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Fruit Physical, Chemical and Molecular Identification of Three Walnuts (*Juglans regia* L.) Genotypes Grown in Egypt

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Abstract

Three genotypes walnut (Juglans regia L.) were investigated during seasons 2019 & 2020 harvests from different selections of trees grown in El Gharbia governorate, Egypt. Physical properties, biochemical and molecular identification of fruit walnut types were examined. Physical properties showed that nut length ranged from 38 to 39.7 mm, nut width from 28.8 to 30.3 mm and nut weight from 11.10 to 12.61g. These genotypes are good quality according to the international walnut descriptor. The chemical analyses showed that total oil ranged from 45.84 to 61.99% while protein ranged from 8.88 to 14.12%. Total carbohydrate was in the range of 44.51-47.35% and the total phenol found between 137.97-147.54mg/g. Palmitoleic, Oleic and Stearic acids were the main fatty acids in Type -1. Also, the polyunsaturated fatty acid especial linoleic acid was found predominant in Type-2 and Type-3. Stigmasterol (18.21%), Nonadecane (30.07%) and Octacosane (17.17%) were the major in Type-1. On the other hand Eicosane, b-Sitosterol, Pentacosane, and Nonadecane were major sterols in Type -2 while Octadecane was main in Type-3. Based on the DNA rbcL gene sequencing the three genotypes were identified as English walnuts and deposited in the Genbank database under accession numbers (MK002719 and MK002720).

Keywords: Walnut; Fruit quality; Nut traits; Kernel oil; Fatty acids; Chemical composition; rbcl gene; Gen Bank.

1. Introduction

Walnut is one of the oldest cultivated fruits in the world; it's a deciduous tree that belongs to the family Juglandaceae and genus Juglans. The genus Juglans includes about 21 species distributed in Asia, Southern Europe, North America, Central America, Western South America, and the West Indies [1, 2]. Species of Juglans are diploid, with akaryo type of 2n = 2x = 32 [3, 4]. Genotypes of walnut are Classified into Rhysocaryon (black walnuts of America), Cardiocaryon (Japanese, Manchurian, and Chinese walnuts), Trachycaryon (butternut of Eastern North America), and Juglans. The Juglans division is widely cultivated in the temperate zone [5]. Cooling requirements for walnut trees are as low as 450-1500 hours of temperatures below 10 ° C during winter [6]. Moreover, cultivated walnut varieties are well adapted to the climatic conditions of different production areas [7].

Walnut has significant economic value, and medicinal importance for human health. It is consumed in large quantities by people; therefore, it has a very important place in the public nutritive habits. The walnut is basic nutritional elements and plays an important role as a promoter of medicine used for heart diseases [8]. Walnuts are a good source of antioxidant activity for the presence of tocopherols and polyphenolics (like ellagic acid and gallic acids) [9]. Walnuts are a rich source of essential polyunsaturated fatty acids (PUFA) such as linolenic,

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olecic and linoleic acids [10]. The high PUFA content of walnuts reduces the risk of heart disease by increasing HDL cholesterol and decreasing LDL cholesterol. It contains a diversity of protein including glutamic acid, arginine, Levine, melatonin and serotonin. It has high vitamins like A, B1, B3, B5, B6, E and folic acid. It also provides minerals such as potassium, calcium, and magnesium. Walnuts have a high nutritional value because they are rich in all this content of compounds. Also hulls, shells and leaves of walnut have industrial potential to produce coloring matter, viral and parasite biocides. Genetics, environmental factors, harvest time, rootstocks, soil quality, processing and storage conditions influence the composition and functionality of walnuts [11]. Nut and kernel quality is strongly affected by genotypes, environment and their interaction [12].

DNA barcodes are DNA fragments that are constant, well-mutated, and easily amplified that can be used to represent the species in living organisms [13]. DNA barcoding is a modern technique for identifying biological organisms [14]. This technology has the advantages of high accuracy, a large detection range, and easy operation. Furthermore, sequence stability is unaffected by individual development. This technology could be effective for species identification in the future. It is recommended that DNA barcodes be used as a tool for species identification and confirmation in the classification framework. The rbcL gene is the barcode DNA for plant species. The rbcL gene is part of the DNA sequence found in cpDNA and has an opportunity to be used as a DNA barcode [15] due to its universality and ease of amplification and analysis [16]. The full length of the gene has approximately 1400 bp, so this provides many characters to study phylogenetic. The sequence of rbcL gene has a lower level of mutation compared to other barcodes in cpDNA, and because this sequence contains a high level of similarity between species. The low level of the mutation in the rbcL gene leads to an in-depth study of genetic changes and developments within the different species of plants.

In this study, it was planned to study the physical parameters related to the quality, biochemical composition and genetic characterization using DNA to estimate the genetic similarity of three walnut genotypes grown under the environmental conditions of El Gharbia governorate, Egypt..

2.1. Plant material

This study was carried out during two successive seasons of 2019 and 2020 to investigate the physical and biochemical properties and molecular identification of three genotypes walnut trees growing in a private commercial orchard located at El Gharbia governorate, Egypt. The studied genotypes were Twenty-years-old cultivated at 6 x 7 m apart in a light loamy soil under surface irrigation system drip. Each genotype represented by 5 trees healthy carefully selected as uniform as possible in growth, vigor, free from pests and diseases and received uniform management practices.

2.2. Physical analysis

Fruits were harvested at fully mature stage for all genotypes in three replicates of 20 fruit in per replicate tree was tested for physical properties as follows:

i. Nut properties

Nut length (mm), nut width (mm), shape index (length/width), nut shape, nut size, nut weight (g), shell roughness and shell breaking.

ii. Kernel properties

Kernel weight (g), kernel ratio (%) and kernel color were determined according to Turkish Standard Institution (TSI) 1275/T1 [17].

iii. Quality nut properties

This index was evaluated as follows

Nut shape; Shape index < 1.25 nut shape is sphere$Nut\ index = \frac{Nut\ length}{(Nut\ diameter + nut\ thickness)\ /\ 2}$, shape index > 1.25 nut shape is oval. Nut size; Extra: Nut width ≥ 27 mm for sphere, nut diameter 26 mm for oval. Class I: Nut width 24-27 mm for sphere, nut diameter 24-26 mm for oval. Class II: Nut width 20-24 mm for sphere and oval. Kernel color: light, medium and dark. Shell firmness: smooth, medium and rough. Shell breaking: medium and ease. Kernel ratio was determined by the formula:

$$\text{Kernel ratio (\%)} = \frac{\text{kernel weight (g)}}{\text{Nut weight (g)}}\ x\ 100$$

2.3. Chemical composition analysis

i. Total carbohydrate (%)
The total carbohydrate content was estimated color metrically by the phenol-sulfuric acid method using D- glucose as a standard [18].

ii. Total protein (%)
Protein was assayed according to AOAC [19]. The nitrogen content of kernels was obtained by Kjeldahl analyses. Then, the protein content was calculated by multiplying the amount of nitrogen by the factor of 6.25.

iii. Total phenol (µg/g)
The total phenol assays were determined by Singleton et al. [20].

iv. Percentage of kernel oil content
Ten grams of dried kernel samples were extracted by adding 100 mL of hexane solvent and extraction in a Soxhlet device for 5 h. After oil extraction, hexane evaporated to dryness under reduced pressure at 40°C. The oils were weighed and stored at -10 until analysis.

v. GC analysis of kernel oil fatty acids composition
The walnut oil methylated in 1.5% sulfuric acid – methanol at 95°C for 2 h. the GC analysis described by El Gengaihi et al. [21].

vi. GC analysis of hydrocarbon and sterols compound
The walnut oil saponified with KOH (20ml, 10%) at 80C for 3h under reflux. Sterols methyl esters were identified by GC. The GC conditions are described by El Gengaihi et al. [21].

2.4. DNA extraction and purification

DNA extraction and purification were carried out according to DNeasy Kit cat no ≠ 69104 (Qiagen Sciences, Maryland, USA) according to the manufacturer's instruction manual.

i. Amplification of rbcL gene

DNA isolation from walnut was amplified by PCR. A PCR reaction mix formed from 1x buffer (Promega), 15Mm $MgCl_2$, 0.2 mM dNTPs, 20pcoml of each primer, 1u of Taq DNA polymerase (GoTaq, Promega), 30 ng DNA. Then, the forward primer rbcl-F (5'-ATGTCACCACAAACAGAGACTAAAGC-3'), and the reverse primer rbcl-R (5'-TCGCATGTACCTGCAGTAGC-3') were added. The rest was added 20 uL ultra-pure water. PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems). The initial program of PCR amplification consisted of 40 cycles at 94ºC for 5 min. Each cycle consisted of a denaturation step at 94ºC for 30 sec., an annealing step at 45ºC for 30 sec, and an elongation step at 72ºC for 30sec. In the final cycle, the primer extension clip was extended to 7 min at 72 °C. Amplifications were run by electrophoresis and resolved in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer at 95 V.. The band size of amplified products was determined using A 100bp DNA ladder standard. Then, amplified products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000). The purified PCR product was subjected to sequences. The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) Sequences were aligned using Align Sequences Nucleotide BLAST and compered with all sequences available at GenBank data base. All sequences were adjusted manually and submitted to GenBank, USA.

2.5. Statistical analysis

Data were submitted for analysis of variance (ANOVA) according to Gomez and Gomez [23], using COSTAT Software version 6.303 (2004), and LSD at a significance level of 0.05 was used to compare means

3. Results and discussion

3.1. Fruit physical properties

Good fruit quality is a desirable and important characteristic of nut cultivars and production [24]. Table 1 presents the physical properties of the three walnut genotypes. Analyses of variance showed that there were statistically significant differences among the genotypes for all traits. The maximum nut length (39.7 mm) was found in Type-1 while the minimum (38 mm) found in the Type-2. The maximum nut width of (30.3 mm) was determined for the Type-3 while a minimum (28.8 mm) was found for Type-2. The shape index was in the range of 1.27 to 1.32 nut shape was determined to be oval for all genotypes.
Regarding the nut weight, results indicated that the highest value showed in Type-1 (12.61g) followed by Type-3 (11.97 g) and Type-2 (11.10 g). The weight of the Kernel ranges from 4.47 g (Type-2) to 5.01 g (Type-1). The kernel weight of type 2 is observed to be low while type 1 and type 3 are not significantly

different. There was no significant difference in kernel/nut ratio between the three genotypes and were lower than 50%. Type-2 recorded the highest percentage of kernel/nut ratio (40.26%), while Type-1 recorded the lowest percentage of kernel/nut ratio (39.70%).

The quality nut traits of the three genotypes are shown in (Table 2). The fruit shape index (fruit length/width) of the all walnut genotypes > 1.25 so the nut shapes were classified to the oval shape. Nut size was determined extra for all genotypes. Kernel colour was light in Type-2 and Type-3, whereas Type-1 was dark kernel. Shell roughness was smooth for Type-1, Type-3 and medium for Type-2. Type-1 was the ease of shell breaking, but other genotypes were Medium (Table 2).

Table 1: Physical properties of some walnut genotypes in Egypt

Genotypes	Nut length (mm)	Nut width (mm)	Shape index	Nut weight (g)	Kernel weight (g)	Kernel/nut ratio (%)
Type-1	39.7^{a}	30.2^{a}	1.31^{ab}	12.61^{a}	5.01^{a}	39.70^{a}
Type-2	38.0^{b}	28.8^{b}	1.32^{a}	11.10^{c}	4.47^{b}	40.26^{a}
Type-3	38.6^{b}	30.3^{a}	1.27^{b}	11.97^{b}	4.77^{a}	39.80^{a}

Means in the same column having different letters are significantly different (P<0.05).

Table 2: Quality traits of some walnut genotypes in Egypt

Genotypes	Nut shape	Nut size	Kernel color	Shell roughness	Shell breaking
Type-1	Oval	Extra	Dark	Smooth	Ease
Type-2	Oval	Extra	Light	Medium	Medium
Type-3	Oval	Extra	Light	Smooth	Medium

Means in the same column having different letters are significantly different (P<0.05).

3.2. Chemical composition

Total carbohydrate, oil, protein and phenol showed a significant difference in all walnut genotypes (Table 3).The highest amount of total oil (61.99%), phenol (14.75mg/g) and protein content (14.12%) were observed inType-2. The maximum carbohydrate content was found in Type-1 (47.35%) followed by Type- 3 (46.08%) and Type-2 (44.51%). The protein results were in agreement with those obtained by Koyuncu and Askin [25] who found that the protein in different walnut genotypes contents varied between 6.30 and 22.0%. Phenol contents were in good agreement with Beyhan et al. [6] who found that the phenol content of different pecans was found to be in the range of 50-2,499 mg GAE/100 g %. USDA [26] determined values between 367- 1,065 mg GAE/100.The total protein, oil and carbohydrate content of walnuts from Portugal were in the range of 14.38 - 18.03%, 68.83 -72.14% and 3.75- 6.10%, respectively [27]. The protein content ranged from 13.77 to 14.92%, oil (62.3–67.3%), carbohydrates (12.84 to 16.67%, ash (2.09 to 2.24%) and fibre (4.2 to 4.6%) in three nuts from Iran [28]. Our results of chemical composition of walnut are comparable to the studies mentioned above. Differences in the chemical composition of nuts can be attributed to the year of harvest and associated environmental conditions such as temperature, precipitation and light

Table 3: Chemical composition of some walnut genotypes in Egypt

Chemical composition	Type-1	Type-2	Type-3
Carbohydrate (%)	47.35^{a}	44.51^{c}	46.08^{b}
Protein (%)	10.54^{b}	14.12^{a}	8.88^{c}
Oil (%)	45.84^{c}	61.99^{a}	59.18^{b}
Phenol (µg/g)	14.15^{b}	14.70^{a}	13.79^{c}

3.2.1. Fatty acids composition

The fatty acids composition of walnut genotypes is illustrated in (Table 4). Type-1 has the highest palmitoleic acid content (30%) followed by Type-2 (11.21%) and Typ-3 (10.64%). It is very interesting that, only Type-1 genotype had odd fatty acid pentadecylic acid (C15:0). Also, the Type-1 has a higher percentage of stearic acid (31.03%) than Type-2 (1.05%). Furthermore, oleic acid is the most

abundant in Type-1 (31.03%) followed by Type-2 (16.9%), and Type-3 (2.04%). While, linoleic (C18:2) appeared greater in Type-3 (74.2%) than Type- 2 (48.69%). Moreover, linolenic acid was found in Type-3 (10%) followed by Type-2 (5.87%) and Type-1(0.2%). Amaral et al. [29] found that the fatty acid composition of walnut oil depending on genetic origin, climate, geography and cultural treatments such as fertilization and irrigation during growth. These results are in agreement with those obtained by Yilmaz and Akca [30] who found that the major fatty acids in some 14 new genotypes walnut were oleic acid, stearic acid and linoleic acid.

Table 4 Fatty acid of some walnut genotypes in Egypt

Fatty acid	Type-1	Type-2	Type-3
Capric acid ($C_{10:0}$)	2.39	2.39	-
Lauric acid ($C_{12:0}$)	0.9	0.9	-
Myristic acid ($C_{14:0}$)	1.94	1.94	12
Pentadecylic acid ($C_{15:0}$)	0.31	-	-
Palmitic acid ($C_{16:0}$)	0.63	10	0.71
Palmitoleic ($C_{16:1}$)	30	11.21	10.64
Stearic acid ($C_{18:0}$)	31.03	-	1.05
Oleic ($C_{8:1}$)	34	16.9	2.04
Linoleic acid($C_{18:2}$)	2.6	48.69	74.2
Linolenic acid ($C_{18:3}$)	0.2	5.87	10
Arachidic acid ($C_{20:0}$)	0.19	2.1	-
Total fatty acid	100	100	100

3.2.2. Hydrocarbon and sterols

Hydrocarbon and sterols of walnut genotypes were identified by GC as shown in (Table 5). The Type-1 was distinguished from the others by having a high content of Nonadecane (30.07%), Stigmasterol (18.21%), Octacosane (17.17%) and Heneicosane (11.94%). While the Type-2 showed the most content of hydrocarbon in Eicosane (26.34%), Nonadecane (11.9%), Pentacosane (11.7%) and b-Sitosterol (10.85%). On the other hand, the Type-3 recorded the largest percentage of Octadecane(35.7%), Docosane (14.41%), Heneicosane (10.64%), and Nonadecane (10.4%). The predominant hydrocarbon and sterol are dependent on the walnut genotypes. The hydrocarbon fraction of Walnut oil from Argentina was a major in C14–C20 and the sterol profile from walnut varieties was β-Sitosterol (85.21–91.78%) [31].

Table 5: Hydrocarbon and sterols of some walnut genotypes in Egypt

Hydrocarbon	Type-1	Type-2	Type-3
Heptadecane (C_{17})	1.4	1.29	3.64
Octadecane (C_{18})	4.06	3.86	35.7
Nonadecane(C_{19})	30.07	11.9	10.4
Eicosane (C_{20})	1.37	26.34	0.05
Heneicosane (C_{21})	11.94	7.87	10.64
Docosane (C_{22})	4.69	4.32	14.41
Tetracosane (C_{23})	3.73	4.71	3.5
Pentacosane (C_{24})	2.61	11.7	4.69
Hexacosane (C_{25})	-	4.18	3.3
Heptacosane (C_{26})	-	4.18	-
Octacosane(C_{28})	17.17	4.19	2.21
Stigmasterol	18.21	4.61	3.46
b-Sitosterol	5.45	10.85	8
Total	100	100	100

3.3. Molecular identification by rbcL barcoding gene

The present study was performed to investigate the possibility of using rbcL DNA barcode for identifying walnuts plants. The rbcL gene was amplified using rbcL-F and rbcL-R primers to generate approximately 600 bp fragments. As shown in (Fig. 1). Amplification and sequencing success rate are the most important criteria to evaluate DNA barcoding for plant identification. Three genotypes of walnuts were subjected to rbcL gene amplification and sequencing the three types were identified as Juglans regia (English walnut) with 99.33% homology with Juglans regia voucher Q310, the second and the third type were genotype identical and identified as Juglans regia (English walnut) with 100% with Juglans regia voucher bal14 and deposited in the genbank under accession numbers (MK002719 and MK002720). These results are agreement with Li et al. [32] who also used DNA barcodes based on chloroplast genes for species identification of Orchidaceae plants, and concluded that DNA barcode technology is a novel molecular recognition technology that uses short and standard DNA fragments for species identification.

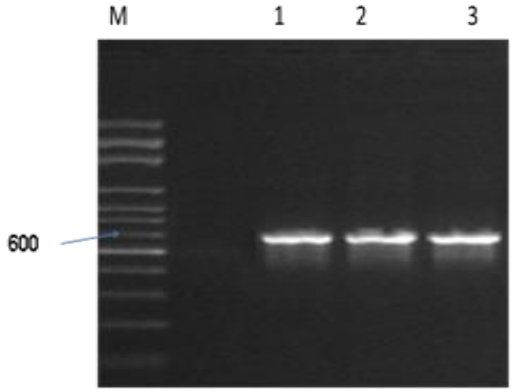

Figure 1: The PCR amplification of rbcL gene of three walnuts genotypes 600bp.

4. Conclusions

The results indicated that these genotypes of walnut (Juglans regia L.) cultivated under Egyptian conditions were characterized by high quality characteristics of nut weight, nut size, kernel weight, shell roughness and breaking, and high content of unsaturated fatty acids, phenols, protein and carbohydrates. Also, it had rich genetic resources in terms of walnut types. Therefore, given their higher yield during cultivation and their high content of polyunsaturated fatty acids, it is possible to indicate that the walnut varieties evaluated have high commercial and nutritional importance. It is clear that molecular genetics data can solve some taxonomic problems, as the data is difficult to access. Based on the research results, it is expected that the obtained results will be useful in determining the characteristics of local walnut varieties and as a reference for the development of walnut tree breeding programs in Egypt.

5. Conflicts of interest

The authors declare that they have no conflict of interest.

6. Formatting of funding sources

No fund available.

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