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Differential Overwintering Performance and Mechanistic Insights in Alfalfa Varieties under Nitrogen Regulation

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Abstract

Background: Overwintering is a critical limiting factor for alfalfa production in northern regions, directly influencing yield in the following year.

Methods: A two-factor split-plot experiment was conducted with two nitrogen rates, 0 and 60 kg N ha^{-1} and ten alfalfa varieties. Winter survival rate, subsequent-year yield, physiological and biochemical traits of root crowns and morphological characteristics were systematically analyzed.

Result: Nitrogen application at 60 kg N ha^{-1} significantly increased winter survival rate by 3.50% to 7.95% across varieties. Variety Chaoxinxing showed the highest survival rate, which was 28.16% higher than that of Caoyuan No.2. Nitrogen application also increased hay yield by 10.57% to 11.63%. Under the same nitrogen rate, Chaoxinxing outperformed other varieties by 52.42% to 140.51%. Physiological analyses revealed that nitrogen enhanced cold resistance: in February, soluble sugars in root crowns increased by 2.55% to 5.89%, soluble proteins by 4.98% to 16.65% and free proline by 11.42% to 12.27%, while malondialdehyde content decreased by 24.06% to 34.8%. Activities of superoxide dismutase, peroxidase and catalase were also elevated. Nitrogen supply improved root crown diameter and burial depth, with Chaoxinxing and Qishi T showing stronger responses than Caoyuan No.2. Path analysis indicated that osmoregulatory substances, antioxidant enzymes and morphological traits were positively correlated with winter survival rate, whereas malondialdehyde content was negatively correlated.In conclusion, this study provides new mechanistic insights into variety-specific overwintering performance under nitrogen regulation. Combining high hardiness varieties such as Chaoxinxing and Qishi T with appropriate nitrogen application synergistically enhances alfalfa cold tolerance through dual pathways: physiological regulation including osmotic adjustment and antioxidant defense and morphological adaptation including root crown enlargement and deeper burial. These findings offer a practical strategy for stable and high yield alfalfa cultivation in cold and arid regions of northern China.

Key words: Alfalfa (*Medicago sativa* L.), Cold tolerance mechanism, Cultivar difference, Nitrogen application rate, Winter survival.

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important legume forage crops worldwide, characterized by high protein content, excellent palatability and strong adaptability, playing a crucial role in animal husbandry and agricultural ecosystems (Wang and Zou, 2019). In recent years, with the structural adjustment of China's livestock industry and the expansion of herbivorous animal breeding scale, the demand for high-quality alfalfa has continued to increase, necessitating the urgent adoption of technical measures to enhance alfalfa yield and quality. Alfalfa cultivation in China is predominantly concentrated in the Northwest and Northeast regions, accounting for 70% of the total national planting area. However, these regions experience long, severe winters, where extreme weather events such as spring freezes and cold waves constitute critical factors affecting successful alfalfa overwintering, thereby severely compromising the sustainable utilization of alfalfa. Consequently, safe overwintering has become fundamental to achieving stable and high-yield alfalfa production in northern China (Ni and Wang, 2024)

Different alfalfa varieties exhibit varying performance in stress resistance (overwintering) due to their distinct genetic backgrounds (Li *et al.*, 2023). Fall dormancy, as a key genetic trait, is closely associated with cold tolerance and yield potential in alfalfa (Jia *et al.*, 2025). For instance, varieties with different fall dormancy levels demonstrate significant differences in osmotic substance accumulation and antioxidant capacity during low-temperature adaptation (Li *et al.*, 2024; Avci *et al.*, 2018). Previous studies have demonstrated that alfalfa cold resistance is closely

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associated with the accumulation of osmotic adjustment substances such as soluble sugars and proline in the root crown, which regulate intracellular osmotic balance to reduce freezing points and effectively alleviate physical damage to membrane systems caused by intracellular ice crystal formation, thereby improving winter survival rates of alfalfa (Smith, 1964). Additionally, winter-hardy alfalfa varieties exhibit certain advantages in plant morphology (Schwab *et al.*, 1996). Through the synergistic effects of physiological regulation and morphological adaptation, alfalfa can collectively withstand low-temperature stress, ensuring safe overwintering and stable, high-yield production in cold regions of northern China.

Nitrogen exerts profound effects on various physiological processes in alfalfa, including photosynthesis, carbon-nitrogen metabolism and stress resistance (Liu *et al.*, 2013). Although alfalfa can acquire atmospheric nitrogen through symbiotic fixation with rhizobium, its nitrogen fixation capacity is constrained by multiple factors such as cultivar characteristics, soil conditions, climatic environments and management practices (Ma *et al.*, 2024), which cannot satisfy the requirements for high yield and superior quality. Consequently, exogenous nitrogen supply has become an important approach for enhancing alfalfa yield and quality (Liu *et al.*, 2024). Different alfalfa varieties exhibit variations in nitrogen fixation, uptake and utilization, as well as in the expression of stress resistance-related genes and physiological response mechanisms, which may lead to divergent responsive patterns to nitrogen fertilizer (Wang *et al.*, 2021). Moreover, studies have revealed that the performance of alfalfa in adapting to low temperature is closely associated with nitrogen metabolism (Li, 2025).

Based on the aforementioned considerations, this study conducted field experiments to systematically analyze the differences in overwintering performance and yield of different alfalfa varieties under varying nitrogen supply levels. Through the analysis of physiological indicators and morphological characteristics during the overwintering period, this study aimed to elucidate the low-temperature adaptation mechanisms underlying the divergent overwintering performance among varieties and to clarify the regulatory effects of nitrogen on winter survival capacity and production potential, thereby providing theoretical basis and technical support for high-yield and high-quality alfalfa production.

MATERIALS AND METHODS

Experimental site description

The field experiment was conducted at the Hailiutu Experimental Base in Tumed Left Banner, Hohhot City, during 2023-2024. The experimental site is located on the Tumed Plain (111°23′ 46″ E, 40°31′ 17″ N, altitude 1008 m) and features a temperate continental monsoon climate. The mean annual temperature is 7.3°C, with an average temperature of 17.6°C during the growing season (May-September). The maximum and minimum annual temperatures are 22.6°C and -11.6°C, respectively. The mean annual sunshine duration is 2744.1 h and the average annual precipitation is approximately 400 mm. The soil physicochemical properties prior to sowing were as follows: total nitrogen 0.99 g kg^{-1}, available phosphorus 28.62 mg kg^{-1}, available potassium 239.67 mg kg^{-1} and pH 8.09.

Experimental materials

Ten alfalfa cultivars were used in this study (Table 1).

Experimental design

This experiment employed a two-factor split-plot design. Factor 1 was nitrogen application rate, comprising two levels: 0 kg N ha^{-1} (N0) and 60 kg N ha^{-1} (N60). Factor 2 was variety, consisting of ten alfalfa varieties (Table 1). Each treatment was replicated three times. Main plots were separated by 0.5-m intervals and subplots were divided by small ridges. The subplot area was 4 m × 5 m, with random distribution of variety subplots within each main plot. Alfalfa was sown by drilling in rows spaced 27 cm apart, at a seeding rate of 0.2 kg ha^{-1}. Phosphorus and potassium fertilizers were applied at 120 kg P_2O_5 ha^{-1} and 120 kg K_2O ha^{-1}, respectively. All fertilizers were applied once annually by trench application after regreening and weeding.

Table 1: Alfalfa cultivars and their sources.

Variety	Code	Source provider
Chaoxinxing	CX	Beijing Baiqingyuan Animal Husbandry Technology Development Co., Ltd.
Qishi T	QS	Beijing Baiqingyuan Animal Husbandry Technology Development Co., Ltd.
Baimu 341	341	Beijing Baiqingyuan Animal Husbandry Technology Development Co., Ltd.
Lüsile	LS	Beijing Baiqingyuan Animal Husbandry Technology Development Co., Ltd.
Zhongmu No. 3	ZM	Beijing Baiqingyuan Animal Husbandry Technology Development Co., Ltd.
Baimu 202	202	Beijing Baiqingyuan Animal Husbandry Technology Development Co., Ltd.
Tiaozhanzhe	TZ	Beijing Baiqingyuan Animal Husbandry Technology Development Co., Ltd.
Aohan	AH	Hohhot Grassland Workstation
Jinhuanghou	JH	Hohhot Grassland Workstation
Caoyuan No. 2	CY	Hohhot Grassland Workstation

Measurement indicators and methods

The indoor experiments for the determination of physiological and biochemical traits as well as morphological characteristics were conducted at the Inner Mongolia Key Laboratory of Soil Quality and Nutrient Resources.

Winter survival rate

On November 15, 2023, three random 1-m row sections were selected within each experimental plot and the number of plants in each section was recorded. The number of surviving plants was surveyed after alfalfa regreening in the following spring. Winter survival rate was calculated using the following formula:

$$\text{Overwintering rate} = \frac{\text{Number of surviving plants after overwintering}}{\text{Number of surviving plants before overwintering}} \times 100\%$$

Alfalfa yield was determined at the early flowering stage. Samples were collected on June 15, 2024 (first cut), August 10, 2024 (second cut) and September 20, 2024 (third cut), with three harvests conducted annually.

Hay yield

Alfalfa was harvested at the early flowering stage. In each plot, three random 1 m × 1 m quadrats were harvested with a stubble height of approximately 5 cm. Fresh herbage was weighed immediately, then subjected to heat inactivation in an oven at 105°C for 30 min, followed by drying at a constant temperature of 65°C until constant weight was achieved. Hay yield per hectare was subsequently calculated.

Physiological indicators

Beginning when plants entered the wintering period during 2023-2024, roots were excavated from the field at 30-day intervals (November 1, December 1, February 1, March 1 and April 1). Three plants were sampled from each plot. After excavation, alfalfa roots were rinsed thoroughly with distilled water, blotted dry with absorbent paper, immediately frozen in liquid nitrogen and stored at -80°C for subsequent determination of root crown morphology and dynamic changes in physiological indicators during the overwintering period.

Soluble sugar content was determined by the anthrone colorimetric method; soluble protein content by the Coomassie brilliant blue G-250 staining method; free proline content by the acid ninhydrin method and malondialdehyde (MDA) content by the thiobarbituric acid method. Superoxide dismutase (SOD) activity was assayed by the nitroblue tetrazolium photochemical reduction method; peroxidase (POD) activity by the guaiacol method and catalase (CAT) activity by the ultraviolet absorption method (Zou, 1995).

Root crown diameter

Measured at the swollen portion of the root crown using vernier calipers.

Root crown burial depth

The vertical distance from the soil surface to the upper end of the root crown.

Data processing and statistical methods

All data were initially organised using Microsoft Office Excel 2019 and two-way analysis of variance (ANOVA) and significance tests were performed using SPSS 27.0. Graphs were produced using Origin Pro 2021.

RESULTS AND DISCUSSION

Winter survival rate and hay yield

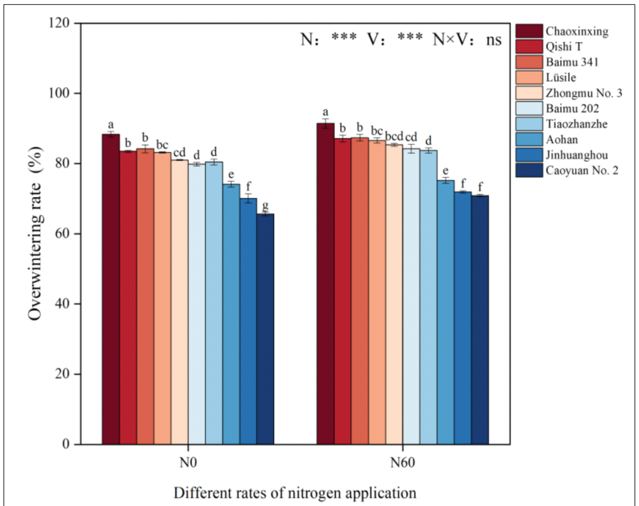
Nitrogen application at 60 kg N ha^{-1} significantly increased winter survival rate of all alfalfa varieties by 3.50% to 7.95% (Fig 1). Variety Chaoxinxing showed the highest survival rate, which was 28.16% higher than that of Caoyuan No.2 under N60 treatment. The interaction between nitrogen and variety was not significant.

Hay yield decreased with cutting frequency. Nitrogen application also significantly increased hay yield by 10.57% to 11.63% across varieties and cuts (Fig 2). Chaoxinxing consistently produced the highest yield, outperforming Caoyuan No. 2 by 52.42% to 140.51% under N60. The interaction between nitrogen fertiliser and variety was significant only at the second cut (August 2024).

Physiological responses to low temperature

Osmotic adjustment substances

Soluble sugars, soluble proteins and free proline in root crowns all exhibited a unimodal trend during winter, peaking



Note: Different lowercase letters indicate significant differences (P<0.05) between varieties under the same nitrogen application rate; '*' denotes a two-way ANOVA for nitrogen and variety, where * indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001 and ns indicates P>0.05; the same applies throughout.

Fig 1: Overwintering rates of different alfalfa varieties under varying nitrogen application rates.

in February when cold stress was most severe (Fig 3-4, Table 2). Nitrogen fertilization significantly increased their accumulation. For soluble sugars, the increase ranged from 2.55% to 5.89% in February. Soluble proteins increased by 4.98% to 16.65% and free proline by 11.42% to 12.27%. High hardiness varieties (Chaoxinxing, Qishi T, Baimu 341) accumulated significantly higher levels of these osmolytes than low hardiness varieties (Aohan, Jinhuanghou, Caoyuan No. 2). For example, under N60, Chaoxinxing had 19.01% higher soluble sugar and 13.58% higher proline than Caoyuan No.2.

Oxidative stress and antioxidant enzymes

Malondialdehyde (MDA) content, an indicator of membrane lipid peroxidation, also peaked in February (Fig 5). Nitrogen application reduced MDA by 24.06% to 34.8%. Low hardiness varieties had significantly higher MDA than high hardiness varieties; under N0, MDA in Aohan was 111.20% higher than in Chaoxinxing.

Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities followed a similar seasonal pattern, reaching maxima in February (Table 3-5). Nitrogen treatment elevated SOD, POD and CAT activities.

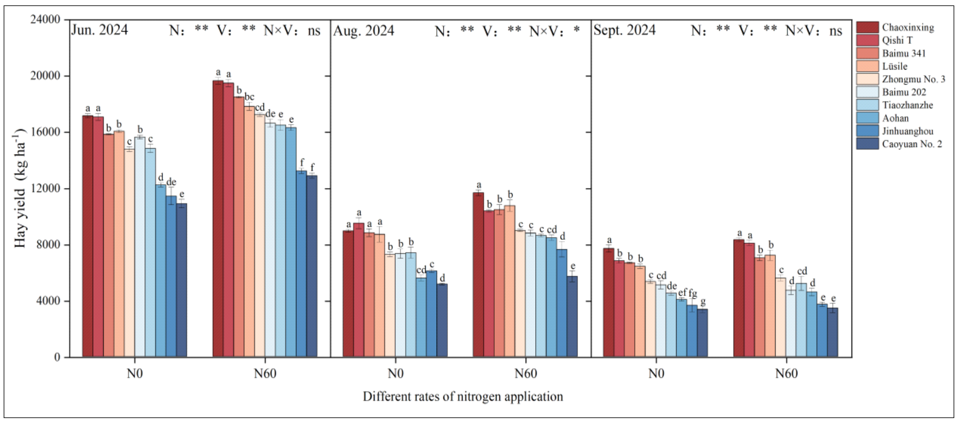


Fig 2: Alfalfa hay yield of different varieties under varying nitrogen application rates.

Table 2: Soluble protein content (μg g^{-1}) in different alfalfa varieties under different nitrogen application rates.

Nitrogen rate	Variety	Soluble protein content (μg g^{-1})				
		Oct. 2023	Nov. 2023	Feb. 2024	Mar. 2024	Apr. 2024
N0	CX	41.88±0.81a	47.98±0.3a	51.9±0.38a	47.39±1.21a	45.5±0.47a
	QS	37.16±1.07bc	42.22±0.09d	48.3±0.3c	46.97±1.11ab	40.42±0.49bc
	341	38.78±0.77b	46.51±0.42b	51.5±0.08a	44.46±1.05abc	44.39±0.71a
	LS	37.65±1.45bc	41.92±0.32d	48.38±0.37c	44.17±0.51abc	45.54±1.02a
	ZM	37.19±1.62bc	46.1±0.37b	49.8±0.11b	43.67±0.1bc	43.99±0.2a
	202	38.94±0.29b	42.44±0.02d	47.92±0.3c	42.81±0.79c	39.69±0.97bc
	TZ	37.74±0.4bc	42.52±0.07d	48.1±0.22c	42.63±1.21cd	40.87±1.61b
	AH	35.15±0.93c	43.46±0.15c	45.66±0.6d	39.03±2.15de	38.34±0.92cd
	JH	35.38±0.53c	42.08±0.36d	45.27±0.8d	38.9±1.85e	38.11±0.35cd
	CY	35.42±0.17c	44.19±0.13c	45.59±0.51d	38.02±1.12e	37.65±0.28d
N60	CX	47.38±0.99a	49.07±0.37b	60.54±1.03a	51.23±0.54a	52.39±0.59a
	QS	46.3±0.58ab	44.42±0.07d	47.35±0.47bc	50.77±0.32ab	47.04±1.22c
	341	47.53±0.71a	50.69±0.41a	59.88±1.62a	49.59±1.24abc	51.84±0.49a
	LS	44.26±0.42bc	43.3±0.14ef	47.96±0.51bc	49.14±0.52bc	51.21±0.53a
	ZM	42.43±0.98cde	45.75±0.05c	47.1±0.2c	48.56±0.37cd	50.12±0.62ab
	202	43.94±0.51cd	44.22±0.35de	60.28±1.09a	46.77±0.2def	47.97±0.32bc
	TZ	43.17±1.43cde	42.96±0.24f	49.99±1.38b	47.66±0.86cde	46.3±1.81c
	AH	41.05±1.02e	44.21±0.51de	47.16±0.6c	46.52±0.35ef	40.72±0.43d
	JH	41.81±1.15cde	43.14±0.08f	47.63±0.26bc	44.31±0.52g	41.41±0.42d
	CY	41.67±0.24de	44.24±0.48de	47.86±0.53bc	45.27±0.98fg	41.27±0.45d
Significance test						
Nitrogen rate (N)		**	**	**	**	**
Variety (V)		**	**	**	**	**
N × V		ns	ns	*	ns	ns

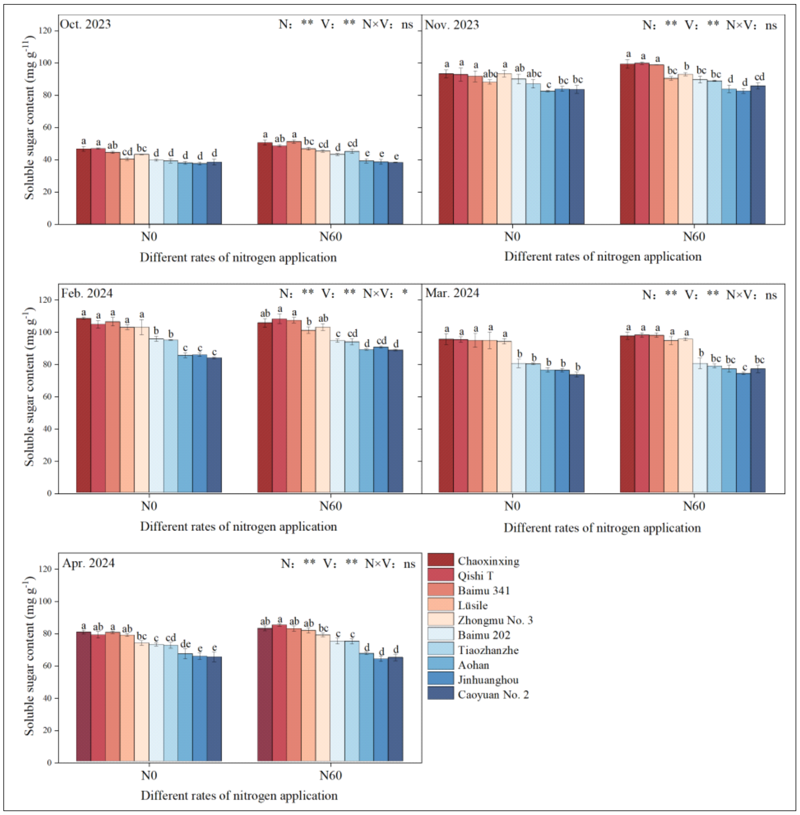


Fig 3: Soluble sugar content in different alfalfa varieties under varying nitrogen application rates.

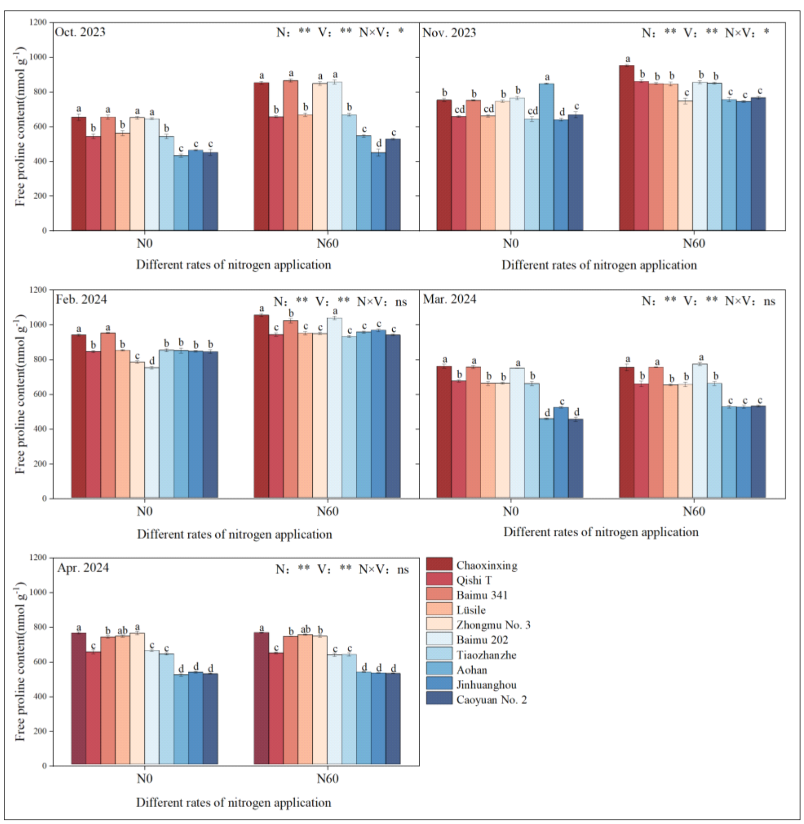


Fig 4: Proline content in different alfalfa varieties under varying nitrogen application rates.

Table 3: SOD activity (U g^{-1} FW) in different alfalfa varieties under varying nitrogen application rates.

Nitrogen rate	Variety	SOD activity (U g^{-1} FW)				
		Oct. 2023	Nov. 2023	Feb. 2024	Mar. 2024	Apr. 2024
N0	CX	45.85±0.3a	54.03±0.38a	65.67±1.15a	60.71±0.62a	51.71±0.58a
	QS	40.08±0.09d	50.43±0.3c	55.37±0.19cd	56.01±0.05b	45.37±0.62bc
	341	44.39±0.42b	53.63±0.08a	57.3±0.64c	60.06±0.79a	50.33±0.89a
	LS	39.79±0.32d	50.51±0.37c	57.89±1.87c	55.71±1.59b	51.77±1.28a
	ZM	43.98±0.37b	51.93±0.11b	55.77±1.1cd	55.12±1.37b	49.82±0.25a
	202	40.31±0.02d	50.05±0.3c	62.13±0.93b	58.82±0.56a	44.45±1.21bcd
	TZ	40.39±0.07d	50.23±0.22c	62.81±1.78ab	54.07±0.25b	45.93±2.02b
	AH	41.33±0.15c	47.79±0.6d	55.76±0.62cd	49.59±0.74c	42.77±1.15cd
	JH	39.95±0.36d	47.4±0.8d	53.43±1.13d	49.46±0.68c	42.47±0.43cd
	CY	42.06±0.13c	47.72±0.51d	52.83±1.28d	51.25±0.92c	41.9±0.34d
N60	CX	46.94±0.37b	65.67±1.03a	66.06±1.27a	64.57±1.01a	56.57±0.74a
	QS	42.29±0.07d	52.48±0.47bc	59.67±0.42b	60.95±0.63b	49.89±1.53c
	341	48.56±0.41a	65.01±1.62a	59.31±1.24b	61.61±0.71ab	55.88±0.61a
	LS	41.17±0.14ef	53.09±0.51bc	53.75±0.88c	59.22±1.18b	55.1±0.66a
	ZM	43.62±0.04c	52.23±0.2c	59.8±1.24b	60.1±0.66b	53.73±0.77ab
	202	42.09±0.35de	65.41±1.09a	64.52±0.59a	60.83±1.15b	51.04±0.39bc
	TZ	40.83±0.24f	55.12±1.38b	64.54±0.4a	60.6±0.97b	48.97±2.26c
	AH	42.08±0.51de	52.29±0.6c	53.5±0.02c	55.79±0.72c	41.99±0.54d
	JH	41.01±0.08f	52.76±0.26bc	55.16±0.55c	54.6±0.94c	42.85±0.52d
	CY	42.1±0.48de	52.99±0.53bc	54.49±0.64c	60.86±2.28b	42.67±0.56d
Significance test						
Nitrogen rate (N)		**	**	**	**	**
Variety (V)		**	**	**	**	**
N × V		ns	ns	*	ns	ns

Table 4: POD activity (U g^{-1} FW min^{-1}) of different alfalfa varieties under varying nitrogen application rates.

Nitrogen rate	Variety	POD activity (U g^{-1} FW min^{-1})				
		Oct. 2023	Nov. 2023	Feb. 2024	Mar. 2024	Apr. 2024
N0	CX	20.8±0.15a	21.16±0.74a	27.04±0.14a	22.08±0.83a	24.91±0.14a
	QS	17.92±0.04d	21.3±0.21a	26.13±0.6a	21.94±1.34a	24±0.6a
	341	20.06±0.21b	20.09±0.34ab	26.52±0.66a	21.59±1.13a	24.39±0.66a
	LS	17.77±0.16d	18.03±0.4cd	25.66±0.36a	20.38±0.45abc	23.53±0.36a
	ZM	19.86±0.19b	19.46±0.11bc	25.68±1.17a	22.13±0.7a	23.55±1.17a
	202	18.03±0.01d	17.69±0.28d	23.87±0.39b	21.04±0.97ab	21.74±0.39b
	TZ	18.06±0.03d	17.46±0.65d	23.69±0.06b	20.02±0.88abc	21.56±0.06b
	AH	18.53±0.08c	16.85±0.44d	21.3±0.37c	18.48±0.16c	19.17±0.37c
	JH	17.85±0.18d	16.63±0.47d	21.39±0.25c	18.94±0.51bc	19.26±0.25c
	CY	18.9±0.06c	17.08±0.96d	20.91±0.13c	18.85±0.81bc	18.78±0.13c
N60	CX	25.6±0.19b	23.11±0.78a	35.13±0.85ab	24.84±0.65a	26.35±0.64ab
	QS	23.27±0.03d	22.11±0.35ab	35.95±0.99a	24.97±0.17a	26.97±0.74a
	341	26.41±0.21a	23.45±0.54a	35.67±0.56a	24.69±0.05a	26.75±0.42a
	LS	22.72±0.07ef	21.21±0.5bc	33.59±0.69b	22.6±0.29bc	25.19±0.52b
	ZM	23.94±0.02c	20.49±0.3cd	34.25±0.7ab	23.26±0.27b	25.69±0.52ab
	202	23.17±0.18de	19.43±0.32d	31.45±0.34c	22.41±0.51bc	23.59±0.26c
	TZ	22.55±0.12f	20.41±0.67cd	31.18±0.62cd	22.22±0.1bc	23.39±0.46cd
	AH	23.17±0.25de	17.47±0.71e	29.6±0.12d	20.95±0.59d	22.2±0.09d
	JH	22.64±0.04f	17.12±0.74e	30.11±0.23cd	20.65±0.37d	22.58±0.17cd
	CY	23.18±0.24de	16.97±0.1e	29.52±0.16d	21.45±0.47cd	22.14±0.12d
Significance test						
Nitrogen rate (N)		**	**	**	**	**
Variety (V)		**	**	**	**	**
N × V		ns	*	ns	ns	ns

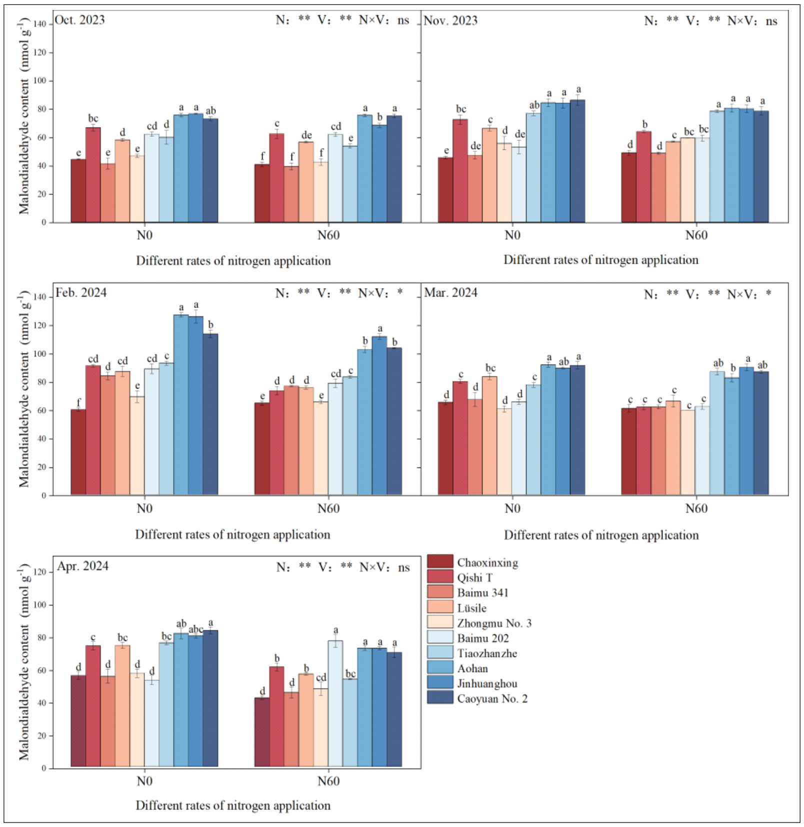


Fig 5: Malondialdehyde content in different alfalfa varieties under varying nitrogen application rates.

Table 5: CAT activity (U g^{-1} FW min^{-1}) of different alfalfa varieties under varying nitrogen application rates.

Nitrogen rate	Variety	CAT activity (U g^{-1} FW min^{-1})				
		Oct. 2023	Nov. 2023	Feb. 2024	Mar. 2024	Apr. 2024
N0	CX	5.09±0.03a	6.75±0.05a	7.3±0.13a	6.55±0.33a	5.75±0.06a
	QS	4.45±0.01d	6.3±0.04c	6.15±0.02cd	6.43±0.13ab	5.04±0.07bc
	341	4.93±0.05b	6.7±0.01a	6.37±0.07c	6.19±0.11abc	5.59±0.1a
	LS	4.42±0.04d	6.31±0.05c	6.43±0.21c	5.9±0.21cd	5.75±0.14a
	ZM	4.89±0.04b	6.49±0.01b	6.2±0.12cd	5.94±0.03bc	5.54±0.03a
	202	4.48±0d	6.26±0.04c	6.9±0.1b	5.31±0.14e	4.94±0.13bcd
	TZ	4.49±0.01d	6.28±0.03c	6.98±0.2ab	5.41±0.19de	5.1±0.22b
	AH	4.59±0.02c	5.97±0.08d	6.2±0.07cd	4.53±0.11f	4.75±0.13cd
	JH	4.44±0.04d	5.93±0.1d	5.94±0.13d	4.8±0.15f	4.72±0.05cd
	CY	4.67±0.01c	5.96±0.06d	5.87±0.14d	4.68±0.15f	4.66±0.04d
N60	CX	5.22±0.04b	6.68±0.05a	8.26±0.16a	6.95±0.16a	6.29±0.08a
	QS	4.7±0.01d	6.25±0.18abc	7.46±0.05b	6.99±0.1a	5.54±0.17c
	341	5.4±0.05a	6.7±0.2a	7.41±0.16b	6.98±0.1a	6.21±0.07a
	LS	4.57±0.02ef	6.42±0.17ab	6.72±0.11c	6.74±0.18a	6.12±0.07a
	ZM	4.85±0.01c	6.15±0.12bcd	7.48±0.15b	6.81±0.07a	5.97±0.09ab
	202	4.68±0.04de	6.04±0.1bcd	8.07±0.07a	5.73±0.24b	5.67±0.04bc
	TZ	4.54±0.03f	6.07±0.25bcd	8.07±0.05a	5.62±0.09bc	5.44±0.25c
	AH	4.68±0.06de	5.78±0.03d	6.69±0c	5.49±0.17bc	4.67±0.06d
	JH	4.56±0.01f	5.89±0.12cd	6.9±0.07c	5.28±0.05c	4.76±0.06d
	CY	4.68±0.05de	5.86±0.19cd	6.81±0.08c	5.48±0.17bc	4.74±0.06d
Significance test						
Nitrogen rate (N)		**	**	**	**	**
Variety (V)		**	**	**	**	**
N × V		ns	ns	ns	ns	ns

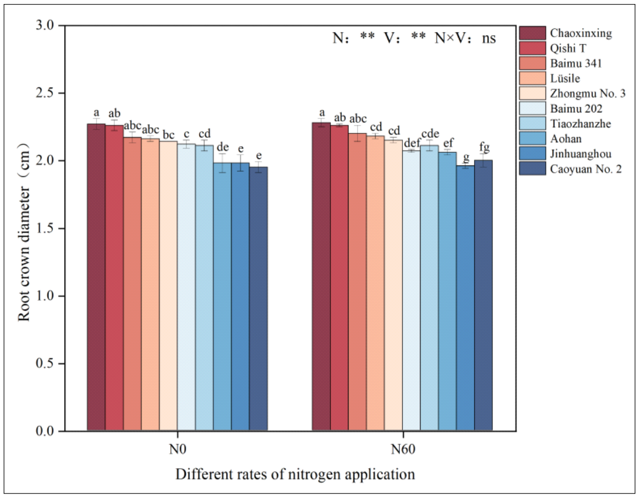


Fig 6: Root collar diameter (cm) of different alfalfa varieties under different nitrogen application rates.

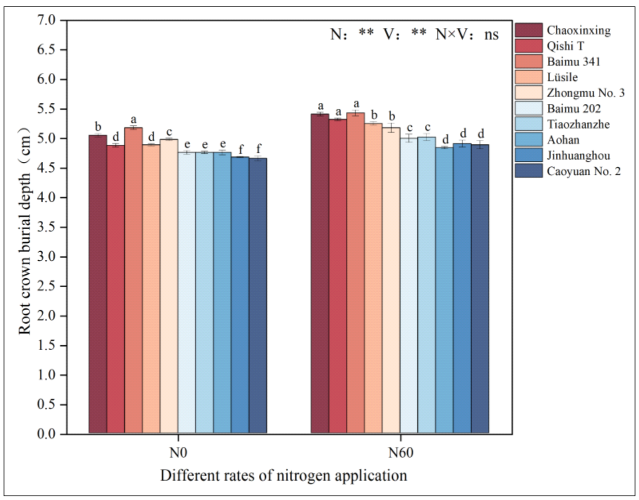


Fig 7: Depth of alfalfa root collars below the soil surface (cm) for different varieties under varying nitrogen application rates.

High hardiness varieties maintained higher enzyme activities throughout winter. For instance, under N60, SOD activity of Chaoxinxing was 21.23% higher than that of Caoyuan No.2 in February. The interaction between nitrogen and variety was occasionally significant for some indicators (*e.g.*, proline in October and November, SOD and POD in February), indicating that high hardiness varieties responded more strongly to nitrogen supply.

Root crown morphology

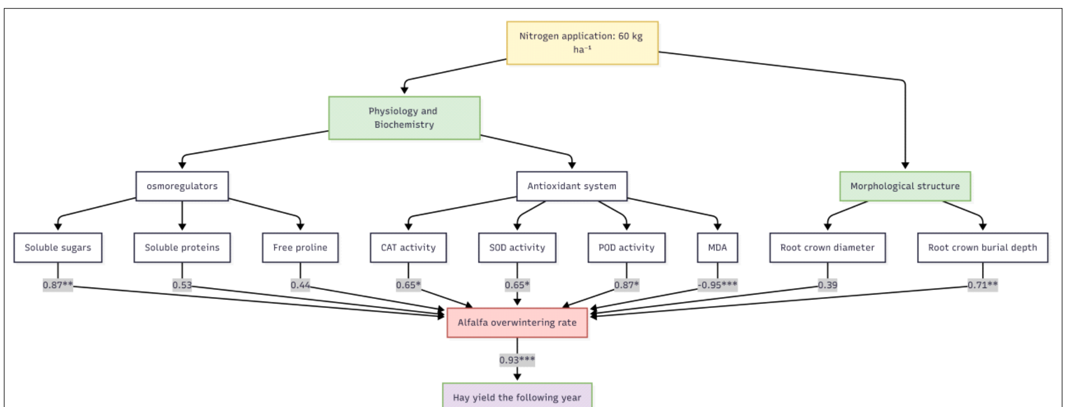
Nitrogen application significantly increased both root crown diameter and burial depth (Fig 6-7). Across varieties, root crown diameter increased by 4.00% to 16.14% under N60 compared to N0. Chaoxinxing and Qishi T had the largest diameters and deepest burial depths, whereas Caoyuan No.2 and Aohan had the smallest values. Burial depth increased by 4.83% to 4.94% under N60 for all varieties.

Path analysis of nitrogen regulation on alfalfa overwintering performance

Path analysis (Fig 8) showed that soluble sugars, soluble proteins, proline, SOD, POD, CAT, root crown diameter and burial depth were all positively correlated with winter survival rate. In contrast, MDA was negatively correlated with winter survival rate. Hay yield was also positively correlated with winter survival rate. These results indicate that nitrogen enhances alfalfa overwintering through coordinated regulation of osmotic adjustment, antioxidant defense and root crown morphology.

Performance of alfalfa in adaptation to low-temperature stress

Low temperature stress is the primary factor limiting alfalfa overwintering in northern regions. Plants adapt by modulating osmotic substances and antioxidant enzymes (Liu *et al*., 2015). In this study, physiological indicators differed significantly among varieties and correlated strongly with winter survival rate.



Note: "*" indicates a correlation analysis between variables; * denotes P<0.05, ** denotes P<0.01 and *** denotes P<0.001.

Fig 8: Analysis of the pathways by which nitrogen regulates the overwintering performance of alfalfa.

Soluble sugars, soluble proteins and free proline are key osmotic regulators. They lower freezing points, maintain homeostasis and stabilise membranes (Yue *et al.*, 2026). All varieties showed a unimodal sugar curve peaking in February. High hardiness varieties (Chaoxinxing, Qishi T, Baimu 341) accumulated significantly more sugars than Aohan and Caoyuan No. 2 (P<0.05), consistent with Liu *et al.* (2019). Moreover, Chaoxinxing displayed more stable sugar consumption after winter, favouring rapid spring regrowth.

Soluble protein and proline trends were similar, both peaking in February. Chaoxinxing, Baimu 341 and Zhongmu No.3 had higher protein levels than Caoyuan No. 2 (P<0.05), indicating more active protective mechanisms. Proline, a cryoprotectant (Wang and Zhuang, 2008), remained high in Chaoxinxing during late winter, while low hardiness varieties showed sharp declines, reducing cellular stability and regrowth

Malondialdehyde (MDA) reflects membrane lipid peroxidation (Liu *et al.*, 2024). MDA peaked in February. Aohan, Jinhuanghou and Caoyuan No. 2 had significantly higher MDA than Chaoxinxing and Zhongmu No. 3 (P<0.05), meaning the latter suffered less oxidative damage. SOD, POD and CAT, key ROS scavengers (Luo *et al.*, 2004), also peaked in February. High hardiness varieties exhibited much higher enzyme activities than low hardiness ones, demonstrating superior ROS elimination capacity.

Effects of alfalfa varieties on winter hardiness

Variety is the intrinsic determinant of overwintering success (Wang *et al.*, 2019). Genetic background leads to differences in cold resistance, physiology and morphology (Wang *et al.*, 2024). In this study, the ten varieties differed significantly in winter survival, yield, physiological traits and root crown morphology (P<0.01). Chaoxinxing, Qishi T and Baimu 341 performed best, while Caoyuan No. 2, Aohan and Jinhuanghou performed worst.

Chaoxinxing had 22.68% higher winter survival than Caoyuan No. 2 under N0, consistent with Wang *et al.* (2015) in eastern Jilin, confirming that variety selection is critical for safe overwintering.

High hardiness varieties accumulated more soluble sugars, proteins and proline, showed higher SOD/POD/CAT activities and lower MDA. These traits collectively alleviate oxidative membrane damage and maintain cellular integrity (Wang *et al.*, 2024). Kang *et al.* (2010) also found that productive varieties possess stronger physiological adaptability.

Root crown diameter and burial depth are important morphological traits (Shi *et al.*, 2009). Chaoxinxing, Qishi T and Baimu 341 had larger diameters and deeper burial than Caoyuan No. 2 and Aohan. Larger root crowns store more carbohydrates and nitrogen, while deeper burial places growing points in thermally stable soil layers, avoiding extreme surface cold (Guo and Shi, 2024; Guo, 2024). These findings align with previous research highlighting the critical role of root crown physiological and morphological dynamics in alfalfa overwintering adaptation (Guo and Shi, 2024).

Effects of nitrogen on alfalfa overwintering

Nitrogen regulates growth, development and stress resistance. Appropriate N supply enhances yield and winter hardiness by optimising physiology and morphology (Zhang *et al.*, 2016). Here, N60 increased winter survival by 3.50-7.95% and hay yield by 10.57-11.63%, with stronger effects on high hardiness varieties.

Physiologically, N promotes accumulation of osmotic substances and antioxidant enzymes (Li *et al.*, 2025). Under N60, soluble sugars, proteins and proline were significantly higher than under N0 (P<0.01), especially in February. This agrees with Liu *et al.* (2013), showing that N supply affects carbon nitrogen balance and cold related substances. N60 also raised SOD, POD and CAT activities and lowered MDA, meaning N alleviates peroxidation by enhancing ROS scavenging. Wan (2023) confirmed that N improves leaf photosynthesis and antioxidant capacity, enhancing cold adaptation.

Morphologically, N significantly increased root crown diameter and burial depth (4.83-4.94% for depth). Larger root crowns store more reserves and deeper burial improves avoidance of surface cold (Chen *et al.*, 2025). Interactions between N and variety were significant for some indicators (proline, SOD, POD) at specific times (*e.g.*, February 2024, P<0.05), indicating differential responses. High hardiness varieties like Chaoxinxing and Qishi T responded more strongly to N than low hardiness ones, possibly due to differences in N metabolism efficiency, carbon N allocation and cold related gene expression (Ma *et al.*, 2026).

CONCLUSION

This study systematically analyzed the overwintering performance and underlying physiological and morphological mechanisms of ten alfalfa varieties under different nitrogen application levels. Nitrogen application significantly enhanced winter survival rate by 3.50-7.95% and hay yield by 8.73-30.31% across varieties. Mechanistically, nitrogen promoted accumulation of osmotic adjustment substances (soluble sugars, soluble proteins, free proline) in root crowns, elevated activities of antioxidant enzymes (SOD, POD, CAT) and reduced malondialdehyde content, thereby alleviating low-temperature oxidative damage. Simultaneously, nitrogen increased root crown diameter and burial depth, optimizing overwintering organ morphology. Significant genetic differences existed among varieties: Chaoxinxing, Qishi T and Baimu 341 exhibited superior low-temperature adaptation with high osmolyte accumulation, strong antioxidant capacity, low MDA and robust root crowns; Caoyuan No. 2, Aohan and Jinhuanghou performed poorly. High hardiness varieties responded more positively to nitrogen supply. In conclusion, variety selection is the foundation for efficient alfalfa overwintering and rational nitrogen application further exploits this potential, offering a synergistic strategy for green and high yield alfalfa production in cold and arid regions of northern China.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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