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Genetic diversity and salt tolerance assessment of 51 alfalfa (*Medicago sativa*) varieties under saline soil conditions

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Introduction: Salt stress significantly impacts plant growth worldwide. Although alfalfa exhibits some tolerance to salt-alkali soils, the specific salt tolerance levels across different alfalfa varieties remain inadequately understood. For this purpose, we conducted a study to investigate the diversity of agronomic traits of alfalfa (*Medicago sativa*) under different soil environments in order to elucidate the salt tolerance of 51 alfalfa varieties.

Methods: We chose three different soil conditions for our study: normal conditions in Yantai as control, saline conditions in Yantai with soil salt concentrations ranging from 0.25 to 0.33%, and saline-alkali conditions in Dongying with soil salt concentrations ranging from 0.28 to 0.32%. We chose 51 alfalfa varieties from different sources as our experimental materials. Planting was conducted following a completely randomized block design with three replicates for each variety. Harvesting was performed when the plants reached 50% flowering, cutting them at a height of 5cm above the ground. Comprehensive assessments encompassed 10 parameters, namely fresh weight, dry weight, stem-leaf ratio, branch count, plant height, crude protein content, crude fat content, acid detergent fiber, neutral detergent fiber, and ash content.

Results: Our findings revealed that the 51 studied alfalfa varieties displayed significant generalized heritability and coefficient of variation, indicating a high level of genetic diversity. Using principal component and cluster analyses, we categorized the varieties into three distinct clusters based on their agronomic traits. Grey correlation degree analysis revealed the commendable performance of Ying st, PI 672734, and Mei zuo across diverse environments. Genotype emerged as a predominant determinant of all parameters, except crude fat, acid detergent fiber, and neutral detergent fiber. Through the application of membership function analysis, PI 672768 emerged as a variety that demonstrated strong tolerance in both saline soil contexts.

Discussion: Despite extensive previous studies indicating Zhong Mu No. 1 as a salt-tolerant alfalfa variety, its performance in this experiment did not distinguish itself. The findings of this study provide a fundamental basis for improving the management of saline-alkali lands and advancing alfalfa cultivation practices.

KEYWORDS

agronomic characters, varietal assessment, forage breeding, salt stress, genotype and environment interaction

1. Introduction

Soil salinity is an abiotic stress that significantly affects plant growth. In recent decades, climate change and concurrent water scarcity have contributed to a gradual increase in soil salinization. This phenomenon has spread to over 100 countries worldwide, causing a growing global concern. According to UNESCO and FAO, the extent of saline soil across the globe has expanded to cover an area totaling 932.2 million hectares (Rengasamy, 2006; Mbarki et al., 2020). The widespread occurrence of soil salinization has resulted in a decrease in available arable land, leading to a decline in both food production and pasture areas. Consequently, lower pasture yields have occurred, leading to increased competition for sustenance between humans and animals. This prevalent predicament of limited land use has prompted the cultivation and careful screening of crop varieties with enhanced salt tolerance. This is particularly significant in the context of shrinking land availability, necessitating the selection of plant varieties capable of thriving in saline-alkali environments to improve land utilization efficiency (Zhang et al., 2008; Lu et al., 2021; Zhang et al., 2023).

Given these challenges, there has been a notable increase in global research focused on the cultivation of alfalfa (*Medicago sativa*). Despite its moderate salt tolerance, alfalfa has shown notable effectiveness in improving saline-alkali soils. Alfalfa, widely distributed in China, has earned the reputation of being the “queen of forage” due to its high protein content, nutritional richness, and prolific yield. However, indigenous alfalfa varieties within China are limited, with a significant proportion being imported from foreign sources. These imported varieties exhibit notable variations in their salt tolerance capabilities. Therefore, it is imperative to investigate the diversity of agronomic traits in the context of saline-alkali soil environments and to identify alfalfa varieties suitable for cultivation under these conditions. These efforts hold the potential to improve the utilization of saline-alkali soils and provide a theoretical foundation for enhancing alfalfa varieties.

Researchers worldwide have made significant progress in unraveling the intricacies of alfalfa's salt tolerance. For instance, Yu et al. (2021) investigated 20 alfalfa varieties subjected to salt stress during the seedling stage and categorized them based on their response: a high-salt tolerance variety, salt-tolerant, medium salt-tolerant, and salt-sensitive. Similarly, Liang et al. (2017) studied the performance of seven traits across 10 alfalfa varieties under salt stress, pinpointing four varieties distinguished by commendable salt tolerance. Wang et al. (2023) examined germination potential and root length diversities among 29 alfalfa varieties during seed germination to discern five varieties endowed with robust salt tolerance. These studies highlight the recognition of salt tolerance variations among alfalfa varieties and hold substantial potential for refining breeding strategies (Al-Khatib et al., 1992). The results of previous studies provide great inspiration and reference for this experiment (Tavakoli et al., 2019; Benabderrahim et al., 2020; Yu et al., 2021).

Building upon the existing knowledge base, this study delves into the phenotypic and quality attributes of alfalfa cultivated under salt stress. It aspires to ascertain the agronomic trait diversity among 51 alfalfa species across three distinct environments. In this study, we investigated the diversity of agronomic traits in 51 alfalfa

species using a gray correlation method with the aim of identifying superior varieties, exploring the relationship between traits, genotypes, and the environment through gene–environment interactions, and finally screening for high-quality alfalfa varieties with pronounced salt tolerance using an affiliation function. The outcomes of this investigation hold the promise of furnishing a theoretical foundation for the selection of salt-tolerant alfalfa varieties, thus advancing the field of alfalfa breeding and variety improvement.

2. Materials and methods

2.1. Test site and test material

The test site for both the Salt Pond and the control group was situated in Zhifu District, Yantai City. The salt pond was irrigated with seawater, resulting in a salt content ranging from 0.25 to 0.33%. In Yantai, the soil's organic matter content accounted for 7.51% of dry weight, with total nitrogen at 1.187 mg/kg, ammonia nitrogen at 6.39 mg/kg, nitrate nitrogen at 4.89 mg/kg, and total phosphorus at 0.25 mg/kg. The saline-alkali land in Dongying is situated in Kenli District, Dongying City. In the Dongying soil, organic matter content accounted for 5.73% of dry weight, with total nitrogen at 1.04 mg/kg, ammonia nitrogen at 5.38 mg/kg, nitrate nitrogen at 5.87 mg/kg, total phosphorus at 0.29 mg/kg, and a salt content ranging from 0.28 to 0.32%. In this study, “control” refers to the natural environment in Yantai, “Yantai” designates the salt field environment in Yantai, and “Dongying” signifies the saline-alkali land environment in Dongying. [Supplementary Table S1](#) provides a summary of the rainfall, minimum temperature, and maximum temperature data during the experimental period.

2.2. Experimental design

In April 2020, we initiated the germination process by sowing three alfalfa seeds in greenhouse pots. After emergence, we carefully selected and retained a single robust alfalfa seedling to ensure consistent growth conditions. Subsequently, in mid-May 2020, we transplanted 51 distinct alfalfa varieties into three different locations: Yantai, the Yantai salt pond, and Dongying's saline-alkali land. The details regarding the varieties can be found in [Supplementary Table S2](#). We planted the alfalfa varieties with a spacing of 30 cm between individual plants. The experimental setup included three replicates for each variety, following a randomized complete block design. After 3 months, we scheduled the harvest when the alfalfa plants reached 50% flowering, maintaining a 5 cm elevation above ground level. The experimental setup did not involve fertilization or artificial irrigation practices. Measurement indices and methodologies are detailed in [Table 1](#).

2.3. Statistical analysis

Statistical analysis of the amassed data was conducted using Excel 2022. For correlation analysis and cluster analysis, SPSS 16 was

TABLE 1 Determination indexes and methods.

Traits	Method
Fresh weight (FW, g)	The cutting height of alfalfa was 5 cm. The fresh weight of alfalfa was measured by electronic balance after cutting.
Dry weight (DW, g)	The cut alfalfa was put into an envelope and baked at 105°C for 30 min, then baked at 75°C to constant weight and weighed to get dry weight.
Fresh/Dry ratio	Fresh weight/Dry weight
Stem/leaf ratio (S/L R)	Stem/leaf dry weight
Plant height (PH, cm)	The straightening length from the base of the plant to the tip of the main stem or inflorescence.
Branch number (BN)	The number of branches of alfalfa per plant was determined.
Crude protein (CP, %)	Kjeldahl method (Han et al., 2022)
Ether extract (EE, %)	Soxhlet extraction method (Han et al., 2022)
Acid detergent fiber (ADF, %)	Van Soest method (Han et al., 2022)
Neutral detergent fiber (NDF, %)	Van Soest method (Han et al., 2022)
Ash (%)	Complete combustion method (Han et al., 2022)

employed. Graphical representation was executed using Origin 9 software.

2.3.1. Genetic diversity analysis

Genetic diversity indices were organized into 10 distinct grades. The initial grade, denoted as $X_i > (X + 2\sigma)$, was juxtaposed with the tenth grade, $X_i > (X + 2\sigma)$. The computation of the genetic diversity index, commonly recognized as the Shannon-Weaver diversity index (H'), adhered to the formula $H' = -\sum P_i \ln P_i$, accommodating a 0.5σ variation between each grade.

Where: X represents the average value of each index, σ signifies the standard deviation of the index, and P_i represents the proportion of specimens within the total cohort displaying grade i characteristics.

2.3.2. Grey relation analysis

Building upon the foundations of grey correlation theory, the study treated the 10 alfalfa traits as a grey system. The construction of the ideal variety, denoted as X_0 , relied on the optimal values encompassing each individual trait. The ensuing correlation coefficient and correlation degree calculations adhered to established equations (Zeng et al., 2020).

$$\epsilon_i(k) = \frac{\min_i \min_k |X_0(k) - X_i(k)| + \rho \max_i \max_k |X_0(k) - X_i(k)|}{|X_0(k) - X_i(k)| + \rho \max_i \max_k |X_0(k) - X_i(k)|}$$

$$R_i = \frac{1}{n} \sum_{k=1}^n \epsilon_i(k)$$

$$w_k = \frac{R_i}{\sum R_j}$$

$$R'_i = \sum_{k=1}^n W_k \epsilon_i(k)$$

where: $|X_0(k) - X_i(k)|$ was the absolute difference between X_0 series and X_i series at k , $\min_k |X_0(k) - X_i(k)|$ was the second level minimum difference, $\max_k |X_0(k) - X_i(k)|$ was the second level maximum difference, ρ was the distinguishing coefficient, the value was considered to be 0.5, W_k was weight, R'_i was weighted relevance.

2.3.3. Heritability calculation

The generalized heritability is calculated according to the following formula (Tian et al., 2023).

$$R = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_{gy}^2 + \frac{\sigma_\epsilon^2}{n_1 n_r}}$$

where σ_g^2 is the genotype, σ_{gy}^2 is the interaction variance, $\frac{\sigma_\epsilon^2}{n_1 n_r}$

is the error, n_1 is the environment, and n_r is the number of replicates.

2.3.4. Salt tolerance index and membership function value

The assessment of salt tolerance was determined through the utilization of the Salt Tolerance Index, calculated as the ratio of the performance of the tested sample (T test) to that of the control sample (Roshdy et al., 2011). To further refine the analysis, the principles of membership function within the realm of fuzzy mathematics were incorporated. This entailed the standardization of the salt tolerance coefficient, enabling a more comprehensive and nuanced evaluation of the salt tolerance characteristics of the examined varieties (Jin et al., 2018).

$$U(X_j) = \frac{X_j - X_{\min}}{X_{\max} - X_{\min}}$$

Where X_j represents the salt tolerance index of j index of the i variety, and X_{\max} and X_{\min} , respectively, represent the maximum and minimum values of the index.

The weight of each comprehensive index:

$$W_j = \frac{P_j}{\sum P_j}$$

Comprehensive salt tolerance:

$$D = \sum_{j=1}^n [U(X_j) \times W_j]$$

where, W_j represents the importance or weight of the j -th comprehensive indicator among all indicators, P_j represents the contribution rate of the j -th comprehensive indicator for each material, and D represents the comprehensive evaluation value of

heat tolerance obtained by evaluating each material under high temperature stress using comprehensive indicators.

3. Results

3.1. Analysis of agronomic trait diversity of alfalfa in different environments

Under controlled conditions, the coefficient of variation for the 51 studied alfalfa varieties ranged from 3.78 to 70.47% (Table 2). The highest coefficient of variation was observed in dry weight (DW) at 70.47%, while the lowest was recorded in CP at 3.78%. Assessing the genetic diversity index, all 10 physiological indices showed values exceeding 1, indicating significant genetic diversity among the alfalfa varieties. Notably, the highest genetic diversity was observed in CP and ADF at 1.98.

Within the Yantai environment, the coefficient of variation across agronomic traits within the 51 alfalfa specimens ranged from 3.91 to 69.92% (Table 3). The maximal coefficient of variation was noted in DW at 69.92%, while the minimum coefficient was witnessed in CP at 3.91%. Genetic diversity exhibited alterations, with increments observed in FW, DW, PH, ADF and ash content. Conversely, reductions were noted in BN, S/L R, CP, and EE. Notably, the genetic diversity of NDF remained constant, with the highest genetic diversity observed in ash content at 2.02.

In Dongying, the coefficient of variation for the 51 agronomic traits of alfalfa ranged from 3.81 to 74.09% (Table 4). The lowest coefficient of variation was found in CP at 3.81%, while the highest was observed in DW at 74.09%. In comparison to the Yantai environment, the genetic diversity index in Dongying showed increased genetic diversity in FW, DW, EE, NDF, and ADF. However, genetic diversity trends diverged, with declines observed in BN, S/L R, CP, and ash content.

In contrast to the control conditions, exposure to the saline environment resulted in significant changes. Specifically, DW, FW, PH, and S/L R exhibited reductions in the salt-affected environment. Conversely, BN, CP, and ash content displayed increases. Conversely, limited fluctuations were observed in EE, ADF, and NDF. Furthermore, compared to the control, the mean values of DW, FW, PH, and S/L R declined by 24.68, 24.45, 19.15, and 15.18%, respectively, in Yantai. At the same time, BN, CP, and ash content exhibited increases of 10.81, 10.04, and 19.94%, respectively. Analogous trends were observed in the Dongying environment, where DW, FW, PH, and S/L R underwent reductions of 24.45, 24.68, 19.15, and 15.18% respectively, while BN,

CP, and ash content experienced increments of 13.51, 10.04, and 19.94%, respectively.

3.2. Correlation analysis of agronomic traits in diverse alfalfa environments

We conducted a comprehensive correlation analysis involving the diverse set of 51 alfalfa species under controlled conditions, revealing intricate relationships among the evaluated traits (Figure 1A). The results revealed significant correlations with FW showing positive associations with BN, S/L R, EE, and ADF. Meanwhile, DW showed positive correlations with FW, BN, S/L R, and ADF, while EE was positively correlated with ADF. Furthermore, CP displayed positive correlations with EE and was as significantly positively correlated with ash content. Notably, a negative correlation was observed between CP and NDF, while EE revealed a notable negative correlation with NDF and positive correlation with Ash content. The ADF content exhibited a positive correlation with NDF, while a significant negative correlation was observed between NDF and ash content.

In the Yantai environment, we presented a thorough correlation analysis among the 51 agronomic traits, providing insights into trait interactions (Figures 1B,C and Figures 2). Importantly, differences from the control environment become apparent, with DW showed a significant positive correlation with EE and ADF content. Conversely, in the saline soil environment of Dongying, BN displayed a positive correlation with ADF. Notably, the content of CP and NDF demonstrated significant negative correlations when contrasted with the control environment, diverging from other correlations which largely mirrored those of the control environment. Additionally, it is worth noting that PH did not show significant correlation with the other measured traits in this context.

3.3. Principal component analysis and cluster analysis

Utilizing advanced analytical techniques, we employed principal component analysis (PCA) and cluster analysis to gain deeper insights into the complex interrelationships among the agronomic traits of diverse alfalfa species across different environments.

In controlled conditions, we enhanced the cluster analysis by converting FW into the Fresh-to-Dry Ratio (F/D R). We then applied the Ward method to cluster the standardized results of the 51 agronomic traits of alfalfa. As a result, the alfalfa varieties coalesced into three distinct groups (Table 5). The primary group comprised 25

TABLE 2 Summary of mean, standard deviation, coefficient of variation, and genetic diversity for 10 agronomic traits of 51 alfalfa species in Yantai's control environment.

Item	FW (g)	DW (g)	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
Mean	139.92	40.93	95.02	31.02	0.74	19.33	2.35	31.13	40.84	9.33
SD	73.43	28.84	14.10	9.98	0.13	0.73	0.13	2.34	1.87	0.52
CV (%)	52.48	70.47	14.83	32.19	17.81	3.78	5.39	7.52	4.58	5.60
H'	1.71	1.40	1.93	1.89	1.93	1.98	1.90	1.98	1.95	1.96

FW, fresh weight; DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H', genetic diversity index.

TABLE 3 Summary of mean, standard deviation, coefficient of variation, and genetic diversity for 10 agronomic traits of 51 alfalfa species in Yantai's salt pond environment.

Item	FW (g)	DW (g)	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
Mean	105.71	30.83	76.82	26.31	0.82	21.27	2.35	31.14	40.77	11.19
SD	53.40	21.56	13.36	8.37	0.14	0.83	0.13	2.33	1.89	0.64
CV (%)	50.51	69.92	17.39	31.79	17.47	3.91	5.45	7.48	4.63	5.70%
H'	1.79	1.43	1.96	1.81	1.90	1.94	1.88	1.99	1.95	2.02

FW, fresh weight; DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H', genetic diversity index.

TABLE 4 Summary of mean, standard deviation, coefficient of variation, and genetic diversity for 10 agronomic traits of 51 alfalfa species in Dongying's saline-alkali land.

Item	FW (g)	DW (g)	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
Mean	106.56	31.53	78.09	26.32	0.84	21.27	2.35	31.14	40.77	11.19
SD	57.98	23.36	12.57	8.56	0.16	0.81	0.13	2.37	1.91	0.63
CV (%)	54.41	74.09	16.10	32.51	19.33	3.81	5.38	7.62	4.68	5.61
H'	1.80	1.45	1.96	1.90	1.80	1.86	1.95	2.00	1.99	1.98

FW, fresh weight; DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H', genetic diversity index.

varieties, characterized by elevated F/D R, PH, Ash content, and NDF. The secondary group consisted of 10 varieties, distinguished by the highest average PH and Ash content. Meanwhile, the tertiary group, encompassing 16 varieties, showcased heightened average values for DW, BN, S/L R, EE, and ADF. The insights obtained from principal component analysis supported these findings. Principal component 1 explained 25.6% of the total trait variation, and principal component 2 accounted for 24.7% of the overall variation in the controlled environment.

In the Yantai environment, similar procedures led to the clustering of the 51 alfalfa species into three distinct groups (Table 6). The primary cluster encompassed 19 varieties distinguished by elevated PH, ADF, and NDF. The secondary cluster, containing 20 varieties, exhibited the highest Fresh-to-Dry Ratio (F/D R) and average CP. Conversely, the tertiary cluster, composed of 12 varieties, exhibited superior average values for DW, BN, S/L R, EE, and Ash content. Principal component analysis reaffirmed these findings, with principal component 1 and 2 explained 25.3 and 24.1% of the total trait variation, respectively.

In the Dongying environment, principal component analysis also revealed intricate patterns. Principal component 1 and 2 collectively expounded 25.1 and 23.9% of the total trait variation, respectively. Cluster analysis led to the categorization of the 51 alfalfa species into three discernible groups (Table 7). The primary group, constituted of 17 varieties, exhibited the highest average values for CP, ADF, NDF, and Ash content. Meanwhile, the secondary group, featuring 21 varieties, was distinguished by superior average F/D R and PH. The tertiary group, housing 13 varieties, displayed heightened average values for DW, BN, S/L R, and EE.

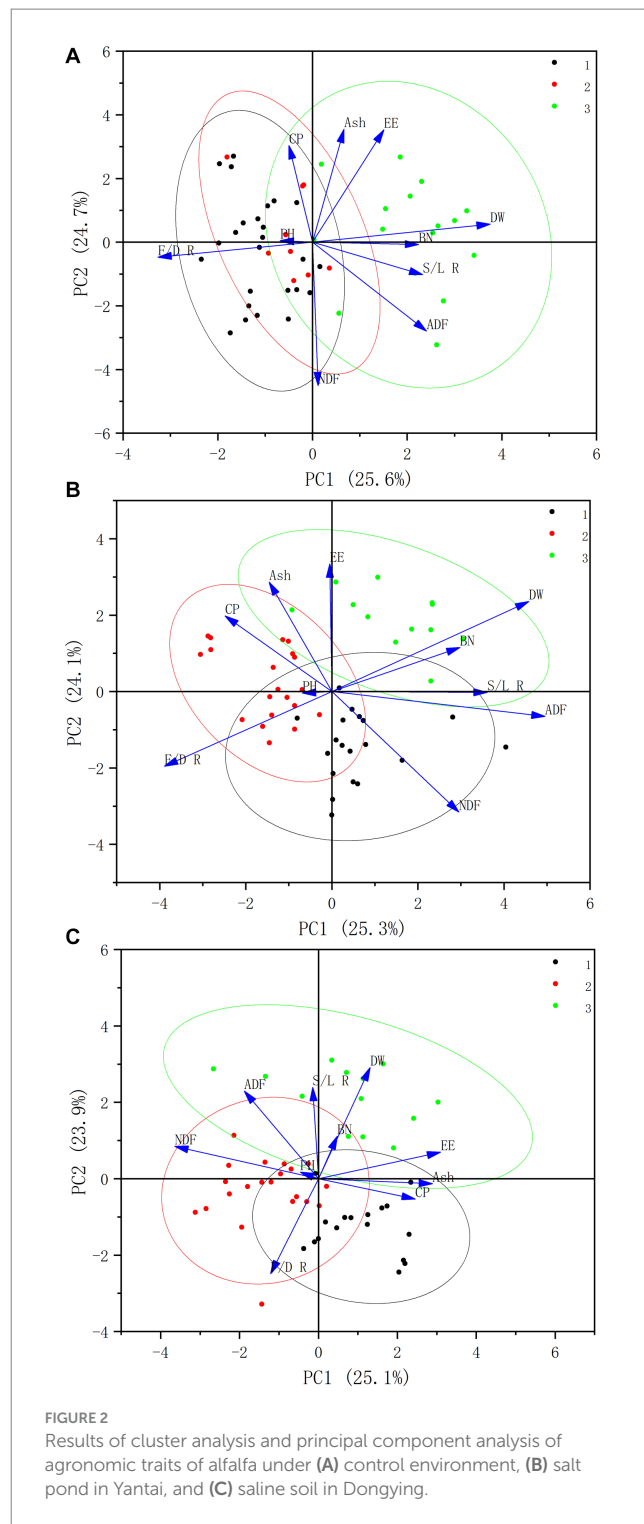
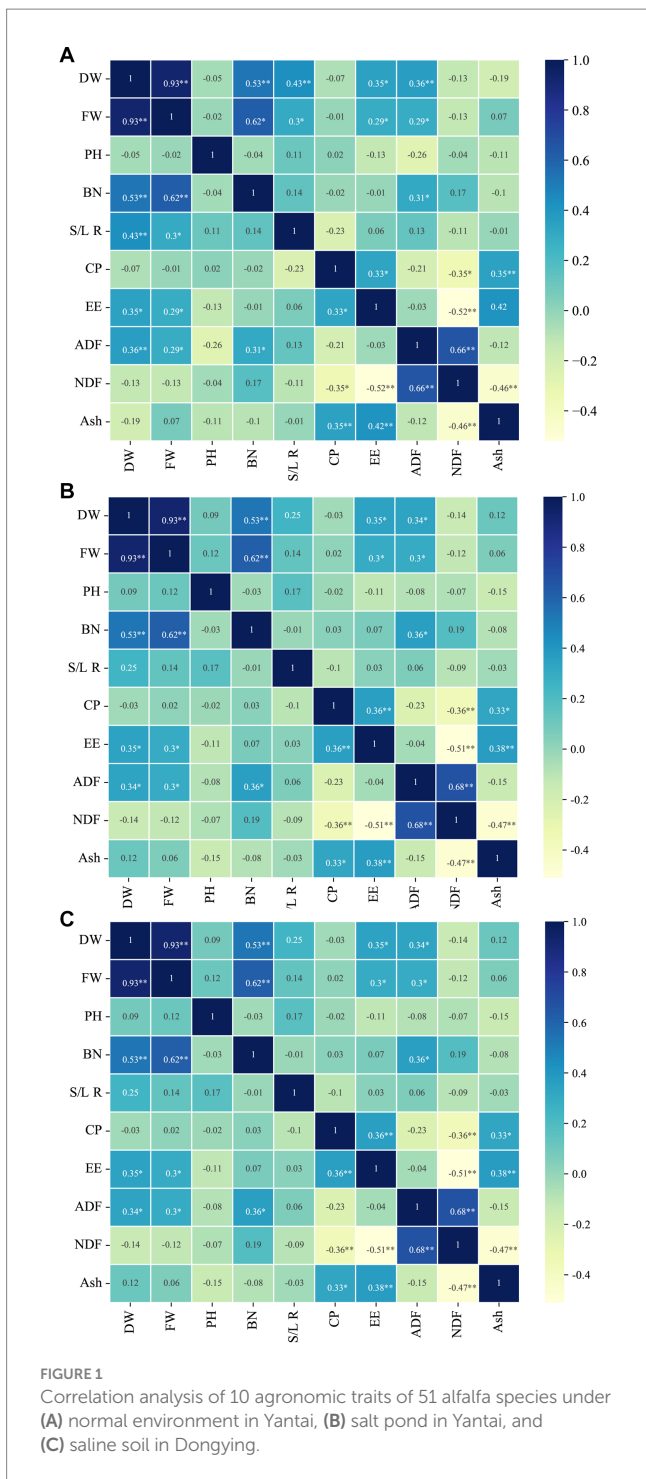
The integration of principal component analysis and cluster analysis elucidated the intricate trait associations and groupings across distinct environments, thereby affording valuable insights into the varietal behavior of alfalfa under differing conditions.

3.4. Screening of excellent forage performance varieties

The process of identifying superior alfalfa varieties, enriched by rigorous analytical methods, aimed to reveal strains that exhibited exceptional performance across various traits and environments. To create a representative ideal variety designated as X_0 , each attribute was optimized to maximize values, except for ADF, NDF, and Ash, where the goal was to minimize values. This approach facilitated the normalization of data through the initial value method, enabling the computation of both $\min_k |X_0(k) - X_i(k)|$ and $\max_k |X_0(k) - X_i(k)|$.

Subsequently, the weighted correlation degree was employed as a discerning criterion for the stratification of the alfalfa varieties (Table 8). The outcomes of this process revealed the top five ranked varieties across the entire spectrum. In a broader context, the Ying st, PI 672734, Mei zuo, Gan nong NO.6 and Nan mu 501 emerged as prominently ranked, indicating their superior quality across diverse traits. This trend persisted within the Yantai environment, where Ying st, PI 672734, Mei zuo, Gan nong No.6 and Xiang yang No.6 garnered the highest acclaim. Likewise, within the Dongying environment, the elite rankings included PI 672734, Ying st, Nan mu 501, Gan Nong 6, and Mei zuo. It is worth noting the consistent excellence exhibited by Ying st, PI 672734, Gan nong No.6, and Mei zuo, confirming their exceptional qualities across all three distinct environments, firmly establishing them as high-caliber alfalfa varieties.

The meticulous process of strain selection, supported by comprehensive analytical techniques, serves to underscore the significance of Yingst, PI 672734, Gan nong No.6 and Mei zuo as robust candidates for continued agricultural propagation and advancement. This discerning approach aligns with the pursuit of enhancing alfalfa cultivation while attaining optimal yields and performance across varying conditions.



3.5. Genotype-by-environment (G × E) analysis and identification of salt-tolerant varieties

A thorough investigation of G × E interactions, supported by meticulous data analysis (Tables 9, 10), formed the basis for assessing 10 distinct agronomic traits in alfalfa. The findings underscored the undeniable influence of genotype on the majority of traits ($p < 0.01$). Nevertheless, traits such as F/D R, EE, ADF, and NDF remained unaffected by the environment, thus indicating the

inherent genetic stability of these traits in the face of environmental variability. Conversely, crucial traits including DW, PH, BN, S/L R, CP and ash content were significantly impacted by environmental conditions. This substantiated that the genetic integrity of F/D R, EE, ADF, and NDF endured regardless of external environmental factors. Interestingly, an interaction between genetic makeup and environmental factors was observed, especially concerning DW and ash content. Notably, the relative significance ranking of the six traits significantly influenced by the genotypic environment emerged as

TABLE 5 Average values of 10 agronomic traits of 51 alfalfa species across three groups in Yantai’s normal environment.

Group	DW (g)	Fresh/Dry ratio	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
1	24.04b	4.38a	99.58a	29.04b	0.71b	19.37a	2.29b	29.86b	41.05a	9.14b
2	25.33b	4.16a	83.67b	27.20b	0.66b	19.45a	2.38a	31.94a	40.69a	9.60a
3	77.06a	2.95b	95.00a	36.50a	0.84a	19.20a	2.43a	32.63a	40.59a	9.46ab

DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H', genetic diversity index. Different lower-case letters indicate a significant difference among treatments.

TABLE 6 Average values of 10 agronomic traits of 51 alfalfa species across three groups in Yantai’s salt pond environment.

Group	DW (g)	Fresh/Dry ratio	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
1	25.43b	4.08a	80.63a	29.65a	0.80b	20.89b	2.26c	32.28a	42.54a	10.74b
2	16.16c	4.43a	71.98a	20.82b	0.77b	21.54a	2.37b	29.45b	39.69b	11.42a
3	63.83a	2.86b	78.88a	30.19a	0.92a	21.41ab	2.47a	32.13a	39.78b	11.52a

DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H', genetic diversity index. Different lower-case letters indicate a significant difference among treatments.

TABLE 7 Average values of 10 agronomic traits of 51 alfalfa species across three groups in Dongying’s saline-alkali land.

Group	DW (g)	Fresh/Dry ratio	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
1	17.18b	4.17a	74.61a	22.53b	0.81b	21.60a	2.39a	28.85b	39.14c	11.47a
2	20.74b	4.50a	81.24a	26.02b	0.81b	21.11a	2.27b	32.02a	42.26a	10.87b
3	67.72a	2.63b	77.54a	31.77a	0.94a	21.09a	2.43a	32.71a	40.50b	11.35a

DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H', genetic diversity index. Different lower-case letters indicate a significant difference among treatments.

follows: Ash > CP > DW > S/L R > PH > BN in Yantai; Ash > DM > CP > S/L R > PH > BN in Dongying.

To holistically evaluate the salt tolerance of alfalfa, the six agronomic traits concurrently influenced by both genotype and environment were harnessed as evaluative indices: DW, PH, BN, S/L R, CP, and Ash. Transmuting these attributes into a salt tolerance index, a membership function was invoked for comparison. The resulting rankings were presented in Table 11, revealing the relative standing of each trait. The hierarchy of traits significantly influenced by the genotypic environment mirrored the following sequence: Ash > DW > CP > S/L R > PH > BN.

Within the Yantai environment, excellent salt-tolerant varieties were Gu yuan zi hua, Ao han, Huang guan, PI 672768, and WL366. Similarly, the ranking of salt-tolerant varieties in the Dongying environment were lead PI 672734, Qian jing, Xiang yang No. 6, PI 672768, and PI 672763. This discerning analysis substantiates the multifaceted nature of salt tolerance within the gamut of alfalfa species, offering invaluable insights into the adaptation and performance of these varieties across contrasting environments.

4. Discussion

4.1. Genetic diversity and environmental adaptation

The phenomenon of long-term natural selection has endowed plants with a relatively stable phenotype and a rich reservoir of genetic

diversity (Wu et al., 2018). As evidenced by the substantial divergence in growth parameters, different varieties of the same species exhibit distinctive responses to varied environments (An et al., 2021). This divergence becomes notably pronounced under the stress of salt-induced conditions, where the growth of salt-sensitive varieties is impeded, ultimately posing a threat of plant mortality (Li et al., 2010). Central to assessing alfalfa quality are agronomic traits encompassing DW, PH, and nutrient composition (Akdeniz et al., 2019). The statistical analysis of 51 alfalfa traits across three distinct environments underscores the elevated genetic diversity and coefficient of variation inherent to alfalfa’s adaptive repertoire across these varied settings.

4.2. Salt stress and genetic diversity

Salt stress stands as a formidable abiotic factor that profoundly influences alfalfa’s growth and development. Prior studies, predominantly conducted under simulated salt stress conditions, offer a myriad of insights into alfalfa’s salt tolerance (Annicchiarico, 1992; Tucak et al., 2008). However, a divergence often exists between simulated stress and actual saline-alkali conditions (Qados, 2011; Mann et al., 2019). Correspondingly, Al-Khatib et al. (1992) observed a heightened genetic diversity in alfalfa under salt stress. In congruence with these findings, our experiment illuminates that the genetic diversity of more than half the traits underwent augmentation in response to salt stress. Generally, the imposition of salt stress leads to escalated osmotic pressure in the soil, impairing water absorption and exerting a detrimental impact on alfalfa growth. Consequently, a

TABLE 8 Ranking of 51 alfalfa species in three environments based on grey correlation degree analysis results.

Variety	Rank			Variety	Rank		
	Control	Yantai	Dongying		Control	Yantai	Dongying
A er gang jin	41	40	39	Qian jing	37	34	31
Biao ba	34	41	32	Qing chun	44	44	34
Ao han	35	29	26	Sa lan na si	23	23	18
Bo la tu	29	33	23	San de li	50	48	46
Bei ni dong	22	24	24	Su lian No.36	42	42	35
Gan nong No.1	24	25	27	Lei ting	32	35	33
Gan nong No.3	15	13	7	Long mu 801	49	49	49
Gan nong No.6	4	3	4	Tiao zhan zhe	38	32	41
Fa duo	43	43	45	PI 672763	27	28	28
Mu xu wang	28	19	30	PI 672767	45	46	48
WL366	26	20	25	PI 672768	12	14	8
WL440HO	11	10	11	Zhong mu No.1	18	18	16
WL-SQT	21	21	14	PI 672734	2	2	1
Gong nong No.1	30	39	38	Kang bao	13	17	19
Gu yuan zi hua	40	27	47	PI 672764	36	38	37
De guo da ye	20	26	17	PI 631976	16	22	29
Huang guan	7	5	12	PI 631975	31	36	36
Jin huang hou	14	7	13	Xi mo	9	12	20
Jin neng	17	16	15	Xiang yang No.6	6	6	6
Kai en	25	30	21	Yan shi	19	11	9
Nan mu 501	5	8	3	Ying st	1	1	2
PI 672741	48	50	44	PI 672759	8	9	10
PI 672755	33	31	42	Zhong mu No.3	10	15	22
Liang mu No.2	51	51	50	Tu ku man	46	45	51
Mei zuo	3	4	5	Wei shen	39	37	40
Long mu No.1	47	47	43				

TABLE 9 Two-factor analysis of variance of 51 alfalfa species in Yantai's normal environment and salt pond environment based on 10 agronomic traits.

Character	F (G)	F (E)	F (G × E)	R
DW(g)	82.69**	170.30**	2.24**	0.96
Fresh/Dry ratio	6.68**	0.16	0.23	
PH(cm)	7.5**	175.20**	0.32	0.87
S/L R	14.00**	48.14**	0.47	0.93
BN	6.49**	24.54**	0.02	0.85
CP (%)	28.60**	2258.21**	0.27	0.97
EE (%)	66.46**	0.60	0.15	
ADF (%)	598.22**	0.001	0.93	
NDF (%)	198.57**	3.18	0.71	
Ash (%)	239.12**	31470.23**	2.85**	0.98

DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber.
**Indicates very significant correlation.

TABLE 10 Two-factor analysis of variance of 51 alfalfa species in Yantai's normal environment and Dongying's saline soil environment based on 10 agronomic traits.

Character	F (G)	F (E)	F (G × E)	R
DW (g)	93.45**	155.60**	1.62*	0.98
Fresh/Dry ratio	5.99**	0.05	0.27	
PH (cm)	7.08**	153.33**	0.40	0.86
S/L R	13.34**	44.79**	0.41	0.93
BN	5.55**	33.15**	0.45	0.82
CP (%)	29.17**	2363.62**	0.24	0.97
EE (%)	58.70**	0.52	0.12	
ADF (%)	582.11**	0.03	0.91	
NDF (%)	207.08**	3.18	0.72	
Ash (%)	316.73**	42387.25**	3.32**	0.99

DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber. NDF: neutral detergent fiber.
*Indicates correlation.
**Indicates very significant correlation.

TABLE 11 Ranking of 51 alfalfa species in two salt environments based on two-factor analysis of variance results and membership function theory.

Variety	Rank		Variety	Rank	
	Yantai	Dongying		Yantai	Dongying
A er gang jin	21	42	Qian jing	37	2
Biao ba	26	46	Qing chun	8	27
Ao han	2	41	Sa lan na si	27	17
Bo la tu	41	44	San de li	31	7
Bei ni dong	33	48	Su lian No.36	19	39
Gan nong No.1	24	16	Lei ting	25	9
Gan nong No.3	6	22	Long mu 801	7	26
Gan nong No.6	10	32	Tiao zhan zhe	38	45
Fa duo	18	24	PI 672763	40	5
Mu xu wang	17	36	PI 672767	34	31
WL366	5	40	PI 672768	4	4
WL440HO	15	23	Zhong mu No.1	36	18
WL-SQT	46	20	PI 672734	43	1
Gong nong No.1	29	47	Kang bao	11	30
Gu yuan zi hua	1	33	PI 672764	20	15
De guo da ye	49	10	PI 631976	51	51
Huang guan	3	49	PI 631975	47	6
Jin huang hou	48	34	Xi mo	35	11
Jin neng	22	25	Xiang yang No.6	13	3
Kai en	50	50	Yan shi	12	13
Nan mu 501	14	14	Ying st	39	12
PI 672741	16	38	PI 672759	30	19
PI 672755	45	43	Zhong mu No.3	42	29
Liang mu No.2	28	8	Tu ku man	32	21
Mei zuo	9	37	Wei shen	23	35
Long mu No.1	44	28			

decrease in FW, DW, and PH is an anticipated outcome. These outcomes are in line with previous research that posits salt stress amplifies the S/L R of alfalfa, thereby enhancing overall quality a trend corroborated by our experiment’s results.

4.3. Nutrient content and salt stress

Reinforcing the intricate relationship between nutrient content and salt stress, Akdeniz et al. (2019) establish a direct correlation between high CP levels and elevated Ash content. Additionally, Ferreira et al. (2015) and Wan et al. (2023) unveil salt stress’s role in augmenting CP content while diminishing ADF and NDF concentrations. Nonetheless, discrepancies persist due to variable factors including alfalfa variety and experimental locale.

4.4. High quality alfalfa varieties

Grey system theory can standardize all kinds of indicators and assign weight coefficients to them, which can not only

effectively overcome the limitations caused by single indicators, but also objectively and accurately conduct comprehensive evaluation of alfalfa varieties (lines), and has been widely used in comprehensive evaluation of forage. Wang et al. (2021) According to the study of 8 kinds of alfalfa quality traits by grey correlation degree, WL358, WL440HQ, WL656HQ, and WL366HQ are excellent alfalfa varieties. According to the study of 8 kinds of alfalfa quality traits by grey correlation degree, WL358, WL440HQ, WL656HQ, and WL366HQ are excellent alfalfa varieties. Lu et al. (2021) also evaluated 12 kinds of alfalfa by grey correlation degree and found that WL656HQ, WL525, and WL440HQ were relatively excellent varieties, indicating that grey correlation degree analysis is a commonly used and reliable evaluation method for alfalfa evaluation. Li et al. (2021) found that Ying st performed better in the study on the introduction of alfalfa in Yunnan province, and Ying st performed better in the three environments in this experiment, while Quan et al. (2023) found that Ying st was not prominent in the variety comparison test in Gansu province. This is different from the results of this study, and the reason for this result is the different environment of the planting site.

4.5. G × E interaction and salt tolerance evaluation

A pivotal facet in plant agronomy lies in comprehending the intricate interplay between genotype and environment. Such interactions significantly impact agronomic traits across varied plant species, including wheat, oats, and alfalfa (Purchase, 1997; Peterson et al., 2005; Qiu et al., 2023; Tian et al., 2023). Distinct acidic soils further underscore genotype's pivotal role in shaping alfalfa traits, as elucidated by Achir et al. (2020), who deem environmental influence on alfalfa yield as paramount. In our study, genotype emerged as the principal driver of diverse agronomic traits in control and saline-alkali soil environments. Conversely, the environment significantly affected multiple traits, excluding F/D R, EE, Acid detergent Fiber, and Neutral detergent Fiber. Interestingly, the traits influenced by genotype and environment generally exhibited higher average generalized heritability, reflecting predominant genetic influence. However, as a comprehensive quantitative trait, salt tolerance is a complex trait influenced by many genetic and non-genetic factors (Ashraf and Foolad, 2013), and it is difficult for a single trait to reflect the salt tolerance of plants, comprehensive salt tolerance evaluation demands the amalgamation of morphological, physiological, and biochemical indices (El-Hendawy et al., 2005; Badran et al., 2015; Guo et al., 2022). In the past, Al-Khatib et al. (1994) believed that branch length could be used as an evaluation index of salt-tolerance of alfalfa, while Tavakoli et al. (2019) and Benabderrahim et al. (2020) believed that reliable results could be obtained only when the results of multiple indexes were comprehensively considered. In this experiment, G × E analysis found that only part of the traits studied were affected by environmental factors. Therefore, we used the membership function method to comprehensively evaluate these traits and finally screened out Gu yuan zi hua, Ao han, Huang guan, PI 672768, WL366, PI 672734, Qian jing, Xiang yang No. 6, and PI 672763 excellent salt-tolerant varieties.

In essence, this study contributes to our understanding of alfalfa's genetic diversity, environmental responsiveness, and salt tolerance. The interplay between genotype and environment profoundly shapes the alfalfa's performance, offering insights into suitable varieties for saline-alkali land improvement. By effectively marrying analytical techniques, this research aligns with the overarching goal of optimizing alfalfa cultivation and bolstering agricultural productivity. The screened high-quality purple alfalfa varieties can provide materials for the breeding of purple alfalfa varieties, and at the same time, high-quality salt-tolerant varieties have value in future saline-alkali land improvement.

5. Conclusion

In this study, an exhaustive exploration of agronomic traits across 51 alfalfa varieties under three distinct environments was conducted, yielding valuable insights into the plant's genetic diversity and adaptive capabilities. The observed richness in genetic diversity across the diverse array of agronomic traits underscores the inherent flexibility of alfalfa to respond to varying conditions. Among the plethora of varieties assessed, four standouts emerged: Yingst, PI 672734, Gan nong No.6 and Mei zuo, all manifesting exceptional traits across the different environments studied.

Furthermore, the assessment of salt tolerance in alfalfa under diverse saline and alkaline conditions revealed intriguing nuances. Contrary to conventional assumptions, the highest-performing alfalfa varieties did not universally exhibit superior salt tolerance. An

exemplary case is PI 672768, which demonstrated remarkable salt tolerance despite not being classified among the highest-performing varieties under normal conditions. This finding underscores the complexity of salt tolerance mechanisms within alfalfa varieties.

The outcomes of this study hold significant implications for the selection and breeding of alfalfa varieties optimized for diverse environments. The elucidation of genetic diversity, coupled with a nuanced understanding of salt tolerance, lays the foundation for more effective alfalfa variety selection and improvement strategies. Ultimately, these findings contribute to the advancement of sustainable agriculture practices by harnessing the genetic potential of alfalfa to thrive in challenging and diverse conditions.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

SF: Investigation, Resources, Writing – review & editing. JC: Data curation, Writing – original draft. JM: Resources, Writing – original draft. MZ: Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2023.1278913/full#supplementary-material>

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