American-Eurasian J. Agric. & Environ. Sci., 12 (6): 807-814, 2012
ISSN 1818-6769

DOI: 10.5829/idosi.aejaes.2012.12.06.1746

Phenylalanin Ammonia-Lyase Activity, Total Phenolic and Flavonoid Content in Flowers, Leaves, Hulls and Kernels of Three Pistachio (*Pistacia vera* L.) Cultivars

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Abstract: Phenylalanin ammonia-lyase (PAL) plays a pivotal role in the production of phenolic compounds, which are responsible for the success of the defense strategies in harsh environments in response to different stimuli. Measurements of the PAL activity, total phenolics, total flavonoids and anthocyanin contents were performed in flowers, leaves and fruits of three pistachio cultivars "Ahmadaghaii", "Ohadi" and "Kallehghuchi". The results showed that PAL activity was different in cultivars and in plants organs of pistachio trees (flowers, leaves and fruits). The highest activity rate of their compounds were observed in Ahmadaghaii cultivar. A positive correlation was observed between PAL activity, total phenolics and total flavonoids in leaves and a negative correlation between PAL activity and anthocyanin contents in leaves and flowers of Ahmadaghaii cultivar. PAL activity and total phenolic in fruits of pistachio suffered a decreased when the maturation processes began. It is suggested that the hulls of the pistachio fruits, contain high level of phenolic compounds (especially in Ahmadaghaii cultivar), may function as a protective layer of defense chemicals against ultraviolet radiation and pathogens. The final concentration of phenolic compounds, flavonoids and antocyanins in the kernel depend on PAL activity in the kernel's cultivar. The results led to the conclusion that increase in PAL activity, phenolic compounds and flavonoids in Ahmadaghaii can help the plant to cope with the stresses better than the other cultivars. Since phenolic compounds are antioxidant and scavenge free oxygen it is postulated that Ahmadaghaii is the most resistant cultivar to the environmental stresses.

Key words: Ahmadaghaii cultivar · Environmental stress · PAL activity · Phenolic compound · Pistachio

INTRODUCTION

Phenylpropanoids are derived from *trans*-cinnamic acid which is formed from L-phenylalanine in a reaction catalyzed by enzyme L-phenylalanine ammonia-lyase. PAL (EC 4.3.1.5) is an extremely sensitive indicator of stress conditions and it is commonly considered as a biochemical marker indicating the synthesis of both structural and protective compounds [1-3]. It plays a pivotal role in phenolic synthesis and many reports emphasize on the correlation between increase in the corresponding PAL gene/protein expression/activity and increase in the phenolic compounds in response to different stimuli [4]. The phenylpropanoid pathway includes a large range of low molecular weight polyphenols. Flavonoids and their derivatives are the largest and most important group of polyphenols [5, 6]. Flavonoids and anthocyanins are remarkably divers group of secondary products with a vast array of biological function, including apparent roles in stress protection [7]. Phenolic compounds one of the most widely occurring groups of phytochemicals are of considerable physiological and morphological importance in plants. It is thought that the molecular basis for the protective action of phenolic compounds in plants is their antioxidant and free radical scavenging properties. The accumulation of phenolic compounds varies strongly with the growth state, development and responses to environmental stresses and is a result of balance between biosynthesis and further catabolism [8, 9].

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Pistachio (*Pistasia vera* L.) tree is an economically important plant which is cultivated in vast areas of arid and semi arid environments of different elevations and precipitation rates in Iran. Hence, the plant is exposed to different environmental stresses e.g drought and biotic factors. The plant is reproduced by grafting different cultivars on rootstocks and the assessment of the best cultivars with respect to their adaptation to the environmental stresses is likely to be necessary [10]. The presence of phenolic compounds in different parts of pistachio tree has been reported and the antioxidant activity and total phenolic compounds of pistachio (*P. vera*) have been determined in hull extracts [11], skin and seed [12, 13].

The main objective of the present study was to assess phenylpropanoid pathway metabolites in flowers, leaves and fruits of three pistachio cultivars that are grafted on the same rootstock *P. mutica*. The assessments were carried out by measuring PAL activity, total phenolics, flavonoids and anthocyanins. The assessments could lead toward the selection of the most suitable and compatible cultivar in order to produce the highest yield with best quality via its resistance to the environmental, biotic and abiotic stress factors.

Reagents and Equipments: Folin-Ciocalteu's phenol reagent was purchased from Merck KGaA (Darmstadt, Germany). Standared gallic acid, quercetin 3-rutinosid (rutin) were purchased from Sigma Chemical CO. (St. Louis, MO). All chemicals were of analytical grade.

Equipments Were Used: UV-visible spectrophotometer, Cary 50 conc VARIAN, Australia, Centrifuge Eppendorf AG 22331 Hamburg Germany.

MATERIALS AND METHODS

Sample Collection and Preparation: Three pistachio cultivars "Ahmadaghaii", "Ohadi" and "Kallehghuchi" were evaluated on rootstock *Pistacia atlantica* sub sp. *Mutica,* in Iran's Pistachio Research Institute (IPRI). The plant materials were collected in April (flower) and July (leaf) in 2010. Pistachio fruits were randomly selected for sampling throughout ripening, from the young to the harvest date, on the following dates: green hull (July), green-red hull (August), red hull (early-September), kernel at harvesting time (late-September). The plant materials were subsequently frozen in liquid nitrogen and stored at -80°C for the analysis. The experiment was conducted under similar condition at irrigation and soil type, using a Randomized Complete Block Design (RCBD) with three blocks. Measurements were carried out on three replicate samples from each block.

Measurements

Phenylalanine Ammonia-Lyase Activity: Phenylalanine ammonium-lyase (PAL) was extracted from fresh cell mass (300 mg fw) with 6.5 ml of 50 mM pH 8.8 Tris-HCl buffer containing 15mM of ß-mercaptoethanol in an ice-cooled mortar, ground with a pestle for about 5 min. The homogenate was centrifuged for 30 min and the supernatant was collected for enzyme assay. PAL activity was determined based on the rate cinnamic acid production. Briefly, 1 ml of the extraction buffer, 0.5 ml of 10 mM L-phenylalanine, 0.4 ml of deionized water and 0.1 ml of enzyme extract were incubated at 37°C for 1h. The reaction was terminated by the addition of 0.5 ml of 6 M HCl and the product was extracted with 15 ml ethyl acetate followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 ml of 0.05 M NaOH and the cinnamic acid concentration was measured spectrophotometrically by the absorbance at 290 nm. One unit of PAL activity is equal to 1 µmol of cinnamic acid produced per min [14]. Protein was estimated according to Bradford [15] using BSA as a standard.

Total Phenolic Compounds: Total phenolic content was determined using the Folin-Ciocalteau method Singleton and Rossi [16] as modified by Velioglu *et al*. [17]. Samples (100 mg) were extracted with 80% methanol containing 1% hydrochloric (5 ml) at room temperature for 2 hr on a shaker. The mixture was centrifuged at 3000g for 10 min. The supernatant was used for to determine total phenolics. One hundred microliter of extract was mixed with 0.75 ml of Folin- Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22°C for 5 min; 0.75 ml of sodium bicarbonate (60 g/L) solution was added to the mixture. After 90 min at 22°C, absorbance was measured at 725 nm. Gallic acid was used for constructing the standard curve. Results were expressed as mg gallic acid (GA) per gram of the fresh weight.

Total Flavonoids: One hundred milligrams of samples were extracted with 10 ml 80% aqueous methanol. The mixture was centrifuged for 10 min at 2000g. Supernatants were used for subsequent analysis**.** The flavonoid content was measured employing the colorimetric assay described by Zhishen *et al*. [18]. 0.5 ml aliquots of extracts were added to 10 ml volumetric flask containing 4.5 distilled water. 0.3 ml 5% sodium nitrite was added to each aliquot after 5 min, then 0.6 ml of 10% aluminum chloride was added. After 6 min, 2 ml of 1 M sodium hydroxide was added to the mixture following by the addition of 2.1 ml distilled water. Absorbance was recorded at 510 nm and flavonoid content was expressed as mg of rutin equivalent per 100 g of fresh weight.

Anthocyanins: Determination of anthocyanin contents was carried out using the method of Wagner [19]. Samples (0.1 g) were soaked in 10 ml acidified methanol [methanol: HCl 99:1 v/v]. The tissues were crushed and kept at 25°C for 24 h in the dark. The extracts were then centrifuged at 4,000*g* for 5 min at room temperature. The absorption rate of the supernatant was read by spectrophotometer at 550 nm. To calculate the amount of anthocyanins, the extinction coefficient 33,000 mol^{-1} cm^{-1} was used and anthocyanin content were expressed as μ mol g^{-1} fw.

Statistical Analysis: Data of each parameter were subjected to two-way ANOVA. Significant differences between the means of treatments were determined with 95% confidence ($p \leq 0.05$) limit by Duncan multiple range test (DMRT) using SPSS. Data are shown as the means of three replicates. Correlations between the level characters were determined at 95% and 99% (Table 2).

RESULTS

Flowers and Leaves: The results showed significant differences in PAL activity, total flavonoids, total phenolic compounds and anthocyanin contents in leaves and flowers among three cultivars. However, total flavonoid contents of the cultivars were not significantly different in the flowers while they were different among their leaves (Table 1-A,B). PAL activity showed a high trend in Ahmadaghaii leaves and flowers compared to the other cultivars. The highest total phenolic content was observed in flowers of Ahmadaghaii and the lowest amount in Kallehghochi, also total phenolic contents was highest in the leaves of Ahmadaghaii and the lowest observed in Ohadi. The highest total flavonoid content was observed in the leaves of Ahmadaghaii but no significant difference was observed between the total flavonoid contents of the flowers in three pistachio cultivars. Anthocyanin contents were lower in Ahmadaghaii cultivar than the other's leaves and were lower than Ohadi and equal to Kallehghochi in their flowers (Table 1). Correlations were found among the examined parameters. Negative correlation existed between PAL activity and anthocyanin contents of leaves and flowers. There was a high positive correlation (99%) between PAL activity and total phenolics and flavonoids in the leaves (Table 2).

Fruits (Hull, Kernel): The results showed significant differences in PAL activity, total flavonoids, total phenolic compounds and anthocyanin contents in hulls (stage green, green-red and red) and kernels among three cultivars (Table 3).

Table 1: PAL activity, total phenolic, total flavonoid and antocyanin content in flowers (A) and leaves (B) of pistachio cultivars

A: flowers				
Cultivar	PAL activity (unit mg^{-1} protein)	Total phenolic (mg GA g^{-1} fw)	Total flavonoid (mg Ru 100 g^{-1}fw)	Antocyanin content (μ mol g^{-1} fw)
Ohadi	0.57±0.09 ^{c}	9.88±0.02 ^{b}	31.48±1.58 ^{a}	25.16±0.67 ^{a}
Kallehghochi	0.87±0.04 ^{b}	6.42±0.08 ^{c}	36.31±2.48 ^{a}	19.6±0.53 ^{b}
Ahmadaghaii	1.15±0.02 ^{c}	12.44±0.07 ^{a}	38.10±3.06 ^{a}	20.69±0.31 ^{b}
B: leaves				
Cultivar	PAL activity (unit mg^{-1} protein)	Total phenolic (mg GA g^{-1} fw)	Total flavonoid (mg Ru 100 g^{-1} fw)	Antocyanin content (μ mol g^{-1} fw)
Ohadi	2.32±0.04 ^{b}	15.63±0.23 ^{c}	63.16±5.40 ^{c}	103.92±1.19 ^{a}
Kallehghochi	2.36±0.01 ^{b}	17.25±0.23 ^{b}	103±6.91 ^{b}	94.33±1.21 ^{b}
Ahmadaghaii	4.52±0.02 ^{a}	23.41±0.17 ^{a}	184.35±4.54 ^{a}	60.90±0.75 ^{c}

Results are mean±SE (n = 9), different letters in the same column represent significant differences at (p=0.05) according to Duncan test. GA: gallic acid, Ru: rutin, unit: (1PAL unit = 1 μmol cinnamic acid produced per min

Table 2: Correlations between characters

	ANf	ANl	TFl	TPHl	PALf	PALl
Anf	1					
ANl		1				
TFl			1			
TPHl			.965(**)	1		
PALf	-.646				1	
PALl		-.965(**)	.942(**)	.976(**)		1

AN: antochyanin, TF: total flavonoid, TPH: total phenolic, PAL : PAL activity, (f: flower, l: leaf)

** Correlation is significant at 0.01 level

Table 3: Total flavonoid (A) and Antocyanin content (B) in green hull, green-red hull, red-hull and kernel of pistachio cultivars (during the fruit ripening)

A: Total flavonoid (mg Ru 100 g^{-1}fw)				
Cultivar	green	green red hull	red hull	kernel
Ohadi	38.90±0.1 [a]	30.90±0.14 [b]	19.89±0.12 [c]	12.34±0.04 [b]
Kallehghochi	32.87±0.16 [b]	34.97±0.16 [a]	22.50±0.15 [b]	12.98 ±0.01 [b]
Ahmadaghaii	30.33±0.14 [c]	28.30±0.14 [c]	52.40±0.51 [a]	15.24±0.05 [a]
B: Antocyanin content (µ mol g^{-1} fw)				
Cultivar	green	green red hull	red hull	kernel
Ohadi	7.05±0.13 [b]	17.05±0.13 [b]	35.87±0.38 [a]	7.34±0.08 [a]
Kallehghochi	7.27±0.1 [a]	17.72±0.1 [a]	22.23±0.14 [b]	5.44±0.07 [c]
Ahmadaghaii	6.51±0.07 [c]	16.65±0.16 [c]	21.37±0.12 [c]	6.22±0.05 [b]

Results are mean±SE (n = 9), different letters in the same column represent significant differences at (p=0.05) according to Duncan test. Ru: rutin, unit: (1PAL unit = 1 µmol cinnamic acid produced per min)

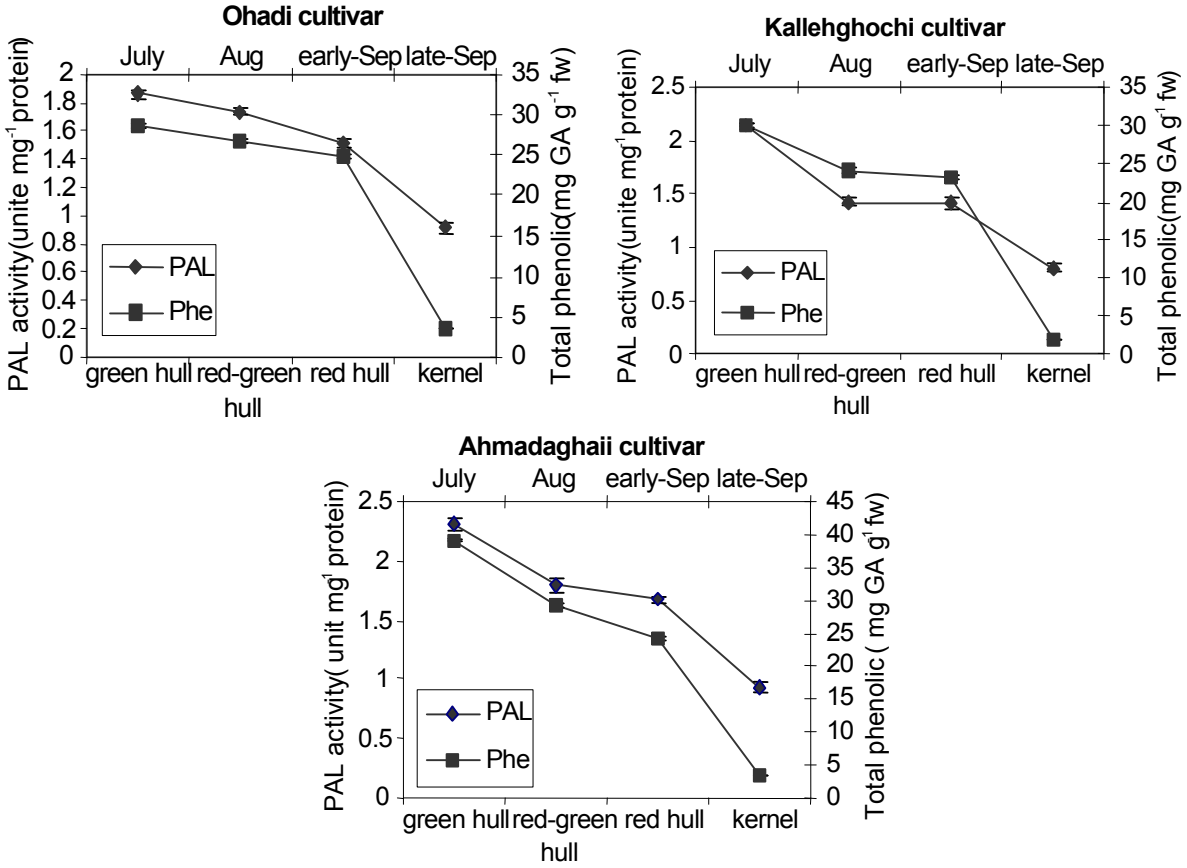

Fig. 1: Changes in total phenolic compounds (■) and PAL activity (◆) in pistachio cultivars during ripening fruits. (GA: gallic acid, unit: 1PAL unit = 1 µmol cinnamic acid produced per min)

Total phenolics and PAL activity of Pistachio fruits from three cultivars studied were different remarkably (Fig 1). The results showed decrease in PAL activity and total phenolics during the fruit ripening in all cultivars and at all parts of the fruits, the highest rate in each stage was observed in Ahmadagaii cultivar. In early stage of fruit ripening, in green hall, total phenolic compounds in all cultivars were high and gradually decrease at the end of ripening. In fruits ripening, in red hull stage the highest anthocyanin content were observed in Ohadi and Ahmadaghaii cultivar detected the highest total flavonoids. The harvest time, kernels showed the lowest rate pal activity and total phenolic compounds. In this stage, total phenolic contents and PAL activity of kernel from three pistachio cultivars were the lowest than the other stages. The highest PAL activity, total phenolics and total flavonoids obtained in kernel of Ahmadaghaii and anthocyanin contents were highest in Ohadi cultivar than the others. Although all cultivars have started accumulating anthocyanin at the first stage, they showed a great increase in anthocyanin content in red hull stage, associated with the ripening process, the process being more obvious mainly in Ohadi cultivar. It was observed that the outer part of Ohadi cultivar fruits is strongly colored.

DISCUSSION

The accumulation of phenolic compounds is a carefully controlled process with both the levels of secondary metabolites and the composition of the phenolic pool varying considerably between organisms, tissues, developmental stages and, in relation to environmental conditions [20]. The PAL activity which is highly sensitive to environmental condition, play a major role in controlling the flux into total phenolics. The main function of phenoilc is maintain the stable concentration of free radical by producting and scavenging them and their physiological function may be shown by regulation of cell redox potential [21].

Hura *et al*. [22] indicated that determination of PAL enzyme activities should be explained during the stress period to obtain a better understanding of how resistant and sensitive varieties differ in their response. In another experiment, Hura *et al*. [23] also reported a correlation between PAL activity and phenolic compounds in leaves of hybrid maize in drought stress and considered the accumulation of phenolic compounds as the indication of activated defense reaction in the drought resistance of that genotype. In our study, positive correlation was observed between the increase of PAL activity and total phenolics in leaves. Increase in PAL activity and the accumulation of high levels of phenolic compounds in Ahmadagaii cultivar in this study can suggest that this cultivar is more resistant to the stresses than the others. Our results also confirm the positive role of phenolic compounds in plant protection again stress condition. It seems the better resistance of pistachio cultivars can be characterized by increase in PAL activity and phenolic level that act as the antioxidants of reactive oxygen species generated under stress influence.

Positive correlation between PAL activity and total flavonoids in this study shows that increase in PAL activity possibly induces the production of flavonoids. In agreement with the present results, Cheng *et al*. [24] showed that the activity of PAL has a direct effect on flavonoid formation and that the long wave light increased the activity of PAL and CHS[1] (the fist enzyme in the flavonoid's biosynthesis) and also enhanced flavonoids accumulation. According to the literature, mutant plants (deficient in CHS) are unable to accumulate flavonoids and were found to be more sensitive to oxidative stresses [25, 26]. It is hypothesized that antioxidant flavonoids have protective function during many stresses and seems that flavonoids accumulation in the leaves and flowers of Ahmadaghaii cultivar could be, more than the others, useful for its resistant to the environmental stresses. Hence, Ahmadaghaii cultivar is considered more resistance than Ohadi and kallehghochi cultivars.

In the present study, negative correlations were observed between PAL activity and anthocyanins in leaves and flowers. However, the contents of both total flavonoids and phenolic compounds were highest in Ahmadaghaii cultivar. It has been indicated that although PAL activity is required for anthocyanin synthesis, it does not necessarily guarantee its synthesis since the cinnamic acid can be diverted into numerous other phenolic or flavonoid compounds [27]. According to Ju *et al*. [28] and Feng *et al*. [29] changes in anthocyanins accumulation can occur independently from changes in PAL activity in apple and pear. Hamouz *et al*. [30] also in agreement to our results showed that the contents of phenolics and anthocyanins were dependent on the plant genotype. It also was observed in this study that different cultivars responded differently in case of the anthocyanin contents. So it can be concluded that PAL activity alone did not regulate anthocyanin synthesis and the other enzyme are involved in their synthesis.

[1]chalcon synthase

Fruits (Hull- Kernel): Regarding to results observed in the study, we have to conclude that the ripening characteristics of the fruits in pistachio cultivars affect both on PAL activity and total phenolic contents (Fig.1). Both suffered a decrease when the maturation process began during pistachio development. Comparative changes in PAL activity and total phenolics of three cultivars show a correlation between the compounds where highest value observed in Ahmadaghaii cultivar. Our results indicated that PAL activity, which is highly sensitive to environmental condition, play a major role in controlling the flux into total phenolics. These data agreed with those of Montero *et al*. [31], who observed that PAL enzyme is involved in biosynthesis of phenolic compounds, which are known to accumulate during early stage of development. During organ growth the phenolic profiles often undergo remarkable changes indicating that their metabolism is integrated into programs of growth and development [32].

Our results indicated that PAL activity and total phenolic compounds present in outer layer of pistachio fruits that highest value was observed in Ahmadaghaii cultivar. High concentration of phenolic compounds in fruits often go parallel with low incident by pathogen [33]. The lowest aflatoxin content of pistachio kernels with hulls compared with hulled kernels is also probably the result of the aflatoxin inhibitory effect of the hulls [34]. There is also evidence that flavonoids, in some plant organs is restricted to epidermal cells, may function in plants to screen harmful radiation [35]. Goli *et al*. [11] also in agreement to our results showed that pistachio hull is a natural source of phenolic compounds and Tomaino *et al*. [13] indicated that these compounds are present higher in skin than in seed. In this study, the outermost layer of the pistachio fruits and nut kernels, the hulls, were showed to contain high level of total flavonoid and phenolic compounds. It seems the hulls of pistachio fruits (especially Ahmadaghaii cultivar) being exposed to the environment may function as a protective layer, thus phenolic compounds tend to accumulation in dermal tissue of plant bodies because of their potential roles of protection against ultraviolet radiation and as defense chemical against pathogens and predators.

Anthocyanin contents in early stages of pistachio fruits development are low and increase during the progress of ripening. The highest rate observed in Ohadi cultivar in the hulls of mature fruits. It seems anthocyanin contents increase in the hulls of mature fruits depend on the variety and the stage of maturity. These findings are consistent with previous studies [36]. It was observed that the outer part of Ohadi cultivar fruits is strongly colored. Although all cultivars have started accumulating anthocyanin at the first stage, mainly in Ohadi cultivar, they showed a great increase in anthocyanin content in red hull stage that associated with the ripening process.

There is no study about the PAL activity in kernel pistachio in the literature. The present results indicate that PAL activity and total phenolics in kernels of pistachio decrease significantly during fruit ripening in harvest time and significant difference between pistachio cultivars exist mainly in Ahmadaghaii cultivar coinciding with higher value on PAL activity. These finding suggested that PAL may be involved in growth and development during seed maturation, also in the final concentration of phenolic compounds and flavonoids and anthocyanins in the kernels. Ballistreri *et al*. [37] reported pistachio kernels contain a remarkable amount of phenolic compounds such anthocyanins and flavonoids. Silva *et al*. [38] determined high correlation between polyphenolic content and the antioxidant activity. The antioxidant activity of pistachio seed was reported by Tomaino *et al*. [13]. Our results either indicated the presence of a number of bioactive compounds in kernel of pistachio, the highest value observed in Ahmadaghaii cultivar.

CONCLUSION

An increase in the activity of PAL can be considered as a biochemical marker for the resistance of the plants to the environmental stresses, given that this enzyme is the key for necessary synthesis of phenolic compounds associated with resistance. Ahmadaghaii cultivar grafted on mutica examined in this research has a high content of PAL activity, phenolics and flavonoids and hence has high antioxidant ability in the flowers, leaves, hulls and kernels. The cultivar can be introduced as a resistant genotype to the environmental stresses and total phenolics and flavonoids and anthocyanins from kernels of this cultivar can play an important role in human health. However, to select the most resistant cultivars to the environmental stresses, further investigations are required to be carried out on the other antioxidants and in many other cultivars grown in pistachio orchards especially when the cultivars grafted on other rootstocks.

ACKNOWLEDGMENTS

The authors acknowledge Shahid Bahonar University for providing research funds and Iran's Pistachio Research Institute (IPRI) for providing the research material.

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