Biochemical Properties of Superior Persian Walnut Genotypes Originated from Southwest of Iran

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Abstract

Evaluation of genetic diversity and identification of superior genotypes is a fundamental step in walnut breeding programs. In addition, information on biochemical properties of superior genotypes can help walnut breeders to release commercial varieties with high kernel quality. To gain more information on superior genotypes, a walnut population located in southwest of Iran was morphologically evaluated from 2010 to 2016. Based on important walnut breeding traits, nine superior walnut genotypes were selected from a total of 612 tested genotypes. These genotypes were characterized by high yield, moderate to late-leafing, lateral bearing, thin shell and large nuts with light and extra-light kernel color. Biochemical traits of the selected superior genotypes were evaluated for two consecutive years (2017 and 2018) and a high variation was observed among genotypes in respect of oil, protein and total phenol contents. Oil, protein and phenol contents of walnut kernels ranged between 57.9 to 69.6%, 13.0 to18.1% and 46.6 to 61.5 mg GAE g^{-1}, respectively. Polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) constituted on average 63.8%, 26.7% and 9.7% of fatty acid content, respectively. There was a negative correlation between some phenological traits and oil and protein contents. Lateral bearing genotypes had darker kernels with higher amounts of saturated fatty acids. In general, the selected walnut genotypes not only are superior in various aspects of phenotypic characteristics, but also have high kernel quality and nutritional value which can be used as a source of desirable genes for future walnut breeding programs.

Keywords: Fatty acid, kernel color, protein, phenol, *Juglans*, germplasm.

Introduction

Persian walnut (*Juglans regia* L.) is one of the domesticated nut crops with valuable kernel being very popular and largely consumed in the world. Among nut crops, walnuts have the highest consumption ranking in middle income economies (29% of total consumption, INC, 2019). Walnut has a beneficial fatty acid profile with high levels of unsaturated fatty acids (UFAs) and a large amount of protein (16-24%), carbohydrates (12-21%), fiber (1.5-2%) (Chatrabnous et al., 2018), melatonin, minerals (1.7-2%), folic acid, potassium, vitamin E (Mao and Hua, 2012), phytosterols (Pereira et al., 2008),

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flavonoids (Martínez et al., 2010), tannins and polyphenols (Li et al., 2006; Khodadadi et al., 2016; Khodadadi et al., 2020). Due to its unique properties and high nutritional value, walnut kernels have been implicated to control heart disease, especially by decreasing the risk of coronary heart disease (Pereira et al., 2008; Ros et al., 2004; Siqueira et al., 2015; Hayes et al., 2016). Walnut proteins have been shown to have anticancer activity by inhibiting the activity of reactive oxygen species (Jahanbani et al., 2016; Jahanbani et al., 2018).

Previous studies have illustrated that depending on the variety, 40-60% of walnut fruit is composed of kernel having a high level of oil content (52-70%) (Labuckas et al., 2008). Linolenic (18:3), oleic (18:1) and linoleic (18:2) acid are the major fatty acids of walnut kernels. Although polyunsaturated fatty acids (PUFAs) are sensitive to oxidation and have a low shelf life, they play an important role in decreasing total and LDL-cholesterol and increasing HDL-cholesterol. Dietary intervention studies have demonstrated that linolenic acid, as a type of omega-3, is able to prevent disorders such as depression, dementia and Alzheimer's disease (Bourre, 2005; Dogan and Akgul, 2005).

Fatty acids and other metabolites content of walnut kernels are largely dependent on the genotype, geolocation, developmental stage and postharvest handling practices (Amaral et al., 2005; Kodad et al., 2016). Pereira et al. (2008) studied the fatty acid profile and bioactive properties of six commercial walnut cultivars in Portugal. They reported that crude protein and total oil content varied between 14.38-16.33% and 68.83-72.14%, respectively. However, different profiles with previous studies in term of oil and protein content have been also reported (Amaral et al., 2003; Pereira et al., 2008), which is probably due to different environmental conditions (Kodad et al., 2016). A study on the biochemical compounds of walnut varieties in Argentina revealed significant differences among varieties for all fatty acid compounds, including palmitic (6.38-8.15%), stearic (0.93-2.16%), oleic (16.1-25.4%), linoleic (52.5-58.9%) and linolenic (11.4-16.5%) acid (Martínez and Maestri, 2008). Ghanbari et al. (2018) reported that oil and protein percentages of kernels of some superior walnut genotypes originating from the northwest of Iran ranged from 43.97 to 63.42% and 6.14 to 13.99%, respectively.

Iran has a long history in walnut production and ranks as the third country, 11.2% of total production, in worldwide walnut production (FAO, 2018). In Iran, walnut has been planted extensively for both nut and timber production, grows in wide naturalized populations, and plays a key role in the country's economy. Iranian walnut populations inhabit areas with widely varying temperature, precipitation, altitude and latitude (Vahdati et al., 2014). Due to the fact that walnuts have been sexually propagated in Iran for many years and the country is one of the main origin centers of walnuts in the world, there is a high genetic diversity in the walnut population of Iran (Vahdati et al., 2019) and it is easy to find superior walnut genotypes with desirable fruit and kernel characteristics. Due to this high genetic diversity, evaluation of this genetic diversity and identification of superior genotypes is the main strategy for achieving commercial varieties (Khorami et al., 2018; Vahdati et al., 2019). Considering the high nutritional value, widely use and emphasis on health care of walnut kernels, this study was conducted with the aim of evaluating the biochemical composition and nutritional value of superior walnut genotypes originating from the southwest of Iran.

Material and Methods

Plant Material

To identify superior walnut genotypes, 612 individual trees were selected based on absence of chilling injury and blight

symptoms from the regions Bavanat (FaBa; 412 genotypes), Eqlid (FaEq; 148 genotypes) and Sepidan (FaSe; 58 genotypes) in the Fars province as one of the main walnut distribution centers in the southwest of Iran (Fig. 1).
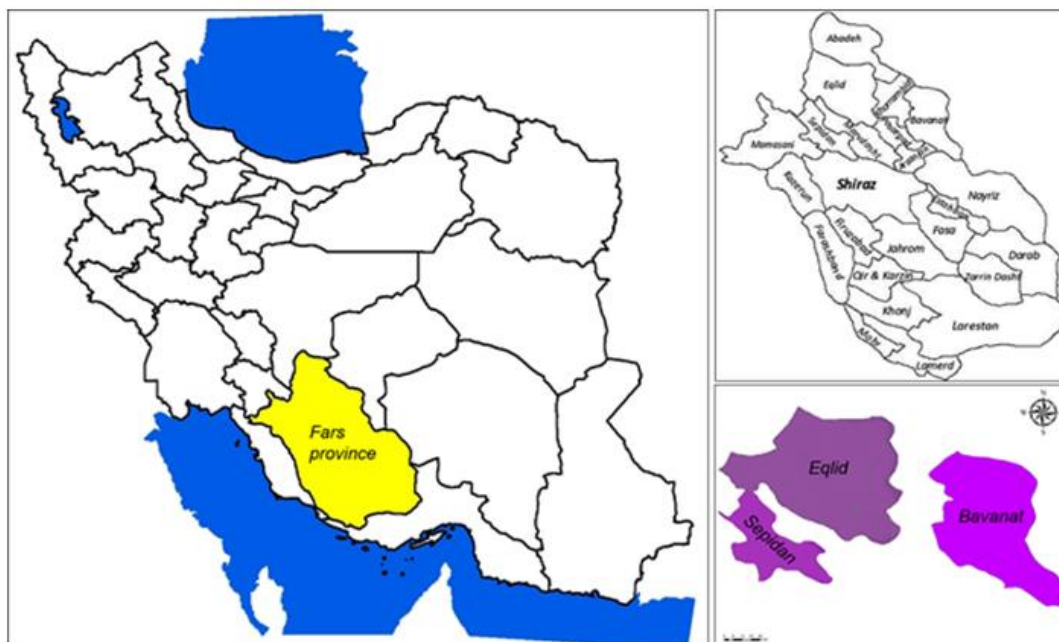

Fig. 1. Geographical location of the areas studied to identify superior walnut genotypes

The selected genotypes were morphologically evaluated at their original site based on IPGRI (International Plant Genetic Resources Institute) descriptors (IPGRI, 1994) for three consecutive years (2011-2013). After primary evaluation, 46 genotypes were selected for further study based on high yield, lateral bearing, heavy and large nut, high kernel percentage, light kernel color. These selected genotypes were morphologically assessed at their original site for four consecutive years (2014-2017) and finally, 9 out of 46 genotypes were selected as superior walnut genotypes. These genotypes were characterized by nut and kernel weight and kernel percentage of more than 13 g, 6.5 g and 49 %, respectively, high yield, lateral bearing, high kernel quality, thin shell, light to extra light kernel color and easy removal in halves.

Phenotypic traits
Phenological traits including budbreak, first and last pollen shedding, first and last pollen receptivity and harvesting date were evaluated by day after reference standards (DARS) with the earliest leafing genotype considered as a reference standard (Arzani et al., 2008). Twenty nuts from each selected genotype were used to assess pomological traits including yield, bearing habit, nut weight and size, kernel weight, percentage and color and shell thickness. After harvesting nuts and removing hulls, nuts (walnut seeds) were kept in the shade at room temperature for one month (Zeneli et al., 2005). Nut and kernel weight was determined by a digital balance. Kernel percentage was calculated by kernel: nut weight ratio. Lateral bearing habit was measured based on the percentage of current season lateral shoots with fruitlet (Akça *et al.,* 2020). Nut size index (NSI) as a parameter of nut size was determined by the average of nut length (NL), width (NW) and thickness (NT) (Khorami et al., 2018). Nut length, width and thickness and shell thickness were measured by a digital caliper. Yield and kernel color were

determined based on IPGRI descriptors (IPGRI 1994).

Biochemical traits

Biochemical traits of the studied superior genotypes were evaluated during two consecutive years (2017-2018). For all biochemical traits, three replications were considered for each superior walnut genotype. For this purpose, nuts were manually shelled, and 20 g of kernel were ground using an electric grinder. Oil was determined from finely chopped kernels by adding 500 mL of hexane solvent and extraction in a Soxhlet apparatus for 5 h. After oil extraction, hexane was removed in a rotary evaporator at 40 °C. The fatty acid profile was determined by gas chromatography (GC, Unicam 4600) with a split/split less injector, flame ionization detector (FID), fused-silica capillary column (BPX70; 0.22μm × 0.25 mm × 30 m) and helium carrier gas. The column and injection port temperatures were 200 °C and 300 °C, respectively. Before injection, the sample was prepared as methyl esters. For this purpose, 1 g of extracted oil was added to 5 mL of NaOH-methanol (2%) and refluxed at 70 °C for 10 min. Subsequently, 2.175 mL of BF_3 (Boron trifluoride) was added to the samples and refluxed at 70 °C for 3 min. After cooling, 1 mL hexane was added and occasionally shaken. During the shaking, 1 mL saturated NaCl solution was added to the mixture. After that 1.2 μL of the supernatant was injected in the GC (Wirasnita et al., 2013).

Protein content of walnut superior genotypes was determined according to AOAC (Association of Official Agricultural Chemists) Official Methods (AOAC 1995). For this purpose, nitrogen content of kernels was evaluated by Kjeldahl analyses. After that, protein content was obtained from multiplying the amount of nitrogen by the factor 6.25. Total phenol content (TPC) was measured with three replications for each superior genotype. For this purpose, 5 g of whole kernel flour was extracted by methanol: water (6:4 v/v) solvent at room temperature in the dark. After extraction and filtration through Whatman paper, the samples were concentrated in a rotary evaporator at 38 °C. Total phenol content was determined by Folin and Ciocalteu's phenol reagent (Labuckas et al., 2008) by adding 100 μL of extracted sample to 500 μL of Folin and Ciocalteu's phenol reagent and 2.5 mL of sodium carbonate. The mixture was adjusted to 10 mL with distilled water and incubated for 1 h at room temperature and the absorbance at 725 nm was measured. A standard curve was made with gallic acid and TPC was expressed as mg of gallic acid equivalents (GAE) per g of extract (mg GAE g^{-1} extract).

Statistical analysis

The biochemical data obtained from the selected superior walnut genotypes were analysed as a completely randomized design (ANOVA) using SAS software (Ver. 9.2). Means comparisons were done using LSD test. Before bivariate correlation analysis, the data was normalized by a specific reference sample. Correlation analysis was performed using the online web tool MetaboAnalyst 3.0 (www.metaboanalyst.ca). Spearman coefficient was used for bivariate correlation analysis.

Results

Phenotypic characteristics of the selected superior walnut genotypes

All superior walnut genotypes were moderate to very late-leafing genotypes. Among the studied superior genotypes, FaBaAg1, FaSeAr7 and FaSeKa8 were very late-leafing (≈ 26-30 DARS) and mid-season ripening genotypes. The results showed that the selected superior genotypes had high to very high yield, light to extra light kernel color and lateral bearing habit (except FaSeAr7 with a lateral bearing of around 50%). Evaluation of walnut superior genotypes showed that nut and kernel

weight and kernel percentage varied between 13.2-18.8 g, 7.2-10.2 g and 48.7-56.5% respectively. The studied superior genotypes had large nuts with average nut size index of 36.7. Shell thickness of superior walnut genotypes was lower than 1.5 mm, which classified them as nut with paper shell (Table 1).

Biochemical traits
The results showed that there was no significant difference between the studied traits in two consecutive years, which was probably due to relatively similar weather conditions during the two years. Based on the obtained biochemical data, a high variation was observed among different walnut genotypes in terms of oil, protein and total phenol content (TPC). Total protein and phenol contents of walnut kernels ranged between 13.0-18.1% and 46.6-61.5 mg GAE g^{-1} extract, respectively. The highest amount of total phenol and protein content were observed in FaBaNs12 genotype. In contrast, FaSeKa8 and FaBaAg1 genotypes had the lowest amount of total phenol and protein content, respectively (Fig. 2).

Table 1. Average phenotypic characteristics of the selected superior walnut genotypes in Southwest of Iran during 2014-2018

| Genotype name	Weight (g)		Kernel percentage	Nut size index	Shell thickness (mm)	Bearing habit	Yield	Kernel color	Leafing date
	Nut	Kernel							
FaBaNs12	18.4 a	8.9 c	48.7 c	38.6 a	1.1 d	Lateral	Very high	Extra light	Moderate
FaBaKr2	17.1 b	8.4 cd	48.8 c	36.3 b	1.4 b	Lateral	Very high	Extra light	Moderate to late
FaBaCh2	14.4 d	8.1 d	56.5 a	34.9 c	1.3 b	Lateral	Very high	Light	Moderate
FaBaAg1	14.2 d	7.9 d	55.5 a	36.7 b	1.2 c	Lateral	Very high	Extra light	Very late
FaBaHm5	15.1 c	8.1 d	53.9 ab	36.3 b	1.2 c	Lateral	High	Extra light	Late
FaEqNs5	13.2 e	7.2 e	54.8 a	35.1 c	1.1 d	Lateral	High	Light	Late
FaEqFm1	18.8 a	9.5 b	50.5 bc	39.1 a	1.5 a	Lateral	Very high	Extra light	Moderate
FaSeAr7	16.4 b	8.4 cd	51.6 bc	37.1 b	1.4 b	Moderate	High	Light	Very late
FaSeKa8	18.4 a	10.2 a	55.2 a	36.3 b	1.5 a	Lateral	Very high	Extra light	Very late

- Means followed by same letter(s) within a column are not significantly different from each other at 0.01 level probability
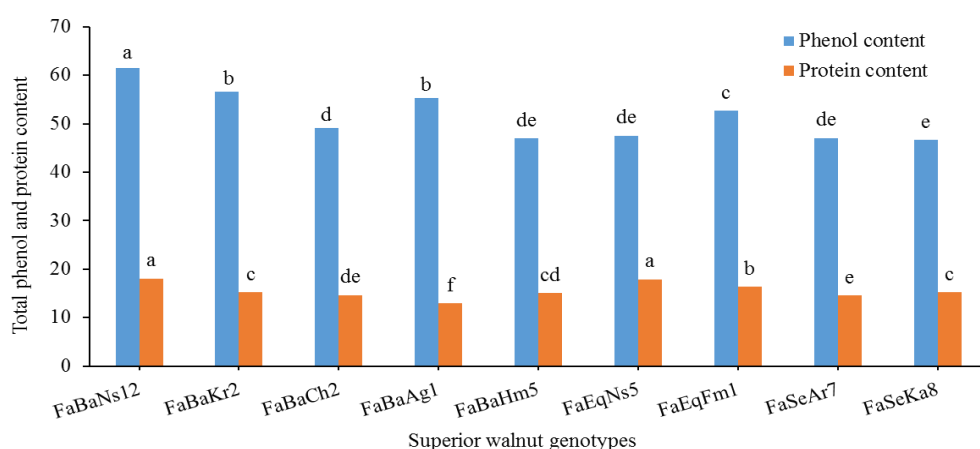
Fig. 2. Total phenol (mg GAES g^{-1}) and protein (%) content of the studied superior walnut genotypes in southwest of Iran

The highest and lowest amounts of oil percentage were observed in FaBaHm5 (57.9%) and FaEqFm1 (69.6%) genotypes, respectively (Fig. 3). Polyunsaturated fatty acids (PUFAs) were the major part of fatty acid content in the studied genotypes with linoleic (C18:2) and linolenic (C18:3) acid constituting more than 55.8% of the fatty acid content. In contrast, saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) varied between 8.7-10.6% and 19.4-37.2%, respectively. The highest amount of PUFA was observed in superior genotypes originated from Eqlid region (FaEqNs5 and FaEqFm1). FaBaNs12, FaBaKr2 and FaSeKaq8 showed the highest amount of saturated fatty acids. In general, PUFA, MUFA and SFA constituted 63.8%, 26.7% and 9.7% of the fatty acid content of superior genotypes, respectively (Fig. 3).
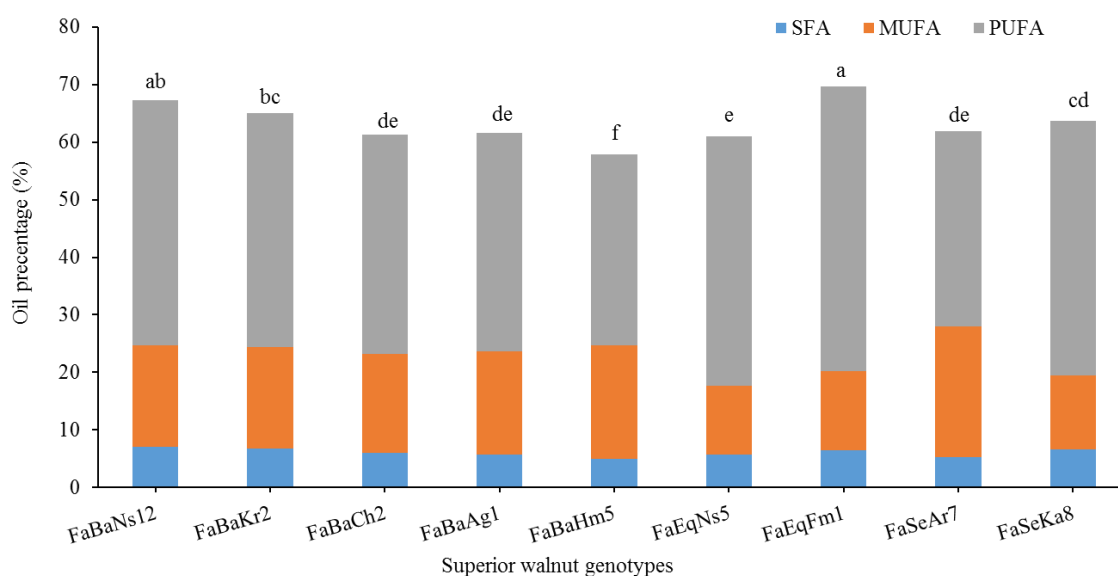
Fig. 3. Oil percentage and fatty acid composition of the studied superior walnut genotypes in Southwest of Iran. Genotypes with same letters did not show significant differences with each other in terms of total oil content.

Oil with a PUFA/SFA ratio of more than 1.5 plays an important role in human health (Bouabdallah et al., 2014). The highest and the lowest levels of PUFA/SFA were 7.7 in FaEqFm1 and 5.9 in FaBaNs12, respectively (Table 2). The fatty acid composition of the studied genotypes is given in Table 2. Palmitic (C16:0), stearic (C18:0), oleic (18:1), linoleic (C18:2) and linolenic (C18:3) acids were the major fatty acids in the studied genotypes. A small amount of arachidonic acid (C20:4) (lower than 0.15%) was observed in FaBaHm5, FaSeAr7 and FaSeKa8. Except for FaBaNs12 and FaBaAg1, small amounts of erucic acid were detected in all superior walnut genotypes. With 44.8 to 59.1%, linoleic acid as a polyunsaturated omega-6 fatty acid was the major fatty acid in all the studied genotypes with the highest amount in FaEqNs5. Palmitic (5.9-7.5%) and stearic (2.4-3.5%) acids were detected as saturated fatty acid in the studied genotypes. The highest and the lowest amounts of palmitic acid were found in FaBaKr2 and FaBaAg1, respectively. Stearic acid was relatively high in FaBaNs12 and low in FaBaHm5. In addition to erucic acid, oleic acid was another monounsaturated fatty acid that was detected in the studied genotypes and varied from 19.3% in FaEqNs5 to 37.1% in FaSeAr7. Linolenic (C18:3) acid as one of the essential fatty acids for human health was ranged from 8.6 to 16.9% (Table 2).

Table 2. Average fatty acid composition (%) and PUFA: SFA ratio of the walnuts from studied superior walnut genotypes in Southwest of Iran during 2017-2018

Genotype	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Arachidonic acid	Erucic acid	PUFA:SFA ratio
FaBaNs12	7.1 b	3.5 a	26.1 f	51.6 d	11.7 de	0.0 c	0.0 d	5.9 d
FaBaKr2	7.5 a	2.9 e	27.0 e	45.5 g	16.9 a	0.0 c	0.2 a	6.0 d
FaBaCh2	6.6 d	3.3 c	27.9 d	53.5 c	8.6 f	0.0 c	0.2 a	6.3 c
FaBaAg1	5.9 f	3.4 b	28.9 c	49.6 e	12.1 d	0.0 c	0.0 d	6.6 b
FaBaHm5	6.3 e	2.4 h	33.8 b	48.3 f	8.9 f	0.1 b	0.1 c	6.6 b
FaEqNs5	6.9 c	2.6 f	19.3 i	59.1 a	12.0 d	0.0 c	0.1 c	7.5 a
FaEqFm1	6.1 e	3.1 d	19.6 h	57.8 b	13.2 c	0.0 c	0.1 c	7.7 a
FaSeAr7	6.3 e	2.5 g	37.1 a	44.8 g	10.9 e	0.1 a	0.1 c	6.4 c
FaSeKa8	7.1 b	3.4 b	19.9 g	53.9 c	15.6 b	0.1 b	0.1 b	6.6 b

- Means followed by same letter(s) within a column are not significantly different from each other at 0.01 level probability

Correlation analysis

One of the objectives of this study was to investigate the correlation between biochemical traits and some important morphological traits. Correlation analysis revealed a negative correlation between oleic acid and linoleic, linolenic and palmitic acid. Total MUFA had a negative correlation with total SFA and PUFA. In contrast, a positive correlation was found between total PUFA and SFA. The results showed a negative correlation between phenological traits including flowering time, harvest date and budbreak date with oil and protein content. The higher levels of oil and protein contents were observed in early leafing and early flowering genotypes. In addition, a negative correlation was found between palmitic acid and total SFA with several phenological traits. In contrast, palmitic acid and saturated fatty acids had a positive correlation with lateral bearing. Based on our results, lateral bearing genotypes had darker kernels with higher amounts of saturated fatty acids. In addition, a negative correlation was observed between lateral bearing and several phenological parameters (Fig 4).
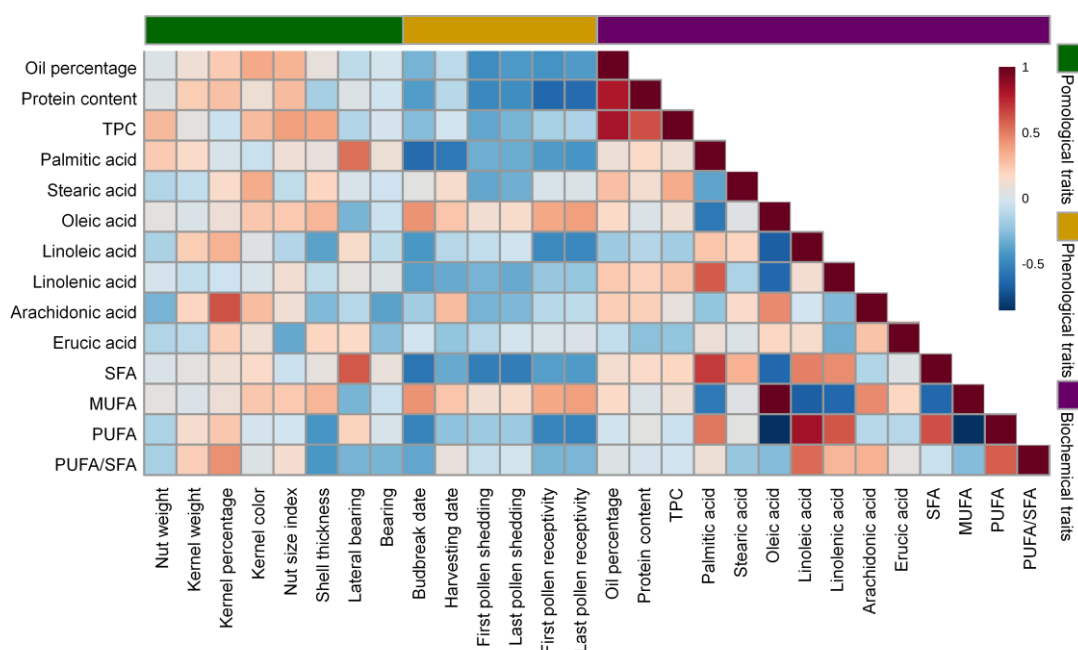
Fig. 4. Spearmen correlation analysis between biochemical traits and phenotypic traits of the studied walnut superior genotypes in the Southwest of Iran

Discussion

Due to protein content as well as high levels of unsaturated fatty acids (UFAs), walnut is considered as a strategic product for human health and nutrition in the FAO list of priority crops (Gandev, 2007). There are large populations of Persian walnut in Iran (Khadivi-Khub et al., 2015; Sarikhani Khorami et al., 2012). This huge and invaluable genetic resource of walnut can be considered as an international capital for walnut breeding programs. In recent years, global warming has severely affected several phenological traits of trees (Korner and Basler, 2010). Early budbreak and flowering as a result of global warming increase the risk of spring frost damage, while late leafing and flowering increase the chance to escape from late-spring frost (Hassankhah et al., 2017). Superior walnut genotypes selected during this study were moderate to very late-leafing genotypes that can be considered as promising and valuable genotypes in future breeding programs. These genotypes are also superior in terms of other important breeding traits such as yield, lateral bearing, nut and kernel weight, shell thickness and kernel color. In all these aspects, the selected genotypes were superior to those identified in previous studies (Aslantas, 2006; Arzani et al., 2008; Simsek et al., 2010; Ebrahimi et al., 2015)

Due to high levels of PUFAs (omega-6 and omega-3), walnut is considered as an essential diet for human health (Pereira et al., 2008).

In addition, a PUFA/SFA ratio of more than 1.5 is directly related to beneficial effects on human health (Bouabdallah et al., 2014). Our results demonstrated that PUFA/SFA ratio of the studied genotypes varied between 5.65 and 7.75. PUFA, MUFA and SFA constituted 63.8%, 26.7% and 9.7% of the fatty acid content of the studied walnut superior genotypes which is consistent with previous studies in Portugal (Pereira et al., 2008), Tunisia (Bouabdallah

et al., 2014), Iran (Akbari et al., 2014) and Serbia (Rabrenovic et al., 2011). The biochemical traits were significantly different in the studied genotypes, which are in line with the results of Pereira et al. (2008) and Akbari et al. (2014) that also found significant differences for the studied biochemical traits. Previous studies reported the effect of environmental conditions on the biochemical composition such as oil content and fatty acids (Amaral et al., 2005; Roozban et al., 2005). In our study, no significant differences were observed between oil and protein content in the two years of the experiment, but the climate data during the fruit developmental stage also indicated similar environmental conditions in two years. Nevertheless, walnut genotypes originating from different regions showed different biochemical properties. In the other words, environmental condition seems to have significant effects on biochemical properties (Amaral et al., 2005).

The fatty acid profile determines storage life, taste, flavor and rancidity of walnut oil (Zwarts et al., 1999). Oleic acid as a main MUFA in walnut kernel has a positive relation with storage life. In the other words, storage life of walnut oil increases with increasing oleic acid concentration. Our selected superior genotypes high levels of oleic acid (Yılmaz and Akça, 2017). Arachidonic acid (C20:4) as an omega-6 fatty acid with four double bonds is not an essential fatty acid for humans. It however has a significant effect on brain performance and can be found in large amounts in the brain. High levels of arachidonic acid are associated with the onset of Alzheimer's disease (Amtul et al., 2012). A small amount of arachidonic acid was detected in FaBaHm5, FaSeAr7 and FaSeKa8. Erucic acid was detected in small amounts in most tested genotypes. Erucic acid is a major component of rapeseed oil. Zhang et al. (1991) reported that the high amount of erucic acid in rapeseed oil increases weight of livers in

rats and decreases hepatic oxidation capacity. However, a small amount of erucic acid as an omega-6 fatty acid can have beneficial effects for health (Zhang et al., 1991).

Total phenol content (TPC) of the kernels of the superior walnut genotypes varied between 46.6 to 61.5 mg GAES g^{-1} extract, which was higher than what was observed in Argentinian genotypes (Labuckas et al., 2008). Protein content ranged between 13.0-18.1% which is in a similar range as in previous studies on genotypes from Turkey and Portugal (Ozcan, 2009; Periera et al., 2008). Recent studies found that walnut seeds have different groups of monomeric and polyphenoilcs compound having strong antioxidant activity (Zhang et al., 2009). Higher levels of phenolic and protein content of walnut kernel directly related to human health (Labuckas et al., 2008; Zhang et al., 2009).

Studying correlations between traits helps breeders to facilitate breeding programs since correlation studies can represent linkage between related genes or multi gene effects (Amiri et al., 2010; Sarikhani Khorami et al., 2014). So far, correlation between phenotypic and biochemical data have not been studied in walnut. Our results illustrated a negative correlation between phenological data (budbreak and harvest date, pollen shedding and receptivity date) and oil and protein content. The higher amounts of oil and protein content were observed in early leafing and flowering genotypes. Lateral bearing genotypes had darker kernels with higher amounts of saturated fatty acids.

Conclusion
In conclusion, we observed a high phenotypic and biochemical variation between superior walnut genotypes from the Southwest of Iran. Evaluation of biochemical characteristics as selection markers can complete phenotypic data to study genetic variation in walnut populations. In addition, knowledge of biochemical characteristics such as oil, protein, fatty acid, and phenolic compounds are very important to introduce a superior genotype with high kernel quality. Based on the results, the selected genotypes were superior in various aspects of phenotypic characteristics as well as kernel quality and had high levels of nutritional value. Therefore, these selected superior genotypes not only have the potential to be introduced as commercial cultivars in the near future, but also can be used as a source of desirable genes for future walnut breeding programs.

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