Phyton-
International Journal of
Experimental Botany

Tech Science Press

http://doi.org/10.32604/phyton.2026.087517

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Evaluation and Screening of Nitrogen Efficiency at Seedling Stage of Rice Germplasm Resources

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Received: 17 June 2026; Accepted: 28 July 2026; Published: 28 August 2026

ABSTRACT: Excessive nitrogen fertilizer application increases production costs and environmental burdens in rice cultivation, highlighting the need to identify low-nitrogen-tolerant germplasm. In this study, 266 rice accessions were evaluated in hydroponic culture for 35 d under normal-nitrogen conditions (NN: 1.6 mM NO_3^- and 1.6 mM NH_4^+) and low-nitrogen conditions (LN: 0.4 mM NO_3^- and 0.4 mM NH_4^+). Fifteen seedling traits related to growth, chlorophyll status, nitrogen accumulation, and nitrogen utilization were measured, and the corresponding low-nitrogen tolerance indices were evaluated using correlation analysis, principal component analysis (PCA), membership-function analysis, and cluster analysis. All measured traits had coefficients of variation greater than 10%, indicating substantial variation among accessions. PCA extracted six principal components that explained 82.533% of the total variation. Relative shoot dry weight, relative total dry weight, and relative aboveground nitrogen accumulation were major indicators of seedling performance under LN. The LNTI-based D-values classified the 266 accessions into four tolerance groups. Comparison of this classification with the treatment-specific comprehensive evaluation identified six overlapping accessions—Q3-2, Q3-3, Q5-1, TD201, Jijing 88, and Jiadao 37—with both high relative low-nitrogen tolerance and high nitrogen-efficiency-related performance under NN and LN. These accessions represent preliminary candidates for further evaluation across nitrogen gradients, reproductive stages, field environments, and years before their breeding value and underlying physiological and molecular mechanisms can be confirmed.

KEYWORDS: Rice; principal component; correlation; membership function; nitrogen

1 Introduction

Rice (*Oryza sativa* L.) is one of the most important staple crops worldwide, particularly in Asia, and maintaining stable rice production is essential for food security. Nitrogen (N) is a major nutrient required for plant growth and yield formation because it participates in the synthesis of amino acids, nucleic acids, proteins, chlorophyll, and other essential cellular compounds [1–4]. However, N availability in paddy systems can decline rapidly after fertilization due to plant uptake, leaching, runoff, ammonia volatilization, and other loss pathways. Insufficient N supply restricts seedling establishment, reduces vegetative growth, impairs chlorophyll formation and photosynthetic capacity, and ultimately limits yield potential [5–7]. To maintain high rice productivity, large quantities of chemical N fertilizers are often applied. Global N fertilizer consumption has increased substantially in recent decades [8], and China produces approximately 20% of global rice while consuming a disproportionately high share of N fertilizer used in rice production [9]. Excessive N input increases production costs and causes environmental problems such as nutrient runoff, nitrate leaching, ammonia volatilization, and greenhouse gas emissions [10–14]. Therefore, improving nitrogen use efficiency (NUE) while maintaining rice productivity is an important goal for sustainable rice production [15].

Genetic improvement of rice NUE depends on the identification and utilization of germplasm with superior performance under reduced N input. Previous studies have demonstrated considerable genotypic variation in rice responses to N availability, indicating that low-N-tolerant and N-efficient germplasm can provide useful donor materials for breeding [16–19]. Different approaches, including hydroponic culture, soil culture, and field trials, have been used to evaluate N efficiency and low-N tolerance in crops [20–25]. Field trials provide the most realistic assessment of agronomic performance, but they are time-consuming, labor-intensive, and strongly affected by soil heterogeneity and environmental variation. Soil culture better reflects the natural root environment than hydroponics, but controlling N availability uniformly across many genotypes remains difficult. In contrast, hydroponic culture allows precise control of nutrient supply and is suitable for rapid and large-scale screening at the seedling stage. Therefore, seedling-stage hydroponic screening is useful as an initial step for identifying promising germplasm, although the selected accessions must be further validated under field conditions and at later growth stages.

The efficiency of low-N tolerance screening also depends on the selection and integration of appropriate evaluation indicators. Biomass accumulation, root traits, chlorophyll content, N accumulation, and N utilization efficiency have been used as indicators of plant responses to N deficiency. Multivariate statistical methods, including principal component analysis, membership function analysis, comprehensive evaluation values, and cluster analysis, have been applied in soybean, maize, wheat, millet, and other crops to integrate multiple traits for stress-tolerance evaluation [26–30]. Similar strategies have also been used in rice for nutrient-stress evaluation, such as low-phosphorus tolerance screening [31]. However, evaluation criteria for low-N tolerance still differ among studies in terms of N treatment levels, growth stages, trait combinations, and weighting methods. Many previous studies were based on limited numbers of genotypes or emphasized single traits, which may restrict the comparability and general applicability of the screening results. Thus, a large-scale evaluation that integrates seedling growth, chlorophyll status, N accumulation, and N-efficiency-related traits can provide a more comprehensive basis for preliminary identification of low-N-tolerant rice germplasm. Compared with previous studies involving fewer genotypes or single-trait evaluation, large-scale germplasm screening can improve the probability of identifying useful candidate materials for nitrogen-efficient rice breeding [32].

In this study, 266 rice germplasm accessions were evaluated under normal nitrogen (NN) and low nitrogen (LN) conditions using a hydroponic system at the seedling stage. A single LN level was used for primary screening because the objective was not to characterize genotype responses across a full N gradient, but to identify accessions that maintained relatively stable growth and N-efficiency-related performance under a clearly discriminating N-deficient condition. Fifteen seedling traits related to plant growth, chlorophyll content, N accumulation, and N utilization were measured and integrated using correlation analysis, principal component analysis, membership-function analysis, and cluster analysis. The objectives of this study were to: (i) characterize phenotypic variation among rice germplasm accessions under low-N stress; (ii) identify key seedling-stage indicators associated with low-N tolerance; and (iii) screen candidate germplasm with superior low-N tolerance and nitrogen-efficiency-related performance. The results provide preliminary candidate materials and an integrated evaluation framework for subsequent field validation, physiological characterization, and genetic studies of nitrogen-efficient rice.

2 Materials and Methods

2.1 Experimental Materials

A total of 266 rice germplasm resources of different genotypes were selected (Table S1), which were provided by the Rice Research Institute of Jilin Agricultural University. To improve the readability of figures, the 266 rice accessions were assigned unique codes from V1 to V266. The correspondence between accession codes and complete germplasm names is provided in Table S1.

2.2 Experimental Design

The experiment was conducted in a greenhouse at Jilin Agricultural University, Changchun, China, between May and October 2022. The mean day/night temperatures were approximately 28/22°C, and the mean relative humidity was approximately 60%. Plants were grown under a natural photoperiod without supplemental artificial lighting. Three temporally separated experimental rounds were conducted in the same greenhouse during 2022. In each round, each nitrogen treatment included three biological replicates. One turnover box was considered one biological replicate and contained ten rice accessions. For each accession, seeds were sown in ten holes, with three seeds per hole, resulting in approximately 30 seedlings per accession in each biological replicate and approximately 300 seedlings per turnover box. Thus, approximately 90 seedlings per accession were included in each nitrogen treatment in each experimental round.

In each experimental round, the 266 accessions were assigned independent random positions within each nitrogen treatment using a computer-generated randomization sequence. Containers were distributed across the available shelf positions, and their positions were rearranged regularly within each chamber to reduce potential effects of light, temperature, airflow, and shelf location. The chamber assignments of the NN and LN treatments were exchanged between successive experimental rounds to reduce confounding between nitrogen treatment and chamber identity. The entire experiment was conducted in three independent rounds using the same growth conditions and experimental procedures.

Rice seeds were surface-disinfected with 0.1% $KMnO_4$, thoroughly rinsed with distilled water, and germinated in Petri dishes at room temperature. After germination, the seeds were transferred to seedling trays filled with vermiculite. The seedlings were initially supplied with distilled water for 3 d, followed by half-strength Kimura B nutrient solution for 4 d and full-strength Kimura B nutrient solution for 7 d. Uniform seedlings were then subjected to different nitrogen treatments. The complete composition of the standard Kimura B nutrient solution is provided in Table S2. Two nitrogen treatments were established: normal nitrogen (NN), containing 1.6 mM NO_3^- and 1.6 mM NH_4^+, and low nitrogen (LN), containing 0.4 mM NO_3^- and 0.4 mM NH_4^+. The NN treatment corresponded to the nitrogen concentration of the standard Kimura B nutrient solution commonly used for rice hydroponic culture. The LN concentration was selected on the basis of preliminary experiments using different nitrogen gradients. This concentration maintained seedling survival while producing clear phenotypic variation in nitrogen responses among the rice accessions and was therefore considered suitable for primary low-nitrogen screening. The concentrations of all nutrients other than nitrogen were identical between the NN and LN treatments. The two treatments were maintained in separate sets of turnover boxes. The nutrient solutions were renewed every 3 d, and the pH was adjusted to 5.5 using HCl or NaOH. Seedlings were harvested after 35 d of nitrogen treatment. Individual plants within each biological replicate were treated as subsamples rather than independent biological replicates. The measurements obtained from the sampled plants were first averaged within each biological replicate, and the three biological-replicate means were then used to calculate the mean for each experimental round.

For correlation analysis, principal component analysis, membership-function analysis, and cluster analysis, accession-level means calculated across the three experimental rounds were used. Experimental rounds were regarded as temporal repetitions conducted in the same greenhouse rather than as independent site-year environments.

2.3 Measurement Indicators and Methods

After 35 d of nitrogen treatment, three seedlings were randomly selected from each biological replicate for trait measurements. Individual plants sampled within the same biological replicate were treated as subsamples. Plant height was measured from the stem base to the tip of the longest leaf, and root length was measured from the stem base to the tip of the longest root. The leaf area were measured, and then they were divided into above-ground and underground parts. After curing in the oven, dry to constant weight, measure the above-ground dry weight, root dry weight and total dry weight of individual plants, and calculate the root-to-shoot ratio.

The nitrogen content was measured according to Zhang [33] method, and the nitrogen content in the sample was measured using the H_2SO_4-H_2O_2 method. The chlorophyll determination method refers to Zhang [33] method, using acetone-ethanol (1:1) extraction and colorimetry.

Nitrogen accumulation (NA) = plant dry matter mass × plant nitrogen content.

Nitrogen utilization efficiency (NUtE) = dry matter mass/nitrogen accumulation.

Low nitrogen tolerance index (LNTI) = measured value under low nitrogen/measured value under normal nitrogen.

2.4 Calculation Method of Comprehensive Value of Nitrogen Efficiency

Because low-nitrogen tolerance is a complex trait that cannot be adequately represented by a single indicator, the membership-function method was used to integrate multiple trait responses into a comprehensive evaluation score. This method transforms indicators with different units and numerical ranges to a common scale ranging from 0 to 1, thereby allowing multiple traits to be combined in a unified evaluation framework. Membership-function approaches have been widely used for the comprehensive evaluation of crop responses to abiotic stress [34–36].

The comprehensive evaluation was based on the low-nitrogen tolerance indices (LNTIs) of the selected traits. All 15 low-nitrogen tolerance indices were retained in the comprehensive evaluation because they represented distinct operational aspects of seedling growth, biomass allocation, chlorophyll status, nitrogen accumulation, and nitrogen utilization. Although some indices were strongly correlated or mathematically related, they were retained to maintain consistency with the predefined trait framework. The potential overrepresentation of correlated information was considered when interpreting the comprehensive scores. For traits positively associated with the maintenance of seedling performance under low-nitrogen conditions, the membership value was calculated as follows:

$$U_{ij} = \frac{X_{ij} - X_{jmin}}{X_{jmax} - X_{jmin}} \tag{1}$$

U_{ij} represents the membership value of the jth low-nitrogen tolerance index for the ith rice accession; X_{ij} represents the corresponding LNTI value; and X_{jmin} and X_{jmax} represent the minimum and maximum values, respectively, of the jth LNTI across the 266 accessions.

E_j represents the weight assigned to the jth LNTI, and C_j represents its coefficient of variation across the 266 accessions. The sum of the weights of all included LNTIs equals 1.

The weight calculation formula is:

$$E_j = \frac{c_j}{\sum c_j} \quad (2)$$

The comprehensive low-nitrogen tolerance score (D-value) of each accession was calculated as follows:

$$D_i = \sum \left(U_{ij} \times E_j\right) \quad (3)$$

where D_i is the comprehensive low-nitrogen tolerance score of the ith accession. A higher D-value indicated a greater overall ability to maintain the selected seedling traits under the specific low-nitrogen treatment used in this study. The D-value represents a relative evaluation within the tested germplasm population and should not be interpreted as direct evidence of field-level nitrogen-use efficiency.

The coefficient-of-variation weighting method was selected because it provides an objective and transparent means of assigning greater weight to indicators showing stronger discriminatory variation within the evaluated population, without requiring subjective expert scoring. However, these weights reflect statistical variability rather than the biological importance of individual traits. No formal comparison with alternative weighting methods was conducted in this study. Therefore, the resulting D-values were interpreted as population-relative screening scores and were evaluated together with the PCA, cluster analysis, and treatment-specific comprehensive classification rather than being regarded as independently validated measures of low-nitrogen tolerance.

Treatment-specific comprehensive performance values were calculated separately under NN and LN conditions using the 15 measured traits: PH, RL, ADW, RDW, AFW, RFW, RSR, TDW, TFW, LA, Ca, Cb, C, ANA, and ANUtE. All traits were treated as positive indicators and standardized to membership values ranging from 0 to 1 using the minimum and maximum values across the 266 accessions within each treatment. Trait weights were determined separately under NN and LN conditions according to their coefficients of variation, with the weights summing to 1 within each treatment. The comprehensive value of each accession was calculated as the weighted sum of its membership values. Higher values indicated better relative seedling performance under the corresponding nitrogen treatment. The population means under NN and LN were used as thresholds to classify the accessions into four types.

2.5 Statistical Analysis

The coefficient of variation (CV) was calculated as the standard deviation divided by the mean and expressed as a percentage. Pearson correlation analysis was performed using accession-level means. Correlations among mathematically related variables were interpreted as descriptive associations rather than as evidence of causal relationships.

Principal component analysis (PCA) was performed using the standardized low-nitrogen tolerance indices to characterize multivariate relationships among traits and identify the major sources of phenotypic variation. The suitability of the dataset for PCA was evaluated using the Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy and Bartlett's test of sphericity. Principal components were extracted from the standardized low-nitrogen tolerance indices. The number of retained principal components was determined using the Kaiser criterion, and only components with eigenvalues greater than 1 were retained. PCA was used as an explanatory analysis and was not used directly to calculate the membership-function-based D-values. Separate PCAs based on standardized trait values under NN and LN conditions were conducted only to visualize treatment-specific relationships among traits.

The comprehensive low-nitrogen tolerance D-value was calculated directly from the membership values of the selected low-nitrogen tolerance indices and their coefficient-of-variation weights, as described in Section 2.4. Treatment-specific comprehensive performance values under NN and LN conditions were calculated separately from the 15 measured traits using treatment-specific membership values and coefficient-of-variation weights, as described in Section 2.4. Hierarchical cluster analysis was subsequently performed based on the D-values using Euclidean distance.

Origin 2022 (OriginLab Corporation, Northampton, MA, USA) was used to generate the PCA plots and other statistical figures. The circular dendrogram was generated in R version 4.3.2 using the "ggtree" package. Statistical significance was defined as $p < 0.05$. Differences between NN and LN for each measured trait were tested using accession-level observations, and the corresponding test statistics and exact p values are reported in Table S3. Because only two nitrogen treatments were compared, no post hoc multiple-comparison test was required. Low-nitrogen tolerance indices were calculated as LN/NN ratios and were therefore not subjected to a separate comparison between nitrogen treatments.

3 Results

3.1 Analysis of Various Indicators of Rice Seedlings

The statistical comparisons between NN and LN are presented in Table S3. LN significantly affected most measured seedling traits, although the magnitude and direction of the response differed among traits. Descriptive means, ranges, standard deviations, and coefficients of variation are presented in Table 1. The coefficient of variation among accessions ranged from 11.07% to 33.82% under NN and from 14.38% to 46.24% under LN, indicating substantial variation among genotypes and supporting the feasibility of screening under LN conditions. Compared with NN, most seedling traits were reduced under LN, except for root length and root-to-shoot ratio, suggesting that low nitrogen stress inhibited overall seedling growth. Under NN conditions, plant height, aboveground dry weight, aboveground fresh weight, root fresh weight, total dry weight, total fresh weight, leaf area, chlorophyll a, chlorophyll b, total chlorophyll, and aboveground nitrogen accumulation were 10%–122% higher than under LN. In contrast, root length, and root-to-shoot ratio were higher under LN, indicating that seedlings prioritize root growth to enhance nitrogen acquisition under limited nitrogen availability. This pattern suggests a possible shift in biomass allocation toward root growth under low-nitrogen conditions; however, nitrogen uptake capacity was not directly measured in this study. A direct comparison between NN and LN is provided in Supplementary Table S3.

The degree of variation of each variety index is an important basis for measuring whether the screening index was appropriate (Table 1). The greater the degree of variation, the more conducive the indicator is to showing the differences between varieties. Under normal nitrogen conditions, the coefficient of variation is expressed as the aboveground nitrogen accumulation > chlorophyll b > root to shoot ratio > chlorophyll a > dry weight above ground > total chlorophyll > fresh weight above ground > leaf area > total plant dry weight > aboveground fresh weight > total fresh weight > underground dry weight > plant height > root length > aboveground nitrogen utilization efficiency. Under low nitrogen conditions, the expression is chlorophyll b > chlorophyll a > total chlorophyll > leaf area > plant height > above ground fresh weight > root to shoot ratio > aboveground nitrogen accumulation > total plant fresh weight > underground fresh weight > underground dry weight > aboveground dry weight > total plant dry weight > root length > aboveground nitrogen utilization efficiency.

Table 1: Traits of 266 rice varieties grown low and normal N environment.

Index	Treatment	Average	Maximum Value	Minimum Value	Standard Deviation	CV/(%)
PH (cm)	NN	30.549	44	14.34	6.418	21.01
	LN	16.859	25.433	5.833	4.484	26.6
RL (cm)	NN	16.204	23.967	8.9	2.543	15.57
	LN	16.923	22.967	7.5	2.71	16.02
ADW (g)	NN	0.178	0.309	0.103	0.054	30.32
	LN	0.113	0.182	0.061	0.022	19.43
RDW (g)	NN	0.04	0.073	0.025	0.009	23.45
	LN	0.034	0.06	0.016	0.007	20.03
AFW (g)	NN	0.643	1.563	0.32	0.157	24.48
	LN	0.581	1.023	0.25	0.153	26.35
RFW (g/plant)	NN	0.333	0.886	0.159	0.093	28.01
	LN	0.301	0.501	0.104	0.072	24.06
RSA	NN	0.24	0.417	0.101	0.077	31.99
	LN	0.31	0.646	0.142	0.08	25.82
TDW (g)	NN	0.218	0.354	0.132	0.056	25.87
	LN	0.147	0.224	0.089	0.024	16
TFW (g)	NN	0.976	2.449	0.49	0.235	24.09
	LN	0.882	1.496	0.361	0.21	24.33
LA (cm^2)	NN	18.487	33.668	7.948	4.972	26.89
	LN	16.157	32.198	5.503	4.846	29.99
Ca (mg/g)	NN	1.388	2.718	0.502	0.425	30.64
	LN	0.976	2.12	0.239	0.403	41.29
Cb (mg/g)	NN	0.298	0.825	0.128	0.097	32.39
	LN	0.198	0.49	0.036	0.082	41.41
C (mg/g)	NN	1.686	3.251	0.666	0.488	28.96
	LN	1.174	2.383	0.343	0.464	39.52
ANA (mg)	NN	0.486	0.968	0.236	0.165	33.82
	LN	0.219	0.347	0.1	0.053	24.39
ANUtE ($g \cdot g^{-1}$)	NN	0.368	0.51	0.293	0.041	11.1
	LN	0.529	0.776	0.35	0.076	14.41

Note: PH: plant height; RL: root length; ADW: aboveground dry weight; RDW: root dry weight; AFW: aboveground fresh weight; RFW: root fresh weight; RSA: root-shoot ratio; TDW: total plant dry weight; TFW: total plant fresh weight; LA: leaf area; Ca: Chlorophyll a; Cb: Chlorophyll b; C: Total chlorophyll; ANA: aboveground nitrogen accumulation; ANUtE: aboveground nitrogen utilization efficiency.

3.2 Analysis of Various Indicators of Low Nitrogen Tolerance Index of Rice Seedlings

In order to eliminate the biological differences between different genotypes, the low nitrogen tolerance ability of rice varieties was determined through the low nitrogen tolerance index. It can be seen from Table 2 that the coefficient of variation of the low nitrogen tolerance coefficient of each index was relative chlorophyll b > relative chlorophyll a > relative total chlorophyll > relative root-shoot ratio > relative aboveground nitrogen accumulation > relative leaf area > relative underground dry weight > relative underground fresh weight > relative aboveground fresh weight > relative total plant fresh weight > relative aboveground dry weight > relative total plant dry weight > relative plant height > relative aboveground nitrogen utilization efficiency > relative root length. Among them, the coefficient of variation of relative chlorophyll b was the largest, 39.94%; the coefficient of variation of relative root length was the smallest, 14.99%.

Table 2: Low nitrogen tolerance index of different rice germplasm.

Index	Average	Maximum Value	Minimum Value	Standard Deviation	CV/(%)
RPH	0.552	0.816	0.282	0.097	17.6
RRL	1.056	1.97	0.525	0.158	14.99
RADW	0.667	1.22	0.334	0.148	22.18
RRDW	0.894	1.559	0.248	0.252	28.18
RAFW	0.923	1.492	0.361	0.228	24.73
RRFW	0.94	1.597	0.346	0.243	25.85
RRSR	1.395	3.298	0.499	0.489	35.05
RTDW	0.701	1.205	0.355	0.14	19.97
RTFW	0.924	1.45	0.402	0.214	23.15
RLA	0.903	1.653	0.313	0.266	29.5
RCa	0.732	1.58	0.203	0.29	39.65
RCb	0.694	1.511	0.134	0.277	39.94
RC	0.719	1.512	0.246	0.266	36.95
RANA	0.48	1.018	0.207	0.144	30.03
RANUtE	1.451	2.261	0.824	0.249	17.19

Note: RPH: relative plant height; RRL: relative root length; RADW: relative aboveground dry weight; RRDW: relative root dry weight; RAFW: relative aboveground fresh weight; RRFW: relative root fresh weight; RRSR: relative root-shoot ratio; RTDW: relative total plant dry weight; RTFW: relative total plant fresh weight; RLA: relative leaf area; RCa: relative Chlorophyll a; RCb: relative Chlorophyll b; RC: relative total chlorophyll; RANA: relative aboveground nitrogen accumulation; RANUtE: relative aboveground nitrogen utilization efficiency.

In order to further select identification indicators for evaluating the low nitrogen tolerance of rice, correlation analysis was performed on the 15 measured indicators (Table S4). Under normal nitrogen levels, there was an extremely significant positive correlation between the nitrogen accumulation in the aboveground and the total dry weight of the plant (r = 0.908**). There was a very significant negative correlation between root-shoot ratio and aboveground dry weight (r = −0.723**). At low nitrogen levels, there was a highly significant positive correlation between aboveground dry weight and total dry weight (r = 0.951**), aboveground nitrogen accumulation (r = 0.815**).

3.3 Comprehensive Evaluation and Screening of Low Nitrogen Tolerant Rice Germplasm Resources

3.3.1 Principal Component Analysis

Principal component analysis was performed on 15 low-nitrogen tolerance indices (Table 3). The KMO value was 0.72, and Bartlett's test of sphericity was significant (χ^2 = 6155.51, df = 105, $p < 0.001$), confirming the suitability of the dataset for PCA. Six principal components with eigenvalues greater than 1 were retained, explaining 82.533% of the total variation. PC1 explained 21.441% of the variation and had high loadings for RADW, RTDW, and RANA, representing relative dry-matter and nitrogen accumulation. PC2 explained 19.301% and was mainly associated with RAFW, RRFW, RRSR, and RTFW, reflecting relative fresh biomass and root–shoot allocation. PC3 accounted for 16.269% and was dominated by RCa, RCb, and RC, representing relative chlorophyll status. PC4 explained 10.033% and was mainly associated with RRDW and RRSR, reflecting relative root biomass and biomass allocation. PC5 accounted for 8.397% and was primarily associated with RANUtE and RRL, whereas PC6 explained 7.093% and was mainly associated with RRL and RLA. Overall, these components summarized the major variation in biomass, nitrogen accumulation and utilization, chlorophyll status, and plant morphology under low-nitrogen conditions.

Table 3: Eigenvalues, component loadings, and explained variance of the six principal components derived from the low-nitrogen tolerance indices of 15 selected seedling traits in 266 rice accessions.

Index	PC1	PC2	PC3	PC4	PC5	PC6
RPH	−0.033	0.199	−0.144	−0.22	−0.056	−0.237
RRL	−0.045	0.132	0.057	0.132	−0.555	0.499
RADW	0.783	−0.519	−0.125	0.125	0.237	0.073
RRDW	0.285	0.438	−0.096	0.831	−0.002	−0.103
RAFW	0.537	0.609	−0.297	−0.343	−0.048	0.07
RRFW	0.536	0.629	−0.237	−0.165	0.039	−0.165
RRSR	−0.262	0.716	0.001	0.591	−0.161	−0.125
RTDW	0.791	−0.375	−0.137	0.362	0.236	0.046
RTFW	0.588	0.674	−0.296	−0.306	−0.019	−0.013
RCa	0.294	0.226	0.857	−0.05	0.112	−0.088
RCb	0.214	0.083	0.757	−0.037	−0.064	0.012
RC	0.304	0.215	0.903	−0.051	0.087	−0.074
RLA	0.205	0.206	0.091	−0.015	0	0.778
RANA	0.75	−0.538	−0.05	0.058	−0.329	−0.089
RANUtE	−0.272	0.26	−0.083	0.066	0.821	0.259
Contribution rates (%)	21.441	19.301	16.269	10.033	8.397	7.093
Cumulative contribution rate (%)	21.441	40.741	57.011	67.043	75.44	82.533

Note: RPH: relative plant height; RRL: relative root length; RADW: relative aboveground dry weight; RRDW: relative root dry weight; RAFW: relative aboveground fresh weight; RRFW: relative root fresh weight; RRSR: relative root-shoot ratio; RTDW: relative total plant dry weight; RTFW: relative total plant fresh weight; RLA: relative leaf area; RCa: relative Chlorophyll a; RCb: relative Chlorophyll b; RC: relative total chlorophyll; RANA: relative aboveground nitrogen accumulation; RANUtE: relative aboveground nitrogen utilization efficiency.

Separate PCA analyses were conducted using standardized trait values under LN and NN conditions, independently of the LNTI-based PCA in Table 3. Under LN, four principal components explained 74.848% of the total variation (Table S5). PC1 explained 37.331% and was mainly associated with biomass and nitrogen accumulation traits, including ADW, AFW, RFW, TDW, TFW, and ANA. PC2 explained 17.589% and was dominated by Ca, Cb, and C. PC3 explained 12.308% and was mainly associated with RDW, RSR, and ANUtE, while PC4 explained 7.620% and was primarily associated with ANUtE and PH.

Under NN, five principal components explained 79.885% of the total variation (Table S6). PC1 explained 31.764% and was mainly associated with ADW, AFW, RFW, TDW, TFW, and SNA. PC2 explained 17.579% and was dominated by Ca, Cb, and C. PC3 explained 15.358% and was mainly associated with RDW, RFW, RSR, and TFW. PC4 explained 8.074% and was associated with PH, RL, LA, and ANUtE, whereas PC5 explained 7.110% and was primarily characterized by ANUtE. These results indicate that biomass and nitrogen accumulation were the main sources of variation under both treatments, while chlorophyll status, root−shoot allocation, morphology, and nitrogen utilization accounted for additional variation.

3.3.2 Membership-Function Analysis and Comprehensive Low-Nitrogen Tolerance Scores

The D-values showed differences among the 266 rice accessions, indicating clear genotypic differences in the maintenance of seedling performance under LN conditions (Table S7). Q3-2, Q5-1, Jijing 88, and Jiadao 37 had D-values greater than 0.70 and ranked among the most low-nitrogen-tolerant accessions. By contrast, Xinnong 999, Jiyudao 8, Qinglin 90, Jijing 826, and Jiyujing had the lowest D-values and were therefore classified as relatively sensitive to LN. These results provided the quantitative basis for the subsequent cluster analysis and classification of the 266 accessions into different low-nitrogen tolerance groups.

3.3.3 Cluster Analysis

Clustering was performed based on the D value (Fig. 1). It can be seen from the cluster analysis diagram that the 266 rice varieties can be divided into 4 categories. The first category included 29 varieties including Q3-2, Q5-1, Jijing 88 and Jiadao 37, which had the strongest tolerance to low nitrogen. The second category included 119 varieties including Songze 318, TD403, Luke 9 and Longjingxiang 7, which had strong low nitrogen tolerance. The third category included 113 varieties including Z2003, Tonghe 865, Z2007 and Jinongda 667, which had average low nitrogen tolerance. The fourth category included five varieties, including Jijing 826, Jiyudao 8, Qinglin 90, Xinnong 999 and Jiyujing, which had poor low nitrogen tolerance.

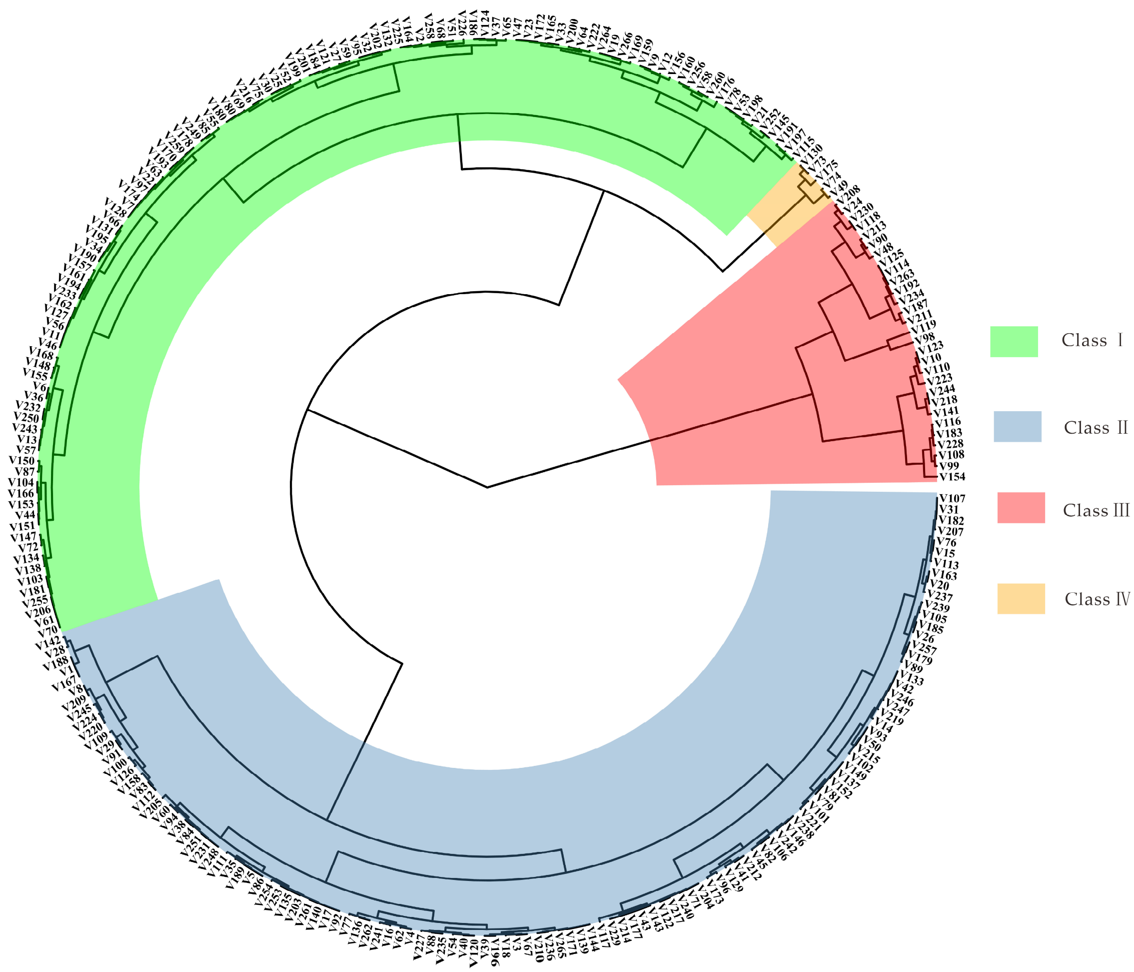

Figure 1: Hierarchical clustering of 266 rice germplasm accessions based on comprehensive low-nitrogen tolerance D-values calculated from low-nitrogen tolerance indices (LNTIs). The D-value represents the relative ability of each accession to maintain seedling performance under LN compared with NN conditions. Accessions were classified into four low-nitrogen tolerance groups: Class I, average low-nitrogen tolerance; Class II, strong low-nitrogen tolerance; Class III, strongest low-nitrogen tolerance; Class IV, poor low-nitrogen tolerance.3.3.4. Classification of rice accessions based on comprehensive performance under NN and LN conditions.

The treatment-specific comprehensive performance values varied among the 266 rice accessions under both NN and LN conditions (Table S8). Based on the population mean values under the two treatments, the accessions were classified into four types (Fig. 2). Type I comprised 51 accessions (19.17%) with above-average comprehensive performance under both NN and LN conditions. Type II comprised 54 accessions (20.30%) with below-average performance under NN but above-average performance under LN. Type III comprised 152 accessions (57.14%) with below-average performance under both treatments, whereas Type IV comprised nine accessions (3.38%) with above-average performance under NN but below-average performance under LN. Type I accessions were therefore considered to have comparatively high nitrogen-efficiency-related seedling performance under both nitrogen conditions.

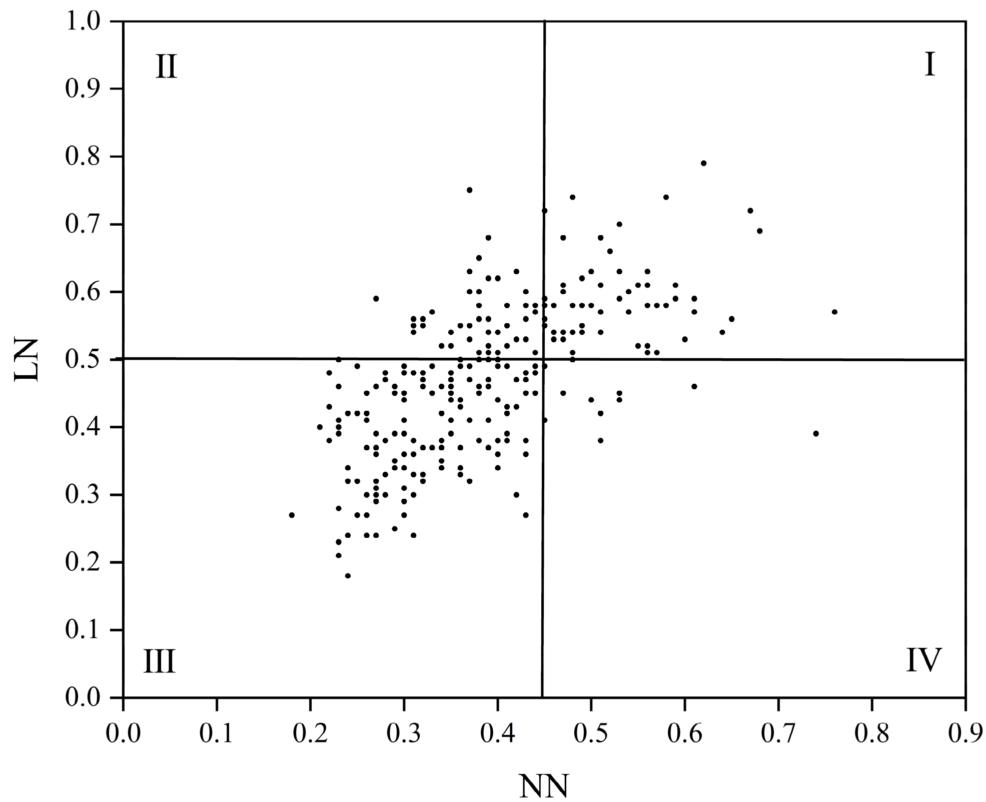

Figure 2: Classification of 266 rice germplasm accessions based on comprehensive nitrogen-efficiency values under normal-nitrogen (NN) and low-nitrogen (LN) conditions. The x-axis represents the comprehensive nitrogen-efficiency value under NN conditions, and the y-axis represents the comprehensive nitrogen-efficiency value under LN conditions. Each point represents one rice accession. The vertical and horizontal lines indicate the population mean values under NN and LN conditions, respectively, and were used as thresholds to classify accessions into four types. Type I accessions showed high nitrogen efficiency under both NN and LN conditions and were considered broadly nitrogen-efficient. Type II accessions showed low nitrogen efficiency under NN but high nitrogen efficiency under LN, suggesting specific adaptation to nitrogen limitation. Type III accessions showed low nitrogen efficiency under both NN and LN conditions. Type IV accessions showed high nitrogen efficiency under NN but low nitrogen efficiency under LN, indicating sensitivity to low-nitrogen stress.

The two screening strategies were compared using a Venn diagram (Fig. 3). Of the 51 Type I accessions showing high comprehensive nitrogen-efficiency values under both NN and LN conditions, 6 were also included among the 29 Class III accessions identified as highly low-nitrogen tolerant based on the D-values. The six overlapping accessions—Q3-2, Q3-3, Q5-1, TD201, Jijing 88, and Jiadao 37—showed both high relative tolerance to low nitrogen and high nitrogen-efficiency-related performance under NN and LN conditions. Therefore, these accessions were identified as preliminary candidates for further field and reproductive-stage validation. The overlap between the two screening strategies provided an internal consistency check for candidate selection, although it did not constitute an independent validation of the coefficient-of-variation weighting method.

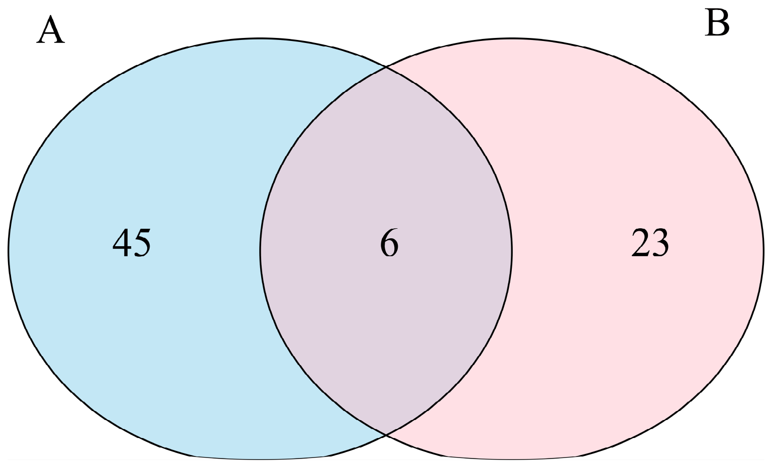

Figure 3: Venn diagram showing the overlap between the two screening strategies used to identify candidate low-nitrogen-tolerant and nitrogen-efficient rice germplasm. Set A represents accessions classified as Type I in Fig. 2, which showed high comprehensive nitrogen-efficiency values under both NN and LN conditions. Set B represents accessions classified as Class III in Fig. 1, which showed the strongest low-nitrogen tolerance based on LNTI-derived D-values. The intersection represents accessions selected by both the absolute nitrogen-efficiency evaluation and the relative low-nitrogen tolerance evaluation.

4 Discussion

Hydroponic screening provides a controlled and efficient approach for the preliminary evaluation of large germplasm populations, whereas field screening is influenced by soil heterogeneity, climatic variation, and spatial differences in nitrogen availability [37,38]. Previous studies have used biomass, agronomic traits, chlorophyll characteristics, and nitrogen-related traits to evaluate crop responses to nitrogen limitation [39,40]. Previous studies have also investigated nitrogen-stress responses from multiple perspectives, including molecular regulation [41], root-growth characteristics associated with nitrogen efficiency [42], and comprehensive screening of low-nitrogen tolerance or nitrogen efficiency at the seedling stage in cotton, rapeseed, and broomcorn millet [43–45]. Consistent with these studies, the present results demonstrate that seedling biomass, chlorophyll status, nitrogen accumulation, and derived nitrogen-utilization traits provide complementary information for distinguishing rice accessions under low-nitrogen conditions. Compared with evaluations based on a single trait or performance under only one nitrogen condition, the present study combined relative low-nitrogen tolerance indices with comprehensive performance under both NN and LN conditions in a panel of 266 rice accessions. This design made it possible to distinguish accessions that maintained their relative performance under LN from those that showed comparatively high performance under both nitrogen treatments. However, the statistical procedures used here, including correlation analysis, PCA, membership-function analysis, coefficient-of-variation weighting, and clustering, are established approaches. Therefore, the contribution of this study is an incremental improvement in large-panel seedling screening and the integration of two complementary evaluation perspectives rather than the development of a new statistical method or the demonstration of a new physiological mechanism.

Most shoot-growth, biomass, chlorophyll, and aboveground nitrogen-accumulation traits were lower under LN than under NN. Root length and the root-to-shoot ratio were higher under LN, whereas root dry weight decreased from 0.040 g under NN to 0.034 g under LN. The higher root-to-shoot ratio should not be interpreted as direct evidence of increased root biomass or active biomass reallocation because it may have resulted primarily from a greater reduction in shoot biomass than in root biomass. Similarly, the greater maximum root length does not demonstrate enhancement of the entire root system. Only the length of the longest root and root dry weight were measured, whereas root number, total root length, root diameter, root surface area, root volume, branching pattern, and root spatial distribution were not evaluated. The present results therefore describe differences in maximum root elongation and relative root-to-shoot biomass allocation but do not establish changes in complete root architecture, root-system development, or nitrogen-acquisition capacity.

Aboveground nitrogen utilization efficiency was higher under LN than under NN, but this result should also be interpreted cautiously. This variable was derived from biomass and aboveground nitrogen accumulation and was therefore affected by the relative changes in both component traits. A higher calculated value under LN does not necessarily indicate enhanced physiological nitrogen metabolism, nitrogen assimilation, or nitrogen-acquisition capacity. Nitrogen uptake kinetics, root nitrogen influx, nitrogen-assimilation enzyme activities, photosynthetic rate, carbon–nitrogen metabolism, gene expression, and molecular responses were not measured. Consequently, the present study cannot determine the physiological or molecular mechanisms responsible for the observed phenotypic differences. The results should be interpreted as a phenotypic assessment of seedling responses under the specified hydroponic nitrogen conditions rather than as evidence of particular root, photosynthetic, metabolic, or molecular adaptation mechanisms.

The correlation and PCA results indicated that the relative indices of shoot dry weight, total dry weight, and aboveground nitrogen accumulation had high loadings on major dimensions of variation among accessions. These variables reflect the relative maintenance of seedling dry matter and aboveground nitrogen status under LN and may therefore be useful indicators for preliminary screening. Chlorophyll-related indices displayed substantial variation and were associated with a separate principal-component dimension, indicating that the maintenance of leaf pigment status differed among accessions. Root dry weight, root-to-shoot ratio, leaf area, and aboveground nitrogen utilization efficiency represented additional dimensions of phenotypic variation. These results confirm that seedling responses to LN cannot be represented adequately by a single trait.

Nevertheless, PCA loadings and correlations do not demonstrate causal importance. Several retained variables were biologically correlated or mathematically related. Total dry weight was calculated from shoot and root dry weights, total chlorophyll was calculated from chlorophyll a and chlorophyll b, and aboveground nitrogen accumulation was derived from shoot dry weight and shoot nitrogen content. The correlations among these traits may therefore partly reflect shared component variables or overlapping biological information. Accordingly, the indicators identified by PCA should be regarded as statistical representations of major dimensions of variation within this germplasm panel, rather than as independent causal determinants of low-nitrogen tolerance.

The integration of multiple traits can provide a broader evaluation than selection based on a single variable, but it also introduces methodological uncertainty. In this study, PCA was used to summarize major sources of variation, while the membership-function D-value was calculated from selected low-nitrogen tolerance indices using coefficient-of-variation weights. This weighting method assigned greater weights to traits with greater variation among the evaluated accessions, thereby increasing their contribution to discrimination within this population. However, high statistical variability does not necessarily correspond to high biological importance. In addition, correlated or mathematically related traits may contribute overlapping information, and alternative weighting methods could produce different accession rankings. Because no sensitivity analysis or comparison with alternative weighting approaches was conducted, the resulting D-values, cluster assignments, and rankings should be considered dependent on the selected traits, evaluated population, and analytical procedure.

The use of only one NN concentration and one LN concentration represents an additional limitation. Although the selected LN treatment produced clear phenotypic variation while allowing the seedlings to survive, the current experiment did not include a nitrogen concentration gradient. It was therefore not possible to determine whether accession rankings remained stable across mild, moderate, and severe nitrogen limitation. Relative indices calculated from the ratio between LN and NN may change with treatment intensity, and different accessions may show nonlinear or concentration-specific responses. An accession ranked highly at the nitrogen concentrations used in this study may not retain the same position under a different LN concentration. Thus, the reported tolerance levels and accession rankings apply only to the specific NN and LN conditions evaluated here and should not be generalized across nitrogen environments without further testing.

The two screening strategies provided complementary but not independent evidence. The LNTI-based D-value described the relative ability of each accession to maintain seedling performance under LN compared with NN, whereas the comprehensive values under the two treatments described relative seedling performance within NN and LN separately. The highest-tolerance D-value cluster contained 29 accessions, and 51 accessions were classified as Type I based on above-average comprehensive performance under both treatments. Six accessions, Q3-2, Q3-3, Q5-1, TD201, Jijing 88, and Jiadao 37, were identified by both approaches. Their agreement indicates that these accessions combined relatively stable performance under LN with comparatively high seedling performance under the two nitrogen treatments used in this experiment. However, both evaluations were derived from the same hydroponic dataset and therefore do not constitute independent biological validation. These six accessions should be regarded only as preliminary candidates for further evaluation, not as confirmed low-nitrogen-tolerant or nitrogen-efficient breeding materials.

The experimental conditions further restrict the applicability of the findings. The accessions were evaluated only at the seedling stage under controlled hydroponic conditions, and the three experimental rounds represented temporal repetitions under the same general environment rather than independent locations or years. Hydroponic culture does not reproduce the heterogeneous nitrogen distribution, soil–root interactions, microbial processes, water fluctuations, and environmental stresses encountered under field conditions. Moreover, seedling biomass and nitrogen-related traits may not predict performance during reproductive growth, grain yield, grain quality, or whole-season nitrogen-use efficiency. Genotype rankings may change with developmental stage, soil type, climate, nitrogen-management regime, and the severity or timing of nitrogen limitation. Therefore, the present experiment cannot establish the field nitrogen efficiency, yield stability, or breeding value of the selected accessions.

Future studies should first evaluate the stability of the rankings across a gradient of nitrogen concentrations and at multiple developmental stages. The candidate accessions should then be tested in replicated field experiments across locations and years using contrasting nitrogen-application rates. Grain yield, yield components, total plant nitrogen accumulation, agronomic nitrogen-use efficiency, nitrogen recovery, and performance stability should be included in these evaluations. Detailed root architectural traits, nitrogen uptake kinetics, nitrogen-assimilation enzyme activities, gas-exchange parameters, and molecular analyses would also be required to determine whether the candidate accessions differ in specific physiological or genetic mechanisms. Until such independent validation is completed, the main value of the present study lies in narrowing a relatively large germplasm panel to a manageable set of candidates for subsequent nitrogen-gradient, field, physiological, and molecular evaluation.

5 Conclusion

Low-nitrogen treatment reduced most growth, biomass, chlorophyll, and nitrogen-accumulation traits in rice seedlings, while substantial genotypic variation was observed among the 266 accessions. Multivariate analysis of 15 selected low-nitrogen tolerance indices identified relative shoot dry weight, total dry weight, and aboveground nitrogen accumulation as key indicators of seedling performance under LN. By integrating the LNTI-based D-value classification with comprehensive performance under NN and LN conditions, six accessions—Q3-2, Q3-3, Q5-1, TD201, Jijing 88, and Jiadao 37—were identified as preliminary candidates with both high relative low-nitrogen tolerance and high nitrogen-efficiency-related performance. Because the evaluation was conducted at the seedling stage under a single hydroponic LN level, these accessions require further validation across nitrogen gradients, reproductive stages, field environments, and years before their breeding value and underlying physiological and molecular mechanisms can be confirmed.

Acknowledgement: Not applicable.

Funding Statement: This study was financially supported by the Jilin Province Key Research and Development Project (2025JLGJ0007NC).

Author Contributions: Conceptualization, Shuting Zhao and Fenglou Ling; methodology, Fenglou Ling and Xiao Han; software, Yuzhuo Yan; validation, Feisal Mohamed Osman; formal analysis, Qiang Zhang and Zexin Qi; investigation, Shuting Zhao, Yuzhuo Yan, Feisal Mohamed Osman, Qiang Zhang, Zexin Qi and Zhian Zhang; resources, Fenglou Ling and Xiao Han; writing—original draft preparation, Shuting Zhao and Yuzhuo Yan; writing—review and editing, Fenglou Ling and Xiao Han; supervision, Fenglou Ling; project administration, Fenglou Ling and Xiao Han; funding acquisition, Fenglou Ling and Xiao Han. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The authors declare that all relevant data generated or analyzed during this study are included in this published article; further inquiries can be directed to the corresponding authors.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Supplementary Materials: The supplementary material is available online at https://www.techscience.com/doi/10.32604/phyton.2026.087517/s1. Table S1: Test rice germplasm; Table S2: Kimura B nutrient solution formula; Table S3: Statistical comparisons of seedling traits between normal-nitrogen and low-nitrogen treatments across 266 rice accessions, including treatment means, test statistics, and exact p values; Table S4: Correlation coefficients of traits of 266 rice germplasm at seedling stage under normal nitrogen (above the diagonal) and low nitrogen (below the diagonal) conditions; Table S5: Component loadings and explained variance of the principal components derived from 15 seedling traits under low-nitrogen conditions; Table S6: Component loadings and explained variance of the principal components derived from 15 seedling traits under normal-nitrogen conditions; Table S7: Membership values, comprehensive low-nitrogen tolerance scores (D-values), rankings, and cluster classifications of 266 rice accessions based on the selected low-nitrogen tolerance indices; Table S8: Trait membership values, coefficient-of-variation weights, and treatment-specific comprehensive performance values of 266 rice accessions under NN and LN conditions.

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