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# Autumn cold acclimation and freezing tolerance of three oak species in semi-Mediterranean Zagros forests

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## Abstract

*Aim of study*: To identify and compare the early frost resistance mechanisms in three oak species (*Quercus brantii*, *Quercus libani* and *Quercus infectoria)*.

*Area of study*: Zagros forests of Iran.

*Material and methods*: The physiological and biochemical variables such as chlorophyll fluorescence, relative water content (RWC), electrolyte leakage, and osmotic metabolite content, such as proline, glucose, and potassium of three oak species seedlings with varying altitudinal and latitudinal ranges were measured under various treatments, including cold treatments (4°C: control, -20°C (1 hour) and -20°C (2 hours)) and four levels of hardening steps with decreasing temperature and photoperiod.

*Main results*: Results showed that decreasing photoperiod and temperature during cold hardening was associated with decreasing $F_v/F_m$, ΦPSII and electron transport rate, increasing (NPQ) measured from chlorophyll fluorescence, as well as increasing osmotic metabolite content and decline of RWC, except the glucose content decreased in *Q. brantii* from lower altitudes and south aspect. On the other hand, *Q. libanii,* which originates from higher altitudes and north aspect showed the strongest cold-resistance and faster developing cold-acclimation capacity using earlier accumulation of osmotic metabolites, diminishing RWC and subsequently lowest EL compared to the other oak species.

*Research highlights*: The physiological and biochemical responses of oak species differed based on origin and there was a positive relation between osmotic metabolite content, NPQ, altitude, and cold stress resistance. These physiological responses, especially NPQ (as a fast and non-invasive tool) provide a quantitative assessment of the risks associated with autumn freezing in different oak species and ecotypes relevant to conservation and reforestation projects of the Zagros forests under changing climatic conditions.

**Additional keywords:** cold hardening; early season frosts; interspecific variations; Mediterranean forests; physiological responses.

**Abbreviations used:** EL (electrolyte leakage); ETR (electron transport rate); $F_v/F_m$ (maximum quantum yield of PSII), K+ (potassium); NPQ (nonphotochemical chlorophyll fluorescence quenching); qP (photochemical quenching); RWC (relative water content); ΦPSII (optimum quantum yield efficiency).

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# Introduction

Cold stress is one of the environmental stress that limits the growth and geographical distribution of forest trees across latitudinal and altitudinal gradients (Alberdi & Corcuera, 1991). In temperate climates, tree survival frequently depends on withstanding extremely low temperatures both before and after winter hardening (Sakai & Larcher, 2012). During autumn, plants are exposed to both chilling and freezing stress (Gao et al., 2020). Chilling stress can be defined as occurring above 2°C and is less harmful than freezing stress for plants. Models of climate simulation showed that despite global warming trends, extreme events – such as early and late season frosts – are more frequent (IPCC, 2013) due to increasing autumn and spring air temperatures that can delay dormancy at the end of the growing season or advance bud break at the start (Gu et al., 2008). Consequently, forest trees and seedlings may be increasingly exposed to chilling and freezing stress due to rapid temperature shifts (Rixen et al., 2012). Cold hardening is an important mechanism for many tree species to withstand low temperatures and cope with changing environmental conditions (Hamilton et al., 2016; Zeps et al., 2017), particularly given predicted shifts in species' geographic ranges to higher altitudes and latitudes especially across the Mediterranean areas (Valavi et al., 2019). Moreover, late flushing species like the oaks are less freezing resistant than earlier flushing species in temperate environments (Vitra et al., 2017), and global warming increases of 1–4 °C are changing the phenology of trees, negatively impacting the development of cold hardiness and autumn freezing resistance (Pagter & Arora, 2013; Zeps et al., 2017) due to insufficient physiological transitions in the fall (Hamilton et al., 2016).

Cold hardening is a gradual process that occurs in autumn when tree species are exposed to low temperatures and declining photoperiods (Beck et al., 2004). Cold hardening processes and freezing tolerance are accompanied by a series of biochemical and physiological changes (Alberdi & Corcuera, 1991; Rixen et al., 2012) and low photosynthetic productivity that causes growth cessation (Rossi et al., 2008; Bauerle et al., 2012). For example, osmotic adjustment through the accumulation of total carbohydrates (Morin et al., 2007) and proline (Hare & Cress, 1997) can allow plants to avoid intracellular freezing in cold hardening tissues of forest species trees (Delauney & Verma, 1993). Sodium and potassium can contribute to the regulation of osmotic potential under environmental stress, including chilling and freezing (Bogeat-Triboulot & Lévy, 1998). Also, chlorophyll fluorescence as a non-invasive tool also is appreciated for the study of tree species hardening such as oak species under low temperatures (Cavender-Bares et al., 2005; Corcuera et al., 2005; Cavender-Bares, 2007). Another reliable physiological indicator for testing the freezing tolerance and cold hardiness of seedlings and trees is electrolyte leakage (EL) (Flint et al., 1967; Koehler et al., 2012). This method is used extensively and shows membrane permeability and degradation of membrane integrity by cold stress (Moshtaghi et al., 2009).

The Zagros forests located in the west of Iran cover an area of about 6 million hectares, representing 44% of the country's forested area (Sagheb-Talebi et al., 2013). Zagros forests are particularly important for water supply, soil conservation, environmental change, and the socio-economical balance of the whole country. Oak species (*Quercus brantii* Lindl., *Quercus infectoria* Oliver and *Quercus libani* Oliver) are dominate in these forests. Also, these species are native to large areas of Mediterranean forests and they are widely distributed in western Asia, namely in Iraq, Syria, Lebanon and Turkey (Mirhashemi et al., 2023).

Seedlings of oak during the first years of their life are very sensitive to environmental stress such as extreme summer drought and low temperatures. Therefore, these unfavorable climatic conditions can limit the distribution of oak species, and also most reforestation projects with these oak species have poor field performance. Some prior research has been conducted on physiological and biochemical responses to low temperatures stress in European or American oak species such as *Quercus robur* L., *Quercus pubescens* Willd., *Quercus ilex* L., *Quercus virginiana*, *Quercus oleoides* (Cavender-Bares et al., 2000; 2005; Cavender-Bares, 2007; Morin et al., 2007; Andivia, 2012b; Koehler et al., 2012), but there is no information for these three oak species (*Q. brantii*, *Q. infectoria* and *Q. libani*) despite the wide distribution of them in Mediterranean forests. On the other hand, understanding the physiological responses to low temperatures and the mechanisms involved in cold acclimation of species originating from altitudinal clines is very limited and it is critical to predicting how species will respond to climate changes (Cheaib et al., 2012). This information may also allow the selection of cold-tolerant ecotypes or populations for reforestation.

Therefore, the objectives of our studies were 1) to determine how cold hardening alters physiological responses to low temperature in these three oak species and the traits that contribute to low-temperature resistance after hardening or cold acclimation, and 2) to predict early season freezing resistance in these three species.

# Material and methods

## Distribution of the oak species studied

Three oak species studies are distributed in the Zagros forests of Iran. The climate of these forests is sub-humid with cold winters according to Emberger's formula (Emberger, 1943) with a long dry season lasting between 5-7 months. These regions are thus characterized by double stresses of summer drought and winter cold like other Mediterranean environments (Mitrakos, 1982). The southern Zagros is both drier and warmer than northern Zagros. These forests are dominated by three oak species, *Q. brantii*, *Q.*

*infectoria*, and *Q. libani*, whose distributions collectively span an area of about 5 million hectares (Fatahi, 1995). *Q. brantii* has a very wide-ranging distribution and spanning northern and southern Zagros forests, while *Q. infectoria* and *Q. libani* are found only in the North of Zagros (Fig. 1). Pure populations of *Q. libani* are found at higher elevations (from 1500 m asl) than the other two oak species and at north aspects due to humidity conditions. In contrast, *Q. brantii* may be more adapted to drought and less cold tolerant given its distribution in lower elevation and south aspects. *Q. infectoria* is located primarily at the middle elevations and north aspects (Sagheb-Talebi et al., 2013).

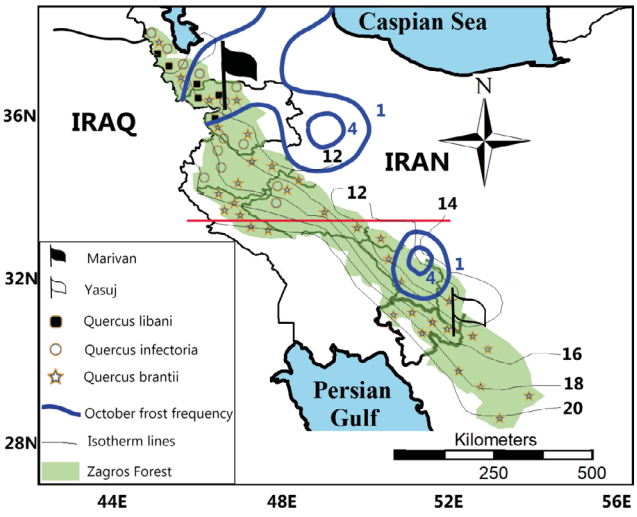


**Figure 1**. Distribution of different oak species (Nazemi et al., 2008) and thermal climatic characteristics in Zagros forests, which are divided into northern and southern sections by the horizontal red line at approximately 33°N (Khalyani & Mayer, 2013). The two days of persistent frost in October in Iran are depicted by the blue lines (Alijani et al., 2010).

## Seedlings preparation

Seedlings of three oak species (*Quercus brantii*, *Q. infectoria*, and *Q. libani*) were raised from seeds sourced locally (Zaribar forests) in the Rikhalan nursery (governmental nursery). The Rikhalan nursery is located 10 km southeast of Marivan (35°25′ N, 46° 0′ E and 1230 m asl), a town in the Kurdistan province (NW of Iran). The annual precipitation is about 756 mm and the mean temperature is 14.3. The extreme minimum and maximum winter temperature is between -14.1 and 14°C and the mean summer temperature is between 7.6 and 37.6°C, respectively. In November 2016, after two seasons of growth, seedlings of each oak species were transferred to Yasouj University nursery (29°52′ N; 49°55′ E, 800 m asl, SW of Iran) and planted in plastic pots (15 × 35 cm dimension) containing forest soil modified by incorporating 20% sand for a final mixture that was 50.6% sand, 24.4% silt, and 25.2% clay. The mean day/night temperatures in the greenhouse were 38/18±5°C and 35-40% relative humidity.

## Hardening induction and cold temperature treatments

The experiment had a completely randomized full factorial design with 15 replicates per each hardening treatment and species. To induce cold hardening, 160 of the 200 seedlings were transferred to the growth chamber in July 2017. Thereafter, 15 seedlings of each of the three oak species were randomly selected and removed at the end of each of the four hardening treatments for chilling and freezing treatments (Fig. 2). The hardening induction conditions were simulated in the growth chamber using shortened photoperiods and decreasing day and night temperatures for two months. The four hardening

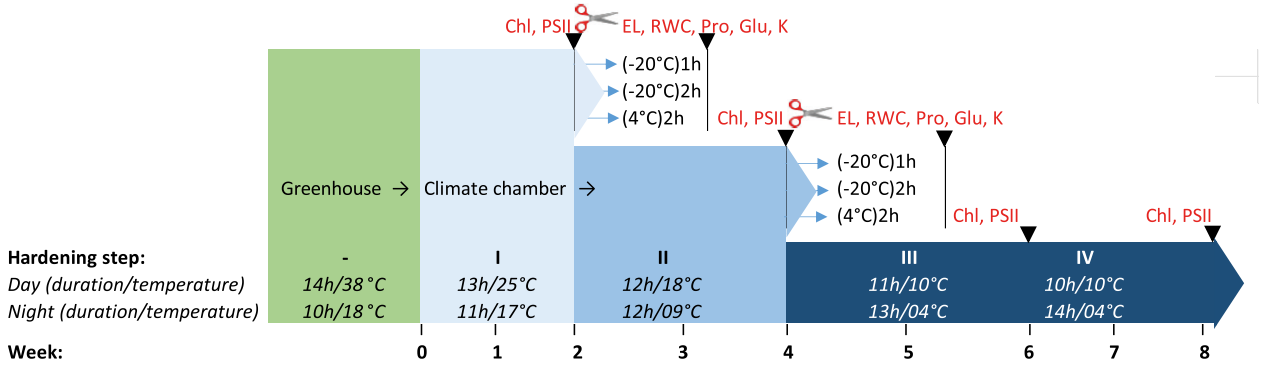


**Figure 2**. Program of cold hardening and chilling or freezing stress treatments. Chl: chlorophyll content. PSII: chlorophyll fluorescence. EL: electrolyte leakage. RWC: relative water content. Pro: proline content. Glu: glucose content. K: potassium content. The scissors show the seedling harvest time following each hardening stage in order to calculate RWC, EL, Pro, Glu, and K.

treatments were designed to match the monthly lowest and highest mean temperature in Marivan, north of Zagros forests, the seed origin, in different months as follows: *hardening 1* – temperatures and photoperiods in September; *hardening 2* – October; *hardening 3* – through November, and *hardening 4* – only decreasing of photoperiod (Fig. 2). The four hardening inductions were reflected in the early autumn conditions before a winter freeze. In the initial phase, seedlings were exposed to a decreasing photoperiod and temperature and then only with a photoperiod below 11 hours.

After each hardening induction (Control, *hardening 1, hardening 2, hardening 3, hardening 4)*, 15 seedlings of each of the three species were moved to dark cold storage at a chilling temperature of 4°C for one night. Then, the chlorophyll content and chlorophyll fluorescence of seedlings were measured. After that, seedlings were divided into three groups, and each group was randomly assigned to one of the three low-temperature treatments (control (4°C for 2 hours), -20°C (1 hour), and -20°C (2 hours)) (Fig. 2). The freezing (−20°C) and thawing rate of samples were 2 hours. Samples were wrapped in paper bags and aluminum foil to prevent desiccation during freezing. After low temperature exposure, leaves and stem segments of each seedling were cut and sampled from the refrigerator and freezing chamber for other physiological and element measurements (RWC, EL, Proline, Glucose, and K+) (8 replicates per treatment and species).

## Measurement of physiological traits

Chlorophyll fluorescence: The efficiency of PSII (both light and dark-acclimated) were evaluated using a portable fluorometer (PSM-2000, H Wals GmbH, Effeltrich, Germany). The maximum quantum yield of PSII ($F_v/F_m$), optimum quantum yield efficiency (ΦPSII), electron transport rate (ETR), photochemical quenching (qP), and non-photochemical quenching (NPQ) were calculated based on Minagawa (2008).

Chlorophyll index value that expresses relative chlorophyll content was measured at three points of the leaves outside the central mid-vein using a chlorophyll meter (SPAD-502, Minolta Co. Japan).

The alcoholic extraction was used for the estimation of soluble sugar and proline content. Leaf frozen samples (100 mg fresh weight) were powder and homogenized by grinding and then mixed in 5 mL of 95% ethanol. The homogenate was centrifuged at 6000 g for 15 min and the supernatant was decanted into a clean tube. After that, the residue was washed again by 2 mL of 70% ethanol and followed by centrifuged at 6000 g for 15 min. The supernatant was once again carefully decanted and pooled with the previous extract. The soluble sugar content was estimated using the anthrone reagent method (Chow & Landhäusser, 2004); 100 µL of alcoholic extraction was mixed with 3 mL of anthrone reagent (200 mg anthrone in 100 mL of 75% sulfuric acid). Then the tubes were boiled in a water bath at 100°C for 10 min. After cooling, the absorbance was measured at 620 nm wavelengths using UV- visible spectrophotometer (Lambda EZ 210 model). The concentration of each sample was determined using a standard curve and expressed as mg/g of fresh weight.

Proline content was measured by the acid ninhydrin method (Bates et al., 1973); 1 mL of alcoholic extraction was mixed with 10 mL of $ddH_2O$ and then 5 mL of acid ninhydrin (0.125 g ninhydrin in 2 mL 6M phosphoric acid and 3 mL glacial acetic acid) was added to the tube. After that, the mixture was incubated in a boiling water bath at 100 °C for 45 min and then the reaction was finished in an ice bath; 10 mL of toluene was added to the reaction mixture and samples were vortexed for 20 s. Then, the concentration of proline in the toluene fraction of each sample was determined by measuring the absorbance at 520 nm using by UV-visible spectrophotometer (Lambda EZ 210 model). Also, the toluene was used as blank and a range of standard L-proline solutions containing 0-0.6 mmol proline/mL were prepared in the same way of plant samples and finally the concentration of each sample was calculated as mg/g of fresh or frozen plant leaf.

EL, RWC, and K+ content were measured in different frozen plant tissues (leaf and stem). Electrolyte leakage was measured to estimate the cellular membrane injury in leaves and stems by cold stress (Flint et al., 1967). EL and RWC were assessed through the method described by Zolfaghari et al. (2022). A conductivity meter (model LF 315/SET, WTW, Arles, France) was used to measure the EL after 24 hours, and in order to obtain 100% electrolyte leakage, the mixture was boiled for 30 min in a water bath. Also, RWC was determined by the weighting of fresh (FW), dry (DW, 48 h at 80°C), and turgid weight (TW) of tissues after floating in $ddH_2O$ for 24h at room temperature in darkness. Finally, EL and RWC were calculated using the formulas below:

EL% = EC24⁄EC × 100 RWC (%) = [(FW - DW) / (TW - DW)] × 100

For determining potassium content in leaf and stem samples of three oak species, 0.1 g of dried plant tissues was ashed in an oven at 500 °C for 4h, and then samples were dissolved in 1 mL of 1 N HCl and diluted up to 15 mL by $ddH_2O$. After filtration, the concentrations of K+ were determined using a flame photometer (Jenway PFP7, UK).

## Statistical analysis

Analysis of variance was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The putative outliers were found by drawing a box-and-whisker plot; values more than 3 times the standard deviation was considered putative outliers. Tests of normality and equality of variances of the residuals were conducted by Kolmogorov-Smirnov and Leven's tests. Chlorophyll content and chlorophyll fluorescence were analyzed by two-way ANOVA to determine the main and the interaction effects of hardening with 5 levels (four level hardening plus

control or greenhouse condition) and species with 3 levels (5×3). EL, RWC, proline, soluble sugar, potassium and sodium content were analyzed by three-way ANOVA to determine significant effects of hardening with 2 levels, species with 3 levels and low-temperature treatment with 3 levels (2×3×3). Significant differences among groups were compared by Duncan's test and Student's t-test at the 0.05 level of confidence. Also, principal component analysis (PCA) as a multivariate analysis was applied for revealing relationships between all traits (physiological and biochemical traits) and treatments (species, *hardening 1* and *2*, cold temperature stress).

# Results

All PSII traits were restricted by progressing hardening, but the effect of species was only significant for NPQ (Tables 1 and 2). *Quercus libani* had the largest value of NPQ compared to the other two species (1.22 vs 1.04). We found a significant interaction effect between hardening and species for chlorophyll content (Table 1). Thus that, despite the general decline of chlorophyll content by progressing hardening in the studied oak species, the rate of reduction was different in different species. *Q. libani* responded to simulated hardening quickly, whereas in *Q. brantii* and *Q. infectoria* chlorophyll declined at a slower rate (Fig. 3A).

The results of the three-way ANOVA test of the other physiological traits showed that the main effect of species was a significant factor in explaining variation in all traits except for K contents of the stem (Table 3). Also, RWC, and proline displayed highly significant differences among cold temperature treatments (Table 3). On the other hand, the interaction between species, cold temperature, and hardening for all of them except for leaf K was significant. The significant interaction between species and cold temperatures of leaf EL showed that *Q. infectoria* had the highest value under the chilling condition (4°C), while leaf EL of the other two oak species showed a larger increase under freezing treatments. The increase of leaf EL under freezing temperature was less pronounced in *Q. libani* and this species showed the lowest leaf EL value under freezing temperature (Fig. 3B). Also, the interaction of cold temperature and hardening showed that stem EL had a lower value under freezing temperature of *hardening 2* compared to *hardening 1* (Fig. 3C). There were no

**Table 1**. Mean square values according to two-way ANOVA showing the effects of species and cold hardening treatments for chlorophyll fluorescence traits. The numbers in table indicate mean square with the p-value (N= 200).

| Chlorophyll fluorescence traits | Species (S) | Hardening (H) | S×H | Error |
|---|---|---|---|---|
| Chlorophyll index | 34.1 | 1382.1** | 112.9** | 11269.3 |
| $F_v/F_m$ | 0.88 | 72.7 ** | 1.35 | 0.264 |
| ΦPSII | 1.65 | 9.43** | 1.3 | 2.4 |
| qP | 2.3 | 8.5** | 0.64 | 11.6 |
| ETR | 1.77 | 12.01** | 0.95 | 171 |
| NPQ | 3.15* | 19.17** | 1.2 | 89.5 |

Fv/Fm: maximum quantum yield of PSII. ΦPSII: optimum quantum yield efficiency. qP: photochemical quenching. ETR: electron transport rate. NPQ: nonphotochemical chlorophyll fluorescence quenching. * $p < 0.05$, ** $p < 0.01$.

**Table 2**. Mean values (±SE) of chlorophyll fluorescence traits in seedlings of three oak species under different cold hardening treatments.

| Chlorophyll fluorescence traits | Control (greenhouse) | Hardening 1 | Hardening 2 | Hardening 3 | Hardening 4 |
|---|---|---|---|---|---|
| Chlorophyll index | 48.3 ± 0.53 A | 44.04 ± 1.4 B | 41.8 ± 1.07 B | 39.3 ± 0.64 C | 38.4 ± 0.98 C |
| $F_v/F_m$ | 0.81 ± 0.002 A | 0.81 ± 0.003 A | 0.77 ± 0.004 B | 0.76 ± 0.004 B | 0.73 ± 0.006 C |
| ΦPSII | 0.58 ± 0.008 B | 0.64 ± 0.01 A | 0.56 ± 0.01 BC | 0.54 ± 0.01 CD | 0.52 ± 0.01 D |
| qP | 0.68 ± 0.01 B | 0.85 ± 0.03 A | 0.59± 0.02 C | 0.7 ± 0.02 B | 0.64 ± 0.02 BC |
| ETR | 207.8 ± 7.8 B | 279.6 ± 14 A | 172.6 ± 9.7 C | 197.3 ± 9.9 BC | 166.3 ± 8.9 C |
| NPQ | 0.89 ± 0.05 B | 0.64 ± 0.08 C | 1.33 ± 0.08 A | 1.5 ± 0.07 A | 1.38 ± 0.04 A |

Fv/Fm: maximum quantum yield of PSII. ΦPSII: optimum quantum yield efficiency. qP: photochemical quenching. ETR: electron transport rate. NPQ: nonphotochemical chlorophyll fluorescence quenching. Different letters in each row indicate significant differences ($p < 0.05$) of mean values.

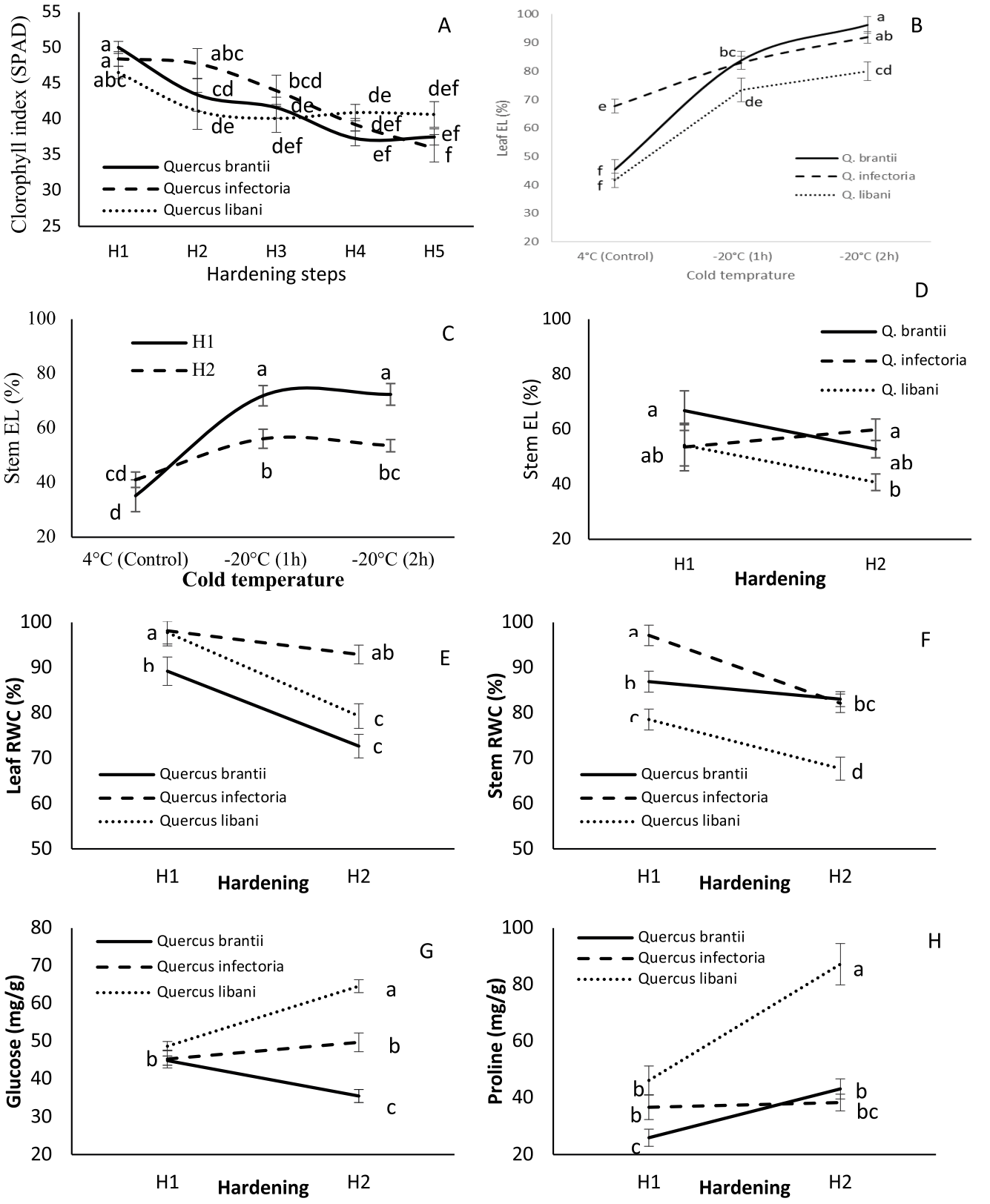


**Figure 3**. The mean (± SE) of interactive effects of treatments (species, cold temperature, and hardening) for measured traits. Vertical bars are standard errors (SE) and different letters indicate significant differences ($p < 0.05$) of mean values. EL: electrolyte leakage. RWC: relative water content.

significant differences between species for stem EL in the *hardening 1* treatment, but *Q. libani* performed significantly better than *Q. infectoria* in the *hardening 2* treatment due to the higher value of stem EL in *Q. infectoria* (Fig. 3D). Leaf RWC showed the lowest value in the *hardening 1* treatment in *Q. brantii* and declined with progressive cold hardening in both *Q. brantii* and *Q. libani*, while leaf RWC was stable between the *hardening 1* and *hardening 2* treatments in *Q. infectoria* (Fig. 3E). Also, stem RWC declined during hardening in *Q. infectoria* and *Q. libani* but did not change in *Q. brantii*. However, the RWC values were lower in *Q. libani* in both hardening treatments (Fig. 3F). Conversely, biochemical traits like proline and glucose increased during hardening treatments except for glucose, which decreased in *Q. brantii*. There was no significant change for glucose and proline with hardening treatment in *Q. infectoria* (Figs. 3G, H).

Comparison of means between species revealed that the leaf K content of *Q. libani* was significantly higher than the other two species (Fig. 4A). Leaf proline content did not change significantly from 4°C to -20°C (1 hour) and then increased after two hours of freezing at -20°C (Fig. 4B). Also, the effect of cold hardening on all traits was significant except for leaf EL and glucose (Table 3). The comparison of *hardening 1* and *2* treatments showed that leaf and stem K content increased in the *hardening 2* treatment (Figs. 4C, D), but the response of species to cold hardening was different for the other physiological and biochemical traits (RWC, EL, glucose, and proline) (Table 3).

Irrespective of the cold hardening and the freezing treatments, significant correlations among all measured traits were analyzed for each species, individually. Most of the traits in *Q. libani* and *Q. brantii* were significantly correlated while in *Q. infectoria* only leaf EL and leaf RWC had a weak direct correlation (r = 0.2).

In *Q. libani,* leaf EL was positively correlated with both leaf and stem RWC and negatively correlated with $F_v/F_m$. Also, K content showed a positive correlation with $F_v/F_m$. Glucose and proline content of *Q. libani* were highly positively correlated with each other, while there was no significant correlation between glucose and proline in *Q. brantii*. Stem RWC of *Q. libani* was also negatively correlated with leaf and stem K content and $F_v/F_m$, but the correlation between leaf RWC and $F_v/F_m$ in *Q. brantii* was positive (Fig. 5).

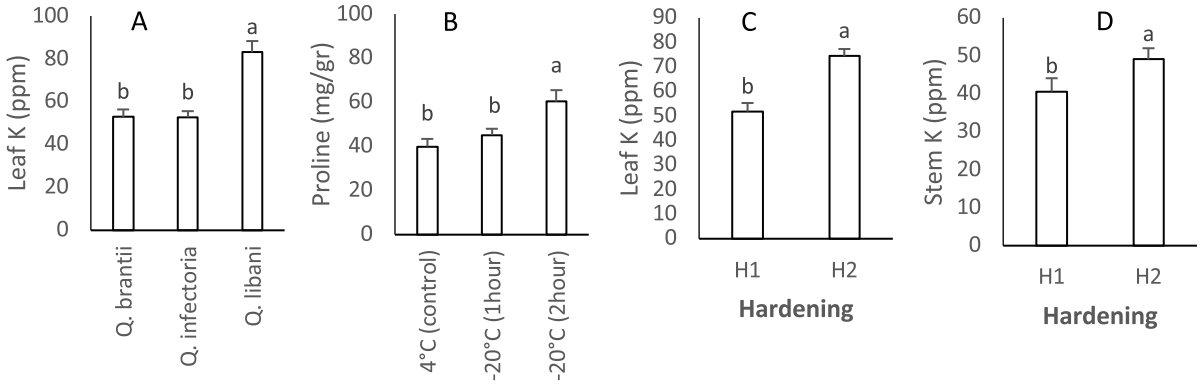


**Figure 4**. Mean values of main effects of treatments (species, cold temperature, and hardening treatments) for measured traits. Vertical bars are standard errors (SE) and different letters indicate significant differences ($p < 0.05$) of mean values.

**Table 3**. Mean square values according to three-way ANOVA showing the effects of species, cold temperature, and hardening treatments (N=90).

| Traits | Species (S) | Coldness (C) | Hardening (H) | S × C | S × H | C × H | S × C × H | Error |
|---|---|---|---|---|---|---|---|---|
| Leaf EL | 1361.7** | 9951** | 152.5 | 463.6** | 122.45 | 140.8 | 114.2 | 4198.2 |
| Stem EL | 979.01* | 5565.7** | 1462** | 175.8 | 832.1** | 911.5* | 225.6 | 11571.9 |
| Leaf RWC | 1247.9** | 879.9** | 3676.6** | 10/2 | 370.1* | 22.5 | 69.9 | 4327.3 |
| Stem RWC | 1830.4** | 665.8** | 2048.2** | 39 | 153.5* | 13.4 | 46.9 | 2518 |
| Glucose | 1638.4 ** | 95.01 | 190 | 148.8 | 993.2** | 113.1 | 38.2 | 4088.4 |
| Proline | 7136.4** | 1667.66** | 6137.5** | 260.1 | 2110.9** | 300.4 | 154.7 | 14727.1 |
| Leaf K | 4459.882** | 196.190 | 5753.141** | 37.888 | 288.934 | 77.608 | 366.079 | 154.646 |
| Stem K | 43.821 | 23.591 | 817.453* | 37.033 | 328.190 | 67.541 | 108.954 | 113.947 |

EL: electrolyte leakage. RWC: relative water content. * $p < 0.05$, ** $p < 0.01$.

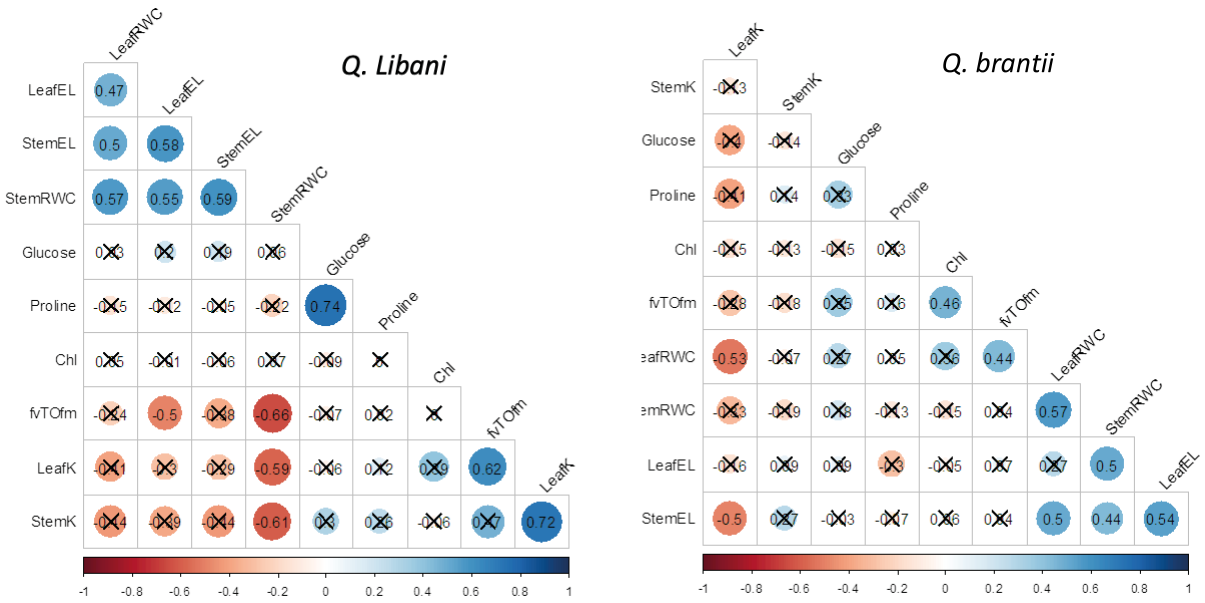


**Figure 5**. Pearson correlation between physiological and biochemical variables in *Quercus libani* and *Quercus brantii*. Non-significant correlations ($p > 0.05$) are marked with a cross. Positive and negative correlations are in blue and red, respectively. The intensity of the color and the size of the circles are proportional to the correlation coefficient. EL: electrolyte leakage. fvTOfm: maximum quantum yield of PSII. RWC: relative water content.

Principal component analysis (PCA) showed that the first two components explained 45.68% of the total variance. An ANOVA and t-test indicated that these two PCs were significant in different treatments. So, there were significant differences among the species and cold stress temperatures by only PC1. While both PC1 and PC2 were significant among *hardening* 1 and 2 (Table 4). The PC1 was contributed by the positive coefficient of physiological traits like EL and RWC content and a negative coefficient of K content (scores > 0.50). *Q. libani* seedlings, chilling stress (4°C), and some extent progressive hardening were aligned on the negative position of PC1 in comparison to the other two species (*Q. brantii* and *Q. infectoria*), freezing temperature (-20°C) and *hardening* 1. High osmolyte concentration, such as glucose and proline, was another characteristic that identified the second PC (Table 4 and Fig. 6).

# Discussion

In temperate regions, cold hardening processes are critical for resistance to cold and freezing stress in forest trees (Burke et al., 2003). In this study, the autumn hardening was stimulated by decreasing photoperiods and temperature for oak seedlings during two months as previously described in other studies (Beck et al., 2004; Hamilton et al., 2016; Homayounfar et al., 2019). During progressive hardening, a reduction in chlorophyll content was observed in the three studied oak species. This result is consistent with other studies that found the preliminary chlorophyll degradation is mostly triggered by the shortening of daylight and further reduction occurrs by decreasing temperature (Fréchette et al., 2016). At the beginning of the hardening period, the amount of leaf chlorophyll content in *Q. brantii* and *Q. libani* seedlings diminished more in comparison to *Q. infectoria* which could provide frost tolerance in these two species by preventing photo-degradation (Oberschelp et al., 2020). All chlorophyll fluorescence traits also progressively declined during hardening, and in contrast, NPQ increased. In many tree species a restriction in photochemical quenching of photosystem II during cold acclimation has been observed (Cavender-Bares et al., 2000; Zhou et al., 2017; Homayounfar et al., 2019) due to a decline in metabolic sink capacity mediated by short photoperiod (Hamilton et al., 2016) or loss of photochemical efficiency of PSII and inhibition of the electron transport as a consequence of low temperature (Cavender-Bares et al., 2005). Furthermore, there were no significant variations across oak species in any of the chlorophyll fluorescence traits except of NPQ. The greater NPQ in *Q. libani* in comparison to the other two species during hardening suggested that *Q. libani,* which occurs at higher altitudes, has more efficient mechanisms of photo-protective energy dissipation (Murchie & Lawson, 2013). Consistent with this result, higher NPQ under chilling temperatures was observed in *Q. virginiana* found in temperate climates compared to *Q. oleoides,* which occurs in tropical climates that lack a cold winter period (Cavender-Bares, 2007).

Also, there was inter-specific variation in physiological responses between studied oak species to freezing stress after hardening. Freezing temperature can impair the integrity of cell membranes, causing an increase in EL (Rihan et

**Table 4**. Coefficients of each trait's first three principal components and a statistical comparison of each PC's mean under different treatments (F-value)

| Treatments | PC1 | PC2 | PC3 |
|---|---|---|---|
| | F-value (sig.) | | |
| Species | 14** | 1.8$^{ns}$ | 0.4$^{ns}$ |
| Cold temperature | 12.3** | 0.7$^{ns}$ | 2.1$^{ns}$ |
| Hardening | 0.2** | 0.4** | 0.08$^{ns}$ |
| **Traits** | **PC coefficients** | | |
| Leaf EL | **0.745** | 0.052 | 0.203 |
| Stem EL | **0.693** | 0.014 | 0.321 |
| Leaf RWC | **0.593** | 0.338 | 0.141 |
| Stem RWC | **0.865** | 0.069 | -0.119 |
| Glucose | -0.089 | **0.894** | 0.002 |
| Proline | -0.448 | **0.762** | -0.034 |
| Leaf K | **-0.735** | -0.214 | 0.158 |
| Stem K | -0.327 | -0.040 | **0.632** |
| Chlorophyll | 0.158 | -0.090 | **0.495** |
| $F_v/F_m$ | -0.201 | 0.125 | **0.583** |

EL: electrolyte leakage. RWC: relative water content. Fv/Fm: maximum quantum yield of PSII. The bold numbers indicate important traits with higher eigenvector for each PC. **: significant ($p < 0.01$). $^{ns}$: not significant.

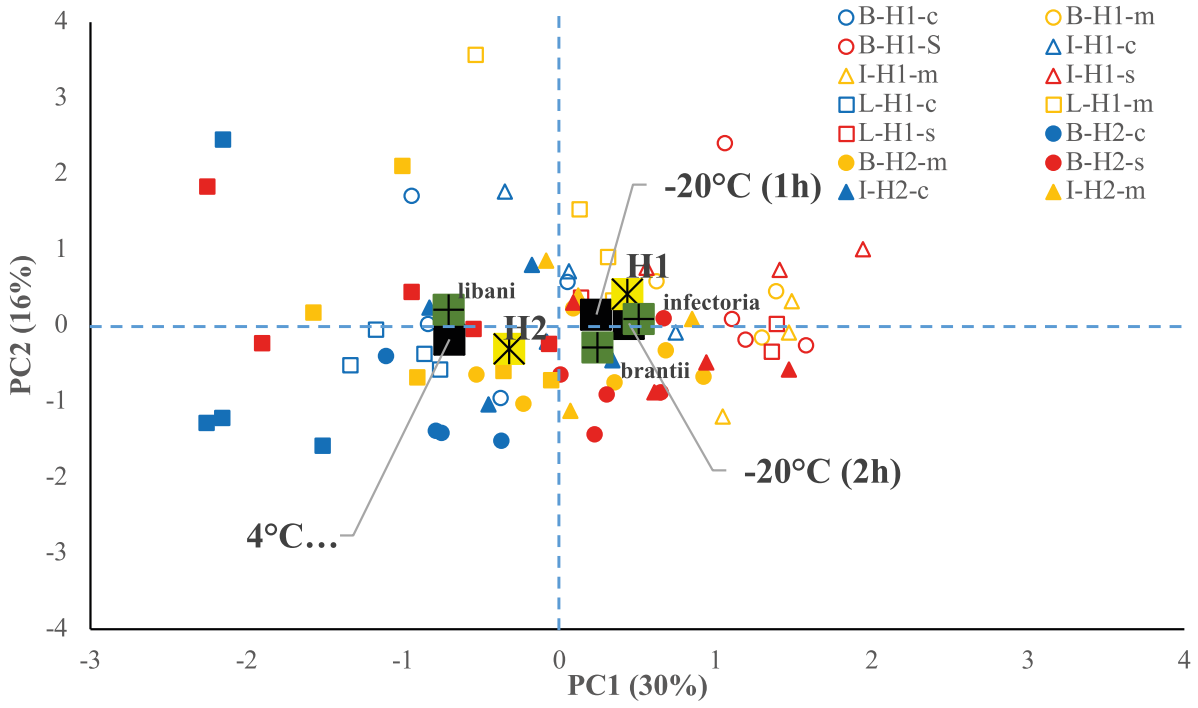


**Figure 6**. PCA-Biplot of the seedlings of different oak species treated by *hardening 1* and *2*, cold temperature. The different species are shown by a circle (*Quercus brantii*), a triangle (*Quercus infectoria*) and a square (*Quercus libani*). The blue, yellow and red symbol indicates the different cold stress (Control: 4°C for 2 hours, mild stress: -20°C (1 hour) and severe stress: -20°C (2 hours), respectively) and empty and filled symbols are related to *hardening 1* (H1) and *hardening 2* (H2), respectively. The centroid of each treatment (species, cold hardening and cold temperature) is also shown.

al., 2017). We observed increases in leaf and stem EL with more negative freezing temperatures, as has been reported by many studies (Azzarello et al., 2009; Homayounfar et al., 2017). Also, freezing stress duration time had an increasingly negative effect on seedling leaves from one hour to two hours and this result is not surprising since the previous study has shown the leaves are more sensitive tissue to cold stress (Shao et al., 2013). Furthermore, the results of ANOVA and PCA demonstrated that different species had different levels of susceptibility to freezing stress. For example, *Q. libani* seedlings, which are native to colder regions and higher elevations than other species, displayed a lower increase in leaf EL during freezing stress. These results suggest that *Q. libani* is more freezing tolerant than *Q. brantii* or *Q. infectoria*. Also, a smaller increase in stem EL under freezing stress was observed by progressing the hardening period. These results revealed that freezing resistance is enhanced upon hardening, as expected in temperate species (Koehler et al., 2012; Homayounfar et al., 2017; Oberschelp et al., 2020). Yet the response of different oak species to hardening treatments was different, and freezing resistance increased earlier in the *Q. libani*. Prior observations also showed a more positive effect of cold acclimation on freezing responses in tree species from climates where freezing occurs earlier and is more intense (Cavender-Bares, 2007; Homayounfar et al., 2017). There was an inverse relationship between leaf RWC, EL, and Fv/Fm. Water status is known to affect cold hardening and cold resistance (Worland, 1996). Indeed, the presence of a large amount of RWC under cold stress caused a cellular injury and was accompanied by a reduction of photosynthesis activity (Balamurugan et al., 2018). We also found that decreasing RWC was accompanied by increasing in K+ content in *Q. libani* and *Q. brantii*. Increasing $K^+$ content, especially in *Q. libani*, could protect seedlings from low temperatures by osmotic adjustment of leaf cells upon hardening (Hsiao & Laüchli, 1986), consistent with the findings of Gleeson et al. (2004) and Andivia et al. (2012a). In addition, an increasing $K^+$, proline, and glucose content, as osmo-protectants or scavengers, accompanied by progress of cold hardening and freezing, has been found by other researchers (Meng et al., 2015). Among species, we found highly significant variation in the accumulation of glucose and proline upon hardening. Because of the higher proline and sugar content of *Q. libani*, the osmotic adjustment ability of this species was stronger than *Q. brantii* and *Q. infectoria,* which originated from milder climates at lower altitudes. Similar results have been reported previously in some other *Quercus* species (Morin et al., 2007; Fernández et al., 2008; Andivia et al., 2012b). A reverse pattern of glucose in *Q. brantii* during progressive hardening and a lower value of PC2, which is associated with osmolyte content, demonstrated that glucose was excessively consumed as energy under conditions of severe low-temperature stress (Meng et al., 2015). *Q. brantii* was still able to increase cell osmotic to some extent concentration by increasing proline content in comparison to *Q. infectoria*.

# Conclusions

Variation in cold tolerance in three studied oak species corresponds well with the habitat of their origin and provides evidence in support of adaptive plasticity in cold and freezing tolerance (Cavender-Bares, 2007; Koehler et al., 2012). *Q. libani,* which originates from colder climates and higher altitudes has evolved a survival strategy of higher tolerance to freezing stress through earlier and more effective cold acclimation and hardening than other species. The accumulation of osmotic metabolites such as glucose, proline, and $K^+$ appears to play an important role in seasonal increases in freezing tolerance in *Q. libani* seedlings. Also, NPQ measurement due to vast variation between species under cold stress is in agreement with the results Hussain et al. (2022) and can be usefully applied as a non-destructive tool to foresters for assessing the development of dormancy status and predicting freezing tolerance in oak species and selecting the genotypes or mother trees of oak species most likely to persist in a changing environment. Moreover, based on PC1 results, measuring EL and RWC could be the next choice for future evaluation, as already reported by other researchers as important indicators of cold tolerance (Wu et al., 2012; Balamurugan et al., 2018).

It appears that *Q. infectoria* and *Q. brantii* are susceptible to early frost based on physiological data. In reality, though, *Q. brantii* is expected to die at a higher rate than *Q. infectoria* because early frost danger is higher in lower altitude species with wider geographic ranges. Fortunately, the PCA bi-plot analysis and other studies (Alikhani et al., 2014) suggested a high intraspecific variation within *Q. brantii*. Therefore, artificial screening for frost tolerance based on physiological responses to cold and freezing stress in *Q. brantii* families and ecotypes is important for the reforestation of Zagros forests.

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