Phytochemistry, 1978, Vol 17, pp 1727–1730 © Pergamon Press Ltd Printed in England. 0031-9422/78/1001-1727 $02.00/0

MONOGENIC BASIS FOR REDUCTION OF (+)-PULEGONE TO (−)-MENTHONE IN *MENTHA* OIL BIOGENESIS

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(*Revised received* 10 *April* 1978)

Key Word Index—*Mentha arvensis*; *M. crispa*; Labiatae; mint; genetics; biosynthesis; monoterpene; (+)-pulegone.

Abstract—A chemotype of *Mentha arvensis*, having the genotype *AA pp rr FF* with 79% (+)-pulegone, less than 6% (−)-menthone and less than 0.1% menthofuran, was hybridized with the menthol-producing cultivar *M. arvensis* L. var. *piperascens*, having the genotype *AA PP RR* (or *Rr*) *FF* with 78% (−)-menthol, 5% (−)-menthone and less than 0.1% (+)-pulegone and menthofuran. All 58 F_1 hybrids had menthone and/or its derived alcohol menthol showing that the *P* gene is dominant or epistatic. Fertile F_1 hybrids (*Pp*) backcrossed to the pulegone parent (*pp*) gave a 1:1 ratio, demonstrating the monogenic nature of the *P* gene in *M. arvensis* which converts (+)-pulegone to (−)-menthone. Data in the F_2 agree with a 3 menthone: 1 pulegone ratio. It appears that the accumulation of 60–90% (+)-pulegone in certain strains and species of *Mentha* which have the *A* gene to convert piperitenone to pulegone occurs because they lack the dominant *P* gene to reduce pulegone to menthone and the *ff* genotype to oxidize pulegone to menthofuran. A residual amount of 0.1–15.0% pulegone is found in 24 S_1 strains of *M. crispa*, some strains of *M. spicata*, *M. X piperita* and *M. arvensis* that have an *A- P- F-* genotype and produce 50–90% (−)-menthone or its products. *Mentha crispa* S_1 strains and perhaps some strains of *M. pulegium* and *M. gattefossei* having nearly equal amounts of pulegone and menthone may be postulated to have a *P-* genotype with the conversion of pulegone influenced by modifying factors, or a *pp* genotype.

INTRODUCTION

In studies of the control of biosynthesis of monoterpenoids, it is necessary to obtain direct proof of the gene control of conversions. In the genus *Mentha* this is particularly difficult as it is a polyploid genus containing allo-tetraploid and allo-octoploid species. Studies of genetic control of mint monoterpenoid biosynthesis have both commercial and scientific values; i.e. there is considerable chemosystematic interest in *Mentha* and related genera of the Labiatae. The *Mentha* oil constituent (+)-pulegone is of paramount importance to an understanding of the biogenesis of 3-oxygenated *p*-menthane monoterpenoids since it is believed to be oxidized to menthofuran or reduced to (+)-isomenthone or (−)-menthone. These ketones are further converted to the corresponding alcohols and esters; i.e. (−)-menthone to (−)-menthol and (−)-menthyl acetate [1–4]. Altogether, these constituents account for about 80% of the oil of peppermint [5] and any increase of menthofuran above the preferred level of 1–3% lowers the oil quality and thus the market value of the oil.

The conversion of pulegone to menthone or menthofuran, in *Mentha*, was hypothesized by Reitsema [4]. This hypothesis has since been extended to include conversion of pulegone to (+)-isomenthone as well as (−)-menthone by Loomis [3]. Subsequent radioactive tracer studies using cell-free systems have confirmed the latter conversions [6, 7]. However, the nature of the genetic control of the conversion of pulegone to (−)-menthone (Fig. 1) has not been examined. Such a study requires the use of species or varieties having menthone and pulegone as their principal oil constituents.

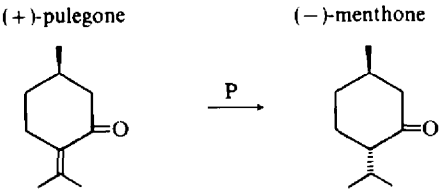

Fig. 1.

Certain strains of *M. arvensis* L. var. *piperascens* Briq. with the genotype *rr* produce oils containing 75–80% (−)-menthone, whereas strains with an *RR* or *Rr* genotype produce oils containing 70–85% (−)-menthol and are the source of natural menthol or of partly dementholized corn mint oil used as a flavoring agent [8]. In contrast, members of the subgenus *Pulegium* of the genus *Mentha* usually have 70–85% (+)-pulegone and very small amounts of (−)-menthone [9]. These species presumably lack the enzyme that allows the conversion of pulegone to menthone in *M. piperita* L. [6, 7, 10]. *Mentha requienii* Benth. (2n = 18) could not be hybridized to *M. spicata* L. (2n = 48) or any species of the subgenus *Menthastrum* having menthone. *Mentha gattefossei* Maire (2n = 48) could be hybridized to *M. arvensis* var. *piperascens* (2n = 96), but the F_1 hybrids having menthone were completely sterile and

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could not be backcrossed to the recessive *M. gattefossei* parent to determine from the genetic ratio whether or not a single gene controlled the enzyme [11].

If a fertile menthone-odored individual had been obtained in a first backcross progeny, further backcrossing of the individual often would have been possible and would allow substitution of the epistatic genes or the dominant allelomorph of a single gene from the *M. arvensis* species into the recurrent parent *M. gattefossei* by 4–6 convergent backcrosses. The gene substitution method has been previously employed in studying *M. aquatica* L. [12–14], but appeared impossible here unless polyploid strains of *M. gattefossei* were produced and hybridized with the F_1 hybrid. The discovery and report [15] of a wild-type strain of *M. arvensis* having 79–92% pulegone allowed the more direct experimental approach of hybridizing interfertile varieties of *M. arvensis* (2n = 96) having pulegone and menthone for a study of F_2 and test cross progenies. Thus, one of the objectives of the present work was to ascertain from intercrosses of fertile allo-octoploid *Mentha* species whether or not the conversion of pulegone to menthone was due to a single gene *P*.

RESULTS AND DISCUSSION

One fertile strain of *M. gattefossei* and the fertile strains of *M. pulegium* having oils containing 60–70% pulegone and 1–15% menthone were self-pollinated. The data in Table 1 show that these strains breed true since all the S_1 individuals had a strong pulegone odor. The odor of a compound which is present in predominant proportions in an essential oil is discernible to an experienced observer. The data from the sterile *M. arvensis* × *M. gattefossei* F_1 hybrids previously reported [11], with the totals repeated in Table 1, indicate that ability to convert pulegone to menthone is due to an epistatic or dominant gene or genes designated 'P'. The postulated recessive *pp* or *pp pp* genotype of *M. gattefossei* apparently does not allow appreciable conversion of pulegone to menthone. *Mentha gattefossei* and *M. pulegium* also must have the dominant *FF* genotype to accumulate pulegone, since the recessive *ff* genotype of *M. aquatica* would allow the rapid oxidation of pulegone to menthofuran [13].

The strain of *M. arvensis* from Saskatoon, Canada whose oil has 79% pulegone and almost no menthone

Table 1. Genetic data and probable genotypes for crosses examining the genetic control of pulegone conversion to (−)-menthone (data based on odor evaluation)

Self or cross	Principal oil constituent: Menthone/menthol	Pulegone	Piperitone	*P* value of χ^2 test
M. gattefossei 66% pulegone *pp AA* selfed	0	61	0	
M. pulegium strain 1 60% pulegone *pp AA* selfed	0	921	0	
M. pulegium strain 2 52% pulegone *pp AA* selfed	0	5036	0	
M. arvensis ms* 78% menthone/menthol *PP AA* × *M. gattefossei* 66% pulegone *pp aa*	208	0	0	
M. arvensis ms 79% pulegone *pp AA* × *M. rotundifolia* 50% piperitenone oxide *PP aa*	9	0	0	—
M. arvensis ms 79% pulegone *pp AA* × 4n *M. longifolia* 60% piperitone oxide *PP PP aa aa*	66	0	0	
M. arvensis ms 79% pulegone *pp AA* × 4n *M. spicata* 53% piperitone *PP PP PP PP aa aa aa aa*	37	0	0	
M. arvensis var. *piperascens* 78% menthone/menthol *PP AA* selfed	402	0	0	
M. arvensis ms 79% pulegone *pp AA* × *M. arvensis* 78% menthone/menthol *PP AA*	58	0	0	
F_1(*M. arvensis* pulegone chemotype × *M. arvensis* menthol chemotype) selfed for F_2	175	53	0	0.5†
M. arvensis ms 79% pulegone *pp AA* × F_1 *Pp AA* of above cross for a test cross	145	120	0	0.1‡
M. aquatica ms menthone/menthofuran *PP AA* × fertile *M. aquatica* menthone/menthofuran *PP AA*	780	0	0	
M. aquatica ms 84% menthofuran/menthone *PP AA* × *M. pulegium* 60% pulegone *pp aa*	28	0	0	
M. crispa S_1 strain 211 43% pulegone *pp A*? selfed	0	16	0	
M. arvensis ms 83% menthone/menthol *PP AA* × *M. crispa* S_1 strain 211 43% pulegone *pp A-*	35	0	0	
M. aquatica ms 84% menthofuran/menthone *PP AA* × *M. crispa* S_1 strains 211 43% pulegone *pp A-*	99	0	0	
M. crispa S_1 strain 213 33% pulegone *pp A*? selfed	0	8	0	
M. arvensis ms 83% menthone/menthol *PP AA* × *M. crispa* S_1 strain 213 33% pulegone *pp A-*	85	0	0	
M. aquatica ms 84% menthofuran/menthone *PP AA* × *M. crispa* S_1 strain 213 33% pulegone *pp A-*	211	0	0	
M. crispa S_1 strain 213 ms high pulegone *pp Aa* × *M. longifolia* 50% piperitone oxide *PP aa*	122	0	101	0.2‡
M. crispa S_1 strain 213 ms high pulegone *pp Aa* × 4n *M. longifolia* 60% piperitone oxide *PP PP aa aa*	33	0	32	0.5‡
M. crispa S_1 strain 213 ms high pulegone *pp Aa* × *M. spicata* S_1 60% menthone *PP AA*	51	0	0	

* ms = genic male sterility; †3:1 ratio; ‡1:1 ratio.

Table 2. Monoterpenoid composition of parental strains (% of total essential oil)

Oil constituent	*M. arvensis* pulegone chemotype	*M. arvensis* menthol chemotype	*M. crispa* S_1 Strain 213
α-Pinene	0.1	0.3	0.7
β-Pinene	0.1	0.3	1.5
Myrcene	0.1	0.5	2.1
Limonene	0.2	3.5	0.7
Cineole + 3-octanol	0.4	0.5	6.0
Sabinene hydrate + linalool	0.5	0.3	—
Piperitenone	0.5	—	—
(−)-Piperitone	2.4	2.4	2.4
(+)-Pulegone	79.3	tr	33.2
Isopulegone	2.4	—	—
(+)-Menthofuran	0.1	tr	0.4
(−)-Menthone	5.5	4.7	43.8
(+)-Isomenthone	5.5	2.4	2.3
(−)-Menthol	0.6	78.4	0.8
(+)-Neomenthol	0.2	1.8	0.1
(−)-Menthyl acetate	0.1	2.8	0.4

(Table 2) is male sterile and cannot be self-pollinated to determine if it is true breeding for the postulated recessive *pp* or *pp pp* genotype. The genotype of this strain must be determined from crosses with known tester genotypes and from the function of related genes.

Wild-type or natural strains of *M. arvensis* from North America and cultivar strains of *M. arvensis* var. *piperascens* grown for menthol production in Japan and Brazil have 96 somatic chromosomes [16, 17], allowing the production of fertile F_1 progeny. Clonally propagated individuals of the high pulegone chemotype of *M. arvensis pp AA* were hybridized with true breeding individuals of the menthone/menthol chemotype *PP AA* (Table 2) and produced 58 F_1 hybrids (*Pp AA*) that had a menthone/menthol odor, confirming previous data that the *P* gene or genes allowing the conversion of pulegone to menthone was epistatic or dominant. About half the individuals were male sterile like the seed parent, the other half had fertile pollen. Fertile F_1 hybrids (*Pp*) backcrossed to the pulegone chemotype of *M. arvensis* (*pp*) gave a ratio of 145 menthone/menthol-odored: 120 pulegone-odored individuals ($P = 0.1$ for a 1:1 ratio), demonstrating the monogenic nature of the *P* gene in *M. arvensis* that converts pulegone to menthone. Chromatographic analyses showed no mistakes in 20 menthone-odored individuals and one mistake in 10 pulegone-odored. F_2 data of 175:53 agree with 3:1 ratio ($P = 0.5$). From these ratios based on odor with verification of 29 out of 30 individuals assayed, it appears that the accumulation of 60–90% (+)-pulegone occurs in certain *M. pulegium*, *M. gattefossei*, and *M. arvensis* strains that have the dominant *A* gene to convert piperitenone → pulegone, but lack the dominant *P* gene to reduce pulegone → menthone or the recessive *ff* genotype to oxidize pulegone to menthofuran.

All F_1 hybrids of the high pulegone *M. arvensis* crossed with either *M. rotundifolia*, or colchicine-induced polyploids of *M. longifolia* and *M. spicata* having recessive *aa* genotypes [18] produce oils with a strong menthone odor (Table 1). In the first cross, these results would occur if pulegone *M. arvensis* had the genotype *AA pp*, *M. rotundifolia aa PP*, and the F_1 hybrid *Aa Pp*. In *M. rotundifolia* the recessive *aa* genotype is epistatic over the *P* gene since pulegone cannot be converted to menthone unless pulegone is made from its presumed precursor piperitenone by the dominant *A* gene. Similarly, the polyploids of *M. longifolia* and *M. spicata* would be homosygous for the *a* and *P* genes, but on tetraploid and octoploid levels, respectively.

All strains of *M. gattefossei*, *M. pulegium*, *M. requienii* and *M. arvensis* that have 60–85% pulegone with 5–10% total (−)-menthone and (+)-isomenthone may be postulated to have the genotype *pp AA*, if one assumes that small amounts of menthone are due to the recessive *p* allelomorph performing the same function as the dominant *P* allelomorph at a much slower rate. The question is whether or not *M. pulegium* strain 2, having 52% (+)-pulegone, 30.8% (−)-menthone and 5.2% (+)-isomenthone, is different and has a *Pp AA* or *PP AA* genotype which allows menthone production reduced by modifying factors.

In *M. piperita*, a sterile allo-hexaploid which is able to convert pulegone to other monoterpenoids, the amounts of these compounds changes with leaf development and is influenced by environmental factors, such as photoperiod and night temperature [19]. Thus, they vary with geographic area and season of harvest [19]. The expected developmental pattern is for reduced amounts of pulegone and increased amounts of its products. The assay for the high pulegone strain of *M. arvensis* (Table 2) was from mature plant material harvested on October 27 to examine this possibility, and had negative results.

The genetic basis for the conversion of pulegone to menthone was also examined in two tetraploid species, *M. spicata* and the closely related *M. crispa* ($2n = 48$). Twenty-eight S_1 inbred strains of *M. crispa* were harvested on 25 June, 1960, and their essential oils had differing proportions of pulegone and menthone plus isomenthone. The proportions of menthone plus isomenthone ranged from 38.7 to 81.0% and pulegone from < 1.0 to 43.4%. Some seasonal variation in the proportions was found, but the strains retained their identity from 1949 to 1972 [20]. Variations in the proportions of pulegone and menthones did not appear to influence the amounts of other constituents. The essential oil of Strain 213 had 19.2% of other components and 49.6% menthone. Strain 192 was also characterized by 19.8% of other components, but had 75.7% of the menthones. Two strains (211 and 213) with the highest pulegone proportions, 43.4 and 31.2% respectively, were useful as tester strains.

The self-pollinated progeny of Strains 211 and 213 seemed to breed true for a high pulegone odor (Table 1), but the numbers were small and should have been assayed to show variation in proportions of pulegone and menthone. Hybrids with cultivar *M. arvensis* and *M. aquatica* were menthone/menthol odored.

A dominant male-sterile gene found in *M. spicata* was substituted into the *M. crispa* S_1 by three convergent crosses to produce male-sterile Strain 213 with a strong pulegone odor. The presence of progeny with a high piperitone (or its oxide) in hybrids with *M. longifolia* show that male-sterile Strain 213 has the genotype *Aa*. The crossing data presented in Table 1 are not sufficient to determine whether the genotype of fertile *M. crispa* S_1 Strains 211 and 213 or male-sterile Strain 213 is *PP*, *Pp* or *pp*. These strains are shown as having *pp*

genotypes on the dubious strength of the S_1 data. Alternatively, they may have *Pp* genotypes in which the conversion of pulegone is reduced by modifying factors. However, 24 of the *M. crispa* S_1 strains seem to have a *PP AA* or *Pp AA* genotype since the 1-15% pulegone found in the oil could be considered as a residual precursor constituent not wholly converted to menthones. Similarly, *M. pulegium* or *M. gattefossei* strains with equal amounts of pulegone and menthone may have either a *P- A- FF* genotype and modifying factors that reduce the conversion of pulegone, or a *pp A- FF* genotype.

EXPERIMENTAL

All parental strains used in this research may be obtained from the Basic *Mentha* Foundation Nursery maintained by the U.S. Dept. of Agriculture at Oregon State University, Corvallis. All crosses were made in a screened greenhouse and progeny grown until large enough for odor analysis. *Mentha crispa* S_1 inbreds were maintained by vegetative propagation from 1949 to 1972 in a field nursery. Gas chromatographic assays were made on hexane extracted oil of greenhouse-grown plants, using a Beckman GLC-4 equipped with a 7.31 m × 3.2 mm o.d. stainless steel column packed with a 3% soln of Silicone DC QF-1 plus 0.2% Co 880 on Chromosorb W High Performance 100–120 mesh, N_2, FID, digital integrator.

Acknowledgements— We would like to thank Prof. J. H. Langenheim for providing comments on the manuscript. A high pulegone strain of *M. arvensis* [15] had not been retained, but Prof. E. von Rudloff of the National Research Council, Prairie Regional Laboratory, Saskatoon, Saskatchewan was kind enough to collect a similar clone from the same Beaver Creek area approximately 10 miles south of Saskatoon. Steam distilled herbage from field plots in 1972 had a similar oil composition to the strain previously reported. We are indebted to R. Hegnauer for *M. aquatica* from Leiden, Netherlands.

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