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Effect of Pre-winter Irrigation on the Overwintering Capacity of Alfalfa Crown Buds under Simulated Animal Digging and Grazing Disturbance

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ABSTRACT

Background: In the high-latitude regions of Northeast China, the overwintering ability of crown buds directly determines the above-ground population density and productivity of alfalfa in the following year and is closely related to soil moisture conditions during the overwintering period.

Methods: This experiment established three irrigation gradients [equal to 0 mm (W1), 20 mm (W2), 40 mm (W3) rainfall] and simulated three animal excavation disturbance depths [0 cm (G1), 3 cm (G2), 6 cm (G3)].

Result: The results showed that pre-winter irrigation promoted alfalfa crown bud growth (vs. W1: length: W2 +3.82%, W3 +12.70%; number: W2 +39.21%, W3 +65.69%). In contrast, disturbance inhibited both bud length and count. Additionally, irrigation reduced the activities of antioxidant enzymes such as superoxide dismutase (vs. W1: W2 -24.97%, W3 -18.03%), peroxidase (vs. W1: W2 -31.50%, W3 -16.04%) and catalase (vs. W1: W2 -19.70%, W3 -42.03%), while increasing the contents of soluble sugar and soluble protein during the overwintering period without grazing disturbance (vs. W1: soluble sugar: W2 + 43.40%, W3 + 60.60%; soluble protein: W2 +14.07%, W3 +17.40%). Under simulated grazing disturbance, irrigation simultaneously increased the contents of antioxidant enzymes, soluble sugar and soluble protein. These findings indicate that alfalfa maintains a stable reserve state in the absence of grazing disturbance but enhances its stress-resistance metabolism to cope effectively when subjected to stress.

Key words: Alfalfa, Crown buds, Disturbance, Irrigation, Overwintering, Physiological regulation.

INTRODUCTION

Northeast China is a key region for livestock production, with a pronounced seasonal climate. It endures long, bitterly cold winters and limited precipitation, which typically create a low-temperature, arid environment (Boren *et al.*, 2024). Alfalfa *(Medicago sativa*), a perennial legume, is valued for its high yield, strong adaptability and substantial nutritional and economic benefits, making it a premier forage crop (Crookston *et al.*, 2025). Nevertheless, the extreme winter cold in this region leads to low overwintering survival rates, which severely constrain alfalfa's production potential (Cetin *et al.*, 2024).

Facing forage shortages before and during winter, herbivores like sheep often use their front hooves to dig through the soil surface of grasslands, feeding on the tender crown buds of alfalfa buried underground (Eltarabily *et al.*, 2024). Although this behavior supplies essential food for the flock, it poses a potential threat to alfalfa overwintering and subsequent yield formation (Donovan *et al.*, 2021). From the plant's perspective, digging and grazing directly damage the crown - the key nutrient storage organ - and its buds. Since the crown serves as a primary reservoir for carbohydrates (*e.g.*, starch, soluble sugars) and nitrogenous compounds (Kamran *et al.*, 2022), its integrity is crucial for withstanding winter low temperature stress, maintaining basal metabolism and supporting spring regrowth. Crown bud loss reduces tillering and shoot initiation, ultimately lowering plant density and productivity (Liu *et al.*, 2023). Moreover, repeated hoof digging disrupts surface vegetation and topsoil structure (Ma *et al.*, 2025a), exposing crown tissues to cold, dry winter air, which causes severe dehydration and necrosis in some

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tissues. Consequently, the cascade of "digging grazing → crown exposure → water stress → low temperature injury" severely hampers alfalfa overwintering, manifesting as delayed spring emergence, a sharp reduction in new buds and diminished productivity (Ma *et al*., 2025b).

Water plays a critical role in plant cold acclimation and pre winter irrigation is known to enhance alfalfa overwintering performance. Moderate increases in soil moisture promote photosynthesis, stimulate crown bud growth and boost the accumulation of osmolytes (*e.g*., proline and soluble sugars) while also increasing membrane lipid unsaturation (Mu *et al*., 2023). These physiological and biochemical changes collectively support alfalfa's cold tolerance, membrane integrity and metabolic activity (Wagle *et al*., 2024). In addition, sufficient water supply maintains cell turgor in exposed crown tissues, reduces dehydration injury and supplies substrates and moisture for wound healing. Nevertheless, the physiological and ecological mechanisms by which irrigation regulates crown bud number and overwintering capacity under digging and grazing disturbance remain unclear (Wang *et al*., 2024a). In this study, we conducted field experiments to examine the effects of winter irrigation and simulated sheep digging/grazing on crown bud number, size and overwintering capacity, with the following hypotheses: (1) increased disturbance intensity significantly inhibits crown bud growth; (2) adequate winter irrigation significantly promotes bud growth, reflected in greater bud length and number and enhanced subsequent yield.

MATERIALS AND METHODS

Study site description

The experiment was conducted at the Jilin Songnen Grassland Ecosystem National Field Scientific Observation and Research Station, which is located in Changling County, Jilin Province, in the southwestern part of the Northeast Songnen Grassland. This area lies between latitudes 43°59′ N and 44°42′ N and longitudes 123°6′ E and 123°48′ E. It has a temperate continental monsoon climate, with annual mean temperatures of 4.7-6.3°C, annual precipitation of 290-470 mm and a frost free period of approximately 140 days.

The region has a typical seasonal climate: dry and windy springs, hot and rainy summers, mild and cool autumns and clear, cold winters. The study site has an elevation of approximately 144 m above sea level. The soils consist mianly of alkali soil and aeolian sandy soil, they are generally saline with a pH of 7.8. Their properties include organic carbon (7.05±0.49 $mg \cdot g^{-1}$), electrical conductivity (1.66 $dS \cdot m^{-1}$) and total nitrogen (0.77±0.00 $mg \cdot g^{-1}$), phosphorus (0.53±0.37 $mg \cdot g^{-1}$) and potassium (0.95±0.22 $mg \cdot g^{-1}$ Ao *et al*., 2025).

Experimental materials

The study used the alfalfa cultivar "Dongmu No.1", which was sown in 2018 at 16 kg ha^{-1} with 30-cm row spacing. The field was managed with three annual cuts and areas with uniform growth were selected as experimental plots (Crookston *et al*., 2025).

Experimental design

Overview of the experiment

We employed a two factor randomized block design with two factors: irrigation (equal to 0, 20, 40 mm rainfall, designated W1-W3) and simulated digging/grazing disturbance depth (0, 3, 6 cm, designated G1-G3). In a flat alfalfa grassland with uniform autumn regrowth, we established 3 m × 3 m plots spaced 0.5 m apart, totaling 36 plots (9 combinations × 4 replicates) arranged randomly. After assigning treatments, we applied irrigation and disturbance on 1 November and conducted sampling and observations on 1 December (early winter) (Zheng *et al*., 2023; Cavero *et al*., 2017).

Experimental observation, sampling and measurement procedures

Before the scheduled crown bud observation, suitable plots were selected within the experimental site. The chosen plots were cleared of debris and the irrigation amounts and disturbance depths for each plot were randomly assigned and marked. Irrigation and simulated disturbance were then applied according to the predetermined protocols. During irrigation, care was taken to ensure uniform water distribution and avoid overflow beyond plot boundaries, thus preventing any effect on adjacent plots.

Simulated animal digging and grazing disturbance was uniformly applied to all plants within each plot. Crown buds were cut or pinched off using scissors or tweezers to simulate animal behaviors such as "biting off" or "pulling". During the simulated disturbance, crown buds were cut obliquely about 2 mm from the main root base, following the prescribed disturbance depth and removal proportion (30%-40%). Cuts were made at random positions.

Three plants with relatively uniform growth were randomly selected from each plot, totaling 108 alfalfa plants across the 36 plots. During sampling, with the main stem as the center and a radius of 15-25 cm, an iron shovel was used to vertically excavate 15-20 cm. Residual soil around the root zone was then cleaned with a soft bristled brush to expose the root system. The rhizome-stem junction (*i.e*., the connection between the above ground stem and root) was measured with a ruler and positions 3 cm (G2) and 6 cm (G3) below it were marked with a marker pen. Crown buds at these positions were collected. The buds were then wrapped in aluminum foil, placed in liquid nitrogen containers and transported to the laboratory for storage at -80°C for subsequent physiological and biochemical assays. Dry hay yield was measured in May and August of the following year.

In each plot, a random 1 m^2 quadrat was clipped at 5 cm stubble height and fresh weight was recorded immediately. A subsample was then taken, weighed and stored in mesh

bags. After oven drying, the subsamples were reweighed to determine dry weight and the hay yield per hectare was calculated using the fresh/dry weight ratio (Zhu *et al*., 2025; Zhang *et al*., 2019; Rauber *et al*., 2021).

Indicator measurements

Crown bud number and length were measured with a steel ruler. For the biochemical assays, SOD activity was measured by the nitroblue tetrazolium photoreduction method; POD activity by the guaiacol colorimetric method; CAT activity by the ultraviolet absorption method; soluble sugar by the anthrone colorimetric method; and soluble protein by the Coomassie Brilliant Blue G 250 staining method (Bai *et al*., 2025; Aili *et al*., 2023).

Data statistical analysis

Statistical analyses were performed using SPSS 27.0 (IBM, Chicago, USA). A two way ANOVA was used to examine treatment effects on crown bud growth, antioxidant enzyme activities and osmotic substances, after confirming normality and homogeneity of variance. Data are expressed as mean ± SE. Correlation was assessed by Pearson's method and figures were created with Origin 2022. Significance was set at $P<0.05$ ($\alpha = 0.05$). Means were separated using an F-protected LSD ($P = 0.05$) as described in reference (Welsby *et al*., 2022).

RESULTS AND DISCUSSION

Effects of irrigation and disturbance on crown buds growth and yield of alfalfa

As shown in Table 1, increasing irrigation promoted crown bud number, length and dry hay yield, while deeper disturbance suppressed these parameters. Specifically, relative to W1, W2 and W3 increased bud length by 3.82% and 12.70%, bud number by 39.21% and 65.69% and hay yield by 36.4% and 79.9%, respectively; W3 further increased length and number by 8.55% and 19.07% over W2. Conversely, relative to G1, G2 and G3 reduced length by 1.67% and 5.15%, number by 6.33% and 10.79% and hay yield by G2 14.6%% vs, G3 22.2% respectively; G3 reduced length and number by 3.52% and 4.75% relative to G2. These results indicate that winter irrigation enhances crown bud growth under arid conditions, whereas grazing disturbance suppresses it. This is biologically meaningful, as crown bud tillering gives rise to branches after overwintering under favorable temperature and moisture (Wang *et al*., 2024b). The superior performance of W3 (greater bud number and length than W1 and W2) suggests that this irrigation level adequately supports crown bud development and improves bud quality, which has practical implications for alfalfa production (Yao *et al*., 2023; Cavero *et al*., 2017; Sharma *et al*., 2025).

Effects of different pre-winter irrigation amounts and disturbance depths on the antioxidant enzyme system of alfalfa

Irrigation amount and disturbance depth significantly affected SOD activity ($P<0.05$) (Table 2), with values across all nine treatment combinations ranging from 954.12 to 1908.07 $U \cdot g^{-1}$ (Fig 1A). As shown in Fig 1A, SOD activity initially decreased and then increased with irrigation level, averaging in the order W1 > W3 > W2. Compared with W1, W2 and W3 reduced activity by 24.97% and 18.03%, respectively; W3 exceeded W2 by 9.24%. All pairwise

Table 1: Changes in length, number of alfalfa crown buds and dry hay yield under irrigation and disturbance stress.

Pre-winter irrigation amount (mm)	Disturbance depth (cm)	Number	Length (cm)	Dry hay yield ($kg \cdot ha^{-1}$)
W1(0)	G1(0)	442	2.48±0.66	2911.26±588.79
	G2(3)	394	2.45±0.70	2706.82±614.51
	G3(6)	388	2.35±0.71	2755.88±437.54
LSD(0.05)		46.40	1.97	883.87
W2(20)	G1(0)	601	2.54±0.57	4440.49±959.78
	G2(3)	552	2.54±0.59	3639.07±843.90
	G3(6)	551	2.50±0.59	3344.68±215.95
LSD(0.05)		58.13	1.88	1197.04
W3(40)	G1(0)	710	2.85±0.52	5609.90±239.91
	G2(3)	695	2.74±0.53	4252.40±566.57
	G3(6)	624	2.62±0.56	5209.19±2134.72
LSD(0.05)		124.36	1.94	2905.14
W1(mean)		102.17	2.43	2791.320
W2(mean)		142.08	2.53	3808.08
W3(mean)		169.08	2.74	5023.83
LSD(0.05) Irrigation amount		39.81	0.08	959.62

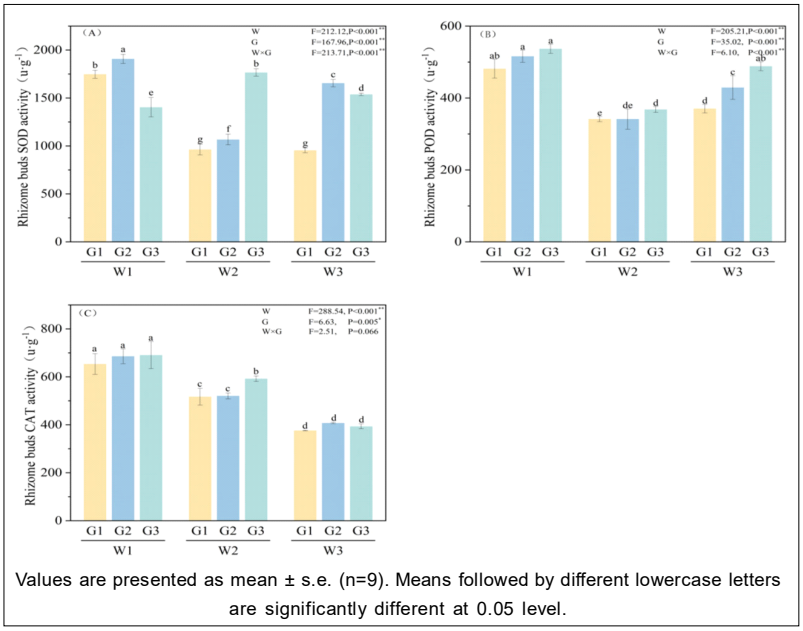
Note: Values are presented as mean ± s.e. (n=9). Different letters indicate significant differences ($P<0.05$) among grazing or irrigation treatments based on multiple comparisons. LSD (0.05) was used to determine the treatment rates of alfalfa crown buds count, length and dry hay yield under different moisture gradients at various interference depths.

comparisons among W1, W2 and W3 were highly significant (P<0.01). Regarding disturbance, SOD activity rose with depth (G3 > G2 > G1). Relative to G1, G2 and G3 increased activity by 26.29% and 28.42%, respectively. G1 differed significantly from both G2 and G3 (P<0.01), whereas G2 and G3 did not differ significantly (P>0.05).

Irrigation amount highly significantly affected POD activity (P<0.01), while disturbance depth had a significant effect (P<0.05) (Table 2). Across all nine treatment combinations, POD activity ranged from 342.13 to 536.35 U·g^{-1} (Fig 1B). Under irrigation, activity decreased then increased with amount (order: W1 > W3 > W2): compared with W1, W2 and W3 decreased by 31.50% and 16.04%, respectively; W3 exceeded W2 by 22.5%. All pairwise differences among W1-W3 were highly significant (P< 0.01). With disturbance depth, activity rose (G1 < G2 < G3): G2 and G3 increased by 7.73% and 16.68% over G1, while G3 was 8.3% higher than G2. The G2 vs. G3 difference was significant (P<0.05) and G1 differed highly significantly from both G2 and G3 (P<0.01).

Irrigation amount highly significantly affected CAT activity (P<0.01), whereas disturbance depth had no significant effect (Table 2). Across all nine treatment combinations, CAT activity ranged from 393.29 to 690.64 U·g^{-1} (Fig 1C). Under irrigation, activity decreased with amount (order: W1 > W2 > W3): compared with W1, W2 and W3 decreased by 19.70% and 42.03%, respectively; W3 decreased by 27.81% relative to W2. All pairwise differences among W1-W3 were highly significant (P<0.01). With disturbance depth, activity generally increased (G1 < G2 < G3): G2 and G3 increased by 4.33% and 8.37% over G1. A significant difference was found between G1 and G3 (P<0.05), but G2 did not differ significantly from either G1 or G3.

SOD, POD and CAT are key antioxidant enzymes in alfalfa that protect cells by scavenging ROS and maintaining membrane integrity (Ming *et al*., 2024; Zhao *et al*., 2022). Specifically, SOD converts O_2^- to H_2O_2 and O_2, while POD and CAT eliminate the resulting H_2O_2 (Maghsoodi *et al*., 2017; Jiang *et al*., 2024). In our study, increasing irrigation



Values are presented as mean ± s.e. (n=9). Means followed by different lowercase letters are significantly different at 0.05 level.

Fig 1: Effects of irrigation and disturbance stress on the activities of antioxidant enzymes SOD (A), POD (B) and CAT (C) in the crown buds of alfalfa.

Table 2: Correlation analysis between pre-winter irrigation and disturbance on the physiological indicators of alfalfa crown buds.

	W	G	SOD	POD	CAT	Soluble sugar	Soluble protein
W	1						
G	0.000	1					
SOD	-0.356*	0.406*	1				
POD	-0.447**	0.363*	0.609**	1			
CAT	0.958**	0.145	-0.209	-0.408*	1		
Soluble sugar	0.690**	-0.438**	-0.578**	-0.695**	0.603**	1	
Soluble protein	0.613**	-0.176	-0.086	-0.489**	0.588**	0.461**	1

Note: * indicates significant correlation (P<0.05); ** indicates extremely significant correlation (P<0.01).

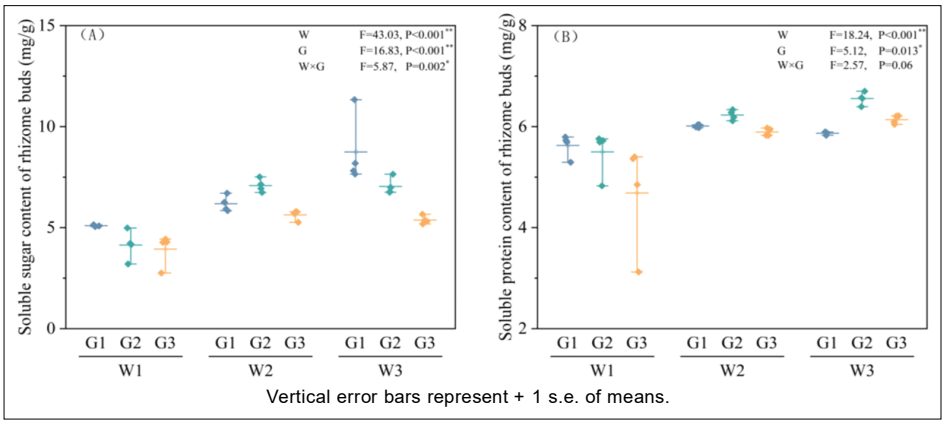


Fig 2: Effects of irrigation and disturbance stress on the soluble sugar (a) and soluble protein (b) content in the crown buds of alfalfa.

generally reduced the activities of all three enzymes. This is likely because the W1 (non irrigated) treatment imposed drought stress, which triggered an elevation in antioxidant enzyme activity. Following irrigation, the stress was relieved, ROS levels declined and consequently, SOD, POD and CAT activities decreased (Kamran *et al.*, 2022).

Effects of different pre-winter irrigation amounts and disturbance depths on the osmotic adjustment system of Alfalfa

Both irrigation and disturbance significantly affected soluble sugar content (P<0.01) (Table 2), with values ranging from 4.14 to 8.75 mg/g across treatments (Fig 2A). Under irrigation, content increased with amount: compared with W1, W2 and W3 increased by 43.40% and 60.60%, respectively; W3 exceeded W2 by 11.98%. Under disturbance, content decreased with depth: relative to G1, G2 and G3 decreased by 22.15% and 33.90%, respectively; G3 was 8.78% lower than G2.

Irrigation amount had a highly significant effect on soluble protein content (P<0.01), while disturbance depth showed no significant impact (Table 2). Among the nine treatment groups combining winter irrigation amounts and disturbance depths, soluble protein content ranged from 4.69 to 6.56 mg·g^{-1} (Fig 2B). Regarding different irrigation levels, soluble protein content generally exhibited an increasing trend with higher irrigation amounts. Compared to the W1 treatment, the average soluble protein contents of the W2 and W3 treatments increased by 14.67% and 17.40%, respectively. In terms of different disturbance depths, the average soluble protein content showed an initial increase followed by a decrease with increasing disturbance depth. Compared to the G1 treatment, the average soluble protein content of the G2 treatment increased by 4.43%, while that of the G3 treatment decreased by 4.72%.

Soluble protein content was highly significantly affected by irrigation (P<0.01) but not by disturbance depth (Table 2), ranging from 4.69 to 6.56 mg·g^{-1} across treatments (Fig 2B). Under irrigation, content increased with amount: compared with W1, W2 and W3 increased by 14.67% and 17.40%, respectively. Under disturbance, content first increased then decreased with depth: relative to G1, G2 increased by 4.43%, while G3 decreased by 4.72%.

Soluble sugars and soluble proteins are key osmotic regulators and cold resistance indicators in plants, reflecting tissue energy storage and metabolic activity. In this study, they showed a highly significant positive correlation with irrigation and a significant negative correlation with disturbance depth (Wagle *et al.*, 2024; Dhaka *et al.*, 2025). Moderate pre winter irrigation creates a favorable moisture environment for alfalfa, promoting root uptake and translocation of nitrogen and other nutrients. Adequate moisture boosts nitrogen metabolizing enzyme activity and thus protein synthesis, while also sustaining high leaf photosynthetic efficiency. During the autumn period with ample light, pre winter irrigation enables the plant to produce more carbohydrates via photosynthesis, which are then transported to crown buds and stored as soluble sugars to support winter dormancy and basal metabolism. Overall, irrigation elevates soluble sugar and protein contents, thereby enhancing cellular water retention, protecting membrane integrity and improving overwintering survival and stress tolerance (Chen *et al.*, 2025; Gamble *et al.*, 2022; Devi *et al.*, 2025).

Correlation analysis of the effects of pre-winter irrigation and disturbance on alfalfa crown buds

As shown in Table 2, the contents of soluble sugars and soluble proteins in alfalfa crown buds exhibited highly significant positive correlations with irrigation amount and highly significant negative correlations with disturbance depth (P<0.01). Conversely, antioxidant enzyme contents were negatively correlated with irrigation amount and positively correlated with disturbance depth.

CONCLUSION

We conclude that: (1) Pre winter digging and grazing of alfalfa crown buds in cold Northeast China significantly impair bud growth, physiology and subsequent biomass cumulation; (2) Pre winter irrigation alleviates these impacts

by improving soil moisture and cold tolerance, enhancing winter survival and providing practical guidance for alfalfa winter grazing to support overwintering and hay production in the following year.

Data availability

Data will be made available on request.

Declaration of funding

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Confict of interest

The authors have no other conflict of interest to declare.

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