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Physiological response to low-nitrogen stress and comprehensive evaluation in four rice varieties

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Abstract

Rice (*Oryza sativa* L.) research has rarely focused on the response to low-nitrogen stress in different subtypes previously and lacked a low-nitrogen tolerance evaluation system. Here, we investigated the physiological characteristics under moderate and low-nitrogen stress conditions in two *japonica* cultivars (NG46 and NG9108) and two *indica* cultivars (LYP9 and 9311). Using subordinate function analysis and principal component analysis, the low-nitrogen tolerance of four rice varieties was comprehensively evaluated; stomatal conductance, total carotenoid content, and nitrate reductase NR activity were taken as the low-nitrogen tolerance evaluation system. Among the four rice cultivars, NG46 and LYP9 had significant advantages in photosynthetic gas-exchange capacity, optimizing the balance between light-harvesting capacity, the ratio of reaction center inactivation, the magnitude of decrease in heat dissipation, and nitrogen-metabolism enzyme activities. The results investigated the physiological mechanisms of rice adaptation to low-nitrogen stress and offered a reliable method for assessing low-nitrogen tolerance in rice.

Keywords: low-nitrogen tolerance evaluation; nitrogen metabolism; photosynthesis; rice; subordinate function analysis.

Introduction

Nitrogen is the primary component of photosynthetic pigments like chlorophyll and is called the 'life element' of plants. It is also necessary for the synthesis of nucleic acids and proteins (Kusano *et al.* 2011). At present, China accounts for 30% of the global chemical N fertilizer use, while the average nitrogen uptake and utilization efficiency of plants is less than half of that of developed countries (Haegele *et al.* 2013, Ju *et al.* 2015). Not only over-applied and insufficient usage rate of nitrogen result in significant financial waste, but also the loss of excess nitrogen through ammonia volatilization, nitrification and denitrification, and nitrate leaching is leading to serious environmental pollution problems (Han *et al.* 2015). Therefore, under the premise of ensuring crop yield, it is of great significance to appropriately reduce the amount of nitrogen fertilizer applied and increase the rate at which nitrogen fertilizer is utilized for the environment's and crops' sustainable development (Dahlin and Marstorp 2012).

Nitrogen metabolism is involved in many aspects of material and energy metabolism in life activities, including uptake, transport, assimilation, and retransfer processes (Tian *et al.* 2022). Plants have evolved two nitrogen uptake and transport mechanisms, NO_3^- and NH_4^+ uptake and transport, which ensure that crops can sense the state

Highlights

- Nitrogen deficiency affected physiological parameters in all four rice cultivars
- The evaluation system of low-N tolerance in rice was established
- NG46 and LYP9 were low-nitrogen-tolerant materials

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Abbreviations: Car – carotenoids; Chl – chlorophyll; C_i – intercellular CO_2 concentration; E – transpiration rate; GDH – glutamate dehydrogenase; GOGAT – glutamate synthase; GS – glutamine synthetase; g_s – stomatal conductance; NR – nitrate reductase; P_N – net photosynthetic rate; RC – reaction center.

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of nitrogen in the soil in the face of different environments and thus adjust their uptake capacity (Xuan *et al*. 2017). Nitrite obtained by nitrate reductase (NR) reduction into chloroplasts or plastids is converted to ammonium by nitrite reductase (NiR) before it can be utilized by plants (Krapp 2015), then NH_4^+ with the help of glutamine synthetase (GS)/glutamate synthetase (GOGAT) cycle to accomplish amino acid and protein metabolism (Maeda *et al*. 2014, Plett *et al*. 2016). Glutamine dehydrogenase (GDH) is also an auxiliary pathway for nitrogen assimilation in plants, and a study has confirmed that when the GS pathway fails to assimilate NH_4^+ in the external environment promptly, the activity of GDH in plants will increase (Skopelitis *et al*. 2006).

Chloroplasts, an important organelle for photosynthesis in plants, and chlorophyll (Chl), a photosynthetic pigment, are mainly constructed through nitrogen, so a lack of nitrogen will seriously affect the photosynthesis of plants (Wen *et al*. 2019). Xu *et al*. (2020) found that the intensity of photosynthesis in rice was linearly related to the nitrogen content of the plant within a certain range, and the net photosynthetic rate of flag leaves tended to increase with the increase of nitrogen content of leaves. When nitrogen deficiency occurred, the Chl content, SPAD value, leaf area index, stomatal conductance (g_s), and Rubisco enzyme activity of rice leaves were reduced, resulting in weakened photosynthesis and impeded carbon-hydride synthesis (Tantray *et al*. 2020). The technique of studying plant photosynthesis using the fluorescence produced by Chl as a reference is sensitive to the plant's physiological state and its relationship with the environment (Kalaji *et al*. 2014a). Zheng *et al*. (2021) found in wheat that nitrogen deficiency caused the photochemical quenching activity and electron transport rate to decrease, but an increase in nonphotochemical quenching.

Crop responses to abiotic stresses are complex and evaluating resistance by only one physiological and biochemical index is one-sided (Zou *et al*. 2022). With the deepening of the concept of comprehensive evaluation of multiple indicators, principal component analysis (PCA) and affiliation function are two multivariate statistical techniques that are increasingly being employed for the thorough assessment of plant resistance (Li *et al*. 2018). For example, low nitrogen in broomcorn millet (Liu *et al*. 2020), high temperature and humidity in melon (Weng *et al*. 2021), and drought in winter wheat (Chen *et al*. 2012). Its advantage lies in using the idea of dimensionality reduction to turn multiple indicators into several composite indicators, and then through the affiliation function analysis for comprehensive evaluation (Sivakumar *et al*. 2020). However, there are few studies that have comprehensively evaluated low-nitrogen tolerance in rice by several indicators.

It is generally believed that nitrogen-use efficiency is lower in *japonica* compared to *indica* and varies considerably among varieties (Hu *et al*. 2015). Therefore, the differences in nitrogen uptake, translocation, enrichment, and molecular physiological mechanisms among *indica* and *japonica* subspecies should be investigated first. On this basis, we should screen the varieties, determine the indexes of low-nitrogen tolerance, and utilize the hybridization between *indica* and *japonica* subspecies to cultivate rice varieties for effectively solving the problem of excessive nitrogen application.

Here, we selected two *japonica* cultivars (NG46 and NG9108) and two *indica* cultivars (LYP9 and 9311) to elucidate the potential physiological adaptation mechanisms of rice in reaction to low-nitrogen stress by contrasting the modifications in physiological characteristics (photosynthesis and nitrogen metabolism) of four varieties. Additionally, based on the physiological and biochemical indicators found in leaves, a thorough assessment of each variety's low-nitrogen tolerance was conducted using principal component analysis, subordinate function analysis, and cluster analysis. This will hopefully enable a timely assessment of rice's low-nitrogen tolerance and establish a theoretical foundation for reducing the amount of nitrogen fertilizer applied.

Materials and methods

Plant materials and growth conditions: The experiment was conducted at the Xianlin Campus, Nanjing Normal University, Nanjing, China (32°03'N, 118°47'E). The soil was clay loam with medium fertility and the total nitrogen content was 1.06 g kg^{-1}. Four high-quality rice varieties NG9108, NG46, LYP9, and 9311 were selected for this project and were sown in mid-May and transplanted to the experimental field. The mean, maximum, and minimum month temperatures during the rice growth period were 26.6 ± 0.18, 35.2 ± 0.17, and 18.00 ± 0.32°C, respectively. Two nitrogen concentrations were set for treatment, low nitrogen (LN, 90 kg ha^{-1} of pure nitrogen during the whole life cycle) and moderate nitrogen (MN, 180 kg ha^{-1} of pure nitrogen during the whole life cycle), respectively, and ridges were constructed between the different nitrogen concentrations to prevent cascading of water and fertilizer. Nitrogen fertilizer (urea) was divided into base fertilizer (1 d before transplanting), tiller fertilizer (7 d after transplanting), flower-promoting fertilizer (30 d before spiking), and flower-protecting fertilizer (15 d before spiking), and applied four times at the ratio of 4:2:2:2.

Sampling was performed at the tillering stage. Leaves were selected with growth and uniformity and then frozen in liquid nitrogen immediately.

Photosynthetic parameters: Measurement was performed according to Li *et al*. (2010). Using a portable photosynthesis system (*LI-6400*, *LI-COR*, Lincoln, Nebraska, USA), Measurements of the net photosynthetic rate (P_N), the stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) of four rice varieties under LN and MN conditions were made in the field. The PAR intensity was 1,200 μmol(photon) m^{-2} s^{-1} and ambient CO_2 concentration was 400 μmol mol^{-1}. The measurements were taken from 8:00 to 11:00 h, with five repeats for each treatment.

Photosynthetic pigments: Determination of photosynthetic pigments in rice leaves was done as described by Arnon

(1949). Pigments of leaves were extracted with acetone: ethanol: water (4.5:4.5:1, v/v/v), and then the absorbance of the supernatant was measured at 470, 645, and 663 nm, respectively, using UV-Vis spectrophotometer (*Genesys 10*, *Thermo Electron*, USA); three replicates were set up for each treatment. Measurements were used to calculate photosynthetic pigment content according to Lichtenthaler (1987).

Chlorophyll *a* fluorescence: Using a portable fluorometer (*Handy PEA*, *Hansatech*, UK), the Chl fluorescence characteristics of four different types of rice were assessed according to the technique outlined in Strasser *et al.* (2004). Leaves were darkly acclimatized with leaf clips for 30 min and were then irradiated with continuous light at a saturation pulse intensity of 3,000 μmol(photon) m^{-2} s^{-1}. We determined 10 replicates for each treatment and analyzed them with *PEA Plus Software* (*Hansatech*, UK). Specific fluorescence parameters are defined as shown in Table 1S (*supplement*).

Nitrogen metabolizing enzyme activities: Using a UV-Vis spectrophotometer (*Genesys 10*, *Thermo Electron*, USA), the activities of nitrate reductase (NR, EC 1.7.99.4) were measured at 520 nm by the techniques of Luo *et al.* (2013) with minor modification. Glutamine synthetase (GS, EC 1.6.6.1) activity was assayed after modification as described by Farnden and Robertson (1980). Extracted with imidazole–HCl (pH 7.5) the absorbance value of the supernatant was determined at 540 nm. Glutamate dehydrogenase (GDH, EC 1.4.1.2) and glutamate synthase (GOGAT, EC 1.4.7.1) activities were measured following the method of Lin and Kao (1996). Leaves were extracted with 20 mM L-glutamine, 20 mM α-ketoglutarate, 10 mM KCl, 3 mM NADH, and 25 mM Tris–HCl, and absorbance values were measured at 30-s intervals for 11 consecutive measurements over 5 min at 340 nm.

Data analysis: The data is displayed as the average (± SD) of a minimum of three biological replicates. With the aid of *SPSS 22.0*, data were analyzed using a two-way analysis of variance (*ANOVA*) followed by *Tukey*'s test ($P<0.05$). All images were created by *Origin Pro 2022*.

To accurately assess the low-nitrogen tolerance of different rice varieties, the coefficients of low-nitrogen tolerance of physiological indicators were analyzed by principal component analysis (PCA), which in turn led to a comprehensive evaluation using the method of affiliation function in fuzzy mathematics. The calculation formula is as follows (Cao *et al.* 2015):

$$\mu X_i = (X_i - X_{min})/(X_{max} - X_{min}) \quad (1)$$

$$W_i = Pi / \sum_{i=1}^{n} Pi \quad (2)$$

$$D = \sum_{i=1}^{n} \left[\mu X_i \times W_i\right] \quad (3)$$

where μX_i is the value of the affiliation function of each indicator, and X_i denotes the i-th composite indicator. X_{max} and X_{min} are the maximum and minimum values of the i-th composite indicator. W_i denotes the i-th composite indicator among all composite indicators in the index weight, Pi is the contribution of the i-th principal component of different varieties contribution rate. D is the overall evaluation value of the low-nitrogen tolerance of each variety.

Results

Photosynthetic characteristics: According to the two-way *ANOVA*, the variety effects were not significant for P_N and *E* but were significant for g_s and C_i, the treatment effects were significant except for C_i, and their interactions were all significant (Table 2S, *supplement*). As illustrated in Fig. 1, the photosynthetic activity of four rice varieties in leaves was measured, and the net photosynthetic rate (P_N) significantly decreased in all varieties. The influences of low-nitrogen stress on leaves' photosynthetic parameters in LYP9 and 9311 were greater than those in NG9108 and NG46 (Fig. 1). Compared with MN condition, N stress reduced the P_N value of NG9108 and NG46 by 12.9 and 12.8%, respectively, while the P_N value was reduced by 21.8 and 28.4% in LYP9 and 9311 (Fig. 1*A*). Similar patterns were followed for the stomatal conductance (g_s) and transpiration rates (*E*) of four varieties, with 9311 showing the most significant decrease, which was 30% lower than MN (Fig. 1*B*,*C*). Overall, the intercellular CO_2 concentration (C_i) under N stress was almost the same as MN conditions except for 9311, whereas the C_i value declined by 3.8% under N stress in 9311 (Fig. 1*D*).

Photosynthetic pigment contents: The photosynthetic pigments of the four rice varieties are shown in Fig. 2. According to two-way *ANOVA*, a significant variety effect, treatment effect, and interaction was observed in total Chl, Chl *a*/*b*, and total Car (Table 2S). A significant decreasing trend in the total Chl contents was observed but the Chl *a*/*b* ratio showed the opposite pattern when treated with N stress (Fig. 2). Total Chl content of NG46 was only 9.5% lower under LN than MN condition, while the other three varieties were all reduced by more than 30% (Fig. 2*A*). Compared to MN condition, the Chl *a*/*b* ratio of 9311 was significantly higher by 28.0% under LN condition; this was followed by a 22.4% increase in LYP9 and more than 10% in NG46 and NG9108 (Fig. 2*B*). Total Car content of NG46 under LN was approximately 10% higher than MN (Fig. 2*C*), indicating that the less affected photosynthetic capacity in NG46 by N deficit may be closely related to its higher Car content for light protection.

Chlorophyll fluorescence: The Chl fluorescence parameters of the four rice varieties (shown in Fig. 3) performed differently under the N stress conditions. All parameters were measured by 11:00 h after the photosynthetic parameters were measured (Fig. 3). Under N stress condition, both the electron transport flux per reaction center (RC) at t = 0 (ET_0/RC) and the electron transport flux per excited leaf cross-section (CS) at t = 0 (ET_0/CS_0) showed substantial decreases. The electron transfer of NG9108 was most affected by nitrogen limitation, except for LYP9, whose ET_0/RC value

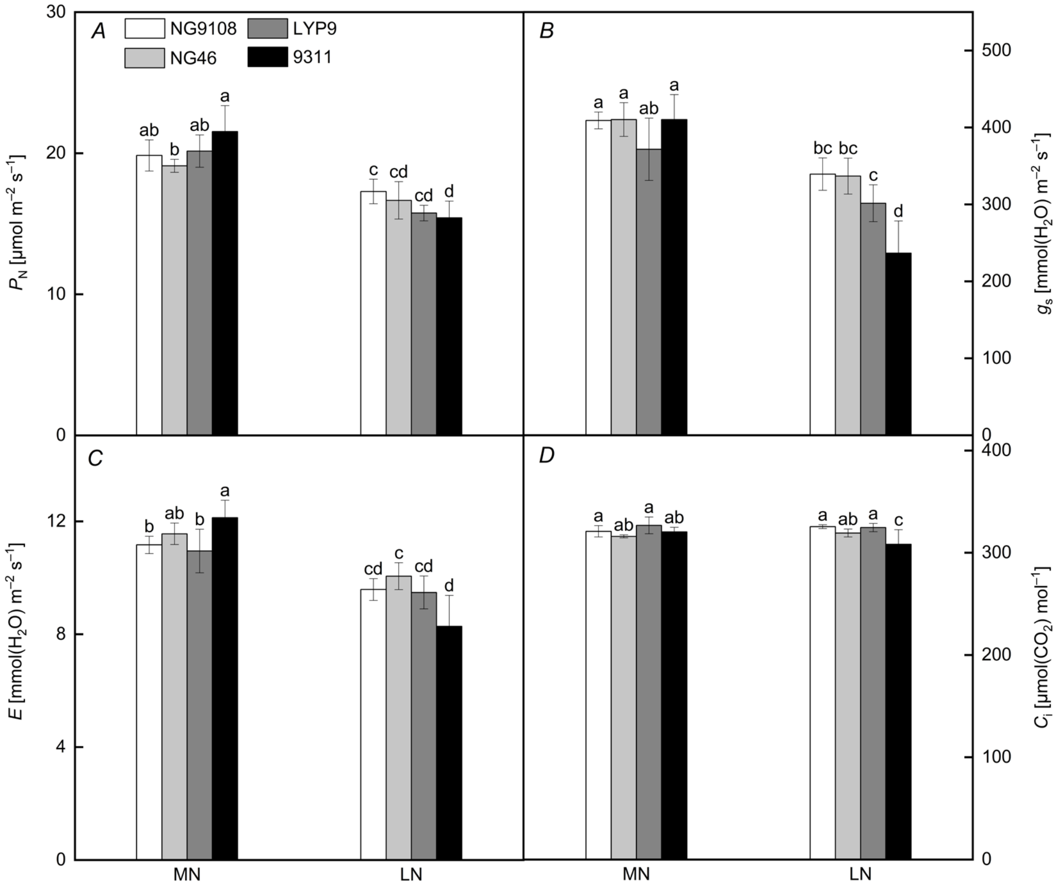

Fig. 1. Photosynthesis parameters of leaves of four rice varieties under different nitrogen treatments. (*A*) Net photosynthetic rate (P_N), (*B*) stomatal conductance (g_s), (*C*) transpiration rate (*E*), (*D*) intercellular CO$_2$ concentration (C_i). MN – moderate nitrogen condition, LN – low nitrogen condition. Data are displayed as mean and standard deviation ($n = 5$). *Different letters* indicate significant differences according to two-way *ANOVA* followed by *Tukey*'s test ($P<0.05$).

was elevated by a factor of 1.04 compared with MN. In contrast, DI$_0$/RC and DI$_0$/CS$_0$ values of NG9108 were about 1.2 and 1.1 times greater, respectively, under N stress conditions than that of MN, suggesting increased energy dissipation. In addition, the trapped energy flux per RC at t = 0 (TR$_0$/RC) and the absorption flux per RC (ABS/RC) of NG9108 showed a similar pattern of increase, both more than 1.1 higher than MN. The maximal quantum yield of PSII photochemistry (F$_v$/F$_m$) of NG9108 was not dominant under the LN condition. ABS/CS$_0$ and TR$_0$/CS$_0$ showed little variation among four rice varieties after reduced nitrogen application. Furthermore, PI$_{total}$ and PI$_{abs}$ values showed substantial increases in NG9108 and 9311; NG9108 performed more prominently, respectively. Thus, NG9108 had superior overall functional activity performance of PSII and PSI under LN conditions.

Enzyme activities related to nitrogen metabolism: As shown in Fig. 4, the activities of enzymes related to nitrogen assimilation were differentially affected by nitrogen limitation among four rice cultivars. According to two-way *ANOVA*, a significant variety effect, treatment effect, and interaction was observed in nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH) activity (Table 2S). The NR activity was drastically reduced by low-nitrogen stress except for NG9108. The data suggested that compared with MN, nitrogen deficiency decreased the NR activity of LYP9, 9311, and NG46 by 61.4, 33.0, and 18.3%, respectively (Fig. 4*A*). While the GS activity of NG9108 and NG46 was 28.5 and 34.2% lower than MN, respectively, the GS activity of LYP9 had opposite pattern and no significant change was found in 9311 (Fig. 4*B*). For GS and NR activities, these four rice materials performed differently under low-nitrogen stress. The GOGAT and GDH activities of rice were heavily influenced by nitrogen deficiency. The GOGAT activities of NG46 and LYP9 under LN condition were significantly higher than those of NG9108 and 9311, and nitrogen deficiency reduced the GOGAT activities of NG9108 and 9311 by 70.4 and 61.2% on average, respectively (Fig. 4*C*). The GDH activities were differently affected by low-nitrogen stress in the four rice varieties. Nitrogen deficiency had a greater effect on GDH activity in NG46 and 9311, which was 47.7 and 72.6% lower than the MN condition, respectively (Fig. 4*D*).

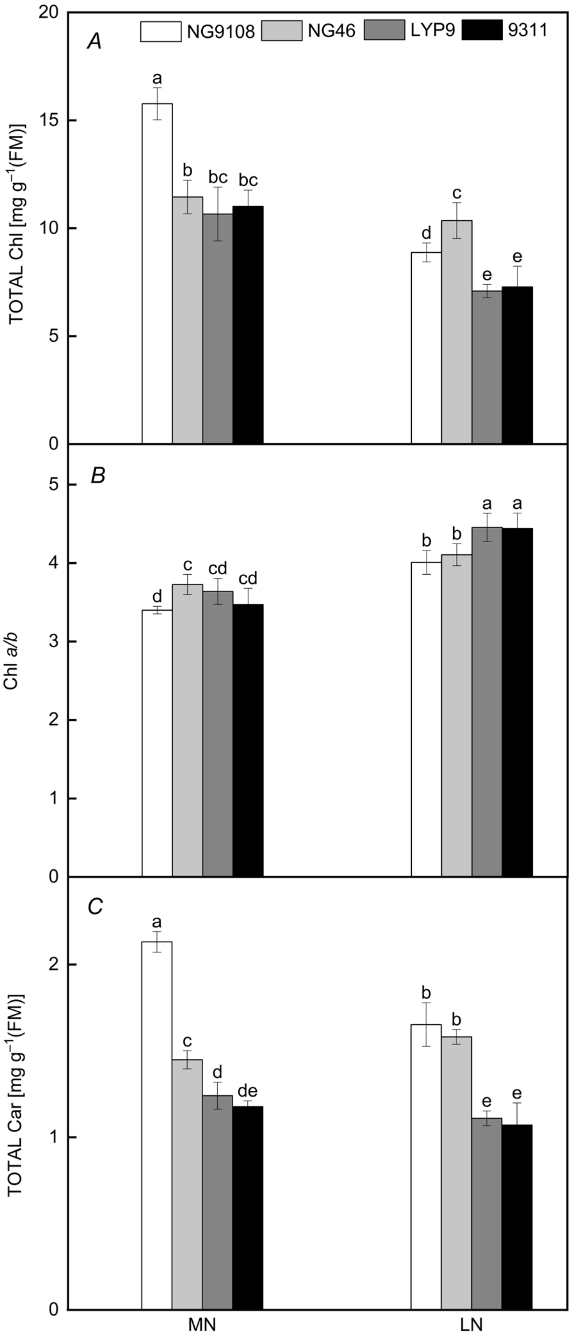

Fig. 2. Photosynthetic pigments of leaves in four rice varieties under different nitrogen treatments. (*A*) Total Chl content, (*B*) Chl *a/b* value, (*C*) total Car content. MN – moderate nitrogen condition, LN – low nitrogen condition. Data are displayed as mean and standard deviation (n = 5). *Different letters* indicate significant differences according to two-way *ANOVA* followed by *Tukey*'s test ($P<0.05$).

Correlation between physiological parameters: *Pearson*'s correlation analysis was conducted to characterize the lower-N-tolerance coefficients of several physiological indicators, and the correlation of each indicator was shown in Fig. 5. Except for the intercellular CO_2 concentration, highly significant positive correlations were observed between the relevant indicators of photosynthetic gas exchange ($P<0.001$), the Chl content also showed highly significant positive correlations with indicators related to photosynthetic gas exchange ($P<0.01$). Contrarily, the Chl *a*/*b* value highly negatively correlated with most physiological indicators ($P<0.001$). The N metabolism-related enzyme activities except for GS were also significantly positively correlated with most photosynthetic parameters and pigments ($P<0.05$). It followed that there was some information overlap among the indicators of lower-N tolerance, therefore, a single indicator could not comprehensively and objectively evaluate the lower-N tolerance of four rice varieties, and a combination of variables would be needed for evaluation.

Principal component analysis (PCA) of physiological parameters: To remove the less influential parameters and transform the above physiological parameters into fewer and more effective evaluation indexes, PCA was performed based on the ratio of physiological parameters to moderate nitrogen of four rice varieties. As illustrated in Table 1, twelve physiological parameters were extracted into three principal components. The PC1 with the largest eigenvalue and contribution was g_s with 0.987 and 46.306%, respectively. Similarly, the PC2 and PC3 of higher eigenvalue were Car and NR, with the contribution of 28.926% and 10.768%, respectively. Thus, PC1 separated the photosynthetic capacity, PC2 indicated the photosynthetic pigmentation profile, and PC3 revealed the effects of nitrogen stress. The cumulative contribution of three principal components attained 86%, which was used to represent most of the physiological parameters with credible information. Therefore, lower-N tolerance characteristics of the four rice varieties could be comprehensively analyzed by three freestanding composite indicators.

Comprehensive evaluation of low-nitrogen tolerance: Subordinate function analysis was performed based on the composite index values derived from the PCA results, which will provide a more objective and comprehensive evaluation of low-N tolerance of four rice varieties. As shown in Table 2, if only X3 would be considered, LYP9 had the maximum value of the affiliation function 1.32686, which was the most tolerant to low-nitrogen, and NG46 had the minimum value of the affiliation function –0.84153, which was the most sensitive to low nitrogen. Therefore, the D value of each variety was calculated by combining the affiliation function and index weight, a high D value indicated a high tolerance to low nitrogen. The D values were further clustered systematically using the squared Euclidean distance method, and the four rice materials were clustered into three classes, with NG46 and LYP9 being the low-N tolerant, 9311 being the low-N sensitive, and NG9108 being the intermediate low-N tolerant materials, respectively (Fig. 6).

Discussion

Physiological response to low-N stress in rice: Photosynthesis is the foundation for crop development and

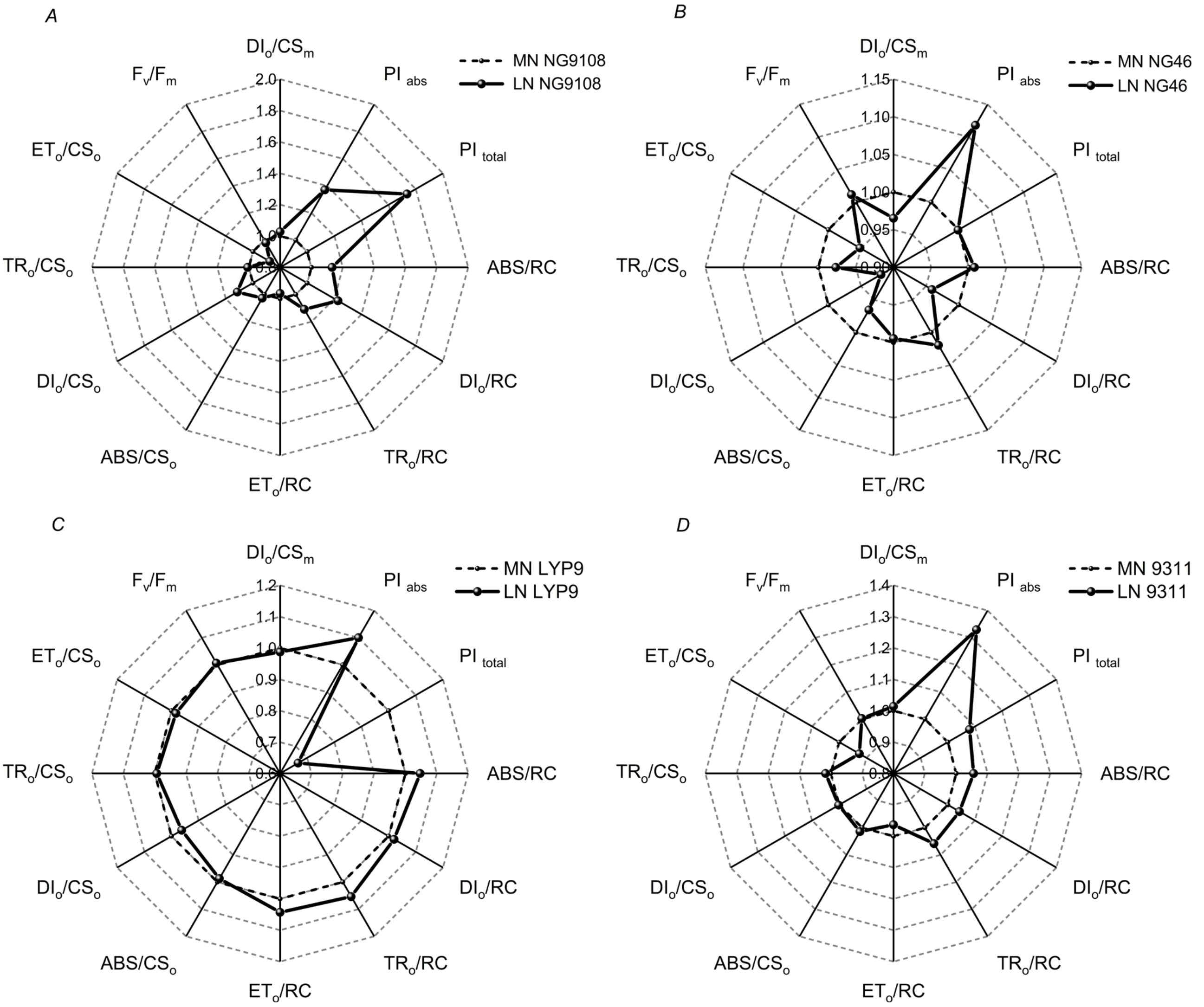

Fig. 3. Spider plots of leaves of four rice varieties under different nitrogen treatments. Each parameter was standardized using moderate nitrogen as a control. The formulae and definitions of each parameter are described in Table 1S. *A*, *B*, *C*, and *D* represent the measurements of NG9108, NG46, LYP9, and 9311, respectively. MN – moderate nitrogen condition, LN – low nitrogen condition.

yield formation (Singh and Thakur 2018). As the material basis of plant photosynthesis, variations in the content and composition of photosynthetic pigments directly affect the net photosynthetic rate (Beckmann *et al*. 2009). Vialet-Chabrand *et al*. (2017) have shown that the Chl *a*/*b* ratio reflected plant stress tolerance and was negatively correlated with photosynthetic efficiency; this view was also confirmed by *Pearson*'s correlation analysis in this study, where the Chl *a*/*b* value showed highly significant negative correlation with photosynthetic parameters (Fig. 5). It was found that elevated Chl *a*/*b* ratio helped plants adapt to high-light conditions (Jin *et al*. 2016), while tolerance to shade was indicated by the low Chl *a*/*b* value (Matsubara *et al*. 2012). In this study, the total Chl content of four rice varieties was significantly reduced under low-nitrogen condition, while the Chl *a*/*b* value increased in all of them (Fig. 2). This may be due to the inhibition of Chl synthesis and osmotic stress caused by low-nitrogen stress in rice, which produced a large amount of reactive oxygen species, leading to the destruction of chloroplast structure and accelerated Chl decomposition (Liang *et al*. 2015). The increase in the Chl *a*/*b* value indicated that the net photosynthetic rate of rice was inhibited under low-N stress and rice resisted photoinhibition by adjusting the balance between phytochrome composition and light-trapping capacity to complete the entire reproductive period (Perrine *et al*. 2012). On the other hand, the Chl *a*/*b* ratio is also implied by N partitioning within the leaf, the proportional allocation of N under low-nitrogen conditions is more in favor of PSII, while LHCII is relatively stable, so the Chl *a*/*b* ratio increases (Kitajima and Hogan 2003). Carotenoids are important pigments in plants against photoinhibition; the total Car content of NG46 in this study was significantly accumulated under low-nitrogen stress, which reflected the photoprotective capacity of NG46 (Zhang *et al*. 2018).

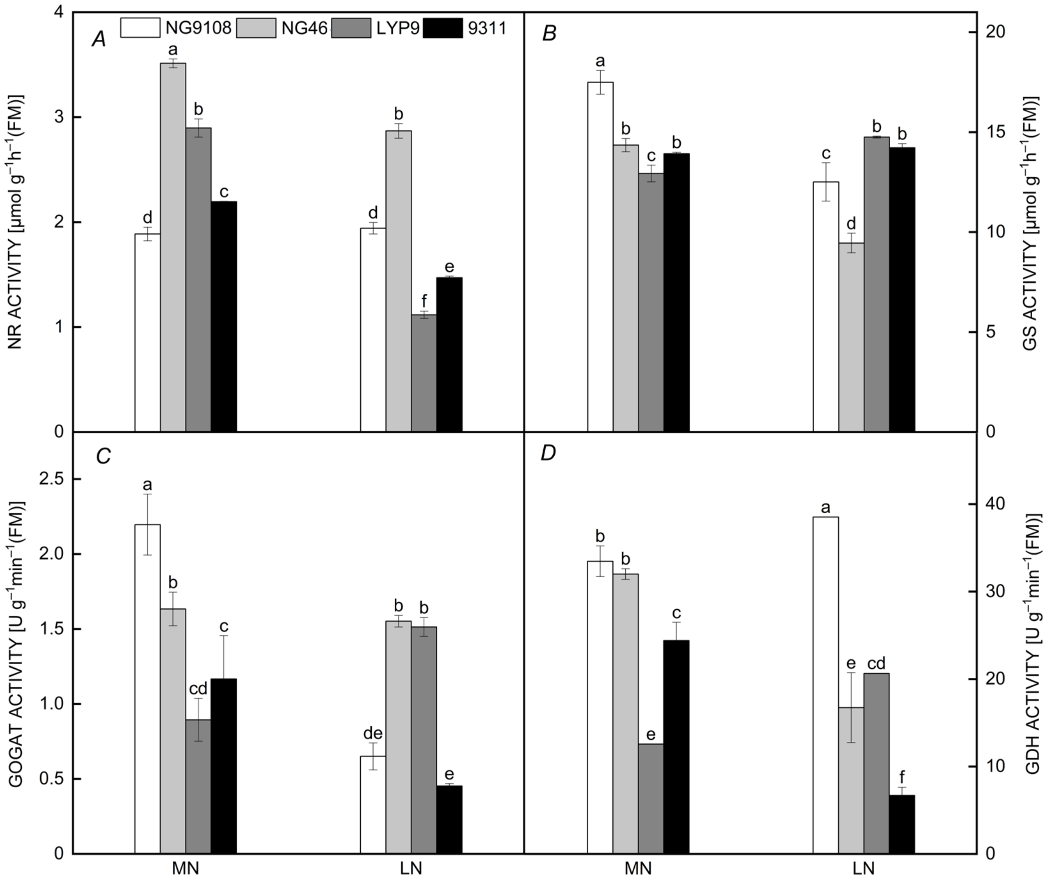

Fig. 4. Nitrogen metabolism enzyme activities of leaves of four rice varieties under different nitrogen treatments. (*A*) Nitrate reductase (NR) activity, (*B*) glutamine synthetase (GS) activity, (*C*) glutamate synthase (GOGAT) activity, (*D*) glutamate dehydrogenase (GDH) activity. MN – moderate nitrogen condition, LN – low nitrogen condition. Data are displayed as mean and standard deviation ($n = 5$). *Different letters* indicate significant differences according to two-way *ANOVA* followed by *Tukey*'s test ($P<0.05$).

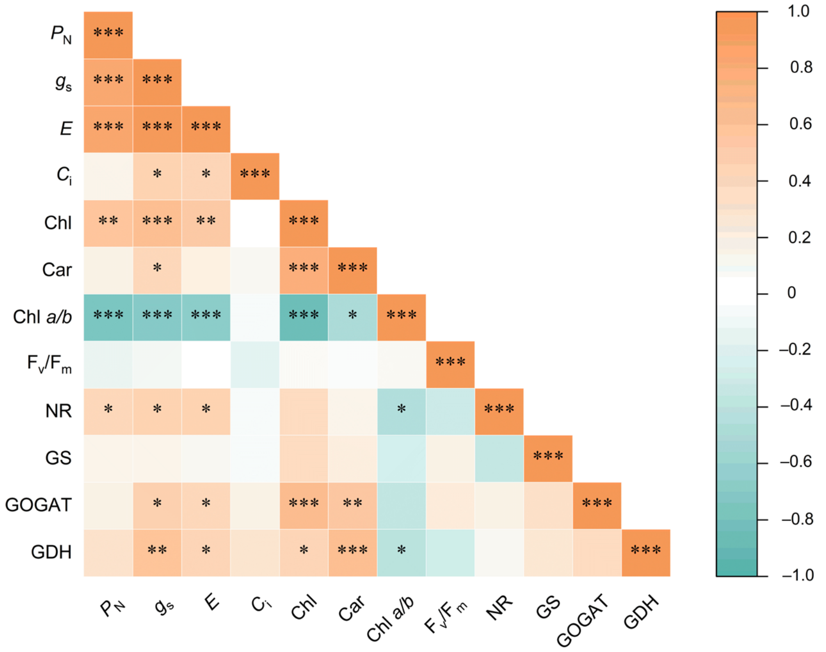

Fig. 5. *Pearson*'s correlation analysis of physiological parameters in four rice varieties. *, **, *** denote correlation levels of 0.05, 0.01, and 0.001, respectively.

Table 1. Coefficients and contribution rate of each indicator by PCA.

	Parameters	Principal component		
		1	2	3
Loading matrix of each component	P_N	0.928	0.059	-0.367
	g_s	0.987	-0.049	0.151
	E	0.975	0.023	0.221
	C_i	0.983	-0.037	-0.178
	Chl	0.210	0.977	-0.043
	Car	0.067	0.998	0.016
	Chl *a*/*b*	-0.833	-0.468	0.293
	F_v/F_m	-0.182	0.870	0.458
	NR	0.331	-0.189	-0.925
	GS	-0.574	-0.216	0.790
	GOGAT	0.285	0.288	0.914
	GDH	0.530	-0.516	0.674
Eigenvalue		5.557	3.471	1.972
Contributive ratio [%]		46.306	28.926	10.768
Cumulative contribution [%]		46.306	75.232	86.000

Table 2. Values for subordinate functions, comprehensive indicators, and comprehensive evaluations.

Varieties	X_1	X_2	X_3	μX_1	μX_2	μX_3	D
NG9108	0.645	-1.338	0.212	0.886	0.000	0.486	0.531
NG46	0.896	0.859	-0.842	1.000	1.000	0.000	0.752
LYP9	-0.226	0.662	1.327	0.493	0.910	1.000	0.739
9311	-1.315	-0.184	-0.697	0.000	0.525	0.066	0.168

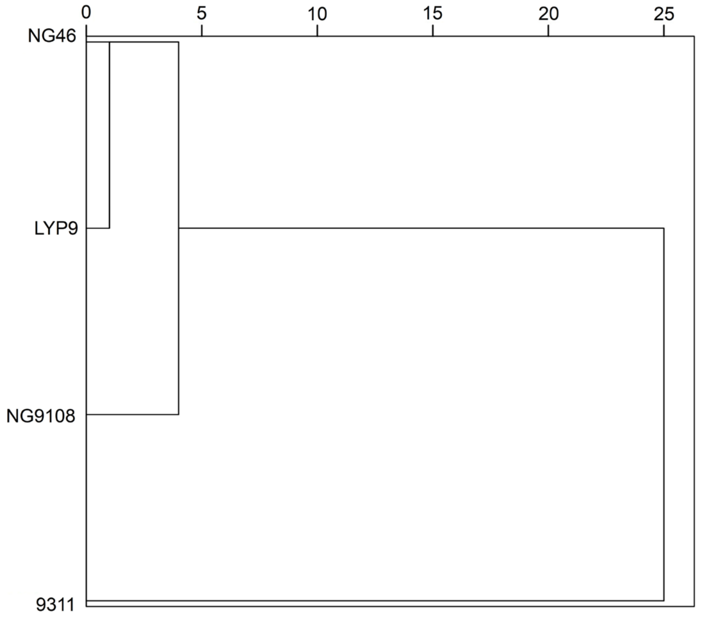

Fig. 6. Distribution of four rice varieties cluster analysis. *The top horizontal coordinate* is Euclidean distance and *the left vertical coordinate* is varieties, and *the nodes* indicate clustering into one category.

The reasons for the decrease in P_N included stomatal and nonstomatal factors, and when the pattern of change in P_N was consistent with C_i, it indicated that photosynthesis in plants was influenced by stomatal factors (Dong *et al.* 2006). In this study, we found that P_N and g_s of NG46 and NG9108 were thoroughly reduced, while C_i slightly increased under low-nitrogen stress (Fig. 1), suggesting that the decrease in their P_N might be a balance between stomatal and nonstomatal limitation (Ma *et al.* 2017, Jiang *et al.* 2021, Chen *et al.* 2021), whereas the reduced C_i of two *indica* may enhance low-nitrogen tolerance through water conservation and protection of photosynthetic organs (Liu *et al.* 2020).

PSII is the main site of photochemical reactions, and the parameters of Chl fluorescence can reveal a lot about how light energy is absorbed, transmitted, dispersed, and distributed by photosystems, *etc*. (Gong *et al*. 2019). It has been shown that nitrogen-deficient plants reduced light energy uptake and decreased the activity of PSII reaction centers (Tantray *et al*. 2020). In this study, ABS/RC, TR_0/RC, and DI_0/RC values were significantly higher in NG9108, LYP9, and 9311 under low-nitrogen conditions (Fig. 3). This suggests that low nitrogen led to the inactivation of the reaction centers in these three varieties and the reduction in their ability to light capture, which was thought to be a downregulation mechanism that dissipated excess light energy absorbed (Kalaji *et al.* 2014b). In this regard, the NG46 was more prominent. In addition, the ET_0/RC and ET_0/CS_0 values in NG9108 and 9311 were significantly reduced under low-nitrogen conditions, suggesting that the electron transport capacity was disrupted on the PSII receptor side of chloroplasts, affecting PSII primary photochemical reactions in NG9108 and 9311 (Akhter *et al*. 2021). PI_{abs} reflects the magnitude of light energy capture and conversion capacity of PSII reaction centers. In contrast to Filacek *et al.* (2022), the present study found that the PI_{abs} values of four rice varieties were elevated under low-nitrogen conditions, with NG9108 and 9311 showing the greatest increase. The F_v/F_m values also showed no significant reduction, indicating that the imbalance of energy capture and utilization and the damage to reaction centers is reversible (Gao *et al*. 2022, Hlahla *et al*. 2022).

NO_3^- in plants must be assimilated into NH_4^+ to participate in nitrogen metabolism, and the enzyme that plays a key role in its assimilation is NR, the first rate-limiting enzyme for nitrogen assimilation (Stitt *et al.* 2002). Moreover, GS is a crucial enzyme for plants' absorption of ammonia. In this study, we found that NR activity was drastically reduced while GS activity increased in two *indica* LYP9 and 9311 under low-nitrogen stress; the opposite trend of GS activity was observed in two *japonica* NG46 and NG9108 (Fig. 4). Relevant study has shown that the GS activity was positively correlated with nitrogen efficiency under low-nitrogen stress and high GS expression increased the efficiency of nitrogen metabolism in plants (Fuentes *et al*. 2001). The GS/GOGAT pathway is a key hub of nitrogen metabolism, and GDH is equally essential in regulation of nitrogen utilization (Kishorekumar *et al.* 2020). In the present study, the elevated GOGAT activity of LYP9 under low-nitrogen stress might be because high expression of GOGAT converted glutamine (Gln) to glutamate (Glu), which consumed a large amount of NH_4^+ and maintained its growth requirements, thus avoiding the toxicity of NH_4^+ accumulation to itself. Low nitrogen had no significant effect on the GOGAT activity of NG46 and no high concentration of NH_4^+ was to stimulate the elevation of GDH activity.

A comprehensive evaluation of low-N tolerance in rice: Resistance coefficient can eliminate the inherent differences between different varieties; comprehensive analysis of resistance coefficients using the affiliation function method has been widely used to assess plant stress tolerance (Pei *et al*. 2007). Miao *et al*. (2022) introduced the use of D value to categorize maize varieties into different low-nitrogen tolerances. Deng *et al*. (2023) analyzed the physiological parameters of nine quinoa species as an affiliation function; they found that aboveground and root biomass, F_v/F_m, total Chl content, nitrogen-metabolizing enzymes, and nitrogen content could be used as indicators for evaluating the low-nitrogen tolerance of quinoa. However, fewer studies have been conducted on the effective evaluation of the response indexes in different rice varieties to low-nitrogen stress. In this study, the physiological parameters of four rice varieties were comprehensively evaluated by subordinate function analysis and principal component analysis; the result was that 12 physiological indicators were extracted into three factors. The maximum eigenvector load was g_s, total Car content, and NR activity (Table 1). These results indicated that low-nitrogen tolerance in rice was affected by multiple factors such as environment and genetic background. It was difficult to comprehensively and accurately assess the low-nitrogen tolerance using a single resistance indicator, therefore, it was necessary to use multiple indicators to make the results of the assessment closer to reality (Bo *et al*. 2017).

In addition, different genotypes of rice were classified based on the comprehensive evaluation value (D) of low-nitrogen tolerance calculated by the affiliation function method. The results showed that NG46 and LYP9 were the low-N tolerant, 9311 was the low-N sensitive, and NG9108 was the intermediate low-N tolerant materials, respectively (Table 2, Fig. 6). These results provided theoretical references for high-yield and high-efficiency rice breeding and low-nitrogen tolerance breeding.

Conclusions: In this study, four rice cultivars were thoroughly assessed for their poor nitrogen tolerance using principal component analysis and subordinate function analysis. The g_s, total Car content, and NR activity were considered in the low-nitrogen tolerance assessment procedure, and the results indicated that NG46 and LYP9 were low-nitrogen tolerant materials. Although nitrogen deficiency affected physiological parameters in all four rice cultivars, NG46 and LYP9 had significant advantages in photosynthetic gas-exchange capacity, adjusting the balance between light-harvesting capacity, ratio of reaction center inactivation, magnitude of decrease in heat dissipation, and nitrogen-metabolism enzyme activities. This powerful physiological strategy may be one of the reasons for the higher resistance to low nitrogen in NG46 and LYP9.

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