Responses of photosynthetic characteristics and enzyme activity of nitrogen metabolism to low nitrogen in maize with different nitrogen tolerance

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Abstract: Understanding the physiological processes associated with leaf photosynthetic characteristics and nitrogen (N) assimilation during grain-filling stage are helpful for enhancing nitrogen utilization efficiency (NUtE) of maize.   In this study, the leaf photosynthetic and N assimilation parameters in maize, including Zhengdan 958 (ZD958), a low-N tolerance cultivar and Huanong 138 (HN138), a low-N sensitive cultivar under different N rates were examined.   Results showed that ZD958 displayed significant increases on grain yield and NUtE than that in HN138.   Analyses on the leaf photosynthetic and N assimilation-associated processes indicated that ZD958 had higher leaf N remobilization (Rem N), net photosynthetic rate (Pn) and photosynthetic N use efficiency (PNUE) with respect to those of HN138 during grain-filling stage.   In addition, ZD958 was also shown to be higher activities of leaf nitrate reductase (NR), glutamine synthetase (GS), nitrate reductase (GDH) and glutamine synthetase (GAGOT) than those of HN138.   The leaf PNUE was significantly positively correlated with NR, GS, GDH, GOGAT suggesting that leaf PNUE and NR, GS, GDH, GOGAT jointly determined the N remobilization efficiency and the leaf N remobilization during post-silking.   These results suggested that ZD958 possessed improved PNUE, NR and GS activities in leaves during grain-filling stage that contributes improve grain weights and yield formation capacities upon under low-N conditions.

Keywords: low-N, photosynthesis, nitrogen assimilation, nitrogen utilization efficiency, maize

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1   Introduction

Nitrogen (N) is the critical limiting nutrient for most crops. Globally, over 110 million tons of N fertilizer is applied annually to maximize crop yields.   Application of N fertilizers contributes greatly to the increase of the grain protein content, biomass and economic yield of maize.   However, excessive nitrogen applications can reduce nitrogen use efficiency (NUE), which pose a potential threat to the ecological environment[1,2].   Increasing maize N use efficiency (NUE) is an important approach to reduce fertilizer application and improve resource utilization efficiency. NUE is contributed by N utilization efficiency and N uptake

efficiency[3].   Differences in N uptake and utilization vary significantly among genotypes[4,5], The selection of N-efficient cultivar varieties (cultivars with higher grain yield under low N condition) plays a significant role in improving the NUE[6,7]. Therefore, improvement on plant NUE through breeding high NUE cultivars together with suitable N fertilizer management can help accomplish the goal for optimized high yield.

In the maize reproductive stage, a portion of N storage in vegetative organs is remobilized to grain, of which, that stored in leaves contributes largely to the grain N accumulation[8].   The N of leaves is also required for photosynthesis to produce dry matter, hence, grain yield[9].   Additionally, excessive leaf N remobilization can lead to early senescence of leaf tissue. Therefore, it is a great challenge to increase N remobilization from leaves while ensuring high photosynthesis during the grain filling stage in maize.   Photosynthetic N use efficiency (PNUE) represents the photosynthetic capacity of N content per unit leaf area, which is an important index indicating whether the photosynthetic capacity of the leaves and N use efficiency are coordinated[8].   Numerous studies have reported that the modern maize cultivars have higher green leaf area duration, leaf N content, and post-silking N uptake[10-12].   Moreover, the maize cultivars to be low-N tolerant display higher net photosynthetic rate (Pn) and PNUE than those to be low-N sensitive under N deprivation conditions[8,13-15].   The N-efficient cultivar (ZD958) had higher Pn and PNUE than N-inefficient cultivar (QS101) at low N application

rates[16].   Simultaneously achieve high yield and N remobilization efficiency, a maize cultivar might maintain the PNUE of lower leaves while increasing the PNUE of upper and middle leaves at the canopy-level[8].

In plants, several physiological studies and recent genomic approaches have identified genes encoding enzymes involved in N metabolism that are specifically activated during N remobilisation[17,18].   Gutamine synthetase (GS) appeared to be one of the most characteristic biochemical events occurring during the transition from N assimilation to N remobilization[19-21].   The GDH is an enzyme capable of catalysing glutamate de-amination as well as glutamate synthesis, carries out the de-amination reaction in source leaves[22].   It has also been shown that GOGAT is still functional in senescing leaves and can participate in glutamate synthesis.   These enzymes represent putative key reactions that may influence grain yield and its components[23].   In some crops, the N-efficiency or low-N tolerant genotype sustained relative higher NR and GS enzyme activities than those of the N-inefficiency or low-N sensitive genotype, such as in maize[24,25], wheat[26] and barley[27].

Currently, although the process related to N uptake and remobilization were performed in maize, the physiological mechanisms associated photosynthetic parameters and N metabolism characteristic in ear-leaf of different low-N tolerances cultivars in response to N application are still largely characterized.   The low-N tolerant maize cultivar possess higher photosynthetic function and much more improved N assimilation characteristic during post-silking stage were hypothesized.   A 2-year field study using two maize cultivars with drastically different low-N tolerant were performed.   The objectives of the study were to: (i) determine the physiological processes difference related to photosynthesis parameters and N metabolism in ear-leaf between low-N tolerant and low-N sensitive cultivars in maize.   (ii) Analyze the correlations between these markers were then evaluated to determine whether or not they are linked to each other regardless of the genetic background, N application conditions, developmental stage.   This study provides insights into the N-associated physiological processes that maize species responds to N deprivation and puts forward guidance for breeding low-N tolerant and high NUtE maize cultivars under low-N conditions.

2 Materials and methods

2.1 Site description

The field experiments were conducted during the 2017 and 2018 growth seasons at Experimental Station of Hebei Agricultural University (43°31′N, 124°48′E), Xinji city, China.   The annual means on air temperature and precipitation from 1980 to 2010 were 13.8°C and 516.4 mm at the station, respectively.   The daily air temperature and precipitation of 2017 and 2018 maize growth seasons were showed in Figure 1.   The soil (depth 20 cm) texture for experiment was clay loam (ISSS classification, International Soil Science Society), with bulk density of 1.47 g/cm^3, and organic matter 18.47 g/kg, total nitrogen 1.25 g/kg, alkali-hydrolyzable N 91.55 g/kg, extractable Olsen-P 27.50 mg/kg, and ammonium acetate extractable K 145.79 mg/kg, pH 7.8.
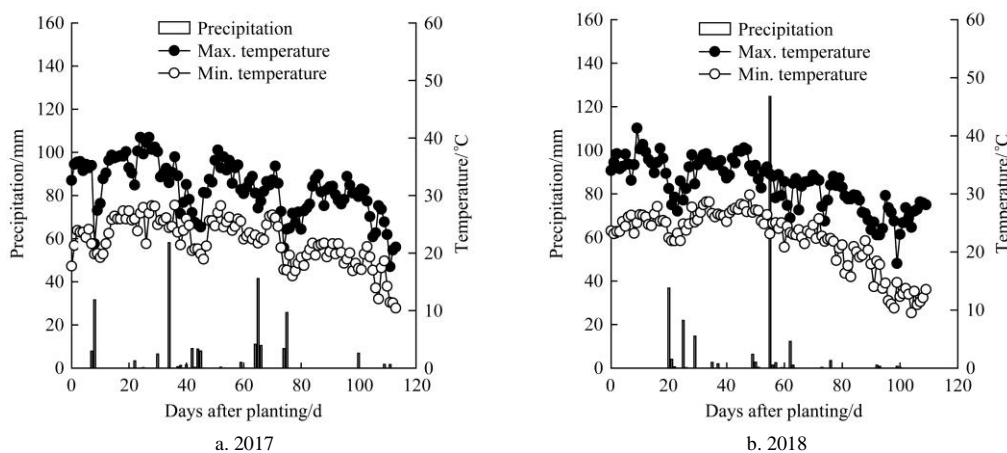

a. 2017   b. 2018

Figure 1   Daily precipitation and temperature during the 2017 and 2018 growing season of summer maize

2.2 Experimental design

Two maize cultivars, including Zhengdan 958 (ZD958) was to be low-N tolerant cultivar[28,29] and Huanong 138 (HN138) was to be low-N sensitive cultivar[28], were used in this study.   Three N application rates, including 0, 120 and 240 kg N/hm² (referred to as N0, N120 and N240, respectively), were established using urea as the N source.   Of which, half was applied as basal and the other half was to topdressing at V9 stage (i.e. the 9th leaf in plants was fully expanded).   The cultivar and N rate were established based on a split plot design with three replicates, using nitrogen rate as main plots and cultivar as sub-plots.   Seeds of maize were sown on June 14th, 2017, and on June 18th, 2018, respectively.   The maize seedlings were thinned at the V5 stage to achieve a standard density of 67 500 plants/hm².   Each cultivar was planted in a distinct plot (20 m in length, with 8 rows).   Basal phosphorus (90 kg/hm² P_2O_5) and potassium (120 kg/hm² K_2O) were used for all of the plots.   Other cultivation practices, such as irrigation (prior to sowing and at the heading stage), removal of weeds, and chemical control of diseases and pests, were performed in accordance with the recommended conventional approaches in North China Plain.   The harvest dates during two growth seasons were October 5th, 2017, and October 7th, 2018.

2.3 Assessment of dry matter amounts, yield components and grain yield

The above-ground tissues of plants were harvested from silking to maturity with interval of 10 day each.   Five plants of medium three rows per plot were sampled, then separated into various tissues, including leaves, stalks, cobs, husks, and grains (leaves and stalks at silking).   All tissue samples were oven dried at 105°C for 30 min and dried to constant weight at 70°C.   Dry mass of the tissues was ground into powder after obtaining weight to assess contents of nutrients.   Briefly, appropriate amount (0.3 g) of the ground materials were used to determine total nitrogen content by a modified Kjeldahl digestion method[30].   At maturity,

the cobs from three center rows of each plot were collected to determine grain yield (moisture content 14%)[31]. One hundred-grain weights were determined, and the grain numbers per ear were recorded. N uptake of plants was calculated based on N concentration and the total above-ground biomass.

2.4   Assessment of photosynthetic parameters in leaf

Leaf area and Pn were measured every 10 d from silking to physiological maturity. Five plants with the same silking date were tagged in each plot. The five plants used for determination of Leaf area by the formula[32]. Leaf area (cm^2) was calculated as by leaf length (cm) ×maximum leaf width (cm) × 0.75. Leaf area index was calculated as by plant leaf area / land area. The ear-leaf of five plants per plot was measured Pn on clear sunny day between 09:00a.m. and 11:00a.m, using a portable photosynthetic system (CIRAS-3 USA) with a controlled light intensity of 1600 $\mu mol/m^2$ s. The CO_2 concentration in leaf chamber was controlled at 400 μmol CO_2/mol air. Using the leaf area, leaf dry weight, and leaf N content, the specific leaf N (SLN, g N/m^2) was calculated as N content (g) per leaf area (m^2). PNUE (μmol CO_2/g N s) was calculated as P_n per SLN (Sinclair et al. 1989). Leaf N content was calculated as leaf dry weight by N concentration.

2.5   Assessment of N assimilation enzymatic activities in leaf

The ear-leaf from five plants was sampled between 09:00 a.m. and 11:00 a.m. from silking to physiological maturity with interval of 10 d each. The fresh samples were immediately frozen in liquid nitrogen and stored at –80 ℃ for measurement the enzymatic activities of nitrate reductase (NR), glutamine synthetase (GS), nitrate reductase (GDH) and glutamine synthetase (GAGOT).

The activity of NR was measured using the method[33]. Fresh leaf samples (0.5 g) were placed in 10 mL of an incubation medium, which was 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 M KNO_3 with 1% (v/v) propanol. Prior to the assay, the buffer solution was purged with N_2 gas for 30 min to remove any dissolved oxygen. The samples were then vacuum infiltrated (two times) and subsequently incubated in a water bath at 30 ℃ for 30 min in the dark. 1 mL samples were withdrawn, and the color reaction was initiated by adding 2 mL of aminobenzenesulfonic acid and 2 mL of a-naphthylamine. After 20 min of incubation, the amount of nitrite (NO_2^-) was determined by absorbance at 520 nm using a standard curve by a spectrophotometer.

To assay the activity of GS, fresh leaf samples (0.5 g) of leaf tissue were homogenized with 3 mL of 10 mM Tris-HCl buffer (pH 7.6, containing 1 mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol) via a chilled mortar and pestle. The homogenate was centrifuged at 15 000×g for 30 min at 4 ℃. The supernatant was subsequently used to determine enzymatic activity. The glutamine synthetase activity was assayed according to the methods[30]. The reaction mixture contained 80 mM Tris-HCl buffer, 40 mM L-glutamic acid, 8 mM ATP, 24 mM $MgSO_4$, and 16 μM NH_2OH in a final volume of 1 mL. The final pH was 8.0. The reaction was initiated by the addition of 0.1 mL of enzyme extract. After incubation for 30 min at 30 ℃, the reaction was stopped by adding 2 mL of 2.5% (w/v) $FeCl_3$ and 5% (w/v) trichloroacetic acid in 1.5 M HCl. After centrifugation at 3000×g for 10 min, the absorbance of the supernatant at 540 nm was recorded by a spectrophotometer.

To assay the activity of GDH, the extraction buffer (pH 7.9) was consisted of 0.05 M imidazole and 5 mM DTT. Fresh leaf samples (1 g) were ground in liquid nitrogen and then centrifuged at 12000×g for 40 min at 4 ℃. The supernatant was used to measure the activity of glutamate dehydrogenase according to the methods[34].

To assay the activity of GOGAT, the extraction buffer contained 10 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM mercaptoethanol. Fresh leaf samples (2 g) were centrifuged at 12000×g for 30 min at 4 ℃. The standard assay mixture was consisted of 40 mM potassium phosphate buffer (pH 7.5), 10 mM L-glutamine, 10 mM 2-oxoglutarate, 0.14 mM NADH, and crude enzyme (final volume of 3 mL). The increase in absorbance was recorded at 340 nm for 3-4 min at room temperature (25 ℃).

2.6   Measurement of soil nitrate nitrogen

At sowing silking and harvest, four soil samples per plot were taken using an auger. In 2018, soil samples to a depth of 60 cm at 20 cm increments were taken. Soil samples were extracted using 0.01 mol/L $CaCl_2$ and analyzed for NO_3^--N using the continuous flow analysis (TRAACS 2000, Bran and Luebbe, Norderstedt, Germany).

2.7   Calculation of N-associated traits

Calculations for leaf N remobilization and the N efficiency parameters were determined as the N content between vegetative and reproductive stages utilizing the next equations[35-37].

Leaf N remobilization (leaf RemN) = leaf N content at silking – leaf N content at sample date after silking

Nitrogen utilization efficiency (NUE) = grain yield/plant N content at maturity.

Nitrogen harvest index (NHI, %) = grain N content/plant N content at maturity × 100

2.8   Statistical analysis

Statistical evaluations were performed using IBM SPSS version 19.0 software. All data in the figures and tables were analyzed by analysis of variance (ANOVA) and were compared using the least significant difference (LSD) test at $p<0.05$ probability level. All figures were constructed using SigmaPlot 10 (Systat Software Inc. San Jose, CA, USA). The relevance relevant analysis was were carried out using the R Studio.

3   Results and discussion

3.1   Grain yield, NUtE and dry matter accumulation

Significant differences were found in grain yield, kernel numbers, NtUE and NHI between the two maize cultivars, whereas no significant differences in the ear numbers and 100-grains weights. Grain yield, kernel numbers, NUtE and NHI were higher in ZD958 than that in HN138 (Tables 1 and 2). Across all N rates, the grain yields were higher than those in HN138 by 2.28 t/hm^2 and 2.56 t/hm^2 in 2017 and 2018, respectively, this due to the higher kernel numbers. The year had significant effects on kernel numbers, NUE and NHI, but not on grain yield. Kernel numbers was higher in 2017 than 2018, while NUE and NHI were higher in 2018 than 2017. N rate had significant effects on grain yield, kernel numbers, NUE and NHI, nitrogen-deficiency (N120 and N0) stress decreased grain yield, kernel numbers, NUE and NHI.

No significant difference was found in dry matter accumulation at before-silking between two maize cultivars (Figure 2), whereas the dry matter accumulation at post-silking and maturity stage showed significant difference between ZD958 and HN138, and the two parameters significantly affected by the year and the values in 2017 higher than that in 2018. The dry matter accumulation at maturity stage of ZD958 were higher than that in HN138, this due to the higher dry matter accumulation at post-silking stage. In 2017 year, the dry matter accumulation at post-silking and maturity stage in ZD958 were higher 6.19% and 7.68% than those in HN138

under high-N rate (N240), whereas higher 10.41% and 8.60% than those in HN138 under low-N rate (N120). Similarly results showed that in 2018 year, the dry matter accumulation at post-silking and maturity stage in ZD958 were higher 2.45% and 3.98% than those in HN138 under high-N rate (N240), whereas higher 2.03% and 3.96% than those in HN138 under low-N rate (N120). These results suggest that ZD958 possess higher dry matter accumulation at post-silking stage under low-N condition.

3.2 Genotypic variation in leaf photosynthetic parameters

Significant differences were found in leaf area index and Pn between cultivar (H), nitrogen (N) and sample date (S) and the interaction of H×N, S×N (Table 3). The significant differences were found in leaf area index and Pn between the two maize cultivars and three N rates (Figure 3), whereas no significant differences in the ear numbers and 100-grains weights. Leaf area

index and Pn were higher in ZD958 than that in HN138 each N rates and sample dates. The leaf area index decreased significantly from 0 DAS to 50 DAS. The effect of cultivar and N rate on the leaf area index was significant. The leaf area index of ZD958 was higher than that of HN138 across two years. In 2017 year, from silking (0 DAS) to maturity (50 DAS), the leaf area index of ZD958 and HN138 decreased by 25.18% and 18% under high-N rate (N240), decreased by 27.29% and 27.78% under low-N rate (N120). Similarly results showed that in 2018 year, the leaf area index of ZD958 and HN138 decreased by 25.96% and 19.43% under high-N rate (N240), decreased by 20.71% and 34.17% under low-N rate (N120). ZD958 rapid down leaf senescence after silking under high-N rate (N240) and slows down leaf senescence after silking under low-N rate (N120), but ZD958 possess higher leaf area index in the whole grain-filling period in two years.

**Table 1   Effects of nitrogen supply on grain yield and its components of ZD958 and HN138 in 2017 and 2018**

Year	N rate	Cultivar	Grain yield/t hm^{-2}	Ear numbers/10^4 ears hm^{-2}	Kernels numbers	100-grains weights/g	NUE/kg kg^{-1}	NHI/%
2017	N240	ZD958	11.17a	6.67a	530.89a	31.58a	57.25a	72.66a
		HN138	10.25b	6.66a	477.44b	34.24a	55.99b	68.55b
	N120	ZD958	10.18a	6.46a	520.91a	30.27a	70.31a	76.16a
		HN138	9.08b	6.31a	449.24b	30.15a	68.99b	70.49b
	N0	ZD958	9.21a	6.41a	479.62a	29.95a	75.09a	79.98a
		HN138	7.95b	6.05b	417.31b	31.51a	70.26b	70.09b
2018	N240	ZD958	11.97a	6.36a	527.47a	35.67a	64.87a	78.21a
		HN138	11.24a	6.18a	495.52b	36.01a	59.80b	73.91b
	N120	ZD958	10.40a	6.03a	515.27a	33.51a	77.23a	78.00a
		HN138	9.01b	5.67b	492.97a	35.29a	72.24b	72.44b
	N0	ZD958	10.16a	5.83a	510.68a	34.12a	83.20a	76.20a
		HN138	8.72b	5.58b	480.37b	35.06a	79.21b	72.46b
Analysis of variance								
Year (Y)			NS	*	*	NS	*	NS
Cultivar (C)			**	NS	**	NS	*	*
Nitrogen (N)			**	*	**	NS	**	NS
Y×C			NS	NS	*	NS	*	NS
Y×N			NS	NS	NS	NS	NS	NS
C×N			NS	NS	NS	NS	NS	NS
Y×C×N			NS	NS	NS	NS	NS	NS

Note: N240, N120 and N0 indicate 240, 120 and 0 kg N ha^{-1}, respectively. The values followed by different lowercase letters in a column are significantly different at the 0.05 level; ** $p < 0.01$; * $p < 0.05$; NS means insignificant.

Table 2   Analysis of variance of years, cultivars and nitrogen rates on LAI, Pn, total N, PNUE and NR, GS, GDH, GAGOT of leaves in ZD958 and HN138

Analysis of variance	LAI	Pn	PNUE	Total N	Rem N	NR	GS	GDH	GAGOT
Year (Y)	NS	NS	*	*	*	NS	NS	NS	NS
Cultivar (C)	**	**	**	**	**	*	*	*	*
Nitrogen (N)	**	**	**	**	**	**	**	**	**
Sample date (S)	**	**	**	**	**	**	**	**	**
Y×C	NS	NS	*	*	*	NS	NS	NS	NS
Y×N	NS	NS	*	*	*	NS	*	NS	*
C×N	**	**	*	*	*	**	**	**	**
Y×S	NS	NS	NS	NS	NS	NS	NS	NS	NS
S×N	**	**	**	**	**	**	**	**	**
Y×C×N	NS	NS	NS	NS	NS	NS	NS	NS	NS
Y×C×S	NS	NS	NS	NS	NS	NS	NS	NS	NS
S×C×N	NS	NS	NS	NS	NS	NS	NS	NS	NS
Y×C×N×S	NS	NS	NS	NS	NS	NS	NS	NS	NS

Note: LAI: leaf area index; Pn: photosynthetic rate; PNUE: photosynthetic nitrogen use efficiency; Rem N: remobilization N; NR: nitrate reductase activity; GS: glutamine synthetase activity;. **, $p < 0.01$; *, $p < 0.05$; NS, not significant.
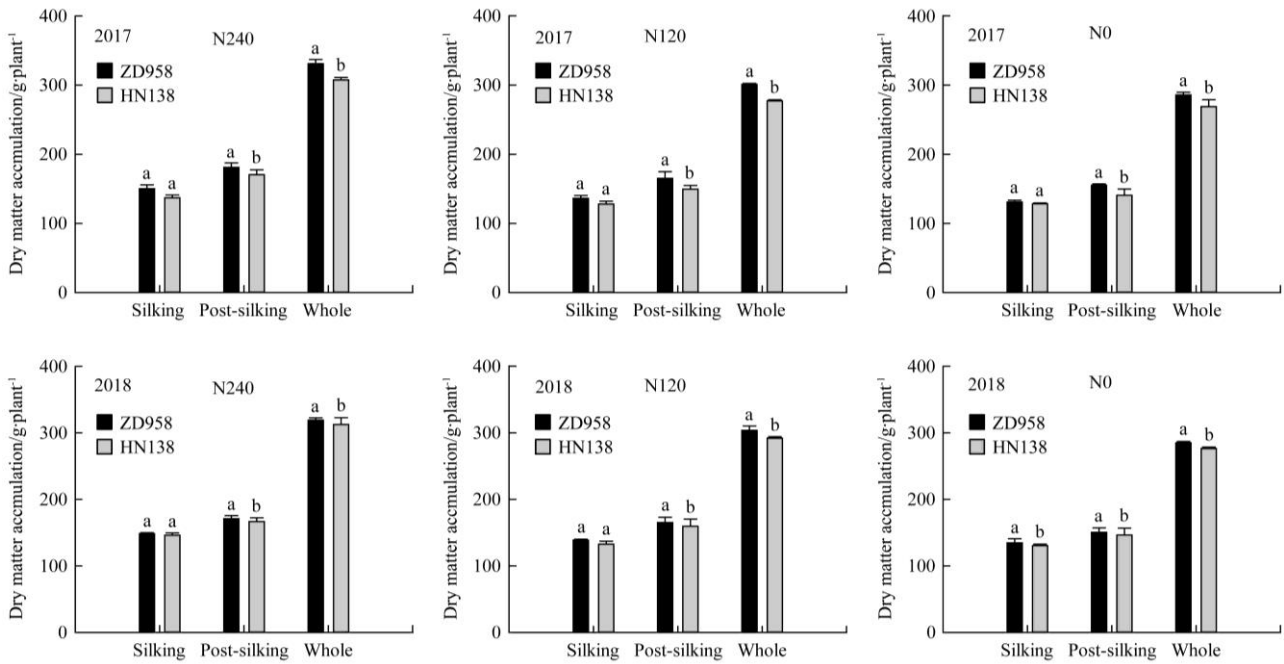
Note: N240, N120 and N0 indicate 240, 120 and 0 kg N/hm², respectively.   The data are presented as the meaNS ± SEs.   The values followed by different lowercase letters are significantly difference at the 0.05 level.

Figure 2   Dry matter accumulation of ZD958 and HN138 under three N rates at the silking, post-silking and whole (maturity) stages
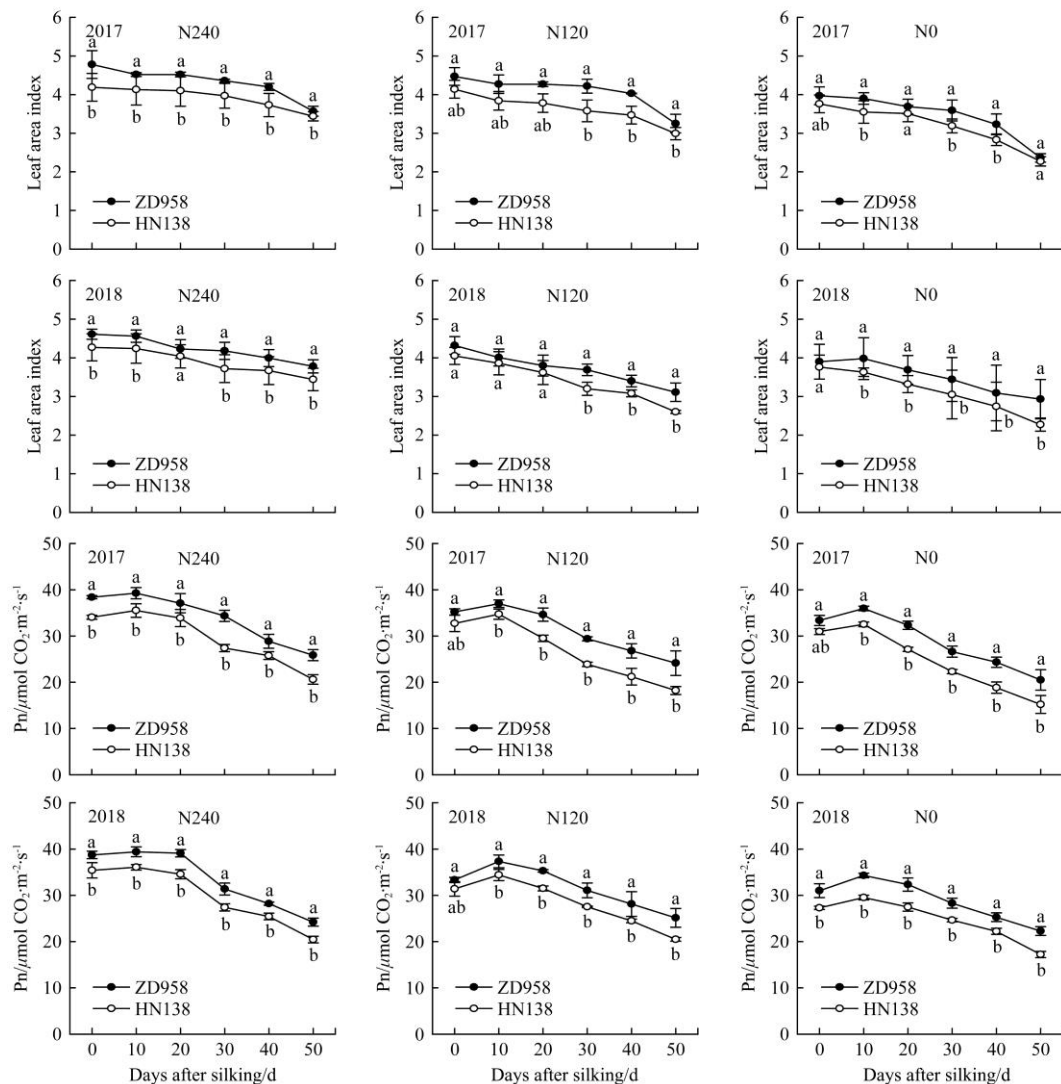
Note: N240, N120 and N0 indicate 240, 120 and 0 kg N/hm², respectively.   The data are presented as the meaNS ± SEs.   The values followed by different lowercase letters are significantly difference at the 0.05 level.   Pn: photosynthetic rate.

Figure 3   Leaf area index and Pn in ear-leaf of ZD958 and HN138 under three N rates from silking to 50 DAS

Table 3   Correlations between leaf Rem N, Pn, PNUE, NR, GS, GDH and GOGAT in maize

Traits	Rem N	Pn	PNUE	NR	GS	GDH	GOGAT
Rem N	–	−0.51**	−0.22*	−0.57*	−0.54*	−0.50*	−0.51*
Pn		–	0.80**	0.62*	−0.54*	−0.52*	−0.54*
PUNE			–	0.35**	0.51**	0.45**	0.50**
NR				–	0.95**	0.32**	0.85**
GS					–	0.85**	0.75**
GDH						–	0.85**
GOGAT							–

Note: Rem N: remobilization N; Pn: photosynthetic rate; PNUE: photosynthetic nitrogen use efficiency; NR: nitrate reductase activity; GS: glutamine synthetase activity; * $p < 0.01$; ** $p < 0.001$

Similarly, the Pn of ZD958 was greatly increased than that in HN138 in each N treatment and each year (Figure 3).   The Pn was higher in ZD958 than HN138 in each N treatment and each year. The Pn was higher in 2017 than 2018.   The Pn decreased significantly from 0 DAS to 50 DAS.   The Pn of ZD958 was higher than that of HN138.   The leaf Pn decreased rapidly at 30 DAS under high-N rate (N240) and decreased rapidly at 20 DAS under low-N rate (N120).   For example, in 2018 year, the leaf Pn of ZD958 and HN138 decreased by 19.02% and 22.60% at 30 DAS under high-N rate (N240), respectively.   The leaf Pn of ZD958 and HN138 decreased by 4.81% and 9.88% at 20 DAS under low-N rate (N120), respectively.   These results suggest that ZD958 possess higher leaf area index and Pn during grain-filling stage under low-N conditions, this could promote accumulation of more photosynthetic products in leaf.

Post-silking dry matter accumulation is related to Pn, leaf area index and the duration of photosynthetic function.   All of these factors are closely correlated to N content in leaf[10,39].   It has been reported that N translocation decreases leaf area index, chlorophyll content, and photosynthetic rate.   The slow decline on chlorophyll content and net photosynthetic rate after silking, together with a longer duration of high photosynthetic activity, is essential for sufficient carbohydrate supplies for grains during the grain-filling stage[10,14,40].   This result indicated ZD958 has a higher leaf area index and Pn than does HN138 under all N rates, the leaf area index and Pn of this cultivar declined slowly under low-N conditions, and displayed prolonged high photosynthetic activity (Figure 3).   These finding are in consistent with previous results that suggested a sufficient carbohydrate supply to be beneficial for grain filling[10].   These results indicated that ZD958 had relative higher green leaf area and possess active photosynthetic under low-N conditions at grain-filling stage.

3.3   Genotypic variation in leaf N contents parameters

Significant differences were found in leaf N contents and N remobilization between year (Y), cultivar (H), nitrogen (N) and sample date (S) and its interaction of Y×N, Y×H, H×N, S×N (Table 3).   Significant differences were found in leaf N contents between the two cultivars and three N rates (Figure 4).   The leaf N contents decreased significantly from 0 DAS to 50 DAS.   The effect of cultivar and N rate on the leaf N contents was significant. From 0 DAS to 40 DAS, the leaf total N of ZD958 was higher than that in HN138 (Figure 4), but at 50 DAS, the leaf total N of ZD958 was lower than that in HN138.   In 2017 year, from 0 DAS to   50 DAS, the leaf total N of ZD958 and HN138 decreased by 75.33% and 63.04% under high-N rate (N240) and decreased by 77.12% and 68.58% under low-N rate (N120), respectively.   Similarly results showed that in 2018 year, the leaf

total N of ZD958 and HN138 decreased by 70% and 65.34% under high-N rate (N240) and decreased by 74.22% and 47.27% under low-N rate (N120), respectively.   The leaf N remobilization occurred at 10 DAS but that there was no significant difference between the two maize cultivars (Figure 4). The difference in leaf N remobilization between the two cultivars could be observed from 30-40 DAS under high-N rate (N240), the difference occurred   10 days earlier, i.e. from 20-30 days after silking under low-N rate (N120).   These results suggest that ZD958 possess higher leaf N remobilization during grain-filling stage, the higher leaf N remobilization could promote N assimilation and utilization.

3.4   Genotypic variation in leaf PNUE

Significant differences were found in leaf N contents and N remobilization between years (Y), cultivar (H), nitrogen (N) and sample date (S) and its interaction of Y×N, Y×H, H×N, S×N (Table 3).   Significant differences were found in leaf PNUE between the two cultivars and three N rates (Figure 4).   From 0 DAS to 30 DAS, the leaf PNUE of ZD958 was similar with HN138 under same N treatment and same years, but from 30 DAS to 50 DAS, the leaf PNUE of ZD958 was higher than that in HN138 (Figure 4).   The PNUE of ear-leaf was higher in 2018 than 2017.   The PNUE of ear-leaf decreased significantly from 0 DAS to 50 DAS.   In 2017 year, from 0 DAS to 50 DAS, the PNUE of ear-leaf in ZD958 and HN138 decreased by 19.22% and 27.60% under high-N rate (N240) and decreased by 18.71% and 35.24% under low-N rate (N120), respectively.   In 2018 year, from 0 DAS to 50 DAS, the leaf PNUE of ZD958 and HN138 decreased by 15.68% and 13.17% under high-N rate (N240) and decreased by16.25% and 23.48% under low-N rate (N120), respectively. These results suggest that ZD958 possess higher leaf PNUE during grain-filling stage, could accumulate more amounts of nitrogen per unit area.

The PNUE of leaves is defined as the photosynthetic capacity per unit leaf N amount and is controlled by SLN and Pn which are determined by stomatal and non-stomatal factors, largely affected by the allocation of leaf N to photosynthetic and non-photosynthetic proteins[41,42].   When compared to HN138, the generally higher PNUE of ZD958 across years and sampling dates is likely related to its higher Pn (Figure 4).   This result also showed that ZD958 exhibited higher leaf N remobilization and Pn during the grain-filling stage under low-N conditions, which ensure a high-level accumulation of photosynthetic products and leaf N remobilization to maintain grain yields and N concentration.   This maybe ZD958 could maintain a high photosynthetic rate when N was translocated from the leaves to the grain.   PUNE is affected by the proportion of nitrogen involved in photosynthetic and photosynthetic enzyme activity in the leaves[43].   The proper application of nitrogen fertilizer is helpful for increasing the leaf PUNE, which could increase the yield of maize[44,45].   This result indicated that under low-N conditions, enhancement of post-silking dry matter accumulation and grain yield is important to improve the leaf PUNE, prolong photosynthetic function, and improve the premature senescence of leaves.

3.5   Genotypic variation in N assimilation enzymes activities

The N assimilation enzymes activities in ear-leaf were further examined.   Significant differences were found in N assimilation enzymes activities between year (Y), cultivar (H), nitrogen (N) and sample date (S) and its interaction of H×N, S×N (Table 3).   The N assimilation enzymes activities, nitrate reductase (NR) activity, glutamine synthetase activity (GS), glucose dehydrogenase activity
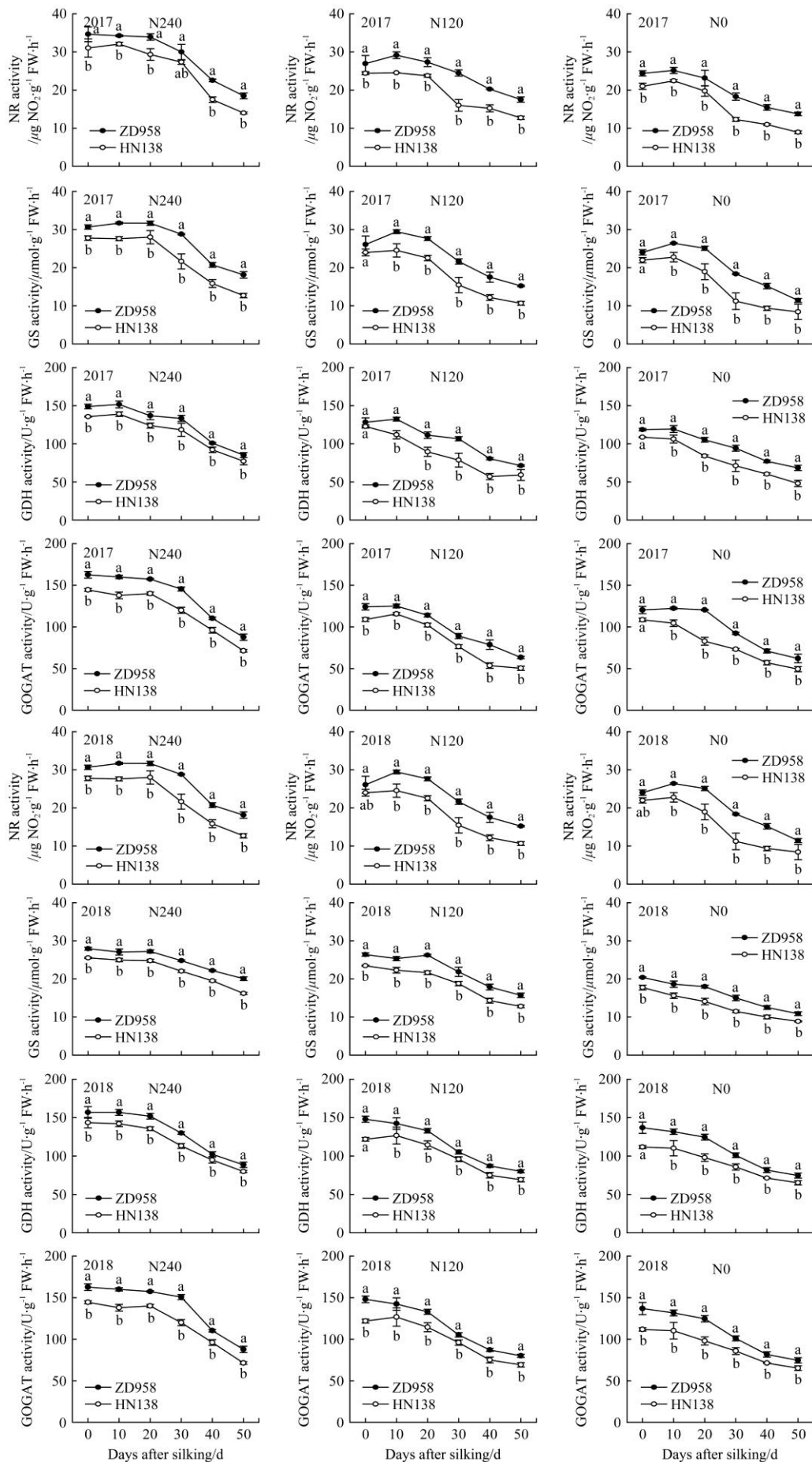
(GDH) and glutamate synthase activity (GOGAT) were higher in 2017 than those in 2018. The values in ZD958 increased significantly compared to that in HN138 under all N rates during the sampling period (Figure 5). Furthermore, The N assimilation enzymes activities increased slowly from 0 DAS to 20DAS or 30 DAS, decreased rapidly beginning at 30 DAS under high-N rate (N240). Whereas decreased rapidly beginning at 20 DAS under low-N rate (N120 and N0).
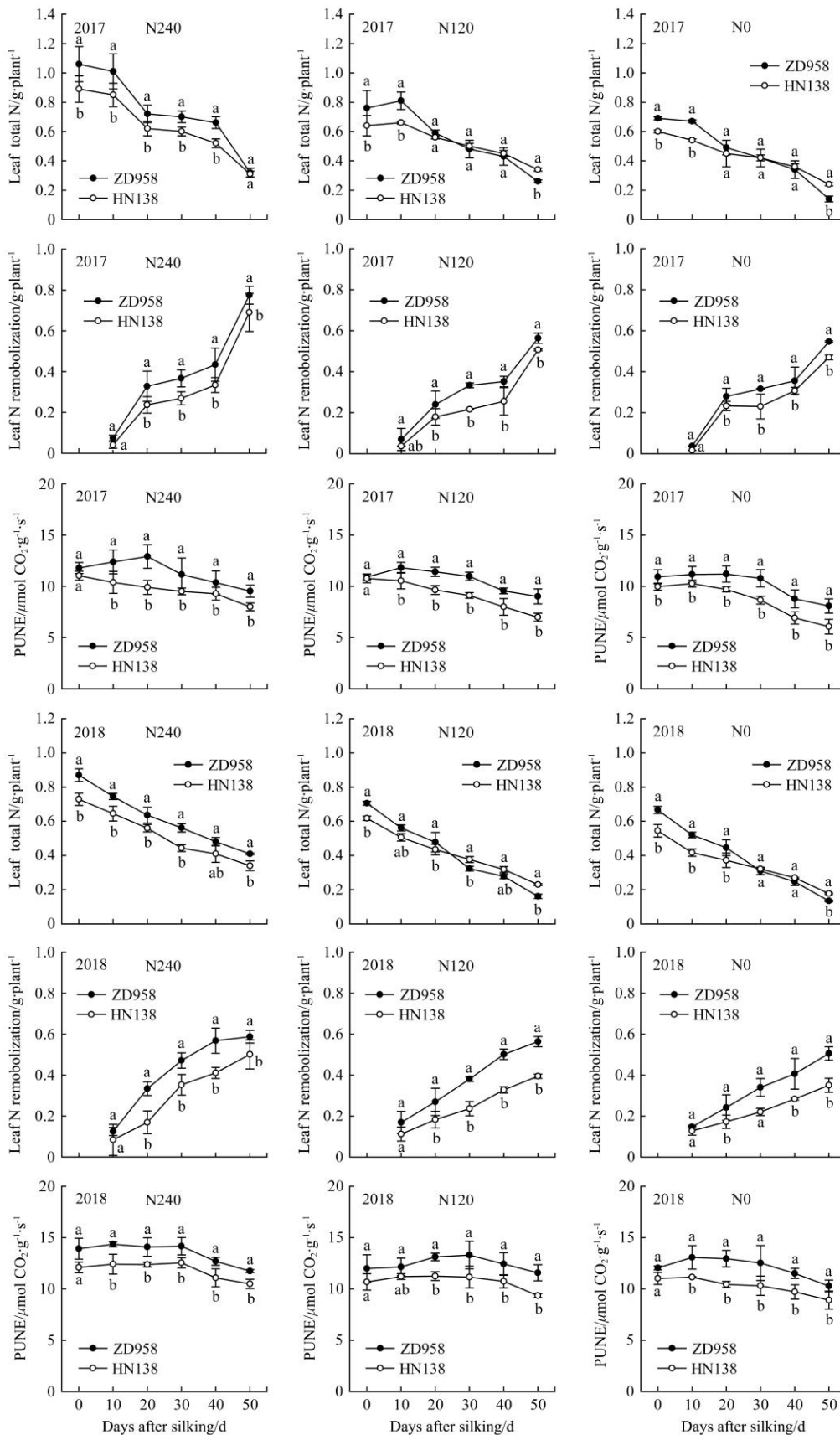
Note: N240, N120 and N0 indicate 240, 120 and 0 kg N/hm², respectively. The data are presented as the mean value ± SEs. The values followed by different lowercase letters are significantly difference at the 0.05 level. Rem N: remobilization N; PNUE: photosynthetic nitrogen use efficiency.

Figure 4　Leaf total N, remobilized N and PNUE of ZD958 and HN138 under three N rates from silking to 50 DAS

The values followed by different lowercase letters are significantly difference at the 0.05 level.   NR: nitrate reductase activity; GS: glutamine synthetase activity; GDH: glucose dehydrogenase activity; GOGAT: glutamate synthase activity.

Figure 5   Leaf NR, GS ,GDH and GAOT activities in ear-leaf of ZD958 and HN138 under three N rates from silking to 50 DAS

Under low-N rate (N120 and N0), the NR activities in ZD958 and HN138 decreased by 17.05% and 35.66% at 20 DAS in 2017, respectively, and decreased by 10.05% and 25.36% at 20 DAS in 2018, respectively.   Similarly results showed that, the GS activities in ZD958 and HN138 decreased by 20.71% and 22.16% in 2017 at 20 DAS under low-N rate (N120), respectively. Whereas, the two maize cultivars decreased by 22.01% and 25.21%, respectively at 20 DAS in 2018.   Similarly results showed that in the GDH and GOGAT activities in ear-leaf between ZD958 and HN138.   These results suggest that ZD958 possess higher leaf NR, GS, GDH and GOGAT activities during grain-filling stage, the higher enzymes activities involved in nitrogen assimilation of ear-leaf could promote N assimilation and utilization.

Glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle is a main pathway of nitrogen assimilation and conversion route for the conversion of inorganic nitrogen to organic nitrogen[46].   Previous studies have shown that genotypes with high NUE exhibit relatively increased amounts of leaf nitrate reductase and glutamine synthetase activity[47,48].   The glutamine synthetase activity, and total nitrogen contents in low-N tolerant cultivars are higher than those in low-N sensitive cultivars[48]. This study showed that the GS activity of leaf in ZD958 was higher than HN138 under low-N conditions (Figure 5).   Moreover, the higher GS activity of leaf was maintained at the late grain-filling stage, suggesting that ZD958 exhibited a strong capability of N assimilation and remobilization with high N uptake and storage. The GDH is an enzyme capable of catalyzing glutamate de-amination as well as glutamate synthesis, carries out the de-amination reaction in source leaves[22].   It has also been shown that GOGAT is still functional in senescing leaves and can participate in glutamate synthesis.   In this case, the origin of 2-oxoglutarate and reducing power remains to be determined. This study showed that the GDH and GOGAT activity of leaf in ZD958 was higher than HN138 under low-N conditions (Figure 5). Moreover, the higher GDH and GOGAT activity of leaf was maintained at the late grain-filling stage, suggesting that ZD958 exhibited a strong capability of N assimilation and remobilization with high N uptake and storage, which was consistent with previous study results[22].

3.6 Soil nitrate concentrations

To determine if the change of soil nitrate depletion, nitrate concentration in the soil profile was investigated.   There were no significant differences between the genotypes at the sowing stage. But the soil nitrate concentrations at the silking and harvest stage were higher in ZD958 than HN138.   There was high variability in soil nitrate levels in three treatments among ZD958.   Under the N240 treatment (Table 4), there were no significant differences between the genotypes.   Under the N120 treatment, the soil nitrate concentration in HN138 declined in the 0-60 cm layer compared with ZD958.

3.7 Correlations between N remobilization, photosynthetic rate and N assimilation enzymes activity

The correlations between N remobilization, photosynthetic rate and enzyme activities involved in N assimilation were examined (Table 3).   A significant correlation coefficient between Rem N, Pn, PNUE and NR, GS, GDH and GOGAT.   Among these correlations, a significant negative correlation was found between Rem N and Pn, PNUE, and NR, GS, GDH, GOGAT.   These results indicated that the increased leaf nitrogen remobilization led to a decrease in other factors.   The PNUE was significantly and positively correlated with NR, GS, GDH, GOGAT suggesting that

PNUE and NR, GS, GDH, GOGAT jointly determined the nitrogen remobilization efficiency and the leaf N remobilization during post-silking.

Table 4   Soil nitrate concentrations in ZD958 and HN138 under different N treatments in 2018

Cultivars	N treatment	Soil depth /cm	Soil nitrate concentrations/mg kg^{-1}		
			Sowing	Silking	Harvest
ZD958	N240	0-20	34.45	69.22	49.38
		20-40	29.25	52.64	17.13
		40-60	21.00	36.01	12.04
	N120	0-20	30.38	46.43	28.95
		20-40	25.84	42.32	12.37
		40-60	18.92	30.47	10.21
	N0	0-20	29.34	36.76	30.28
		20-40	24.78	27.03	14.39
		40-60	17.68	25.85	8.65
HN138	N240	0-20	34.32	57.92	44.89
		20-40	29.32	41.34	18.31
		40-60	20.00	24.71	8.68
	N120	0-20	27.38	35.13	7.99
		20-40	24.30	31.02	13.99
		40-60	17.92	22.17	6.14
	N0	0-20	26.34	25.46	4.70
		20-40	23.00	21.73	11.70
		40-60	16.68	17.55	1.52

Leaf N remobilization is negatively correlated to PNUE and all the N assimilation enzymes (Table 3).   The high Pn of ZD958 is consistent with its higher leaf area index, PNUE and N assimilation enzyme, but also inconsistent with its N remobilization in leaves (Table 3).   This is in agreement with the study results[1], who found that although modern maize stay-green high-yield breeding significantly increased the green leaf area and the N content of leaves.   The PNUE may be improved, if the N of leaves is channeled into the critical limiting photosynthetic proteins[49] to enhance their efficiency[50].   Enhance the NR and GS activity of leaf could contribute to possess higher PNUE, this due to the increased GS activity can improve nitrogen remobilization efficiency and nitrogen assimilation efficiency[27,51].

4 Conclusions

In this study, the analysis of two maize cultivars with contrasting low-N tolerant revealed that, to simultaneously achieve high grain yield and high NUtE, a maize cultivar should maintain the higher PNUE while increasing the N assimilation enzyme activity of the ear-leaf during the grain-filling stage.   Firstly, it is worth mentioning that enhance GS activity can improve N remobilization and assimilation, this is an important physiological mechanism of high NUtE in maize.   In addition, the PNUE was significantly and positively correlated with NR, GS, GDH, GOGAT, suggesting that the PNUE and NR, GS, GDH, GOGAT jointly determined the leaf N remobilization.   The plant traits comprising a higher PNUE and N assimilation enzyme activities of the ear-leaf can be used as selection criteria to select low-N tolerance cultivars for growth under low-N conditions.   Finally, this investigation provides insights into the N-associated physiological processes that maize species responds to N deprivation and puts forward guidance for breeding low-N tolerant and high NUE maize cultivars under low-N conditions.

Acknowledgements

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