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CHANGES IN MONOTERPENE COMPOSITION OF *MENTHA AQUATICA* PRODUCED BY GENE SUBSTITUTION FROM *M. ARVENSIS*

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Abstract—*Mentha arvensis* var. *piperascens* ($2n = 96$) genotype *cc AA PP RR FF* has 70–75% L-menthol and less than 0·1% menthofuran whereas *M. aquatica* ($2n = 96$) has 60–80% menthofuran and less than 1% menthol. Each of two cultivars of *M. arvensis* hybridized with either the Dutch or German strains of *M. aquatica* produced 780 F_1 hybrid individuals with a menthol herbage odor and assays show that these hybrids may have 34·2–72·1% L-menthol and 0·4–25% menthofuran. Backcrosses of the F_1 hybrids to the *M. aquatica* parent gave a total of 2076 menthol-odored individuals genotype *cc AA PP RR Ff* to 2106 menthofuran-odored individuals genotype *cc AA PP RR ff* with the odor of the parental *M. aquatica* strains. The 1 : 1 ratio shows that the single incompletely dominant gene *F* controls production of substantial amounts of menthol or menthofuran. Oil from menthol-odored individuals of the 4th backcross progenies contains 1·5% menthofuran, 10·5% menthones, 47·8% menthols, and 14·3% menthyl esters as compared that of *M. aquatica* with 66·4% menthofuran and only 0·3% menthone and its products. The *FF* genotype allows less than 0·1% menthofuran, the *Ff* genotype 0·4–25%, and the *ff* genotype 60–80%, with the oxidation of pulegone to menthofuran taking precedence over the reduction of pulegone to menthone.

INTRODUCTION

THE OBJECTIVE of the convergent backcrossing program was to substitute the gene or genes that cause 70–75% L-menthol in *Mentha arvensis* L. var. *piperascens* Briq. into *M. aquatica* L. which usually has 60–80% menthofuran and less than 1% L-menthol. This chemogenetic study should determine the inherited basis for the major differences in chemical composition between the two octoploid species ($2n = 96$) and establish the genic basis for the oxidation of pulegone to menthofuran.

RESULTS

Analyses of commercial samples of *M. arvensis* oil have been reported by Smith and Levi.[1] Oil from a first generation inbred strain of *M. arvensis* grown at South Bend, Indiana had about 3% piperitone, 0·1% pulegone, 0·04% menthofuran, 10% menthone, 73% menthol, and 3% menthyl acetate. This strain has the truebreeding genotype *cc AA PP RR FF* since all its self-pollinated progeny have 60–80% L-menthol. The *M. arvensis* genotype must be *cc AA* since the dominant gene *C* causes carvone and the double recessive *cc aa* piperitone.[2] The genotype *cc AA* causes pulegone formation and the dominant gene or genes

[1] D. M. SMITH and L. LEVI, *J. Agric. Food Chem.* **9**, 230 (1961).
[2] M. J. MURRAY, *Genetics* **45**, 931 (1960).

P allow the conversion of pulegone to menthone.[3] The dominant *R* gene allows the conversion of menthone to menthol, whereas the recessive *r* gene allows very little conversion[4] as shown in columns 3 and 4 of Table 3.[3]

The true-breeding genotype of both the Dutch (donated by R. Hegnauer) and German (donated by S. R. Baquar) strains of *M. aquatica* is *cc AA PP RR ff* as the following genetic results will demonstrate. The substitution of the dominant gene *C* causes carvone formation in *M. aquatica*.[5] The cross of *M. aquatica* to a fertile polyploid of *M. spicata* having the genotype *cc cc aa aa* and high piperitone gave 710 peppermint-odored hybrids with evident menthol. This shows clearly that both *M. aquatica* strains have the genotype *cc AA*, not *cc Aa* or *cc aa* since any *cc aa* hybrid of the above cross would have a musty piperitone odor, not the hot odor of menthone and the cool odor of its alcohol menthol. The cross of *M. aquatica* to a strain of *M. arvensis* having 80% menthone (see column 4 of Table 3) and genotype *cc AA PP rr FF* produced 171 F_1 hybrids having high menthol. This shows that *M. aquatica* has the genotype *RR* even though the oil of the species has very little menthol.[5,6] While the monogenic basis of the *P* gene has not been established, the recessive gene or genes found in *M. gattefossei*,[3] certain wild-type *M. arvensis*, and *M. pulegium* largely prevent the conversion of pulegone to menthone and result in the accumulation of pulegone.[7,8]

The oil from mature herbage of the two parental strains of *M. aquatica* had 60–80% menthofuran, with the lower very mature leaves having less menthofuran than the flowers and upper younger leaves. Significant differences in oil composition during plant development were not observed in material grown at Freiburg.[6] Strains grown on the mineral soil in Freiburg (48°) did not differ in menthofuran content from the same strains grown on organic soil at South Bend, Ind. (43°20′) if harvested at approximately the same stage of maturity. The *M. aquatica* strains breed true for a high menthofuran content. This statement is supported by the fact that over 7000 self-pollinated progeny had a strong menthofuran herbage odor and by assays of more than 30 individuals.

A self-pollinated selection of the standard cultivar strain of *M. arvensis* var. *piperascens* and the Northern Progress variety from Japan breed true for 60–80% menthol. Thus over 1000 self-pollinated individuals had a strong menthol herbage odor and assays of 50 individuals showed 60–80% menthol and no more than 0·1% menthofuran. Unlike *M. piperita*, maturity has little effect on the menthol content of the *M. arvensis* parental strains.

When either strain of *M. aquatica* was hybridized with either strain of *M. arvensis*, all 780 F_1 hybrids of the four hybrid combinations had a menthol herbage odor and exhibited differences in plant maturity. GLC assays of the oil of 50 F_1 individuals for 12 principal oil constituents showed that the F_1 hybrids varied in menthol and menthofuran content without any obvious relationship to inherited differences in plant maturity. Upon the basis of these preliminary assays, the three F_1 hybrids (for which detailed assays are given in Table 1) were selected to illustrate the quantitative variation found in the menthone, menthol, and menthofuran content of F_1 individuals. These differences are largely the result of the segregation of modifying genes present in the *M. arvensis* parent which determines whether a self-pollinated *M. arvensis* individual has 60, 72, 80, or 88% menthol. It is also possible that there are some modifying genes in the *M. aquatica* parent.

[3] M. J. MURRAY, P. M. MARBLE and D. E. LINCOLN, *J. Hered.* **62**, 363 (1971).
[4] M. J. MURRAY, *Genetics* **45**, 925 (1960).
[5] F. W. HEFENDEHL and M. J. MURRAY, *Phytochem.* **11**, 189 (1972).
[6] F. W. HEFENDEHL, *Arch. Pharmazie* **300**, 438 (1967).
[7] E. VON RUDLOFF and F. W. HEFENDEHL, *Can. J. Chem.* **44**, 2015 (1966).
[8] F. W. HEFENDEHL, *Phytochem.* **9**, 1985 (1970).

TABLE 1. QUANTITATIVE COMPOSITION OF THREE *M. arvensis* × *M. aquatica* F_1 HYBRIDS ILLUSTRATING THE VARIABILITY FOUND IN THE MENTHONE, MENTHOL, AND MENTHOFURAN CONTENT OF F_1 INDIVIDUALS

	Oil constituents	Hybrid 1 (%)	Hybrid 2 (%)	Hybrid 3 (%)
1	α-Pinene	0·5	1·1	0·7
2	β-Pinene	0·7	1·1	0·8
3	Sabinene	0·3	0·4	0·3
4	Myrcene	0·6	0·2	0·6
5	Limonene	14·6	10·7	7·7
6	Cineole	0·3	0·3	0·2
6a	β-Phellandrene	Trace	Trace	Trace
7	*cis*-Ocimene	0·1	Trace	0·3
8	*trans*-Ocimene	Trace	Trace	0·1
9	Unknown	0·2	0·1	Trace
10	Menthone	13·2	4·6	0·9
11	Menthofuran	0·4	24·1	0·4
12	Isomenthone	0·9	2·0	2·4
13	Sesquiterpene-HC	0·1	Trace	0·1
14	Neomenthyl acetate	0·1	0·1	Trace
15	Menthyl acetate	3·6	5·6	4·0
15a	Isomenthyl acetate	Trace	Trace	Trace
16	Neomenthol	2·7	1·1	1·8
17	Unknown	0·4	0·9	0·3
17a	Unknown			
18	Caryophyllene	2·2	1·0	3·3
19	Neoisomenthol	0·2	0·3	1·6
20	Menthol	55·4	34·2	72·1
21	Pulegone	0·2	11·4	0·3
22	Isomenthol	Trace	Trace	0·1
23	Sesquiterpene-HC	0·4	0·1	1·0
24	Piperitone	2·2	0·1	0·4
Trace peaks, numbered and unnumbered		0·7	0·6	0·6

Analyses done on (1) PEG 4000 3 m × 3 mm; (2) QF-1 3 m × 3 mm; (3) Capillary-column 100 m × 0·25 mm PEG 1540.

Each backcross of an F_1 hybrid to the *M. aquatica* parental strain had a ratio of 1 individual having a menthol herbage odor like the *M. arvensis* parent to 1 individual having a menthofuran herbage odor like the *M. aquatica* parent. The total for first to fourth backcross progenies for all four hybrid combinations was 2076 menthol-odored to 2106 menthofuran-odored with $P = 0{\cdot}6$ for a 1 : 1 ratio. These data show that a single gene, designated as *F*, controls whether an individual has substantial amounts of menthol or menthofuran. Fertile octoploid species having bivalent pairing and four genomes could have recessive genes on similar chromosomes in two or more genomes without changing genetic ratios, but the presence of a heterozygous dominant gene on each of two genomes (*F/f F/f*) would have given a duplicate gene ratio of 3 menthol-odored to 1 menthofuran-odored in the first backcross progeny. This was not found.

Menthol-odored F_1 hybrids of each combination were crossed to *M. aquatica* four times to substitute the dominant gene *F* controlling menthol production into *M. aquatica*. The fourth backcross individuals were very uniform in morphological appearance and closely resembled the recurrent *M. aquatica* parent. Individuals with the *ff* genotype and menthofuran were uniform in oil composition and not distinguishable from the *M. aquatica* parent.

TABLE 2. OIL COMPOSITION OF MENTHOL-ODORED *M. aquatica* HYBRIDS HAVING THE GENOTYPE *Ff* SUBSTITUTED BY 4 BACKCROSSES FOR THE GENOTYPE *ff* OF NORMAL MENTHOFURAN-ODORED *M. aquatica**

	Oil constituents	%†	Method of identification A‡	IR	UV	$[\alpha]^{28}_D$§
1	α-Pinene	0·4	+	+		
2	Camphene	Trace	+			
3	β-Pinene	0·8	+	+		
4	Sabinene	0·4	+	+		
5	Myrcene	0·6	+		+	
6	α-Phellandrene	Trace	+			
7	α-Terpinene	Trace	+			
8	Limonene	5·1	+	+		
9	β-Phellandrene	Trace	+		+	
9a	Cineole	6·0	+	+		
10	*cis*-Ocimene	0·8	+		+	
11	*trans*-Ocimene	0·3	+		+	
	and γ-Terpinene		+			
12	ρ-Cymene	0·3	+		+	
13	Terpinolene	Trace	+			
14	L-Menthone	6·8	+	+		−25·7°
15	Menthofuran	1·5	+	+		
16	Isomenthone	3·7	+	+		
17	Sesquiterpene-HC	0·2				
17a	Neomenthyl acetate	0·3	+	+		
18	Sesquiterpene-HC	0·2				
19	Sesquiterpene-HC	0·3				
20	Isomenthyl acetate‖	Trace	+			
20a	Menthyl acetate	12·2	+	+		
21	Neoisomenthyl acetate	1·8	+	+		
22	D-Neomenthol	3·4	+	+		+15·8°
23	Sesquiterpene-HC	0·2				
24	Caryophyllene	5·7	+	+		
25	D-Neoisomenthol	3·3	+	+		+ 2·9°
26	L-Menthol	40·6	+	+		−42·2°
27	Pulegone	0·4	+	+		
28	Sesquiterpene-HC	0·2				
29	Isomenthol	0·5	+	+		
30	Unknown	0·2				
31	Unknown	0·2				
32	Sesquiterpene-HC	0·5				
33	Sesquiterpene-HC	0·2				
34	Sesquiterpene-HC	0·2				
35	Unknown	0·4				
36	Unknown	0·2				
37	Piperitone	0·6	+	+		
38	Carvone	0·5	+	+		
39	Sesquiterpene-HC	0·6				
40	Sesquiterpene-HC	0·1				

* The identification as sesquiterpene-hydrocarbons (Sesquiterpene-HC) is based on chromatographic behavior.

† Related to total oil; the trace components and unnumbered compounds make up 0·3%.

‡ Addition analysis with reference compounds on SCOT and thin-film capillary columns (for columns used see Experimental).

§ The relatively high deviation from the theoretical value is due to the low amount of substance (10–15 mg) available for use.

‖ Identification rechecked after saponification by Ret. value of the resulting alcohol.

GLC of the hybrid oil from menthol-producing fourth backcross individuals gave the results shown in Table 2.

The assays were of oils obtained from herbage harvested shortly before blossoming. The fourth backcross individuals having menthol were similar to the *M. aquatica* parent in having low oil yields. The small amount of oil (no more than 1·5 ml) was insufficient to allow the identification of some of the oxygenated compounds by spectroscopic methods. The sesquiterpenes, except caryophyllene, could not be separated by preparative chromatography and their identification is based solely on chromatographic behavior and IR analysis of the mixture.

TABLE 3. SUMMARY OF MAJOR EFFECTS OF GENES *F* AND *R* ON OIL COMPOSITION

Genotypes	*M. aquatica* Normal *ff RR*	*M. aquatica* Hybrid from gene substitution *Ff RR*	*M. arvensis* S_1 with high menthol* *FF Rr*	*M. arvensis* S_1 with high menthone* *FF rr*
Limonene	4·9	5·1	3·5	8·3
Cineole	7·7	6·0	0·5	0·9
Piperitone		0·6	2·4	3·2
Pulegone	Trace	0·4	<0·1†	<0·1†
Menthofuran	66·4	1·5	0·01	0·04
Menthones	0·1	10·5	7·1	83·2
Menthols	0·2	47·8	80·2	1·1
Menthyl esters	Trace	14·3	2·8	0·3

* Data are G.C. area percentages from.[3]

† β-Caryophyllene interfered with accurate measurement but 0·01% definitely present.

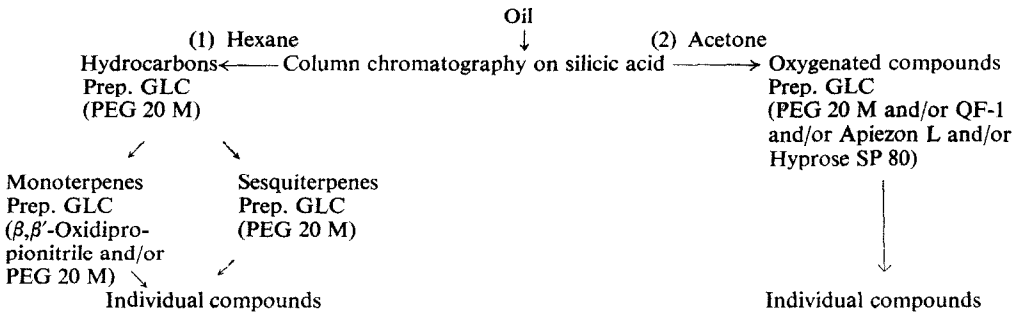
The principal differences between the menthol-producing hybrid and the control natural strain of *M. aquatica* are given in columns 1 and 2 of Table 3. The single incompletely dominant gene *F* must be assumed to cause these major differences in oil composition, but the present data cannot totally eliminate the possibility that the recessive gene *f* is closely linked with the recessive *p* gene in *M. aquatica* (f-p/f-p). However, coupling phase genetic linkage F-P/f-p in the *M. arvensis—M. aquatica* hybrids would have to be very great with less than 0·001% crossing-over since recombinant individuals having high pulegone were not found in a total progeny of 1598 first backcross individuals or in a combined total of 4182 backcross progeny. The postulated *PP* genotype of *M. aquatica* is also supported by the fact that all *M. aquatica* F_1 hybrids with *M. longifolia* (L.) Huds. having a *cc aa* genotype and piperitone produce menthol.

DISCUSSION

The oxidation of pulegone to menthofuran as postulated in several biogenetic designs[9–12] is controlled by the single gene *F* that is not completely dominant, whereas the reduction of pulegone to menthone is controlled by the gene or genes designated as *P*. The monogenic basis of *P* has not been established, but the dominant *P* gene, or genes, allows reduction and

[9] R. H. REITSEMA, *J. Am. Pharm. Assoc. Sci. Ed.* **47**, 267 (1958).
[10] Y. FUJITA, *Koryo* **59**, 41 (1960).
[11] W. D. LOOMIS, in *Terpenoids in Plants* (edited by J. B. PRIDHAM), p. 59, Academic Press, New York (1967).
[12] F. W. HEFENDEHL, E. W. UNDERHILL and E. VON RUDLOFF, *Phytochem.* **6**, 823 (1967).

the recessive *p* gene, or genes, largely prevents reduction. Since there is no evidence that the genes *F* and *P* are closely linked, one may assume that the genotype of *M. aquatica* is *ff PP* and that the oxidation reaction takes precedence over the reduction reaction and thus indirectly controls the amount of conversion of pulegone to menthone. The *FF PP* genotype of *M. arvensis* produces 0·01–0·1% menthofuran with 80–90% menthone and its products, the *ff PP* genotype of *M. aquatica* 60–80% menthofuran with less than 1% menthone and its products, and the *Ff PP* genotype of the menthol-odored *M. aquatica* hybrids 0·4–25·0% menthofuran with an intermediate amount of 45–83% menthone and its products. The Mitcham peppermint cultivar (*M. piperita* L.) which has 1–18% menthofuran with 70–80% menthone and products presumably has an *Ff PP* genotype derived from hybridization of *M. aquatica* (*ff PP*) and *M. spicata* L. (*FF PP*).



SCHEME 1. PREFRACTIONATION OF THE OIL.

EXPERIMENTAL

Plant material, oil isolation and prefractionation. The breeding program was done in Kalamazoo, Michigan (U.S.A.). Plants for genetic analyses and oil isolation were cultivated partly on a field near South Bend, Indiana, and partly on the experimental field of the Institute of Pharmacognosy, Freiburg (Germany). All propagations were done vegetatively. The oil isolation was performed by H_2O–H_2O steam distillation.[13] For prefractionation of the oil, see Scheme and Ref. 5.

Gas chromatography and quantitative analysis. Preparative analysis: GC 5754 A, Hewlett-Packard with TC. Columns: SS Steel, deactivated,[5] 3 m × 6 mm (15% liquid phase). For refractionations: 3 m × 3 mm (10% liquid phase). Carrier: Chromosorb W, DMCS treated 60/80 mesh. Liquid phases: PEG 20 M Merck, β,β′-Oxidipropionitrile Varian, QF-1 Varian, Apiezon L Varian, Hyprose SP 80 Varian. Analytic analysis: (1) Scott-Column PEG 20 M 15 m × 0·5 mm; (2) Thin-film capillary columns 100 m × 0·25 mm PEG 1540 and Apiezon L. Quantitative analysis: Digital-Integrator Mod. 477 Varian MAT. Internal normalization; values corrected.[5]

Identification of isolated compounds. The identifications were done as mentioned in Table 2. All esters were re-analyzed after saponification by the resulting alcohols. For details also for difference analysis (reaction chromatography) see.[5]

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[13] F. W. HEFENDEHL, *Planta Med.* **10**, 241 (1962).