

Article

Comprehensive Evaluation of Waterlogging Tolerance in 51 Safflower (*Carthamus tinctorius* L.) Germplasms at the Seedling Stage

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Abstract

Waterlogging stress severely restricts the growth and stable yield of *Carthamus tinctorius* L., an important medicinal and oilseed crop. At present, systematic phenotypic evaluation of safflower seedling waterlogging tolerance across diverse germplasm resources remains insufficient. In this study, 15 seedling agronomic indicators of 51 safflower accessions under waterlogging treatment and normal control conditions were measured. Descriptive statistics, correlation analysis, principal component analysis, fuzzy membership function transformation and hierarchical cluster analysis were combined to conduct phenotypic evaluation of waterlogging response. Obvious phenotypic differentiation was detected among tested germplasms; the coefficient of variation in most traits under waterlogging ranged from 18.84% to 63.48%, far higher than the 2.44–4.83% observed under normal growth conditions. Four principal components with eigenvalues greater than 1 were retained, collectively explaining 79.64% of total phenotypic variation. Comprehensive waterlogging tolerance D-values were calculated by weighting principal component contribution rates, and the 51 germplasms were statistically divided into five tolerance grades based on D-value intervals, with six materials exhibiting superior seedling waterlogging performance screened out. Correlation analysis showed shoot fresh weight (SFW), stem diameter (SD), root diameter (RD) and leaf area (LA) had strong positive correlations with D-value. Nine agronomic traits with close correlation to overall waterlogging performance were identified as candidate indicators for preliminary phenotypic screening. It should be noted that the D-value only provides statistical ranking based on measured seedling phenotypes and cannot directly interpret the physiological regulatory mechanisms of waterlogging tolerance. This study adopted a well-established multi-index statistical pipeline to characterize phenotypic responses of safflower seedlings under 7-day waterlogging stress at the six-leaf stage. Notably, all phenotypic data were derived from a single greenhouse trial under controlled waterlogging conditions; multi-environment field validation is required to confirm the broader applicability of the identified indicators and germplasms.

Keywords: *Carthamus tinctorius* L.; seedling stage; agronomic traits; waterlogging tolerance; membership function



Academic Editor: Junhua Peng

Received: 15 June 2026

Revised: 22 July 2026

Accepted: 23 July 2026

Published: 24 July 2026

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1. Introduction

Safflower (*Carthamus tinctorius* L.) is an important dual-purpose oil and medicinal crop widely planted across temperate and semi-arid regions, with its phytochemical constituents and pharmacological activities comprehensively summarized in previous reviews [1,2]. Though it shows moderate tolerance to drought and saline soil, safflower seedlings are extremely sensitive to waterlogging stress. Short-term flooding creates rhizosphere hypoxia, inhibits root respiration and photosynthate transport, restricts seedling biomass accumulation, and even causes widespread seedling death under severe waterlogging conditions. With the frequent occurrence of seasonal heavy rainfall in recent years, waterlogging has become a major abiotic barrier limiting stable safflower yield and large-scale germplasm promotion.

Flooding-induced hypoxic stress severely disrupts plant morphological and physiological metabolism, and the adaptive mechanisms of diverse crops under waterlogging have been systematically reviewed [3]. To cope with frequent waterlogging disasters under climate change, developing waterlogging-tolerant crop varieties via germplasm screening has become a core breeding target [4]. Traditional waterlogging tolerance evaluation mostly relies on a single growth indicator such as plant height or root weight, which cannot fully reflect the comprehensive stress performance of different germplasms. This limitation is particularly relevant for safflower breeding, as waterlogging disrupts seedling establishment and causes severe phenotypic distortion, which hinders reliable germplasm screening and thus impedes the development of waterlogging-tolerant varieties [5]. Waterlogging tolerance is a complex quantitative trait jointly regulated by multiple morphological attributes; evaluation relying on only one trait often leads to biased screening results. Multivariate statistical methods such as principal component analysis (PCA) combined with fuzzy membership functions integrate multiple phenotypic traits, remove redundant trait information, and generate a unified comprehensive tolerance index. This integrated analytical framework has become a standard tool for germplasm stress tolerance screening in oil crops, yet systematic multi-trait assessment focusing on safflower seedling waterlogging response remains insufficient. Similar multi-trait phenotypic identification strategies at the seedling stage have been successfully adopted in chili, hot pepper, kiwifruit, wild chrysanthemum and wheat to screen stress-tolerant germplasms [6–10].

In this study, 15 seedling phenotypic traits of 51 safflower accessions under controlled waterlogging treatment were measured and analyzed. The objective of this study was to quantify the waterlogging tolerance of safflower accessions using multi-trait phenotypic data and establish a preliminary screening standard for tolerant germplasms. To achieve this, we adopted PCA combined with the fuzzy membership function to integrate dozens of phenotypic indicators, eliminate overlapping information among traits, and convert multi-dimensional stress responses into a unified comprehensive score. The phenotypic data and preliminary classification standard obtained from greenhouse seedling trials can only serve as a reference for safflower waterlogging-tolerant germplasm pre-screening. Multi-year field verification under diverse ecological environments is still required before wide application in breeding programs.

2. Materials and Methods

2.1. Plant Materials

A total of 51 safflower (*Carthamus tinctorius* L.) cultivars (designated Ct01–Ct51) were used in this study. All germplasms were provided by the Germplasm Resource Bank of the Institute of Chinese Herbal Medicines, Henan Academy of Agricultural Sciences, Zhengzhou, China. These accessions represent modern improved varieties widely cultivated in major safflower-producing regions across China (e.g., Gansu, Inner Mongolia,

Xinjiang) between 2020 and 2022. Seeds were air-dried to approximately 8% moisture content, placed in sealed paper bags, and stored at 4 °C in the dark until use.

2.2. Plant Cultivation and Waterlogging Stress Treatment

The experiment was conducted in a naturally lit greenhouse at the experimental farm of the Institute of Chinese Herbal Medicines, Henan Academy of Agricultural Sciences (34.78° N, 113.65° E) from March to May 2025. The average daytime temperature was 25 ± 3 °C, and the average nighttime temperature was 18 ± 2 °C, with a relative humidity of 60–70%.

To minimize phenotypic variation arising from genotypic differences in growth rate, a uniform cultivation protocol was strictly followed. Safflower seeds were initially sown in germination boxes filled with sterilized vermiculite. After 10 days, robust seedlings at the six-leaf stage with consistent growth (uniform height and leaf number) were selected for transplanting.

Seedlings were transplanted into plastic pots (20 cm top diameter, 15 cm bottom diameter, 17 cm height) filled with a homogenized substrate mixture consisting of field soil, vermiculite, and organic compost (3:1:1, *v/v/v*). The physical and chemical properties of the substrate were as follows: pH 7.2, organic matter content 18.6 g/kg, available nitrogen 92.3 mg/kg, available phosphorus 24.7 mg/kg, and available potassium 156.2 mg/kg.

Experimental Design and Replication: To clarify the experimental unit, each pot served as one biological replicate containing three uniformly growing seedlings. For each accession, four biological replicates (i.e., four independent pots) were arranged randomly.

After 15 days of acclimatization period (during which plants were watered normally to maintain soil moisture at 60–70% of field capacity), waterlogging stress was imposed using the double-pot method. Pots assigned to the waterlogging treatment were placed inside larger water-holding containers, and tap water was added to maintain a water level 3 cm above the soil surface, which was kept constant by daily replenishment. Plants in the control group were watered normally. The duration of the waterlogging treatment was 7 days. Throughout the experiment, pots were rearranged randomly every two days to minimize positional effects.

2.3. Measurement of Morphological and Physiological Traits

To minimize phenotypic variation arising from inherent genotypic differences in growth rates, uniform seedlings at the six-leaf stage were specifically selected for measurement seven days after the onset of waterlogging treatment. From each biological replicate, three seedlings exhibiting consistent growth (uniform height and leaf number) were sampled. The following 15 traits were then determined:

Number of leaves (NL), number of green leaves (NGL), plant height (PH), stem diameter (SD), shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), total root length (TRL), root diameter (RD), chlorophyll content (Chl), leaf length (LL), leaf width (LW), leaf area (LA), and leaf perimeter (LP).

NL and NGL were counted manually; PH and TRL were measured using a ruler; SD and RD were measured using a digital caliper (Mitutoyo, Kawasaki, Japan); SDWs and RFWs were determined using an electronic balance (Sartorius, Göttingen, Germany) with an accuracy of 0.001 g; dry weights were obtained after drying samples in a forced-air oven at 80 °C for 8 h to constant weight; LL, LW, LA, and LP were measured using a portable leaf area meter (LI-3100C, LI-COR, Lincoln, NE, USA); and Chl was measured using a SPAD-502 chlorophyll meter (Konica Minolta, Osaka, Japan).

2.4. Data Standardization and Membership Function Analysis

To quantify genotypic responses to waterlogging, two coefficients (Stress Coefficient, CS; Waterlogging Coefficient, WC) were calculated, followed by membership function transformation and PCA-based comprehensive evaluation.

Stress Coefficient (CS): The Stress Coefficient (CS) reflects the absolute change rate of a trait under waterlogging (vs. the control group):

$$CS(\%) = \frac{X_c - X_w}{X_c} \times 100\%$$

where X_c is the mean value of a specific trait under the control condition, and X_w is the mean value under waterlogging stress. A larger CS indicates a greater reduction in the trait (more severe stress damage). For traits where values increase under stress (e.g., SDW, RDW), the CS yields negative values, reflecting biomass accumulation or reallocation rather than reduction.

Waterlogging Coefficient (WC): The Waterlogging Coefficient (WC) standardizes trait values across all 51 accessions to enable cross-genotype comparison. For most growth/biomass traits (e.g., plant height, leaf area), higher WC = better stress tolerance (positive indicator). For “negative indicators” (e.g., root/shoot dry weight ratio, where lower values = better tolerance), the formula is reversed:

$$WC = \frac{X_w}{X_c} \times 100\%$$

This ensures higher WC values consistently indicate stronger tolerance.

Coefficient of Variation (CV): The coefficient of variation (CV) was calculated to assess the degree of phenotypic dispersion among the 51 accessions for each trait. It was defined as

$$CV(\%) = \frac{SD}{\bar{X}} \times 100\%$$

where SD represents the standard deviation of a specific trait across all tested germplasms, and $\bar{X}$ is the mean value of that trait. A higher CV value indicates greater genetic diversity and wider phenotypic variation within the germplasm collection, suggesting a higher potential for selecting waterlogging-tolerant genotypes. This metric was crucial for identifying traits that exhibit substantial variability under stress, which served as the basis for screening effective evaluation indicators.

Membership Function Transformation: To integrate multi-trait data, WC values were normalized to [0, 1] via a fuzzy membership function (capturing “partial tolerance”):

$$U(X_{ij}) = \frac{X_i - X_{i(min)}}{X_{i(max)} - X_{i(min)}}, i = 1, 2, 3, \dots, n$$

where $U(X_{ij})$ is the membership value of accession i for trait j , and $X_{i(min)}$ and $X_{i(max)}$ are the minimum and maximum WC values for trait j (across all accessions). For negative indicators, the membership function was adjusted to ensure consistency (higher $U(X_{ij})$ = better tolerance).

2.5. Principal Component Analysis (PCA) and Comprehensive Evaluation

PCA was performed on membership values to reduce dimensionality and derive a comprehensive waterlogging tolerance index (D-value). Principal components (PCs) with eigenvalues > 1 (Kaiser criterion) were retained.

Weight Assignment: The weight of each PC (W_k) was calculated as

$$W_k = \frac{\text{Variance Contribution of PC}_k}{\sum \text{Variance Contributions of All Retained PC}_s}$$

This weights PCs by their ability to explain phenotypic variation.

The comprehensive D-value integrates membership values (from membership function transformation) and PC weights:

$$D = \sum_{k=1}^n [U(X_k) \times W_k]$$

where n is the number of retained PCs. A higher D-value indicates stronger overall waterlogging tolerance.

2.6. Cluster Analysis and Statistical Validation

To validate genotypic differences and visualize tolerance groups, cluster analysis and statistical tests were performed.

Cluster Analysis: Accessions were clustered by their D-values using the UPGMA method (Euclidean distance). This grouped genotypes with similar tolerance profiles, facilitating interpretation of phenotypic variation.

Descriptive Tests: Mean, standard deviation (SD), and coefficient of variation (CV) were calculated for each trait (Table 1).

Table 1. Descriptive statistics and ANOVA multiple comparison results of seedling traits of 51 safflower accessions.

Trait	Treatment	Min	Max	Mean	SD	CV (%)	CS (%)
NL	Stress	10.67	26.00	18.35 a	3.60	19.64	4.23
	CK	18.00	22.00	19.16 b	0.92	4.83	-
NGL	Stress	6.33	25.00	14.51 a	3.78	26.06	23.31
	CK	18.00	22.00	18.92 b	0.87	4.59	-
PH (cm)	Stress	9.00	32.75	16.92 a	6.85	19.62	26.66
	CK	21.96	24.09	23.07 b	0.67	2.92	-
SD (mm)	Stress	2.77	9.42	6.22 a	1.34	21.58	31.42
	CK	8.65	9.54	9.07 b	0.26	2.87	-
SFW (g)	Stress	3.81	23.86	10.50 a	4.22	40.19	69.38
	CK	32.51	38.02	34.29 b	1.14	3.32	-
SDW (g)	Stress	1.60	8.51	4.66 b	1.82	39.09	−66.43
	CK	2.65	3.09	2.80 a	0.09	3.26	-
RFW (g)	Stress	0.47	6.88	2.22 a	1.42	63.48	0.45
	CK	2.13	2.34	2.23 b	0.06	2.89	-
RDW (g)	Stress	0.23	1.87	0.77 b	0.38	49.76	−285
	CK	0.20	0.21	0.20 a	0.01	2.46	-
TRL (cm)	Stress	3.23	16.50	9.68 a	2.69	27.79	19.97
	CK	11.18	12.33	11.80 b	0.36	3.03	-
RD (mm)	Stress	2.69	7.49	5.05 a	0.96	19.02	15.97
	CK	5.73	6.62	6.01 b	0.21	3.53	-
Chl	Stress	16.78	77.10	57.75 a	10.88	18.84	11.87
	CK	61.73	67.99	65.53 b	1.99	3.03	-
LL (cm)	Stress	6.07	21.73	15.14 a	3.33	21.99	45.13
	CK	26.13	28.83	27.59 b	0.78	2.82	-
LW (cm)	Stress	1.55	19.72	4.36 a	2.38	54.59	7.23
	CK	4.47	4.96	4.70 b	0.14	3.05	-
LA (cm ²)	Stress	6.21	52.70	29.76 a	10.08	33.85	38.93
	CK	46.83	51.58	48.73 b	1.19	2.44	-
LP (cm)	Stress	13.56	48.56	34.95 a	8.17	23.37	41.53
	CK	57.03	62.64	59.77 b	1.74	2.91	-

Different lowercase letters within the same trait indicate significant differences between CK and stress at $p < 0.05$.

ANOVA and Duncan's Test: ANOVA assessed trait differences across accessions, and Duncan's test ($p < 0.05$) identified significant differences (Table 1).

All analyses (descriptive stats, ANOVA, PCA, membership function, clustering) were conducted in IBM SPSS Statistics 26.0 (IBM, Chicago, IL, USA). Figures were constructed in Origin 2025 (OriginLab, Northampton, MA, USA).

3. Results

3.1. Descriptive Statistical Analysis of Safflower Seedling Traits

Descriptive statistical analysis was conducted on 15 seedling traits of 51 safflower varieties (lines) after flooding stress treatment (Table 1). Compared with the control, waterlogging stress significantly affected most agronomic traits, but the responses varied distinctly among traits.

For the majority of growth-related traits—including shoot fresh weight (SFW), plant height (PH), and leaf area (LA)—the mean values under waterlogging stress were markedly lower than those under normal conditions. Specifically, SFW exhibited the most pronounced reduction, with a Stress Coefficient (CS) of 69.38%, indicating high sensitivity to waterlogging stress.

Conversely, dry matter-related traits showed an opposite trend. Both shoot dry weight (SDW) and root dry weight (RDW) increased significantly under waterlogging stress, with CS values reaching 66.43% and 285.00%, respectively.

In terms of phenotypic diversity, the coefficient of variation (CV) for most traits under waterlogging stress ranged from 18.84% to 63.48%, which was substantially higher than that of the control group (2.44–4.83%). This elevated CV indicated that waterlogging stress amplified phenotypic differentiation among the 51 tested accessions, and similar inter-genotype differentiation under waterlogging conditions has also been documented in other plant germplasm resources [11].

3.2. Correlation Analysis of Seedling Traits

Correlation analysis was conducted on the seedling trait indicators of safflower under waterlogging stress treatment and control, respectively, and the results are shown in Table 2.

Table 2. Pearson correlation coefficients of seedling phenotypic traits of safflower under control and waterlogging stress treatments.

Treatment	Trait	NL	NGL	PH	SD	SFW	SDW	RFW	RDW	TRL	RD	Chl	LL	LW	LA	LP
Control	NL	1.00														
	NGL	0.14	1.00													
	PH	−0.18	−0.03	1.00												
	SD	0.04	0.02	−0.03	1.00											
	SFW	−0.05	0.30 *	−0.01	−0.11	1.00										
	SDW	0.12	0.32 *	−0.15	−0.14	0.34 *	1.00									
	RFW	0.08	−0.04	0.15	0.00	−0.30 *	−0.04	1.00								
	RDW	−0.03	−0.05	−0.16	−0.04	−0.29 *	−0.13	0.16	1.00							
	TRL	−0.17	−0.17	0.02	−0.24	0.02	0.17	−0.10	−0.22	1.00						
	RD	0.09	0.32 *	0.04	−0.07	0.37 **	0.20	−0.17	0.12	0.01	1.00					
	Chl	0.35 *	0.14	−0.06	−0.05	−0.08	−0.04	−0.15	0.07	−0.06	−0.06	1.00				
	LL	0.10	0.07	−0.12	−0.22	−0.06	−0.10	−0.15	−0.19	−0.04	−0.27	−0.10	1.00			
	LW	−0.20	−0.07	0.12	−0.12	−0.27	−0.30 *	0.02	0.16	−0.06	0.18	−0.02	−0.01	1.00		
	LA	−0.05	0.06	−0.09	−0.19	0.02	0.01	−0.03	0.22	0.15	0.06	0.05	0.11	0.14	1.00	
	LP	0.11	−0.15	−0.06	0.09	−0.07	0.21	−0.00	−0.01	−0.17	−0.03	0.06	−0.22	0.09	−0.30 *	1.00
Waterlogging stress	NL	1.000														
	NGL	0.80 **	1.00													
	PH	0.61 **	0.50 **	1.00												
	SD	0.10	0.28 *	−0.31 *	1.00											
	SFW	0.50 **	0.59 **	0.09	0.76 **	1.00										
	SDW	0.58 **	0.45 **	0.48 **	0.27	0.67 **	1.00									
	RFW	0.17	0.13	−0.11	0.43 **	0.55 **	0.35 *	1.00								
	RDW	0.32 *	0.29 *	−0.07	0.46 **	0.65 **	0.62 **	0.80 **	1.00							
	TRL	0.13	0.20	0.17	0.02	0.08	0.07	0.26	0.24	1.00						
	RD	0.16	0.26	−0.30 *	0.73 **	0.74 **	0.36 **	0.73 **	0.75 **	0.26	1.00					
	Chl	0.11	0.34 *	−0.04	0.28 *	0.27	0.04	0.29 *	0.29 *	0.36	0.32 *	1.00				
	LL	−0.12	0.10	−0.40 **	0.83 **	0.55 **	0.15	0.33 *	0.33 *	0.20	0.68 **	0.21	1.00			
	LW	0.18	0.11	0.10	0.38 **	0.33 *	0.20	0.12	0.13	0.04	0.33 *	−0.09	0.34 *	1.00		
	LA	0.09	0.32 *	−0.15	0.79 **	0.74 **	0.30 *	0.39 **	0.41 **	0.18	0.70 **	0.19	0.82 **	0.40 **	1.00	
	LP	−0.05	0.13	−0.37 **	0.84 **	0.60 **	0.19	0.39 **	0.38 **	0.19	0.71 **	0.23	0.97 **	0.40 **	0.86 **	1.00

* indicates significant correlation at 0.05 level, ** indicates extremely significant correlation at 0.01 level.

Under the normal growth environment (control), the number of significant correlations among traits was limited. Significant positive linear correlations at the 0.05 alpha level were detected between NGL and SFW, SDW, and RD, as well as between NL and Chl. Additionally, highly significant positive correlations ($p < 0.01$) were observed between SFW and SDW, as well as between SFW and RD. Conversely, SDW showed a significant negative correlation with LW ($p < 0.05$). The majority of other trait combinations did not exhibit significant linear associations.

Under a waterlogging stress environment, the number of correlated trait pairs increased markedly. Most of these correlations were statistically significant or extremely significant. Specifically, root-related traits (RFW, RDW, and RD) were extremely significantly positively correlated with each other. Furthermore, these root traits also exhibited extremely significant positive correlations with aboveground traits, including SD, SFW, and LA. TRL was found to be significantly positively correlated with Chl.

3.3. Principal Component Analysis (PCA) of Waterlogging Coefficients

To further explore the multi-trait variations in safflower responses to waterlogging, principal component analysis was conducted based on the waterlogging tolerance coefficients (WC) of 15 agronomic traits. Four principal components (PCs) with eigenvalues >1 (Kaiser criterion) were retained, accounting for a cumulative contribution rate of 79.64% of the total phenotypic variation (Table 3).

Table 3. Characteristic load values and contribution rates of each comprehensive index principal component.

Trait	Principal Component 1	Principal Component 2	Principal Component 3	Principal Component 4
NL	0.32	0.84	0.10	−0.07
NGL	0.48	0.67	0.34	0.19
PH	−0.03	0.85	0.30	0.02
SD	0.86	−0.29	0.21	−0.08
SFW	0.87	0.20	0.18	−0.07
SDW	0.50	0.62	−0.15	−0.30
RFW	0.61	0.04	−0.69	0.02
RDW	0.64	0.21	−0.68	−0.07
TRL	0.32	0.13	−0.08	0.64
RD	0.85	−0.15	−0.36	−0.05
Chl	0.40	0.05	0.01	0.74
LL	0.77	−0.45	0.34	0.08
LW	0.40	−0.00	0.21	−0.49
LA	0.85	−0.22	0.22	−0.10
Perimeter	0.83	−0.43	0.25	0.01
Eigenvalue	6.02	2.89	1.66	1.38
Contribution rate/%	40.14	19.24	11.04	9.22
Cumulative contribution rate/%	40.14	59.38	70.42	79.64
Factor weight	0.53	0.20	0.15	0.12

PC1 explained 40.14% of total variation, with high positive loading values for aboveground and root biomass traits: shoot fresh weight (SFW, 0.87), shoot dry weight (SDW, 0.86), leaf area (LA, 0.85), plant perimeter (0.83), leaf length (LL, 0.77), root dry weight (RDW, 0.85), and root fresh weight (RFW, 0.61).

PC2 accounted for 19.24% of variation, with large positive loadings on plant height (PH, 0.85) and node number (NL, 0.84), while leaf width (LW), leaf area (LA), and plant perimeter exhibited negative loading values.

PC3 contributed 11.04% to total variation; chlorophyll content (Chl, 0.74) showed the highest positive loading, whereas node number per leaf (NGL) displayed negative loading.

PC4 explained 9.12% of variation, with the maximum loading value observed for total root length (TRL, 0.64).

3.4. Comprehensive Evaluation of Waterlogging Tolerance of Safflower Germplasm Resources at Seedling Stage

The membership-function-weighted scores were used to calculate the comprehensive waterlogging tolerance coefficient (D-value; Table 4). Based on the D-values, hierarchical cluster analysis (Euclidean distance, UPGMA) was performed to group the 51 accessions. The optimal number of clusters (five) was determined using the elbow method based on the cluster dendrogram (Figure 1), which generated distinct intervals of D-values for phenotypic grouping. The five tolerance grades were classified strictly according to the D-value interval ranges obtained from hierarchical clustering.

Table 4. Comprehensive waterlogging tolerance coefficient of 51 safflower germplasm resources seedlings under waterlogging stress.

Germplasm Number	D-Value (Comprehensive Waterlogging Tolerance Coefficient)	Ranking	Germplasm Number	D-Value (Comprehensive Waterlogging Tolerance Coefficient)	Ranking	Germplasm Number	D-Value (Comprehensive Waterlogging Tolerance Coefficient)	Ranking
Ct01	1.19	37	Ct18	1.68	23	Ct35	0.95	46
Ct02	0.88	49	Ct19	1.96	12	Ct36	2.68	3
Ct03	1.24	35	Ct20	1.82	18	Ct37	0.93	47
Ct04	2.03	9	Ct21	1.15	39	Ct38	3.48	1
Ct05	1.21	36	Ct22	2.441	7	Ct39	1.81	19
Ct06	1.16	38	Ct23	0.99	43	Ct40	0.90	48
Ct07	1.88	15	Ct24	2.03	10	Ct41	2.59	4
Ct08	1.49	28	Ct25	1.57	27	Ct42	1.45	30
Ct09	1.74	21	Ct26	2.54	6	Ct43	1.76	20
Ct10	1.98	11	Ct27	1.43	31	Ct44	2.55	5
Ct11	1.68	25	Ct28	0.97	44	Ct45	1.86	16
Ct12	1.48	29	Ct29	2.06	8	Ct46	0.86	50
Ct13	1.41	32	Ct30	3.03	2	Ct47	0.96	45
Ct14	1.27	34	Ct31	1.94	13	Ct48	1.14	40
Ct15	1.82	17	Ct32	1.58	26	Ct49	1.68	24
Ct16	0.59	51	Ct33	1.33	33	Ct50	1.00	42
Ct17	1.93	14	Ct34	1.71	22	Ct51	1.11	41

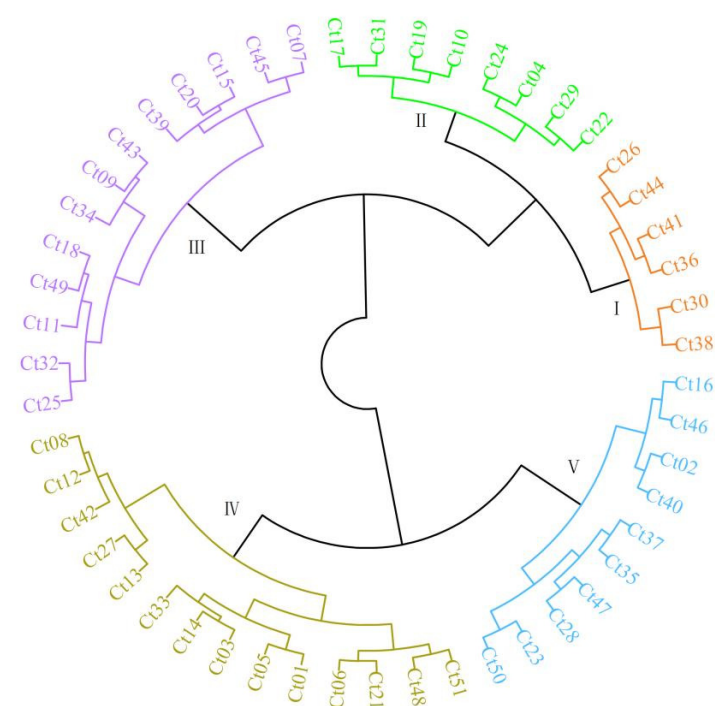


Figure 1. Cluster analysis of comprehensive waterlogging tolerance D-values of 51 safflower germplasm resources. The D-values were calculated by membership function analysis based on 15 agronomic traits ($n = 3$ biological replicates per genotype). Group I (orange branch), highly tolerant (HT); Group II (green branch), tolerant (T); Group III (purple branch), moderately tolerant (MT); Group IV (yellow branch), sensitive (S); Group V (light-blue branch), highly sensitive (HS).

The five clusters are presented as five groups with separate D-value ranges:

Group I (orange branch), highly tolerant (HT): six accessions (Ct26, Ct30, Ct36, Ct38, Ct41, Ct44). This cluster diverged first on the dendrogram and had D-values of 2.54–3.48, accounting for 11.76% of all tested accessions.

Group II (green branch), tolerant (T): eight accessions, adjacent to Group I on the dendrogram, with D-values 1.93–2.44.

Group III (purple branch), moderately tolerant (MT): 13 accessions, D-values 1.57–1.88.

Group IV (yellow branch), sensitive (S): 14 accessions, D-values 1.11–1.49.

Group V (light-blue branch), highly sensitive (HS): 10 accessions, D-values 0.59–1.00, accounting for 19.61% of the panel.

To compare trait performance across clustering groups, multiple comparisons of Waterlogging Coefficient (WC) values were conducted among the five clusters (Table 5). Most agronomic traits showed a general downward trend in WC values from Group I to Group V, while PH, TRL and LW exhibited irregular variation trends across tolerance grades. Significant WC differences between Group I/II and Group V were observed for NL, NGL, SD, SFW, SDW, RFW, RDW, RD, LL, LW, LA and LP; no significant inter-group difference was detected for PH and TRL. For SFW, significant differences existed not only between high-tolerant/tolerant/moderately tolerant groups and sensitive/highly sensitive groups, but also between Group IV and Group V.

Table 5. Waterlogging tolerance coefficient (WTC) of 15 seedling agronomic traits of safflower under five waterlogging tolerance grades.

Trait	Group I	Group II	Group III	Group IV	Group V
	Highly Tolerant	Tolerant	Moderately Tolerant	Sensitive	Highly Sensitive
NL	1.16 ± 0.06 a	1.08 ± 0.05 ab	0.96 ± 0.04 ab	0.89 ± 0.03 ab	0.87 ± 0.08 b
NGL	0.98 ± 0.04 a	0.88 ± 0.07 ab	0.79 ± 0.05 ab	0.69 ± 0.03 ab	0.60 ± 0.07 b
PH	0.69 ± 0.12 a	0.77 ± 0.06 a	0.84 ± 0.08 a	0.71 ± 0.10 a	0.64 ± 0.09 a
SD	0.91 ± 0.04 a	0.77 ± 0.02 ab	0.69 ± 0.02 bc	0.63 ± 0.03 cd	0.55 ± 0.04 d
SFW	0.56 ± 0.05 a	0.39 ± 0.01 b	0.33 ± 0.01 b	0.24 ± 0.01 c	0.17 ± 0.01 d
SDW	1.20 ± 0.11 a	1.08 ± 0.10 a	0.85 ± 0.08 a	0.77 ± 0.07 ab	0.53 ± 0.07 b
RFW	1.76 ± 0.40 a	0.96 ± 0.10 ab	1.12 ± 0.16 ab	0.90 ± 0.18 ab	0.54 ± 0.06 b
RDW	3.15 ± 0.63 a	2.24 ± 0.26 a	1.85 ± 0.20 ab	1.71 ± 0.19 ab	1.07 ± 0.14 b
TRL	0.85 ± 0.05 a	0.85 ± 0.05 a	0.87 ± 0.07 a	0.84 ± 0.05 a	0.71 ± 0.10 a
RD	1.08 ± 0.07 a	0.93 ± 0.03 ab	0.85 ± 0.03 b	0.78 ± 0.03 bc	0.69 ± 0.03 c
Chl	1.00 ± 0.06 a	0.90 ± 0.05 a	0.90 ± 0.04 a	0.90 ± 0.04 a	0.79 ± 0.07 a
LL	0.66 ± 0.07 a	0.64 ± 0.02 ab	0.57 ± 0.02 abc	0.52 ± 0.03 bc	0.44 ± 0.04 c
LW	1.08 ± 0.08 ab	1.38 ± 0.40 a	0.93 ± 0.04 ab	0.78 ± 0.03 b	0.64 ± 0.04 b
LA	0.82 ± 0.05 a	0.70 ± 0.05 ab	0.64 ± 0.03 b	0.49 ± 0.03 c	0.36 ± 0.04 c
LP	0.73 ± 0.04 a	0.68 ± 0.02 ab	0.61 ± 0.02 ab	0.54 ± 0.04 bc	0.45 ± 0.04 c

Values represent mean ± standard error. Different lowercase letters within the same row indicate significant differences at $p < 0.05$.

Correlation between single agronomic traits WC and the comprehensive D-value is shown in Table 6. SDW, RDW, LP, NGL, LL, RFW, and LN exhibited moderate to highly significant positive correlations with D-value ($0.5 < r < 0.7$, $p < 0.01$). Chl and LW showed weak significant correlations, while PH and TRL had non-significant, near-zero correlation coefficients with D-value.

Table 6. Correlation analysis between comprehensive waterlogging tolerance coefficient (D-value) and seedling traits.

Indicator	NL	NGL	PH	SD	SFW	SDW	RFW	RDW	TRL	RD	Chl	LL	LW	LA	LP
D-value	0.50 **	0.59 **	0.10	0.77 **	0.10 **	0.69 **	0.58 **	0.69 **	0.13	0.76 **	0.29 *	0.58 **	0.35 *	0.76 **	0.64 **

* indicates significant correlation at 0.05 level, ** indicates extremely significant correlation at 0.01 level.

4. Discussion

4.1. Effects of Flooding Stress at Seedling Stage on Safflower Agronomic Traits

Environmental factors including light, temperature and water jointly regulate plant growth and metabolism; previous reviews have fully clarified plant morphological, physiological and molecular adaptations to waterlogging [12,13]. Flooding stress constitutes a paramount abiotic constraint that induces tissue hypoxia, impairs gas exchange and assimilate translocation, thereby disrupting normal growth and leading to substantial yield penalties [14]. In response to such adversity, plants actively orchestrate sophisticated stress response mechanisms, adjusting morphological architecture, biomass allocation, and physiological metabolism to acclimate to hypoxic conditions and mitigate growth inhibition [15]. Consistent with our multi-group comparison results across five tolerance clusters, waterlogging stress exerted stronger growth inhibition on sensitive genotypes, and the phenotypic gap between highly tolerant and highly sensitive accessions was amplified under prolonged waterlogging treatment [16]. For instance, seedling growth is typically retarded under waterlogging [16], characterized by a progressive decline in shoot dry weight (SDW) concomitant with an elevated root/shoot ratio, alongside marked inhibition of chlorophyll biosynthesis [17]. Regarding maize seedlings, while waterlogging primarily suppresses root biomass accumulation, plants exhibit remarkable plasticity by reallocating resources to stems and leaves to sustain vital functions [18].

Complementing these physiological insights, Zhao et al. [19] conducted a genome-wide association study (GWAS) on safflower germplasm, evaluating grain yield (YP), days to flowering (DF), plant height (PH), 100-seed weight (SW), seed oil content (OL), and crude protein content (PR) post-flooding. Significant phenotypic variations were detected across all traits. Consistent with observations in field-grown cotton [20], these findings underscore that seedling-stage agronomic traits serve as robust proxies for predicting adult waterlogging tolerance, thereby facilitating early germplasm screening and expediting the breeding pipeline for waterlogging-resilient varieties.

Waterlogging stress triggered extensive phenotypic differentiation across the tested safflower germplasms and drastically reshaped the balance of vegetative and underground growth under hypoxic environments. Severe suppression of aboveground biomass under flooding is a conserved adaptive phenotype shared by many oil and horticultural crop seedlings, which has been fully documented in previous germplasm evaluation research [11,21–24]. Unlike existing safflower studies that mainly focused on yield and drought responses of mature plants, this research systematically uncovered seedling-stage trait variation under short-term waterlogging, which fills the gap in early flooding phenotypic research for safflower and supplies a rich material basis for subsequent tolerant germplasm screening.

Correlation analysis further elucidated the shift in trait coordination under stress. Under normal conditions, trait correlations were weak and sparse; however, under waterlogging, these correlations intensified significantly, with most traits exhibiting significant or extremely significant associations. Particularly, root biomass (RFW, RDW) and RD showed extremely significant positive correlations with each other and with shoot traits (SD, LA, LP). This synergy suggests that root thickening and dry matter accumulation enhance aeration capacity and nutrient uptake efficiency, thereby sustaining aboveground growth. Morphological and anatomical remodeling of roots represents the primary adaptive strategy for plants to survive hypoxic waterlogging environments [25]. Consistent with this observation, coordinated adjustment of root architecture and aboveground growth performance is a conserved stress response across oilseed crops; relevant research on soybean further confirmed that maintaining balanced root–shoot development is critical for improving plant resilience under abnormal water conditions [26].

Correlation results only indicated that root morphological traits are reliable phenotypic markers to judge safflower waterlogging tolerance. However, these statistical associations cannot directly confirm intrinsic physiological regulatory pathways; further physiological and molecular tests are required to verify root-mediated stress adaptation mechanisms [27]. Relevant studies have verified that alterations in root morphology and anatomy act as core adaptive strategies to strengthen internal ventilation under hypoxia [28,29].

Notably, elevated root and shoot dry weight under short-term waterlogging observed in safflower differs from most long-term flooding reports of field crops. This unique seedling adaptation may arise from three coordinated physiological adjustments: First, rhizosphere hypoxia triggers root cell lignification and cell wall thickening, increasing root dry matter accumulation per unit volume; second, fixed photosynthate is preferentially transported to underground tissues to strengthen root aeration structure; third, young safflower seedlings adopt a carbon allocation strategy prioritizing root structural remodeling to cope with transient hypoxic stress. Similar biomass redistribution patterns under mild waterlogging have been summarized in previous plant flooding adaptation reviews [3].

To comprehensively dissect this complexity, fifteen seedling traits were synthesized via principal component analysis (PCA). Principal component analysis is a widely recognized statistical tool to decompose multi-dimensional phenotypic data for stress tolerance evaluation, and its standardized application workflow has been systematically summarized in methodological reviews [30]. This analytical paradigm has been echoed across diverse crops utilizing PCA-based multi-trait frameworks [5,31]. Using membership-function-weighted scores, a comprehensive waterlogging tolerance coefficient (D-value) was derived.

To comprehensively dissect the complex multi-trait response of safflower under flooding, we integrated PCA and fuzzy membership functions to quantify germplasm waterlogging tolerance. Most existing safflower stress evaluation systems rely on only one or two simple agronomic indicators and lack systematic multi-index screening at the seedling stage. The combined PCA-D-value system constructed in this work can integrate multiple interrelated morphological traits to eliminate evaluation bias caused by a single-indicator limitation.

Through comprehensive phenotypic discrimination, we screened out a set of stable representative morphological markers that can distinguish germplasm with contrasting flooding tolerance, and obtained candidate seedling materials with strong waterlogging adaptability, which can serve as preliminary resources for safflower tolerance breeding pre-selection under greenhouse conditions.

Correlation analysis between single-trait WC values and the comprehensive D-values further revealed the hierarchical correlation strength of each indicator to overall waterlogging tolerance. It should be noted that the strong correlation between SFW and D-value originates from the mathematical circular relationship, as SFW was incorporated into the construction of D-value, so SFW cannot be simply defined as the absolute dominant predictive indicator without independent verification trials. Collectively, these correlative patterns indicate that safflower seedling waterlogging tolerance is predominantly driven by the maintenance of biomass accumulation coupled with structural robustness. Most existing safflower germplasm screening relies on scattered single indicators without unified simplified core trait combinations. Based on the differential correlation performance of all measured traits, we refined a compact set of nine representative morphological markers that closely reflect seedling flooding adaptability. This streamlined core index system greatly reduces measurement workload during large-scale germplasm pre-screening under greenhouse seedling conditions, offering an operable reference for rapid preliminary identification of waterlogging-tolerant safflower materials in early breeding stages.

Collectively, these results corroborate the prevailing paradigm that root diameter and biomass preservation, coupled with the maintenance of leaf surface (LA/perimeter) and shoot growth, constitute the primary discriminators for genotypic ranking under waterlogging [32,33]. While previous studies on safflower have largely focused on physiological and yield-related traits under water-related abiotic stresses (e.g., drought or soil moisture fluctuations) at later growth stages [34], systematic evaluations of waterlogging tolerance at the seedling stage remain scarce. This study bridges that critical knowledge gap by establishing a robust, seedling-stage evaluation framework across a diverse panel of 51 accessions, offering a high-throughput, trait-based pre-screening tool to prioritize elite germplasms prior to costly and time-consuming field trials.

4.2. Comprehensive Evaluation of Waterlogging Tolerance at Seedling Stage Based on Membership Function Method

Crop stress resistance is a polygenic quantitative trait; evaluation relying on a single index often produces biased results that cannot fully reflect the actual stress tolerance of germplasms [35,36]. Therefore, multi-trait comprehensive evaluation based on the fuzzy membership function method has become a widely recognized standard analytical workflow for stress tolerance identification.

The fuzzy membership function combined with PCA is a mature multi-index tool to convert complex stress phenotypes into unified comprehensive scores for germplasm grading [8,10,37–39]. Most existing safflower stress evaluations rely on single growth indicators only. This study built a targeted evaluation system based on 15 seedling morphological traits, further screening 9 stable representative core indicators for rapid seedling germplasm pre-screening. The five-grade tolerance classification standard derived from D-values provides an operable reference for safflower waterlogging germplasm identification under greenhouse conditions.

Consistent with the above research paradigms, the present study integrated 15 seedling phenotypic WC values to calculate the comprehensive D-value via fuzzy membership function weighting, and further classified 51 safflower accessions into five tolerance grades through hierarchical cluster analysis [32,33,40]. This multi-index system avoids the one-sidedness of single-trait screening, and the five-grade classification standard based on D-value intervals provides an operable phenotypic grading scheme for rapid seedling-stage identification of safflower waterlogging tolerance. The nine core indicators screened from correlation analysis can simplify the evaluation workload of large germplasm panels, which has practical application value for safflower stress-resistance breeding.

Notably, the D-value only ranks germplasm phenotypically based on 15 seedling traits and cannot reveal the underlying physiological, biochemical or molecular regulatory pathways of safflower waterlogging tolerance.

5. Conclusions

This study applied a combined analytical workflow integrating the established fuzzy membership function, principal component analysis and hierarchical cluster analysis to assess waterlogging tolerance of safflower seedlings. Based on the comprehensive waterlogging tolerance coefficient (D-value) calculated from 15 seedling phenotypic traits, the 51 tested safflower accessions were grouped into five distinct tolerance grades via statistical stratification. The present analysis demonstrated the capacity of this multi-index statistical pipeline to classify the phenotypic performance of the tested germplasm panel and provides preliminary phenotypic evidence for targeted screening of waterlogging-tolerant materials.

To our knowledge, this work represents a systematic seedling-stage waterlogging tolerance assessment of *Carthamus tinctorius* using this combined multi-trait statistical pipeline. The analytical workflow effectively distinguished phenotypic differences among genotypes under waterlogging, characterized divergent phenotypic response patterns to hypoxic stress, and screened a simplified set of nine core phenotypic indicators for seedling evaluation. Six germplasm accessions (Ct38, Ct30, Ct36, Ct44, Ct41, Ct26) with superior waterlogging performance were identified from the panel, which can serve as phenotypic materials for subsequent stress-resistance breeding research.

Collectively, waterlogging stress imposes strong inhibitory effects on most seedling growth traits of safflower, and obvious phenotypic differentiation exists across the tested germplasm collection. The multi-trait analytical pipeline presented herein delivers a preliminary seedling pre-screening method only suitable for greenhouse seedling tests under the current experimental conditions. Further independent multi-environment validation trials will be required to confirm the broader applicability and stability of this evaluation pipeline. This study supplies greenhouse seedling-stage phenotypic data, core diagnostic indicators and candidate waterlogging-tolerant germplasm materials, which can only act as preliminary phenotypic references for later safflower waterlogging tolerance breeding exploration after multi-environment verification.

Notably, all findings are confined to a single short-term waterlogging trial in a controlled greenhouse; multi-year, multi-location field trials are indispensable to validate the stability of the five-grade tolerance classification and the nine core indicators before large-scale breeding applications.

Author Contributions: Conceptualization, S.A. and Y.Y.; methodology, S.A. and L.X.; software, L.X.; validation, S.A., Y.Y. and X.W.; formal analysis, S.A.; investigation, J.F., Y.L. and H.Y.; resources, Q.Y. and W.D.; data curation, S.A. and L.X.; writing—original draft preparation, S.A.; writing—review and editing, Y.Y. and H.L.; visualization, S.A. and L.X.; supervision, Y.Y. and H.L.; project administration, Y.Y.; funding acquisition, H.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the China Agriculture Research System of MOF and MARA (Grant No. CARS-21); the Henan Agriculture Research System (Grant No. HARS-22-11-G1); the Zhongyuan Scholar Program (Grant No. 264000510011); the Key Research and Development Program of Henan (Grant Nos. 241111310200 and 231111110800); the Henan Center for Overseas Scientists (Grant No. GZS2024025); the Henan Province Science and Technology Research Project (Grant Nos. 252102110283 and 262102110285); the Doctoral Scientific Research Start-up Fund Project of the Henan Academy of Agricultural Sciences (Grant No. 2026BX119); and the Special Fund for Independent Innovation of the Henan Academy of Agricultural Sciences (Grant No. 2026ZC160). The APC was funded by the China Agriculture Research System of MOF and MARA (CARS-21).

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Acknowledgments: We acknowledge the technical support provided by the Experimental Farm of the Institute of Chinese Herbal Medicines, Henan Academy of Agricultural Sciences. During the preparation of this manuscript, the authors used ChatGPT (OpenAI, Version 4.0) for language polishing and formatting assistance. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

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

Abbreviations

The following abbreviations are used in this manuscript:

NL	Number of leaves
NGL	Number of green leaves
PH	Plant height
SD	Stem diameter
SFW	Shoot fresh weight
SDW	Shoot dry weight
RFW	Root fresh weight
RDW	Root dry weight
TRL	Total root length
RD	Root diameter
Chl	Chlorophyll content
LL	Leaf length
LW	Leaf width
LA	Leaf area
LP	Leaf perimeter

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