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MOROCCAN WALNUT ACCESSIONS RESEARCH USING MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR ANALYSES

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ABSTRACT

The application of different methods to assess genetic diversity is crucial for plant breeding and conservation programs. In this study, the morphological traits, the biochemical parameters and 11 ISSR molecular marker were used to investigate 11 Moroccan walnut accessions. The results of the morphological study revealed an important variation of the analyzed traits. The largest variation revealed in the position of the pad on the suture (CV=45.02%), whereas for leaflet margin no variation was found. The matrix of the Euclidean distance coefficient exhibited a large morphological variation between accessions, ranging from 3.1 to 12.1. In addition, the Mantel test showed no correlation between morphological and geographical distance of the accessions (r=0.159, p=0.84). Regarding the biochemical results, the parameters revealed a large variation. The coefficient of variation of energy value was the lowest (3.68%) while that of total flavonoids was the highest (62.02%). For the molecular analysis, the results indicated a high percentage of polymorphism (89%) as well as efficiency of the primers UBC836 and UBC841 in the research of the genetic diversity of this species. The principal component analysis divided the 66 trees into three groups independently of geographic origin and mountain range type. In addition, the altitude provided a weak effect on the structuring of the accessions (1.8%). Finally, the mantel test showed no significant correlation between the molecular and morphological markers (r= 0.591; P value= 0.998), molecular and biochemical markers (r=0.38; P=0.475), and morphological and biochemical markers (r=0.602, P=0.784). The results of this work can be useful for the conservation and improvement program of walnut in Morocco.

1.Introduction

The conservation and development of plant genetic resources are crucial for increasing agricultural productivity and sustainability (FAO, 1997). Genetic diversity is the fundamental basis for durability since it constitutes the raw material for the adaptation, evolution and survival of species and individuals, especially under modified environmental, social and pathological conditions (Hammer, 2003). The evaluation of genetic diversity has been greatly facilitated by the availability of a number of marker systems (Beyene *et al*., 2005). The morphological and biochemical markers are widely used to assess the genetic diversity, even if they are influenced by environmental factors (Zhang *et al*., 2011). In addition, DNA markers (RAPD, RFLP, AFLP, SSR, ISSR, SNP, ...) are also applied and were considered the efficient systems for genetic diversity analysis (Zhang *et al*., 2011). The choice of technic depends on the objective of the study, financial constraints, skills and available resources (Beyene *et al*., 2005).

Walnut (*Juglans regia* L.) belongs to the family *Juglandacea*e. Its natural origin is extended from the Carpathian Mountains of Eastern Europe to the Southern Caucasus, northern Turkey, Iran, Tian Shan province of western China, Himalayan states of India, Sikkim, and Bhutan (Angmo *et al*., 2013). In 2017, the global walnut production reached 3.829.626 tons for an area of 1.18 million hectares. The main nut-producing countries are China which is the first producer with 51.15% of the world production on an area of 487.007 ha. The United States of America occupies the second place with 15.18%, followed by Iran (9.27%) and Turkey (5.57%) (FAOSTAT, 2018). The walnut kernel consists of about 60% of lipids and represents a good source of macronutrients, micronutrients and other bioactive (Souci *et al*., 2008; Bolling *et al*., 2010; USDA, 2018; Yerlikaya *et al*., 2012). Indeed, previous studies revealed its richness in phosphorus, potassium, magnesium, iron, zinc, sodium, calcium and natural antioxidants such as polyphenols, folates, tannins (Li *et al*., 2006, Cosmulescu, *et al*., 2009; Tapia *et al*., 2013). As a result of this composition, the walnut has beneficial effects on human health, leading to an increasing demand in the market (Carvalho *et al*, 2010).

In this context, studying the genetic diversity and biochemical composition of the walnut provides useful information for genetic

resource management and the development of new high-yielding cultivars better adapted to drought conditions (Shamasbi *et al*., 2018). The objectives of this study are the evaluation the biochemical composition and genetic diversity of 11 walnut accessions as well as their structuration and relationships. Finally, the evaluation of the level of correlation between the morphological and molecular distance.

2. Material and methods

2.1. Plant Material

The plant material used in this study collected in September 2014 from 11 Moroccan accessions covering the main walnut-growing area. Table 1 and Figure 1 present the accessions studied with their geographical origins and ecological factors. In fact, developed leaves and healthy nuts were collected randomly from different sides and at different elevation of tree.

2.2. Morphological analysis

Based on the instructions provided by the IPGRI and UPOV descriptors (IPGRI, 1994; UPOV, 1999), a total of 31 leaf and fruit traits were considered (Table 2). The measurements were carried out on ten fresh leaves and 20 nuts after one month of harvest when the moisture content was less than 8%. (UPOV, 1999).

2.3. Biochemical analysis

The following chemical parameters are performed on walnut kernel from three trees of each accession:

The moisture was calculated on the basis of dry weight and fresh weight (Mikdat, 2010). The Ash was determined by incineration of 5 g of kernels at 600 °C for 240 minutes using a muffle furnace (AOAC, 1995). Total oil was extracted by Soxhlet apparatus using 5 g of ground kernels with N-hexane at 55-60 °C for 8 hours (AOCS, 1998). The protein content was obtained by Kjeldahl method (AOAC, 1995). The carbohydrates content and the energy value were estimated applying the following formulas: Carbohydrate content (%) = 100 % - (moisture (%) + protein (%) + oil (%) + ash (%) (Grosso *et al*., 2000) and Energy kcal = 4 x (protein g + carbohydrate g) + 9 x (lipid g) (Pereira *et al*., 2008) respectively. The crude fiber was determined with 5 g of ground samples which were digested in H_2SO_4 (1.25 %) for 45 min. Then the mixture was filtered and washed with hot distilled water before a second digestion in 100 ml of 1.25 % NaOH solution for 60 min. The resulting product was filtered and washed with hot deionized water, followed by over drying and measurement. The residue is incinerated in a furnace at 550 °C for 3 hours. Weight loss represents the amount of crude fiber. (Aryapak and Ziarati, 2014).

In order to determine the phenolic and flavonoids content as well as the scavenging activity of walnut, 5 g of ground kernel were macerated for 48 hours using 50 ml of 80 % methanol (Jacki *et al*., 2011). The Phenolic compounds was estimated according to Singleton and Rossi (1965) method. Briefly, 1 ml of extracts was mixed with 1 ml of Folin and Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture and adjusted to 10 ml with distilled water. The absorbance measured at 725 nm after 90 minutes in the dark and room temperature. The results are expressed as mg of gallic acid equivalents per 100 g of DM. The flavonoids content quantified using a modified colorimetric method of Yang (2009). Briefly, 1:10 diluted extracts were mixed with distilled water and then with 0.07 ml of sodium nitrite solution (5%). Afterwards, 0.15 ml of aluminum chloride (10%) was added and allowed to react for another 6 minutes before the addition of 0.5 ml of one molar sodium hydroxide. Finally, distilled water was added to all samples in 1 ml portions. The absorbance was immediately measured at 510 nm. The results are expressed as mg rutin equivalents per 100g of DM

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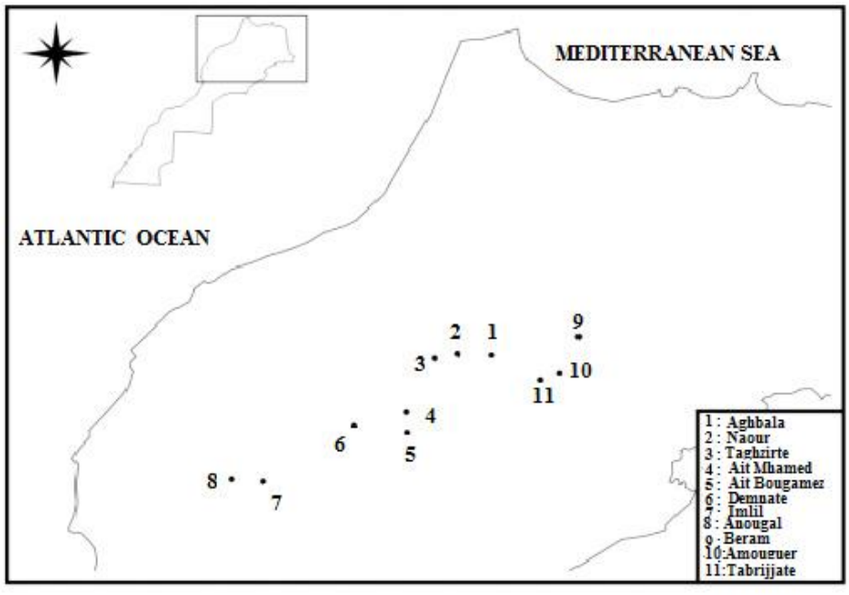

Figure 1. Geographical location of the 11 accessions studied

Table 1. Geographic and ecological parameters of walnut accessions studied

Accessions	Code	Geographic origin	Altitude (m)	Latitude N	Longitude W	Zone	Rainfall average (mm)
Aghbala	AGH	32 Km North east of Aghbala	1673	32°32’	5°39’	Middle Atlas	450
Naour	NAO	Central Naour	1300	32°29’	5°58’	Middle Atlas	600
Taghzirte	TAG	12 Km East of Tagzirte	650	32 26	6° 12’	Middle Atlas	700
Ait Bougamez	ABZ	Ait Bougamez Centre	1996	31°38’	6° 28’	High Atlas	580
Ait Mhamed	AMD	20 Km South east of Azilal	1728	31° 25’	2° 28’	High Atlas	450
Demnate	DEM	3 km South east of Demnate	932	31° 43’	6° 58’	High Atlas	350
Imlil	IML	17 km South of Asni	1763	31° 8’	7° 55’	High Atlas	459
Anougal	ANG	40 km South of Amzmiz	1569	31° 9’	8° 15’	High Atlas	681
Beram	BER	5 km South of Midelt	1521	32° 40’	4° 44’	High Atlas	210
Amouguer	AMG	40 km West of Rich	1569	32° 12’	5° 8’	High Atlas	250
Tabrijjate	TBR	70 km East of Imilchil	1831	32° 16’	4° 56’	High Atlas	319

Table 2. Morphological traits analyzed in Moroccan walnut accessions.

Leaf	Width of Pad on Suture
Leaf Length	Prominence of Pad on Suture
Leaf Width	Shape of Base Perpendicular to Suture
Number of Leaflet	Shape of Apex Perpendicular to Suture
Leaflet Shape	Prominence of Apical Tip
Leaflet Width	Structure of Surface of Shell
Leaflet Length	Shell Color
Leaflet Margin	Shell Strength
Leaf Color	Adherence of Two Halves of Shell
Rachis Color	Thickness of Shell
Nut	**Kernel**
Nut Shape in Longitudinal section through Suture	Difficulty of Removal of Kernel
The nut Shape in Longitudinal section Perpendicular to Suture	Kernel Weight
Nut Width	Kernel Percentage*
Nut Length	Kernel Fill
Nut Weight	Kernel Color
Nut: Position of Pad on Suture	Kernel Flavor

*Kernel percentage = Kernel weigh/nut weight ∗100

Regarding the scavenging activity of kernel extracts, using the free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH), it was monitored according to a method reported by Hatano *et al.* (1988). The amount of 0.3 ml of sample extracts were mixed with 2.7 ml of methanolic solution of DPPH (6.10^{-5}mol.l^{-1}). The mixture incubated in the dark for 30 min, before the absorbance measurement at 517 nm. The radical scavenging activity was calculated by the following formula.

$$\text{Scavenging (\%)} = \frac{Ab_{517\ control} - Ab_{517\ sample}}{Ab_{517\ control\ nm}} \times 100 \qquad (1)$$

Where:
$Ab_{517\ control}$: control absorbance.
$Ab_{517sample}$: control sample absorbance.

The results were expressed as µmol Trolox equivalent. g^{-1} DW.

2.4. Molecular analysis

For each accession, the young leaves belonging to six trees randomly selected, were collected. The extraction of DNA was carried out using the method described by Doyle and Doyle (1990). Then, a concentration of 10 ng/µl was prepared for PCR amplification. Indeed, 66 PCR amplification of the DNA were carried out using 11 ISSR primers, which are previously tested (Christopoulos *et al.*, 2010; Aiqing *et al.*, 2014; Shamasbi *et al.*, 2018) (Table 5). The final volume of the reaction was 12.5µl, contained: 15 ng of DNA template, 1x reaction buffer, 1 mM of $Mgcl_2$, 0.8 mM of dNTPs, 0.8 µM of each primer and 0.75 U of My Taq™ DNA polymerase. PCRs

were conducted in a Multigene gradient thermocycler (Labnet, NJ. USA). The DNA amplification is performed according to the following program: a pre-denaturation at 94 °C for 5 min, followed by 45 cycles of amplification. Each cycle includes a denaturation step at 94 °C for 45 s, a hybridization step for 45 s and an elongation step at 72 °C for 2 min. A final elongation at 72 °C for 7 min is programmed. The amplification products are separated by electrophoresis on 0.7 % agarose gel submerged in 0.5 x TBE buffer and then stained with 1 µg/µl of ethidium bromide. The DNAs were visualized under UV light using the Gel Doc system (EnduroTM GDS, Labnet). A 1 Kb DNA HyperLader™-Bioline was used for molecular weight estimation of PCR product.

2.5. Statistical analysis

Biochemical data. Average value, standard deviation, minima, maxima and coefficients of variation were determined for the studied parameters. The coefficient of variation (CV) was calculated using the following formula: CV (%) $=\frac{SD}{\bar{X}} \times 100$.

Morphological data. Average value, standard deviation and coefficients of variation were calculated for the analyzed traits. Moreover, the morphological divergence among the 11 accessions determined by the STATISTICA (Statistica StatSoft, 2011) using the UPGMA method. A Mantel test was used to research any correlation between the morphological and the geographic distance of the accession using Mx Comp of NTSys-pc software verion 2.02g.

Molecular data. The ISSR binary matrix obtained was used to carried out the principal component analysis (PCA) with the XLSTAT, (2014). The analysis of molecular variance (AMOVA) was applied to estimate the amount of difference between three altitude groups of accessions: Very low (Demnate and Taghzirte accessions), low (Naour and Anougal accessions) and moderate (Aghbala, Imlil, Ait Mhamed, Ait Bougamez, Amouguer and Beram accessions). Finally, the correlation between the Euclidean distance matrix based on morphology and the F_{ST} pairwise distance matrices obtained with ISSR markers were analyzed using the approach developed by Mantel (1967). The data analyses were performed using Mx Comp of NTSys-pc software version 2.02g.

3. Results and discussion

3.1. Variation of morphological traits

The description and coefficient of variation values related to leaf, nut and kernel trait were summarized in Table 3. The majority of examined traits showed an important value of coefficient of variation, indicting a high level of morphological variation. Indeed, the coefficient of variation varied from 45.02 % to 00.00%. In general, the large variation in leaf characters was registered in leaflets shape (CV=24.59 %), while the leaflets margins reveled with CV=00.00 % meaning no variation between accessions for this character. Concerning the leaf length and leaf width of Moroccan walnut tree, they have shown a CV of 12.29 % and 14.24 % respectively. Regarding the traits related to the nut, the highest variation was observed for the position of pad on suture, with a CV value of 45.02 %, followed by Shell Color (CV=40.50 %). However, the width, length and weight of nut are characterized by a moderate variation with a CV of 9.68 %, 10.28 %, and 26,27 % respectively. In addition, the kernel weight indicted an important variation with a CV value of 36.63 %, followed by the kernel color (CV=27.41 %), while the kernel percentage recorded a CV of 25.97%.

Morphological divergence of the 11 accessions was determined by calculating a morphological distance matrix (Table 4). The Euclidean distance coefficient matrix showed a large morphological divergence among accessions ranging from 3.1-12.1. Indeed, the Demnate and Amouguer accessions, separated by a geographical distance of 204 km, registered a coefficient of 12.1, which means that these accessions are the most divergent. Whereas, the Ait Mhamed and Anougal accessions were found to be less divergent (3.1), even though a geographic separation of 186 km. This finding is in agreement with the Mantel test, which indicated no correlation between morphological and geographical distance (r=0.159, p=0.84) for the accessions studied.

3.2. Variation of biochemical parameters

Among all biochemical parameters (Table 5), the maximum values of moisture, Ash, oil, protein, carbohydrates, energetic value, crude fiber, total phenols, total flavonoids and scavenging activity were 4 %, 2.7 %, 70.58 %, 22.75 %, 32.27 %, 734.06 Kcal, 7.52 %, 52 mg GAE 100 g^{-1}, 84.17 mg RE 100 g^{-1} and 87.3 %, while the minimum values were 0.2 %, 1.12 %, 53.72 %, 5.25 %, 641.9 Kcal, 3.32 %, 6.17 mg GAE 100 g^{-1}, 2.15 mg RE 100 g^{-1} and 72.23 %, respectively. Based on the values obtained, the oil has the abundant component. Concerning the coefficients of variation of the same parameters, they were 37.64 %, 17.31 %, 7.3 %, 25.59 %, 30.5 %, 3.68 %, 19.78 %, 56.02 %, 62.02 % and 5.48 %, respectively. The coefficient of variation of energetic value was the smallest (3.68 %) and the coefficient of variation of total flavonoids was the largest (62.02 %).

3.3. Molecular analysis

The electrophoretic gels of 66 trees using 11 ISSR primers, exhibited a high

polymorphism level in Moroccan walnut. The ISSR primers generated 135 bands, including 123 polymorphic and 12 monomorphic. These data have allowed to record a high percentage of polymorphism of 89% (Table 6). Although the results confirmed the ability of these primers to reveal the molecular polymorphism of Moroccan walnut accessions, no primers produced a specific band, thus no specific markers were revealed in the ISSR assay.

Table 3. Descriptive statistics of morphological characters analyzed

Character	Description	CV (%)
Leaf		
Leaf Length (cm)	41.47±5.10	12.29
Leaf Width (cm)	25.95±3.70	14.24
Leaflet Width (cm)	4.9±0.79	16.13
Leaflet Length(cm)	10.57±5.101.63	15.45
Leaflets color	Green	18.12
Leaflets shape	Broad elliptic	24.59
Leaflets margins	Entire	0
Rachis colour	Green	18.12
Number of Leaflet	8±1	12.3
Nut		
Nut Width (mm)	30.24±2.93	9.68
Nut Length (mm)	35.69±3.67	10.28
Nut Weight (g)	9.47±2.49	26.27
Thickness of Shell (mm)	1.6±0.42	26.33
Nut Shape in Longitudinal section through Suture	Broad elliptic	33.11
The nut Shape in Longitudinal section Perpendicular to Suture	Broad elliptic	30.43
Nut: Position of Pad on Suture	On upper 2/3 of nut	45.02
Width of Pad on Suture	Medium	26.35
Prominence of Pad on Suture	Medium	21.41
Shape of Base Perpendicular to Suture	Rounded	37.91
Shape of Apex Perpendicular to Suture	Truncate	28.36
Prominence of Apical Tip	Weak	27.87
Structure of Surface of Shell	Moderately grooved	27.67
Shell Color	Medium	40.5
Shell Strength	Intermediate	21.55
Adherence of Two Halves of Shell	Medium	23.01
Kernel		
Kernel Weight (g)	3.7±1.36	36.63
Kernel Percentage (%)	38.29±9.95	25.97
Difficulty of Removal of Kernel	Medium	26.56
Kernel Fill	Well	24.08
Kernel Color	Light amber	27.41
Kernel Flavor	Satisfactory	27.09

CV: Coefficient of variation

Table 4. Morphological distance among the accessions

	Tagzirte	**Tbrijjate**	**Amouguer**	**Anougal**	**Ait Mhamed**	**Imlil**	**Beram**	**Aghbala**	**Naour**	**Ait bougamez**	**Demnate**
Tagzirte	0										
Tbrijjate	6.2	0									
Amouguer	6.3	7.41	0								
Anougal	8.11	4.9	10.4	0							
Ait Mhamed	6.6	3.84	9.2	**3.1**	0						
Imlil	8.42	5.64	7.3	6.9	5.82	0					
Beram	5.89	3.73	6.4	5	4.28	5.4	0				
Aghbala	7.33	8.18	10.9	7.6	7.4	11.2	7.95	0			
Naour	5.59	5.51	7.2	7.3	5.52	7.7	5.53	6.2	0		
Ait bougamez	5.48	5.85	3.9	8.5	6.98	5.9	5.38	9.3	5.88	0	
Demnate	8.98	8.32	**12.1**	6.6	7.4	11.1	6.95	8	9.3	11.2	0

Table 5. Minima, maxima, average value, standard deviation and coefficients of variation of biochemical parameters

Parameters	Min	Max	Mean	SD	CV (%)
Moisture (%)	0.2	4	2.52	0.95	37.64
Ash (%)	1.12	2.7	2.08	0.36	17.31
Oil (%)	53.72	70.58	60.86	4.45	7.3
Protein (%)	5.25	22.75	15.59	3.99	25.59
Carbohydrates (%)	5.36	32.27	18.92	5.77	30.5
Energetic value (Kcal)	641.9	734.06	685.85	25.24	3.68
Crude fiber (%)	3.32	7.52	5.51	1.09	19.78
Total phenols (mg GAE 100 g^{-1})	6.17	52	25.79	14.58	56.02
Total flavonoids (mg RE 100 g^{-1})	2.15	84.17	37.18	23.08	62.09
DPPH (%)	72.23	87.3	79.73	4.37	5.48

Min: Minima, Max: Maxima, SD: Standard deviation, CV: coefficient of variation

Furthermore, the PCA was performed to determine the main primers contributing to the classification of walnut accessions. The first two components explained 15.78% of the observed variation (Table 7). The PC1, with an Eigen value of 9.76, contributed 8.07% of the total variability, while PC2, with an Eigen value of 9.08, accounted for 7.51% of the total variability. In PC1, primers USB811, USB834, USB836, USB841, and UBC889 with the eigenvalues of 24.55, 20.05, 18.79, 10.94, and 17.15, respectively, were the most efficient. Whereas the main primers contributing to PC2 were UBC818, UBC836, UBC841 and UBC889 with eigenvalues of 18.48, 29.85, 16.47 and 10.35, respectively. This study indicates that primers UBC836 and UBC841 may be the most suitable for investigating molecular variation in this species in future studies, while the USB855 with low contribution is the least informative. In addition, the PCA biplot divided the 66 trees into three groups. The first one formed by one accession (DEM2) from the Haut Atlas and the second group composed specifically by trees belonging to ANG, TBR, IML and BER from Haut Atlas Mountain as well as some trees from ABZ, AMG, DEM and AGH. The last group comprised by all trees of NAO and TAG belong to Middle Atlas Mountain, which were added to the trees from ABZ, AGH, AMG and DEM (Figure 2). According to the results obtained, the 66 trees are structured independently of their origin geographic and mountain type.

For hierarchical AMOVA, it was applied to examine the structuration of 66 trees according to altitude level (Table 8). The result showed a low percentage of genetic variation among the altitude groups (1.8 %). Therefore, altitude has no significant effect on the structuration of these accessions.

Table 6. Properties of 11 ISSR primers used in this study.

ISSR Loci	Sequence (5'-3')	Simple size	Number of bands amplified				Polymorphism (%)	Monomorphism (%)
			Total band	Polymorphic band	Monomorphic band	Unique loci		
UBC 807	(AG) 8T	66	9	9	0	0	100	0
UBC 810	(GA)8T	66	10	7	3	0	70	30
UBC 811	GA(AG)7C	66	10	10	0	0	100	0
UBC 814	(CT) 8A	66	10	8	2	0	80	20
UBC 818	(CA) 8 G	66	7	6	1	0	85	15
UBC 834	(AG) 8YT	66	15	14	1	0	93	7
UBC 836	(AG) 8YA	66	17	17	0	0	100	0
UBC 840	(GA) 8YT	66	14	12	2	0	85	15
UBC 841	(GA)8YC	66	19	19	0	0	100	0
UBC 855	(AC) 8YT	66	9	6	3	0	66	34
UBC 889	(AC) 7	66	15	15	0	0	100	0
Total		726	135	123	12	0		
Average			12,27	11,18	1,09	0	89	11

Note. Y = (C, T).

Table 7. Eigen-vectors on the two principal components

Primers	PC1	PC2
UBC807	2.73	5.64
UBC810	2.67	1.65
UBC 811	24.55	1.78
UBC 814	1.45	3.63
UBC 818	0.27	18.42
UBC 834	20.05	3.95
UBC 836	18.79	29.85
UBC 840	1.19	4.97
UBC 841	10.94	16.47
UBC 855	0.2	3.28
UBC 889	17.15	10.35
Eigen value	9.762	9.088
Variance (%)	8.068	7.51
Cumulative	8.068	15.578

Table 8. Analysis of molecular variance (AMOVA) for 11 walnut accessions

Source of variation	d.f	Sum squares	Variance component	Percentage of variation	*F Statistic*
Among altitude groups	2	34.083	0.11995 Va	1.89	FCT : 0.018
Among accessions Within groups	8	119.583	1.74132 Vb	27.37	FSC : 0.279***
Within accessions	55	247.500	4.50000 Vc	70.74	FST : 0.2926***
Total	65	401.167	6.36127		

Signification level (p<0,05), *** Very high significant.

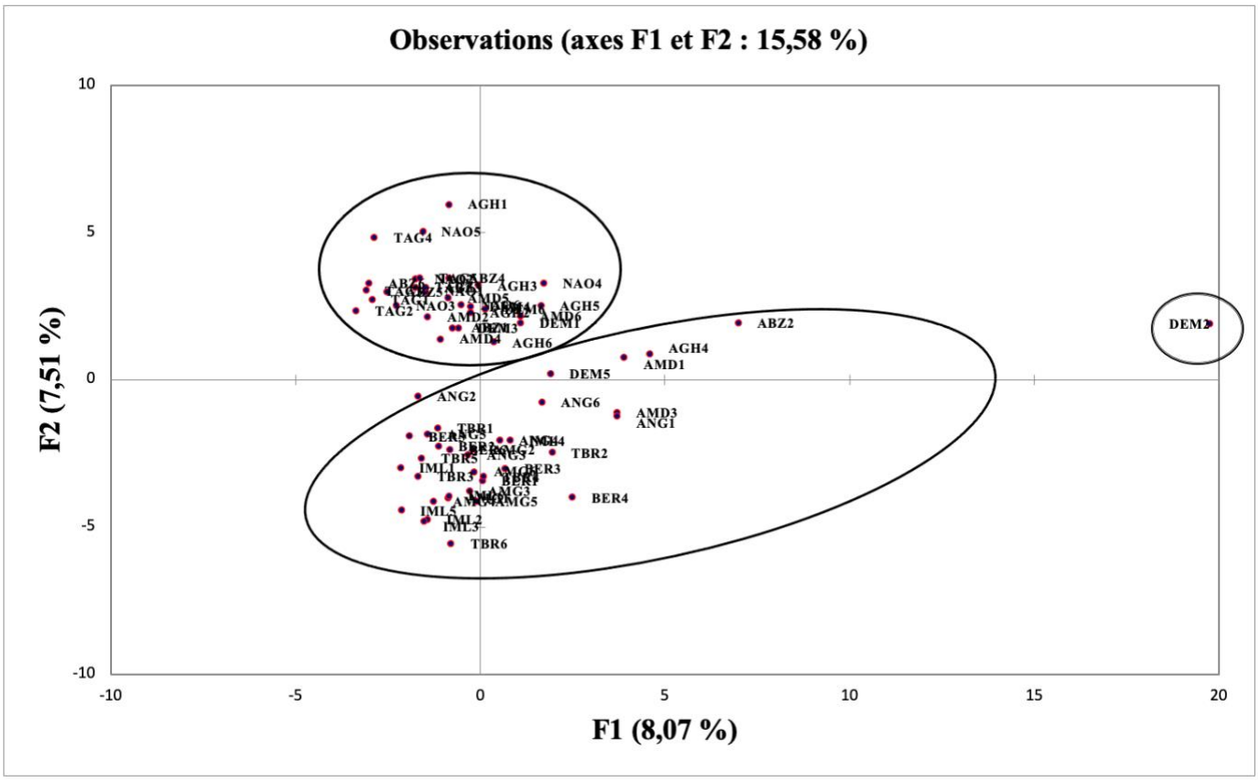

Figure 2. The plot of the 11 accessions examined on ISSR marker

3.4. Correlation between molecular and morphological analysis

The congruence between morphological and molecular markers is analyzed by the Mantel test. The result of correlation between morphological and molecular distance matrices, molecular and biochemical distance matrices, and morphological and biochemical distance matrices exhibited no correlation between these three markers (r= 0.591; P value= 0.998; r=0.38; P=0.475; r=0.602, P=0.784 respectively).

3.5. Discussion

The morphological results showed considerable variation specially for the position of pad on suture and shell color, which showed the highest coefficient of variation (45.02 and 40.50 % respectively), while the leaflets margin was Entire for all accessions (00.00 %). In

addition, the result obtained revealed a great variation of leaf length and width (12.29 and 14.24 % respectively). These values are higher than that registered by Ghanbari *et al.* (2018), in Iranian walnut (4.41 and 0.625 % respectively). Regarding the length, width and weight of the nut, they recorded a CV of 9.68, 10.28 and 26.27 % respectively. Similarly, Khadivi-Khub *et al.* (2015) published the coefficients of variation of nut length of 12.87%, nut width of 11.62% and nut weight of 20.49% in Iranian walnut. Other similar results were revealed in Romanian walnut by Cosmulescu, (2013) (9.53, 11.42 and 19.18% respectively). For the kernel, presenting the edible part, revealed with a CV of 36.63% for kernel weight, 25.97% for kernel percentage and 27.41% for kernel color. These values are higher of those reported by Khadivi-Khub *et al.* (2015) (25.76, 11.99 and 24.19% respectively) and by Cosmulescu, (2013) (22.34 and 12.19% respectively). These finding provides large combinations of traits in order to obtain a genotype with desired traits to satisfy the requests of breeders, farmers, consumers and industry. Demnate and Amouguer accessions, both belonging to the High Atlas and separated by 204 km, revealed the great morphological divergence while the weak divergence is found between Angual and Ait Mhamed of the High Atlas and distant by 186 km. This result suggests that the long distance between accessions leads to large differentiation. In contrast, the Mantel test, revealed no significant correlation between geographic and morphological distances (r=0.159, p=0.84), indicating that geographic distance is not the main factor in the morphological differentiation of walnut accessions. Indeed, the same climatic conditions have an equal effect on morphological traits (Khadivi-Khub *et al.* 2015). Nevertheless, a large geographic distance may limit gene flow (Sefc *et al.* 2000), as well as the small population effect, genetic drift and geographic isolation may be the main factors causing genetic differentiation among populations (Li *et al.* 2018). These random factors could be an important reason for the absence of correlation between geographic and genetic distances (Zhang *et al.*, 2015).

The results of the biochemical parameters of Moroccan walnut revealed large level of moisture (0.2-4 %), Ash (1.12-2.7 %), oil (53.72-70.58 %), protein (5.25-22.75 %), carbohydrates (5.25-32.27 %), energetic value (641.9-734.06 Kcal), crude fiber (3.32-7.52 %), total phenols (6.17-52 mg GAE 100 g^{-1}), total flavonoids (2.15 -84.17 mg RE 100 g^{-1}) and scavenging activity (72.23-87.3 %). These results are in agreement with the ranges recorded by Erdoğan *et al.* (2021) which are 53.75-71.43 % for total oil, 10.21-20.71 % for protein, 14.31-27.52 % for carbohydrates and 1.64-3.32% for Ash content. Regarding the total phenol content obtained in this study, it is comparable to that registered in the superior genotypes of Iranian walnuts (46.6 to 61.5 mg GAE g-1) (Sarikhani *et al.*, 2021). In fact, recent studies on walnut kernels showed different groups of monomeric and polyphenolic compounds with great antioxidant activity (Zhang *et al.*, 2009). High content of phenolic compounds and protein in walnut kernels had positive effect on human health (Labuckas *et al.*, 2008; Zhang *et al.*, 2009). According to the coefficient of variation ranging from 3.68 to 62.09 %, there are a great variation of biochemical parameters between the different accessions, providing a huge breeding selection potential.

The efficiency of a molecular marker technic is determined by the percentage of polymorphism demonstrated among the accessions under investigation. The results obtained in Moroccan walnut registered a polymorphism level of 89 %. Similarly, several studied reported a high polymorphism rate in the walnut tree (Malvolti *et al.*, 2010, 73.8 %; Christopoulos et al., 2010, 82.8 %; Aiqing *et al.*, 2014, 92.31 %). This finding is consistent with the common observation of high variation levels detected in long-lived, wind-pollinated tree species (Streiff *et al.*, 1998; Victory *et al.*, 2006). Effectively, the ISSR primers used in this work appears very useful, especially the UBC836 and UBC841 primers, which revealed the high eigenvalues in two first PC. This result can be confirmed by the values of PIC, MI and Rp (Kabiri *et al.*, 2019).

Concerning the classification of walnut trees into three groups, it was carried out undependably of the geographic origin and the mountain range with a weak effect of altitude (1.8%). These finding is in addition to the low genetic differentiation between regional (13.24%) and bioclimatic groups (1.31%) of 11 Moroccan walnut accessions (Kabiri *et al.*, 2019). These low rates of differentiation indicated limited adaptation of Moroccan walnut accessions to the local environment. Similarly, Farrokhi Toolir and Mozaffari, (2020) reported that ten genotypes of wild walnuts originating from Iran, are grouped independently of the geographical distances between them. Whereas, Geethanjali *et al.* (2017) indicated a clustering of genotypes into two main groups corresponding to the geographic origins.

The result of correlation analysis between morphological and molecular matrix was not significant (r= 0.591; P value= 0.998). The same result was observed in walnut trees grown in Iran (Ebrahimi *et al.*, 2011), as well as in an olive cultivars from Campania (Corrado *et al.*, 2009). Moreover, the comparison of ISSR and morpho-agronomic traits similarity matrices was not significant in the study of genetic variation among cumin accessions from Iran, Syria and Afghanistan (Rostami-Ahmadvandi

et al., 2013). There are two reasons why there is little or no correspondence between molecular and morphological variation. On the hand, molecular markers generally cover a large genome, including both coding and non-coding regions. On the other hand, non-specific molecular markers (such as ISSR) are generally used to measure genetic diversity and are not subject to artificial selection (Semagn, 2002).

4. Conclusions

Walnut with its various benefices, occupies a great place in nutritional history and habits of Moroccan people. These studies have shown a biochemical richness as well as the efficiency of morphological and molecular markers in the study of genetic diversity of walnut. Moreover, the Mantel test showed no significate correlation between the morphological and molecular markers. These finding would permit the selection of a high-performance genotype with the desired characteristics to improve yield and quality of Moroccan walnut.

6.References

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