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Research article

Low-nitrogen tolerance comprehensive evaluation and physiological response to nitrogen stress in broomcorn millet (*Panicum miliaceum* L.) seedling

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ABSTRACT

Developing the new crop varieties with high productivity under low nitrogen (N) input is an important access to facilitate modern agricultural sustainability. In the present study, 20 broomcorn millet (*Panicum miliaceum* L.) varieties were characterized for their morphological and nutrient parameters to different low N levels in seedling. The results showed that 0.25 mM NH_4NO_3 was the standard concentration for the evaluation and identification of low-N tolerance. Through pearson's correlation analysis, principal component analysis, and subordinate function analysis, the tolerance of 20 varieties under N stress was evaluated and plant height, root length, shoot biomass, and shoot and root N content were considered as the evaluation system of low-N tolerance. Although leaves photosynthetic capacities and activities of N metabolism related enzymes showed the decreasing tendency to N stress, low-N tolerant varieties had higher activities in both leaves and roots as compared to low-N sensitive varieties. The work provides a reliable and comprehensive method for evaluating low-N tolerance in broomcorn millet and our data elucidate possible physiological adaptive mechanisms by which broomcorn millet tolerates N stress.

1. Introduction

Nitrogen (N) is essential for plant growth and strongly required in the synthesis of nucleic acids, proteins, chlorophyll, lipids, and other metabolites (Kusano et al., 2011). Generally, it is fundamental for most plants to produce 1 kg of dry biomass with consuming 50 g of N (Xu et al., 2014). During the past 50 years, triple increase in N fertilizer application has contributed up to 70% increase in grain productivity in China, which simultaneously led to negative effects on the ecological environment due to lower N uptake and utilization efficiencies of plants, such as soil acidification, emission of greenhouse gases and water eutrophication (Wei et al., 2013; Shah et al., 2017; Undurraga et al., 2017). Although the government made attempts to repair the agricultural ecosystem by reducing fertilizers input and effective tillage measures in recent years, this phenomenon is still generally existing. Meanwhile, soil erosion is also major factors constraining agricultural production in many areas, such as the Loess Plateau in China (Fu et al., 2004, 2019). Therefore, breeding of varieties with low-N tolerance and reducing N requirements plays the pivotal role in developing green agriculture and sustainable development.

In plants, the processes of N metabolism involve the mobilization, transport, absorption, and assimilation of amino acids, ultimately promoting growth (Nunes-Nesi et al., 2010; Luo et al., 2013). Ammonium (NH_4^+) and nitrate (NO_3^-), the most abundant forms in the soil, are the two main inorganic N forms directly absorbed by plants (Xuan et al., 2017). After absorption via several membrane transporters located in the root cell membrane, the remaining NH_4^+ and NO_3^- are transported to the leaves or other parts of the plant (Xu et al., 2012). In the assimilation process, NO_3^- is converted to NH_4^+ by nitrate reductase (NR) in the cytoplasm and then transported to the chloroplast (Rennenberg et al., 2010). Subsequently, NH_4^+ is assimilated into glutamine produced by glutamine synthetase (GS). The formation of glutamine requires glutamine and 2-oxoglutarate by the catalysis of glutamate synthase (GOGAT) (Maeda et al., 2014; Plett et al., 2016). Glutamate dehydrogenase (GDH) synthesizes glutamate under the action of NH_4^+ and 2-oxoglutarate, which releases ammonium as

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substrate for GS in the veins and during leaf senescence (McAllister et al., 2012). In general, when N supply is insufficient, NR, GS, GOGAT, GDH and other enzymes activities are affected by aging and stress (Balotf et al., 2016; Wen et al., 2019).

Chloroplasts serve as storage units for N, and more than 70% of N in plants are stored in organelles. Therefore, since chlorophyll is synthesized from compounds containing N, leaves photosynthetic characteristics would be directly inhibited under N-limiting conditions (Wen et al., 2019). Zhu et al. (2014) found that leaf net photosynthesis rate and total chlorophyll concentration in switchgrass significantly decreased under low N treatments compared with those in full strength nutrient conditions. Zhao et al. also (2005) reported that N deficiency significantly reduced leaf chlorophyll concentration and leaf net photosynthesis rate of sorghum, resulting in lower biomass production. Furthermore, the decrease of leaf net photosynthesis rate caused by N deficiency was mainly related to the lower stomatal conductance. Chlorophyll fluorescence is closely associated with the photosynthesis process and can be applied as an internal probe to investigate the relationship between stress conditions and photosynthesis (Ren et al., 2018). Wei et al. (2016) pointed out that N deficiency significantly reduced the maximum efficiency of PSII photochemistry (Fv/Fm) of maize leaves under dark adaptation conditions, which affected the photochemical activity of PSII and decreased photosynthesis, and these responses were associated with leaf senescence. Therefore, chlorophyll fluorescence of leaf, to a certain extent, is strongly influenced when plants are subjected to stress.

Broomcorn millet (*Panicum miliaceum* L.), which has a short growing cycle of 6–12 weeks, is an important food source and forage species that is grown along the Great Wall of China (Zhang et al., 2019a). Featured with low requirements for water and fertilizers, it is also grown in Mongolia, Korea, Russia, Afghanistan, Pakistan, India, and Japan (Vetriventhan and Upadhyaya, 2018). Extensive geographical distribution and long-term domestication lead to abundant and varied germplasm resources of broomcorn millet that more than 8700 are collected and preserved by China nowadays (Zhang et al., 2019a). Till date however, most of the studies on broomcorn millet focused on tillage cultivations and physiochemical properties (Zhang et al., 2017b; Yang et al., 2018), and only a few studies focused on abiotic stress (Zhang et al., 2019b). Cultivating N tolerant varieties employing these germplasm resources can not only effectively improve the use efficiencies of barren land but also reduce fertilizers input. Therefore, as N plays important roles in growth and physiology, screening varieties and confirming the indicators of low-N tolerant, and further understanding the physiological mechanisms underlying broomcorn millet low-N tolerance will help breeders genotype improvement and develop best management practices for poor land problems.

Subordinate function analysis is one of effective methods used in the evaluation of abiotic stresses. Nevertheless, using only dependent subordinate function analysis in the evaluation is onefold due to comprehensive characteristics of abiotic stresses on crops (Han et al., 2006; Shi et al., 2010). Principal component analysis (PCA) is an analyzing method by reducing the dimensionality when mass of multivariate datasets is analyzed. One of its strengths is that more original indicators would be changed into several new relatively independent comprehensive indicators. Following PCA, subordinate function analysis is suitable for the comprehensive evaluation of low-N tolerance. This comprehensive method has been applied for other abiotic stresses, such as drought in winter wheat (Chen et al., 2012), chilling in tomato (Cao et al., 2015), and salt in Fraxinus mandshurica (Zeng et al., 2015), but not in broomcorn millet.

Therefore, in this study, seedlings of 20 broomcorn millet core collections were used to compare their differences in low-N tolerance capability. PCA, subordinate function analysis, and cluster analysis were used together to comprehensively evaluate and classify low-N tolerance of broomcorn millet varieties. The purposes of this work were to (i) identify the optimal N concentration for the evaluating low-N tolerance, establish the evaluation system of low-N tolerance, and further screen for low-N tolerant and sensitive broomcorn millet varieties by investigating morphological (plant height and root characteristics), nutrient (N accumulation and use efficiency of shoot and root) parameters; (ii) compare the changes in physiological characteristics (photosynthetic and N metabolism) of N tolerant and N sensitive varieties, and elucidate the potential physiological adaptive mechanisms by which broomcorn millet tolerates N stress. Our study would provide a comprehensive and dependable method for evaluating low-N tolerance in broomcorn millet as well as the theoretical basis for improvements in N application on different low-N tolerant varieties in the hilly area of China.

2. Materials and methods

2.1. Plant materials

A total of 20 broomcorn millet core collections (Table S1) were used as materials, including landraces and cultivars, of which 18 varieties came from China and 2 varieties came from America. All materials were provided by the College of Agronomy, Northwest A & F University.

2.2. Experimental design

Seeds of similar sizes and appearance were sterilized with 2.5% sodium hypochlorite (NaClO) for 10 min, rinsed with sterile water three times, and neatly placed on a double-layered, moistened filter paper in Petri dishes for 1 day at 30 °C in the dark. Germinated seeds were sown in a seedling identification instrument (12.4 cm × 17.5 cm × 13.5 cm) and cultured in whole nutrient solution when the seedlings had one leaf in a greenhouse at the College of Agronomy, Northwest A & F University (Yangling, China, 108°240 E, 34°200 N, altitude 521 m) under controlled conditions (30 ± 1 °C day/18 ± 1 °C night temperature, 24,000 lx illumination intensity, 14 h light/10 h dark cycle, and 55–60% relative humidity). The whole nutrient solution (as control) consisted of: 5 mM NH_4NO_3, 0.25 mM KH_2PO_4, 0.75 mM K_2SO_4, 0.1 mM KCl, 2 mM $CaCl_2$, 0.65 mM $MgSO_4$, 0.2 mM Fe-EDTA, 1×10^{-3} mM $MnSO_4$, 1×10^{-3} mM $ZnSO_4$, 1×10^{-4} mM $CuSO_4$, 5×10^{-6} mM $(NH_4)_6Mo_7O_{24}$, and 1×10^{-3} mM H_3BO_3. The nutrient solution was renewed every 2 days with a regular intermittent supply of air. Consistent and uniform seedlings having two fully expanded leaves were transferred into N limitation medium including 0, 0.10, 0.25, 0.50, 0.75, 1.00, and 2.50 mM with NH_4NO_3 to evaluate the optimal N deficiency concentration, and 5.00 mM was set as control. After the treatment of 21 days, seedlings were harvested with 4 biological replicates when the morphological changes between treatments could be distinguished. The shoot and root tissues were separated and the samples of 5 plants from each group were mixed as one replicate. The leaves and roots were sampled for physiological analysis, rapidly frozen in liquid nitrogen, and then stored at −80 °C.

2.3. Measurements of plant height and root structure

Plant height was measured with a ruler. The roots were sampled and washed with water until the nutrient solution could be clearly removed. The clean root fractions were separated and placed on a glass dish that contained water, after which the roots were scanned using an Epson Perfection V700 Pro scanner (Seiko Epson Corp., Suwa, Japan). The root length (cm), root surface area (cm^2), and root volume (cm^3) were analyzed using WinRHIZO software (Reagent Instruments Inc., Quebec, Canada).

2.4. Measurements of biomass and N nutrition

The shoot and root tissues with 4 biological replicates (each replicate for 5 plants) were harvested and baked at 105 °C for 30 min and

dried in an oven at 75 °C for a constant weight to determine the shoot and root biomass (g) (Zhang et al., 2017b). After measurements, the shoot and root tissues were smashed with high speed pulverizer (Wuxi Jiuping Instrument Co. Ltd., Jiangsu, China), respectively. Then, samples were digested in H_2SO_4–H_2O_2 and N content was determined by the Kjeltec 2300 analyzer unit (Foss Tecator AB, Hoganas, Sweden). N accumulation and use efficiency, including the shoot and root tissues, were calculated according to the method of Gong et al. (2020).

2.5. Measurements of photosynthesis parameters and chlorophyll fluorescence

The photosynthesis parameters of first fully expanded leaf blades, including net photosynthetic rate (A), transpiration rate (E), intercellular CO_2 concentration (Ci), and stomatal conductance (gs) were determined with a CIRAS-3 portable photosynthesis system (PP Systems, Amesbury, Massachusetts, USA). The measurements were conducted under photosynthetically active radiation of 1200 μmol m^{-2} s^{-1}, a CO_2 concentration in the atmosphere of 400 μmol mol^{-1}, leaf temperature of 30 ± 1 °C. Four plants from every treatment were measured. The pigments were extracted with 80% acetone until the tissue was completely bleached. The samples were analyzed at 645 and 663 nm using a spectrophotometer (UV-2550, Shimadzu, Japan). The chlorophyll concentration (mg g^{-1} FW) was then calculated by following the protocol described in Yaryura et al. (2009).

Chlorophyll fluorescence was analyzed using a MINI-PAM-II fluorometer (Imaging PAM, Walz, Effeltrich, Germany) by the method of Xu et al. (2014). The first fully expanded leaf was dark-adapted for 30 min and then the maximal quantum yield of PSII photochemistry (Fv/Fm) was measured. Four plants were analyzed per treatment.

2.6. Measurements of enzyme activities

Activities of Nitrate reductase (NR, EC 1.7.99.4) were determined in roots and leaves according to the methods of Luo et al. (2013) at 540 nm using a spectrophotometer; glutamine synthetase (GS, EC 1.6.6.1) activity was extracted as proposed by Gong et al. (2019) with slight modification. Frozen leaves and roots were cut into small pieces, fully ground in liquid nitrogen, and extracted in 1 mL of Tris-HCl (pH 7.8). Enzyme activity was determined by measuring the formation of glutamyl hydroxamate in the supernatant at 540 nm; activities of glutamate dehydrogenase (GDH, EC 1.4.1.2) and glutamate synthase (GOGAT, EC 1.4.7.1) were assayed in roots and leaves based on the method of Lin and Kao (1996) with some modification. Samples (leaves and roots) (0.1 g) was extracted with 10 mM TRIS-HCl buffer (pH 7.6), 1 mM $MgC1_2$, 1 mM EDTA, and 1 mM β-mercaptoethanol at 4 °C. After centrifugation (15,000 g, 4 °C, 30 min), the supernatant was used for determination of enzyme activities. Both enzyme activities were recorded spectrophotometrically at 340 nm.

2.7. Data analysis

All results are represented as means (± SE) of at least four replications. Data were analyzed with SPSS statistics software (Version 19.0 for Windows, SPSS, Chicago, IL, USA). Differences between mean values were determined using the least significant difference (LSD) test. All analyses of significance were conducted at the $P < 0.05$ level. Different uppercase letters indicate significant differences among different varieties ($P < 0.05$) under N deficiency treatment while different lowercase letters indicate significant differences among different varieties ($P < 0.05$) under the normal N supply level. The effects of varieties and N stress on variables were analyzed by two-way ANOVAs. In order to accurately assess physiological responses to N stress, most of parameters in this paper, including the plant height, root structure, the biomass, N content and N accumulation as well as N use efficiency, were expressed in relative terms of the treatment receiving 5.00 mM NH_4NO_3 $plant^{-1}$, according to the methods of Chen et al. (2016) and Hu et al. (2016).

Principle component analysis (PCA) and cluster analysis were performed using the relative values of physiological traits to evaluate the differences among 20 broomcorn millet varieties and the final total score was calculated by subordinate function analysis to represent physiological responses. Pearson correlations were calculated to determine the relationship between their N-tolerance capabilities and their physiological parameters or total scores of physiological responses. All figures were drawn by Origin pro 2018.

According to the membership function formula of fuzzy mathematics, the parameters and indicators in the test process are quantitatively converted, and the membership function value and its average value are obtained, which was used to compare the resistance of different species. At present, subordinate function analysis is one common method used in the evaluation of abiotic resistance and calculated using the following formulas (Cao et al., 2015):

$$\mu\,(Xj) = (Xj–Xmin)/(Xmax–Xmin)\ j = 1, 2, 3, \mathrm{L}, \ldots \ldots, n \quad (1)$$

where $\mu\,(Xj)$ is the subordinate function value of the j-th comprehensive indicator, Xj is the j-th comprehensive indicator value, $Xmax$ is the maximum value of the j-th comprehensive indicator, and $Xmin$ is the minimum value of the j-th comprehensive indicator.

$$Wj = \mathrm{Pj}/\sum_{j=1}^{m} Pj \quad j = 1, 2, 3, \mathrm{L},\&\&, n \quad (2)$$

where Wj is the index weight of the j-th comprehensive indicator among all comprehensive indicators and Pj is the contribution rate of the j-th comprehensive indicator.

$$D = \sum_{j=1}^{m} [\mu(Xj) \times Wj] \quad j = 1, 2, 3, \mathrm{L},\&\&,n \quad (3)$$

where D is the comprehensive evaluation value of low-N tolerance of broomcorn millet varieties under N stress. The higher the D value, the stronger the resistance to low N stress of broomcorn millet varieties; the lower the D value, the weaker the resistance to low N stress of broomcorn millet varieties.

3. Results

3.1. Analysis of lower-N tolerance coefficients among traits of different varieties and concentrations

There were significant changes in each trait of 20 broomcorn millet varieties under different N concentrations. In order to avoid the inherent biological differences of crops, lower N tolerance coefficient (relative value) that accurately reflected the variety's low-N tolerance was adopted in this study (Table 1). Coefficients of lower N tolerance of root biomass, root N content, and shoot and root N use efficiency were greater than 1 for any N concentration. Similarly, most varieties had greater coefficients of lower N tolerance of root length, root surface area, and root volume than 1, indicating that N stress significantly affects the root growth and development. In contrast, coefficients of plant height, shoot N content, and shoot N accumulation for most varieties to N stress were lower than 1. This result integrally reflected that the effects of N stress on the growth of belowground traits were far greater than those of aboveground. In addition, in terms of N concentration, we found that excepting for root length, shoot N content, and shoot and root N use efficiency, the remaining coefficients had the largest coefficients of variation (CV) at 0.25 mM. The same result could be obtained by averaging the data of all indicators (Fig. S1). Meanwhile, the distribution of broomcorn millet varieties was relatively scattered at this concentration (Fig. 2A). Consequently, we determined that 0.25 mM NH_4NO_3 was the standard concentration for the evaluation and

Table 1
Coefficient and variation for tolerance of growth characters to N stress in broomcorn millet. Data were expressed in relative terms of the treatment receiving 5.00 mM NH_4NO_3 plant^{-1}.

Traits	N concentration (mmol L^{-1})	Variation range (%)	Average (%)	SD	CV (%)	Traits	N concentration (mmol L^{-1})	Variation range (%)	Average (%)	SD	CV (%)
Plant height	0	27.85–57.83	46.92	0.08	16.98	Shoot N content	0	33.38–59.85	44.76	0.08	18.49
	0.10	33.90–76.86	52.31	0.11	21.42		0.10	29.92–50.93	42.01	0.06	14.16
	0.25	62.00–155.68	82.66	0.22	26.27		0.25	39.60–74.61	47.99	0.08	16.88
	0.50	45.38–90.90	68.74	0.11	16.53		0.50	31.81–56.92	44.08	0.07	14.77
	0.75	45.21–104.27	74.83	0.14	18.34		0.75	50.63–85.31	70.42	0.09	13.35
	1.00	43.24–86.48	63.16	0.13	19.95		1.00	57.23–78.19	62.58	0.05	8.16
	2.50	54.32–127.07	89.76	0.18	20.03		2.50	60.65–91.59	77.29	0.08	10.36
Root length	0	16.88–74.59	41.12	0.14	34.14	Root N content	0	41.64–103.91	63.17	0.17	26.18
	0.10	45.00–189.10	106.04	0.44	41.75		0.10	36.59–100.06	57.76	0.17	29.78
	0.25	24.92–172.26	80.11	0.36	44.72		0.25	29.09–131.94	54.41	0.25	45.87
	0.50	41.68–183.71	109.10	0.43	39.62		0.50	54.21–127.87	81.32	0.22	26.67
	0.75	22.46–207.70	108.81	0.49	45.10		0.75	53.52–192.74	90.77	0.32	35.07
	1.00	40.92–92.87	68.50	0.15	22.53		1.00	50.80–160.95	78.49	0.27	34.83
	2.50	38.78–186.06	92.05	0.39	42.53		2.50	66.54–148.55	89.96	0.20	22.07
Root surface area	0	11.47–95.65	31.58	0.19	60.23	Shoot N accumulation	0	7.79–33.66	16.64	0.06	39.05
	0.10	32.37–279.94	92.90	0.57	61.10		0.10	14.59–65.49	31.64	0.15	46.97
	0.25	17.30–212.50	70.25	0.48	68.52		0.25	25.23–213.84	52.28	0.41	78.90
	0.50	28.66–179.76	95.26	0.43	45.28		0.50	13.16–66.14	39.73	0.15	36.65
	0.75	16.35–228.75	100.42	0.52	51.95		0.75	19.46–114.80	51.68	0.24	46.78
	1.00	43.66–211.15	73.90	0.36	48.64		1.00	8.80–54.03	22.95	0.12	53.79
	2.50	30.32–290.48	105.34	0.66	62.98		2.50	25.27–166.19	91.22	0.40	43.35
Root volume	0	7.86–57.30	19.25	0.12	61.31	Root N accumulation	0	12.87–91.03	37.84	0.20	52.38
	0.10	19.71–175.85	70.99	0.40	57.00		0.10	14.21–113.26	49.67	0.25	50.16
	0.25	11.42–147.92	48.24	0.34	70.38		0.25	0.42–127.34	22.90	0.40	175.58
	0.50	23.32–127.92	63.49	0.32	49.67		0.50	1.84–100.28	55.32	0.31	55.30
	0.75	11.89–163.14	75.35	0.39	51.28		0.75	20.29–162.62	84.24	0.41	48.93
	1.00	33.29–120.57	62.97	0.22	34.53		1.00	27.36–198.27	70.26	0.46	65.55
	2.50	23.66–168.44	89.97	0.41	45.03		2.50	14.87–262.73	101.50	0.59	58.29
Shoot biomass	0	13.01–78.04	38.34	0.16	40.70	Shoot N use efficiency	0	21.74–180.95	90.93	0.43	47.57
	0.10	20.39–115.83	54.92	0.28	51.53		0.10	47.22–286.33	134.07	0.71	52.90
	0.25	38.83–229.54	78.92	0.46	58.49		0.25	65.51–441.52	175.61	1.09	61.90
	0.50	26.08–142.77	85.87	0.36	41.84		0.50	40.18–345.87	188.43	0.92	48.69
	0.75	22.81–164.47	71.17	0.33	46.07		0.75	26.74–235.63	102.57	0.54	52.61
	1.00	30.91–170.05	73.79	0.39	52.99		1.00	54.77–410.93	130.49	0.86	65.69
	2.50	27.59–204.81	116.36	0.47	40.44		2.50	30.12–340.98	158.71	0.76	48.20
Root biomass	0	25.64–170.37	76.51	0.37	47.84	Root N use efficiency	0	27.00–353.51	173.53	1.04	59.78
	0.10	49.72–240.00	110.67	0.57	51.62		0.10	28.16–447.94	181.04	1.12	62.06
	0.25	43.33–300.00	93.94	0.60	63.56		0.25	60.27–555.32	209.63	1.39	66.33
	0.50	26.67–197.30	112.18	0.47	41.87		0.50	38.54–363.98	159.98	0.93	58.14
	0.75	31.25–228.57	100.06	0.55	55.36		0.75	20.30–427.08	137.71	0.98	71.10
	1.00	20.00–283.33	94.16	0.58	61.72		1.00	32.08–373.85	138.19	0.86	62.46
	2.50	29.63–266.67	116.84	0.62	53.27		2.50	59.03–371.85	150.88	0.91	60.10

SD, standard deviation; CV, coefficient of variance.

identification of low-N tolerance in broomcorn millet seedling.

3.2. Pearson's correlation analysis of lower-N tolerance coefficients among traits of different varieties and concentrations

Pearson's correlation analysis was carried out to better understand the characteristics of lower N tolerance coefficients (Fig. 1). Excepting

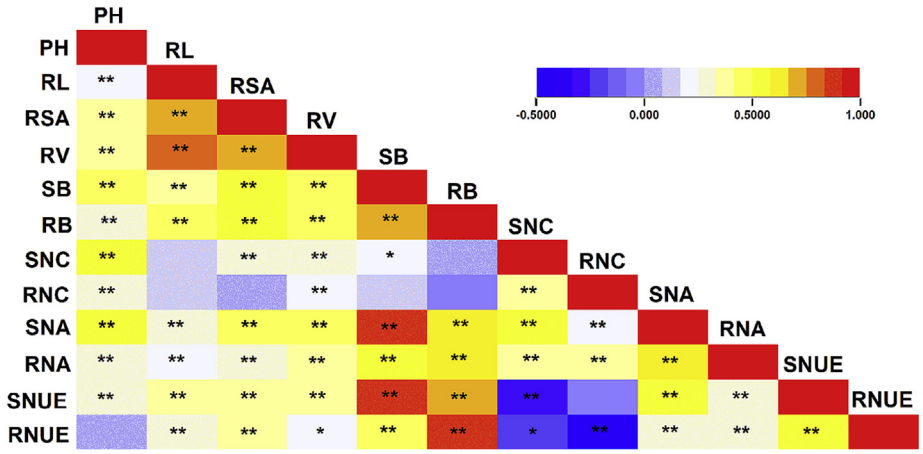

Fig. 1. Pearson's correlation coefficients of all traits of broomcorn millet under N stress. * Correlation is significant at the 0.05 level. ** Correlation is significant at the 0.01 level. PH, plant height; RL, root length; RSA, root surface area; RV, root volume; SB, shoot biomass; RB, root biomass; SNC, shoot N content; RNC, root N content; SNA, shoot N accumulation, RNA, root N accumulation; SNUE, shoot N use efficiency; RNUE, root N use efficiency.

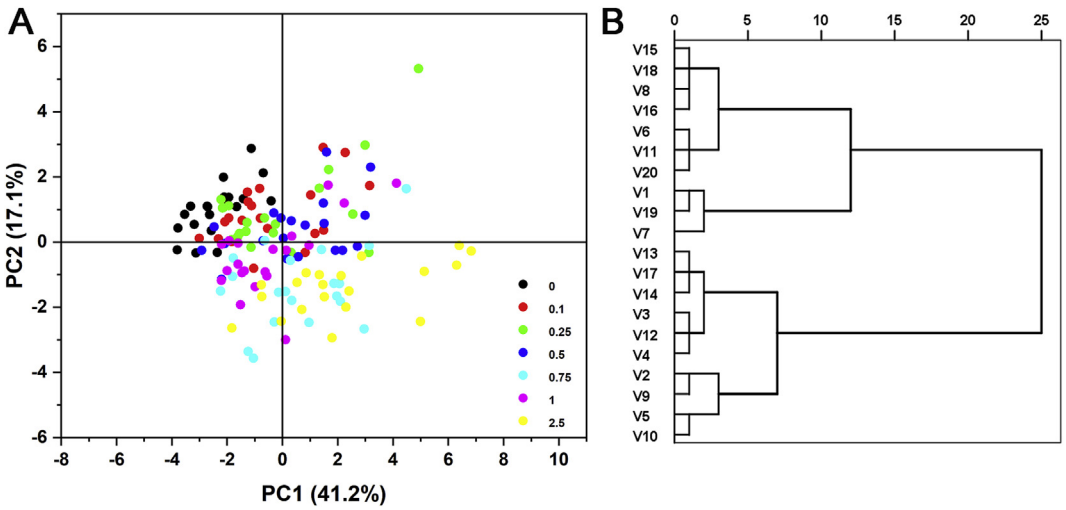

Fig. 2. Distribution of 20 broomcorn millet varieties under different N concentration (A) and cluster analysis based on D value (B).

for the root N use efficiency, plant height was highly positively correlated with all measured biological indicators ($P < 0.01$). Meanwhile, similar trends were observed among root structure and the plant biomass with other most indicators, and the shoot biomass was positively correlated with shoot N use efficiency and root N efficiency ($P < 0.01$), taking that both correlation coefficients were above 0.8. In contrast, the root N accumulation was highly negatively correlated with root N use efficiency ($P < 0.01$), indicating that increased N accumulation may reduce N utilization to some extent. It follows that there may be information overlap between different indicators, and comprehensive variable indicators would be more effective to screen low-N tolerance germplasm resources.

3.3. PCA of lower-N tolerance coefficients among traits of different varieties and concentrations

In order to eliminate factors with less influence and greater interference, and improve the accuracy of measurement data analysis, the above introduced single indicators were converted into a reduced number of more effective indicators, and thus PCA was performed based on the low-N tolerance coefficients of 20 broomcorn millet varieties. As shown in Table 2, the first principal component, featured with the largest contribution rate and eigenvalue, was shoot biomass, which was 41.23% and 4.95, respectively. Analogously, the second, third, fourth, and fifth principal components of higher eigenvalue was root N content, root length, plant height, and shoot N content, with the contribution rate of 17.10%, 11.88%, 7.85%, and 7.19%, respectively. The cumulative contributions to total variation of population from first to fifth of principal component reached over 85.25%, which was enough to represent a large part of the information of the original indicators. Therefore, low-N tolerance characteristics of broomcorn millet could be objectively analyzed by five independent comprehensive indicators.

3.4. Comprehensive evaluation of lower-N tolerance

Based on PCA results, the scores of the comprehensive indexes were obtained and were then used in subordinate function analysis. Using comprehensive index Z1 as an example, the maximum subordinate function value was 2.8415 for V7, while the minimum was −3.2343 for V2. This suggested that when only considering Z1, V7 showed the highest level of low-N tolerance, while V2 had the weakest low-N tolerance. Combined with subordinate function values, $\mu(Xj)$, we calculated index weight (Wj) of each comprehensive indicator, which was 48.36%, 20.05%, 13.94%, 9.23%, and 8.44% of the first, second, third, fourth, and fifth principal components, respectively. Furthermore, the comprehensive evaluation values (D) of low-N tolerance of broomcorn millet varieties were calculated and ranked (Table 3) according to the formula given in the Material and methods section. D value of the top ranked varieties was higher, which indicated that they have a high

Table 2
Eigenvalue and contribution of each comprehensive index and loading matrix of each component.

Items	Traits	Principal component				
		1	2	3	4	5
Eigenvalue		4.9476	2.0516	1.4257	0.9420	0.8633
Contributive ratio (%)		41.2300	17.0968	11.8810	7.8496	7.1941
Cumulative contribution (%)		41.2300	58.3267	70.2077	78.0572	85.2513
Loading matrix of each component	Plant height	0.2446	−0.2096	−0.214	0.5743	−0.1533
	Root length	0.2747	−0.1129	0.561	−0.0202	−0.1648
	Root surface area	0.3247	−0.0609	0.4252	0.1229	0.0616
	Root volume	0.273	−0.2613	0.3968	−0.0571	−0.1854
	Shoot biomass	0.3873	0.088	−0.2793	0.0943	−0.1962
	Root biomass	0.3463	0.2653	0.0043	−0.2204	0.2813
	Shoot N content	0.1433	−0.4861	−0.1421	0.2338	0.5504
	Root N content	0.0733	−0.4832	−0.2645	−0.4399	−0.273
	Shoot N accumulation	0.3726	−0.0029	−0.2481	0.1878	0.0812
	Root N accumulation	0.3008	−0.1741	−0.1604	−0.5537	0.2091
	Shoot N use efficiency	0.3129	0.3007	−0.2128	−0.0363	−0.482
	Root N use efficiency	0.2484	0.4503	0.0221	−0.0512	0.3652

Table 3
Value of each comprehensive indicators (*Z*), subordinate function values, *μ(X)*, comprehensive evaluation value (*D*) and order.

Variety	*Z1*	*Z2*	*Z3*	*Z4*	*Z5*	*μ(X1)*	*μ(X2)*	*μ(X3)*	*μ(X4)*	*μ(X5)*	*D*	Order
V1	1.0607	−0.5814	−0.3903	−1.4056	−1.7555	0.5627	1.0000	0.4085	0.5248	0.0000	0.5779	2
V2	−3.2343	0.3557	−0.3114	−0.3544	−0.1673	0.0024	0.3895	0.0004	0.1312	0.1190	0.1015	19
V3	−1.8160	−0.5586	1.7857	0.1534	0.7235	0.0000	0.3375	0.8513	0.0000	0.8101	0.2547	15
V4	−2.0098	−0.3752	−0.4269	0.1664	−0.2012	0.0893	0.4296	0.0928	0.3357	0.5727	0.2215	16
V5	−2.1128	0.7084	0.0310	0.0283	−0.6681	0.0980	0.3242	0.2468	0.3143	0.0791	0.1824	18
V6	−0.2220	1.2068	0.1223	−0.3924	0.4407	0.3631	0.2515	0.3482	0.3317	0.4615	0.3441	10
V7	2.8415	3.8690	−2.3946	0.0929	0.7149	1.0000	0.2432	0.2308	0.4861	0.3099	0.6355	1
V8	−0.0206	−1.1964	0.4049	0.4894	−0.4069	0.0267	0.8394	0.6246	1.0000	1.0000	0.4448	6
V9	−3.0759	0.5679	−0.3747	−0.1119	0.1282	0.0060	0.2854	0.0000	0.1699	0.2797	0.0994	20
V10	−2.1291	−0.3159	0.4526	0.2490	0.1557	0.0152	0.2788	0.3138	0.2266	0.7205	0.1887	17
V11	−1.0105	0.3968	0.3952	0.9036	0.6244	0.2956	0.0000	0.7218	0.3479	0.9948	0.3595	9
V12	−0.8372	0.7410	0.5197	0.2022	−0.3895	0.1531	0.2558	0.4503	0.4125	0.4034	0.2601	14
V13	0.7317	0.8596	1.4654	−0.7605	1.7607	0.1386	0.1257	0.7548	0.3582	0.8405	0.3013	12
V14	−0.5645	0.5535	0.1830	−0.2822	−0.8087	0.2827	0.3189	0.5084	0.4539	0.0439	0.3170	11
V15	1.8339	1.5573	0.5866	0.7670	0.7744	0.6295	0.0823	0.3627	0.5635	0.5650	0.4711	4
V16	0.9036	−0.2436	0.9607	−0.0324	−0.4998	0.2611	0.4961	0.7001	0.6692	0.5705	0.4331	7
V17	−0.7422	−0.0016	0.4277	0.2088	−0.1677	0.0955	0.4017	0.4801	0.6804	0.3724	0.2877	13
V18	−0.5382	0.0630	−0.1463	−0.4253	−0.8485	0.2675	0.9726	0.5374	0.3341	0.4759	0.4702	5
V19	0.2432	0.3016	−0.1761	−0.5065	−1.5505	0.5560	0.7658	0.7317	0.3638	0.2172	0.5763	3
V20	1.8445	1.3924	2.1260	0.2129	−0.7869	0.4035	0.0012	1.0000	0.5252	0.1040	0.3919	8

"Z" values were determined by principal component analysis (PCA) and the scores of the comprehensive indicators.

tolerance to N stress. On the contrary, *D* value of the later ranked varieties was lower, which indicated that they have low resistance to N stress. Therefore, two representative high N tolerant varieties (V7, V1) and low-N sensitive varieties (V2, V9) were selected for physiological metabolism analyses. The accuracy of varieties selections was supported by cluster analysis at a Euclidean distance of 5 (Fig. 2B).

3.5. Response of photosynthetic characteristics and chlorophyll fluorescence in leaves of different varieties to N stress

The effects of N stress on leaves photosynthetic characteristics in V2 and V9 occurred stronger than those in V7 and V1 (Fig. 3). N stress decreased net photosynthetic rate (A) of V2 and V9 to 78.4% and 71.0% of those at 5.00 mM NH_4NO_3, respectively, whereas the A value declined by 41.8% and 54.9% in V7 and V1 (Fig. 3A). Similar patterns were observed for the transpiration rates (E) and stomatal conductance (gs) of low-N tolerant and low-N sensitive varieties. The E and gs values of all varieties were observably inhibited when treated with N stress, but those in V2 and V9 were lower than those in V7 and V1 (Fig. 3B and D). Furthermore, the intercellular CO_2 concentrations (Ci) of V1 is significantly higher than that of V7, V2, and V9 under control condition. However, compared with the controls, the Ci values of low-N sensitive varieties significantly increased and the highest Ci (217.25 μmol mol^{-1}) was observed in N stressed V2 (Fig. 3C). Meanwhile, the Ci values in the two N tolerant varieties were improved by lower N treatment, and changing trends was the same in Ci value between the two varieties.

As shown in Fig. 4A, lower N treatment significantly decreased the maximum efficiency of PSII photochemistry (Fv/Fm) in V9, although this effect varied inconsiderably among other varieties. The chlorophyll concentration was declined by N stress, and the degree of this reduction was significantly affected by varieties as well as by the interaction between varieties and treatment history. Although the chlorophyll concentration of the two low-N tolerant varieties were higher than those of the two low-N sensitive varieties under N stress treatment, the effect was not significant ($P < 0.05$) (Fig. 4B).

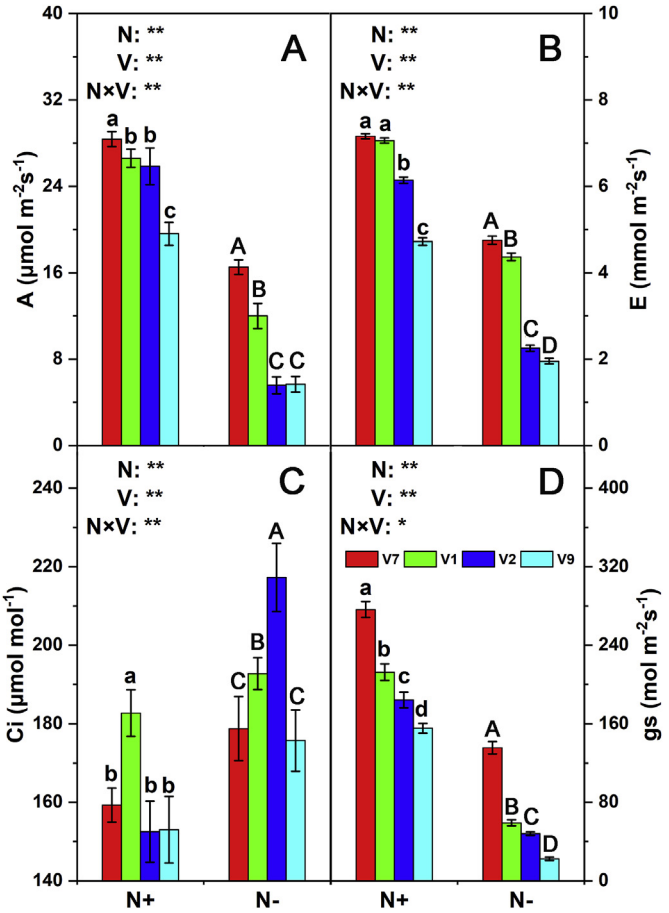

Fig. 3. Changes in leaves net photosynthetic rate (A), transpiration rate (B), intercellular CO_2 concentration (C), and stomatal conductance (D) of different low-N tolerance varieties in broomcorn millet seedling. N+ represents the normal N supply level (5 mM NH_4NO_3) and N- represents N deficiency treatment (0.25 mM NH_4NO_3). Different uppercase letters indicate significant differences among different varieties ($P < 0.05$) under N deficiency treatment while different lowercase letters indicate significant differences among different varieties ($P < 0.05$) under the normal N supply level. P-values of the ANOVAs of N treatment, varieties, and their interaction are indicated. *$P < 0.05$; **$P < 0.01$.

3.6. Response of N metabolism in leaves and roots of different varieties to N stress

Limiting N supply also influenced activities of enzymes implicated in N assimilation in low-N tolerant and sensitive varieties. The activities

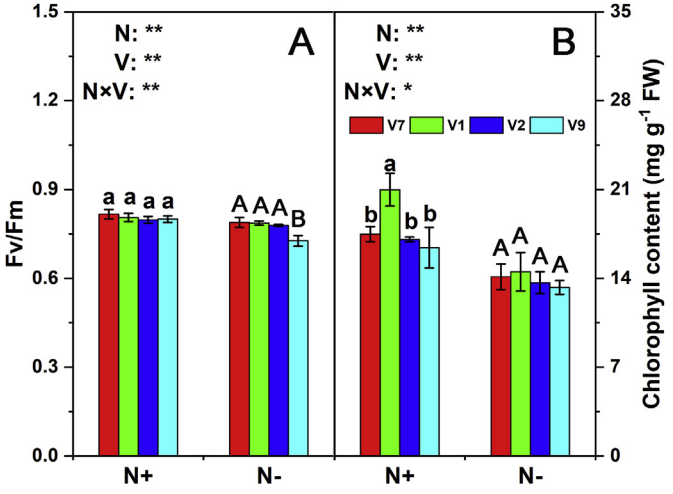

Fig. 4. Changes in leaves Fv/Fm (A) and chlorophyll concentration (B) of different low-N tolerance varieties in broomcorn millet seedling. N+ represents the normal N supply level (5 mM NH_4NO_3) and N- represents N deficiency treatment (0.25 mM NH_4NO_3). Different uppercase letters indicate significant differences among different varieties ($P < 0.05$) under N deficiency treatment while different lowercase letters indicate significant differences among different varieties ($P < 0.05$) under the normal N supply level. P-values of the ANOVAs of N treatment, varieties, and their interaction are indicated. *$P < 0.05$; **$P < 0.01$.

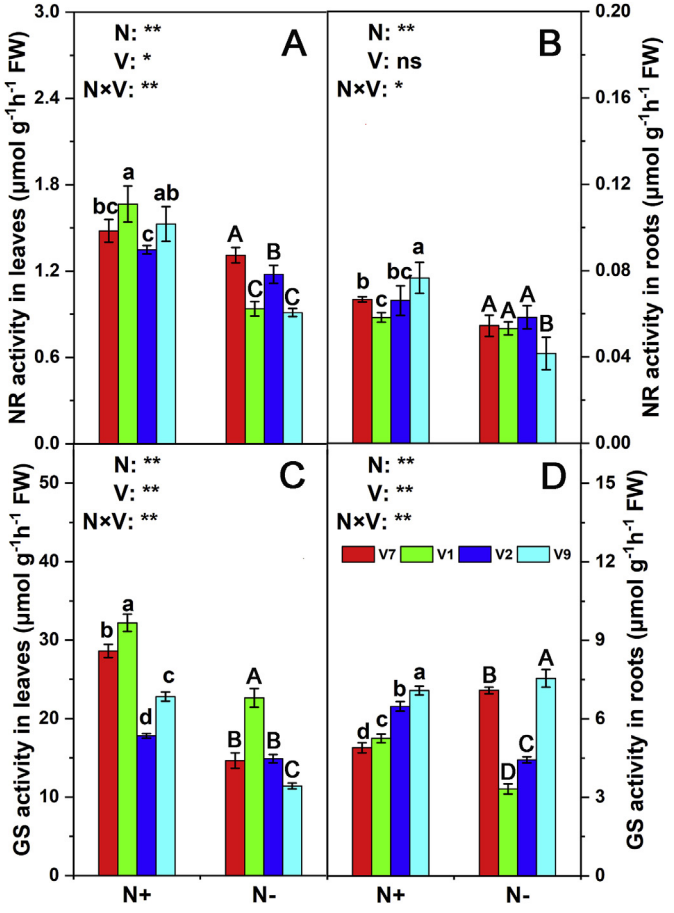

Fig. 5. Changes in NR and GS activities in leaves (A and C) and roots (B and D) of different low-N tolerance varieties in broomcorn millet seedling. N+ represents the normal N supply level (5 mM NH_4NO_3) and N- represents N deficiency treatment (0.25 mM NH_4NO_3). Different uppercase letters indicate significant differences among different varieties ($P < 0.05$) under N deficiency treatment while different lowercase letters indicate significant differences among different varieties ($P < 0.05$) under the normal N supply level. P-values of the ANOVAs of N treatment, varieties, and their interaction are indicated. *$P < 0.05$; **$P < 0.01$; ns, not significant.

of nitrate reductase (NR) and glutamine synthetase (GS) were inhibited in response to N deficiency in leaves. The data show that compared with those of the controls, 0.25 mM NH_4NO_3 decreased the NR and GS activities of V9 by 40.3% and 50.0%, respectively. However, the activities of NR and GS were only decreased by 12.6% and 16.4% in V2 under N stress (Fig. 5A and C), although both they were classified as N sensitive varieties. By contrast, there were no marked differences among the activities of NR of the four varieties in roots under N stress, whereas lower N treatment significantly restrained the GS activities of V1 and V2 (Fig. 5B and D). For all these parameters, the different varieties exhibited different responses to N stress.

Activities of glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) in leaves and roots were significantly affected by low N level. Average GDH activities of the two low-N tolerant varieties were significantly higher than those of the two low-N sensitive varieties, and N stress decreased the GDH activities of the two low-N sensitive varieties in leaves and roots by 54.5% and 47.4% on average, respectively (Fig. 6A and B). Similar patterns were observed for the GOGAT activities of low-N tolerant and low-N sensitive varieties in response to N stress (Fig. 6C and D). Although the activities of all varieties were significantly inhibited in leaves and roots when treated with 0.25 mM NH_4NO_3, but decrease of roots in V2 and V9 were more than those in V7 and V1.

3.7. PCA of physiological parameters

To uncover the traits responding to N stress, PCA was performed by physiological parameters related to photosynthesis and N metabolism. PC1 and PC2 accounted for 61.2% and 17.4% of the total variance, respectively (Fig. 7). PC1 clearly separated the variation of varieties effects, and PC2 uncovered the effects of N stress. Net photosynthetic rate, transpiration rate, and GDH activity in leaves were the main contributors to PC1. GS in roots and Ci were essential factors for PC2 (Table S2). In both low-N and normal condition, the greater distances between symbols were due to differences from the physiological parameters.

4. Discussion

4.1. Evaluation of low-N tolerance in broomcorn millet seedling

In recent years, broomcorn millet is known for its multiple resistances to salt, alkali, drought, and barren (Liu et al., 2015; Zhang et al., 2019b). However, there are not many systematic studies on broomcorn millet low-N tolerance, and the response of different broomcorn millet varieties to N stress and the effective evaluation of indicators are issues to be explored. Although there are differences in the selection of low-N tolerance indicators in different crops, the response of crops to stress are largely morphological and physiological characteristics. Jiang et al. (2019) found that the relative N uptake and the relative N physiological utilization efficiency could be used as indicators for the evaluation of low-N tolerance of barley germplasm by comparing 19 local barley varieties. Zhang et al. (2017a) screened 8 indicators, respectively called plant height, stem diameter, leaf area, root-shoot ratio, chlorophyll concentration, maximum chlorophyll fluorescence (Fm), superoxide dismutase activity in root and nitrogen use efficiency, which can be used for rapid identification of low-N tolerance tartary buckwheat genotypes via the subordinate function analysis. Similar results were also reflected in the current study. Yet the various varieties tended to exhibit similar patterns of morphological

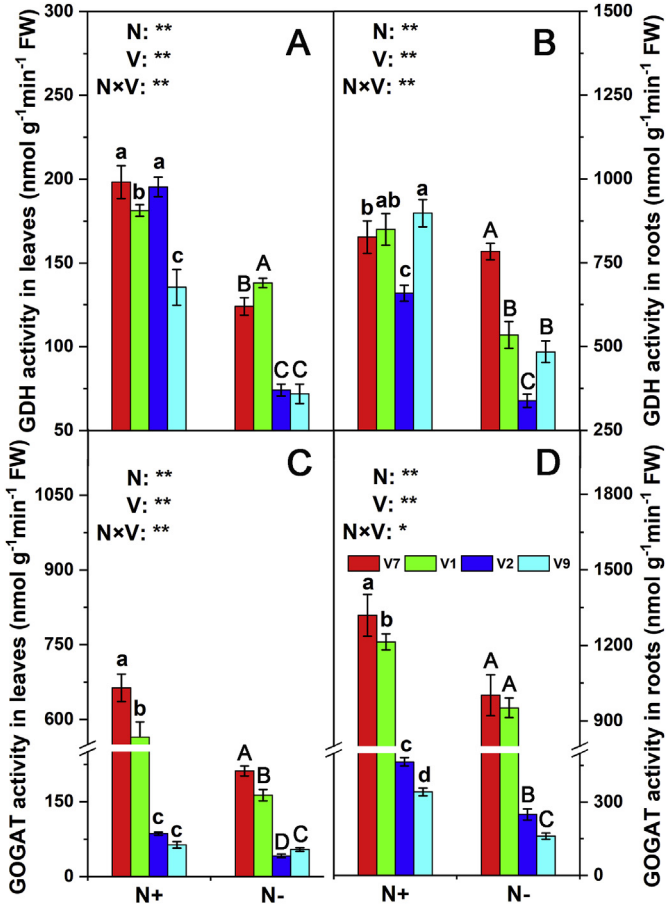

Fig. 6. Changes in GDH and GOGAT activities in leaves (A and C) and roots (B and D) of different low-N tolerance varieties in broomcorn millet seedling. N+ represents the normal N supply level (5 mM NH_4NO_3) and N- represents N deficiency treatment (0.25 mM NH_4NO_3). Different uppercase letters indicate significant differences among different varieties ($P < 0.05$) under N deficiency treatment while different lowercase letters indicate significant differences among different varieties ($P < 0.05$) under the normal N supply level. P-values of the ANOVAs of N treatment, varieties, and their interaction are indicated. *$P < 0.05$; **$P < 0.01$.

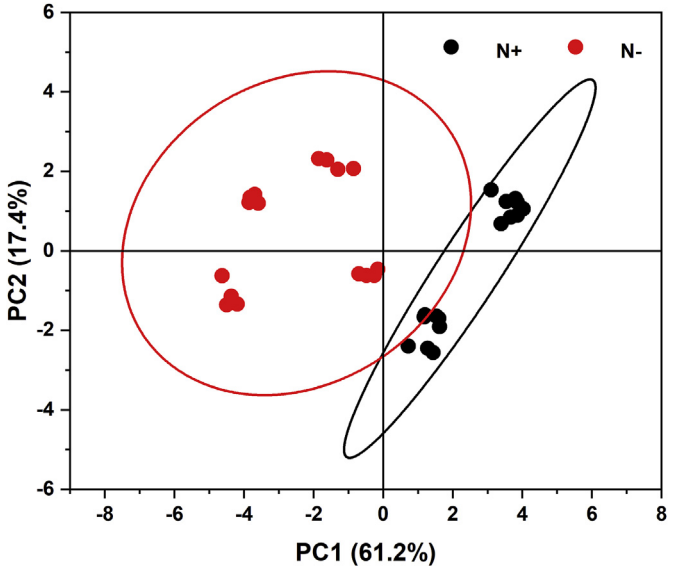

Fig. 7. PCA plots of physiological parameters of broomcorn millet. N+ represents the normal N supply level (5 mM NH_4NO_3) and N- represents N deficiency treatment (0.25 mM NH_4NO_3).

and nutrient changes under N stress, the amplitudes of these changes varied among the varieties. Through pearson's correlation analysis, principal component analysis, and subordinate function analysis, the tolerance of 20 broomcorn millet varieties under N stress was comprehensively evaluated and 12 identification indicators were classified into 5 factors, and the maximum eigenvector load was plant height, root length, shoot biomass, and shoot and root N content, respectively. Furthermore, these five indicators were positively corrected with the comprehensive value (D) for low-N tolerance (Table S3), which reached significant level ($P < 0.01$). These results indicated that low-N tolerance of plant is a complex trait that is determined by both genetic and environmental factors. Therefore, indicators of many aspects should be taken into account in the evaluation of low-N tolerance. Through the combination of multiple methods, the complicated traits were simplified and visualized to reflect the low-N tolerance information of broomcorn millet germplasm resources in detail, and the results were more convincing (Cao et al., 2015; Bo et al., 2017).

In addition, in terms of N concentration, we found that excepting for root length, shoot N content, and shoot and root N use efficiency, the remaining coefficients had the largest coefficients of variation (CV) at 0.25 mM, indicating that plant growth was strongly affected by N at this concentration. When N concentration is greater than 0.25 mM, the plant would be not in stress by absorbing more nutrients; on the contrary, too little nutrient could not meet the growth requirements, which would induce broomcorn millet death. The same result could be obtained by averaging the data of all indicators (Fig. S1). Meanwhile, the distribution of broomcorn millet varieties was relatively scattered (Fig. 2A) and consequently, we determined that 0.25 mM NH_4NO_3 was the standard concentration for the evaluation and identification of low-N tolerance in broomcorn millet seedling.

Firstly, physiological changes were detected when plants suffered stress before injury symptoms became visible (Zong and Shanguan, 2014). It is of great significance to understand the growth and development of plants to verify the damage state of plants by detecting the change of physiological indicators. Therefore, according to the comprehensive value (D) for low-N tolerance, we selected the first two varieties, V7 and V1, as low-N tolerance varieties; correspondingly, selected the last two varieties, V2 and V9, as sensitive varieties, which would be explored the physiological mechanism of broomcorn millet under N stress.

4.2. Physiological response to N stress in broomcorn millet seeding

The improvement of plant growth under the external environment is often correlated with physiological metabolism (Naeem et al., 2010). Photosynthetic capacity can be determined through gas exchange, reflected that A indicating the carbon assimilation capacity per unit leaf area, E closely associated with changes in environmental water potential, and Ci manifesting the assimilation ability of mesophyll cells for CO_2 in plants (Dong et al., 2006). In present study, A, E, and gs were significantly declined in all varieties under N stress, whereas Ci was increased, showing that the increment in N sensitive varieties was greater than that in N tolerant varieties (Fig. 3A). These results comprehensively imply that the mainly reason for the decreased photosynthesis was nonstomatal factors, such as limited the ability to capture CO_2, altered chloroplast structure, and damaged photosynthetic organs. In addition, reduced chlorophyll concentration under N deficiency supported this speculation (Fig. 4B). A similar result was reported by Huang et al. (2004) in rice. Genotypes displaying the less increases of Ci might have higher tolerance, particularly under long and severe stress conditions, because they would conserve water and preserve their photosynthetic organs (Mauro et al., 2011; Hafeez et al., 2019). Chlorophyll fluorescence measurements provide a good way to assess the effects of environment on plants and to gain insight into the

behavior of photosynthetic mechanism (Gong et al., 2019). In the present study, the Fv/Fm decreased under low-N stress in all varieties, indicating that low-N stress led to photoinhibition, which potentially damaged the active center of PSII and injured further photosynthetic structures, losing the ability to transfer the excitation energy of the light-collecting complex to PSII. In addition, compared to low-N sensitive varieties, the decrement of Fv/Fm in low-N tolerant varieties was less, further demonstrating that low-N tolerant varieties could maintain a stronger ability to harvest and transfer light, improve the light utilization of PSII and enhance the stress tolerance ability.

The adjustment of N metabolism is parallel to the growth adaptation to N fluctuations. NR is a key enzyme that regulates the rate-limiting step in NO_3^- assimilation pathway. In our study, NR activities in leaves and roots decreased for all the varieties under N stress and the decrement in roots was less than in leaves, especially for low-N tolerant varieties (Fig. 5A and B), indicating that roots were more resistant to N stress than leaves. Another factor between these varieties may be related to biological characteristics, for instance the difference in regulation of N transporter genes or N fluxes in roots (Britto and Kronzucker, 2002). Meanwhile, higher GS activities of V7 in roots were also observed under N stress (Fig. 5D), which was conducive to plant ammonia assimilation and N transfer from roots to leaves, producing more metabolites for growth and development. It is well known that NO_3^- is converted into NH_4^+ in plants, and NH_4^+ is assimilated primarily through the GS/GOGAT pathway. GOGAT catalyzes the synthesis of two glutamate molecules from the transamination of glutamine and 2-oxoglutarate; conversely, GDH is involved in glutamate catabolism, which releases ammonium as substrate for GS in the veins, therefore, these two enzymes are also closely associated with N metabolism in plants (Pageau et al., 2006; Krapp, 2015). In this study, GDH and GOGAT activities decreased under N deficiency, and the decreased extent varied greatly over the varieties. Low-N tolerant varieties (V7 and V1) had greater activities in both leaves and roots as compared to low-N sensitive varieties (V2 and V9). Similar results were obtained by previous research, which reported that under low N condition, high N efficient genotypes had more enzyme activities than low N inefficient genotypes in barely (Shah et al., 2017). Thus, stronger N metabolism related enzyme activities may be beneficial for the absorption, assimilation and allocation of N within plants. In addition, PCA was performed by the physiological parameters related to photosynthesis and N metabolism, greater distances between symbols associated with N treatment levels suggested a stronger responsiveness of physiological parameters to changes in N supply levels (Fig. 7). Taken together, our data suggest that the application of N fertilizer to broomcorn millet should take the N fertilization response capacities of different varieties into account.

5. Conclusions

In this study, 0.25 mM NH_4NO_3 was the standard concentration for the evaluation and identification of low-N tolerance in broomcorn millet seedling. Through pearson's correlation analysis, principal component analysis, and subordinate function analysis, the tolerance of 20 varieties under N stress was comprehensively evaluated and plant height, root length, shoot biomass, and shoot and root N content were taken into account in the evaluation system of low-N tolerance. Although leaves photosynthetic capacities and N metabolism related enzymes in leaves and roots were decreased for all the varieties, low-N tolerant varieties had greater activities as compared to low-N sensitive varieties, including the higher net photosynthetic rate, chlorophyll concentration, NR, GS, GOGAT and GDH. This strong physiological metabolism strategy may be one of the mechanisms explaining why the low-N tolerant varieties maintained higher low-N resistance levels than the low-N sensitive varieties did.

Author contributions

C.J. Liu and X.W. Gong performed the experiments and drafted the manuscript. X.P. Deng and B.L. Feng designed the project and guided the experiments. C.J. Liu, X.W. Gong, H.L. Wang and K. Dang investigated the material characteristics. C.J. Liu and X.W. Gong analyzed the data and planted the material. C.J. Liu, X.P Deng and B.L. Feng organized and coordinated the whole project.

Declaration of competing interest

No conflict of interest exists in the submission of this manuscript, and the manuscript has been approved by all authors for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.03.027.

References

Balotf, S., Kavoosi, G.K., Kholdebarin, B., 2016. Nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthase expression and activity in response to different nitrogen sources in nitrogen-starved wheat seedlings. Biotechnol. Appl. Biochem. 63 (2), 220–229. https://doi.org/10.1002/bab.1362.

Bo, W., Fu, B., Qin, G., Xing, G., Wang, Y., 2017. Evaluation of drought resistance in Iris germanica L. based on subordination function and principal component analysis. Emir. J. Food Agric. 29 (10), 770–778. https://doi.org/10.9755/ejfa.2017.v29.i10.1260.

Britto, D.T., Kronzucker, H.J., 2002. NH_4^+ toxicity in higher plants: a critical review. J. Plant Physiol. 159, 567–584. https://doi.org/10.1078/0176-1617-0774.

Cao, X., Jiang, F., Wang, X., Zang, Y., Wu, Z., 2015. Comprehensive evaluation and screening for chilling-tolerance in tomato lines at the seedling stage. Euphytica 205, 569–584. https://doi.org/10.1007/s10681-015-1433-0.

Chen, D., Wang, S., Cao, B., Cao, D., Leng, G., Li, H., Yin, L., Shan, L., Deng, X., 2016. Genotypic variation in growth and physiological response to drought stress and re-watering reveals the critical role of recovery in drought adaptation in maize seedlings. Front. Plant Sci. 6, 1241. https://doi.org/10.3389/fpls.2015.01241.

Chen, X., Min, D., Yasir, T.A., Hu, Y., 2012. Evaluation of 14 morphological, yield-related and physiological traits as indicators of drought tolerance in Chinese winter bread wheat revealed by analysis of the membership function value of drought tolerance (MFVD). Field Crop. Res. 137, 195–201. https://doi.org/10.1016/j.fcr.2012.09.008.

Dong, H.Z., Li, W.J., Tang, W., Li, Z.H., Zhang, D.M., 2006. Effects of genotypes and plant density on yield, yield components and photosynthesis in Bt transgenic cotton. J. Agron. Crop Sci. 192 (2), 132–139. https://doi.org/10.1111/j.1439-037X.2006.00196.x.

Fu, B.J., Meng, Q.H., Qiu, Y., Zhao, Y., Zhang, Q.J., Davidson, D.J., 2004. Effects of land use on soil erosion and nitrogen loss in the hilly area of the Loess Plateau, China. Land Degrad. Dev. 15 (1), 87–96. https://doi.org/10.1002/ldr.572.

Fu, X., Wang, J., Sainju, U.M., Liu, W., 2019. Soil nitrogen fractions under long-term crop rotations in the Loess Plateau of China. Soil Till. Res. 186, 42–51. https://doi.org/10.1016/j.still.2018.10.004.

Gong, X., Li, J., Ma, H., Chen, G., Dang, K., Yang, P., Wang, M., Feng, B., 2020. Nitrogen deficiency induced a decrease in grain yield related to photosynthetic characteristics, carbon–nitrogen balance and nitrogen use efficiency in proso millet (*Panicum miliaceum* L.). Arch. Agron Soil Sci. 66 (3), 398–413. https://doi.org/10.1080/03650340.2019.1619077.

Gong, X., Liu, C., Ferdinand, U., Dang, K., Zhao, G., Yang, P., Feng, B., 2019. Effect of intercropping on leaf senescence related to physiological metabolism in proso millet (*Panicum miliaceum* L.). Photosynthetica 57 (4), 993–1006. https://doi.org/10.32615/ps.2019.112.

Hafeez, A., Ali, S., Ma, X., Tung, S.A., Shah, A.N., Ahmad, S., Chattha, M.S., Souliyanonh, B., Zhang, Z., Yang, G., 2019. Photosynthetic characteristics of boll subtending leaves are substantially influenced by applied K to N ratio under the new planting model for cotton in the Yangtze River Valley. Field Crop. Res. 237, 43–52. https://doi.org/10.1016/j.fcr.2019.04.015.

Han, R., Lu, X., Gao, G., Yang, X., 2006. Analysis of the principal components and the subordinate function of alfalfa drought resistance. Acta Agrestia Sin 14 (2), 142–146. https://doi.org/10.11733/j.issn.1007-0435.2006.02.010.

Hu, W., Zhao, W., Yang, J., Oosterhuis, D., Loka, D., Zhou, Z., 2016. Relationship between potassium fertilization and nitrogen metabolism in the leaf subtending the cotton (*Gossypium hirsutum* L.) boll during the boll development stage. Plant Physiol. Biochem. 101, 113–123. https://doi.org/10.1016/j.plaphy.2016.01.019.

Huang, Z.A., Jiang, D.A., Yang, Y., Sun, J.W., Jin, S.H., 2004. Effects of nitrogen deficiency on gas exchange, chlorophyll fluorescence, and antioxidant enzymes in leaves of rice plants. Photosynthetica 42, 357–364. https://doi.org/10.1023/B:PHOT.0000046153.08935.4c.

Jiang, Q., Chen, Z., Liu, C., He, T., Guo, G., Gao, R., Xu, H., Li, Y., Lu, R., Huang, J., 2019. Screening and identification indices of low-nitrogen tolerance for barley landraces at seedling stage. Acta Agriculturae Boreali–Sinica 34 (1), 148–155. https://doi.org/10.7668/hbnxb.201751103.

Krapp, A., 2015. Plant nitrogen assimilation and its regulation: a complex puzzle with missing pieces. Curr. Opin. Plant Biol. 25, 115–122. https://doi.org/10.1016/j.pbi.2015.05.010.

Kusano, M., Fukushima, A., Redestig, H., Saito, K., 2011. Metabolomic approaches toward understanding nitrogen metabolism in plants. J. Exp. Bot. 62, 1439–1453. https://doi.org/10.1093/jxb/erq417.

Lin, C.C., Kao, C.H., 1996. Disturbed ammonium assimilation is associated with growth inhibition of roots in rice seedlings caused by NaCl. Plant Growth Regul. 18, 233–238. https://doi.org/10.1007/BF00024387.

Liu, M., Qiao, Z., Zhang, S., Wang, Y., Lu, P., 2015. Response of broomcorn millet (*Panicum miliaceum* L.) genotypes from semiarid regions of China to salt stress. Crop J. 3, 57–66. https://doi.org/10.1016/j.cj.2014.08.006.

Luo, J., Li, H., Liu, T., Polle, A., Peng, C., Luo, Z., 2013. Nitrogen metabolism of two contrasting poplar species during acclimation to limiting nitrogen availability. J. Exp. Bot. 64, 4207–4224. https://doi.org/10.1093/jxb/ert234.

Maeda, S., Konishi, M., Yanagisawa, S., Omata, T., 2014. Nitrite transport activity of a novel HPP family protein conserved in cyanobacteria and chloroplasts. Plant Cell Physiol. 55 (7), 1311–1324. https://doi.org/10.1093/pcp/pcu075.

Mauro, R.P., Occhipinti, A., Longo, A.M.G., Mauromicale, G., 2011. Effects of shading on chlorophyll content, chlorophyll fluorescence and photosynthesis of subterranean clover. J. Agron. Crop Sci. 197 (1), 57–66. https://doi.org/10.1111/j.1439-037X.2010.00436.x.

McAllister, C.H., Beatty, P.H., Good, A.G., 2012. Engineering nitrogen use efficient crop plants: the current status. Plant Biotechnol. J. 10, 1011–1025. https://doi.org/10.1111/j.1467-7652.2012.00700.x.

Naeem, M.S., Jin, Z.L., Wan, G.L., Liu, D., Liu, H.B., Yoneyama, K., Zhou, W.J., 2010. 5-Aminolevulinic acid improves photosynthetic gas exchange capacity and ion uptake under salinity stress in oilseed rape (*Brassica napus* L.). Plant Soil 332, 405–415. https://doi.org/10.1007/s11104-010-0306-5.

Nunes-Nesi, A., Fernie, A.R., Stitt, M., 2010. Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. Mol. Plant 3, 973–996. https://doi.org/10.1093/mp/ssq049.

Pageau, K., Reisdorf-Cren, M., Morot-Gaudry, J.F., Masclaux-Daubresse, C., 2006. The two senescence-related markers, GS1 (cytosolic glutamine synthetase) and GDH (glutamate dehydrogenase), involved in nitrogen mobilization, are differentially regulated during pathogen attack and by stress hormones and reactive oxygen species in Nicotiana tabacum L. Leaves. J. Exp. Bot. 57, 547–557. https://doi.org/10.1093/jxb/erj035.

Plett, D., Baumann, U., Schreiber, A.W., Holtham, L., Kalashyan, Elena, Toubia, J., Nau, J., Beatty, M., Rafalski, A., Dhugga, K.S., Tester, M., Garnett, T., Kaiser, B.N., 2016. Maize maintains growth in response to decreased nitrate supply through a highly dynamic and developmental stage-specific transcriptional response. Plant Biotechnol. J. 14, 342–353. https://doi.org/10.1111/pbi.12388.

Ren, B., Zhang, J., Dong, S., Liu, P., Zhao, B., 2018. Exogenous 6-benzyladenine improves antioxidative system and carbon metabolism of summer maize waterlogged in the field. J. Agron. Crop Sci. 204, 175–184. https://doi.org/10.1111/jac.12253.

Rennenberg, H., Wildhagen, H., Ehlting, B., 2010. Nitrogen nutrition of poplar trees. Plant Biol. 12, 275–291. https://doi.org/10.1111/j.1438-8677.2009.00309.x.

Shah, J.M., Bukhari, S.A.H., Zeng, J.B., Quan, X.Y., Ali, E., Muhammad, N., Zhang, G.Q., 2017. Nitrogen (N) metabolism related enzyme activities, cell ultrastructure and nutrient contents as affected by N level and barley genotype. J. Integr. Agric. 16 (1), 190–198. https://doi.org/10.1016/S2095-3119(15)61308-9.

Shi, Y., Wan, L., Liu, J., Wang, Y., Guo, R., Wu, X., Li, X., 2010. Analysis of the principal components and the subordinate function of *Lolium perenne* drought resistance. Acta Agrestia Sin 18 (5), 669–672. https://doi.org/10.3724/SP.J.1077.2010.01263.

Undurraga, S.F., Ibarra-Henríquez, C., Fredes, I., Jm, Á., Gutiérrez, R.A., 2017. Nitrate signaling and early responses in Arabidopsis roots. J. Exp. Bot. 68, 2541–2551. https://doi.org/10.1093/jxb/erx041.

Vetriventhan, M., Upadhyaya, H.D., 2018. Diversity and trait-specific sources for productivity and nutritional traits in the global proso millet (*Panicum miliaceum* L.) germplasm collection. Crop J, 6, 451–463. https://doi.org/10.1016/j.cj.2018.04.002.

Wei, H.R., Yordanov, Y.S., Georgieva, T., Li, X., Busov, V., 2013. Nitrogen deprivation promotes Populus root growth through global transcriptome reprogramming and activation of hierarchical genetic networks. New Phytol. 200, 483–497. https://doi.org/10.1111/nph.12375.

Wei, S., Wang, X., Shi, D., Li, Y., Zhang, J., Liu, P., Zhao, B., Dong, S., 2016. The mechanisms of low nitrogen induced weakened photosynthesis in summer maize (*Zea mays* L.) under field conditions. Plant Physiol. Biochem. 105, 118–128. https://doi.org/10.1016/j.plaphy.2016.04.007.

Wen, B., Li, C., Fu, X., Li, D., Li, L., Chen, X., Wu, H., Cui, X., Zhang, X., Shen, H., Zhang, W., Xiao, W., Gao, D., 2019. Effects of nitrate deficiency on nitrate assimilation and chlorophyll synthesis of detached apple leaves. Plant Physiol. Biochem. 142, 363–371. https://doi.org/10.1016/j.plaphy.2019.07.007.

Xu, G.H., Fan, X.R., Miller, A.J., 2012. Plant nitrogen assimilation and use efficiency. Annu. Rev. Plant Biol. 63, 153–182. https://doi.org/10.1146/annurev-arplant-042811-105532.

Xu, W.Z., Deng, X.P., Xu, B.C., Gao, Z.J., Ding, W.L., 2014. Photosynthetic activity and efficiency of Bothriochloa ischaemum and Lespedeza davurica in mixtures across growth periods under water stress. Acta Physiol. Plant. 36, 1033–1044. https://doi.org/10.1007/s11738-013-1481-9.

Xuan, W., Beeckman, T., Xu, G., 2017. Plant nitrogen nutrition: sensing and signaling. Curr. Opin. Plant Biol. 39, 57–65. https://doi.org/10.1016/j.pbi.2017.05.010.

Yang, Q., Zhang, P., Qu, Y., Gao, X., Liang, J., Yang, P., Feng, B., 2018. Comparison of physicochemical properties and cooking edibility of waxy and non-waxy proso millet (*Panicum miliaceum* L.). Food Chem. 257, 271–278. https://doi.org/10.1016/j.foodchem.2018.03.009.

Yaryura, P., Cordon, G., Leon, M., Kerber, N., Pucheu, N., Rubio, G., García, A., Lagorio, M.G., 2009. Effect of Phosphorus Deficiency on reflectance and chlorophyll fluorescence of cotyledons of oilseed rape (*Brassica napus* L.). J. Agron. Crop Sci. 195 (3), 186–196. https://doi.org/10.1111/j.1439-037X.2008.00359.x.

Zeng, F., Li, L., Liang, N., Wang, X., Li, X., Zhan, Y., 2015. Salt tolerance and alterations in cytosine methylation in the interspecific hybrids of Fraxinus velutina and Fraxinus mandshurica. Euphytica 205, 721–737. https://doi.org/10.1007/s10681-015-1432-1.

Zhang, C., Zhang, Y., Lu, Z., Liu, L., 2017a. Screening fagopyrum tataricum genotypes tolerant to low nitrogen stress at seedling stage and its evaluating indices. Acta Agron. Sin. 43 (8), 1205–1215. https://doi.org/10.3724/SP.J.1006.2017.01205.

Zhang, D., Panhwar, R.B., Liu, J., Gong, X., Liang, J., Liu, M., Lu, P., Gao, X., Feng, B., 2019a. Morphological diversity and correlation analysis of phenotypes and quality traits of proso millet (*Panicum miliaceum* L.) core collections. J. Integr. Agric. 18 (5), 958–969. https://doi.org/10.1016/S2095-3119(18)61997-5.

Zhang, Y., Gao, X., Li, J., Gong, X., Yang, P., Gao, J., Wang, P., Feng, B., 2019b. Comparative analysis of proso millet (*Panicum miliaceum* L.) leaf transcriptomes for insight into drought tolerance mechanisms. BMC Plant Biol. 19, 397. https://doi.org/10.1186/s12870-019-2001-x.

Zhang, Y., Han, H., Zhang, D., Li, J., Gong, X., Feng, B., Xue, Z., Yang, P., 2017b. Effects of ridging and mulching combined practices on proso millet growth and yield in semi-arid regions of China. Field Crop. Res. 213, 65–74. https://doi.org/10.1016/j.fcr.2017.06.015.

Zhao, L., Reddy, K.R., Kakani, V.G., Reddy, V.R., 2005. Nitrogen deficiency effects on plant growth, leaf photosynthesis, and hyperspectral reflectance properties of sorghum. Eur. J. Agron. 22, 391–403. https://doi.org/10.1016/j.eja.2004.06.005.

Zhu, Y., Fan, X., Hou, X., Wu, J., Wang, T., 2014. Effect of different levels of nitrogen deficiency on switchgrass seedling growth. Crop J. 2, 223–234. https://doi.org/10.1016/j.cj.2014.04.005.

Zong, Y., Shangguan, Z.P., 2014. Nitrogen deficiency limited the improvement of photosynthesis in maize by elevated CO_2 under drought. J. Integr. Agric. 13 (1), 73–81. https://doi.org/10.1016/S2095-3119(13)60349-4.