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CHANGES IN MONOTERPENE COMPOSITION IN *MENTHA AQUATICA* PRODUCED BY GENE SUBSTITUTION

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Abstract—The dominant gene *C* that causes 49 5% carvone and 17 9% dihydrocarvone in *Mentha crispa* L. was substituted into *M aquatica* L by five convergent backcrosses The natural strain of *M aquatica* had 7 7% cineole, 4 9% limonene, a trace of terpinolene and pulegone, 0 1% menthone, 0 2% menthol, 66 4% menthofuran, and 18 9% of 12 different hydrocarbons. The modified hybrid strain with the dominant gene *C* had 11 8% cineole, 19 0% limonene, 0·1% terpinolene, 1 6% carvone, 0 4% carveol, 9 1% carveyl acetate, a trace of dihydrocarvone, 0 1% dihydrocarveol, and 11 0% dihydrocarveyl acetate with only small amounts of pulegone, menthone, menthol, menthyl acetate, menthofuran, and 40 1% of 13 different hydrocarbons The multiple changes in oil composition are probably due to the difference in a single gene These results support the concept that menthofuran is derived from pulegone or a product of it The dominant *R* gene which converts menthone → menthol is shown here to convert carvone → carveol and dihydrocarvone → dihydrocarveol with the genetic basis for high ester formation unknown. Identification of the alcohols and esters of carvone and dihydrocarvone is also reported

INTRODUCTION

BIOGENETIC designs[1–4] to explain the origin of *Mentha* oil constituents usually assume that the hydrocarbon limonene produces either the 2-oxygenated compounds carvone and dihydrocarvone or via intermediate compounds (terpinolene, isopiperitenone, and piperitenone) the 3-oxygenated compounds pulegone and menthone with pulegone oxidized to menthofuran Murray and Reitsema[5] showed that the dominant gene *C* caused carvone and the 2-oxygenated compounds while the recessive gene *c* caused the formation of the 3-oxygenated compounds In *M spicata* L and *M crispa*, Murray[6] further found that the double recessive *cc aa* genotype produced piperitone which was mostly converted to piperitone oxide, and that the *cc AA* genotype produced pulegone which in this material was always partly converted to menthone Any individual having the dominant *C* gene produced carvone without regard to whether the other gene was *AA*, *Aa*, or *aa* This genetic work does not prove that piperitone rather than piperitenone is the precursor of pulegone, but it does show that in the presence of certain other true-breeding genes, the double recessive *cc aa* type has piperitone. The *cc AA* genotype produces pulegone, but varying amounts of the pulegone are immediately converted to menthone by the dominant *P* gene or genes that are true breeding in these strains [7] The menthone strains of *M spicata* or *M crispa* have the recessive genotype *rr* and can reduce only enough menthone to form 1–10% menthol,

[1] R H REITSEMA, *J Am Pharm Assoc*, *Sci Ed* **47**, 267 (1958)
[2] Y FUJITA, *Koryo* **59**, 41 (1960)
[3] F W HEFENDEHL, E W UNDERHILL and E VON RUDLOFF, *Phytochem* **6**, 823 (1967).
[4] W D LOOMIS, in *Terpenoids in Plants* (edited by J B PRIDHAM), p 59, Academic Press, New York (1967).
[5] M J MURRAY and R H REITSEMA, *J Am Pharm Assoc Sci Ed* **43**, 612 (1954)
[6] M J. MURRAY, *Genetics* **45**, 931 (1960)
[7] M. J MURRAY, D E LINCOLN and P M MARBLE To be published.

whereas *M arvensis* L var *piperascens* Briq strains having menthone and the dominant *R* gene produce 60–80% l-menthol [8] Interspecific hybrids with *M aquatica* indicate that this species has the genotype *cc AA RR* and would produce 50–70% menthol if it did not also have the recessive genotype *ff* allowing menthofuran development From a chemical viewpoint, Nigam and Levi[9] found 0 05% menthofuran in *M sylvestris* L although this species has 4 5% piperitone, 66 3% piperitone oxide, 2 5% piperitenone, 2·7% piperitenone oxide, and no pulegone or menthone [10] In *M aquatica*, Handa *et al* [10] reported 1 8% pulegone and 51 3% menthofuran, whereas Hefendehl[11] found no measurable amount of pulegone in strains having 60–80% menthofuran The oxidation of pulegone to menthofuran either must be almost complete in *M aquatica* and very incomplete in *M piperita* L , or menthofuran must originate in some other manner

The most direct genetic test of these alternatives is to substitute the dominant gene *C* into *M aquatica* by five or more convergent backcrosses The dominant gene *C* should almost entirely prevent the formation of pulegone and thus menthofuran, if menthofuran is derived from pulegone Of equal importance, the modified strain of *M aquatica* should establish whether the dominant *R* gene that causes reduction of menthone to menthol can also reduce carvone to carveol and dihydrocarvone to dihydrocarveol Since the total alcohol and ester content of the cultivar spearmint species, *M spicata* and *M cardiaca* Baker, is 3–7%, the alcohols and esters have received very little study by modern methods [12]

RESULTS AND DISCUSSION

Genetic Data on Gene Substitution

A specific clonal strain of *M crispa* with the genotype *Cc Aa* has 2 6% cineole, 5 2% limonene, 1 0% menthone, 49 5% carvone, 17 9% dihydrocarvone, and less than 10% unidentified alcohols and esters The self-pollinated progeny of *M crispa* consisted of 841 carvone, 201 pulegone and 87 piperitone individuals which is a ratio of 12 carvone/dihydrocarvone-odored individuals (*C- A-* or *C- aa*) to 3 menthone/pulegone-odored individuals (*cc A-*) to 1 musty piperitone-odored individual (*cc aa*) [6] The colchicine-induced polyploid strain of *M. crispa* made without self-pollination is fertile and should have the genotype *Cc Cc Aa Aa* The self-pollinated progeny of polyploid *M crispa* consisting of 146 carvone, 35 pulegone, and 2 piperitone-odored individuals is inadequate to establish a precise 4*n* ratio, but shows that the polyploid strain segregates for both the *C* and *A* genes *Mentha aquatica* is true breeding for a menthofuran odor with no exceptions in over 5000 progeny from selfed fertile individuals or sibbed male-sterile by fertile individuals

The hybridization of *M aquatica* having a *cc cc AA aa ff ff RR rr* genotype with polyploid *M crispa* having a *Cc Cc Aa Aa FF FF rr rr* genotype should and does give a duplicate gene ratio of 3 carvone/dihydrocarvone-odored individuals (312 observed) to 1 pulegone/menthone-odored (93 observed) All non-carvone individuals have the recessive genes *cc cc*, the dominant genes *A*, *F*, and *R* thus allowing the formation of pulegone, menthone and menthol but not menthofuran The 2 1 1 ratio in the first backcross progeny of 38 carvone, 14 pulegone, and 15 menthofuran-odored indicates that the carvone-odored F_1 hybrid used in the cross had the genotype *CC cc A- Ff ff* while the *M aquatica* parent had

[8] M J MURRAY, *Genetics* **45**, 925 (1960)
[9] I C NIGAM and L LEVI, *J Pharm Sci* **53**, 1008 (1964)
[10] K L HANDA, D M SMITH, I C NIGAM and L LEVI, *J Pharm Sci* **53**, 1407 (1964)
[11] F W HEFENDEHL, *Arch der Pharm* **300**, 438 (1967)
[12] D M SMITH, W SKAKUM and L LEVI, *J Agric Food Chem* **11**, 268 (1963)

the genotype *cc cc AA ff ff* All individuals in the second to fifth backcross progenies were definitely either carvone- or menthofuran-odored in a 1 1 ratio as shown by the total of 606 carvone to 641 menthofuran odored (P = 0 3) In the fifth backcross progeny, individuals with a carvone odor and the genotype *Cc cc AA aa RR rr ff ff* are morphologically indistinguishable from the recurrent parental strain of *M aquatica* having a menthofuran odor and the genotype *cc cc AA aa RR rr ff ff* The substitution of the gene *C* into *M aquatica* was accomplished without more than a 25 per cent loss of vigor due to inbreeding.

Chemical Composition of M. aquatica *Hybrid and Control*

The data on hydrocarbon oil constituents of both hybrid and control *M. aquatica* are given in Table 1 The data on the oxygenated compounds of both hybrid and control are given in Table 2. The summarized assays were of oils obtained from herbage harvested shortly before blossoming No qualitative differences in oil composition were observed in oils from earlier harvested leaves, but the quantitative amounts of oil constituents of both the hybrid and the control varied during ontogenesis [11,13] The analysis of the *M aquatica* oil was a recheck of an earlier examination [11] Using capillary columns, we were able to obtain some new observations, especially for the minor oxygenated compounds, which are

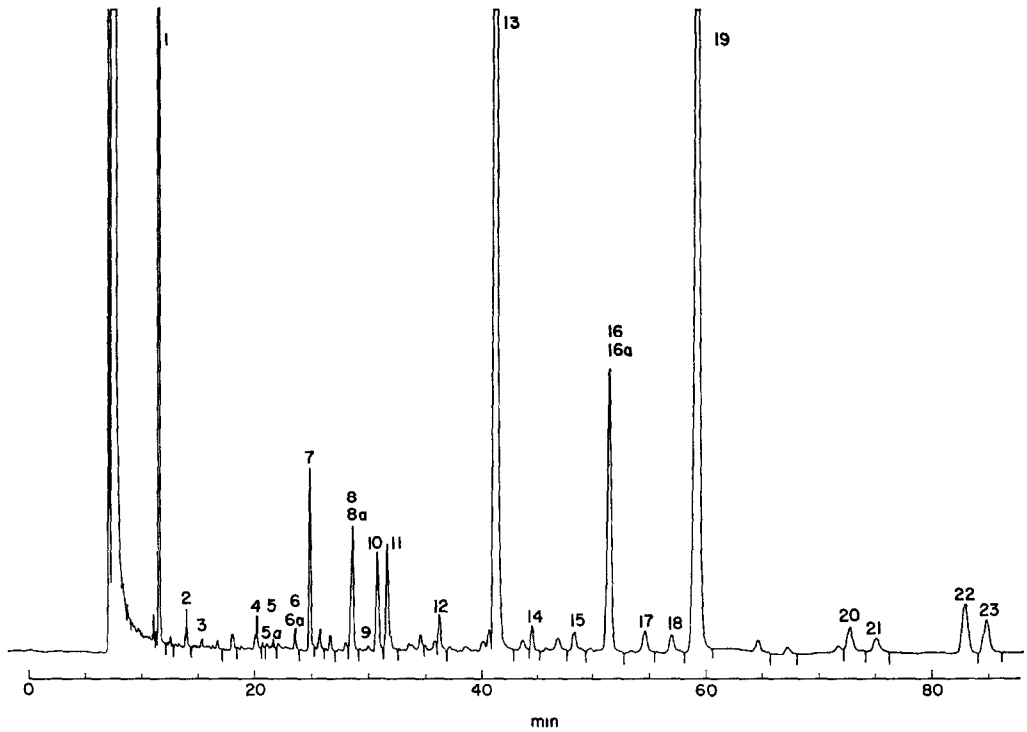

FIG 1 CHROMATOGRAM OF OXYGENATED OIL CONSTITUENTS OF *M aquatica* HYBRID

The peak numbers are those of the constituents in Table 2 Capillary column PEG 1540 100 m × 0 25 mm, 130°, gas flow 1 8 ml/min N_2, detector FID $10^{-12} \times 8$, 220°, modified injector 140°, amount of oil 1 μl (solution 1 100) split 1 10

[13] F. W HEFENDEHL, *Phytochem* 9, 1985 (1970)

TABLE 1. QUANTITATIVE AMOUNTS OF HYDROCARBON OIL CONSTITUENTS IDENTIFIED IN *M aquatica* HYBRID AND CONTROL

	Mentha aquatica—Hybrid				*Mentha aquatica*			
		Identification				Identification		
Hydrocarbons	%*	A†	IR	UV	%*	A†	IR	UV
α-Pinene	1 6	+	+		1 8	+	+	
Camphene	trace	+			trace	+		
β-Pinene	2 5	+	+		1 9	+	+	
Sabinene	1 4	+	+		0 8	+		
Myrcene	3 5	+	+	+	1 3	+	+	+
Limonene	19 0	+	+		4 9	+	+	
β-Phellandrene	0 3	+		+	0 1	+		+
cis-Ocimene	6 3	+	+	+	1 8	+		+
trans-Ocimene	2 1	+		+	0 5	+		+
ρ-Cymene	1 4	+	+	+	0 1	+	+	
Terpinolene	0 1	+		+	trace	+		
β-Bourbonene	1 8	+	+					
Caryophyllene	12 7	+	+		7 0	+	+	
(Humulene)‡	0 8				0 4			
ϵ-Muurolene	5 7		+		3 2		+	
Other compounds, all lower than 0 1%	1 8				0 8			
Total hydrocarbons	61 0				24 6			

* Related to total oil

† Addition-analysis with reference compounds on 6 different columns (Nos. 3, 4, 5, and 6 a–c, see Experimental)

‡ Identification only by comparison of retention value

thought to be precursors of menthofuran Certain other minor compounds of biogenetic interest were evaluated by addition analysis (Table 2) All results, which were achieved by comparison of retention values or addition analysis should be considered as tentative identifications Since the oil yields of *M. aquatica* are always low and the convergent strains are less vigorous due to inbreeding, there was an insufficient amount of oil to confirm these results by other methods.

Biogenetic Interpretation of Differences in Oil Composition

The data in Table 1 show that the modified *M aquatica* strain and the *M. aquatica* control have the same hydrocarbon constituents (except possibly β-bourbonene), but that the amounts of all constituents except α-pinene, camphene, and terpinolene are greater in the hybrid Why the hybrid should have 61 0% total hydrocarbons and the control 24·6% is not understood, but two thirds of the difference is due to the higher amounts of limonene, ocimene, and caryophyllene in the hybrid.

The total of all oxygenated compounds in Table 2 is 75 4% for the control and 39 0% for the hybrid. The control and hybrid are alike in having small amounts of the ketones pulegone, menthone, isomenthone, and their alcohols and esters (menthol, menthyl acetate, isomenthyl acetate, and neomenthyl acetate) The control had 0 3% linalool and the hybrid 0 6% Residual amounts of this acyclic compound might be expected since it is a possible precursor of cyclic monoterpenes [1,14]

[14] M J MURRAY and D E LINCOLN, *Genetics* **65**, 457 (1970)

TABLE 2 QUANTITATIVE AMOUNTS OF OXYGENATED OIL CONSTITUENTS IDENTIFIED IN *M aquatica* HYBRID AND CONTROL

	Mentha aquatica—Hybrid				*Mentha aquatica*			
		Identification				Identification		
Oxygenated compounds	%*	A†	IR	RA‡	%*	A†	IR	RA‡
1 Cineole	11 8	+	+		7 7	+	+	
2 Octyl acetate	trace	+		+	trace	+		+
3 Octanol	trace	+		+	trace	+		+
4 Unknown	0 1							
5 Menthone	trace	+	+		0 1	+	+	
5a Menthofuran	trace	+	+	+	66 4	+	+	+
6 Isomenthone	trace	+			trace	+	+	
6a Ester	trace			+				
7 Linalool	0 6	+	+	+	0 3	+	+	+
8 Menthyl acetate }	0 5	+	+	+	trace	+	+	+
8a Isomenthyl acetate }		+						
9 Dihydrocarvone	trace	+						
10 Neomenthyl acetate	0 4	+	+	+				
11 Ester	0 5			+				
12 Menthol	0 1	+	+	+	0 2	+	+	+
13 Dihydrocarveyl acetate	11 0	+	+	+				
14 Alcohol	0 1			+				
15 Ester	0 1			+				
16 *trans*-Carveyl acetate	0 3	+	+	+				
16a Carvone	1 6	+	+					
17 Dihydrocarveol	0 1	+	+	+				
18 Ester	0 1			+				
19 *cis*-Carveyl acetate	8 8	+	+	+				
20 Ester	0 2			+				
21 *trans*-Carveol	0 1	+	+	+				
22 Ester	0 5			+				
23 *cis*-Carveol	0 3	+	+	+				
1-Octen-3-ol					0 3	+	+	+
Pulegone					trace	+		
Other compounds, all lower than 0 1%	1 8				0 4			
Total	39 0				75 4			

* See Table 1
† See Table 1 (without Column No. 5).
‡ Difference analyses (see Experimental).

Most *Mentha* biogenetic designs assume that alpha-terpineol is converted to either cineole or limonene and that limonene is converted either to carvone/dihydrocarvone or to terpinolene/piperitenone/pulegone/menthone/menthol/menthyl acetate. The principal distinguishing differences listed in Table 3 are explained by the design if the *Cc AA* genotype allows limonene → carvone → dihydrocarvone and the *cc AA* genotype produces pulegone which is oxidized to menthofuran The formation of carvone limits but does not totally prevent the formation of pulegone and its products. The exact effects of the gene *C* on biogenesis are unknown. Residual amounts of the postulated intermediates α-terpineol and piperitenone were not found The presence of 0 8% piperitone and 0 3% carvone reported for a different strain of *M aquatica*[10] was not confirmed.

TABLE 3 SUMMARY OF PRINCIPAL DIFFERENCES BETWEEN *M aquatica* HYBRID AND CONTROL

	Hydrocarbons		Oxygenated compounds			
Mentha aquatica	Limonene	Terpinolene	Cineole	Carvone	Carveol	Carveyl acetate
Hybrid	19 0	0 1	11 8	1 6	0 4	9 1
Control	4 9	trace	7 7	—	—	—

	Oxygenated compounds				
Mentha aquatica	Dihydrocarvone	Dihydrocarveol	Dihydrocarveyl acetate	Pulegone Menthone	Menthofuran
Hybrid	trace	0 1	11 0	trace	trace
Control	—	—	—	0 1	66 4

CONCLUSIONS

M aquatica with the gene *C* produces carvone and dihydrocarvone with only trace amounts of pulegone and menthofuran This is evidence that menthofuran is an oxidation product of pulegone The fact that *M aquatica* strains with 60–80% menthofuran have almost no residual pulegone must be ascribed to the completeness of the conversion The dominant gene *R* which reduces menthone → menthol is shown to also reduce carvone → carveol and dihydrocarvone → dihydrocarveol. *M aquatica* probably has a gene or genes that promote rapid alcohol → ester conversion in strains having a terpenoid alcohol

EXPERIMENTAL

Plant material and oil isolation The breeding program was done in Kalamazoo (U S A) The plants for oil isolation were cultivated on the experimental field of the Institute of Pharmacognosy, Freiburg (Germany) All propagations were done vegetatively The oil isolation was done by water–water steam distillation [15]

Column chromatography Hydrocarbons were separated from oxygenated compounds by column chromatography on silic acid Merck 0 2–0 3 mm Silic acid was deactivated by treatment with polyethylene glycol 4000 [13] (Ratio silic acid oil hexane (acetone) = 80 g 1 ml 350 ml (700 ml) Under these conditions, menthofuran appears partially in the hexane fraction

Gas chromatography GC Varian Aerograph 1860–4 with TC (preparative analyses) and FID (analytic analyses) The injector was modified so that evaporation occurred in an all-glass system Column material SS-Steel, washed with detergent, $CHCl_3$, MEOH, acetone To avoid catalytic rest activities, all columns were treated with a solution of 2% PEG 20 M in CH_2Cl_2 and washed with the same solvent afterwards Columns (1) Preparative 3 m × 6 mm (15% liquid phase), (2) Analytic (a) 3 m × 3 mm (8% liquid phase), (b) capillary or SCOT-columns Stationary phases were all on Chromosorb W, DMCS-treated, 60–80 or 80–100 mesh (1) PEG 4000 Merck, (2) Apiezon L, (3) QF-1, (4) Hyprose SP 80, (5) β,β′-Oxidipropionitril, (6) Capillary columns (a) PEG 1540 100 m × 0 25 mm, (b) Apiezon L 100 m × 0 25 mm, (c) PEG 20 M 50 ft × 0 02 in SCOT

Quantitative analysis Digital-Integrator Mod 477 Varian MAT Internal normalization [16] Values corrected corresponding to their molecular weight or structure [17]

Individual compounds Aliquots of the prefractionated terpenes were repeatedly injected on preparative PEG columns and each peak collected in cooled capillaries (−70°) at the instrument exit The results were tested on analytical columns and, if necessary, the separation was repeated on QF-1 columns The fractionation of monoterpene hydrocarbons was achieved on β,β′-Oxidipropionitrile columns The menthone-isomenthone-menthofuran fraction was separated on small silic acid columns with hexane–acetone Menthofuran appears in the hexane fraction as detected by TLC on silic acid (benzene ethylacetate 95 5, vanillin-H_2SO_4)

After saponification and refractionation on QF-1, the resulting alcohols of compounds 8, 13, 16 and 20 (Table 2) were run on IR IR and UV analyses by IR Grating-spectrophotometer Mod 257 Perkin–Elmer measured as film (AgCl-plates, NaCl-plates, Reflection plates Wilks MIR-15, GC-IR analyzer) and UV Zeiss-Spectrophotometer PMQ II

[15] F W HEFENDEHL, *Planta Med* **10**, 241 (1962)
[16] E BAYER, *Gaschromatographie*, Springer-Verlag, Berlin (1959)
[17] R G ACKMAN, *J Gaschromatog* **2**, 173 (1964)

Difference analyses (Reaction chromatography) for (1) *Alcohols* (a) Boric acid reaction chromatography,[18] (b) Reaction with methoxalic acid anhydride Merck (20 mg oil, 0 8 ml methoxalic acid anhydride, 4% in benzene, 0 08 ml pyridine for 30 min 20° and 30 min 4°), (2) *Esters* Saponification in 0 5 N KOH (EtOH) and g c of resulting alcohols, (3) *Ketones* Reaction with semicarbazide,[19] (4) *Menthofuran* Reaction with maleic acid anhydride [20]

Acknowledgment—One of us (F W H) thanks the Deutsche Forschungsgemeinschaft for financial support.

[18] F W HEFENDEHL, *Naturwissenschaften* **51,** 138 (1964)
[19] E GILDEMEISTER and FR HOFFMANN, *Die Ätherischen Ole*, Vol II, Akademie Verlag, Berlin (1960)
[20] E GILDEMEISTER and FR HOFFMANN, *Die Atherischen Ole*, Vol IIId, Akademie Verlag, Berlin (1960)

Key Word Index—Mentha aquatica, Labiatae, biosynthesis, monoterpene, menthofuran, gene substitution