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ORIGINAL RESEARCH

Physiologia Plantarum

Different metabolic adaptation strategies after overwintering in *Eutrema* sp. and *Arabidopsis* accessions under field conditions in Germany

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Abstract

Successful overwintering is a prerequisite for high fitness in temperate perennials and winter annuals and is highly dependent on increased freezing tolerance and timely balancing of deacclimation with growth resumption in spring. To assess fitness costs associated with overwintering and elucidate metabolic mechanisms underlying winter survival and the switch from acclimated freezing tolerance to growth resumption, we performed a comparative field study using 14 *Eutrema salsugineum* accessions, *E. halophilum*, *E. botschantzevii* and 11 *Arabidopsis thaliana* accessions differing in freezing tolerance. Winter survival and reproductive fitness parameters were recorded and correlated with phenological stage and metabolite status during growth resumption in spring. The results revealed considerable intraspecific variation in winter survival, but survival rates of the extremophyte *Eutrema* were not inherently better. In both *Eutrema* and *A. thaliana*, improved winter survival was associated with reduced reproductive fitness. Metabolic analysis by GC–MS revealed intrinsic differences in the primary metabolism of the two genera during deacclimation. *Eutrema* contained higher levels of several amino and chlorogenic acids, while *Arabidopsis* had higher levels of several sugars and sugar conjugates. In both genera, increased levels of several soluble sugars were associated with increased winter survival, whereas *myo*-inositol has different roles in overwintering of *Eutrema* and *A. thaliana*. In addition, differences in amino acid metabolism and polyhydroxy acids levels after winter survival were found. The results provide strong evidence for a trade-off between increased winter survival and reproductive fitness in both *Eutrema* and *Arabidopsis* and document inherent differences in their metabolic strategies to survive winter.

1 | INTRODUCTION

Successful overwintering is a prerequisite for high fitness in temperate perennial plants and winter annuals. Plant winter survival is affected by a multitude of factors associated with genotype, plant vigour and the occurrence of biotic and abiotic stress factors (Bergjord Olsen et al., 2018; Wagner et al., 2021). Low temperatures represent a crucial abiotic factor determining winter survival as well as the geographical distribution, growth and reproductive fitness of plants (Boinot et al., 2022; Kreyling et al., 2015; Li et al., 2015; Weiser, 1970). To overcome the constraints of low temperatures, plants native to temperate climates show natural low-temperature acclimation during

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autumn in preparation for winter frost. This process is termed cold acclimation. Maximum freezing tolerance is reached midwinter. Upon exposure to warmer temperatures in spring, plants lose the freezing tolerance acquired during acclimation by deacclimation while they resume growth and development (Xin & Browse, 2000). Cold acclimated freezing tolerance has been shown to vary greatly among genotypes with different geographical origins (Bahrani et al., 2021; Hannah et al., 2006; Zuther et al., 2012;), and freezing tolerance is a highly adaptive trait contributing to geographic differentiation, especially in colder climates (Ågren & Schemske, 2012). However, increased freezing tolerance and winter survival may entail a fitness cost for the plants. A recent study monitoring fitness parameters and winter survival in field experiments or freezing tolerance in controlled chamber experiments of 11 *Arabidopsis thaliana* accessions found lower fitness, expressed as 1000-seed mass, in more freezing-tolerant accessions than freezing-sensitive accessions (Boinot et al., 2022).

While climate warming reduces the occurrence of frost events and the date of the last spring frost is shifting earlier in many regions (IPCC 2021), the warming-induced advancement of the growing season of plants in the Northern Hemisphere may induce more frequent frost days during deacclimation, when organs and tissues are becoming increasingly vulnerable to freezing temperatures (Liu et al., 2018; Zohner et al., 2020). Hence, balancing the deacclimation rate in spring with the initiation of growth and development is likely becoming increasingly important for winter survival and fitness (Zuther et al., 2015). In addition, as the climate warms, the frequence and severity of unseasonable warm spells in late winter and spring might increase, leading to repeated deacclimation and acclimation cycles and impaired winter survival (Pagter et al., 2023; Pagter & Arora, 2013; Vitasse et al., 2014; Vyse et al., 2019).

Plants in natural environments are exposed to a greater range of day lengths and greater variation in temperature, humidity and light quality across all timescales than those typically encountered under controlled conditions (Bergelson & Roux, 2010). Consequently, phenotypes observed in controlled environments do not necessarily correlate to the phenotypes observed under natural conditions with regard to fitness assessments (Köhl & Laitinen, 2015) and the leaf metabolome observed under recurring environmental conditions in climate chambers may be remarkably different from the metabolome observed under natural conditions (Annunziata et al., 2018; Buckley et al., 2019). Also, phenotypic analyses under natural conditions reveal strong genotype–environment interactions for numerous traits (Alonso-Blanco et al., 2021). Field or common garden experiments are therefore highly needed in order to capture metabolic responses that are important under ecologically realistic conditions.

The low-temperature acclimation response is a multigenic, quantitative trait involving massive re-programming of the metabolome (see Fürtauer et al., 2019; Guy et al., 2007; Hincha et al., 2012 for reviews). Similarly, deacclimation involves extensive metabolic regulation of the developmental switch, resulting in reduced freezing tolerance and the resumption of growth (Pagter et al., 2023; Pagter et al., 2017; Rathore et al., 2021; Vyse et al., 2019). In particular, carbohydrate metabolism plays an exceptionally important role in overwintering and growth resumption of perennials and winter annuals (Pagter et al., 2017; Rathore et al., 2021). The two processes additionally involve changes in the metabolism of amino acids, storage lipids and cell wall components (Kjær et al., 2018; Pagter et al., 2017).

Arabidopsis thaliana is widely distributed in the Northern Hemisphere, and several studies have shown significant natural variation in the response of *Arabidopsis* accessions to low temperatures (Hannah et al., 2006; Korn et al., 2008; Zuther et al., 2012). In addition, specific deacclimation patterns were shown for 12 *Arabidopsis* accessions under controlled conditions (Juszczak et al., 2016; Zuther et al., 2015). For 11 *Arabidopsis* accessions, winter survival was strongly correlated with their respective freezing tolerance after cold acclimation (Boinot et al., 2022), indicating high importance of cold acclimation capacity for winter survival of *A. thaliana*. *Eutrema* (tribe *Eutremeae*) is an extremophyte emerging as a model plant for stress resistance (Bressan et al., 2001). It is a close relative to *Arabidopsis* but with a closer relation to agronomically important *Brassica* species (Bailey et al., 2006; Wong et al., 2005). Although some accessions of *Eutrema* are not extremophile with regard to freezing tolerance, others show significantly higher freezing tolerance than any *Arabidopsis* accession under controlled conditions (Lee et al., 2012). Additionally, *E. salsugineum* shows a remarkable long-term acclimation capacity, with a significant increase in freezing tolerance after three weeks of cold acclimation compared to two weeks. Such an increase was not observed in *A. thaliana* (Khanal et al., 2015). Metabolite data indicate different metabolic adaptation strategies to freezing between *Eutrema* and *Arabidopsis* (Lee et al., 2012). However, an understanding of metabolic mechanisms underlying deacclimation and growth resumption of *Eutrema* is completely lacking. Differences between the model species *A. thaliana*, with limited stress tolerance traits, and its more stress tolerant relative *Eutrema* provide possibilities for the discovery of novel metabolic mechanisms underlying winter survival and the switch from acclimated freezing tolerance to growth resumption in the spring (Zuther et al., 2018).

Thus far, most studies investigating metabolism during deacclimation and growth resumption used individual genotypes, and results could therefore not be correlated quantitatively with differences in phenology or winter survival. Here, we performed a comparative study using 14 *E. salsugineum* accessions, the two closely related species *E. halophilum* and *E. botschantzevii* and 11 accessions of *A. thaliana* for a complete analysis of phenotypic variation in winter survival, developmental stage, reproductive fitness and metabolite status, and a correlation analysis of differences at the metabolic and whole-plant level. We hypothesize that *Eutrema* accessions generally have a higher winter survival rate than *A. thaliana* accessions but that improved winter survival entails a fitness cost in both species. We further expect that the two species share some general metabolomic responses to successfully survive winter, but also accumulate divergent metabolites that may partly explain differences in winter survival and/or spring phenology. In addition to interspecific variation in metabolic profiles, it is hypothesized that there is intraspecific variation in the regulation of primary metabolism during deacclimation and growth resumption between accessions originating from different climates.

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2 | MATERIALS AND METHODS

2.1 | Plant material and experimental set-up

The experiment included *E. halophilum*, *E. botschantzevii* and 14 natural accessions of *E. salsugineum*, spanning a large geographical range from the USA and Canada through China and Russia, and 11 natural accessions of *Arabidopsis thaliana* originating from Canada, Cape Verde, Portugal, Spain, Poland, Russia or India. The accessions of both *Eutrema* and *A. thaliana* have previously been shown to vary in cold-acclimated freezing tolerance, ranging from very tolerant to very sensitive (Lee et al. 2012; Zuther et al. 2012). Seeds of the *Eutrema salsugineum* (Pall.) Al-Shehbaz & Warwick, accessions Colorado, Cracker Creek, Dillibrough, Hebei, Henan, Jiangsu, Shandong, Xinjiang and Yukon were kindly provided in 2011 by Prof. Ray A. Bressan (Purdue University, West Lafayette, IN). Seeds of further *E. salsugineum* accessions (Altai 1, Altai 2, Buriatia, Tuva and Yakutsk), *E. halophilum* (C.A.Mey.) Al-Shehbaz & Warwick (Bayanual) and *E. botschantzevii* (D.A. German), Al-Shehbaz & Warwick (Saratov) were collected in Russia and Kazakhstan and kindly provided in 2011 by Alexei Babakov (All-Russia Research Institute of Agricultural Biotechnology RAAS, Moscow, Russia). The geographical origins (latitude and longitude) of the *E. salsugineum* accessions, *E. halophilum* and *E. botschantzevii* and mean minimum habitat temperature recorded during the coldest month of the growing season at the climate station nearest to the collection site were previously published by Lee et al. (2012). Despite being different species, *E. halophilum* and *E. botschantzevii* will hereafter be referred to as accessions along with the *E. salsugineum* accessions. Seeds of the *A. thaliana* accessions were originally obtained from Nottingham Arabidopsis Stock Center (NASC; University of Nottingham, Loughborough, UK). The geographical origins (latitude and longitude) of the *A. thaliana* accessions were previously published by Zuther et al. (2012). Seeds of both *Eutrema* and *A. thaliana* were further propagated at the Max Planck Institute of Molecular Plant Physiology.

For the experiment, 100 seeds per accession were sown on October 25, 2013 in plastic boxes with holes (50 x 40 x 15 cm; W x L x H) with sandy substrate "Haufen B" supplemented with 1 g Osmocote Start (ICL Group Ltd.)/1 L substrate that was sterilized prior to use. Five replicate boxes were prepared for each accession. Two weeks after sowing on November 7, 2013, boxes were transferred from the polytunnel to the field in Potsdam - Golm (52° 24′ N 13° 04′ E) following a random design and were aligned with a water spirit level to avoid uneven distribution of rain (Köhl & Laitinen, 2015). Plants were grown under natural weather conditions except for some additional watering when necessary. Weather conditions during the experimental period (November 7, 2013 to May 12, 2014) were previously described in detail (Boinot et al., 2022). In brief, the mean air temperature 5 cm above ground ± SE was 0.6 ± 0.3°C, while the absolute minimum temperature was −16.5°C. The number of days with snow cover was 17. In May of the following year, boxes were transferred back to the polytunnel and inflorescences bagged in groups for seed harvest. After 2 months, ripe seeds were collected and weighed for determination of total seed yield per plant.

2.2 | Visual phenotyping

On March 28, 2014 field grown plants were visually scored for absolute number (winter survival) and on April 4 for phenological developmental stage. The developmental stage was determined according to the BBCH scale (Meier 1997) adopted for *A. thaliana* (Schwachtje et al., 2011). For *Arabidopsis*, the results of the visual phenotyping have previously been published by Boinot et al. (2022). The non-numeric BBCH scores for each species were ranked in Excel with the function Rank.avg and further statistical analysis was done in RStudio.

2.3 | Seed harvest and weighing

After harvesting, seeds were weighed to obtain the total seed yield per box for each accession. Seed mass per plant was obtained by dividing the total seed mass by the number of survivors per box. For *A. thaliana*, 1000 seeds of each accession and replicate were counted at the Institute of Applied Genetics at the Free University of Berlin with an Elmor C3 seed-counting machine (Elmor AH). 1000-seed mass was weighed with a Sartorius LE244S precision balance (240 g x 0.0001 g). For *Arabidopsis*, the results concerning seed yield per plant and 1000-seed mass have previously been published by Boinot et al. (2022).

2.4 | GC–MS metabolite profiling

Pools of one to nine plants from five boxes per accession were harvested for metabolite profiling on April 3 between 12:30 and 13:30. Polar metabolites were extracted from 80 mg (fresh weight) with a tolerance of ∼5–10% and processed as described previously (Erban et al. 2020) by dual, sequential injections using splitless-mode and split-modes at a split flow ratio of at least 1:30 optimized to the linear quantification range of abundant metabolites. Gas chromatography coupled to electron impact ionization-time of flight-mass spectrometry (GC/EI-TOF-MS) was performed and metabolites were identified as described previously (Erban et al. 2020). Metabolite intensities were normalized to sample fresh weight and the internal standard $^{13}C_6$-sorbitol. In total, 129 primary metabolites were annotated, including known and yet unknown compounds that are archived by the Golm Metabolome Database, http://gmd.mpimp-golm.mpg.de/ (Kopka et al. 2005). Acidic metabolites were named as 'acids'. We consider this naming as synonymous to their respective metabolic anion, according to the naming and list of synonyms used by the KEGG and BioCyc databases. Metabolites with more than 50% missing values over all samples within a species and known contaminations, annotated reagents and internal standards were removed from further analysis, resulting in 102 and 101 metabolites for *Eutrema* and *A. thaliana*, respectively. Missing values of the remaining metabolites were imputed by half of the minimum value per metabolite over all samples within a species. A complete list of normalized metabolite levels can be found in Table S1.

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2.5 | Statistical data analysis and visualization

Data analysis and figure generation were performed in R v4.1.0 and RStudio. To examine whether winter survival and seed yield per plant differed between *Eutrema* and *A. thaliana* and between accessions within a species data were analyzed using two-way ANOVA in R. Heterogeneities of variance were tested using Bartlett's test and differences between individual means were identified using Tukey's Studentized Range Test at the 5% significance level. A non-parametric Kruskall–Wallis test was also used to test differences in winter survival between *Eutrema* and *A. thaliana* and between accessions. This test was done to supplement the results from the ANOVA, as the assumption concerning homogeneity of variance was not fulfilled.

Outliers in the metabolite dataset were identified using the Rosner Test (rosnerTest) included in R-package EnvStats (Millard, 2014) and replaced by 'NA', if values much higher or lower than the rest of the data were not related to genotype. In *E. salsugineum*, the compound classified 'similar to Glycerolaldopyranosid' was identified as an outlier in four out of five samples of Altai 2 and all replicate samples of Saratov. However, the metabolite remained in the dataset, as this was interpreted as a genotype-specific high concentration. Melibiose and putrescine were identified as outliers in two and three samples of Saratov and Bayanaul, respectively. The concentrations of these metabolites were, however, also high in the other replicates of Saratov and Bayanaul, and therefore remained in the dataset. In *A. thaliana*, xylose and ribonic acid were identified as outliers in four out of five samples of N13, but both metabolites remained in the dataset as these were interpreted as genotype-specific high concentrations. Normalized metabolite levels were compared between *Eutrema* and *A. thaliana* using a *t*-test in R with correction for multiple testing using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). Metabolic differences between *Eutrema* and *A. thaliana* were also investigated by Principal Components Analysis (PCA) applied to the normalized metabolite levels using the R-package pcaMethods (Stacklies et al., 2007). The PCA indicated different metabolic compositions of the two species and the metabolite data were subsequently analyzed separately for *Eutrema* and *A. thaliana*. For statistical analysis only, the remaining metabolite intensities were divided by the median intensity across all measurements and resulting fold changes were $\log_2$-transformed to approximate normal distribution, i.e. $\log_2$-median transformation. To detect metabolites whose levels differed significantly between accessions, the $\log_2$-transformed metabolites levels were analyzed using a one-way ANOVA with correction for multiple testing using the Benjamini-Hochberg method.

Spearman rank order correlation was performed with $\log_2$-median transformed data and the survival percentage, ranked BBCH scores or total seed yield per plant utilizing the function rcorr in the Hmisc package and a significance threshold of $P < 0.05$ following correction for multiple testing using the Benjamini-Hochberg method. Heatmaps were generated using the package pheatmap (https://cran.r-project.org/package=pheatmap). Heatmap rows and columns were hierarchically clustered using the pheatmap function and an Euclidian distance measure.

3 | RESULTS

3.1 | Winter survival and phenological development

Winter survival, analyzed in late March after the cold in winter, did not vary significantly between *Eutrema* and *A. thaliana*, indicating that winter survival of *Eutrema* is not consistently greater than that of *A. thaliana*. In contrast, winter survival varied strongly between accessions ($P = 6.029e\text{-}11$). The average winter survival of *Eutrema* accessions ranged from 13% (Xinjiang) to 80% (*E. botschantzevii* Saratov) (Figure 1A). In *A. thaliana*, the average winter survival rate varied less, ranging from 33% (Sah-0 and C24) to 61% (N13) (Figure 1B). The winter survival of the *A. thaliana* accessions in the current field trial has previously been shown to correlate with the cold-acclimated

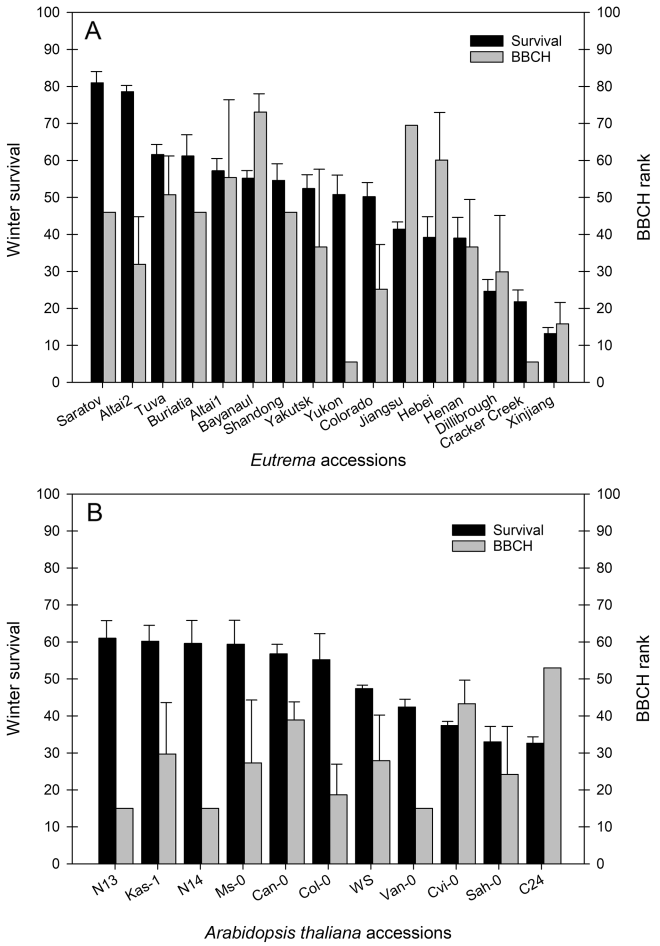

FIGURE 1 Winter survival (%) and the developmental stage of plants in 16 *Eutrema* accessions (A) and 11 *Arabidopsis thaliana* accessions (B) under field conditions. Winter survival and developmental stages were assessed in early April and developmental stages are expressed as ranked BBCH indexes. Bars represent the average of five biological replicates ± SE. Accessions are ordered from the highest (Saratov/N13) to the lowest (Xinjiang/C24) average winter survival rate. For *Arabidopsis*, winter survival and ranked BBCH indexes have previously been published by Boinot et al. (2012).

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freezing tolerance (LT_{50}ACC) determined for plants grown under controlled conditions (Boinot et al. 2022). An analogous correlation analysis for *Eutrema* between the winter survival of the accessions in the current field trial and cold-acclimated freezing tolerance (LT_{50}ACC) previously determined for plants grown under controlled conditions (Lee et al. 2012) confirmed that cold acclimation capacity is also of high importance for field winter survival of *Eutrema* (Figure 2A).

The plants' phenological development was recorded in early April, concurrent with sampling for metabolite profiling, using the BBCH scale for plant development. In *Arabidopsis*, some accessions are rapid-cycling summer annuals, whereas others are vernalization responsive and thus behave as winter annuals (Michaels et al., 2003). In the current study, all *Arabidopsis* accessions performed as winter annuals, which germinate in autumn, overwinter and then bolt and flower the following spring. Compared to *A. thaliana*, a longer vernalization period is required for flowering in *Eutrema* (Batelli et al., 2014). Accordingly, the *Eutrema* accessions in the current study also performed as winter annuals. Spring phenology was advanced in *A. thaliana* compared to *Eutrema*. Among the *Eutrema* accessions, *E. halophilum* Bayanaul had reached BBCH growth stage 58 (first flower petals visible), while the two latest developing accessions Cracker Creek and Yukon had reached BBCH stage 50 (vegetative plant parts have reached final size). In *A. thaliana*, average BBCH growth stage ranged from 63 (30% of flowers open) for N13, N14 and Van-0 to 73 (30% of fruits have reached final size) for C24. For the *Eutrema* accessions, the ranked BBCH index (Figure 1A) was weakly positively correlated with winter survival ($r_s = 0.31$, $P = 0.009$, Table 1). For the *A. thaliana* accessions, no significant correlation was found between the ranked BBCH index (Figure 1B) and winter survival ($r_s = -0.22$, $P = 0.18$, Table 2).

3.2 | Reproductive output

The average seed yield per plant differed significantly between the two species (P < 2e-16), with the seed yield per plant over all accessions being on average six times greater in *A. thaliana* than in *Eutrema*. The average seed yield per plant also differed between accessions within a species ($P = 4.55e-11$ for *Eutrema* and $P = 0.00608$ for *A. thaliana*). In *Eutrema*, it ranged from 0.01147 g per plant for *E. halophilum* Bayanaul to 0.0558 g per plant for Xinjiang, while in *A. thaliana* it ranged from 0.1184 g per plant for Cvi-0 to 0.3033 g per plant for Van-0. To investigate if increased winter survival entailed a fitness cost for the plants, correlation analyses of the seed yield per plant, and for *A. thaliana* also 1000-seed mass, with the winter survival rate of the respective accessions were done. In *Eutrema*, a significant negative correlation between seed yield per plant and winter survival was obtained (Figure 2B), representing a lower fitness for accessions with higher winter survival. No significant correlation was found between winter survival and seed yield per plant in *A. thaliana* ($r_s = -0.28$, $P = 0.08$, Figure S1A); however, winter survival was negatively correlated with the 1000-seed mass ($r_s = -0.54$, $P = 0.0001$, Figure S1B), revealing lower seed mass with increasing winter survival. In addition, 1000-seed mass was positively correlated with the BBCH index ($r_s = 0.45$, $P = 0.0024$, Table 2), indicating higher seed mass with earlier phenological development.

According to Boinot et al. (2022), there was no correlation between the seed yield of the *A. thaliana* accessions in the current field trial and their acclimated freezing tolerance (LT_{50}ACC), whereas the 1000-seed mass was positively correlated with LT_{50}ACC, revealing higher seed mass for accessions with lower freezing tolerance. In comparison, we found a significant positive correlation between the seed yield of the *Eutrema* accessions in the current field trial and LT_{50}ACC previously determined for plants grown under controlled

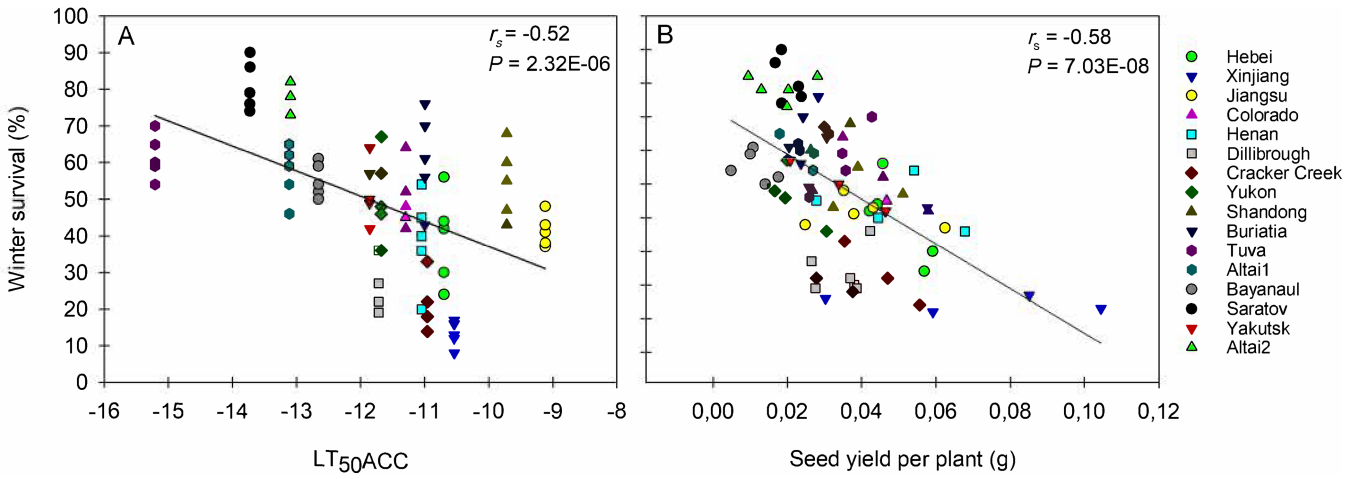

FIGURE 2 Correlation analysis of the relation between cold-acclimated freezing tolerance (LT_{50}ACC) and winter survival (A) or seed yield per plant and winter survival (B) of 16 accessions of *Eutrema* under field conditions. LT_{50}ACC was previously determined by Lee et al. 2012 (Figure 1 in Lee et al.), while winter survival and seed yield were scored using plants grown in the field until bagging of inflorescences. Data points represent five biological replicates for each accession (differently coloured, n = 80). The Spearman rank order correlation coefficients (r_s) and *p* values are indicated.

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TABLE 1 Correlation analysis between winter survival, seed yield per plant or ranked BBCH scores and metabolites that showed significantly different levels between 16 *Eutrema* accessions. Plants were grown in the field from sowing in October until May of the following year. The numbers indicate Spearman rank order correlation coefficients. From top to bottom, metabolites are ordered from the highest positive correlation coefficient between winter survival rates and metabolite levels to the highest negative correlation coefficient. Significant correlations following correction for multiple testing are indicated.

Parameter	Cluster	Survival	Seed yield	BBCH
Survival		NA	-0.5855***	0.3082**
Seed yield		-0.5855***	NA	-0.1487
BBCH		0.3082**	-0.1487	NA
NA213001	III	0.7664***	-0.4793***	0.4083***
similar to glycerolaldopyranosid	II	0.7447***	-0.4459***	0.1568
Glucose	II	0.6682***	-0.3162**	0.4558***
NA211001	III	0.6626***	-0.3938***	0.4138***
Inositol, *myo*-	II	0.6604***	-0.2080	0.2544*
A250001-101	III	0.6568***	-0.3744**	0.4643***
Sucrose	II	0.6558***	-0.2024	0.3321**
A214004-101	III	0.6346***	-0.3800**	0.3659**
Phosphate	III	0.6257***	-0.3879**	0.1965
A214003-101	III	0.6205***	-0.3146**	0.3795**
A185003-101	II	0.6160***	-0.2731*	0.4554***
Glycerophosphoglycerol	III	0.5910***	-0.1734	0.4142***
Raffinose	II	0.5875***	-0.1927	0.1808
Galactose	II	0.5862***	-0.2318	0.2394*
myo-Inositol-1-phosphate	III	0.5588***	-0.2361	0.3720**
A311002-101	II	0.5432***	-0.1500	0.2419*
A196004-101	II	0.5382***	-0.3325**	0.2969*
A217004-101	III	0.5310***	-0.1458	0.3355**
Fructose	II	0.5126***	-0.1721	0.5429***
Glucose-6-phosphate	III	0.4988***	-0.2450*	0.2765*
Glutaric acid, 2-hydroxy-	II	0.4952***	-0.2077	0.3541**
Trehalose, alpha,alpha'-	II	0.4873***	-0.1024	0.2437*
Isoleucine	III	0.4848***	-0.1534	0.2690*
Maltose	III	0.4819***	-0.1439	0.2203
Ribose	III	0.3890***	-0.2820*	0.3331**
NA176001	III	0.3656	-0.0518	0.3461**
Dehydroascorbic acid dimer	III	0.3469	-0.1506	0.2775*
Valine	I	0.2919*	-0.4023***	0.1805
Ribitol	I	0.2623*	-0.1947	-0.0950
A228001-101	IV	0.1521	-0.1055	0.2791*
Citric acid	I	0.0785	-0.0841	-0.2651*
Erythronic acid	IV	0.0135	0.1940	0.2098
Threonic acid	IV	-0.0064	0.2872*	0.2135
NA298001	IV	-0.0290	0.2382*	0.1629
Glutaric acid, 2-oxo-	IV	-0.0981	0.0544	0.0462
Quinic acid, 3-caffeoyl-, trans-	IV	-0.1976	0.2853*	0.1624
A304001-101	V	-0.3967***	0.2735*	-0.1044
Glutamic acid	V	-0.4130***	0.0980	-0.0949
Serine	V	-0.4165***	0.0440	-0.3271**
Glyceric acid	V	-0.4273***	0.1468	0.0261

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TABLE 1 (Continued)

Parameter	Cluster	Survival	Seed yield	BBCH
Succinic acid	V	-0.4327***	0.2648*	-0.1863
Aspartic acid	V	-0.4376***	0.2121	-0.2662*
Pyroglutamic acid	V	-0.4486***	0.0775	-0.2544*
A240004-101	IV	-0.4595***	0.3150**	0.0443
Quinic acid	IV	-0.5414***	0.4000***	0.0182
Alanine	V	-0.5791***	0.1080	-0.1790
Glycine	V	-0.6588***	0.2463*	-0.3517**

*$P \leq 0.05$,
**$P \leq 0.01$,
***$P \leq 0.001$. The numerical *P*-values are listed in Table S4. The 'Cluster' column indicates which of the five clusters shown in Figure 5 the metabolites belong to.

conditions (Lee et al., 2012) ($r_s = 0.43$, $P = 0.00015$, data not shown), representing a higher seed yield for accessions with lower freezing tolerance (higher LT_{50}ACC).

3.3 | Metabolite profiling

The measurement of the relative metabolite levels in the close-to-simultaneously sampled (within a 1-h-period) *Eutrema* accessions and *A. thaliana* accessions showed substantial differences between the two species, as shown by PCA (Figure S2). The normalized metabolite levels in *Eutrema* and *A. thaliana*, averaged across all accessions, are compared in Figure 3. The relative levels of 67 out of 94 common metabolites differed between the two species. *Eutrema* had significantly higher levels of 2-methylmalic acid, quinic acid and several amino acids, including alanine, glutamic acid, glycine, proline, pyroglutamic acid, threonine, valine, β-alanine and O-acetylserine (OAS) and its derivative N-acetylserine. The latter three non-proteinogenic amino acids were not detected in *A. thaliana* under field sampling conditions. *Eutrema* also contained putrescine, trans-ferulic acid and the chlorogenic acids 3-*O*-caffeoylquinic acid (3-CQA) and 5-*O*-caffeoylquinic acid (5-CQA), which were not detected in *A. thaliana*. Another observation was the significantly higher levels of several phosphates in *Eutrema*, including glyceric acid-3-phosphate, myo-inositol-1-phosphate, phosphate and phosphate acid monomethyl ester. *Arabidopsis*, on the other hand, had significantly higher levels of fumaric acid, threonic acid-1,-4-lactone, arabitol and the sugar and sugar conjugates galactinol, galactose, glucose, maltose, melibiose, raffinose and trehalose. Threonic acid-1,4-lactone, arabitol and galactinol were not detected in *Eutrema*. Levels of several unknown compounds also varied significantly between *Eutrema* and *A. thaliana* accessions (unknown compounds shown in Figures 5 and 6).

In order to detect metabolites or pathways of importance for winter survival and/or growth resumption within *A. thaliana*, with limited stress tolerance traits, and its more stress-tolerant relative *Eutrema*, we then analyzed the metabolite data for each species separately. According to PCA, winter survival was the dominant source of variance underlying PC1 for both *Eutrema* and *A. thaliana*, explaining 37.0% and 29.2% of the variance for *Eutrema* and *A. thaliana*, respectively (Figure 4). For *Eutrema*, the profiles of accessions with lowest winter survival (Cracker Creek, Dillibrough and Xinjiang) and accessions with highest winter survival (*E. botschantzevii* Saratov and Altai2) were distributed furthest apart, while profiles of accessions with intermediate winter survival were located in between. PC2 explained an additional 22.9% variability and tended to separate the species *E. botschantzevii* Saratov, *E. halophila* Bayanaul and *E. salsugineum* Altai2 with high winter survival from the other *E. salsugineum* accessions (Figure 4A). For *A. thaliana*, PC1 separated the profiles of C24, Sah-0 and Cvi-0 with low winter survival (33–37%) from profiles of seven accessions with intermediate-high winter survival (42–61%); the profile of Can-0, having an early phenological development and a survival rate of 57%, was located in between (Figure 4B). Neither PC2 nor PC3, which explained 14.9% and 13.8% of the total variance, respectively, yielded any clear separation with respect to winter survival, phenological development or seed yield.

A total of 47 metabolites in *Eutrema* showed significantly different pool sizes between accessions. In accordance with the PCA, hierarchical clustering of these metabolites separated the accessions into four clusters according to winter survival, highlighting the importance of metabolic reprogramming in the winter survival of *Eutrema* (Figure 5). Cluster A included *E. botschantzevii* Saratov and Altai2 with the highest winter survival rates (79–81%). Cluster B contained accessions with high-to-intermediate winter survival rates (50–62%, Altai1, Buriatia, Tuva, Colorado, Shandong). Cluster C contained accessions with intermediate-to-low survival rates (39–55%, *E. halophilum* Bayanaul, Henan, Hebei, Jiangsu, Yakutsk, Yukon), while cluster D contained Xinjiang, Cracker Creek and Dillibrough with the lowest winter survival rates (13–24%). Clustering further grouped the metabolites into five clusters of varying sizes, corresponding to different metabolite levels (Table S2). (I) Metabolites with quite varied accumulation patterns. This cluster was small, containing only valine, citric acid and ribitol. (II) and (III) Metabolites decreasing in abundance with decreasing winter survival. Cluster II contained six sugars, four unknown compounds, one acid and one polyol. Cluster III was dominated by four phosphates and 6 unknown compounds. Pool sizes of metabolites in (IV) were highest in accessions with intermediate-high winter survival and

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TABLE 2 Correlation analysis between winter survival, seed yield per plant, ranked BBCH scores or 1000-seed mass and metabolites that showed significantly different levels between 11 *A. thaliana* accessions. Plants were grown in the field from sowing in October until May of the following year. The numbers indicate Spearman rank order correlation coefficients. From top to bottom, metabolites are ordered from the highest positive correlation coefficient between winter survival rates and metabolite levels to the highest negative correlation coefficient. Significant correlations following correction for multiple testing are indicated.

Parameter	Cluster	Survival	Seed yield	BBCH	1000 seed mass
Survival		1.0000	-0.2848	-0.2239	-0.5442***
Seed yield		-0.2848	1.0000	-0.1087	-0.0259
BBCH		-0.2239	-0.1087	1.0000	0.4507**
1000 seed mass		-0.5442***	-0.0259	0.4507**	1.0000
Galactose	II	0.5260***	-0.2953	-0.4279**	-0.5240***
Fructose	II	0.4763**	-0.3248*	-0.2890	-0.4099**
Glucose	II	0.4653**	-0.1675	-0.2790	-0.4179**
Maltose	I	0.4592**	-0.4473**	-0.2008	-0.2730
Galactinol	IV	0.4446**	-0.2831	-0.5757***	-0.4514**
Raffinose	II	0.4404**	-0.2474	-0.5383***	-0.4670**
Melibiose	II	0.4366**	-0.1038	-0.4906***	-0.5053***
Sucrose	II	0.4348**	-0.3971*	-0.2231	-0.3531*
Ascorbic acid	II	0.4215**	-0.0147	-0.3359*	-0.4716**
similar to Glycerolaldopyranosid	II	0.4097**	-0.4293**	-0.2902	-0.3596*
Xylose	IV	0.3989**	-0.3395*	-0.4790**	-0.6126***
Threonic acid	II	0.3857*	0.0081	-0.2936	-0.3725*
A185003-101	II	0.3694*	-0.2784	-0.2977	-0.3645*
NA213001	II	0.3592*	-0.1750	-0.2623	-0.2686
Erythronic acid	II	0.3580*	0.0351	-0.3833*	-0.5719***
NA298001	IV	0.3556*	-0.2178	-0.5128***	-0.3556*
Galactonic acid	II	0.3277*	-0.3270*	-0.3529	-0.3370*
A214004-101	IV	0.3118	-0.3934*	-0.1661	-0.3730*
Arabinose	II	0.2935	-0.1877	-0.4809**	-0.3905*
A228001-101	II	0.2932	-0.3230	-0.3365	-0.2656
Glucose-1-phosphate	III	0.2806	-0.4071**	-0.2875	-0.1168
A311002-101	IV	0.2738	-0.2312	-0.4958***	-0.4832**
Glutaric acid, 2-hydroxy-	I	0.2563	-0.2474	-0.0484	-0.1410
A250001-101	IV	0.2456	0.0790	-0.2155	-0.5916***
Trehalose, alpha,alpha'-	I	0.2267	-0.2307	-0.3041	-0.0956
Glucose-6-phosphate	I	0.1971	-0.4764**	-0.2483	-0.0496
Fructose-6-phosphate	I	0.1757	-0.4822**	-0.1705	-0.0807
A251003-101	IV	0.1698	0.2298	-0.4227**	-0.4425**
A203003-101	I	0.1510	0.0585	0.0195	-0.0057
A236005-101	III	0.1322	-0.3355*	-0.2934	-0.2884
A148006-101	II	0.1252	0.0669	-0.3983*	-0.1106
Ribonic acid	IV	0.1181	-0.2701	-0.2466	-0.3346*
Pyroglutamic acid	III	0.1133	-0.0990	-0.2691	0.0507
A199004-101	III	0.0903	-0.6079***	-0.2777	0.0647
A300001-101	III	0.0875	-0.2179	-0.3872*	-0.1096
Ethanolaminephosphate	III	0.0493	-0.1560	-0.1154	0.0611
Gluconic acid	III	0.0276	-0.4044**	-0.2500	0.2245
A240004-101	III	-0.0006	-0.3436*	-0.1668	-0.0972
Citric acid	IV	-0.0396	-0.0762	0.0035	0.0061

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TABLE 2 (Continued)

Parameter	Cluster	Survival	Seed yield	BBCH	1000 seed mass
A304001-101	III	-0.0602	-0.3573*	-0.2177	0.1357
NA176001	IV	-0.0750	0.0612	-0.2268	-0.0280
D155405	III	-0.0878	-0.1865	-0.2578	0.0824
Glutamic acid	III	-0.1104	-0.3244*	-0.2063	0.1385
Valine	III	-0.1370	-0.2008	0.0754	0.4291**
Glycerophosphoglycerol	III	-0.1507	-0.2302	-0.3371*	0.0821
Threonine	IV	-0.1649	0.2194	-0.1775	0.1450
A175008-101	IV	-0.1897	-0.0668	0.1736	0.0544
Malic acid	IV	-0.2188	0.3433*	0.4404**	0.1073
Inositol, *myo*-	IV	-0.2330	-0.0493	-0.1964	0.1671
Serine	IV	-0.2693	0.0573	0.0560	0.0978
Fumaric acid	IV	-0.3517*	0.1054	0.0574	0.1099
Succinic acid	IV	-0.3875*	0.1317	-0.0037	0.2257

*$P \leq 0.05$,
**$P \leq 0.01$,
***$P \leq 0.001$. The numerical *P*-values are listed in Table S5. The 'Cluster' column indicates which of the four clusters shown in Figure 6 the metabolites belong to.

lowest in the accessions with lowest and highest winter survival. This cluster included two polyhydroxy acids, two acids, one phenylpropanoid and three unknown compounds. Metabolites in cluster (V) were found in the highest amounts in accessions with the lowest winter survival and in the lowest amounts in the accessions with the highest winter survival. This cluster was dominated by six amino acids.

To identify individual metabolites that may contribute to winter survival, growth resumption in spring or reproductive fitness of *Eutrema*, we correlated metabolite levels with winter survival, ranked BBCH index and seed yield per plant. All metabolites in clusters II and III showed moderate or strong positive correlations with the winter survival of all replicates of the 16 *Eutrema* accessions (Table 1). Most metabolites in clusters II and III were additionally weakly or moderately positively significantly correlated with the ranked BBCH index. In contrast, levels of all metabolites in cluster V were negatively significantly correlated with the winter survival of field grown *Eutrema* accessions, and four of them were also negatively, although weakly, correlated with the ranked BBCH index. Few correlations were found between levels of metabolites in clusters I or IV and winter survival or ranked BBCH index. Seed yield of the *Eutrema* accessions was significantly positively correlated with levels of five and three metabolites in clusters IV and V, respectively, and significantly negatively correlated with altogether 12 metabolites in clusters II or III. However, most correlations between individual metabolites and seed yield were weak (r_s <0.4).

In *A. thaliana*, a total of 52 metabolites showed significantly different pool sizes between accessions. Between the accessions, it was clear that C24, with low winter survival and advanced phenological development, was most unlike the other 10 accessions as it formed its own cluster following hierarchical clustering of significant metabolites (Figure 6 cluster A). Cluster B corresponded to N13, Van-0, Col-0 and N14, which were characterized by intermediate winter survival (42–61%) and less advanced phenological development (BBCH 63) than the four accessions in cluster C (Ms-0, Kas-1, WS, Can-0), which also had intermediate winter survival rates (47–60%) but more advanced spring phenology (BBCH 65–66). Cluster D consisted of Sah-0 and Cvi-0 characterized by low winter survival rates (33–37%). Clustering of the metabolites revealed four major metabolite accumulation patterns (Table S3). The major clusters included (I) metabolites whose pool sizes were highest in cluster C accessions with intermediate winter survival rates and relatively advanced phenological development. This cluster included two phosphates, two sugars, one unknown compound and one acid. (II) Metabolites found in the lowest amounts in the accessions with lowest winter survival. This cluster was dominated by seven sugars and four polyhydroxy acids. Metabolites in cluster (III) were found in the highest pool sizes in Sah-0 and Cvi-0 and the lowest pool sizes in C24, despite all three accessions being characterized by low winter survival rates. This cluster contained three phosphates, three amino acids, one polyhydroxy acid and six unknown compounds. (IV) Metabolites present in the highest amounts in C24 and accessions with intermediate-high winter survival and relatively late phenological development (cluster B). This was a diverse cluster including, among others, four acids and two amino acids.

The potential significance of the metabolites in winter survival, phenological development and reproductive output of *A. thaliana* were explored by correlation analysis (Table 2). In cluster I, the levels of fructose-6-phosphate, glucose-6-phosphate and maltose were significantly negatively correlated with seed yield, and maltose was additionally positively correlated with winter survival. None of the metabolites in cluster I were correlated with the ranked BBCH index. Except for arabinose and one unknown compound, levels of all metabolites in cluster II were positively significantly correlated with the winter survival of all replicates of the 11 *A. thaliana* accessions. Most of these metabolites were additionally negatively significantly correlated with the 1000-seed mass and ranked BBCH index and less frequently with seed yield per plant.

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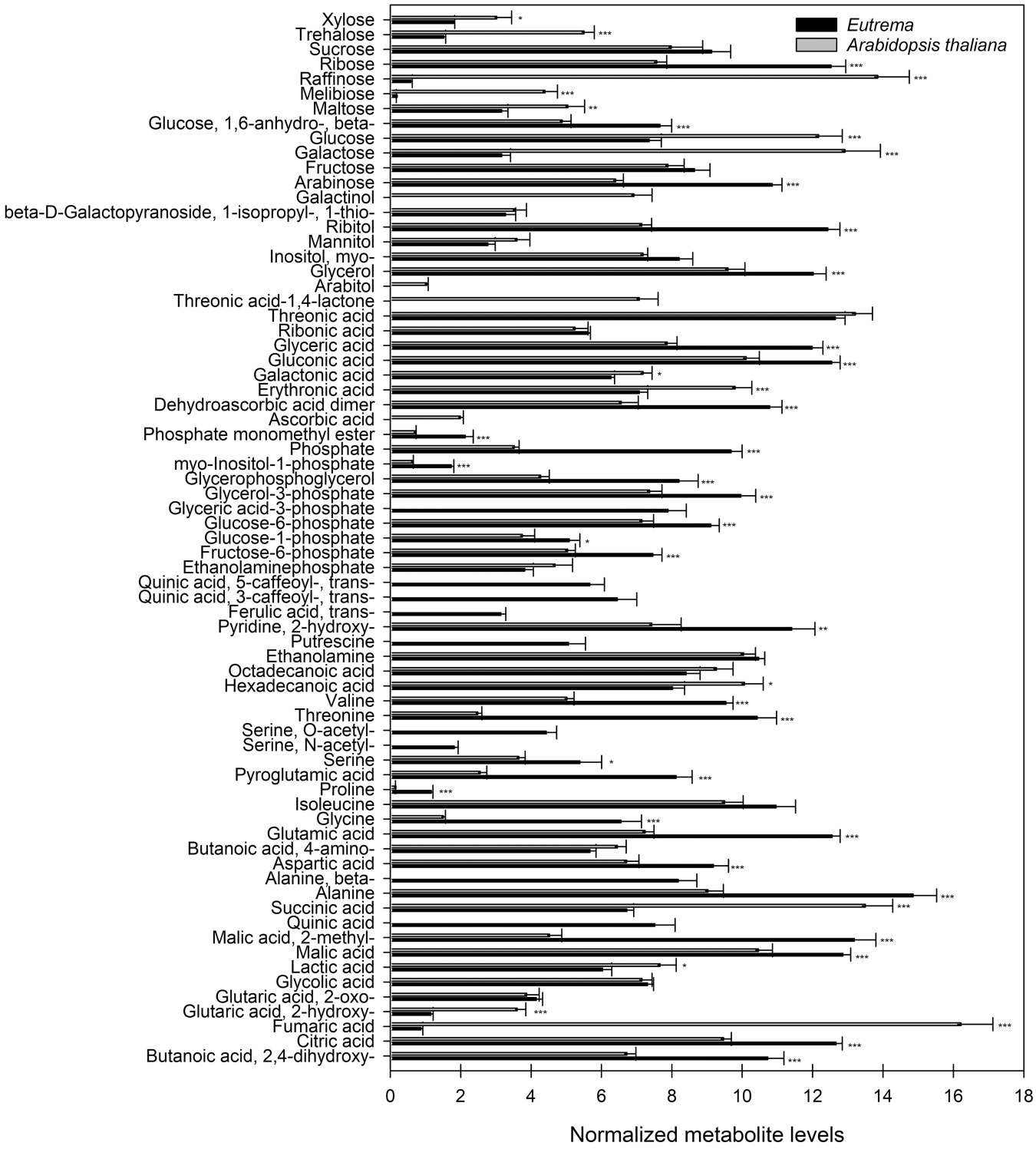

FIGURE 3 Comparison of levels of identified metabolites averaged across 16 accessions of *Eutrema* (grey) and 11 accessions of *A. thaliana* (black). Plants were grown in the field from sowing in October and until sampling in early April the following year. Normalized metabolite levels are presented as mean ± SE of n = 80 for *Eutrema* and n = 55 for *A. thaliana*. Metabolite levels that are significantly different between *Eutrema* and *A. thaliana* according to a *t*-test at FDR $P < 0.05$ are indicated. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

Metabolites in cluster III appeared functionally most related to seed yield as seven metabolites, including four unknows, were negatively significantly correlated with seed yield per plant. Only a few significant correlations were found between winter survival or ranked BBCH index and metabolites in cluster III. In cluster IV, five metabolites showed a significant negative correlation with the ranked BBCH index. Few significant but generally weak correlations were found between metabolites in cluster IV and winter survival or seed yield per plant.

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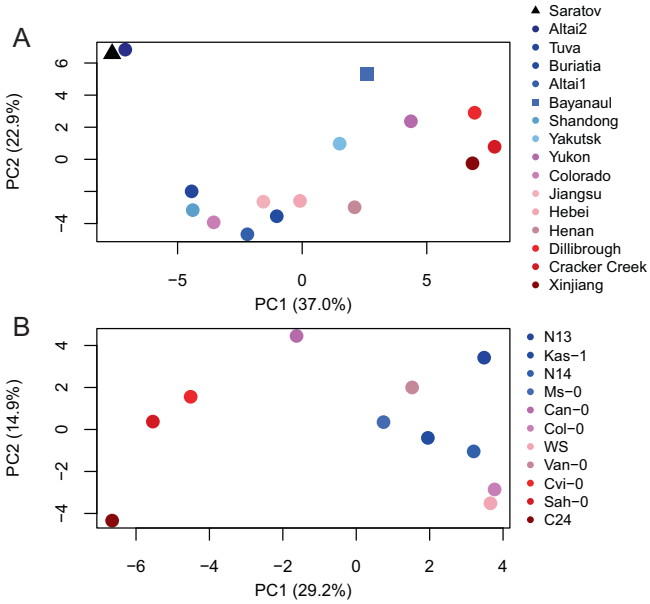

FIGURE 4 Principal component analyses (PCAs) for $\log_2$-median transformed metabolite abundances of leaf rosettes from 16 *Eutrema* accessions (A) and 11 *Arabidopsis thaliana* accessions (B) grown in the field from October until sampling in early April the following year. Each color-coded data point represents the mean of five biological replicates of each accession. The first and second components are plotted, comprising 59.9% (top) and 44.1% (bottom) of the total variance of each data sub-set, respectively. Accessions are ordered from the highest (Saratov/N13) to the lowest (Xinjiang/C24) average winter survival rate. On the top figure, *E. botschantzevii* Saratov is indicated by a triangle, *E. halophilum* Bayanual is indicated by a square and the 14 *E. salsugineum* accessions are indicated by circles.

4 | DISCUSSION

4.1 | Natural variation in winter survival and phenological development of *Eutrema* and *A. thaliana* accessions

Our survey of 16 and 11 geographically diverse *Eutrema* and *A. thaliana* accessions identified considerable natural inter- and intra-specific variation in winter survival. Winter survival varied more among *Eutrema* than *A. thaliana* accessions, with some of the *Eutrema* accessions having higher (Saratov, Altai2) or lower (Xinjiang, Cracker Creek and Dillibrough) average winter survival than any of the investigated *A. thaliana* accessions. Low winter survival rates of some of the *Eutrema* accessions disagree with our first hypothesis as the *Eutrema* accessions have previously been shown to have overlapping or greater cold acclimated freezing tolerance than 54 *A. thaliana* accessions when cold acclimated for two weeks under controlled conditions (Lee et al., 2012), and *Eutrema* remarkably outperforms *Arabidopsis* in long-term (three weeks) acclimation capacity (Khanal et al., 2015). Nonetheless, it has previously been shown that cold acclimation capacity is of high importance for successful overwintering of *Arabidopsis* (Boinot et al., 2022). Following that, we could show that the winter survival of the *Eutrema* accessions was strongly correlated with their respective cold acclimated freezing tolerance, determined as LT_{50} in electrolyte leakage assays under controlled conditions (Lee et al., 2012). Hence, cold acclimation capacity is also very important for winter survival of *Eutrema*. Large variation in winter survival rates highlights the importance of other factors than acclimated freezing tolerance for successful winter survival. Other factors influencing overwintering of winter annuals include, for instance, deacclimation resistance in response to intermittent warm periods, snow cover depth and duration and soil water content (Bergjord Olsen et al., 2018; Rapacz et al., 2017). Variation in winter survival rates may additionally be due to accessions being adapted to their local environment, resulting in a fitness trade-off when grown in a different environment (Oakley et al., 2014). Nevertheless, all the *Eutrema* accessions were from rather distant origins, and in *A. thaliana*, accessions from more distant origins (N13, Kas-1) showed similar or higher winter survival than e.g. Col-0 from Poland.

The timing of flowering affects the environmental conditions in which fertilization and seed maturation occur. Consequently, flowering time influences fecundity rate and plant fitness. Flowering time is controlled by various environmental signals experienced at both the seed and rosette stages (Debieu et al., 2013). Advanced spring phenology in *A. thaliana* compared to *Eutrema* is in accordance with previous observations of later flowering in *Eutrema* than in *Arabidopsis* (Amtmann, 2009) and may be due to a greater vernalization requirement of *Eutrema* (Guo et al., 2012). Natural variation in bolting and flowering time in *A. thaliana* is well documented and likely reflects adaptation to different environmental conditions depending on geographical site of origin. In some studies, latitudinal clines in flowering time across accessions have been demonstrated (Stinchcombe et al., 2004; Shindo et al., 2006). In accordance, we found a significant negative relationship between latitude of origin and BBCH index recorded in early April ($r_s = -0.66$, $P = 0.026$, data not shown), as determined by Spearman rank order correlation, indicating that accessions from southern latitudes showed more advanced spring phenology than accessions from northern latitudes. Little is known about variation in bolting and flowering time within *Eutrema*. According to our results, *Eutrema* also show genetic variation in early-season phenology; however, we found no empirical evidence in support of a latitudinal cline in the spring phenology of *Eutrema* ($r_s = -0.35$, $P = 0.2$, data not shown). Similarly, Lee et al. (2012) found no significant correlation between the latitude of the geographical origin of the accessions and their freezing tolerance before or after cold acclimation.

4.2 | Improved winter survival reduces reproductive fitness in both *Eutrema* and *A. thaliana*

There was a large divergence in seed yield per plant between *Eutrema* and *Arabidopsis*, but also considerable variation in seed yield within *A. thaliana* and even more within *Eutrema*. In accordance with Boinot et al. (2022), the lower reproductive fitness in *Arabidopsis* accessions

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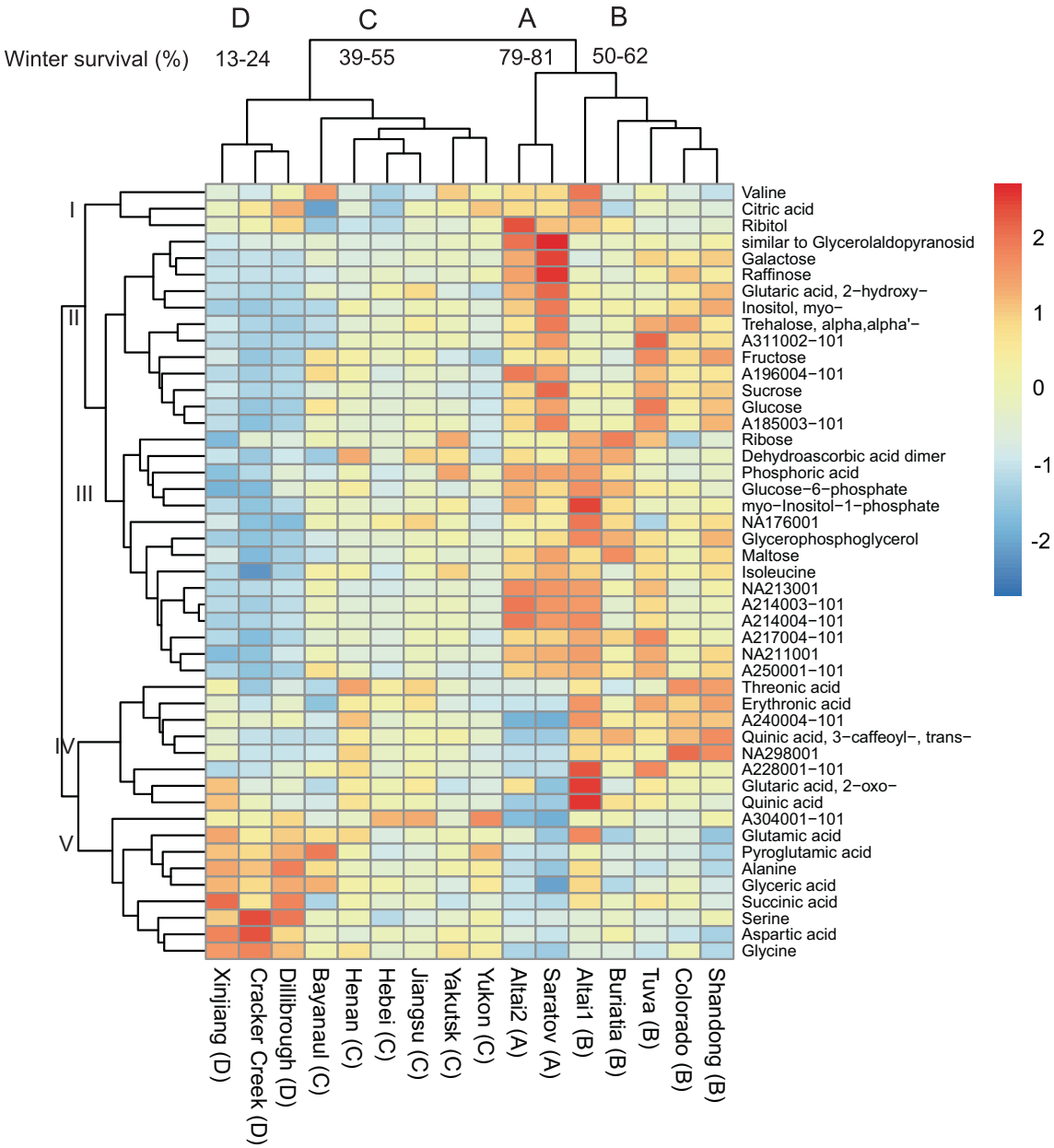

FIGURE 5 The metabolic profiles of leaves of 16 *Eutrema* accessions were statistically analyzed using a one-way ANOVA at FDR $P < 0.05$ by comparing all accessions against each other. Metabolites that showed significantly different levels between accessions were clustered using Euclidean distance, resulting in separation of the accessions into four clusters (A-D) according to winter survival. The panel shows row-scaled normalized metabolite intensities as indicated by the different colors.

was associated with a higher winter survival rate and a higher freezing tolerance, as evidenced by the lower 1000-seed mass in accessions with higher winter survival rates compared to accessions with lower winter survival rates. Plants can increase their fitness by producing larger and/or heavier seeds, which support the embryo and the seedling with more resources. Heavier seeds in more southern accessions may be a result of earlier flowering, which allows for a longer development time of seeds and larger seeds (Bolmgren and Cowan, 2008). Hence, the 1000-seed mass was positively correlated with the BBCH index, indicating higher seed mass with earlier phenological development. In contrast, earlier flowering onset can also imply fewer resources allocated for maternal plant growth, smaller size at the time of reproduction, and thus fewer resources available for seed production (Bolmgren and Cowan, 2008).

In *Eutrema*, increased winter survival and higher acclimated freezing tolerance were also associated with reduced reproductive fitness, as evidenced by a negative correlation between seed yield per plant and winter survival and a positive correlation between seed yield per plant and LT_{50}ACC previously determined for plants grown under controlled conditions. This is in accordance with the allocation cost theory, stating that the attribution of energy and metabolites into defense and acclimation limits growth and reproduction

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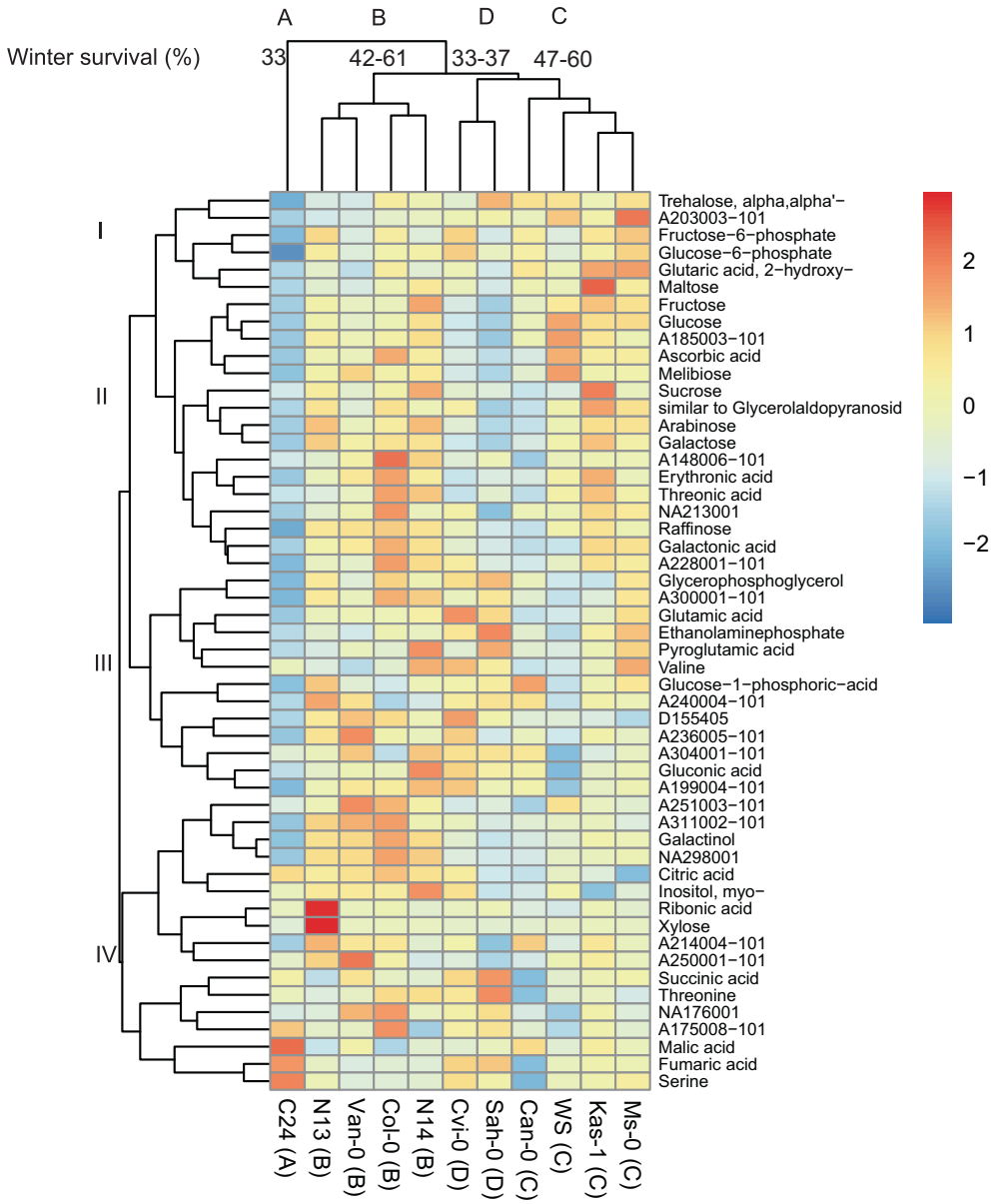

FIGURE 6 The metabolic profiles of leaves of 16 *Arabidopsis thaliana* accessions were statistically analyzed using a one-way ANOVA at FDR $P < 0.05$ by comparing all accessions against each other. Metabolites that showed significantly different levels between accessions were clustered using Euclidean distance, resulting in separation of the accessions into four clusters (A-D) according to winter survival and phenological development. The panel shows row-scaled normalized metabolite intensities as indicated by the different colors.

(Wan et al., 2017). Increased tolerance to abiotic stressors has previously been associated with reduced fitness parameters in other plant species; i.e. more frost-tolerant *A. lyrata* populations from higher latitudes depicted smaller plant size (Wos & Willi, 2015) and transgenic *A. thaliana* plants constitutively expressing cold tolerance genes had lower fitness, as determined by fruit number, than the wildtype (Jackson et al., 2004).

4.3 | *Eutrema* and *A. thaliana* have distinct metabolic profiles under field conditions

The two species have very distinct metabolic profiles, suggesting different basal metabolic compositions and/or different metabolic adaptation strategies for overwintering. This is in accordance with previous studies investigating metabolic differences between the two species both in the absence of stress and in response to different abiotic stressors under controlled conditions (Benina et al., 2013; Lee et al., 2012; Pinheiro et al., 2019; Zuther et al., 2018), but has not previously been shown under natural conditions. Interestingly, several of the metabolites found at different levels in the two species were also among the most important metabolites distinguishing the *E. salsugineum* accessions Tuva and Yukon from 10 *A. thaliana* accessions under controlled non-acclimating and cold acclimating conditions (Zuther et al., 2018). Unique features of the *Eutrema* metabolome under field conditions, averaged across all accessions, were higher levels of amino acids, in particular glycine, proline, pyroglutamic acid, serine, threonine, β-alanine, O-acetylserine (OAS) and its derivative N-acetylserine. Proline is a well-documented osmoprotectant with a prominent role in freezing tolerance, which is known to accumulate to a much larger extent in *Eutrema* than in *Arabidopsis* in response to cold (Lee et al., 2012; Benina et al., 2013). Pyroglutamic acid and, therefore, glutamic acid can serve as precursors for proline synthesis (Mazelis & Pratt, 1976). Accordingly, glutamic acid was also found in higher levels in *Eutrema* than in

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A. thaliana. Increased levels of serine and glycine may be a sign of high photorespiratory activity during deacclimation and growth resumption in *Eutrema*. Photorespiration can act as an electron sink, especially under stress conditions such as high light and cold, by consuming reducing equivalents during the refixation of released ammonia and by exporting reduced components from the chloroplast to the mitochondrion (Wingler et al., 2000; Peterhansel et al., 2010). However, these free amino acids may also contribute to osmoregulation in response to osmotic stress (Di Martino et al., 2003). OAS is a signaling molecule regulating the expression of a common set of six genes in response to environmental factors (Apodiakou & Hoefgen, 2023), while beta-alanine is involved in multiple stress responses in plants and may additionally function in lignin biosynthesis (Parthasarathy et al., 2019). Benina et al. (2013) also found higher levels of β-alanine in *Eutrema* than in *A. thaliana*.

The chlorogenic acids 3-*O*-caffeoylquinic acid (3-CQA) and 5-*O*-caffeoylquinic acid (5-CQA) and their precursors quinic acid and ferulic acid were solely found in *Eutrema* after overwintering in the field. CQAs are specialized secondary metabolites derived from the phenylpropanoid biosynthesis pathway and are often induced by stress. They serve specific roles in plant protection, i.e. chemical defense against herbivores and pathogens, ultraviolet screening, or structural components of the cell wall (Soviguidi et al., 2022). In addition, 3-CQA and 5-CQA may function as antioxidants serving to mitigate the effects of oxidative stress (Grace & Logan, 2000; Yamasaki & Grace, 1998), further pointing to increased photoprotection in *Eutrema*.

Sugar metabolism, in particular, plays a major role in several types of abiotic stress, especially in tolerance against osmotic stressors such as drought, salt and freezing temperatures. *Arabidopsis* was revealed to possess unique sugar metabolism with specific sugars and sugar conjugates at much higher levels than *Eutrema*. Particularly, much higher levels of galactinol, galactose, raffinose and melibiose suggest that the raffinose family oligosaccharides (RFOs) play a central role in overwintering of *A. thaliana*. The biosynthesis of RFOs begins with the galactosylation of *myo*-inositol to produce galactinol. Raffinose is synthesized from sucrose by the subsequent addition of activated galactose moieties donated by galactinol (Sengupta et al., 2015). RFOs have been shown to accumulate during cold acclimation of several plant species and function as compatible solutes (Hincha et al., 2006; Yan et al., 2022). The much higher level of trehalose in *A. thaliana* than in *Eutrema* implies a different role of this disaccharide in the overwintering of the two species. Trehalose is implicated in responses to cold and salinity (Lunn et al., 2014) and can serve as an osmoprotectant of biological membranes and can stabilize macromolecular structures (Crowe et al., 1998). Higher levels of raffinose and trehalose in *A. thaliana* than in *Eutrema* are consistent with findings by Zuther et al. (2018).

4.4 | Common metabolic strategies of winter survival within *Eutrema* and *A. thaliana*

Despite substantial differences in the levels of a range of metabolites, common metabolic adaptation strategies to survive winter were also observed when correlating metabolite pool sizes with winter survival rates or phenological development within *Eutrema* or *A. thaliana*. Sampling for metabolite profiling was carried out in early April, at which time all accessions had initiated spring growth and presumably lost some or all of their acclimated freezing tolerance, although nights were still cold, with the average minimum temperature 5 cm above ground being 0.6°C 7 days before harvesting. Considering the advanced developmental state of the plants, it is somewhat surprising that we, in both *Eutrema* and *A. thaliana*, mostly identified metabolites that appear functionally related to winter survival. This may reflect the high metabolic interconnection between deacclimation and ontogenetic development (Pagter et al., 2017) or may be an indication that the extent of primary metabolism response to changes in growth temperature is connected to the geographical site of origin, similarly to the winter survival rate. Thus, the metabolic response of *A. thaliana* to a change in growth temperature differs significantly between accessions (Weiszmann et al., 2023). Also, there is intraspecific variation in the metabolic reprogramming underlying cold acclimation and deacclimation of *A. thaliana* (Korn et al., 2008; Zuther et al., 2015) and cold acclimation of *Eutrema* (Lee et al., 2012).

The most obvious common response was the positive correlations between winter survival rates and pool sizes of fructose, galactose, glucose, raffinose and sucrose in both species. The contents of glucose, fructose, sucrose and raffinose in leaves have previously been shown to be linearly correlated with leaf freezing tolerance in a larger collection of *A. thaliana* accessions under controlled conditions (Lee et al., 2012; Zuther et al., 2012), and these sugars were also identified as important predictors of freezing tolerance in *A. thaliana* (Korn et al., 2008). In *Eutrema*, on the other hand, biochemical data for raffinose, sucrose, glucose and fructose only found a significant positive correlation between leaf sucrose and freezing tolerance under controlled conditions (Lee et al., 2012). However, carbohydrate reserves may increase winter survival in other ways than via increased freezing tolerance, i.e. as substrates for respiration and regrowth (Bertrand, 2003). Accordingly, contents of fructose and glucose were also positively correlated with the ranked BBCH index in *Eutrema*, but not in *A. thaliana*. In both species, maltose, a degradation product of starch, was also positively correlated with the winter survival rate.

4.5 | Myo-inositol has different functional roles in overwintering of *Eutrema* and *A. thaliana*

Increased winter survival of accessions with increased contents of *myo*-inositol and its precursors glucose-6-phosphate and *myo*-inositol-1-phosphate suggests an important role of the *myo*-inositol biosynthetic pathway in overwintering of *Eutrema* and highlights the inherent differences in the metabolomes and/or strategies to survive winter in the two species. *Myo*-inositol has been proposed to enhance cold stress tolerance in several plant species (Li et al., 2021; Zhuo et al., 2013) and *myo*-inositol phosphate synthase, the rate-limiting enzyme for *myo*-inositol biosynthesis, is upregulated when plants encounter cold, drought and salt stress (Li et al., 2021). *Myo*-inositol is a versatile compound that can act as a compatible solute to balance

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the cell turgor and as a regulator of ROS-induced programmed cell death (Loewus & Murthy. 2000; Meng et al., 2009). In *A. thaliana*, no correlation between winter survival and *myo*-inositol was observed. Instead, *myo*-inositol was likely conjugated with UDP-D-galactose to form galactinol, a precursor for RFO biosynthesis, as indicated by the much higher levels of galactinol, galactose, raffinose and melibiose (a raffinose degradation product) in *A. thaliana*, as discussed above. In accordance, winter survival of *A. thaliana* was also positively correlated with galactinol, galactose and melibiose. Conversely, galactinol was not detected in *Eutrema* and levels of galactose and melibiose did not differ between *Eutrema* accessions. Interestingly, both galactinol, raffinose and melibiose were negatively correlated with the ranked BBCH index in *A. thaliana*, suggesting that the persistence of the acclimated state may compete for these RFOs with growth resumption in the spring.

4.6 | Species-specific metabolic responses of *Eutrema*

In addition to the sugars discussed above, the winter survival rate of *Eutrema* was positively correlated with ribose. Similarly, in flower buds of *Ribes nigrum*, seasonal changes in levels of ribose correlated with various sugars and transcript levels of several genes putatively associated with freezing tolerance (Andersen et al., 2017).

The functional significance of negative correlations between winter survival rates and several amino acids (cluster V) in the *Eutrema* accessions is unclear. As has been mentioned before, pyroglutamic acid is a reservoir of glutamate, which is the predominant amino donor for transamination reactions in the cell. In addition, glutamic acid is the precursor for the important osmoprotectants proline and gamma-aminobutyric acid (GABA) and chlorophyll synthesis in developing leaves (Liao et al., 2022). The α-amino group of glutamate may be transferred to oxaloacetate to form aspartate, which is a precursor of asparagine and the aspartate family of amino acids, but also associated with multiple metabolic pathways, such as protein synthesis, nucleotide metabolism, TCA cycle, glycolysis and hormone biosynthesis (Han et al., 2021). The α-amino group of glutamate may also be transferred to pyruvate to form alanine. Deacclimation and growth resumption involve major changes in protein and amino acid metabolism, including degradation of freezing tolerance-related proteins (Pagter et al., 2014), increased respiratory energy production and carbon depletion for the biosynthesis of the pyruvate, the aspartate and glutamate families of amino acids and increased protein biosynthesis (Pagter et al., 2017). Depending on the time of sampling, these changes may be reflected in higher or lower levels of specific amino acids. Alternatively, both glutamic acid, aspartic acid and alanine are glucogenic; thus, their synthesis is dependent on the carbohydrate scaffolds and higher levels in accessions with the lowest winter survival rates and generally lowest accumulation of carbohydrates could be involved in the regulation of photosynthesis intensity. Using the example of *Arabidopsis*, it was proved that, during low-temperature treatment, most of the carbon assimilated in photosynthesis was inserted into amino acids, preventing the accumulation of carbohydrates, which, on the way of feedback, could stop photosynthesis (Bocian et al., 2015).

4.7 | Species-specific metabolic responses of *A. thaliana*

In *A. thaliana*, four polyhydroxy acids (ascorbic acid, erythronic acid, galactonic acid and threonic acid) were positively correlated with winter survival rates and negatively correlated with phenological development. Ascorbic acid is a universal antioxidant involved in the ascorbate-glutathione cycle but may also be implicated in regulating processes such as photosynthesis, floral induction and senescence (Akram et al., 2017). Exposure of *A. thaliana* to cold acclimating conditions has previously been shown to induce increasing levels of polyhydroxy acids in leaves (Vyse et al., 2022) and exogenous application of ascorbic acid can increase plant freezing tolerance (Min et al., 2020). Galactonic acid is an intermediate compound in one proposed biosynthetic pathway of ascorbic acid (Agius et al., 2003), while threonic acid is an ascorbic acid degradation product (Loewus, 1999).

In *Arabidopsis*, arabinose and xylose were negatively correlated with the phenological development of the accessions (ranked BBCH index), suggesting that modification of cell wall properties may be involved in growth resumption. Both xylose and arabinose are associated with hemicellulose component of the cell wall and their contents have previously been shown to change during deacclimation of *Arabidopsis* (Pagter et al., 2017). Changes in cell wall composition are known to be a component of deacclimation and growth resumption (Kutsuno et al., 2023).

The TCA cycle intermediates citric acid, succinic acid, fumaric acid and malic acid, which were all found in cluster IV, were not quantitatively related to winter survival rates, phenological development or reproductive fitness in a consistent way, suggesting that their accumulation patterns mostly reflected intraspecific variation in TCA cycle activity at the time of sampling.

5 | CONCLUSIONS

In conclusion, freezing tolerance is an important, but not the sole, component of winter survival of *Eutrema*, which is not an extremophile with regard to winter survival when tested under temperate conditions. In both *Eutrema* and *A. thaliana*, increased winter survival entails a fitness cost, expressed either as reduced seed yield or smaller seed size. In *A. thaliana* accessions, flowering time and seed mass covary with the latitude of origin, whereas in *Eutrema*, intraspecific variation in reproductive fitness traits is not related to the latitudinal site of origin. The first metabolite profiling of *Eutrema* and *A. thaliana* after overwintering under field conditions verifies previous findings from controlled chamber experiments showing that the two species have distinct metabolic profiles, including differences in metabolism of amino acids, chlorogenic acids and sugars. In both species, however,

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increased winter survival is associated with increased pool sizes of several soluble carbohydrates. The *myo*-inositol biosynthetic pathway appears to play an important role in *Eutrema*'s overwintering, whereas *myo*-inositol likely predominantly contributes to RFO biosynthesis in *A. thaliana*. Other unique metabolic responses during deacclimation include amino acid metabolism in *Eutrema* and levels of polyhydroxy acids and cell wall constituents in *A. thaliana*. Altogether, the results enable a better understanding of different inter- and intraspecific metabolic mechanisms in determining the ability to survive winter, which aids our understanding of the complex genetic and environmental factors underlying plant metabolic adaptations.

AUTHOR CONTRIBUTIONS

Ellen Zuther and Majken Pagter designed and performed the experiment. Alexander Erban and Joachim Kopka measured and annotated the metabolites. Majken Pagter analyzed the data and Ellen Zuther and Majken Pagter wrote the manuscript with contributions of Alexander Erban and Joachim Kopka.

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DATA AVAILABILITY STATEMENT

All data generated during this study are included in this published article and its supplementary information files.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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