \tag{1}$$

The grain-filling rate (G) was calculated according to Eq. (2), which is the derivative of Eq. (1):

$$G = \frac{AkBe^{-kt}}{N(1 + Be^{-kt})^{\left(\frac{N+1}{N}\right)}} \tag{2}$$

($W$, kernel weight (mg); $A$, final kernel weight (mg); $t$, time after anthesis (d); $B$, $k$ and $N$, coefficients determined by regression).

The active grain-filling period was defined as the period when $W$ was between 5% ($t_1$) and 95% ($t_2$) of $A$. The mean grain-filling rate during this period was accordingly calculated from $t_1$ to $t_2$.

#### 2.2.4. Hormones

Using 80% (v/v) methanol, endogenous ABA was extracted following a previous study [35]. ABA was quantified by enzyme-linked immunosorbent assay [35]. The recovery rate for ABA was 90.7% ± 6.5%.

The ETH generated by kernels was determined following Liu et al. [15]. ETH was assayed by gas chromatography (Trace GC Ultra, Thermo Fisher Scientific, Waltham, MA, USA) following our previous report [20].

#### 2.2.5. Sucrose and starch contents in grain and non-structural carbohydrate (NSC) contents in the stems

Using 80% ethyl alcohol, sucrose was extracted from kernels and measured by the resorcinol–HCl method [35]. After sucrose extraction, the residue was extracted with 36 mol $L^{-1}$ perchloric acid (PCA) and then with 18 mol $L^{-1}$ PCA. All of the extraction solutions were combined and used for starch measurement. Starch concentration was measured by the anthrone method [35], and the NSC concentration in the stems was measured as described previously [35].

#### 2.2.6. SPAD (soil and plant analyzer development) values of flag leaves

At 10, 20 and 30 days after anthesis, 20 flag leaves from labeled plants (that had flowered on the same day) were selected, and SPAD values were measured with a SPAD-502 Chlorophyll Meter (Minolta Co. Ltd., Osaka, Japan).

### 2.3. Statistical analyses

Statistical product and service solutions (SPSS 24.0) was used to fit an analysis of variance. The data from each sampling were analyzed separately. The means were tested by Tukey HSD test at $P$=0.05.

## 3. Results

### 3.1. Grain yield and grain yield components

Both N application and planting density not only independently and significantly affected wheat grain yield, but also exhibited a significant interaction (Table 1). For DM, high N application rate had no effect on grain yield in 2015–2016, but a significant reducing effect on grain yield in 2016–2017. For DH, high N treatment significantly reduced grain yield in both growing seasons.

DH significantly increased spike density in two growing seasons. However, with increasing planting density, spikelet number per spike and 1000-kernel weight tended to decrease. The effects of nitrogen showed a similar trend. With increase in N application rate, spike number per area increased, while spikelet number per spike and 1000-kernel weight decreased.

**Table 1 – Effect of nitrogen fertilizer and plant density on grain yield and grain yield components of wheat.**

| Plant density | Nitrogen | Spikes per hectare ×$10^6$ | Kernel number per spike | 1000-kernel weight (g) | Grain yield (t $ha^{-1}$) |
|---|---|---|---|---|---|
| 2015–2016 | | | | | |
| DM | NM1 | 5.86 ± 0.13 e | 35.89 ± 1.13 a | 42.25 ± 0.88 ab | 7.14 ± 0.17 a |
| | NM2 | 5.56 ± 0.24 e | 36.15 ± 0.66 a | 42.82 ± 1.12 a | 7.21 ± 0.28 a |
| | NH1 | 6.31 ± 0.26 d | 32.96 ± 0.94 b | 40.28 ± 0.49 c | 7.04 ± 0.20 a |
| | NH2 | 6.46 ± 0.04 cd | 33.03 ± 0.26 b | 41.06 ± 0.74 abc | 7.09 ± 0.36 a |
| DH | NM1 | 6.88 ± 0.22 ab | 34.74 ± 0.25 a | 40.30 ± 0.64 c | 7.29 ± 0.21 a |
| | NM2 | 6.73 ± 0.30 bc | 35.59 ± 0.67 a | 40.63 ± 0.70 bc | 7.32 ± 0.24 a |
| | NH1 | 7.25 ± 0.22 a | 28.52 ± 1.37 d | 35.72 ± 0.81 e | 5.86 ± 0.33 c |
| | NH2 | 7.19 ± 0.31 a | 31.38 ± 0.66 c | 38.07 ± 2.28 d | 6.54 ± 0.27 b |
| 2016–2017 | | | | | |
| DM | NM1 | 5.68 ± 0.24 f | 36.22 ± 0.89 a | 43.46 ± 1.30 a | 7.03 ± 0.28 a |
| | NM2 | 5.30 ± 0.11 g | 36.86 ± 1.12 a | 43.66 ± 2.34 a | 6.90 ± 0.24 ab |
| | NH1 | 5.99 ± 0.23 ef | 31.68 ± 1.44 c | 40.80 ± 1.31 b | 6.31 ± 0.26 cd |
| | NH2 | 6.19 ± 0.14 de | 33.66 ± 1.29 b | 41.27 ± 2.63 b | 6.50 ± 0.37 bcd |
| DH | NM1 | 6.55 ± 0.33 bc | 33.72 ± 1.13 b | 40.26 ± 0.54 b | 6.71 ± 0.51 abcd |
| | NM2 | 6.44 ± 0.21 cd | 33.85 ± 1.67 b | 40.15 ± 0.97 b | 6.73 ± 0.49 abc |
| | NH1 | 6.94 ± 0.18 a | 27.71 ± 1.12 d | 34.04 ± 0.70 d | 5.28 ± 0.31 e |
| | NH2 | 6.90 ± 0.16 ab | 32.09 ± 0.66 c | 36.37 ± 0.40 c | 6.22 ± 0.48 d |
| *F*-value | | | | | |
| | Year (Y) | ** | ns | ns | ** |
| | Density (D) | ** | ** | ** | ** |
| | Ntrogen (N) | ** | ** | ** | ** |
| | Y × D | ns | ns | * | ns |
| | Y × N | ns | ns | ns | ns |
| | D × N | ns | ** | * | ** |
| | Y × D × N | ns | ns | ns | ns |

Values within a column and the same year followed by different letters are significantly different at $P = 0.05$.
ns, no significant difference ($P > 0.05$). DM, plant density of 3.00 × $10^6$ seedlings $ha^{-1}$; DH, plant density of 5.25 × $10^6$ seedlings $ha^{-1}$; NM, N level of 180 kg N $ha^{-1}$; NH, N level of 270 kg N $ha^{-1}$; 1, N fertilizer was all applied as basal application; 2, N fertilizer was applied half as basal application and half at jointing stage as topdressing.
* $P < 0.05$.
** $P < 0.01$.

### *3.2. Kernel number per spikelet and weight of the grain of different spikelets*

Kernel number per spikelet and weight of the grain of the middle spikelet were significantly greater than those of the top and bottom spikelets (Fig. 1). Overall, high nitrogen application and high planting density tended to reduce kernel number per spikelet and weight of grain of the top, middle, and bottom spikelets. Nitrogen and planting density showed a significant interaction effect on both kernel number per spikelet and grain weight.

High plant density significantly reduced the kernel number per spikelet of top spikelets in the NM and NH treatments. Under NM, the kernel number of bottom spikelets was significantly lower under DH than under DM. However, the kernel number per spikelet of the middle spikelets was significantly lower under DH than under DM under the NH treatment. Under DM, the high nitrogen fertilizer had no significant effect on the kernel number per spikelet of the middle spikelets, but reduced the kernel number per spikelet of the top and bottom spikelets. Under DH, the high nitrogen application significantly reduced the kernel number per spikelet in the middle, top and bottom spikelets. For NM, nitrogen topdressing had no significant effect on kernel number per spikelet. However, nitrogen topdressing significantly increased kernel number per spikelet of the top and bottom spikelets.

High planting density significantly reduced the grain weight of the top, middle, and bottom spikelets in the NH treatment. Under NM, DH significantly reduced the grain weight of the middle spikelets; however, planting density had no significant effect on the grain weight of the top and bottom spikelets. Under NH, DH significantly reduced the grain weight of the top, middle, and bottom spikelets. For DM, high nitrogen had no significant effect on the grain weight of the top and bottom spikelets. However, high nitrogen application significantly reduced the grain weight of the middle spikelets under DM. For DH, the high nitrogen application significantly reduced the grain weight of the top, middle, and bottom spikelets. Nitrogen topdressing increased grain weight at the middle position of spikelets in the DH and NH treatments.

### *3.3. Filling of the grain located on the middle spikelet*

The weight of superior grain was significantly greater than that of inferior grain (Fig. 2). Interaction between nitrogen and plant density affected grain filling. High plant density reduced the grain-filling rate and the weight of superior grain under NH, but had no significant effect on the filling of superior grain under NM (Figs. 2, 3). Under DM, high nitrogen application had no significant effect on grain-filling rate, active grain-filling period, or weight of superior grain. Under DH, high nitrogen significantly inhibited grain filling and reduced both grain-filling rate and weight of superior grain.

High plant density and high nitrogen application significantly reduced the grain filling rate and weight of the inferior grain, and NH and DH showed additive effects on the decrease in both parameters. The high planting density significantly

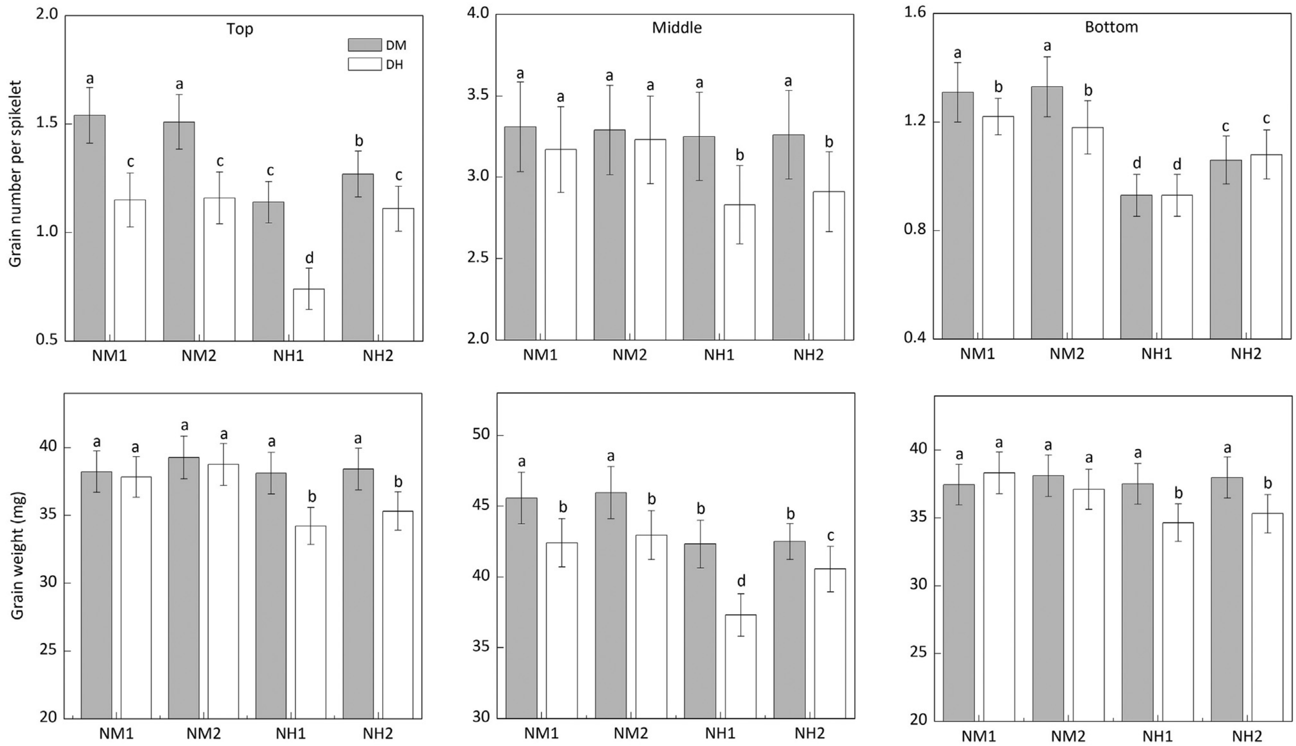



**Fig. 1 – Effect of nitrogen fertilizer and plant density on kernel number and grain weight of spikelets. Values are means of two years. Vertical bars represent standard deviation. Values within a column followed by different letters are significantly different at $P = 0.05$. DM, plant density of $3.00 \times 10^6$ seedlings $ha^{-1}$; DH, plant density of $5.25 \times 10^6$ seedlings $ha^{-1}$; NM, N level of 180 kg N $ha^{-1}$; NH, N level of 270 kg N $ha^{-1}$; 1, N fertilizer was all applied as basal application; 2, N fertilizer was applied half as basal application and half at jointing stage as topdressing.**

reduced the active grain-filling period of the inferior grain. The nitrogen topdressing significantly increased the grain-filling rate and weight of the inferior grain in the DHNH treatment.

### 3.4. *Carbohydrate contents*

When nitrogen fertilizer was held constant, NSC accumulation in stems was significantly higher in the DM than in the DH treatment (Fig. 4). Under constant planting density, NH significantly reduced NSC concentration in stems compared with NM. Across the treatments, DHNH displayed the lowest value of NSC concentration in the stems, and nitrogen topdressing significantly increased stem NSC concentration in the DHNH.

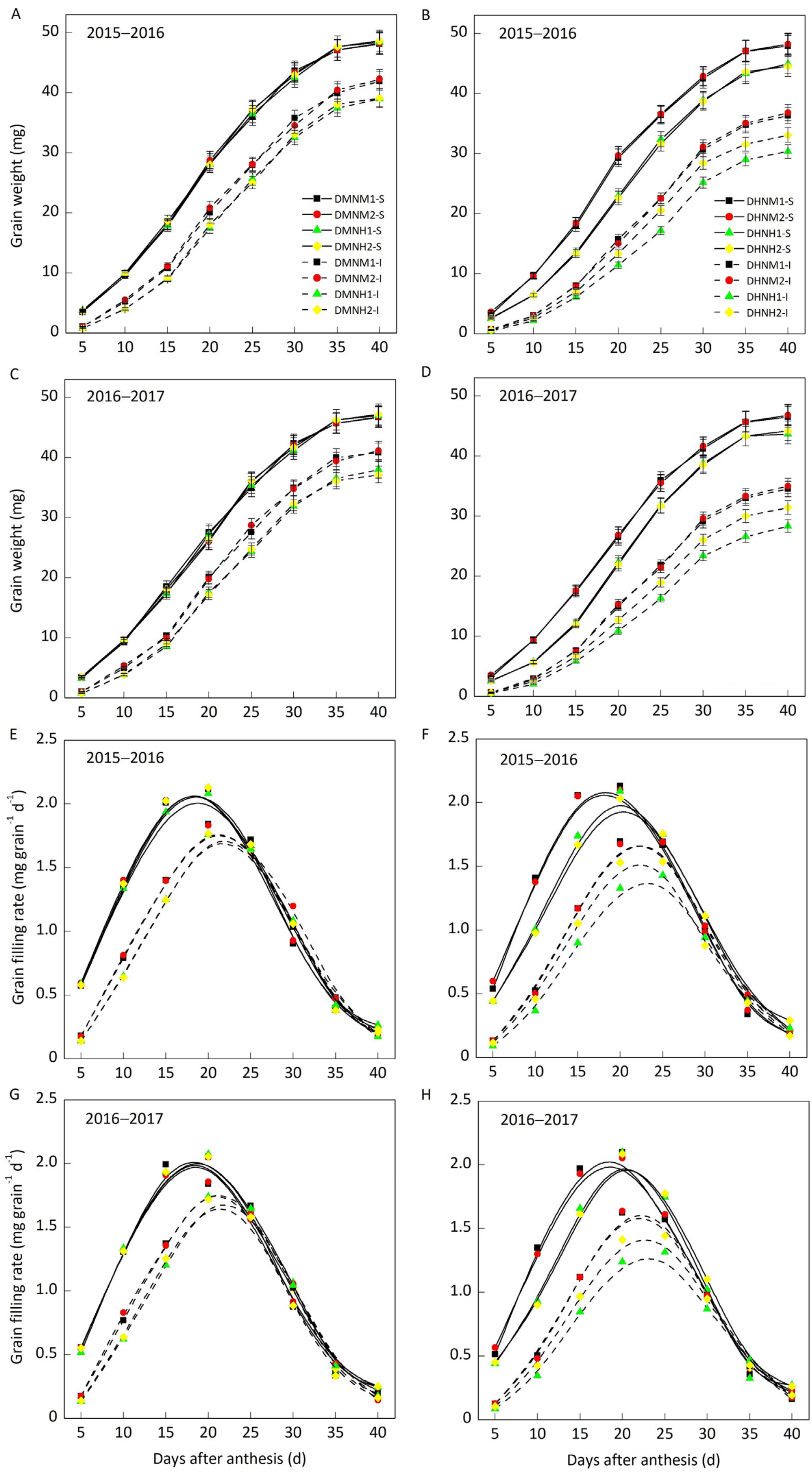
The sucrose content in superior grain was significantly higher than that in inferior grain (Fig. 5). Compared with DM, DH significantly reduced the sucrose content in the superior and inferior grain at 10 days after anthesis (DAA). Under DM, NH had no significant effect on the sucrose content in superior grain, but significantly reduced the sucrose content in inferior grain at 10 days after anthesis. Under DH, NH significantly reduced sucrose content in both superior and inferior grain at 10 days after anthesis. Nitrogen topdressing significantly increased sucrose content in inferior grain at 10 days after anthesis in the DHNH treatment.

Planting density had no significant effect on starch content in superior grain under NM, but DH significantly reduced starch content in superior grain under NH (Fig. 6). High nitrogen significantly reduced starch content in inferior grain under DH. However, NH had no significant effect on starch content in superior grain under DM.

### 3.5. *ABA and ETH*

The ABA content in superior grain was significantly higher than that in inferior grain at 10 days after anthesis. However, the ETH evolution rate of superior grain was significantly lower than that of inferior grain at 10 days after anthesis (Fig. 7). DH increased the ABA content and the ETH evolution rate of superior and inferior grain at 10 days after anthesis, but nitrogen application rate had no significant effect on ABA content or ETH evolution of superior grain. However, NH significantly reduced ABA content and ETH evolution rate of inferior grain at 10 days after anthesis.

ABA content and ETH evolution rate of grain were not significantly correlated with grain-filling rate, active grain filling period, or grain weight (Fig. S3). However, the ABA/ETH ratio was significantly and positively correlated with grain-filling rate and grain weight.


A
2015–2016
B
2015–2016
C
2016–2017
D
2016–2017
E
2015–2016
F
2015–2016
G
2016–2017
H
2016–2017
Grain weight (mg)
Grain filling rate (mg grain⁻¹ d⁻¹)
Days after anthesis (d)
DMNM1-S
DMNM2-S
DMNH1-S
DMNH2-S
DMNM1-I
DMNM2-I
DMNH1-I
DMNH2-I
DHNM1-S
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DHNH1-I
DHNH2-I

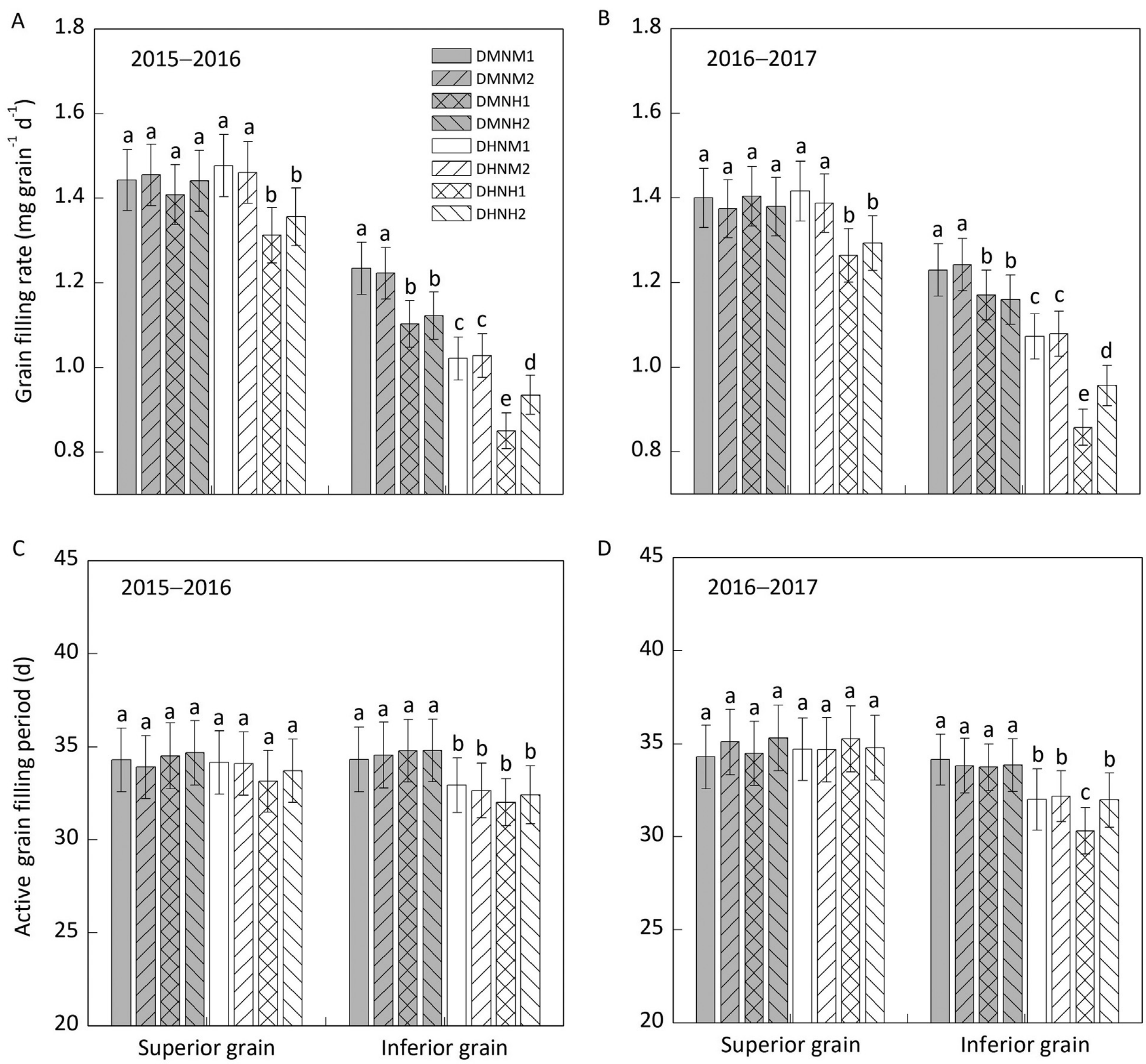



**Fig. 3 – Effect of nitrogen fertilizer and plant density on mean grain-filling rate and active grain-filling period of superior and inferior grain during grain filling period. Vertical bars represent standard deviation. Values for the same grain type followed by different letters are significantly different at P = 0.05. DM, plant density of 3.00 × $10^6$ seedlings $ha^{-1}$; DH, plant density of 5.25 × $10^6$ seedlings $ha^{-1}$; NM, N level of 180 kg N $ha^{-1}$; NH, N level of 270 kg N $ha^{-1}$; 1, N fertilizer was all applied as basal application; 2, N fertilizer was applied half as basal application and half at jointing stage as topdressing.**

### *3.6. SPAD values of flag leaves*

The SPAD values of flag leaves decreased during the grain-filling period (Fig. 8). DH reduced the SPAD values of flag leaves at 20 and 30 days after anthesis and NH increased them during the grain-filling period. Nitrogen topdressing had no significant effect on the SPAD values of flag leaves during the grain-filling period.

**Fig. 2 – Effect of nitrogen fertilizer and plant density on grain weight and grain filling rate of superior and inferior grain during the wheat grain-filling period. Vertical bars represent standard deviation. DM, plant density of 3.00 × $10^6$ seedlings $ha^{-1}$; DH, plant density of 5.25 × $10^6$ seedlings $ha^{-1}$; NM, N level of 180 kg N $ha^{-1}$; NH, N level of 270 kg N $ha^{-1}$; 1, N fertilizer was all applied as basal application; 2, N fertilizer was applied half as basal application and half at jointing stage as topdressing. S, superior grain; I, inferior grain.**

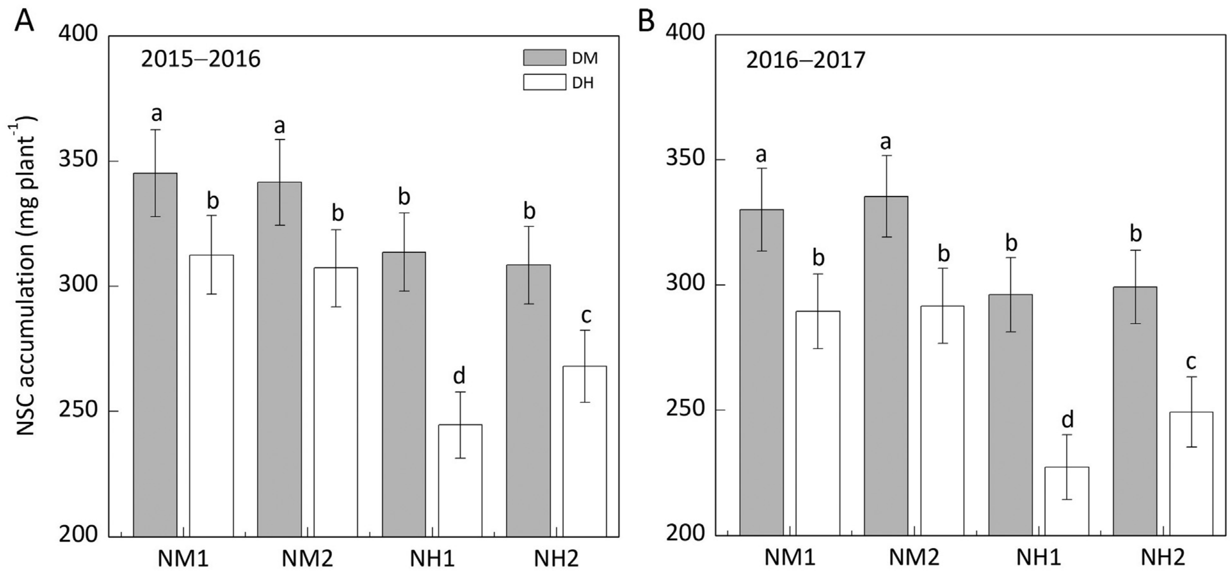



**Fig. 4 – Effect of nitrogen fertilizer and plant density on NSC accumulation in stem at anthesis stage. Vertical bars represent standard deviation. Values followed by different letters are significantly different at $P = 0.05$. DM, plant density of $3.00 \times 10^6$ seedlings $ha^{-1}$; DH, plant density of $5.25 \times 10^6$ seedlings $ha^{-1}$; NM, N level of 180 kg N $ha^{-1}$; NH, N level of 270 kg N $ha^{-1}$; 1, N fertilizer was all applied as basal application; 2, N fertilizer was applied half as basal application and half at jointing stage as topdressing.**

## 4. Discussion

### *4.1. Effects of high nitrogen and high planting density on kernel number per spikelet and grain weight*

In our study, high nitrogen application with high planting density significantly increased wheat spike number per unit area, but reduced kernel number per spike and 1000-kernel weight. This tendency is consistent with findings of previous studies [4,5]. Kernel development and filling in wheat corresponds closely to position on the spike. Feng et al. [36] reported that in wheat, kernel number per spikelet and weight of the grain of middle spikelets were significantly greater than those of top and bottom spikelets, and that the contribution of inferior grain to an increase in wheat yield was greater than that of superior grain. In the present study, kernel number per spikelet and grain weight in spikelets responded differently to nitrogen fertilizer and planting density. With respect to kernel number per spike, both high nitrogen application and planting density reduced the grain number per spikelet of the top and bottom spikelets. The coefficients of variation of the kernel number per spikelet of the top and bottom spikelets were respectively 20.9% and 13.8% over all treatments, values significantly greater than that for the middle spikelets, 5.8%. Thus, the effects of nitrogen fertilizer and planting density on kernel number per spike were strongly governed by the kernel number per spikelet of the top and bottom spikelets. In contrast, both high nitrogen application and high planting density significantly reduced the weight of the grain of the middle spikelets. The coefficient of variation of the grain weight of the middle spikelets over all treatments was 7.43%, which was greater than the values for the top and bottom spikelets (3.72% and 3.09%, respectively). These results indicate that high nitrogen and high plant density reduced 1000-kernel weight mainly by reducing the weight of the grain of the middle spikelet. Thus, nitrogen fertilizer and planting density exerted a grain-position effect on the regulation of kernel number per spike and 1000-kernel weight, whereas the number and weight of kernels at different positions on spikes of wheat responded differently to nitrogen and plant density.

Previous studies have shown that high planting density increases wheat population numbers and intensifies competition between individual plants, while the gain of assimilates is significantly reduced compared with that under low-density conditions [37]. Also, high nitrogen application promotes the development of vegetative organs, reducing the amount of assimilates transported to organs such as spikes [38]. These findings indicate that high nitrogen and high planting density are not conducive to the accumulation of assimilates in spike organs during differentiation. During spike development in wheat, the middle spikelets differentiate first, followed by the top and bottom spikelets [39]. Thus, the middle spikelet has the “master advantage” and a stronger ability to acquire nutrients, while the top and bottom spikelets lag behind. The top and bottom spikelets have relatively weak ability to acquire nutrients and are more sensitive to environmental change because of their delayed differentiation [40]. When the supply of assimilates is restricted, the middle spikelet can obtain a greater assimilate supply because its development of that occurs earlier and is thus more complete, while the top and

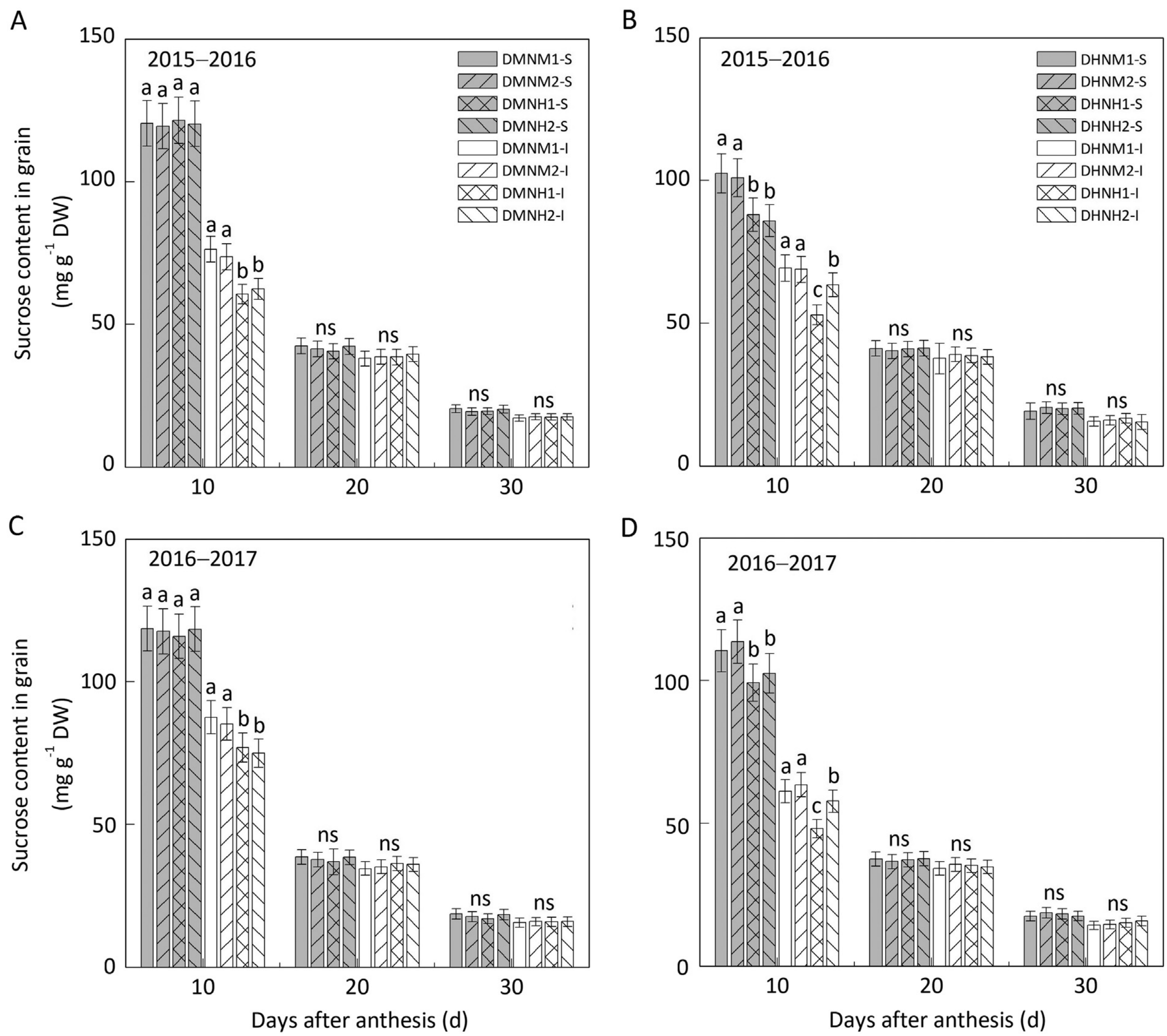



**Fig. 5 – Effect of nitrogen fertilizer and plant density on sucrose content in grain during grain filling period. Vertical bars represent standard deviation. Values for the same grain type and the same day followed by different letters are significantly different at $P$ = 0.05. DM, plant density of 3.00 × $10^6$ seedlings $ha^{-1}$; DH, plant density of 5.25 × $10^6$ seedlings $ha^{-1}$; NM, N level of 180 kg N $ha^{-1}$; NH, N level of 270 kg N $ha^{-1}$; 1, N fertilizer was all applied as basal application; 2, N fertilizer was applied half as basal application and half at jointing stage as topdressing. S, superior grain; I, inferior grain.**

bottom spikelets are more restricted by the supply of assimilates due to later development [41]. Thus, the response of the top and bottom spikelets to high nitrogen and high planting density was more sensitive than that of the middle spikelet, and kernel number per spikelet decreased more on the top and bottom spikelets than the middle spikelet under high nitrogen and high planting density.

Unlike the changes in kernel number per spikelet, the response to nitrogen and planting density of the grain weight of the middle spikelets was more sensitive than that of the upper and lower spikelets. This different sensitivity may be explained by the kernel number per spikelet. A previous study [36] has shown that the kernel number per spikelet on middle spikelets is greater than that on upper and lower spikelets. In our study, the mean kernel number per spikelet on the middle spikelets was between 2.5 and 3.5, while the mean kernel number per spikelet on the upper and lower spikelets was less than 1.6. In addition, the assimilates available to the grain were markedly decreased under high nitrogen application and high planting density. Although the early-developing middle spikelet may be able to accumulate a relatively high amount of assimilates [40], the increased number of kernels on the middle spikelet intensified the competition for assimilates among the kernels at the different positions. As a result, the variation in grain weight of the middle spikelets was significantly greater than that of the upper and middle spikelets, although the grain weight of the middle spikelets was greater than that of the upper and lower spikelets. The superior grain located at the bottom of the middle spikelet and the inferior grain located at

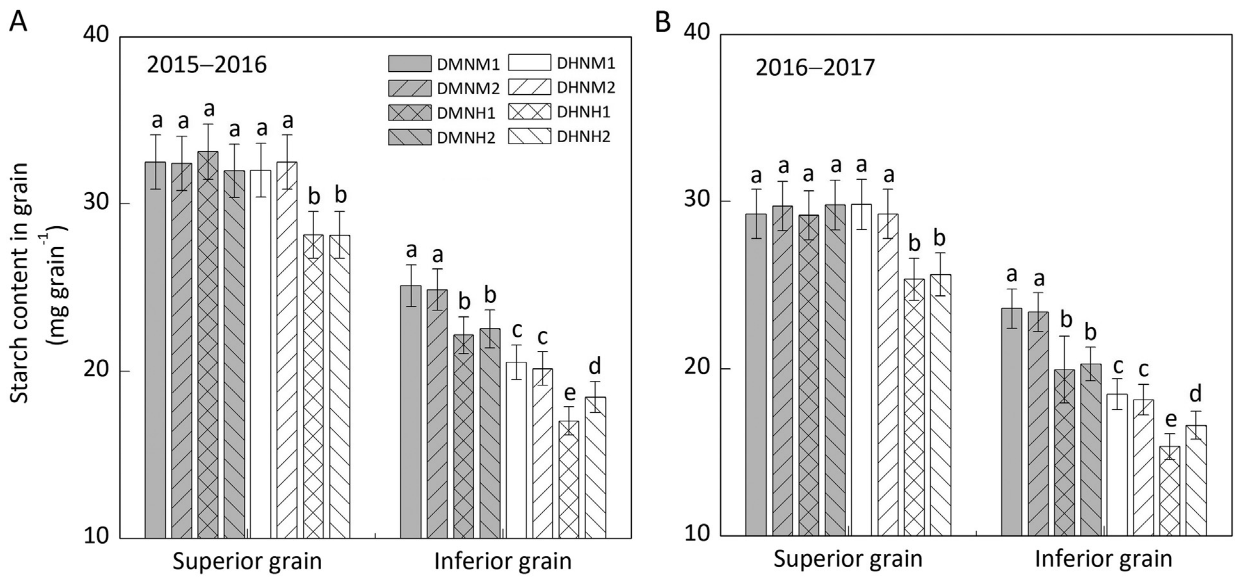



**Fig. 6 – Effect of nitrogen fertilizer and plant density on starch content in grain. Vertical bars represent standard deviation. Values for the same grain type followed by different letters are significantly different at $P$ = 0.05. DM, plant density of 3.00 × $10^6$ seedlings $ha^{-1}$; DH, plant density of 5.25 × $10^6$ seedlings $ha^{-1}$; NM, N level of 180 kg N $ha^{-1}$; NH, N level of 270 kg N $ha^{-1}$; 1, N fertilizer was all applied as basal application; 2, N fertilizer was applied half as basal application and half at jointing stage as topdressing.**

the top of the middle spikelet responded differently to nitrogen and density. The coefficients of variation of the superior grain weight in response to the different nitrogen fertilizer treatments and plant densities in the two growing seasons were 3.47% and 2.99%, respectively, and the coefficients of variation of the inferior grain weight were 11.07% and 12.43%. Thus, the inferior grain weight is more sensitive to nitrogen and density. Previous studies [15,17,30,42] have shown that drought, high temperature, cultivation techniques, and exogenous hormones have greater impacts on the filling of inferior grain than on that of superior grain. Thus, inferior grain is more sensitive to the environment and to agronomic practices. Our results confirmed this and further indicated that high nitrogen and high planting density reduced wheat grain weight mainly by inhibiting the filling of inferior grain on the middle spikelets. Excessive nitrogen application and planting density could not increase grain yield because of the decrease in kernel number per spike and grain weight. A planting density of 3.00 × $10^6$ seedlings $ha^{-1}$ and 180 kg $ha^{-1}$ of N appear to be suitable for local wheat production.

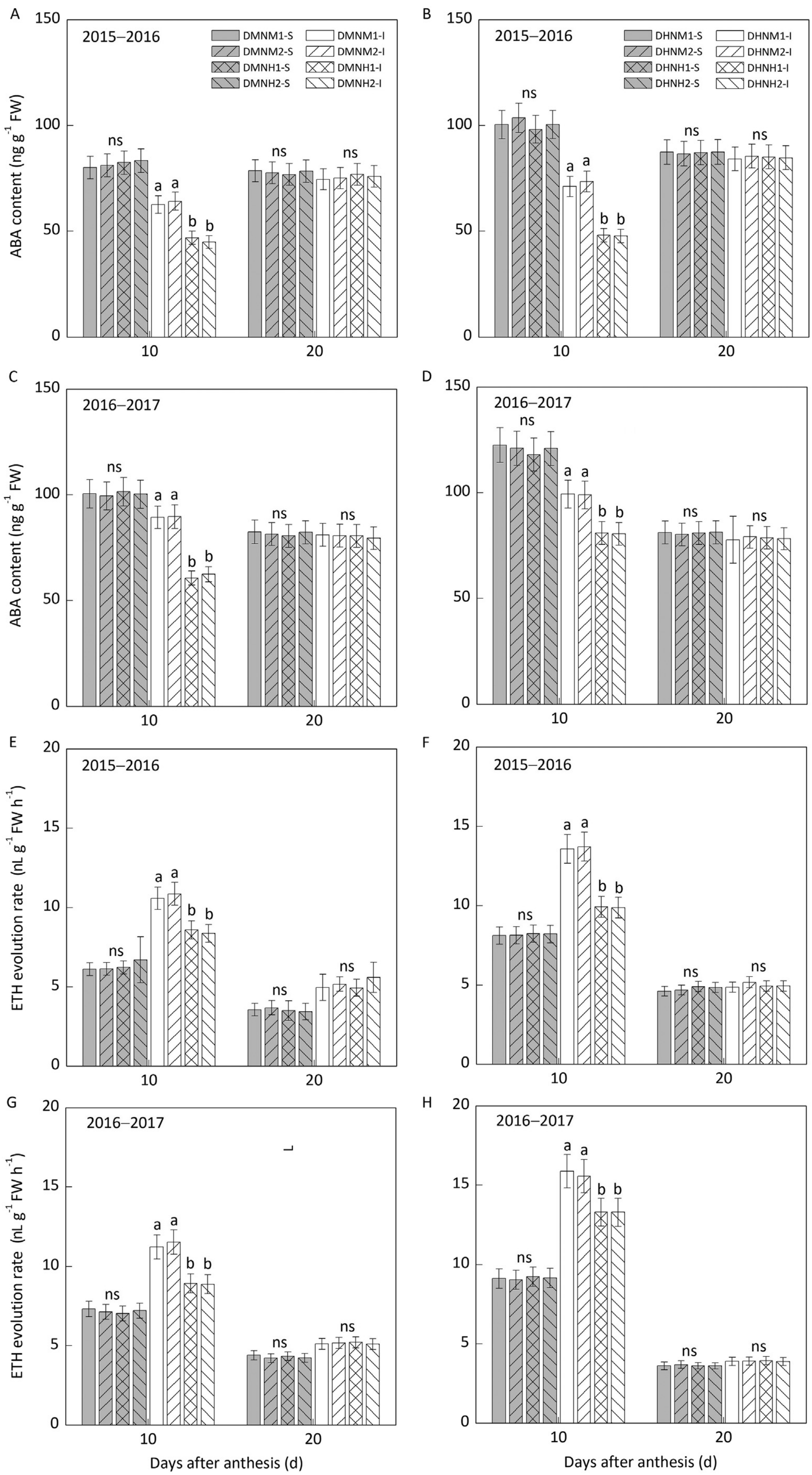
### 4.2. *Effects of high nitrogen and high planting density on the grain-filling characteristics of different types of grain*

Wheat grain weight is determined by grain-filling rate and filling time. There was no significant correlation between the active grain filling period and grain weight (Table S1), indicating that the reducing effect of nitrogen fertilizer and density on grain weight did not correspond to the active filling period. Previous studies have shown that a high plant density leads to premature senescence and a correspondingly reduced number of lower leaves [1,3], reducing the grain-filling time [9]. However, nitrogen application can be expected to postpone the premature senescence of plants and prolong the grain-filling time [19]. Thus, the effects of high nitrogen and high planting density on grain-filling time are inconsistent across studies. The changes in SPAD values indicated that nitrogen fertilizer and density differentially affected leaf senescence. The high plant density aggravated, and the high nitrogen fertilizer reduced, leaf senescence. Thus, interaction between fertilizer and density may cause an inapparent difference between the active filling period and grain weight.

The grain-filling rate is a critical parameter affecting wheat grain weight. The filling of inferior grain was inhibited by high nitrogen application and high planting density, and the grain-filling rate and grain weight showed similar trends, with a significant positive correlation between the two (Table S1). These results indicate that the inhibitory effect of high nitrogen and high planting density on grain weight is caused mainly by the reduction in the filling rate of inferior grain.

Starch is the main component of wheat grain, accounting for approximately 70% of grain weight. The transport of

**Fig. 7 – Effect of nitrogen fertilizer and plant density on ABA content and ETH evolution rate in wheat grain during grain-filling period. Vertical bars represent standard deviation. Values for the same grain type and the same day followed by different letters are significantly different at $P$ = 0.05. DM, plant density of 3.00 × $10^6$ seedlings $ha^{-1}$; DH, plant density of 5.25 × $10^6$ seedlings $ha^{-1}$; NM, N level of 180 kg N $ha^{-1}$; NH, N level of 270 kg N $ha^{-1}$; 1, N fertilizer was all applied as basal application; 2, N fertilizer was applied half as basal application and half at jointing stage as topdressing; S, superior grain; I, inferior grain.**



A
B
C
D
E
F
G
H
2015–2016
2016–2017
DMNM1-S
DMNM2-S
DMNH1-S
DMNH2-S
DMNM1-I
DMNM2-I
DMNH1-I
DMNH2-I
DHNM1-S
DHNM2-S
DHNH1-S
DHNH2-S
DHNM1-I
DHNM2-I
DHNH1-I
DHNH2-I
ABA content (ng g-1 FW)
ETH evolution rate (nL g-1 FW h-1)
ns
a
b
10
20
Days after anthesis (d)

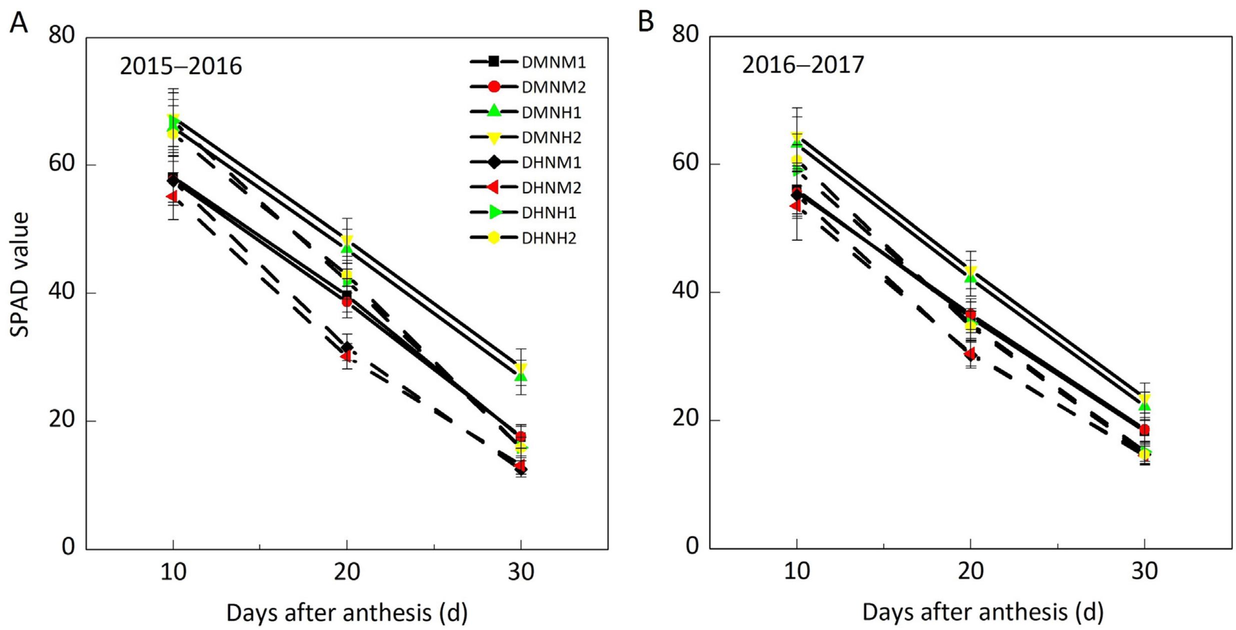



**Fig. 8 – Effect of nitrogen fertilizer and plant density on SPAD value of flag leaves during grain filling period. Vertical bars represent standard deviation. DM, plant density of 3.00 × $10^6$ seedlings $ha^{-1}$; DH, plant density of 5.25 × $10^6$ seedlings $ha^{-1}$; NM, N level of 180 kg N $ha^{-1}$; NH, N level of 270 kg N $ha^{-1}$; 1, N fertilizer was all applied as basal application; 2, N fertilizer was applied half as basal application and half at jointing stage as topdressing.**

sucrose from vegetative organs to the grain and the starch synthesis process in the grain strongly influence the grain filling and grain weight of wheat [18]. In general, the weak ability of inferior grain to acquire sucrose often results in insufficient amounts of substrate for grain filling, poor grain filling, and low grain weight [17,19]. High nitrogen and high planting density significantly reduced the sucrose content in the inferior grain at 10 days after flowering, indicating that these treatments inhibited the transfer of sucrose from the stems and sheaths to the inferior grain. In agreement with a previous finding [5], high nitrogen and high planting density markedly decreased the percentage of tillers with ears (Fig. S1). Thus, high nitrogen application and high planting density increases competition within the wheat population and tillers with ears may accumulate relatively low amounts of carbohydrates. This may lead to a reduction in the amount of carbohydrates transported to the grain.

High nitrogen increased the SPAD values of flag leaves, which may in turn promote carbohydrate synthesis post-anthesis. However, high nitrogen did not promote wheat grain filling. Balotf et al. [43] stated that nitrogen promotes the synthesis of carbohydrates and their transport to the grain by promoting photosynthesis,. However, Liang et al. [6] showed that the amount of carbohydrates transported to the grain decreased under high-nitrogen conditions, owing to the large amount of carbohydrates consumed for the vigorous growth of vegetative organs such as stems and leaves under high nitrogen conditions [38]. Thus, the effects of nitrogen on carbohydrate transport can vary. In the present study, high nitrogen increased the SPAD values of the flag leaves. However, high nitrogen also promotes the growth of vegetative organs such as leaves, and this consumes many carbohydrates and may reduce their transport to the grain. In the present study, high nitrogen and high plant density reduced the harvest index (Fig. S2). Thus, under high nitrogen and high plant density, less sucrose accumulated in the grain. Owing to the reduction in the amount of substrates caused by the decrease in sucrose content in the grain, the synthesis of starch is inhibited, and the grain weight decreases.

### *4.3. Association between hormone activity and grain filling in response to high nitrogen and high plant density*

Hormones play an important role in the regulation of grain filling of crop plants. ABA and ETH affect wheat grain filling [44]. However, in the present study, ABA and ETH levels in grain were not significantly correlated with grain-filling rate, active grain-filling period, or grain weight (Fig. S3). This does not mean that ABA and ETH have no effect on grain filling in wheat. High plant density significantly increased ABA content and ETH evolution rate of inferior grain, but high nitrogen fertilizer reduced these properties. Thus, high plant density and high nitrogen fertilizers exerted opposite regulatory effects on ABA and ETH. This antagonism may account for the absence of correlations between ABA and ETH levels and grain filling, but the levels are regulated by nitrogen and plant density. A previous study [19] has suggested that the regulatory effects of hormones on grain filling depend not on the content of individual hormones but rather on the balance of hormones. Yang et al. [44] reported that, under drought stress, grain filling of wheat was affected by the ABA/ETH ratio, and there was a significant positive correlation between the ABA/ETH ratio and the grain filling rate. Lyu et al. [45] suggested that the regulatory effects of potassium on wheat grain filling were relate to ETH, CTK, and other hormones. In the present study, the ABA/ETH ratio was positively correlated with grain-filling rate and grain weight, suggesting that the regulation by nitrogen fertilizer and plant density of wheat grain filling may depend on the balance between these two hormones. The ABA/ETH ratio was positively correlated with grain-filling rate but not the active filling stage. Thus, ABA and ETH may participate in the regulation of grain filling by affecting grain-filling rate. Nitrogen and plant density had no significant effect on ABA content and ETH evolution rate of superior grain. Thus, the balance between ABA and ETH may regulate the grain-filling rate of inferior grain, in turn affecting the grain filling of wheat in response to nitrogen and plant density. Previous studies [46,47] have shown that ABA and ETH are involved in regulation of sucrose transport and starch synthesis in crop plants; ABA promotes sucrose transport and starch synthesis, while ETH plays an opposite role. Combined with these results, our results suggest that, under various nitrogen and plant density treatments, the balance between ABA and ETH is involved in the regulation of sucrose transport and starch synthesis. A greater ABA/ETH value is beneficial to the transport of sucrose to the grain and the synthesis of starch within the grain, thus improving the grain-filling rate, promoting grain filling, and increasing grain weight. However, the specific regulatory mechanism by which ABA and ETH affect wheat grain filling under high nitrogen and high plant density awaits further study.

In this study, we used one cultivar, Xinong 979, for our experiments. Xinong 979 is one of the main wheat cultivars grown on the northern plain, the most important wheat production region in China. However, there were significant genotypic differences in wheat growth in response to nitrogen fertilizer and plant density [1,5]. A single cultivar may not display the full response of wheat grain filling to nitrogen and plant density. Studies with more cultivars will be needed to elucidate the physiological mechanisms underlying the effects of nitrogen and plant density on wheat grain filling.

## 5. Conclusions

High nitrogen and high planting density increased spike number per unit area but reduced kernel number per spike and 1000-kernel weight. A planting density of $3.00 \times 10^6$ seedlings $ha^{-1}$ and an N application rate of 180 kg $ha^{-1}$ appear to be an effective practice for local wheat production. The inhibitory effect of high nitrogen application and high planting density on kernel number per spike was achieved mainly by a reduction in kernel number per spike on top and bottom spikelets. High nitrogen application and high planting density reduced 1000-kernel weight mainly by reducing the weight of the grain of middle spikelets. High nitrogen and planting density reduced the grain-filling rate of the inferior (top) grain in the middle spikelets during the early and middle grain-filling periods, inhibiting grain filling and reducing grain weight. There was no relationship between grain weight and the active filling period of wheat. High nitrogen and planting density inhibited sucrose transport and starch accumulation in inferior grain of middle spikelets by reducing the ABA/ETH ratio. This reduction may be a physiological mechanism underlying the inhibitory effects of high nitrogen and high planting density on the filling of inferior wheat grain.

## CRediT authorship contribution statement

Wenzhao Liu and Yuncheng Liao conceived and designed the experiments; Yang Liu performed the experiments and analyzed the data. Yang Liu and Wenzhao Liu wrote the paper.

## Declaration of competing interest

Authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2020.06.013.

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