Screening of almond cultivars and genotypes in relation to frost stress tolerance

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Abstract

Almond is one of the most important horticultural crops of Iran, which has been of interest to Iranian growers for a long time owing to its unique characteristics. An investigation was carried out in the Horticultural Sciences Researches Institute under laboratory conditions to screen cultivars or genotypes of almonds from almond germplasm in terms of frost tolerance. Cuttings containing flower buds of 50 genotypes and varieties were used in the study. Genotypes were investigated in a factorial layout using a completely random design with 3 replications. The factors of the study included two levels of cold (normal temperature or without frost stress and -3 ℃) and 50 levels of genotypes. Two tolerant (Shokofeh and A43D99), two sensitive (Peerless and Rabie), and two mid-way varieties and genotypes (Nanpareil and A47MS3) were selected for further investigation. The selected genotypes in this study are the result of breeding programs, and further research is required to introduce some of these genotypes as resistant to frost stress with a potential to use in breeding and cultivation programs.

Keywords: enzyme, morphological traits, physiological traits, pomology, *Prunus dulcis*

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Introduction

Almond is one of the most important horticultural crops because of its unique characteristics for fruit production such as high efficiency in water consumption (Socias I Company and Gradziel, 2017; Imani, 2019) and drought-resistance, i.e., appropriate tolerance against water deficiency. Iran is located in the arid and semi-arid region of the world which means water deficiency for crops. Because of the ease of harvesting, transporting, and storing its fruit and also considerable employment, this species has long been of interest to Iranian growers (Imani, 2019). However, one of the major problems of the almond growers in the region is irregular production and annual fluctuations in yields, which is primarily due to the early flowering of native genotypes, when their flowering time coincides with cold spring conditions. Since almonds are self-incompatible, the resulting frost also disrupts pollination in the absence of pollinating insects during flowering. Lack of simultaneous flowering in late-flowering varieties and the deficiency in technical and horticultural knowledge lead to the crop loss with the damage estimated by 60% to 100% percent in years with spring frost conditions. Nowadays, these problems have been addressed in research,

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and the literature has presented solutions along the lines of novel knowledge and practices of proper horticulture management, e.g. the introduction of late-flowering and cold-tolerant cultivars, selection of suitable pollinizers, and pollination management (Kester et al., 1996; 2009; Imani, 2019). The development of improved late-flowering almond cultivars can be economically rewarding. The investigation of cold tolerance based on some index traits related to frost tolerance showed that frost damages flowers. Freshly formed fruits are particularly vulnerable to frost and are one of the limiting factors of almond production in most of the world's almond growing regions (Imani, 2019). Although almonds are resistant to low temperatures in winter, certain temperatures and duration of cold in late spring are fatal for most reproductive organs at the flowering stage. In some years, even cold-resistant cultivars are damaged by low temperatures. The period of the main risk of frost starts from the beginning of flowering and extends well into active growth (of branches and fruits). The temperature at which flower buds are damaged depends mainly on their growth stages. When the buds are completely at rest during winter, they have the highest resistance to frost (Miranda et al., 2005). As soon as the buds begin to swell and bloom, their resistance to freezing decreases. Therefore, in temperate climates, frost damage during flowering is much more important than damage due to low temperatures in winter. Cold resistance in flower buds is probably the result of several factors including structural, physiological and morphological traits. It has been reported that factors such as genotype, development stages, ice formation, moisture level, and material nutrition status have a lot to do with the sensitivity or tolerance of flowers to spring frost. Frost resistance varies among the buds of different cultivars of the same species (Kester et al., 1996; Socias and Gradziel, 2017). For this reason, the critical index temperature for almonds in different stages of phenology has been defined separately and led to the achievement of a criterion for selecting cultivars with high resistance to basic freezing (-4 ℃). Freezing temperatures can seriously damage plant tissue. The effects of spring frost on almond reproductive organs are very different and depend on the characteristics of cold stress and plant condition. Different responses to frost among plant genotypes, tissues of the same plant, and different seasons led to the study of damage mechanism. In some cases, accumulation of organic osmolyte substances such as proline and glycine betaine can be investigated as the level of these substances increases under stress conditions (Storey et al., 1977). It seems that proline has different roles in osmotic stress, e.g. stability of proteins, membranes, and cellular microstructures and protection of cellular function by inhibiting active oxygen species. Also, studies show that the severity of frost damage is influenced by genotype. Genotypes that are more resistant to frost damage contain a higher level of proline (Trovato et al., 2008). It is suggested that measuring the level of proline as an index of frost tolerance should be taken into consideration in almond breeding programs and the selection of proper genotypes (Kester et al., 1996; Szabados and Savoure, 2010; Socias and Gradziel, 2017). Planting techniques such as irrigation, reducing nitrogen consumption, soil protection system, and placing heaters are used in commercial orchards to reduce frost damage (Kester et al., 1996; Socias and Gradziel, 2017). Therefore, the most effective way to prevent frost damage is to use spring frost-resistant cultivars such as late blooming cultivars with less sensitivity among cultivars at a similar phenological stage (Kester et al., 1996). Cold is one of the important abiotic factors that limits the growth, production, and distribution of plants. Low temperature reduces biosynthetic activities, limits the normal functioning of physiological processes, and causes permanent damage that will eventually lead to necrosis (Kester et al., 1996; Masip et al., 2018).

Frost damages are due to a general disorder in the metabolic and cellular process and a change in the properties of the membrane. Cold-tolerant and frost-sensitive plants can survive in temperatures slightly below zero, but they are severely damaged by the formation of ice in their tissues. The actual degree of cold tolerance depends on the species, developmental stage, and duration of cold stress (Kester et al., 1996; Miranda et al., 2005). There is evidence that the proteins produced as a result of stress during wintering of the plant have multiple

functions and it is very important to identify the cold adaptation mechanisms through them in the plant (Sanghera et al., 2011) .

Winter metabolism is related to intermittent biochemical changes in plants. Commonly observed changes include alternation in gene expression, changes in hormone levels, increase in soluble sugars, accumulation of osmotic and cellular protective proteins, and changes in membrane lipid compositions. The relationship between these changes in order to increase cold tolerance is not yet known. But different genes and regulators play a role in this process (Chen and Murata, 2002, 2008). The process of adaptation to low temperature leads to many biochemical changes inside the cell. There is a great correlation between soluble protein contents and cold tolerance and increasing the amount of mRNA for protein synthesis such as cold regulatory proteins or COR-Protein (Chen and Murata, 2002, 2008). The plasma membrane is the first site of injury during freezing; therefore, most of the changes are made in order to increase the tolerance to freezing, with the aim of maintaining the cell membrane health (Allen, 1995; Rao et al., 1996). A change in the lipid contents of the plasma membrane and chloroplast leads to a maximum reduction of damage in cold-resistant plants compared to cold-sensitive plants. This function is caused by the increase in the fluidity of the membrane, which is the result of the change in the lipid content and the increase in the fatty acids of the plasma membrane. Soluble substances such as proline accumulate in cold-resistant plants when facing cold. In general, soluble substances lead to maintaining turgescence and preventing cell dehydration. They also have protective effects on macromolecules (Sanser and Beck, 1979; Stephen et al., 1989). Cold also causes stress to plants by creating active oxygen species. Therefore, the activity of antioxidant enzymes (catalase, peroxidase, ascorbate peroxidase, glutathione reductase, superoxide dismutase, etc.) plays an important role in scavenging free radicals caused by cold. In the present study, almond cultivars and genotypes were first screened in terms of cold tolerance. Second, the selected genotypes were examined for further investigation, especially in relation to cold tolerance traits.

Materials and Methods

This experiment was carried out at the Horticultural Research Station of Meshkin Abad, Temperate Fruits Research Center, the Institute of Horticultural Sciences (51° East longitude; 35° 48’ North latitude; 1320 m above sea level). The average annual temperature is 13.7 ℃. The average rainfall of the region is 254.5 mm per year, the highest in April with 26.6 mm and the lowest in March and August, equal to zero mm (Karaj Synoptic Station, 2015).

Screening almond cultivars and genotypes

The tolerance level of 45 promising almond genotypes along with 5 commercial cultivars as controls was determined under laboratory conditions by the method of Miranda et al. (2005). In order to compare the resistance to cold stress in the flowers of the cultivars and genotypes under investigation, a completely random basic design was arranged with 3 replications under the temperature condition of -3 ℃. Plant samples in the form of branches with a length of 20 to 50 cm and with flower buds in the open flower stage were prepared from three directions (one sample from each direction). Branches were cut a few centimeters from the lower part, and in order to increase their life span and provide energy, they were placed in containers supplied with water + 5% sucrose and transported to the laboratory. A room equipped with temperature and light control was used to apply the treatment at -3 ℃. The cut branches were kept for 30 minutes at 7 ℃, 60 minutes at a 5°C, 60 minutes at 3 ℃, 60 minutes at 1 ℃, 60 minutes at - 1.5 ℃, and finally, 60 minutes at -3 ℃ (Miranda et al., 2005). In order to evaluate the amount of damage to the pistils of the flowers, after removing the temperature stressed branches containing the flower buds from the chiller, they were placed at room temperature for 24 hours for recovery. In the next step, 10 flower buds were separated from different parts of the branches and the changes in pistil color were inspected visually. The pistils with a change in color as well as the pistils showing no color change to the naked eye were counted. Then, a vertical cut was made on the surface of the pistil and the damage and change in color were observed using optical binoculars. Finally, the

percentage of damage was recorded, based on the 10 evaluated pistils (Miranda et al., 2005).

Physiological traits of cultivars and genotypes

Following visual evaluations and determination of the percentage of frost damage in flowers (pistils), a completely random basic design was arranged with three replications to probe into the frost-related physiological and biochemical parameters in two tolerant, two sensitive, and two intermediate genotypes using factorial experiment. The first factor of study was cold in two levels (normal temperature or without cold stress and -3 ℃), and the second factor included genotype in six levels (due to the large number of cultivars and genotypes in this research after screening). Traits related to frost tolerance including chlorophyll fluorescence index, proline, catalase enzyme, and glycine betaine were assayed before and after cold treatment using methods as described by Wang et al. (2018), Bates et al. (1973), Hasanuzzaman et al. (2021), and Min et al. (2021) respectively.

Finally, the results obtained from this research were analyzed with the help of SAS (9.4) software and the mean effects of treatments were compared using Minitab-17 software and Tukey's test, to select the most tolerant genotypes and cultivars for further exploitation.

Table 1
The ANOVA results of the effect of freezing stress on 50 almond genotypes at -3 °C under controlled conditions

Sources Changes	df	Freezing (-3°C)
Genotype	49	78.92**
error	98	14.29
CV	-	10.34

**significant at the 1% level

Results

Screening the almond genotypes for frost stress tolerance showed that flower frosting was significantly affected (P≤0.01) by almond genotype and cold stress (Table 1). Different almond genotypes showed significant differences from each other, and in all studied genotypes, the frost resistance of the control plants was less than that of stressed plants. Table 2 presents the comparison of means, suggesting that the effect of frost stress on almond genotypes was different. The results of the comparison of means showed that freezing stress had a significant effect on

Table 2 - Percentage of frost damage at -3°C temperature in controlled conditions

Genotype/ Variety Cod	Frost Damage (%)	Genotype/ Variety Code	Frost Damage (%)
A1	100a	B16	80c
A2	100a	B17	96ab
A6	98ab	B18	95ab
A7	100a	B19	94ab
A9	100a	B20	93ab
SAHAND	90b	B21	100a
A12	100a	B23	100a
A13	99a	C3	100a
Super Nova	94ab	Rabie	95ab
A18	98a	C5	90b
A19	90b	C9	100a
A20	100a	C10	100a
Azar	100a	C12	100a
A200	93ab	C14	90b
A23	80c	C17	90b
B1	100a	C18	97a
B2	97a	D3	91b
B3	90b	D5	30f
B4	96ab	D7	65d
B5	93ab	D10	93ab
B6	90b	D15	93ab
B7	93ab	D19	84c
B10	90b	D20	88bc
B12	99a	D21	59e
B15	95ab	D22	93ab

Code of genotype/variety with common letters does not have a significant difference at the 5% probability level of the LSD test.

flower freezing in the almond genotypes under study.

Results of the freezing rate of the almond cultivars and genotypes in relation to the traits related to frost tolerance are presented in Table 3. As the table shows, almond cultivars and genotypes under study were significantly different in terms of the traits related to frost tolerance (P≤0.01).

Examining the flowers of the flowering branches subjected to -3 ℃ cold treatment showed that most of the genotypes suffered frost damage (Fig. I). Moreover, the cultivars and genotypes experienced different levels of damage under -3°C (Fig. II).

Results of the chlorophyll fluorescence assays before and after freezing showed that the ratio of variable to maximum fluorescence (F_v/F_m) decreased with a drop in the temperature in all cultivars and genotypes under study. The greatest decrease in F_v/F_m was observed in Peerless and Rabi cultivars under freezing stress, especially at -3 ℃ (Fig. III).

According to the obtained results, cultivars and genotypes differed significantly in terms of proline content (Table 3), so that the highest and lowest proline contents were observed in A45D99 genotype and Peerless cut branches, respectively. A decreasing trend in flower proline content was observed in the cultivars at -3 ℃ (Fig. IV). However, A45D99 genotype showed a significant increase in flower proline compared to other genotypes under -3 ℃ treatment.

According to the obtained results (Table 3), cultivars and genotypes differed significantly in terms of glycine betaine content under frost condition. The maximum glycine betaine level was observed in A45D99 genotype and the lowest glycine betaine content belonged to Rabie variety. A significant increase in flower glycine betaine was observed in A45D99 genotype compared to other genotypes when the temperature was decreased to -3 ℃. A decreasing trend in glycine betaine contents of flowers was observed in all cultivars under -3 ℃ condition (Fig. V).

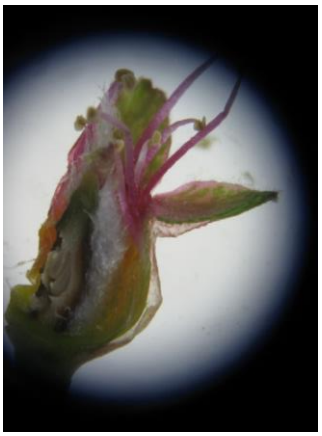

Fig. I. Assessing the amount of damage to the flowers of the branches under temperature treatment of -3 °C using optical binoculars.

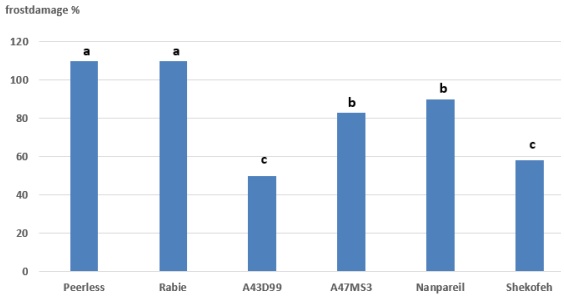

Fig. II. The degree of frost damage of selected almond cultivars and genotypes at -3 °C; means that do not share a letter are significantly different.

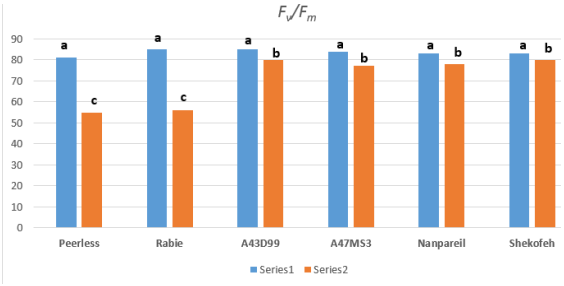

Fig. III. The effect of cold stress on the F_v/F_m in selected cultivars and genotypes of almonds (Series1: without cold stress; Series2: with cold stress: -3 °C); means that do not share a letter are significantly different.

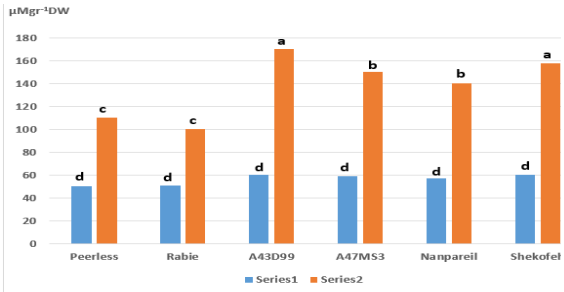

Fig. IV. The effect of cold stress on the proline content in selected cultivars and genotypes of almonds (Series1: without cold stress; Series2: with cold stress: -3 ˚C)

Table 3
The ANOVA results of the effect of freezing stress on 6 selected cultivars and genotypes in terms of the frost tolerance-related traits

Sources of Change	DF	Frost Damage %	F_v/F_m (Post-frost)	F_v/F_m (Pre-frost)	Glycine (control)	Glycine -3 °C	Catalase -3 °C	Catalase (control)	Proline control	Proline -3 °C
Genotype × Frost	5	1277.39**	255.44**	0.6000 ns	34.107 ns	942.0**	47.149**	109.42 ns	1353.31 ns	1531.6 **
Error	22	1.06	55	0.0004	0.378	10.3	0.281	0.2	1.98	7.2
CV	-	11.15	3.11	3.34	5.67	23.91	13.15	7.14	10.15	12.03

**; significant at the 1% level; ns not significant

The activity of catalase enzyme in almond cultivars and genotypes under cold stress also showed variations compared to the control (Table 3). In fact, the cut branches of the different cultivars and genotypes of almond under normal conditions (control) and those under frost stress contained different concentrations of catalase (Fig. VI).

Discussion

In this research, after screening 50 varieties and genotypes of almonds for tolerance to freezing stress, two varieties and genotypes, namely Shokofeh and A43D99, were found as tolerant and two varieties, namely Peerless and Rabie, were considered as sensitive, and two varieties and genotypes, namely Nanpareil and A47MS3, were designated as intermediates. These varieties were selected for further investigation, especially in terms of frost-related physiological traits. It is noted that the selected genotypes in this research were the result of breeding programs, and with further research, some of these genotypes can be introduced as genotypes resistant to frost stress and used in breeding and cultivation programs.

This study suggests that despite the new methods of identifying the physiological dimensions of frost damage in plants, the observation technique is still a useful approach to better understand the cold tolerance mechanism and traits in almond cultivars and genotypes (Rodrigo, 2000).

Phenological stage can be very important in relation to frost damage. Trees in the flowering and petal fall stage are more affected by low temperature conditions and are more sensitive (Pakkish et al., 2011). Miranda et al. (2005) reported that Prunus species, including almonds, are resistant to cold before flowering, but in the

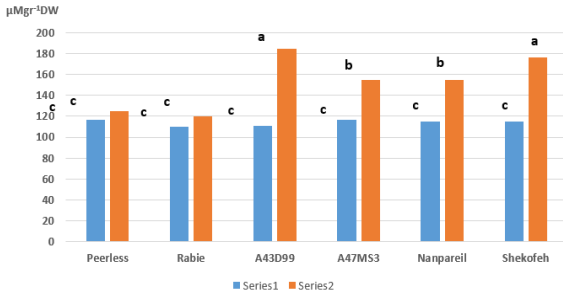

Fig. V. The effect of cold stress on the amount of glycine betaine content in selected cultivars and genotypes of almonds (Series 1: without cold stress; Series 2: with cold stress: -3 °C)

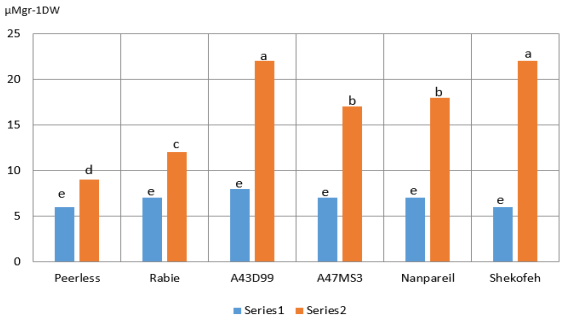

Fig. VI. The effect of cold stress on the amount of catalase in selected cultivars and genotypes of almonds (Series 1: without cold stress; Series 2: with cold stress: -3 °C)

full flower and later stages compared to the previous stages (deep rest stage of the flower bud), they are sensitive to cold. Finally, as Socias and Gradziel (2017) argue, under the same phenological conditions, the type of genotype is a decisive factor.

Small almond fruits are very sensitive organs to cold (Bigdali et al., 2018). Also, identification of the highest and lowest temperature limits for each of the almond organs has been reported (Miranda et al., 2005). According to our results, while the flowers lose their color slightly at -1.5 ℃, and

some areas turn brown, freezing damage at this temperature does not cause significant economic damage (Magenau et al., 2023). By lowering the temperature to -3 ℃, the flowers changed in color (Fig. I) very clearly, and the flowers were severely damaged (Table 2, Fig. II). Similar results have been reported by Megenau and coworkers (Magenau et al., 2023).

In the present study, the F_v/F_m of the samples decreased with decreasing temperature (Fig. III). In fact, F_v/F_m decreased by 25% and 30% in Peerless and Rabi cultivars compared to the control, respectively (Fig. III). Maximum quantum efficiency shows the capacity to use radiation energy absorbed by PSII in a photochemical process in the dark-adapted state (Oxburg, 2004). The maximum quantum yields consistently decreased from 0.83 before chilling to 0.57 (~37% reduction) under low temperature treatment (Fig. III). High temperature has no effect on photosynthetic activity compared to low temperature in frosting of almond flowers. The significant decrease in F_v/F_m at low temperature after cold treatment indicated that the almond flowers were under severe stress and the photochemical efficiency of PSII was severely impaired, which indicated that low temperature had significant effects on PSII photochemistry and led to light inhibition. PSII photochemistry and photo-inhibition have been linked to conformational modification of PSII, particularly the D1 protein (Haldiman and Feller, 2004; Fu et al., 2012; Yang *et al.*, 2017). Furthermore, the sharp decrease in F_v/F_m under low temperature was due to the increase in the minimal fluorescence (Fo) under stress conditions. A decrease in F_v/F_m involves an increase in Fo (Yamada et al., 1996). Our result is in agreement with the results reported by Partelli et al. (2009), who recorded a continuous decrease in F_v/F_m in coffee grown at low temperatures. Also, the results of the present study agree with those reported by Hogwoning and Harbinson (2007) who observed photo-inhibition in the leaves of *Calathea makoyana* during frost stress. It seems that chlorophyll fluorescence and especially determination of photochemical capacity after freezing is a suitable method to determine cold resistance in plants. A decrease in the F_v/F_m ratio after freezing indicates a shift of photosystem II reaction centers from functional to downregulated or nonfunctional centers (Powles, 1984; Krause, 1988). This indicates impaired photosynthetic performance, as photochemical capacity (F_v/F_m) is experimentally correlated with photosystem II quantum efficiency (Adams and Perkins, 1993). However, the reduction of photochemical capacity does not necessarily mean a direct rupture of the thylakoid membrane after freezing. The thylakoid membrane is reportedly not the primary site of freezing damage (Adams and Perkins, 1993), but its inactivation is due to the inhibition of photosynthetic CO2 uptake after freezing. Inhibition of CO2 absorption appears to be due to the reduced activation of light-phase enzymes of the Calvin cycle, which may be caused by changes in the characteristics of the chloroplast envelope during freezing (Powles et al., 1988). Stephen et al. (1989) showed that following cold or heat stress, initial damages occur in the plasmalemma. The subsequent leakage of ions or toxic compounds from the cell leads to changes in the cellular environment, which in turn may disrupt the function of other cell organelles such as chloroplasts and mitochondria (Senser and Beck, 1979; Stephen et al., 1989). In most plant species, F_v/F_m between 0.750 and 0.850 indicates normal photosynthetic conditions (Maxwell and Johnson, 2000). While lower values indicate stress conditions. Technically, F_v/F_m is used as a reliable indicator of stress tolerance.

It was also found in the present study that, with decreasing temperature, there was an increase in flower proline content of all almond cultivars under investigation. Proline, a major adaptive solute (Sabados and Savor, 2010) is found in large amounts under all stress conditions (Trovato et al., 2008; Verslues and Sharma, 2010). In this experiment, proline accumulated in all cultivars under cold stress conditions (Fig. IV) because it plays an important role in plant growth processes in different ways and is found in plants even in non-stress conditions (Trovato et al., 2008). However, the highest concentration of proline was observed under cold stress condition followed by normal temperature. These results show that flowers of almond trees exposed to low temperature experience high stress levels,

because proline accumulation is considered as a strong indicator of biotic and abiotic stress (Trovato et al., 2008).

Photosynthetic responses, morphological characteristics, proline concentration, and F_v/F_m in flowers of almond trees were affected differently by temperature conditions. Fluorescence parameters, such as F_v/F_m, were affected significantly at low temperature compared to normal temperature conditions. In addition, proline accumulation showed the greatest response to low temperature. Research to use possible temperature treatments to identify fast responses to temperature by the photosynthetic performance of almond flowers is beneficial. On the other hand, it was found in the present study that the activity of glycine betaine in almond cultivars and genotypes under cold stress increased rapidly compared to the control, and it was 156.6% and 170% in the control and under stress for almond genotypes and cultivars, respectively. Following the difference between almond cultivars and genotypes, glycine betaine activity increased much more in the resistant cultivars than in the sensitive cultivars (Fig. V). Glycine betaine is a quaternary ammonium compound that accumulates to significant osmotic levels in many salt-tolerant plants (Rhodes and Hanson, (1993) and salt-tolerant cyanobacteria (Chen and Murata, 2008). Biochemical and biophysical studies showed that glycine betaine protects membranes against heat and freezing. Glycine betaine levels vary significantly among species and plant organs, as shown in Fig. (V). Almonds normally have low levels of glycine betaine under normal conditions, but accumulate higher amounts of glycine betaine when subjected to abiotic stresses (Storey et al., 1977). In many other species, no glycine betaine is found under normal conditions. or undetectable stressors There is now strong evidence that glycine betaine plays an important role in abiotic stress tolerance.

Following the difference between almond cultivars and genotypes, catalase activity increased much more in the resistant cultivar than in the sensitive cultivar (Fig. VI). However, catalase is generally considered to be the most dominant method of ROS scavenging in plant systems (Allen, 1995; Rao et al., 1996). Fluctuations in catalase enzyme activity were an important index of frost stress tolerance in almond cultivars in our study (Fig. VI). Consistent with previous studies (Prasad, 1997; Prasad et al., 1994), the freezing tolerance of both tolerant cultivars was closely related to the activity of antioxidant enzymes (Fig. VI). Our results showed that catalase increased during the cold acclimation period (Scalabrelli et al., 1991).

Conclusion

This study showed that freezing tolerance in flowers of almond genotypes is associated with increased capacity to remove or detoxify by enzymatic and nonenzymatic antioxidant systems.Enzymatic activity of catalase is especially at work during the processes of rest and growth. Also, the antioxidant system, including proline and betaine glycine, all of which accumulate especially during environmental stress processes. A comparison of almond genotypes under normal and cold stress conditions showed a dominant strategy in antioxidant defense systems to create tolerance to freezing. In sum, the cultivars and genotypes in this study showed great variation in all the examined traits. Also, investigation of cold tolerance of flowers of almond genotypes in the present study showed that flower tolerance was different among cultivars and genotypes. After screening 50 almond cultivars and genotypes, two tolerant (Shokofeh and A43D99), two sensitive, (Peerless and Rabie), and two intermediate cultivars and genotypes (Nanpareil and A47MS3) were selected as frost tolerant, all which being the result of breeding programs. Finally, further research is required to determine some of these genotypes as resistant to frost stress and introduce them for breeding and cultivation programs.

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