

## ORIGINAL RESEARCH



# Biochemical changes after cold acclimation in Nordic red clover (*Trifolium pratense* L.) accessions with contrasting levels of freezing tolerance

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## Abstract

The ability to tolerate low freezing temperatures is an important component of winter survival and persistence of red clover. Cold acclimation (CA) allows plants to acquire higher levels of freezing tolerance. However, the biochemical responses to cold and the importance of such changes for the plant to acquire adequate freezing tolerance have not been investigated in red clover of Nordic origin, which has a distinct genetic background. To shed light on this, we selected five freezing tolerant (FT) and five freezing susceptible (FS) accessions and studied the effect of CA on the contents of carbohydrates, amino acids, and phenolic compounds in the crowns. Among those compounds which increased during CA, FT accessions had higher contents of raffinose, pinitol, arginine, serine, alanine, valine, phenylalanine, and one phenolic compound (a pinocembrin hexoside derivative) than FS accessions, suggesting a role for these compounds in the freezing tolerance in the selected accessions. These findings, together with a description of the phenolic profile of red clover crowns, significantly add to the current knowledge of the biochemical changes during CA and their role in freezing tolerance in Nordic red clover.

## 1 | INTRODUCTION

Red clover (*Trifolium pratense* L.) is widely cultivated in temperate regions and is the main forage legume grown in Northern Europe, where it is mainly used in mixtures with grasses to produce silage (Annicchiarico et al., 2015; Helgadóttir et al., 2014). Red clover is a protein-rich feed, particularly suited for ensiling and use in ruminants' rations because of its high polyphenol oxidase (PPO) activity, which reduces nitrogen losses (Hart et al., 2016; Lee, 2014). Through their symbiosis with rhizobia, legumes can fix atmospheric nitrogen, resulting in decreased application of nitrogen fertilizers and more sustainable agricultural systems (Jensen et al., 2012; Lüscher et al., 2014). The adoption of forage legumes in crop rotations is particularly

important in regions characterized by cold climates, such as parts of Northern Europe and Canada, where low temperatures and short growing seasons may hamper the cultivation of grain legumes. In such areas, the utilization of red clover may be limited due to its poor persistence, mainly because of the high mortality during winter (Abberton & Marshall, 2005).

Winter survival is a complex trait affected by many biotic and abiotic factors (Bélanger et al., 2006). Freezing temperature is a main cause of winter mortality at continental locations characterized by harsh winters, and the ability of plants to acquire a high level of freezing tolerance is a key trait for winter survival at these locations. Freezing tolerance is achieved during a period of cold acclimation (CA) at low above-freezing temperatures and can be improved by selection

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(Bertrand et al., 2016). Despite some similarities between the climatic conditions that crops in Canada and the Nordic area are exposed to, a unique feature of the Nordic area is the extension of cultivation to much higher latitudes, where plants experience a very high variation in the photoperiod during the year. With global warming, plants are increasingly exposed to shorter photoperiods and less light during the time of the year when the process of CA takes place (Ergon, 2017).

Nordic red clover constitutes a unique group with a distinct genetic background (Nay et al., 2022), which suggests that the development of specific characteristics at the phenotypic level may have resulted from exposure to the Nordic climate. In Nordic red clover gene bank material (wild populations and landraces), freezing tolerance was recently shown to be correlated with low annual temperatures at their place of origin, suggesting that selection pressure has favored more freezing-tolerant plants (Zanotto et al., 2021). Therefore, red clovers of Nordic origin may possess specific mechanisms in response to CA, resulting in different biochemical characteristics than those observed in Canadian red clovers by Bertrand et al. (2020).

CA in winter annual and perennial herbaceous plants adapted to temperate climates is associated with huge changes at the transcriptional, proteomic, and metabolomic levels (Fürtauer et al., 2019; John et al., 2016; Theocharis et al., 2012). Metabolic changes during CA (as well as acclimation to other types of abiotic stress) typically include an increase in the content (on a dry matter basis) of soluble sugars, sugar alcohols, organic acids, amino acids, and amino acid derivatives, and other substrates for secondary metabolites. Soluble carbohydrates are thought to have multiple functions in freezing tolerance, such as stabilization of membranes and proteins, lowering of the freezing temperature within the cell, and quenching or scavenging of reactive oxygen species (Pommerrenig et al., 2018). Amino acids such as proline and glycine betaine have been shown to accumulate under stress in a wide range of species (Giri, 2011; Szabados & Savouré, 2010). In addition to functioning as osmolytes, glycine betaine protects photosystem II under stress, while proline stabilizes membranes, protects against oxidative stress, and acts as a signaling molecule (Szabados & Savouré, 2010). The amino acid arginine is a precursor in the synthesis of polyamines, which play roles in abiotic and biotic stress resistance by stabilizing membranes, acting as scavengers of reactive oxygen species, and by participating in complex signaling networks that regulate stress responses (Alcázar et al., 2011; Menéndez et al., 2019; Pál et al., 2015). Phenolic compounds play important roles in the defense toward abiotic and biotic stress and have been shown to act as antioxidants (Agati et al., 2012). A study of mutant lines with defects in the first steps of flavonoid biosynthesis showed that flavonoids are important for freezing tolerance in *Arabidopsis thaliana* (Schulz et al., 2016). Red clover is rich in phenolic compounds, particularly in the sub-group of isoflavones, which have been shown to be involved in oxidative stress response in both roots and leaves (Saviranta et al., 2010a, 2010b).

Proteome analyses of red clover crowns showed that CA induced marked changes in protein abundance. Proteins involved in carbohydrate metabolism, energy production, and amino acid metabolism were upregulated in response to CA but varied in abundance between

different genetic backgrounds (Bertrand et al., 2016). Biochemical analyses of populations recurrently selected for freezing tolerance showed that the accumulation of soluble sugars and specific amino acids in plant crowns (transition between shoot and root) after a prolonged period of CA is associated with the acquisition of freezing tolerance in red clover and alfalfa (Bertrand et al., 2020; Castonguay et al., 2011). Some responses seem to be more general as they were similar for all genetic backgrounds within the two species. Particularly sucrose increased markedly during CA and reached higher concentrations in populations with higher levels of freezing tolerance in most genetic backgrounds of both species. Among the amino acids, the content of proline, arginine, and asparagine showed the largest increase in response to CA, but the effect of selection for improved freezing tolerance on the relative amounts of these amino acids differed between the genetic backgrounds tested. In addition, the identification of up- and down-regulated proteins involved in the biosynthesis of phenolic compounds after CA (Bertrand et al., 2016), such as the isoflavone-reductase family proteins, suggests that phenolic compounds may play a role in the acquisition of higher freezing tolerance in red clover. However, up to date, a study of the possible involvement of phenolic compounds in FT of red clover is lacking in the literature.

The aim of the current study was to better understand the large differences in freezing tolerance that we previously observed among red clover accessions of Nordic origin (LT<sub>50</sub>-values ranging from −6.73°C to −13.09°C). To reach this goal, we characterized the biochemical changes induced by short-term CA in red clover accessions differing in their levels of freezing tolerance. We studied plant crowns as this is the most critical tissue for winter survival, giving rise to shoot regrowth after winter. We hypothesized that: (1) significant changes in the content of specific carbohydrates, amino acids, and phenolic compounds occur in crowns of Nordic wild populations and landraces of red clover during short-term CA; and (2) red clover accessions with different levels of freezing tolerance differ in these responses to short-term CA. This is, to our knowledge, the first study of associations between freezing tolerance and biochemical changes occurring during CA of Nordic red clover.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant materials, growing conditions, and experimental treatments

Ten red clover accessions of Nordic origin were selected from the NordGen (the Nordic Genetic Resource Centre) collection; five freezing tolerant (FT) accessions and five freezing susceptible (FS) accessions (Table 1). These accessions, all originating from locations with a rather continental climate, had the most extreme freezing tolerance (high or low), assessed as LT<sub>50</sub> (temperature required to kill 50% of the plants), in our previous screening of 48 red clover gene bank accessions of Nordic origin (Zanotto et al., 2021). In the same study, we found that the level of freezing tolerance was significantly

**TABLE 1** Information about the origin, freezing tolerance, and winter survival of 10 Nordic red clover accessions used in the study.

| Accession              | NordGen ID <sup>a</sup> | Type     | Country of origin | Latitude  | Longitude | Altitude (m a.s.l.) | Freezing tolerance (LT50) <sup>b</sup> | %WS <sup>c</sup> | Group |
|------------------------|-------------------------|----------|-------------------|-----------|-----------|---------------------|----------------------------------------|------------------|-------|
| HIETAMA AP0201         | NGB2844                 | Landrace | Finland           | N 62° 35' | E 25° 36' | 130                 | −6.73                                  | 0.17             | FS    |
| VUOTJÄRVI HM0201       | NGB14442                | Wild     | Finland           | N 63° 15' | E 28° 18' | 152                 | −8.58                                  | 0.33             | FS    |
| NOLGÅRDEN NÄS FO0501   | NGB16965                | Wild     | Sweden            | N 58° 5'  | E 13° 42' | 224                 | −9.08                                  | 0.37             | FS    |
| SAUKKAVAARA ME0201     | NGB1139                 | Landrace | Finland           | N 64° 25' | E 28° 11' | 197                 | −9.11                                  | 0.14             | FS    |
| ULTUNA                 | NGB4089                 | Landrace | Sweden            | N 59° 55' | E 17° 29' | 43                  | −9.18                                  | 0.00             | FS    |
| TØEN 09-6-48-1         | NGB1571                 | Wild     | Norway            | N 62° 25' | E 10° 52' | 890                 | −12.15                                 | 0.70             | FT    |
| SÖDRA SUNDERBYN PH0102 | NGB1022                 | Landrace | Sweden            | N 65° 40' | E 21° 52' | 22                  | −12.16                                 | 0.42             | FT    |
| ROLIGHETEN 09-6-54-1   | NGB6736                 | Wild     | Norway            | N 59° 13' | E 9° 40'  | 319                 | −12.33                                 | 0.80             | FT    |
| RAUNAVAARA AK0402      | NGB1011                 | Wild     | Sweden            | N 66° 30' | E 23° 37' | 163                 | −12.42                                 | 0.95             | FT    |
| HYRKÄS ME0101          | NGB1146                 | Landrace | Finland           | N 64° 48' | E 26° 7'  | 65                  | −13.09                                 | 0.61             | FT    |

Note: FI, Finland; FS, freezing susceptible; FT, freezing tolerant; NO, Norway; SE, Sweden.

<sup>a</sup>ID code in NordGen (the Nordic Genetic Resource Centre).

<sup>b</sup>Freezing tolerance as determined by Zanotto et al. (2021).

<sup>c</sup>Winter survival at Jokioinen, Finland, in 2016 as determined by Zanotto et al. (2021).

correlated with the winter survival recorded in a field experiment after a winter characterized by severe temperatures. FT and FS accessions were significantly different in their level of freezing tolerance and winter survival according to analyses of variance (ANOVA) ( $p < 0.05$ ).

Seeds were scarified with sandpaper, and sown in peat soil ("Gartnerjord," pH 5.5–6.5, L.O.G. AS) in September 2019. After germination, seedlings (three per pot) were transplanted into pots (420 cm<sup>3</sup>; 7.5 cm wide, 7.5 cm height) with the same peat soil. The plants were grown in a greenhouse at Ås, Norway (59°40' N, 10°47' E) at 16°C, supplied with light from metal halide lamps (HQI lamps, OSRAM GmbH) providing a photosynthetic photon flux density (PPFD) of approximately 100–125  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in the top of the canopy under a photoperiod of 12 h. When the plants were 8-weeks-old, half of them were moved to cold acclimating conditions in a separate cold room at 3–4°C, 12 h photoperiod, and a PPFD of 110  $\mu\text{mol m}^{-2} \text{s}^{-1}$  from metal halide lamps (HQI lamps, OSRAM) without natural light, for 3 weeks (cold acclimation treatment, hereafter referred to as CA), while the others continued to grow in the same conditions as described above for two more weeks (no acclimation treatment, hereafter referred to as NA). The NA treatment was given for 1 week less in order to compensate for the faster growth experienced by plants in NA conditions. The experiment had a completely randomized design with four replicated blocks that were spatially separated within the greenhouse where plants were grown. Within each block, there were 10 pots per accession with three plants per pot, giving a total of 2400 plants (30 plants  $\times$  10 accessions  $\times$  4 blocks  $\times$  2 treatments).

## 2.2 | Sampling of crown tissue

Plant crowns, a 2-cm transition zone between shoots and roots, were separated from the roots and shoots after washing and flash-frozen in

liquid nitrogen. Crowns of the 30 plants of each accession per treatment were pooled after harvesting, resulting in a total of 80 samples (10 accessions  $\times$  4 blocks  $\times$  2 treatments). Samples were freeze-dried with an LYOQUEST −55 freeze dryer (Telstar) and milled to a fine powder with a Cyclotec 1093 sample mill (Foss A/S) with a 1-mm sieve. The samples were stored at −20°C until the analyses described below.

## 2.3 | Determination of carbohydrate and amino acid content and composition

Soluble sugars and amino acids were extracted from 0.2 g freeze-dried crown tissue per sample with 7 mL of methanol:chloroform:water (MCE, 12:5:3, v:v:v). The extracts were heated for 20 min at 65°C to stop enzymatic activity, cooled on ice, and left overnight at 4°C for optimal extraction. After a 10-min centrifugation at 4°C and 1500 g, 1-mL of the supernatant was collected, and 0.25 mL of water was added to the tubes. After homogenization on a vortex mixer, the samples were left to rest for 10 min and then centrifuged at 13,000 g for 3 min to complete the phase separation. A 0.7-mL subsample of the aqueous phase was collected and evaporated to dryness overnight using a centrifugal evaporator (Savant Speedvac plus SC210A, Thermo Fisher Scientific). Pellets were dissolved in water (0.7 mL), homogenized on a vortex mixer, and centrifuged for 3 min at 13,000 g prior to HPLC analyses.

Soluble sugars were separated and quantified on a Waters HPLC analytical system controlled by the Empower II software (Waters), as described in Bertrand et al. (2020). An HPX-87P column (Bio-Rad) was used for sugar separation at 80°C at a flow rate of 0.5 mL min<sup>−1</sup> with water as the mobile phase and detected on a Refractive Index Detector (Waters, Model 2414). Peak identities and quantities of raffinose, stachyose, sucrose, pinitol, glucose, and fructose were determined by

comparison to standards obtained from the following suppliers: Stachyose (Sigma-Aldrich S4001), Raffinose (Sigma-Aldrich R0250), Sucrose (ACP S-7500), Glucose (Sigma-Aldrich G8270), Pinitol (Sigma-Aldrich 441252), Fructose (Sigma-Aldrich F0127). Sugar standards ranged from 1 to 5  $\mu\text{g mL}^{-1}$ . The quantity of total soluble sugars (Tot.SS) was calculated as the sum of raffinose, stachyose, sucrose, pinitol, glucose, and fructose. Starch was hydrolyzed by adding 3 mL of digestion buffer (200 mM sodium acetate, pH 4.5) with amyloglucosidase (AGS, 15  $\text{U mL}^{-1}$ , Sigma-Aldrich) into the tubes containing the remaining supernatant and pellet after extraction of soluble carbohydrates. Potato starch (Sigma-Aldrich S2630) quantities of 0, 2.5, 5.0, 7.5, and 10 mg were included in the hydrolysis batch to establish the linear regression between the quantity of glucose yielding from starch hydrolysis ( $R^2 = 0.9997$ ). The difference in glucose concentration obtained after and before AGS hydrolysis was fitted into the regression equation to calculate the starch content of each sample expressed on a 105°C DM basis ( $\text{mg g}^{-1}$  DM).

Twenty-one amino acids (alanine, arginine, asparagine, aspartate, glutamate, glutamine, glycine,  $\gamma$ -aminobutyric acid (GABA),  $\alpha$ -aminobutyric acid (AABA), histidine, proline, methionine, lysine, serine, leucine, isoleucine, ornithine, phenylalanine, threonine, tyrosine, and valine) were separated and quantified using Waters ACQUITY UPLC analytical system controlled by the Empower II software (Waters). Amino acid derivatization was performed using AccQ Tag Ultra reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, Cohen, 2003). Derivatives were separated on an AccQ Tag Ultra column ( $2.1 \times 100$  mm; Waters) and detected with Waters ACQUITY Tunable UV detector at 260 nm. Peak identity and amino acid quantity were determined by comparison to a standard mix containing the 21 amino acids with a range of detection from 2.5 to 40  $\text{pmol } \mu\text{L}^{-1}$  and an  $R^2$  of 0.999. The content of total amino acids (Tot.AA) was calculated as the sum of the 21 amino acids.

## 2.4 | Determination of polyphenol content and composition

Red clover crown extracts were prepared using a procedure developed by Mirali et al. (2014) and modified by Elessawy et al. (2020). However, since this is an untargeted study (previous studies were targeted), an important modification was that internal standards were not used. Instead, as is common for untargeted analyses, a quality control (QC) sample, which is a mixture containing an equal portion (15  $\mu\text{L}$ ) of each sample, was used for retention time alignment and to enable relative quantification of the metabolites. A volume of 1 mL of the acetone:water (70:30 v/v) extraction solvent was added to 0.2 g of freeze-dried, powdered crown tissue (per sample), and samples were subsequently shaken for 1 h at 23°C on a Thermo-mixer C (Eppendorf) at a speed of 3.3 g. The samples were centrifuged with a Fresco 21 centrifuge (Thermo Fisher) at a speed of 16,200 g for 15 min, and each supernatant was transferred into a new-labeled tube and centrifuged at 16,200 g for 5 min to ensure removal of all pellet material. A 200  $\mu\text{L}$  aliquot of each extract was

dried with a Speed Vac SPD111V (Thermo Fisher), and the dried samples were kept at  $-20^\circ\text{C}$  until analysis. Blank samples were prepared similarly using empty 2 mL microtubes (Sarstedt). Dried samples were reconstituted in 1000  $\mu\text{L}$  of MilliQ-water:methanol (90:10 v/v) just prior to analysis.

The untargeted workflow used liquid chromatography (LC) and high-resolution mass spectrometry. The LC system was a Dionex 3000 LC equipped with a diode array detector (DAD) that was placed in-line between the column outlet and the inlet to the ion source of the high-resolution Thermo Fisher Q-Exactive mass spectrometer. For LC separation, a Waters Acquity HSST3 column ( $2.1 \times 50$  mm,  $1.8 \mu\text{m}$ ) was used; the temperature of the column chamber was set to  $30^\circ\text{C}$  and the liquid flow rate was  $0.35 \text{ mL min}^{-1}$ . The run time was 35 min with an injection volume of 4  $\mu\text{L}$ , the gradient is given in Table S1. Eluent exiting the column passed through the DAD, which acquired absorbance data from 200 to 600 nm at 5 Hz before being introduced to the HESI (heated ESI) source of the mass spectrometer. The Q-Exactive was used to acquire full scan data for the crown samples using a mass resolution (full width at half maximum, FWHM, @  $m/z$  200) of 140,000 in positive mode and a mass range of 120–1800  $m/z$ . A QC (quality control) sample and four ID (identification samples) were also prepared. The QC sample contained equal amounts of all 78 red clover samples, whereas each ID sample contained equal amounts of samples within the groups NA-FT (ID1), NA-FS (ID2), CA-FT (D3), and CA-FS (ID4). The QC sample was injected every 9–10 runs to account for changes in retention time and/or signal intensity, thereby enabling relative quantification of the polyphenols. The ID samples were used to obtain fragmentation (MS/MS) spectra using the “Full scan/DDMS2” scan function. DDMS2 (data-dependent MS/MS) acquired fragmentation data on the most abundant ions detected in full-scan mode. Mass resolution of the full scan analysis was 140,000 (FWHM @  $m/z$  200) and MS/MS was carried out on the top (most abundant) seven peaks at a resolution of 17,500 (FWHM @  $m/z$  200) from each scan using a stepped collision energy fragmentation (15, 35, and 55 eV). The MS/MS acquisition used an exclusion list ( $m/z$  values) of the most intense ions detected from the blank sample. Additional MS/MS spectra were also acquired using stepped collision energies of 10/15 and 70/90 eV to assist in compound identification.

## 2.5 | Data analyses

The contents of carbohydrates and amino acids were subjected to ANOVA, performed with treatment (CA vs. NA), accession group (FT vs. FS), and the interaction between them as factors in the analyses. The above function of the package Stats (R Core Team, 2021) was used for the analyses after the homogeneity of variance was assessed with Levene's test and the normality of the data with the Shapiro-Wilk test, respectively. A Multi-Factor Analysis (MFA) was performed with the package FactorMineR (Le et al., 2008) to summarize the contribution of the amino acid and carbohydrate contents to the biochemical changes induced by the CA treatment in red clover crowns. This

multivariate analysis allows for simultaneous analysis of quantitative and qualitative variables accounting for a group structure in the data. In this case, the analysis was used to summarize the contribution of the amino acid and carbohydrate contents to the biochemical changes induced by the acclimation treatment in red clover crowns. The contents of amino acids and carbohydrates were used as active quantitative variables and classified into two different groups, while the acclimation treatment and accession groups were used as categorical variables (active and supplementary, respectively). Visualization of the results of the MFA was obtained with the R package *factoextra* (Kassambara & Mundt, 2020).

For the analyses of polyphenols, a customized untargeted workflow was developed by adapting an existing workflow in the Compound Discoverer 3.1 software (Thermo Fisher) to process LC-MS raw data. The workflow is similar to the one reported previously, with some modifications (Elessawy et al., 2021). The Compound Discoverer 3.1 parameters used in generating the analysis are incorporated in a "Summaries" window by the software. Tabs within this summary include "Workflow," "Study," "Grouping & Ratio," and "Filters." These tabs are in text format and the outputs for this study are given in supplementary file. Separate analyses were carried out for comparing FT vs FS and NA vs CA, but only the "Grouping & Ratio" tab changed between these analyses. Thus, in the supplementary information, there are two different summaries for "Grouping & Ratio," one for NA/CA and the other for FT/FS. The Compound Discoverer workflow uses the full-scan accurate mass data to determine the possible molecular formula for each  $m/z$  value and MS/MS spectra from ID spectra to help identify compounds. In addition to using Thermo's *mzCloud* library, which contains fragmentation data of over 19,000 compounds analyzed with Thermo Orbitrap instrumentation ([www.mzcloud.org](http://www.mzcloud.org)), the MS/MS spectra were also compared (using the  $m/z$  vault node) to an in-house library at the Core Mass Spectrometry Facility (UofS). Fragmentation spectra from several other libraries were also used offline, including libraries available in public databases, such as FoodB ([foodb.ca](http://foodb.ca)) and the human metabolome database ([hmdb.ca](http://hmdb.ca)). The primary focus included the differences between the CA and NA treatment and between the FT and FS accession groups. Two samples did not yield enough tissue for all the analyses, therefore, only 78 samples were analyzed for their phenolic profile. The identification levels of the identified polyphenols follow that used in the work by Elessawy et al. (2021), which was derived from Sumner et al. (2007) with the addition of level 2/3 indicating isomeric compounds, including *cis/trans*. For simplicity, we used similar nomenclature for identification as Saviranta et al. (2010a); (2010b), that is, A = aglycone, G = glucoside or galactoside, M = malonate. We did not have authentic standards for any of the compounds and therefore, no compound identities were confirmed, although several assignments are putative or isomeric. Several of the peaks show characteristic losses of  $m/z$  162 (–G), 248 (–G-M), and 334 (–G-M-M) in their MS/MS spectra leaving the aglycone. In many cases, the comparison of fragmentation of the aglycone to MS/MS libraries was pivotal in identification.

### 3 | RESULTS

#### 3.1 | Carbohydrate and amino acid content

The treatment effect (CA vs. NA) accounted for most of the variation (73%) in carbohydrate and amino acid content among samples (Figure 1). The CA treatment clearly affected all the analyzed carbohydrates and amino acids except glucose (Figures 2 and 3 and Tables S2, S3). CA samples had a higher content of most metabolites, and only starch, GABA, and tyrosine were more concentrated in NA samples. Among the soluble carbohydrates, sucrose had the strongest absolute increase (in  $\text{mg g}^{-1}$  DW) in response to CA. However, raffinose showed the greatest relative change, with 12 times as high content in the CA samples as in the NA samples, where this sugar was almost absent. Stachyose remained at a low, undetectable level throughout the experiment. Among the amino acids, proline, arginine, and asparagine had the strongest increases in response to CA and ended up as the most abundant amino acids in CA crowns. Proline and arginine accounted for around 40% of the total amino acid content in the CA crowns, while they represented only 10% of the amino acids in the NA crowns.

The group effect (FT vs. FS) accounted for 15% of the variation in carbohydrate and amino acid content among samples (Figure 1). The FS group had a significantly higher content of starch, Tot.SS, fructose, and glucose than the FS group, while the FT group had a higher content of pinitol and raffinose. Sucrose did not differ significantly between the FT and FS accession groups. The largest differences were seen for fructose, which had an 80% higher concentration in the FS group, and raffinose, which had a 50% higher concentration in the FT group. The effect of the accession group was weaker for the amino acids than for carbohydrates. However, serine, arginine, alanine, GABA, tyrosine, valine, and phenylalanine differed between accession groups, all being significantly more concentrated in the FT than in the FS group. Phenylalanine and tyrosine showed the highest difference of about 40%, while the other amino acids generally differed by about 10%. The interaction effect between the treatment and accession group was significant only for raffinose and fructose among the carbohydrates and for valine among the amino acids. The increase in the content of raffinose and valine after CA was more pronounced in the FT group, with 7% and 13% higher content in the FT compared to the FS, respectively, while that of fructose was 14% higher in the FS group than in the FT group. A summary of the differences in carbohydrate and amino acid contents between the FS and the FT accessions in the NA and CA treatments is shown in Figure 4.

#### 3.2 | Polyphenol content

The phenolic compounds with the highest intensities across samples were identified as pseudobaptigenin hexoside malonate (G-M) (peak 23), formononetin-G-M (peak 24), and maackiain-G-M (peak 26) (Table 2, Figure 5. Supplementary file 2, Figure S2).

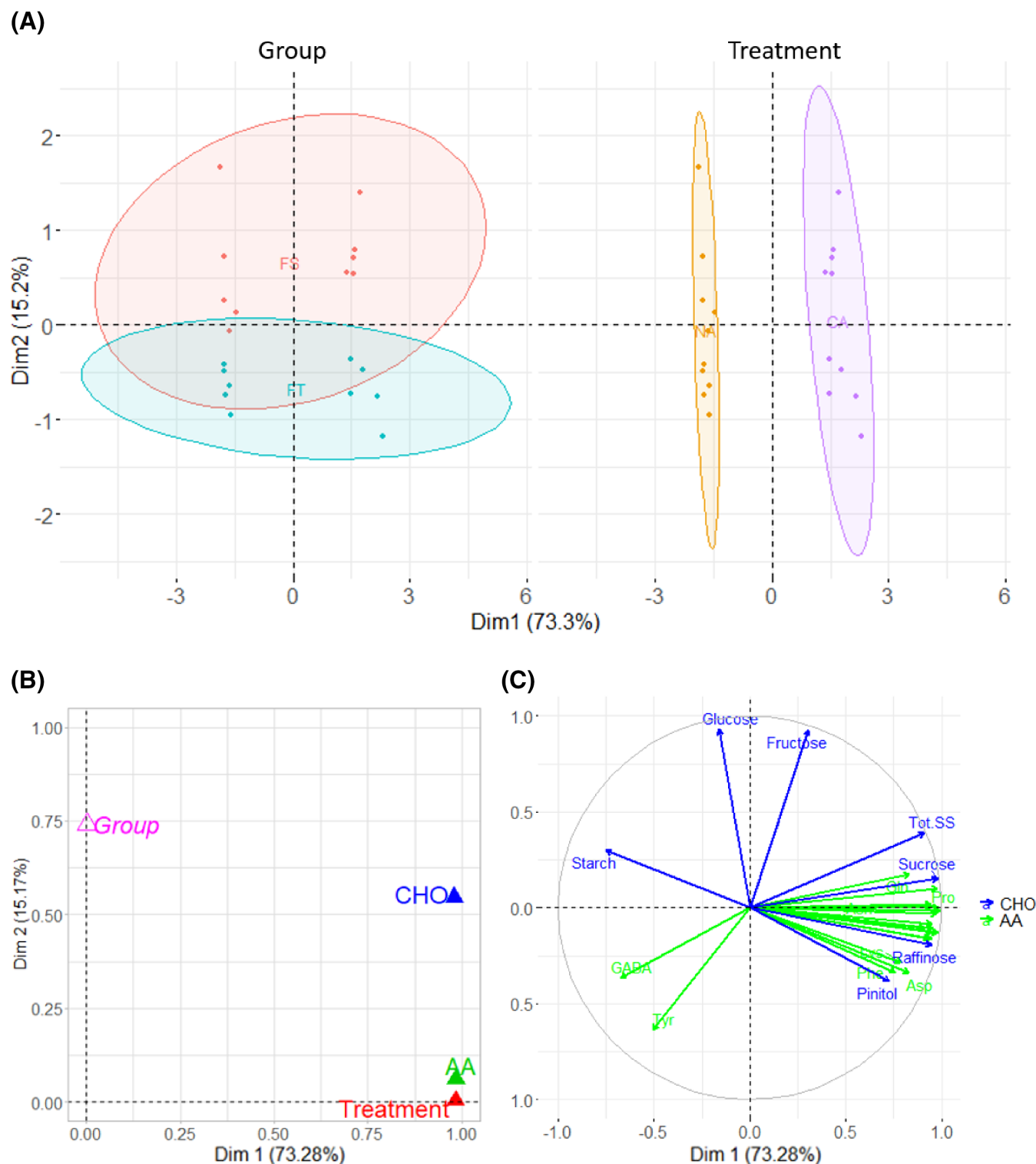


**FIGURE 1** Multi-factor analysis (MFA) of carbohydrate (CHO) and amino acid (AA) content in crowns of five freezing sensitive (FS) and five freezing tolerant (FT) red clover accessions after being either cold-acclimated (CA) or non-acclimated (NA). (A) Representation of the accessions in the space defined by the first two dimensions of the MFA. Ninety-five percent confidence ellipses are shown. (B) Projection of the variable groups in the first two dimensions of the MFA. (C) Correlation of quantitative variables belonging to the CHO and AA variable groups with the first two dimensions of the MFA; only variables significantly correlated ( $p < 0.05$ ) with the first and/or second dimension are shown.

Twelve and four peaks had significantly different intensities (of at least 2X at  $p < 0.05$ ) between the acclimation treatments and the accession groups, respectively (Figures 6, S3, and Table 3). Only three

of these 16 peaks, peak 1 (L- tryptophan), 4 (deoxyclovamide), and 7 (dideoxyclovamide), were among the 31 most intense peaks initially identified (Figure 5); the first was more intense in the CA samples and

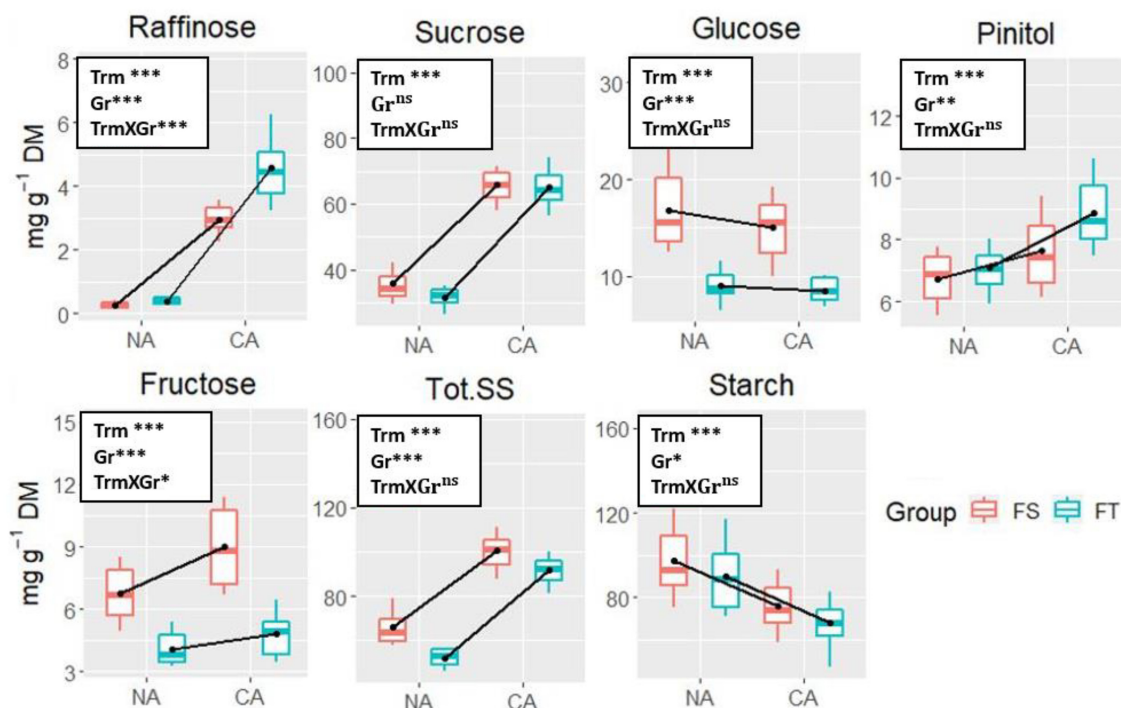


**FIGURE 2** Content of individual and total soluble sugars as well as starch in crowns of five freezing sensitive (FS) and five freezing tolerant (FT) red clover accessions that had been either cold-acclimated (CA) or non-acclimated (NA). Interaction effects are shown by the black lines connecting the means (black dots) of the two treatments within accession group. Results from analysis of variance shown in each panel indicate which factors had a significant effect. Trm; treatment (CA or NA), Gr; accession group (FS or FT), TrmXGr (treatment X accession group interaction, \*\*\*,  $p < 0.001$ , \*\*,  $p < 0.01$ , \*,  $p < 0.05$ ). Note that the scale on the y-axes varies among the different metabolites.

the latter two more intense in the NA samples. The other compounds were detected only at relatively low intensities. An irilone derivative, daidzein, biochanin A isomer, L-phenylalanine, genistein, and N-(phenylacetyl) aspartic acid had higher peak intensities in CA than in NA, while a formononetin derivative, tetrahydro-carboline-3-carboxylic acid, and an irilone derivative had higher intensities in NA than in CA samples. Among the compounds that distinguished the FT from the FS accessions, a pinocembrin-G derivative was found with higher peak intensity in the FT group, while dihydrokaempferol, biochanin A-G-M-M, and a phloretin isomer had higher peak intensities in the FS accessions.

## 4 | DISCUSSION

In agreement with our hypotheses, short-term CA induced a clear response in terms of the content of specific carbohydrates, amino acids, and polyphenols, and this response differed to some degree between accessions with different levels of freezing tolerance.

### 4.1 | Variation in carbohydrate and amino acid content

We observed a sharp increase in soluble sugars, mainly sucrose, after short-term CA. This was likely due to the breakdown of starch, which

declined. Starch is the primary carbohydrate reserve in forage legumes and its breakdown, resulting in the accumulation of soluble sugars, is a well-known mechanism involved in the acquisition of freezing tolerance during CA (Bélanger et al., 2006). In particular, cold stress is known to trigger starch degradation through increased expression of  $\beta$ -amylase (Dong & Beckles, 2019). Since the increase in Tot.SS in  $\text{mg g}^{-1}$  DW was higher than the corresponding decrease in starch, it is likely that during CA, plant crowns function as sinks, accumulating sugars photosynthesized in other tissues, in addition to those arising from starch breakdown. Sucrose accumulation in the CA plants could also be due to reduced catabolism, as emphasized in a proteome analysis of red clover crowns, showing that the abundance of the sucrose-cleaving enzyme sucrose synthase decreased in response to CA (Bertrand et al., 2016). The higher content of starch and Tot.SS in the FS than in the FT accession groups is inconsistent with previous findings by Bertrand et al. (2020), who studied Canadian red clover cultivars after long-term CA under semi-natural conditions. In that study, subpopulations recurrently selected for improved freezing tolerance had higher starch levels in the autumn before CA and accumulated higher total soluble sugar and sucrose levels during CA, than the non-selected cultivars. In line with this, (Svenning et al., 1997) found a correlation between freezing tolerance and the content of both sucrose and Tot.SS within two cultivars. In alfalfa, the dynamic changes of starch and soluble sugar content in response to CA varied among studies and the genetic backgrounds of the material. While Castonguay et al. (1995)

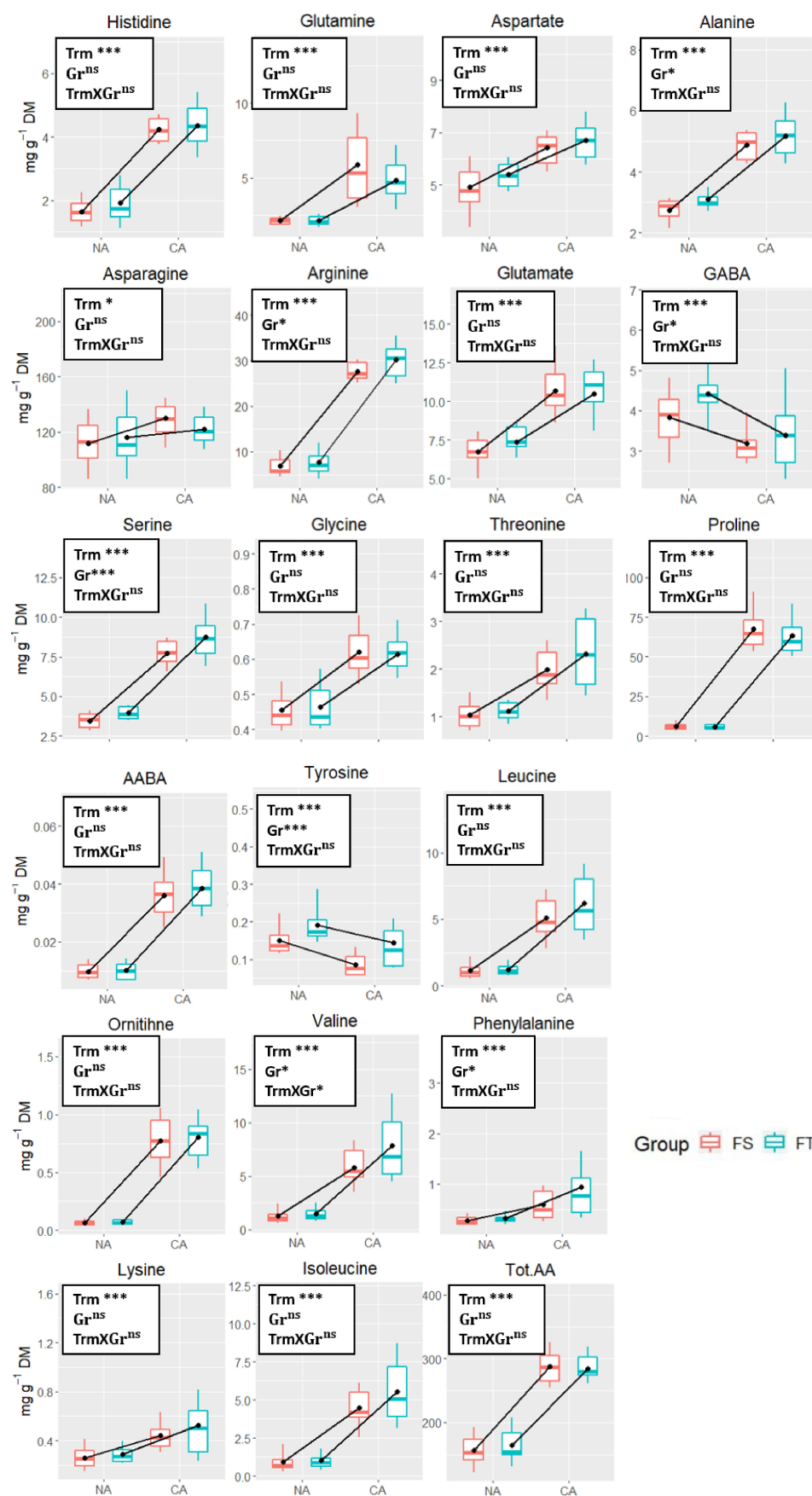


**FIGURE 3** Content of individual and total amino acids in red clover crowns of five freezing sensitive (FS) and five freezing tolerant (FT) red clover accessions that had been either cold-acclimated (CA) or non-acclimated (NA). Interaction effect is also shown by the black lines connecting the means (black dots) of the two treatments within accession group. Results from analysis of variance shown in each panel indicate which factors had a significant effect. Trm; treatment (CA or NA), Gr; accession group (FS or FT), TrmXGr (treatment X accession group interaction), \*\*\*,  $p < 0.001$ , \*\*,  $p < 0.01$ , \*,  $p < 0.05$ . Note that the scale on the y-axes varies among the different metabolites. † A zoom-in of a representative panel with the results of the analyses of variance is provided to help the reader to understand the text and symbols in the smaller panels.

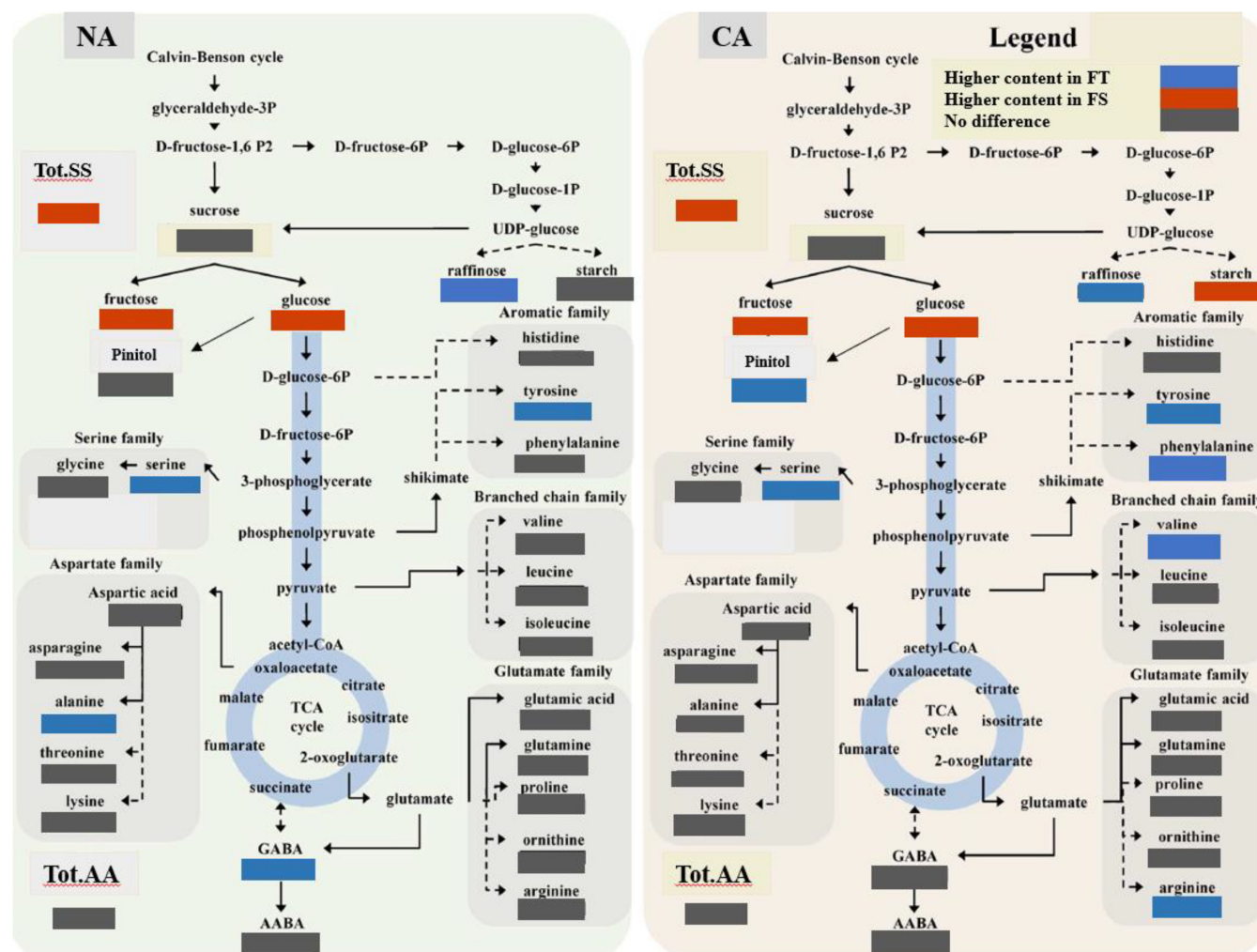


**FIGURE 4** Metabolic pathway maps of differences in carbohydrate and amino acid content between freezing susceptible (FS) and freezing tolerant (FT) accessions which were either non-acclimated (NA) or cold-acclimated (CA) (adapted from Gagné-Bourque et al., 2016). Metabolites are color-coded to indicate differences between FT and FS accessions, according to results of two samples t-test within acclimation treatments (Table S4). Dashed arrows symbolize multistep reactions and solid arrows one-step reactions.

found no association between sucrose accumulation and freezing tolerance of the cultivars, a later study found that two non-selected alfalfa cultivars accumulated less sucrose during CA than populations obtained after several cycles of recurrent selection for the improvement of freezing tolerance, which was indeed quite high (improvement of freezing tolerance of 5°C) (Castonguay et al., 2011). The lack of a significant difference in sucrose content between the FT and FS accession groups in our study may be due to a rather low light intensity during CA, possibly preventing FT accessions from accumulating to the same extent as they might accumulate under more natural light conditions. Nevertheless, the fact that freezing tolerance was determined after CA under similar light conditions (Zanotto et al., 2021), suggests that sucrose is not related to the difference in freezing tolerance after short-term CA that we have observed in the accessions under study. The strong association between freezing susceptibility and a high content of glucose and fructose, suggests that these monosaccharides are not linked with higher levels of freezing tolerance and that they are probably used as building

blocks for the production of other valuable carbohydrates by the FT accessions, of which some are discussed below.

The stronger increase in raffinose and pinitol content during CA in the FT than in the FS group and the sharper increase in FT than in FS (significant for raffinose only), suggest that these carbohydrates play a role in the freezing tolerance of red clover. Bertrand et al. (2020) reported an increased content of pinitol with CA, which was maintained during the whole winter, and proposed that pinitol enhances freezing tolerance in red clover through its osmoprotective function. The trisaccharide raffinose is the smallest molecule of the raffinose family oligosaccharides (RFOs), which are  $\alpha$ -1,6-galactosyl extensions of sucrose (Sengupta et al., 2015), produced by adding galactinol molecules to an initial molecule of sucrose. RFOs are thought to have several functions, such as carbohydrate storage and transport, signaling, and abiotic stress response (Sengupta et al., 2015; van den Ende, 2013; Vinson et al., 2020), including protection against chilling stress (Nishizawa et al., 2008) and freezing (Knaupp

**TABLE 2** Identities of the 31 most abundant polyphenols identified.

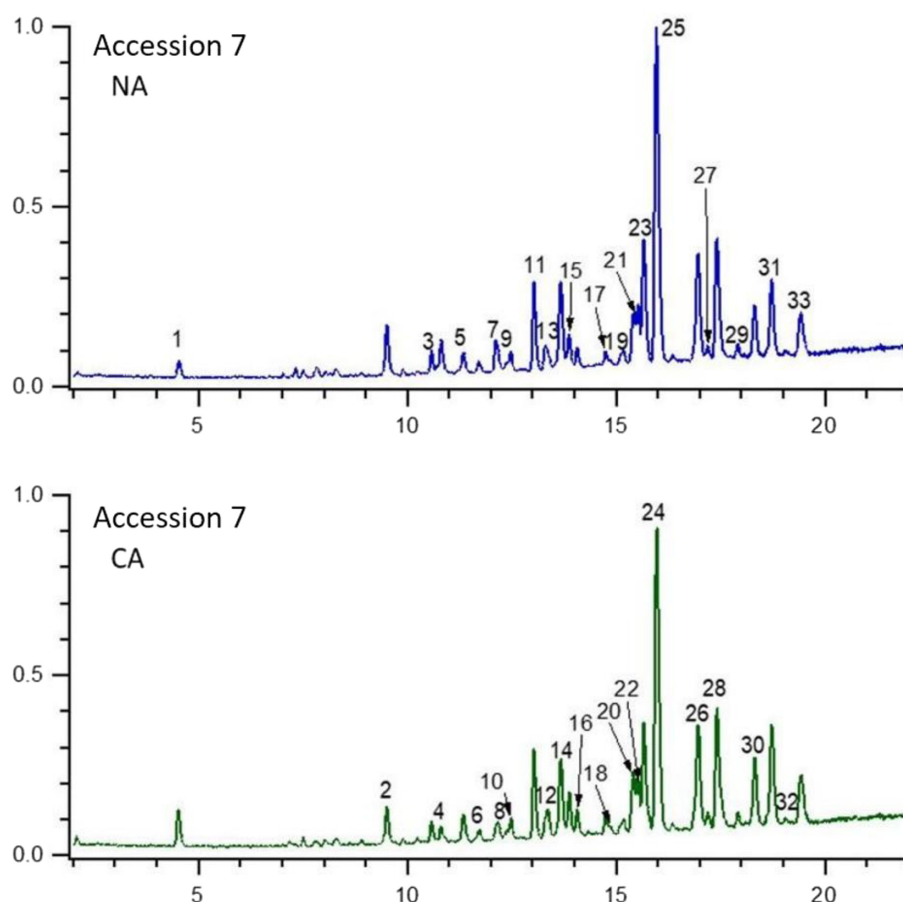
| Peak # | Retention time (min) | Polyphenol <sup>a</sup>                              | Chemical formula                                              | Measured molecular weight (Da) <sup>b</sup> | Mass error (ppm) <sup>c</sup> | $\lambda_{\max}$ (nm) <sup>d</sup> | ID level <sup>e</sup> |
|--------|----------------------|------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------|-------------------------------|------------------------------------|-----------------------|
| 1      | 4.53                 | L-Tryptophan                                         | C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub> | 204.08958                                   | −1.45                         | 280                                | 2                     |
| 2      | 9.54                 | cis-clovamide                                        | C <sub>18</sub> H <sub>17</sub> NO <sub>7</sub>               | 359.09971                                   | −2.21                         | 290,320                            | 2/3                   |
| 3      | 10.63                | Calycosin-G                                          | C <sub>22</sub> H <sub>22</sub> O <sub>10</sub>               | 446.12068                                   | −1.38                         | 250,260,285                        | 2/3                   |
| 4      | 10.82                | Deoxyclovamide (caffeoyl tyrosine)                   | C <sub>18</sub> H <sub>17</sub> NO <sub>6</sub>               | 343.10559                                   | −1.50                         | 295,320                            | 2/3                   |
| 5      | 11.39                | Genistin                                             | C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>               | 432.10510                                   | −1.27                         | 260,318                            | 2                     |
| 6      | 11.75                | Pratensein 7-O- $\beta$ -D-glucoside                 | C <sub>22</sub> H <sub>22</sub> O <sub>11</sub>               | 462.11555                                   | −1.43                         | 263,328                            | 2                     |
| 7      | 12.18                | Dideoxyclovamide                                     | C <sub>18</sub> H <sub>17</sub> NO <sub>5</sub>               | 327.11026                                   | −1.26                         | 299,308                            | 2/3                   |
| 8      | 12.24                | Daidzein G-M                                         | C <sub>24</sub> H <sub>22</sub> O <sub>12</sub>               | 502.11028                                   | −1.68                         | 262,308                            | 2/3                   |
| 9      | 12.41                | Calycosin-G-M                                        | C <sub>25</sub> H <sub>24</sub> O <sub>13</sub>               | 532.12097                                   | −1.35                         | 260,290w                           | 2/3                   |
| 10     | 12.51                | Calycosin-G-M                                        | C <sub>25</sub> H <sub>24</sub> O <sub>13</sub>               | 532.12106                                   | −1.18                         | 260,290w                           | 2/3                   |
| 11     | 13.04                | Calycosin-G-M                                        | C <sub>25</sub> H <sub>24</sub> O <sub>13</sub>               | 532.12104                                   | −1.22                         | 260,286                            | 2/3                   |
| 12     | 13.36                | Pseudobaptigenin-G                                   | C <sub>22</sub> H <sub>20</sub> O <sub>10</sub>               | 444.10518                                   | −1.05                         | 250,262,292                        | 2/3                   |
| 13     | 13.42                | Irilin B/methylorobol-G-M                            | C <sub>25</sub> H <sub>24</sub> O <sub>14</sub>               | 548.11661                                   | −1.21                         | 263,334w                           | 2/3                   |
| 14     | 13.71                | Ononin (formononetin 7-O- $\beta$ -D-glucoside)      | C <sub>22</sub> H <sub>22</sub> O <sub>9</sub>                | 430.12602                                   | −0.85                         | 260,300                            | 2                     |
| 15     | 13.91                | Genistin 6''-O-malonate                              | C <sub>24</sub> H <sub>22</sub> O <sub>13</sub>               | 518.10562                                   | −0.82                         | 260,330w                           | 2                     |
| 16     | 14.09                | Pratensein 7-O- $\beta$ -D-glucoside-6''-O-malonate  | C <sub>25</sub> H <sub>24</sub> O <sub>14</sub>               | 548.11595                                   | −1.19                         | 264,330                            | 2                     |
| 17     | 14.75                | Irilin B/methylorobol-G-M                            | C <sub>25</sub> H <sub>24</sub> O <sub>14</sub>               | 548.11595                                   | −1.20                         | 260,290                            | 2/3                   |
| 18     | 14.90                | Calycosin-G-M-M                                      | C <sub>28</sub> H <sub>26</sub> O <sub>16</sub>               | 618.12108                                   | −1.62                         | 250,285w                           | 2/3                   |
| 19     | 15.22                | Pseudobaptigenin-G-M                                 | C <sub>25</sub> H <sub>22</sub> O <sub>13</sub>               | 530.10559                                   | −0.85                         | 260w,290                           | 2/3                   |
| 20     | 15.41                | Calycosin                                            | C <sub>16</sub> H <sub>12</sub> O <sub>5</sub>                | 284.06817                                   | −1.07                         | a                                  | 2                     |
| 21     | 15.49                | Irilone/afroformosin-G                               | C <sub>22</sub> H <sub>20</sub> O <sub>11</sub>               | 460.10004                                   | −1.13                         | 268,340w                           | 2/3                   |
| 22     | 15.56                | Formononetin-G-M                                     | C <sub>25</sub> H <sub>24</sub> O <sub>12</sub>               | 516.12640                                   | −0.73                         | 260,305                            | 2/3                   |
| 23     | 15.69                | Pseudobaptigenin-G-M                                 | C <sub>25</sub> H <sub>22</sub> O <sub>13</sub>               | 530.10556                                   | −0.91                         | 260,290                            | 2/3                   |
| 24     | 16.01                | Formononetin-G-M                                     | C <sub>25</sub> H <sub>24</sub> O <sub>12</sub>               | 516.12638                                   | −0.78                         | 260,305                            | 2/3                   |
| 25     | 16.98                | Maackiain-G-M + NH <sub>3</sub>                      | C <sub>25</sub> H <sub>27</sub> NO <sub>13</sub>              | 549.14736                                   | −1.60                         | 284,309 s                          | 3                     |
| 26     | 16.98                | Maackiain-G-M                                        | C <sub>25</sub> H <sub>24</sub> O <sub>13</sub>               | 532.12092                                   | −1.45                         | 284,309 s                          | 2/3                   |
| 27     | 17.24                | Irilone/afroformosin-G-M                             | C <sub>25</sub> H <sub>22</sub> O <sub>14</sub>               | 546.10044                                   | −0.95                         | 266,334w                           | 2/3                   |
| 28     | 17.46                | Irilone/afroformosin-G-M                             | C <sub>25</sub> H <sub>22</sub> O <sub>14</sub>               | 546.10044                                   | −0.94                         | 268,338w                           | 2/3                   |
| 29     | 17.95                | Formononetin-G-M-M                                   | C <sub>28</sub> H <sub>26</sub> O <sub>15</sub>               | 602.12636                                   | −1.35                         | 250,300w                           | 2/3                   |
| 30     | 18.35                | Biochanin A 7-O- $\beta$ -D-glucoside-6''-O-malonate | C <sub>25</sub> H <sub>24</sub> O <sub>13</sub>               | 532.12121                                   | −0.91                         | 260,322                            | 2                     |
| 31     | 18.76                | Prunetin-G-M                                         | C <sub>25</sub> H <sub>24</sub> O <sub>13</sub>               | 532.12123                                   | −0.86                         | 260,325w                           | 2/3                   |
| 32     | 19.08                | Pseudobaptigenin                                     | C <sub>16</sub> H <sub>10</sub> O <sub>5</sub>                | 282.05276                                   | −0.21                         | 250,293                            | 2                     |
| 33     | 19.47                | Formononetin                                         | C <sub>16</sub> H <sub>12</sub> O <sub>4</sub>                | 268.07317                                   | −1.45                         | 250,301                            | 2                     |

<sup>a</sup>A = aglycone, G = glucoside or galactoside, M = malonate.<sup>b</sup>Mass of the neutral compound for the most abundant isotope based on the measured accurate mass (Da).<sup>c</sup>Mass error = ((measured accurate mass – calculated exact mass)/(calculated exact mass))  $\times 10^6$  in parts per million (ppm).<sup>d</sup>a = too small to measure accurately, w = weak, s = strong.<sup>e</sup>Identification level as described in Sumner et al., (2007) with the addition of the 2/3 level in Ellessawy et al., (2021).

et al., 2011). Bertrand et al. (2020) reported an increase in raffinose content during the CA of red clover but reported that the positive effect of selection for freezing tolerance on the accumulation of raffinose after CA was only significant in one of the two genetic backgrounds under study. On the other hand, Castonguay et al. (1995) found that differences in the maximum level of freezing tolerance between non-hardy and winter-hardy cultivars of alfalfa were more related to the capacity of the plants to accumulate the RFOs

stachyose and raffinose than sucrose. Bertrand et al. (2016) did not find enzymes specifically involved in the biosynthesis of raffinose to be up- or down-regulated during CA in their proteome analyses of red clover crowns. However, in alfalfa crowns, a several-fold increase in the expression level of galactinol synthase was reported after exposure to low temperatures (Bertrand et al., 2017). Galactinol is involved in the synthesis of raffinose from sucrose (Sengupta et al., 2015). Taken together, these reports hint at a role for RFOs in the freezing

**FIGURE 5** Representative LC–MS trace using accession 7, SÖDRA SUNDERBYN PH0102 (FT), where NA indicates non acclimated, and CA indicates cold acclimated. For clarity, the odd-numbered peaks are labeled in the upper trace and the even-numbered peaks are labeled in the lower trace. The peak numbers correspond to the peak numbers given in Table 1.



tolerance of red clover. However, it seems that this legume species has a limited capacity to accumulate raffinose in some genetic backgrounds, and an inability to accumulate stachyose. This may explain its lower capacity to reach high levels of freezing tolerance as compared to alfalfa.

The increased accumulation of free amino acids during CA is a well-known plant stress response. Arginine, asparagine, and proline, which had the highest increase after CA, were all previously found to be involved in the cold stress response in red clover and alfalfa (Bertrand et al., 2020; Castonguay et al., 2011), while arginine and proline have been shown to be involved in white clover (Svenning et al., 1997). In our study, only arginine among these was more concentrated in the FT than the FS group, suggesting that arginine may play a role in determining the difference in freezing tolerance between the two groups. Arginine is a key storage compound in higher plants and the close relationship between its abundance in taproots of alfalfa during overwintering and shoot regrowth in the spring suggests that this amino acid may play a determinant role as an *N* reserve in perennial plants (Dhont et al., 2003). Arginine is also a precursor of polyamines, which in turn can be catabolized into GABA. An alternative precursor of GABA is glutamate. Both polyamines and GABA are involved in stress response and tolerance in legumes (Menéndez et al., 2019), for example, polyamines are involved in freezing tolerance in *M. falcata* (Zhuo et al., 2018) and drought tolerance in *T. repens* (Li et al., 2018; Yong et al., 2017). The decrease in GABA

concentration in CA relative to NA crowns, in spite of the concomitant increase in the concentration of both glutamate and arginine, in our study, might suggest that the synthesis of other compounds, such as polyamines, was prioritized. However, this is in contrast with the results of Bertrand et al. (2020), who found that GABA was at its highest level in December and, as such, could be involved in acquiring maximum freezing tolerance in red clover. This discrepancy may be due to the different experimental conditions with a short-term acclimation treatment at an above-zero temperature or the different materials used in our study. The higher content of serine, valine, tyrosine, alanine, and phenylalanine in the FT than in the FS group suggests that these are also involved in the differences in freezing tolerance between the two groups. The role of phenylalanine in acquiring freezing tolerance, as a precursor of the phenylpropanoid pathway, is discussed below.

## 4.2 | Variation in polyphenol content

To our knowledge, the current study is the first to investigate the phenolic profile in crowns of red clover, while several other studies focused on roots and aerial tissue (Chiriac et al., 2020; Lin et al., 2000; Saviranta et al., 2008, 2010a, 2010b; Sivesind & Seguin, 2005; Tsao et al., 2006). Crowns are the meristematic tissues providing root and shoot regrowth in the spring and, as such, are vital for the winter

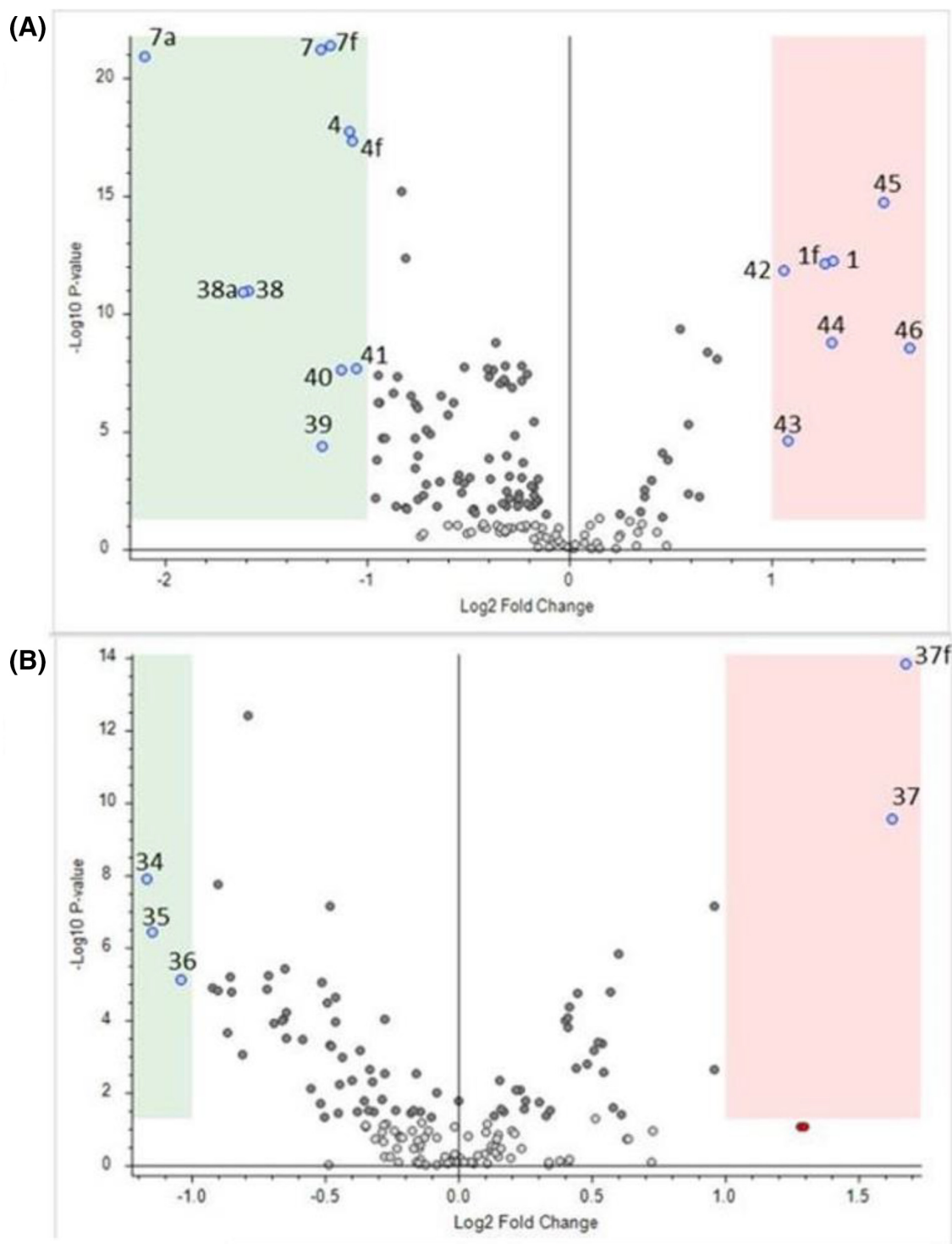


**FIGURE 6** Volcano plots showing phenolic compounds of (A) CA versus NA and (B) FT (freezing tolerant) versus FS (freezing susceptible) accessions. The red and green areas show the compounds that have significantly higher and lower intensities, for the two comparisons (CA vs. NA and FT vs. FS), respectively. The numbers correspond to the assignments in Tables 1 and 2, however, numbers with a suffix are not in the tables as they are redundant. The suffix “a” indicates an adduct ion and the suffix “f” indicates a fragment ion.

survival of red clover. Most of the detected compounds belong to the isoflavones, a class of phenolic compounds particularly abundant in legumes (Mazur et al., 1998). The finding of 31 major phenolic compounds in red clover crowns (for a description of the isoflavone

biosynthetic pathway see Dinkins et al. (2021)) is consistent with previous studies that identified 28 and 31 phenolic compounds in red clover roots and leaves, respectively (Saviranta et al., 2010a, 2010b). The most abundant phenolic compounds that we found, pseudobaptigenin-G-M,

**TABLE 3** Compounds identified as having significantly different intensities in cold-acclimated (CA) versus non-acclimated (NA) red clover crowns or freezing tolerant (FT) versus freezing susceptible (FS) accessions (see Figure 6), listed with increasing log<sub>2</sub> fold change.

| Peak #       | Retention time (min) | Polyphenol <sup>a</sup>                | Chemical formula                                               | Measured molecular weight (Da) <sup>b</sup> | Mass error (ppm) <sup>c</sup> | Log <sub>2</sub> fold change | p-value | ID level <sup>d</sup> |
|--------------|----------------------|----------------------------------------|----------------------------------------------------------------|---------------------------------------------|-------------------------------|------------------------------|---------|-----------------------|
| CA versus NA |                      |                                        |                                                                |                                             |                               |                              |         |                       |
| 38           | 10.60                | Formononetin derivative                | C <sub>31</sub> H <sub>38</sub> N <sub>2</sub> O <sub>10</sub> | 598.25155                                   | −1.83                         | −1.59                        | 1.1e-11 | 3                     |
| 7            | 12.18                | Dideoxyclovamide                       | C <sub>18</sub> H <sub>17</sub> NO <sub>5</sub>                | 327.11026                                   | −1.26                         | −1.23                        | 6.3e-22 | 2/3                   |
| 39           | 6.00                 | Tetrahydro-carboline-3-carboxylic acid | C <sub>12</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>  | 216.08944                                   | −2.03                         | −1.22                        | 4.1e-5  | 2/3                   |
| 40           | 15.29                | Irilone derivative                     | C <sub>29</sub> H <sub>31</sub> NO <sub>12</sub>               | 585.18364                                   | −1.69                         | −1.12                        | 2.5e-8  | 3                     |
| 4            | 10.82                | Deoxyclovamide (caffeoyl tyrosine)     | C <sub>18</sub> H <sub>17</sub> NO <sub>6</sub>                | 343.10559                                   | −1.50                         | −1.09                        | 1.8e-18 | 2/3                   |
| 41           | 15.08                | Irilone derivative                     | C <sub>29</sub> H <sub>31</sub> NO <sub>12</sub>               | 585.18364                                   | −1.69                         | −1.05                        | 2.1e-8  | 3                     |
| 42           | 14.41                | Daidzein                               | C <sub>15</sub> H <sub>10</sub> O <sub>4</sub>                 | 254.05789                                   | −0.07                         | 1.06                         | 1.5e-12 | 2                     |
| 43           | 22.85                | Biochanin A isomer                     | C <sub>16</sub> H <sub>12</sub> O <sub>5</sub>                 | 284.06827                                   | −0.73                         | 1.08                         | 2.4e-5  | 3                     |
| 44           | 2.11                 | L-phenylalanine                        | C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>                 | 165.07881                                   | −1.04                         | 1.30                         | 1.8e-9  | 2                     |
| 1            | 4.55                 | L-tryptophan                           | C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>  | 204.08958                                   | −1.45                         | 1.31                         | 5.5e-13 | 2                     |
| 45           | 17.24                | Genistein                              | C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>                 | 270.05274                                   | −0.30                         | 1.56                         | 1.9e-15 | 2                     |
| 46           | 7.21                 | N-(phenylacetyl)aspartic acid          | C <sub>12</sub> H <sub>13</sub> NO <sub>5</sub>                | 251.07915                                   | −0.87                         | 1.68                         | 2.8e-9  | 2/3                   |
| FT versus FS |                      |                                        |                                                                |                                             |                               |                              |         |                       |
| 34           | 9.47                 | Phloretin isomer                       | C <sub>15</sub> H <sub>14</sub> O <sub>5</sub>                 | 274.08375                                   | −1.35                         | −1.17                        | 1.2e-8  | 3                     |
| 35           | 8.66                 | Dihydrokaempferol                      | C <sub>15</sub> H <sub>12</sub> O <sub>6</sub>                 | 288.06302                                   | −1.27                         | −1.15                        | 3.8e-7  | 2                     |
| 36           | 20.42                | Biochanin A-G-M-M                      | C <sub>28</sub> H <sub>26</sub> O <sub>16</sub>                | 618.12121                                   | −1.42                         | −1.04                        | 7.7e-6  | 2/3                   |
| 37           | 7.88                 | Pinocembrin-G derivative               | C <sub>21</sub> H <sub>27</sub> NO <sub>10</sub>               | 453.16272                                   | −1.71                         | 1.63                         | 3.0e-10 | 3                     |

<sup>a</sup>A = aglycone, G = glucoside or galactoside, M = malonate;

<sup>b</sup>Mass of the neutral compound for the most abundant isotope based on the measured accurate mass (Da).

<sup>c</sup>Mass error = ((measured accurate mass – calculated exact mass)/(calculated exact mass)) × 10<sup>6</sup> in parts per million (ppm).

<sup>d</sup>Identification level as described in Sumner et al., (2007) with the addition of the 2/3 level in Elessawy et al., (2021);

formononetin-G-M, and maackiain-G-M, were also the most abundant compounds found in roots of field-grown red clover by Saviranta et al. (2010a). Apart from this, the roots of field-grown plants, and leaves had a somewhat different profile, with higher levels of formononetin aglycone (A) and biochanin A in roots of pot-grown plants (Saviranta et al., 2010a), and higher levels of biochanin A-G-M and phaseolic acid in leaves (Saviranta et al., 2010b). Lin et al. (2000) found that flowers contained flavones, whereas the leaves had isoflavones as the major flavonoids. Among these, they identified formononetin-G-M, which we also found in red clover crowns. Biochanin A and formononetin were the predominant isoflavones in all cultivars (13) and all plant tissues (leaf, flower, petiole, and stem) considered in the study by Tsao et al. (2006), in addition, pseudobaptigenin was also identified in minor amounts in all plant tissues. Formononetin and biochanin A were also the major isoflavones identified in all plant parts independently of the maturity stage in field-grown red clover (Sivesind & Seguin, 2005). In our study, biochanin A was not among the most abundant compounds, which is somehow in contrast with the above-mentioned studies. However, since none of those considered the crowns for the analyses, it is possible that this compound is accumulated in higher amounts in roots and aerial tissues and to a lower extent in the plant crown. On the other hand, formononetin-G-M was among the three most abundant compounds in our study and thus, it seems to be more evenly accumulated in high amounts in all plant parts.

In general, the higher total number of peaks distinguishing the CA versus NA plants and the fact that some of the major peaks were significantly different between NA and CA, indicate that the CA treatment had a stronger effect than accession group in the accumulation of phenolic compounds in red clover crowns. Among the compounds that increased with CA, we found two aromatic amino acids, L-tryptophan and L-phenylalanine plus a third compound with an amino acid-like structure (N-(phenylacetyl) aspartic acid). Phenylalanine is a precursor of the phenylpropanoid pathway, leading to the production of flavones, isoflavones, anthocyanins, tannins, and lignin, compounds whose synthesis is often affected by both biotic and abiotic stresses (Dinkins et al., 2021; Moura et al., 2010). The three other phenolic compounds showing a significantly higher abundance in CA than NA crowns, the isoflavones daidzein, genistein, and an isomer of biochanin A, are also products of the phenylpropanoid pathway (Dinkins et al., 2021). Indeed, calchone isomerase, an enzyme involved in the synthesis of these isoflavones in the phenylpropanoid pathway, is upregulated by cold (Bertrand et al., 2016). Daidzein, genistein, and biochanin A were all previously identified in red clover roots (Saviranta et al., 2008), and were suggested to play a role in the defense in the rhizosphere (Dinkins et al., 2021). Among the major phenolics in our study were deoxidated forms of clovamide, a phenolic compound from the class of hydroxy-cinnamic acid amides previously found in red clover roots after treatment with jasmonic acid and

that was proposed to be involved in the plant defense system (Tebayashi et al., 2000). In *Arabidopsis thaliana* a decreased biosynthesis of lignin was associated with higher FT, likely because a lower accumulation of lignin in the cell wall results in higher flexibility and better tolerance to damages due to freezing (Ji et al., 2015).

In line with the general trend that we observed, Schulz et al. (2015) found significantly higher amounts of phenolic compounds in the classes of flavonols and anthocyanins in *A. thaliana* leaves after 14 days of CA at 4°C. The response differed between accessions with different genetic backgrounds, which is similar to the situation in red clover grown in a multi-year multi-location field trial (Sivesind & Seguin, 2005). These observations indicate a strong genetic control of the accumulation of phenolic compounds under CA. Furthermore, Schulz et al. (2015) showed that the levels of only a few phenolic compounds were significantly correlated to freezing tolerance. A subsequent study (Schulz et al., 2016) employing plants mutated in different genes in the phenolic compound biosynthesis pathway, suggested a redundancy effect of different phenolics on the level of freezing tolerance. These authors also suggested that a combined contribution of a set of different phenolic compounds is more likely than the specific action of a particular compound. Furthermore, Saviranta et al. (2010a) found only a minor effect of ozone stress in the accumulation of single phenolic compounds in red clover roots. Similar results were reported in a study on red clover leaves (Saviranta et al., 2010b), where ozone stress significantly increased the total phenolic content, while single compounds were only marginally affected. Furthermore, Saviranta et al. (2008) showed that the concentration of four different isoflavones in red clover was tissue-specific and that these differences also depended on the developmental stage, something which was also confirmed in a later study (Saviranta et al., 2010b).

## 5 | CONCLUSIONS

Overall, this work constitutes a comprehensive study of the biochemical changes during CA and their possible role in freezing tolerance in red clover accessions with a Nordic origin. The decrease of starch and the accumulation of soluble sugars and free amino acids were the most evident responses to cold. Raffinose, pinitol, arginine, phenylalanine, alanine, valine, and serine had higher concentration in CA than in NA crowns and at the same time, they had higher concentrations in FT than in FS accessions, suggesting a role for these metabolites in the freezing tolerance of Nordic red clover. This study also provides the first description of the phenolic profile of red clover crowns before and after CA. Specific phenolic compounds significantly changed in concentration in response to CA and others were differentially accumulated in FT versus FS accessions, suggesting a role of these secondary metabolites in cold stress response and freezing tolerance of red clover.

## AUTHOR CONTRIBUTIONS

Stefano Zanotto conceived the idea for this study. Stefano Zanotto, Åshild Ergon, and Annick Bertrand planned the experiment. Stefano

Zanotto conducted the growth experiment, extracted phenolic compounds, and prepared samples for carbohydrate extraction and amino acid extraction. Annick Bertrand conducted analysis of carbohydrate and amino acid content. Fatma M. Elessawy and Randy W. Purves conducted the analysis of phenolic compounds. Stefano Zanotto and Randy W. Purve produced figures and tables. Stefano Zanotto wrote the paper under supervision and co-writing by Åshild Ergon and Jorunn E. Olsen. Annick Bertrand and Randy W. Purve contributed to the writing of the paper. All authors read and approved the final version.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NMBU Open Research Data at <https://doi.org/10.18710/3OTFVO>.

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## SUPPORTING INFORMATION

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