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The effect of cold stress on H_2O_2 and MDA contents in barely genotypes

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Abstract

During the course of cold acclimation, a key process associated with plant cold tolerance, some biochemical components may be up- or down-regulated. In carrying out this experiment, the objective was to investigate the correlation between acclimation and hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) production under monitored conditions (regular chamber and cold regime) in 20 barely genotypes through in a split plot experiment, with temperatures as main plot and genotypes as a sub plot, based on a complete randomized blocks design with three replicates. According to the results, there was a significant difference in the amount of H_2O_2 and MDA between the two temperature conditions, so was the difference between genotypes regarding MDA content. However, no significant difference was observed between genotypes concerning H_2O_2 content. Further analysis of variance, for MDA content before and after acclimation as well as discrepancies between the two conditions, was conducted using a complete randomized block design, which robustly discriminated the genotypes performance after cold acclimation, and revealed the significant difference between the two temperature conditions. Some genotypes (EC83-12 and Schulyer) possessed the least amount of H_2O_2 and MDA, hence designated as tolerant, and genotypes (Kavir and Aths) with highest amount of H_2O_2 and MDA were considered cold sensitive. There also existed a positive, significant correlation between MDA and H_2O_2 contents in the event of difference between control and post- acclimation treatment. Finally, cluster analysis of the mean contents of MDA and H_2O_2 in genotypes, using "linkage between groups" method, resulted in four groups as sensitive, semi-sensitive, semi-tolerant and tolerant. Present study has been planned to examine the role of MDA and H_2O_2 related to cold stress of tolerant and sensitive barley.

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Introduction

During their life span, plants are constantly subjected to a wide range of abiotic stresses, which have adverse effects on the survival and growth as well as quality and quantity of agricultural products (Knight and Knight, 2001). Among all environmental stresses, cold and freezing temperatures are considered the most harmful of all during the autumn, winter and early spring, restricting crop production in many areas. According to the reports, ice-free surface only accounts for one third of the total land on earth, as opposed to the 42 percent where temperatures below -20°C (Ramankutty *et al.*, 2008). Low temperature stress can be seen in either forms of cold temperatures (5- 15°C) or freezing (below zero). In cold regions, temperatures below zero during vegetative period result in limitation to plants' cultivation and growth. In some years, the occurrence of severe cold temperatures may cause irreparable damage to plants, and in some cases it may even lead to the death of plants (Warmund *et al.*, 2008). Therefore, identifying cold tolerant varieties and introducing them to be cultivated in candidate areas can play a prominent role in reducing the risk of cold-induced damage to agriculture.

Autumn grains spend one part of their vegetative period exposed to cold condition. Among grains, rye is the most tolerant to cold, followed by wheat, barley and oats (Sleper and poehlman, 2006). Barley production in the world is placed fourth, after wheat, rice and corn. Autumn barley yield is significantly higher than spring varieties. To avoid heat and drought stress, spring varieties need to be planted early in season, so producing cold tolerant varieties of barley in most countries in the world constitutes the main goal in barely improvement programs (Kóti *et al.*, 2006).

Plants originating from temperate regions possess different degrees of cold tolerance and are capable of acquiring freezing tolerance following their exposure to a mild cold pretreatment, a process known as cold acclimation (Thomashow, 1999). Cold acclimation is a cumulative process that can be stopped, reversed or resumed (Fowler *et al*, 1977). During this stage, a variety of chemical and physical reactions associated with freezing tolerance take place, enabling the plant to adapt to the cold temperatures at morphological, physiological, anatomical and biochemical levels (Zhang *et al.*, 2002). Afterwards, the plants face temperatures below zero, namely, freezing stress. If plants can survive these temperatures, it can mean they have acquired freezing tolerance (Palva *et al.*, 2001).

Many strategies have been proposed accounting for the physiological and biochemical basis of changes associated with freezing tolerance. Studies have shown that the enhanced production of reactive oxygen species (ROS) in plant systems contribute to some of the effects of freezing stress (McKersie *et al.*, 2000). The cellular components susceptible to damage by ROS are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation of enzymes), carbohydrates (breaking up of polysaccharides), and nucleic acids (Scandalios, 1993, beak and skinner, 2012), escalating the situation into a secondary stress, namely, oxidative stress (Desikan *et al.*, 2004). It is said that production of H2O2, one of ROS molecules, within sub-cellular compartments to be responsible for the molecular damage ensuing plants exposure to stress conditions (Nyuk *et al.*, 2013]. H2O2 is a small molecule that can be transported through different cell compartments. At a basal level of production, H2O2 maintains an important role in metabolic activities [Halliwell, 2006]. Upon experiencing stress conditions by plant, overproduction of H2O2 occurs which may lead to oxidative damage and cell death [Gadjev, *et al.*, 2008, Nyuk *et al.*, 2013].

It seems that the cellular membrane degradation is at the forefront of freezing-induced injury mediated by H_2O_2. Studies have suggested a correlation between H2O2 content and cold tolerance in plants. Guo *et al.* (2006) showed that the cold tolerant varieties of rice displayed low concentration of hydrogen peroxide.

Öktem *et al.* (2008) also stated that increase in oxidative damage caused by cold stress in lentils was due to high production of H_2O_2.

On the other hand, cold stress-induced peroxidation of lipid molecules of the cell membrane, mediated by oxygen molecules, leads to an impaired membrane permeability and stability index, which in turn paves the way for the injury to the membrane (Dhindsa, 1991).

Under stress condition, peroxidation of the cell membrane gives rise to malondialdehyde production, imposed by shrinking the stability and increasing the permeability of the cell membrane (Sairam *et al*., 2001), contributing to the peroxidation of unsaturated fatty acids by ROS in plant cells (Stewart and Bewley, 1980).

Accordingly, this experiment aimed to determining the relationship between freezing and some physiological and biochemical traits.

Materials and methods

Plant material and experimental treatments

Plant material consisted of 20 Barely (*Hordeum vulgare L.*) genotypes obtained from Seed and Plant Improvement Institute (SPII) of Karaj (table 1). Barely genotypes were evaluated in a split plot design experiment, with temperatures as main plot and genotypes as a sub plot, based on a complete randomized blocks design with three replicates, within greenhouse and growth chamber in faculty of agriculture of University of Tabriz. Temperature was applied in five levels (-8, -10, -12, -14 and -16) as the main factor and genotype constituted the subplots.

Seeds were sown in rectangular 50 × 40 cm plastic pots containing crop soil, after being sterilized in Mancozeb 2 ppt. Four rows were arranged in each pot, and 25 seeds planted in each row, dipped 2cm down the soil. Irrigation performed when necessary. The greenhouse temperature was kept at 21 °C during the days and 18 °C for the nights. After reaching three, four leaf stage, the seedlings were transferred to a growth chamber for four weeks at 4°C during the day and at 2 °C at nights, under a 12-h day length at 250 µmol $m^{-2}s^{-1}$ photosynthetically active radiations, to acclimatize to low temperatures. In order to conduct biochemical variables measurements before and after acclimation, the third developed leaves were sampled.

Determine of the lipid peroxidation

To determine the amount of lipid peroxidation a method devloped by of Stepien *et al* (2005) were used. The amount of malondialdehyde (MDA) was measured as an indicator of the damage to the plasma membrane. Here, after homogenization of leaf samples with TCA one percent, centrifugation was performed. Then, TBA 0.05% and TCA 20% were added to the supernatant. The mixture was heated at 95 ° C for 30 minutes. Afterwards, the amount of absorption was measured at 532 nm. Non-specific absorption at 600 nm was deducted from this amount.

Measurement of hydrogenperoxid content

The amount of hydrogen peroxide was measured using a Gong *et al.* (2005) method. Leaf samples were homogenized in TCA 1%, centrifuged, then buffer phosphate 10 mMol (PH=7.5) and potassium iodide (KI) 1 Mol were added to the supernatant. The absorption was read at 390 nm.

Statistical analysis

Prior to analysis, the assumptions of homogeneity of variance and normality of errors were examined. Since the biochemical variables were measured before and after the acclimation stage, variance analysis of the data based on randomized complete block design with three replications was conducted for traits for which genotypes showed significant differences. The means of the genotypes were compared by Duncan test. Data analysis was performed using the software SPSS19 and MATATC.

Results and discussion

However, Due to the nature the experiments, the

variables were analyzed in split plot but due to the not significant main plot error, variables were analyzed using factorial.Variance analysis of the biochemical traits conducted as a factorial experiment using a complete randomized complete block design (Table 2).

The results revealed a significant difference between Barely genotypes in respect of MDA content ($p<0.05$), but for H_2O_2 content the difference was not significant at this level. However, for the aforesaid traits, there was a significant difference between the two temperature conditions, control vs. cold temperatures ($p<0.01$). Interaction of temperature and genotype in MDA and H_2O_2 was not significant, that is genotype and temperatures were affected independently regarding these traits.

The amount of H_2O_2 after acclimation to cold temperature had significantly increased compared with control ($p<0.01$) (Fig.1). Likewise, the MDA content had experienced a rise after acclimation to cold, compared to control (Fig. 2).

Table 1. Code/name and pedigree of barley genotypes used in evaluation of cold stress.

Genotype No	Genotype Code/Cultivar Name	Pedigree
1	EC79-10	Walfajre/Miraj 1
4	EC80-7	YEA389.3/YEA475.4
5	EC80-11	ALGER/(CI10117/CHOYO
9	EC82-5	Alger/(CI10117/Choyo
11	EC82-11	Np106/Minn14133-Gvaxduois//Gi10143
14	EC83-10	GkOmega
15	EC83-12	K-096M3
16	EC83-15	SCHUYLER//(M.RNB89.80/NB1905//L.527)
18	A1C84-7	Star/Dundy
20	A1C84-12	Kozir/330
21	A1C84-14	Astrix(C)/3/Mal/OWB753328-5H//Perga/Boyer
22	A1C84-15	Monolit/Plaisant
28	A2C84-14	Cyclone/Arar
29	A2C84-18	Mal/OWB753328-5H//11840-76/3/Radical
31	Makouee	Makouee
33	Rihane	Rihane
34	Kavir	Kavir
35	73M4-C	73M4-30
36	Schulyer	Schulyer
38	Aths	Aths

Table 2. Analysis of variance (mean squares) of MDA and H_2O_2 contents .

		Mean Square	
Source of Variation	Degree of Freedom	MDA	H_2O_2
Replication	2	1/504 ^{ns}	0/166 ^{ns}
Temperature	1	118/576 **	7/437**
Genotype	19	1/908*	0/113 ^{ns}
T×G	19	1/059 ^{ns}	0/065 ^{ns}
Error	79	0/910	0/143
CV%		27/43	52

ns: not significant, * significant at 0.05% and ** significant at 0.01.

Fig. 3, showed the difference of means for H_2O_2 contents before and after acclimation was maximum in genotypes number 38 and 34, and lowest in genotypes 5, 15, 36 and 9, respectively. From the fig. 4, we can see that the difference of mean for MDA contents, before acclimation vs. after acclimation, was highest in cultivars 34, 38, 1 and 21, and lowest in 5, 9 and 15, respectively.

Table 3. Analysis of variance of MDA content in barley genotypes Leaves.

		Mean square		
Source of Variance	Degree of Freedom	control	acclimation	Difference between control and acclimation
Replication	2	2/377^{ns}	^{ns}0/180	1/712^{ns}
Genotype	19	2/427^{ns}	2/677 *	2/201*
Error	38	0/888	1/279	1/505
Non-additivity	1	0/273 ^{ns}	0/233 ^{ns}	3/322 ^{ns}
Residual	37	0/905	1/307	1/456
CV%		36/01	23/56	56/20

To delve more into the difference observed between genotypes before and after acclimation stage and the difference between the two conditions, an analysis of variance for MDA conducted as a complete randomized blocks design (Table. 3). The results revealed that, between genotypes studied, the MDA content was significantly different at 5%, so was the difference between before and after acclimation. After acclimation, the MDA content ranged from 2.60 to 5.89 nmol per gram fresh weight of leaves.

Table 4. Pearson correlation between the MDA and H_2O_2 content of barley leaves in the control, acclimation and difference between control and acclimation conditions.

Trait	MDA: control	MDA: acclimation	MDA: Difference between control and acclimation	H_2O_2: control	H_2O_2: acclimation	H_2O_2:Difference between control and acclimation
MDA: control	1					
MDA: acclimation	0.570**	1				
MDA: Difference between control and acclimation	-0.422	0.505*	1			
H_2O_2: control	0.010	- 0.298	-0.339	1		
H_2O_2: acclimation	0.373	0.273	0.692**	0.293	1	
H_2O_2:Difference between control and acclimation	-0.370	0.459*	0.895**	-0.360	0.787**	1

ns: not significant, * significant at 0.05% and ** significant at 0.01%.

On the contrary, the MDA content before acclimation ranged from 2 to 4 nmol per gram fresh leaf for all genotypes. The highest discrepancy belonged to Kavir and Aths, the more susceptible cultivars, and the least amount was measured in Schulyer as a tolerant one (Fig. 4).

H_2O_2 is an ROS, which is synthesized as a byproduct of some of vital cellular pathways including photo-respiration, photosynthesis and cellular respiration (Krebs cycle), in small amounts, acts as signal messenger. However, under extreme conditions like freezing, its production increases deeply, beyond the plants' ability to scavenge the excess (Neil *et al.*, 2002). H_2O_2 overproduction harms cellular vital processes directly (Vaidianatan *et al.*, 2003), peroxidation of unsaturated fatty acids, among others, in plasma membrane lipids, as well their permeability is highly affected by H_2O_2 production in excess plants' needs (Yamazaki *et al.*, 2003).

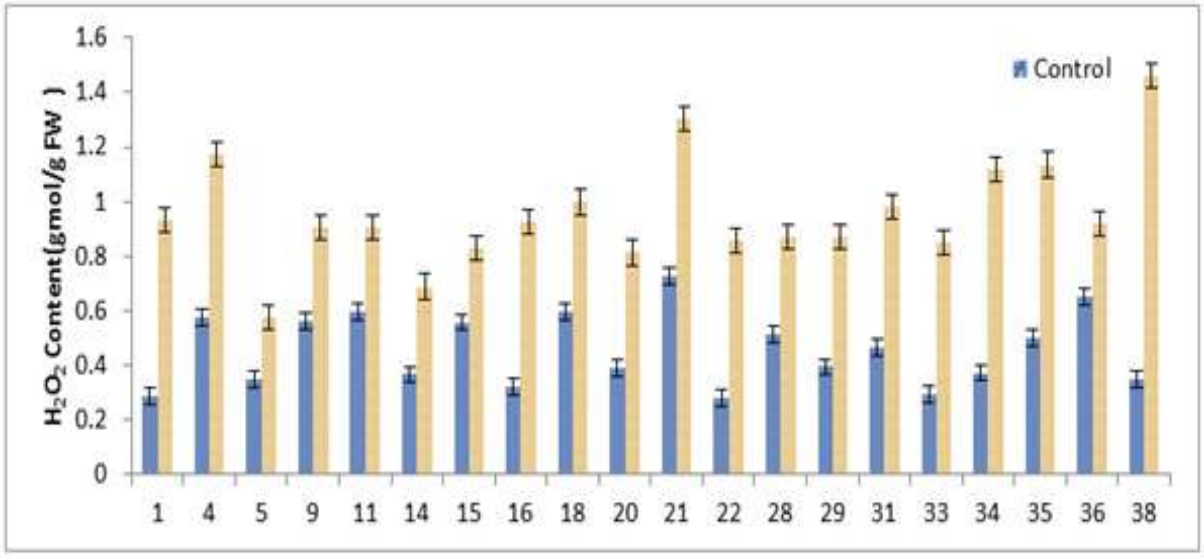

Fig. 1. Changes in the content of H_2O_2 in the leaves of barley genotypes under control and cold conditions.

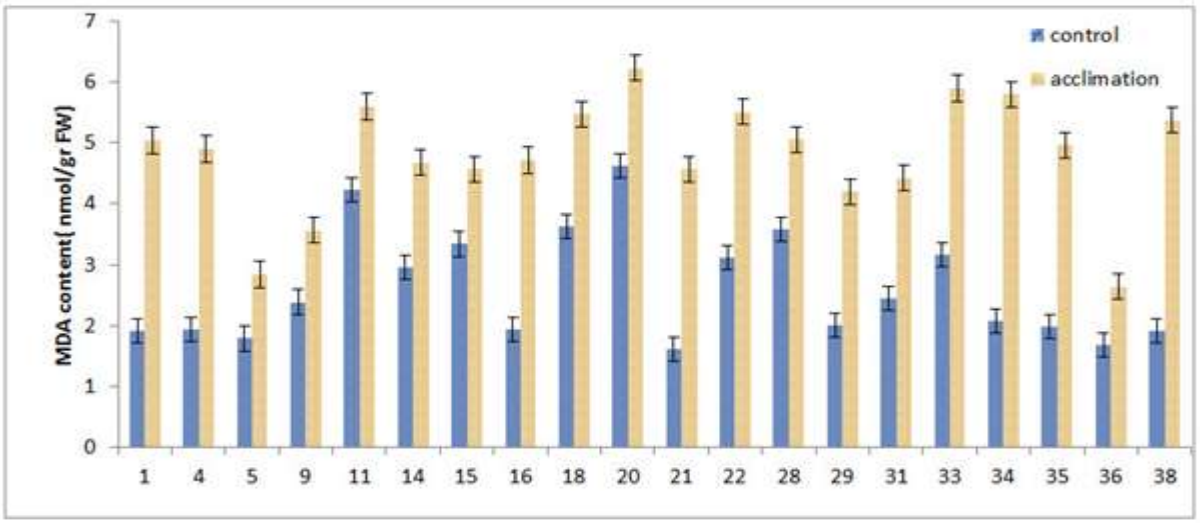

Fig. 2. Changes in the content of MDA in the leaves of barley genotypes under control and cold conditions.

MDA is produced as a consequence of cell membrane peroxidation, induced by drop in stability index and increase in membrane permeability (Sairam *et al*, 2001). The positive correlation observed at 1 % between H_2O_2 and MDA indicates that increase in aggregation of H_2O_2 gives rise to lipid peroxidation (Table. 4).

Besides imposing direct lipid peroxidation, raised level of hydrogen peroxide can indirectly accelerate Haber-Weiss reaction, which is involved in the production of hydroxyl radicals responsible for lipid peroxidation (Loggini *et al*., 1999). These radicals attack the double bonds found in unsaturated lipids of the cell membrane (Blokhina *et al*., 2003). Apostolova *et al*. (2008) reported a 40% and 100% increase in the content of H_2O_2 in the leaves of winter wheat and spring wheat, respectively. In this study, the production of hydrogen peroxide in susceptible spring cultivars, Aths and Kavir, amounted 111% and 75%, respectively, and in the autumn tolerant Schulyer and 5, 15 genotypes measured 27%, 22% and 26%, respectively. Wang *et al* (2009) in a study of two varieties of alfalfa and Guo *et al* (2006) in a study of four rice cultivars reported that the cold tolerant cultivars showed less accumulation of hydrogenperoxid.

The findings, here again, match those obtained by previous researches (Huang and Guo, 2005; Apostolova *et al.*, 2008 and Fahimirad *et al*, 2013), reporting maximum production of MDA in sensitive cultivars.

Cluster analysis

Cluster analysis was carried out using "between group linkage methods" according to the Euclidean distance on standardized data (Fig. 5). At a cut off 10 the, dendrogram revealed four clusters. Group one includes 6 genotypes (4, 21, 35, 1, 16, and 33) with more than average value for H_2O_2. Considering the characteristics, the group can be considered as semi-sensitive genotypes based on the investigated characteristics under cold stress.

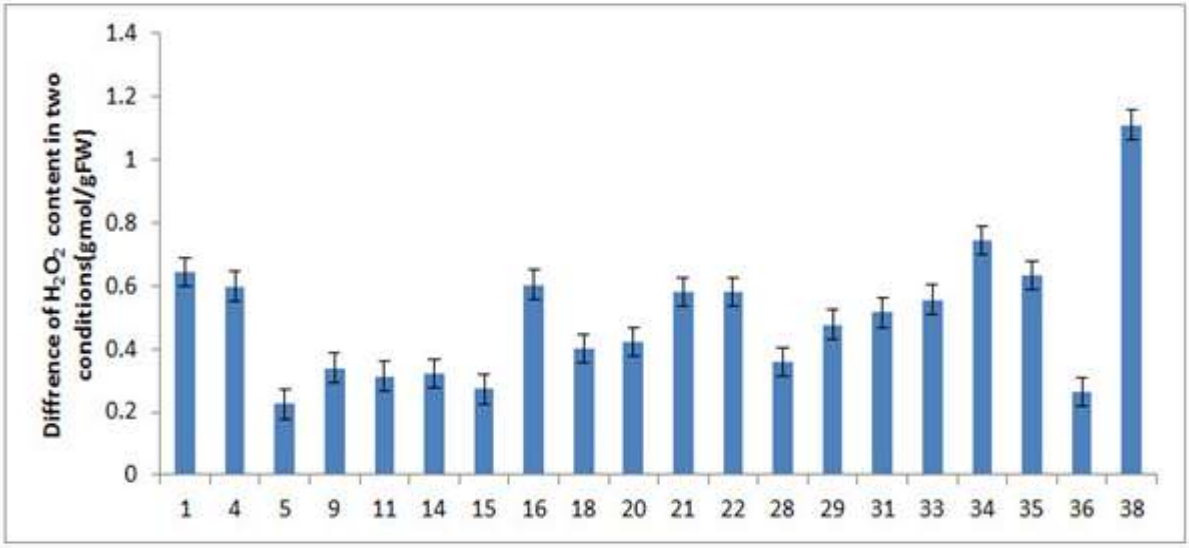

Fig. 3. Difference of H_2O_2 content in the leaves of barley genotypes in two conditions.

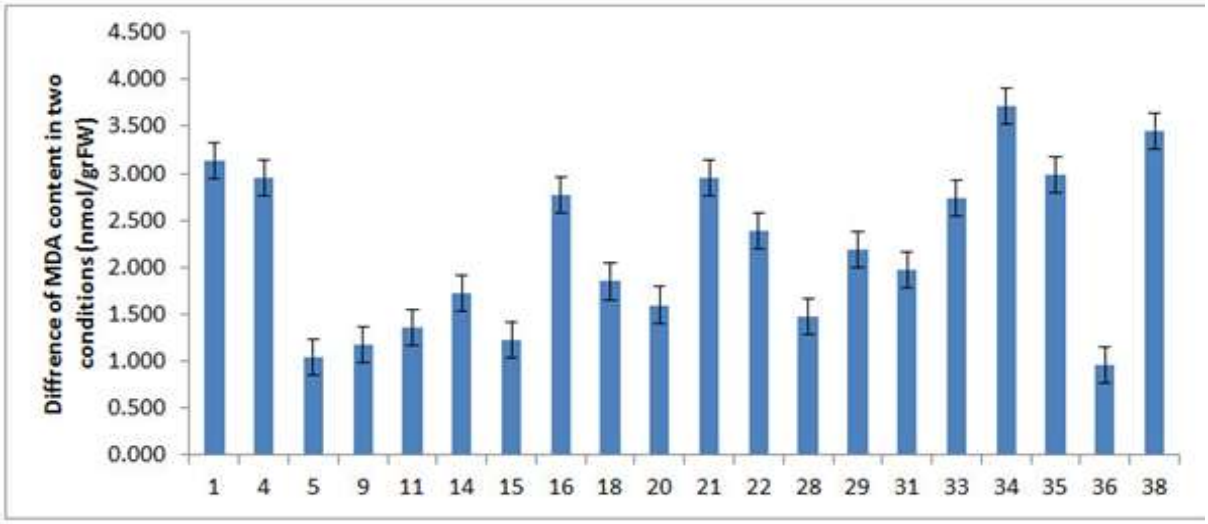

Fig. 4. Difference of MDA content in the leaves of barley genotypes in two conditions.

Second group includes (29, 31, 22) has less than average value for traits. This group can be considered as semi- tolerant genotypes. Group three includes (34,38) with higher mean for MDA and H_2O_2, ranked as sensitive genotypes to cold stress and fourth group (9,15,5,36,14,18,11,28,20) has lower mean for the traits and this group considered as tolerant to cold stress.

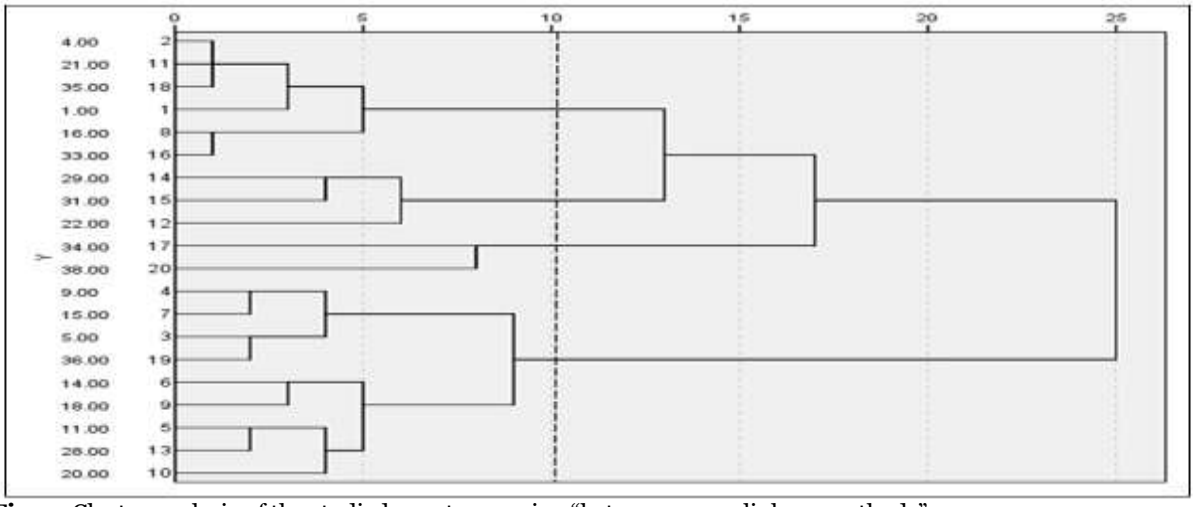

Fig. 5. Cluster analysis of the studied genotypes using "between group linkage methods"

Conclusion

Given the findings of this research, in summary, we can draw the conclusion that H_2O_2 and MDA contents increase under cold temperature stress, with sensitive cultivars display the highest increase significantly in MDA contents.

Although the cluster analysis was not necessary, but the analysis using MDA content trait could be divided genotypes into four groups properly. Therefore, the tolerance of barely genotypes to freezing can be predicted through investigating the MDA content.

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