Nitrogen fertigation effect on photosynthesis, grain yield and water use efficiency of winter wheat

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Abstract: Excessive fertilization is common in North China Plain. It is essential to determine the extent to which reduction of nitrogen application rates has little impact on yield and understand its physiological basis. Two seasons of field experiments were conducted in a winter wheat field with three nitrogen (N) fertilization treatments, i.e. N3, traditional N application rates (290 kg N hm⁻²), N2, ~65% N application rates of N3 (190 kg N hm⁻²) and N1, ~40% N application rates of N3 (110 kg N hm⁻²). Yield (*Y*) and water use efficiency (WUE) of N2 didn't reduce significantly in both seasons despite that the instantaneous net photosynthetic rates (*A_n*) and stomatal conductance (*g_s*) of N2 were significantly lower than those of N3. *Y* and WUE of N1 treatment reduced significantly in the second season. The reduction of *Y* was better indicated by photosynthetic capacity (*A_{max}*) in the grain filling stage (around 48-50 days after the reviving stage irrigation, Dari 48-50) because *A_{max}* decrease in this stage was proportionally to *Y*. The *A_{max}* and apparent quantum efficiency (*α*) of N2 measured on Dari 48-50 were not significantly lower than those of N3, which could be the reason of no significant reduction of *Y* for N2. Thus, the fertilization amount of N2 (190 kg N hm⁻²) can be applied in this area to keep *Y* steady for at least two years. Differences of the relationships between *A_{max}* and biological factors among treatments and non-stomatal limitation of photosynthesis were also discussed. These results are important for understanding the mechanisms of yield reduction by reducing N application and provide scientific basis for application of fertigation.

Keywords: Nitrogen fertilization; Photosynthetic capacity; Leaf nitrogen content; Water use efficiency; Yield; Winter wheat

1. Introduction

The North China Plain (NCP) is the main grain-producing area with over one-fourth of the total grain yield in China. The grain yield is affected by irrigation regimes and yield-water relationships in this area has been intensively studied (Qiu et al., 2008; Li et al., 2015), optimal irrigation scheduling has been recommended in some studies (Li et al., 2005; Dong et al., 2011; Liu et al., 2011). Besides water, applying nitrogen (N) fertilizer is still a main measure for increasing crop yield. The amount from 60 up to 385 kg N hm⁻² per season (October to June of next year) is applied to winter wheat field according to a survey in the NCP area (Zhang et al 2008). Within this fertilization range, the yield ranged from 4100 to 6500 kg hm⁻² in this area with adequate irrigation (Wang et al., 2006; Zhang et al., 2016). However, the N uptake of winter wheat with yield of 6000 kg hm⁻² was about 190 kg N hm⁻² (Zhang and Ju, 2002). The traditional applied dosage is 290 kg N hm⁻² per season in this area, which is much higher than crop requirement. Some researches demonstrated that N application rates in wheat fields may be significantly reduced compared to common farmers' practice, without negatively affecting grain yield, and the fertilizer leaching and residual will be much less (Hartmann et al., 2015; Sui et al., 2015). Determination of the optimal N fertilization is as much important as water management because yield may not increase linearly with N fertilizer (Shi et al., 2013; 2014).

Some researchers have tried to explain the mechanism of crop yield increase from the application of N fertilizer through the photosynthetic regulation (Bindraban, 1999; Miranzadeh et al., 2011). Regulation of the photosynthetic physiological processes of plants by N fertilizer, will ultimately effect on yield and water use efficiency (Ruiz et al., 2008). Therefore, understanding the photosynthetic regulation on yield reduction by reduce N fertilization would give us important references for N management.

The photosynthetic capacity is closely related to the structural properties and biochemical composition of leaves (Wright et al., 2004). Among the traits which underlined these properties, specific leaf area and leaf N content, have been identified as two of the key characters determining photosynthetic capacity (Hikosaka, 2004; Poorter et al., 2009). The relationship between photosynthesis and leaf N content has been investigated in many studies (Evans, 1989). The application of N fertilizer increases the leaf N content. The higher leaf N content is correlated with high chlorophyll content and it also increases the activities of chloroplast, thereby increasing the photosynthetic rate (Li et al., 2013). Conversely, when the N application decreases, the leaf N content decreases significantly and the photosynthetic capacity also declines (Pal et al., 2005). Evans (1983) concluded in one of his

review that leaf N is uniquely related to photosynthesis rate, irrespective of nutrient status, season, or leaf age. It has been shown that 50 % of N is distributed to the photosynthetic organs in order to maintain natural photosynthesis in crops (Saibo et al., 2009). Hikosaka (2004) discussed that the interspecific variation in the relationship between photosynthesis and nitrogen content in leaves and explained the physiological causes of the interspecific difference. Yet, we still do not know, for one species, if different N treatments will affect the relationships between photosynthesis and leaf N content.

Carbon isotope (^{13}C) discrimination (Δ) provides a time integrated index of photosynthetic activity and is related to water use or transpiration efficiency (Farquhar et al., 1982; Evans et al., 1986). In many researches, Δ is usually determined as an inherent factor to assess water status of crops in high yield and water use efficiency varieties selection (Xue et al., 2002; Misra et al., 2010). Furthermore, Δ is also affected by N supply (Clay et al., 2001; Wang et al., 2013), which affects N status of the crop, thus interlinking photosynthesis and yield (Cabrera-Bosquet et al., 2007). Further study is also need on this subject.

Photosynthetic products accumulated in the latest emerging leaves - namely the flag leaf- is the primary source of nutrients in the grain filling period (Kichey et al., 2006). Therefore, researches on the photosynthetic characteristics of the wheat flag leaf, especially on the physiological response to N fertilizer and on the regulation of gas exchange are essential in improving yield and the efficiency of water and fertilizer use (Cabrera-Bosquet et al., 2009). Most studies of the photosynthesis of winter wheat flag leaves under different N treatments are only focused on the descriptions of the changes in a particular growth period of crops or the daily variations of the photosynthetic rate (Pal et al., 2005). Analyzing the interactions of gas exchange parameters would explain the decrease of flag leaf photosynthetic rate caused by stomatal or non-stomatal limitations. Such research had been conducted on potted wheat (Li et al., 2013), which may be different in temperature and light conditions to that in the field. At the same time, related biochemical properties of flag leaves need to be determined to identify the sources that cause the differences in photosynthetic parameters. Such researches would give us a better understanding on the photosynthetic regulation of yield-fertilizer-relationships in winter wheat field.

In this study, we are trying to determine the extent to which reduction of nitrogen application rates has little impact on yield and understand its physiological basis. Two seasons of field experiments focusing the nitrogen fertigation and yield relationship in a winter wheat field in North China Plain were conducted. In addition to the traditional N fertilization amount, two lower levels were set according to literatures. Optimal water supply was

applied to minimize the interaction effects between irrigation and fertilization. The response of crop yield to the reduction of N fertilization comparing to the traditional amount were studied. The photosynthetic parameters were measured to explain the physiological basis of yield changing. Leaf N content and Δ were also determined to give an explicit explanation of the relationships between yield and photosynthetic parameters.

2. Materials and methods

2.1. Research site

The experiment was conducted in the Agricultural Water Conservation Irrigation Experiment Station of the Chinese Institute of Water Resources and Hydropower Research in the Daxing District of Beijing, China (39°39'N, 116°15'E). The local climate of the site belongs to the semi-arid continental monsoon climate. The mean annual precipitation is about 540 mm, with only less than 100 mm occur during the winter wheat growing season. The soil texture of 100 cm depth of the experimental plot is loam, with average field capacity, organic matter content and bulk density of 0.306 cm³ cm⁻³, 3.25 g kg⁻¹ and 1.58 g cm⁻³, respectively. The initial nitrate nitrogen content was 5.43 mg kg⁻¹ in 2012.

2.2. Experimental design

Field experiments with winter wheat (*Triticum aestivum* L.) were conducted for two successive seasons (2012/2013 and 2013/2014). Sowing dates, the reviving stage started dates and harvest dates, and irrigation managements were listed in detail in Table 1 for the two seasons. Winter wheat were sowed in Octobers using a sowing machine set to a row spacing of 12.5 cm. Experimental field was irrigated 75 mm before sowing in order to ensure wheat germination and healthy seedling. 50 mm of winter irrigation was applied in November of the same year by sprinkler irrigation. The reviving stages started in April of next years and the harvest dates were in the middle days of June. Drip irrigation was selected during the period from reviving stage to harvest for a precision control of the irrigation amount. The irrigation amount during the reviving stage was 24 mm. Afterwards, the irrigation scheduling was developed with reference to the cumulative evaporation (E) from a standard evaporation pan with a diameter of 20 cm. Irrigation was applied when cumulative E was up to 40 mm, and the irrigation quota was 0.8E, i.e. 32 mm (Liu et al, 2007). During the reviving stage to harvest stage, there were only twice rains with $P > 5$ mm occurring in July (the harvest stage) of 2013. The rain distribution of the same period of 2014 was more even than that of 2013, with six rains with $P > 5$ mm occurring 3 times in April, twice in May and once in July. Thus, there were 5 irrigations in 2013 and 4 irrigations in 2014 during the period from reviving stage to harvest stage, for

the precipitation in the same period of 2013 is less than that of 2014 (Table 1). The Irrigation dates and amounts are Apr. 10 (24 mm), Apr. 21 (24 mm), May. 4 (32 mm), May. 16 (32 mm) and May.30 (32 mm) for 2013, Apr. 3 (24 mm), Apr. 23 (32 mm), May. 7 (32 mm) and May. 23 (32 mm) for 2014.

Three N treatments were set as 290 (N3, traditional N application rates in this area), 190 (N2, N requirement for a yield of 6000 kg hm⁻², ~65% of N3, Zhang and Ju, 2002), 120 (N1, ~40% of N3) kg N hm⁻². Base fertilizer of 83 kg N hm⁻² was applied with compound fertilizer (N-P₂O₅-K₂O, 15-15-15) during sowing. Treatments were set by applying different amounts of urea (containing 46 % N) as the top dressing fertilizers in this study, of 207, 107 and 37 kg N hm⁻², respectively. Top dressing fertilizer was applied once during the irrigation at the reviving stage using the Venturi injector for the two seasons. When applying the top dressing fertilization, the urea was dissolved into water according to the same urea/water ratio for all the treatments until the complete dissolution of fertilizer to keep the same N concentration among treatments. Each treatment had three replications, and nine plots were assigned randomly. Six drip lines were arranged in each plot at the beginning of the reviving stage with a length of 20 m and space of 0.5 m. The emitters (Netafim Ltd., Tel Aviv, Israel) were spaced 30 cm apart, with a 1.2 L·h⁻¹ flow rate at 1 bar operating pressure.

2.3. Measurement of the photosynthetic parameters of flag leaves

During the heading stage, a Li-Cor 6400 portable photosynthesis system was used to measure the gas exchange and the photosynthesis rates (A_n) to light (photosynthetic active radiation, PAR) response curves (A_n -PAR curves) of fully extending flag leaves on clear days. In 2013, gas exchange of all the treatments were measured twice on May 1st and June 3rd. For each N treatment, two leaves were selected to measure the daily dynamics of gas exchange parameters, including A_n , stomatal conductance (g_s) and intercellular CO₂ concentration (C_i). The measurements were started at 8:00 am, and were taken out every two hours until 16:00. In 2014, three measurements of A_n -PAR curves were carried out during May 4th – 5th, May 22nd – 23rd, and June 2nd – 3rd. Two light curves were measured for every treatment, except for May 4th – 5th, during which dates only one light curve for every treatments were measured.

The temperature during the A_n -PAR curves measurement was controlled as 30 °C and the CO₂ concentration of the reference chamber (CO₂-R) was set as 400 μmol mol⁻¹. The PAR was set at 2500, 2200, 2000, 1750, 1400, 1100, 800, 500, 200, 150, 120, 80, 40 and 0 μmol m⁻² s⁻¹, respectively. Light inducement were done by keeping the leaves in the chamber under PAR = 2000 μmol m⁻² s⁻¹ until parameters readings were stable. Linear fitting was used for the

light data at 0–150 $\mu\text{mol m}^{-2} \text{s}^{-1}$, the slope of the line was the apparent quantum efficiency (α), the intercept was the respiration rate (R_d) and the intersection of the regression line and the x-axis (PAR) was the light compensation point (PAR_c). After the determination of the above parameters, the maximum photosynthetic rate (A_{max}), also known as the photosynthetic capacity, was derived by fitting the non-rectangular hyperbola model (Lu and Yu, 2001). The expression of the non-rectangular hyperbola model (Thornley, 1976) is as Eq. (1):

$$A = A_n + R_d = \frac{\alpha I + A_{\text{max}} - \sqrt{(\alpha I + A_{\text{max}})^2 - 4 * \theta * \alpha I A_{\text{max}}}}{2\theta} \quad (1)$$

Where, A is the total photosynthesis rate, A_n is the net photosynthesis rate, θ is the convexity of the curve. The larger the convexity is, the greater the curvature of the curve will be. In the figure of A_n -PAR, when $0 < \text{PAR} < 150 \mu\text{mol m}^{-2} \text{s}^{-1}$, the PAR value that corresponds to the intersection of the linear fitting of A_n and PAR and $y = A_{\text{max}}$ is the light saturation point (PAR_s).

The instantaneous A_n , g_s and the non-stomatal limitation factor (Ci/Ca) of flag leaves were compared among N treatments for the two seasons firstly. Three largest values of the above variables from the daily dynamics for 2013, and from the light saturation stage ($1000 < \text{PAR} < 2500 \mu\text{mol m}^{-2} \text{s}^{-1}$) of each light-curve for 2014 were selected for the comparing. Then, the intrinsic photosynthetic parameters such as A_{max} , α , R_d , PAR_c and PAR_s were compared among N treatments for the year of 2014.

In this study, g_s was measured as the conductance to H_2O for the Li-Cor 6400 system. For the future convenience, g_s was converted to the conductance to CO_2 , by dividing by 1.57 according to the differences of diffusion rates of CO_2 and H_2O across stomata. For comparison of the two years, and to other studies, we referred the measuring dates as *days after the irrigation of reviving stage* (Dari). Thereafter, the two measuring dates in 2013 were corresponding to Dari 22 and Dari 55, respectively, and the three measuring dates are corresponding to Dari 31–32, Dari 48–50 and Dari 60–61, respectively.

2.4. Determination of flag leaf nitrogen content and carbon isotope discrimination

In the season of 2013/2014, after the measurement of the light curves the flag leaves were removed and scanned for the leaf area. After scanning, the leaves were dried at 105 °C for 20 minutes, then dried at 65 °C for constant mass. The ratio of the dry mass to leaf area is the specific leaf mass (SLM, g m^{-2}). After the determination of SLM, the flag leaves were ground and passed through a 100 mesh sieve. The dried leaf samples were then sent to the Stable Isotope Ratio Mass Spectrometry Laboratory of the Chinese Academy of Forestry Sciences to determine

the carbon isotope abundance (δ). The ratio of ^{13}C to ^{12}C for these samples were determined using a stable isotope ratio mass spectrometer (DELTA V Advantage isotope ratio mass spectrometer, Thermo Fisher Inc., USA), and compared using international standards (Pee Dee Belnite, PDB) to calculate the $\delta^{13}\text{C}_{\text{sample}}$ value. Using the known $\delta^{13}\text{C}_{\text{air}} = -8\text{‰}$, the ^{13}C discrimination of sample (Δ) can be calculated with Eq. (2):

$$\Delta = \frac{(\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{sample}}) * 1000}{1 + \delta^{13}\text{C}_{\text{sample}}} \quad (2)$$

The nitrogen content of the flag leaf was determined by using a mass spectrometer to analyze the N combustion at high temperatures during the determination of $\delta^{13}\text{C}_{\text{sample}}$ using a stable isotope ratio mass spectrometer. The N content per unit of leaf dry mass was expressed as the N -mass (g N kg^{-1} dry mass). The N content per unit of leaf area was presented as N -area (g N m^{-2} leaf area). N -area is the product of N -mass and SLM.

2.5. Determination of water use efficiency

Water use efficiency (WUE) at the field scale, were determined as the ratio of the yield (Y) to evapotranspiration (ET_a) of the field (Eq. 3) and compared among different N treatments.

$$\text{WUE} = Y / ET_a \quad (3)$$

Six sample plots of 1 m^2 were harvested from each replication to determine the yield (Y , kg hm^{-2}) of that replication. The evapotranspiration of winter wheat fields during the experimental period was calculated based on the water balance method (Eq. 4):

$$ET_a = I + P - \Delta S - R - L \quad (4)$$

Where, ET_a is the total evapotranspiration (mm) during the experimental period; I is the amount of irrigation (mm); P is the effective precipitation (mm); ΔS is the difference in soil moisture between the end and beginning of the experiment; R is the surface runoff loss (in this study, surface drip irrigation was used, there was no surface water accumulation, so R can be ignored); D is the drainage loss (in this study, irrigation system was controlled by a water meter, and the irrigation amount was low, so there was no drainage).

Determination of soil water content is the key to determine ET_a (Eq. 4) using the water balance method. In this study, soil water content was determined in the field by collecting soil samples with a soil auger and drying method. The wet and dry zones (separated by the drip lines, areas under the drip lines are wet zones, and areas between drip lines are dry zones) in each plot were selected before reviving irrigation and on the harvesting day for the two seasons, and soil samples were collected at different depths (0–10, 10–20, 20–40, 40–60, 60–80, 80–100 cm). ET_a

between reviving and harvesting stages were calculated with Eq. 4. The total ET_a of the whole growing season (October to July of next year) was determined based on the five year study of winter wheat in North China Plain (Liu et al., 2002), in which the ET_a between reviving and harvest stages was determined as 65 % of the total ET_a .

2.6 Data analysis

SPSS 13.0 (SPSS Inc., United States) was used in the calculation of a custom nonlinear regression model for photosynthesis light curves of non-rectangular hyperbola fitting. One-way-analysis of variance using SPSS13.0 and the multiple comparisons with Duncan's test was used to determine the mean difference among the photosynthetic parameters. Σ Plot 12.5 (SPSS Inc., United States) was employed in the linear regression analysis of the 0–150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthesis-light data and linear regression analysis between flag leaf photosynthetic and biological parameters. Σ Plot 12.5 was also used to make the figures in this manuscript.

3 Results

3.1 Comparison of instantaneous photosynthetic parameters among N treatments

In 2013, both the daily dynamics showed single-peak curves (Fig. 1). In the measurement of Dari 22, except at 16:00, when A_n value dropped down to below 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$, the rest of the time A_n was fluctuated between 21–26 $\mu\text{mol m}^{-2} \text{s}^{-1}$. In the measurement of Dari 55, except at 16:00, when A_n value of N2 was higher than that of N1 and N3, the ranking of A_n value for the remaining hours were $N3 > N2 > N1$. Throughout the day, A_n values fluctuated between 10–16 $\mu\text{mol m}^{-2} \text{s}^{-1}$, obviously less than that measured in Dari 22. In addition to the leaf senescence, the reason may be that the average air temperature on Dari 55 (36.5°C) was quite high, and a lot higher than that on Dari 22 (27°C), the activity of photosynthetic organ was diminished. In 2014, all the three measurements of light curves were conducted under the same leaf chamber conditions, the seasonal variations of photosynthetic parameters were not very large and mainly because of the leaf senescence. The light curves can be well fitted by non - rectangular hyperbolic equation (Fig. 2). From Dari31-32 (the heading stage) to Dari 60-61 (the maturity stage), the differences of the A_n values among treatments became more apparent. The plateau of the A_n -PAR fit curves reached earlier and decreased gradually. The slopes of the beginning liner increase part of the A_n -PAR fit curves of N1 and N2 were smaller than that of N3 (the inserts of Fig. 2).

The comparison of instantaneous A_n showed significantly differences among N treatments for most measurements (Fig. 3), with an exception of the measurement of Dari 22 in 2013, on which, there was no significant

difference of A_n among treatments with an average of $22.95 \mu\text{mol m}^{-2} \text{s}^{-1}$. In the measurement of Dari 55, A_n of N2 treatment ($16.06 \mu\text{mol m}^{-2} \text{s}^{-1}$) was not significantly reduced, while A_n of N1 ($14.30 \mu\text{mol m}^{-2} \text{s}^{-1}$) was significantly less than that of N2 and N3 ($P = 0.047$). In 2014, the difference among treatments became larger. In the measurement of Dari 31-32, the reduction of A_n values of N2 ($23.71 \mu\text{mol m}^{-2} \text{s}^{-1}$) and N1 treatments ($23.61 \mu\text{mol m}^{-2} \text{s}^{-1}$) were marginally significantly comparing to that of N3 ($25.21 \mu\text{mol m}^{-2} \text{s}^{-1}$, $P = 0.067$). Dari 48-50, A_n values of N2 ($20.16 \mu\text{mol m}^{-2} \text{s}^{-1}$) and N1 ($19.10 \mu\text{mol m}^{-2} \text{s}^{-1}$) were not significantly different, but both were significantly lower than that of N3 ($24.14 \mu\text{mol m}^{-2} \text{s}^{-1}$, $P < 0.001$). At the late growing season, Dari 60-61, A_n of N1 ($13.71 \mu\text{mol m}^{-2} \text{s}^{-1}$) treatment further reduced and was significantly lower than that of N2 ($16.50 \mu\text{mol m}^{-2} \text{s}^{-1}$), both with A_n values significantly lower than that of N3 ($21.21 \mu\text{mol m}^{-2} \text{s}^{-1}$, $P < 0.001$).

In the measurement of Dari 22 of 2013, there were no significant differences of g_s and Ci/Ca among N treatments (Fig.4a and 5a). In the measurement of Dari 55, the difference of g_s among treatments was marginally significant ($P = 0.054$). The g_s of N1 ($0.27 \text{ mol m}^{-2} \text{s}^{-1}$) was lower than that of N3 ($0.30 \text{ mol m}^{-2} \text{s}^{-1}$), while g_s of N2 ($0.29 \text{ mol m}^{-2} \text{s}^{-1}$) was between the two (Fig.4a). The difference of Ci/Ca among treatment was significant ($P=0.044$). The Ci/Ca of N1 (0.85) was significantly higher than that of N3 (0.73), while Ci/Ca of N2 (0.78) was between the values of N3 and N1, but with no significant difference between values of N2 and N3 or N2 and N1 (Fig.5a).

In 2014, comparison of g_s under different N treatments from the three measurement dates showed different patterns. The g_s of N1 measured on Dari 32-33 were significantly higher than those of N3, and both were significantly higher than those of N2. On Dari 48-50, g_s of N3 were significantly higher than those of N2 and N1. On Dari 60-61, g_s of N3 and N2 were significantly higher than those of N1. When comparing the three measurement dates the mean values of g_s measured on Dari 48-50 were the highest (Fig. 4b). The mean values of g_s from all three measurements varied from 0.16 and $0.29 \text{ mol m}^{-2} \text{s}^{-1}$. Fig. 5b showed that, except for the no significant differences among treatments for Ci/Ca values measured on Dari 60-61, in the other two measurements, Ci/Ca of N1 were significantly higher than those of N3 and N2.

3.2 Comparison of intrinsic photosynthesis parameters among N treatments

The A_{max} , α and PAR_s were affected significantly by different N fertilization applications, but there were no significant differences on R_d or PAR_c among treatments. Fig. 6 illustrates the results of the comparison of light-curve parameters of the three dates in 2014.

The $A_{\max}$ values gradually decreased over the duration of the field trial. The $A_{\max}$ of N3, N2 and N1 treatments on Dari 60-61 were 14.0, 28.9 and 39.9 % lower than the $A_{\max}$ values measured on Dari 31-32, respectively (Fig. 6a). The $A_{\max}$ of N1 treatment on Dari 48-50 ($26.60 \mu\text{mol m}^{-2} \text{s}^{-1}$) was significantly lower than that of N3 treatment ($30.78 \mu\text{mol m}^{-2} \text{s}^{-1}$). $A_{\max}$ of N2 ($28.08 \mu\text{mol m}^{-2} \text{s}^{-1}$) was between N3 and N1, but the differences were not statistically significant between N2 and N3 or N2 and N1. The differences of the $A_{\max}$ values among treatments on Dari 60-61 were significant. The $A_{\max}$ of N3 was significantly higher than that of N2 and N1, and $A_{\max}$ of N2 was significantly higher than that of N1. The $A_{\max}$ of N1 was lower than that of N3 by 4.42, 13.57 and 33.19% for the three measurement dates, respectively.

α value didn't change significantly as the leaves aged, but the α values were significantly different among the treatments (Fig. 6b). α values of N3 (0.048) and N2 (0.046) were significantly higher than that of N1 (0.041) when measured on Dari 48-50. α values of N3 (0.053) were significantly higher than that of N1 (0.044) on Dari 60-61, while, the α value of N2 (0.046) was between that of N3 and N1, but with no significant difference between α values of N2 and N3 or N2 and N1.

The values of R_d and PAR_c were relatively low during the Dari 31-32 measurement dates. R_d and PAR_c showed no significant differences among treatments for the measurements on Dari 48-50 and Dari 60-61 (Fig. 6c and 6d). The mean values of R_d and PAR_c of the three measurements were 1.54 , 1.98 and $1.90 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 35.5 , 44.9 and $40.9 \mu\text{mol m}^{-2} \text{s}^{-1}$ respectively. The PAR_s values measured on Dari 48-50 were not significantly different among N treatments, with a mean value of $680.7 \mu\text{mol m}^{-2} \text{s}^{-1}$. On Dari 60-61, PAR_s of N1 ($477.7 \mu\text{mol m}^{-2} \text{s}^{-1}$) was significantly lower than that of N3 ($592.3 \mu\text{mol m}^{-2} \text{s}^{-1}$). Again, PAR_s of N2 ($552.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) fell between the PAR_s values of N3 and N1, but with no significant difference between values of N2 and N3 or N2 and N1.

3.3 Relationships between photosynthetic capacity and biological factors

3.3.1 Each measurement analyzed separately

We found that there was no significant correlation between $A_{\max}$ and N-mass for the three measurements (Fig. 7). For data collected on Dari 31-32, there was significant correlation only between $A_{\max}$ and N-area. For data collected on Dari 48-50, $A_{\max}$ was significantly correlated with Δ . For data collected on Dari 60-61 there was significant correlation between $A_{\max}$ and N-area. The statistical parameters of the significant correlations are given in Fig. 7.

3.3.2 All measurements analyzed together

Except that $A_{\max}$ was marginally significant linear correlation with Δ ($P = 0.057$) for N1 treatment, there were significant linear relationships between $A_{\max}$ and N-mass, N-area and Δ under all other treatments. N treatments affected those relationships (slopes and interceptions of the liner regressions) significantly. With the decreased N level, absolute values of slopes of the regression lines were increased (Fig. 8). The regression coefficients and the statistical parameters of all correlations are listed in Table 2.

3.4 Comparison of yield and water use efficiency among N treatments

ET_a during the reviving to harvest stages was calculated using the water balance method of Eq. 4. Water balance components, such as I , P , ΔS , and ET_a , during the reviving stage to harvest stage for the two seasons were listed in Table 3. ΔS of all the N treatments for the two seasons was similar, varying from 78.4 to 94.2mm. There were no significant differences of ET_a during the reviving to harvest stages among N treatments. ET_a of the whole growing season was estimated with the ratio of the ET_a during the reviving stage to harvest stage to the ET_a of the whole growing season (0.65, get from a reference with 5 years experiment of the same area), and list in Table 4 to calculate the WUE.

The Y values of winter wheat for the two seasons were variation between 5000 to 6000 kg hm^{-2} . Although there were no significant differences of Y , ET_a and WUE among N treatments for the season of 2012/2013, significant differences of Y and WUE among N treatments were found for the season of 2013/2014 (Table 4). For N1 treatments, in which N fertilizer was reduced to ~20% of the highest level, the Y reduced significantly only in the season of 2013/2014. Comparing to N3 treatment, Y of N2 treatment, in which the fertilizer was reduced to about 50%, was not affected significantly for the two seasons. In 2012/2013, Y of N1 and N2 treatments was 6.23% and 1.46% lower than that of N3. In 2013/2014, Y of N1 was 12.6 % lower than that of N3 with significant difference. Y of N2 was 5.18% lower than that of N3. There were no significant differences between Y of N2 and N3 or N2 and N1. Different N treatments did not significantly affect ET_a when adequate water was supplied. The mean value of ET_a was 440.7mm and 469.2 mm for all N treatments for the season of 2012/2013 and 2013/2014, respectively. The WUE values of all the treatments were variation between 11.03 to 12.90 kg $hm^{-2} mm^{-1}$. The means comparison results of WUE were similar to those of Y for the two seasons.

4 Discussion

4.1 Comparison of yield, water use efficiency and photosynthetic parameters among N treatments

In 2013, even if we could not find significant reduction of Y , photosynthesis was affected significantly by N deficit. If N deficit was not removed, as we observed in 2014, photosynthetic parameters reduced further for N1 treatment (a reduction of ~60% of traditional N application rates), and the Y also reduced significantly. For N2 treatment, with sufficient water supply and the reduction of ~35% of traditional N application rates, there was no significantly reduction of Y for the two seasons (Table 4), suggesting that we could lower the N fertilization by about 35% at least for a couple seasons without decreasing grain yield. Similar results were also found by Hartmann et al. (2015). The fertilization level of N2, i.e. 190 kg N hm^{-2} is within the reported optimal N application rates based on 2-3 seasons of experiments of winter wheat in the same area (Zhao et al, 2009; Liu et al, 2013).

Photosynthesis is the key factor affecting crop yield and the effects of N nutrition on yield may be driven primarily by photosynthesis (Makino, 2011). Low N supply generally decreases photosynthesis in crops (Boussadia et al., 2010; Miranzadeh et al., 2011), which was confirmed in this study. Both the instantaneously A_n and the photosynthetic capacity ($A_{\max}$) of flag leaves did decrease under conditions of lower N treatments for the two seasons (Fig. 1-3, Fig. 6). Integrated the Y difference among treatments, it seems that the $A_{\max}$ was a better indicator of yield than instantaneously A_n was. Although the A_n of N1 reduced significantly on Dari 55 in 2013, the reduction of Y for N1 was not significantly, suggesting that plant photosynthetic parameters were more sensitive to N treatment comparing to dry mass factors. The similar situation occurred in 2014 for N2 treatment. Even though A_n was significantly lower than that of N3 and significant reduction of $A_{\max}$ comparing to N3 was only found on Dari 60-61 measurement, which did not result significant yield drop of N2 treatment.

Since there were only three levels of N treatments in this study, it was not easy to draw a regression line between yield and photosynthetic capacity with statistics significant. However, the comparisons among N treatments showed that grain yield and photosynthetic parameters both decreased remarkably with the reduction of N application (Fig 3 and 6, Table 4), suggesting the closing relationships between them. Yield of N1 reduced by 12.6 % compared to N3 in the season of 2013/2014, and $A_{\max}$ of N1 measured on Dari 48-50 the same season was approx. proportionally decreased (13.6 %) with grain yield, suggesting that $A_{\max}$ measured during this period would predict the yield differences among N treatments. This agrees with the results of higher yield related with higher flag leaf photosynthesis rates during the grain filling period (Shi et al., 2013). $A_{\max}$ of N2 on Dari 48-50 was not significantly

different from that of N3 (Fig.6a), which probably be one reason of no differ of the yield between the two treatments. On the other hand, according to the conclusion of Gaju and colleagues' research, delaying the onset of post-anthesis senescence may be an important trait for increasing grain yield of wheat (Gaju et al., 2011). Similar results were found in our study. Under N3 treatment, A_{max} of flag leaf kept relative higher at the end of the grain filling stage for the season of 2013/2014 (A_{max} of N3 treatment on Dari 60-61 reduced 14.0 % of A_{max} on Dari 31-32 VS. 39.9 % of N1) was also beneficial for grain filling and increased the crop yield.

Other intrinsic photosynthetic parameters reducing significantly by N application rates reduction included α and PAR_s in the season of 2013/2014. Lu and Yu (2001) have pointed out that under field conditions, the value of α is between 0.05 – 0.07 for wheat. In our study, winter wheat had α value of about 0.05 when treated with N3. Significantly different of α was only found between N1 and N3 (Fig. 6c). These results indicated that low N fertigation decreased α , which could also be a reason of Y difference. The PAR_s values over the three measurements dates gradually decreased, suggesting the decreased ability of the flag leaf to utilize the high intensity light over time. Significantly decrease of PAR_s was only found between N1 and N3 on Dari 60-61 (Fig. 6d), PAR_s of N2 did not drop much comparing to N3. Higher PAR_s is also benefit for yield formatting. N treatments did not have a significantly effect on PAR_c value, which was consistent with the results of pot-grown wheat experiment (Hall et al., 1984).

There were no significant differences in ET_a among N treatments in our study, which probably was because of the same irrigation amount for all treatments. The actual ET_a has two components, i.e. crop transpiration and soil evaporation. When adequate water is supplied, high N treatment can significantly increase leaf transpiration (Cabrera-Bosquet et al., 2009). Yet, because of the higher leaf area index, soil surface is more shaded and the soil evaporation is reduced. Consequently, the ET_a value remains almost the same despite of significant differences in transpiration rates among N treatments (Liu et al., 2012). In this case, lower N treatment resulting in lower yield in our study corresponded to lower WUE.

4.2 Interactions between photosynthetic parameters

In addition to analyzing the N treatment effects on photosynthetic parameters directly, interactions among the parameters, especially for the stomatal or non-stomatal limitation on photosynthesis, are discussed below.

The reduction in A_n with lower N application may be partially due to lowered g_s . However, non-stomatal limitations on A_n might also occur in leaves under stressful conditions (Li et al., 2013). It should be noted that, in our

study, the g_s data collected from plots treated with N3 treatments were not always the highest measured over different data collection dates (Fig. 4). For example, on Dari 48-50 of 2014, g_s of the N3 was lower than that of N1. Under growth conditions with adequate water supply, a moderately higher application of N can improve g_s value of a crop (Shangguan et al., 2000). But if the soil water was not adequate, the water demand of high N fertilizer treated plants should be higher. Consequently, the water deficiency would increase in plots with high N treatment, which increased the stomatal limitation of the plants, thereby making g_s reduction (Choi et al., 2005). In this study, the measurements conducted on Dari 48-50 of 2014 were performed at the late stage of the irrigation cycle, thus there may have been a mild water deficiency. This might be the reason for the low g_s value measured under high N treatment. On the other two measurements, g_s were lower in plots treated with lower N, which is consistent with the lower A_n and Tr.

The C_i/C_a value reflects the restriction of photosynthesis created by processes of the electron transport chain and Rubisco enzyme regeneration. Research conducted by Xu (2002) and Farquhar and colleagues (1982) described that if the decrease of the photosynthetic rate was accompanied with an increase in C_i , then the main limiting factors of photosynthesis are non-stomatal factors such as the decline of the photosynthetic activity in the mesophyll cells. Therefore, we compared the changes of C_i when the photosynthetic rates of the flag leaf decreased with the decrease of light intensity under different N treatments. It showed that, under high N (N3 and N2) treatment, when the light intensity decreased, the C_i value measured on Dari 31-32 and Dari 48-50 of 2014 decreased first then increased, while under low N treatment (N1) the C_i value increased right away with the A_n decreasing (data not shown), indicating that non-stomatal limitations occurred early under N1 treatment. With the decrease of light intensity the photosynthetic activity of mesophyll cells was lower under N1 treatment compared with N3 and N2 treatment. This result is consistent with results of the higher C_i/C_a values of N1 treatment (Fig. 5). Higher non-stomatal limitations were not found in N2 treatment. Those results suggested that low N treatment affects the photosynthetic gas exchange process due to the increase of the non-stomatal limitation. Therefore, it would be a disadvantage to the formation of photosynthetic product and the improvement of water use efficiency.

4.3 Relationships between photosynthetic parameters and biological factors of flag leaves

Statistical significantly correlations were found between A_{max} and leaf biological factors, such as leaf nitrogen content (N-mass and N-area) and ^{13}C isotope discrimination (Δ) for the season of 2013/2014. Those correlations were analyzed in two levels with different purposes.

Firstly, in order to determine the biological factors that caused the differences in $A_{\max}$ with different N treatments, we analyzed the correlations of $A_{\max}$ to N-mass, N-area and Δ for each measurement. N-mass failed to explain the variation of $A_{\max}$ for all the three measurements, however N-area can explain the differences of $A_{\max}$ in two measurement dates (Fig. 7), which indicates that using the N-area to explain the effects of different N treatments on the photosynthetic parameters is a more useful measurement in this study. The way $A_{\max}$ and leaf N content are related differs depending on whether N is expressed on a mass (N-mass) or an area (N-area) basis had also been found in other studies. Since $A_{\max}$ is expressed on an area basis, using N-area as an indicator of $A_{\max}$ is more popular (Hikosaka, 2004).

The other level are when the parameters from the three measurement periods are combined, the biological factors that correlated with the seasonal variations of $A_{\max}$ can be analyzed. It also can be determined whether the correlation is affected by different N treatments. The seasonal dynamics of $A_{\max}$ were also driven by the biological factors and the linear regressions between $A_{\max}$ and the biological factors were affected by N treatments (Fig. 8). This is different to the conclusion that the relationship between $A_{\max}$ and leaf N content is irrespective of nutrient status (Evans, 1983). The $A_{\max}$ of higher N treatment were higher at a given leaf N content, both at the mass and area basis (Fig. 8a and b). This increase in $A_{\max}$ has previously been reported in wheat grown under different amounts of N fertilization (Cabrera-Bosquet et al., 2007; Cabrera-Bosquet et al., 2009) and also in wheat and barley grown under different N types (Lopes et al., 2004; Lopes et al., 2006). Without more related data, we couldn't give more explanations of the higher $A_{\max}$, possible ones could either be a result of increase in nitrogen partitioning in the photosynthetic apparatus (Evans, 1989), or the less carbohydrate accumulation and repression of photosynthesis for the higher N treatment (Takao et al., 2010).

The quantitative correlation of $A_{\max}$ and leaf N is linear when the leaf N content is low. When the leaf N content is high and the changes are broad, the correlation is a curve with exponential growth (Bindraban, 1999). Viewed from the regression coefficient (R^2), there were better correlations between $A_{\max}$ and N-mass and N-area in the N1 treatment. This is reasonable since the range of leaf N content is narrower in this treatment. It has previously been reported that photosynthesis increases almost linearly up to an N-area value of about 1.5g m^{-2} , while beyond this value, the amount of N accumulated in the leaves is in excess for photosynthesis (Tambussi et al., 2005). If all the data in our study were pooled together, an exponential increase to maximum curve can be fit between $A_{\max}$ and N-

mass or N-area and $A_{\max}$. This supports the hypothesis that there is a critical value above which the $A_{\max}$ increase much less than that when leaf N blow the critical value.

The data from this study showed a seasonal variation of Δ . Leaf Δ increases as the value of $A_{\max}$ decreases during leaf senescence (Fig. 7c). These two variables exhibited a negative correlation (Fig. 7c and 8c). This conclusion is consistent with the conclusions of Gentsch and colleagues from their study of the seasonal variation of Δ (Gentsch et al., 2014). In previous literature, studies that employed ^{13}C stable isotope focused on the relationship between Δ in leaves or seeds at certain stages (anthesis or ripening) and photosynthetic rate, yield or water use efficiency for different species or varieties (Merah et al., 2001; Wahbi and Shaaban, 2011; Wang et al., 2013). Due to limited observational tools, fewer studies have been conducted on seasonal variations of Δ . In the present study, through repeated sampling and analysis from heading to grain filling stages, the relationship between $A_{\max}$ and Δ were found to be different from the above studies, but consistent with the findings from Xue and colleagues (Xue et al., 2002).

5 Conclusions

Y and WUE of N1 treatment (low N treatment, with ~40% N fertilizer of the highest level) reduced significantly in the season of 2013/2014. Unlike N1 treatments, Y and WUE of N2 (middle N treatment, with ~60% N fertilizer of the highest level) didn't reduce significantly in both seasons, suggesting that we could apply 190 kg N hm^{-2} per season in winter field of this area and keep the grain yield steady for at least a couple years.

We explained the yield differences by photosynthetic parameters. Significant reduction of *instantaneous* and *intrinsic* photosynthetic parameters (A_n , g_s , α , PAR_s and $A_{\max}$) of lower N treatments were found in both seasons. Intrinsic photosynthetic parameters, such as α , PAR_s and $A_{\max}$, were better indicators of yield than instantaneously photosynthetic parameters (A_n and g_s). For N2 treatments, even though A_n was significantly lower than that of N3 for the season of 2013/2014, the reduction of $A_{\max}$ was not significantly for two measurements, with the only exception of data on Dari 60-61. These results, together with α and PAR_s of N2 were not different significantly with N3, supported that Y of N2 didn't reduce significantly.

The variation of $A_{\max}$ could be explained by leaf N content and the ^{13}C discrimination of sample (Δ). Significant correlations between $A_{\max}$ and leaf N content or Δ were found in this study. These relationships were affected by N treatments, suggesting a different partitioning of leaf N to photosynthetic organs. Moreover, non-stomatal limitation was higher and occurred earlier in N1 treatment comparing to N3, but not in N2 treatment, which

would also be a reason to the difference of photosynthetic parameters among N treatments. These results are important for understanding the mechanisms of yield reduction by reducing N application rates and provide the scientific basis for application of fertigation in this area.

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Figure captions

Fig. 1. Daily dynamics of net photosynthesis rates of flag leaves of all the treatments measured on (a) Dari 22 and (b) Dari55 in 2013.

Notes: Error bars in the figure indicate the standard deviations; for comparison of the two years, and to other studies, we referred the measuring dates as *days after the irrigation of reviving stage* (Dari), such notes are the same in Fig. 2.

Fig. 2. Non-rectangular hyperbola fits of the net photosynthetic rates (A_n) and the photosynthetic active radiation (PAR) under different N treatments. The inset figures are the linear fits between A_n and PAR when $0 < \text{PAR} < 150 \mu\text{mol m}^{-2} \text{s}^{-1}$. Panel a, b and c are the response curves from the three data collection times, i.e. Dari 31-32, Dari 48-50 and Dari 60-61, respectively, in 2014

Fig. 3. Comparison of the effects of N treatments on flag leaf instantaneously net photosynthesis rates, A_n in (a) 2013 and (b) 2014.

Notes: Error bars in the figure indicate the standard deviations. Different lower-case and upper-case letters in the figure indicate statistically significant differences at the level of 0.05 and 0.1, respectively; *ns* indicates no significant difference among treatments, such notes are the same in Fig. 4 and 5

Fig. 4. Comparison of the effects of N treatments on flag leaf stomatal conductance g_s in (a) 2013 and (b) 2014.

Fig. 5. Comparison of the effects of N treatments on flag leaf non stomatal limitation C_i/C_a in (a) 2013 and (b) 2014.

Fig. 6. Comparison of the effects of N treatments on flag leaf photosynthesis parameters (a, maximum photosynthetic rate A_{max} ; b, apparent quantum efficiency α ; c, respiration rate R_d ; d, light compensation point PAR_c and light saturation point PAR_s) measured on the three dates in 2014.

Notes: Error bars in the figure indicate the standard deviations. Different lower-case and upper-case letters in the figure indicate statistically significant differences at the level of 0.05 and 0.1, respectively; *ns* indicates no significant difference among treatments.

Fig. 7. Correlations between the maximum photosynthetic rate ($A_{\max}$) and (a) N content of unit leaf weight (N-mass), (b) N content per unit leaf area (N-area) and (c) ^{13}C isotope discrimination (Δ) of each measuring dates in 2014.

Note: Only the significantly fit linear regressions were shown.

Fig. 8. Correlations between the maximum photosynthetic rate ($A_{\max}$) and (a) N content of unit leaf weight (N-mass), (b) N content per unit leaf area (N-area) and (c) ^{13}C isotope discrimination (Δ) of the winter wheat flag leaves under different N treatments in 2014.

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649 Table 1 Dates of sowing, reviving stage (SR) started and harvest, and irrigation managements for the two seasons.

Seasons	Dates of sowing	Dates of winter irrigation (WI)	Irrigation amounts of WI	Dates of reviving stage (SR) started	Dates of harvest	Dates of fertigation	Irrigation times during the SR to harvest
2012/2013	Oct. 12, 2012	Nov. 22, 2012	50 mm	Apr. 5, 2013	Jun. 19, 2013	Apr.10,2013	5
2013/2014	Oct. 11, 2013	Nov. 20, 2013	50 mm	Apr. 1, 2014	Jun. 15, 2014	Apr. 4, 2014	4

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653 Table 2 Parameters of liner regression ($Y = y_0 + aX$) between the maximum photosynthetic rate ($A_{\max}$)
654 and N content of unit leaf weight (N-mass), N content per unit leaf area (N-area) and ^{13}C isotope
655 discrimination (Δ) of each measurement in 2014.

Dependent variable	Independent variables	Treatments	Regression coefficients		Statistical parameters	
			y_0	a	R^2	P
$A_{\max}$ ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	N-mass (g kg^{-1})	N3	24.15	0.19	0.794	0.043
		N2	13.63	0.41	0.790	0.044
		N1	6.42	0.63	0.985	0.001
	N-area (g m^{-2})	N3	25.15	2.42	0.841	0.028
		N2	15.51	5.40	0.812	0.037
		N1	8.90	8.66	0.979	0.001
	$\Delta(\text{‰})$	N3	76.05	-2.19	0.960	0.003
		N2	125.79	-4.67	0.856	0.024
		N1	185.31	-7.54	0.753	0.057

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Table 3 Water balance components, such as irrigation amount (I), precipitation (P), change in soil water storage (ΔS), and evapotranspiration (ET_a), during the reviving stage (SR) to harvest stage for the two seasons.

Water balance calculating period (reviving stage, SR to harvest)	Treatments	Irrigation amount I (mm)	Precipitation P (mm)	Soil water storage change ΔS (mm)	ET_a during the SR to harvest (mm)
Apr. 5-June. 19, 2013	N3	144	59	-83.9	286.9
	N2	144	59	-88.0	291.0
	N1	144	59	-78.4	281.4
Apr. 1-June. 15, 2014	N3	120	94	-87.2	301.2
	N2	120	94	-91.5	305.5
	N1	120	94	-94.2	308.2

Table 4 Comparison of crop yield (Y), evapotranspiration (ET_a) and water use efficiency (WUE) under different N treatments for the two seasons.

Year	Treatments	Yield (Y , kg hm ⁻²)	Evapotranspiration* (ET_a , mm)	Water use efficiency (WUE, kg hm ⁻² mm ⁻¹)
2013	N3	5375.8(338.6) a	441.5(9.6) a	12.18(0.58) a
	N2	5297.4(402.5) a	447.7(7.2) a	11.83(0.72) a
	N1	5040.8(399.6) a	433.0(8.3) a	11.64(0.71) a
2014	N3	5981.8(443.9) a	463.4(7.6) a	12.90(0.68) a
	N2	5672.0(496.1) ab	470.0(14.2) a	12.07(0.73) ab
	N1	5229.2(325.8) b	474.3(10.2) a	11.03(0.50) b

* Evapotranspiration (ET_a) of this table was for the whole growing season (from October to July of next year) and was estimated with the ratio of the ET_a during the reviving stage to harvest stage (from the water balance method, Table 3) to the ET_a of the whole growing season, 0.65, get from a reference with 5 years experiment of the same area.