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Harvest date and genotype influences growth characters and essential oil production and composition of *Petroselinum crispum* plants

Hussein A. H. Said-Al Ahl[1]*, Mohamed Abou-Ellail[2] and Elsayed A Omer[1]

[1]*Medicinal and Aromatic Plants Researches Department, National Research Centre, 33 El-Bohouth St., (former El-Tahrir St.,) Dokki, Giza, Egypt. Postal Code: 12622*
[2]*Department of Genetics, Faculty of Agriculture & Natural Resources, University of Aswan, Aswan, Egypt*

ABSTRACT

A field experiment was conducted to compare growth characters, herb fresh weight and essential oil content and composition among four parsley cultivars at five harvest times in addition to evaluate the variability at the DNA level in these cultivars. Results showed that Plain Italian Giant cultivar was the best in number of branches, herb fresh weight and essential oil yield; while Moss curled no.2 cultivar was the best in the essential oil percentage and Local cultivar was the tallest and lowest in essential oil percentage. On the other hand, Moss curled no.2 cultivar was least in plant height, number of branches, herb fresh weight and essential oil yield. For the harvest time, 3rd cut gave the highest values of the plant height and weight of fresh herb, while the highest values of branches number were obtained from 4th cut. Generally, 3rd and 4th cuts gave the highest oil yield values in all cultivars. Volatile oil % increased gradually from 1st to 5th cuts, which gave the highest essential oil % in all cultivars. Four major compounds exist in local cultivar, and three major compounds exist in European cultivars but with different percentages. β-myrcene > β-phellandrene > myristcin > 1,3,8-p-menthatriene (Petroselinum crispum cv. Local); β-phellandrene > 1,3,8-p-menthatriene > myristcin (Petroselinum crispum cvs. Moss curled no.2, Plain Italian Giant and Plian). The cultivars plain Italian Giant and plain presented the greatest genetic similarity, while Moss curled no.2 and Local cultivars were the most divergent. However, Plain Italian Giant and Plain genotypes are closely related. However, Moss curled no.2 and Local are genetically distinct. The genotypes (Plain Italian Giant and Plain) are closed in cluster and the genotype. Local cultivar was nearest to two genotypes (Plain Italian Giant and Plain), while the genotype Moss curled no.2 cultivar was separated far away from all the other three genotypes (Plain Italian Giant, Plain and Local cultivars).

Key words: Parsley, Cultivar (cv.), Essential oil, β-myrcene, β-phellandrene, myristcin, 1,3,8-p-menthatriene, RAPD-PCR

INTRODUCTION

Petroselinum crispum (parsley, Apiaceae family) is an important aromatic and medicinal herb native to the countries of the Mediterranean region. It is cultivated for its use as a fresh or dry herb, edible roots and as a source of essential oils, pharmaceutical, perfume, cosmetic and food industries attributed to its a wide array of phytochemicals such as essential oils, fixed oil, flavonoids, coumarins, furanocoumarins, oleoresins, tannins, glycosides, vitamins A,B, C and minerals (iron and calcium) [1-5]. It is used in folk medicine as a digestive, colic, for relief of bladder inflammation and to treat kidney ailments, increase lactation, resume menstruation, lessen gum and dental pains and for treatment of skin diseases [6]. Earlier studies demonstrated

that parsley had pharmacological activities such as hepatoprotective, hypoglycemic activity, anti-diabetic, analgesic, spasmolytic, immunosuppressant, anti-platelet, gastroprotective, laxative, estrogenic, diuretic, antihyperlipidemic, antioxidant, and anti-inflammatory properties[7-16]. In view of the great diversity, the various species and varieties or cultivars of parsley have been classified tot three main types as follows: the plain leaf type and the curly leaf type which are cultivated for their foliage, and the turnip-rooted type primarily grown for its roots. These types differ in plant morphology and the content and chemical composition of essential oil and are currently cultivated worldwide [17-19]. The existence of this morphological and chemical variability creates great possibilities for growing different cultivars of parsley. Beside genetic variation, environmental conditions, agronomic practices and type of processing are other factors strongly influence the parsley yield and quality. Previous studies established that the time of harvest is very important and influential factor in the quantity and quality of essential oil [20-22].

Genetic diversity within a species is crucial for stating any breeding program [23].The selection of varied superior genotypes that could be used in breeding program is usually accomplished with molecular markers. Moreover, genetic markers are a useful method for the identification of the genetic variability available in natural populations and germplasm collections [24]. However, introducing new species or varieties of parsley to be cultivated in Egypt and through selecting the superior cultivar is required to produce plants with the desired production and quality of compounds needed for pharmaceutical and industrial products.

In addition, DNA markers have numerous applications in plant breeding such as (i) marker assisted evaluation of breeding materials like assessing the level of genetic diversity, parental selection, cultivar identity and assessment of cultivar purity [25, 26], study of heterosis, and identification of genomic regions under selection, (ii) marker assisted backcrossing, and (iii) marker assisted pyramiding [27]. Molecular marker techniques overcome many of the limitations of morphological and biochemical techniques and can detect variation at the DNA level [28]. Furthermore, genetic markers based on DNA polymorphisms have been developed and became routinely common tool employed for germplasm characterization, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites or simple sequence repeat (SSR) [29].

Selecting the superior cultivar through introducing new cultivars of parsley to be cultivated in Egypt and with respect to lack of information about the growth, yield and essential oil content of parsley cultivars, this research was conducted to evaluate the yield and essential oil content as well as to determine the harvesting times optimal for maximizing yield and essential oil production in different cultivars of parsley. Moreover, investigate the molecular characterization of different parsley cultivars that to increase the gene pool of the Egyptian cultivar and raising the production and the quality of certain medicinal compounds.

EXPERIMENTAL SECTION

Parsley plant materials and optimization of growing conditions

A field experiment was conducted at the Experimental Farm of the Faculty of Pharmacy, Cairo University, Giza, Egypt, during two successive seasons (2010/2011 and 2011/2012). The physical and chemical properties of the soil sample were determined according to Jackson [30] to indicate that the field soil is sandy loam, having a physical composition as follows: 51.1% sand, 25% silt, 23.9 % clay and 0.47% organic matter. Soil chemical analysis was as follows: E.C (ds/m) = 4.9; pH= 8.05 and available N, P and K =0.07, 0.53 and 2.8 mg/kg, respectively.

Seeds of three parsley cultivars; *Petroselinum crispum* cv. parsley plain (plain leaf type); *Petroselinum crispum* cv. parsley plain Italian Giant (plain leaf type) and *Petroselinum crispum* cv. parsley Moss curled no.2 (curly leaf type) were obtained from the HEM ZADEN B.V- P.O. Box 4-1606 ZG Venhuizen-The Netherlands. Besides, seeds of the Local cultivar (Egyptian, plain leaf type) were obtained from Medicinal and Aromatic Research Dep., Ministry of Agriculture, Egypt. The seeds of the fourth cultivars were sown on 15th October in the two seasons into 3 x 3.5 m plots on rows, with 60cm a part and 5 cm between the seeds on both sides of the row. The experimental layout was a complete randomized block design with three replications.

Growth, yield and essential oil production

Sample preparation

During each growing season (before flowering), the plants were harvested 5 times on1st February (105 days), 1st March (135 days), 1st April (165 days), 1th May (195 days) and 1st June (225 days) respectively, after sowing. The

fresh non flowering plant materials from each harvest date were harvested at 5 cm above the soil and immediately transferred to the laboratory to extract the volatile oil. Plant height, number of branches/plant, fresh material of different samples (whole aerial parts g/plant) and essential oil content of the fresh samples of each collection were determined.

Isolation of essential oils

Representative plant samples were hydro distillated using a Clevenger-type apparatus according to the method described in the British Pharmacopoeia [31]. Essential oil yield was expressed as ml/100 g fresh material, while essential oil yield per plant was expressed as ml plant^{-1}. The essential oils were collected and dehydrated over anhydrous sodium sulphate and kept in refrigerator until GC-MS analyses.

Gas chromatography/mass spectrometry (GC-MS) analysis

The volatile oil of eight cultivars was analyzed with gas chromatography-mass spectrometry (GC-MS) instrument stands with the following specifications. Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer).The GC/MS system was equipped with a TG-WAX MS column (30 m x 0.25 mm i.d., 0.25 µm film thickness). The carrier gas was helium at a flow rate of 1.0 mL/min and a split ratio of 1:10 using the following temperature program: 40 ºC for 1 min; rising at 4.0 ºC/min to 160 ºC and held for 6 min; rising at 6 ºC/min to 210 C and held for 1min. The injector and detector temperatures were held at 210 ºC. Diluted samples (1:10 hexane, v/v) of 0.2 µL of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450. Most of the compounds were identified using mass spectra (authentic chemicals, Wiley spectral library collection and NIST library).

DNA extraction, primers and DNA amplification

Genomic DNA was extracted and purified from fresh young leaves using a Biospain Plant Genomic DNA Extraction Kit (BioFlux). Ten primers (Table 1) were used to determine the genetic biodiversity among studied parsley genotypes. The primers were obtained from Pharmacia Biotech. (Amersham Pharmacia Biotech UK Limited, Ebgland HP79 NA). PCR amplifications were performed as described [29].

Table1. Ten primer sequences used in identification of different parsley cultivars

Primer code	Sequence	Primer code	Sequence
OP-A01	5’ CAGGCCCTTC 3’	OP-A11	5’ CAATCGCCGT 3’
OP-A02	5’ TGCCGAGCTG 3’	OP-A16	5’ AGCCAGCGAA 3’
OP-A04	5’ AATCGGGCTG 3’	OP-A19	5’CCTTGACGCA 3’
OP-A05	5’ AGGGGTCTTG 3’	OP-B15	5’ GGAGGGTGTT 3’
OP-A09	5’ GGGTAACGCC 3’	OP-B18	5’ CCACAGCAGT 3’

Statistical analysis

Growth characters, fresh herb and essential oil in this study were analyzed with the analysis of variance (ANOVA) using JMP 10 program (SAS Institute, NC, USA). The mean values of treatments were compared using Tukey’s HSD test. Values accompanied by different letters are significantly different at $p \leq 0.05$.

RAPD-PCR data analysis

Data were scored for computer analysis on the basis of the presence or absence of the amplified products for each primer. If a product was present in a genotype, it was designated as “1”, if absent it was designated as “0” after excluding the unreproducible bands. Genetic similarity coefficients were computed [32]. The similarity coefficients were used to construct the Unweighted Pair Group Method with Arithmetic averages (UPGMA) dendogram using the PAST (PAleontological STatistics) software program. Primer efficiency was calculated by dividing the number of bands generated by a primer by the total number of bands generated by all primers. Polymorphism percentages were calculated by dividing the number of polymorphic bands amplified by a primer by the total number of bands amplified using the same primer [33]. Discrimination power for each primer was calculated by dividing the number of polymorphic bands amplified by a primer by the total number of polymorphic bands obtained [33].

RESULTS AND DISCUSSION

Effect of harvesting date and genotype on growth characteristics

Table 2 shows that there was a significant difference among the cutting intervals dates (cutting numbers), and the plants harvested at third date (165 days after sowing) gave the highest values of plant height and fresh weight of herb, while plants harvested at fifth date (225 days after sowing) gave the lowest values of plant height and fresh weight of herb in all cultivars in the two seasons. The maximum number of branches was obtained by harvesting at the fourth cut date (195 days after sowing), while the first cut date (105 days after sowing) gave the lowest branches/plant in all cultivars in the two seasons. Also, there are significant differences among the cultivars under study, where cv. Local gave the tallest plants followed by cv. plain Italian Giant and plain cultivar then cv. Moss curled no.2 at all cuts in both seasons. Plain Italian Giant cultivar gave the highest values of number of branches and herb fresh weight in the first and second seasons of all cuts followed by cv. Local and cv. plain then cv. Moss curled no.2.

Effect of harvesting date and genotype on essential oil production

For the essential oil %, the maximum values were obtained from plants harvested in 1st June (5th cut) and the lowest values were obtained from harvested plants in February (1st cut) in all cultivars in the two seasons. Similarly, the lowest oil yield values were obtained from plants harvested in February (1st cut) in all cultivars in the two seasons. It is well known that herb fresh weight of each cut influences the oil yield, so generally, the highest values of oil yield in parsley cultivars were obtained from plants harvested from both 3rd and 4th cuts. The genotype also influenced the essential oil production. For example, Moss curled no.2 gave the highest essential oil % followed by Plain Italian Giant cultivar and plain cultivar then Local cultivar. Whereas, cv. plain Italian Giant gave the highest essential oil yield followed by Plain cultivar and Local cultivar and then cv. Moss curled no.2 in all cuts in both seasons.

Table 2. Plant height, branches number and fresh herb of the vegetative herb of different 4 parsley cultivars harvested fifth times under Egyptian conditions at the first and second seasons

Cultivar	Cuts number	1St Season			2nd Season		
		Plant height	Branches No	Fresh Weight	Plant height	Branches No	Fresh Weight
Moss curled no.2	1st cut	16.1±0.6* ^{k}	3.33±0.3 ^{i}	0.56±0.03 ^{i}	14.1±0.7 ^{j}	2.67±0.3 ^{i}	0.49±0.04 ^{k}
	2nd cut	18.5±0.5 ^{jk}	6.67±0.7f-i	1.18±0.04gh	19.6±0.4hi	7.00±0.6g	1.03±0.09ij
	3rd cut	20.6±0.7 ^{ij}	9.00±0.0e-g	1.37±0.06fg	23.7±1.5e-h	9.33±0.3ef	1.31±0.01hi
	4th cut	19.3±0.8 ^{jk}	10.0±0.6ef	0.79±0.03hi	17.6±0.5ij	9.67±0.3e	0.89±0.0j
	5th cut	11.1±0.5 ^{l}	4.67±0.3hi	0.41±0.04i	13.9±0.2j	3.33±0.3i	0.48±0.02k
Plain Italian Giant	1st cut	31.4±1.6d-g	9.0±0.6e-g	1.99±0.05de	28.0±0.9d-f	10.33±0.3de	1.95±0.04ef
	2nd cut	32.9±0.6d-f	14.7±0.3cd	2.7±0.03ab	33.3±0.4bc	15.3±0.3c	2.8±0.05cd
	3rd cut	39.4±0.2ab	18.7±0.7b	3.2±0.09a	37.0±0.4ab	21.7±0.3a	3.4±0.07a
	4th cut	33.1±0.8de	23.3±1.3a	2.9±0.08ab	30.9±1.1cd	22.0±0.0a	3.1±0.08bc
	5th cut	27.6±0.9gh	15.0±1.2cd	1.4±0.05fg	24.8±1.1e-g	12.0±0.6d	1.6±0.0gh
Plain	1st cut	24.5±1.5hi	5.00±0.6hi	1.39±0.03fg	22.4±0.6g-i	4.33±0.3hi	1.26±0.03i
	2nd cut	27.3±0.5gh	7.67±0.3f-h	2.02±0.04de	28.7±1.5c-e	7.33±0.3fg	1.96±0.01ef
	3rd cut	30.8±0.5d-g	10.0±0.0ef	2.6±0.03bc	32.4±1.1b-d	10.7±0.3de	2.2±0.06e
	4th cut	29.4±0.8e-g	11.7±0.7de	2.2±0.28c-e	27.4±1.0d-g	14.7±0.7c	2.0±0.0ef
	5th cut	20.4±0.3i-k	6.00±0.6g-i	1.19±0.07gh	23.2±1.1f-h	6.67±0.3g	1.06±0.02ij
Local	1st cut	34.4±1.4cd	5.67±0.7g-i	1.81±0.03ef	31.3±0.9cd	6.33±0.7gh	1.67±0.09fg
	2nd cut	38.7±0.3b	12.3±0.9de	2.4±0.10b-d	37.3±0.5ab	11.0±0.6de	2.6±0.0d
	3rd cut	43.3±0.3a	14.3±0.7cd	2.9±0.10ab	38.7±1.2a	12.3±0.3d	3.2±0.10ab
	4th cut	37.4±0.4bc	16.3±0.9bc	2.7±0.10ab	36.7±0.3ab	18.7±0.3b	2.9±0.11bc
	5th cut	28.7±0.7fg	12.0±0.6de	1.3±0.08g	30.0±1.7cd	10.3±0.3de	1.1±0.07ij

**Numbers accompanied by different letters within each column are significantly different at p≤0.05 using Tukey HSD test. Each number is the mean of three replicates ±SE*

With respect to essential oil %. From Tables [2, 3], it is clear that the interaction between cultivar and harvest intervals dates (cutting numbers) has a significant impact on the studied characters such as, plant height, number of branches, herb fresh weight and volatile oil % as well as volatile oil yield in both seasons. For example, the tallest plants (43.3 cm) were obtained from Local cultivar harvested at the 3rd cut, whereas, Plain Italian Giant cultivar gave the highest number of branches/plant (23.3) and essential oil yield (3.81ml/m^2) in the 1st May (4th cut). However, Plain Italian Giant cultivar gave the highest values of fresh herb weight (3.2 kg/m^2) in the 1st of April (3rd cut). Moreover, Moss curled no.2 was superior in essential oil % (0.19%) when plants were cut in the 1st of June (5th cut).

Our results are in agreement with some previous studies. For instance, Petropoulos et al. [17] observed that herb fresh weight of curly-leafed ranged from 52.6- 86.3 g/plant and in Plain-leafed 62.3-80.7 g/plant in the first and second seasons, respectively. For essential oil content, curly-leafed cultivar was higher (o.05 ml per 100 g fresh weight) than Plain-leafed cultivar (0.04 ml per 100 g fresh weight). Similarly, oil yield of the leaves was found to be higher in curly-leafed cultivar (1.17 ml per m2) than Plain-leafed cultivar (0.87 ml per m2). In another study, Najla et al. [34] found significant differences in the productivity of parsley plain and parsley curly leafed cultivars. Plain leafed was higher in leaf area, fresh and dry weight and stem length and diameter than curly-leafed parsley. Sabry et al. [35] demonstrated that Plain leaf cultivar was superior in plant height, number of branches/plant, fresh herb yield (ton/ha) and essential oil content compared to the Clause (Italian) and curly cultivars. In addition, cutting date influenced the productivity and the essential oil yield, where the 3rd cut produced the highest values in all tested cultivars. Kmiecik and Lisiewska [36] also found significant differences between the productivity of plain and curly leafed parsley cultivars. In a trial on Curly cultivar in Egypt, Aziz et al. [37] concluded that plant height and number of branches/plant were higher in the second cut than first cut, while the fresh herb/plant was lower in the second cut than first cut. However, the essential oil % was higher in second cut (0.19-0.20%) than first cut (0.16-0.18%) in the first and second seasons respectively.

Table 3. Essential oil production (% and yield) of the vegetative herb of different 4 parsley cultivars harvested fifth times under Egyptian conditions at the first and second seasons

Cultivar	Cuts number	1St Season		2nd Season	
		Oil%	Oil Yield	Oil%	Oil Yield
Moss curled no.2	1st cut	0.043±0.003jk	0.24±0.02jk	0.057±0.003hi	0.28±0.02g
	2nd cut	0.080±0.006gh	0.94±0.03f-h	0.085±0.003fg	0.87±0.04d-g
	3rd cut	0.117±0.007c-e	1.60±0.09c-e	0.107±0.003de	1.39±0.05c-e
	4th cut	0.143±0.007b	1.13±0.09e-h	0.133±0.003bc	1.18±0.027c-f
	5th cut	0.190±0.010a	0.78±0.06g-j	0.160±0.0a	0.77±0.032e-g
Plain Italian Giant	1st cut	0.037±0.009jk	0.82±0.12g-j	0.032±0.002k	0.62±0.04fg
	2nd cut	0.073±0.003g-i	2.01±0.09b-d	0.052±0.002ij	1.77±0.38bc
	3rd cut	0.11±0.006d-f	3.46±0.09a	0.093±0.007ef	3.19±0.24a
	4th cut	0.133±0.003b-d	3.81±0.20a	0.127±0.003c	3.91±0.18a
	5th cut	0.173±0.003a	2.47±0.13b	0.147±0.007ab	2.35±0.11b
Plain	1st cut	0.022±0.002kl	0.30±0.02i-k	0.018±0.002k	0.23±0.02g
	2nd cut	0.042±0.002jk	0.84±0.03g-i	0.033±0.003jk	0.65±0.07e-g
	3rd cut	0.090±0.0fg	2.33±0.02b	0.070±0.0g-i	1.55±0.04cd
	4th cut	0.098±0.002e-g	2.12±0.29bc	0.085±0.0fg	1.72±0.06bc
	5th cut	0.140±0.006bc	1.67±0.15c-e	0.120±0.006cd	1.28±0.08c-f
Local	1st cut	0.010±0.0l	0.18±0.003k	0.017±0.003	0.28±0.067g
	2nd cut	0.027±0.003kl	0.65±0.10h-k	0.030±0.0	0.78±0.0e-g
	3rd cut	0.047±0.003jk	1.35±0.09e-g	0.057±0.003	1.56±0.33cd
	4th cut	0.053±0.003ij	1.45±0.13d-f	0.063±0.003	1.66±0.09bc
	5th cut	0.060±0.0h-j	0.80±0.05g-j	0.073±0.003	0.83±0.033d-g

**Numbers accompanied by different letters within each column are significantly different at p≤0.05 using Tukey HSD test. Each number is the mean of three replicates ±SE*

It is well established that genetic and environmental conditions are strongly influence the secondary metabolites biosynthesis also in addition to agronomic conditions and the type of processing [38, 39]. Harvest time is very important and influential factor in the quantity and quality of oil [22]. Long days and high light intensities are required during the maturation period for maximum oil production [40]. This was confirmed by Court et al. [20] and Murray et al. [22], where the time of harvest was a major factor for determining the quality of essential oil in mint plants. In a study on 104 accessions of *Petroselinum crispum*, including curly and flat leaf and hamburg types, essential oil content of fresh leaves ranged from 0.00 to 0.16% [41]. Melchior and Kastner [42] found that parsley leaves contain (0.1–0.7%) essential oil, while the % can be much lower (0.03%) in other study [4].

GC/MS analysis of essential oil

The relative percentage of main constituents of the essential oil extracted from the herb before flowering stage of parsley cultivars are shown in Tables [4, 5]. The identified compounds of essential oil in five harvests (105, 135, 165, 195 and 225 days after sowing) were grouped into three items i.e., major compounds (more than 10%), minor compounds (less than 10% and more than 1%) and trace ones (less than 1%).

It is evident that, β-myrcene, β-phellandrene, 1,3,8-p-menthatriene and myristcin exhibited as majors of Local cultivar. However, β-phellandrene, 1,3,8-p-menthatriene and myristcin were the major components in Moss curled no.2, Plain and Plain Italian Giant cultivars. Local cultivar had the highest percentage of β-myrcene (27.1%) and 1,3,8-p-menthatriene (20.4%) at third harvest. Whereas, the highest % of β-phellandrene (22.94%) and myristcin (34.4%) were obtained from fifth and fourth harvest, respectively. The lowest percentage of β-myrcene (14.34%), β-phellandrene (13.94%) was obtained from plants harvested at first harvest, while harvesting plants at the third and fifth cuts gave the lowest percentage of myristcin (6.8%) and 1,3,8-p-menthatriene (7.89%) respectively. In Moss curled no.2 cultivar, plants harvested at 2nd cut, 1st cut and 5th cut gave the highest % of β-phellandrene (32.19%), 1,3,8-p-menthatriene (20.96%) and myristcin (67.9%), respectively, whereas, the lowest % (6.09; 7.81 and 30%) from these compounds, were obtained when plants harvested at 4th cut, 5th cut and 2nd consecutive.

From Table (5), we found that plain and Plain Italian Giant cultivars have β-phellandrene, 1,3,8-p-menthatriene and myristcin as major constituents . Harvesting at 5th cut gave the highest % of 1,3,8-p-menthatriene (30.75%) and (47.8%), respectively. However, the lowest % from these compounds was obtained when harvested at 1st cut in plain and Plain Italian Giant cultivars. Plain and Plain Italian Giant cultivars gave the highest % of β-phellandrene when harvested at 5th cut (31.2%) and 3rd cut (32.2%), respectively, while harvesting at 4th cut gave the lowest % in plain and Plain Italian Giant cultivars. As for myristcin, plain cultivar gave the highest % (26.6) at 4th cut, whereas, the highest % in Plain Italian Giant cultivar was obtained when harvested at 3rd cut (16.4%). The lowest % of myristcin in both plain and Plain Italian Giant cultivars was obtained when harvested at 5th cut. When comparing cultivars, there are clear differences between the four cultivars (Table 6). *Petroselinum crispum* cv. Local showed the highest % of β-myrcene followed by cv. plain and Italian then Moss curled no.2. On the other hand, *Petroselinum crispum* cv. Plain Italian Giant showed the highest % of β-phellandrene and 1,3,8-p-menthatriene followed by cv. plain and Local then Moss curled no.2. Petroselinum crispum cv. Moss curled no.2 gave the highest % of myristcin followed by Local and plain then Plain Italian Giant. This indicates that the genotype has a significant influence on the chemical composition of parsley essential oil.

Table 4. Essential oil composition of parsley cultivars herb during five cuts of 2011/2012 season

Compound	*Petroselinum crispum* cv. Local					*Petroselinum crispum* cv. Moss curled no.2				
	1st cut	2nd cut	3rd cut	4th cut	5th cut	1st cut	2nd cut	3rd cut	4th cut	5th cut
α-pinene	3.20	2.50	6.4	0.90	1.0	0.80	3.86	1.40	-	0.90
β -thujene	0.10	-	0.03	-	0.14	0.04	0.12	-	-	-
β-pinene	2.10	2.30	3.90	0.8	1.70	0.22	-	0.77	-	0.34
sabinene	0.57	0.40	0.11	0.02	0.10	-	0.06	-	-	0.09
β-myrcene	14.34	19.60	27.1	26.9	24.88	0.88	1.70	7.94	2.25	3.65
α-phellandrene	4.06	3.80	3.35	0.29	0.50	0.16	3.81	0.50	0.14	0.21
limonene	6.02	4.90	2.38	1.89	1.64	0.33	0.72	1.60	0.62	0.88
β-phellandrene	13.94	20.80	19.3	16.7	22.94	12.78	32.19	21.24	6.09	9.99
β-ocimene	0.75	-	0.12	0.03	-	0.12	-	0.27	-	0.13
α-terpinolene	7.0	3.80	1.13	0.52	1.78	0.13	3.90	4.76	0.87	2.43
p-cymene	0.77	1.90	2.42	0.55	2.22	0.44	0.65	1.28	1.11	0.44
1,3,8-p-menthatriene	17.89	19.0	20.4	10.5	7.89	20.96	14.39	14.2	11.0	7.81
p-cymenene	2.91	-	-	1.15	-	0.10	-	0.13	-	0.04
α-copaene	0.20	0.11	-	-	0.05	-	0.12	0.05	-	-
caryophyllene	2.05	0.50	-	0.08	-	0.08	-	0.11	-	0.06
germacrene D	2.23	0.85	0.13	0.3	0.34	0.09	-	-	-	0.10
α - farnesene	0.22	-	0.11	0.04	0.07	0.12	0.11	0.22	0.63	0.17
β-sesquiphellandrene	1.84	0.70	0.65	0.60	0.25	0.41	2.43	0.87	3.42	0.51
β-citronellol	0.32	0.10	-	0.15	-	-	0.11	0.11	-	0.04
β -elemene	0.54	-	0.10	-	0.20	0.45	0.40	0.22	0.74	0.04
caryophyllene oxide	0.19	0.10	-	-	0.05	0.23	-	-	0.15	0.04
α-cadinol	0.19	0.12	-	0.30	0.45	0.15	0.30	0.33	0.21	0.35
myristcin	8.34	12.62	6.8	34.4	29.52	55.60	30.00	38.95	63.70	67.9
elemicin	0.18	-	-	0.13	0.11	2.49	1.45	1.08	2.25	0.36
apiol	6.77	3.50	3.80	1.90	1.98	2.02	2.34	0.40	3.56	2.06

Table 5. Essential oil composition of parsley cultivars herb during five cuts of 2011/2012 season

Compound	*Petroselinum crispum* cv. Plain					*Petroselinum crispum* cv. Plain Italian Giant				
	1st cut	2nd cut	3rd cut	4th cut	5th cut	1st cut	2nd cut	3rd cut	4th cut	5th cut
α-pinene	3.50	3.67	3.88	0.77	2.80	2.95	2.11	3.26	1.77	3.19
β -thujene	0.24	0.16	-	0.12	0.10	0.21	0.22	0.19	0.10	0.19
β-pinene	2.13	1.44	1.57	0.45	3.15	1.87	1.12	1.40	0.91	1.67
sabinene	0.63	0.11	0.10	0.07	0.34	0.52	0.06	0.09	0.10	0.08
β-myrcene	9.62	8.45	8.13	4.69	6.57	9.75	8.98	6.98	6.39	3.55
α-phellandrene	4.29	3.89	0.72	0.46	1.05	4.24	2.90	1.60	0.61	0.54
limonene	5.71	4.89	1.47	1.3	1.43	6.19	3.89	1.29	1.99	1.23
β-phellandrene	19.72	25.60	27.8	17.6	31.2	23.65	27.70	32.8	23.1	25.1
β-ocimene	0.72	0.25	1.62	0.66	0.39	0.72	0.88	0.90	1.12	0.89
α-terpinolene	6.87	4.45	2.3	3.76	3.70	7.72	4.66	1.87	4.39	2.46
p-cymene	1.0	1.90	1.74	1.29	0.77	1.27	1.29	1.95	1.06	0.60
1,3,8-p-menthatriene	18.56	22.30	23.98	26.33	30.75	14.85	20.20	16.6	30.4	47.8
p-cymenene	2.71	2.40	2.23	1.22	1.30	4.29	-	-	1.39	-
α-copaene	0.17	0.10	0.10	0.03	0.11	0.16	0.27	-	-	0.15
caryophyllene	1.53	0.78	0.10	0.12	0.17	1.44	0.34	0.18	0.10	-
germacrene D	1.98	1.49	0.13	0.32	0.17	1.29	-	0.24	0.31	0.12
α - farnesene	0.19	-	-	0.03	0.09	0.24	0.11	0.07	-	0.09
β-sesquiphellandrene	1.79	1.39	0.13	0.41	0.38	1.97	1.0	0.13	0.25	-
β-citronellol	0.34	0.22	-	-	0.12	0.20	-	-	0.13	-
β-elemene	0.53	0.56	0.23	-	0.48	0.77	0.35	0.08	-	-
caryophyllene oxide	0.20	0.18	0.45	0.13	0.44	0.38	0.21	0.33	-	-
α-cadinol	0.15	-	0.13	0.18	-	0.40	-	-	0.23	-
myristcin	7.43	8.90	16.3	28.6	8.61	9.29	12.82	19.4	14.1	7.00
elemicin	0.09	0.11	0.55	0.56	0.12	-	0.18	0.31	0.09	-
apiol	6.23	3.78	4.54	8.6	4.08	3.90	8.78	9.7	8.91	3.75

However, essential oil quantity and chemical composition varies depending on numerous factors, such as climate, cultivar, seeding date, management practices, plant parts and the developing stage of the plant at harvest time [43]. The amount of aroma constituents of *Petroselinum crispum* (whole herb) varied widely depending on harvesting time. These results are in agreement with Aziz et al. [37] who found that myristcin (28.65 and 33.61 %), followed by β-phellandrene (16.42 and 12.46 %), β-myrcene (9.81and 10.37 %), 1,3,8-menthatriene (4.58 and 0.86 %) were the major constituents of curly-leafed parsley in the first and second cuts, respectively.

Previous results indicate that genetic, physiological and environmental factors as well as processing conditions play an important role on essential oil quality [44-46]. The genetic variability had the major effect on essential oil constituents in a germplasm collection of parsley [41]. Our results indicated that different chemotypes of parsley are exist in parsley populations, which are widely influenced with both the genetic variation and the environmental conditions in agreement with Bernath [47] who concluded that the composition of essential oil is influenced by the plant genetic base and development and environmental conditions. Simon and Quinn [41] showed significant variability in essential oil constituents according to parsley accessions collection (country of origion). They identified various chemotypes based on the dominance of a particular marker constituent, such as 1,3,8-p-menthatriene, β-phellandrene, myristicin, and myrcene. They concluded that individual accessions varied greatly in essential oil composition due to genetic variation. Parsley herb oil composition revealed differences in the main compounds. Myristicin (30.7–42.7%), β-phellandrene (21.8–35.9%), p-1,3,8-menthatriene (5.4–10.0%), and β-myrcene (4.5–8.7%) were identified in parsley leaves as the major constituents [48]. Furthermore, Pino et al. [49] found Myristicin (63.9%) and apiole (14.4%) were the major components of parsley herb oil. Myristcin (25.70 to 46.41%), followed by β-phellandrene (8.77 to 16.42%), β-myrcene (6.72 to 11.44%), 1,3,8-menthatriene (0.89 to 12.83%), p-cymene (5.14 to 8.16%) and α-terpinolene (3.32 to 7.91%) were the major constituents of curly-leafed parsley [37]. Similarly, in a study of five cultivars of parsley [35] β-phellandrene, 1,3,8-menthatriene, bisabolene, myristicin and carotol were the major components in oil Plain and soft leaf and Clause (Italian) cultivars, respectively; while myristicin, 1,3,8- menthatriene and β-phellandrene were the major in Curly leaf. In the same time, myristicin , 1,3,8- menthatriene and β-phellandrene were the major in Rough leaf cultivar. Mangkoltriluk et al. [50] found that 1,3,8-p-Menthatriene, pinene, myrcene, phellandrene and apiole were found in fresh parsley leaves as the major components. The main compounds identified by Orav et al. [51] were p-1,3,8-menthatriene, β-phellandrene, myristicin , and myrcene were major components in parsley leave oil. Terpinolene, α-phellandrene, limonene, 1-methyl-4-isopropenylbenzene, β-pinene and α-pinene were found in quantities from 0.6% to 4.2% and

the other constituents below 0.8%. The same compounds were found in the parsley leaves and roots as the main components by other investigators [41, 52–56]. In the current study, the minor compounds such as α-pinene, β-pinene, α-phellandrene, limonene, α-terpinolene , p-cymene, p-cymenene, caryophyllene, germacrene D, β-sesquiphellandrene and apiol in Local; α-pinene, β-myrcene, α-phellandrene, limonene, α-terpinolene , p-cymene, β-sesquiphellandrene , elemicin and apiol in Curly; α-pinene, β-pinene, β-myrcene, α-phellandrene, limonene, β-ocimene, α-terpinolene , p-cymene, p-cymenene, caryophyllene, germacrene D, β-sesquiphellandrene and apiol in plain and Italian cultivars (Tables 4 and 5). In Local cultivar, α-phellandrene, limonene, α-terpinolene, p-cymenene, caryophyllene, germacrene D, β-sesquiphellandrene and apiol were the highest % when plants harvested at first cut and α-pinene, β-pinene and p-cymene were the highest when plants harvested at third cut. In the curly cultivar, the highest % of elemicin; α-pinene and α-phellandrene; β-myrcene limonene, α-terpinolene and p-cymenene in the third cut as well as β-sesquiphellandrene and apiol were obtained in the first, second , third and furth cuts, respectively. The similarity between the plain and italian cultivars was found in the behavior of many minor compounds such as β-myrcene, α-phellandrene, limonene α-terpinolene, p-cymenene, caryophyllene, germacrene D and β-sesquiphellandrene, which have higher percentage when harvested at first cut; as well as α-pinene from third cut in the two cultivars (plain and Italian). However, some exceptions were noted as the highest percentage of β-pinene from 5th cut in plain and 1st cut in Italian; β-ocimene and apiol from 3rd cut in plain and 4th cut in Italian and p-cymene from 2nd cut in plain and 3rd cut in Italian. Local cultivar gave the highest % of α-pinene, β-pinene at 3rd cut as well as caryophylleneand germacrene D at 1st cut. However, Curly cultivar gave the highest % of β-sesquiphellandrene and elemicin at 4th cut and 1st cut, respectively. While, Plain cultivar gave the highest % of α-phellandrene and β-ocimene at 1st cut cut and 3rd cut, respectively. Eventually, Italian cultivar gave the highest % of limonene, α-terpinolene and p-cymenene at 1st cut and p-cymene at 3rd cut. This indicates that chemical composition of the essential oil in parsley not only is affected by genotype, but also by the cut date. It is expected that at a certain age, plants generate molecular and biochemical pathways that lead to the biogenesis of particular compounds at specific tissues. This synthesis process presumably will be influenced by the various environmental conditions. This might explain the differences in essential oil accumulation by cut date. In this regard, temperature and other environmental conditions could favor the synthesis of particular compounds at each phenological stage.

When comparing the four cultivars in their content of the minor compounds, we found that Local cultivar followed by Plain and Italian then Curly had the highest % of β-pinene, α-phellandrene limonene and p-cymene. Plain followed by Local and Italian then Curly had the highest % of α-pinene caryophyllene and germacrene D. Also, Italian followed by Plain and Local then Curly contained the highest % of β-ocimene, α-terpinolene and apiol. However, Curly followed by Plian and Local then Italian had the highest % of β-sesquiphellandrene.. Plain followed by Italian and Local then Curly contained the highest % of p-cymenene. This confirms the idea that the interaction been the environmental conditions and the genetic factors determines the makeup of the essential oil, and therefore the chemotype dominating in particular location.

Other compounds were considered as traces, such as β –thujene, sabinene, β-ocimene, α-copaene, α-farnesene, β-citronellol, β-elemene, caryophyllene oxide, α-cadinol and elemicin in Local cultivar; β-thujene, β-pinene, sabinene, β-ocimene, α-copaene, α- farnesene, β-citronellol, β-elemene, caryophyllene oxide, α-cadinol, p-cymenene, caryophyllene and germacrene D in Curly cultivar; β-thujene, sabinene, α-copaene; α-farnesene; β-citronellol; β-elemene; caryophyllene oxide; α-cadinol and elemicin in plain and Italian cultivars.

Table 6. The main differences in major compounds (more than 10%) of different parsley cultivars essential oils

Compound	cultivar			
	Moss curled no.2	Plain Italian Giant	Plain	Local
	% (mean of five cuts)			
β-myrcene	3.28	7.13	7.49	22.56
β-phellandrene	16.45	26.47	24.38	18.73
1,3,8-p-menthatriene	13.67	25.77	24.38	15.13
myristcin	51.23	11.92	13.56	18.33

Results of GC/MS analysis of the essential oil obtained from four parsley cultivars in the five harvests revealed both qualitative and quantitative changes (Table 6). The major compounds were as follows: β-myrcene > β-phellandrene > myristcin > 1,3,8-p-menthatriene (*Petroselinum crispum* cv. Local); β-phellandrene > 1,3,8-p-menthatriene > myristcin (*Petroselinum crispum* cvs. Curly, Italian and Plian). These differences are mainly attributed to the genetic variation, but there are other factors, which may potentially affect the essential oil percentage determined, such as

the age and organ of the plant used for study and the environmental conditions under which the plants have been grown[43]. The plant cultivars used for the present study were grown under the same conditions, so that differences in the chemical profiles should reflect genetical differences between the various cultivars. Classification of parsley cultivars based on the accumulation of specific components could be an important approach for phytotaxonomy. In addition, studying the composition of essential oil enables the identification of marker compounds that are responsible for exerting the characteristic aroma of parsley. Based on Grayer et al. [57], the chemotype classification system is based on the chemicals combination of its major components rather than the sole dominant compound or a major component as one with content close to 20%. As it is clear from the Table (4) within essential oil constituents of the four parsley cultivar, the chemotypes can be summarized as chemotype 1) β-myrcene > β-phellandrene > myristcin > 1,3,8-p-menthatriene in Local cultivar and chemotype 2) β-phellandrene > 1,3,8-p-menthatriene > myristcin in all European cultivars (Curly, Italian and Plain).

Molecular genetic identification by randomly amplified polymorphic DNA (RAPD) markers

RAPD-PCR technique was used to identify the genetic distance among four different genotypes and ten random primers were used for this identification (Table 1). All primers successfully amplified high polymorphism among the four genotypes (Fig.1). As shown in Table 7, PCR amplification with 10 RAPD primers gave totally 182 RAPD fragments of different molecular weight; involving 50 monomorphic fragments with percentage about 27.5% while; 132 fragments (with an average of 72.5%) were polymorphic. Only 5 out of 10 primers showed less than 75% polymorphism. The number of amplified fragments per cultivar varied from 11 bands for the primers OPA02 and OPA04 showing the lowest primer efficiency (3.3 and 3.9%, respectively) to 24 bands for the primer OPA01 showing the highest primer efficiency (11.5%) with an average of 18.2 fragments per primer, and which varied in size from 200bp to 1500bp. The discriminatory power of the various primers varied greatly. The discriminatory power percentage of 10 RAPDs primers ranged from 4.55 (OPA04) to 15.91 (OPA01) (Table 7).

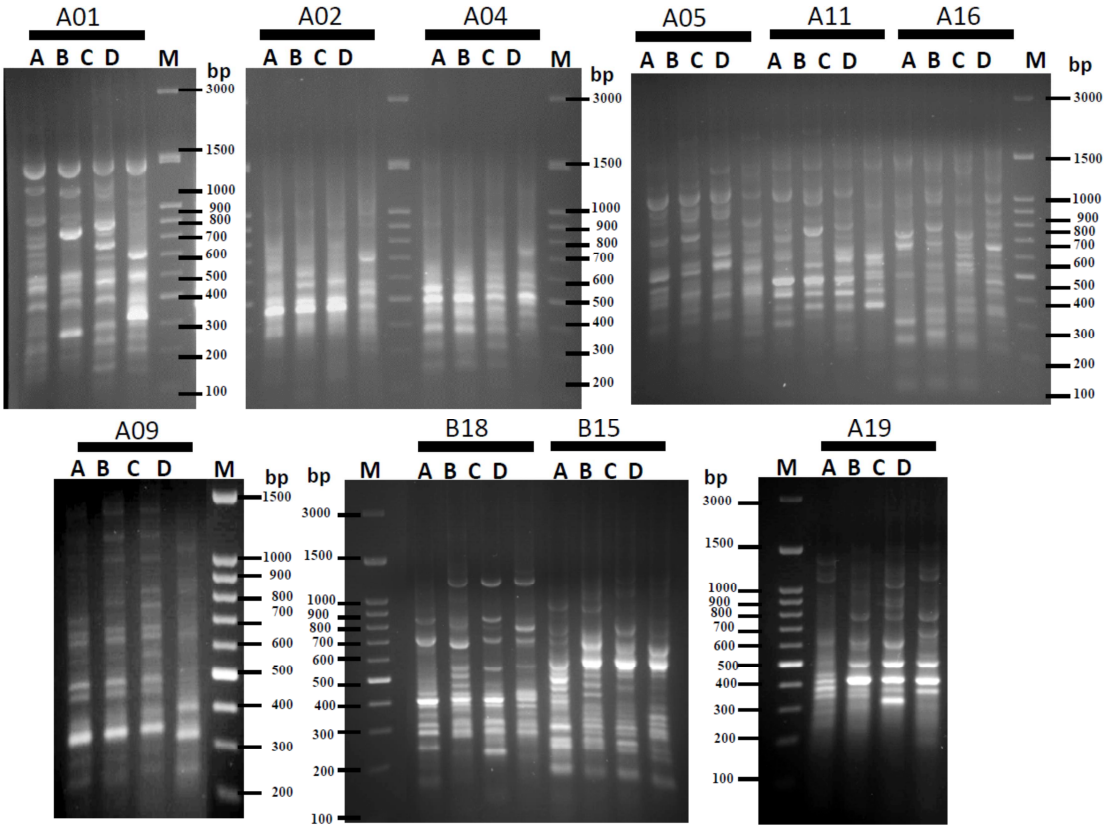

Figure 1: RABD-PCR analysis of four parsley cultivars with ten random primers (A01, A02, A04, A05, A11, A16, A09, B18, B15 and A19). Lane M = 100 bp DNA ladder. Lane A: Moss curled no.2, lane B: Plain Italian Giant, lane C: Plain and lane D: Local

Table 7. Number of bands amplified, polymorphic bands, primer efficiency and discrimination power of the ten primers used for RAPD-PCR analysis in four parsley cvs

Primers	Total bands	Monomorphic bands	Polymorphic bands	Polymorphism %	Efficiency %	discriminatory power %
A01	24	3	21	87.5	11.5	15.91
A02	11	4	7	63.64	3.9	5.30
A04	11	5	6	54.55	3.3	4.55
A05	20	7	13	65.00	7.1	9.85
A11	17	4	13	76.47	7.1	9.85
A16	20	5	15	75.00	8.2	11.36
A09	17	3	14	82.35	7.7	10.61
B18	20	8	12	60.00	6.6	9.09
B15	23	9	14	60.87	7.7	10.61
A19	19	2	17	89.47	9.3	12.88
All primers	182	50	132	72.53	0.725	~100

Jaccard's similarity index was calculated according to Jaccard [32] based on the presence or absence of bands by RAPD analysis for 4 genotypes (Table 8). It ranged from as low as 0.435 between genotypes, Petroselinum crispum cv. Moss curled no.2 (A) and Petroselinum crispum cv. Local (D) to as high as between genotypes Petroselinum crispum cv. Plain Italian Giant (B) and Petroselinum crispum cv. Plain (C). High similarity values mean that the genotypes are closely related. While the low values mean that, the genotypes are genetically distinct.

Table 8. Similarity indices among the four parsley cultivars as estimated using RAPD-PCR data

Cultivar	Moss curled no.2	Plain Italian Giant	Plain	Local
Moss curled no.2	1,000			
plain Italian Giant	0,510	1,000		
Plain	0,510	0,603	1,000	
Local	0,435	0,516	0,516	1,000

The genetic relatedness among parsley genotypes were illustrated through UPGMA dendrogram based on Jaccard's similarity analysis of RAPD-PCR data as shown in Figure 2. The dendrogram exhibits that the genotypes Plain Italian Giant and Plain are closed in cluster and the genotype Local was nearest to two genotypes plain Italian Giant and Plain while the genotype Moss curled no.2 was separated far away from all the other three genotypes (Plain Italian Giant, Plain and Local).

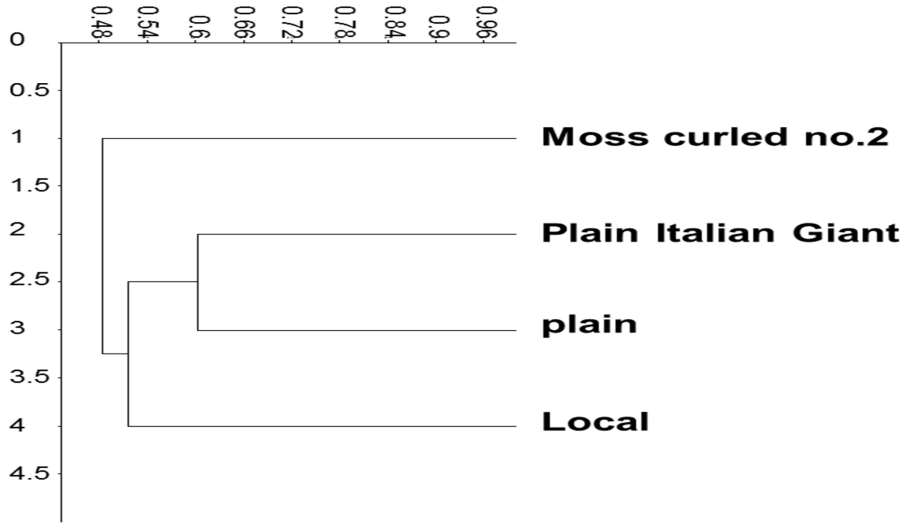

Figure 2. Dendrogram, revealing the genetic distance among four parsley species using RAPD-PCR data by UPGMA algorithm using Jaccard's similarity coefficient

Parsley is an important medicinal plant that contains several of pharmaceutical and nutritional compounds. The quantity, quality and activity of these compounds could vary between the different variants of parsley. Reports

indicated that genetic background, environmental factors and developmental stage, influences the synthesis of natural compounds [55]. Moreover, environmental disturbance can influence genetic diversity via biological and demographic processes, spatial and temporal variation in habitat suitability, and natural selection and evolution [56]. Adaptation to challenging environments presents by enhancing and/or suppression of gene expression or existing new genotypes by excitation the mutations [58]. The present study, demonstrated a marked genetic variability between the four cultivars. That in turn, the genetic variations is necessary to increase the gene pool of parsley and improvement quantity and quality of natural compounds through recruitment these cultivars in breeding program. In addition, these foreign genetic materials could be required as a source for genes that are involved in tolerance and /or resistance to abiotic and biotic stresses. Moreover, The RAPD markers technique has been reported to be an efficient tool to discriminate genetically isolated species and to verify the existence of spices that presented as a result of genetic drift or natural selection [59]. The RAPD is useful, rapid and accurate technique for studying genetic diversity and germplasm characterization of some fig [29] and parsley cultivars [60]. RAPD-PCR is a useful technique for detecting the genetic variability in our four cultivars. This genetic variability is required for plant breeding in order to increase the frequencies of favorable alleles and genetic combinations.

CONCLUSION

The results clearly demonstrate the superiority of plain Italian Giant cultivars in herb fresh weight and volatile oil yield. Also, Local and plain Italian Giant cultivars were more superior in β-myrcene (27.1%) and β-phellandrene (32.8%), respectively when harvested in April (3rd cut). plain Italian Giant and Moss curled no.2 cultivars were more superior in 1,3,8-p-menthatriene (47.8%) and myristcin (67.9%) when harvested in June (5th cut), respectively. DNA genotyping exhibited genetic variation among the four cultivars. These results support that a very well characterized parsley cultivars will be available for further breeding purposes to increase the gene pool of the Egyptian cultivars.

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