Study on Genetic Diversity of Some of the Barley (*Hordeum Vulgare* L.) Cultivars Using Morphophysiological Traits under the Chilling Stress

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Abstract
Plants are invariably exposed to a broad range of non-biological stresses, such as cold stress. These stresses have undesirable effects on the survival, growth, quality, and quantity of crops. This research has applied the genetic diversity of 20 cultivars of barley using physiological traits related to chilling tolerance, amino acid proline, pigments (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids). Some yield indicators (spike length, weight of the main spike, 1000-grain weight, number of fertile tillers, and grain weight per plant) were tested in conditions controlled by the in-situ composite analysis model based on a completely randomized design, including four locations (Control temperature levels (+8˚C), +2˚C, 0˚C, and -2˚C) and three replication. The results of the analysis of variance indicated that the interaction of cultivar in cold was significant for all traits at the level of 1% probability. Cluster analysis divided the cultivars of control and severe stress levels (-2˚C) into 5 groups. The biplot obtained from GGbiplot illustrated a considerable phenotypic variation in the studied germplasm.
Keywords: barley, genetic diversity, chilling stress

1. Introduction

Today, cereals have dedicated about 70% of the area under cultivation of crops. Approximately, half of human food needs, especially in Asia, are met directly from cereals. *Hordeum vulgare* L. barley with a global area under cultivation of about 50 million hectares and a production of about 133 million tons per year in terms of area under cultivation is the second most important crop in the world after wheat. According to the recent statistics, the area under barley cultivation in the 2014-15 crop year in Iran is equal to 1.7 million hectares with a production of about 3.4 million tons per year. Moreover, the area under cultivation of irrigated barley is 650.000 hectares with a production of about 2.1 million tons per year (Ahmadi et al., 2019). Low temperature is one of the major agricultural risks and one of the important factors, which limits the life and geographical distribution of plants in many parts of the world that can cause plant death as temperature stress (Esra and Sulun, 2010). Leaf chlorophyll is one of the most important indicators of environmental stress on the plant. The amount of chlorophyll in plants under stress decreases, which changes the light absorption and thus reduces the total light absorption by the plant (Hartmut and Babani, 2000). The use of osmolytes increases the adaptability of plants, while not harming it. One of the most important organic osmolytes is soluble and insoluble sugars and quaternary amino acids, such as proline (Nuccio et al, 1999). Proline is an amino acid that acts as a protector against stress in addition to osmotic regulation, which interacts directly and indirectly with macromolecules, thereby helping to maintain their natural shape and structure against stress conditions (Koc et al., 2011). Yield is a quantitative and complex trait, which is controlled by a large number of genes that are strongly affected by the environment. The selection of optimal genotypes based on yield is less useful. This selection is more useful when it is based on traits that directly or indirectly contribute to the yield (Farshadfar, 2010). Multivariate analysis is an efficient tool to measure genetic diversity in plant sample collections, determine the share of each trait in the total diversity of genotypes selection to be used in a breeding program, the relationships between yield components and morphological structures, key yield components, the relationship between geographic diversity and genetic diversity, and reduce the dimensions of the data. Multivariate methods, such as principal component analysis, factor analysis, cluster analysis, path analysis, and multivariate regression, have been used to estimate diversity, as well as determining proper genotypes for breeding purposes (Bhatt,1973; Pecetti and Nachit, 1993; Yau et al., 1989; Zobel et al., 1988). Accordingly, the purpose of the present research was to evaluate the late stress tolerance of barley cultivars through measuring physiological traits and some yield indicators.

2. Materials and Methods

The experiment was performed using the in-situ composite analysis model based on a completely randomized design, including 20 barley cultivars (Bahman (code 1), Dasht (code 2), Fajr 30 (code 3), Zarjoo (code 4), Zahak (code 5), Khatam (Code 6), Jolgeh (Code 7), Sahra (Code 8), Jonoob (Code 9), Nosrat (Code 10), Behrokh (Code 11), Yousef (Code 12), Reyhan (Code 13), Goharan (Code 14), Valfajr (Code 15), Kavir (Code 16), Nimruz (Code 17), Reyhan 03 (Code 18), Makoui (Code 19), and Nik (Code 20) at four temperature levels in locations (Control temperature levels (+8˚C), +2˚C, 0˚C, and -2˚C) and three replication in the Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran in 2016. Initially, the seeds were put in a germination device for four days (temperature 25°C and humidity 70%) after disinfection with 5% sodium hypochlorite (v/v). the Petri dishes were refrigerated for six weeks at 1±3°C to provide the chilling when the shoots reach 2cm. At the end of the allotted time, 10-15 seedlings were planted in pots. The seedlings were transferred to the cold chamber to apply the cold treatment by modeling late conditions from nature at three levels (+2, 0, -2) and control treatment +8˚C two months after they reached the reproductive stage (Zadox scale 48 to 60) (Zadoks et al., 1974). Lichtenthaler method was used to determine the concentration of plant pigments (Lichtenthaler, 1987). Finally, the concentrations of chlorophyll a, b, total chlorophyll, and carotenoids in $mg.g^{-1}$ of fresh weight were obtained through the following equations:

$$C_a = 16.72A_{665} - 9.16A_{652} \quad (1)$$

$$C_b = 34.09A_{652} - 15.28A_{665} \quad (2)$$

$$C_{a+b} = 1.44A_{665} - 24.93A_{652} \quad (3)$$

$$C_c = \frac{(1000A470 - 1/63Ca - 104/96Cb)}{221} \quad (4)$$

Bates method was applied to measure proline (Bates et al., 1973) and the amount of proline was determined based on the following equation in µg.g^{-1}.

$$\mu M \text{ Proline on g fresh leaves} = \frac{(\mu g \text{ Proline} / ml \times ml \text{ Toluen})}{(115/17\mu g/mol)/(g \text{ of sample}/5)} \quad (5)$$

Agronomic traits including spike length, 1000-grain weight, number of fertile tillers, weight of the main spike, and grain weight per plant were calculated from each pot after applying stress levels.

Given that one of the assumptions of analysis of variance is that errors must have a normal distribution, the normality test of experimental error distribution of data for each of the traits was performed, and then statistical analysis was performed on the data. Univariate analysis of variance was performed and cluster analysis of cultivars was done through Ward's method using Euclidean distance square for standardized variables using SPSS software ver18. Furthermore, G*E biplot was performed using GGE Biplot software ver6.3.

3. Results and discussion

The results of the combined analysis of variance (ANOVA) of the studied traits showed a significant difference (p<0.0001) between cultivars, which indicates the genetic diversity in terms of measured traits (Table 1). The genetic diversity effective in chilling stress tolerance can be very useful to selection high yield stress-tolerant cultivars and a better understanding of physiological mechanisms related to chilling stress tolerance in barley. Moreover, a significant difference was observed between the studied environments and the interaction of cultivars in the environment was significant for most traits. The statistically significant difference between the cultivars indicates the high genetic diversity between the evaluated plant materials. Probably, different mechanisms between them in response to chilling stress can be used to select proper parents and produce dispersing populations for local gene positioning. The significant cultivar interaction in the environment shows different responses of cultivars in various environments.

Table1. Mean squares of barley cultivars under chilling stress for physiological and morphological traits.

Mean of square											
S.O.V	df	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotenoids	Proline	Spike length	1000-grain weight	Weight of the main spike	Number of fertile tillers	Grain weight per plant
Environment	3	7.620*	1.558*	16.058	7.743*	0.005	22.5	501.3	6.69	614.1	226561

		*	*	**	*	**	59**	66**	1**	17**	7.7**
Replication(Environment)	8	0.088 ^{ns}	0.002 ^{ns}	^{ns} 0.098	^{ns} 0.034	0.00014*	^{ns} 0.376	^{ns} 2.007	^{ns} 0.082	8.063*	2258.867 ^{ns}
Cultivars	19	1.748**	0.377**	3.398**	0.768**	0.00036**	8.823**	172.462**	0.715**	38.017**	355389.708**
Cultivar×Environment	57	0.705**	0.113**	1.246**	0.207**	0.00038**	2.093**	130.029**	0.469**	7.169**	99167.031**
Error	152	0.263	0.014	0.332	0.052	0.000069	0.56	3.005	0.078	3.374	4178.910
(%)cv		9.12	9.79	8.43	9.83	1.3	13.5	5	23.84	24.24	14.75

***, * and ^{ns}, respectively significance at the level of probability of 1% and 5% and non-significance*

4. Cluster analysis

Cluster analysis showed that the studied cultivars were divided into five groups at the control temperature level (Figure 1). The results confirmed the analysis of the detection function of cultivars belonging to each group. Cultivars are distributed in 5 groups in cluster analysis for control temperature level (+8˚C). The third group had the highest value for the traits, including the weight of the main spike and the amount of proline. The fourth group had the highest values for the traits, including the fertile tillers, chlorophyll a, total chlorophyll, and carotenoids. The fifth group had the highest value for the traits, including chlorophyll b. Accordingly, it can be observed that the cultivars in the fourth group had a higher value than the traits of chlorophyll a, total chlorophyll, and carotenoids, as well as the number of fertile tillers, which can be desirable choices in some breed programs that are done by examining the relevant traits. The results of cluster analysis for the last stress level (-2˚C) showed (Figure 2) that the studied traits were able to compare the 5 groups more accurately. The study of desirable cultivars in the cluster analysis section at stress temperature (-2˚C) showed that the traits of proline, 1000-grain weight, grain weight per plant in the third group had the highest value, which includes cultivars (Jolgeh, Kavir, and Zahak). The second group includes the highest amount of these traits regarding the studied plant pigments; Yousef and Fajr 30 are in this group. The results of the genetic distance matrix using the Euclidean distance method showed that Nosrat and Khatam cultivars had the highest genetic distance (58.65) from each other for control temperature level (Table 2). Behrokh and Nimruz cultivars were the closest cultivars in terms of the lowest distance (2.607). Khatam and Kavir cultivars had the highest genetic distance (61.89) from each other regarding the extreme stress temperature levels (-2˚C). It was found that Nosrat and Makoui cultivars were the closest cultivars to this level of temperature stress regarding the lowest distance (4.45) (Table 2). With regard to the maximum genetic distance between these two cultivars at both levels, the maximum heterosis is expected by crossing these two cultivars at both control and extreme temperature stress levels. The results can be used as a raw material to improve cultivars (Farshadfar, 1998).

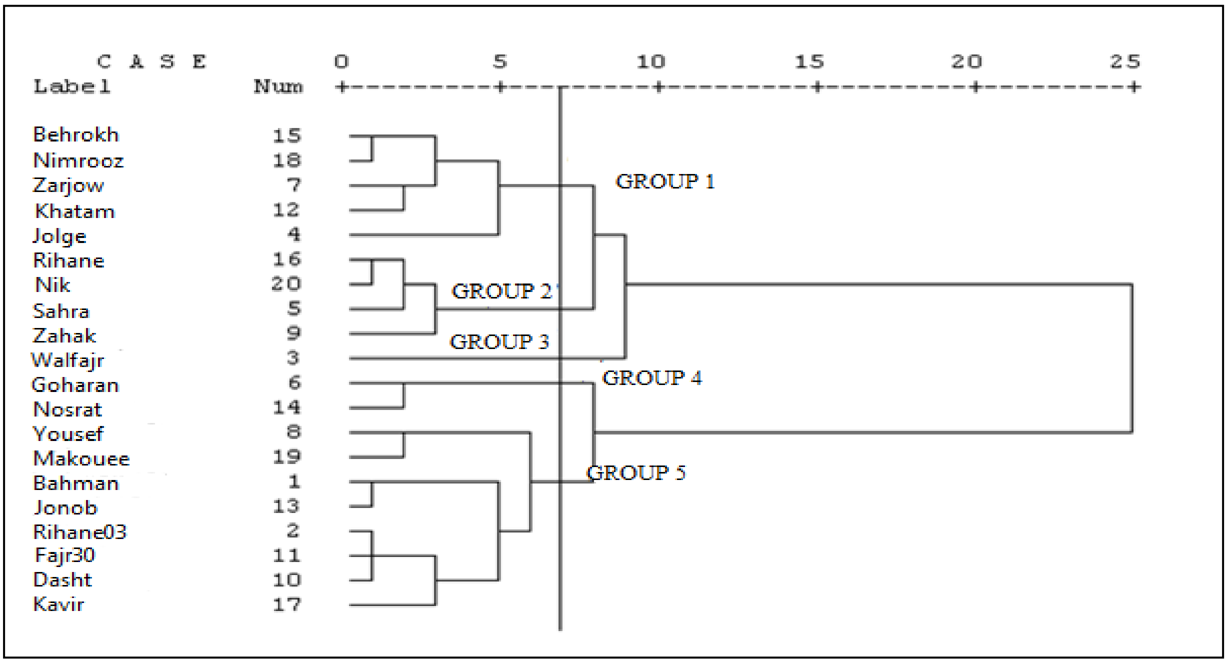


Figure1. Dendrogram obtained from cluster analysis of 20 barley cultivars based on Ward method under control conditions (+8 ℃) for physiological traits.

Figure2. Dendrogram obtained from cluster analysis of 20 barley cultivars based on Ward method under severe stress conditions (-2 ℃) for physiological traits.

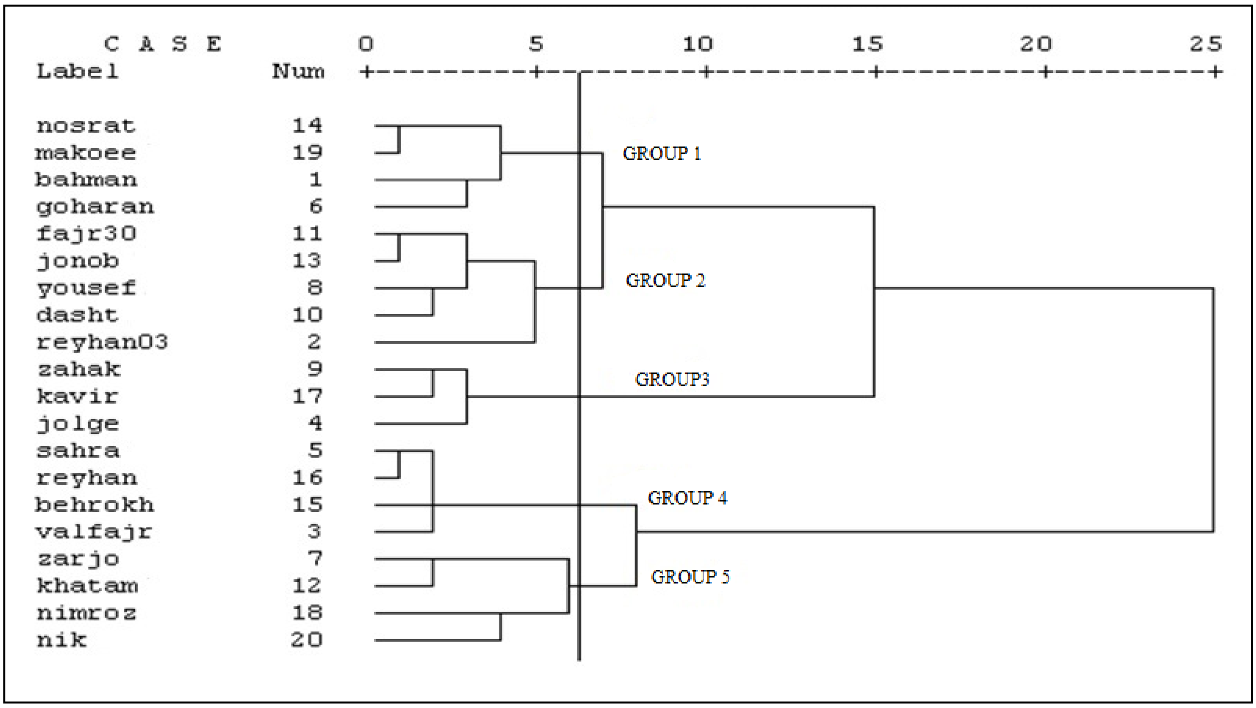


Table 2- Determination of genetic distance of cultivars at control temperature (+8ºC) (bottom diameter) and severe stress (-2ºC) (up diameter)

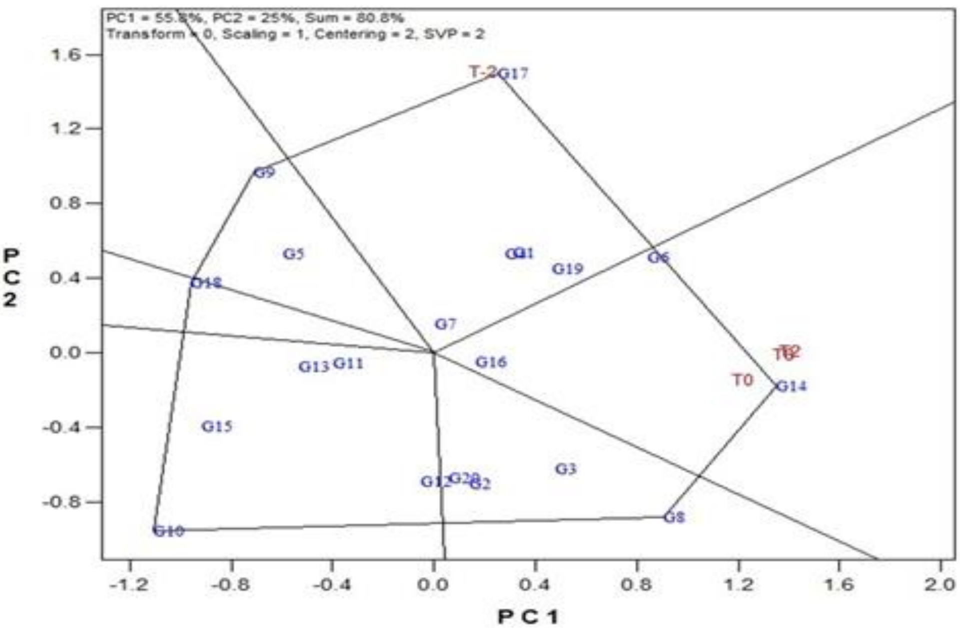
	Bahman	Reyhan 03	Valfajr	Jolgeh	Sahra	Goharan	Zarjoo	Yousef	Zahak	Dasht	Fajr 30	Khatam	Jonoob	Nosrat	Behrokh	Reyhan	Kavir	Nimruz	Makoui	Nik
Bahman	0	17.382	17.12	23.309	11.344	12.081	21.354	16.458	24.441	15.312	11.265	35.993	11.415	11.239	25.327	17.148	24.831	13.937	9.389	20.175
Reyhan 03	8.57	0	19.753	22.761	13.324	12.419	27.797	17.567	26.023	17.709	14.399	39.512	10.523	13.817	31.035	15.42	21.046	30.248	23.119	25.047
Valfajr	35.362	24.746	0	16.997	11.342	24.363	10.912	20.227	28.975	12.576	20.246	15.107	24.022	10.699	10.529	6.961	36.263	26.35	16.379	20.606
Jolgeh	21.305	17.906	31.614	0	7.792	14.856	27.085	35.027	10.006	22.106	26.166	46.223	20.503	17.433	14.865	13.699	14.473	30.277	15.426	38.872
Sahra	10.141	11.452	23.81	18.022	0	11.184	15.843	17.721	7.367	12.887	9.469	27.726	9.349	6.492	9.264	5.072	9.211	16.29	8.35	15.479
Goharan	15.446	9.736	28.306	25.128	16.754	0	23.287	29.524	11.057	22.779	19.689	47.887	16.483	13.306	28.032	21.396	11.433	15.473	13.18	32.92
Zarjoo	29.906	17.756	21.367	14.557	25.668	23.394	0	29.816	24.374	15.49	29.4	8.517	26.634	15.197	12.367	14.286	36.499	13.514	25.863	20.145
Yousef	13.789	10.26	23.65	24.297	21.226	26.112	33.483	0	39.399	8.743	7.888	41.591	11.223	6.667	30.402	26.079	31.005	35.637	13.648	27.753
Zahak	17.045	16.842	40.759	26.564	10.063	14.069	23.541	44.301	0	25.234	24.622	44.063	19.408	19.226	14.324	17.397	8.333	14.734	18.335	28.074
Dasht	10.158	5.494	38.329	17.601	8.66	18.533	31.514	15.916	18.337	0	11.644	27.234	8.155	7.054	13.837	17.994	30.06	19.811	14.54	20.492
Fajr30	8.82	4.431	26.511	30.289	8.445	10.99	30.554	15.666	15.858	6.702	0	40.58	6.921	6.073	25.512	18.301	17.728	25.291	8.723	16.487
Khatam	29.648	25.55	32.357	18.115	19.15	35.507	10.657	44.405	15.981	30.467	33.912	0	41.422	31.324	16.719	15.172	61.886	26.479	46.346	14.642
Jonoob	5.44	9.616	35.813	28.593	9.062	17.698	37.919	17.073	15.405	9.597	8.127	30.715	0	10.418	23.893	17.932	15.866	21.532	15.717	20.389
Nosrat	13.566	15.389	44.67	35.556	32.076	10.269	42.405	19.541	36.026	24.463	17.746	58.653	20.997	0	17.222	15.344	15.553	21.255	4.446	22.589
Behrokh	27.798	15.486	33.929	20.543	21.328	19.659	9.93	38.644	13.016	24.229	24.743	8.05	26.412	39.354	0	8.081	31.448	16.775	21.015	16.236
Reyhan	18.246	15.252	15.794	16.828	8.811	16.143	8.652	29.224	10.927	22.734	17.535	7.583	21.803	35.676	9.45	0	25.19	21.106	20.873	10.894
Kavir	16.468	7.602	24.224	19.636	16.871	11.836	21.704	13.091	28.881	12.629	10.064	35.649	24.445	15.128	23.392	17.146	0	30.698	15.259	39.144
Nimruz	19.425	8.975	33.998	13.061	13.962	15.599	10.693	29.822	10.234	12.482	16.816	9.963	20.207	32.064	2.607	9.325	15.188	0	22.851	15.225
Makoui	14.554	13.381	23.271	12.798	12.22	22.92	30.505	9.9	33.691	11.206	16.317	39.122	20.094	27.157	40.397	24.252	14.001	27.241	0	30.182
nik	12.756	12.419	20.435	11.323	5.654	11.872	15.446	25.206	10.526	13.354	12.498	14.51	17.593	26.156	14.563	4.317	11.135	9.386	14.939	0

5. Biplot analysis

Detection of genotypes with desirable yield in both stress-free and environmental stress conditions seems complex due to the significant interaction between the genotype and the environment (Shiri et al., 2010). Various methods have been presented to investigate the interaction of genotype × environment, as well as determining stable genotypes (Becker and Leon, 1988). Biplot method is one of these methods, which was provided based on the analysis of principal components (Yan et al.,2000). Genotype evaluation has been reported in multi-environments experiments in wheat (Changizi et al., 2014) and barley (Dehghani et al., 2006; Mortazavian et al., 2014).

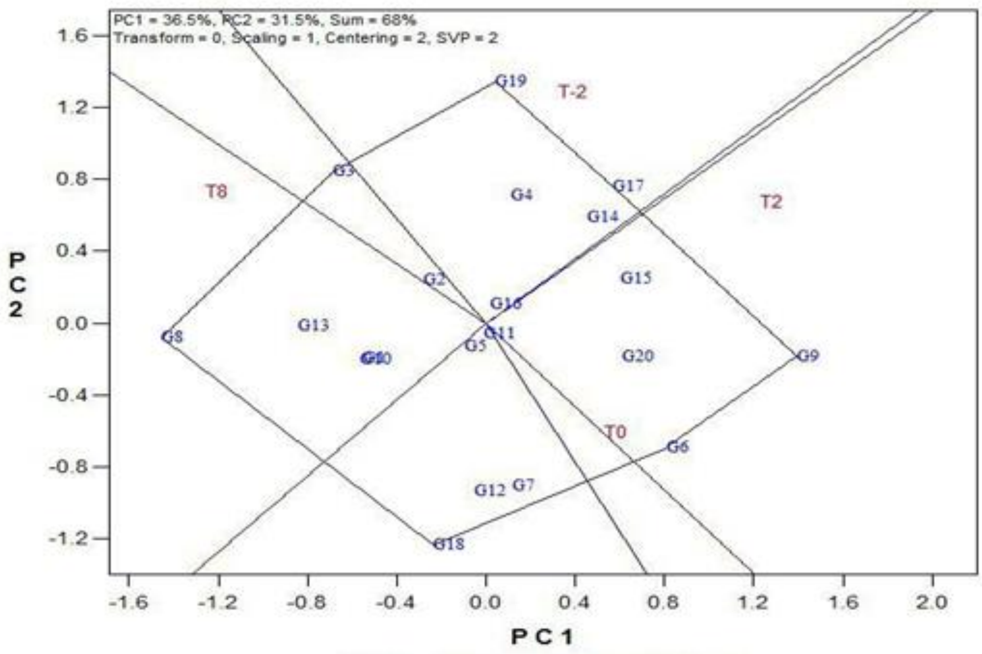
According to the polygonal biplot for the trait of grain weight per plant in 4 temperature levels, it is observed that control, +2˚C, and 0˚C environments are along with each other (Figure 3). In this figure, the cultivars with maximum distance from the inception of the biplot are connected by straight lines to make a polygon. Then, lines perpendicular to the vertices of these polygons are drawn from the inception of the coordinates, and large perimeters are specified. The cultivars at the top of this polygon are the superior cultivars of that perimeter. Therefore, the polygons showed that the changes in grain weight per plant in these three environment are close to each other and did not change much due to stress up to 0˚C. However, the grain yield (grain weight per plant) reduced from 0˚C to -2˚C. Nosrat (code 14) cultivar was the best for these three environment, and Kavir (code 17) was the best cultivar regarding the level of severe stress (-2˚C), which can be mentioned as a cold-tolerant cultivar.

Figure3. Polygonal schema of biplot cultivar in the environment for grain yield



Regarding the proline trait, the environments are far apart, which indicates the difference between the values of proline at stress levels. In other words, proline changes do not have a constant trend and fluctuate. Therefore, environments are dispersed based on this trait. Yousef (code 8) cultivar in control environment (+8˚C), Zahak (code 9) cultivar in +2˚C environment, Goharan (code 6) cultivar in 0˚C environment, Makoui (code 19) cultivar in -2˚C environment had the highest amount of proline (Figure 4).

Figure4. Polygonal schema of biplot cultivar in the environment for proline

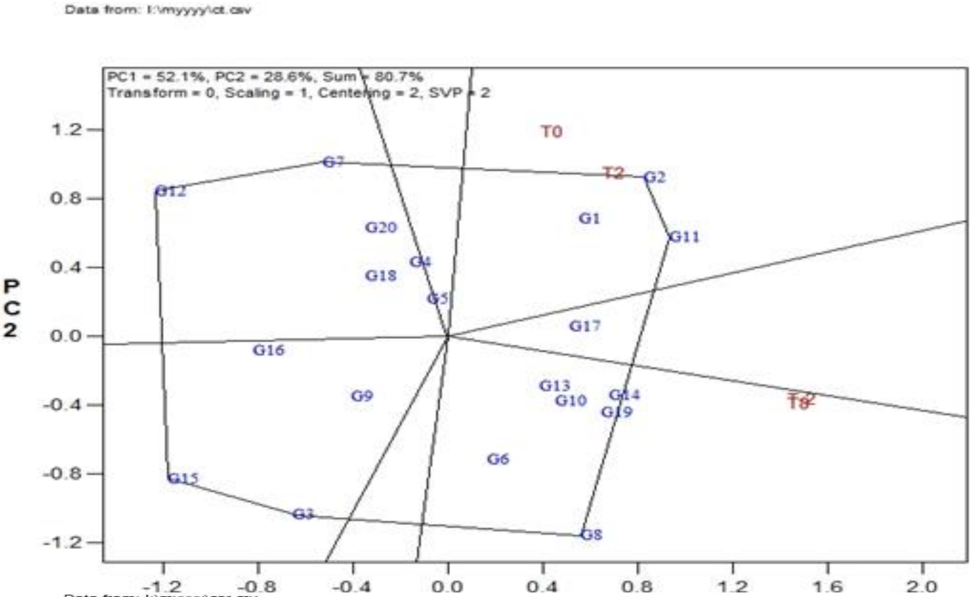


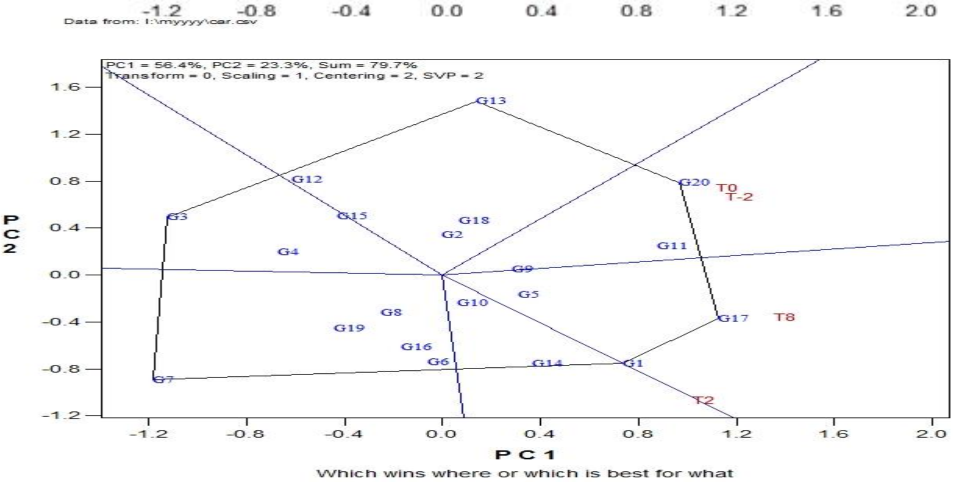
The polygon biplot, related to the trait of total chlorophyll showed that cultivars located in a particular section or environment have a good yield in that environment. For example, the Yousef cultivar was introduced as the best cultivar for control levels (+8˚C) for total chlorophyll and extreme temperature stress (-2˚C). Reyhan 03 and Fajr 30 cultivars were recognized as the best cultivars at the other two levels of +2˚C and 0˚C. Therefore, the best cultivar in each section is the cultivar that is at the top of the polyhedron. On the other hand, cultivars that are close to the inception do not respond well to changes in the environment. Therefore, Jolgeh, Sahra, Nimruz, and Zahak cultivars were generally stable and did not react much to changes in the environment. Valfajr, Behrokh, Khatam, and Zarjoo cultivars are very far from the inception, and their distance from the mean axis of the environments was long, which shows the high range of variation in these cultivars. On the other hand, there was no environment in the sections where these cultivars were located, which means that these cultivars did not have good yield in any of the studied environments. This result is not a reason that these cultivars are not good because they may have good yield and stability in other environments, which are not investigated in this research (Figure 5).

Regarding the physiological trait of carotenoids, Nick cultivar in -2˚C and 0˚C, and Kavir and Bahman cultivars in control and +2˚C, were determined as the best cultivars. Moreover, Valfajr, Jonoob, Zarjoo, and Goharan were not placed in any environment.

Therefore, they did not have a good yield in the studied environments. The rest of the cultivars also often had a general adaptation to changes in environments (Figure 6).

Figure5. Polygonal schema of biplot cultivar in the environment for total chlorophyll





6. **Conclusion**

The mentioned traits were evaluated, and their polygenicity was determined. Another valuable point is that some cases of spring barley cultivars were observed, which had higher values of the studied morphophysiological traits in tolerating chilling stress than winter cultivars. In other words, these cultivars do not have a constant reaction process in the face of a gradual increase in cold stress. It seems that this situation is due to the specific abilities and potentials of each cultivar that are proportional to changes in environmental conditions or through the stability of elements effective in tolerance such as maintaining the amount of chlorophyll or decomposing physiological compounds and providing to the plant to create tolerance (proline production), and finally through the synthesis of new metabolites (related polypeptides). In addition, some yield indicators were high. Since the plant has many possibilities in this regard, thus, each cultivar uses a group of material in facing the changes of environmental conditions, which are different in size and strength.

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