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INVESTIGATING THE EFFECT OF COLD TEMPERATURE STRESS ON UNOPENED MALE CATKINS AND INOCULATED FEMALE FLOWERS OF IRANIAN NATIVE HAZELNUT CULTIVARS

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ABSTRACT

In many low-temperature areas, the environmental factor is an important limiting factor for the production and distribution of horticultural plants. This study aimed to investigate the cold tolerance of the male catkins and inoculated female flowers to screen the popular native hazelnut cultivars in Qazvin under low-temperature stress. A completely randomized factorial block design with three replications was used in this experiment with eight cultivars (Nakhnroud, Khandan, Mish-Pestan, South of Qarabagh, Asl-e-Qarabagh, Rasmi, and Gerdashkevar). After removing each of the treated samples at the end of the experiment, the samples were examined morphologically (appearance) and compared with the control. The changes were recorded as qualitative traits. To understand the influence of cold stress on reproductive organs, hydrogen peroxide and proline were measured. The results showed the onset of freezing in unopened male catkins at -7 and -9 °C and in inoculated female flowers at -3 °C. Damage to unopened male catkins' tissue occurred at -11 °C and in female flowers at -5 °C. The highest value observed among cultivars in the case for proline content of male catkins was in Mish-Pestan and Khandan cultivars with 0.816 and 0.660 µmol/ g FW, respectively. In inoculated female flowers, Mish-Pestan and Tabestaneh cultivars with 0.185 and 0.168 µmol/ g FW, respectively, showed the highest statistically significant increase in proline content. Interestingly, the cultivars with the highest proline content in male catkins indicated the most increase in H_2O_2; Mish-Pestan and Khnadan with 0.569 and 0.541 ug/g FW, respectively. Asl-e-Qarabagh was observed to have the least H_2O_2 content (0.042 ug/g FW) among cultivars. Again, in inoculated female flowers, those with the highest concentration of proline (Mish-Pestan and Tabestaneh) were found to have the highest H_2O_2 content (0.335 and 0.331 ug/g FW, respectively.

Keywords: *Low-temperature stress, Proline, Hydrogen peroxide, Discoloration, Morphological traits*

1. INTRODUCTION:

Plants face various environmental stresses during their lifetime, limiting the chances of plants growing and surviving. In many parts of the world, good growing conditions last only for a short time. In some places where the growing conditions are suitable, increasing the density and number of plants is a factor in creating competition for plants to obtain water and nutrients (Beck *et al.*, 2004; Peng *et al.*, 1996). Low temperature is the most important factor determining the distribution of plant species on the earth. And it can limit the yield and distribution of crops and orchards. Today, many plants are not native to their cultivated áreas or derived from a cross between species or cultivars that are not native to the area. The importance of low temperatures in horticulture has been known since the beginning of agriculture; for example, the Romans selected species to be cultivated in certain areas with cold weather (An *et al.*, 2019; Peng *et al.*, 1996).

Low temperatures in winter and the risk of spring frosts as important factors which nearly two thousand years ago, in the first century AD, efforts were made to protect crops from frost damage, but despite the various methods of frost protection that have been invented over time, frost damage to plants has been a major issue and a problem of great economic importance. This problem exists even in the subtropical regions (Molnar *et al.*, 2017).

Frost damage reduction has been devoted to addressing this important problem, not only through the development of conservation methods or delayed flowering but also through the study of physiological mechanisms involved in frost stress. However, this remarkable effort has only led to a relatively small improvement. The ultimate reasons determining how plants can withstand the cold stress are still unclear to a large degree (Cristofori *et al*., 2017; Kosenko *et al*., 2019; Wanjiku and Bohne, 2015).

Therefore, frost is still one of the biggest causes of loss of agricultural products among all environmental and biological harmful factors (Saielli *et al*., 2012; Sajadian *et al*., 2019). Hazelnut is one of the nut trees that is of great economic value. The plant is mostly shrubby and is rarely seen as a tree. The main hazelnut growing areas around lakes and seas have mild winters and cool summers (Gönenc *et al*., 2006; Linaldeddu *et al*., 2016). The three major hazelnut growing areas in the world include Turkey (under the influence of the Black Sea), Italy, and Spain (Affected by the Pacific) (Saydut *et al*., 2016; Silvestri *et al*., 2020). The world production of hazelnuts in 2019 is estimated at 1037,500 tons. With a production of 780,000 tons, Turkey was the top producer of hazelnuts and produced 60% of the world's hazelnuts. Italy has 130,000 tons and the United States, with 30,000 tons, Spain with 25,000 tons.

In comparison, Iran has a production quantity of 18000 tons per year harvested from 20459 thousand hectares (Çetİn *et al*. The yield of hazelnuts in Iran is very low and up to one ton per hectare, while in the major producer countries, it reaches 4 to 4.5 tons per hectare (Guliyev *et al*., 2019; Mostashari-Rad *et al*., 2020). Cold and frost are natural phenomena that cause a lot of damage to orchards, including hazelnuts, in some years. Cold damage to deciduous trees varies depending on the species and cultivar, and this action is often due to early spring frosts. Decreasing the temperature from the minimum tolerance threshold of the plant can be harmful. Gardeners are fully aware of the detrimental consequences of the trees' lack of resistance to winter cold (Çetinbaş-Genç *et al*., 2020; Kosenko *et al*., 2019; Silvestri *et al*., 2020).

Thus, understanding how cold stress and frost occurs and its symptoms in each area by examining the hazelnuts trees in the area can guide and help agricultural planners and gardeners reduce frost damage. Additionally, screening native hazelnut cultivars to find the most resistance ones is a significant step in assisting the farmers to cultivate the cold stress tolerance cultivars to minimize the economic consequence of frost damage. The two outcomes mentioned above are the main reasons for carrying out this study.

2. MATERIALS AND METHODS:

2.1. Plant materials and treatments

To perform this experiment, 20-year-old hazelnut trees are located at the Agricultural and Natural Resources Research Center of Qazvin Province (East of Alamut). Factors in this experiment were: first, native hazelnut cultivars (Nakhnroud, Khandan, Mish-Pestan, South of Qarabagh, Asl-e-Qarabagh, Rasmi, and Gerdashkevar), the second: cold intensity in ten temperature levels (+3, +1, -1, -3, -5, -7, -9, -11, -13, -15 ^{o}C), using an incubator and the third; the cooling time was one hour in the flowering stage (unopened male catkins and female flower inoculated). Samples prepared from each cultivar were placed in the incubator for one hour at each mentioned temperature. After applying cold treatments, the samples were taken out of the incubator, and the extent of damage to each sample was examined (Figure 1).

2.2. Morphological traits

Morphology, including (superficial tissue discoloration, inner tissue discoloration, tissue margin burn, weak freezing, complete tissue freezing, tissue loss after 24 hours), were examined. Then the changes were performed as a qualitative scoring table at level five. Score: zero (no change), one (low change), three (medium change), five (high change), seven (very high change), each of which includes a series of morphological changes at each level.

2.3. Determination Hydrogen peroxide

The H_2O_2 content was estimated via Çatav *et al*. (2020) method. Fresh tissue (0.3 g) was homogenized in 0.1% TCA and centrifuged at 12000 rpm for 15 min. The supernatant (0.5 mL) was added to 0.5 ml potassium phosphate buffer (pH 7.0), and 1 ml potassium iodide (1 M) and absorbance were recorded by spectrophotometer at 390 nm.

2.4. Determination of Proline

Proline content was measured, according to Rathika *et al*. (2020). Fresh material was homogenized in 5 mL sulfosalicylic acid (3%) and then was centrifuged at 13000 rpm for 20 min. Two mL of supernatant was mixed with acid ninhydrin (2 mL) and glacial acetic acid (2 mL) and then was boiled at 100°C for one hour. The reaction mixture was extracted with 4 mL toluene, and the absorbance was recorded at 520 nm.

2.5. Statistical analysis

The statistical design of the factorial experiment in a completely randomized block design with three replications (two samples in each experimental unit) was used. SAS software was used for statistical analysis of data. A comparison of the means was performed by Duncan test at the level of 1% and 5% probability. EXCEL software was used to draw the graphs.

3. RESULTS AND DISCUSSION:

Examination of the morphology of unopened catkins (Figure 2) showed that at 5 ° C, the male catkins had shown sensitivity to cold stress. In unopened male catkins, the onset of freezing is often at -5 and -7 °C, while complete freezing and tissue loss occurs at -11 °C. Among the cultivars studied for unopened male catkins, Mish-Pestan with a freezing point at -9 °C and complete tissue destruction at -11 °C showed more resistance than other cultivars. The morphology study results of inoculated female flowers (Table 2) showed that the onset of freezing occurred at -3 °C, and complete freezing occurred at -5 and -7 °C, which among the studied cultivars, Tabestaneh showed the highest tolerance.

The comparison of the mean data in unopened male catkins and inoculated female flowers have been presented in Figures 2 and 3, respectively, indicated that with time and increasing cold intensity, the concentration of hydrogen peroxide and proline increased in most cultivars. However, the level of hydrogen peroxide did not enhance due to cold stress as much as the protective amino acid proline. In the unopened male catkins and inoculated female flowers, respectively, proline has reached its maximum at the temperature where the serious injury took place. That is, over time and with increasing cold intensity, the proline has increased further. The highest value observed among cultivars in the case for proline content of male catkins was in Mish-Pestan and Khandan cultivar with 0.816 and 0.660 µmol/ g FW, respectively, however proline content of Tabestaneh cultivar (0.562) did not show a significant difference at 5% level of Duncan's test with Khandan.

Proline content in the Asl-e-Qarabagh cultivar was found to be the lowest (0.336 µmol/ g FW) compared to other cultivars (Figure 2). In inoculated female flowers, Mish-Pestan and Tabestaneh cultivars with 0.185 and 0.168 µmol/ g FW, respectively, showed the highest statistically significant increase in proline content when exposed to cold stress, whereas Rasmi cultivar with 0.077 µmol/ g FW showed the least proline content (Figure 3). The accumulation of this amino acid in perennial plants from mid-autumn to mid-winter is a natural physiological event in nitrogen storage metabolism. It was reported that pistachio cultivars, Akbari and Ahmad Aghaei under cold stress, to maintain the water potential of their tissues increasing proline was a significant approach (Sajadian *et al*., 2019), similar results were observed in our study.

Barand *et al*. (2020) showed that the proline content of leaves and fruits of pistachios cultivars exposed to cold stress significantly increased compared to the control. The amount of free proline in many plants in response to environmental stresses such as cold stress and drought increases to a large extent and stabilizes the membrane (Mansour and Salama, 2020). Research on winter wheat has also revealed a positive correlation between an increase in cold stress intensity and proline content, and often, resistant cultivars observed to have more proline than susceptible ones (Ignatenko *et al*., 2019; Pál *et al*., 2018; Venzhik *et al*., 2016). This case has also been seen in citrus so that the rate of increase proline in Trifoliate orange leaves, known as a cold-resistant plant, was about 4 times higher than that of found in rough lemon leaves after cold treatment (Liu *et al*., 2017; Mohammadrezakhani *et al*., 2019).

The proline content in soybean increased by a decrease in temperature, and the maximum amount was in winter (Yadegari *et al*., 2007; Yildiztugay *et al*., 2017). In cold-sensitive plants, the increase in cellular proline is insufficient to increase resistance unless high proline levels occur before stress. (Savouré *et al*., 1997). It can be concluded that proline in the flowering stage compared to its previous periods (from defoliation to the beginning of growth in buds) experienced a significant upward trend due to the conversion of proline from the storage phase to non-storage form and also other forms, especially the consumable form for the plant (Charest *et al*.,1990) which is consistent with our research. The concentration of sugar and proline increases during cold resistance, while starch concentration decreases (Patton *et al*., 2007).

As shown in Figures 4 and 5, in the unopened male catkins and the inoculated female flower, respectively, the enzyme hydrogen peroxide levels increased in some cultivars and decreased in others. This research indicated that the excessive increase of hydrogen peroxide increases free radicals and active single oxygen species, which in turn impose significant damage. Interestingly, the cultivars with the highest proline content in male catkins indicated the most increase in H_2O_2; Mish-Pestan and Khnadan with 0.569 and 0.541 ug/g FW, respectively. Asl-e-Qarabagh was observed to have the least H_2O_2 content (0.042 ug/g FW) among cultivars. Again, in inoculated female flowers, those with the highest concentration of proline (Mish-Pestan and Tabestaneh) were found to have the highest H_2O_2 content (0.335 and 0.331 ug/g FW, respectively, Figure 5). Hydrogen peroxide increases are due to initiate signaling systems as a messenger to activate the cold resistance genes, however, excessive increase of this enzyme triggers an enhancement in catalase and peroxidase to quench it. When plants are exposed to cold stress, due to disturbance in plant metabolism, the production of oxygen radicals such as superoxide ($-O_2$), hydrogen peroxide, and hydroxyl (-OH) increase (Fasih and Afshari, 2018; Rasoulnia *et al*., 2011).

Klíma *et al*. (2012) investigated the reaction of different parts of *Larix europea* under different temperature treatments. The result indicated a different pattern of peroxidation activity in leaf, seed, and branch samples, and peroxidation activity of leaf samples were several folds higher than branch samples. Research on Quercus robur has shown that with seasonal changes, peroxidase activity fluctuates so that its activity increases at the beginning of the cold season (Morecroft *et al*., 2003) and with the approach of the cold season, peroxidase activity has increased and in December, several times higher than in summer. However, its amount has decreased slightly compared to September. Castillo (1986) and Hung and Kao (2004) showed that peroxidase is the most sensitive plant enzyme to environmental stresses. Examination of black pine branches showed that peroxidase activity was not the same in different seasons and was several times higher in the winter (Chen *et al*., 2006).

Peroxidase activity was not the same in different seasons of the year, its maximum activity is in the cold seasons of the year while it was the least in the summer (Siqueira *et al*., 2007). Investigating the relationship between peroxidase activity and phloem vascularization in pine, spruce, and birch stem during the growing season showed that peroxidase activity increases at the beginning of the growing and lignification period (Marjamaa *et al*., 2003). The earlier the small amount of this enzyme increases at the beginning of the cold season, the greater its resistance to early frosts, and also the less it decreases in early spring compared to winter, indicating its higher resistance to the occurrence of early spring frosts (Fischer and Höll, 1991; Zolfaghari *et al*., 2005). At low temperatures, hydrogen peroxide accumulates without decomposing due to the inactivity of the enzyme catalase. During the freezing stages, respiration often increases and then begins to decrease (Prasad *et al*., 1994; Purvis and Shewfelt, 1993).

4. CONCLUSIONS:

In the seasonal changes of proline observed in this study, the accumulation of this amino acid during the cold adaptation period increased the resistance. The aggregation of this amino acid in Mish-Pestan and Tabestaneh cultivars were found to be the highest. Generally, it can be inferred that Mish-Pestan and Tabestaneh owe their cold resistance to a large extent to the accumulation of proline. The fluctuation of hydrogen peroxide concentration under cold treatments observed in this study was not significant compared to proline. Therefore, with very small changes in this enzyme, particularly in Mish-Pestan and Tabestaneh cultivars, an increase in cold resistance in those two cultivars was observed. In areas where there is a risk of freezing, hazelnut-resistant cultivars should be cultivated, which among the eight cultivars studied, Mish-Pestan and Tabestaneh cultivars were more resistant. Given the importance of this crop and its vulnerability to freezing, it is highly recommended that other cultivars be comprehensively investigated to find the cold stress tolerance cultivars and realize the underlying physiological mechanisms involved.

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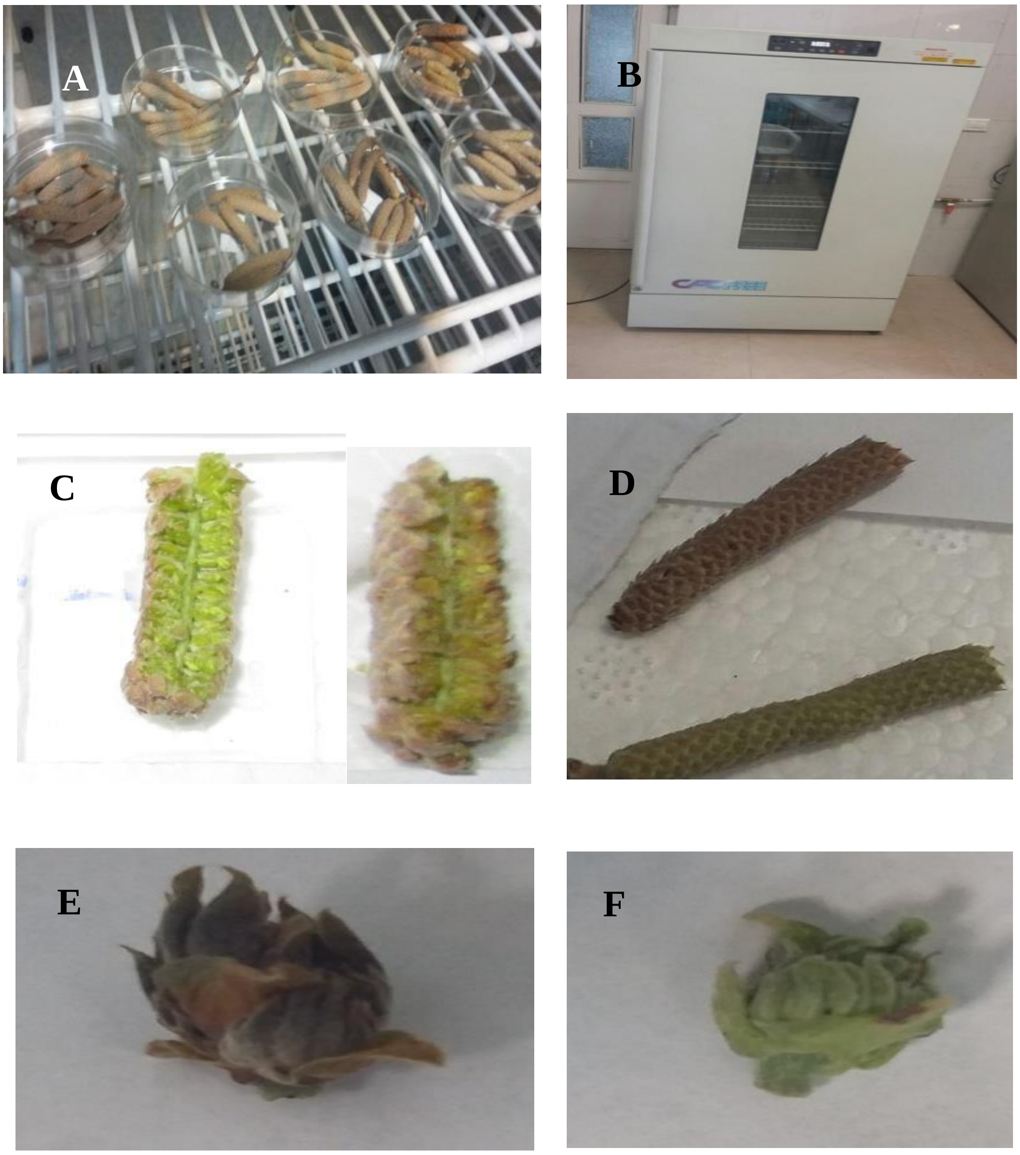

***Figure 1**. (A and B) Samples of unopened male catkins and inoculated female flowers of eight hazelnut cultivars prepared and placed in the incubator to be exposed to low temperature. (C and D) male catkins under normal temperature and injured by cold stress, respectively. (E and F) inoculated female flowers as control and damaged by cold stress, respectively.*

Table 1. Scoring qualitative morphological traits of male catkins exposed to low-temperature stress.

	zero (no change)	one (low change)	three (medium change)	five (high change)	seven (very high change)
Cultivars	No changes	The decreased freshness of tissues, no external or internal discoloration	Reduction in tissue hardiness, the onset of freezing, no external or internal discoloration	Reduction in tissue hardiness, freezing of tissues, external discoloration from green to dark green	Reduction in tissue hardiness, complete freezing, and external and internal discoloration to brown
Nakhnroud	+1،+3	-1	-3	-5	-7
Khandan	+1،+3	-1	-3	-5	-7
Tabestaneh	-1،+1،+3	-3	-5	-7	-7
South of Qarabagh	+1،+3	-1	-3	-5	-5
Asl-e-Qarabagh	+1،+3	-1	-3	-5	-7
Rasmi	+1،+3	-1	-3	-5	-5
Gerdashkevar	+1،+3	-1	-3	-5	-5
Mish-Pestan	+1،+3	*	-1،-3	-5	-5

Table 2. *Scoring qualitative morphological traits of inoculated female flowers exposed to low-temperature stress.*

	Zero (no change)	one (low change)	three (medium change)	five (high change)	seven (very high change)
Cultivars	No changes	Weak external discoloration	Weak external discoloration, the onset of freezing	Weak external browning, freezing, weak internal discoloration	Freezing, external and internal discoloration (Dark green), and necrosis
Nakhnroud	-1،+1،+3	-3	-5	-7،-9	-11
Khandan	+1،+3	-1	-3	-7،-5	-11،-9
Tabestaneh	+1،+3	-1	-5،-3	-9،-7	-11
South of Qarabagh	+1،+3	-3،-1	-5	-9،-7	-11
Asl-e-Qarabagh	+1،+3	-1	-5،-3	-7	-9
Rasmi	+1،+3	-1	-5،-3	-9،-7	-11
Gerd-eh-ashkevar	+1،+3	-1	-3	-7،-5	-9
Mish-Pestan	-3،-1،+1،+3	-5	-7	-9	-11

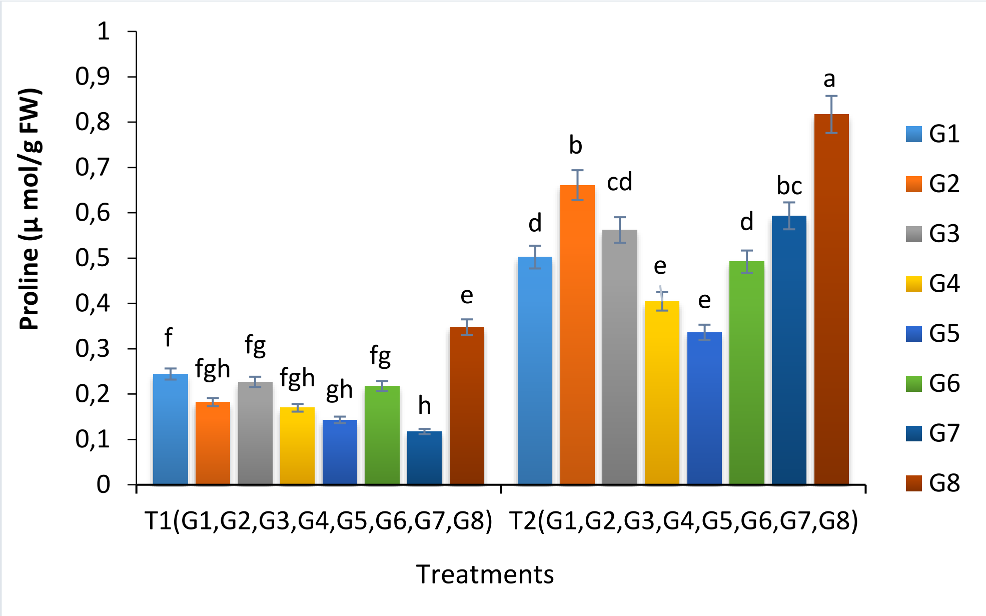

Figure 2. *The effect of cold stress treatments on proline accumulation in male catkins of hazelnut cultivars.* ***Note:*** *Cultivars: G1; Nakhnroud, G2; Khandan, G3; Tabestaneh, G4; South of Qarabagh, G5; Asl-e-Qarabagh, G6; Rasmi, G7; Gerdashkevar, and G8; Mish-Pestan*

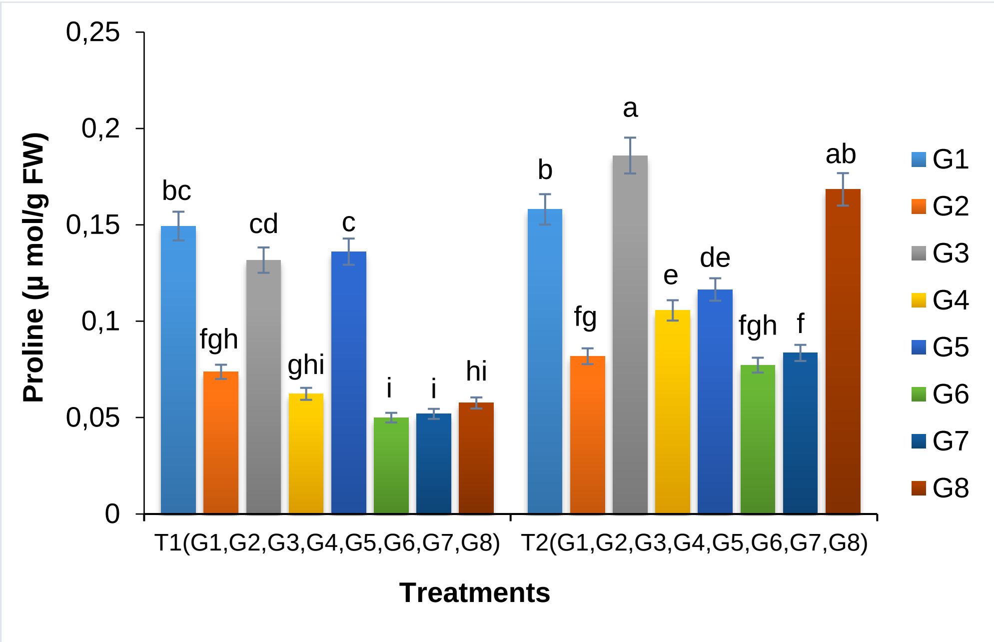

Figure 3. *The effect of cold stress treatments on proline accumulation in inoculated female flowers of hazelnut cultivars.* ***Note:*** *Cultivars: G1; Nakhnroud, G2; Khandan, G3; Tabestaneh, G4; South of Qarabagh, G5; Asl-e-Qarabagh, G6; Rasmi, G7; Gerdashkevar, and G8; Mish-Pestan*

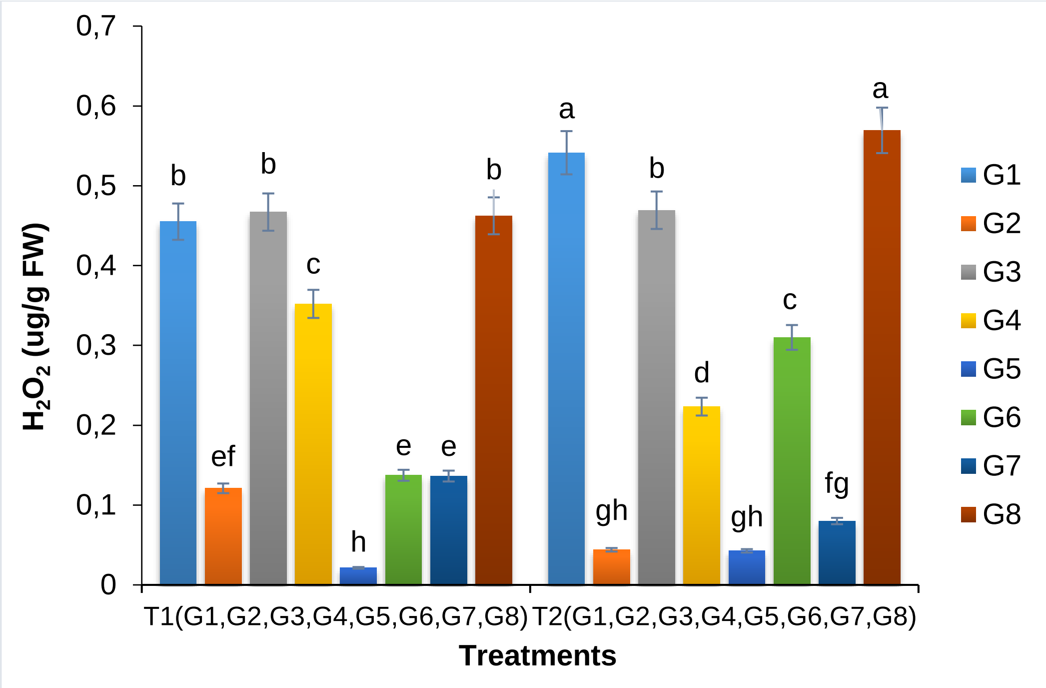

Figure 4. *The effect of cold stress treatments on H_2O_2 accumulation in male catkins of hazelnut cultivars.* ***Note:*** *Cultivars: G1; Nakhnroud, G2; Khandan, G3; Tabestaneh, G4; South of Qarabagh, G5; Asl-e-Qarabagh, G6; Rasmi, G7; Gerdashkevar, and G8; Mish-Pestan.*

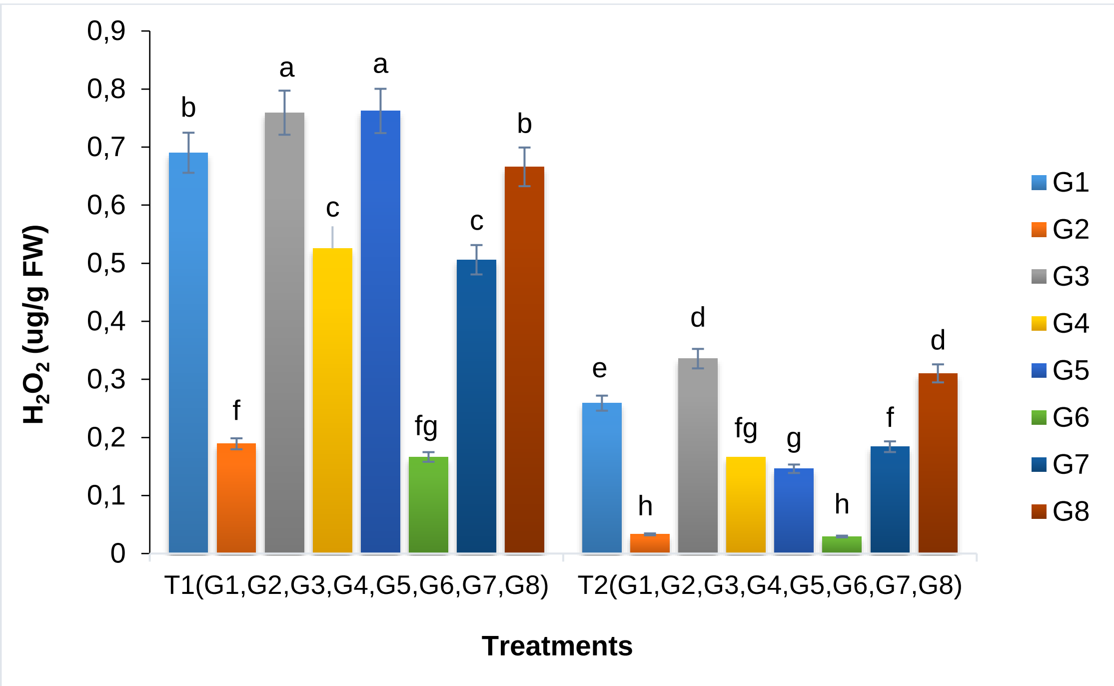

Figure 5. *The effect of cold stress treatments on H_2O_2 accumulation in inoculated female flowers of hazelnut cultivars.* ***Note:*** *Cultivars: G1; Nakhnroud, G2; Khandan, G3; Tabestaneh, G4; South of Qarabagh, G5; Asl-e-Qarabagh, G6; Rasmi, G7; Gerdashkevar, and G8; Mish-Pestan*