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Dynamic Analysis of Physiological and Biochemical Substances in Two Types of Root Type Alfalfa during Overwintering Period

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ABSTRACT

Background: Cold resistance in alfalfa *(Medicago sativa* L.) is significantly influenced by root system type. The root system plays a crucial role in water absorption and soil stress response. This study investigates the physiological and biochemical responses of creeping-rooted and taprooted alfalfa to cold stress during early winter and mid-winter periods.

Methods: Samples were collected on November 3,2023 and January 7,2024, from the experimental plot. Roots at a depth of 20 cm were cleaned with distilled water and stored in cryopreservation tubes at ultra-low temperatures. Free proline, soluble sugars, soluble proteins, malondialdehyde (MDA), superoxide dismutase (SOD) activity and catalase (CAT) activity were measured using standard biochemical methods.

Result: Results indicated that with decreasing temperatures, the contents of soluble sugars, soluble proteins, free proline, MDA and CAT activity increased, whereas SOD activity decreased. In the early overwintering stage, creeping-rooted alfalfa exhibited higher soluble sugar content and SOD activity compared to taprooted alfalfa. During the overwintering period, creeping-rooted alfalfa maintained higher levels of soluble sugars, soluble proteins, MDA and CAT and SOD activities. Principal component analysis identified CAT, MDA, soluble sugars and soluble proteins as key indicators for evaluating cold resistance in alfalfa.

Key words: Creeping rooted alfalfa, Physiological character, Taprooted alfalfa, Winter resistance.

INTRODUCTION

Alfalfa (*Medicago sativa* L.), often known as the “king of forage grass”, is celebrated for its drought tolerance, cold tolerance, salt tolerance, strong adaptability, high yield, excellent quality, high economic value and benefits in soil and water conservation and soil improvement through fertilization. These attributes have garnered worldwide attention (Yang, 2003). The root systems of alfalfa can be classified into four types: tap-rooted, branch-rooted, creeping-rooted and rhizomatous (Liu *et al*., 2003). Alfalfa thrives in warm and humid climates, but low temperatures significantly limit its distribution and promotion. Studies have shown that under freezing stress, alfalfa activates osmotic regulation and antioxidant mechanisms. Varieties with strong cold resistance have higher contents of free proline (Pro), soluble sugar (SS) and soluble protein (SP), enhancing their osmotic regulation and antioxidant capacity (Ma *et al*., 2016). Delauney *et al*. (1993) and Zhou *et al*. (2018) demonstrated that free proline is a crucial osmotic regulator in plants, maintaining osmotic balance between protoplasts and the environment. Zhao Yihang *et al*. (2021) and Song (2016) found that soluble sugar content in plants significantly increases under low temperatures. Qi (2017) noted that alfalfa varieties with stronger cold resistance have higher soluble protein content. Deng Xueke *et al*. (2005) and Feng Changjun *et al*. (2005); Pamungkas *et al.* (2022) showed that superoxide dismutase (SOD) activity in alfalfa plants rises significantly under low-temperature stress to scavenge free radical damage. Li *et al*. (2021) found that free proline, soluble sugar, soluble protein and SOD activity in alfalfa increase as temperature decreases, while malondialdehyde (MDA) content also rises.

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In this experiment, the contents of proline, soluble sugar, soluble protein and malondialdehyde, as well as the activities of catalase and superoxide dismutase, were measured in the roots of creeping-rooted and taprooted alfalfa at the end of autumn and the beginning of winter (early overwintering period) and in the middle of winter (overwintering period). The differences in cold resistance between the two root types of alfalfa were compared, providing a theoretical basis for the scientific cultivation of creeping-rooted alfalfa in production. Additionally, the anatomical structure of the root necks of both root types was observed to compare their cold resistance. This comparison provides a theoretical foundation for the scientific cultivation of these alfalfa root types in production.

MATERIALS AND METHODS

Materials and experimental location

The experiment was conducted at the experimental base of Inner Mongolia Agricultural University in Hailiutu Village, Tumote Left Banner, Hohhot City, Inner Mongolia Autonomous Region. The site is located at an altitude of 1008 meters with alkaline soil (pH 8.6 to 9.1). The soil composition includes 0.072% total nitrogen, 0.057% total phosphorus, 1.89% total potassium, 58.8 mg/kg alkali-hydrolyzable nitrogen, 26.1 mg/kg available phosphorus and 175.9 mg/kg available potassium. The region experiences a mid-temperate continental monsoon climate, with distinct seasonal changes. The average annual temperature is 6.3°C, with an average annual precipitation of 400 mm and a frost-free period of about 130-140 days. The hottest month, July, has an average temperature of 23.7°C, while the coldest month, January, averages -10.5°C, with extreme temperatures ranging from 37.5°C to -35.1°C. Samples were collected on November 3, 2023 and January 7, 2024. Roots at a depth of 20 cm were cleaned with distilled water, placed in cryopreservation tubes and stored in an ultra-low temperature refrigerator.

Osmotic adjustment substance

The accumulation of osmotic regulators can also improve the stability of cell membranes and the activity of proteins and enzymes (Vanhove *et al*., 2012). Proline is one of the most important organic solutes, maintaining water content under pressure conditions by acting as an osmotic protective agent in the membrane. Free proline (Pro) was determined using the ninhydrin method. Soluble sugar (SS) was determined using the anthrone method and soluble protein (SP) was determined using the Coomassie brilliant blue method (Zhang, 2019). Based on the membership function value, a comprehensive evaluation of the test materials during the overwintering period was conducted. The formula is as follows:

$$UXi = \frac{Xi - Xmin}{Xmax - Xmin}$$

In the formula:

Xi= Measured value of a certain index of the variety.

Xmin= Minimum value of a certain index of all varieties.

Xmax= Maximum value of a certain index of all varieties.

The average value of the membership values of the relevant indicators of each material is accumulated. The larger the value, the better the comprehensive performance.

Antioxidant enzyme activity

The activity of SOD was measured using the NBT photochemical reduction method (Sun, 2007), with 50% inhibition of NBT photochemical reduction per gram of fresh weight as the enzyme activity unit. CAT activity was determined by ultraviolet spectrophotometry (Sun, 2007), with enzyme activity defined as the amount of enzyme that decreases optical density by 0.1 at 240 nm per minute per gram of fresh weight. POD activity was measured using the guaiacol method (Sun, 2007), with enzyme activity defined as the amount of enzyme that changes A470 nm by 0.01 per minute per gram of fresh weight.

Cell membrane lipid peroxidation

The content of MDA was determined using the thiobarbituric acid (TBA) method. Membrane lipid peroxidation, a critical factor in membrane structure and function destruction, produces malondialdehyde (MDA) as a result of the peroxidation of unsaturated fatty acids in phospholipids. MDA is a key product of membrane lipid peroxidation under drought stress conditions. The extent of membrane lipid peroxidation serves as an indicator of cell membrane free radical damage. Therefore, MDA content is an essential indicator for assessing plant membrane system damage (Wang, 2020).

Statistical analysis

One-way analysis of variance (One-way ANOVA) was employed to compare the physiological index differences between the two root types of alfalfa across different periods. Calculations were performed using Origin 2021 and graphs were created with GraphPad Prism 8.0.

RESULTS AND DISCUSSION

Soluble sugar

Soluble sugars function as osmotic regulators in plant cells, helping to maintain water balance and prevent excessive water loss that could seriously damage the plant (Wang, 2021). In the early overwintering stage, the soluble sugar content in creeping-rooted alfalfa was 24.56 $mg \cdot g^{-1}$, while in taprooted alfalfa, it was 15.69 $mg \cdot g^{-1}$ (Fig 1a). During the overwintering period, the soluble sugar content of the two root types increased and the soluble sugar content of the creeping-rooted alfalfa was 2.5 times that of the axial root type alfalfa. Tao (2008) measured the soluble sugar content in the roots of 22 alfalfa varieties and found significant differences between them, with a strong positive correlation between soluble sugar content and overwintering survival rates. This indicates that the cold resistance of alfalfa varieties is closely related to the soluble sugar content accumulated in the roots during autumn. Therefore, soluble sugar content can serve as an important index for evaluating the cold resistance of alfalfa varieties (Wang, 2023). Bertrand *et al*. (2003) found that the accumulation of soluble sugars in alfalfa roots before overwintering is closely related to the safe overwintering and regreening of alfalfa in spring. Additionally, studies by Chen *et al*. (1996) and Zhu *et al.* (2018); Angotra *et al.* (2020) have shown that the transformation between starch and soluble sugar in the root neck is crucial for alfalfa's overwintering and regreening in the following spring. The performance of different alfalfa varieties varies significantly in this regard (Liu, 2023).

Soluble protein

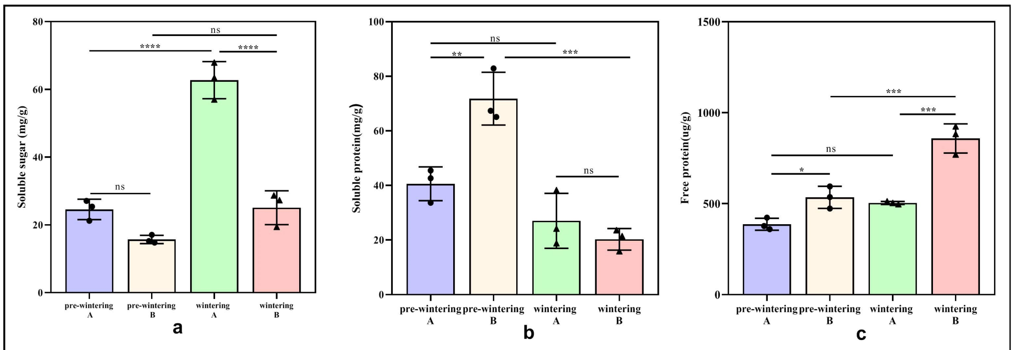
Proteins are the fundamental material basis of life. Under low temperature stress, plants enhance cold resistance by increasing the synthesis of soluble proteins (Nan *et al*., 2011). In the early overwintering stage, the soluble protein content differed significantly between the two root types of alfalfa, with creeping-rooted alfalfa having higher levels than taprooted alfalfa. During the overwintering period, the soluble protein content showed a downward trend; however, creeping-rooted alfalfa maintained higher levels than taprooted alfalfa (Fig 1b). Studies have shown that soluble proteins are crucial osmotic regulators in plants and their content is closely related to plant cold resistance (Zhao, 2014). Schwab *et al.* (1996) demonstrated that the soluble protein content in plants changes significantly under low temperature stress. They found that when soluble protein content in alfalfa roots increased significantly, specific proteins related to cold resistance were produced to combat cold damage (Wang, 2023). Soluble glycoproteins are also important osmoregulatory substances in the cytoplasm. Gao *et al*. (2020) reported that the soluble glycoprotein levels in alfalfa seedlings were significantly higher under low temperature conditions compared to the control group, indicating their effectiveness in resisting cold damage. Meng (2018) observed eight alfalfa varieties from September to the following May, finding that soluble protein content exhibited an inverted V-shaped pattern with temperature, initially increasing and then decreasing (Tian, 2023).

Free protein

The increase in free proline content improves the ability of plant cells to retain water and enhances the adaptability of plants to low temperatures (Wang, 2021). In the early overwintering stage, the free proline content of the two root types of alfalfa ranged from 386.58 to 534.793 nmol·g^{-1}. As temperatures decreased, the proline content of the tested materials showed an increasing trend. During the overwintering period, the proline content in taprooted alfalfa was higher, indicating greater sensitivity to low temperatures compared to creeping-rooted alfalfa (p<0.05) (Fig 1c). At the same temperature, higher free proline content in alfalfa correlates with stronger water retention in plant cells and greater cold tolerance of the corresponding varieties. Conversely, varieties with low proline content exhibit weaker cold tolerance (Wang, 2021). Liu *et al*. (2020) found that cold environments damage alfalfa roots, reducing respiratory function and increasing proline levels, thereby forming an inherent cold resistance mechanism. In this study, free proline content was higher in taprooted alfalfa than in creeping-rooted alfalfa during both periods. Proline, as an osmoregulatory substance, helps maintain intracellular stability and protects cells (Tao *et al*., 2009). However, cold resistance is a complex quantitative trait and alfalfa's resistance to low temperatures cannot be determined by a single index (Wang, 2023). Some scholars have found that while stress can lead to increased proline content, it does not necessarily correlate with cold resistance (Wang *et al*., 2001). Alfalfa varieties and growth periods differ and proline content in roots will vary accordingly. Proline content increases as temperature decreases, showing a negative correlation (Zhang, 2006, Atta *et al*., 2022). Some foreign scholars believe that in cold environments, the increase in free proline content in plants is not the main reason for enhanced cold resistance but rather a response to low temperature stress (Bertrand, 1991).

Malonaldehyde

Malondialdehyde content is closely related to the degree of cell membrane system damage. As a product of plant cell membrane lipid peroxidation, it can directly reflect the extent of cell damage. In the early overwintering stage, the MDA content of creeping-rooted alfalfa was 61.65 nmol·g^{-1}, while in taprooted alfalfa, it was 100.2 nmol·g^{-1}. During the overwintering period, the MDA content increased to 195.90 nmol·g^{-1} in creeping-rooted alfalfa and 156.2 nmol·g^{-1} in taprooted alfalfa (Fig 2a). It can be observed that with the decrease in temperature, the MDA content in both root types of alfalfa increased gradually. The significant difference in MDA content between the different root types at various temperatures indicates that taprooted alfalfa had higher



Letter A stands for creeping-rooted alfalfa; Letter B stands for taprooted alfalfa.ns means no statistical significance, * * * * means p<0.0001, * * * means p<0.001, * * means p<0.01, * means p<0.05.

Fig 1: Analysis of physiological indexes of two root types of alfalfa in early overwintering and overwintering period.

MDA levels than creeping-rooted alfalfa in early winter (P<0.05). As temperatures continued to drop, MDA content increased significantly during the overwintering period. The MDA content in taprooted alfalfa was markedly higher than in the early overwintering period ($p<0.0001$), indicating a significant increase in membrane lipid peroxidation. In cold environments, the permeability of cell membranes and the accumulation of MDA in plants increase (Ma *et al*., 2006). It was found that the MDA content of alfalfa samples was significantly higher than that of the control group under 4°C low temperature treatment, indicating that 4°C low temperature stress caused considerable damage to the cell membranes of alfalfa (Yang, 2018). Sun *et al*. (2017) treated alfalfa seedlings with low temperature stress at -6°C, -7°C and -8°C. They observed that MDA content in the roots and leaves of different alfalfa varieties was higher than in their respective control groups and the MDA content gradually increased as temperatures decreased. In this study, the MDA content in creeping-rooted alfalfa was lower than in taprooted alfalfa during the early stage of overwintering. As temperatures decreased, MDA content increased, indicating that cold tolerance in these varieties is closely related to the degree of membrane lipid peroxidation. The reason is that the antioxidant system cannot effectively remove excessive reactive oxygen species at low temperatures. The continuously increasing reactive oxygen species (O^{-1} and H_2O_2) are more likely to accumulate in the roots, causing an aggravation of root membrane lipid peroxidation (Wang, 2023).

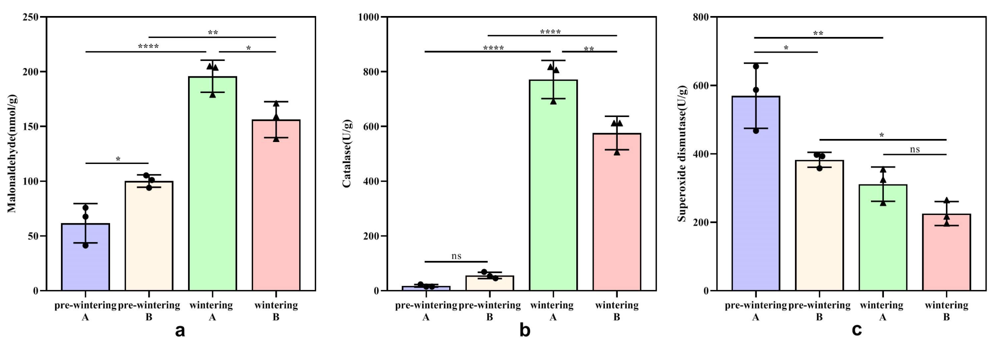
Catalase

Catalase is a protective enzyme in plants, present in all plant tissues and closely related to stress resistance (Liu and Zhang, 1994). Cold-resistant varieties exhibit higher CAT activity. The CAT activity in the roots of the two alfalfa root types showed an increasing trend with decreasing temperatures, indicating an inverse relationship between CAT activity and temperature changes.

With the decrease in temperature, the CAT activity of taprooted alfalfa increased rapidly. In the early overwintering stage, the CAT activity of creeping-rooted alfalfa was lower than that of taprooted alfalfa. During the overwintering period, as temperatures continued to drop, the CAT activity in creeping-rooted alfalfa began to increase, significantly surpassing that of taprooted alfalfa ($p<0.05$) (Fig 2b). At low temperatures, H_2O_2 is readily produced in plant cells and the significant increase in CAT activity helps decompose accumulated peroxides, thereby reducing membrane lipid damage. The activity of CAT is related to the biological characteristics of different alfalfa root types and its persistence can better reflect cold resistance. Wang *et al*. (2022) found that when alfalfa was under low temperature stress, the activities of SOD, POD and CAT in alfalfa increased initially and then decreased as temperatures dropped, with significantly higher levels than in control samples (Hao, 2023). CAT can decompose H_2O_2 into water under low temperature stress (Shen 2016, Saleem *et al.,* 2021). There are few studies on the changes in CAT activity in the root neck of alfalfa under low temperature stress. Yang (2006) studied the effects of natural low temperature stress in the field and found that CAT activity in the root necks of two alfalfa varieties increased initially, reached a maximum in early winter and then fluctuated and decreased significantly in deep winter. This experiment was conducted to study the changes in CAT activity in the roots of creeping-rooted and taprooted alfalfa in a natural environment during early and overwintering periods. Contrary to previous results, the CAT activity of alfalfa showed an increasing trend with decreasing temperatures, with the highest activity observed during the overwintering period. Zhang (2021) showed that CAT activity in the alfalfa root neck is significantly positively correlated with cold resistance under low temperature treatments at -10°C and -30°C, suggesting that CAT activity under these conditions can serve as an index for identifying cold resistance in alfalfa.

Superoxide dismutase

Superoxide dismutase (SOD) can scavenge free radicals and reactive oxygen species, thereby improving the



Letter A stands for creeping-rooted alfalfa; Letter B stands for taprooted alfalfa.ns means no statistical significance, * * * * means $p<0.0001$, * * * means $p<0.001$, * * means $p<0.01$, * means $p<0.05$.

Fig 2: Analysis of physiological indexes of two root types of alfalfa in early overwintering and overwintering period.

antioxidant capacity and resistance of plant tissues. It is generally believed that varieties with strong cold resistance have higher SOD activity. In early winter, the SOD activity in the roots of the two alfalfa root types showed a decreasing trend with seasonal temperature changes (Sun *et al*., 2017). The SOD activity of the tested materials increased rapidly during this period, eliminating superoxide anion free radicals through disproportionation reactions to balance active oxygen metabolism and maintain membrane system stability.In the early and mid-overwintering stages, the SOD activity of creeping-rooted alfalfa increased, whereas the SOD activity of taprooted alfalfa decreased. In the early overwintering stage, the SOD activity of creeping-rooted alfalfa was significantly higher than that of taprooted alfalfa (p<0.05), indicating less damage from superoxide anion free radicals to its membrane lipids (Fig 2c). Generally, at the same temperature, the SOD activity of varieties with stronger cold resistance is higher, reflecting their enhanced ability to decompose peroxides. Conversely, varieties with weaker cold resistance exhibit lower SOD activity. The higher SOD activity observed in creeping-rooted alfalfa during both the early and mid-overwintering periods indicates its strong cold resistance. When alfalfa is under low temperature stress, SOD protects the plant from the damage caused by active oxygen to membrane lipids.In this study, the SOD activity in the roots of creeping-rooted alfalfa was higher than in taprooted alfalfa, which was significantly different (p<0.05). This finding is consistent with the results of (Deng *et al.*, 2005), indicating that varieties with higher SOD activity under the same low temperature conditions have relatively strong cold tolerance.

Principal component analysis

Principal component analysis (PCA) can transform multiple correlated indices into a few uncorrelated comprehensive indices through numerical analysis and calculation. This process avoids information overlap and achieves the goal of dimension reduction. By encompassing most of the original indices, the comprehensive index more intuitively reflects the internal response of plants under stress, making it widely used in the study of plant stress resistance (Yang *et al*., 2021; Hou *et al*., 2022). The results of the PCA showed that the contribution rates of the first three principal components (PC1, PC2 and PC3) were 63.70%, 23.57% and 10.06%, respectively. The characteristic value of the first principal component (PC1) was 3.822, with the major contributing indicators in the corresponding feature vector being catalase, malondialdehyde and soluble sugar, with contributions of 0.502, 0.474 and 0.346, respectively. The characteristic value of the second principal component (PC2) was 1.414, primarily composed of soluble sugar (0.602). The characteristic value of the third principal component (PC3) was 0.604, with the larger feature vector being soluble protein (0.852). Therefore, six individual indicators were transformed into three comprehensive indicators, with a cumulative contribution rate of 97.33% (Table 1). Catalase, malondialdehyde, soluble sugar and soluble protein can be used as the main indexes to evaluate cold resistance through PCA.

According to the results of principal component analysis of physiological indexes of two root types (Fig 3). There were significant differences in physiological indexes and there was no obvious overlap between the two root-type materials. Among them, WA1, WA2, WA3 gathered in quadrant one, PA1, PA2, PA3 gathered in the junction of quadrant two and quadrant three; pB1, PB2, PB3 gathered in quadrant three; wB1, WB2, WB3 are gathered in quadrant four.SS, MDA and CAT gathered in the first quadrant, SOD gathered in the second quadrant, SP gathered in the third quadrant, Pro gathered in the fourth quadrant. SOD and SP were significantly negatively correlated with SS, MDA, CAT and Pro and SS, MDA and CAT were significantly positively correlated with Pro.

Comprehensive evaluation of cold resistance of test materials

During the overwintering period, the soluble sugar content, soluble protein content, free proline content, malondialdehyde content, superoxide dismutase activity and catalase activity of the tested alfalfa materials were used as indicators to comprehensively evaluate the cold resistance of the two root types of alfalfa using the membership function method. The results are shown in Table 2. As illustrated in Table 2, the D-value of creeping-rooted alfalfa and taprooted alfalfa during the overwintering period is ranked as follows: creeping-rooted > taprooted alfalfa.

Table 1: Principal component analysis.

Principal component number	Eigenvalue	Percentage of variance (%)	Cumulative (%)	Eigenvectors					
				SS	MDA	CAT	Pro	SOD	SP
PC1	3.822	63.70	63.70	0.35	0.47	0.50	0.31	-0.41	-0.38
PC2	1.414	23.57	87.27	0.60	0.14	0.11	-0.66	0.41	-0.10
PC3	0.604	10.06	97.33	0.14	0.30	0.05	-0.16	-0.37	0.85

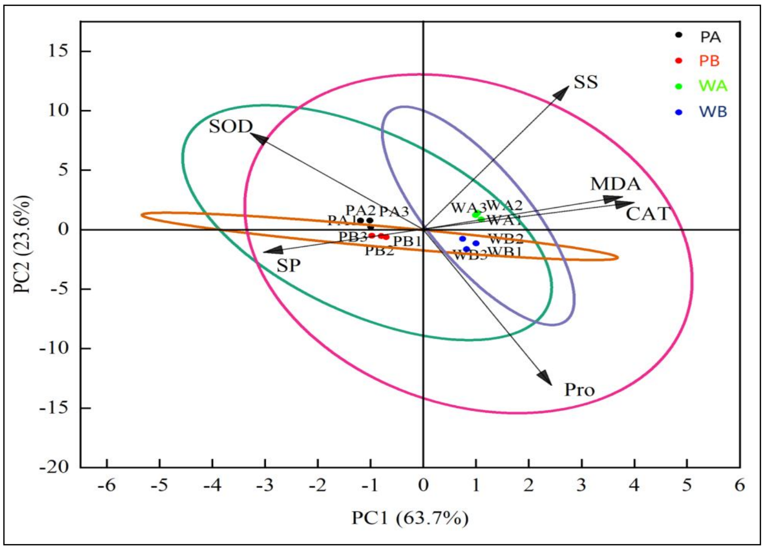


Fig 3: Principal component analysis of physiological indexes of two root types.

Table 2: Membership function analysis evaluation table.

Alfalfa root type	Period	Subordinate function values		D-value	Cold resistance ranking
		μ1	μ2		
Creeping-rooted alfalfa	The pre-winter period	0	0.76	0.20	2
taprooted alfalfa		0.12	0.25	0.16	1
Creeping-rooted alfalfa	The wintering period	1.44	1.00	1.33	2
taprooted alfalfa		0.93	0.00	0.69	1

CONCLUSION

In the early stage of overwintering, the free proline content and catalase activity of creeping-rooted alfalfa were low but stable. As the temperature decreases, these levels begin to accumulate rapidly. During the overwintering period, the protective enzyme activity of creeping-rooted alfalfa was stronger and the response to low temperature was faster. The results of principal component analysis showed that the cold resistance of alfalfa was controlled by many factors. Catalase, malondialdehyde, soluble sugar and soluble protein could be used as the main indexes to evaluate the cold resistance of creeping-rooted alfalfa and taprooted alfalfa. The comprehensive evaluation of membership function showed that the cold resistance of creeping-rooted alfalfa was stronger than that of taprooted alfalfa.

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Conflict of interest

This article is original and has not been published previously. It is not under consideration for publication elsewhere and if accepted, it will not be published elsewhere in the same form, in English or any other language. The submission of the article has been approved by all the authors and the authorities of the host institute where the work was carried out. All the authors have made substantive and intellectual contributions to the article and assume full responsibility for all opinions, conclusions and statements expressed in it.

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