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Evaluation of freezing stress tolerance in promising durum wheat and its relationship with physiological traits and molecular markers

Fatemeh Mohammadi Azar[1], Omid Sofalian[1*], Ali Asghari[1], Asghar Ebadi[1], Rahmatollah Karimizadeh[2]

[1]Department of Agronomy and Plant Breeding, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran.
[2]Gachsaran Agricultural Research Institute, Kohgiluyeh and Boyerahmad Agricultural and Natural Resources Research and Education Center, Gachsaran, Iran.
[*]Corresponding author, 0000-0001-5003-0492. Email: sofalian@uma.ac.ir.

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ABSTRACT

Cold tolerance in wheat is one of the most important factors effective in the field of winter damage in Iran. Freezing and laboratory tests were carried out in the greenhouse and plant breeding laboratory of Mohaghegh Ardabili University in 2018-20 to achieve the set goals. The plant materials used included 45 promising durum wheat lines. Durum wheat genotypes were planted in a randomized complete block design with three stress levels. The results of the variance analysis of LT_{50} showed a significant difference between the genotypes at the probability level of 1%. LT_{50} varied between -0.754 and -26.609 values. The survival percentage of plants decreased with increasing stress. In clustering based on the LT_{50}, the genotypes were divided into 5 groups. The dendrogram obtained from the cluster analysis based on all the traits divided lines in the control level, -8 °C, -10 °C, and -12 °C into 8, 6, 9 and 7 different groups. Four factors were identified in the control level, 5 in the first stress, 6 in the second stress, and 5 in the third stress level. To evaluate the relationship between the measured traits and RAPD molecular markers, stepwise multiple regression analysis was performed and significant relationships were observed. LT_{50} showed a correlation with 9 markers. Finally, according to the tests conducted, lines 1, 2, 3, 4, 5, 6, 7, 8, 10, and 23 were recognized sensitive lines and lines 11, 12, 13, 14, 15, 17, 18, 19, 21, 29, 31 and 27 were recognized as resistant.

Key words: Durum wheat, Freezing stress, LT_{50}, Molecular marker.

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INTRODUCTION

Bread wheat and durum wheat are the most consumed crops in the world. Durum wheat is one of the most important crops in areas where the weather conditions are unsuitable for the production of common wheat (bread) (Sadeghzadeh Ahari *et al.,* 2010). Durum wheat is one of the most basic cereal types and is cultivated on about 17 million hectares worldwide, with a global production of 1.38 million tons in 2019 (Agriculture and Food Canada, 2019). Wheat needs an optimal temperature range for ideal growth and performance, and any deviation from it affects the natural growth process (Hassan *et al.,* 2021). Due to extensive compatibility with various weather conditions, wheat has a broader distribution range than any crop plant. Environmental stresses, including cold stress, are effective in not allowing wheat reaching its performance potential (Rezaei *et al.,* 2022).

Cold stress is harmful to winter wheat during winter (Nowak *et al.,* 2010). One of the main factors in the field of winter damage in Iran is cold tolerance in wheat (Mahfoozi *et al.,* 2001). Most wheat-growing regions in the world are often under low-temperature stress (Zheng *et al.,* 2015; CRP-wheat, 2016). Frost damage occurs when canopy temperature fall below 0 °C or Stevenson screen air temperature below 2 °C (Frederiks *et al.,* 2015). The degree of tolerance of plant species to freezing in different growth stages is diverse (Meyer and Badarudin, 2001). Crop plants of temperate regions, including wheat, tend to overcome cold stress through cold acclimation (Theocharis *et al.,* 2012; Li *et al.,* 2014).

Cold stress causes a series of changes in various biological and biochemical processes in wheat plant cells (Hasan *et al.,* 2021). Physiological and biochemical responses of wheat plant are more vulnerable under low temperature stress (Yadav, 2010). The content of chlorophyll a and b in leaves is essential for photosynthesis in chloroplast and plays a role in the absorption and utilization of light energy (Koc *et al.,* 2020). Ivanov *et al.* (2013) showed a positive correlation between chlorophyll content and the amount of photosynthesis under cold stress. Cold stress inhibits physiological and biochemical reactions in plant cells, which leads to leaf chlorosis, wilting, and even plant cell necrosis (Ruelland and Zuchowski, 2010). In cereals such as wheat, the base resources for cereal production and vital physiological processes in levels of crop growth are photosynthesis and biomass accumulation. These processes are highly vulnerable to low-temperature stress (Rinalducci *et al.,* 2011; Khan *et al.,* 2017). Karimi *et al.* (2011) reported that cold stress causes a decrease in final yield, which is associated with a decline in spike number, spike length, biomass, leaf area, size, and carbohydrate metabolic reactions. Morphological and physiological changes are associated with reduced photosynthetic efficiency (Theocharis *et al.,* 2012; Valluru *et al.,* 2012). Photosynthetic activity in cold-sensitive cultivars is more sensitive to cold stress than in cold-tolerant cultivars (Yamori *et al.,* 2009). Burning flag leaf as a result of freezing stops the photosynthetic activity, leading to a 100% yield reduction (Rajkan and Swanton, 2001). LT_{50} which is a lethal temperature, at which 50% of the tested seedlings die, is considered a suitable trait for laboratory evaluation of cold tolerance (Bridger *et al.,* 1996). Skinner and Garland-Kempel (2008) reported a significant linear relationship between LT_{50} survival percentage at -5 °C for 15 and 20 weeks in wheat. Fowler *et al.* (1999) suggested the survival percentage of LT_{50} as a suitable index to evaluate cold stress.

Examining different physiological changes due to cold in sensitive and tolerant cultivars can be useful in identifying cold tolerance mechanisms. Chlorophyll is the main pigment of the chloroplast of leaf cells, which activates the light reactions of photosynthesis by absorbing the energy of light photons. Due to the special importance of these pigments, the cell tries to use special mechanisms to protect them (Yang *et al.,* 2006). Today, this index is used to select cold-resistant cultivars (Esfandiari *et al.,* 2019). It is possible to observe the imbalance between the metabolic process and production using the chlorophyll fluorescence technique. The study of chlorophyll fluorescence parameters is a simple, non-destructive, and quick technique (Malakoti *et al.,* 2013). At F_0 (minimal fluorescence), the photochemical utilization of the excited energy is maximum, so the photochemical reduction of fluorescence is maximum. When the light intensity is sufficient, the fluorescence increases from F_0 to F_m (maximum fluorescence). When all reaction centers are closed, photochemical reactions do not occur, and chlorophyll fluorescence reaches its maximum value. The fluorometer shows the F_v/F_m ratio and the corresponding curve (Maxwell and Johnson, 2000). The value of F_v/F_m indicates the maximum quantum efficiency of photosystem II and is a measure of the functioning of plant photosynthesis (Fracheboud, 2006). Therefore, in different genotypes, the amount of decrease in quantum performance or changes in fluorescence ($F_v = F_m - F_0$) over some time has been used as a measure of tolerance and resistance to stress (Eshghizadeh and Ehsanzadeh, 2009).

Cold acclimation is a complex phenomenon which occurs with a wide range of physiological, biochemical and molecular changes (Theocharis *et al.,* 2012). Evaluation of genetic diversity in agricultural products has a significant role in advancing reform programs and supporting genetic resources (Pearce *et al.,* 2000). The RAPD marker is a random marker and reinforces random areas in the genome. Many evaluations have been performed by RAPD markers on the wheat. These markers are exerting in studies of genetic diversity, phylogenic, gene labeling, genomic mapping and evolutionary biology (Landkhoest-Klein *et al.,* 1991). In the research carried out by Rahmani *et al.* (2021), four RAPD primers produced 19 polymorphic bands with an average of 4.75 polymorphic bands for each primer, and the average polymorphism percentage was 67.85%. Wu *et al.* (2004) investigated the genetic diversity of 14 wild species rice populations using 26 RAPD primers, the created polymorphic bands were equal to 56.73%, which indicates the high similarity and detection power of this index. Akcura *et al.* (2006) studied 13 durum wheat populations using 15 RAPD primer pairs, which identified 80 polymorphic gene loci in total. They showed that the genetic parameters in native samples were more than cultivated varieties, which include the number of effective alleles, observed heterozygosity, ratio of polymorphic gene loci and gene diversity. Chabane *et al.* (2007) investigated the genetic diversity of 82 samples of bread and durum wheat using 18 pairs of EST primers and a total of 101 loci with an average number of 6.31 alleles were identified.

This paper reviews current research findings on how cold stress negatively affects wheat physiological traits. In addition, it explains how wheat reacts to cold stress by expressing different species of adaptive reactions. This research examines the genetic diversity of several durum wheat lines in terms of cold tolerance through RAPD molecular markers and states its relationship with physiological traits under stress conditions. Finally, cold-tolerant lines are presented according to the tests.

MATERIALS AND METHODS

To achieve the goals set in this research, freezing and laboratory tests were carried out in the greenhouse and plant breeding laboratory of Mohaghegh Ardabili University in 2018-20. Forty-five promising lines of durum wheat were planted in a randomized complete block design with three replications (Table 1). The plants were planted in pots and placed in the greenhouse settings including relative humidity of 40%, a light period temperature of 20±3 °C and dark period temperature of 16±3 °C, and day and night lengths of 16 and 8 h. The plants were kept until the stage of 3 to 5 leaves. The pots were taken to the cold room to create coolness. The chamber temperature was set at 4 °C. The photoperiod was considered to be 11 h light and 13 h darkness. Lighting was provided by one 400 (w/m^2) fluorescent lamp. The incubation period was three weeks and, the plants were watered when needed. The cold treatments included no hypothermia or hypothermia. The temperature of the cold room decreased to 2 °C per h. This situation provides the conditions for redistributing water to plant tissues and preventing the formation of frost inside the cells, which rarely happens in nature (Murray *et al.,* 1998). At the temperature of -3 °C the temperature was kept constant for 12 h, to prevent the phenomenon of super cooling and the creation of ice nuclei in the seedling and to make sure that the mechanism is of the type of tolerance and not avoidance, and after that temperature decreased at a rate of 2 °C per h (Bridger *et al.,* 1996). Four control temperature treatments were considered, including -8, -10, and -12 °C. The plants were kept for one h at each desired temperature to balance the ambient temperature (Auld *et al.,* 1983). The temperature was set at 4 °C and held at this temperature for 24 h to decrease melting speed (Bridger *et al.,* 1996). Then, the pots were returned to the greenhouse. Survival percentage was evaluated after 21 days of recovery.

Measurement of physiological traits

OSI 30 device (ADC Bioscietific Company) was used to measure chlorophyll fluorescence. All measurements were made between 10:00 and 13:00 to minimize daily changes. The youngest whole leaf was used. Plant leaves were placed in the dark for 30 min using special clamps. After this time, the clamps were connected to the optical fiber of the device, and the valve of the clamps was opened. The parameters of initial fluorescence F_0, F_m, F_v/F_m, and F_v were measured.

The amount of chlorophyll (greenness) was measured using a SPAD-502 chlorophyll meter from Konica Minolta. The middle part of the leaf of a developed and mature leaf was placed between the clamp of the device, and by pressing the button device, the amount of chlorophyll was measured in SPAD units.

DNA extraction and PCR

DNA extracted using the CTAB method with a few changes according to the Saqai Maarouf *et al.* method (1984). The quantity and quality of the DNA samples were checked using the Nanodrop device. The sequence of primers used in RAPD analysis is listed in Table 2.

Table 1. Pedigree of durum wheat lines.

Line	Pedigree
1	Dehdasht
2	ALTAR84/STINT//SILVER_45/3/LLARETAINIACDSS99 Y00376S-0M-0Y-13Y-0M-0Y-2M-0Y
3	SIMETO/3/SORA/2*PLATA_12//SRN_3/NIGRIS_4/5/TOSKA_26/RASCON_37//SNITAN/4/ARMENT//SRN_3/NIGRIS_4/3/CANELO_9.1CDSS06B00488T-099Y-099M-11Y-0M-04Y-0B
4	BCRIS/BICUM//LLARETA INIA/3/DUKEM_12/2*RASCON _21/5/1A. 1D 5+1-06/3*MOJO//RCOL/4/ARMENT//SRN_3/NIGRIS_4/3/ CANELO_9 .1CDSS07Y00068S-099Y-099M-4Y-3M-04Y-0B
5	Icarasha2-ICD99-0091-T-3AP-AP-10AP-AP
6	BELLAROI/5/1A.1D 5+1-06/3*MOJO//RCOL/4/ARMENT//SRN_3/ NIGRIS_4/3/CANELO_9. 1CDSS07Y00444S-099Y-099M-8Y-2M-04Y-0B
7	E90040/MFOWL_13//LOTAIL_6/3/PROZANA/ARLIN//MUSK_6/9/USDA595/3/D67.3/RABI//CRA/4/ALO/5/HUI/YAV_1/6/ARDENTE/7/HUI/YAV79/8/POD_9/10/TOSKA_26/RASCON_37//SNITAN/4/ARMENT//SRN_3/NIGRIS_4/3/CANELO_9.1CDSS06Y00497S-11Y-0M-1Y-2M-0Y
8	1A.1D 5+1-06/3*MOJO//RCOL/4/ARMENT//SRN_3/NIGRIS_4/3/ CANELO _9.1/8/ SHAG_21/DIPPER_2//PATA_2/6/ARAM_7 //CREX/ ALLA/5/ENTE/ MEXI_2//HUI/4/YAV_1/3/LD357E/2*TC60//JO69/7/ ARMENT// SRN_3/ NIGRIS_4/3/CANELO_9.1CDSS07Y00151S-099Y-099M-19Y-2M-04Y-0B
9	YAV79/4/ARMENT//SRN_3/NIGRIS_4/3/CANELO_9.1/10/INRAM_1805/9/USDA595/3/D67.3/RABI//CRA/4/ALO/5/HUI/YAV_1/6/ARDENTE/7/HUI/YAV79/8/POD_9CDSS05B00936D-7Y-0M-3Y-4M-0Y
10	JUPARE C 2001*2/IM/6/ADAMAR_15//ALBIA_1/ALTAR 84/3/ SNITAN /4/SOMAT_4/INTER_8/5/SOOTY_9/RASCON_37/7/ GUAYACAN INIA/ KUCUK/4/ARMENT//SRN_3/NIGRIS_4/3/ CANELO_9.1CDSS07Y00533T-099Y-099M-2Y-2M-04Y-0B
11	ICAMOR-TA04-1/Quabrach-1//Adnan-1ICD06-0877-0AP-4AP-0AP-5AP-0THTD -0TR
12	CandocrossH25/Ysf1//CM829/CandocrossH25ICD07-497-BLMSD-0AP-0Tr-2AP-0Tr-1AP-0THT-0AP -0TR
13	Ossl1/Stj5/5/Bicrederaa1/4/BezaizSHF//SD19539/Waha/3/Stj/Mrb3/6/Icajihan12 ICD07-094-BLMSD-0AP-6AP-0Tr-1AP-0THT-0AP -0TR
14	Sebatel-2//Wdz6/Gil4-ICD02-0992-C-12AP-0AP-7AP-0AP-7AP-0AP-1AP-0AP
15	Mgnl3/Ainzen-1/4/Aghrass-1/3/Mrf1//Mrb16/Ru-ICD06-1620-0AP-3AP-0AP-2AP-0THTD
16	ALTAR84/STINT//SILVER_45/3/LLARETAINIACDSS99 Y00376S-0M-0Y-13Y-0M-0Y-2M-0Y
17	OROBEL//BUSHEN_4/2*GREEN_18/8/GEDIZ/FGO//GTA/3/SRN_1/4/TOTUS/5/ENTE/MEXI_2//HUI/4/YAV_1/3/LD357E/2*TC60//JO69/6/SOMBRA_20/7/JUPARE C 2001CDSS07Y00746T-099Y-099M-5Y-3M-04Y-0B
18	Bezajihan*Ossl1/Stj5/5/Bicrederaa1/4/BezaizSHF//SD19539/Waha/3/Stj/Mrb3/6/Icajihan12
19	BCRIS/BICUM//LLARETA INIA/3/DUKEM_12/2*RASCON_21/5/1A.1D 5+1 -06/3*MOJO// RCOL/4/ARMENT//SRN_3/NIGRIS_4/3/CANELO_9.1 CDSS07Y00068S-099Y-099M-4Y-3M-04Y-0B
20	MÂALI/6/MUSK_1//ACO89/FNFOOT_2/4/MUSK_4/3/PLATA_3//CREX/ALLA/5/OLUS*2/ILBOR//PATKA_7/YAZI_1/10/SELIM/9/ALTAR 84/860137 //YAZI _1/4/LIS_8/FILLO_6/3/FUUT// HORA/JOR/8/GEDIZ/FGO//GTA/3/ SRN_1/4/ TOTUS/5/ENTE/MEXI_2//HUI/4/YAV_1/3/LD357E/2*TC60// JO69/6/SOMBRACDSS07Y00784D-2B-07Y-07M-8Y-1B-04Y-0B
21	WID22202/4/SORA/2*PLATA_12//SOMAT_3/3/AJAIA_12/F3LOCAL(SEL.ETHIO.135.85)//PLATA_13/5/CF4-JS 21//TECA96/TILO_1CDSS07B00 683T-0TOPY-099Y-014M-20Y-1M-0Y
22	EXELDUR/8/GEDIZ/FGO//GTA/3/SRN_1/4/TOTUS/5/ENTE/MEXI_2//HUI/4/YAV_1/3/LD357E/2*TC60//JO69/6/SOMBRA_20/7/JUPARE C 2001/9/ SOMAT_3/ PHAX_1//TILO_1/ LOTUS_ 4/3/RASCON_22/RASCON_21// MOJO_2 CDSS08Y00900T-0TOPB-099Y-07M-13Y-3M-0Y
23	PLATA_6/GREEN_17//SNITAN/4/ARMENT//SRN_3/NIGRIS_4/3/CANELO_9.1/11/ARTICO/AJAIA_3//HUALITA/10/PLATA_10/6/MQUE/4/USDA573//QFN/AA_7/3/ALBA-D/5/AVO/HUI /7/PLATA _13/8/THKNEE_11/9/CHEN/ ALTAR 84/3/HUI/ POC//BUB/RUFO/4/FNFOOT CDSS08Y00518S-099Y-025M-11Y-1M-0Y
24	SOMAT_3/GREEN_22/4/GODRIN/GUTROS//DUKEM/3/THKNEE_11/7/CMH83.2578/4/D88059//WARD/YAV79/3/ACO89/5/2*SOOTY_9/RASCON_37/6/1A.1D 5+1-06/3*MOJO/3/AJAIA _12/F3LOCAL(SEL.ETHIO.135.85)// PLATA_13 CDSS08Y00394S-099Y-025M-9Y-2M-0Y
25	TOPDY_18/FOCHA_1//ALTAR 84/3/AJAIA_12/F3LOCAL(SEL.ETHIO. 135.85)// PLATA_13/4/ SOMAT_3/GREEN_22/6/LAHN/HCN//PATA_2/3/ SOMAT_4/INTER_8/5/CREX//BOY/ YAV_1/3/PLATA_6/4/PORRON_11CDSS07B00051S-099Y-018M-1Y-2M-0Y

Table 1 (Continued). Pedigree of durum wheat lines.

Line	Pedigree
26	Mrf1/Stj2/3/1718/BT24//Karim = Icajihan*ICD01-0251-T-8AP-TR-8AP-0AP-5AP-0AP-2AP-0AP-2AP-0AP-0TR
27	Terbol975/Geruftel2*ICD06-1790-0AP-4AP-0AP-4AP-0THTD-0TR
28	Maamouri1/5/IcamorTA0462/4/Stj3//Bcr/Lks4/3/Icamor"s"/6/Mgnl3/Ainzen1*ICD06-0367-BLMSD-0AP-2AP-0Tr-2AP-0Tr-4AP-0THT-0AP-0TR
29	Mgnl3/Ainzen1/3/IcamorTA0463//H.mouline/Sbl2/4/Mgnl3/Ainzen1*ICD06-0261-BLMSD-0AP-1AP-0Tr-4AP-0Tr-2AP-0THT-0AP-0TR
30	PH896-21/5/BRAK_2/AJAIA_2//SOLGA_8/3/CANELO_8//SORA/2*PLATA_ 12/4/YAZI_1/AKAKI_4// SOMAT_3/3/AUK/GUIL//GREEN/6/HUBEI// SOOTY_9/RASCON_37/3/2*SOOTY_9/RASCON_ 37/4/SOOTY_9/ RASCON_37CDSS07Y00461T-099Y-099M-1Y-3M-04Y-0B
31	CBC 509 CHILE/4/SKEST//HUI/TUB/3/SILVER/5/GREEN_14//YAV_10/AUK
32	STOT//ALTAR 84/ALD*2/3/AUK/GUIL//GREEN
33	AINZEN-1/3/SNTURKMI83-84503/LOTUS_4//MUSK_4/6/CMH82A.1062/3/GGOVZ394//SBA81/PLC/4/AAZ_1/CREX/5/HUI//CIT71/CII
34	TRN//21563/AA/3/BD2080/4/BD2339/5/RASCON_37/TARRO_2//RASCON_37/6/AUK/GUIL//GREEN,CDSS00B00364T-0TOPY-0B-2Y-0M-0Y-1B-0Y
35	TRN//21563/AA/3/BD2080/4/BD2339/5/RASCON_37/TARRO_2//RASCON_37/6/AUK/GUIL//GREEN,CDSS00B00364T-0TOPY-0B-33Y-0M-0Y-1B-0Y
36	STOT//ALTAR 84/ALD*2/3/YAV79/CROC_1
37	SNITAN/3/RASCON_37/TARRO_2//RASCON_37/4/STOT//ALTAR 84/ALD
38	HAAHKA_1/SNITAN/9/USDA595/3/D67.3/RABI//CRA/4/ALO/5/HUI/YAV_1/6/ARDENTE/7/HUI/YAV79/8/POD_9
39	KUCUK_2/PATA_2//AJAIA_13/YAZI/4/YAZI_1/AKAKI_4//SOMAT_3/3/AUK/GUIL//GREEN
40	AKAKI_7/BEJAH_7//BUSCA_3/3/STOT//ALTAR 84/ALD/4/AKAKI_7/BEJAH_7//BUSCA_3
41	SOOTY_9/RASCON_37//STORLOM
42	AJAIA_12/F3LOCAL(SEL.ETHIO.135.85)//PLATA_13/3/SOMAT_3/4/SOOTY_9/RASCON_37,CDSS97Y00729S-0TOPM-2Y-0M-0Y-0B-0B-1Y-0BLR-4Y-0B
43	GEDIZ/FGO//GTA/3/SRN_1/4/TOTUS/5/ENTE/MEXI_2//HUI/3/YAV_1/GEDIZ/6/SOMBRA_20/7/STOT//ALTAR 84/ALD
44	GODRIN/GUTROS//DUKEM/3/DF900.83/2*RASCON_37/4/ARMENT//SRN_3/NIGRIS_4/3/CANELO_9.1
45	Stk/Hau//Heca-1/3/1536-OGDOI

Table 2. The sequence and annealing temperature of RAPD primers used.

Number	Primer sequence	Annealing temperature	GC (%)	Number	Primer sequence	Annealing temperature	GC (%)
1	CCT GGG CTT C	34	70	9	CCT GCG CTT A	32	60
2	CCT GGG CTT G	34	70	10	GGG GGG ATT A	32	60
3	CCT GGG CTT A	32	60	11	CCC CCC TTT A	32	60
4	CCT GGG CTG G	36	80	25	ACA GGG CTC A	32	60
5	CCT GGG TTC C	34	70	27	TTT GGG GGG A	32	60
٦	CCT GGG CCT A	34	70	28	CCG GCC TTA A	32	60
7	CCT GGG GGT T	34	70	29	CCG GCC TTA C	34	70
8	CCT GGG GGT A	34	70				

PCR reaction for RAPD primers in the form of heat program, initial annealing (94 °C, 5 min), annealing (94 °C, 1 min), primer binding (37 °C, 1 min), primer extension (72 °C, 2 min) and the final extension (72 °C, 5 min) was performed by a thermocycler made by TECHNE. 1.5% agarose gel electrophoresis and ethidium bromide staining were used to reveal the PCR products resulting from RAPD analysis. PCR reaction components included primer (5 µmol) 1.6 µL, DNA (25 ng/µL) 4 µL, purified water 4.4 µL, and master mix

10 µL. The final volume of each tube was 20 µl. Two µl of X6 loading color solution (0.25% xylene cyanol, 0.25% bromophenol blue and 30% glycerol) was added to each tube containing the PCR product. The entire PCR product and added dye were loaded inside a well. Electrophoresis was run for 2.5 h with a constant voltage set at 100. The gel was photographed by the gel doc, after the completion of electrophoresis. RAPD molecular primers were used to detect and evaluate the diversity between genotypes of the durum wheat plants. Multiple stepwise regression analysis was performed. Significant relationships were observed to evaluate the relationship between measured traits and molecular markers. The examined traits were entered into the model as function variables and molecular data (zero and one) as fixed variables, based on the regression analysis.

Analysis of physiological data

Analyses of variance were accomplished based on the experimental design as a randomized complete block design with three replications. The normality test was performed by the Kolmogorov-Smirnov Test method for all traits before performing the analysis of variance. The mean comparison was done with the LSD method at the five percent probability level. Ward's cluster analysis method was used using the square measure of the Euclidean distance to group the lines. Several different temperatures were used to calculate LT_{50}, then the LT_{50} of each genotype was determined by probit analysis, and the most tolerant genotype was identified. Statistical analyses were carried out with MSTATC and SPSS16 software, and graphs were drawn using Excel and NCSS12 software.

Analysis of molecular data

Each DNA fragment produced as a discrete variable was scored as one to indicate the presence and zero to indicate the absence of the band in each sample. The relationship between the molecular markers of RAPD and the studied physiological data was investigated using the stepwise multiple regression method. In this way, each quantitative trait was considered by a dependent variable and molecular markers as independent variables (Nakhaii badrabadi *et al.,* 2011, Sepehri *et al.,* 2014). SPSS v.19, GenAelex 6.4, NTSYS 2.2, and NCSS12 software were used to perform the above analysis.

RESULTS AND DISCUSSION

A fifty percent lethality tolerance threshold (LT_{50}) was determined using probit analysis. The results of LT_{50} variance analysis showed a significant difference between the genotypes at the probability level of 1%, indicating the considerable genetic diversity among the genotypes in this trait (Table 3). Considering the different reactions of genotypes in terms of LT_{50}, it concluded that LT_{50} is a suitable trait for evaluating frost resistance (Bridger *et al.,* 1996). The results of the LT_{50} variance analysis of wheat genotypes in the experiments of Rustai *et al.* (2009) showed that the difference between the genotypes in terms of cold tolerance was significant at the probability level of 1%. The results of analysis of variance showed that the investigated genotypes had significant differences in all traits (Table 4). Significant differences were observed in all traits in terms of stress levels. The interaction effect of line and stress was significant in all traits. The significance of the interaction effect shows that the process of changes of genotypes in traits in normal conditions and stress conditions is not the same, and the superior cultivars in normal conditions are not recommended for stress conditions.

Table 3. Analysis variance of LT_{50}.

Source	df	LT_{50}
R	2	0.076**
line	44	0.077**
Error	88	0.001
Total	134	3.545

The highest amount of the mean comparison about the LT_{50} was observed in genotype 44 (-0.754), and the lowest amount was observed in genotype 36 (-26.609) (Figure 1). The lower the LT_{50} value in the line, the higher its stress resistance. Genotypes' ability to survive the winter cold is considered one of the final factors in selection of genotypes (Sio-Se Mardeh *et al.,* 2009). A comparison of the average survival percentage of lines in 4 stress levels showed that the survival percentage of plants decreased with increasing stress. In severe stress (-12 °C), the highest survival percentage was related to lines 11, 13, 14, 15, 17, 18, 27, 33, 34, 35, 36, 37, and 41. The lowest survival percentage was related to lines 1, 4, 5, 7, 8, 9, 10, 23, 24, 25, 43, and 45 (Table 5). According to the experiments carried out by Si-Seh Mardeh *et al.* (2009), cold stress and genotype had a significant effect on cold resistance, and Augusta had the highest cold resistance among the genotypes.

The comparison of the traits means showed that the SPAD chlorophyll was associated with a decrease in stress conditions. The highest amount of SPAD chlorophyll was related to lines 30, 37, and 40 in the control level, lines 1 and 34 in stress level -8, lines 28 and 39 in stress level -10, and lines 2 and 34 in stress level -12.

Table 4. Analysis variance of physiological traits.

Source	df	SPAD	F_t	F_v	F_0	F_m	F_v/F_m	Chlorophyll a	Chlorophyll b	Chlorophyll total	Carotenoid
Replication	2	368.865**	202.539**	1392.891**	133.106**	376.906^{ns}	0.053^{ns}	3.211**	0.953^{ns}	2.709**	752.580**
Factor A	3	1035.487**	1014.910**	15335.740**	8173.987**	16073.962**	11.127**	35.542**	70.386**	48.476**	1090.855**
Factor B	44	85.483**	2522.442**	11142.307**	4484.568**	7709.972**	3.412**	1.764**	1.643**	1.740**	79.336**
AB	132	54.958**	1599.308**	3914.864**	2398.830**	3655.047**	0.835**	1.347**	2.471**	1.761**	57.435**
Error	358	36.603	39.433	109.394	8.202	138.056	0.064	0.143	0.358	0.178	12.650
Total	539	23.98	12.12	15.17	5.06	11.30	4.90	5.62	9.88	5.91	14.76

SPAD: Chlorophyll content, F_t: Terminal fluorescence, F_v: Variable fluorescence, F_0: Primary fluorescence, F_m: Fluorescence max, F_v/F_m: Quantum functionality.

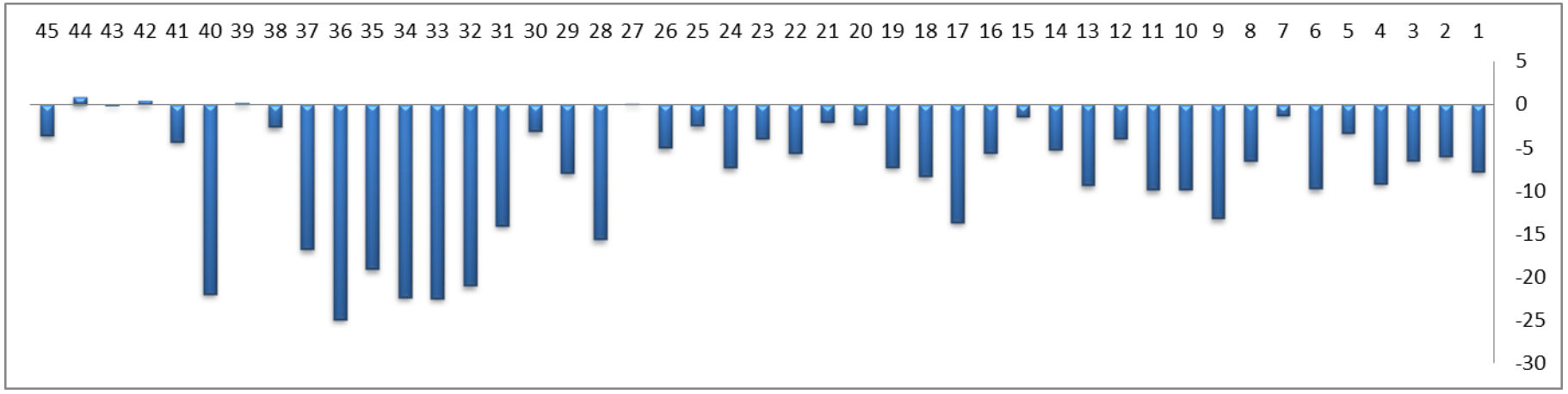

Figure 1. Comparison of the mean trait LT_{50} in durum wheat lines.

Table 5. Survival percentage of durum wheat lines at different levels of cold stress.

	Norm	-8 stress	-10 stress	-12 stress
1	80	56.81818	48.57143	9.090909
2	90.90909	86.84211	36.11111	25
3	81.25	67.85714	44	16.66667
4	89.47368	83.33333	53.84615	13.33333
5	90	58.82353	43.47826	10
6	90.90909	85.18519	42.10526	16.66667
7	82.6087	55.55556	53.33333	5.263158
8	91.30435	76.08696	41.37931	8.333333
9	94.73684	97.2973	60.86957	8.333333
10	94.33962	75.4717	58.06452	7.142857
11	85.71429	73.17073	33.33333	63.63636
12	76.19048	55.26316	38.88889	37.5
13	78.26087	50	48.14815	66.66667
14	94.73684	31.57895	48.71795	46.66667
15	92.59259	24.07407	37.83784	47.36842
16	84.61538	82.75862	36	30.76923
17	86.95652	86.11111	41.17647	54.54545
18	79.16667	73.68421	35.48387	60
19	73.07692	61.76471	43.75	33.33333
20	79.16667	85	25.71429	28.57143
21	85.71429	80	25.80645	20
22	60	51.85185	51.28205	38.46154
23	85.71429	27.27273	50	9.090909
24	78.26087	64.51613	73.68421	6.666667
25	68.96552	56.41026	60	10
26	87.5	60	44.73684	23.52941
27	83.33333	56.41026	24.13793	50
28	81.81818	81.13208	36	33.33333
29	88	93.75	35.89744	25
30	96	85.71429	38.46154	33.33333
31	72.41379	66.66667	52.63158	23.07692
32	90.32258	79.06977	75.86207	33.33333
33	79.41176	52.83019	73.68421	52
34	83.33333	73.17073	67.85714	59.18367
35	74.28571	58.13953	80	51.42857
36	87.87879	69.81132	66.66667	59.25926
37	82.85714	78.43137	68.18182	70.90909
38	84.84848	81.81818	38.23529	21.05263
39	66.66667	52.5	47.36842	35.08772
40	75	66.66667	68.18182	43.13725
41	72.97297	64.40678	51.72414	57.44681
42	69.44444	49.01961	40	38.70968
43	75	70.58824	50	8.333333
44	75.67568	38.88889	41.66667	36.84211
45	69.44444	60.31746	59.45946	12.5

The lowest amount of this trait in the control level corresponds to lines 1 and 17 respectively, lines 38, 44, and 10 in stress -8, lines 20 in stress -10, and line 30 in stress 12. Investigating different physiological changes due to cold in sensitive and tolerant cultivars can be beneficial in identifying cold tolerance mechanisms. One of these physiological changes is the relative amount of leaf chlorophyll (Neto *et al.,* 2005). Mahfoozi *et al.* (1994) and Wulf *et al.* (1994) have benefited from the method of measuring chlorophyll stability in their evaluations of cold stress. Jahanbakhsh *et al.* (2009) investigated the effect of cold on two resistant and sensitive varieties of bread wheat. They reported that the amount of chlorophyll decreased in the sensitive variety and increased in the resistant variety. These results show that in the resistant wheat lines, the photosynthetic system suffers less damage than the sensitive lines during the cold stress. The highest amount of terminal fluorescence was related to lines 18 and 38 in the control level, lines 31 and 45 in stress level -8, lines 6 and 29 in stress level -10, and lanes 29 and 33 in stress level 12. The lowest amount of this trait was related to lines 17 and 16 in control level, lines 17 and 37 in stress -8, and lines 17 and 16 in stress -10 and -12. The highest amount of variable fluorescence was related to lines 15 and 16 in the control level, lines 18 and 13 in stress level -8, lines 22 and 27 in stress level 10, and lines 17 and 16 in stress level -12. The lowest amount of this attribute was related to line 1 of all stress levels. The highest amount of primary fluorescence is related to line 36 in the control level, lines 11, 26, 27, and 45 in stress level -8, lines 36 and 37 in stress level -10, and lines 17 and 18 in stress level -12. The lowest level of this trait was related to line 34 in the control level, lines 1 and 16 in stress -8, lines 34 and 16 in stress -10, and line 2 in stress -12. Today, the chlorophyll fluorescence index was used to select cold-resistant cultivars. The use of this index allows the researchers to evaluate many cultivars and genotypes in the shortest possible time without destroying the plant structure and using chemicals (Esfandiari *et al.,* 2010). Ling *et al.* (1997) reported that the initial fluorescence value was the highest in higher stress levels, which indicated the destruction of photosystem II reaction centers in stress conditions. An increase in the initial fluorescence value and a decrease in the maximum fluorescence disrupt the activity of photosystem II (Anonymous, 1993). The maximum amount of fluorescence was related to line 37 in the control level, line 18 in stress level -8 stress, lines 12 and 10 level -10, and line 17 in stress level -12. The lowest amount of this trait was related to lines 25 and 45 in the control level, line 1 in stress -8, line 25 in stress -10, and lines 2 and 21 in stress -12 (Figures 4-6). The increase in maximum fluorescence under drought stress conditions indicates the oxidation of the electron acceptor (QA) under drought stress conditions. Drought stress has a negative effect on carbon synthesis, reducing the capacity of accepting and transferring electrons, and as the result, the system

quickly reaches F_m. The highest amount of F_v/F_m was related to line 42 in the control level, line 31 in stress level -8, line 24 in stress level -10 and line 10 in stress level -12. The lowest amount of this trait was related to line 17 in the control level and stress -12, lines 13 and 18 in stress -8, and lines 13, 17 and 19 in stress -10. By using the chlorophyll fluorescence technique, it is possible to observe the imbalance between the metabolic process and the production. The study of chlorophyll fluorescence parameters is a simple, non-destructive, and quick technique (Malakoti *et al.,* 2004). Investigating the state of photosynthesis is a reliable criterion for evaluating the degree of adaptation of plants to their surrounding environment. What the fluorometer shows are the F_v/F_m ratio and the corresponding curve (Maxwell and Johnson, 2000). The value of F_v/F_m indicates the maximum quantum efficiency of photosystem II and is a measure of the functioning of plant photosynthesis. This parameter is about 0.83 for most plant species in normal environmental conditions. Lower values are observed when the plant has faced stress, which indicates the phenomenon of photoinhibition (Franchboub, 2006). Ramzi and Morales (1994) reported that tolerant cultivars have a higher F_v/F_m ratio than susceptible cultivars. In other words, the efficiency of optical system II was higher in the resistant variety. The highest amount of chlorophyll a was related to lines 33 and 23 in the control level, lines 32 and 22 in stress level -8, line 43 in stress level -10, and lines 13, 3, and 40 in stress level -12. The lowest amount of this attribute was related to lines 2 and 20 in the control level, lines 1, 9, 7, and 17 in stress -8, lines 2, 9, and 13 in stress -10, and lines 29 in stress level -12. The highest amount of chlorophyll b was related to lines 13, 23, and 16 in the control level, line 33 in stress level -8, line 21 in stress level -10, and lines 13 and 40 in stress level -12. The lowest amount of this trait was related to lines 2 and 17 in the control level, line 7 in stress -8, line 2 in stress 10, and line 26 in stress level -12 .The decrease in the amount of chlorophyll in the stress condition may be due to the increase in the production of oxygen radicals, which cause peroxidation and reduction of this pigment. Chlorophyll a is the dominant photosynthetic pigment, while chlorophyll b is a secondary pigment and constitutes about one-third or less of the total content of chlorophyll in the leaf (Lefsrud *et al.,* 2006). Chen *et al.* (2006) have reported that the concentration of chlorophyll in leaf cells increases gradually with the increase in the duration of chilling and the further reduction of the relative water content of leaf cells. This process can occur by the slow growth of seedlings in the cold and the decrease in cell division, which increases the amount of chlorophyll per surface unit. In addition, types of active oxygen also attack chlorophylls and decompose them. Therefore, the lack of reduction of chlorophyll under stress indicates the plant's tolerance to chloroplast photo damage (Yang *et al.,* 2006). The highest amount of total chlorophyll was related to lines 13 and 23 in the control level, lines 32, 33, and 22 in stress level -8, line 21 in stress level -10, and lines 13 and 40 in stress level -12. The lowest amount of this trait was related to lines 2, 17, and 20 in the control level, lines 9, 7, and 17 in stress -8, line 2 in stress -10, and line 29 in stress level -12. The carotenoid trait was affected by cold conditions, and its amount decreased. The highest amount of carotenoid was related to lines 33 and 23 in the control level, lines 32, 33, and 22 in stress level -8, line 43 in stress level -10, and lines 13 and 40 in stress level -12. The lowest amount of this trait was related to lines 2 and 20 in the control level, lines 9 and 7 in stress -8, line 2 in stress -10, and line 44 in stress level -12. Mohsenzadeh *et al.* (2003) showed a significant decrease in the amount of chlorophyll and carotenoid in cold stress compared to the control condition. The amount of carotenoid synthesis in leaves increases due to their role in protection against free radicals at the beginning of environmental stress. But its amount decreases with time and when the plant adapts to the stresses (Grupa and Benavides, 2008). The usage of the correlation between traits in breeding is of specific importance. The degree of correlation may indicate the degree of a genetic connection between two or more traits (Falconer, 1996). In other words, the estimated values as phenotypic correlation can be divided into two parts, genetic and environmental. The correlation between traits was measured in four stress levels separately.

F_m and chlorophyll b traits had the lowest correlation in the control level, F_t with total chlorophyll traits at -8 stress, carotenoid with LT_{50} traits and carotenoids with F_0 traits. The carotenoid trait had the highest correlation with chlorophyll at all levels. Correlation of traits with LT_{50} showed that this trait had the highest correlation with the F_m at the control level and -8 stresses and the highest correlation with the SPAD trait at -10 and -12 stresses. The lowest correlation was observed in LT_{50} with the carotenoid trait at the control level and -10 stresses, with the chlorophyll trait at -8 stress and -12 with the F_v trait (Figures 2, 3, 4, and 5).

The multivariate statistical methods are important strategies for classifying germplasm, sorting variability in a large number of samples, or evaluating genetic relationships between studied materials.

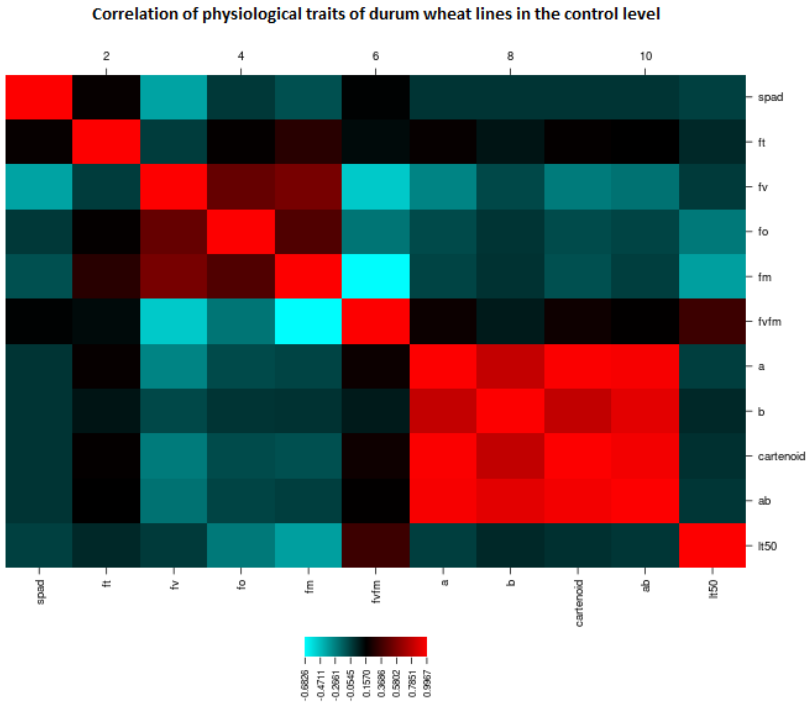

Figure 2. Correlation of durum wheat traits at control levels.

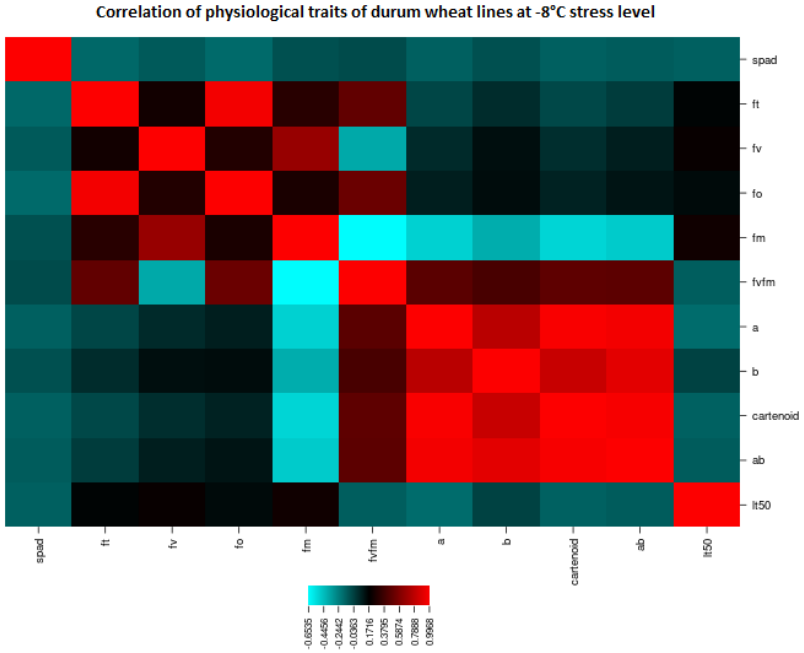

Figure 3. Correlation of durum wheat traits at -8 °C stress levels.

These methods are widely used in the evaluation of genetic diversity, regardless of the type of data (Arcade *et al.,* 2000). In cluster analysis, if the grouping is successful, the components or individuals within the cluster are genetically closer to each other, and the distant clusters will be more different (Bonato *et al.,* 2006). Therefore, instead of carrying out random crossings between genotypes, crossing between the representatives of the created groups is done, as the maximum possible diversity created in the progeny and the probability of selecting superior genotypes increases. For this purpose, for all four stress levels,

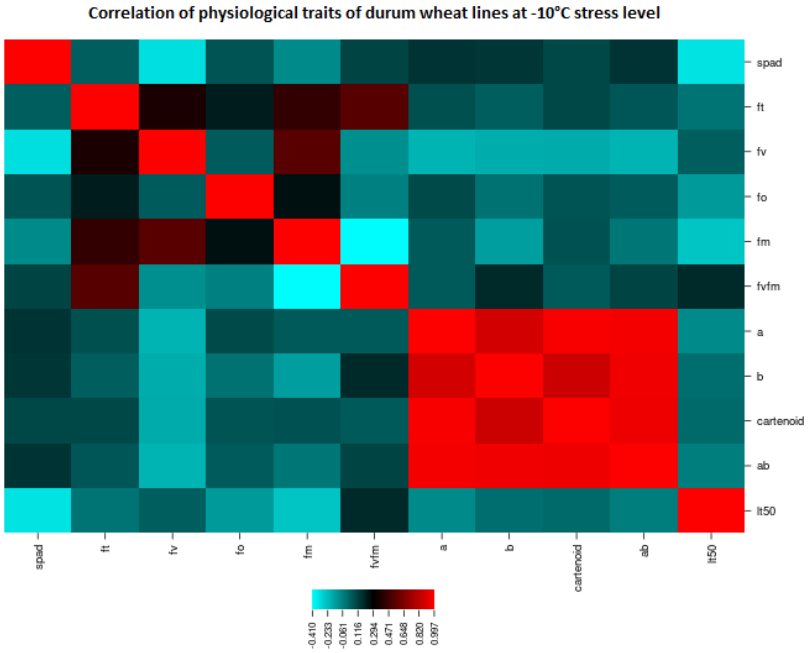

Figure 4. Correlation of durum wheat traits at -10 °C stress levels.

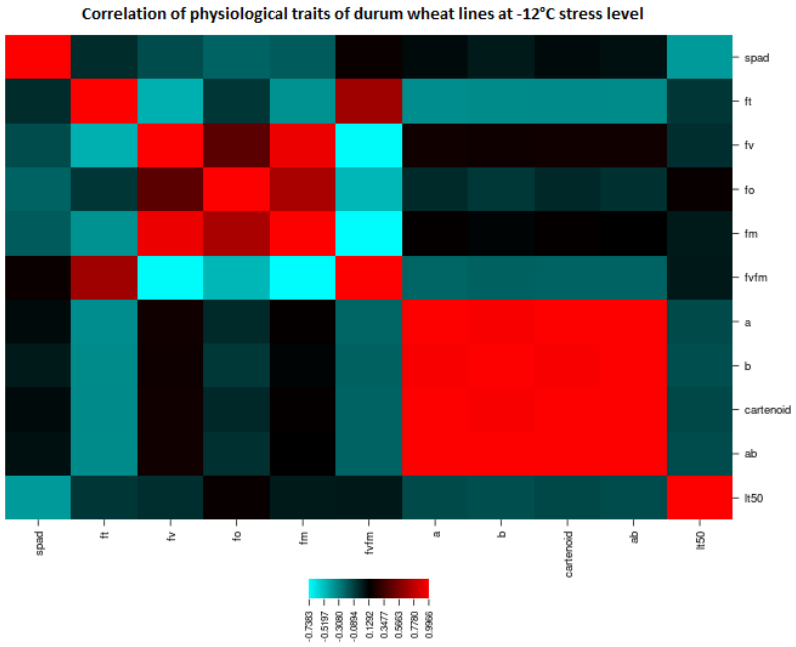

Figure 5. Correlation of durum wheat traits at -12 °C stress levels.

according to all the measured traits, in order to examine and group the cultivars, cluster analysis was performed using the Ward method using the Euclidean distance criterion based on the standardized mean of the traits. The genotypes were divided into five groups in clustering analysis based on the trait LT_{50}. The fifth group with 23 members was recognized as the superior and more resistant group, according to the average of each group in traits, which had the lowest value of LT_{50} in terms of the overall average. The fourth group had the highest value of LT_{50} (Figure 6, Table 6).

The dendrogram obtained from the cluster analysis of the lines has been divided into 8 different groups based on all the traits at the control level. The first group included lines 23, 25, and 26. Genotypes 3, 4, 5,

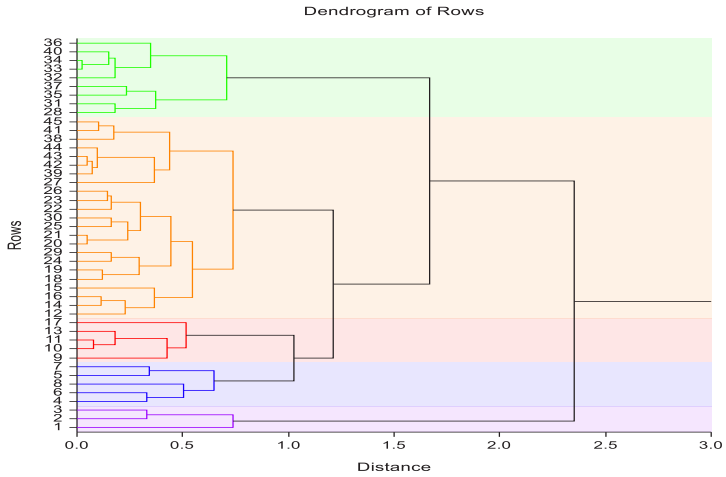

Figure 6. Grouping of durum wheat lines in terms of LT_{50} traits in stress level.

Table 6. The average LT_{50} in clusters resulting from cluster analysis.

	1	2	3	4	5
Line number	9,10,11,13, 17	4,5,6,7,8	1,2,3	12,14,15,16,18,19,20,21,22,23,24,25, 26,27,29,30,38,39,41,42,43,44,45	28,31,32,33,34, 35,36,37,40
Mean of group	-14.695	-8.691	-4.873	-0.943	-21.999

6, 21, and 22 were placed in the second group and had the highest amount of chlorophyll SPAD trait. The third group included lines 8, 9, 10, and 20. Lines 24, 28, 29, 30, 42, and 44 were placed in the fourth group, which had a high mean F_v/F_m trait .The fifth group included lines 13, 19, 18, and 35 and had a high average in terms of F_t, chlorophyll a, and carotenoid traits. The sixth group with the high average in chlorophyll b and total chlorophyll traits included lines 7, 11, 12, 14, 15, 31, and 32. Lines 27, 38, 40, and 41 were classified in the seventh group. The rest of the lines were grouped in the eighth group, which had the highest mean in terms of F_v, F_0, F_m traits and in terms of the overall mean of the traits.

The dendrogram obtained from cluster analysis divided the lines into 6 different groups based on the traits evaluated under -8 stress conditions. In the control level, the first group included genotypes 14 and 20. The second group included genotypes 7, 8, 9, 10, and 11 and had a high average for the chlorophyll SPAD trait. Genotypes 5, 12, 13, 15, 16, 17, 18, and 19 were placed in the third group; this group had the highest average in terms of F_t, F_v, and F_0 traits. The fourth group with the highest F_m trait had lines 23, 26, 27, 28, 29, 30, 31, 43, and 45. Lines 1, 2, 3, 4, and 6 were in the sixth group with the highest mean for chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids. The rest of the lines were placed in the fifth group with a high average for the F_v/F_m trait. Groups 3 and 6 with the highest mean for the traits of the most resistant groups were recognized.

The dendrogram obtained from the cluster analysis at the stress level of -10 has divided the cultivars into 9 different groups based on all the traits. The third group included lines 2, 9, 13, 17, and 18, which had a high average for the F_v/F_m trait. The seventh group, including lines 4, 5, 6, 7, 8, 11, and 19, had a high mean of chlorophyll SPAD and F_t. The eighth group had the highest average of all traits. This group had a high average for F_v, F_0, and F_m traits. Lines 29, 30, 31, 32, 33, 37, 42, 43, 44, and 45 were grouped in group 8. Group 9 had a high average for chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids. Group 9 included lines 10 and 12.

At the stress level of -12, the genotypes were divided into seven groups. According to the average of each group in traits, the third group with lines 25, 32, 33, 34, 35, 36, 37, 38, 39, and 45 was recognized as the superior group. The third group had a high average for F_v, F_0, F_m, and F_v/F_m traits. The seventh group included lines 22, 23, 24, 26, 27, 28, 29, 31, 41, 42, 43, and 44, which had a high average for chlorophyll

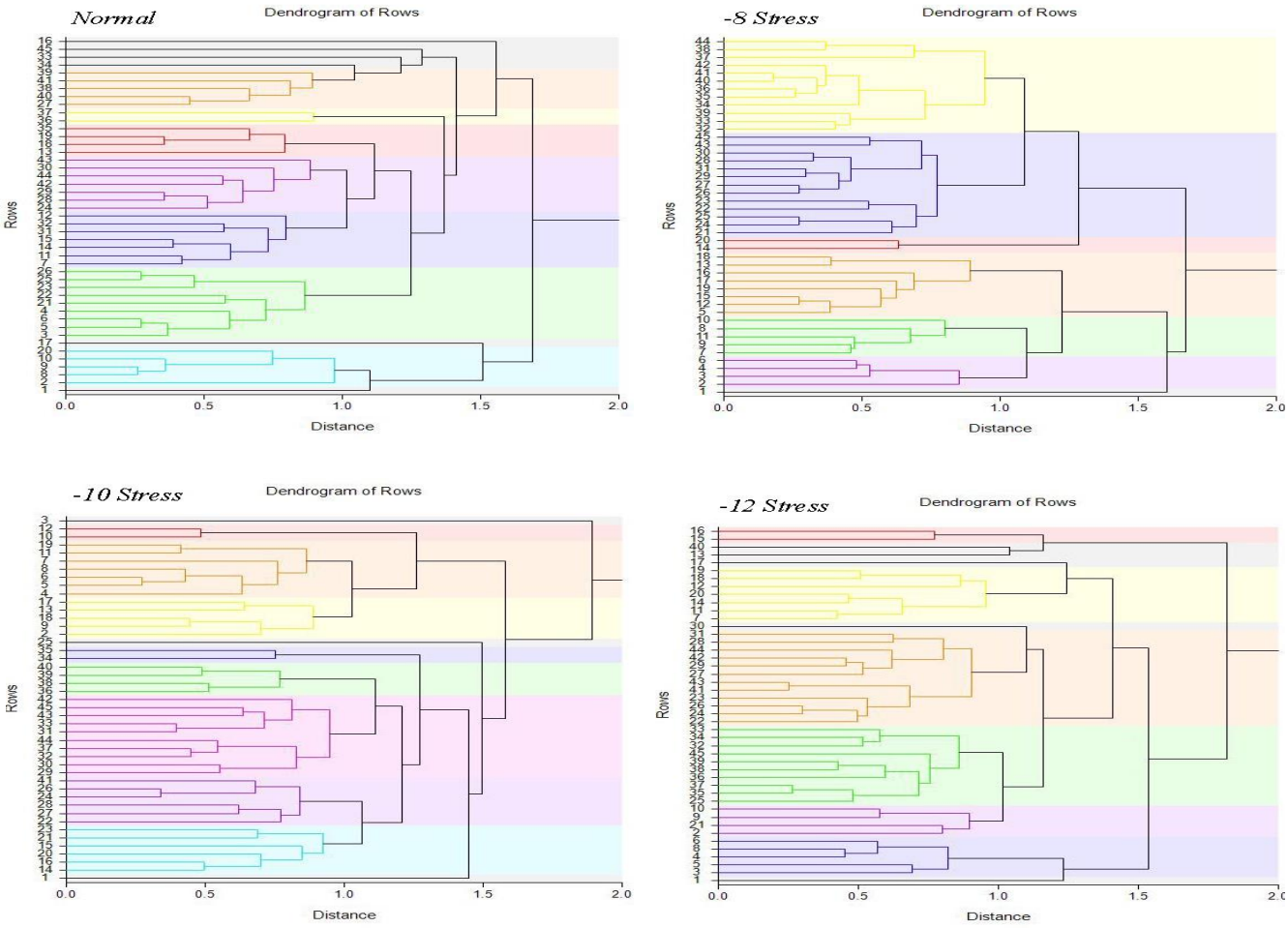

Figure 7. Grouping of durum wheat lines in terms of studied traits in stress levels.

a, chlorophyll b, total chlorophyll, and carotenoids. According to the final results in the cluster analysis of lines in all levels, lines 28, 29, 30, 31, 32, 33, 34, 35, 36, and 37 were recognized as the best lines. Lines 3, 4, 5, 6, and 8 were placed in groups with a lower average (Figure 7, Table 7).

Traits were assigned to different factors based on the values of the factor coefficients after rotating the factors using the Varimax method. Factor analysis provides more information compared to a simple matrix. Groups of variables and the percentage of each factor's contribution are shown in this method (Seiler and Stafford, 1985). Groups of variables that have the highest intra-group correlation and display the lowest correlation with other groups are known in this method. Decomposition into factors at different stress levels was carried out, separately. Four factors were selected to interpret the data based on eigenvalues greater than 1 for all levels. Four factors were identified in the control level, 5 in the first stress, 6 in the second stress, and 5 in the third stress level. These factors are justified by 69.58, 81.08, 84.47, and 81.79 percent of total data in the control level, the stress at -8 °C, the stress of -10 °C and stress at -12 °C.

The first factor with the largest contribution (27.80%) had large and positive factor coefficients for total chlorophyll, chlorophyll a, chlorophyll b, and carotenoid traits, in the control level (Table 8). In the first stress level, the first factor with the largest contribution (31.49%) had large and positive factor coefficients for total chlorophyll, carotenoid, chlorophyll a, chlorophyll b, chlorophyll fluorescence index F_v/F_m, and F_m (Table 9). At the second stress level, the first factor with the largest contribution (27.16%) had large and positive factor coefficients for total chlorophyll, chlorophyll a, carotenoid, and chlorophyll b traits (Table 10). In the third stress level, the first factor with the largest contribution (27.66%) had large and positive factor coefficients for total chlorophyll, chlorophyll b, carotenoid, and chlorophyll a traits (Table 11).

Table 7. The average of all traits in clusters resulting from cluster analysis.

	Lines number	Normal									
		SPAD	F_t	F_v	F_0	F_m	F_v/F_m	chlorophyll a	chlorophyll b	chlorophyll total	Carotenoid
Cluster1	23, 25,26	32.59394	90.54546	135.2424	103.7576	239.5152	0.5529252	9.555503	6.889385	16.44489	26.82794
Cluster2	3,4,5,6,21,22	40.11905	44.42857	44.61905	44.09524	76.33334	0.6167119	10.79857	4.764176	15.56275	27.32377
Cluster3	8,9,10,20	39.01667	66.08334	40.75	43.5	291.1667	0.1410292	8.597642	4.291633	12.88927	25.87008
Cluster4	24,28,29,30,42,44	35.71333	50.4	92	95.8	91.46667	1.017519	12.21016	5.023267	17.23343	28.95953
Cluster5	13,18,19,35	34.47778	119.7778	33.77778	37.44444	272.1111	0.1205556	17.67337	5.8191	23.49247	34.9458
Cluster6	7,11,12,14,15,31,32	31.14167	34.875	79.70834	37.08333	106.2083	0.6891946	15.23445	10.16099	25.39544	32.50688
Cluster7	27,38,40,41	26.98056	42.77778	62.33333	37.11111	98.88889	0.5930811	4.605061	3.215878	7.820939	21.87749
Cluster8	1,2,16,17,33,34,36,37,39,43,45	20.83333	13.33333	301	142.3333	446.6667	0.67384	4.46856	2.803	7.27156	21.74099
		-8 stress									
Cluster1	20, 14	29.38333	50.66667	28.16667	45.44444	73.61111	0.3684694	2.930128	1.884244	4.811472	17.56034
Cluster2	7, 8, 9, 10, 11	29.925	35.95833	46.33333	43.29167	53.54167	0.8780521	10.90109	6.426175	17.3269	25.71432
Cluster3	5, 12, 13,15, 16,17, 18, 19	26.24286	84.2381	89.66666	94.71429	96.52381	0.9287434	8.714319	5.912428	14.62675	22.89333
Cluster4	22,23,24,25,26,27,28,29,30,31,43,45	24.10833	50.69444	76.77778	54.69444	131.4722	0.5870728	2.614868	1.507625	4.119593	17.21819
Cluster5	1, 2, 3, 4, 6	15.95833	42.66667	46.66667	48.66667	50	0.9337884	12.17989	6.496058	18.67595	27.08116
Cluster6	21,32,33,34, 35,36,37,38,39,40,41,42,44	23.3125	55.625	68.625	65.25	78.54166	0.8842658	15.68192	8.835709	24.51763	30.1167
							-10 stress				
Cluster1	22, 24,26,27,28,41	14.67778	29.18518	68.88889	24.25926	96.88889	0.71455	1.755844	0.8390256	2.594869	19.20613
Cluster2	1, 3, 25	11.35952	41.38095	68.66666	77.52381	152.5238	0.4338467	2.390114	1.257229	3.647343	19.95082
Cluster3	2, 9, 13, 17, 18	9.141666	19.41667	202.6667	41.66667	250.6667	0.8013833	4.679542	3.168975	7.848516	22.28964
Cluster4	14, 15, 16, 20, 21,23	12.08095	38.95238	15.2381	36.52381	60.23809	0.2645919	1.528361	0.7643795	2.292741	19.112
Cluster5	36, 38, 39, 40	15.78667	51.66667	42.06667	19.86667	73.06667	0.56517	5.932106	3.80146	9.733566	23.55825
Cluster6	34, 35	8.546667	65.46667	44.86666	45.66667	100.6667	0.4375847	1.148893	0.6185	1.767393	18.75651
Cluster7	4, 5, 6, 7, 8, 11, 19	16.14167	73.16666	88.66666	38.16667	133.1667	0.6670716	1.98025	0.7382166	2.718467	19.50392
Cluster8	29, 30, 31, 32,33,37,42,43,44,45	13.73333	24	215.6667	125.6667	347.6667	0.6111778	5.827678	3.312133	9.139812	23.43778
Cluster9	10, 12	12.03333	20.88889	70.66666	28.44444	104.3333	0.6807711	14.82537	10.36627	25.19163	32.3511
							-12 stress				
Cluster1	3, 4, 5, 6, 8	15.4	30	57.48148	22.14815	83	0.677233	1.662636	0.8314996	2.494136	19.14995
Cluster2	1, 13, 17, 30, 40	9.816667	33.61111	127.5	63.33333	197.1667	0.6398989	2.154472	1.197633	3.352106	19.68021
Cluster3	25, 32,33,34,35,36,37,38,39,45	12.42667	18.46667	222.0667	86.26667	314.6667	0.7121207	6.273674	3.98364	10.25731	23.88377
Cluster4	15, 16	12.07381	38.52381	32.76191	64.95238	103.0952	0.3026147	2.423638	1.206256	3.629894	20.04343
Cluster5	7,11,12,14,18,19,20	9.844444	59.88889	37.88889	42	91.11111	0.3867211	1.117803	0.5404926	1.658296	18.68811
Cluster6	9, 10, 21, 2	15.85833	55.66667	52.33333	20.16667	82.83334	0.6021861	5.419	3.33255	8.75155	23.00029
Cluster7	22, 23,24,26,27,28,29,31,41,42,43,44	12.03333	20.88889	70.66666	28.44444	104.3333	0.6807711	14.82537	10.36627	25.19163	32.3511

Table 8. The matrix of coefficients of the factors after varimax rotation in normal stress conditions.

	Component			
	1	2	3	4
Chlorophyll total	0.981	-0.075	0.102	0.025
Chlorophyll a	0.965	-0.118	0.132	-0.019
Carotenoid	0.959	-0.125	0.106	-0.038
Chlorophyll b	0.881	0.016	0.031	0.106
F_v	-0.170	0.840	-0.116	0.054
F_m	-0.036	0.803	0.334	-0.046
F_v/f_m	0.118	-0.755	0.088	-0.076
F_0	-0.099	0.539	0.418	0.161
F_t	0.092	0.057	0.623	-0.237
SPAD	-0.183	-0.486	0.524	-0.013
Total	4.170	2.755	1.867	1.645
Variance (%)	27.802	18.364	12.447	10.969
Cumulative (%)	27.802	46.166	58.613	69.582

Table 9. The matrix of coefficients of the factors after varimax rotation in -8 °C stress conditions.

	Component				
	1	2	3	4	5
Chlorophyll total	0.936	-0.143	0.249	0.043	0.013
Carotenoid	0.923	-0.202	0.223	0.009	-0.017
Chlorophyll a	0.909	-0.167	0.201	0.108	-0.006
Chlorophyll b	0.845	-0.085	0.287	-0.065	0.041
F_v/f_m	0.710	0.245	-0.590	-0.217	-0.039
F_m	-0.614	0.507	0.554	0.013	-0.028
F_t	0.154	0.922	-0.188	-0.208	-0.064
F_0	0.280	0.907	-0.126	-0.162	-0.051
F_v	-0.031	0.486	0.795	0.157	0.138
SPAD	-0.142	-0.208	-0.194	-0.72	0.849
Total	4.725	2.798	2.089	1.497	1.054
Variance (%)	31.499	18.651	13.927	9.978	7.027
Cumulative (%)	31.499	50.150	64.077	74.054	81.082

Table 10. The matrix of coefficients of the factors after varimax rotation in -10 °C stress conditions.

	Component					
	1	2	3	4	5	6
Chlorophyll total	0.987	0.096	-0.054	0.035	0.078	0.001
Chlorophyll a	0.978	0.025	-0.007	0.059	0.104	0.027
Carotenoid	0.972	0.007	0.036	0.015	0.091	0.002
Chlorophyll b	0.932	0.178	-0.108	0.003	0.040	-0.032
F_v/f_m	0.021	0.953	-0.008	-0.004	-0.071	-0.095
F_t	0.023	0.541	0.754	0.051	0.159	0.004
F_m	0.029	-0.396	0.819	0.029	0.114	0.206
F_v	-0.148	-0.022	0.762	0.043	-0.286	0.004
F_0	0.027	-0.102	0.201	0.838	0.123	0.209
SPAD	0.027	0.212	-0.271	0.106	0.723	0.591
Total	4.074	2.278	2.028	1.589	1.445	1.256
Variance (%)	27.163	15.158	13.522	10.592	9.633	8.371
Cumulative (%)	27.163	42.348	55.57	66.462	76.095	84.465

The RAPD bands with high and significant explanatory coefficients were discussed and investigated according to the entered traits. Fifteen primers produced a suitable and scorable band pattern among the initial 100 primers after the screening. A total of 117 bands were created in the studied genotypes. An example of the banding pattern of initiator number 2 is presented in Figure 8. The number of changes explained by markers in trait LT_{50} showed a value of 0.704, and this trait showed correlation with nine markers, the highest positive correlation value was found with marker 5d and the highest negative

correlation was found with marker 2i (Table 12). Today, the use of linkage between molecular markers and genes controlling quantitative traits has accelerated the process of plant breeding. So instead of evaluating traits, the indirect selection is carried out with the help of continuous markers. Identifying the chromosomal regions involved in the changes is carried out by two main methods: linkage analysis and analysis of the relationship between genotype and phenotype (Gupta *et al.,* 2000). According to the multiple regression coefficients between traits and markers, all traits at all stress levels showed a significant relationship with several RAPD markers, except the SPAD trait at -8 and -10 °C stress. The lowest corrected explanatory coefficient was related to the total chlorophyll trait, and the highest corrected explanatory coefficient was related to the F_v/F_m chlorophyll trait at all levels.

Table 11. The matrix of coefficients of the factors after varimax rotation in -12 °C stress conditions.

	Component				
	1	2	3	4	5
Chlorophyll total	0.981	0.047	-0.175	-0.045	0.020
Chlorophyll b	0.977	0.030	-0.162	-0.054	-0.005
Carotenoid	0.977	0.066	-0.176	-0.020	0.047
Chlorophyll a	0.976	0.059	-0.183	-0.038	0.038
F_m	0.046	0.947	-0.188	-0.065	-0.023
F_v	0.082	0.825	-0.268	-0.247	0.031
F_0	-0.052	0.810	0.032	0.294	-0.141
F_v/f_m	-0.174	-0.794	-0.028	0.457	-0.007
F_t	-0.301	-0.361	0.746	0.694	-0.199
SPAD	0.049	-0.251	-0.052	-0.691	0.831
Total	4.150	3.312	2.009	1.518	1.282
Variance (%)	27.664	22.077	13.391	10.117	8.545
Cumulative (%)	27.664	49.741	63.132	73.249	81.794

The values of the corrected explanatory coefficients in the control level showed that the F_0 chlorophyll trait had the highest and the total chlorophyll trait had the least amount of changes explained by the markers. The chlorophyll F_v/F_m had the most, and the chlorophyll F_t had the least amount of changes explained by the markers in the -8 °C stress. F_m had the highest, and chlorophyll b had the least amount of changes explained by the markers at the -10 °C stress. Chlorophyll b had the highest and F_t had the least amount of changes explained by the markers in -12 °C stress. SPAD chlorophyll, F_0, and F_v/F_m had the highest number of markers with 9 markers in the control level. The total chlorophyll trait had the highest number of markers by 11 markers in -8 °C stress. The highest number of markers was related to the trait F_m by ten markers in the -10 °C stress. The highest number of markers was related to chlorophyll a by 11 markers in the -12 °C stress. The highest positive correlation was found between chlorophyll trait F_m and marker 28c and the highest negative correlation was found between

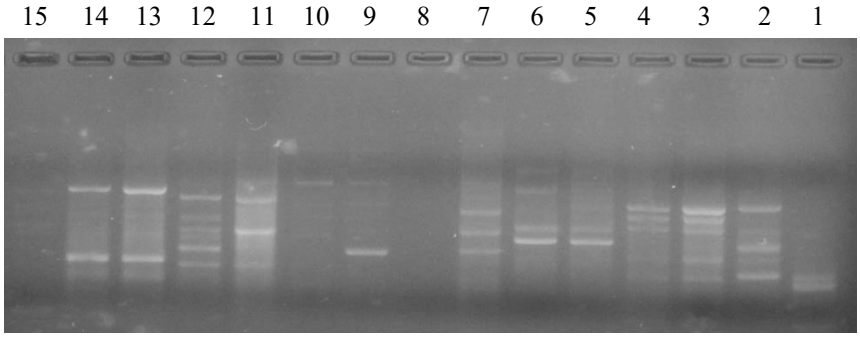

Figure 8. Banding pattern of primer number 2 in the 15 studied lines of durum wheat.

Table 12. Regression coefficients between LT_{50} and RAPD markers.

Width from the origin	Markers associated with the trait LT_{50}									
	1f	2b	2d	2i	3c	5d	9d	27a	27b	R2
-8.37	-0.245	-0.236	-0.405	-0.497	0.283	0.389	0.257	o.28	-0.381	0.704

Table 13. Regression coefficients between traits and RAPD markers in stress levels.

Trait	Stress	T	R^2max (%)	R^2T (%)
SPAD	Normal	9	0.378	0.873
	-8	0	-	-
	-10	0	-	-
	-12	3	0.453	0.644
F_t	Normal	7	-0.351	0.854
	-8	4	0.486	0.419
	-10	5	0.709	0.519
	-12	4	0.484	0.368
F_v	Normal	6	0.36	0.573
	-8	4	-0.425	0.655
	-10	9	-0.614	0.744
	-12	7	0.359	0.68
F_0	Normal	9	-0.224	0.948
	-8	8	-0.402	0.664
	-10	9	-0.485	0.722
	-12	4	0.495	0.625
F_m	Normal	5	0.528	0.473
	-8	5	0.347	0.813
	-10	10	-0.43	0.837
	-12	5	0.347	0.734
F_v/F_m	Normal	9	-0.385	0.854
	-8	8	0.129	0992
	-10	8	-0.348	0.733
	-12	9	0.307	0.897
Chlorophyll a	Normal	5	-0.534	0.526
	-8	6	0.319	0.868
	-10	7	0.483	0.747
	-12	11	-0.43	0.838
Chlorophyll b	Normal	4	-0.552	0.51
	-8	7	-0.38	0.759
	-10	5	0.432	0.493
	-12	9	0.348	0.929
Carotenoid	Normal	7	-0.527	0.511
	-8	4	-0.424	0.713
	-10	9	0.58	0.791
	-12	11	-0.399	0.967
Chlorophyll total	Normal	2	-0.437	0.281
	-8	11	-0.274	0.895
	-10	9	0.454	0.742
	-12	10	0.466	0.729

T: Number of markers; R^2max (%): The highest explanatory coefficient related to an indicator for a quantitative trait (percentage); R^2T (%):The total sum of the coefficient of explanation of indicators for quantitative traits (percentage).

chlorophyll b and marker 6d in the control level. The highest positive correlation was found between trait F_t and marker 25b, and the highest negative correlation was found between trait F_v and marker 2a at the -8 stress. The highest positive correlation was found between chlorophyll F_t and marker 4g, and the highest negative correlation was found between F_v and marker 1b at the -10 stress. The highest positive correlation was observed between F_0 and marker 9e and the highest negative correlation was observed between trait chlorophyll a and marker 27d at the -12 stress (Table 13). Mohammadi *et al.* (2013) studied the relationship between each of 5 physiological traits and 40 polymorphic markers using the stepwise multiple regression method, and the results of regression showed a significant relationship between two markers with five physiological traits in the control temperature and the significant relationship of 6 indicators with 5 traits under severe stress showed that they can be used in the preliminary selection in the correctional programs.

CONCLUSION

Wheat, the most important crop in the world, has many genotypes in breeding programs. Therefore, it is necessary to use them effectively and correctly to identify the relationships of genotypes to determine the level of available diversity (Zhang *et al.,* 2002). According to the results of this test, choosing the correct method of cluster analysis and the index used in examining genetic diversity and grouping genotypes is very important. The results showed that freezing stress caused different reactions among the lines. Lines 29, 31, 37, and 44 were the most tolerant, and lines 3, 4, 5, and 6 were recognized as the most sensitive lines. In general, the results of this research showed that freezing tests in controlled conditions and measuring the percentage of survival and morphological traits can provide an acceptable criterion to estimate the damage caused by cold. Finally, to ensure the cold tolerance of the mentioned genotypes, it is suggested to experiment with field conditions.

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