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Comparison and Evaluation of Low-Temperature Tolerance of Different Soybean Cultivars during the Early-Growth Stage

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Abstract: Low temperatures have seriously affected crop growth owing to climate change and frequent extreme weather. Low-temperature disasters easily affect the early-growth stages of planted soybeans in Northeast China. In the present study, the comprehensive evaluation method using low-temperature (4 °C) simulation at soybean germination and seedling stages was used to compare soybean cultivars. The results revealed that low temperatures inhibited the germination ability of soybean seeds and prolonged the average germination time (about 7–13 days under low temperatures). Simultaneously, low-temperature stress at the seedling stage decreased plant height and dry weight, but accumulated proline and soluble sugar. The soluble protein content of most cultivars decreased at low temperatures. Peroxidase activity was significantly decreased in henong70, suinong82, and heinong83, and opposite in the other cultivars. Additionally, MDA content increased in cultivars heinong69, dongnong42, and dongnong55. The final comprehensive evaluation showed that Suinong42 had better low-temperature tolerance, whereas Kendou40 was more sensitive to low temperatures. The grey correlation analysis also showed that dry weight and proline can be used as the target traits for cultivar improvement.

Keywords: soybean; low temperature; breeding; crop improvement

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1. Introduction

Soybean (*Glycine max* (Linn.) Merr.) is an important crop for grain and oil, and a source of plant protein and vegetable oil for humans [1,2]. Soybeans are being grown worldwide, with global production reaching approximately 372 million tons in 2021 [3]. However, the current soybean production level cannot meet the global demand owing to various abiotic stress factors, such as droughts, floods, and low temperatures [4,5]. Furthermore, with the increasing complexity of climate change, the impact of extreme weather conditions on crop production is severe. Therefore, improving soybean yield by cultivating cultivars adaptable to adverse environmental conditions in a region is important [6].

Northeast China is an important soybean-producing area with considerable temperature fluctuations in spring (April–May) [7]. Crop seeds are sown on the farmland under optimal temperatures. However, a sudden cold airflow after sowing may result in an unpredictable drop in air temperature owing to global climate change and the uncertainty of meteorological conditions. Therefore, early crop growth stages (from seed germination to the seedling stage) may be affected by low-temperature stress.

Generally, the response and sensitivity of crops to low temperatures depend on the growth stage [8,9]. The suitable germination temperature for soybean is approximately 30 °C, but the germination rate (GR) can be 50% at 17 °C [10]. When the temperature is <10 °C, it can be considered a low-temperature disaster. Cell-membrane repair is important during seed germination. When the water content of mature and dry seeds is low, the cell membranes are dehydrated, and the cytosol leaks after water absorption. When the

cell content leaks excessively, the seed loses germination ability. Therefore, the rate of repair of the cell membrane determines seed germination [11]. Additionally, damaged structure of the mitochondria of dry seeds reduces seed respiration rate, which is ameliorated by repairing the structure of the membrane and the binding of protein complexes. However, low temperature considerably affects seed respiration and imbibition rates [12], affecting the membrane repair; therefore, low-temperature-sensitive seeds display low germination ability.

At the seedling stage, low temperatures inhibit crop growth and produce reactive oxygen species (ROS) [13,14]. The increase in ROS levels induced by low temperatures upsets the balance between ROS production and cell clearance. Excessive ROS oxidizes membrane lipids, leading to protein degradation, DNA damage, and then lead to cell structure and function damage, and even death [15]. Correspondingly, plants scavenge ROS by upregulating the activity of antioxidant enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and other antioxidant enzymes reported in different plants [16,17]. Additionally, low-temperature stress considerably affect the chlorophyll content and electron-transport rate during photosynthesis [18,19]. Reduced light-transformation efficiency are important factors for plant-growth retardation. Wang et al. reported that the pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (α-KGDH) activities in rice leaves were considerably downregulated under low-temperature stress [20]. These two enzymes regulate mitochondrial respiration. In summary, low-temperature stress at the seedling stage considerably affects photosynthesis, respiration, and cell defense. Previous researchers have found that adverse environments at the seedling stage of plants affects subsequent reproductive growth and yield [21,22]. Therefore, tolerance to low-temperature stress at the seedling stage is critical for soybean growth and yield.

To obtain soybean cultivars adaptable to areas with frequent low temperatures, 60 soybean cultivars were collected from Northeast China. First, we subjected seeds to low temperatures and screened tolerant cultivars germinating under low-temperature stress conditions. Next, a low-temperature simulation was conducted at the seedling stage, and physiological indices, protective enzymes, and osmotic substances were determined, and the low-temperature tolerant and sensitive types were identified by a membership function and comprehensive evaluation. The grey correlation analysis revealed the importance of different indices for soybean cold tolerance at the seedling stage. The obtained cultivars can be used for subsequent mechanism analysis or for popularizing planting in areas with a suitable climate to address future fluctuations in soybean yield under a changing climate.

2. Materials and Methods

A total of 60 soybean cultivars in Northeast China were collected (Table 1). All these varieties were bred for planting in the northeast region, so they have similar growth environments and may be subjected to low-temperature disaster in the early stage of growth.

Table 1. Experimental cultivars and numbers.

No.	Cultivars	Sources
I	Hefeng26(1), Hefeng34(2), Hefeng35(3), Hefeng36(4), Hefeng39(5), Hefeng40(6), Hefeng46(7), Hefeng55(8), Hefeng56(9) Hefeng60(10) Henong67(11), Henong70(12), Henong75(13), Henong76(14)	Jiamusi Branch of Heilongjiang Academy of Agricultural Sciences

Table 1. *Cont.*

No.	Cultivars	Sources
II	Heinong35(15), Heinong37(16), Heinong45(17) Heinong48(18), Heinong49(19), Heinong52(20), Heinong63(21), Heinong64(22), Heinong69(23) Heinong83(24), Heinong85(25)	Soybean Research Institute of Heilongjiang Academy of Agricultural Sciences
III	Suinong4(26), Suinong26(27), Suinong27(28) Suinong33(29), Suinong34(30), Suinong42(31) Suinong44(32), Suinong71(33), Suinong81(34) Suinong82(35), Suinong94(36), Suinong119(37)	Suihua Branch of Heilongjiang Academy of Agricultural Sciences
IV	Heike59(38), Heike60(39), Heike71(40) Heihe43(41) Heihe49(42)	Heihe Branch of Heilongjiang Academy of Agricultural Sciences
V	Kenfeng6(43), Kenfeng8(44), Kenfeng14(45) Kenfeng16(46), Kenfeng17(47) Kenfeng20(48) Kendou40(49)	Heilongjiang Academy of Land Reclamation Sciences
VI	Dongnong42(50), Dongnong55(51), Dongnong65(52), Dongnong69(53) Dongnong70(54)	Soybean Research Institute of Northeast Agricultural University
VII	Kennong34(55), Kennong36(56) Kennong38(57), Kennong46(58)	Heilongjiang Bayi Agricultural Reclamation University
VIII	Dongsheng3(59)	Northeast Institute of Geography and Agroecology, Chinese Academy of Science
IX	Mufeng7(60)	Mudanjiang Branch of Heilongjiang Academy of Agricultural Sciences

The seeds were disinfected using 5% NaClO for 30 s and washed using distilled water. The between-paper germination method was used for the germination experiment using a wet filter paper. The control and treatment groups were germinated at room temperature (25 °C) and 4 °C, respectively. Each processing set was repeated thrice, and 15 seeds were used for each treatment. The filter paper was changed regularly during the experiment. The low-temperature treatment and control groups were subjected to treatment for 14 and 7 days, respectively. The number of germinated seeds was recorded daily. The germination potential after 5 days (GP), the final germination rate (GR), the average germination time (GT), and the germination index (GI) were calculated using the following formulae:

$$\text{GP}\ (\%) = \frac{n_{5d}}{n_T} \times 100 \quad (1)$$

where n_{5d} is the number of germinated seeds at day 5 after establishment of the experiment, and n_T is the total number of seeds.

$$\text{GR}\ (\%) = \frac{n}{n_T} \times 100 \quad (2)$$

where n is the number of germinated seeds, and n_T is the total number of seeds.

$$\text{GT} = \sum(\frac{ni \times di}{n}) \quad (3)$$

where n represents the number of seeds germinated on day i, d denotes the number of days, and n is the total number of germinated seeds.

$$\text{GI} = \sum \frac{Gt}{dt} \quad (4)$$

where Gt is the number of germinated seeds, and dt represents the number of experimental days.

2.1. Low-Temperature Treatment and Recovery at the Seedling Stage

A mixture of vermiculite and perlite at a ratio of 4:1 was used as the substrate in a plastic pot (diameter, 13 cm; height, 11 cm). Two seedlings were sown per pot. The control group was cultured at normal temperature (25 °C, 275 μmol·m^{-2}·s^{-1}, 16 h of light and 8 h of dark), and the low-temperature treatment group was transferred to the low-temperature incubator (4 °C, 275 μmol·m^{-2}·s^{-1}, 16 h of light and 8 h of dark) after the first compound leaves were expanded. The seedlings were irrigated daily with 200 mL Hoagland nutrient solution [23]. The plants were randomly placed in the incubator, and the position of the pot was randomly changed every 24 h. After 72 h of low-temperature treatment, the control and low-temperature treatment groups were sampled simultaneously, and the leaves were stored in the refrigerator at −80 °C. Each three pots of seedling leaves were mixed as a repetition, and each treatment was repeated three times.

For the temperature recovery treatment, the seedlings were grown at 4 °C for three days, and then transferred into a 25 °C incubator. After three days of growth at 25 °C, the survival rate was counted.

2.2. Determination of Plant Dry Weight

The plants were killed for 30 min at 105 °C, dried at 65 °C for 72 h, and the dry weight was determined.

2.3. Malondialdehyde Content Determination

Using the method by Zhang [24], malonaldehyde (MDA) content was determined using the modified thiobarbituric acid (TBA) method. The leaves were ground in an ice bath with phosphate buffer (0.1 g), the supernatant was added to a water bath with a TBA-mixture, and a spectrophotometer was used to compare the colors at the corresponding wavelength.

2.4. Determination of Peroxidae Activity

The guaiacol colorimetric method was used to determine peroxidase (POD) activity, as described by Wang [25]. Fresh plant leaves (0.1 g) were placed in a mortar, ground with phosphate buffer of pH = 6.0, and the supernatant was the crude extract after centrifugation at 4 °C. Phosphate buffer, guaiacol, and H_2O_2 were mixed according to the prescribed concentrations as the reaction solution. Finally, 3 mL reaction solution and 40 μL crude extract were mixed, and the activity of POD was determined using the colorimetric method.

2.5. Proline Content Determination

Using the method described by Bates [26], the proline content(Pro) was determined using ninhydrin colorimetry. Leaves (0.1 g) were ground with sulfosalicylic acid and extracted in a boiling water bath. The extract was cooled, the supernatant was added to glacial acetic acid and a chromogenic solution (ninhydrin dissolved in a mixture of glacial acetic acid and phosphoric acid), and placed in a boiling water bath for 40 min. After cooling, toluene was extracted, and the toluene layer was used for colorimetric determination.

2.6. Soluble Protein Content Determination

The Coomassie brilliant blue method was used to determine the soluble protein content, according to the method by Li [27], and ethanol and phosphoric acid were used to configure the Coomassie brilliant blue solution. After grinding the plant leaves using a mortar, the supernatant was centrifuged, Coomassie brilliant blue solution was added, and the color was compared using an ultraviolet spectrophotometer after mixing thoroughly.

2.7. Soluble Sugar Content Determination

Soluble sugar content was determined using the anthrone method according to the description by Li [27]. Briefly, 0.1 g fresh plant leaves were ground using a mortar, distilled water is used as the extracting solution. the supernatant was extracted after centrifugation,

anthrone reagent was added, bathed in boiling water for 10 min, and the colorimetric method was used in a spectrophotometer after cooling.

2.8. Comprehensive Evaluation and Grey Relational Degree Analysis

A relative value was used for the analysis to eliminate the influence of basic characteristics of the cultivar. The formulae used is as follows:

$$\text{Relative value} = \text{index value after low-temperature treatment} / \text{index value of the control condition} \quad (5)$$

The formula of the comprehensive index is as follows:

$$Z_i = \sum_{i=1}^{n} a_i X_i \quad (6)$$

where Z_i is the comprehensive index of the ith index, a_i is the eigenvector corresponding to the ith index eigenvalue, and X_i is the relative value of the ith index.

Subsequently, the membership function method was used to standardize the data as follows:

$$U(Z_i) = \frac{Z_i - Z_{imin}}{Z_{imax} - Z_{imin}} \quad (7)$$

Z_{imin} represents the minimum value of the ith comprehensive index. Z_{imax} represents the maximum value of the ith comprehensive index.

The weight formula is as follows:

$$W_i = P_i / \sum_{1}^{n} p_i \quad (8)$$

P_i is the contribution rate of the ith comprehensive index of each cultivar.

The final comprehensive evaluation value (D value) formula is as follows:

$$\mathrm{D} = \sum_{i=1}^{n} (U(Z_i) \times W_i) \quad (9)$$

where $U(Z_i)$ is a comprehensive index standardized using the membership function method. W_i is the weight.

Grey correlation analysis was used to reveal the index that contributed the most to low-temperature resistance. The formula used is as follows:

$$\zeta_i(k) = \frac{\min(i)\min(k)|x_{0(k)} - x_{i(k)}| + \rho\max(i)\max(k)|x_{0(k)} - x_{i(k)}|}{|x_{0(k)} - x_{i(k)}| + \rho\max(i)\max(k)|x_{0(k)} - x_{i(k)}|} \quad (10)$$

where $X_i(k)$ is the relative value of cultivar k on index i, $X_0(k)$ represents the D value of cultivar k, $\zeta_i(k)$ represents the correlation coefficient between $X_i(k)$ and the D value, and ρ is the resolution coefficient, $\rho \in (0, 1)$. In this test, ρ is taken as 0.5 [28].

We also performed stepwise regression analysis and calculated the regression equation to simplify the follow-up evaluation work.

2.9. Statistical Analysis

All data were processed using Microsoft Office Excel 2021, statistical analysis was performed using IBM SPSS software (version 23.0; IBM Corporation, Armonk, NY, USA), and figures were produced using OriginPro 2021 (OriginLab Corp., Northampton, MA, USA).

3. Results

3.1. Effect of Low-Temperature Stress on Seed Germination

As shown in Supplementary Table S1, low-temperature stress during germination inhibited soybean seed germination. The GR and GP were calculated using Equations (1) and (2). The results revealed that the germination rate of all cultivars in the control group was higher than 85%, and the germination potential was higher than 80% after 5 days but

decreased significantly at low temperatures. The GP of cultivar No. 31 exceeded 30% in 5 days. The GP of most cultivars was zero in 5 days, proving that the germination ability of these cultivars was weak at low temperatures. The average GT of all the experimental cultivars was calculated using Equation (3). The GT of each cultivar in the control group was almost similar, with most cultivars germinating at 2–3 days, whereas the GT of each cultivar was prolonged by the low-temperature treatment. Most varieties of GT between 8–10 days, but the GT of cultivars No. 11 and No. 12 were >12 days. In agricultural production, long germination periods can lead to crop seed rot owing to the influence of the soil environment resulting in loss of seedlings in the field. Although the degree of low-temperature stress simulated in this experiment was more severe than that of natural low-temperature weather, the responses of the cultivars revealed that they were susceptible to low temperatures.

The germination index of each cultivar at low temperatures was calculated according to Equation (4), and a systematic cluster analysis was conducted based on the germination index, as shown in Figure 1. The clustering results revealed that cultivar No. 6 was independent of the other cultivars. Additionally, nine cultivars, including No. 6, were grouped and were considered low-temperature-tolerant cultivars at the germination stage, with strong germination ability at low temperatures. However, the remaining 51 cultivars were not apparently separated in cluster analysis. Therefore, we selected nine cultivars in Cluster I and nine cultivars with the lowest germination index for further screening at the seedling stage.

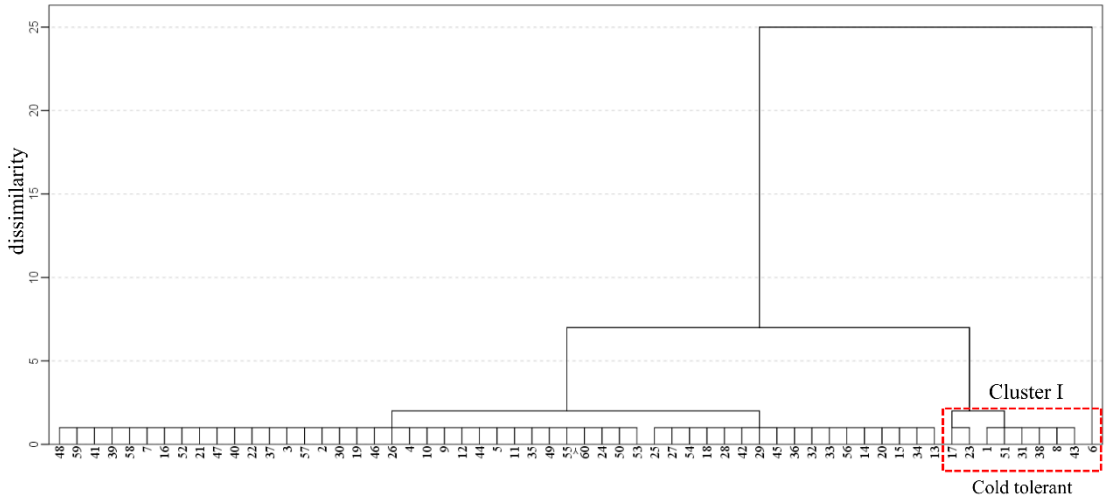

Figure 1. Cluster analysis of germination index at germination.

3.2. Effects of Low-Temperature Stress on Plant Height and Dry Matter Weight of Different Soybean Cultivars in the Seedling Stage

Among the 18 experimental cultivars, the effect of low-temperature stress on the plant height of cultivars No. 23, No. 51, No. 12, and No. 11 had no significant effect. Low-temperature treatment considerably decreased the plant height of the other cultivars, with decreases ranging between 15.81 and 37.73% compared to the control (Figure 2). Furthermore, cultivars No. 24 and No. 46 had the biggest decreases, of >30%, compared with those of other cultivars.

The shoot dry weight of the 18 cultivars subjected to low-temperature treatment decreased compared to the control group, ranging between 7.4–56.17%. However, the performance differed among different cultivars. For example, the performance of cultivar No. 50 decreased by 7.4%, whereas the performances of cultivars No. 31, No. 38, No. 5, and No. 49 decreased by >50%, proving that low temperature had a considerable effect on the growth of these cultivars.

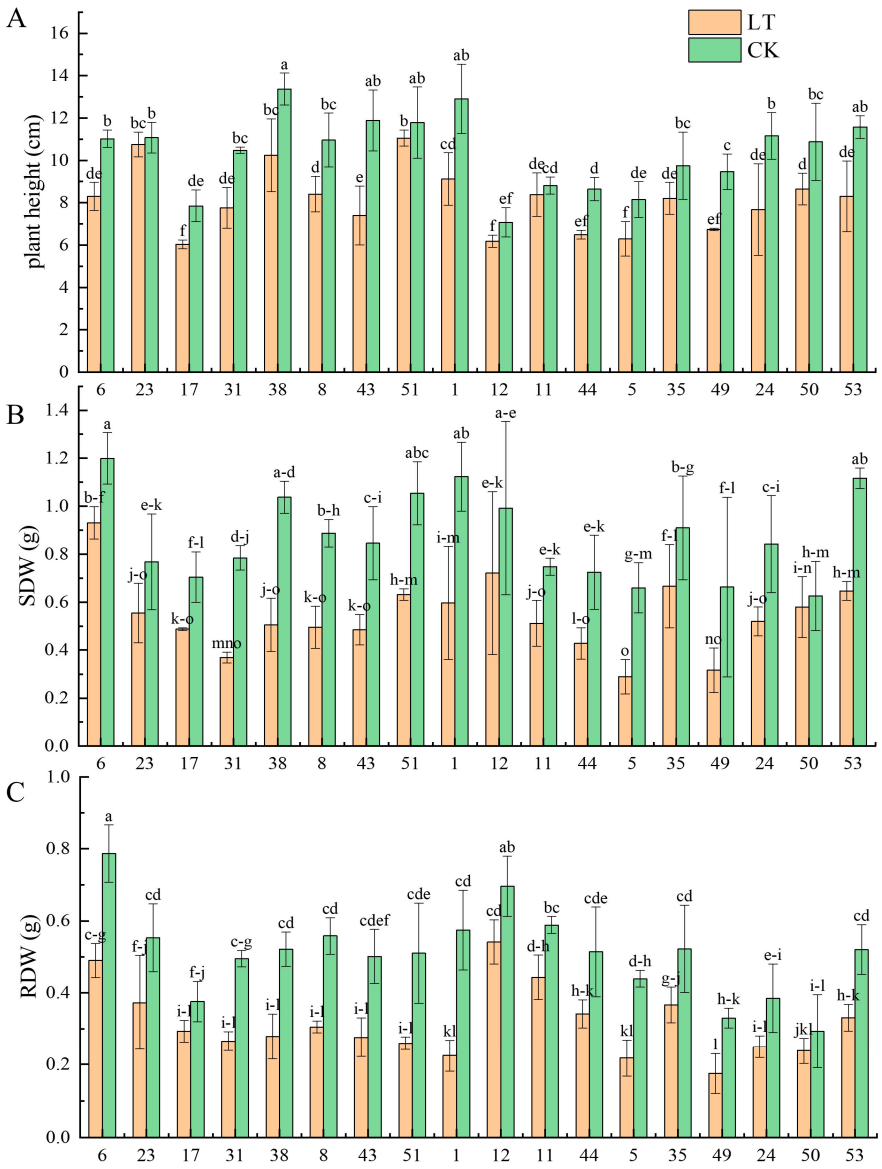

Figure 2. The plant height and dry weight changes of different soybean cultivars under low-temperature stress at the seedling stage: (**A**) plant height, (**B**) shoot dry weight, and (**C**) root dry weight. Different letters indicate significant differences according to Duncan's single-factor variance test at the 5% level of significance, and data are presented as mean ± standard error (n = 3). LT (low-temperature treatment) and CK (control group). Different letters in treatments indicate significant differences according to Duncan's single factor variance test at the 5% level.

Under low-temperature treatment, the change trend of root dry weight was similar to that of shoot dry weight. The root dry weight of all the tested cultivars decreased at low temperatures, ranging between 18.59–60.92%. Cultivars No. 13 and No. 9 had the biggest root dry weight decreases when compared with the control group, decreasing by 50.41 and 60.92%, respectively.

Shoot and root dry weights decreased under low temperatures, but the degree was different in the two parts in the different cultivars, resulting in differences in the root–shoot ratio. Among them, the root–shoot ratios of No. 17, No. 31, No. 38, No. 12, No. 11, No. 44, No. 35, No. 49, No. 24, and No. 53 cultivars increased under low temperature, indicating that their shoots were more affected by low temperature than the roots. Contrastingly, the root–shoot ratio of the other cultivars decreased under low temperatures, indicating that the roots were more affected by low temperatures than the shoots.

3.3. Effects of Low Temperature on Oxidative Stress of Different Soybean Cultivars

Low-temperature stress considerably affected the POD activity and MDA content of soybean seedlings (Figure 3). Compared with the control group, the POD activities of most cultivars was upregulated at low temperatures, whereas the POD activities of No. 12, No. 35, and No. 24 cultivars decreased. Among all the cultivars, the POD activity of cultivar No. 23 was more upregulated (>109.59%).

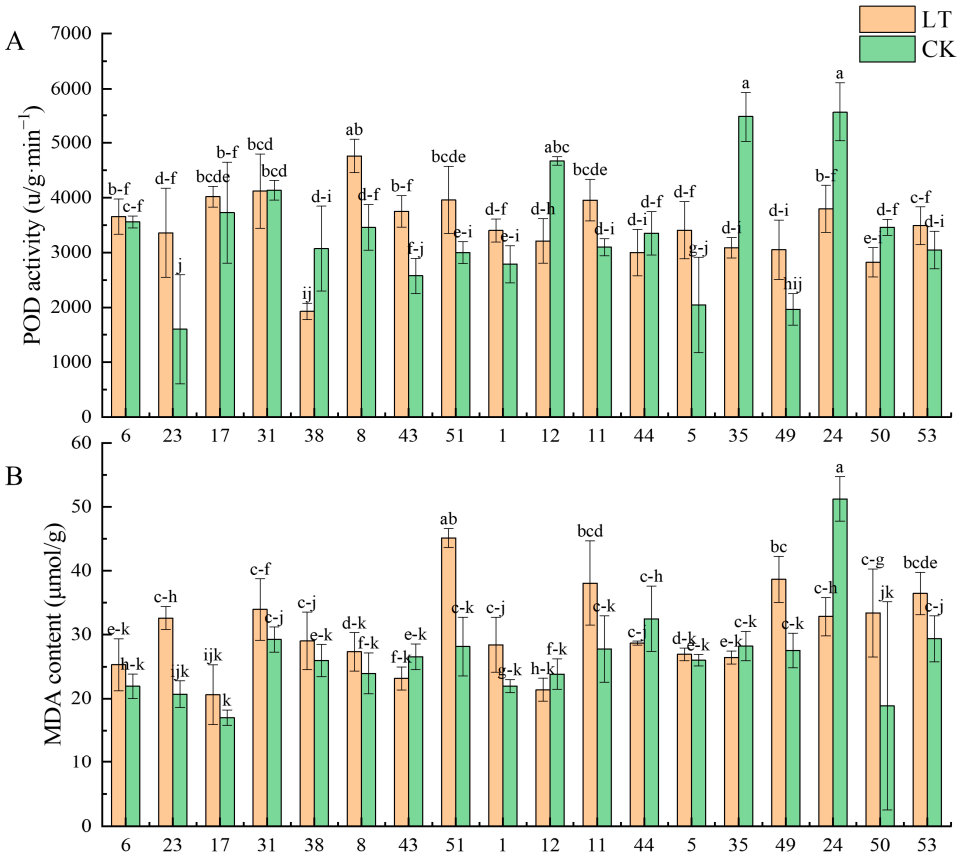

Figure 3. The peroxidase (POD) activities (**A**) and malonaldehyde (MDA) contents (**B**) of different soybean seedlings under low-temperature stress. LT (low-temperature treatment) and CK (control group). Different letters in treatments indicate significant differences according to Duncan's single factor variance test at the 5% level.

MDA is a product of membrane lipid oxidation, and its level in leaves can reflect the degree of oxidative stress in crops. Among the 18 tested cultivars, the MDA contents of No. 23, No. 51, and No. 50 increased considerably, proving that these cultivars were severely damaged by oxidation. Correspondingly, under low temperature, the POD activities of No. 23 and No. 51 varieties were higher than that of the control. Contrastingly, low POD activity and high MDA content was observed in No. 50. After comparison, we found that the reactions of MDA and POD in different varieties was different. Therefore, we determined the correlation between MDA and POD (Table 2). The results showed that there was no significant correlation between POD and MDA. Additionally, the MDA content of No. 24 decreased considerably, and its POD activity was lower than that of the control. Therefore, from the oxidative stress perspective, we found that No. 24 cultivar may tolerate low temperatures.

Table 2. Correlation analysis between POD and MDA.

		MDA
POD	Pearson correlation	0.146
	Significance	0.086
	Number of cases	18

3.4. Effect of Low Temperature on the Content of Osmotic Regulating Substances in Different Soybean Cultivars

The proline content varied under the influence of low temperatures, but the performance of each cultivar was inconsistent (Figure 4). The proline content in No. 44 had the greatest increase (31.67%) compared with the control. The proline contents of No. 17, No. 31, No. 44, No. 5, No. 49, No. 50, and No. 53 increased considerably, but those in other cultivars did not change. Under low temperatures, soluble sugars increased considerably compared with the control in all tested cultivars (ranging between 87.45–280.59%) (Figure 4B). The performance of soluble proteins was in contrast with that of soluble sugars. After low-temperature treatment, the soluble protein content of most cultivars decreased considerably; cultivars No. 17, No. 31, and No. 53 had no significant changes in soluble protein contents compared to those of the control group.

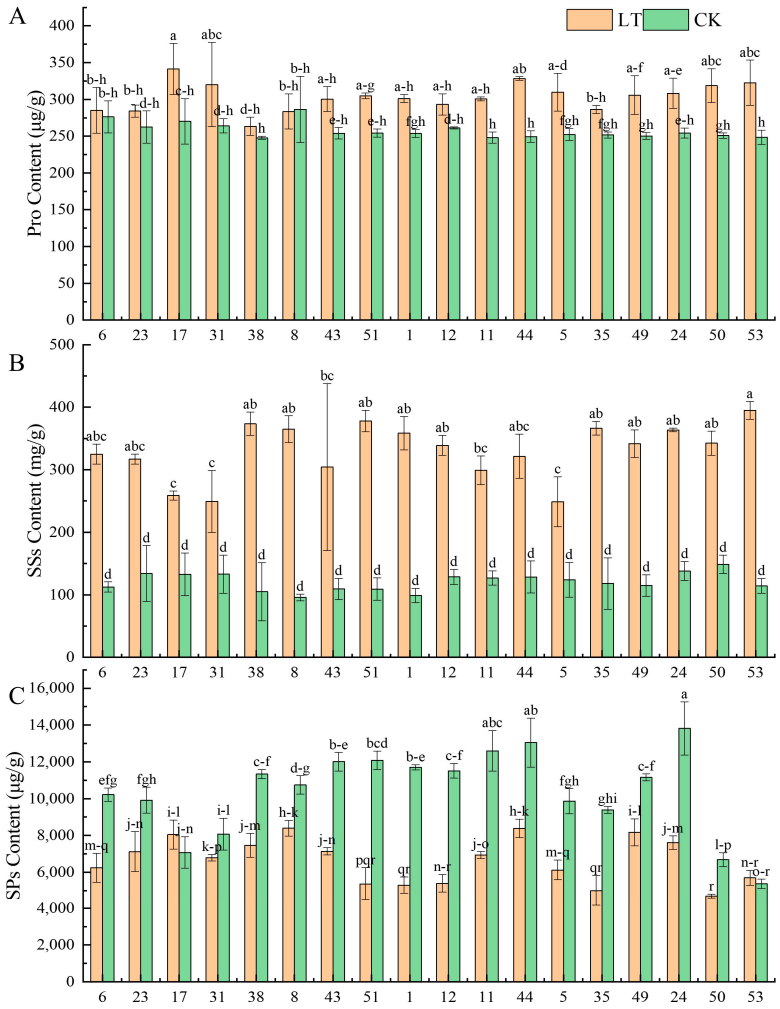

Figure 4. The proline contents (**A**), the soluble sugar contents (**B**), and the soluble protein contents (**C**) of different soybean seedlings under low-temperature stress. LT (low-temperature treatment) and CK (control group). Different letters in treatments indicate significant differences according to Duncan's single factor variance test at the 5% level.

3.5. Comprehensive Analysis

To eliminate the differences caused by the basic traits of the cultivars, relative values were used to describe the responses of plants to low temperatures (Equation (5)). Based on Equation (6), all physiological indices were integrated into three comprehensive indices (PC-1, PC-2, and PC-3). Each comprehensive index contains all the physiological indices, but different physiological indices have different effects on them, which is reflected in the load (value) and direction (±) of physiological indices (Table 3). The larger the value, the more similar the comprehensive index is to the physiological index. In the first principal component, shoot dry weight, root dry weight, and plant height contributed considerably, indicating that the first principal component reflected the growth of seedlings at low temperatures. The second principal component contributed more to proline, soluble protein, and root–shoot ratio than other parameters, indicating that the second principal component reflected seedling protein accumulation. The third principal component accounted for the largest proportion of POD and MDA, indicating that the third principal component was closely related to the antioxidant capacity of seedlings. The contribution values of the three comprehensive indicators were 27.74, 26.69, and 15.87%, respectively. According to Formula (8), the weights of the three indicators are 0.3945, 0.3797, and 0.2257, respectively.

Table 3. Coefficient, eigenvalues, variance contribution rates, and accumulated contribution rates of the principal components.

Traits	PC-1	PC-2	PC-3
POD	−0.04473	−0.34415	0.62143
MDA	0.28397	−0.3722	0.35187
SSs	−0.35347	−0.24344	−0.33836
SPs	0.04621	0.27383	0.37371
PRO	0.14732	0.41636	0.32963
H	0.40699	−0.28496	0.01509
SDW	0.54221	0.24179	−0.1644
RDW	0.54667	−0.05584	−0.30231
R/S	−0.10045	0.54096	0.09252
Eigenvalue	2.49696	2.40266	1.42817
Percentage of Variance (%)	27.74	26.69	15.87
Cumulative (%)	27.74	54.44	70.31

POD, peroxidase; MDA, malondialdehyde; SS, soluble sugar; SP, soluble protein; Pro, proline; H, plant height; SDW, shoot dry weight; RDW, root dry weight; R/S, root–shoot ratio.

To eliminate the influence of different orders of magnitude on the evaluation results, the comprehensive index was standardized based on the membership function method (Formula (7) (μ1, μ2, and μ3 represents the standardized value of the comprehensive index)), and the results are presented in Table 4. The D value was calculated using Formula (9). As a comprehensive index value of seedling tolerance to low-temperature stress, the higher the D value, the better the tolerance to low temperatures. Simultaneously, 18 cultivars were analyzed using cluster analysis according to the D value (Figure 5). All cultivars were divided into three categories. Cluster I contained nine cultivars considered moderately tolerant to low temperatures. Cluster II included six low-temperature-tolerant cultivars, namely, Heinong45, Suinong42, Henong67, Kenfeng8, Hefeng39, and Dongnong42. Three cultivars in Cluster III were sensitive to low temperatures: Hefeng55, Hefeng26, and Kendou40.

Considering the D value as the dependent variable and the relative value of each index as an independent variable, stepwise regression analysis was carried out, in which the root–shoot ratio did not follow a normal distribution; thus, it was excluded and calculated using the other eight indices. Finally, the regression equation was established as follows:

$$D = 0.237 - 0.253\ X_1 + 0.465\ X_2 + 0.477\ X_3 + 0.164\ X_4\ (R^2 = 0.987) \quad (11)$$

where X_1, X_2, X_3, and X_4 represent soluble sugar, root dry weight, proline, and soluble proteins, respectively. We used the D value results obtained using this equation for hierarchical clustering, which was consistent with the original results (Figure 5), confirming the reliability of the regression equation.

Table 4. Weight (*Wi*), membership function values, and D values ranking.

Cultivars	Subordinate Function Value			D Value
	μ1	μ2	μ3	
6	0.5034	0.4477	0.3158	0.4399
23	0.7433	0	1	0.5189
17	0.7999	1	0.7636	0.8676
31	0.5851	0.9494	0.7235	0.7546
38	0.2555	0.3601	0.6954	0.3945
8	0.0244	0.2472	0.3258	0.1770
43	0.2608	0.5582	0.5246	0.4332
51	0.5114	0.1661	0.5789	0.3955
1	0	0.0828	0.2512	0.0881
12	0.5738	0.8279	0.1536	0.5754
11	0.7542	0.5646	0.6311	0.6544
44	0.5036	0.949	0.4125	0.6521
5	0.4774	0.6554	0.8961	0.6394
35	0.3947	0.7288	0.0332	0.4399
49	0.0426	0.489	0	0.2025
24	0.3439	0.953	0.1422	0.5296
50	1	0.5942	0.5239	0.7384
53	0.233	0.5768	0.4447	0.4113
weight	0.3945	0.3797	0.2257	

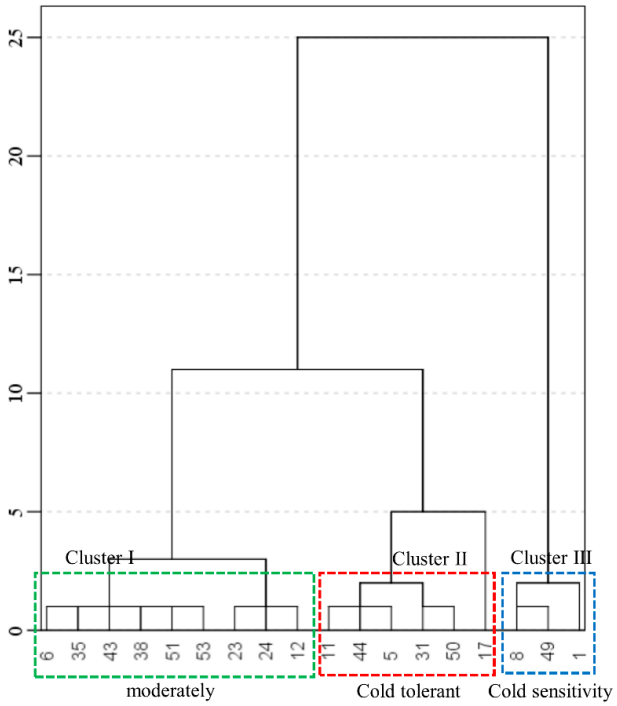

Figure 5. Cluster analysis of comprehensive evaluation results at the seedling stage.

3.6. Grey Correlation Degree Analysis

We used grey correlation analysis (Formula (10)) to analyze the correlation of several physiological indices with the D value (Table 5). The results showed that root dry weight, shoot dry weight, and proline had the greatest influence on the D value, proving their

importance in soybean seedling tolerance to low-temperature stress, whereas POD, soluble sugar, and MDA had the least influence on the D value, indicating that the above indices have little influence on soybean resistance to low temperatures. Therefore, they may not be the best traits for low-temperature improvement.

Table 5. Grey correlation analysis results.

Index	POD	MDA	SSs	SPs	Pro	H	RDW	SDW	R/S
Correlation degree	0.5902	0.6387	0.5926	0.6755	0.7036	0.6892	0.7506	0.7181	0.7072
Correlation order	9	7	8	6	4	5	1	2	3

POD, peroxidae; Melonaldehyde, MDA; SS, soluble sugars; SP, soluble proteins; Pro, proline; H, plant height; RDW, root dry weight; SDW, shoot dry weight; R/S, root–shoot ratio.

3.7. Correlation Analysis between the Germination and Seedling Stages

Comparing the cluster analysis results at the seedling stage (Figure 5) with those at the germination stage (Figure 1), we found that some cultivars were tolerant to low temperature at the germination stage and sensitive to low temperature at the seedling stage. Therefore, a correlation analysis was conducted using the germination index GI at the germination stage and the comprehensive index D value of the seedling experiment (Table 6). The results revealed that the significance was 0.739 and the correlation coefficient was −0.085. There was no correlation between the performance at low temperatures in the germination stage and that of the seedling stage. Therefore, the evaluation of the low-temperature tolerance of soybeans should be divided into different periods rather than a single stage.

Table 6. Correlation analysis between D value and GI.

		GI
D value	Pearson correlation	−0.085
	Significance	0.739
	Number of cases	18

3.8. Temperature-Recovery Treatment

The temperature-recovery treatment was carried out on the low-temperature-tolerant and -sensitive cultivars obtained by comprehensive evaluation (the low-temperature treatment cultivar was transferred to a 25 °C incubator after 72 h, and the survival rate was counted after growing for 72 h). The sensitive and cold-tolerant cultivars exhibited different survival rates (Figure 6). Cultivar No. 31 (Suinong42) had the best survival rate (79.63%), whereas No. 49 (Kendou40) performed the worst after temperature recovery. The survival rate was only 29.63%.

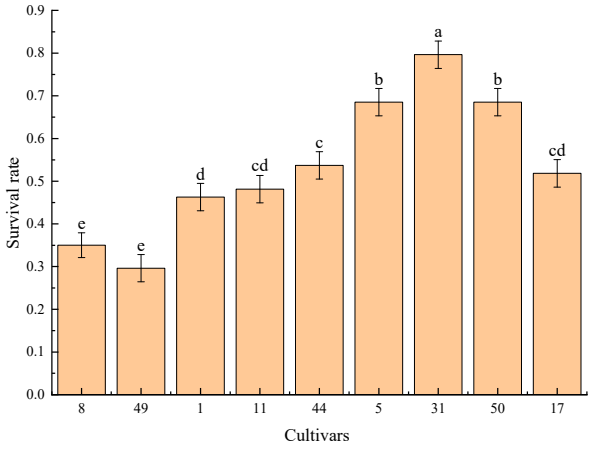

Figure 6. Seedling survival rate after temperature recovery. Different letters in treatments indicate significant differences according to Duncan's single factor variance test at the 5% level.

4. Discussion

4.1. Identification and Improvement of Plant Cold Tolerance

With the increasing complexity of climate change and population growth, there is a greater demand for crop cultivars that adapt and produce food in adverse environments [29]. From the perspective of crop-stress-resistant breeding, obtaining potential key resistance genes and stress-resistant mechanisms that can be used as potential targets and directions for molecular breeding to cultivate cultivars that meet human needs is important [30]. Generally, scholars have obtained related cultivars through cultivar screening and analyzed their physiological mechanisms [31,32]; however, different screening methods lead to different results. Current studies often evaluate cultivar resistance based on performance at the germination or seedling stages [33,34]. Based on the correlation analysis, the present study found no correlation between low-temperature resistance at the germination and seedling stages. Specifically, during the life cycle of a soybean crop, resistance could not be defined by the performance of a particular growth stage, as this may mislead future studies. The performance of crops in a period vulnerable to natural disasters must be analyzed considering the climatic conditions of a particular region.

In the agricultural production system, crops must have a certain tolerance to low temperatures, and seedlings must quickly restore growth after temperature-recovery to ensure a normal production cycle. Therefore, this implies that the "ideal breed" we pursue can quickly change from "defense against low temperature" to a "rapid growth state" after temperature recovery. However, most studies on the balance between growth and defense have focused on defense against herbivores [35] and few studies on the mechanisms of low-temperature and temperature recovery. This study found that different soybean varieties showed different survival rates after temperature recovery. By overexpressing Glycosyltransferase OsUGT90A1 [36], researchers improved the plasma membrane integrity and improved the freezing resistance of seedlings, but the low-temperature seedling survivability of seedlings was not improved. The low-temperature seedling survivability is a polygenic traits [37]. The omnigenic theory previously proposed shows that almost any gene can influence a complex trait [38], but their contributions are different. Therefore, in the current study, if the knockout or overexpression of a gene can have a significant impact on the indicators we care about (such as antioxidant enzyme activity, reactive oxygen species content, plasma membrane integrity, etc.), it can be considered as a potential improvement target. When multiple genes are integrated, it is very likely to have an ideal impact on the composite traits, so as to obtain the varieties we want to survive after adversity.

In the present study, cultivars obtained by simulating low temperatures can be used as experimental materials in future studies on the physiological mechanisms of crops under temperature fluctuations and to improve the study of crops to cope with temperature changes.

4.2. Changes in MDA and Antioxidant Enzymes

When plants are exposed to natural disasters, such as drought and low temperature, ROS production leads to cell damage [39]. Therefore, as a product of the ROS oxidation of membrane lipids, MDA is also considered to be related to stress resistance in plants. According to a previous study, MDA levels are negatively correlated with stress resistance [40]. In the present study, the MDA content of most cultivars increased considerably under low temperatures, which confirmed the damage to soybean leaves caused by low temperatures and the change in antioxidant enzyme activity represented by POD. However, the changing POD activity and MDA content trends differed in all cultivars. Various ROS may be produced in cells at low temperatures, such as $O^{2-}\cdot$, $\cdot OH$, and H_2O_2 [41], that may trigger membrane lipid peroxidation to produce MDA, corresponding to various enzyme decomposition free radicals, such as SOD and CAT. While POD catalyzes the decomposition of H_2O_2, the ROS produced in some cultivars may not be decomposed by POD but can still oxidize membrane lipids, resulting in the accumulation of MDA and no considerable effect on POD content. Additionally, antioxidant enzymes affect the survival

and growth of plants after temperature recovery. According to Buchanan et al. [42], the duration of low temperatures is not the most damaging for crops, and the critical moment to determine whether plants can survive does not come until the temperature is restored. Studies consider that this is related to whether plants can remove ROS in time because the overexpression of antioxidant enzyme-related genes through genetic engineering considerably improves the tolerance of plants to low temperature [43]. The current methods to improve the low-temperature tolerance of plants by chemical regulation are correlated with the activity of antioxidant enzymes. Wu et al. [44] found that exogenous NO improved the low-temperature tolerance of plants by improving the ascorbate–glutathione cycle. Exogenous zeaxanthin improved the survival ability of pepper at low temperatures by increasing antioxidant enzymes such as POD and CAT [45].

4.3. Accumulation of Compatible Solutes

A comparison of the physiological indices of 18 soybean cultivars revealed that the soluble sugar content of all soybean cultivars increased considerably under low-temperature stress and the proline content increased simultaneously. Under abiotic stress, plants accumulate soluble sugars and proline [46,47]; this may be the result of changes in enzyme activity. The *APL1* gene (encoding glucose pyrophosphorylase) cloned from grape plants was ectopically expressed in Arabidopsis and tomato, which considerably improved the survival rate of plants at low temperatures owing to the conversion of starch to soluble sugar, which reduces cell osmotic potential and provides energy [48]. Additionally, molecular chaperone proteins, represented by proline, protect the integrity of cell membranes and protein folding and maintain the advanced structure of proteins and other physiological activities [49,50]. Therefore, these compounds are used as indicators of cold tolerance in crops [51]. However, in the present study, no soluble protein accumulation was observed in the tested cultivars, which is different from the well-known resistance of plants to abiotic stress [40]. According to Zheng et al. [52], the soluble protein content in *Jatropha carcass* leaves decreased at low temperatures, whereas the rate of photosynthesis decreased, and >50% of the soluble protein in plants was Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco [53]). Therefore, we speculated that the decrease in Rubisco at low temperatures decreased soluble protein content.

4.4. Screening of Cold Tolerance Indices at the Seedling Stage

Low temperatures considerably affect most crop physiological indices. Based on the grey correlation analysis, shoot dry weight, root dry weight, and the root–shoot ratio of soybean seedlings had the greatest contribution to the D value, proving that the above indices were sensitive to low temperatures. At the seedling stage (vegetative growth stage), the roots and shoots grow simultaneously; the roots absorb water, nitrogen, and other mineral nutrients [54], and the shoots fix C through photosynthesis, whereas after experiencing abiotic stress, the above-ground and underground parts display different responses. Walne et al. [55] found that biomass resources are prioritized in the root system at low temperatures. Moses et al. found that low temperatures in sweet pepper increased nitrogen distribution in the roots, decreasing the C/N ratio [56]. Based on previous conclusions, we believe that plants tend to ensure root growth and better absorb nutrients to help them resist adverse environmental factors. This inference was supported by Jiang et al. [57], they found that low temperatures increase underground biomass of alfalfa seedlings, while plants with higher biomass had a higher growth rate after the restoration of conducive temperature than those with lower biomass. In the present study, the decrease in root dry weight of the cultivars tolerant to low temperatures at the seedling stage was less than that in sensitive cultivars, and showed a higher root–shoot ratio. Contrastingly, the root dry weights of sensitive cultivars decreased considerably. Maintaining good root traits may promote resistance to low-temperature stress in soybeans.

However, the analysis of physiological indices in the present study was not comprehensive, and there was no analysis of the photosynthetic characteristics of soybean seedlings, which is one of the limitations of the present study.

5. Conclusions

By determining the performance at the germination stage and the physiological indices at the seedling stage, we obtained the soybean cultivars Suinong42 and Kendou40, which are tolerant and sensitive to low temperatures in the early-growth stage of soybean. In addition, root dry weight, shoot dry weight, and proline content can be used as key indicators to measure the cold tolerance of soybean seedlings. Simultaneously, our study also found no correlation between germination and seedling stages responses to low temperatures. Therefore, plant resistance cannot be evaluated by the performance of a period alone.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13071716/s1, Table S1: Germination parameters of different soybean genotypes under low-temperature.

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