




Freezing stress induces changes in the morphophysiological of chickpea and wild mustard seedlings

Mehdi Rastgoo¹  | Ahmad Nezami^{1,2}  | Alireza Hasanfard¹ | Jafar Nabati² | Mohammad Javad Ahmadi-Lahijani¹ 

¹Department of Agrotechnology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

²Research Center for Plant Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

Correspondence

Mehdi Rastgoo, Department of Agrotechnology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.

Email: m.rastgoo@um.ac.ir

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Abstract

Wild mustard is one of the common and troublesome winter weeds of chickpea fields and a great competitor to reduce the chickpea productivity. Plant species (chickpeas cv. Saral and wild mustard) were compared at freezing temperatures (+4 as a control, 0, −4, −8, −12, −16, and −20°C) based on the morphophysiological traits and their recovery ability. Chickpea chlorophyll fluorescence was more sensitive to low temperatures than wild mustard. Chickpea and wild mustard F_v'/F_m' (light-adapted maximum efficiency of photosystem II [PSII] photochemistry) decrease 33% and 11% exposed to −16°C, respectively, compared with +4°C. Particularly at lower temperatures, wild mustard electrolyte leakage was smaller than that of chickpea; the temperature drop had a greater impact on the stems than the leaves. Per temperature degree drop from −12 to −20°C, the survival probability decreased by 12.5%. Wild mustard had a greater root dry matter (RDM) compared with chickpea plants. 50% dry matter depression temperature (RDMT₅₀) could better distinguish among the species freezing response; wild mustard RDMT₅₀ was ~1°C higher than chickpea. Plant survival and F_v'/F_m' correlation suggested the reliability of chlorophyll fluorescence measurements to assay plants freezing tolerance. The important contribution of a more powerful root system to wild mustard survival under adverse circumstances may be suggested by the positive association between plant survival and RDM. Higher tolerance of wild mustard to freezing stress ultimately leads to greater survival, regeneration, continued growth, and geographical distribution. Therefore, the wild mustard invasion will be possible in chickpea fields after freezing stress, especially in the cold climates and high-altitude regions.

KEYWORDS

chlorophyll fluorescence, instantaneous WUE, mesophyll conductance, photochemical quenching, stomatal conductance, survival

1 | INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the most important sources of proteins, carbohydrates, vitamins, and nutrients (Venkidasamy et al., 2019). Chickpea planting is widely popular in many parts of the world, especially in West Asia and North Africa, due to the low-input requirement. Chickpeas are grown in more than 50 countries worldwide, and India is known as the world's largest producer, with 73% (FAOSTAT, 2020). Iran, the United States, and Pakistan are among the largest producers of this product; each produces about 2% of the world's chickpeas (FAOSTAT, 2020). This plant is the main option in providing protein for the human body worldwide, especially in developing countries (Shafaei et al., 2016).

In Iran, with about 40% of production, chickpea is in the first place among pulse crops (Agricultural Statistics, 2018). According to Loke et al. (2016), the main reasons for consuming legumes are low fat and sodium and the lack of cholesterol and gluten in these products. Besides, because legumes are rich sources of iron, protein, fibers, and potassium, they can be used as important sources to meet the body's nutritional needs.

Weeds are one of the most critical factors that seriously reduce the chickpea yield in Iran on average by 50% (Nosratti et al., 2017). Annual winter weeds can interfere directly with crops and serve as alternative hosts for important pests, especially in reduced tillage systems (Hayden et al., 2012). However, one of the crucial limitations of the successful production of chickpeas is the presence of weeds. Due to dwarfism, slow establishment, and limited vegetative growth, chickpea plants have little ability to compete with weeds during the growing season (Toker et al., 2012).

Wild mustard (*Sinapsis arvensis* L.) is a broadleaf and annual winter weed that belongs to the *Brassicaceae* plant family. Wild mustard has indeterminate upright growth and may reach a height of more than two and a half meters (Siyahpoosh et al., 2012). This weed proliferates extreme spreading by producing thousands of seeds. Wild mustard is one of the most critical weeds in chickpea fields, distributed in most parts of Iran, and causes damage to autumn crops such as chickpeas (Shahbazi et al., 2019). Increasing the wild mustard density reduces the leaf area, canopy structure, plant height, and crop plant yield (Shahbazi et al., 2019). Besides, the stable seed bank, high competitiveness, high regeneration, and herbicide resistance are among the most critical problems of wild mustard control (Warwick et al., 2005), making it necessary to study it in various fields.

One of the most important factors that affected the growth and geographical distribution of plant species (crops and weeds) and crop-weed interference is freezing stress. Plant tolerance to freezing stress is considered a factor determining their success after winter (Interrante et al., 2020; Nabati et al., 2021). On the other hand, examining the ability of weeds to compete with crops after the recovery period and adopting appropriate management methods to control them is considered one of the new strategies in weed control in autumn crops. Mittler (2002) defined "freezing" or "chilling" stress as damage to plant sensitive organs due to a sudden decline in temperature during the growing season, which can occur even at

temperatures above 0°C (0 to 5°C). At this temperature range, the ice nucleus does not form in the cell.

After freezing stress, recovery capacity is one of the consequences of high tolerance to freezing stress in weeds, leading to more remarkable survival, continued growth, regeneration, and geographical distribution. Working on 12 weed species in different regions of Canada showed significant variations in freezing tolerance among the species. According to the experiment results, due to their high tolerance to cold stress, autumn weed species were more successful regarding their period and spatial distribution in the fields (Cici & Van Acker, 2011).

Freezing stress should be considered an important indicator in assessing plant suitability, including weeds and crops, in cold regions. In other words, freezing stress tolerance is probably influential in the establishment and competitiveness of competing species. Therefore, according to the definition of fitness, that is, weed ability to establish, survive, and successfully regenerate under non-applying herbicides conditions, freezing tolerance is considered one component of fitness (Park et al., 2004). The high density of winter weeds such as wild mustard in spring in Iranian winter crops indicates the overwintering potential of these weeds (Hasanfard et al., 2021). Therefore, the lack of proper cold acclimation of winter crops in future climate change scenarios increases the likelihood of winter weed dispersal and invasion.

Stomatal factors are considered one of the limiting factors of photosynthesis, which reduce carbon dioxide entrance into the inter-cellular space due to reducing stomatal conductance. This will disrupt the process of carbon fixation and photosynthesis (Ahmadi-Lahijani et al., 2018). Any environmental stress, biotic or abiotic, directly or indirectly, would affect the leaf stomatal aperture, which, in turn, interferes with the leaf photo assimilation rate. The plant cell membrane fluidity is decreased exposed to low temperatures, resulting in damage to the proteins and other membrane components. Besides, low temperatures reduce the activity of some enzymes, especially those involved in the photosynthetic procedure. The leaf chlorophyll fluorescence study can determine the value of damage to photosystem II (PSII) (Murchie & Lawson, 2013). Therefore, gas exchange variables and chlorophyll fluorescence will help to determine the plant stress tolerance.

In general, one of the determining factors in the geographical distribution and invasion ability of weeds is the threshold of freezing stress tolerance, the study of which, while improving basic information, will predict their distribution pattern and provide appropriate management strategies. Freezing and chilling stresses threaten chickpea fall cultivation. Wild mustard is one of the common winter weeds of chickpea fields and a great competitor to reduce chickpea productivity. Due to limited information on the competing ability of wild mustard with chickpeas under freezing stress conditions, this study was carried out aimed to (1) obtain basic information about the freezing tolerance threshold in wild mustard, (2) investigate the chlorophyll fluorescence and photosynthetic parameters of wild mustard and chickpea exposed to freezing stress, (3) compare the freezing tolerance of wild mustard compared to chickpeas to freezing stress,

(4) assess the ability of wild mustard to compete with chickpeas after the recovery period, and (5) predict the distribution and possible invasion of wild mustard in cold regions according to its freezing tolerance threshold.

2 | MATERIAL AND METHODS

2.1 | Plant materials and growth conditions

This study was performed under a natural condition at the Faculty of Agriculture, Ferdowsi University of Mashhad (35.74°N, 57.57°E; altitude 985 m above sea level). Experimental factors included plant species (chickpeas cv. Saral and wild mustard) and freezing temperatures (in seven levels including +4 as control, 0, −4, −8, −12, −16, and −20°C).

Chickpea seeds were prepared from the Mashhad Chickpea Collection (MCC) at the Research Center for Plant Sciences, Ferdowsi University of Mashhad. Wild mustard seeds were collected from about 500 mature plants from infested farms around Mashhad-Iran in June 2019. Because dormancy was found in wild mustard seeds in an initial germination experiment, before sowing, dormancy was broken after being immersed in a 0.2% potassium nitrate (KNO₃) solution for 3 days at 5°C. The seeds were then placed in 9 cm Petri dishes on moist filter papers and kept at 20°C for 72 h to germinate. Both species seeds were sown in mid-November in plastic pots (12 cm diameter) containing farm soil, leaf mold, and sand (v:v 1:1:1) in a glasshouse (22/16 ± 2°C day/night and the photoperiod ~10 h). Ten seeds of each plant species were sown in each pot separately and were thinned to five after establishment. The plants were normally irrigated during the growing periods. The last irrigation was done 24 h before the application of freezing treatments.

The pots were kept in natural conditions (Figure 1) outside the glasshouse to the 2–4 leaf stage to adapt to the low temperatures for

cold acclimation. Then, pots were moved to a thermogradient freezer to expose the freezing temperatures. First, the pots were placed at a temperature of 5°C. Then, the temperature decreased at a rate of 2°C per hour to reach the intended temperature. To prevent the ice nuclei formation, that is, the supercooling phenomenon, a thin layer of INAB (ice nucleation active bacteria) was sprayed on the seedlings to produce ice nuclei (Zhang & Liu, 2018). The plants were kept at the intended temperature for 1 h and then taken out of the thermogradient freezer and immediately placed in a growth chamber at 5 ± 1°C for 24 h to decrease the speed of ice melting. The pots were then placed in a pre-cold acclimation condition for 3 weeks to recover.

2.2 | Measurements

2.2.1 | Gas exchange variables

Leaf photosynthetic parameters were measured 1 week after freezing stress. The youngest fully developed leaves were used to measure the photosynthetic parameters between 10:00 a.m. and 2:00 p.m. three times for each treatment. Net photosynthetic rate (A_N), transpiration rate (E), and intercellular CO₂ concentration (C_i) were measured using a portable photosynthesis system (ADC Bio Scientific Ltd, UK) at approximately 1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PAR (photosynthetically active radiation), relative humidity of 40% ± 5%, the ambient CO₂ concentration, and a 25°C leaf temperature. Stomatal conductance (g_s) was measured using a portable leaf porometer (SC-1, USA). Instantaneous (WUE_i) and intrinsic (A_N/g_s) water use efficiency were calculated by dividing A_N by E and g_s , respectively. Mesophyll conductance (g_m) was also calculated by dividing A_N by C_i (Ahmadi-Lahijani & Emam, 2016). At the same time, leaf chlorophyll content (SPAD value) was measured on the same leaves using a handheld chlorophyll meter (SPAD 502, Spectrum Technologies, Inc.).

2.2.2 | Leaf chlorophyll fluorescence

Leaf chlorophyll fluorescence parameters, including light-adapted maximum efficiency of PSII photochemistry (F_v'/F_m'), photochemical quenching (qP, also known as F_q'/F_v'), and the quantum efficiency of PSII electron transport (ΦPSII , also known as F_q'/F_m'), were recorded from the youngest fully developed leaf by a fluorometer (Opti Science, Inc.). The glass fiber was set at a 1 mm distance from the leaf and a saturation pulse PPFD (photosynthetic photon flux density) at ~7500 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 0.8 s. Chlorophyll fluorescence was measured before transferring the plants to the freezing temperatures. The trend of chlorophyll fluorescence changes was recorded 6, 12, 24, 48, and 72 h after freezing stress (AFS) during a recovery period.

The linear electron transport rate (J) described as in Equation (1) (Genty et al., 1989):

$$J = \Phi\text{PSII} \times \text{PPFD} \times (0.5) \quad (1)$$

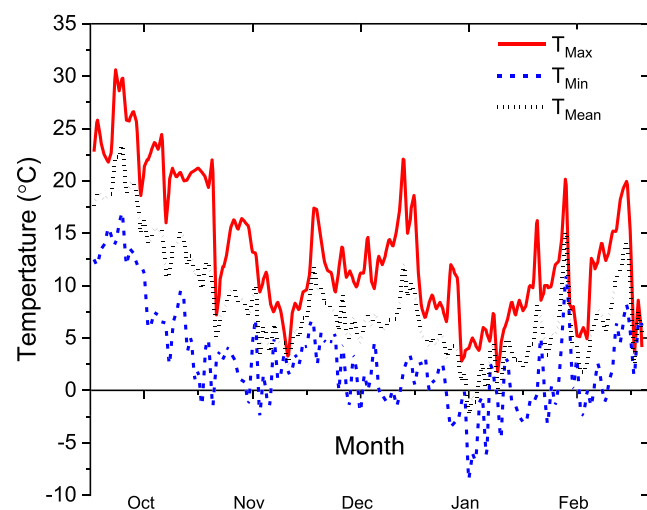


FIGURE 1 Maximum, minimum, and mean air temperatures during the experiment

PFDa (absorbed photon flux density) is the absorbed light ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), and 0.5 is a factor (accounts for the partitioning of energy between PSII and PSI). It is generally not practical to measure the light absorbed by a leaf; therefore, relative changes of J can usefully be monitored by simply multiplying ΦPSII by incident light (Maxwell & Johnson, 2000). Therefore, Equation (2) can be modified as

$$J = \Phi\text{PSII} \times \text{PFDi} \quad (2)$$

2.2.3 | Electrolyte leakage (EL)

A complete plant with two fully developed true leaves and its stems was separated and placed in vials containing 50 ml of deionized water from each pot. The vials were placed in the laboratory temperature for 24 h, after which the electrolyte leakage was measured using an electrical conductivity meter (Jenway Model 4510) and recorded as EC_1 . The vials were transferred to an autoclave (110°C and 1.2 atm) for 30 min. They were then placed in a laboratory temperature for 24 h, and the electrical conductivity was again measured and considered as EC_2 . The percentage of electrolyte leakage (EL%) was calculated using Equation (3) (Ghoulam et al., 2002).

$$\text{EL}(\%) = \left(\frac{\text{EC}_1}{\text{EC}_2} \right) \times 100 \quad (3)$$

2.2.4 | Plant growth parameters

Growth parameters were measured after 21 days of recovery. Leaf area (LA) was measured by a leaf area meter (Delta-T, Type WDIGC-2). Above- and below-media dry matter (leaf + stem; shoot dry matter [SHDM] and root; root dry matter [RDM], respectively) was determined after being oven-dried at 80°C temperature to constant weight. Plant height (PH), leaf number (LN), and branch number (BN) per plant were also recorded.

2.2.5 | Plant survival (SU)

To determine the survival percentage and plant regrowth, the pots were transferred to the greenhouse at $\sim 20^\circ\text{C}$ and kept for 21 d under a natural photoperiod. The percentage of plant survival was measured by counting the number of alive plants before frost stress and 21 days after the freezing stress using Equation (4).

$$\text{Survival}\% = \left(\frac{A}{B} \right) 100 \quad (4)$$

Here, B and A are the number of alive plants before and after freezing stress, respectively.

2.2.6 | 50% lethal temperature (LT_{50})

To determine the lethal temperature of 50% of the plants based on the 50% dry matter depression temperature (RDMT_{50}) and the survival percentage ($\text{LT}_{50\text{su}}$), the logistic equation for the data of each species at different temperatures was fitted using Equations (5) and (6), respectively (Eizenberg et al., 2005):

$$y = \frac{a}{1 + e^{-k(x-xc)}} \quad (5)$$

$$y = \frac{a}{1 + be^{-kx}} \quad (6)$$

In this equation, y represents the survival percentage, x represents the freezing temperature, a is one of the coefficients of the equation and represents the maximum survival percentage, b is another coefficient of the equation and represents the slope of the curve at point x , and xc represents the point of x at which y is equal to 50% of its maximum value (LT_{50}). 50% dry matter depression temperature was determined by drawing the dry matter diagrams of plants against freezing temperatures and determining each curve midpoint.

2.2.7 | Statistical analysis

Data were subjected to a two-way analysis of variance (ANOVA), followed by the calculation of the Tukey's Studentized Range (HSD) test at the $p \leq 0.05$ probability level using the SAS software (v.9.1, SAS Institute Inc., Cary, NC, USA). Experiments for traits such as electrolyte leakage and plant growth parameters were performed as a factorial arrangement in a completely randomized design and for chlorophyll fluorescence as a factorial experiment in a randomized complete block design with four replications. The origin software was used to determine the LT_{50} .

3 | RESULTS

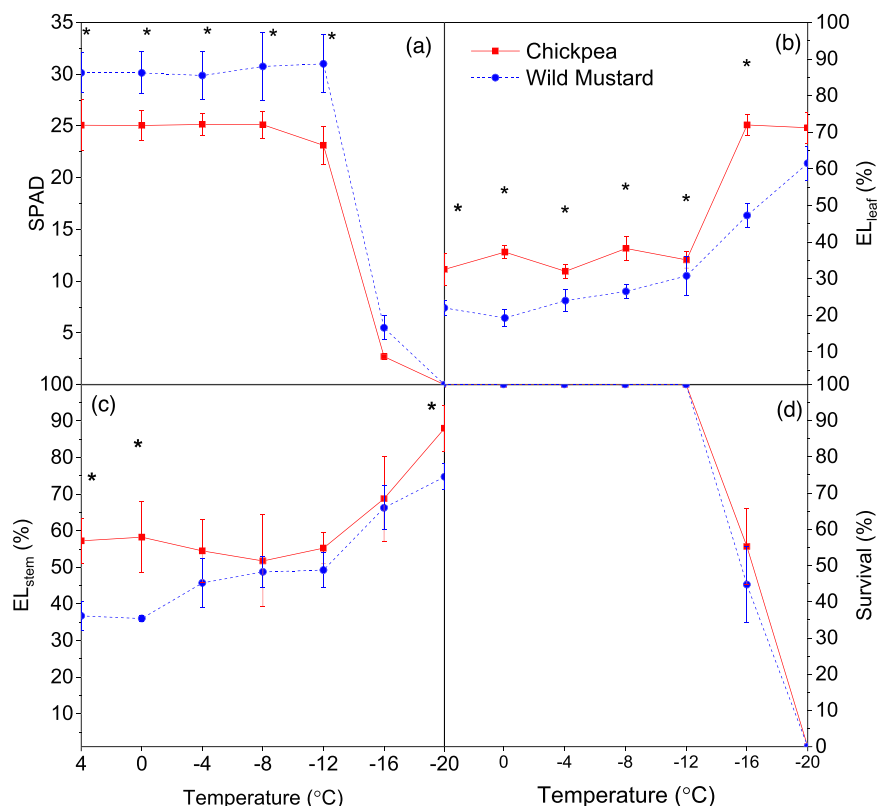
3.1 | Leaf chlorophyll content (SPAD value)

According to Figure 2a, the SPAD value decreased with a sharp slope after a stable trend up to -12°C and ultimately reached zero at -20°C . Wild mustard SPAD was higher at all temperatures compared with chickpea plants. Decreasing temperature to -16°C decreased SPAD by 4.8 and 4.6 times in chickpea and wild mustard, respectively, compared with -12°C (Figure 2a).

3.1.1 | Electrolyte leakage (EL)

The damage to cell membranes was significantly affected by temperature and plant species (Figure 2). With decreasing

FIGURE 2 Changes in leaf chlorophyll content (SPAD) (a), leaf (b) and stem electrolyte leakage (c), and survival percentage (d) of chickpea and wild mustard plants under freezing temperatures. Each point is average, and vertical bars indicate \pm SD ($n = 4$). Asterisk represents significant differences between the plants at $p \leq 0.05$.



temperature, the EL percentage increased, in which chickpea plants were more susceptible than wild mustard; that is, wild mustard EL was lower than that of chickpea in most temperatures. Stems were more affected by the temperature decline; for example, the percentages of chickpea and wild mustard EL_{stem} were 88% and 75% at -20°C , respectively, whereas the EL_{leaf} was 70% and 60% chickpea and wild mustard, respectively, at -20°C (Figure 2b,c). Up to -12°C , there was a relatively constant trend in leaf and stem EL of the species; however, it significantly increased at -16°C thereafter.

3.1.2 | Plant survival

Freezing temperatures had significant impacts on the survival percentage of plant species. A decline in temperature from 0 to -12°C did not affect the plant SU, but it showed a sharp decline after -12°C (Figure 2d); that is, survival percentage decreased by 12.5% each temperature degree depression from -12 to -20°C .

3.1.3 | Gas exchange variables

Gas exchange variables of both plant species statistically remained unaltered to -12°C , but -16°C dramatically decreased those parameters compared with the higher temperatures. Wild mustard showed higher A_N before the stress onset than chickpea at all temperatures; however, it was more affected by freezing temperatures (Table 1).

With decreasing the freezing temperatures, A_N was declined in both plant species. Up to -12°C , no significant decrease was observed in the plant species A_N compared with temperature 4°C ; but decreasing temperature to -16°C diminished chickpea and wild mustard A_N 1.3 and 1.5 times, respectively, compared with 4°C . Nevertheless, both plants A_N reached zero at -20°C . The same trend was observed for g_s ; for instance, chickpea and wild mustard g_s were decreased by 1.3 and 3.3 times, respectively, compared with 4°C . Although chickpea WUE_i decreased by declining temperature to -12°C compared with before stress, at -16°C , WUE_i showed a higher value compared with the higher temperatures. Wild mustard had a higher WUE_i at all temperatures compared with chickpea (Table 1).

3.1.4 | Leaf chlorophyll fluorescence

Chlorophyll-a fluorescence was recorded just before the onset of and after 6, 12, 24, and 48, and 72 h after freezing stress (AFS) at different temperatures (Figure 3). Generally, chickpea leaf chlorophyll fluorescence was more sensitive to low temperatures compared with that of wild mustard. The maximum light-adapted quantum yield of PSII photochemistry, F_v'/F_m' , was recorded at 4°C (Figure 3a,b). Chickpea F_v'/F_m' exposed to -16 and -20°C decrease 33% and 43%, respectively, compared with 4°C and continued to decrease to reach zero at 48 h. Whereas in wild mustard, F_v'/F_m' decreased only by 11% at both -16 and -20°C compared with 4°C and reached zero at 72 h.

TABLE 1 Photosynthetic variables of chickpea and wild mustard plants before and after exposure to freezing temperatures

Temperature (°C)		A_N ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	g_m ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	g_s ($\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	C_i ($\text{mol}\cdot\text{mol}^{-1}$)	E ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	WUE_i ($\mu\text{mol}\cdot\text{mmol}^{-1}$)
4	CBS	10.9 (2.18) ^a	0.02 (0.004)	0.22 (0.06)	348 (23)	1.75 (0.30)	6.3 (0.7)
	CAS	8.1 (3.01)	0.02 (0.006)	0.17 (0.09)	380 (22)	1.70 (0.16)	4.8 (1.7)
	MBS	14.6 (0.95)	0.03 (0.002)	0.34 (0.02)	418 (2)	2.22 (0.20)	6.6 (0.3)
	MAS	13.6 (1.03)	0.03 (0.003)	0.34 (0.02)	409 (16)	1.72 (0.23)	7.9 (0.6)
0	CBS	10.8 (2.0)	0.03 (0.004)	0.21 (0.05)	365 (35)	1.85 (0.35)	5.9 (0.8)
	CAS	8.1 (2.4)	0.02 (0.004)	0.17 (0.07)	362 (37)	1.60 (0.18)	5.0 (1.4)
	MBS	14.0 (0.7)	0.03 (0.001)	0.29 (0.03)	409 (32)	2.02 (0.27)	7.0 (0.5)
	MAS	11.8 (1.9)	0.03 (0.003)	0.26 (0.08)	405 (34)	1.53 (0.07)	7.7 (0.1)
−4	CBS	10.3 (1.9)	0.02 (0.005)	0.20 (0.04)	401 (38)	1.81 (0.33)	6.0 (1.3)
	CAS	7.9 (1.4)	0.02 (0.004)	0.17 (0.01)	399 (25)	1.67 (0.20)	4.7 (1.7)
	MBS	13.9 (0.3)	0.03 (0.004)	0.28 (0.02)	411 (39)	1.90 (0.17)	7.3 (0.5)
	MAS	10.5 (0.7)	0.02 (0.002)	0.19 (0.01)	384 (11)	1.31 (0.07)	8.0 (0.2)
−8	CBS	11.8 (1.7)	0.03 (0.005)	0.24 (0.05)	415 (41)	1.95 (0.49)	6.6 (3.2)
	CAS	7.8 (1.3)	0.02 (0.003)	0.18 (0.01)	399 (28)	1.64 (0.26)	4.7 (1.7)
	MBS	13.9 (1.1)	0.03 (0.003)	0.28 (0.06)	402 (18)	1.89 (0.46)	7.6 (1.4)
	MAS	9.6 (0.4)	0.02 (0.001)	0.17 (0.01)	396 (2)	1.17 (0.05)	8.1 (0.2)
−12	CBS	11.0 (2.1)	0.03 (0.005)	0.22 (0.05)	376 (29)	1.80 (0.27)	6.1 (1.4)
	CAS	7.9 (1.3)	0.02 (0.003)	0.17 (0.02)	384 (17)	1.63 (0.26)	4.8 (1.9)
	MBS	13.7 (0.9)	0.03 (0.001)	0.27 (0.04)	400 (16)	1.84 (0.40)	7.7 (1.4)
	MAS	9.9 (0.4)	0.02 (0.002)	0.19 (0.01)	382 (13)	1.19 (0.02)	8.3 (0.3)
−16	CBS	11.0 (1.7)	0.03 (0.004)	0.22 (0.05)	381 (26)	1.87 (0.46)	6.2 (1.7)
	CAS	4.7 (0.8)	0.01 (0.003)	0.09 (0.01)	360 (32)	0.69 (0.15)	7.0 (1.2)
	MBS	13.8 (1.0)	0.03 (0.001)	0.28 (0.05)	396 (29)	1.85 (0.45)	7.7 (1.4)
	MAS	5.4 (0.8)	0.01 (0.002)	0.09 (0.01)	320 (32)	0.70 (0.11)	7.7 (0.3)
−20	CBS	11.2 (2.2)	0.03 (0.007)	0.19 (0.05)	392 (28)	1.93 (0.22)	5.9 (1.2)
	CAS	0.0 (0.0)	0.00 (0.000)	0.00 (0.00)	0 (0)	0.00 (0.00)	0.0 (0.0)
	MBS	13.9 (0.8)	0.03 (0.005)	0.27 (0.03)	394 (39)	1.91 (0.18)	7.3 (0.3)
	MAS	0.0 (0.0)	0.00 (0.000)	0.00 (0.00)	0 (0)	0.00 (0.00)	0.0 (0.0)
ANOVA							
SOV							
P		**	**	**	ns	ns	**
T		**	**	**	**	**	**
Ti		**	**	**	**	**	**
P × T		**	ns	**	*	ns	ns
P × Ti		ns	ns	**	ns	**	**
T × Ti		**	**	**	**	**	**
P × T × Ti		ns	ns	ns	ns	ns	*
CV		10.6	13.4	15.9	7.8	18.2	17.1

Note: ns, not significant at $p > 0.05$.

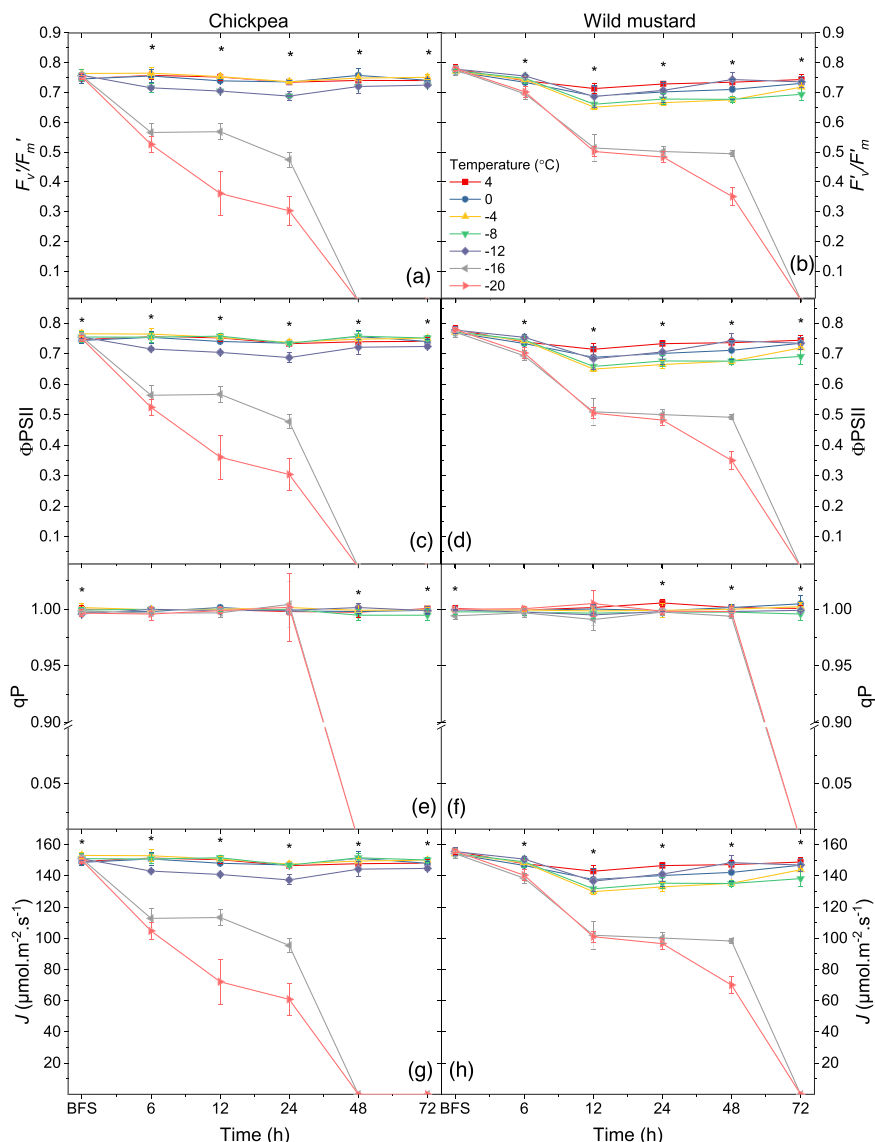
Abbreviations: A_N , net photosynthetic rate; ANOVA, analysis of variance; C_i , substomatal CO_2 concentration; CAS, chickpea after freezing stress; CBS, chickpea before freezing stress; CV, coefficient of variation; E , transpiration rate; g_m , mesophyll conductance; g_s , stomatal conductance; MAS, wild mustard after freezing stress; MBS, wild mustard before freezing stress; P, plant species; SOV, source of variation; T, temperature; Ti, time of measurement; WUE_i , water use efficiency.

^aValues in parentheses are mean (\pm SD) of four replicates ($n = 4$).

*Significant at $p \leq 0.05$.

**Significant at $p \leq 0.01$.

FIGURE 3 Changes in light-adapted maximum efficiency of photosystem II (PSII) (a, b), quantum efficiency of PSII (c, d), photochemical quenching (e, f), and linear electron transport rate (g, h) of chickpea and wild mustard plants under freezing temperatures. BFS, before freezing stress onset; Time, hours after freezing stress during the recovery period. Each point is average, and vertical bars indicate \pm SD ($n = 4$). * Significant at $p \leq 0.05$.



The maximum quantum yield of PSII, Φ_{PSII} , was affected differently in the plant species. Chickpea Φ_{PSII} decreased 33% and 43% in -16°C and -20°C treated plants, respectively, 6 h AFS compared with 4°C . Nevertheless, wild mustard Φ_{PSII} only decreased by 10% at both -16°C and -20°C 6 h AFS compared with 4°C (Figure 3c,d). Leaf photochemical quenching showed the same trend in both species, and it was not affected by temperatures to -16°C ; however, qP showed different behaviors in plants exposed to -20°C . Wild mustard qP started to decrease with a sharp slope 48 h AFS, but Chickpea qP decrease started 24 h AFS (Figure 3e,f). The same trend as F_v/F_m' and Φ_{PSII} was observed for J , where wild mustard J remained to about 72 h AFS (Figure 3g,h).

3.1.5 | Plant growth parameters

Plant species and temperature interacted to affect the growth parameters. Although chickpea plants had a greater SHDM than wild

mustard, a decrease in temperature from zero to -12°C reduced chickpea SHDM by 31% compared with -8°C , whereas wild mustard SHDM remained unaltered to -12°C . However, a further decrease in temperature to -16°C decreased wild mustard SHDM as well, but with a gentler slope (Figure 4a). RDM showed a contrariwise behavior; wild mustard had a greater RDM compared with chickpea plants, but the same sensitivity trend was observed as for SHDM (Figure 4b). However, both plant species SHDM and RDM reached zero at -20°C .

Both species LA showed a similar trend. No significant LA decrease was observed to -12°C , although wild mustard had a greater LA than chickpeas (Figure 4c). LA per plant decreased 68% and 130% in chickpea and wild mustard, respectively, at -16°C compared with -12°C . Chickpea plants, on the other hand, had a greater LN per plant than wild mustard but with a higher sensitivity to low temperatures (Figure 4d). There were no changes in wild mustard LN to -12°C , whereas chickpea LN decreased by 37% by a 4°C temperature decline from -8 to -12°C .

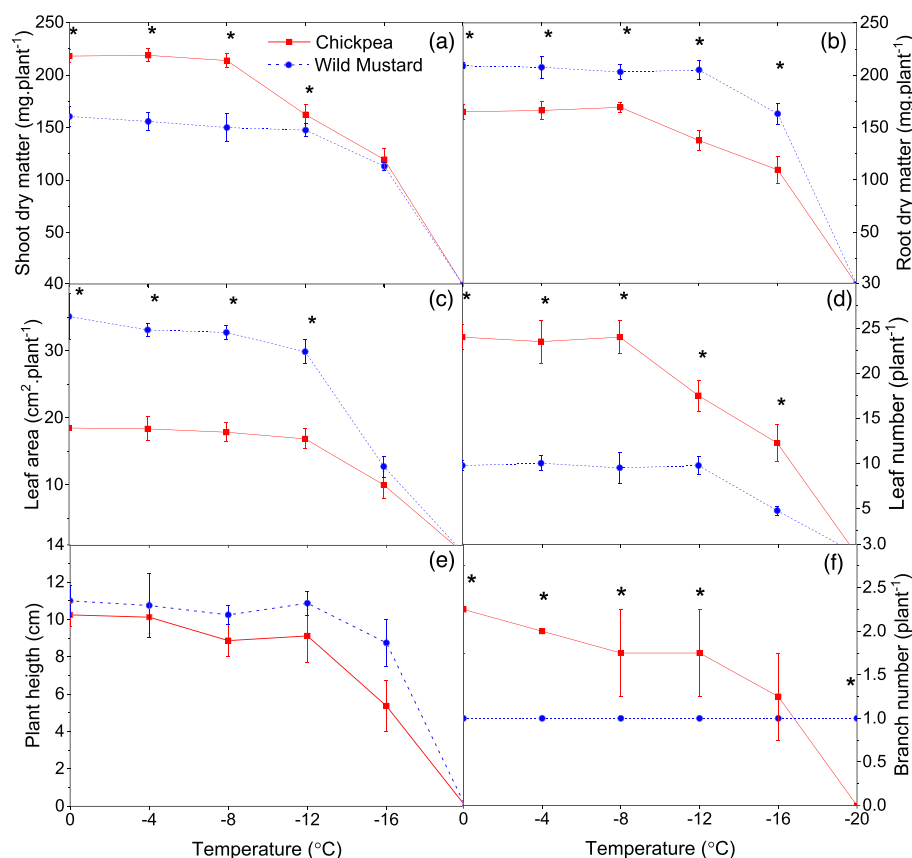


FIGURE 4 Changes in the shoot (a) and root dry matter (b), leaf area (c), leaf number (d), plant height (e), and branch number (f) of chickpea and wild mustard plants under freezing temperatures. Each point is average, and vertical bars indicate \pm SD ($n = 4$). Asterisk represents significant differences between the plants at $p \leq 0.05$.

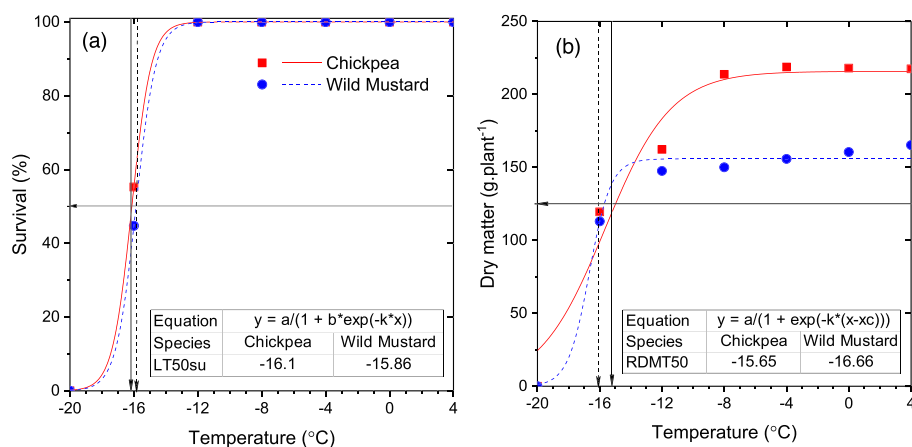


FIGURE 5 50% lethal temperature based on survival percentage (LT_{50su}) (a) and 50% dry matter depression temperature ($RDMT_{50}$) (b) of chickpea and wild mustard plants under freezing temperatures

Plant height showed a similar trend in both plant species. To -12°C , no decline was observed in neither plant species; however, further temperature decreases to -16°C decreased chickpea plant height by 70% compared with -12°C (Figure 4e). Chickpea plants had greater branches than wild mustard; wild mustard plants had no branches (single stem) that remained to -20°C . Chickpea branch number decreased with a gentle slope by temperature decline and reached zero at -20°C (Figure 4f).

3.1.6 | LT_{50su} and $RDMT_{50}$

Both species remained LT_{50su} up to -12°C , but the SU was declined by increasing the freezing temperature intensity (Figure 5a). Chickpea lost 50% of plant survival at -16.1°C , whereas wild mustard lost 50% of plant survival at -15.9°C . A significant difference (1.01°C) was observed between the plant species in terms of $RDMT_{50}$. Chickpea with an $RDMT_{50}$ of -15.7°C showed a lower rate of regrowth after

the recovery period compared with wild mustard, which means that in chickpea, a decrease in temperature to less than -15.7°C caused a 50% decrease in DM (dry matter) at the end of the recovery period, whereas decreasing the temperature to less than -16.7°C reduced wild mustard DM by 50% (Figure 5b).

4 | DISCUSSION

The freezing tolerance of a plant varies significantly among different tissues. For instance, stem, meristems, the lower and upper leaves of the plant canopy, and roots have different freezing tolerance (Herzog, 1987; Herzog & Olszewski, 1998). Antifreeze proteins and ice nuclei control the initial formation of ice. Such mechanisms as membrane fluidity and osmotic regulation are often associated with freezing tolerance at the cellular level. Both metabolic and physiological alterations in the plant in response to low temperatures highly affect the cold acclimation or hardening process. Alteration in cellular and metabolic status, including greater sugars, soluble proteins, proline, organic acids, and altered lipid membrane composition, may lead to cold acclimation (Hughes & Dunn, 1990, 1996).

Plant DM decreased as temperature declined in both species. The results indicated that both leaf and stem EL were increased, but the enhancement of EL and plant DM depletion was greater in chickpea plants than wild mustard. A negative correlation was observed between the EL with survival and plant DM. Previous findings also indicated the adverse effect of freezing stress on cell membrane stability (Bertin et al., 1996; Kaur et al., 2008). The freezing stress tolerance evaluation of *Trifolium hirtum* showed that the leaf EL increased with declining temperature from -6 to -14°C (Nunes & Smith, 2003).

An investigation on the effect of cold stress on two wheat (*Triticum aestivum*) cultivars at the seedling stage showed that ion leakage levels were not affected by temperatures above 0°C , whereas EL of plants was increased with decreasing temperatures below zero (Apostolova et al., 2008). However, in the present study, it was observed that even temperatures below 0°C did not affect the leaf EL in wild mustard to -12°C , a sign of greater tolerance of wild mustard. High EL values indicate the membrane's lack of ability to retain intracellular compounds; more electrolytes leak from the membrane and damage the cell membrane. Studies showed that the unsaturated fatty acids present in cell membranes are essential in membrane fluidity. Low temperatures change the fluidity of these membrane fatty acids from semi-liquid to crystalline (Mahajan & Tuteja, 2005), and subsequently, ionic leakage increases. Reactive oxygen species (ROS) produced under low temperatures can react with the membrane lipids and cause lipid peroxidation, leading to cellular content leakage and rapid cell dehydration and cell death (Takac, 2004). These changes give rise to other effects of chilling or freezing on the plant and cell levels (Blum, 2018).

Although differently, freezing stress decreased the F_v'/F_m' of both plant species; chickpea plants showed more sensitivity than wild mustard. The leaf F_v'/F_m' of chickpea plants exposed to -16°C significantly decreased 6 h AFS and reached zero after 48 h of the recovery

period. Accordingly, ΦPSII and J also showed a similar trend. None of the plant species recovered their fluorescence parameters during the recovery period. Hasanfard et al. (2021) indicated that the leaf F_v'/F_m' decreased in turnipweed (*Rapistrum rugosum* (L.) All.) with a decrease in temperature from -12°C and during the first 24 h after the freezing treatment; the F_v'/F_m' levels decreased by 28% compared to before the freezing stress. They found that a temperature decline from -12°C disrupted the carbon exchange and PSII electron transport. Chlorophyll fluorescence measurement is a reliable and appropriate tool to evaluate plant tolerance to low temperatures (Ehlert & Hinch, 2008; Rizza et al., 2001). This method can reveal the susceptibility of the PSII electron transport chain (Maxwell & Johnson, 2000) and provide a non-destructive and faster diagnostic tool for evaluating the effect of freezing stress on plants than the destructive methods such as the EL (Christen et al., 2007; Su et al., 2015).

Plants are more sensitive to low temperatures in the autotrophic than the heterotrophic stage. Freezing temperatures mainly impact the growing seedlings by the cell membrane damage and cause the respiration and photosynthesis to decrease. Besides, plant wilting due to loss of leaf turgor results in temperature-induced drought stress. In the present study, temperatures below -12°C significantly reduced A_N and photosynthetic variables, likely due to the cell membrane damage and electron transport chain disruption. Photosynthesis is regulated by stomatal and non-stomatal factors, depending on plant species and the environmental conditions (Ahmadi-Lahijani et al., 2018). Freezing and chilling temperatures induce water loss through a slow stomatal aperture, increased membrane permeability, lower root hydraulic conductivity, and root water uptake (McWilliam et al., 1982; Wolk & Herner, 1982).

Wild mustard A_N/g_s showed an increasing trend by decreasing the temperature up to -16°C , with the greatest value at -16°C (Figure 6b). The species behaved differently; freezing-stressed chickpeas had relatively lower A_N/g_s compared with the unstressed plants, whereas it was vice versa in wild mustard, relatively higher A_N/g_s in freezing-stressed plants compared with the unstressed (Figure 6a,b). It may indicate that lower chickpea A_N was due to a greater stomatal limitation under relatively lower temperatures. Plants vary in their capacity to regulate how much water they lose per unit carbon gained, determining by "intrinsic WUE," A_N/g_s (Condon et al., 2002). Although wild mustard is a C_3 species, its extensive root system and large photosynthetic capacity make it a very competitive weed (Szmigielski et al., 2015). Besides, wild mustard's higher stomatal density than many other broadleaves weed species leads to a higher photosynthetic rate, faster plant (re)growth, and lower stomatal closure effect on the leaf photosynthesis. However, the greater decrease in wild mustard $C_i:C_a$ by decreasing temperature (e.g., -16°C) indicating the greater role of stomatal factors in photosynthetic regulation even at extremely low temperatures (Figure 6d).

A_N was significantly diminished in both species at -16°C , indicating the damages imposed to photosynthetic apparatus at low temperatures. Wild mustard A_N was higher compared with chickpea up to -16°C . This, along with a relatively lower E , led to a rise in wild mustard WUE_i compared with chickpea. Water use efficiency is

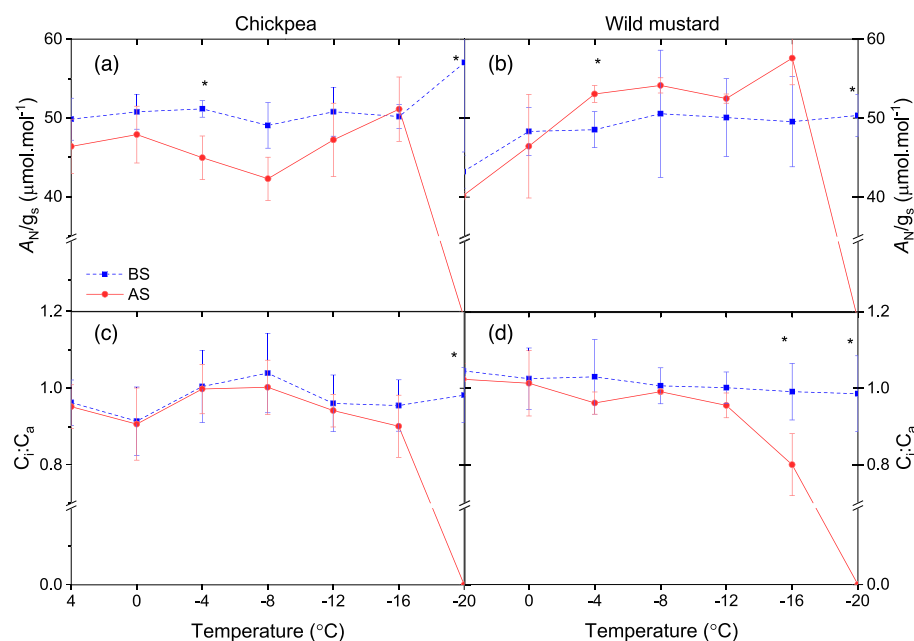


FIGURE 6 Changes in the net photosynthesis rate to stomatal conductance ratio in chickpea (a) and wild mustard (b), and substomatal to ambient CO_2 concentration ratio in chickpea (c), and wild mustard (d) plants under freezing temperatures. BS, before stress; AS, after stress. Each point is average, and vertical bars indicate \pm SD (n = 4). Asterisk represents significant differences between the time of measurement at $p \leq 0.05$.

considered an important factor in crop yield determination and one of the parameters to assay plant freezing tolerance (Navarrete-Campos et al., 2013). Low temperature (-5°C for 12 h) reduced tea (*Camellia sinensis* L.) plantlet g_s by 88% compared with the control (Li et al., 2018). Gupta et al. (2016) also found a decrease in g_s in plants exposed to various low temperatures. Positive correlations were observed between A_N with g_s and g_m (Figure 7), suggesting that these variables were coupled. Chilling and freezing stress reduces the conductivity of the tonoplast and plasmalemma of the guard cells, resulting in the stomata less responsive to leaf water potential alterations in sensitive species. Under conditions of continued evaporative demand in the light, slow closure of stomata and reduced water uptake reduce water potential, leading to tissue dehydration (McWilliam et al., 1982).

Chickpea SPAD value started to decline with a gentle slope after a stable trend at -8°C , whereas wild mustard SPAD value did not change to -12°C compared with $+4^\circ\text{C}$. However, both species experienced a sharp decrease afterward. Cold-acclimated rapeseed plants showed a higher SPAD value by 41% than the unacclimated plants (Nezami et al., 2009). SPAD and A_N were positively correlated (Figure 6), indicating the close relationship between leaf pigment content and photosynthetic activities.

Plant survival after freezing stress in crops and their common weeds sheds more light on the dynamics of their distribution and competitiveness in areas with low temperatures. Plant survival remained unaffected to -12°C in both species; however, reducing the temperature to -16°C decreased the plant SU. The exponential set curve showed that both species survived above 50% to $\sim -16^\circ\text{C}$ (Figure 5a). However, all plants died at the temperature of -20°C . A highly positive correlation coefficient ($r = +0.97^{**}$) was observed between the plant survival percentage and A_N (Figure 7). The linear regression between plant SU and A_N suggested that the survival rate

increased with increasing the rate of A_N . $LT_{50\text{SU}}$ has been reported as a suitable indicator of cold tolerance in plants (Fowler et al., 1996; Liang et al., 2003). Nezami et al. (2016) observed that 50% of chickpea plant lethal temperature was about 6°C lower in the tolerant than the susceptible species. Wery (1990) found that although some genotypes tolerate -12°C in the vegetative stage after emergence, the minimum temperature at which chickpeas generally survive is -8°C . Increasing autumn temperatures resulting from climate change in Iran led to reduced freezing tolerance of autumn crops (Hasanfarid et al., 2021). In other words, due to the lack of optimal cold acclimation in autumn, chickpeas will not tolerate freezing stress. As a result, they will be damaged by decreasing temperatures during winter. However, weeds generally have a higher ability to tolerate freezing stress (Cici & Van Acker, 2011). Based on this, it can be inferred that climate change is adversely shifting weed flora in an Iranian cropping system.

As temperatures declined below -12°C , both species suffered severe damage; at -16°C , the LA was dramatically diminished due to plant death. A similar study found that turnipweed LA decreased less at -12°C compared with that of wild oat (*Avena ludoviciana* Durieu.) (Hasanfarid et al., 2021). Higher A_N was correlated with a greater SHDM (Figure 7). Cold acclimation requires the energy supplied by photosynthetic activities. However, during cold acclimation, the chloroplast properties are changed (Huner et al., 1998). Experiments to assay freezing tolerance of chickpea and grass species revealed that plant dry matter was decreased by temperature decline to -8°C (Nezami et al., 2007; Nezami et al., 2016). Plant growth reduction might associate with a slower rate of food reserve transfer and reduced photoassimilate mobilization due to reduced enzyme activity (Powell & Matthews, 1978).

Wild mustard had a greater RDM than chickpea, which remained longer and tolerated freezing temperature to -12°C without any

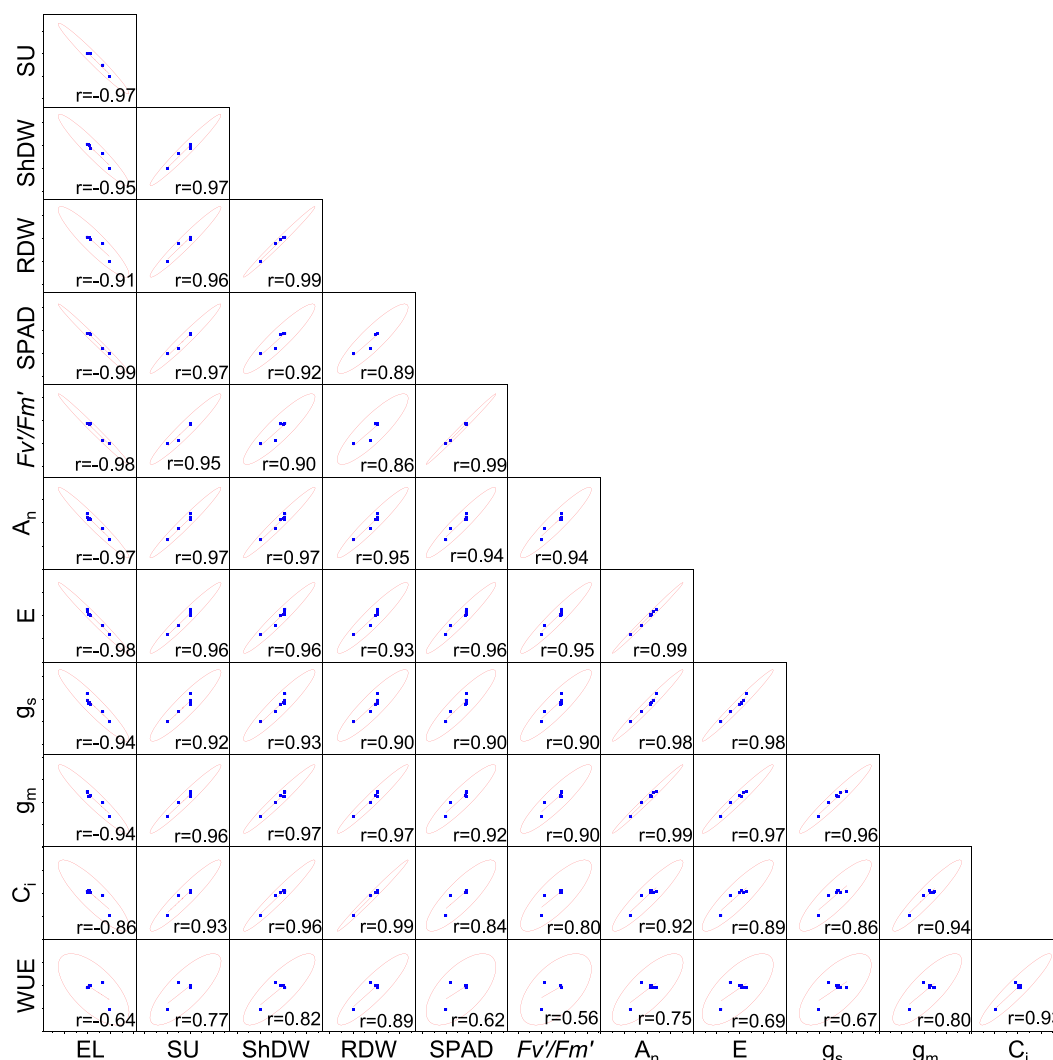


FIGURE 7 Pearson's correlation between the experimental parameters in chickpea and wild mustard plants under freezing temperatures. A_N, net photosynthetic rate; C_i, substomatal CO₂ concentration; E, transpiration rate; EL, electrolyte leakage; F_v/F_m', light-adapted maximum efficiency of PSII; g_m, mesophyll conductance; g_s, stomatal conductance; RDW, root dry weight; ShDW, shoot dry weight; SPAD, leaf chlorophyll content; SU, survival percentage; WUE, water use efficiency

significant changes. However, wild mustard RDM started to reduce in plants exposed to -16°C ; nevertheless, it was still greater than chickpea RDM. The greater survival of wild mustard might be due to the greater RDM. A highly positive correlation between plant SU and RDM ($r = +0.96^{**}$) (Figure 7) may indicate the bold role of a more robust root system in plant survival under stressful conditions. Weeds have always been a strong competitor to crops for environmental resources; the greater underground parts of weeds might be their secret of better survival.

Studies showed that sometimes freezing stress may not kill some plants, although it affects plant regrowth during recovery. Therefore, plants might not fully recover and resume their regrowth properly under such conditions. The index RDMT₅₀ can distinguish between the plants for freezing tolerance and provide more precious discrimination. In the present study, the plant species differed in RDMT₅₀.

Although there was no significant difference in LT_{50su} of plant species, wild mustard showed a better regrowth with a higher RDMT₅₀ (-15.6 vs. -16.6°C in chickpea and wild mustard, respectively) during the recovery period (Figure 5b). Plant dry matter is related to physiological responses and decreases during the recovery period due to freezing damage to plant physiological processes and regrowth ability (Azizi et al., 2007). Izadi Darbandi et al. (2020) also reported that the seedlings of wild barley (*Hordeum spontaneum* Koch.) and feral rye (*Secale cereale* L.) with RDMT₅₀ of -8.1 and -11.6°C , respectively, were the most susceptible and most tolerant plants under freezing stress. Nezami et al. (2007) observed a high correlation between LT₅₀ and RDMT₅₀ in chickpea genotypes. They found RDMT₅₀ was lower in the tolerant than susceptible genotypes. Hekneby et al. (2006) also found that forage legume tolerant species had better regrowth than susceptible species.

5 | CONCLUSIONS

Due to the increasing cultivation area of autumn chickpea in Iran, it is predicted that wild mustard interruption, especially in cold regions, will increase in the future. Plant species show various levels of tolerance and sensitivity to environmental stresses. Therefore, evaluating the freezing tolerance of wild mustard helps us better understand how this weed will distribute and invade. The significant correlation between F_v/F_m' and plant survival suggested the reliability of chlorophyll fluorescence measurements to assay plant freezing tolerance. The results showed that temperatures below -12°C decreased both species' photosynthetic variables. Freezing stress reduced plant survival percentage and the shoot dry matter at the end of the recovery period, although the species response varied depending on the intensity of stress. Although LT_{50su} of plant species did not significantly differ, $RDMT_{50}$ could distinguish between the species freezing response; wild mustard $RDMT_{50}$ was $\sim 1^{\circ}\text{C}$ higher than chickpea. Accordingly, chickpea showed more sensitivity to freezing stress than wild mustard in this experiment. The positive correlation between plant survival and root dry matter may indicate the significant role of a more robust root system in wild mustard survival under stressful conditions. Overall, due to climate change and temperature fluctuations in winter, it seems that wild mustard has a high ability to adapt to these conditions and, if not controlled, can lead to more damage, especially in Iranian chickpea fields. Obtained data from this study can be used in other similar climates and other cropping systems. Our findings predict that moving away from winter weed management, such as lack of crop rotation, will encourage wild mustard dispersal under harsh winter conditions.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

None.

ORCID

Mehdi Rastgoo  <https://orcid.org/0000-0002-1974-8727>

Ahmad Nezami  <https://orcid.org/0000-0001-9490-6935>

Mohammad Javad Ahmadi-Lahijani  <https://orcid.org/0000-0001-7356-7276>

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