

Article

Comprehensive Evaluation of 65 Leafy Mustard Cultivars for Chilling Tolerance to Low Temperature Stress at the Seedling Stage

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Abstract: Mustard is an important cash crop of the genus *Brassica* in the family Cruciferae. Low temperature is an important environmental factor limiting the growth of mustard. In this study, 65 leafy mustard cultivars were used as experimental materials, 25 °C was set as the control temperature, and 5 °C was set as chilling stress temperature to investigate the physiological response of chlorophyll (Chl) content, soluble sugar (SS) content, proline (Pro) content, antioxidant enzyme activity, malondialdehyde (MDA) content, and chlorophyll fluorescence to chilling injury. The chilling tolerance coefficients of each individual index were measured and correlation analysis, principal component analysis (PCA), the membership function method, and cluster analysis were applied to evaluate chilling tolerance. In a comprehensive analysis, the most chilling-tolerant cultivar was SJTKJ, the least chilling-tolerant cultivar was DX. Stepwise regression was used to establish a mathematical model for evaluating the chilling tolerance of mustard, and four chilling tolerance identification indices, including Fv/Fm, ΦPSII, POD activity, and Rfd were screened. This study provides a reference for the evaluation of the chilling tolerance of mustard and the breeding of new chilling-tolerant cultivars.

Keywords: *Brassica*; chilling injury; antioxidant enzyme activity; chlorophyll fluorescence; comprehensive analysis



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1. Introduction

Mustard (*Brassica juncea* L.), an annual herbaceous plant of the genus *Brassica* in the family Cruciferae, is a speciality vegetable of China and is rarely cultivated in Europe and the United States [1,2]. China is one of the primary origin centres of mustard; Northwest China is the origin of mustard in China, and the Sichuan Basin is the secondary origin centre of mustard [2]. Mustard has a long history of cultivation in China, is rich in germplasm resources and is widely cultivated; mustard is grown all over the country, except in arid and alpine areas [1]. China divides mustard into root mustard, stem mustard, leaf mustard, and carex mustard, representing four categories of 16 varieties [3], of which leaf mustard is mainly distributed in the southern region of China. Mustard is rich in nutritional value, and its leaves are rich in chlorophyll, β-carotene, ascorbic acid, and minerals such as potassium and calcium [4]. Mustard seeds contain proteins, carbohydrates, dietary fibres and fats, and a variety of vitamins and minerals [5,6]. Mustard prefers cool and moist environments where the growth temperature is 15–22 °C, and plant growth is inhibited when the temperature is lower than 12 °C [7,8]. Mustard has a short growth cycle and can be cultivated all year round, but low temperature and chilling injury in the fall, winter and early spring hinder the growth and development of mustard, especially low temperatures in the seedling stage, which have a greater impact on leafy mustard and can lead to weakening

of the plant, cessation of growth and even death. Thus, screening chilling-tolerant mustard cultivars is highly important for guiding the cultivation and production of mustard and the genetic improvement of chilling tolerance. Currently, most of the research on mustard has focused on cultivation technology, disease resistance, drought resistance, breeding of sterile male lines, and gene function studies. In contrast, less research on the chilling tolerance and leaf photosynthetic phenotypic characteristics of leafy mustard has been reported.

Low temperature is one of the major abiotic stresses affecting plant growth and development and crop species distribution, and low-temperature stress is categorized into chilling and freezing stress [9]; exposure to temperatures below 0 °C results in freezing injury, whereas chilling injury occurs as a consequence of exposure to low but non-freezing temperatures (above 0 °C). Low temperature can have significant negative effects on plant nutrient metabolism and physiology, leading to the inhibition of plant growth and development, decreasing yield and quality, and in severe cases even leading to plant death [10,11]. Under low-temperature stress, plant cell membrane structure and lipid composition change, and intracellular electrolyte and amino acid leakage occurs. In addition, protoplasm, protein content, enzyme activity, and the ultrastructure of various cellular components change. In order to cope with non-freezing temperatures, plants trigger a series of “cold acclimation” response mechanisms [12]. To overcome the stress generated by exposure to low non-freezing temperatures, plants can trigger a series of events leading to changes in gene expression that induce biochemical and physiological modifications that enhance their tolerance. Genes responsive to low temperatures include those encoding soluble sugars and proline, xanthophylls and carotenoids; late embryogenesis-enriched proteins; heat shock proteins; cold shock proteins; and dehydrins [13]. Low temperature increases the relative conductivity (REC), malondialdehyde (MDA) content, soluble protein (SP) content, proline (Pro) content, and defence enzyme activities of plant leaves [14,15]. Low temperature affects the uptake and distribution of mineral elements in plants, leading to physiological disorders such as plant malnutrition [16]. Low temperature affects the formation of internal structures in plant leaves; destroys the integrity and function of chloroplasts; and significantly reduces the maximum fluorescence (Fm), maximum photochemical efficiency (Fv/Fm), and actual photochemical and quantum efficiency (ΦPSII), leading to a decrease in the rate of plant photosynthesis [17,18].

The physiological and biochemical characteristics of different plants of different species and their chilling tolerance performance under low-temperature stress are different, and most of the previous studies used a single approach to evaluate chilling tolerance and lacked a systematic study to assess chilling tolerance; comprehensive evaluation and comparison play an important role in physiological response and resilience assessment [19]. A comprehensive evaluation method combining principal component analysis (PCA), membership function method, and cluster analysis has been applied to evaluate the chilling tolerance of *Arachis hypogaea* [20], *Cucumis melo* [21], *Solanum lycopersicum* [22], *Vitis vinifera* [23], and other plants. At present, there are few reports on the physiological characteristics and chlorophyll fluorescence characteristics of mustard under low-temperature stress, and comprehensive evaluations of the chilling tolerance of mustard cultivars are rare.

In this study, 65 leafy mustard cultivars were treated at 5 °C and 25 °C, and the chlorophyll (Chl) content, soluble sugar (SS) content, proline (Pro) content, antioxidant enzyme activity, and malondialdehyde (MDA) content of mustard leaves were determined under different treatments. Additionally, chlorophyll fluorescence technology was used to determine the photosynthetic performance of leaves. Furthermore, correlation analysis, principal component analysis (PCA), membership function method, and cluster analysis were used to comprehensively evaluate the chilling tolerance of mustard cultivars. The objective of this study was to clarify the tolerance of different leafy mustard cultivars to chilling injury, select cultivars with strong chilling tolerance, establish a mathematical model for evaluating the chilling tolerance of mustard and screen the main indicators for evaluating chilling tolerance. The results will provide a reference basis for the selection

and breeding of chilling-tolerant cultivars of mustard, and lay a research foundation for the comprehensive evaluation of chilling tolerance of mustard cultivars.

2. Materials and Methods

2.1. Plant Materials and Treatments

The test materials included 65 local varieties, breeding materials, and mustard cultivars, which were provided by two units, namely, the Crop Research Institute of Fujian Academy of Agricultural Sciences and the Longyan Institute of Agricultural Science (Table 1).

Table 1. Mustard cultivars obtained across China.

No.	Cultivar	Origin	No.	Cultivar	Origin
1	TCKJ	Shenzhen, Guangdong	34	ZJS	Beijing, China
2	MQD	Fuzhou, Fujian	35	XYJT	Shanghai, China
3	MQDG	Fuzhou, Fujian	36	TCDNN	Yibin, Sichuan
4	KYHR	Xingning, Guangdong	37	ZHQC	Mianyang, Sichuan
5	XYXLH	Wuhan, Hubei	38	KJ2	Beijing, China
6	KROK	Nanning, Guangxi	39	HYQC	Beijing, China
7	TW	Taiwan, China	40	YYGG	Guangzhou, Guangdong
8	ZZ1	Zhangzhou, Fujian	41	DJCZ	Guangzhou, Guangdong
9	LYCJ	Zhuzhou, Hunan	42	JXSD	Guangzhou, Guangdong
10	LJ3	Longyan, Fujian	43	ZY	Linyi, Shandong
11	LJ2	Longyan, Fujian	44	GLTJT	Nanning, Guangxi
12	LJ1	Longyan, Fujian	45	GLHBT	Nanning, Guangxi
13	LYCJ2	Longyan, Fujian	46	JS2	Shantou, Guangdong
14	PH	Zhangzhou, Fujian	47	CTNS1	Longyan, Fujian
15	ZZ2	Zhangzhou, Fujian	48	CTNS2	Longyan, Fujian
16	FZKG	Fuzhou, Fujian	49	AJQT	Fuzhou, Fujian
17	588	Guangzhou, Guangdong	50	HNHBT	Nanning, Guangxi
18	ZBTJY	Longyan, Fujian	51	GCKRD	Nanning, Guangxi
19	GDJN	Guangzhou, Guangdong	52	H19-10	Wuhan, Hubei
20	YDJY	Longyan, Fujian	53	JC2	Longyan, Fujian
21	YDKJQ	Longyan, Fujian	54	WHYL	Wuhan, Hubei
22	LCXQZ	Longyan, Fujian	55	QC	Huanggang, Hubei
23	LCXQ	Longyan, Fujian	56	YSJY1	Longyan, Fujian
24	2951	Longyan, Fujian	57	YSJY2	Longyan, Fujian
25	SS1	Longyan, Fujian	58	DX	Taizhou, Zhejiang
26	SC	Zigong, Sichuan	59	YSJY3	Longyan, Fujian
27	2952	Longyan, Fujian	60	PK2	Longyan, Fujian
28	MBDX	Shaoguan, Guangdong	61	CSJY1	Chishui, Guizhou
29	SS2	Longyan, Fujian	62	CSJY2	Chishui, Guizhou
30	ZYJ	Longyan, Fujian	63	XS	Longyan, Fujian
31	YDKJT	Longyan, Fujian	64	XY	Longyan, Fujian
32	AJKD	Nanning, Guangxi	65	SJTKJ	Xingning, Guangdong
33	GR	Chongqing, Sichuan			

This experiment was carried out in a small thin-film greenhouse and a containerized plant factory at the Digital Institute of Fujian Academy of Agricultural Sciences. Seedlings were raised on tidal seedbeds and sown in 128-well trays with Hoagland's nutrient solution (EC 1.0 mS/cm, pH 6.5) [24]. After the seedlings were grown to the four-leaf stage and transplanted into nutrient pots (aperture of 25 cm, height of 21 cm) for one week of conventional culture, the plants were moved to an artificial climate chamber for cultivation

with a relative humidity of 70–80%; the light condition was LED light with the ratio of red and blue light of 7:3, and the light intensity was set at $250 \mu\text{mol}/(\text{m}^2 \cdot \text{s})$. Two different temperature treatments were employed: 25°C (CK) and 5°C (chilling stress). After 21 d of treatment, the physiological and biochemical indices of the leaves were collected. Each treatment was repeated three times, and three plants were used for each replicate. Representative plants were selected for chlorophyll fluorescence imaging and parameter collection.

2.2. Determination of Chlorophyll Content

Chlorophyll (Chl) content was determined using the acetone-ethanol mixture method [25]. First, fresh plant leaves (0.2 g) were weighed, 10 mL acetone-ethanol mixture was added, and the mixture was placed in the dark with a stopper. After immersion until the leaf tissue had completely turned white, the mixture of the extraction solution was filtered and adjusted to a volume of 25 mL. The acetone-ethanol mixture was used as a blank control, and the absorbance was determined using a UV spectrophotometer (T2600, Yoke, Shanghai, China) at wavelengths of 663 nm, 645 nm, and 440 nm.

2.3. Determination of Soluble Sugar Content

Soluble sugar (SS) content was determined using the anthrone colorimetric method [26]. First, fresh plant leaves (0.3 g) were weighed and 10 mL distilled water was added. The leaves were sealed with plastic film, extracted in boiling water for 30 min (two extractions), and filtered. The final volume was adjusted to 25 mL. Then, 0.5 mL sample extract, 1.5 mL distilled water, 0.5 mL ethyl acetate reagent anthrone, and 5 mL concentrated sulphuric acid were added, and the mixture was shaken thoroughly. The test tube was immediately placed in a boiling water bath for 1 min, and the tube was removed and allowed to cool naturally to room temperature. The blank was used as a reference, and its absorbance was determined using a spectrophotometer (T2600, Yoke, Shanghai, China) at 630 nm.

2.4. Determination of Malondialdehyde Content

Malondialdehyde (MDA) content was determined using the thiobarbituric acid method [26]. Plant material (0.5 g) was weighed, 5 mL trichloroacetic acid (5%) was added, and the mixture was ground to a homogenate and centrifuged at $1006 \times g$ for 10 min. The supernatant (2 mL) and 2 mL thiobarbituric acid (0.67%) were mixed and heated in the boiling water bath for 30 min, cooled and then centrifuged once more. The absorbance values of the supernatant at 450 nm, 532 nm and 600 nm were determined using a spectrophotometer (T2600, Yoke, Shanghai, China).

2.5. Determination of Proline Content

Proline (Pro) content was determined using the sulfosalicylic acid method [26]. Plant leaves (0.5 g) and 5 mL sulfosalicylic acid (3%) were mixed up, and then extracted in a boiling water bath for 10 min, shaken frequently during the extraction process, and filtered after cooling. Then, the extract (2 mL), 2 mL glacial acetic acid and 2 mL acid ninhydrin reagent were added. The mixture was heated in a boiling water bath for 30 min to make the solution appear red. After cooling, toluene (4 mL) was added, and the mixture was shaken for 30 s. Then, the upper layer of liquid was transferred to a 10 mL centrifuge tube and centrifuged at $1006 \times g$ for 5 min. A pipette was used to gently aspirate the upper layer of the proline red toluene solution in a cuvette, with toluene serving as a blank control, and the absorbance at 520 nm was determined using a spectrophotometer (T2600, Yoke, Shanghai, China).

2.6. Determination of Defence Enzyme Activities

Superoxide dismutase activity (SOD, EC 1.15.1.1) was determined using the nitrogen blue tetrazolium (NBT) photoreduction method [26]. Plant leaves (0.5 g) were removed, 1 mL of precooled phosphate buffer (pH 7.8) was added, the mixture was ground into a pulp on an ice bath, and buffer was added to a final volume of 5 mL. Then, the homogenate was centrifuged for 10 min at $1789\times g$ at 4 °C, and the resulting supernatant was obtained as the crude SOD extract. First, 1.5 mL phosphate buffer (0.05 mol/L), 0.3 mL Met (methionine, 130 mmol/L), 0.3 mL NBT (750 $\mu\text{mol/L}$), 0.3 mL EDTA- Na_2 (disodium ethylenediaminetetraacetate, 100 $\mu\text{mol/L}$), 0.3 mL riboflavin (20 $\mu\text{mol/L}$), 0.05 mL enzyme extract, and 0.25 mL distilled water were added to a transparent test tube. The control tube was used to replace the enzyme extract with buffer. After mixing, one control tube was placed in the dark, and the other tubes were allowed to react for 20 min under 55 $\mu\text{mol}/(\text{m}^2\cdot\text{s})$ white light. After the reaction was complete, the control tube without light was used as a blank, and the absorbance of the other tubes was measured at 560 nm. SOD activity units were expressed as one enzyme activity unit (U) for 50% inhibition of NBT photochemical reduction.

Peroxidase activity (POD, EC 1.11.1.X) was determined using the guaiacol method [26]. Plant leaves (0.5 g) were weighed and ground into a homogenate with an appropriate amount of phosphate buffer (pH 5.5). The homogenate was centrifuged at $1006\times g$ for 10 min, after which the supernatant was transferred to a 25 mL volumetric flask. The precipitate was extracted twice with phosphate buffer and the supernatant was fixed at 25 mL and stored at low temperature for further use. The following reaction mixture was used for the enzyme activity assay: 2.9 mL phosphate buffer (0.05 mol/L), 1.0 mL H_2O_2 (2%), 1.0 mL guaiacol (0.05 mol/L), and 0.1 mL enzyme extract were added sequentially. The enzyme extract, which was heated and boiled for 5 min, was used as the control, and the reaction system was kept warm in a 37 °C water bath for 15 min immediately after the enzyme extract was added. Then, the mixture was quickly transferred to an ice bath, and the reaction was terminated by adding 2.0 mL trichloroacetic acid (20%). The mixture was centrifuged at $2795\times g$ for 10 min and appropriately diluted, and the absorbance was measured at 470 nm.

Catalase activity (CAT, EC 1.11.1.6) was determined using the UV absorption method [26]. First, fresh plant leaves (0.5 g) were ground into a homogenate with 3 mL precooled phosphate buffer (pH 7.8) and the homogenate was fixed in a 25 mL volumetric flask. The upper part of the clarified solution was taken and centrifuged at $1789\times g$ for 15 min, and the supernatant was collected as the crude peroxidase extract and stored at 5 °C for further use. First, 0.2 mL enzyme extract, 1.5 mL phosphate buffer (pH 7.8), and 1.0 mL distilled water were added, and the control blank tubes were cooked in a boiling water bath for 1 min to stop enzymatic activity. Then, all the test tubes were preheated at 25 °C. Next, 0.3 mL H_2O_2 (0.1 mol/L) was added to the tubes. The time was recorded immediately after each addition, and the samples were quickly poured into a quartz cuvette. Then, the absorbance was measured at 240 nm, and the readings were taken once every 1 min for a total of 4 min.

2.7. Determination of Chlorophyll Fluorescence Parameters

A chlorophyll fluorescence imager (FC800-D, Photon Systems Instruments (PSI), Drásov, Czech Republic) was used for the determination. First, the samples to be tested (whole live plants) were dark-adapted for 30 min in a dark environment in the greenhouse, and the value of the saturating pulse was set to 5250 $\mu\text{mol}/(\text{m}^2\cdot\text{s})$ on a Fluorcam10 system. Then, the samples to be tested were placed in the instrument at a distance of 30 cm from the lens, and the real-time initial fluorescence was determined based on an actinic light 2 value of 422 $\mu\text{mol}/(\text{m}^2\cdot\text{s})$ on the Fluorcam10 system. Then, the samples to be tested were placed into the instrument with a distance between the samples and the lens of 30 cm, and the real-time initial fluorescence value (F_0), the maximum photon efficiency of PSII (F_v/F_m), the effective photon yield of PSII (F_v'/F_m'), the actual photon efficiency of PSII (ΦPSII), the

nonphotochemical quenching coefficient (NPQ), and the steady-state fluorescence decay rate (Rfd) were obtained.

2.8. Chilling Tolerance Evaluation and Data Analysis

All measurements were repeated three times in this experiment. Fluorcam 7 was used to analyse the chlorophyll fluorescence imaging parameters. Excel 2021 software was used to process the raw data and make relevant graphs. SPSS 27.0 was used to perform principal component analysis and correlation analysis, and Origin 2021 was used to perform cluster analysis.

In order to eliminate the differences between the basic traits of different cultivars, chilling tolerance was evaluated using a single index of chilling tolerance coefficient (CTC). The chilling tolerance coefficient of a single index was calculated as follows:

$$CTC = \frac{\text{treatment measurements}}{\text{control measurements}} \quad (1)$$

According to fuzzy mathematics principles, the membership function method was used to convert various indicators into membership function values:

$$U(X_j) = \frac{(X_j - X_{\min})}{(X_{\max} - X_{\min})}, j = 1, 2, 3, \dots, n \quad (2)$$

$$W_j = P_j / \sum_{j=1}^n P_j, j = 1, 2, 3, \dots, n \quad (3)$$

In the formula, X_j denotes the j indicator; $X_{\min}$ and $X_{\max}$ denote the minimum and maximum values of indicator j , respectively; W_j denotes the importance of indicator j among all the indicators, i.e., weight; and P_j denotes the contribution of indicator j to the mustard cultivars obtained using principal component analysis.

$$D = P_j / \sum_{j=1}^n [U(X_j) \times W_j], j = 1, 2, 3, \dots, n \quad (4)$$

In the formula, D represents the integrated chilling tolerance assessment value of mustard cultivars; the larger the integrated assessment value is, the stronger the chilling tolerance is.

3. Results

3.1. Effect of Chilling Stress on the Physiological Indices of Leafy Mustard

As shown in Table 2, the mean Chl content, SS content, Pro content, SOD activity, CAT activity, MDA content, NPQ, and Rfd of mustard were greater under low-temperature treatment at 5 °C compared to the control (25 °C), with the average values of each index increasing by 21.12%, 82.40%, 108.39%, 87.58%, 49.17%, 103.72%, 29.37%, and 7.78%, respectively. The mean POD activity, F_v/F_m , F_v'/F_m' , $\Phi PSII$, and qP decreased, with average decreases of 25.00%, 1.13%, 3.85%, 15.56%, and 11.48%, respectively. Among them, SS, Pro, and MDA showed greater variation and greater coefficients of variation, at 70.58%, 108.06%, and 226.85%, respectively. F_v/F_m and F_v'/F_m' showed less variation and smaller coefficients of variation at 5.22% and 6.53%, respectively.

Table 2. Analysis of variation physiological indices of leafy mustard under chilling stress.

Index	Treatment	Minimum	Maximum	Mean	Standard Deviation	Coefficient of Variation%
Chl	5 °C	0.37	2.65	1.14	0.47	41.07
	25 °C	0.08	2.23	0.94	0.45	47.89
SS	5 °C	0.17	3.42	0.99	0.70	70.58
	25 °C	0.07	2.51	0.54	0.51	92.93
Pro	5 °C	17.01	303.53	56.84	61.42	108.06
	25 °C	16.38	253.47	27.28	29.41	107.81
SOD	5 °C	155.04	350.83	251.39	42.26	16.81
	25 °C	54.78	300.2	134.02	60.05	44.81
POD	5 °C	1027.39	4327.89	1953.05	702.05	35.95
	25 °C	1316.39	3843.67	2604.16	681.63	26.17
CAT	5 °C	66.42	861.33	379.14	194.27	51.24
	25 °C	78.42	790	254.16	151.65	59.67
MDA	5 °C	0.73	68.92	6.00	13.60	226.85
	25 °C	0.42	26.88	2.94	4.48	152.36
Fv/Fm	5 °C	0.60	0.84	0.79	0.04	5.22
	25 °C	0.66	0.85	0.80	0.04	4.71
Fv'/Fm'	5 °C	0.53	0.79	0.70	0.05	6.53
	25 °C	0.61	0.79	0.73	0.04	5.73
ΦPSII	5 °C	0.07	0.54	0.25	0.10	40.42
	25 °C	0.06	0.45	0.30	0.09	29.52
NPQ	5 °C	0.17	1.67	0.66	0.32	48.26
	25 °C	0.18	1.54	0.51	0.20	39.18
qP	5 °C	0.11	0.71	0.36	0.14	39.42
	25 °C	0.09	0.60	0.41	0.11	27.23
Rfd	5 °C	0.40	2.95	1.30	0.59	45.02
	25 °C	0.39	2.10	1.21	0.35	29.03

3.2. Chilling Tolerance Coefficients of Various Indices and Correlation Analysis in Different Leafy Mustard Cultivars

The chilling tolerance coefficients (CTC) of different leafy mustard cultivars for each index are shown in Table 3, from which it can be seen that 46 cultivars showed an increase in Chl content (CTC > 1) under chilling stress, of which No. 23 showed the highest increase in Chl content, which was 14.98 times that of the control. Forty-seven cultivars showed an increase in SS content, among which No. 5 showed the highest increase in SS content, which was 24.43 times more than the control. Fifty-one cultivars showed an increase in Pro content, with No. 54 showing the highest increase in Pro content, which was 14.30 times that of the control. Forty-five cultivars showed an increase in MDA content, of which No. 41 showed the highest increase, with its content being 9.19 times that of the control. Sixty-three mustard cultivars showed elevated SOD activity under chilling stress, of which No. 64 showed the greatest change in enzyme activity, with a change in SOD enzyme activity that was 4.51 times that of the control; No. 8 showed the least change in SOD enzyme activity. Twelve mustard cultivars showed elevated POD activity with chilling tolerance coefficients ranging from 0.36 to 1.58, with No. 31 being the highest and No. 16 the lowest. Forty-six mustard cultivars showed elevated CAT activity, of which No. 57 showed the highest change in enzyme activity, with a change in CAT enzyme activity of 9.49 times that of the control; No. 2 showed the lowest change in CAT enzyme activity. In addition, under chilling stress, Fv/Fm and Fv'/Fm' varied little, and the highest chilling tolerance coefficients were found in No. 19 and No. 56, with 1.15 and 1.11, respectively. The chilling tolerance coefficients of ΦPSII and qP ranged from 0.31 to 2.67, with No. 65 being the highest and No. 58 the lowest, and the chilling tolerance coefficients of NPQ and Rfd were in the range of 0.27–3.27, with No. 4 and 19 being the highest, and No. 12 the lowest.

Table 3. Chilling tolerance coefficients of physiological indexes of different leafy mustard cultivars.

No.	Chl	SS	Pro	SOD	POD	CAT	MDA	Fv/Fm	Fv/Fm'	ΦPSII	NPQ	qP	Rfd
1	1.08	1.35	1.58	2.06	1.06	1.36	2.33	1.03	0.96	1.62	2.00	1.67	1.88
2	1.04	0.91	1.01	3.36	0.60	0.20	1.41	0.95	0.95	1.07	0.90	1.16	0.97
3	1.14	0.95	5.44	1.28	0.75	1.14	0.70	0.90	0.92	0.37	0.67	0.42	0.53
4	0.65	10.18	1.19	3.75	0.59	0.45	0.54	0.99	0.87	0.76	3.26	0.91	2.41
5	0.33	24.43	2.87	4.22	0.58	0.49	2.10	0.90	0.77	0.85	2.04	1.09	1.65
6	0.65	4.64	1.41	2.99	0.60	0.95	0.89	1.00	0.91	0.38	2.76	0.43	1.17
7	1.73	6.13	1.10	3.22	0.41	1.14	0.44	1.00	0.97	0.70	1.92	0.73	1.27
8	0.79	7.94	1.21	1.86	0.67	1.55	1.10	1.01	0.92	0.85	2.17	0.97	1.56
9	1.08	8.99	1.52	1.44	0.68	3.49	1.30	1.09	1.04	0.76	2.23	0.73	0.96
10	1.29	10.53	1.31	3.38	0.63	2.22	1.71	1.01	1.00	0.63	1.31	0.64	0.86
11	0.42	17.43	1.13	3.44	0.40	0.92	2.06	0.95	0.92	0.36	1.09	0.39	0.61
12	2.27	1.52	1.33	1.38	0.70	2.47	0.77	1.01	1.10	0.46	0.34	0.41	0.27
13	0.99	1.01	2.31	1.41	0.70	1.55	0.76	1.01	1.06	0.55	0.53	0.52	0.51
14	1.27	1.10	1.37	1.05	0.72	0.50	0.61	0.95	1.04	0.59	0.40	0.57	0.53
15	1.06	11.21	0.99	1.39	0.85	1.29	0.58	0.94	0.90	0.60	1.27	0.65	0.74
16	0.78	5.07	1.01	1.15	0.36	1.42	1.03	0.95	0.93	0.83	0.99	0.87	0.86
17	1.22	17.19	1.51	1.08	1.02	1.91	0.96	0.95	0.87	0.70	2.18	0.79	1.28
18	1.22	7.71	1.70	0.89	0.47	0.89	0.62	0.98	0.99	1.23	0.75	1.25	1.27
19	0.84	1.36	1.33	1.46	0.52	0.48	1.31	1.15	1.10	1.26	2.55	1.16	3.27
20	1.31	2.84	2.18	1.57	0.38	0.46	0.88	0.98	0.96	0.59	0.95	0.60	0.62
21	3.62	9.16	1.34	1.57	0.65	1.11	1.25	0.98	0.90	0.69	2.29	0.77	1.24
22	1.12	4.30	1.81	1.51	0.77	0.93	4.43	0.95	0.87	0.87	2.07	1.00	1.44
23	14.98	0.65	1.25	1.27	1.15	3.39	0.54	0.95	0.99	0.66	0.59	0.66	0.77
24	4.80	2.58	1.63	1.62	0.88	1.68	1.04	0.99	0.99	0.77	0.92	0.78	0.90
25	0.70	1.03	2.87	1.47	0.71	3.10	0.45	1.05	1.01	0.63	1.63	0.62	0.80
26	2.16	1.45	2.42	1.23	0.81	1.87	1.32	0.99	0.99	0.97	0.88	0.96	0.99
27	1.72	4.27	1.44	0.95	1.07	2.37	0.80	0.99	0.97	1.57	1.18	1.60	1.61
28	1.25	1.60	1.34	1.38	0.67	1.92	1.16	0.99	0.88	0.82	2.49	0.95	1.34
29	1.11	1.49	1.31	2.10	0.81	1.18	0.79	0.99	0.99	1.23	0.85	1.21	1.00
30	0.91	1.09	13.24	1.94	0.66	0.46	4.45	1.00	1.00	1.12	0.93	1.11	0.98
31	1.15	1.43	1.56	1.68	1.58	2.32	2.37	1.05	1.06	1.04	1.26	1.00	1.24
32	0.88	0.74	0.98	1.06	0.90	1.01	1.26	0.98	0.97	0.59	1.02	0.63	0.63
33	1.28	0.48	1.58	1.91	0.41	0.73	3.13	1.08	1.10	1.27	1.19	1.19	1.35
34	1.65	9.32	8.08	1.50	0.48	1.99	3.23	0.96	0.96	1.20	0.81	1.23	1.13
35	0.49	0.91	6.95	1.44	0.63	0.38	1.38	0.93	0.88	0.75	1.07	0.88	0.83
36	0.88	9.59	1.52	2.11	0.61	0.51	1.26	0.98	0.81	1.00	2.53	1.20	2.02
37	1.17	9.26	2.79	2.28	0.85	0.50	2.60	0.99	0.92	1.11	1.56	1.21	1.81
38	2.14	2.44	0.94	2.73	0.66	1.43	1.08	0.99	0.92	1.75	2.19	2.00	2.57
39	3.06	3.80	8.74	3.32	0.75	3.82	1.26	0.98	0.93	1.03	1.40	1.13	1.60
40	1.19	2.29	9.52	4.12	1.03	3.77	1.50	0.99	0.90	1.06	1.77	1.20	2.16
41	0.59	11.06	1.32	3.26	0.39	4.42	9.19	0.99	0.87	0.62	2.07	0.71	1.27
42	0.63	1.63	1.82	4.03	0.94	1.28	1.61	0.99	0.92	0.84	1.86	0.90	1.08
43	1.12	1.09	5.94	1.83	0.67	0.43	3.57	0.90	0.85	1.29	1.35	1.50	1.39
44	0.79	1.40	3.74	1.57	0.78	4.69	2.80	0.98	0.86	0.70	2.18	0.80	1.46
45	0.77	0.49	3.07	1.30	1.36	4.56	1.29	0.98	0.96	0.93	1.06	0.97	0.83
46	1.10	0.36	1.44	2.59	1.15	1.50	1.79	1.01	1.04	0.50	0.80	0.50	0.56
47	2.49	0.39	1.21	3.78	1.50	4.89	3.64	1.03	1.07	0.55	0.67	0.52	0.53
48	1.29	0.53	1.51	1.53	0.62	2.47	1.75	0.97	0.95	0.68	1.52	0.71	0.81
49	3.81	1.64	4.86	2.19	0.94	1.93	1.21	1.05	1.00	1.15	1.95	1.18	2.62
50	1.79	8.26	1.32	3.24	0.76	1.94	2.23	1.05	1.03	1.09	1.31	1.04	1.21
51	0.93	1.55	0.55	2.28	0.58	4.24	1.88	1.00	1.03	0.70	0.96	0.70	0.70
52	1.90	0.49	0.54	1.27	0.71	3.33	1.90	1.01	1.04	0.70	0.71	0.65	0.56
53	1.16	0.34	0.73	3.01	0.95	0.65	1.11	1.00	1.06	0.50	0.49	0.48	0.38
54	1.40	2.52	14.30	2.89	0.55	1.09	1.33	1.01	0.97	0.90	1.43	0.91	1.01
55	1.59	0.47	4.44	2.42	0.43	1.08	0.51	1.14	1.09	0.72	2.34	0.67	1.07
56	2.51	0.88	0.50	1.74	0.96	0.74	1.47	1.11	1.11	1.11	1.19	1.00	0.67
57	3.77	0.34	0.58	4.16	0.95	9.49	1.39	0.88	0.87	0.35	0.83	0.38	0.53
58	1.31	0.98	0.63	1.80	0.79	1.44	0.80	0.92	0.95	0.31	0.52	0.33	0.40
59	0.77	0.70	5.22	3.96	1.04	1.40	1.08	0.88	0.87	0.68	0.94	0.78	0.74
60	1.12	4.51	0.36	2.15	0.61	1.51	0.56	1.04	0.97	1.00	2.35	1.03	1.20
61	1.80	0.51	0.52	2.37	1.17	3.88	0.53	0.85	0.85	1.40	0.78	1.70	0.87
62	1.00	3.24	1.46	2.00	0.92	0.53	0.42	1.07	1.10	1.17	1.05	1.05	1.48
63	1.60	12.94	0.90	3.88	0.78	4.62	1.83	0.94	1.00	1.14	0.44	1.16	0.60
64	1.40	2.24	0.90	4.51	1.47	7.26	1.29	0.99	0.99	1.17	1.12	1.15	0.91
65	1.44	1.09	0.69	2.90	0.94	2.48	1.32	1.04	1.00	2.67	1.95	2.44	1.87

Correlation analysis of the chilling tolerance coefficients of 13 indices in 65 leafy mustard cultivars is shown in Figure 1. As the correlation coefficient values approaches 1 or -1 , it indicates a strong relationship between the variables. Specifically, there was a significant negative correlation between SS content and POD activity, and a highly significant negative correlation between SS content and F_v'/F_m' , with correlation coefficients of -0.30 and -0.42 , respectively. Meanwhile, there was a significant positive correlation between SS content and NPQ, with a correlation coefficient of 0.31 . SOD activity showed a significant positive correlation with CAT activity, with a correlation coefficient of 0.31 . POD activity showed a highly significant positive correlation with CAT activity and a significant negative correlation with NPQ, with correlation coefficients of 0.43 and -0.25 , respectively. F_v/F_m showed a significant positive correlation with F_v'/F_m' and NPQ, with correlation coefficients of 0.72 and 0.32 , respectively. Additionally, it was significantly positively correlated with Rfd, with a correlation coefficient of 0.31 . F_v'/F_m' was highly significantly negatively correlated with NPQ, with a correlation coefficient of -0.37 . $\Phi PSII$ was highly significantly positively correlated with qP and Rfd, with correlation coefficients of 0.97 and 0.57 , respectively. NPQ was highly significantly positively correlated with Rfd and significantly positively correlated with qP, with correlation coefficients of 0.71 and 0.24 , respectively. Finally, qP was highly significantly positively correlated with Rfd, with a correlation coefficient of 0.62 .

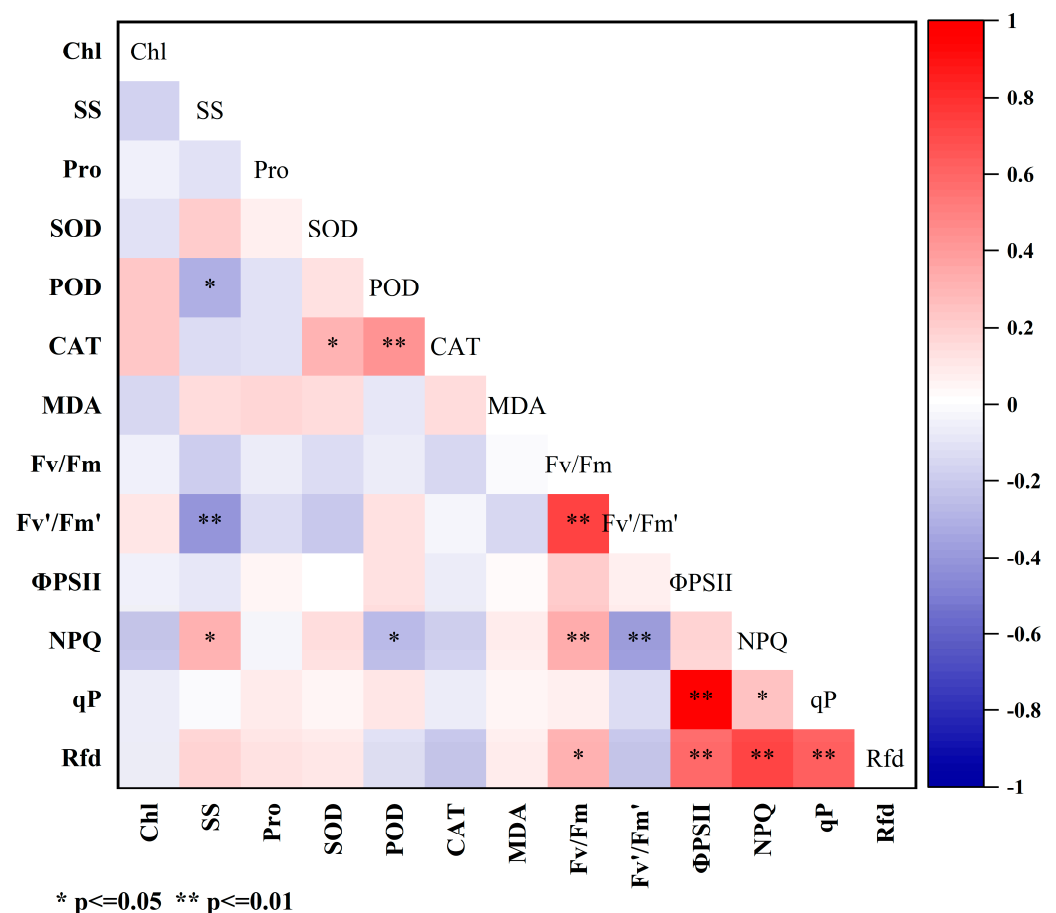


Figure 1. Correlation analysis of chilling tolerance coefficients. Note: red (+1) indicates a positive correlation between the different indicators, while blue (-1) indicates a negative correlation. ** indicates a significant correlation at the 0.01 level; * indicates a significant correlation at the 0.05 level.

3.3. Principal Component Analysis of the Chilling Tolerance Coefficients of the Indicators

Principal component analysis (PCA) of the chilling tolerance coefficients of the 13 indices (Table 4) revealed that the contributions of the first six indices were 22.784%,

17.2%, 14.217%, 9.718%, 9.175%, and 6.963%, respectively, and the cumulative contributions were 22.784%, 39.984%, 54.201%, 63.918%, 73.093%, and 80.056%, respectively. When the cumulative contribution rate of the principal components was >80%, these six principal components represented most of the information of the original 13 indices for the comprehensive evaluation of the chilling tolerance of mustard.

Table 4. Principal component analysis of chilling tolerance coefficients of indicators.

Index	Principal Component						Total Load	Ranking of Best Indicators
	1	2	3	4	5	6		
Chl	−0.096	0.121	0.156	−0.052	−0.291	0.713	0.039	12
SS	0.096	−0.291	−0.063	0.162	−0.181	−0.143	−0.048	13
Pro	0.048	−0.050	0.024	−0.353	0.539	0.490	0.055	10
SOD	0.047	−0.146	0.248	0.372	0.167	−0.061	0.068	7
POD	−0.073	0.169	0.358	0.099	−0.091	−0.051	0.061	8
CAT	−0.100	0.008	0.370	0.332	0.056	0.102	0.076	5
MDA	0.055	−0.119	0.083	0.163	0.550	−0.013	0.069	6
Fv/Fm	0.088	0.283	−0.251	0.391	0.148	0.128	0.094	2
Fv′/Fm′	−0.097	0.353	−0.172	0.198	0.175	−0.111	0.042	11
ΦPSII	0.246	0.211	0.177	−0.166	0.010	−0.244	0.085	3
NPQ	0.243	−0.099	−0.112	0.287	−0.160	0.323	0.058	9
qP	0.264	0.143	0.207	−0.214	−0.026	−0.216	0.076	4
Rfd	0.300	0.040	0.005	0.076	−0.095	0.282	0.094	1
Eigenvalue	2.962	2.236	1.848	1.263	1.193	0.905		
Variance contribution%	22.784	17.2	14.217	9.718	9.175	6.963		
Cumulative contribution rate%	22.784	39.984	54.201	63.918	73.093	80.056		

As shown in the table, the indicators that had a negative impact on the first principal component included Chl, POD, CAT, and Fv′/Fm′, with relatively low loadings ranging from −0.100 to −0.073. Here, ΦPSII, NPQ, qP, and Rfd were high positive load indicators with loads of 0.246, 0.243, 0.264, and 0.300, respectively. The indicators that had a negative impact on the second principal component included SS, Pro, SOD, MDA, and NPQ, with loadings ranging from −0.291 to −0.050. Fv/Fm and Fv′/Fm′ were the highest positive load indicators, with loads of 0.283 and 0.353, respectively. The indicators that had a negative impact on the third principal component included SS, Fv/Fm, Fv′/Fm′, and NPQ, with loadings ranging from −0.251 to −0.063. POD and CAT were high positive load indicators, with loads of 0.358 and 0.370, respectively. The indicators that had a negative impact on the fourth principal component included Chl, Pro, ΦPSII, and qP, with a load range of −0.353 to −0.052. SOD, CAT, and Fv/Fm were high positive load indicators, with loads of 0.372, 0.332, and 0.391, respectively. The indicators that had a negative impact on the fifth principal component included Chl, SS, POD, NPQ, qP, and Rfd, with loadings ranging from −0.291 to −0.026. Pro and MDA were high positive load indicators, with loads above 0.5. The indicators that had a negative impact on the sixth principal component included SS, SOD, POD, MDA, Fv′/Fm′, ΦPSII, and qP, with a load range of −0.244 to −0.013. Chl was a relatively high positive load indicator, with a load of 0.713.

The loadings corresponding to the 13 indicators were multiplied by their corresponding variance contributions in the six principal components and finally summed and calculated. The larger the calculated result is, the stronger the positive effect on the chilling tolerance of mustard. This information indicates that the evaluation of the chilling tolerance indices of mustard is superior, and the indices of chilling tolerance can be ranked. The top ten chilling tolerance indicators were Rfd, Fv/Fm, ΦPSII, qP, CAT, MDA, SOD, POD, NPQ, and Pro.

3.4. Membership Function Analysis and Cluster Analysis of Comprehensive Index Chilling Tolerance Coefficient

As shown in Table 5, the range of variation in the value of the membership function for the same composite index is between 0 and 1. For the principal component 1, the U (X1) value of No. 65 is the largest, and the U (X1) value of No. 23 is the smallest. These findings indicate that the chilling tolerance of No. 65 is the strongest and that of No. 23 is the weakest under principal component 1. Similarly, the strongest cultivars in principal components 2, 3, 4, 5, and 6 were No.65, No.64, No.41, No.30, and No.23, respectively, whereas the weakest cultivars were No.5, No.55, No.43, No.23, and No.63, respectively. According to the membership function values and the weight of each composite index, the composite evaluation value of the chilling tolerance (D) of each variety is calculated, and the value represents its chilling tolerance ability. The maximum D-value of the No. 65 was 0.73, and the minimum D-value of the No. 58 was 0.28. These findings indicate that the No. 65 had the strongest chilling tolerance and that the No. 58 exhibited the weakest chilling tolerance.

To better classify the chilling tolerance of mustard, systematic cluster analysis was performed on the D-value, and the chilling tolerance of 65 mustard cultivars was classified into four categories (Figure 2). The blue part indicates that the first category belongs to the highly chilling-tolerant type, including one mustard cultivar (No. 65), with a maximum D-value of 0.73. The red part indicates that the second category belongs to the moderately chilling-tolerant type, with D-values ranging from 0.60 to 0.63, including six mustard cultivars. The green part belongs to the third category, which belongs to the mildly chilling-tolerant type, with D-values ranging from 0.39 to 0.55, including 45 mustard cultivars. The purple part belongs to the fourth category, which is not a chilling-tolerant type, with D-values ranging from 0.28 to 0.38, including 13 mustard cultivars.

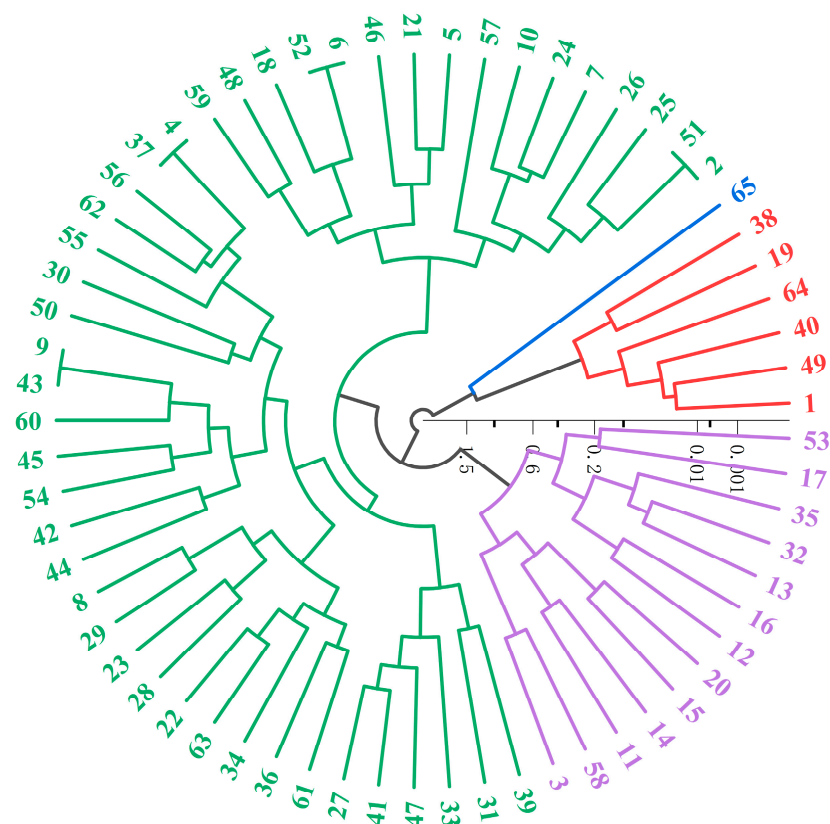


Figure 2. Cluster analysis of D-value for comprehensive evaluation of chilling tolerance in mustard.

Table 5. Membership function values, weights, D-values, and sorting of 65 leafy mustard cultivars.

Ranking	U (X1)	U (X2)	U (X3)	U (X4)	U (X5)	U (X6)	D	No.
1	1.00	1.00	0.76	0.41	0.37	0.01	0.73	65
2	0.87	0.93	0.03	0.77	0.41	0.41	0.63	19
3	0.93	0.73	0.58	0.38	0.26	0.25	0.62	38
4	0.65	0.55	0.73	0.50	0.63	0.51	0.61	40
5	0.77	0.80	0.55	0.47	0.42	0.18	0.61	1
6	0.68	0.80	0.45	0.53	0.40	0.58	0.61	49
7	0.34	0.72	1.00	0.90	0.44	0.13	0.60	64
8	0.39	0.89	0.55	0.66	0.48	0.18	0.55	31
9	0.52	0.56	0.63	0.39	0.57	0.54	0.54	39
10	0.10	0.74	0.73	0.97	0.64	0.24	0.53	47
11	0.56	0.16	0.50	1.00	0.87	0.28	0.53	41
12	0.61	0.82	0.57	0.27	0.28	0.14	0.53	27
13	0.55	0.84	0.18	0.54	0.62	0.17	0.52	33
14	0.50	0.66	0.40	0.73	0.48	0.19	0.51	50
15	0.51	0.61	0.37	0.08	1.00	0.42	0.50	30
16	0.37	0.95	0.25	0.63	0.44	0.19	0.50	56
17	0.48	0.89	0.26	0.54	0.40	0.14	0.50	62
18	0.79	0.34	0.28	0.75	0.21	0.39	0.50	4
19	0.65	0.53	0.42	0.44	0.43	0.23	0.49	37
20	0.46	0.79	0.00	0.75	0.53	0.46	0.49	55
21	0.47	0.46	0.50	0.56	0.49	0.42	0.49	44
22	0.47	0.52	0.50	0.64	0.45	0.21	0.48	42
23	0.49	0.55	0.33	0.17	0.81	0.57	0.48	54
24	0.29	0.72	0.66	0.45	0.43	0.20	0.48	45
25	0.44	0.66	0.19	0.83	0.39	0.30	0.47	9
26	0.64	0.47	0.56	0.00	0.59	0.25	0.47	43
27	0.56	0.64	0.25	0.62	0.29	0.25	0.47	60
28	0.76	0.38	0.34	0.44	0.23	0.31	0.46	36
29	0.43	0.59	0.94	0.11	0.22	0.03	0.46	61
30	0.57	0.43	0.41	0.42	0.49	0.28	0.46	22
31	0.35	0.50	0.70	0.63	0.44	0.00	0.46	63
32	0.53	0.51	0.42	0.15	0.67	0.29	0.45	34
33	0.58	0.51	0.28	0.56	0.29	0.27	0.45	8
34	0.44	0.73	0.43	0.32	0.38	0.09	0.45	29
35	0.53	0.53	0.32	0.46	0.29	0.35	0.44	28
36	0.00	0.85	0.75	0.31	0.00	1.00	0.44	23
37	0.01	0.37	0.99	0.81	0.36	0.40	0.43	57
38	0.35	0.71	0.39	0.31	0.41	0.25	0.43	26
39	0.45	0.57	0.43	0.33	0.43	0.08	0.42	2
40	0.24	0.63	0.38	0.66	0.49	0.17	0.42	51
41	0.30	0.69	0.23	0.59	0.43	0.31	0.42	25
42	0.35	0.47	0.32	0.75	0.44	0.20	0.42	10
43	0.46	0.49	0.23	0.63	0.32	0.30	0.42	7
44	0.26	0.70	0.42	0.39	0.30	0.40	0.41	24
45	0.16	0.69	0.39	0.62	0.50	0.18	0.41	46
46	0.73	0.00	0.48	0.53	0.29	0.16	0.41	5
47	0.47	0.45	0.28	0.49	0.21	0.45	0.41	21
48	0.44	0.39	0.18	0.72	0.32	0.38	0.40	6
49	0.18	0.72	0.33	0.53	0.46	0.20	0.40	52
50	0.50	0.66	0.26	0.19	0.32	0.10	0.40	18
51	0.29	0.39	0.66	0.27	0.49	0.22	0.39	59
52	0.32	0.56	0.33	0.44	0.42	0.27	0.39	48
53	0.49	0.35	0.38	0.50	0.16	0.26	0.38	17
54	0.12	0.68	0.31	0.56	0.47	0.11	0.37	53
55	0.38	0.46	0.33	0.02	0.53	0.30	0.36	35
56	0.23	0.63	0.28	0.36	0.38	0.17	0.35	32
57	0.15	0.70	0.19	0.42	0.47	0.18	0.35	13
58	0.04	0.74	0.22	0.50	0.44	0.22	0.34	12
59	0.36	0.50	0.23	0.25	0.34	0.14	0.33	16
60	0.31	0.41	0.33	0.38	0.22	0.16	0.32	15
61	0.27	0.53	0.14	0.30	0.40	0.23	0.32	20
62	0.14	0.66	0.21	0.24	0.38	0.13	0.30	14
63	0.31	0.18	0.22	0.64	0.41	0.11	0.30	11
64	0.13	0.45	0.32	0.10	0.45	0.30	0.28	3
65	0.06	0.49	0.33	0.34	0.35	0.17	0.28	58
Wj	0.28	0.21	0.18	0.12	0.11	0.09	-	-

3.5. Establishment of Regression Equations and Screening of Identification Indices

As shown in Table 6, the correlations of SOD activity, Fv/Fm, Φ PSII, NPQ, qP, and Rfd with the overall evaluation value of chilling tolerance (D) were highly significant, and the correlations of POD activity and CAT activity with the overall evaluation value (D) were significant. The correlation coefficients between the chilling tolerance coefficients of Φ PSII, qP, and Rfd and the overall evaluation value (D) were greater, with correlation coefficients of 0.737, 0.703, and 0.693, respectively. The optimal regression equation was established using the value of D as the dependent variable and the chilling tolerance coefficients of the individual indices as the independent variables. The optimal regression equation was established as follows: $Y = -0.333 + 0.004 \times X_1 - 0.002 \times X_2 + 0.003 \times X_3 + 0.019 \times X_4 + 0.06 \times X_5 + 0.011 \times X_6 + 0.012 \times X_7 + 0.479 \times X_8 + 0.11 \times X_9 + 0.018 \times X_{10} + 0.044 \times X_{11}$, where Y is the predicted value of the overall evaluation of chilling tolerance, X_1 represents chlorophyll, X_2 represents soluble sugar, X_3 represents proline, X_4 represents SOD activity, X_5 represents POD activity, X_6 represents CAT activity, X_7 represents MDA, X_8 represents Fv/Fm, X_9 represents Φ PSII, X_{10} represents NPQ, and X_{11} represents Rfd. The coefficients of each indicator represent the weight of their impact on the comprehensive evaluation value (D) of chilling tolerance. The determination coefficient of the regression equation is $R^2 = 0.999$ ($p < 0.001$), there is no multicollinearity problem (tolerance > 0.2 , VIF < 5), and these 11 indicators have an impact on the comprehensive evaluation value (D) of chilling tolerance. The correlation analysis and regression analysis results between the chilling tolerance index and the comprehensive evaluation value (D) of chilling tolerance indicate that the Fv/Fm, Φ PSII, POD activity, and Rfd represent the preferred indicators for identifying chilling tolerance in mustard.

Table 6. Correlation between the indicators and the overall evaluation value (D) of chilling tolerance.

Index	Correlation Coefficient	p-Value
Chl	0.069	0.585
SS	−0.133	0.292
Pro	0.095	0.452
SOD	0.324	0.008
POD	0.319	0.010
CAT	0.258	0.038
MDA	0.242	0.052
Fv/Fm	0.486	0.001
Fv'/Fm'	0.136	0.279
Φ PSII	0.737	0.001
NPQ	0.435	0.001
qP	0.703	0.001
Rfd	0.693	0.001

3.6. Physiological Differences between Most Chilling-Tolerant and Least Chilling-Tolerant Cultivars of Mustard

The comprehensive analysis showed that No. 65 (SJTKJ) was the most chilling-tolerant cultivar and No. 58 (DX) was the least chilling-tolerant cultivar. As shown in Table 7, all the indices (except Pro and Fv'/Fm') of SJTKJ were greater than those at 25 °C under the 5 °C treatment, whereas only the Chl content, SOD activity, and CAT activity of DX were greater than those under the 25 °C treatment. All of the other indices were less than those under the 25 °C treatment, and all the indices (except Pro content and CAT activity) of SJTKJ were greater than those of DX under the 5 °C treatment. As shown in Figure 3, under 5 °C treatment, the new leaf portion of SJTKJ showed orange colour, indicating that the low temperature had less effect on the PSII maximum light quantum efficiency (Fv/Fm) of the new leaf, suggesting that the new leaf was more photosynthetically capable. whereas the steady-state fluorescence decay rate (Rfd) was mostly red at the leaf margins. The middle portion of DX leaves under the 5 °C treatment was largely green and blue in colour, with larger decreases in Fv/Fm and Rfd. The corresponding values of all fluorescence

parameters of DX leaves under 5 °C treatment were lower than those under 25 °C treatment, indicating that DX was severely damaged by stress. These results also indicate that its photosynthetic capacity was reduced and that the photosynthetic ability of DX was weaker than that of SJTKJ. This finding indicates that the screening of chilling-tolerant cultivars using multivariate evaluation methods is reliable.

Table 7. Physiological differences between most chilling-tolerant and least tolerant mustard cultivars.

Index	SJTKJ		DX	
	5 °C	25 °C	5 °C	25 °C
Chl content mg/g	1.50	1.04	0.93	0.71
SS content %	0.29	0.27	0.25	0.27
Pro content µg/g	17.89	25.84	23.90	38.14
SOD activity U/g	288.69	99.80	257.44	143.65
POD activity U/(g·min)	3068.11	3264.39	2963.72	3776.50
CAT activity U/(g·min)	558.92	225.67	607.58	422.92
MDA content nmol/g	1.91	1.46	1.37	1.72
Fv/Fm	0.79	0.76	0.68	0.74
Fv'/Fm'	0.72	0.72	0.63	0.66
ΦPSII	0.16	0.06	0.08	0.26
NPQ	0.43	0.22	0.23	0.44
qP	0.22	0.09	0.13	0.39
Rfd	0.73	0.39	0.40	1.01

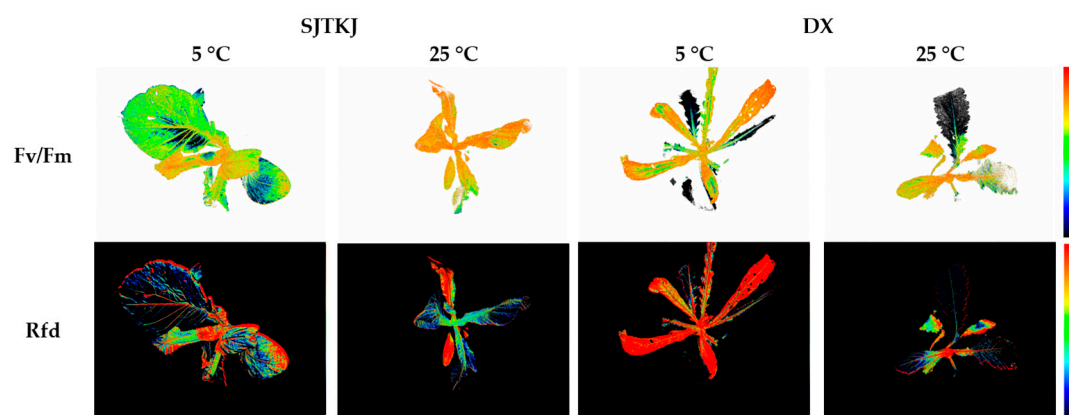


Figure 3. Chlorophyll fluorescence imaging images of most chilling-tolerant and least chilling-tolerant mustard cultivars. Note: The maximum optical quantum efficiency (Fv/Fm) of PSII is 0.6–0.9 from blue to red, and the steady-state fluorescence decay rate (Rfd) is 0.3–1.1 from blue to red.

4. Discussion

4.1. Response of Leafy Mustard to Chilling Stress

Under low-temperature stress, the osmotic pressure inside the plant changes, the membrane system is disrupted, and the cell fluid leaks, leading to an imbalance between the inside and outside of the cell. Plants maintain osmotic pressure balance by regulating the content of osmoregulatory substances, reducing the damage caused by low temperature [27,28]. The accumulation of soluble sugars increases the concentration of cell fluid, decreases the freezing point of the intracellular solution, decreases the content of free water, enhances the water retention ability of cells, prevents the cytoplasmic matrix from freezing and solidifying, and increases the stability of cell membranes [29]. In this study, the soluble sugar content of most mustard cultivars increased under 5 °C treatment. The soluble sugar content of the different mustard cultivars ranged from 0.17 to 1.42%, with an average value of 0.99%, which was 0.82 times greater than that of the 25 °C treatment. This finding is similar to the research results of Zhou et al. [21] on melon seedlings. Under chilling stress, the hydrolysis of mustard leaves is enhanced, and macromolecular compounds such as

starch and protein are degraded into soluble sugars, which increases the concentration of cell fluid, increases cell osmotic pressure, and enhances chilling tolerance [30].

Proline is an amino acid involved in the composition of proteins and can rapidly accumulate in low-temperature environments to help proteins transport water efficiently, enhance the tolerance of plants to low-temperature stress [31], and alleviate intracellular redox reactions [32]. Studies have shown that proline content is positively correlated with the chilling tolerance of plants under low-temperature stress [33]. In this study, the proline content of most mustard cultivars increased under 5 °C, and the proline content of different mustard cultivars ranged from 17.01 to 303.53 µg/g, with a mean value 1.08 times greater than that under 25 °C. This finding indicates that during low-temperature stress, membrane lipid peroxidation is enhanced in mustard leaves and excessive oxidant production leads to oxidative damage of cellular biomolecules; proline acts as a reactive oxygen species (ROS) scavenger to reduce the oxidative damage and maintain a balanced state between oxidants and antioxidants. In addition, mustard maintains membrane stability and protein structure by accumulating a large amount of proline, which can act as a nonenzymatic antioxidant to scavenge ROS, protect cell membrane structure, and improve plant resilience [34].

Under low-temperature stress, a large amount of ROS accumulate and cause membrane lipid peroxidation, leading to an increase in the content of the membrane lipid peroxidation product malondialdehyde (MDA). MDA reflects the degree of cell membrane damage and is an important indicator of the degree of plant oxidative stress [35]. In this study, the MDA content of mustard showed an increase under 5 °C, with a range of 0.73 to 68.92 nmol/g and an average value 1.04 times greater than that of the control. These results are consistent with those of Sun et al. [14]. Chilling stress can increase the content of ROS and free radicals in mustard leaves and enhance membrane lipid peroxidation, leading to increased cell membrane permeability, electrolyte leakage, and damage to the cell membrane system, resulting in the accumulation of a large amount of MDA [36].

Excessive accumulation of ROS in plants under low-temperature stress leads to oxidative damage in plant cells [37]. Plants remove excess ROS by regulating their own enzymatic reaction system (SOD, CAT, and POD), where SOD catalyses the disproportionation of superoxide anion radicals to produce O₂ and H₂O₂; POD works synergistically with CAT to decompose H₂O₂ into H₂O and O₂, thereby reducing the toxic effects of hydrogen peroxide on cells [38]. In this study, the SOD and CAT activities of most mustard cultivars were increased, whereas POD activity were decreased. The results suggest that chilling stress activates the antioxidant enzyme system to scavenge excess reactive oxygen radicals in the cells by increasing enzyme activity, thus reducing the damage caused to the cells and improving the chilling tolerance of mustard [39]. SOD and CAT activities play major roles in the ability of mustard to protect against oxidative damage caused by chilling stress, indicating that most mustard has a strong ability to scavenge ROS.

Chlorophyll is one of the most important physiological indicators of chilling tolerance in plants. High levels of polyunsaturated fatty acids in chloroplast lipids help to maintain plant survival and normal formation of chloroplast membranes under low-temperature stress [40]. In this study, more than half of the mustard cultivars exhibited an increase in chlorophyll content at 5 °C, and the chlorophyll content of the different mustard cultivars ranged from 0.37 to 2.65 mg/g; this finding may be due to changes in endogenous hormone levels, information transfer, and plant physiological responses under short-term low-temperature stress. Plants construct a low-temperature defence system to actively increase chlorophyll content and prevent the reduction in photosynthesis and energy production caused by the gradual decrease in chlorophyll content [18].

Chlorophyll fluorescence technology can be used to determine the degree of harm caused by chilling stress in plants [41]. Chlorophyll fluorescence parameters can be used to evaluate the function of photosynthetic mechanisms and the response of plants to low-temperature stress. Chlorophyll fluorescence parameters reflect the photosynthetic potential of plants, their ability to convert light energy into chemical energy, and their level of photosynthetic activity. In this study, the Fv/Fm, Fv'/Fm', ΦPSII, and qP exhibited an

decrease in most mustard cultivars under 5 °C treatment, indicating reductions in plant photosynthetic capacity, the efficiency of the photosynthetic electron transfer process, and photosynthetic activity, which is consistent with the results of Zhang et al. [18]. Under low-temperature conditions, the metabolism of leaf cells is severely inhibited mainly due to the effects of chilling stress on reducing carbon dioxide fixation or delaying the photosynthetic cycle as well as changes in sugar formation and distribution, which reduce the ability of plants to recover from stress damage [41].

4.2. Evaluation of the Chilling Tolerance of Leafy Mustard Cultivars

These findings indicated that the variation trends of the various physiological indices of the different mustard cultivars were not completely consistent, and the trends of the various physiological indices varied greatly. The correlation analysis revealed different degrees of correlation among the indices and close correlation among the chlorophyll fluorescence parameters. Given that the different degrees of correlation of the indicators resulted in overlapping information and given the different roles played by each indicator in the chilling stress response of mustard, it is impossible to accurately evaluate the chilling tolerance of mustard cultivars using these indicators, and a comprehensive analysis is needed. The chilling tolerance of plants is affected by their own genetic factors and external environmental factors; using a single index to evaluate the chilling tolerance of plants limits the knowledge obtained [42,43]. It was necessary to analyse multiple physiological indices in an integrated manner to comprehensively and accurately evaluate the chilling tolerance ability of mustard.

PCA is a method for reducing the dimensionality of a particular dataset. It does this by creating new covariates that are uncorrelated with each other, which are called principal components, and will reduce the problem of solving eigenvalues/eigenvectors [44]. In this study, PCA was used to transform the 13 indicators into six more representative composite indicators, and these indicators focus on the information of the original indicators with nonsignificant differences. The membership function method utilizes the principles of fuzzy mathematics to provide a comprehensive and scientific evaluation of things or phenomena [45]. In this study, the membership function value method in fuzzy mathematics was utilized to calculate the comprehensive evaluation value (D-value) of the chilling tolerance of different cultivars, and the D-value reflects the difference in chilling tolerance among different cultivars. The results showed that SJTKJ Mustard had the strongest chilling tolerance, and DX Mustard had the weakest chilling tolerance. Cluster analysis refers to the classification of any sample category or other prior knowledge in a batch of samples based on the characteristics of the samples. Identical or similar characteristics are classified into one category based on some measure of similarity [46]. In this study, 65 mustard cultivars were divided into four categories according to the strength of chilling tolerance and chilling tolerance evaluation models were established using stepwise regression analysis for non-chilling-tolerant, mildly chilling-tolerant, moderately chilling-tolerant and highly chilling-tolerant cultivars. Four chilling tolerance indices, namely, F_v/F_m , $\Phi PSII$, POD activity, and Rfd, were selected based on correlation analysis of each trait index and regression analysis of the D-value of the comprehensive evaluation index. However, in future studies, in addition to physiological indicators and fluorescence characteristics, we should further consider collecting more biomass and leaf, petal, seed phenotypic characteristics, and other indicators of mustard at different reproductive stages, because this is more conducive to a comprehensive understanding of the correlation between the whole life cycle of chilling tolerance in mustard from different germplasm resources, and make a more comprehensive and integrated analysis.

5. Conclusions

This study investigated the physiological responses of 65 leafy mustard cultivars to chilling stress. We found that the trends of various physiological indexes of different mustard cultivars are inconsistent, and it is difficult to use a single index to accurately

evaluate the adaptive ability of mustard to chilling stress. Therefore, correlation analysis, PCA, membership function method, cluster analysis, and regression analysis were used to reduce the data dimensionality and simplify the evaluation indicators. The 65 leafy mustard cultivars were divided into four chilling tolerance categories, and the strongest chilling tolerance mustard cultivar was SJTKJ. A model for evaluating the chilling tolerance of mustard was established and Fv/Fm, ΦPSII, POD activity, and Rfd were selected as the best indices for comprehensively evaluating the chilling tolerance of mustard. This finding indicates that using chlorophyll fluorescence technology to identify the chilling tolerance of mustard is feasible. This study provides a basis for the cultivation of new mustard cultivars and the accurate and non-destructive evaluation of chilling tolerance.

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