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Role of Osmotic Regulation and Cryoprotectant Substances in the Freezing Tolerance of Alfalfa in Cold, Dry Conditions

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ABSTRACT

Background: Freezing temperatures are a limiting factor hindering the survival of alfalfa (*Medicago sativa* L.) through winter. Soil drought also damages alfalfa crowns and can affect their regeneration. Therefore, it is important to determine the effects of cold, dry conditions on alfalfa to develop effective water management strategies in winter.

Methods: Cold, dry conditions were established in the laboratory and the freezing tolerance of crowns was compared between the alfalfa cultivars 'WL440HQ' and 'ZhaoDong'. The degree of freezing tolerance was estimated on the basis of relative electrolyte leakage and semi-lethal temperature. The effects of cold, dry conditions on alfalfa crowns were determined by investigating the rate of water loss and the concentrations of soluble sugars (which function as cryoprotectants) and betaine and proline (which function as osmoregulation substances).

Result: Compared with crowns of 'ZhaoDong', those of 'WL440HQ' showed a higher water loss rate in cold, dry conditions and were irreversibly damaged and their freezing tolerance was severely affected. In comparison, crowns from 'ZhaoDong' showed a lower water loss rate and lower relative electrolyte leakage under cold, dry conditions and their freezing tolerance was not significantly affected. The higher water loss rate and lower freezing tolerance were the direct causes of death of 'WL440HQ', the cultivar with a higher fall dormancy score. In cold, dry conditions, the content of proline, an osmotic regulation substance, increased in response to soil water deficit and may have improved the ability to withstand freezing temperatures by preventing rapid water loss. In addition, sucrose, fructose, galactose and stachyose contents in the crown increased under cold, dry conditions and may have enhanced freezing tolerance because of their function as cryoprotectants. The results of this study indicate that maintaining soil moisture within the range suitable for the growth of crowns is important for the successful overwintering of alfalfa.

Key words: Alfalfa, Freeze-drying, Freezing tolerance, Water management, Winter survival.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is an excellent forage grass that is widely cultivated in China. It is mainly cultivated in northern China, where very cold temperatures can lead to the death of overwintering alfalfa crops in winter. As early as 1973, Lyons described the effect of cold temperatures on plant cells and proposed the hypothesis of "membrane lipid phase change under chilling injury" (James, 1973). Cold and freezing temperatures have negative effects on alfalfa, specifically on its cell membranes, the initial site of sensing stimulation and of injury (Song *et al.*, 2016). Cold stress causes cell membranes to become rigid and to change in shape and thickness. This results in cracking, increased membrane permeability, cytoplasmic leakage and eventually, intracellular ion imbalance (Seo *et al.*, 2010). Brief exposure to low temperatures may cause temporary changes that plants can generally survive, while prolonged exposure to cold stress can cause plant necrosis or death. In research on plants' responses to cold temperatures, ion leakage resulting from decreased membrane integrity of freeze-damaged tissues can be measured to estimate freezing tolerance (Sulc *et al.*, 1991). For instance, the semi-lethal temperature (LT_{50}, the temperature corresponding to 50% cytoplasmic leakage) has often been used as an index of the freezing tolerance of plants (Thapa *et al.*, 2008).

Low temperatures have resulted in severe losses in yield of alfalfa in the northern United States and western Canada, where alfalfa is cultivated across large areas (Keane *et al.*, 2018). Northern China not only has cold winters, but also a dry and windy climate, which causes alfalfa to suffer from a combination of cold and drought stress from winter to early spring. Previous studies have shown that favorable moisture conditions in winter are conducive to the successful overwintering of forage (Naydenova *et al.*, 2013). Water has a high heat capacity and thermal conductivity. Therefore, in winter and spring, the surface temperature of soil changes more gradually when the water content is high. This means that soil freezing and thawing

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occur more slowly. Because there are not sudden changes in temperature, cryogenic stress is relatively light. This buffering effect can mitigate damage from sudden large changes in air temperature, thereby improving the overwintering survival rate (Sun *et al.*, 2004; Li *et al.,* 2020).

In alfalfa cultivation management, antifreeze-water is usually irrigated at the start of winter. However, the water infiltration rate into soil can vary depending on the soil texture or soil structure (Li *et al.*, 2015). For example, the water content is often low near the surface of sandy soil because water readily infiltrates (Liu *et al.*, 1995) and this means that alfalfa crowns are subjected to cold and dry conditions throughout the whole winter. The crown is the transitional structure connecting the shoots and roots of alfalfa and is associated with the persistence and productivity of the crop (Márquez-Ortiz *et al.*, 1999). In winter, the crown is the tissue closest to the soil surface. Under cold and dry conditions, alfalfa crowns are likely to be damaged by the combination of drought and freezing temperatures. Therefore, it is important to understand the effects of these stresses on alfalfa and its freezing tolerance so that appropriate water management strategies can be developed.

Plants under cold stress undergo numerous biochemical/physiological changes, such as increases in the concentrations of proline (Wanner and Junttila, 1999), betaine (Sakamoto and Murata, 2002) and other substances that function as cryoprotectants (*e.g.* soluble sugars) to maintain freezing tolerance (Patton *et al.*, 2007). A key function of such compounds is to stabilize membranes against freezing-induced cellular dehydration (Thomashow, 1999). As osmotic regulatory substances, they can also reduce water loss by increasing the osmotic potential inside and outside the cell, causing the cell to absorb water from its surroundings to maintain its own osmotic pressure, thereby maintaining viability (He *et al.*, 2012). Thus, it is useful to study the effects of these osmotic regulators and small molecule soluble sugar on water loss and freezing tolerance of the crowns of alfalfa plants subjected to cold and dry conditions. In this study, therefore, we simulated winter freezing and dry conditions by controlling temperature and soil moisture content. We conducted analyses to determine (i) the effect of freezing and dry conditions on alfalfa in terms of membrane permeability, freezing resistance and membrane lipid peroxidation; (ii) the mechanism by which freezing and dry conditions affect the freezing tolerance of alfalfa crowns, *i.e.*, how water loss and cryoprotectant substances, such as soluble sugars, affect freezing tolerance and how osmotic regulatory substances affect water loss. The results of this study provide a reference for the water management of alfalfa crops during winter.

MATERIALS AND METHODS

Materials

Alfalfa 'WL440HQ' (WL, fall dormancy score 6) and 'ZhaoDong' (ZD, fall dormancy score 2) were selected for these analyses. Both cultivars are commonly cultivated in northern China and represent the range of fall dormancy scores of cultivars grown in this region. Seeds of WL and ZD were purchased from Zhengdao Ecological Technology Co. (Beijing, China) and Jiuquan Daye Forage Seed Co. Ltd. (Gansu, China), respectively. Alfalfa seeds were sown in sandy soil in May 2021 at the Experimental Station of the Chinese Academy of Agricultural Sciences (Hebei, China). After sowing, soil moisture was maintained by irrigation every second day and weeds and pests were suppressed. After 3 months, uniform plants were selected and transplanted into polyvinyl chloride pipes (pipe diameter, 10 cm; pipe height, 15 cm; four plants per pipe) filled with a 100:30:55 (w/w/w) mixture of soil, perlite and vermiculite. The water holding capacity (WHC) of the mixed substrate was 151% [(wet weight-dry weight)/dry weight]. The following experiments were conducted in the laboratory and included four phases and two water-controlled treatments, which were replicated four times. A total of 48 pipes (192 plants) were prepared. After transplantation, alfalfa plants were cultivated in a growth room for 2 weeks under the following conditions: 24°C/20°C (day/night), 12 h/12 h (day/night) photoperiod, photosynthetic photon flux density of 300-400 μmol m^{-2} s^{-1} and substrate moisture content of 75% WHC.

Experimental treatment

After restorative growth in the growth room, all the plants were transferred into a low-temperature light incubator LRH-200-GD (Tai Hong Medical Instruments, Guangdong, China) randomly for experiments. The four phases established in this study are depicted in Fig 1 and can be summarized as follows:

Phase 1, acclimated growth. First, plants were allowed to acclimate for 1 week in the growth chamber. The substrate moisture content was maintained at 75% WHC during this phase and the other conditions were as follows: 24°C/20°C (day/night), 12 h/12 h (light/dark) photoperiod, photosynthetic photon flux density of 300-400 μmol m^{-2} s^{-1}. After 1 week of acclimation, plants were sampled randomly to represent the initial growth state. The samples of WL and ZD from this phase were designated as WL-F and ZD-F, respectively.

Phase 2, cold acclimation. After phase 1, the temperature in the incubator was decreased from 24°C/20°C (day/night) to 4°C/0°C (day/night) to simulate winter temperatures. In this phase, the substrate moisture content was maintained at 75%. Plants were randomly sampled after 1 week of cold acclimation. The above-ground parts of alfalfa plants were collected for analysis.

Phases 3 and 4, freezing and dry conditions. For these phases, the surface substrate was replaced around some plants before the temperature was reduced to freezing. For each cultivar, half of the potted plants were randomly selected and 5 cm of the surface substrate was removed and replaced with dried substrate. For the other half of the potted plants, the moisture content of the surface substrate

was maintained at 75% WHC. The plants of WL and ZD whose surface substrate had been replaced were designated as WL-D and ZD-D, respectively. The incubator light was turned off after replacing the surface substrate and the incubator temperature was decreased from 4°C/0°C (day/night) to -2°C (night) to simulate freezing. In phases 3 and 4, sampling was conducted randomly after 5 and 7 days.

Sampling

In each treatment, four biological replicates were selected randomly at the end of each phase as shown in Fig 1 and samples from the four plants in each pipe were pooled as one biological replicate. At time points S1 to S4, sampling was conducted as early as possible during the day. The aboveground parts (shoots), crowns with buds (5-cm transition zone between shoots and roots) (Liu *et al.*, 2016) and belowground parts (roots) were separated. The crowns collected from four plants were cut into small pieces (2-3 mm) to obtain homogeneous samples for the determination of indexes. All the sliced crowns from four plants in one pipe were divided into several segments for the determination of membrane permeability, LT_{50} and proline and soluble sugars contents. Fresh samples were used for the determination of membrane permeability and LT_{50}. The samples used for the determination of proline and soluble sugars contents were frozen in liquid nitrogen and stored at -80°C until analysis.

Determination of membrane permeability and semi-lethal temperature

Relative electrolyte leakage was determined to characterize the membrane permeability of crowns. The crowns were considered to be dead when permeability exceeded 50% (Anower *et al.*, 2016). For these analyses, about 0.2~0.3 g crowns and 5 mL deionized water were placed in a 15-mL centrifuge tube; the mixture was shaken on a HZQ-A gyratory platform shaker (Hengrui Instrument and Equipment, Changzhou, Jiangsu, China) at 120 rpm for 12 h and then the electrolyte conductivity value was recorded as EL_1. After the crown samples in the centrifuge tubes were autoclaved at 121°C for 30 min and shaken again, the electrical conductivity value was measured and recorded as EL_2. The conductivity of deionized water was determined as the blank control (EL). The relative electrolyte leakage of crown samples was calculated using the following formula:

$$\text{Relative electrolyte leakage (\%)} = \frac{(EL_1\text{-}EL)}{(EL_2\text{-}EL)} \times 100$$

The freezing tolerance of crowns was estimated on the basis of the LT_{50} value, which was calculated as described by Xu *et al.* (2020). In freezing tests, the freezing temperatures for crown samples collected from S1 were set to 0, -2, -4, -6, -8, -10, -12, -14 and -16°C; for the crowns collected from S2, the freezing temperatures were set to -6, -9, -12, -14, -16, -18, -20, -22 and -25°C and for the crowns collected from S3 and S4, the freezing temperatures were set to -5, -7, -9, -11, -13, -15, -17, -19 and -22°C.

Quantification of biochemical substances

To determine the proline and betaine contents, lyophilized crown samples (each 0.1 g) were analyzed as described by Troll and Lindsley (1955) and Walker and Erlandsen (1951), as per the instructions of appropriate commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The contents of soluble sugars galactose, glucose, sucrose, raffinose, stachyose, fructose and maltose were also determined for four biological replicates. Lyophilized crown samples (each 0.1 g) were used for these analyses, which were conducted in accordance with the methods of Xu and Tong (2020). The total soluble sugars content was calculated as the sum of galactose, glucose, sucrose, raffinose, stachyose, fructose and maltose contents.

Data analysis

The results are presented as mean ± standard error. Data were analyzed using SPSS software. The significance of difference among phases or among four samples was determined using the least significant differences test and differences were considered as significant at $P<0.05$. Pearson's correlation coefficients were calculated to explore the relationship between LT_{50} and soluble sugars contents and correlations were considered significant at $P<0.05$ and $P<0.01$.

RESULTS AND DISCUSSION

Relative electrolyte and LT_{50} of alfalfa

After phases 3 and 4, the relative electrolyte leakage of WL-D was 51.61% and 88.53%, respectively and the crowns were irreversibly damaged (Fig 2a). Compared with WL-D, WL-F showed less severe damage. The relative electrolyte leakage of ZD-F and ZD-D did not change significantly between phases 3 and 4.

In phase 2, the LT_{50} of crowns decreased significantly for ZD, but not for WL (Fig 2b). In phases 3 and 4, WL-D died; therefore, the LT_{50} could not be calculated. The LT_{50} values of WL-F, ZD-F and ZD-D did not differ significantly between phases 3 and 4 (Fig 2b). These results indicate that the cold, dry conditions had a serious negative impact on the freezing tolerance of WL crowns, but little impact on that of ZD.

Soil moisture and its effect on freezing tolerance

In phases 3 and 4, ZD-D crowns showed moisture loss rates of 2.32%/d and 5.39%/d, respectively and those of WL-D crowns were 8.96%/d and 5.85%/d, respectively (Fig 3). However, the moisture content did not change obviously for ZD-F in phases 3 and 4. For WL-F, it only decreased significantly in phase 3 at a rate of 2.46%/d. Among the four samples, WL-D crowns had the highest water loss rate and died after two freezing phases. The moisture content decreased more slowly in WL-F, ZD-F and ZD-D crowns and their freezing tolerance was not significantly affected. Thus, the death of WL-D crowns was closely related to rapid water loss.

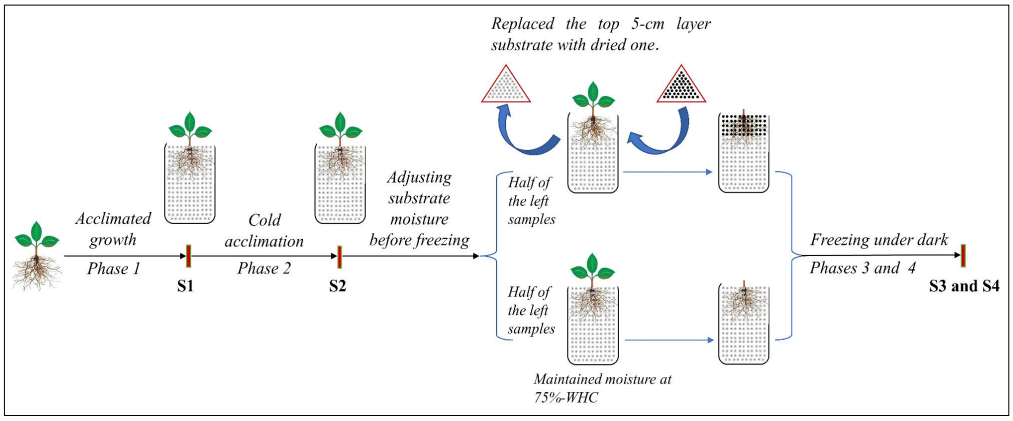


Fig 1: Experimental process in this study.
S1, S2, S3 and S4 represent sampling time points at phases 1, 2, 3 and 4, respectively.

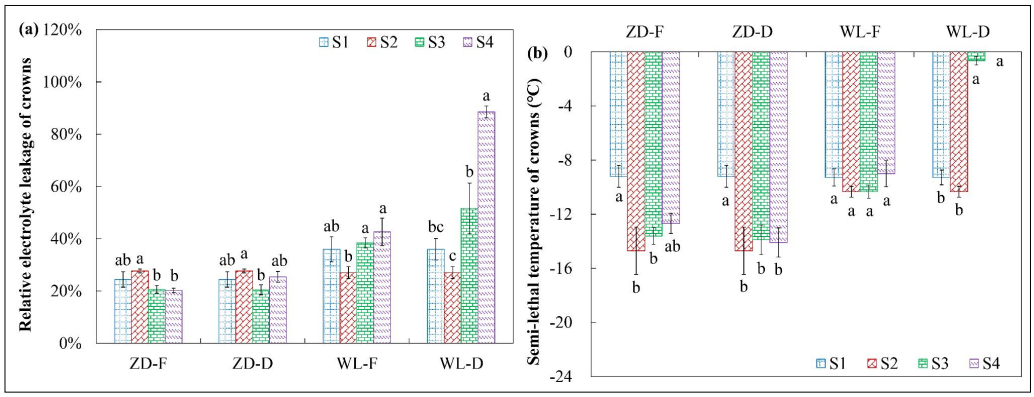


Fig 2: Membrane permeability and semi-lethal temperature of crowns at sampling time points. Figures a and b show the comparison among different time points. Different letters indicate significant differences at $P < 0.05$.

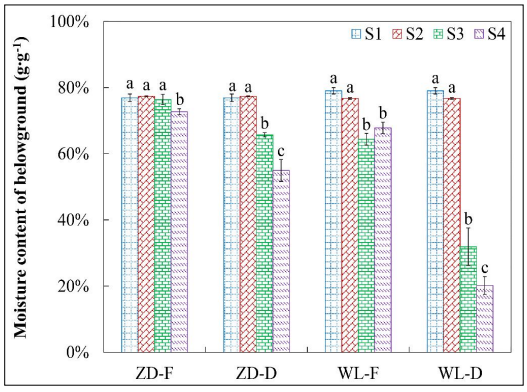


Fig 3: Moisture contents of belowground parts at four sampling time points. In this study, moisture content of the belowground part (root) was considered to be equal to that of the crown. Different letters indicate significant differences in moisture content of belowground parts among time points ($P < 0.05$).

On the basis of the relative electrolyte leakage and LT_{50} values, we concluded that cold, dry conditions had strong adverse effects on the freezing resistance of alfalfa, especially for the cultivar with the higher fall dormancy score. Moisture is important for freezing resistance in alfalfa. Soil moisture has a buffering effect on the soil cooling rate because of its high heat capacity and thermal conductivity and this can mitigate the damage caused by drastic changes in temperature during winter (Li *et al.*, 2020). When alfalfa overwinters, its degree of frost resistance might be affected by soil water deficit caused by seepage or evaporation. For field alfalfa production, it is suggested that adequate water should be applied before winter and soil moisture should be monitored and supplemented in winter. Providing irrigation water during freezing periods is an energy intensive process. Glasshouses are an important agricultural facility for cold protection and water conservation. For areas with water shortages, a one-time investment in glasshouse construction may be a good strategy to meet the demand for alfalfa in small quantities.

Contents of proline and betaine and effect on water loss

Proline is an important osmotic regulator in plant cells (Girousse *et al.*, 1996). In phases 2 and 3, the proline content decreased in ZD-F and ZD-D (Fig 4b). In phases S3 and S4, the proline content was significantly higher in ZD-D than in ZD-F (Fig 4a) and significantly decreased in WL-F. In phase 4, the proline content markedly increased in WL-D (Fig 4b) to a level significantly higher than that in WL-F

(Fig 4a). For each cultivar of alfalfa, the proline content was significantly higher in the water-deficit treatment (-D) than in the fully watered treatment (-F) after the freezing period. Comparing the two cultivars of alfalfa, WL responded more strongly to cold, dry conditions. The results show that freezing tended to decrease the proline content in WL crowns, while water deficit tended to increase it. The role of proline in the freezing tolerance of alfalfa is still uncertain (Castonguay *et al.*, 2011), but it is probably favorable for withstanding freezing because it prevents rapid water loss. The proline content varied between the two cultivars under cold, dry conditions and this may have been dependent on whether low temperature or water deficit was the stronger stimulus. This might explain why the proline content in WL-D increased markedly in phase 4.

Betaine is another important osmotic regulator in plant cells (Sakamoto and Murata, 2002). At the four time points, there was no significant difference in betaine content between ZD-D and ZD-F (Fig 5a). The betaine content decreased significantly in WL-D in phase 4 (Fig 5b) and was significantly lower than that in WL-F at S4 (Fig 5a). The betaine content did not increase under water-deficit treatment, so its role in reducing water loss remained uncertain. The betaine content decreased in all four samples from S1 to S4 (Fig 5b), indicating that decreasing temperature resulted in decreased betaine content. From these results, we could not conclude whether betaine had a positive role in resistance to water loss and freezing temperature.

Contents of soluble sugars and effect on freezing tolerance

During the experiment, the contents of galactose, sucrose, stachyose, fructose and total soluble sugars in alfalfa crowns gradually increased in ZD-F, ZD-D, WL-F and WL-D, with the largest increases during phase 2. The contents of galactose, stachyose and fructose in ZD-F, ZD-D, WL-F and WL-D crowns also increased significantly in phases 3 and 4 (Fig 6b, d, f, h, j). For all the soluble sugars examined, there was no significant difference in the contents between the water-deficit treatment and fully watered treatment in each cultivar at phases 3 and 4 (Fig 6a, c, e, g, i). Thus, the soluble sugars content increased in response to cold temperatures, but not in response to water deficit.

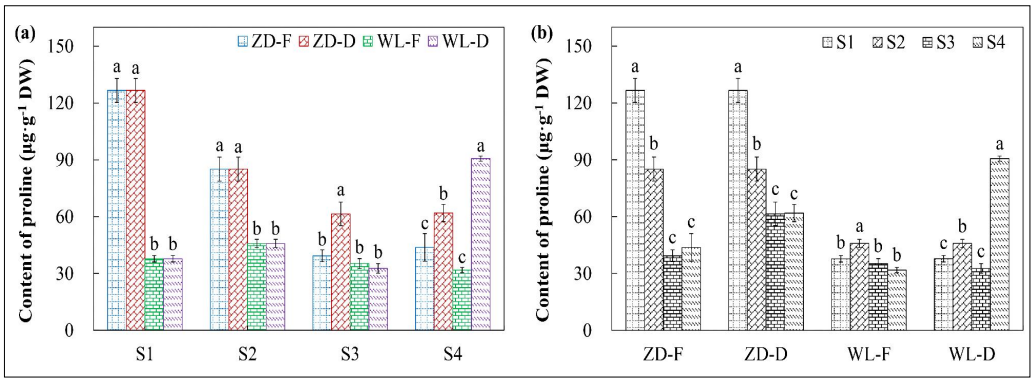


Fig 4: Proline contents in crowns at different sampling time points.
Fig a shows the comparison among four samples, Fig b shows the comparison among different time points. Different letters indicate significant differences ($P < 0.05$).

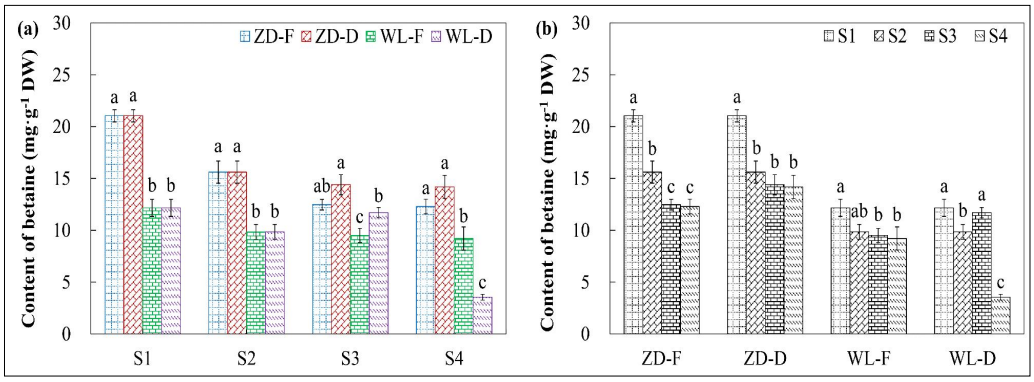


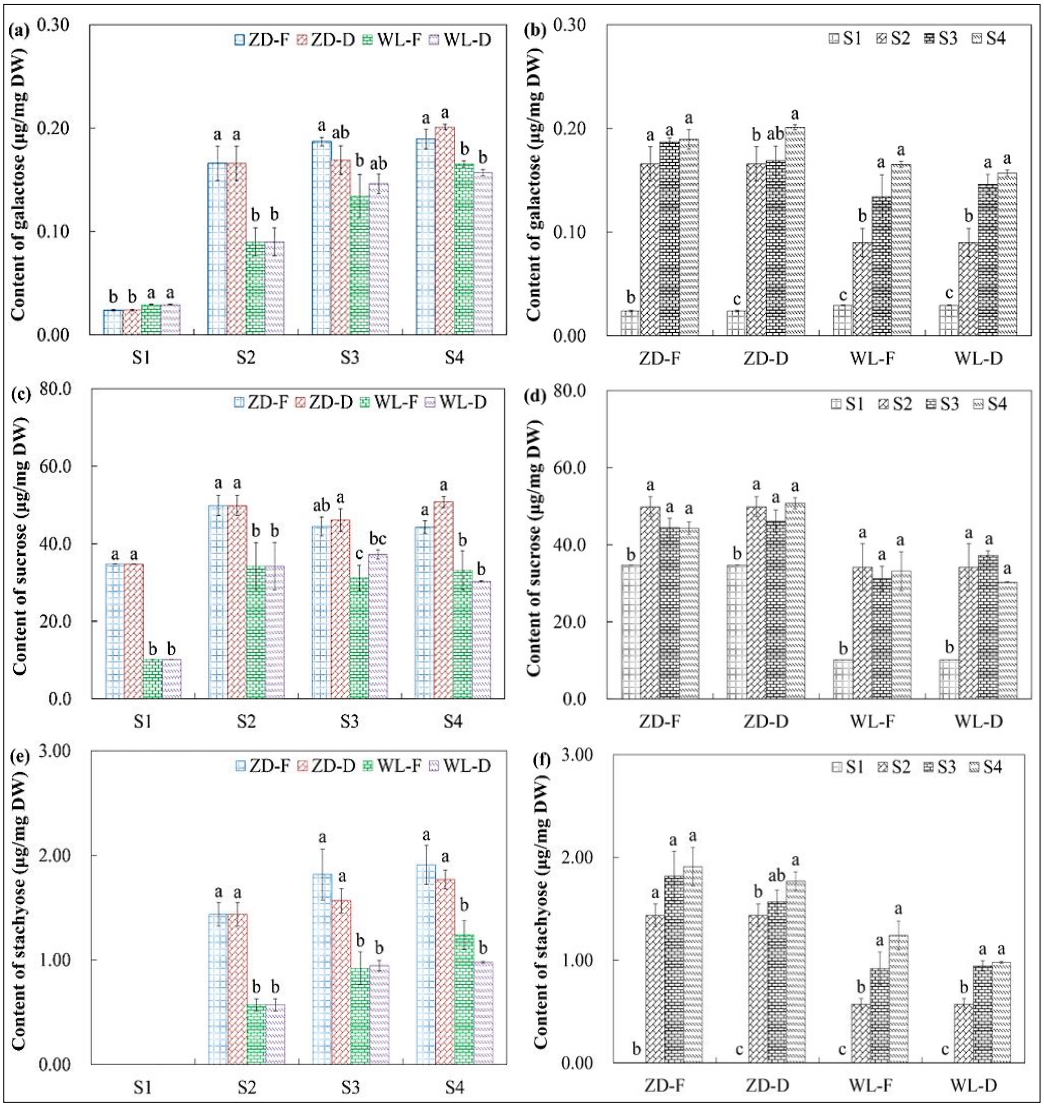
Fig 5: Betaine contents in crowns at sampling time points.
Fig a shows the comparison among four samples, Fig b shows the comparison among different time points. Different letters indicate significant differences ($P < 0.05$).

Previous studies have shown that soluble sugars not only function as osmotic regulators, but also as cryoprotectants, which can protect biological membranes and biomacromolecules. Hincha *et al.* (2002) showed that fructose molecules are inserted into the head region of lipid molecules, thereby stabilizing phosphatidylethanolamine in the liquid crystal phase. This reduces the temperature at which the lipid transforms from the crystal phase to the gel phase; thus, fructose enhances the stability of biological membranes. Other soluble sugars such as raffinose and stachyose can also positively affect the freezing tolerance of alfalfa (Castonguay *et al.*, 2011). In the present study, the contents of fructose, sucrose, galactose, stachyose and total soluble sugars in alfalfa crowns increased in response to decreasing temperature. After phases 2, 3 and 4, their contents were higher in ZD-F and ZD-D (significantly or not significantly) than in WL-F and WL-D (Fig 6a, c, e, g, i). This was consistent with the trends in the change in freezing

Table 1: Correlation between LT_{50} and soluble sugars contents.

	Galactose	Glucose	Sucrose	Raffinose	Stachyose	Fructose	Maltose	Total soluble sugar
ZD-F	-0.78**	0.40	-0.87**	0.63**	-0.57*	-0.54*	0.68**	-0.84**
ZD-D	-0.74**	0.53*	-0.86**	0.72**	-0.70**	-0.58**	0.71**	-0.84**
WL-F	-0.02	0.06	-0.27	0.18	-0.11	-0.06	-0.08	-0.27

Note: "*" indicates significant correlation and "**" indicates highly significant correlation at $P < 0.05$ and $P < 0.01$, respectively. At S3 and S4, relative electrolyte leakage of WL-D crowns was >50.0%, indicative of crown death.



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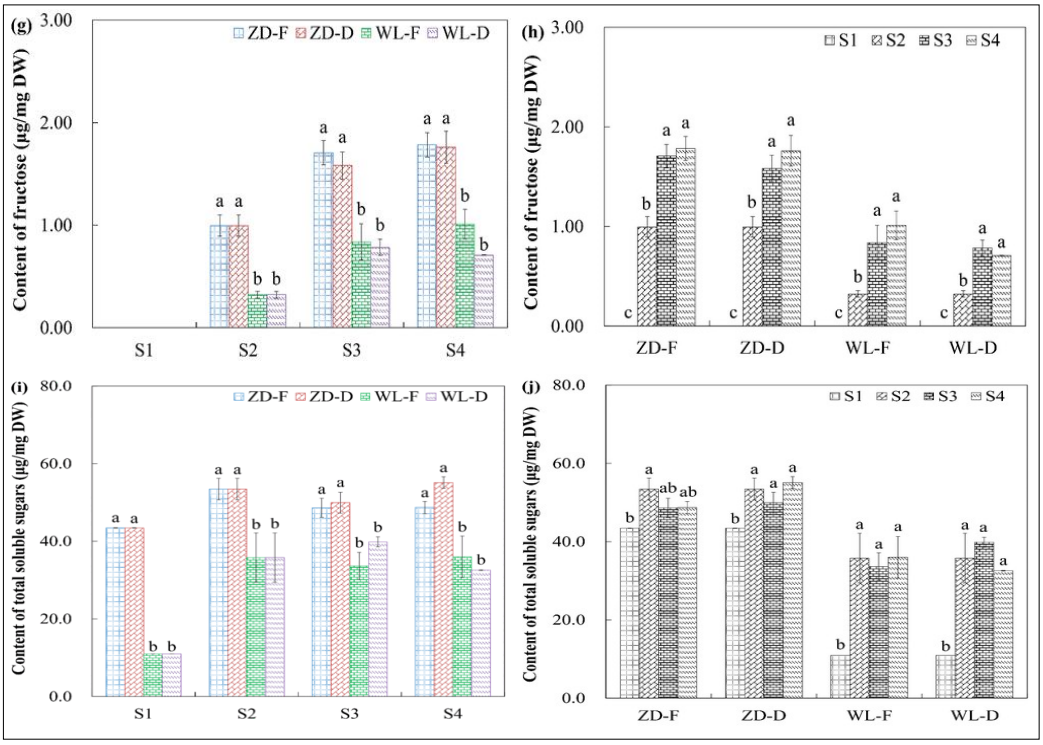

Fig 6: Sugar contents in two cultivars of alfalfa at different timepoints. Figures a, c, e, g and i show comparisons among four samples; figures b, d, f, h and j show comparisons among different time points. Different letters indicate significant differences at $P < 0.05$.

tolerance after cold acclimation and indicative of a positive relationship between freezing tolerance and sugar content (Castonguay *et al*., 2006). Combined with the results of the correlation analysis between soluble sugars and LT_{50} (Table 1), our results confirmed that soluble sugars have a positive effect on the freezing tolerance of alfalfa.

Effect of priming measures on freezing tolerance

Plants growing under field conditions are constantly exposed, either simultaneously or sequentially, to more than one abiotic stress factor. In 1975, Boussiba theorized that plants that encounter stress show better tolerance to a subsequent simultaneous stress and thereby adapt better (Boussiba *et al*., 1975). In agricultural production, priming measures may be an effective way to increase the freezing tolerance of crops (Sinha *et al*., 2021; Çirka *et al*., 2021). For example, exposing alfalfa to water deficit could increase its winter survival by enhancing its freezing tolerance (Paquin *et al*., 1980). In this study, increased contents of proline and soluble sugars enhanced the freezing tolerance of alfalfa because of their osmotic regulation and cryoprotectant properties. Further studies should explore whether measures such as spraying with plant hormones or exposure to other stresses (Revathi *et al*., 2021) can trigger proline and sugars accumulation in alfalfa, thereby increasing its resistance to winter (cold and dry) conditions.

CONCLUSION

After being subjected to cold, dry conditions, the relative electrolyte leakage of crowns of 'WL440HQ' was higher than 50%, the crowns were irreparably damaged and their freezing tolerance was negatively affected. In contrast, for the crowns of 'ZhaoDong', the cold, dry treatment resulted in lower relative electrolyte leakage and it did not significantly affect their freezing tolerance. Compared with 'ZhaoDong', 'WL440HQ' showed greater water loss and lower freezing resistance under cold, dry conditions, resulting in death.

In cold, dry conditions, proline promoted the ability of alfalfa to withstand freezing temperature by preventing rapid water loss. In addition, the contents of the soluble sugars sucrose, fructose, galactose and stachyose increased in response to decreasing temperature and these sugars functioned to enhance the freezing tolerance of alfalfa as a result of their cryoprotectant properties. According to the results of this study, maintaining soil water at a sufficient level during the freezing period is important for successful overwintering of alfalfa crops.

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Conflict of interest: None.

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