Gas exchange variables as promising criteria for screening freezing-tolerant faba bean (Vicia faba L.) landraces at early growth stages

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Abstract
Photosynthesis is one of the parameters first affected by freezing stress. So studying the efficiency of photosynthetic parameters could be an effective strategy in screening freezing-tolerant genotypes. An experiment was conducted to assess freezing temperature effects (0°C, −4°C, −8°C, −12°C, −16°C, −20°C, and −24°C) on two faba bean landraces (Borujerd and Neyshabur). Leaf chlorophyll and carotenoid content were decreased as the temperature declined below 0°C. Net photosynthetic and transpiration rate declined earlier in Neyshabur, whereas a relatively constant trend was observed in Borujerd at temperatures between 0°C and −12°C. A 43% greater $N_p$ was recorded in Borujerd than Neyshabur at −12°C. $N_p$ recovered by 30% and 36% in Borujerd and Neyshabur, respectively, on Day 14 compared with the second day of the recovery period. Except for $C_i$, which showed a relatively constant trend, the other parameters showed a decreasing trend till 7 days after freezing stress (DAF). WUE$_i$ increased more significantly in Neyshabur than in Borujerd after the seventh day of the recovery period. With a subtle evaluation of the landraces’ freezing tolerance, both landraces LT$_{50su}$ and RDMT$_{50}$ remained up to −10°C and −12.5°C, respectively, indicating faba bean plants (the landraces here) can tolerate freezing stress up to −10°C (by enduring minor damages). The positive relationship between the survival percentage and $N_p$ indicated that $N_p$ could be a reliable criterion to screen freezing-tolerant faba beans at early growth stages.

KEYWORDS
chlorophyll, instantaneous WUE, mesophyll conductance, stomatal conductance, survival, transpiration

Abbreviations: Chla, chlorophyll a; Chlb, chlorophyll b; Chlt, total chlorophyll; $C_i$, intercellular CO$_2$ concentration; $C_{i:C_a}$, intercellular to ambient CO$_2$ concentration; DAF, days after freezing stress; $g_m$, mesophyll conductance; $g_s$, stomatal conductance; INAB, ice nucleation active bacteria; LT$_{50su}$, the lethal temperature of 50% of the plants; $N_p$, net photosynthetic rate; PAR, photosynthetically active radiations; RDMT$_{50}$, 50% dry weight depression temperature; ROS, reactive oxygen species; SU, survival percentage; $T_r$, transpiration rate; WUE$_i$, instantaneous water use efficiency.
1 | INTRODUCTION

As a part of sustainable food production, legumes play an essential role in the human diet. The legume importance is such that 2016 was declared the International Year of Pulses (Rubiales et al., 2018). This was to heighten the public awareness of the nutritional benefits of legumes aiming toward future food security. Faba bean (Vicia faba L.), also known as the broad bean or fava bean, is a Fabaceae family species. Faba bean is widely cultivated as a crop for human consumption and a cover crop. Faba bean plays a significant contribution in maintaining agricultural sustainability and is efficiently high in the symbiotic fixation of atmospheric nitrogen (Karkanis et al., 2018). With a production of 17,500 tonnes, Iran is ranked as the fourth producer of faba beans in Asia (FAOSTAT, 2018). Faba bean productivity has increased mainly due to reduced susceptibility to abiotic and biotic stresses in recent years (Ali et al., 2016; Link et al., 2010; Sillero et al., 2010; Singh et al., 2012). Faba beans are highly nutritious because of their high protein content (~35% in dry seeds), mineral nutrients, and also bioactive compounds, such as polyphenols, carotenoids, and carbohydrates (Landry et al., 2016; Longobardi et al., 2015; Neme et al., 2015; Turco et al., 2016). However, variety and environmental conditions influence the chemical composition (Cazzato et al., 2012; Witten et al., 2015).

Low temperature is one of the most common limiting factors for many plant species’ geographical distribution and production (Heidarvand & Amiri, 2010). Low temperatures induce reactive oxygen species (ROS) accumulation and reduce the photochemical efficiency of PSII and photosynthesis (Hájek et al., 2016). Net photosynthetic and transpiration rate, stomatal conductance, and instantaneous water use efficiency have been reported as essential plant tolerance parameters to environmental stresses (Xiao et al., 2005). Zhou et al. (2018) found a reduction in chlorophyll fluorescence of faba bean (V. faba L.) plants exposed to cold stress. Assessment of limiting factors of photosynthetic procedures under unfavorable environmental conditions, such as low temperatures, is a promising method in plant screening for cold and freezing stress (Pedranzani et al., 2015).

Both stomatal and nonstomatal factors can affect the photosynthesis at low temperatures; that is, cold stress could affect the stomatal activity and carbon metabolism enzymes. Although low temperatures could denature the photosynthetic proteins, the expression of those proteins is enhanced in the cold-tolerant plants (Zhou et al., 2017). Photosynthesis reduction in sensitive plants is depended on the rate and duration of cold temperature (Lukatkin et al., 2012), which is related to reducing chloroplast efficiency, the quantum yield of PSI and PSII, and ATP synthase (Allen & Ort, 2001). Reduction of chlorophyll a (Chla) and chlorophyll b (Chlb) is another result of cold stress, leading to photosynthetic disruption (Wise & Naylor, 1987). Liu et al. (2013) observed that the decrease in naked oat (Avena nuda L.) leaf chlorophyll content started from 1°C, and the lowest chlorophyll content was found at −10°C. They also stated that the low temperature was an important factor in reducing photosynthesis and light use efficiency.

Assessment of plant survival after freezing stress is also considered for screening freezing-tolerant plants (Kim & Anderson, 2006). Therefore, if a plant’s physiological processes, such as photosynthesis, show a high correlation with survival percentage (SU), they could be used as reliable indices in plant screening for cold tolerance. Any changes in plant survival and dry matter after a recovery period could represent plants’ freezing tolerance level. The lethal temperature of 50% of the plants (LT50) and 50% dry weight depression temperature (RDMDT50) could help to screen the freezing-tolerant plants. The percentage of plant survival under low temperature and determination of the temperature(s) that causes the death of 50% of plants (LT50su) and reducing 50% of plant dry weight (RDMDT50) can be mentioned among the most reliable parameters to recognize the freezing-tolerant plants (Kendall & McKersie, 1989; Nagao et al., 2005; Perry & Herrick, 1996; Rogers et al., 1977). Studying such traits as leaf area, plant dry matter, and SPAD after the recovery period could be used as suitable criteria for evaluating plant tolerance to cold stress (Fowler et al., 1981).

Climate change leads to an increase in the area utilized for winter types of faba bean (V. faba L.) (Ali et al., 2016); therefore, screening the genotypes with greater tolerance to extreme temperatures, that is, freezing and heat, would be of great importance. A variety of field and controlled-assessment methods have been performed to identify cold-tolerant cultivars (Ali et al., 2016; Coopman et al., 2010; Rashed Mohassel et al., 2009; Zhou et al., 2018). In general, recognizing the freezing-tolerant plants and improving basic information about unknown landraces helps to understand the freezing tolerance mechanisms and their geographical distribution. Therefore, the present experiment aimed to investigate (1) the efficiency of gas exchange variables as promising criteria for distinguishing the freezing-tolerant faba bean landraces, (2) the relationship between the net photosynthetic rate and freezing tolerance, and (3) the freezing tolerance level of the landraces.

2 | MATERIALS AND METHODS

2.1 | Experimental procedure

2.1.1 | Freezing temperatures

The experiment was conducted at the research greenhouse of the Research Center for Plant Sciences, the Ferdowsi University of Mashhad, in 2016. Faba bean landraces (Boroujerd and Neyshabur) and freezing temperatures (0°C as control, −4°C, −8°C, −12°C, −16°C, −20°C, and −24°C) were considered as the experimental factors. Both landraces are fall-cultivated (winter-sown), and the seeds of Neishabour and Boroujerd landraces are large and small, respectively. Neishabour and Boroujerd regions are cold; Neyshabur is cold and dry, and Boroujerd is cold and high, and severely cold temperatures occur in those regions. Therefore, the seeds that evolved in those regions may have cold tolerance characteristics than the temperate-originated genotypes.
Ten seeds were sown in plastic pots (12-cm diameter) containing equal portions of soil, leaf mold, and sand in mid-November. Plants were grown in a natural environment to four to six leaf stages, thinned to five plants per pot, and were then exposed to cold temperatures. The plants were normally irrigated during the growing periods. Plants were irrigated 24 h before they were transferred to a thermogradient freezer in February. The climatic conditions of the experimental site are indicated in Figure 1. The freezer temperature was set to 5 °C at the beginning of the experiment and declined by 2 °C per hour until −24 °C. A thin layer of ice nucleation active bacteria (INAB) was sprayed on plants at −2.5 °C to prevent the supercooling phenomenon and formation of ice nuclei in plants and ensure that the resistance of plants to freezing stress is due to tolerance (not avoidance).

2.1.2 | Recovery

Plants were kept at each freezing temperature for 2 h and were then immediately transferred to a growth chamber with a temperature of 5 °C ± 2 °C to prevent instant ice melting and kept there for 24 h before transferring to the greenhouse. Plants were kept in a greenhouse under natural day length, the temperature of 25/18 ± 3 day/night, approximately light intensity of 1000 μmol m⁻² s⁻¹ PAR, a relative humidity of 40 ± 5%, at the ambient CO₂ concentration, and leaf temperature of 25 °C. The measurements were carried out between 10:00 and 14:00 three times for each treatment. Instantaneous water use efficiency (WUEi) was calculated as the ratio of $N_p$ to $T_r$. At the same time, total leaf pigment content (SPAD) was measured on the same leaves using a handheld chlorophyll meter (SPAD 502, Spectrum Technologies, Inc.).

2.2 | Measurements

2.2.1 | Gas exchange variable

The youngest fully expanded leaves were selected to measure the photosynthetic parameters. Net photosynthetic rate ($N_p$), transpiration rate ($T_r$), stomatal conductance ($g_s$), mesophyll conductance ($g_m$), intercellular CO₂ concentration ($C_i$), and intercellular to ambient CO₂ concentration ($C_i:C_a$) were measured immediately after taking the plants out of each freezing temperature treatment (1 h after transferring to the recovery conditions [ATR]) and at 2, 4, 7, 14, and 21 days after freezing (DAF) stress using a portable photosynthesis system (ADC Bio Scientific Ltd, UK) at approximately 1000 μmol m⁻² s⁻¹ PAR, a relative humidity of 40 ± 5%, at the ambient CO₂ concentration, and leaf temperature of 25 °C. The measurements were carried out between 10:00 and 14:00 three times for each treatment. Instantaneous water use efficiency (WUEi) was calculated as the ratio of $N_p$ to $T_r$. At the same time, total leaf pigment content (SPAD) was measured on the same leaves using a handheld chlorophyll meter (SPAD 502, Spectrum Technologies, Inc.).

2.2.2 | Leaf photosynthetic pigments

The photosynthetic pigments were evaluated immediately after transferring plants to the greenhouse (1 h ATR). Leaf photosynthetic pigment content including Chla, Chlb, total chlorophyll (Chlt), and carotenoid content were measured based on the method described by Sükran et al. (1998). One hundred-milligram fresh weight of fully expanded young leaves were extracted using 96% ethanol. Absorption was measured in 470, 653, and 666 nm spectrophotometrically (Jenway UV-Visible, Model 6305). Leaf Chla, Chlb, and carotenoid content were calculated using Equations 1–3.

\[
Chl \, a = 13.36 \times A664 - 5.19 \times A648, \tag{1}
\]

\[
Chl \, b = 27.43 \times A648 - 8.12 A664, \tag{2}
\]

\[
C_{(x+c)} = \frac{1000 \times A470 - 2.13 \times Ca - 97.64 \times Cb}{209}. \tag{3}
\]

2.2.3 | Growth parameters

Growth parameters were measured 21 days ATR. Leaf area was measured by a leaf area meter (Delta-T, Type WDIGC-2). Above-ground dry weight (leaf and stem) was determined after being oven-dried at 80 °C temperature to constant weight. The number of alive plants before (B) and after (A) freezing stress were counted and used to calculate the SU according to Equation 4:

\[
\text{Survival} \% = \left( \frac{A}{B} \right) \times 100. \tag{4}
\]

2.2.4 | LT₅₀su and RDMT₅₀

To determine the lethal temperature of 50% of plants, the logistic equation for the data of each faba bean landrace at different temperatures was fitted (Eizenberg et al., 2005) using Equation 5:

\[
\text{FIGURE 1} \quad \text{Maximum and minimum air temperatures during the experimental procedure}
\]
\[
Y = \frac{a}{1 + \exp(-k \times (x-x_c))},
\]

In this equation, \(Y\), \(x\), \(a\), and \(x_c\) represent the SU, freezing temperature, one of the coefficients of the equation and represents the maximum SU, and the point of \(x\) at which \(Y\) is equal to 50% of its maximum value (LT50), respectively. Fifty percent dry weight depression temperature was determined by drawing the dry weight diagrams of plants against freezing temperatures and determining each curve midpoint.

### 2.3 Statistical analysis

The experiment was conducted as a factorial arrangement based on a completely randomized design with four replications. Data residuals were normalized by the Kolmogorov–Smirnov test using Minitab v.17. Analysis of variance was made by SAS v.9.1 software, and means were compared by the least significant difference (LSD) test at \(P \leq 0.05\) significance level. Origin Pro v.9.5 software was used to draw graphs and determine LT50, and RDFT50.

### 3 RESULTS

#### 3.1 Freezing temperatures experiment

##### 3.1.1 Leaf photosynthetic pigments

Freezing temperatures significantly affected the leaf Chla content (Table 1). Overall, the highest photosynthetic pigment content (Chla, Chlb, Chlt, and carotenoids) were observed at the control temperature (Figure 2). Although freezing temperatures decreased leaf Chla content, the reduction percentage and trend were different between the two landraces. Declining the temperature to \(-4^\circ\) decreased Borujerd leaf Chla content by 21%, but no specific trend was found in temperatures lower than \(-4^\circ\). In Neyshabur, however, leaf Chla content decreased as the temperature declined to \(-12^\circ\), but an ascending trend was observed at lower temperatures (Figure 2a). Freezing temperature and fava bean landrace interacted to affect the leaf Chlb content (Table 1). The highest leaf Chlb content in Borujerd was recorded at 0°C, which was 52% greater than Neyshabur (Figure 1b). Leaf Chlb content tended to increase in both landraces up to \(-16^\circ\), but it decreased at lower temperatures. Leaf Chlt content was affected by the interaction of freezing temperature \(\times\) landrace (Table 1). A similar trend as for Chlb was also observed for Chlt (Figure 2c).

Leaf carotenoids content was affected by freezing temperature \(\times\) fava bean landrace interaction (Table 1). The greatest leaf carotenoids content was observed in the control plants (Figure 2d). In Borujerd, although a temperature decline to \(-8^\circ\) did not significantly decrease leaf carotenoids content, the temperature decline from \(-12^\circ\) to \(-24^\circ\) significantly decreased the leaf carotenoids content. In Neyshabur, the lowest leaf carotenoid content was found at \(-20^\circ\), 48% lower than 0°C. A temperature decline from 0 to \(-4^\circ\) also led to a 45% decrease in the leaf carotenoids content of Neyshabur, whereas the temperature decline to \(-12^\circ\) increased leaf carotenoids content by 16% over \(-4^\circ\).

#### 3.1.2 Gas exchange variables

The landraces \(N_p\) responded differently to low temperatures. Generally, Borujerd showed a higher \(N_p\) at all temperatures (except for the control) compared with Neyshabur (Figure 3a). At \(-12^\circ\), however, \(N_p\) of Borujerd was 43% greater than that of Neyshabur. Borujerd \(N_p\) was stimulated as temperature declined to \(-4^\circ\); however, it showed a relatively constant trend between \(-4^\circ\) and \(-12^\circ\). In Neyshabur, on the other hand, \(N_p\) showed a declining trend with a gentle slope as the temperature declined to \(-8^\circ\), but the reduction was more pronounced when the temperature declined from \(-8^\circ\) to \(-12^\circ\) (37% over \(-8^\circ\) to \(-12^\circ\)) (Figure 3a). A somewhat similar trend of changes was observed in \(N_p\) and \(T_s\) in both landraces at temperatures lower than \(-4^\circ\) (Figure 3a,b). Transpiration rate was also increased as temperature declined from 0°C to \(-4^\circ\) (Figure 3b). The results showed that \(N_p\) and \(T_s\) were coupled. A relatively constant trend was observed in temperatures between \(-4^\circ\) and \(-12^\circ\) in Borujerd. Unlike, a more significant temperature decline to \(-12^\circ\) led to a reduction in Neyshabur \(T_s\), and ultimately, reached zero at \(-16^\circ\).

There was no difference in \(g_s\) between the landraces. In both landraces, although \(g_s\) was stimulated as temperature declined to \(-8^\circ\), it showed a descending trend thereafter and reached zero at \(-16^\circ\) (Figure 3c). Nevertheless, freezing temperature and fava bean landrace interacted to affect \(g_m\) (Figure 3d). A greater \(g_m\) was recorded in Borujerd at \(-12^\circ\) and \(-16^\circ\) by 67% and 100%, respectively, compared with Neyshabur. At 0°C, Neyshabur had a 26% higher \(g_m\) than Borujerd, but different trends were observed after that. Borujerd \(g_m\) increased by 41% at \(-12^\circ\) compared with 0°C and significantly decreased to zero at \(-20^\circ\).

As shown in Figure 4a, no significant changes in \(C_i\) were found in temperatures ranged from 0°C to \(-12^\circ\). In comparison, more temperature decline significantly diminished \(C_i\) in both landraces to zero at \(-16^\circ\) and \(-20^\circ\) in Neyshabur and Borujerd, respectively. The \(C_i\) \(C_s\) ratio showed a relatively constant trend up to \(-12^\circ\) in both landraces; however, it declined with a sharp slope thereafter. At \(-16^\circ\),
**FIGURE 2** Changes in leaf (a) chlorophyll a, (b) chlorophyll b, (c) total chlorophyll, and (d) carotenoids content of faba bean landraces under freezing temperatures. Means with the same letters are not significantly different at 5% level of probability based on the least significant difference (LSD) test. $n = 12$.

**FIGURE 3** Changes in leaf (a) net photosynthetic rate, (b) transpiration rate, (c) stomatal conductance, and (d) mesophyll conductance of faba bean landraces under freezing temperatures. Colored areas indicate ±SE. $n = 12$. Asterisk represents significant differences between the landraces.
Borujerd had significantly higher Ci:Ca than Neyshabur (0.3 against 0 in Borujerd and Neyshabur, respectively) (Figure 4b). WUEi reduction started at 0°C and continued to −12°C with a relatively gentle slope in both landraces but severely decreased thereafter (Figure 4c). At −12°C, WUEi decreased 31% and 36% in Borujerd and Neyshabur, respectively, compared with 0°C; however, it reached zero in Neyshabur at −16°C.

Undesirable effects of freezing temperatures on SPAD readings were more evident in temperatures lower than −12°C, with the lowest mean at −16°C, although the landraces responded differently to freezing temperatures (Figure 4d). In Borujerd, SPAD readings increased as the temperature declined to −8°C; Borujerd SPAD readings were 35% and 12% greater than that of Neyshabur but decreased by 84% at −16°C compared with −8°C. In Neyshabur, however, the reduction of SPAD readings started at −4°C (by 30% compared with 0°C). Although Neyshabur SPAD readings improved by 23% from −4°C to −12°C, it reduced again in temperatures lower than −12°C (Figure 4d).

### 3.2 Recovery experiment

#### 3.2.1 Gas exchange variables

$N_p$, $g_s$, and $g_m$ significantly differed between the landraces during the recovery period (Figure 5a,c,d). $N_p$ severely decreased from Day 2 ATR and reached the lowest on the seventh day of the recovery, but it tended to increase from the seventh to 21st days ATR with the highest on Day 14 (Figure 5a). For instance, Borujerd and Neyshabur $N_p$ diminished 3.5 and four times, respectively, on Day 7 ATR, compared with the second day of the recovery. However, $N_p$ peaked on Day 14 by 30% and 36% increases in Borujerd and Neyshabur, respectively, compared with the second day of the recovery period. Stomatal and mesophyll conductance had a similar trend to $N_p$. They decreased during the recovery days of second, fourth, and seventh, and then, the plants experienced an increase in $g_s$ and $g_m$ (Figure 5c,d).

Recovery time and faba bean landrace interacted to affect $T_r$ (Figure 5b). Similar to $N_p$ and $g_s$, $T_r$ also decreased to Day 7 ATR and increased thereafter. Borujerd $T_r$ increased by a sharper slope from Days 7 to 14 than Neyshabur. On a percent basis, Borujerd $T_r$ was significantly higher than Neyshabur $T_r$ on Days 14 and 21 by 86% and 43%, respectively (Figure 5b).

Although the faba bean landraces $C_i$ fluctuated less than other traits during the recovery period, they showed different trends. Borujerd $C_i$ had a downward trend until Day 7 ATR and then increased with a gentle slope, whereas Neyshabur $C_i$ showed an almost constant decreasing trend (Figure 6a). On the other hand, $C_i:Ca$ differed between the landraces and recovery time. The same trend as $N_p$ was observed for $C_i:Ca$; however, unlike $N_p$, significant differences were observed between the landraces on Days 7 and 21 ATR (Figure 6b). Instantaneous water use efficiency decreased to Day 7 of

![Figure 4](image-url)

**Figure 4** Changes in leaf (a) intercellular CO$_2$ concentration, (b) Ci:Ca ratio, (c) instantaneous water use efficiency, and (d) SPAD of faba bean landraces under freezing temperatures. Colored areas indicate ±SE. $n = 12$. Asterisk represents significant differences between the landraces.
FIGURE 5 Changes in leaf (a) net photosynthetic rate, (b) transpiration rate, (c) stomatal conductance, and (d) mesophyll conductance of faba bean landraces during the recovery period; 2, 4, 7, 14, and 21 days after freezing stress (DAF). Colored areas indicate ±SE. \( n = 12 \). Asterisk represents significant differences between the landraces.

FIGURE 6 Changes in leaf (a) intercellular \( \text{CO}_2 \) concentration, (b) \( \text{C}_i : \text{C}_a \) ratio, (c) instantaneous water use efficiency, and (d) SPAD of faba bean landraces during the recovery period; 2, 4, 7, 14, and 21 days after freezing stress (DAF). Colored areas indicate ±SE. \( n = 12 \). Asterisk represents significant differences between the landraces.
the recovery period (Figure 6c). Nevertheless, the landraces showed different behavior thereafter. Although WUEi was relatively lower in Neyshabur compared with Borujerd to Day 7 ATR, it sharply increased in Neyshabur and reached a level higher than in Borujerd. For instance, WUEi increased from Day 7 ATR with a sharper slope in Neyshabur to the highest level on Day 14 (2.8 times higher than that in Borujerd) (Figure 6c).

Despite the significant decrease in SPAD in poststress days, its trend experienced low changes during the recovery period. According to Figure 6d, the SPAD value decreased with a gentle slope after a relatively stable trend up to Day 7 ATR. However, SPAD ultimately decreased by 33% and 24% in Borujerd and Neyshabur, respectively, on Day 21 compared with Day 2 of the recovery period.

3.2.2 | Leaf area and above-media dry weight

In Borujerd and Neyshabur, a decrease in temperature from 0°C to −4°C reduced the LA by 20% and 10%, respectively (Figure 7a). Borujerd LA was more sensitive to freezing temperatures than Neyshabur, that is, the damage to Borujerd plants started at −4°C, whereas Neyshabur LA was not significantly affected to −12°C. Although Neyshabur had a greater LA at all temperatures over Borujerd, however, Neyshabur LA was significantly decreased with a sharper slope (a 97% decrease) from −12°C to −16°C (Figure 7a). The faba beans above-media DW was significantly affected by the interaction of the landrace and freezing temperature. Similar to the plant LA, Neyshabur had a greater DW at all temperatures over Borujerd. A 4°C decline in temperature (0°C to −4°C) diminished above-media DW by 22% and 18% in Borujerd and Neyshabur, respectively (Figure 7b). Further temperature decline beyond −12°C sharply decreased above-media DW in both landraces, where dry weight reached zero at −16°C.

3.2.3 | Plant SU

Even a 4°C decline in temperature (0°C to −4°C) adversely affected the plant SU (Figure 7c). Temperature decline from 0°C to −12°C reduced the plant SU by 17%, that is, SU decreased by 1.4% per each temperature degree depression (Figure 7c). Ultimately, no plant remained survived at −20°C.

3.2.4 | LT50su and RDMT50

A significant difference was observed between the landraces in terms of LT50su. Both landraces remained their LT50su up to −10°C, but the SU declined by increasing the freezing temperature intensity (Figure 8a). Borujerd lost 50% of their plants at −11.2°C, making it a more tolerant faba bean landrace, whereas Neyshabur lost 50% of survival at −10.8°C; accordingly, Neyshabur showed more sensitivity.
to freezing stress than Borujerd. However, Borujerd with an RDMT$_{50}$ of $-12.9$ $^\circ$C showed a lower rate of regrowth after the recovery period compared with Neyshabur, which means a decrease in temperature to less than $-12.8$ $^\circ$C caused a 50% decrease in Borujerd above-media DW while decreasing the temperature to less than $-13.2$ $^\circ$C reduced the above-media DW of Neyshabur by 50% (Figure 8b).

4 DISCUSSION

Although both landraces were originated from cold and high-latitude regions, significant differences were observed between them regarding the photosynthetic performance, plant survival, and above-media DW. The results of Zhou et al. (2018) also revealed that the geographical origin could not directly predict the genotype climate resilience. They treated 127 faba bean genotypes by single cold stress or a combination of a single episode of cold or heat stress and found a wide range of responses to the treatments. They clustered the genotypes into four groups according to cold and heat stresses and distinguished faba beans’ susceptibilities under temperature stress. They found the photosynthetic parameters useful for detecting the extreme temperature damages to photosystem II and identifying tolerant faba bean genotypes.

Here, the net photosynthetic rate decreased with a sharp slope from the second day of the recovery period and finally reached a minimum value on Day 7 ATR. Afterward, the trend of the changes indicated that the living plants were able to improve $N_p$ during the recovery period, in which the highest $N_p$ was recorded on Day 14 ATR. Although a severe decline in $N_p$ occurred at the same temperature for both landraces ($-12$ $^\circ$C), Neyshabur was more sensitive due to a faster onset of $N_p$ reduction. Given that the plant processes, such as photosynthesis, require different appropriate temperatures depending on the species origin and genetic background, it indicates that Borujerd probably could maintain its photosynthesis and optimal growth up to $-12$ $^\circ$C at the early growth stages.

Hasanfard et al. (2020) found that a temperature decline beyond $-12$ $^\circ$C disrupted PSII electron transport. In their experiment, turnipweed ($Rapistrum rugosum$ (L.) All.) leaf $F_v/F_m$ decreased at temperatures lower than $-12$ $^\circ$C during the first 24 h of the recovery period. The photosynthetic process is always affected by temperatures beyond the plant tolerance range, ultimately reducing plant growth. One possible reason for the decrease in $N_p$ in this experiment is the disruption of chlorophyll synthesis and damage to chloroplast membranes. A further reduction of freezing temperatures completely damaged the leaf structure and finally ceased the photosynthesis. Low temperature is one of the leading causes of chloroplast structure destruction, disrupting photosynthesis in plants (Kingston-Smith et al., 1997). Zhou et al. (2017) also showed that cold stress damaged the photosynthetic apparatus of $Rhododendron chrysanthemum$ Pall plants and ceased photochemical activities.

A somewhat similar trend of changes was observed in $N_p$ and $T_r$ in both landraces at temperatures lower than $-4$ $^\circ$C. Considering the role of stomata in both processes, it seems that decreasing CO$_2$ inflow and water outflow from the leaves is due to stomatal closure during freezing stress. Unlike Neyshabur, a relatively constant $N_p$ and $T_r$ trend was observed in temperatures of $-4$ $^\circ$C to $-12$ $^\circ$C in Borujerd. Because freezing-affected plants show symptoms similar to the drought-stressed, lower transpiration at low temperatures is probably due to stomatal closure. Evaluation of several plant species indicated that temperature reduction from 25 $^\circ$C to 0 $^\circ$C resulted in lower transpiration in most species (Gupta et al., 2016). The results showed that $g_m$ and $N_p$ were coupled during the recovery period (Figure 9a). In response to cold stress, stomatal closure occurs due to decreased leaf turgor, and atmospheric pressure ultimately alters photosynthesis and mesophyll metabolism (Medici et al., 2007; Xu & Zhou, 2008). Photosynthesis is more affected by stomatal closure because the diffusion
of CO₂ is more inhibited relative to H₂O (Medici et al., 2007). Stomatal conductance improvement and consequently, Np from the seventh day of recovery, indicated that gₛ might consider an important factor for rapid and reversible plant reaction.

Instantaneous water use efficiency decreased in both landraces after the seventh day of the recovery period, which was more significant in Neyshabur and increased with a sharper slope to Day 14 compared with Borujerd. WUEₛ is an important factor in determining crop yield and is considered one of the parameters in the determination of plant tolerance to freezing stress (Navarrete-Campos et al., 2013), which is decreased in low temperatures as shown by Gupta et al. (2016). Given that WUEₛ is obtained from dividing net photosynthetic by transpiration rate, Neyshabur had less transpiration during the recovery days compared with Borujerd, whereas Np was higher in Borujerd compared with Neyshabur, which indicated that the rate of transpiration in the determination of the WUEₛ was more plausible in these two landraces. Stomatal closure reduces WUEₛ mainly due to the higher effects of gₛ on CO₂ inflow than H₂O outflow the leaves. The higher Borujerd Tₛ during the recovery period than Neyshabur, especially from the seventh day, indicates a higher stomatal conductance of Borujerd. In other words, the Tₛ trend is highly consistent with photosynthesis and stomatal conductance, which could indicate that higher gₛ led to higher Np and Tₛ (Figure 9b).

Here, at all freezing temperatures except for −24°C, Neyshabur had lower leaf Chl content than Borujerd, which could be attributed to the higher ability of Borujerd to cold acclimation. Temperature decline from 0°C to −4°C also led to a 45% reduction in leaf carotenoids content in Neyshabur. In contrast, more temperature decline beyond −16°C increased leaf carotenoids content, which probably could be due to an acclimate to freezing temperatures to minimize the adverse effects of freezing temperatures on the photosynthetic process. However, the rate of leaf carotenoids reduction was landrace dependent. Relative ability to maintain photosynthetic pigments is probably due to plant strategies to absorb light at low temperatures and maintain the metabolic processes. In an experiment by Hurry et al. (1995), it was found that autumn rapeseed (Brassica napus L.) had higher chlorophyll content than the spring cultivar, which resulted from a better cold acclimation of this cultivar. Studying the effect of low temperature on chickpea (Cicer arietinum L.) revealed that low temperatures decreased the genotypes Chla content differently (Nezami et al., 2007).

As temperatures declined below −12°C, both landraces suffered from severe damage, and at −16°C, the LA was almost zero in both landraces due to plant death. Accordingly, Khorsandi et al. (2015) observed that although a decrease of temperature below −6°C had no specific effect on the greenness level of Nigella sativa L. (Gonabad ecotype), a decrease of temperature below −9°C led to a 92% reduction in the greenness level compared with the temperature of −6°C. In the present experiment, Neyshabur and Borujerd maintained their green LA up to −3°C; however, temperature reduction to −4°C led to 10% and 20% decreases in the LA, respectively, compared with the temperature of 0°C.

A higher Np was associated with a greater above-media DW. High photosynthetic efficiency supplies the energy required for cold tolerance (Huner et al., 1998). In a study, plant DW of freezing-stressed grass species decreased after a recovery period (Nezami et al., 2016). They also observed different plant SU in grass species; that is, the SU ranged from 15% to 50% at −12°C in various species. However, in the present experiment, plant SU decreased by 80% when the temperature declined from −12°C to −16°C. In other words, each degree reduction in temperature in this range reduced the survival rate by 20%. However, all plants died at a temperature of −20°C. The results suggested that the survival rate increased with increasing the rate of Np in faba bean plants. In a study by Nezami et al. (2016), the lethal temperature of 50% of the tolerant species was about 6°C lower than the susceptible species, that is, lower LT50su indicates more tolerance of plants to freezing temperatures and more prolonged survival. Other studies have also denoted the LT50su as a suitable indicator to assess plants’ cold tolerance (Fowler et al., 1996; Liang et al., 2003). It was also found that freezing-tolerant species of forage legumes had a better regrowth than susceptible species, and a positive correlation was observed between LT50su and plant regrowth rate after stress (Hekney et al., 2006).

**FIGURE 9** Linear regression relationship and Pearson’s correlation coefficient (Pearson’s r = r) between net photosynthetic rate and (a) mesophyll conductance and (b) transpiration rate in faba bean landraces under freezing temperatures.
Although the SU and LT50 are suitable indices to assess the plant tolerance to freezing stress, sometimes freezing stress may not kill some plants but affect their regrowth after the freezing relief (Hekneby et al., 2006). Under such conditions, the plants will not be able to recover and resume their regrowth properly. RDMT50 will be able to make such a distinction between the plants in terms of freezing tolerance. Although Borujerd showed a relatively higher SU during the recovery period in the present study, Neyshabur had a better regrowth ability with a higher RDMT50 (−12.9°C vs. −13.2°C in Borujerd and Neyshabur, respectively) during such conditions (Figure 8b). In their study on three grass species, Perry and Herrick (1996) reported that Dianthus deltoides had a better regrowth than Aquilegia vulgaris and Lavandula (L.) by declining the temperature to −14°C. In a study by Hekneby et al. (2006), which was performed to determine the freezing tolerance of several forage legumes, it was also found that tolerant species had better regrowth than susceptible species. Studies have attributed the decline in plant dry weight during the recovery period to the effects of freezing damage on plants and their shoot regrowth ability (Azizi et al., 2007). Nezami et al. (2007) found a high correlation between LT50 and RDMT50 of chickpea genotypes and denoted that RDMT50 of the tolerant genotypes was lower than susceptible genotypes.

5 | CONCLUSIONS

Freezing stress reduced SU and above-media DW at the end of the recovery period. However, the response of the parameters varied depending on the intensity of stress and the landrace. Although the study results showed that the temperature decline to −12°C mostly imposed no significant change in photosynthetic apparatus despite the fluctuations of the other parameters, temperatures below −12°C disrupted the photosynthetic system of both landraces and reduced Np efficiency. During the recovery period, photosynthetic efficiency was minimized until the seventh day due to the freezing damage induced on plants. Faba bean plants showed a recovery ability to improve freezing damage induced after the seventh day of the recovery period, which indicates the high ability of this plant in repairing the photosynthetic system and continuing carbon assimilation. According to the results of the relationship between Np, SU, and Np, can be a valuable indicator for evaluating the plant tolerance to freezing stress. The temperatures up to −10°C and −12.5°C, respectively, were obtained as LT50, and RDMT50 in both landraces. According to the results, these faba bean landraces may tolerate freezing stress up to −10°C (by enduring minor damages).

CONFLICT OF INTEREST
There is no conflict of interest.

AUTHOR CONTRIBUTIONS
Jafar Nabati: Supervision, conceptualization, methodology, investigation, project administration, review and editing, and validation. Alireza Hasanfard: Data collecting, data curation, and resources. Ahmad Nezami: Validation and supervision. Mohammad Javad Ahmadi-Lahijani: Software, formal analysis, writing—original draft, and writing—review and editing. Elaheh Boroumand Rezaadzeh: Investigation and data curation. The first draft of the manuscript was written by Mohammad Javad Ahmadi-Lahijani, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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None.

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All authors consented for participation.

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