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Authors: Short, William, and Wolyn, David J.

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William Short and David J. Wolyn

Department of Plant Agriculture, University of Guelph, Guelph, ON N1G 2W1, Canada

Corresponding author: **David J. Wolyn** (email: dwolyn@uoguelph.ca)

Abstract

Cold acclimation induces freezing tolerance in asparagus (*Asparagus officinalis* L.), which is important for survival and longevity in temperate climates. Previous studies indicated that cultivar "Guelph Millennium" (GM), adapted to Southern Ontario, acquired freezing tolerance earlier in the fall and maintained it later in the spring than "UC157" (UC), a cultivar commonly grown in desert climates. As the drought and cold acclimation metabolic pathways overlap, the objective of this research was to determine whether drought alone induced freezing tolerance or interacted synergistically with cold temperatures. Seedlings of GM and UC were subjected to control, drought, or cold acclimating conditions for 6 weeks. Additional treatments included 6 weeks of drought or control conditions followed by 6 weeks of cold acclimation, and plants grown for 12 weeks under control conditions. LT_{50}, the temperature at which 50% of plants die, and metabolites or parameters associated with freezing tolerance were measured. GM had decreased LT_{50} levels (increased freezing tolerance) compared with UC under both drought and cold acclimating conditions, and no cultivar differences were observed under control conditions. Drought stress before cold acclimation resulted in an increased LT_{50} (reduced freezing tolerance) for UC compared with GM. A high root:shoot ratio and low crown water percentage were associated with increased freezing tolerance in GM, while a high crown sucrose concentration in UC was related to decreased freezing tolerance for the drought treatment. Overall, drought stress induced cultivar-specific adaptations causing differences in freezing tolerance, thus confirming the interrelationship between these two physiological pathways.

Key words: asparagus, LT_{50}, cross-adaptation, freezing tolerance, metabolites

Résumé

L'acclimatation au froid permet à l'asperge (*Asparagus officinalis* L.) de tolérer le gel, ce qui est capital pour assurer la survie et la longévité de la plante en climat tempéré. Selon des études antérieures, le cultivar "Guelph Millennium" (GM), acclimaté au sud de l'Ontario, développerait sa tolérance au gel plus tôt à l'automne et la garderait plus longtemps au printemps que la variété "UC157" (UC), couramment cultivée dans les climats désertiques. Comme la sécheresse et l'acclimatation au froid sont des voies qui se chevauchent dans le métabolisme, les auteurs voulaient établir si la privation d'eau peut induire la tolérance au gel à elle seule ou si le mécanisme est synergique avec la chute des températures. Pour le savoir, ils ont soumis des plantules de GM et d'UC à des conditions normales (témoin), à la sécheresse ou à l'endurcissement au froid pendant six semaines; les autres traitements consistaient en six semaines de sécheresse ou de conditions normales, suivies par six semaines d'acclimatation au froid, ou la culture des deux espèces dans des conditions normales pendant douze semaines. Les auteurs ont ensuite mesuré la TL50, c'est-à-dire la température à laquelle la moitié des plants périssent. Ils ont aussi dosé les métabolites ou paramètres liés à la tolérance au gel. La TL50 de GM était plus faible (meilleure tolérance au gel) que celle d'UC dans les conditions de sécheresse et d'acclimatation au froid, mais aucune différence n'a été relevée entre les deux cultivars dans les conditions normales. La sécheresse suivie par l'endurcissement au froid augmente la TL50 (réduit la tolérance au gel) d'UC, comparativement à celle de GM. Un rapport racines:pousses élevé et une faible teneur en eau dans le collet ont été associés à la meilleure tolérance de GM au gel, alors que la plus faible tolérance au gel d'UC notée après la sécheresse semble liée à une plus forte concentration de sucrose dans le collet. En général, la sécheresse entraîne des adaptations spécifiques à la variété, ce qui explique la tolérance variable au gel et confirme les liens entre ces deux mécanismes physiologiques. [Traduit par la Rédaction]

Mots-clés : asperge, TL_{50}, adaptation croisée, tolérance au gel, métabolites

Introduction

Asparagus (*Asparagus officinalis* L.) is a herbaceous perennial grown for its tender emerging shoots in a variety of climates around the world. When grown in southern Ontario, Canada, cultivars must induce dormancy and cold acclimation in the fall before freezing events, maintain freezing tolerance throughout the winter, and de-acclimate in the spring after late spring freezing events have passed.

Nonfreezing cold temperatures are thought to be the main signal used by asparagus to initiate the cold acclimation process (Krug 1996; Yamaguchi 2012; Kim and Wolyn 2015), although this may be impacted by other environmental factors such as drought stress. Experiments in a wide range of species other than asparagus indicate that plants use similar adaptive mechanisms for both drought and freezing stress, including the decrease or cessation of growth, production of antioxidants, hormones and dehydrins, and the accumulation of various osmolytes as reviewed in Hussain et al. (2018). As both cold and drought stress share similar signaling pathways and cellular changes, exposure to one may cause tolerance to the other through cross-adaptation (Hoffman et al. 2012). Moderate drought stress improved cold tolerance both alone and in combination with cold temperatures in one of two perennial ryegrass cultivars. Drought stress also increased crown fructan, proline, and soluble protein content. In strawberry, full freezing tolerance was only achieved when both drought and cold stress were applied simultaneously (Rajashekar and Panda 2014).

"Guelph Millennium" (GM) is an asparagus variety known for possessing high sustained yields and longevity, compared with "UC157" (UC), a cultivar adapted to warm climates that typically dies after 3–4 years in southern Ontario. In a field study, freezing tolerance of GM was greater than that of UC in early October, whereas cultivars did not differ in late fall (Panjtandoust and Wolyn 2016). Therefore, early cold acclimation by the perception of environmental signals may be critical for continued longevity of cultivars in Southern Ontario. In an asparagus seedling assay, the freezing tolerance of GM was elevated compared with UC after 6 weeks, mirroring trends observed for field-grown plants, and was highly correlated with increased crown proline, high-molecular-weight (HMW) fructan and sucrose concentrations, and decreased crown water percentage (Kim and Wolyn 2015). The ability of seedlings under controlled conditions to mimic field fall acclimation patterns suggests they could be used to understand the complexities of the cold acclimation process.

The impact of drought on freezing tolerance in asparagus is unclear, although this stress can impact the concentrations of crown metabolites, including fructan, sucrose, and proline, which typically are correlated positively with freezing tolerance (Pressman et al. 1989; Kim and Wolyn 2015; Nolet and Wolyn 2020). Intense drought through water-withholding for 24 days in asparagus appeared to decrease crown fructan and sucrose concentrations compared with an irrigated control (Pressman et al. 1989). When drought was applied simultaneously with cold acclimating temperatures for 55 days, plants appeared to have a synergistic response as crown sucrose was elevated compared with the cold acclimation only treatment.

Nolet and Wolyn (2020) observed increased crown proline concentrations in a dry growing season compared with one that was wet. A defoliation treatment also increased freezing tolerance and rhizome sucrose concentrations in the dry year.

Investigating the impact of drought stress on freezing tolerance in asparagus could be useful for gaining insight into the complexity of cold acclimation and in the selection of new cultivars, as the incidences of drought (Mishra and Singh 2010; Hatfield et al. 2013) and freezing stress (Easterling et al. 2000; Henry 2008) in temperate climates are expected to rise in the future. The objective of this study was to determine the effects that drought stress alone and drought followed by cold acclimation have on the induction of freezing tolerance and related crown characteristics. Two varieties with different adaptations to Southern Ontario, UC and GM were compared.

Materials and methods

Plant culture

Twenty-four "cone-tainer" flats (SC10, Stuewe and Sons Inc.; Corvallis, OR) each with 96 cells (164 mL volume), were filled with an inert, washed, pure silica beach sand (KING Play Sand). The sand grain size was sieved to 0.08–1.25 mm, with most particles falling between 0.16 and 0.32 mm (50%–70%). Sand was supported by 5 cm of horticultural rockwool (HollandBasics, Holland Industry). Flats were flushed with deionized (DI) water five times before planting. Seed for UC was obtained from Walker Brothers (Pittsgrove, New Jersey) and for GM from Fox Seeds (Simcoe, Ontario). After imbibition in water for 1 day at room temperature, one seed was planted per cone-tainer. In each tray, 49 plants for each cultivar were established as split-plots. Replicate experiments were planted on 8 March 2019 and 7 March 2020.

Twelve random flats were placed in each of the two replicate greenhouse zones. Plants were grown at 23/18 °C (day/night), under natural light supplemented with a 16 h photoperiod from halogen lighting with a photosynthetic photon flux density (PPFD) of ∼80 μmol m^{-2} s^{-1}. Half-strength modified Hoagland's solution (PhytoTechnology Laboratories, Product ID: H353), pH 6.5, was used to water plants as needed and 0.1 g L^{-1} iron chelate (13.2%, Plant Products Co. Ltd.) was applied on 10 and 17 May 2019 and 9 and 16 May 2020. Sand was flushed with DI water weekly to prevent salt accumulation.

Growth chamber treatments

Ten-week-old seedlings were subjected to six treatments (Table 1). Ten randomly chosen flats were placed into each of two replicate "warm" growth chambers (Conviron Model-PGW 36) at 23/18 °C (day/night) and 60% relative humidity and two flats were placed into each of two replicate "cold" growth chambers at 10/5 °C (day/night) and 80% relative humidity. All growth chambers had a 14 h photoperiod provided by alternating strips of fluorescent grow lights and far-red LED bulbs with a total PPFD of 400 μmol m^{-2} s^{-1} measured 56 cm above the bottom of the flat. All flats were

Table 1. Summary of treatment levels applied to 10-week-old asparagus seedlings.

Treatment no.	Stress	Abbreviation	Duration	Temperature (day/night)
1	Control	–	6 week	23/18 °C
2	Drought	–	6 week	23/18 °C
3	Cold	–	6 week	10/5 °C
4	Control	(Con)	12 week	23/18 °C
5	Control→Cold	(Con/Cold)	6 + 6 week	23/18 °C→10/5 °C
6	Drought→Cold	(Dro/Cold)	6 + 6 week	23/18 °C→10/5 °C

watered initially with DI water, after which four flats from each replicate warm growth chamber were chosen randomly to be drought-stressed with incremental applications of 5 g L^{-1}, 25 g L^{-1}, and 50 g L^{-1} PEG6000 (Sigma–Aldrich, Product ID: 81260), where the latter treatment had a water potential of −0.14 MPa, determined by a thermocouple psychrometer (PSY1 Stem Psychrometer; ICT Intl., Armidale, NSW, Australia). The first two concentrations were applied once, 3 days apart. Thereafter, flats were watered with 50 g L^{-1} PEG6000 at 3-day intervals for the duration of the treatment. Control flats were watered at the same time using DI water. All solutions were adjusted to pH 6.5 using dilute HCl and NaOH. At every third watering, both the PEG6000 solution and DI water were made to half-strength Hoagland's (PhytoTechnology Laboratories, Product ID: H353) for fertilization. Plants in cold chambers were watered with DI water as needed.

After 6 weeks, two flats were chosen randomly for each of the control (Table 1; treatment no. 1), drought stress (treatment no. 2), and cold stress (treatment no. 3) treatments from each replicate growth chamber for analysis as described below. For the remaining flats, controls were removed from each warm chamber and transferred to each of two replicate cold chambers (treatment no. 5). Likewise, the two remaining drought-treated flats from each warm chamber were transferred to two replicate cold chambers (treatment no. 6). The remaining two control flats remained in the warm chambers (treatment no. 4). After an additional 6 weeks of growth, plants for treatments 4, 5, and 6 were analyzed as described below.

Sampling and LT_{50}

For each cultivar, crowns from seven randomly chosen seedlings in each flat were bulked for metabolite analyses as described below. The remaining 42 plants, per cultivar, in each flat were used for LT_{50} analysis. Ferns were removed 3 cm above the soil, and flats were flushed with DI water five times to remove PEG6000. One replicate flat for each treatment was placed randomly into each of four chest freezers. Temperature was maintained at 3 °C for 2 h, 0 °C for 3 h, and then lowered to −3 °C for 9 h to allow for ice nucleation. Additional freezing treatments of −6 °C, −9 °C, −12 °C, −15 °C, and −18 °C were achieved by decreasing the temperature 3 °C h^{-1} and maintaining it for 1 h. A Hobo thermocouple (Pocasset, MA) inserted 2.5 cm below the soil surface was used to monitor temperature changes. After each freezing treatment (−3 °C to −18 °C), seven random plants for each cultivar replicate were thawed at 4 °C for 24 h and two replicates per treat-

ment were placed in each of two greenhouse zones and watered with DI water as needed. Plants were grown for 4 weeks to allow for regrowth and rated as dead or alive to estimate LT_{50} values.

Tissue preparation and fresh/dry weight

Selected plants for metabolite/physiological analysis were cut to remove fern tissue, and the remaining crowns were washed thoroughly using cold tap water and blotted dry with a paper towel. Fresh weight (FW) was determined for fern and crown tissue. Ferns were then dried in a convection oven at 60 °C for 1 week, and dry weight (DW) was recorded. Crowns were placed in aluminum bags and immediately frozen in liquid nitrogen. Samples were stored at −80 °C, and then freeze-dried (FreeZone 4.5 L Freeze Dry System, Model 77510, LABCONCO; Kansas City, MO, USA). Crown tissue was weighed, and then homogenized using a Waring blender (Model 7011S, Waring, New Hartford, CT, USA). The powder was passed through a 60-mesh sieve to remove epidermal tissue, transferred to 50 mL falcon tubes, and stored at −80 °C. The water percentage was determined as [(FW − DW)/FW] × 100.

Proline assay

Proline was measured using a modified acid ninhydrin method (Shabnam et al. 2016). Tissue (30 mg DW) was homogenized with 1 mL of EtOH solution (3% (w/v) sulfosalicylic acid in 80% EtOH) in a 2 mL microcentrifuge tube and incubated for 20 min at 70 °C followed by cooling to room temperature on an orbital shaker (150 rpm) for 15 min. Tubes were vortexed for 15 s to suspend tissues and then centrifuged at 20 000g_n for 10 min. For proline determination, a 50 μL aliquot of the supernatant was combined with 950 μL double-deionized water (ddH_2O) and 2 mL of 1.25% ninhydrin in glacial acetic acid in a 15 mL centrifuge tube. Tubes were vortexed for 15 s and placed in a 100 °C water bath for 30 min. The reaction was stopped by placing tubes in an ice bath. Once cooled, the resulting reaction mixture was transferred into a sealed semimicro cuvette and absorbance was measured at 508 nm with a spectrophotometer (Epoch™ 2 Microplate Spectrophotometer from BioTek, Winooski Vermont). Proline concentration was estimated as mg g^{-1} DW using a 0–31 μg mL^{-1} L-proline standard curve.

Sucrose/glucose assay

Glucose and sucrose were quantified using a commercially available kit (K-SUCGL; Megazyme International Ireland, Bray, Ireland). Carbohydrates were extracted from tis-

sue (100 mg DW) with 20 mL ddH$_2$O at 70 °C for 30 min. Two millilitres of solution was transferred to 2 mL microcentrifuge tubes and centrifuged at 20 000g$_n$ at room temperature for 5 min. From each extract, 200 μL of the supernatant was added to each of four separate 20 mL borosilicate glass test tubes. Two hundred microliters of supplied sodium acetate buffer were added to each of the two duplicate test tubes for D-glucose determination, while 200 μL of β-fructosidase was added to each of the remaining two duplicate test tubes for sucrose and D-glucose determination. All tubes were incubated at 50 °C for 20 min in a hot water bath. Subsequently, 3 mL of glucose oxidase/peroxide reagent was added to all tubes which were incubated for an additional 20 min at 50 °C. Absorbances were measured at 510 nm in disposable semimicro plastic cuvettes and compared with a D-glucose control of known concentration. Sucrose concentration was estimated using the difference in absorbance between D-sucrose + D-glucose and D-glucose. The Mega–Calc™ Excel-based tool was used to calculate the concentrations (g 100 g^{-1}) of glucose and sucrose (https://www.megazyme.com/documents/Data_Calculator/K-SUCGL_CALC.xlsx).

Fructan assay

Low-molecular-weight (LMW) and HMW fructans were determined for crown tissues using a commercial fructan analysis kit (K-FRUC; Megazyme International Ireland) (McCleary et al. 2019). Tissue (100 mg DW) was extracted with 25 mL of 90% ETOH at 70 °C for 20 min, centrifuged at 20 000g$_n$, and the supernatant was collected. The remaining residue was extracted with 25 mL double-deionized water at 80 °C for 20 min and centrifuged. A 100 μL sample of the LMW ethanol extraction was evaporated to dryness in a test tube and rehydrated with 200 μL ddH$_2$0. All other steps were followed as per the manufacturer's instructions. Concentrations of D-fructose and D-glucose, derived from fructan hydrolysis, were estimated through a reaction with parahydroxybenzoic acid hydrazide. The color produced at 410 nm was compared with a D-fructose standard of known concentration as the response for both D-fructose and D-glucose is equivalent. The Mega–Calc™ Excel-based tool was used to calculate the fructan concentration (g 100 g^{-1}) in a sample (https://www.megazyme.com/documents/Data_Calculator/K-FRUC_CALC.xlsx).

Statistical analysis

Data analyses were conducted with SAS version 9.4 (SAS Institute, Cary, NC, USA). A split-plot design was used, where treatments (nos. 1–3) or (nos. 4–6) served as whole plots in separate analyses, and cultivars (GM, UC) were subplots. Studentized residual plots were analyzed for homogeneity, and normality was tested using a Shapiro–Wilk test with PROC UNIVARIATE. Proline data for treatment nos. 1–3 were lognormal transformed in SAS, and back-transformed results are shown. Restricted maximum likelihood (REML) covariance parameter estimates were performed on parameters using PROC GLIMMIX. The random effects of year, chamber(year), and chamber × treatment(year) were included in the model. Simple effect comparisons of treatment and cultivar were made using treatment × cultivar least square means with

the "slicediff" option for a Tukey's Honestly Significant Difference (HSD) adjustment ($P \leq 0.05$). PROC CORR was used to generate correlation coefficients between analyzed variables, and LT$_{50}$ was predicted using PROC PROBIT.

Results

Statistical analysis

The effects of year, chamber within year, and interactions with fixed effects were nonsignificant for all parameters. Therefore, data were pooled over years 2019 and 2020 for each treatment. The fixed effects of treatment, variety, and treatment × cultivar for each parameter are summarized (Table 2).

Freezing tolerance

GM and UC did not differ for either the 6-week or 12-week control treatments (Figs. 1A and 1B). GM had decreased LT$_{50}$ values (increased freezing tolerance) compared with UC after 6 weeks of drought or cold acclimation by approximately 1 °C and 2 °C, respectively; however, only cold acclimation treatments differed from controls for both cultivars (Fig. 1A). Drought followed by cold acclimation did not have a synergistic effect on LT$_{50}$; values for cultivars did not differ from those for cold acclimation alone (Fig. 1B). LT$_{50}$ was higher for UC than GM in the drought followed by cold treatment by approximately 2 °C (Fig. 1B), suggesting a cultivar-specific effect, also seen in the drought-only treatment (Fig. 1A).

Physiological parameters

Drought treatment increased the root:shoot ratio only for GM compared with the control, and values for both cultivars after cold acclimation were greater than those for drought (Fig. 2A). GM had an elevated root:shoot ratio compared with UC for both the drought and cold acclimation treatments. Drought followed by cold acclimation appeared to have a synergistic effect only for GM (Fig. 2B).

The fern water percentage generally decreased after 6 weeks of drought treatment or cold acclimation; cultivars differed only for the latter, and the water percentage for GM was lower than that for UC (Fig. 2C). The fern water percentage appeared to decrease as control plants matured in the 6- and 12-week treatment groups; however, no differences were detected between cultivars or treatments within cultivars at the latter sampling time (Figs. 2C and 2D).

Only cold acclimation decreased the crown water percentage for both cultivars and values for GM were lower than those for UC by approximately 3% (Fig. 2E). Drought stress decreased water percentage by 1.5% in GM compared with UC. The crown water percentage appeared to decrease with plant age for controls, comparing the 6- and 12-week treatment groups (Figs. 2E and 2F). For the 12-week treatments, values were generally similar for cultivars and environmental conditions; however, the water percentage was lower for GM than UC when plants were untreated for 6 weeks followed by 6 weeks of cold acclimation (Fig. 2F).

Table 2. Significance of fixed effects for experiments over 2 years studying drought and cold acclimation in asparagus.

Parameter	Six week			Twelve week		
	T	C	T × C	T	C	T × C
LT$_{50}$	*	*	*	*	*	NS
Proline	*	*	NS	*	*	*
Sucrose	*	NS	NS	*	*	NS
Glucose	NS	NS	*	NS	*	*
Fructan (total)	*	NS	NS	NS	NS	NS
Fructan (LMW)	*	NS	NS	NS	*	NS
Fructan (HMW)	*	*	*	NS	*	NS
Root:shoot	*	*	NS	NS	*	*
Fern %H$_2$O	*	*	NS	NS	NS	*
Crown %H$_2$O	*	*	NS	NS	*	*

Note: T, treatment; C, cultivar; T × C = treatment × cultivar interaction; LMW, low-molecular-weight; HMW, high-molecular-weight; %H$_2$O = water percentage goo; NS = nonsignificant. *Significant for $P \leq 0.05$.

Fig. 1. Freezing tolerance (LT$_{50}$) of asparagus cultivars Guelph Millennium (GM) and UC157 (UC) grown under (A) 6 weeks of control, drought, and cold-acclimating conditions or (B) 12 weeks of control (con), control/cold (con/cold), or drought/cold (dro/cold) conditions. Data were pooled over years (2019 and 2020). Mean ± SE, $n = 8$. Upper- and lower-case letters denote differences between treatments for GM and UC, respectively, for each subfigure, determined by Tukey's Honestly Significant Difference (HSD) test, $P \leq 0.05$. Differences between cultivars within treatments were determined by a Tukey's HSD test and denoted by **$P \leq 0.01$; ***$P \leq 0.001$.
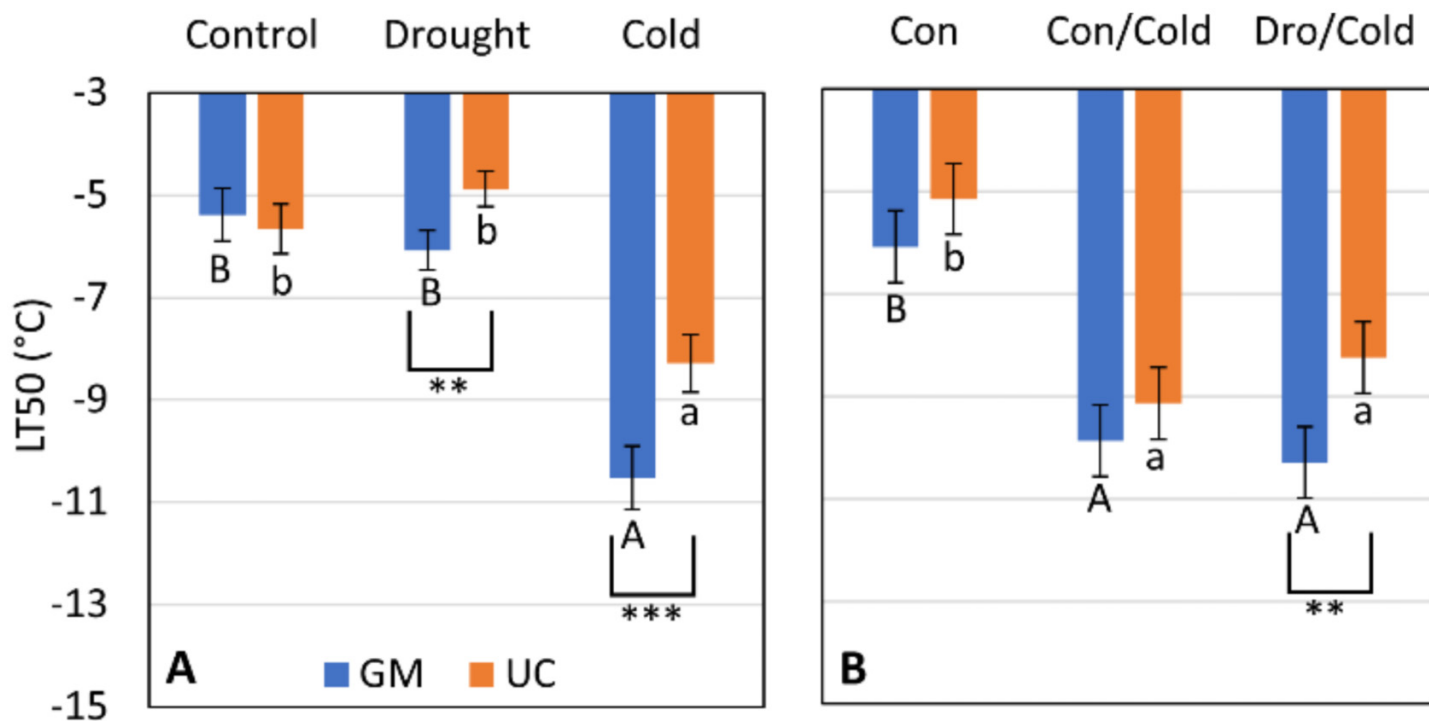

Metabolites

The crown proline concentration increased under both drought stress and cold acclimation within each cultivar compared with the control, although the magnitude of response was greatest for the latter (Fig. 3A). Proline concentration was greater for UC than GM under cold acclimation. Drought followed by cold acclimation did not have a synergistic effect (Fig. 3B). The levels increased for both cultivars when plants were grown under control or drought conditions followed by cold acclimation, and proline concentrations were greater in UC than GM for both treatments.

Crown sucrose concentrations decreased after cold acclimation and cultivars did not differ (Fig. 3C). Levels increased for UC after drought treatment by 30% while GM did not respond. Concentrations for controls appeared lower for those grown with the 12-week compared with the 6-week treatments (Figs. 3C and 3D). The sucrose concentration increased similarly for untreated or drought-stressed plants

Fig. 2. Root:shoot ratio (A, B), fern water percentage (C, D), and crown water percentage (E, F) of asparagus cultivars GM and UC grown under 6 weeks of control, drought, and cold-acclimating conditions (A, C, E) or 12 weeks of control (con), control/cold (con/cold), or drought/cold (dro/cold) conditions (B, D, F). Data were pooled over years (2019 and 2020). Mean ± SE, $n = 8$. Upper- and lower-case letters denote differences between treatments for GM and UC, respectively, for each subfigure, determined by Tukey's HSD test, $P \leq 0.05$. Differences between cultivars within treatments were determined by Tukey's HSD test and denoted by *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. LSM = least square means.
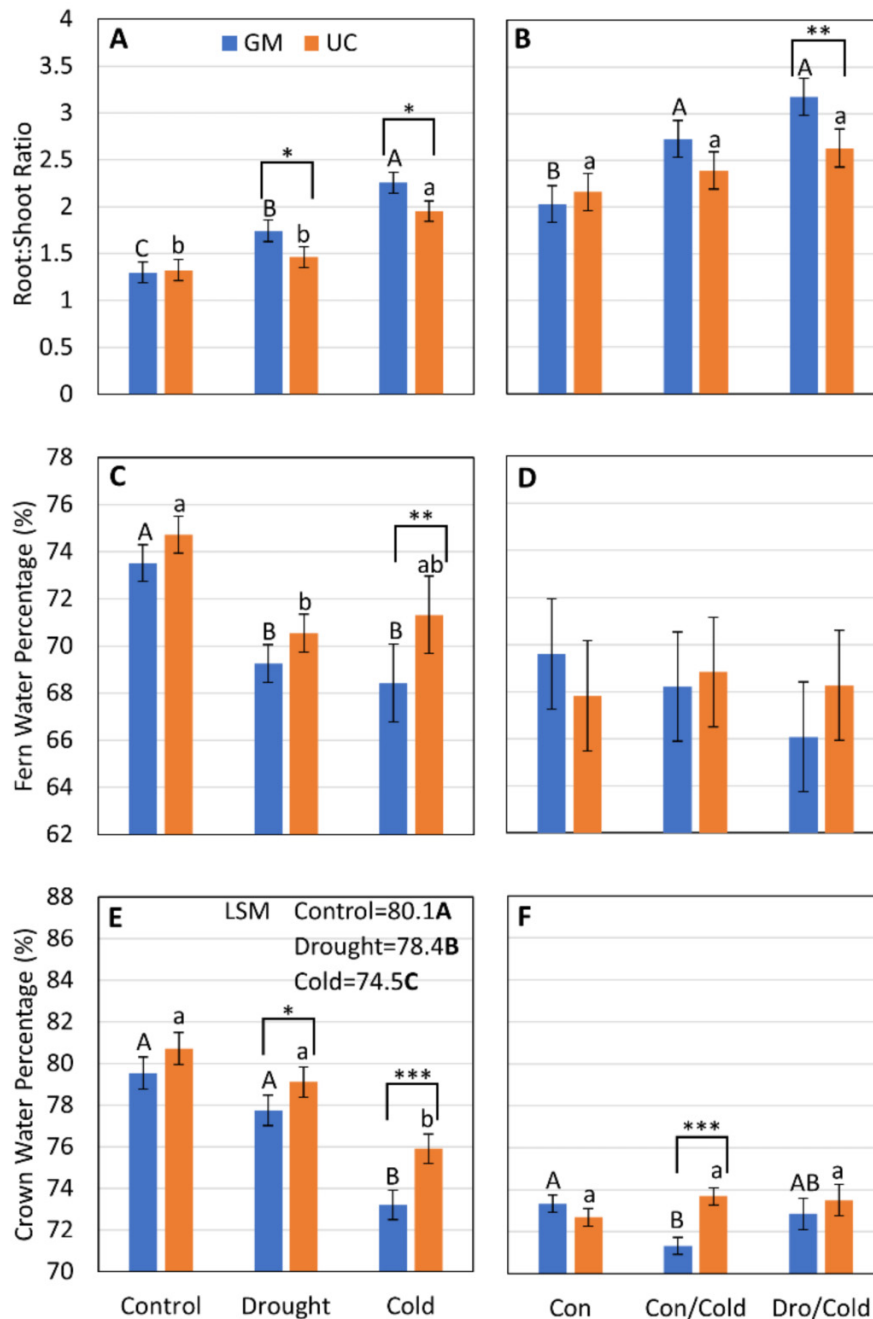

subsequently subjected to cold acclimation, but cultivars did not differ (Fig. 3D).

Crown glucose concentrations were generally not affected by drought or cold acclimation for both cultivars, although levels for UC decreased under cold acclimation (Fig. 3E). A synergistic response was observed for UC plants that were drought stressed followed by cold acclimation; GM was unre-

sponsive to cold acclimation alone or drought stress followed by cold acclimation (Fig. 3F).

Total fructan concentrations increased for UC after both drought and cold acclimation, whereas GM did not respond (Fig. 4A). GM had a higher concentration under control conditions compared with UC (Fig. 4A). The total fructan concentration was not affected by drought or control conditions

Fig. 3. Proline (A, B), sucrose (C, D), and glucose (E, F) concentrations of asparagus crowns in cultivars GM and UC grown under 6 weeks of control, drought, and cold acclimating conditions (A, C, E) or 12 weeks of control (con), control/cold (con/cold), or drought/cold (dro/cold) conditions (B, D, F). Data were pooled over years (2019 and 2020). Mean ± SE, $n = 8$. Upper- and lower-case letters denote differences between treatments for GM and UC, respectively, for each subfigure, determined by Tukey's HSD test, $P \leq 0.05$. Differences between cultivars within treatments were determined by Tukey's HSD test and denoted by **$P \leq 0.01$; ***$P \leq 0.001$.
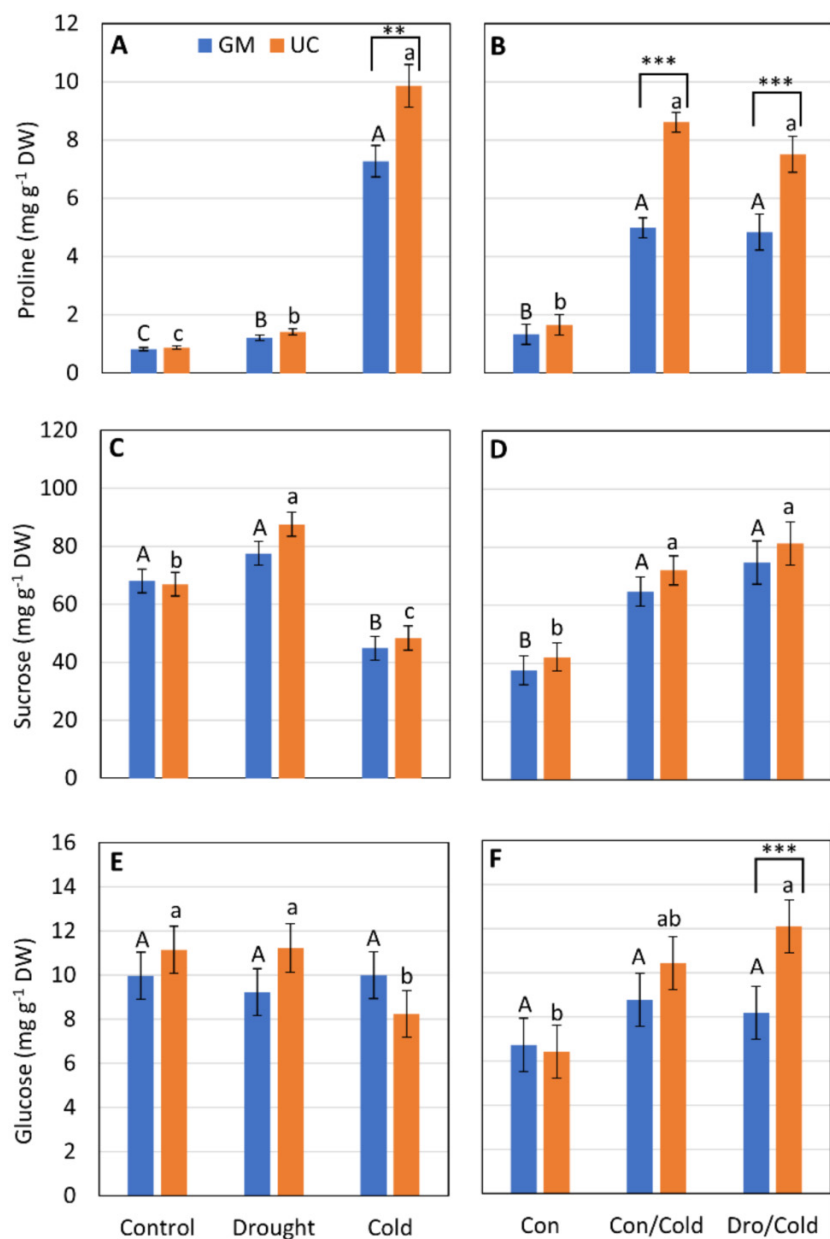
followed by cold (Fig. 4B). The LMW fructan concentration decreased similarly for both cultivars after cold acclimation, and drought had no effect (Fig. 4C). Control conditions or drought stress followed by cold acclimation generally did not impact the trait within cultivar, although values for UC were greater than those for GM in both treatments (Fig. 4D).

HMW fructan concentrations increased only in response to cold acclimation for both cultivars by approximately 150%; however, the level for GM was greater than that for UC (Fig. 4E). Concentrations appeared greater for controls with the 12-week compared with 6-week treatment group (Figs. 4E

and 4F). Both cultivars did not respond to the control or drought-stress treatments followed by cold acclimation, although levels across both treatments were lower for UC than GM (Fig. 4F).

Correlations

LT_{50} was correlated positively with crown sucrose and LMW fructan concentrations and crown water percentage, and negatively with proline and HMW fructan concentrations and root:shoot ratio for the 6-week control, drought, and cold acclimation treatments (Table 3A). For the 12-week treatments,

Fig. 4. Total crown fructan (A, B), low-molecular-weight (LMW) fructan (C, D), and high-molecular-weight (HMW) fructan (E, F) concentrations in asparagus cultivars GM, and UC grown under 6-week control, drought, and cold acclimating conditions (A, C, E) or 12-week control (con), control/cold (con/cold), or drought/cold (dro/cold) conditions (B, D, F). Data were pooled over years (2019 and 2020). Mean $\pm$ SE, $n = 8$. Upper- and lower-case letters denote differences between treatments for GM and UC, respectively, for each subfigure, determined by Tukey's HSD test, $P \leq 0.05$. Differences between cultivars within treatments were determined by Tukey's HSD test and denoted by $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.
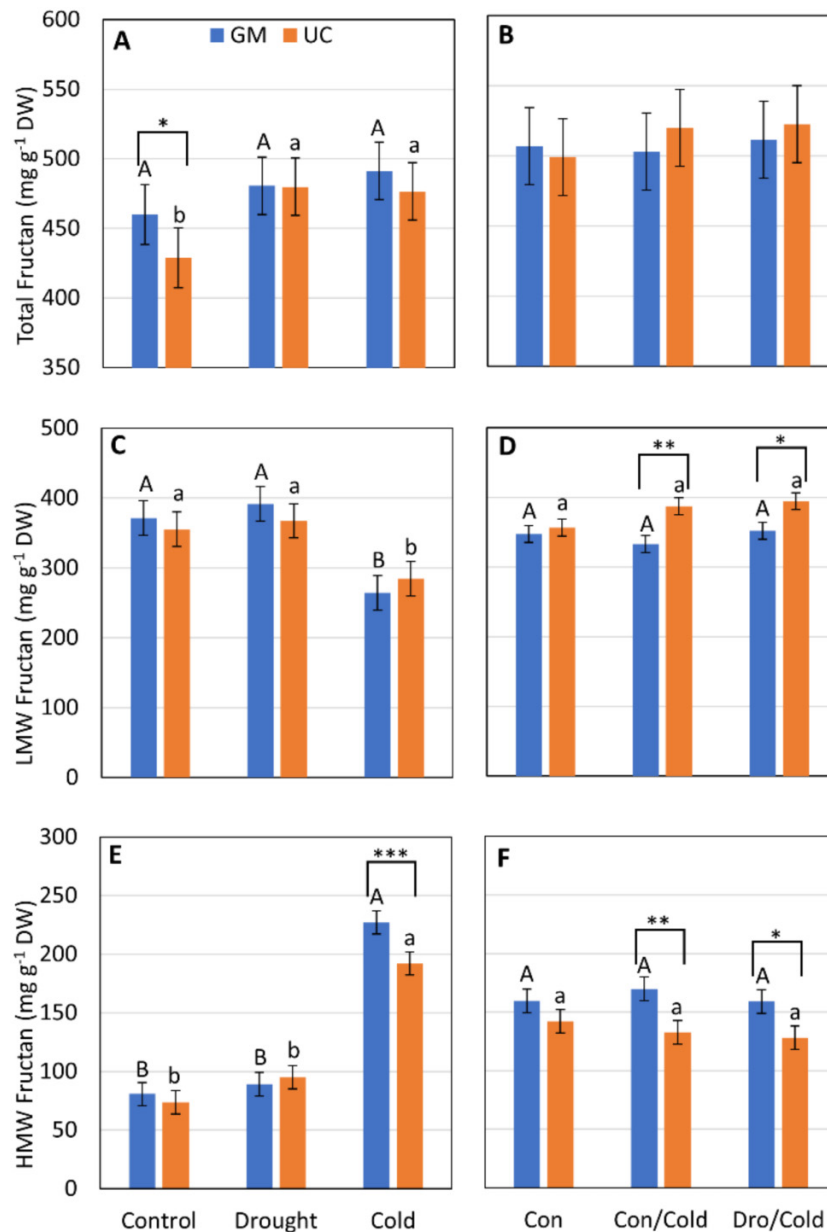
control or control and drought stress followed by cold acclimation, proline and sucrose concentrations, and root:shoot ratios were correlated negatively with LT_{50} (Table 3B). Comparing significant correlation coefficients between the 6-week and 12-week treatment groups, some were unique within a group and others were similar or changed from positive to negative or negative to positive. Similar correlations among treatment groups included the negative correlations of sucrose with HMW fructan, LMW fructan with HMW fructan, and crown% water with HMW fructan (Tables 3A and 3B). For the 6-week treatments, sucrose concentration was correlated positively with LT_{50} and negatively with proline concentration, while negative and positive correlations were observed, respectively, for the 12-week treatment group.

Discussion

Drought stress impacted the acquisition of freezing tolerance and altered the metabolite concentrations and physiological parameters of asparagus seedlings. GM was more freezing tolerant than UC after drought stress, and drought stress followed by cold acclimation. Increased freezing

Table 3. Pearson correlation coefficients between metabolic and physiological parameters analyzed for asparagus cultivars Guelph Millennium and UC157 after (A) 6 weeks of control, drought, and cold acclimating conditions or (B) 12 weeks of control, control/cold, and drought/cold treatments.

A	Proline	Sucrose	Glucose	Fructan (total)	Fructan (LMW)	Fructan (HMW)	Root:shoot	Fern %H$_2$O	Crown %H$_2$O
LT$_{50}$[a]	**−0.82*****	**0.74*****	0.19	−0.24	**0.75*****	**−0.91*****	**−0.77*****	0.32*	**0.79*****
Proline		**−0.64*****	−0.26	0.19	**−0.74*****	**0.85*****	**0.62*****	−0.22	**−0.65*****
Sucrose			**0.38****	0.05	**0.66*****	**−0.71*****	**−0.56*****	0.27	**0.55*****
Glucose				−0.11	0.12	−0.18	**−0.43****	0.26	0.34*
Fructan (total)					-	-	0.34*	−0.22	**−0.59*****
Fructan (LMW)[b]						**−0.71*****	**−0.44****	0.24	**0.39****
Fructan (HMW)[c]							**0.70*****	**−0.40****	**−0.83*****
Root:shoot								**−0.53*****	**−0.79*****
Fern %H$_2$O[d]									**0.53*****

B	Proline	Sucrose	Glucose	Fructan (total)	Fructan (LMW)	Fructan (HMW)	Root:shoot	Fern %H$_2$O	Crown %H$_2$O
LT$_{50}$	**−0.69*****	**−0.60*****	−0.28	−0.10	−0.04	−0.06	**−0.53*****	0.10	0.11
Proline		**0.81*****	**0.40****	−0.08	0.25	−0.34*	0.18	−0.23	0.34*
Sucrose			0.21	−0.24	0.26	**−0.52*****	0.19	−0.24	**0.47*****
Glucose				**0.45*****	0.25	0.21	−0.03	**0.36****	0.09
Fructan (total)					-	-	0.12	**0.36****	**−0.43****
Fructan (LMW)						**−0.47*****	−0.13	0.21	0.07
Fructan (HMW)							0.26	0.15	**−0.52*****
Root:shoot								−0.20	−0.33*
Fern %H$_2$O									−0.17

Note: Significant correlations are indicated by *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$; highly significant correlations (**$P \leq 0.01$, ***$P \leq 0.001$) are highlighted in bold. A single underline indicates the same highly correlated interaction occurred in both (A) and (B), whereas a double underline indicates the opposite highly correlated interaction occurred.
[a]LT$_{50}$ = temperature at which 50% of the population dies
[b]LMW = low-molecular-weight
[c]HMW = high-molecular-weight
[d]%H$_2$O = water percentage

tolerance under drought stress was associated with a high root:shoot ratio, low crown water percentage, and low sucrose levels, whereas the trait was correlated with low glucose and a high root:shoot ratio for the drought followed by cold acclimation treatment.

Cold-acclimated plants had the greatest increase in freezing tolerance, which was similar to that observed in previous seedling studies (Landry and Wolyn 2012; Kim and Wolyn 2015). The higher level of freezing tolerance in GM compared with UC after drought stress may be caused by contrasting adaptative strategies between cultivars. GM may have avoided drought stress through reduced fern growth and crown water concentrations, in contrast to UC, which tolerated drought and continued growth through increased crown sucrose concentrations. The applied drought stress was mild; after 6 weeks, plants had green ferns, although they appeared less dense than those of the control. Differences in drought adaptation between asparagus cultivars have been described previously. For example, "Gijnlim" tolerated drought through water-withholding compared with "Grolim," which avoided drought stress with greater stomatal regulation (Schaller and Paschold 2009). In this study, GM and UC had similar adaptative mechanisms for drought, including increased crown proline concentration and the dehydration of ferns. A fall defoliation study by Nolet and Wolyn (2020) also found general increases in proline levels for both

rhizome and storage root tissues in a dry year, compared with one that was wet.

Freezing tolerance was greater for GM compared with UC in the drought followed by cold acclimation treatment, whereas no difference was found between cultivars for the control followed by cold acclimation treatment. Visually, ferns of UC were greener than those of GM in the former treatment, whereas both cultivars yellowed similarly for the latter. Since flats were flushed with water after the drought treatment, stress was removed and replaced with well-watered cold acclimating conditions. UC, a cultivar typically grown under desert conditions with periods of water withholding followed by irrigation (Toledo 1990), may have reacted to soil flushing as a signal to grow despite cold acclimating conditions. This trait may contribute to UC's poor adaptation to Southern Ontario as late fern growth and cold acclimation have been associated with both decreased freezing tolerance and longevity in asparagus (Landry and Wolyn 2012; Panjtandoust and Wolyn 2016). Interestingly, Wilson et al. (1996) found a similar response in UC where irrigation during the summer and fall negatively impacted spear yields the following season, possibly by causing late fern growth.

Drought followed by cold acclimation appeared to have a synergistic effect on root:shoot ratio for GM and glucose concentration for UC. The high glucose concentration and low root:shoot ratio in UC crowns may be indicators of increased

metabolism and growth, respectively, under cold conditions. For example, in faba (*Vicia faba* L.) cotyledons, the most mitotically active cells had the greatest glucose levels, compared with the most differentiated cells that contained the lowest glucose concentrations (Borisjuk et al. 1998), and adding glucose to sliced potato can increase respiration rates (Geiger et al. 1998). Glucose may have also indirectly impacted freezing tolerance through moderating ethylene, as many ethylene biosynthetic processes are repressed by glucose (Zhou et al. 1998; Yanagisawa et al. 2003). High ethylene levels can increase cold hardiness in peach buds (Liu et al. 2021) and apple (Wang et al. 2021) trees.

In this study, plants from the 12-week control treatment were most likely stressed by being root bound, as ferns were visibly yellowed, and crown DW was approximately twice that for the 6-week control treatment (data not shown). This unintentional "stress" treatment was valuable to show how attributing changes in one parameter to increases in freezing tolerance should be done with caution, as cold acclimation can be a multigenic, quantitative trait associated with many processes (Kazemi-Shahandashti and Maali-Amiri 2018). For example, LT_{50} was positively correlated with crown LMW and negatively correlated with HMW fructan and water percentage in 6-week treatments but not 12-week treatments. These parameters may be indicators of growth cessation, caused by unfavorable growing conditions, rather than increased freezing tolerance. For example, in woody perennials, dormancy is a prerequisite for cold acclimation and the development of freezing tolerance (Weiser 1970).

Negative correlations between HMW fructan with sucrose, LMW fructan, and crown water percentage were observed for both 6- and 12-week treatments. These correlations are consistent with an asparagus seedling study where increased sucrose metabolism was correlated with elevated HMW fructan levels (Witzel and Matros 2020), and field studies where a decrease in LMW fructan and crown water percentage was observed as HMW fructan increased for storage roots in the fall (Panjtandoust and Wolyn 2016; Nolet and Wolyn 2020). In general, LMW fructans constitute the majority of crown fructans observed in this study even under cold acclimating conditions, which was similar to previous fructan composition analysis studies in field-grown asparagus (Shiomi 1992; Suzuki et al. 2011).

The decrease in crown sucrose concentrations in 10-week-old seedlings cold acclimated for 6 weeks compared with controls was unexpected, as previous seedling studies generally found the opposite effect (Landry and Wolyn 2012; Kim and Wolyn 2015). Seedlings in this experiment were sown in early March, compared with early May, thus, seasonal changes in greenhouse irradiance may have impacted the physiological state of plants. Interestingly, plants from the 12-week cold acclimation treatments accumulated high levels of sucrose, suggesting that age may have impacted sucrose metabolism and the cold acclimation process.

In summary, the interaction between drought and cold acclimation was confirmed in asparagus. Drought stress alone caused GM to have a greater freezing tolerance than UC and was correlated with an increased root:shoot ratio, a decreased crown water percentage in GM, and an elevated sucrose concentration in UC. Drought followed by cold acclimation led to decreased freezing tolerance in UC compared with GM, which was correlated with a decreased root:shoot ratio and high crown glucose levels in UC. These findings highlight the importance of investigating multiple stress factors, similar to those observed in nature, while selecting for asparagus cultivars suitable for temperate climates.

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