EVALUATION OF FREEZING TOLERANCE OF CUMIN (*CUMINUM CYMINUM* L.) UNDER CONTROLLED CONDITIONS

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Abstract. *Freezing tolerance of cumin ecotypes was investigated under controlled condition. Six cumin ecotypes (India Rajestan and five Iranian: Ghoochan, Sabzevar, Khaf, Ghayen and Torbate Heydarieh) were subjected to six temperature treatments including 0, -3, -6, -9, -12 and -15 °C. Treatments were arranged in a completely randomized design with three replications. Following three weeks of acclimation plants were transferred to a thermogradient freezer and treated with the above temperatures. The cell membrane integrity was determined after freezing through electrolyte leakage (EL) and the temperature causing 50% leakage (LT_{50el}) was evaluated. For recovery plants transferred for three weeks to glasshouse and at the end of recovery period for each ecotype survival percentage, lethal temperature causing 50% mortality (LT_{50su}), plant height, leaf area and dry matter were determined. The effect of ecotypes and freezing temperatures was significant for most traits. The minimum (20.1%) and maximum (85.3%) EL was observed at 0 °C and -15°C, respectively. Decreasing the freezing temperature reduced plant survival. Ghoochan and Ghaen had greater survival compared to other ecotypes. Plant height decreased significantly under lower temperatures and greater plant height observed for Iranian ecotypes. Plant dry matter and leaf area decreased by 90% and 86% at -12 °C, respectively as compared to non frozen control (0 °C). However, there were no significant differences among cumin ecotypes for dry matter and leaf area. Based on this study, EL could be an easy and efficient method in evaluating the freezing tolerance of cumin ecotypes.*

Keywords: Acclimation, plant survival, lethal temperature, electrolyte leakage.

INTRODUCTION

Cumin (*Cuminum cyminum* L.) is an annual plant, belongs to the *umbelliferae* family, native to the Mediterranean region. Cumin is primary cultivated in Asia, Middle East and North Africa. It is widely cultivated in Iran, China, India, Morocco, South Russia, Japan, Indonesia, Algeria and Turkey; specially, in arid and semi-arid regions (Tuncturk and Tuncturk, 2006). Iran is one of the most important cumin producers and exporters in the world market (Kafi, 2002). Cumin is a valuable medicinal plant and its essential oils have antibacterial effect and are widely used for cosmetics, health and food industry (Balandari, 1994).

Iran is located within an arid and semi-arid belt and limited precipitation is mainly occurred in cold and winter month (Bannayan *et al*. 2010). Therefore, plants which are cultivated in winter such as cumin that is a valuable medicinal plant with drought tolerance is more profitable than other crop.

Cumin has been well adapted to temperate climates and the minimum and maximum temperatures for its growth have been found to be 9 °C and 26 °C, respectively

(Raychaudhuri, 1992). November or early December was found as the best planting dates for cumin (Kafi, 1991). Therefore, it is likely that a cold weather or frost during winter waste the cumin plants, so that it is necessary to identify cold tolerant ecotypes of cumin and determine the range of low temperatures that cumin may withstand without any considerable damage.

Freezing is one the most important abiotic stress limiting the growth and production of plants in temperate regions. Cold stress, similar to drought and salinity, affect the water relations of a plant at the cellular level, as well as the whole plant level, causing damages and adaptation reactions (Beck et al., 2007). According to Beck, cold stress imposes a considerable negative effect on crop productivity, quality, and survival of plants. Low temperature stress can cause serious damages at the physiological, cellular, and molecular level, which is expressed by various phenotypic symptoms on plants. For instance, under cold condition the green leaves of cumin became purple where a longer cold duration leads to death of plant (Agrawal, 1993).

There are two main reasons for conducting cold experiments under controlled and artificial conditions whereby the identification of frost and cold tolerant cultivars is addressed. Firstly, temperature could not be controlled under field natural condition and secondly, weather condition is thought as a stochastic phenomenon that varies temporally and spatially, thus one may not encounter such an appropriate cold condition required for the selection of cold tolerant cultivars (Nazeri *et al*. 2006; Nezami *et al*. 2007).

Since, disruption of cellular membranes, particularly the cytoplasm membrane is the most common damage to cells that cause freezing injury in plants, the degree of this damage could be determined by measuring the EL of injured tissue. The more sensitive is plants to cold, the greater amount of ions will leakage from its cells (Beirami zade *et al*. 2006). Measuring the cytoplasm electrolyte leakage (EL) or electrical conductivity (EC) is one of the common methods to assess the level of cell damage by low temperature (Mirzai-Asl *et al*. 2002). The electrolyte leakage method (EL) is relatively easy, quick, and cheap and utilizes small quantities of tissue compared to the other methods, thus it is a high effective method to determine the cold tolerance of plants (Lyons and Raison, 1990). Several studies using electrolyte leakage method have been carried out on different plants under controlled condition in order to evaluate the freezing tolerance. Coursolle *et al*. 2000 using EL method for assessment of root freezing damage of two-year-old White Spruce, Black Spruce and Jack Pine Seedlings. Tamura (2000) using this method to evaluate the freezing tolerance of Komatsuna (*Brassica campestris* L.) and Spinach (*Spinacia oleracea* L.) found that the freezing tolerance correlated well with electrolyte leakage from leaf tissues, especially with the leaf area that was damaged by freezing during cold acclimation. Nezami *et al* (2010) evaluated freezing tolerance of different triticale (*X Triticosecale Wittmack*) genotypes under controlled condition, found a negative significant correlation between the EL% plant and survival parameters after the recovery period from freezing.

Evaluation of plant regrowth characteristics or survival after exposure to freezing temperature is a reliable method for assessment of freezing tolerance of plants (Steponkus, 1978; Fowler and Carlers, 1979). Azizi *et al.* (2007) found a positive significant correlation between leaf area and seedling dry weight with LT_{50su}. Nezami *et al.* (2007) showed that the regrowth of plants during recovery period could be a good criterion for selecting cold resistant and cold susceptible chickpea genotypes. In their study the dry weight of cold resistant genotypes reduced by 55%-60% at -12 ^{o}C while that of susceptible ones reduced by 90% as compared to unfrozen control. Similarly, Nezami *et al.* (2010)

reported that in all Triticale genotype dry weight and leaf area decreased by 48% and 42% respectively compared to non-frozen control plants.

The objective of this experiment was to evaluate and compare the response of cumin ecotypes to freezing temperatures under controlled conditions.

MATERIAL AND METHOD

Plant material and growth conditions

Five native cumin ecotypes named Ghoochan, Sabzevar, Khaf, Ghayen and Torbate Heydarieh and an Indian ecotype (India Rajestan) were subjected to six different temperatures (0, -3, -6, -9, -12 and -15 °C). At the beginning of the experiment, cumin seeds were germinated in the petri dishes using moistened filter papers. The germinated seeds were then sown in plastic pots to a depth of 1 cm with a density of 10 seedlings per pot. The pots were primarily filled with an equivalent ratio of sand, compost and soil. Plants were grown in a growth chamber at 20/15 °C day/night and 12.5 h photoperiod until second-leaf stage.

Cold acclimation

The plants were acclimated to low temperatures for three weeks as described below. They were kept at 10/8 °C and 11.5 h photoperiod for one week, then transferred to 7/5 °C with 10.5 h photoperiod during the second week and finally subjected to 5/2 °C and 10.5 h photoperiod for the last week. Pots were irrigated during the acclimation period as needed. Following the termination of acclimation, plants were transferred to a thermogradient freezer. The initial temperature of the freezing chamber was 5°C; but gradually decreased at a constant rate of 2 °C h^{-1}. When the temperature cooled down to -3 °C, the seedlings were sprayed with the Ice Nucleation Active Bacteria (INAB) to help the formation of ice nuclei in the seedlings. Cumin plants were kept at the nominated freezing temperature for 1 h. Following the freezing treatments, all samples were kept at 5±1°C for 24 h to decrease the rate of thawing.

Electrolyte leakage evaluation

The integrity of cytoplasmic membrane was determined using the EL method. From each pot three plants were selected and placed into vials filled with double-distilled water. Samples were placed on a shaker and the EL was measured after 6 h using an EC-meter (Jenway Model). To evaluate the total electrolyte leakage of the dead cells, the samples were frozen at -70 °C for 12h in a cold chamber. Subsequently the samples were transferred to the laboratory and exposed to the natural temperature to defreeze. The second EL measurement was made after placing the samples on the shaker for 6 h. The EL% was calculated using Equation (1):

$$EL\% = (EL_1/EL_2) \times 100 \quad (1)$$

Where EL= the relative electrolyte leakage; EL_1= the primary electrolyte leakage for a given freezing temperature and EL_2= the total electrolyte leakage obtained from the super-freezing temperature of -70 °C.

Percent of Lethal temperature 50 (LT_{50}) calculations

Percent of lethal temperature 50 (LT_{50}) according to the electrolyte leakage was determined using the following model (Equation 2) developed for electrolyte leakage data (Anderson *et al*. 1988; Ingram, 1985).

$$EL_p = El_l + [(El_m - El_l) / (1 + e^{-B(T-Tm)})] \qquad (2)$$

Where EL_p =predicted EL value, El_l =lower bound EL value, El_m = higher bound EL value, e= 2.714, B= the rate of increase of the slope of the curve, T=absolute value of the treatment temperature and Tm= inflection point of the curve.

Survival evaluation

Survival of treated seedlings was evaluated by other remained seven plants re-growth as determined three weeks later. The numbers of alive plants were counted at the end of recovery period and the survival percentage index was calculated (Equation 3):

$$PSI\% = (A/B) \times 100 \qquad (3)$$

Where PSI= Survival Percentage Index, A= number of alive plants at the end of recovery period and B= number of plants before freezing treatment.

From each pot five plants were clipped from the soil surface to measure plant height (from the soil surface to terminal node), leaf area and individual plant dry weight. LT_{50su} were estimated based on PSI by plotting these values against the freezing temperatures.

Statistical analysis

The experiment was arranged as factorial on the basis of completely randomized design with three replications. All data were subjected to ANOVA and significant means were separated using LSD test at $P \leq 0.05$. The percentage data were transformed to arcsine prior to analysis.

RESULTS AND DISCUSSION

Electrolyte leakage percentage

Results showed that there were significant differences ($p \leq 0.01$) among freezing temperatures, ecotypes, and interaction between them for electrolyte leakage percentage (EL%). The highest and lowest relative EL values were obtained from 0 °C (20.1%) and -15 °C (85.4%), respectively (Fig.1. a). The EL increased markedly at temperatures lower than -9 °C where at -12°C and -15 °C the EL was 2.8 and 4.2 times higher than control (0 °C), respectively (Fig.1.a). Eugenia *et al*. (2003) found the EL method as a practical and easy measure in evaluating the freezing tolerance of rose clover (*Trifolium hirtum* All.). Tyey found higher EL from leaf at -18 °C compared to -6 °C.

The EL% ranged from 39.9% to 46.4% within the cumin ecotypes. Ecotypes difference in EL% in response to the freezing temperature was found only for the Rajestan. It had the highest EL (46.4%) that was significantly ($p \leq 0.01$) different from other ecotypes however there was no statistical different among other ecotypes (Fig.1.b). Evaluation the cold tolerance of some grasses, Nezami *et al*. (2010) found significant differences among freezing temperatures and grasses where the EL from leaf increased about four times at -16.5 °C as compared to unfrozen control.

With decreasing temperature, a clear increase of the electrolyte leakage was observed in all ecotypes (Fig. 1c). The EL of Rajestan increased markedly at -6 °C although the peak of

EL for the other ecotypes was started at -9°C. In the other hand when the plants were subjected to temperatures lower than -6°C and -9°C, too much cellular damage occurred to Ragestan and other ecotypes, respectively.

The slope of EL curve vs. freezing temperatures was lower for resistant cultivars than susceptible ones in the study of Nezami *et al*. (2007) and Cardona *et al*. (1997). This indicates that under cold stress conditions the resistant cultivars have lower rate of EL. They suggested that the slope of EL curve could be considered as an index of cold tolerance in plant species.

Lethal temperature (LT50)

The temperature resulting in 50% leakage has been known as LT50 i.e. freezing temperature that causes 50% mortality (Gusta *et al*. 1982). There was significant difference ($P \leq 0.01$) among cumin ecotypes in terms of LT_{50el}. Based on the results from LT_{50el}, freezing tolerance varied among ecotypes with India Rajestan (LT_{50el} = -9 °C) from tropical region having the least resistance and the Ghoochan ecotype (LT_{50el} = -12.1 °C) having the most resistance to freezing injury (Table 1). The same results were found by Azizi et al., (2007). They reported that wheat cultivars were different in LT50el. Since, the LT50el ranged from -3.7 °C to -15.8 °C. Such differences in LT_{50el} under cold stress were reported for other plants too (Nezami *et al*., 2007; Tai *et al*., 2003).

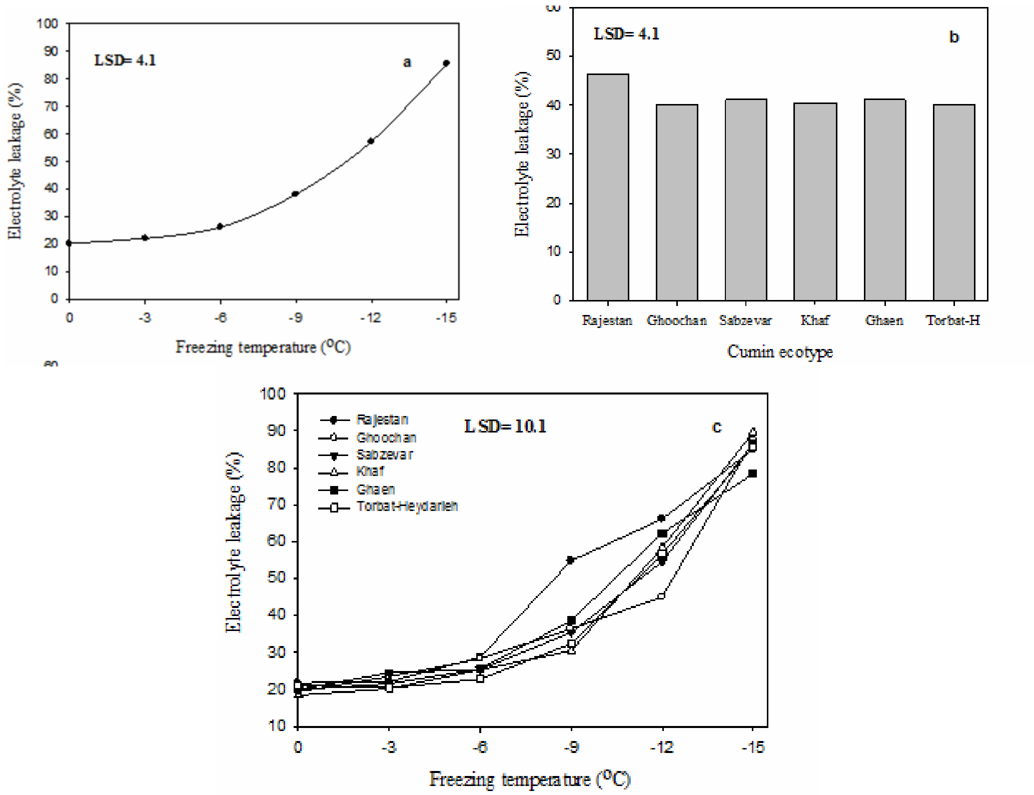

Fig.1. Effect of freezing temperature (a), ecotype (b), and interaction effects of ecotype and temperature (c) on electrolyte leakage percentage.

Table 1.
Variations in LT_{50el}, percent survival, LT_{50su}, plant height, leaf area (LA) and dry weight (DW) among different cumin ecotypes.

Ecotype	LT_{50el} (°C)	Survival (%)	LT_{50su} (°C)	Plant height (cm)	LA (cm^2 $plant^{-1}$)	DW (mg $plant^{-1}$)
Rajestan (India)	-9	58.9	-9.2	1.4	1.2	8.6
Ghoochan	-12.1	71.5	-11.8	2.1	1.3	9.5
Sabzevar	-11.3	61.4	-9.8	2.2	1.5	9.8
Khaf	-11.5	60.5	-9.7	2.0	1.4	10.3
Ghaen	-10.6	68.0	-10.8	2.1	1.4	9.3
Torbat-Heydarieh	-11.4	58.8	-9.3	2.0	1.3	9.5
LSD (0.05)	0.9	4.7	1.3	0.3	ns	ns

Plant re-growth

Plant height

Plant height was significantly affected by freezing temperature ($P \leq 0.01$). In surviving individuals the plant height was significantly less at lower temperature than control. The greatest plant height (3.5 cm) was observed at 0°C while the least (0.8 cm) occurred at -12 °C by 77% reduction in plant height (Table 2). It seems that cold stress interfere with plant growth leading to decreased plant height. Triticale plant height was also decreased by 21% at -12 °C (Nezami et al., 2010). Plant height varied significantly ($P \leq 0.01$) among cumin ecotypes. All Iranian ecotypes (Ghoochan, Ghaen, Sabevar, Khaf and Torbat- Heydarieh) were taller than the Indiain Rajestan (Table 1). Azizi *et al.*, (2007) reported that wheat cultivars were significantly different in plant height while the plant height ranged between 2.9 cm to 4.7 cm. Temperature by ecotype interaction had significant ($P \leq 0.01$) effect on plant height. Although, plant height of all ecotypes decreased with reductions in freezing temperatures, the percentage reduction varied among them. For example, at -12 °C, the height reduction for Goochan was only 55%, however, that of Sabzevar was as great as 85% at the same temperature (Table 3).

Leaf area

Leaf area was significantly ($P \leq 0.01$) influenced by freezing temperatures. The highest and the lowest leaf area for surviving plants were obtained from 0°C with 3 cm^2 and -12°C with 0.4 cm^2, respectively (Table 2). Decreasing temperature less than -6 °C caused clear reduction in leaf area where at -9 °C and -12 °C leaf area was reduced 60% and 87% in comparison with 0°C, respectively (Table 3). The interaction of ecotype by temperature was also significant for leaf area measurements (Table 2). Khaf ecotype at 0 °C (3.6 cm^2) and Sabzevar ecotype at -12 °C (0.1 cm^2) had the highest and lowest leaf area, respectively (Table 3). None of the seedlings from Torbat-heydarieh and Khaf ecotypes survived after -12 °C treatment to produce any leaf. Leaf area reduction in Ghoochan ecotype was 32% where the temperature decreased from 0 °C to -12 °C. However, the greater leaf area reduction was observed for Sabzevar ecotype with 96% decrease. Nezami *et al*., (2007) showed that rapeseed leaf area was affected by freezing stress where the leaf area production in control (0 ^{o}C) was 26% more than -12 ^{o}C.

Plant dry weight

The freezing temperatures were significantly (P ≤ 0.01) effective on plant dry weight. The surviving plants accumulated the highest (19.9 mg) and lowest (2.1 mg) dry matter when subjected to 0 ^{o}C and -12 ^{o}C, respectively (Table 2). Drastic reductions in plant dry weight occurred at freezing temperatures beyond -6 ^{o}C. For example, dry weight reduced by 53% and 89% (relative to control) when cumin plants were subjected to temperatures as cold as -9 ^{o}C and -12 ^{o}C, respectively. This reduction might be the result of adverse effect of freezing stress on regrowth capability of plants over the recovery period. Studies on chickpea showed significant effect of freezing temperatures on plant dry weight where Nezami *et al.,* (2007) reported 76% in dry weight reduction as temperature decreased from -3 ^{o}C to -12 ^{o}C. The same negative effect of cold stress on fennel dry weight was found by Rashed *et al*., (Fennel USA) who reported 36% and 7% reduction in dry matter production of cold acclimated plants at -9 ^{o}C and -12 ^{o}C, respectively in comparison with control plants. The regrowth of bufalograss (*Buchloe dactyloides* (Nutt.) Engelm.] cultivars was highly affected by freezing temperatures with 60% dry weight reduction in the most sensitive cultivar as the temperature decreased from -8 ^{o}C to -12 ^{o}C (Qian *et al.,* 2001).

Table 2.

The effect of different freezing temperatures plant height, leaf area (LA), dry weight (DW) and survival (%), in cumin.

Freezing temperature (°C)	Plant Height (cm)	LA (cm^{2} plant $^{-1}$)	DW (mg plant $^{-1}$)	Survival (%)
0	3.5	3.0	19.9	100.0
-3	3.0	2.2	14.6	100.0
-6	2.6	17	11.5	91.0
-9	2.3	1.2	9.3	74.6
-12	0.8	0.4	2.1	13.8
-15	0.0	0.0	0.0	0.0
LSD (0.05)	0.3	0.3	3.4	4.7

Plant survival

There were significant differences (P ≤ 0.01) among freezing temperatures for the survival percentage. No plant mortality was observed at temperatures up to -3 ^{o}C, however, the survival percentage declined continuously with decreasing temperature. Only 13.8% of plants survived at -12 ^{o}C while no plant stayed alive at -15 ^{o}C (Table 2). In the study of Rife and Zinali (2003) the mean survival percentage for three oilseed rape cultivars was 64, 27, 9 and 7% at -6, -8, -10 and -12 ^{o}C, respectively.

Survival percentage varied (P ≤ 0.01) among cumin ecotypes. Ghoochan ecotype (71.5%) showed the highest and Ghaen ecotype (68.0%) had the lowest survived seedling (Table 1). The survival percentage was affected (P ≤ 0.01) by the ecotype and temperature interaction (Table 2). The greatest survival was observed at 0 ^{o}C for all cumin ecotypes, while very few plants (6.7%) from Sabzevar ecotype survived at -12 ^{o}C (Table 3). Although, survival was 100% for Ghoochan and Ghaen ecotypes at temperatures up to -6 ^{o}C, the proportion of surviving plants decreased noticeably in other ecotypes (e.g. 13 or 20% reductions in Sabzevar and Khaf, respectively). Ghoochan survival percentage was even 45% at -12 ^{o}C followed by Ghaen with 21.6% survival.

Cumin ecotypes also varied ($P \leq 0.01$) with $LT_{50su.}$ Based on LT_{50su}, Ghoochan (LT_{50su}: 11.8) and Ghaen (LT_{50su}: -10.8) were found as the most tolerant ecotypes while India Rajestan and Torbat-heydarieh (LT_{50su} = -9.3) had the lowest tolerance to freezing (Table 1). Similarly, the LT_{50su} was also variable among annual bluegrass (*Poa annua* L.) ecotypes in the study of Dionne *et al.*, (2001).

Table 3.

The interaction of temperature and ecotypes between plants' height, leaf area (LA) and survival (%) after different freezing temperature and recovery in glasshouse.

Treatment	Temperature	Height (cm)	LA (cm^2)	Survival (%)	Treatment	Temperature	Height (cm)	LA (cm^2)	Survival (%)
Rajestan (India)	0	2.7	2.3	100.0	Khaf	0	3.9	3.6	100.0
	-3	2.3	1.9	100.0		-3	3.4	2.3	100.0
	-6	1.5	1.7	88.9		-6	2.9	1.6	80.4
	-9	1.4	1.5	55.7		-9	2.3	1.4	83.0
	-12	1.0	0.3	9.4		-12	0.0	0.0	0.0
	-15	0.0	0.0	0.0		-15	0.0	0.0	0.0
Ghoochan	0	3.8	2.5	100.0	Ghayen	0	3.4	3.0	100.0
	-3	2.8	1.8	100.0		-3	3.0.	2.2	100.0
	-6	2.4	1.5	100.0		-6	2.8	1.6	100.0
	-9	2.2	0.9	84.1		-9	2.5	1.3	87.0
	-12	1.7	1.7	45.0		-12	1.4	0.4	21.6
	-15	0.0	0.0	0.0		-15	0.0	0.0	0.0
Sabzevar	0	3.9	3.5	100.0	Torbat-e-Heydarie	0	3.6	3.2	100.0
	-3	3.4	2.5	100.0		-3	3.2	2.3	100.0
	-6	2.9	1.9	86.7		-6	2.9	1.9	89.8
	-9	2.3	1.4	75.2		-9	2.7	0.5	63.5
	-12	0.5	0.1	6.7		-12	0.0	0.0	0.0
	-15	0.0	0.0	0.0		-15	0.0	0.0	0.0
LSD (0.05)		0.8	0.8	11.7			0.8	0.8	11.7

Correlation Analysis

The results of correlation analysis demonstrated a negative, significant correlation between EL% and survival percentage (r = -0.90**). The correlation between EL and other traits was also negative and significant (Table 6). Survival percentage was highly correlated with plant height (r = 0.90**). The same positive correlations was obtained with dry matter (r = 0.57**) and leaf area production (r = 0.81**) (Table 4).

Table 4.
Pearson's correlation coefficients between leaf area (LA), plant height (Height), dry weight (DW), percent survival and electrolyte leakage (EL) in cumin.

Plant trait	1	2	3	4	5
1- LA	1				
2- Height	0.82**	1			
3- DW	0.55**	0.54**	1		
4- Survival	0.81**	0.90**	0.57**	1	
5- EL	-0.80**	-0.86**	-0.54**	-0.90**	1

CONCLUSIONS

The results of this experiment showed significant effect of freezing temperatures on EL ($P \leq 0.01$) where EL increased as temperature decreased. For example, the EL from plants at -15 °C was 4.24 times higher than control (0 °C). A close relationship was observed between LT_{50el} and LT_{50su} . Therefore, the EL method could be used as a fast and efficient method in evaluating the cold tolerance of cumin ecotypes. Freezing temperatures affected plant height, dry weight and leaf area. However, there were no significant differences among cumin ecotypes with plant dry weight or leaf area. Plant height was different between Iranian ecotypes and India Rajestan where all Iranian ecotypes were taller in height than India Rajestan.

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