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The effect of cold acclimation on the low molecular weight carbohydrate composition of safflower

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Abstract

Understanding cold acclimation and identifying the low molecular weight carbohydrates that support the development of freezing tolerant safflower seedlings will aid in breeding winter-hardy cultivars for temperate cropping systems. Three field selected lines of winter safflower (WSRC01: PI 651878; WSRC02: PI 651879; WSRC03: PI 651880) were cold acclimated for four weeks at 4 °C and compared to seedlings grown for two weeks at 20 °C. The commercial spring-type cultivar, Olé, served as a non-hardy check. Leaf, stem, and root fructose, glucose, sucrose, raffinose, and stachyose concentrations all increased to variable extents across the PI accessions after cold acclimation. In comparison with Olé, winter safflower accessions tended to be more responsive to cold acclimation by increasing metabolite concentration. Verbascose was only recovered within leaf tissue and PI 651880 was the only entry to show a substantial alteration in verbascose concentration due to cold acclimation. Based on these data, no specific low molecular carbohydrate was responsive or responsible for the accumulation of freezing tolerance, but a concert of metabolites and their responsiveness may help explain the observed differences in development, freezing tolerance, and ultimately winterhardiness among safflower germplasm.

Keywords: Agriculture, Plant biology

1. Introduction

Safflower (*Carthamus tinctorius* L.) is an annual dicot used for the extraction of high-quality edible and industrial oil, and for bird feed [24]. The cultivation of winter-hardy safflower could provide a number of benefits in a winter grain crop rotation. However, safflower continues to be limited by its susceptibility to low winter temperatures [22, 50, 10, 45]. Safflower collections from Iran [10] and China [28] which display freezing tolerance normally exhibit a long rosette period or winter habit. Compared with normal spring-sown types, genotypes with this prolonged rosette character tend to have increased cold tolerance and are considered winter types. The increase in freezing tolerance could be in part anatomical, i.e., the result of multiple layers of young leaves and leaf primordia protecting the apical meristem, which is the main growing point of the plant. However, not all genotypes with a prostrate growth habit are freezing tolerant [22, 1].

Yazdi-Samadi and Zali [45] found at a minimum temperature of −4.4 °C, autumn-sown winter types survived better and yielded more than spring types. It remains unclear what the limit to freezing tolerance is among winter-type safflower germplasm, however, during the rosette stage, temperatures from −7 °C [22, 32] and −15 °C [50] to −26 °C [20] are tolerated. Further, if meristems survive, plants are able to recover from injury as regrowth resumes and new leaves replace the older injured leaves. Tolerance does decline precipitously, however, once the stem elongation phase commences, where even a light frost can damage the main apical meristem or main stem, resulting in plant mortality. Therefore, safflower accessions with a low rosette habit; that is, minimal stem elongation during autumn and winter, is an essential character for over-winter survival [22].

In addition to the low rosette trait, metabolic adaptation of safflower in preparation for freezing temperatures is suspected to occur through the cold acclimation process. Plants exposed to a period of non-lethal near freezing temperature initiate a series of events such as an increase in soluble sugars, cold stress proteins, and proline [8, 26, 44], and an increase in both the unsaturated-to-saturated fatty acid ratio and phospholipids of the plasma membrane [34, 2]. Soluble sugars such as sucrose, fructose, and the raffinose family oligosaccharides (RFOs) can function to reduce cellular membrane damage by replacing cell water content with a glassy or vitreous state that minimizes ice crystal formation and freeze induced dehydration [44, 41, 43]. Sugars can also provide energy to maintain low temperature respiration in living cells, allow cell metabolism to recover after freezing, and supply resources for subsequent spring regrowth [39, 13, 46, 35]. The cryoprotectant action of RFOs (raffinose, stachyose, and verbascose) remains less clear but they appear to protect lipid headgroups in cellular membranes from frost injury through complex structural interactions [17]. Evidence suggests that as the

degree of polymerization of RFOs increases they become progressively better at stabilizing liposomes and preventing membrane fusion after rehydration [17].

One of the few studies that reported on metabolic adaptation of safflower to cold acclimating conditions suggested that tolerant genotypes maintained cell membrane integrity and had a high carbohydrate and protein concentration, and low relative water content when exposed to 2–6 °C [11]. Given the moderately warm winter temperatures of that study, it remains uncertain how these results would translate under colder freezing conditions. In other winter annual crops such as winter pea, the greater sucrose accumulation (20–60 mg g^{-1} dw) compared to spring types (5–18 mg g^{-1} dw) after cold acclimation corresponded to significantly greater winter survival rates [4]. Winter-hardy rape cultivars also stored larger amounts of soluble sugars, acclimated faster, and to a lower lethal temperature of −13 °C compared to −7 °C for acclimated spring cultivars [35].

For winter-type safflower, it is unknown how soluble sugar concentrations across different tissue types respond during the cold acclimation period. Therefore, the objective of this study was to evaluate the relationship between cold acclimation and the accumulation of soluble sugars in contrasting populations of winter and spring safflower accessions. Understanding the metabolic response of safflower to cold acclimation will help to determine any link to freezing tolerance, enabling the selection of superior genotypes.

2. Methods

2.1. Cold acclimation protocol

A controlled cold acclimation experiment was conducted in 2012 with five germplasm accessions of safflower (*Carthamus tinctorius* L.). PI accessions 651880, 651879, and 651878 are winter-hardy selections [21] and PI 537695 is a commercial cultivar (Olé) serving as the spring-type non-hardy check. Seed from each entry was sown into four 48 cell (3.8 cm × 6 cm × 5.7 cm) trays (26.7 cm × 53.3) filled with Sunshine® Mix #3 (Sun Gro Horticulture; Agawam, MA). Each tray was separated into 4 blocks consisting of three plants from each of the four entries. Two trays were grown for four weeks under cold acclimation conditions (4 °C, 10 h photoperiod) and the remaining two were grown for two weeks at 20 °C, 12 h photoperiod (Model PGR15; Controlled Environments Limited; Winnipeg, Manitoba). Non-acclimated trays were started two weeks after the cold acclimation treatment to align development.

2.2. Tissue harvest and analysis

Seedlings were harvested at the two true leaf stage and soilless substrate rinsed from roots with distilled water. Plants were separated into roots, leaves, and stem

segments, lyophilized and finely ground. For each entry within a block, three seedlings were combined as one sample.

Replicate leaf and root tissue (100 mg) and stem tissue (10–100 mg) were used to determine the low molecular weight carbohydrates (LMWC): monosaccharides glucose and fructose, disaccharide sucrose, and the raffinose series oligosaccharides (verbascose, raffinose and stachyose) following Knudsen and Li [25]. Tissue materials were extracted twice in 5 ml of 50% EtOH, with 0.5 ml ribitol (1 mg/ml) added as an internal standard, for 30 min at room temperature, stirring every 10 min on a vortex. Cell wall material was then removed by centrifugation (3000 g, 10 min @ room temperature). From the combined supernatant, an aliquot of 200 µl was diluted with an equal volume of ethanol (90%, v/v), stored for 30 min at −20 °C and centrifuged (10,000 *g* for 10 min @ 24 °C) to precipitate proteins. The supernatant was dried at 25 °C (Thermo Scientific DNA 110 Savant™ SpeedVac™, Waltham, MA, U.S.A.) and redissolved in 1 mL of ultrapure water (EMD Milipore Co.; Billerica, MA, U.S.A.). Low-density particles were removed by filtration through a 0.45 µl Millex-HV filter (Millipore Corporation, Bedford, USA) in preparation for high performance liquid chromatography (HPLC) analysis.

A portion (10 µl) of the sample was analyzed for individual LMWCs using an Agilent 1100HPLC with 1260 Agilent RI detector (Agilent Technologies, Santa Clara, CA, U.S.A.) at 35 °C. The mobile phase was water and the flow rate 0.5 ml/min. An Aminex HPX–87 N (300 × 7.8 mm) resin-based column in the sodium form (Bio-Rad Laboratories, Inc.; Hercules, CA, U.S.A.) preceded by a Cation H Bio-Rad Micro-Guard column (304.6 mm) was used at 85 °C to separate sucrose, raffinose, stachyose, verbascose, fructose, glucose, and *myo*-inositol. Galactose was not measured due to a lack of resolution between galactose and glucose.

Identification and assignment of peaks was accomplished by using the retention time (min) of the peaks for the different sugars. Pure standards of D-glucose, sucrose, verbascose, stachyose, raffinose, and ribitol were used (Sigma-Aldrich; St. Louis, MO). Other reagents were of analytical grade.

The experiment was a split block with four replications repeated twice (runs). Main blocks were cold acclimation or no cold acclimation and the sub-blocks were the germplasm entries. The experiments were nested within the two runs. Data were analyzed using proc mixed (SAS Institute 2008) with acclimation and germplasm entries as fixed effects and the main blocks and subplots as random effects. For the acclimation treatment, differences were assessed with the F-test at $P < 0.05$ using the main plot error term. For the germplasm and the acclimation by germplasm interaction, the residual error was used as the error term for the f-test at $P < 0.05$. In the presence of a significant F-value associated with germplasm entries, multiple comparisons were conducted using the LSD at $P < 0.05$ and $P < 0.01$.

3. Results

Leaf (Fig. 1), stem (Fig. 2), and root (Fig. 3) low molecular weight carbohydrates were measured on safflower seedlings following either a cold acclimation or no cold acclimation treatment. An overall trend was observed across winter-type PI accessions as compared to the spring-type cultivar Olé. Sucrose, glucose, fructose, raffinose, and stachyose all showed variable increases across all tissue types in winter-type PIs for acclimated plants compared to the non-acclimated control treatment. An appreciable amount of verbascose was present only in leaf tissues, the concentration of which increased only for PI 651880 in response to acclimation.
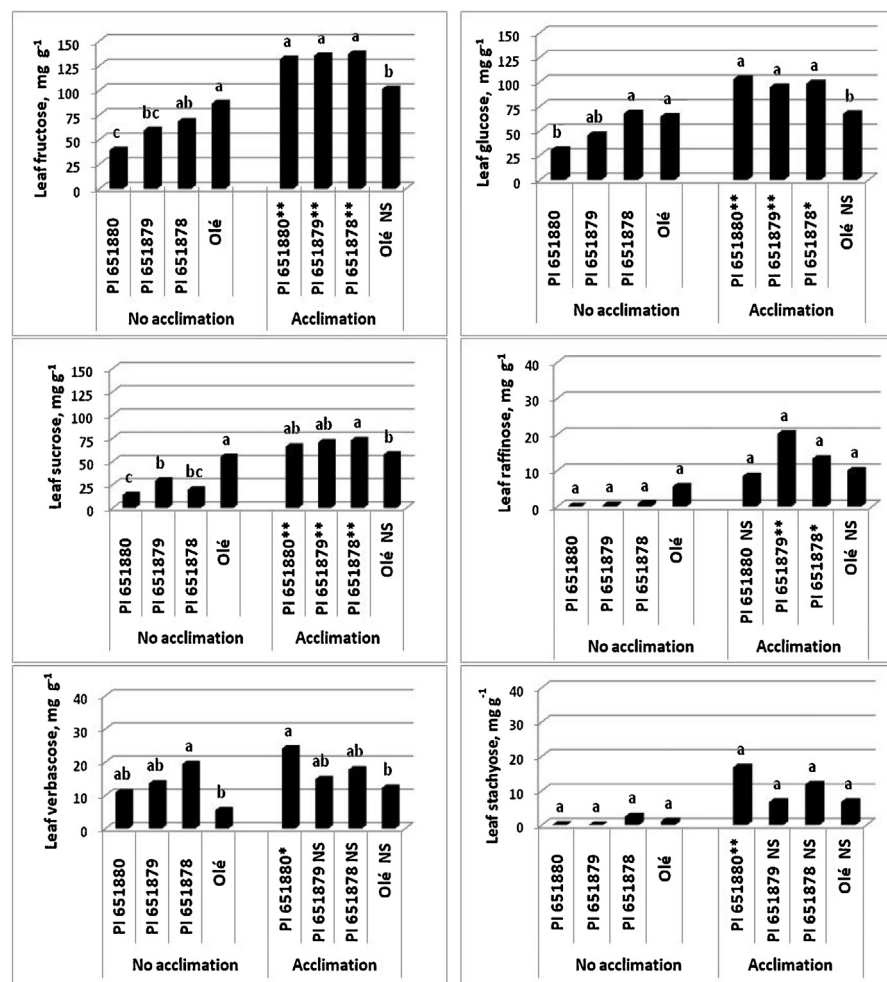
Fig 1. Leaf carbohydrates for acclimated and non-acclimated plants in winter adapted safflower accessions and the spring cultivar Olé. Within each acclimation treatment, accessions sharing letters do not differ at $P < 0.05$ using the LSD. A significant response to acclimation for a given accession is denoted as * or ** for differences at $P < 0.05$ and $P < 0.01$, respectively.
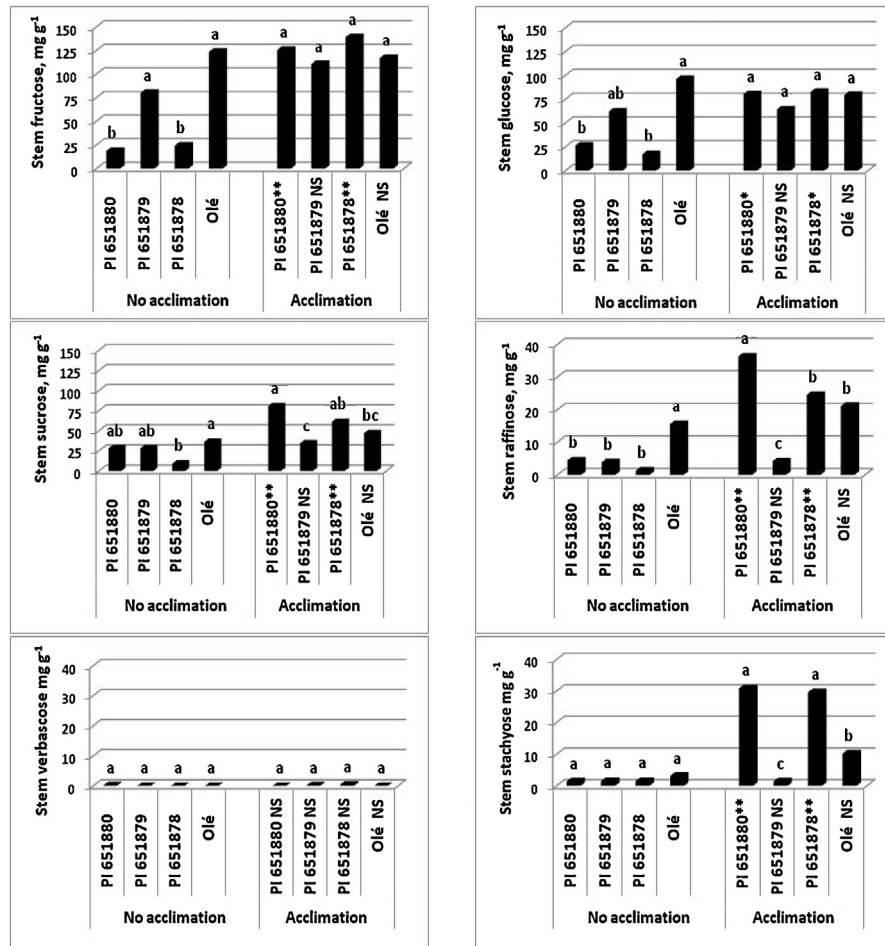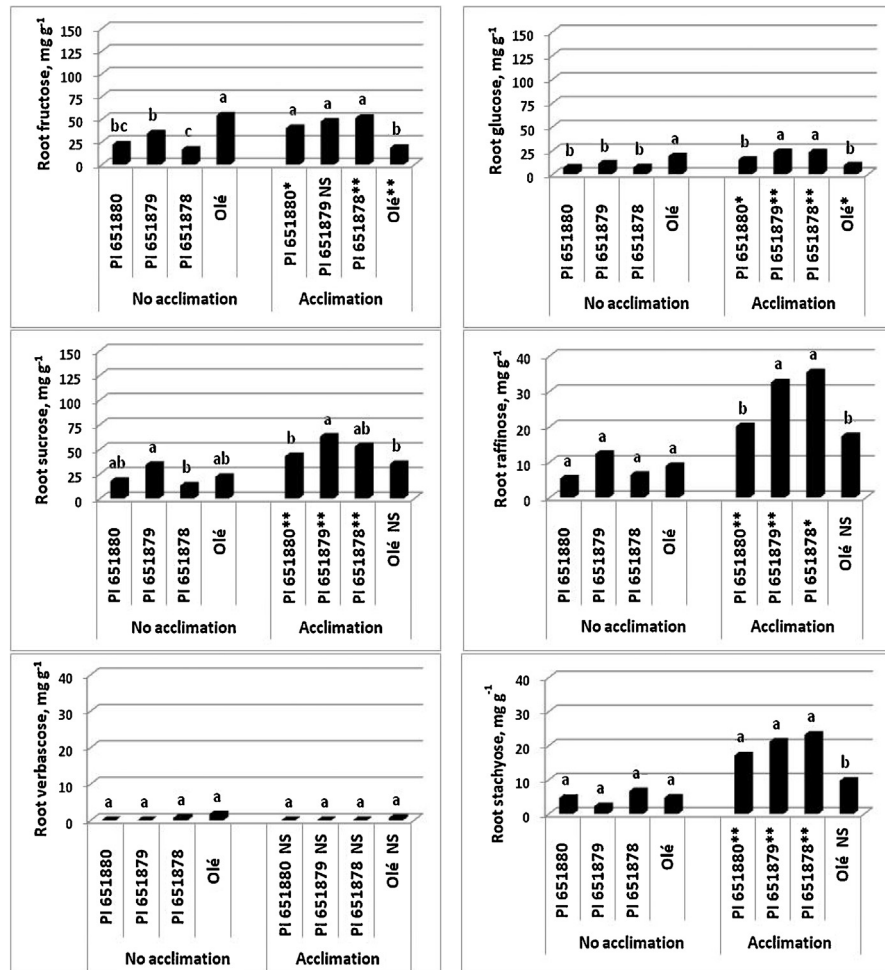
Fig. 2. Stem carbohydrates for acclimated and non-acclimated plants in winter adapted safflower accessions and the spring cultivar Olé. Within each acclimation treatment, accessions sharing letters do not differ at P < 0.05 using the LSD. A significant response to acclimation for a given accession is denoted as * or ** for differences at P < 0.05 and P < 0.01, respectively.

The general increase in carbohydrate concentration was typically more noticeable for winter-type PI accessions as compared to Olé. Notably for leaves, the winter-types had a consistent increase in fructose and glucose with acclimation that was absent for Olé. However, specific inconsistencies were noted. For example, PI 651880 and PI 651878 showed an increase in stem sucrose, raffinose, and stachyose concentrations, whereas PI 651879 did not. Further, the concentration of fructose and glucose within the stem was higher for non-acclimated PI 651879 as compared to the other two PIs and approached the concentration of Olé. For both PI 651879 and Olé, stem fructose and glucose concentrations were not different between acclimated and non-acclimated treatments. Further, there was a small but significant reduction in the concentration of fructose and glucose as a response to acclimation in Olé root tissue, whereas the PIs all showed increases in low molecular weight

Fig 3. Root carbohydrates for acclimated and non-acclimated plants in winter adapted safflower accessions and the spring cultivar Olé. Within each acclimation treatment, accessions sharing letters do not differ at P < 0.05 using the LSD. A significant response to acclimation for a given accession is denoted as * or ** for differences at P < 0.05 and P < 0.01, respectively.

carbohydrates except for fructose of PI 651879, which was not substantially altered due to the cold acclimation treatment.

The leaf tissue was the least discerning at drawing out differences among winter-type PIs for the constituent LMWCs. Across all winter-type PIs, leaf fructose concentration after cold acclimation reached above 125 mg g^{-1} DW and glucose, sucrose, and verbascose concentrations too were the highest across tissue types, whereas Olé showed no response. Leaves are the source and perhaps the primary, at least initially, storage reserve for these energy reserves during cold acclimation. The winter-type accessions appeared to respond by increasing the concentration of low molecular weight carbohydrates, while Olé remained less responsive.

The low molecular weight carbohydrate profile of stem tissue fell somewhere in-between that of the leaf and root, and showed distinct differences of constituents between winter-type accessions as noted earlier in this section. These differences in metabolite accumulation among winter-type stem tissue may hold the most valuable information for understanding freezing tolerance and ultimately identifying the most freezing tolerant genotypes.

4. Discussion

PIs 651880, 651879, and 651878 are germplasm lines released by the USDA-ARS, Western Regional Plant Introduction Station, as WSRC01, WSRC02, and WSRC03, respectively [21]. Average overwinter survival at two field sites in 2004–2005 for WSRC01, WSRC02, and WSRC03 was 88%, 84%, and 78%, respectively, whereas spring-type cultivars failed to overwinter.

A general metabolic response of winter-type safflower seedlings to the controlled cold acclimation treatment was the increase in low molecular weight carbohydrate concentration. There was no appreciable metabolic response of the commercial spring check variety Olé to cold acclimating conditions except for the reduction in the concentration of root fructose and glucose. This lack of response may explain the limited freezing tolerance/winterhardiness of this cultivar. The response of winter-type safflower accessions mirrors the general "sweetening" effect or osmoprotection that is quite ubiquitous among freezing tolerant winter annuals [14, 31, 38] and cold tolerant crops [30, 47] when exposed to temperatures <10 °C. Increasing concentrations of low molecular weight carbohydrates are generally linked to increasing freezing tolerance in response to cold acclimation but are not the only physiological adaptations concurrently taking place [6, 8, 31, 29].

Our low molecular weight carbohydrate data on controlled cold acclimated safflower aligned well with that of Castonguay et al. [6] and Cunningham et al. [8] who found alfalfa (*Medicago sativa* L.) accumulates raffinose, stachyose, and sucrose in leaf and root tissue in response to cold temperatures. They also discovered that over the winter, sucrose content slowly declined, while RFOs and freezing tolerance remained stable, indicating at least a correlative role to winter hardiness. Unfortunately, we do not know the low molecular weight carbohydrate response of field acclimated winter safflower that has been overwintered, and if it would change our conclusion that RFOs are not the main determinant of freezing tolerance in safflower.

Some studies have shown RFOs play a minor role in freezing tolerance. Knaupp et al. [23] reported that raffinose was not involved in protecting electrolyte leakage from leaf cells of *Arabidopsis*, and Zuther et al. [51] reported that freezing tolerance was not reduced in *Arabidopsis* when raffinose production was inhibited. Further, no improvement in freezing tolerance of rosette leaves were observed in

cold acclimated transgenic lines of common beans with artificially induced high levels of stachyose [19], nor in grapevines (*Vitis vinifera*) where neither raffinose nor stachyose were effective in suppressing intra- and extra-cellular ice formation [3].

Leaf fructose and glucose are the two metabolites that had consistent results regarding acclimation and should be validated in future studies. All winter types showed consistent increases in these two metabolites, while Olé showed no response. Also within the cold acclimation treatment, Olé always had less fructose and glucose in leaves than the winter-type PIs. What role these sugars play in freezing tolerance remains to be adequately tested, although in other species, a positive correlation is present [37, 18]. Similarly to the results of Livingston et al. [31] in winter annual grasses, we have observed that freezing tolerance of safflower seedlings is distinctly different depending on tissue, increasing from root to leaf to stem possibly as a result, at the very least, of differences in low molecular weight carbohydrate concentration. It would appear the function of these sugars within root tissue may be more as energy reserves rather than cryoprotection since fructose, glucose, and sucrose are relatively low, but larger molecular weight sugars such as raffinose and stachyose, and possibly starch, are higher than in other tissues. Whereas, source production of glucose, fructose, and sucrose in winter-type leaf tissue (e.g. higher freezing tolerance) results in a loading effect increasing osmotic concentration and supporting storage of larger molecular weight sugars within the root system. This suspected accumulation and transfer of metabolites was more efficient for the winter-type safflower lines compared to Olé.

In conjunction with increasing the concentration of soluble sugars, winter annuals typically exhibit some delay in flowering, their most frost sensitive developmental stage, and exhibit minimal stem elongation and leaf growth in response to cold [4]. A prostrate habit in conjunction with vernalization helps to maintain the storage reserves acquired during the cold acclimation process [18]. Vernalization is a quantitative low-temperature response and flowering is abated through epigenetic mechanisms in response to cold as shown in *Arabidopsis* [48]. Further, vernalization is an important component of freezing tolerance. For example, rape (*Brassica napus*) varieties that exhibited rapid elongation and leaf growth were prone to freezing because of a competition for photoassimilates between growth and acclimation when exposed to a cycle of acclimation/deacclimation [35]. For safflower, however, not all genotypes exhibiting a prolonged prostrate habit are freezing tolerant [22, 1]. Although there is apparently Chinese germplasm with an obligate vernalization requirement [28], the three winter-type PIs tested here and the majority of safflower germplasm on which there are data, have a facultative vernalization response where reproduction occurs whether fall or spring sown [20].

Based on our research and the available literature, the increase in soluble sugars in response to cold acclimation contribute to but are likely not exclusively responsible

for freezing tolerance. In many cases, other factors including hundreds of other metabolites [40, 49, 9], like phenolics, hormones, dehydrin proteins, antioxidants, nitric oxide, and proline and the cell membrane fatty acid composition and amino acid content, which affect water relations between the intra- and intercellular environments [5], along with crop development [22] contribute to freezing tolerance and further winterhardiness in crops [16, 42]. The interplay and complexity of metabolic alterations in response to cold necessitates a systems approach [33] to fully appreciate these dynamics in winter safflower. Possibly, examining a complete metabolic profile [7] in response to cold acclimation across all tissue types including the apical meristem [28] of freezing tolerant and susceptible genotypes across the range in duration of prostrate habits would help to identify specific constituents within each tissue type that contribute to freezing tolerance by assessing the phenology variable. A multivariate statistical analysis could help to evaluate many metabolites by establishing metabolomic fingerprints for cold-sensitive and cold-tolerant accessions as shown in Arabidopsis [50].

Finally, it is well understood that the duration and specific conditions of cold acclimation can affect a plant's tolerance to freezing [15, 12, 4], as well as the genetic background [31, 27, 36]. Longer duration of cold acclimation (i.e., 15 °C/5 °C day/night with a 10 h photoperiod) of the winter-hardy safflower cultivar N-8 showed increasing survival after a freezing stress of −15 °C for 4 h from 28 to 32 to 60 percent after 1, 2, and 3 weeks, respectively [51]. Longer durations of freezing also decrease survival [52]. It remains unclear if alterations in the conditions of artificial cold acclimation would affect the low molecular weight carbohydrate profile or freezing tolerance of these PIs.

In conclusion, four weeks of cold acclimation resulted in a general increase in low molecular weight carbohydrates in the winter types, but not the spring-type check. There was a consistent difference between winter-type PIs and the spring-type Olé for leaf fructose and glucose suggesting a probable role in acclimation of winter-types to freezing temperatures. However, that consistency was not observed in stems and roots. Additional studies are needed to clarify the specific role of low molecular weight carbohydrate accumulation in various tissue types and how that relates to the array of metabolic processes likely involved in freezing tolerance within winter-type safflower germplasm.

Declarations

Author contribution statement

Erik J. Landry, Vicki L. Bradley, R.C. Johnson: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

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