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CHEMICAL COMPOSITION AND GENETIC BASIS FOR THE ISOPINOCAMPHONE CHEMOTYPE OF *MENTHA CITRATA* HYBRIDS

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Key Word Index—*Mentha citrata*; Labiatae; mint; genetic; monoterpenoid; isopinocamphone.

Abstract—The dominant *I* gene found in *Mentha citrata* causes only 1.0–1.5% isopinocamphone and 0.5–2.5% β-pinene in this species that is characterized by 60–90% linalool/linalyl acetate. If the gene *Is* is separated from the linked *I* gene that causes linalool and is substituted into *M. aquatica* having 66.5% menthofuran and the recessive *ii* genotype, the resulting hybrids will have 22.3% β-pinene, 29.9% isopinocamphone and a number of related monoterpenoids seldom observed in *Mentha* oils. Crosses with tester strains show that the *Is* gene almost entirely prevents the development of monoterpenes with a *p*-menthane carbon skeleton substituted at the 2- and/or 3-position which commonly occur in *Mentha* species. A generalized biogenetic scheme is used to hypothesize the position of the action of the *Is* gene in biosynthesis and its interaction with other genes controlling monoterpenoid formation in *Mentha* species.

INTRODUCTION

In the genus *Mentha*, the section *Capitatae* of the subgenus *Menthastrum* consists of two species, *M. aquatica* L. and *M. citrata* Ehrh. These closely related interfertile 8*n* species having 96 somatic chromosomes are sometimes considered to be varieties of a single species *M. aquatica* [1, 2]. Both species have the allo-octoploid habit of coarse, stiff, heavily vascularized stems and stolons; large, thick leaves; and capitate or button-shaped flower heads in contrast to the elongate, cylindrical flower heads of the double diploid or allotetraploid species *M. spicata* L. ($2n = 48$) or the diploid species *M. sauveolens* (Ehrh.) Harley ($2n = 24$) [previously *M. rotundifolia* (L.) Huds.] and *M. longifolia* (L.) Huds. ($2n = 24$). The oil composition and genetic basis for chemotypes containing high proportions of linalool, limonene or menthofuran have been reported for the 8*n* capitate *Menthastrum* species [3–7] and are reported here for a fourth isopinocamphone chemotype.

To recapitulate the previous research, the lavender odour of *M. citrata* is caused by 84–90% linalool and linalyl acetate. All individuals of the linalool chemotype must have one dominant *I* gene to produce linalool but four dominants are possible since this gene is present on two different homeologous pairs of chromosomes [3]. A considerable amount of the linalool can be converted to linalyl acetate in any individual that also has the dominant *E* gene. The dominant *I* gene is epistatic to the expression of other genes controlling the production of monoterpenoids in *Mentha* [3]. A chemotype having 57–94% limonene/cineole found in exceptional *M. citrata* × *M. aquatica* or *M. citrata* × *M. crispa* hybrids is due to a dominant gene *Lm* that is present on two different pairs of chromosomes [4]. All individuals belonging to the high limonene/cineole chemotype must have the recessive *ii* genotype to prevent the formation of linalool, the recessive *cc* genotype to prevent the conversion of limonene into carvone, and the dominant *Lm* gene to prevent the conversion of the precursor of limonene toward terpinolene/piperitenone/pulegone/menthone. Individuals having 50–80% menthofuran found in the typical chemotype of *M. aquatica* must have the recessive *ii*, *cc* and *lm lm* genes in order to produce pulegone which is rapidly oxidized to menthofuran by the recessive *ff* genotype [5–7] or reduced to menthone [8, 9].

The purpose of the present research was to determine the genetic basis for exceptional *M. citratd* × *M. aquatica* individuals having 20–50% isopinocamphone and secondly, to identify all major oil constituents that are associated with the production and accumulation of isopinocamphone. Shimizu *et al.* [10] reported a chemotype of *M. aquatica* whose principal compound was 49% isopinocamphone, but the identity of other constituents was not given.

RESULTS AND DISCUSSION

Monogenic basis for isopinocamphone production

The isopinocamphone chemotype was known only in *M. aquatica* [8], *M. citrata* × *M. crispa* F1 hybrids [4], and *M. citrata* × *M. aquatica* F1 hybrids [3]. It is rare since only 18 individuals were found in 10000 *M. citrata* × *M. crispa* F1 hybrids [4]. These allohexaploid hybrids having 72 somatic chromosomes were as sterile as *M. piperita* L. or *M. aquatica* × *M. spicata* F1 hybrids [7] and were not suitable for genetic study.

M. citrata × *M. aquatica* F1 hybrids are fully fertile with the exception of those individuals that have male sterility [3]. Strain 3 of *M. citrata* having an oil composition of 29.5% linalool, 58.7% linalyl acetate and 1.4% isopinocamphone and the genotype $I_1I_1\ I_2i_2$ should

Table 1. Oil composition (% of total) of first, second, and third backcross individuals having 20–30% isopinocamphone from the cross F_1 (*M. citrata* Strain 3 × *M. aquatica* Strain 1) × *M. aquatica* Strain 1 once, twice or thrice. Compounds listed in order of elution on a Carbowax 6000 column

Peak	Compound	1st* backcross	2nd backcross†		3rd backcross†	
			Mean	Range	Mean	Range
1	α-Pinene	1.3	1.4	1.1–1.5	1.4	0.6–2.0
2	β-Pinene	22.3	22.8	24.5–29.4	27.6	18.1–38.64
3	Sabinene	2.9				
4	Myrcene	0.5				
5	Limonene	17.2	29.1	24.8–33.4	33.0	25.9–28.8
6	1,8-Cineole	1.2	0.5	0.3–0.6	0.4	0.2–0.6
7	*cis*-Ocimene	0.7				
8	γ-Terpinene	0.1				
9	*p*-Cymene	0.5				
10	(3-Octanol)	0.1				
11	Myrtenyl methyl ether	0.1				
12	1-Octen-3-ol	0.1				
13	*trans*-Sabinene hydrate	0.1	tr		tr	
14	Menthone	0.3				
15	Menthofuran	0.1	0.4	0.2–0.8	1.5	0.3–4.1
16	(β-Bourbonene)	1.2				
17	Linalool	0.3	tr		tr	
18	α-Gurjunene	0.1				
19	Isopinocamphone	29.9	22.9	21.2–24.1	20.6	16.4–28.6
20	Menthyl acetate	0.3				
21	Pinocamphone	1.2				
22	Caryophyllene	3.0	3.8	3.0–5.6	3.5	1.4–8.2
23	Terpinen-4-ol	0.2				
24	Menthol	0.8	0.1	tr–0.3	0.2	0.1–1.0
25	Myrtenol	0.3				
26	*trans*-Pinocarveol	0.2				
27	*trans*-Pinocarvyl acetate	1.6				
28	Isomethyl acetate	0.3				
29	(*trans*-β-Farnesene)	0.3				
30	Myrtenyl acetate	3.5				
31	α-Terpineol	0.2				
32	(Germacrene D)	0.3	1.3	0.2–2.9	0.6	0.1–1.0
33	Carvone	1.1	3.8	1.8–4.9	2.5	1.4–4.9
34	Myrtanyl acetate	1.6				
35	Mytenol	0.3				
36	Caryophyllene oxide	1.2				
37	Ledol	2.2	0.8	0.4–1.5	0.6	0.1–1.1

*Constituents associated with isopinocamphone identified using IR by Lawrence and associates.

†Identification by Lincoln based solely on retention time and addition analysis.

breed true for linalool/linalyl acetate except for rare segregants derived from quadrivalent pairing and recombination. As previously reported [3], strain 3 *M. citrata* hybrids with strain 1 *M. aquatica* consisted of 336 lavender-odoured to four menthofuran-odoured individuals. One F_1 individual from this progeny had 40% isopinocamphone. This fertile F_1 individual was hybridized to a male-sterile individual of strain 1 *M. aquatica* and gave 425 isopinocamphone-odoured to 400 menthofuran-odoured individuals in the first test cross progeny. A male-sterile individual from the first test cross hybridized with *M. aquatica* gave a second backcross ratio of 110 isopinocamphone-odoured to 102 menthofuran-odoured. The third convergent backcross to the strain 1 *M. aquatica* had 928 isopinocamphone: 952 menthofuran. The total for first to third backcross progenies is 1463 camphoraceous or isopinocamphone-odoured to 1454 menthofuran or aquatica-odoured.

These test cross data based on herbage odour were confirmed by GC assays of about 30 individuals of each class for major constituents. These data show that the substitution of a single dominant gene *Is* from *M. citrata* into the *M. aquatica* genotype causes the production of 20–40% isopinocamphone. The gene *Is* in the *M. citrata* genotype having one or more dominant *I* genes produces only 1–1.5% isopinocamphone.

Oil composition

Detailed assay data for a bulked oil sample from seven first backcross individuals (*M. citrata* × *M. aquatica*) × *M. aquatica*, having similar isopinocamphone dominated assays are given in Table 1. The constituents of the essential oil of these plants is dominated by the bicyclic monoterpenoids isopinocamphone (22.9%) and β-pinene (22.3%), with significant amounts of limonene (17.2%). Among the monoterpenoids present in smaller amounts are several myrtenol derivatives which are structurally closely related to isopinocamphone (5.8% total).

The oxygenated monoterpenoids with *p*-menthane carbon skeletons which are characteristic of many *Mentha* species are present only in small quantity (5.2% total). When the dominant *Is* gene is substituted into *M. aquatica* it leads to an increase in the amount of β-pinene and its products, e.g. isopinocamphone, from 1.9 to 55.3% while decreasing the 3-oxygenated compounds, mainly menthofuran, from 66.7 to 1.8%. Substitution of the dominant gene *Is* into *M. aquatica* by two or three convergent backcrosses does not substantially change the amounts of the major constituents from the values observed for the first backcross individuals (Table 1).

The major oil constituents and their proportions in the four chemotypes of the 8*n* species *M. aquatica* and *M. citrata* are given in Table 2. Individuals belonging to the isopinocamphone chemotype have 5–12 times the usual amount of β-pinene without any increase in α-pinene. *M. citrata* oil usually has less limonene and cineole than *M. aquatica* oil, but neither the linalool or methofuran chemotypes has as much limonene as any isopinocamphone strain.

Isopinocamphone F_1 hybrids with other principal Menthastrum *species*

The limonene chemotype of *M. aquatica* was hybridized with an isopinocamphone strain and gave 129 isopinocamphone-odoured individuals to 163 individuals that had some other odour (cross 1, Table 3). Three isopinocamphone-odoured individuals were assayed and had an average of 20.3% limonene, 23.3% β-pinene and 29.7% isopinocamphone. All crosses in Table 3 were between first backcross isopinocamphone-odoured individuals having the heterozygous *Isis* genotype and different tester strains having the recessive *isis* genotype. One half of the F1 hybrids in each cross should have isopinocamphone if the *Is* gene can determine development in the hybrid genotype. This is true in all crosses except cross 8 (Table 3) having the polyploid *M. cardiaca* parent. The slight deficiency of the isopinocamphone class in other crosses probably can be ascribed to our reluctance to include individuals in this class unless their herbage odour was unmistakable.

These data show that the gene *Is* prevents the development of large amounts of the 3-oxygenated compounds piperitenone, piperitone, pulegone, isopulegone, 1-menthone, isomenthone, menthol, menthyl acetate and menthofuran (crosses 9–17, Table 3). These data also show that the gene *Is* prevents development of large amounts of the 2-oxygenated ketones carvone and dihydrocarvone, as well as their alcohols and esters (crosses 4–8, Table 3).

In crosses 16 and 17 of Table 3, hybrids with the menthol or isopulegone chemotypes of *M. arvensis* (2*n* = 96) have only 2.3–2.5% isopinocamphone with the 4–6% β-pinene and 72–85% limonene found in the limonene chemotype of *M. aquatica*. The assays of 17 F_1 hybrids with cultivar *M. arvensis* show that the *Is* gene inhibits development of menthone/menthol/menthyl acetate. The range for the 17 measurements was 0.3–1.5% α-pinene, 3.8–8.1% β-pinene, 0.4–4.5% isopinocamphone and 68–87% limonene. Fourteen individuals had less that 1% cineole, while the others had 4.0, 9.1 and 12.0%. Linalool and linalyl acetate were not found but trace amounts of menthofuran were noted in a few individuals.

The dominant gene *I* causing linalool also largely prevents the development of isopinocamphone, limonene, cineole, carvone, dihydrocarvone and all 3-oxygenated compounds [3]. Very small amounts, however, of the pinenes, cineole, 3-octanol, piperitone, pulegone, menthol,

Table 2. Major constituents of the four chemotypes of allo-octoploid species *M. citrata* and *M. aquatica*

	Limonene	Cineole	β-Pinene	Isopino-camphone	Linalool	Linalyl acetate	Mentho-furan
M. aquatica							
Strain 1*	4.9	7.7	1.9	—	0.3	—	66.4
Strain 1	5.6	4.3	0.7	—	—	—	83.0
Strain 2	6.4	22.4	2.3	—	—	—	51.3
Handa Strain	8.7	7.2	1.9	—	—	—	71.4
Strain 3	9.1	9.4	1.9	—	—	—	71.0
M. citrata							
Strain 2	1.9	1.3	0.6	1.3	30.0	58.5	—
Handa Strain	1.1	0.2	0.9	—	32.4	45.0	0.1
High limonene hybrid							
72-7501	72.0	9.1	4.8	0.5	—	—	tr
72-7547	72.8	0.4	6.6	3.0	—	—	tr
72-7587	83.7	0.2	4.8	2.2	—	—	tr
High isopinocamphone hybrid							
72-7689	26.4	0.4	25.0	29.9	—	—	0.1
72-7681	17.5	0.7	25.2	29.3	—	—	0.2

*Strain used by Hefendehl and Murray, 1973.

Table 3. Monoterpenoid composition (% of total volatiles) of strains hybridized to isopinocamphone chemotype and of resulting hybrids having camphoraceous odour

Species hybridized	Strain (chromosome no.)	Compounds largely inhibited by *Is* gene	Number hybrids having		Composition of hybrids with camphor odour		
			iso	not iso	limonene	β-pinene	isopinocamphone
1. *M. aquatica*	Dutch ($2n = 96$)	67% limonene/ 24% cineole	129	163	20.3	23.3	29.7
2. *M. arvensis*	($2n = 96$)	47% cineole/ 16% *cis*-ocimene	121	121	18.7	32.7	6.8
3. *M. citrata*	($2n = 96$)	30% linalool/ 58.5% linalyl acetate	59	91			
Tester strains with 2-oxygenated *p*-menthane monoterpenoids							
4. *M. spicata*	Line 1 ($2n = 48$)	48% carvone/ 16% dihydrocarvone	42	46	32.3	19.5	13.7
5. *M. spicata*	Dutch 17-4 ($2n = 48$)	70% carvone/ 2% dihydrocarvone	246	293	29.3	24.0	18.8
6. *M. crispa*	S_0* ($2n = 48$)	58%carvone/ 11% dihydrocarvone	24	30	29.5	22.0	21.6
7. *M. cordifolia*	Thomas 198 ($2n = 48$)	56% carvone/ 6% dihydrocarvone	96	147	38.7	22.2	14.6
8. *M. cardiaca*	polyploid S_0 ($4n = 144$)	65% carvone/ 2% dihydrocarvone	19	59	85.5	6.0	1.4
Tester strains with 3-oxygenated *p*-menthane monoterpenoids							
9. *M. crispa*	S_1 69-221 ($2n = 48$)	40% piperitenone	20	15	32.1	24.4	19.8
10. *M. rotundifolia*	Fester ($4n = 48$)	55% piperitenone oxide	38	48	52.4	17.1	17.6
11. *M. longifolia*	($4n = 48$)	60% piperitone oxide/ 3% piperitone	103	130	48.0	24.1	13.0
12. *M. crispa*	S_1 69-211 ($2n = 48$)	43% pulegone	107	113	29.2	23.1	22.0
13. *M. crispa*	S_1 70-296 ($2n = 48$)	40% isomenthone	38	44	33.3	21.5	19.4
14. *M. crispa*	S_1 ($2n = 48$)	50–70% menthone	28	37	37.7	20.7	15.2
15. *M. piperita*	polyploid S_0 ($4n = 144$)	22% menthone/ 50% menthol	19	22	45.6	20.7	12.6
16. *M. arvensis*	cultivar ($2n = 96$)	72% menthol/ 10% menthone	380	408	78.8	5.4	2.5
17. *M. arvensis*	Wisconsin ($2n = 96$)	75% isopulegone			82.0	5.9	2.3

*Often named *M. spicata* var. crispata Schrad.

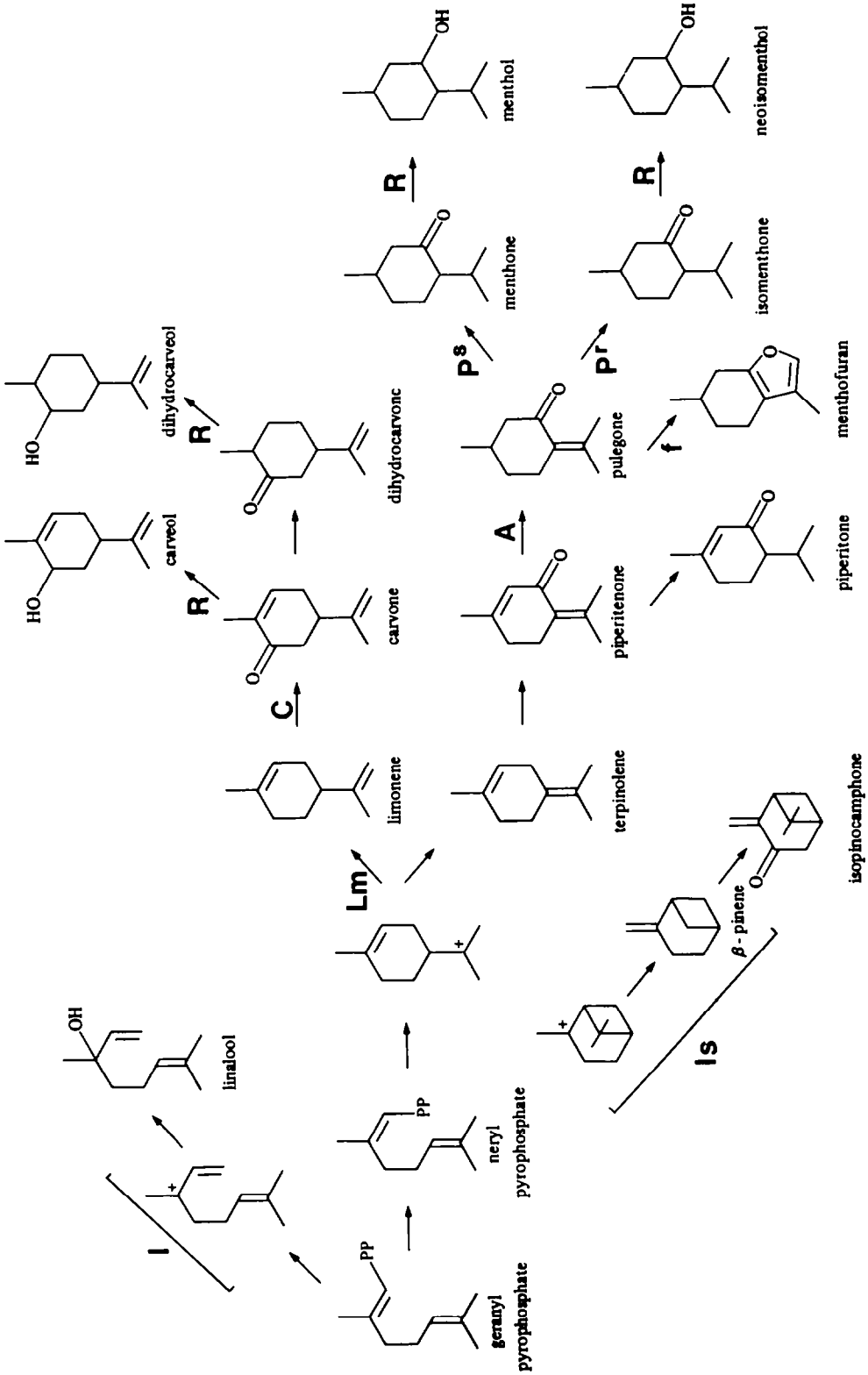

Fig. 1. Hypothesized biosynthetic pathway for some principal monoterpenoids of *Mentha* species and proposed gene actions.

menthyl acetate and menthofuran were reported by Handa *et al.* [11] in the linalool chemotype of *M. citrata*.

The high terpene content of certain *M. arvensis* chemotypes [12] and the unusual oil composition of *M. arvensis* hybrids with either linalool or isopinocamphone chemotypes suggest that the genotype controlling early stages of biogenesis in species of the axillary-flowered or verticillatae group must differ from that of species in the terminal-flowered spicatae and capitatae groups of the subgenus *Menthastrum*. Note that hybrids with the standard strain of *M. arvensis* having the cineole/ocimene chemotype produce almost the same amount of β-pinene but less isopinocamphone that similar hybrids with *M. aquatica*. Certain *M. citrata* hybrids with wild-type *M. arvensis* may have only 15% linalool/linalyl acetate. Strain 2 of *M. citrata* in Table 3 had 30.0% linalool, 58.5% linalyl acetate and 1.9% limonene but two of its hybrids with the *M. arvensis* cultivar had 6.6–7.0% linalool, 25.7–30.1% linalyl acetate and 39.5–41.1% limonene. Other tester strains with affinities to the verticillatae groups follow a similar pattern. The *M. cardiaca* cultivar with a carvone chemotype is believed to be a *M. arvensis* ($2n = 96$) × *M. spicata* ($2n = 48$) F_1 hybrid. The assayed polyploid *M. cardiaca* hybrid had only 1.4% isopinocamphone, 85.5% limonene, 0.6% cineole and no appreciable amount of carvone.

Oil biogenesis

A hypothetical biosynthetic pathway (Fig. 1) is presented in an attempt to explain oil biogenesis in terms of genetic control of specific reactions and gene interactions. Some aspects of the pathway are in dispute, e.g. the roles of geranyl, neryl and linalyl pyrophosphates as initial precursors [13], but general agreement has been reached on many of the monoterpenoid interconversions based on the results of enzymatic isolations (14–16). The dominant *I* gene of *M. citrata* is hypothesized to utilize the precursor to all other monoterpenoids and consequently prevent the formation of any substantial amount of them, despite the possible presence of many of the dominant genes controlling interconversion. Thus, the dominant *I* gene is epistatic to the expression of the other genes. An individual with an *ii* genotype would not produce significant amounts of linalool, but would contain monoterpenoids determined by the other gene products controlling the pathway. A plant containing large amounts of isopinocamphone would have the *ii* genes to prevent formation of linalool and the *Isis* or *IsIs* genotype causing formation of isopinocamphone. This plant could obviously contain either dominant or recessive alleles for the *Lm*, *C*, *A*, *P*, *R* or *f* loci without effect on the presence or absence of the major constituents listed in Table 1. The proposed actions of genes on biosynthesis is consistent with the gene interactions described above and biosynthetic pathways proposed from tracer and enzymatic studies [16, 17].

CONCLUSIONS

The interaction of at least four linked genes in coupling phase (*I Is Lm C/i is lm c*) would seem to be required to determine the presence of initial carbonium ion and its four initial products. Further research is needed to determine whether a fifth gene, *At* or *at*, controlling α-terpineol exists and belongs to this linkage group. Additionally, the genes *A*, *P*, *R* and *E* appear to be independently inherited whereas *P* and *F* that control the conversion of pulegone to menthone or pulegone to menthofuran are possibly linked [9, 18]. The effect of any one gene depends upon the other genes in the genotype and pinpointing the effects of a single gene becomes more definite as other related genes are understood.

The chemical composition of *M. aquatica* $\frac{i\ is\ lm\ c}{i\ is\ lm\ c}$ has been compared to the limonene chemotype [4] $\frac{i\ is\ Lm\ c}{i\ is\ lm\ c}$, carvone substitution strain [5] $\frac{i\ is\ Lm\ C}{i\ is\ lm\ c}$, isopinocamphone chemotype $\frac{i\ Is\ Lm\ c}{i\ is\ lm\ c}$ and *M. citrata* [3] $\frac{I\ Is\ Lm\ C}{I\ is\ lm\ c}$.

EXPERIMENTAL

Plant material and oil isolation. The parental strains and hybrids were grown on organic soil at Mentha, Michigan (42° 33'). The mature herbage was harvested in early September, air dried in a greenhouse and distilled in the 19.9-l. (21 qt.) pressure cooker of an experimental still designed by Reitsema and Baarman [19].

Oil analysis. The bulked oil from seven first backcross individuals was analysed by a combination of chromatographic techniques [20–22]. All compounds isolated during the analysis were characterized by comparing their IR spectrum with standard or previously published spectra. The GLC quantitative assays reported in Tables 2 and 3 were made using a 7.31 mm × 3.2 mm o.d. stainless steel column packed with a 3% soln of silicone SP-2401 plus 0.2% Emulphor ON-870 on Chromosorb W High Performance 100–120 mesh. A Varian Aerograph 1400 was operated with the column flow at 44 ml/min and the temp. programmed from 100 to 160° at 4°/min. All % are uncorrected area % calculated from electronic integration measurements of FID. The compound identities were compared with the above analysis using a peak enrichment technique.

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