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Freezing tolerance of chickpea genotypes in controlled conditions

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Abstract

Lack of chickpea cultivars tolerant to extreme freezing is a limiting factor for autumn planting of this crop in cold areas. In this study, 58 Kabuli-type chickpea genotypes and a control (an international sensitive genotype) were planted in pots and first exposed to -10°C and then to -15°C after being acclimated in natural conditions. No destructive effect of -10°C was observed in plants. So, acclimation was repeated and plants were transferred to -15°C. Survival percentage was measured after three-week recovery period. In addition, 19 genotypes with higher survival percentage along with the control were exposed to temperatures of -16, -18 and -20°C after they were acclimated in controlled conditions. The experiments were arranged as completely randomized design with three replications. Results indicated that seven genotypes had survival percentage more than 80%, 24 genotypes more than 25% and 25 genotypes could not survive in -15°C. MCC803 had the highest and MCC808 and MCC510 the lowest survival percentage. All the genotypes were killed in -18 and -20°C. Among the 19 studied genotypes, eight were able to tolerate -16°C. Among factors affecting cold tolerance such as soluble carbohydrates, proline, total phenol, photosynthetic pigments, DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity and malondialdehyde, soluble carbohydrates showed significant positive correlation with chickpea survival percentage. Generally, among the studied genotypes four genotypes (MCC53, MCC770, MCC776, MCC809 and MCC815) are recommended as promising genotypes for freezing tolerance.

Keywords: Acclimation, Autumn planting, Metabolites, Soluble carbohydrates, Survival

Introduction

Legumes are the second largest family of plants after grasses and are the key components of natural and agricultural ecosystems (Gepts *et al*., 2005). Plants of this family fix atmospheric nitrogen, which is needed to maintain soil structure and fertility (Dwivedi *et al*., 2015). Many legumes known as pulses are consumed as grains and are produced to provide protein for human nutrition and livestock feed (Gepts *et al*., 2005). Food and Agriculture Organization (FAO) named 2016 as the year for pulses in honor of these plants.

Air temperature has increased on a global scale and it is expected to be continued until the end of this century (Stocker, 2014). These changes will be accompanied by a change in the pattern of rainfall as the rainfall decreases in the spring in the Mediterranean regions (Giorgi and Lionello, 2008).

Drought stress is one of the most important limiting factors in crop production and decreasing the amount of spring rainfalls has increased the frequency of severe drought conditions (Giorgi and Lionello, 2008; Homer *et al*., 2016). Crop autumn planting is one of the ways to make optimum use of precipitation and escape from the late season droughts. Compared to spring cultivation, it leads to higher yield and yield stability. Also water use efficiency is improved and soil erosion is reduced due to better soil cover and protection in autumn planting (Armoniene *et al*., 2013).

Chickpea in Iran is usually cultivated under dry farming conditions in spring (late February to mid-April). The most sensitive growth stage in chickpea (pod formation and seed filling) is usually affected by late season drought, which is caused by irregular pattern of rainfall in Iran and end of rainfall in June resulting in yield reduction. Studies have indicated a yield increase of about 70% due to utilization of soil water and so the success of chickpea fall planting in the Mediterranean regions (Nezami and Bagheri, 2005; Najibniya *et al*., 2008). Chickpea autumn cultivation improves plant height, which is suitable for providing the possibility of mechanized harvesting. Also a nitrogen fixation of 80-120 kg ha^{-1} has been observed in fall chickpea cultivations against only 25-45 kg ha^{-1} in spring cultivation (Singh, 1993). With all these benefits, freezing stress usually occurs during the seedling and early growth stages of crops in these regions (Pescador *et al*., 2017; Nezami and Bagheri, 2005).

Processes involved in freezing tolerance include a series of biological and physiological changes which lead to an increase in abscisic acid (ABA) (Shi and

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Yang, 2014; Zhang *et al*., 2016), changes in cell membrane lipid compounds (Welti *et al*., 2002) as well as changes in osmolytes and increase in antioxidants (Yancey, 2005). Study on biochemical and physiological process not only is used in cold-tolerant plant selection, but also helps the breeders to improve cold tolerance in other cultivars. Evaluation of the possibility of chickpea autumn cultivation in highland regions of Iran has been started since early 1990 at Ferdowsi University of Mashhad and results have indicated suitable variations in cold tolerance of about 1000 genotypes available in the seed bank collection of Research Center for Plant Sciences of Ferdowsi University of Mashhad (Porsa *et al*., 2002; Nezami and Bagheri, 2005; Najibniya *et al*., 2008).

The aim of this research in continuation of previous studies is the identification of cold tolerant cultivars for autumn cultivation in highlands and cold regions under controlled conditions and to introduce the metabolite markers related to this tolerance.

Materials and Methods

Plant materials: Chickpea seeds were provided from Mashhad Chickpea Collection at Research Center for Plant Sciences, Ferdowsi University of Mashhad, Mashhad, Iran. In this study, 58 Kabuli-type chickpea genotypes along with an international cold sensitive genotype (ILC533 (International Line of Chickpea) or MCC505) as control were evaluated in three experiments.

Plant growth and treatment conditions (Experiment 1 and 2: -10 and -15˚C): In late October 2015, six seeds of each genotype were planted in 10-diameter plastic pots containing equal proportions of soil, sand and leaf mold (GVV, 1:1:1), after disinfection by fungicide Benomyl. Seedlings were kept in a natural environment for three months and were transferred to a thermogradiant freezer with an initial temperature of 5˚C. The temperature dropped 2˚C per hour till it reached to -10˚C. At -2.5˚C, Ice Nucleation Active Bacteria (INAB) were sprayed as a thin layer on leaves to form ice nuclei in plants and prevent super cooling. Plants were kept at -10˚C for an hour (Wisniewski *et al.*, 2002). They were taken out and transferred to greenhouse and kept there with a temperature of 22/16˚C day/night for 21 days. Survival percentage was measured three weeks after freezing stress. Since no genotype was affected by freezing stress (100% survival), all the plants were kept again in a natural environment for three weeks to be acclimated (Fig. 1) and then were transferred to the thermogradiant freezer. Some plant metabolites such as soluble carbohydrates, photosynthetic pigments, total phenol and DPPH radical scavenging activity were measured before freezing stress. Freezing conditions were the same as the previous stage with the difference in final temperature, which was -15˚C and plant recovery was also measured as mentioned before. Damage to the cell membrane was determined after freezing (over night), by electrolyte leakage test. Survival percentage, plant height and fresh and dry weight were measured three weeks after freezing stress.

Plant growth and treatment conditions (Experiment 3: -16 to -20˚C): Out of 58 primary genotypes, 19 genotypes with survival percentage more than 33% were selected and studied in the third experiment with freezing temperatures of -16, -18 and -20˚C. The international sensitive genotype was also considered as control. Six seeds of each genotype were sown in 3-4 cm depth in plastic pots after they were disinfected. Since natural environmental conditions was not appropriate for cold acclimation, pots were kept in growth chambers with 22/16±2°C day/night, photoperiod of 14/10 hrs. light/dark and light intensity of 400 µmol photon.m^{-2}.s^{-1} on 10 cm of surface pot until 4-6 leaf stage. To be acclimatized, plants were kept in a 6 weeks cold treatment as follows: 14 days in 7/5±1°C day/night, photoperiod of 11/13 hrs. day/night, 14 days at 2/5±1°C day/night, photoperiod of 10/14 hrs. day/night, 14 days at 2/0±1°C day/night, photoperiod of 9/15 hrs. day/night with light intensity of 400 µmol photon.m^{-2}.s^{-1}.

Measurements: Plant metabolites such as soluble carbohydrates, photosynthetic pigments, total phenol, DPPH radical scavenging activity, malondialdehyde and proline (Bates *et al*., 1973) and also plant height, number of lateral branches and number of leaves per plant were measured before freezing stress. Freezing process was applied as the same as previuse experiment with the difference in final temperatures (-16, -18 and -20°C). Electrolyte leakage measurement and recovery period was also the same as mentioned before. Survival percentage, plant height and plant fresh and dry weight were determined three weeks after freezing stress.

Soluble carbohydrates was determined based on the modified method of phenolsulforic (Dubois *et al*., 1956) and photosynthetic pigments were measured according to the procedure described by Dere *et al*. (1998). Total phenol (Singleton and Rossi, 1965), DPPH (Abe *et al*., 1998), malondialdehyde (MDA) (Heath and Parker, 1968) and Proline concentration (Bates *et al*., 1973) were also measured.

To measure electrolyte leakage (EL) (Gepts *et al*., 2005), the youngest fully expanded leaves of a selected plant from each pot were seperated and placed in vials containing 50 ml double-distilled water and kept in the lab for 24 hours. Electrolyte leakage measured by EC meter (Jenway Model) after 24 hours was considered as EC_1. In order to determine full electrolyte leakage caused by plant death, the vials were autoclaved at 110˚C and ½ atmosphere for 30 minutes. Then the vials were kept in the lab and their electrolyte leakage was measured again after 24 hours and were considered as EC_2. Electrolyte leakage percentage (EL%) was calculated using the following equation:

Eq 1: $EL\% = (EC_1/EC_2) \times 100$

Data analysis: The experimental design was completely randomized design with three replications.

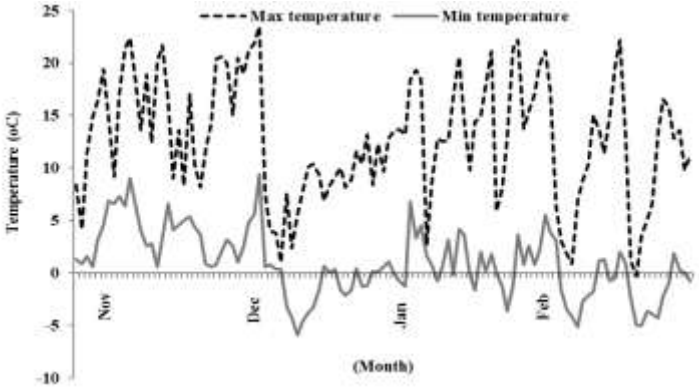

Fig. 1. Air minimum and maximum temperatures in autumn and winter 2015 in Mashhad

Minitab 16, JMP 4 and Excel softwares were used for statistical analysis, cluster anslysis and mean comparison was carried out using Least Significant Difference (LSD) test ($P \leq 0.05$).

Results

Experiment 1: All 58 studied genotypes tolerated freezing temperature of -10°C for one hour and survived at this temperature. For this reason, the plants were again acclimatized for three weeks and exposed to -15°C then after.

Experiment 2: There was a significant difference ($P \leq 0.05$) among genotypes in -15°C three weeks after freezing stress according to survival percentage. Thirty one genotypes survived in these conditions, although the survival range varied from 5 to 95%. Among these, seven genotypes showed the survival percentage of 80%. The highest survival was found in MCC803 and the lowest in MCC808 and MCC510 (Table 1). Twenty seven remaining genotypes, including the international sensitive genotype (ILC533 or MCC505) could not tolerate the freezing stress (Fig. 2).

The highest concentration of photosynthetic pigments was found in MCC753, MCC808 and MCC477 and the lowest in MCC463, MCC803 and MCC771, showing 43% difference between MCC753 and MCC753 (Table 2). The negative correlation (r^2=-0.39*) between the total concentration of photosynthesis pigments and survival percentage was noticeable.

A significant variation was found among genotypes according to soluble carbohydrates as an effective metabolite in freezing tolerance. The highest amount of soluble carbohydrates was found in MCC803, MCC741, MCC 774 and MCC53 which also showed higher survival. On the other hand, MCC477, MCC791, MCC786 and MCC761 accumulated the lowest levels of soluble carbohydrates after cold acclimation and before freezing stress (Table 2). A significant positive correlation (r^2=0.95**) was found between soluble carbohydrates and survival percentage. Increasing trend of survival with higher levels of soluble carbohydrates indicates the effective role of this metabolite in freezing tolerance of chickpea.

There was a high variation among the studied genotypes in terms of total phenol with the highest and lowest level in MCC741 and MCC815, respectively (Table 2). The correlation of survival percentage and total phenol revealed positive but non-significant relationship (r^2= 0.18) between these two parameters. A 13% difference was found between MCC770 and MCC776 as the genotypes showing the highest and lowest DPPH radical scavenging activity before freezing stress in -15°C, respectively (Table 2). Although cold acclimation prepares the plant to cope with further damages of freezing stress, no correlation was found between DPPH radical scavenging activity and survival percentage of the genotypes. It seems that reactive oxygen radicals are activated after freezing stress, which causes plant antioxidant activity to be increased.

Electrolyte leakage was decreased as survival percentage increased and the lowest and highest electrolyte leakage were found in MCC803 and MCC761 with 95 and 5% survival, respectively (Table 1). Also, the significant negative correlation (r^2=-0.92**) between the survival percentage and electrolyte leakage indicated the negative corelation between these two traits. In contrast to electrolyte leakage, the percentage of membrane stability as an indicator of cell membrane integrity had a direct positive relationship (r^2=0.92**) with survival percentage. The highest and lowest membrane stability was found in MCC803 and MCC761, respectively with a difference of 22%.

Plant height measured three weeks after freezing stress varied among genotypes. Although the highest plant height was observed in MCC803 (which also had the highest survival) and there was a significant positive correlation (r^2=0.42) between plant height and survival, but some of the genotypes with lower survival percentages like MCC761 and MCC788 did not show significant difference with MCC803. On the other way, plant height in MCC741 and MCC733 genotypes which didn't have significant difference with MCC803, was significantly different from MCC803 (Table 1).

The highest plant fresh weight was found in MCC803, MCC53 and MCC814 and the lowest in

Table 1- Electrolyte leakage and membrane stability just after freezing stress and survival percentage, plant height, fresh and dry weight and dry matter percentage per plant, three weeks after freezing stress in 31 Kabuli-type chickpea genotypes survived at -15°C.

Genotype MCC*	Survival	Electrolyte leakage	Membrane stability	Plant height	Fresh weight	Dry weight	Dry Matter
	%	%	%	(cm)	(g plant^{-1})	(g plant^{-1})	%
803	95	53	47	15.00	3.10	0.57	18
741	91	60	40	10.78	1.49	0.30	20
774	91	59	41	13.61	2.08	0.42	20
53	90	60	40	15.11	3.03	0.59	19
733	88	61	39	7.89	0.92	0.24	26
809	85	60	40	11.06	1.93	0.45	23
776	81	63	37	12.33	2.34	0.44	19
463	75	62	38	7.11	1.01	0.24	24
814	75	62	38	14.94	3.07	0.57	19
770	74	66	34	14.83	2.33	0.49	21
736	67	65	35	12.25	1.41	0.30	21
775	63	63	37	14.11	2.15	0.44	20
815	60	63	37	12.33	2.18	0.46	21
797	52	65	35	10.78	1.36	0.34	25
771	52	66	34	14.50	2.52	0.51	20
495	50	66	34	9.50	1.51	0.32	21
426	43	68	32	7.50	1.09	0.27	25
498	33	65	35	5.25	0.61	0.19	31
758	33	66	34	9.83	1.23	0.32	26
769	33	67	33	11.92	1.66	0.46	28
780	33	67	33	7.50	1.07	0.28	26
85	32	68	32	12.54	1.40	0.31	22
510	25	69	31	11.83	1.67	0.45	27
808	25	68	32	8.50	1.48	0.33	22
753	22	68	32	7.00	1.49	0.44	30
488	20	72	28	6.50	0.71	0.17	24
788	20	70	30	12.83	2.20	0.49	22
477	17	69	31	10.00	2.26	0.58	26
791	11	74	26	9.25	1.39	0.38	27
786	8	73	27	7.50	1.19	0.33	28
761	5	75	25	14.00	1.59	0.38	24
LSD (0.05)**	27	11	11	3.31	0.95	0.15	4.5

*** Mashhad Chickpea Collection, ** LSD: Least Significant Difference in P≤0.05 probability level**

MCC498, MCC488 and MCC733. The difference between the highest and the lowest mean of this parameter was 80% (Table 1). In terms of plant dry weight, the highest mean was found in MCC53, MCC477 and MCC803 and the lowest in MCC488, MCC498 and MCC733 with 71% difference between the highest and the lowest.

Although the highest plant fresh weight was observed in MCC803, MCC53 and MCC814, the highest plant dry weight was found in MCC53, MCC477 and MCC803, respectively (Table 1).

MCC803 and MCC498 had the lowest and the highest dry matter percentage, respectively (Table 1). A significant and negative correlation (r^2= -0.65**) was found between the dry matter percentage and survival. Freezing temperatures caused a reduction in plant regrowth. The plant's ability to produce more dry matter after freezing stress can indicate greater tolerance of the plant.

Based on cluster anslysis, genotypes divided in three groups in -15°C (Fig. 3) Ten genotypes in first (MCC803, MCC774, MCC809, MCC53, MCC814, MCC771, MCC770, MCC775, MCC815 and MCC776) and second (MCC741, MCC733, MCC463, MCC736,

Table 2- Leaf photosynthetic pigments, soluble carbohydrates, total phenol and DPPH radical scavenging activity measured before freezing stress, in 31 Kabuli-type chickpea genotypes survived at -15°C

Genotype MCC*	Total pigment	Soluble sugar	Total phenol	DPPH
	(mg gfw^{-1})	(mg gfw^{-1})	(mg gfw^{-1})	(mg gfw^{-1})
803	0.849	9.255	4.099	0.891
741	1.059	8.869	5.385	0.908
774	1.311	8.051	3.812	0.889
53	1.174	8.142	4.011	0.847
733	1.122	7.755	4.678	0.856
809	1.049	7.633	4.421	0.900
776	1.058	7.240	4.557	0.769
463	0.832	6.893	4.462	0.876
814	1.060	6.985	3.848	0.899
770	1.029	6.759	4.352	0.920
736	1.225	6.711	4.500	0.890
775	1.185	5.953	4.311	0.884
815	1.009	6.122	2.735	0.882
797	1.047	6.659	4.152	0.882
771	0.927	6.634	3.690	0.881
495	1.261	5.890	4.384	0.856
426	1.235	5.790	4.199	0.859
498	1.113	5.949	4.600	0.864
758	0.997	5.818	5.331	0.900
769	1.122	5.671	3.989	0.862
780	0.992	5.625	3.949	0.854
85	1.043	5.581	3.598	0.867
510	1.063	4.817	3.617	0.881
808	1.426	5.407	3.997	0.874
753	1.454	4.846	4.676	0.888
488	1.257	5.251	3.708	0.873
788	1.316	5.461	4.100	0.862
477	1.397	4.691	4.273	0.853
791	1.186	4.650	4.108	0.845
786	1.161	3.876	3.877	0.882
761	1.090	4.510	4.270	0.890
LSD (0.05)**	0.413	2.611	0.743	0.052

*** Mashhad Chickpea Collection,** LSD: Least Significant Difference in P≤0.05 probability level**

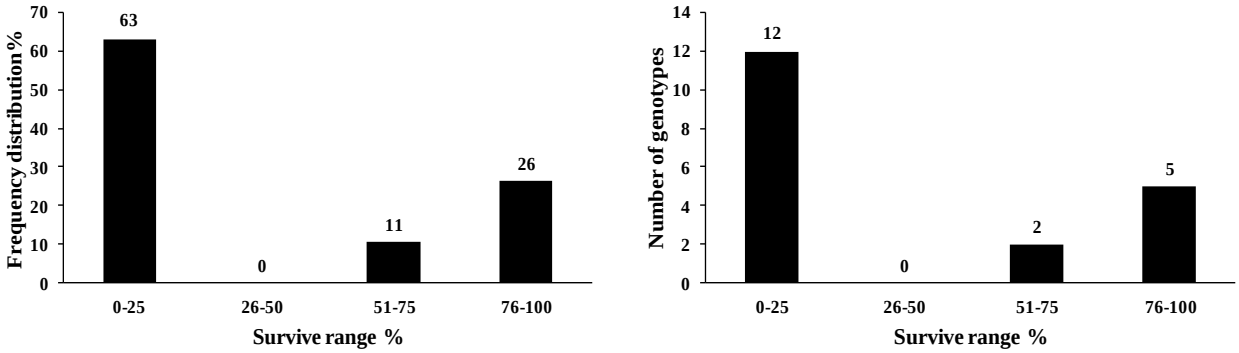

Fig. 2- Number and frequency distributions of chickpea genotypes in different survival range in -15°C

MCC797, MCC758, MCC495, MCC426, MCC498 and MCC780) groupe and 11 genotypes (MCC769, MCC510, MCC85, MCC761, MCC488, MCC791, MCC786, MCC808, MCC 753, MCC788 and MCC477)

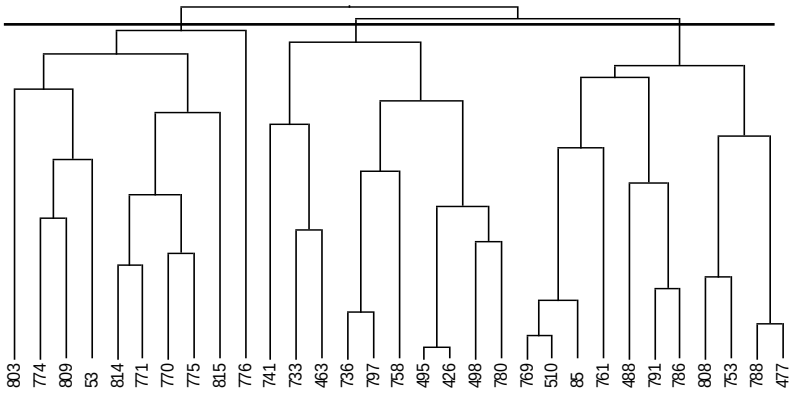

Fig. 3- Cluster grouping of chickpea genotypes based on studied characteristic in -15°C.

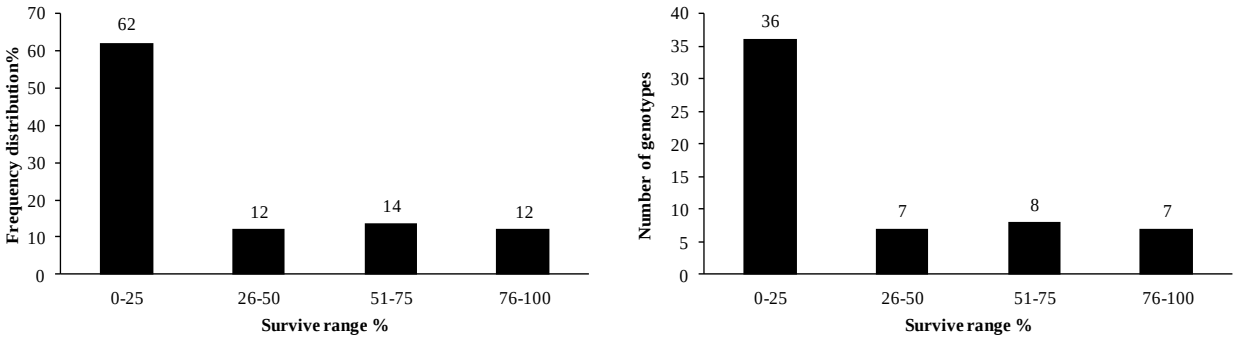

Fig. 4- Number and frequency of chickpea genotypes in different survival range in -16°C

in the third group (Fig. 3).

Experiment 3: -16 to -20°C: Six weeks after acclimation, three freezing temperatures of -16, -18 and -20°C were imposed on 19 chickpea genotypes selected from the previous experiment. All the seedlings were killed at -18 and -20°C, whereas there was a significant difference ($p \leq 0.01$) among genotypes in terms of survival percentage, three weeks after freezing stress at -16°C. The frequency of the studied genotypes in four survival range in -16°C indicated that 63% (12 genotypes) of genotypes (12 genotypes) had a survival range of 0-25%, of which 11 genotypes were killed and one (MCC774) showed 20% survival. None of the genotypes were in the range of 26-50% survival and 11% (2 genotypes) had survival percentage of 51-75%. Twenty six percentages (Fig. 4) (Five genotypes, including MCC53, MCC770, MCC776, MCC809 and MCC815) were in the range of 76-95% survival (Table 3).

Significant difference was observed among genotypes according to total photosynthetic pigments (Table 4) with the highest content in MCC741 and MCC774 which had the lowest survival percentage after freezing stress in -16°C (Table 3). On the other hand, genotypes with higher survival, e.g. MCC809 and MCC815 had the lower photosynthetic pigment content (Table 4). The difference between the highest and lowest content in survived genotypes was 42% (Table 3).

Genotypes with higher survival percentage, also had higher leaf soluble carbohydrates compared to the others and a significant difference was found in this way. The highest and lowest content of soluble carbohydrates before freezing stress were observed in MCC770 and MCC741. A positive significant correlation was found between the survival percentage and leaf soluble carbohydrates ($r^2=0.66^{**}$).

No significant difference was found among genotypes according to leaf proline content (Table 4).

MDA content of leaves as an indicator of cell membrane damage revealed that MDA content was increased as survival percentage decreased. However, the lowest and highest mean of this parameter was observed in MCC776 and MCC495 with 95 and 75% survival, respectively (Table 4).

DPPH radical scavenging activity was increased as survival percentage decreased and a significant negative correlation ($r^2=-0.75^{**}$) was found between these two parameters. A difference of 52% was observed between MCC774 and MCC53 genotypes according to DPPH radical scavenging activity (Table 4).

Chickpea genotypes were different according to total leaf phenol content (Table 4) in a way that MCC53 and MCC770 had the highest and MCC815 and MCC809, the lowest phenol content in their leaves. High survival percentage in both groups of genotypes (with high and low phenol content) were remarkable (Table 3). In this study, decreasing the survival percentage and decreasing membrane stability led to a higher electrolyte leakage (Table 4). MCC53 and MCC770 with the lowest electrolyte leakage and the highest membrane stability showed 95% survival and MCC774

Table 3- Plant height and leaf number per plant before freezing stress, electrolyte leakage and membrane stability just after freezing stress and survival percentage three weeks after freezing stress, in eight Kabuli-type chickpea genotypes survived at -16°C

Genotype MCC*	Plant height	Leaves	Eectrolyte leakage	Membrane stability	Survival
	(cm)	(no.)	%	%	%
53	9	12	69	31	95
770	15	8	64	36	95
776	11	8	74	26	95
809	15	10	75	25	95
815	14	10	83	17	95
495	13	8	87	13	75
741	10	8	94	6	70
774	7	6	84	16	20
LSD (0.05)**	1.66	1.27	8.89	3.87	7.95

*** Mashhad Chickpea Collection**

**** LSD: Least Significant Difference in P≤0.05 probability level**

Table 4- Leaf photosynthetic pigments, soluble carbohydrates, proline content, malondialdehyde (MDA), DPPH radical scavenging activityand and total phenol measured before freezing stress and plant height and leaf number per plant measured three weeks after freezing stress in eight Kabuli-type chickpea genotypes survived at -16°C

Genotype MCC*	Total pigment	Soluble sugar	Proline	MDA	DPPH	Total phenol	Plant Height	Leaves.
	(mg gfw^{-1})	(mg gfw^{-1})	(mg gfw^{-1})	(mg gfw^{-1})	(mg gfw^{-1})	(mg gfw^{-1})	(cm)	(no.)
53	1.264	16.1	4.8	15.3	0.476	7.22	7	6
770	1.601	20.0	7.0	16.7	0.492	6.00	5	6
776	1.370	14.5	6.4	13.5	0.749	5.86	6	6
809	1.108	14.0	4.2	21.1	0.623	4.43	9	7
815	1.236	13.8	3.4	25.0	0.679	4.39	6	8
495	1.327	10.1	3.1	31.4	0.575	5.90	8	8
741	1.903	8.6	2.7	22.6	0.926	5.69	7	7
774	1.669	9.6	4.1	19.2	0.989	4.71	6	6
LSD (0.05)**	0.387	3.63	4.37	14.53	0.212	1.27	2.4	1.58

*** Mashhad Chickpea Collection**

**** LSD: Least Significant Difference in P≤0.05 probability level**

with 20% survival percentage had the highest electrolyte leakage and the lowest membrane stability (Table 3 and Table 4).

Plant height and biomass of autumn cultivated plants are important factors in cold tolerance. High plant height imposes the plant to cold weather and tolerant plants usually spend autumn and winter as rosette. In this study, genotypes varied according to plant height before freezing stress and the highest and the lowest plant height were observed in MCC809 and MCC770 (Table 4). Three weeks after freezing stress, MCC770 was the highest among genotypes and reached to a height threefold more than it was before freezing stress, indicating high growth and recovery ability of this genotype (Table 3). On the other hand, the lowest height measured three weeks after freezing stress, was found in MCC774 which was possibly due to plant tissue damages during freezing stress (Table 3). Studying changes in plant leaf number before and three weeks after freezing stress showed a two-times increase in leaf number of MCC53 which had the most leaf numbers among genotypes. No changes were observed in leaf number of MCC774 and MCC495 after and before freezing stress and only one more leaf was found in MCC741 after freezing stress (Table 3 and Table 4).

Based on cluster anslysis, genotypes were divided in three groups at -16°C (Fig. 5). Three genotypes in first (MCC53, MCC776 and MCC770) and second (MCC495, MCC815, MCC809) groupe and two genotypes (MCC741 and MCC774) in the third groupe (Fig. 5).

Discussion

Autumn-planted crops are exposed to severe environmental conditions such as low temperatures. Low temperature, which is the main limiting factor for growth, production and distribution of agricultural crops (Zhu *et al*., 2007; Zhang *et al*., 2016) has been widely studied and different methods for cold tolerance selection in crops have been reported (Mantri *et al*.,

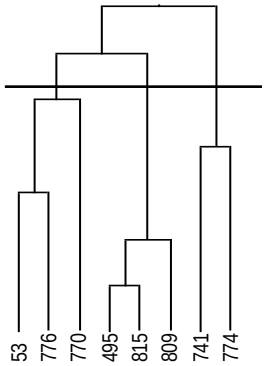

Fig. 5- Cluster grouping of chickpea genotypes based on studied characteristic in -16°C

2012). Some literatures are published on cold tolerance in chickpea (Heidarvand and Maali Amiri, 2013). A controlled method was used for cold stress assessment in 114 faba bean masses (Inci and Toker, 2011) in seedling stage to determine cold tolerant lines. Also, physiological, morphological, biochemical and protein changes have been studied in short-term cold stress in chickpea (Heidarvand and Maali Amiri, 2013).

Chickpea sensitivity to cold stress varies in different growth stages and it is more sensitive to cold stress in the early growth stages (Warner and Johnson, 1972) in a way that some genotypes and cultivars are able to tolerate temperatures below zero (Clarke and Siddique, 2004). In this study, 58 Kabuli-type chickpea genotypes were able to tolerate -10°C which confirms freezing tolerance of chickpea genotypes in the early growth stages (Clarke and Siddique, 2004). Of course, it should be noted that cold acclimation in natural environmental conditions lasted for three months (22 Oct. to 20 Jan.) which possibly led to 100% survival in studied genotypes. Twenty seven genotypes were killed as the temperature dropped to -15°C and only seven genotypes had survival percentage above 80%. In the third experiment in which freezing temperature reduced to -16 to -20°C, only eight genotypes were able to tolerate -16°C. Since cold acclimation in this experiment was performed in controlled growth chamber, seedlings hardiness might not be induced similar to natural conditions. Although, some genotypes could tolerate -16°C.

In this study, genotypes including MCC53, MCC741, MCC770, MCC774 and MCC803 with higher survival percentage also had higher leaf soluble carbohydrates content (Table 1 and 3). Starch biosynthesis increases after a temporary decrease in autumn. Reduction in starch content is a result of several α-amylase and a specific β-amylase gene induction during cold acclimation. The conversion of starch to soluble hexose sugars is a key metabolic process in autumn acclimation, because these sugars act as energy sources and also protective agents against low temperatures (Dauwe *et al*., 2012).

It has been approved that low temperature induces hydrogen peroxide production in plants which could damage cell membrane by peroxidating membrane lipids (Kocsy *et al*., 2001). Furthermore, if photosynthetic capacity reduces, photosystem may be damaged by reactive oxygen radical formation due to excitation. In the present study, higher content of DPPH, total phenol and proline did not result in higher freezing tolerance and no higher levels of antioxidants were found in genotypes with higher survival capacity (Table 2 and 4). The cell membrane is the first place damaged by freezing stress and the main role of cold acclimation is to activate mechanisms that keep the cell membrane stable and protect them against damages caused by freezing stress. So, electrolyte leakage as an indicator of cell membrane damages is used to assay freezing stress effects (Armoniene *et al*., 2013).

Results of a study on chickpea showed increases in electrolyte leakage in lower temperatures (Wenaei *et al*., 2011). In the present study, an inverse relationship was found between electrolyte leakage and survival percentage in a way that electrolyte leakage was decreased as the survival percentage was increased. On the other hand, the membrane stability trend was different from electrolyte leakage and in both experiments, the survival percentage in some genotypes e.g. MCC803, MCC770 and MCC53 was increased as membrane stability increased (Table 1 and 3). Remarkable point was reduction in membrane stability in experiment 2 (-15°C) compared to experiment 3 (-16 to -20°C). It seems that three-months cold acclimation in natural conditions in experiment 2 led to higher seedling hardiness and cold tolerance compared to the shorter periods of cold acclimation in controlled conditions of growth chamber in experiment 3. One degree Celsius lower freezing temperature in the experiment 3 should also be considered that -16°C may possibly be the threshold of increase in cell membrane damage. Growth reduction due to freezing temperatures appears as lower height and ultimately less plant biomass. Although the plant height from ground level is related to growth form (rosette or erects types) and genetic criteria of each genotype, higher plant height after freezing stress in late winter and early spring leads to better chickpea competition with weeds. In experiment 3, MCC770 revealed a rapid growth ability and highly suitable height increase after freezing stress, which could be considered as a proper attribute in

selecting and for transfer to other genotypes.

Conclusion

In the present study, high variation was found among Kabuli-type chickpea genotypes as some genotypes could tolerate freezing temperatures as low as -16°C. Among metabolites that may be introduced as markers in freezing tolerance, total soluble carbohydrates showed positive correlation with survival percentage and could be used in the chickpea genotype selection for freezing tolerance. Since the survival percentage of chickpea genotypes were different in two experiments with freezing temperatures of -15 and -16°C, it seems that cold acclimation conditions affected freezing tolerance in chickpea. Based on the results of the present study, MCC803, MCC741, MCC774, MCC53 and MCC733 acclimated in natural conditions and MCC53, MCC770, MCC776, MCC809 and MCC815 acclimated in controlled conditions could tolerate -15 and -16°C temperatures, respectively. Further studies are needed to use these genotypes as candidates for cold regions at temperatures between -10°C to -16°C in the winter.

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