Int. J. Med. Arom. Plants, ISSN 2249 – 4340
Vol. 2, No. 1, pp. 41-52, March 2012

RESEARCH ARTICLE

Genetic characterization of *Rosa damascena* species growing in different regions of Syria and its relationship to the quality of the essential oils

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Article History: Received 15th December 2011, Revised 16th February 2012, Accepted 17th February 2012.

Abstract: Seven *Rosa damascena* samples and two check samples were collected and studied for both of the chemical oil and the molecular genetics composition. Results of oil chemical analyses of the different samples showed a great deal of resemblance among essential oils resultant from rose samples collected from the same region. The molecular genetics analyses for the same samples using both RAPD and ISSR techniques showed the same trend in the dendrogram. When the data were combined and put into one tree, the samples fell into two main clusters: The first combined samples 1 and 2 (Damascus) as the most closely genetically related followed by 3 and 5, and lastly the two checks were entered 4 (*Rosa centifolia*) and then 9 (*Crataegus*). And the second cluster contained three samples: the two closely related 7 and 8 (Aleppo) followed by the most distant sample of all 6. The study concluded that there were a high degree of conformity between the two dendrograms based on the chemical composition of essential oil on one hand and on the DNA level on the other hand, stressing the role of environment (mostly annual rainfall) on oil characteristics quality. On the other hand, based on these results, the authors suggest that sample 6 may not belong to *Rosa damascena* but to another species, and this needs further confirmation using cytoplasmic DNA techniques that target the variability at the species level.

Keywords: *Rosa damascene*; essential oil; RAPDs; ISSRs.

Introduction

The genus *Rosa* is considered the most important genus in the *Rosaceae* family comprising approximately 200 species and more than 18000 cultivars (Gudin 2000). It is widely distributed throughout the temperate and subtropical habitats of the northern hemisphere. Among all *Rosa* species, *Rosa damascena* Mill. is the most important commercially grown species, producing a high-value aromatic oil, which is used in the pharmaceutical, flavorings and fragrance industries, and for rose water production (Douglas 1993; Narayan and Kumar 2003). Moreover, it is grown as garden roses known as the Damask rose (*Rosa damascena*). *Rosa damascene* first grow wild and it is still self-growing in Caucasus, Syria, Morocco and Andalusia, Iran has also been mentioned as one of its origins (Chevallier 1996). It is generally accepted that Damask rose was introduced from the Middle East into western Europe and re-introduced later in the 16th century (Beales et al. 1998). Some varieties of *Rosa damascena* Mill. are of great importance for rose oil production, others are widely cultivated as garden roses (Rusanov et al. 2005).The world main producers of rose oil are Bulgaria, Turkey and Iran and to a less extend India, China and Northern Africa (Rusanov et al. 2009).

Although, its name is associated with Damascus and the damascene culture where it is reported that every traditional damascene house used to have a Damask rose in its backyard, it was gradually diminished with the extension of modern flats which lack the possibility of growing the Damask rose.

Damask rose preparations are used internally in Ayurveda, traditional Indian medicine, for soothing various complaints (Grieve 1971). Rose oil is used typically for all types of skin conditions from dry skin to aging skin as it softens and enhances tone and texture. Cold cream was originally known as 'ointment of rose water' because it contained rose oil and rose water. External application of rose essential oil is

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useful in soothing irritated skin. In aromatherapy, rose essential oil is utilized to counter depression, anxiety, grief, and negative feelings (Grieve 1971; Bown 2001).The scent alone is said to have an uplifting and stabilizing effect (Schnaubelt 1998). In recent years, antioxidant, antibacterial and antimicrobial activities of *R. damascena* essential oil have been demonstrated by Achuthan et al. (2003), Basim and Basim (2003), Özkan et al. (2004) and Buckle (2003). It is good for heart diseases, for eczema and also it contains high percentage of vitamin C which is necessary for collagen synthesis (Lawless 1995; Ardogan et al. 2002; Achuthan et al. 2003).

Very recently, the Syrian first lady gave special attention to the Damask rose through her appearance in a farm growing Damask rose encouraging the farmers to keep growing this unique tree. In Syria, the Damask rose can be found in Damascus and many of its suburbs and in Aleppo, and despite its aforementioned importance, there is a lack of information about its genetic diversity and the characteristics of it essential oil. Thus, the aim of this study was to document all related information regarding the genetic diversity and the essential oil characteristics of Damask rose in all Syrian regions that were found to grow this unique Shrub.

Material and methods

Plant material and DNA extraction

A set of 7 rose genotypes *Rosa damascena* Mill and one *Rosa centifolia* were collected during flowering time from various regions in Syria. From each genotype, samples from 3-5 plants were bulked and used as one sample for the chemical and the molecular analyses. Table 1 shows the general characteristics and the geographic distribution for these genotypes. A distant check that belongs to a different genus was also studied to verify the distance from the studied species.

Table 1: Genotypes used in this study, the tree characteristics, altitude, and annual.

No	Region	Species	Tree characteristics	Altitude (m)	Annual rainfall (mm)
1	Damascus , Abu-Jarash	*Rosa damascena*	upright bushes, bent thorns	730	189.8
2	Damascus , Abu-Jarash	*Rosa damascena*	upright bushes, bent thorns	730	189.8
3	Damascus , Misraba	*Rosa damascena*	fence, bent thorns	650-750	210
4	Damascus , Abu-Jarash	*Rosa centifolia*	upright bushes, little thorns	730	189.8
5	Qalamoun, Al-Mrahh	*Rosa damascena*	upright bushes, bent thorns	1550	131-250
6	Ourneh	*Rosa damascena*	fence, dense thorns	1600	800
7	Aleppo	*Rosa damascena*	fence ,little thorn	392	350-450
8	Aleppo	*Rosa damascena*	fence ,little thorns	392	350-450
9	Latakia(Costal Mountain region)	*Crataegus L.*	Upright tree 2.5m, yellow fruit,	600-700	700-800

Essential oil extraction

Essential oil was extracted from fresh flowers that were collected in the early morning of the same day according to the standard protocols (British Pharmacopoeia 1993). Two hundred g of flowers representative of each sample (air-dried at ambient temperature) were added to 2000 ml of distilled water and hydro distilled using a Clevenger-type apparatus for 3.5- 4 h where most of the essential oil was extracted and kept in dark colored glass bottles and to which an anhydrous sodium sulphate was added and stored until use at 4-6 °C.

Essential oil components

Qualitative analysis was carried out using Gas chromatography/mass spectrometry (GC/MS) analysis using an Agilent GC/MS 5937 model 5937 equipped with a DP35 capillary column (30m x 0.32 mm, 0.5um film thickness) with argon as the carrier gas at a flow rate of 0.9 ml/min and split rate at 440 ml/min. Oven temperature was performed as follows:50 °C (2 min) to 190 °C at 2 °C/min, then to 250 °C at 4 °C/min. Injector temperature was 225 °C, and interface Temp was 295 °C.; detector temperature, 260 °C; carrier gas, He (0.9 ml/min).

Quantitative analysis was carried out using an Agilent GC Model 6890 N equipped with DP35 capillary column (30m x 0.32 mm, 0.5um film thickness) with helium as the carrier gas (0.6 ml/min). Detector used was Flame Ionization Detector (FID) at 250 °C. Oven temperature used for quantitative analysis was similar to the above mentioned qualitative analysis. The components of the oil were identified by comparison of their mass spectra and retention indices (RI) with those given in literature.

Molecular analyses

DNA preparation

Collected young leaves from 3-5 shrubs representing the genotype of each location were treated as one sample. The samples were washed several times in distilled water, put in liquid nitrogen and stored at -60°C until use.

The DNA was isolated by quick method (Dorokhov and Klocke 1997). 0.4 grams of young leaves were ground to powder in liquid nitrogen using a mortar and pestle. The powder was transformed into 2ml Eppendorf tubes and mixed with 800µl of extraction buffer [200mM Tris-HCl (pH=7.5), 250mM NaCl, 25mM EDTA (Ethylenediamine tetra acetic acid), and 0.5% SDS]. The tubes were homogenized and vortexed for 5 seconds then incubated at 65 ^{o}C for 15 min. A 400µl of cold 5M Potassium acetate were added to each tube and put on ice for 10 min. The tubes were then centrifuged at 15000 rpm for 15 min. and the upper aqueous phase (about 500µl) was recovered to a new tube and mixed with the same volume of isopropanol and left for 10 min. The tubes were then centrifuged at 15000 rpm for 10 min. and the nucleic acid precipitate was washed twice with cold 65% ethanol, then centrifuged at 15000 rpm for 10 min., dried under the laminar flow and resuspended in 150µl of 0.1 X TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Integrity of DNAs was proved by agarose gel electrophoresis, and DNA concentrations were determined using a Gene Quant spectrometer (Amersham Biociences). A working concentration of all DNA samples was set at 10 ng/µl.

RAPD analysis

PCR reactions were carried out in 12.5 µl volumes containing [100 mM Tris-HCl [pH 8.8 (at 25 ^{o}C)], 50 mM (NH4)2SO4, 5X10-5Bovine syrum albumin,2X10-7 Tween20], 3.2 mM MgSO4, 0.25 mM of each dNTP (Roache), 1 unit of Taq polymerase (Fermentas), 10 ng of genomic DNA and 60 ng primer [27 primers (OPERON) and 5 primers (Amersham) (Table 2). Amplification was performed in a Genius Hybaid Thermal Cycler (Techne, UK) with the following program: 94 ^{o}C for 1 min, then 94 ^{o}C for 10 sec, 35 ^{o}C for 10 sec and 72 ^{o}C for 70 sec for 45 cycles. A final extension cycle was performed at 72 ^{o}C for 2 min. Amplification products were stored at 4oC until visualization on agarose gel electrophoresis. The PCR products were electrophoresed on 1.2% ethidium bromide (Fluka)-stained agarose (Q. BIOgene) gels in 0.5X Tris Borate EDTA (TBE). A 100 bp ladder (Vivantis) was used to estimate the approximate molecular weight of amplification products.

ISSR analysis

PCR reactions were carried out in 12.5 µl volumes containing [100 mM Tris-HCl [pH 8.8 (at 25 oC)], 50 mM (NH4)2SO4, 5X10-5Bovine syrum albumin,2X10-7 Tween20], 3.2 mM MgSO4, 0.25 mM of each dNTP (Roache), 1 unit of Taq polymerase (Fermentas), 20 ng of genomic DNA and 150 Pmol of each primer (Alfa and Invitrogen) (Table 3).

Amplification was performed in an Eppendorf Cycler with the following program: 94 oC for 5 min, then 94 ^{o}C for 10 sec, 50 ^{o}C for 10 sec and 72 ^{o}C for 10 sec for 40 cycles. A final extension cycle was performed at 72 ^{o}C for 7 min. Amplification products were stored at 4^{o}C until visualization on agarose gel electrophoresis.

The PCR products were electrophoresed on 1.8% ethidium bromide (Fluka)-stained agarose (Q. BIOgene) gels in 0.5X Tris Borate EDTA (TBE). A 100 bp ladder (Vivantis) was used to estimate the approximate molecular weight of amplification products.

Table 2: Number of RAPD amplified bands scored per primer

Primer name	Sequence	Total no. of lines	Total no. of polymorphic lines	%Polymorphic lines	No. of fragments amplified	No. of polymorphic fragments
Amersham 2	GTTTCGCTCC	17	17	100	35	35
Amersham 3	GTAGACCCGT	21	21	100	56	56
Amersham 4	AAGAGCCCGT	10	10	100	20	20
Amersham 5	AACGCGCAAC	3	2	67	13	4
Amersham 6	CCCGTCAGCA	6	3	50	33	6
OP-D08	GTGTGCCCCA	8	6	75	37	19
OP-E01	CCCAAGGTCC	16	16	100	38	38
OP-E05	TCAGGGAGGT	9	9	100	16	16
OP-E06	AAGACCCCTC	13	13	100	27	27
OP-E07	AGATGCAGCC	12	12	100	37	37
OP-E08	TCACCACGGT	6	4	67	27	9
OP-E09	CTTCACCCGA	7	7	100	19	19
OP-i08	TTTGCCCGGT	9	9	100	17	17
OP-i11	ACATGCCGTG	17	16	94	62	53
OP-i12	AGAGGGCACA	15	15	100	49	49
OP-i15	TCATCCGAGG	8	8	100	17	17
OP-i17	GGTGGTGATG	1	0	0	9	0
OP-N03	GGTACTCCCC	10	9	90	35	26
OP-N04	GACCGACCCA	14	14	100	38	38
OP-N05	ACTGAACGCC	12	11	92	45	36
OP-N14	TCGTGCGGGT	10	10	100	30	30
OP-N18	GGTGAGGTCA	13	13	100	33	33
OP-R04	CCCGTAGCAC	20	20	100	52	52
OP-R06	GTCTACGGCA	14	14	100	33	33
OP-R08	CCCGTTGCCT	6	6	100	17	17
OP-R09	TGAGCACGAG	14	14	100	47	47
OP-R13	GGACGACAAG	11	9	82	50	32
OP-R15	GGACAACGAG	9	7	78	38	20
OP-R17	CCGTACGTAG	2	1	50	10	1
OP-Z04	AGGCTGTGCT	16	16	100	54	54
OP-Z07	CCAGGAGGAC	17	17	100	53	53
OP-Z11	CTCAGTCGCA	18	18	100	49	49
Sum		**364**	**347**		**1096**	**943**

Statistical analysis

Results of essential oil analyses for the studied Rosa damascena biotypes were statistically analyzed using GenStat exectable program to construct the phylogenetic cluster based on the chemical analyses of essential oils. The same data was also introduced to M-STAT program to obtain the least significant difference (LSD) between all tested biotypes.

Resultant bands were screened and photographed under UV light. All reactions were repeated at least twice and only bands that were bright, reproducible were scored for the analysis. Bands were scored either 1 as present or 0 as absent, and the unweighted pair group method with arithmetic averages (UPGMA) (Sneath et al. 1973) and percent disagreement values of the STATISTICA program (STATSOFT, Inc. 2003) were used to construct the matrix and the phylogenetic trees.

Results and discussion

Table 4 shows the percentages of the 18 essential oil components determined from all rose samples tested using both GC and GC-MS techniques. The total percentage of all components ranged from 52.5 to 78.6% for *Rosa damascena* samples whereas it was 51.1% for *Rosa centifolia*. The five major components were: B- citronellol (9.8- 46.9%), eugenol methyl ether (1.1-22.6%), nonadecane (0.8-23%), henicosane (0.4-15.2%), phenyl –ethyl alcohol (1.4-10.4%). Significant differences were recorded

among the major components according to the geographical locations, indicating that there are more than one chemical type of essential oil in Damascus and its suburbs (1, 2, and 3), Ourneh (6), Qalamoun (5), and Aleppo (7 and 8). Damascus samples (1, 2 and 3) were characterized by having significantly lower levels of B- citronellol, geraniol, phenyl –ethyl alcohol compared to other locations, whereas, significantly higher levels of nonadecane, Z5-nonadecane, eicosane, henicosane and tricosane were realized compared to the samples of other locations. It should be noted that Damascus samples (1 and 2) were also distinct from that of its suburb (3). As for Aleppo samples (7 and 8), eugenol methyl ether attained highest percentage (21.8- 22.6%) followed by citronellol (21.7- 22.1%), but were characterized by having low levels of nonadecane, eicosane, henicosane and tricosane compared to the samples collected from other locations. On the other hand, Qalamoun biotype (5) was characterized by having statistically significant higher levels of citronellol and geraniol, and lower levels of nonadecane, eicosane, henicosane and tricosane compared to the samples collected from other locations.

Table 3: Number of ISSR amplified bands scored per primer

Primer name	Sequence	Total no. of lines	Total no. of polymorphic lines	%Polymorphic lines	No. of fragments amplified	No. of polymorphic fragments
A1	(CA)6RR	13	12	92	56	47
A4	(CA)6RY	9	9	100	28	28
A7	(CA)6RM	15	15	100	36	36
A10	(CA)6RK	3	0	0	27	0
A13	(CA)6RS	5	1	20	37	1
A16	(CA)6R	7	4	57	35	8
A26	(CA)6K	9	8	89	27	18
A31	(AGC)4R	8	8	100	19	19
A34	(AGC)4Y	10	7	70	57	30
A38	(AGC)4M	7	5	71	37	19
A41	(AGC)4K	14	11	79	72	45
A44	(AGC)4S	11	8	73	61	34
B3	(CT)8TG	17	17	100	41	41
B4	(CA)6 GG	5	2	40	38	11
B7	(GTG)3 GC	6	6	100	22	22
B10	(CAG)5	4	1	25	32	5
B13	(CAA)5	18	17	94	61	52
C22	(AG)8T	6	4	67	36	18
D4	(GATA)4	13	13	100	50	50
164/2	(AG)10 C	7	6	86	35	35
164/4	(GACA)4	10	7	70	54	27
Sum		**197**	**161**		**861**	**546**

Ourneh sample (6) was characterized by having highest level of Phenyl ethyl Alcohol (10.4%) and reasonably high levels of B- citronellol and eugenol methyl ether (28% and 13.8% respectively) among all its essential oil components.

Indirect environmental factors especially rainfall and altitude seems to have a big role in the determination of components ratio. Thus the percentage of B- citronellol reached an enormous 47% followed by 8.2% of geraniol whereas none of the other components reach 2% in sample 5 (Qalamoun). Damascus samples 1 and 2 and that of its suburb 3 contained the lowest levels of B- citronellol (9.6- 13%), whereas the percentages of nonadecane, eicosane, henicosane and tricosane were highest among all *Rosa damascena* samples. As for Aleppo biotypes 7 and 8 B- citronellol level was around 22% and

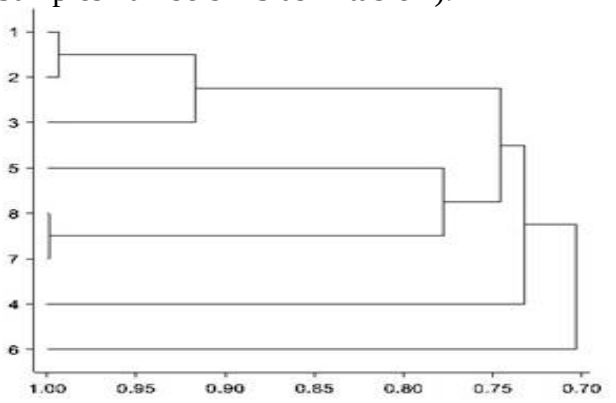
that of geraniol around 4.7% whereas nonadecane level was very low (1.2%).

The variation in the percentage of the essential oil components among the studied samples may be due to the specific environmental conditions in each area that have formulated these biotypes through the years, with any change in the environmental conditions would probably lead to a change in the selection pressure, and thus a differential gene selection would be expected from one sample to another within the entire region (Yousefi et al. 2009).

Using Genestat program, the different essential components were included in the similarity matrix to establish the dendrogram based on the chemical analyses (Figure 1A). The rose samples were clustered into two main clusters: The first contains only Ourneh type (6), and the second contains two subclusters. The first subcluster contained the *Rosa centifolia* check (4), and the second was divided into two groups. The first group contained Damascus region samples (1, 2 and 3), whereas, the second group contained two subgroups. The first contained Aleppo region samples (7 and 8), and the second contained Al-Qalamoun sample (5).

Figure 1A shows clearly that the Aleppo samples 7 and 8 were almost identical in their essential oil components and its concentration (99%). It should be noted that these two samples were collected from places that have similar altitude (316 and 369m), and the same applies to Damascus samples 1 and 2 which also attained high similarity percentage (97.3%). Figure 1A also shows that Ourneh sample (6) was the outgroup in the cluster, even more than the *R. centifolia* check (4). Quantitative and qualitative differences have been reported between the chemical composition and the aroma constituents of essential oil extracted from *R. damascena* and *R. centifolia*. Thus, Khan and Rehman (2005) found that the recovery of essential oil from petals of *R. damascena* was higher (0.24%) than *R. centifolia* (0.22%) on fresh weight basis and that oil color of the latter was yellowish brown while the color of absolute oil of the former was yellowish. Our results showed that except for farnesol and 9 nonadecane, all other essential oil components of *R. centifolia* were lower than that of *R. damascena* (Table 4). These results are contradictory to those reported by Khan and Rehman (2005) who mentioned that the only component that was higher in *R. damascena* compared to *R. centifolia* was phenyl ethyl alcohol. It is evident that the components of *R. damascena* essential oil are favorable to those of the other rose species oils. Among *R. damascena* essential oil components B-citronellol is one of the most important and which gives it the relative preference compared to other rose oils. The percentage of B-citronellol was highest in Al-Qalamoun sample (5) reaching 46.9% followed by Ourneh sample (6) 28%, whereas Damascus sample (3) attained the lowest percentage among all *R. damascena* studied samples (9.65%) which is still considered as high compared to B-citronellol percentage attained in R. centifolia (sample 4) (2.95%).

Figure 1: Dendrogram showing the degree of relatedness among the rose samples based on:
(A) The chemical analysis of essential oil (Samples numbers 1-9 as in table 1).



(B) Combined RAPDs and ISSR data.

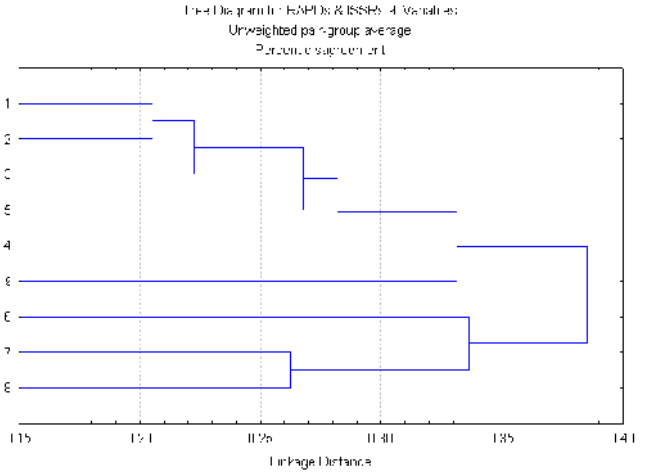


Table 4: Essential oil components of studied rose samples

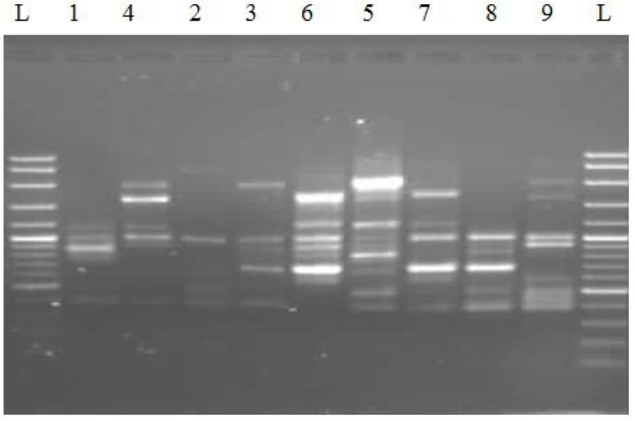
Component / Sample	1	2	3	4	5	6	7	8	LSD5 %
Linalool	E -	E -	E -	E -	C 1.9	D 1.47	A 2.36	B 2.26	0.013
Rose oxide	A 3.42	A 3.38	E 1.06	F 0.88	G -	D 1.77	B 2.49	C 2.38	0.049
Phenyle ethyle Alcohol	E 1.57	F 1.4	D 1.85	G 1.33	B -	A 10.38	C 8.48	b 9.01	0.067
a- terpiniol	E -	E -	E -	E -	C 0.56	D 0.47	A 1.12	B 1.01	0.012
B-Citronellol	E 12.95	F 11.84	G 9.64	H 2.95	A 46.9	B 27.95	D 21.65	C 22.1	0.066
Citral	D -	D -	D -	D -	B 0.66	C 0.36	A 1.36	A 1.3	0.013
Trans-Geraneol	F 1.91	E 2.54	G 1.35	H -	A 8.19	D 3.59	B 4.82	C 4.65	0.017
Eugenol methyle ether	E 4.95	D 5.37	F 2.68	F 2.72	G 1.11	C 13.83	A 22.57	B 21.8	0.019
a- Muurolene	B -	B -	B -	B -	A 0.26	B -	B -	B -	0.006
Pentadecane	B -	B -	A 0.51	B -	B -	B -	B -	B -	0.006
a-Cadinene	B -	B -	B -	B -	B -	A 0.49	B -	B -	0.006
Farnesol	C -	C -	C -	A 0.95	B 0.37	C -	C -	C -	0.008
9-Nonadecan	F -	F -	F -	A 9.8	E 0.16	B 2.44	D 0.53	C 0.69	0.015
Z5-Nonadecan	A 6.56	B 6.54	C 4.03	D 0.74	G -	G -	E 0.5	F 0.44	0.015
Nonadecan	B 22.58	A 22.98	D 15.01	C 17.55	B 0.76	E 5.45	F 1.24	G 1.18	0.017
Eicosan	A 4.08	B 3.87	C 2.35	D 2.26	G 0.29	E 1.04	G 0.28	F 0.4	0.013
Henicosan	A 15.22	B 14.96	D 10.39	C 11.9	F 1.85	E 2.87	G 0.66	H 0.43	0.015
Tricosan	B 5.37	A 5.54	C 3.6	F -	D 1.81	E 0.75	F -	F -	0.061
Sum	**78.61**	**78.42**	**52.46**	**51.08**	**64.82**	**72.86**	**68.07**	**67.7**	

It should be noted that Ourneh location is distinguished by its high altitude (1600m) and high annual rainfall (800mm) compared to other locations. This result is in conformity with those reported by Tabaei-Aghdaei et al. (2007) regarding the clustering of their landraces collected from different regions in Iran. They have shown that a landrace (no 12) collected from a cold and humid region was separated from those collected from all 13 other regions. On the other hand, it seems that the oil quality of Ourneh sample (6) in our study is reasonably good (28%) since it has twice the minimum B-citronellol percentage reported by Khouzani et al. (2007).

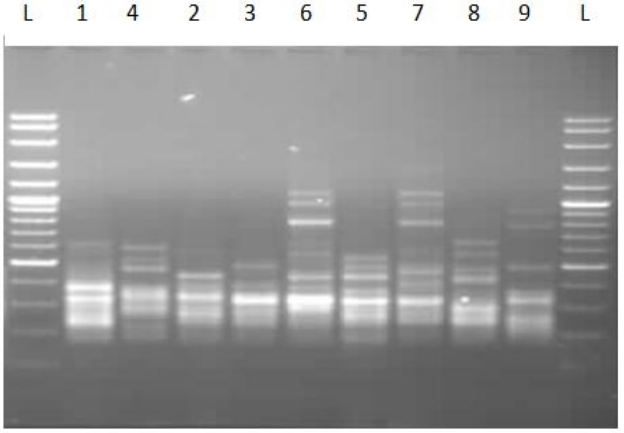
*RAPD analysis*The 32 RAPD primers applied on the tested rose samples resulted in 364 lines, out of which 347 lines were polymorphic (95%). Figure 2A shows the highly polymorphic banding patterns resultant from the use of primer OP-N05. The primers showed polymorphic lines that ranged from 0 (OP-I17) to 100% in 21 primers (Table 2). The total number of unambiguous DNA fragments was 1096 with 943 of which being polymorphic (86%). This percentage is higher than that obtained by Tabaei-Aghdaei et al (2006) who studied 12 Iranian rose landraces using 22 RAPD primers (67%), and the 55.4% obtained by Kiani et al. (2008) who studied 41 Iranian lines using 31 RAPD

primers. In a previous study using 13 RAPD primers on 10 *R. damascena* cultivars and 9 wild species from three Rose groups, Debener et al. (1996) showed that cultivated rose varieties contain larger genetic diversity than the wild species. They concluded that cultivated roses display a high level of genetic variability despite the fact that single morphological and physiological characters may be less polymorphic within rose groups.

Figure 2. (A) Polymorphism generated from the use of RAPD primer OP-N05 on rose samples studied (L: 100bp DNA ladder, Samples numbers 1-9 as in table 1)



(B) Polymorphism generated from the use of ISSR primer B3 on rose samples studied. (L: 100bp DNA ladder, Samples numbers 1-9 as in table 1)



The size of amplified fragments ranged between 300 and 3000bp. Table 5 shows the 5 rose samples that were distinguished by a unique RAPD band from applying different primers. The highest MW was obtained with OP-E09 and sample 8 (2500bp) and the lowest was obtained with OP-E07 and sample 1 (650bp). Notably OP-E09characterized two samples whereas Amer 3, Amer 4, Amer 5, OP-E07, OP-N03, OP-N18 and OP-R15 characterized one sample.

Table 5: Approximate molecular weights for unique bands using RAPD and ISSR

Sample No.	Region	Primer	Unique band MW
	RAPD unique bands		
1	Damascus , Abu-Jarash	OP-E07	650
1	Damascus , Abu-Jarash	OP-N03	1200
1	Damascus , Abu-Jarash	Amersham4	1500-2000
4	*R. centifolia*	Amersham3	850
5	Qalamoun, Al-Mrahh	OP-E09	700
6	Ourneh	OP-R15	1000
6	Ourneh	OP-E09	2500
7	Aleppo	OP-N18	800
8	Aleppo	Amersham5	1500
	ISSR unique bands		
6	Ourneh	C26	1000
6	Ourneh	164-4	500-400
7	Aleppo	A26	600
7	Aleppo	A26	700
7	Aleppo	A26	1000
8	Aleppo	B7	600

In order to estimate the degree of relatedness among the studied rose cultivars based on common amplified fragments, a percent disagreement values (PDV) matrix was established. In the matrix, higher values denotes to the existence of large genetic diversity which may be due to the occurrence of different crosses and selections of segregates under variable environmental pressures, leading to rose genomes comprising mosaics of different species genomes (Debener et al. 1996).

The RAPD matrix (data not shown) showed that percent disagreement values (PDV) were lowest between samples 1 and 2 (from Damascus) (0.24) and highest between sample 6 (Ourneh) and the *Crataegus* check (no 9) (0.49), followed by sample 6 (Ourneh) and sample 5 (Qalamoun), and between sample 6 (Ourneh) and the *R. centifolia* (no 4) sample (0.45). This indicates that sample 6 (Ourneh) is quite distal from both checks (*Crataegus* no 9 and *R. centifolia* no 4) as well as from sample 5 (Qalamoun). The matrix data was put in a dendrogram (data not shown) which shows a close representation of the values obtained in the PDV matrix. The rose samples were split into two main clusters: The first contained the Damascus and its suburb samples and was characterized by its low PDV, the most closely related samples of all were 1

and 2 (PDV=0.24) followed by sample 3 (Damascus suburb) and with *R. centifolia* (no 4), then with sample 5 (Qalamoun). The second cluster included samples 7 and 8 (Aleppo) at (PDV=0.25) which were combined with sample 6 (Ourneh) at (PDV=0.31 and 0.36 respectively). It should be noted that the dendrogram divided the samples according to the annual rainfall rates, thus all samples in cluster 1 had lesser than 250mm whereas those of cluster 2 had more than 350mm (Table1).

ISSR analysis

To further evaluate the genetic relatedness among the collected rose samples, They were subjected to 21 ISSR primers ranging between 13 and 21bases. Table 3 shows the primer sequences, number of lines per primer, number of polymorphic lines and number of polymorphic bands, and Figure 2B shows the amplification products resultant from the use of primer B1 on the studied samples.

The use of the 21 primers for DNA amplification on the studied samples generated 197 lines of bands of which 161 (81.7%) were polymorphic. Only 6 primers generated polymorphism in

all lines of bands (100%). The polymorphism in the remaining 15 primers ranged from 0% for primer A10 to to 94% for primer B13. The total number of bands generated from all primers used was 861 bands of which 546 were Polymorphic (63.4%)(Table 3). It should be noted that Crespel et al. (2009) were the first to report that ISSR was an efficient technique for varietal identification and for the estimation of genetic relatedness among rose cultivars, they were able to discriminate among 33 rose cultivar, and obtained a high polymorphism percentage (93.7%). On the other hand, Jabbarzadeh et al. (2010) recommended the use of ISSR to discriminate among *Rosa* species since the technique does not need any prior primer sequence information in addition to being very simple, fast, cost effective, highly discriminative, reliable and requires small quantity of sample DNA.

The PDV matrix established and the resultant dendrogram (data not shown) indicated that highest PDV obtained was between *R. centifolia* no 4 and sample 6 (Ourneh) (0.46) and the lowest between samples 1 and 2 (from Damascus) and between 2 and 3 (0.14).

The rose samples were split into two main clusters: The first contained the samples 7 and 8 (Aleppo) and sample 6 (Ourneh) and the second cluster contained two subclusters, the first included Crataegus no 9 and the second contained two groups *R. centifolia* no 4 in one and all Damascus samples 1, 2 and 3 and sample 5 (Qalamoun). ISSR divided the samples in a similar way to that of RAPD except that *R. centifolia* no 4 is distal to all *R. damascena* samples in the cluster. The other difference was that the degree of variance among the samples was, as expected, lower in ISSR than that of RAPD, due to lower number of amplification fragments in the former compared to the latter (data not shown).

The sizes of DNA bands shown in amplification products ranged from 400–1000 bp. Unique bands were observed for two samples (sizes 300 to 1250 bp). Table 5 shows the rose samples that were distinguished by a unique band from applying different primers. Sample 1 was distinguished by 3 bands and primer A26 discriminated this biotype by two unique bands at MW 600 and 700bp. sample 6 (Ourneh) was also distinguished by two unique bands when the two primers used C26 and 164-4.

Combined RAPD & ISSR analyses

Since RAPD and ISSR amplify different parts of the genome, the data of the two applied marker techniques were combined to obtain more balanced values for genetic similarity among cultivars and an equilibrated dendrogram representation of the relationships among the studied rose samples. The resultant matrix showed that the highest PDV obtained was 0.47 (between sample 6 (Ourneh) and the *Crataegus* check no 9), and the lowest PDV was 0.21 found between sample 1 and 2 (Damascus) and between sample 2 and 3 (data not shown).

The dendrogram resultant from the PDV matrix of RAPD and ISSR data is shown in Figure 1B. The rose samples were split into two main clusters: The first contained two subclusters, the first included *Crataegus* no 9 and the second contained a group including Damascus samples 1, 2 sample 5 (Qalamoun) and *R. centifolia* no 4, and the second main cluster con-

tained samples 7 and 8 (Aleppo) and sample 6 (Ourneh) . The combination of both markers allowed us to explore the DNA polymorphism in the collection of samples analyzed, and generate many polymorphic markers ensuring a good coverage of the genome. These results suggest that the RAPD and ISSR procedures are viable approaches for the examination of the DNA diversity in *R. damascena*.

The relationship between chemical and molecular characterization

In general, the results of both the chemical and molecular studies showed that studied samples were, to a large extent, clustered in a similar way according to the essential oils characteristics and molecular genetics characteristics. In both cases, most of the samples belonging to *R. damascena*, except for sample 6, were close to each other. It should be noted that sample 6 belonged to Ourneh region which is characterized by being located on high altitude area (1600m) with significantly higher annual rainfall rate (800mm) than all other regions.

The effect of environmental conditions on oil quality is not surprising, Younis et al. (2007) reported that the quantity and the quality of distilled petals rose oil are strongly affected by each of the environmental conditions, petals harvest time, the techniques used in industry and distillation, and by the cultivars. What is surprising that variability whether in oil characteristics or on the molecular genetics level being larger within the same species than that of among species. In fact, it is quite difficult to interpret the result that sample 4 which belong to a different species *R. centifolia* is closer to the other samples that belong to *R. damascena* than sample 6 from Ourneh region which also belongs to *R. damascena*, especially that *R. damascena* is the only species characterized by its unique quality in essential oil. This partially explains the low level of genetic diversity in Damask rose in Bulgaria and Turkey, the two main producers of Damask rose essential oil worldwide. Rusanov et al. (2005) showed no mutated alleles in 33 microsatellite loci after analysis of 31 *R. damascena* accessions originating from common ancestor genotype (phenotype trigintipetala), which have been vegetatively propagated for a long time (at least one to four centuries) in different geographic regions of Europe and Asia. Therefore, It is suggested that sample 6 may not belong to *R. damascena* but to another species. To definitely resolve this issue, further analyses using cytoplasmic DNA techniques aiming at detecting variations at the species level are required.

In a recent review paper, Rusanov et al. (2009) reported that Damask rose oil world production depends on only one or very few genotypes. Turkish scientists Agaoglu et al. (2000a 2000b) and Baydar et al. (2004) did not find any differences among their local lines using several molecular techniques (RAPD, AFLP and SSR). The Bulgarian scientists reached the same result when they analyzed lines from Iran, India and Turkey. Furthermore, they did not find any statistical differences among old Damask rose lines grown in Western Europe for essential oil production (Rusanov et al. 2005). However, genetic diversity studies carried out in Iran revealed high levels of polymorphism among rose genotypes collected from the most important cultivation regions for this species (Emadpour et al. 2009, Pirseyedi et al. 2005, Babaei et al. 2007, Kiani et al. 2008) suggesting that Iran is a center of diversity of Damask rose.

Our results are in conformity with those from Iran, since our samples showed a large genetic diversity which may be due to the fact that these two regions represent centers of diversity of Damask rose, not ruling out possible other diversity centers for *R. damascena*. It should be stressed that the narrow genetic base in Damask rose reported in Bulgaria and Turkey is most probably due to relying on one phenotype (trigintipetala) that has been vegetatively propagated for a long time in different geographic regions of Europe and Asia because of the strict parameters of the rose oil that has been governed by the international standard ISO9842 which specifies certain characteristics of the oil of rose (*Rosa x damascena* Miller) to facilitate assessment of its quality.

Conclusions

The present study concluded that there were a high degree of conformity between the two dendrograms based on the chemical composi-

tion of essential oil on one hand and on the DNA level on the other hand, stressing the role of environment (mostly annual rainfall) on oil characteristics quality. On the basis of these results, it was suggested that sample 6 may not belong to Rosa damascena but to another species. However, this needs further confirmation using cytoplasmic DNA techniques that target the variability at the species level.

Acknowledgements: The authors would like to thank Prof. I. Othman, Director General of AECS for his support throughout the period of this research. The technical assistance of Mr. A. Oudeh in the chemical analysis and Mrs Q. Ganimeh in the molecular analysis is much appreciated.

References

Achuthan, C.R., Babu, B.H., Padikkala, J. 2003. Antioxidant and hepatoprotective effects of *Rosa damascena*. *Pharmaceutical Biology*, 41: 357–361.

Agaoglu, Y. S. 2000a. Molecular analysis of genetic diversity of oil rose (*Rosa damascena* Mill.) grown in Isparta (Turkey). *Biotechnology and Biotechnological Equipment*, 2: 16-18.

Agaoglu, Y. S. 2000b. Rose oil industry and the production of oil rose (*Rosa damascena* Mill.) in Turkey. *Biotechnology and Biotechnological Equipment*, 2: 8-15.

Ardogan, B.C., Baydar, H., Kaya, S., Demirci, M., Ozbasar, D., Mumcu, E. 2002 . Antimicrobal activity and chemical composition of some essential oils. *Archives of Pharmacal Research*, 25: 860–864.

Babaei, A., Tabaei-Aghdaei, S., Khosh-Khui, M., Omidbaigi, R., Naghavi,M.,Esselink,G., Smulders, M. 2007. Microsatellite analysis of Damask rose (*Rosa damascena* Mill.) accessions from various regions in Iran reveals multiple genotypes. *BMC Plant Biology*. (doi:10.1186/1471-2229-7-12).

Basim, E., Basim,H. 2003. Antibacterial activity of *Rosa damascena* essential oil. *Fitoterapia*,74: 394–396.

Baydar, N., Baydar, H., Debener, T. 2004. Analysis of genetic relationships among Rosa damascena plants grown in Turkey by using AFLP and microsatellite markers. *Journal of Biotechnology* 3 : 263-267.

Beales, P., Cairns, T.,Duncan, W.,*Fagan, G.;Grant, W.,Grapes, K.;Harkness, P.;Hughes, K.; Mattock, J.; Ruston, D.;Sutherland, P., Williams, T. 1998. The encyclopedia of roses. Botanica's roses.*Random House, Australia,.

Bown, D. 2001. The Herb Society of America New Encyclopedia of Herbs and Their Uses. London: Dorling Kindersley Ltd,.

British Pharmacopoeia.1993. British Pharmacopoeia Commission. Publisher Stationery Office Books (12 pages). ISBN: 9780113219827.

Buckle, J. 2003. Clinical aromatherapy: Essential oils in practice, 2nd Ed. Edinburgh: Churchill Livingstone.

Chevallier, A. 1996. *The Encyclopedia of Medicinal Plants.* Dorling Kindersely, London, UK,.

Crespel, L., Pernet, A., LeBris, M., Gudin, S.; and Hibrand Saint Oyant L. 2009. Application of ISSRs for cultivar identification and assessment of genetic relationships in rose. *Plant Breeding*,. 128: 501-506.

Debener, T., Bartels, C., Mattiesch, L. 1996. RAPD analysis of genetic variation between a group of rose cultivars and selected wild rose species. *Molecular Breeding* 119: 71- 74.

Dorokhov, D. B., Klocke, E. 1997. A rapid and economic technique for RAPD analysis of plant genomes. *Russian Journal of Genetics*, 33: 358-365.

Douglas, M. 1993. Rose: Rosa damascena 'Trigintipetala' Redbank Research Station. Central Otago, New Zealand Institute for Crop and Food Research, pp. 1-5.

Emadpour, M., Hosseini, R., Beiramizadeh, E. 2009. A high genetic diversity revealed between commercial rose cultivars by RAPD-PCR technique. Int. . *Journal of Plant Production*. 4: 61-66.

Grieve, M. 1971. A Modern Herbal: The Medicinal, Culinary, Cosmetic and Economic

Properties, Cultivation and Folk-Lore of Herbs, Grasses, Fungi, Shrubs & Trees with their Modern Scientific Uses. Dover Publications, Inc. New York. ISBN: 0-486-22798-7.

Gudin, S. 2000. Rose: genetics and breeding. Plant Breeding Reviews, (J. Janick Ed.), John Wiley and Sons, Inc., 17, 159-189.

Jabbarzadeh, Z M., Khosh-khui, H., Salehi A., 2010. Inter simple sequence repeat (ISSR) markers as reproducible and specific tools for genetic diversity analysis of rose species. . *African Journal of Biotechnology*, 9 (37): 6091-6095.

Khan , M. A., Rehman, S.U. 2005. Extraction and Analysis of Essential Oil of Rosa species. *International Journal of Agricultral Biology*, 7: 973-974.

Khouzani, H. L., Fini, S. O., Safari, J. 2007. Essential Oil Composition of *Rosa damascena* Mill Cultivated in Central Iran. *Scientia Iranica*, 14: 316-319.

Kiani, M., Zamani, Z., Khalighi, A., Fatahi, R. And Byrne, D.H. 2008. Wide genetic diversity of *Rosa damascene* Mill. Germplasm in Iran as revealed by RAPD analysis. . *Scientia Horticulturae*, 115: 386-392.

Lawless, J. The Illustrated Encyclopedia of Essential Oils . Element Books, Inc. Rockport, MA 1995;132

Narayan, K. U. D. P. 2003. Agro's Dictionary of Medicinal Plants. p. 292. Publishers Agrobios. India. ISBN: 8177541730.

Özkan, G., Sagdiç, N. G., Baydar, N. , Baydar, H. 2004. Antioxidant and Antibacterial Activities of *Rosa Damascena* Flower Extracts. *Food Science and Technology International*, 10: 277-281.

Pirseyedi, M., Mardi, M., Davazdahemami, S., Kermani, M.; Mohammadi, A. 2005. Analysis of the genetic diversity 12 Iranian Damask rose (*Rosa damascena* Mill.) genotypes using amplified fragment length polymorphism markers. *Iranian Journal of Biotechnology*, 3: 225-230.

Rusanov, K., Kovacheva, N., Atanassov, A. Atanassov, I. 2009. *Rosa damascene* – Genetics of a complex allotetraploid species and perspectives for molecular breeding. *Biotechnology & Biotechnological Equipment* 23/2009/SE SPECIAL EDITION/ON-LINE 23: 594- 596.

Rusanov, R., Kovacheva, N., Vosman, B., Zhag, L., Rajapakse, S., Atavassov, A. and Atanassov, I. 2005. Microsatellite analysis of *Rosa damascene* Mill. Accessions reveal genetic similarity between genotypes used for rose oil production and oil Damask rose varieties *Theoretical and Applied Genetics* 111: 804-809.

Schnaubelt, K. 1998. Advanced Aromatherapy: The Science of Essential Oil Therapy. Rochester, VT: Healing Arts Press. ISBN-13: 9780892817436

Sneath PHA and Sokal RR, 1973. Numerical Taxonomy, the principles and practice of numerical classification. San Francisco: Freeman, 573 pp.

STATSOFT, Inc. 2003. STATISTICA (data analysis software system), version 6, www.statsoft.com..

Tabaei -Aghdaei , S.R., Babei, A., Khosh-Khui, M., Jaimand, K. Rezaee, M.B.; Assareh, M.H., and Naghavi, M.R., 2007. Morphological and oil content variations amongst Damask rose (*Rosa damascene* Mill.) landraces from different regions of Iran. *Scientia Horticulturae* , 113: 44-48.

Tabaei , S., Hosseini, H., Fahimi, H., *et al.* 2006. Genetic variation analysis of different populations of *Rosa damascena* in NW. Iran using RAPD Markers. . *Iranian Journal of Botany*. 12: 121-127.

Younis A., Khan M.A., Khan A.A., Riaz A., Pervez M.A. 2007. *Floriculture Ornamental Biotechnol*, 1: 73-76.

Yousefi, B., Tabaei -Aghdaei, S.R., Darvish, F. and Assareh, M.H. 2009. Flower yield performance and stability of various *Rosa damascene* Mill. Landraces under different ecological conditions. *Scientia Horticulturae*, 121: 333-339.

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