The Inheritance of Leaf Oil Composition in *Clausena anisum-olens* (Blanco) Merr.

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Abstract

The essential oil from leaves of *Clausena anisum-olens* (Blanco) Merr. var. *anisum-olens* (Rutaceae) is studied individually on 91 cultivated and wild plants. Main compounds in the oil were (E)-anethole and/or methyl chavicol, and their respective percentages were stable through time and cultivation for each plant. Variations in oil contents between individuals showed a distribution pattern of apparent genetic origin, with three chemovariants: "pure anethole" oil, "pure methyl chavicol" oil, and "mixed" oil (about 90% anethole - 10% methyl chavicol).

Key Word Index

Clausena anisum-olens, Rutaceae, essential oil composition, (E)-anethole, methyl chavicol.

Introduction

Clausena anisum-olens (Blanco) Merr. var. *anisum-olens* (Rutaceae), an endemic small tree of the Philippine forests (1,2), is used as a multipurpose folk medicine (3) as well as a condiment. Such uses are related to the strong anise-like smell of the whole plant, which is called "Anis" in Batangas Province [other Tagalog names are Kayumanis, Kalomata, Kamangianis, Maisipaisi (3)]. Although roots and fruit are sometimes used in medicinal preparations, leaves are the most aromatic and the most frequently used part of the plant (3). Former studies reported that the oil is easily isolated from leaves by steam distillation (1-3% oil from fresh weight), and consists either of methylchavicol (90-95 %) (4,5) or anethole (75-90%) (6,7).

In 1990 and early 1991, a first study on a sample consisting of 32 seedlings collected in the Philippines and grown in a greenhouse in France suggested the presence of at least three different chemovariants for the leaf oil composition. Later, larger samples were taken, in order to verify this hypothesis and to study the variation in the oil composition.

Experimental

A total number of 91 individuals were studied. Two were small trees (numbered 138 & 139), of unknown origin cultivated in Quezon City, in the collections of the College of Pharmacy, University of the Philippines, Manila. Two other trees grew spontaneously in cultivated areas of the Batangas Province (Luzon Island), where they were kept as medicinal plants. The first is hereafter referred as BI (for Barangay Inosluban, the locality where it has been found), while the second is called MK (for Mataas na Kahooy, another village). Among the numerous natural seedlings growing at the foot of the latter, we

studied a total number of 87, and brought 32 to France. Voucher specimens were collected from trees MK (*J-F. Molino 201*) and BI (*J-F. Molino 202*) and deposited in the Herbarium of the Institut de Botanique, Montpellier (MPU).

A small leaf sample was collected on each plant and immediately weighed. Weights varied from 0.03 g, for the smallest seedling, to 2.2 g, for a sample collected on tree BI. Each sample was then introduced in a precise volume of solvent (5, 10, 15, 16 or 17 mL, depending on the size of the leaf sample) and sealed. Solvents are Dichloromethane (Table I) or Ethanol absolute (Tables II-V). After a few days static extraction the solvent was submitted to GC/MS analysis. GC/MS was performed using a 5890 Series II Hewlett-Packard Gas Chromatograph coupled via an interface to a Magnetic Sector Mass Spectrometer (Profile, Kratos), using the following conditions: split/splitless injector at 250°C, FFAP (Hewlett-Packard) capillary column (50 m x 0.32 mm; film thickness 0.53 μm). Oven was first kept isothermal at 50°C for 3 min, then temperature program rate was 3°C/min up to 120°C, 2°C/min up to 220°C. Samples (1 μL) were injected using splitless mode. Mass spectrometer setup: source and interface temperature were 200°C and 250°C respectively; source vacuum was 2-10-5 Torr and the electron energy 70 eV. The magnet was calibrated from mass 20 to mass 350 with PFK.

Only four major compounds (in some cases only two) were quantified. The total of all analyzed compounds varies in most cases between 15 and 30 g/kg. It is similar to or higher than the percentage of oil obtained through steam distillation (4-6), and is therefore considered as a good approximation to the total oil content. In all cases, methyl chavicol and (E)-anethole, accounted for 97-100% of the oil. The two other constituents were (Z)-anethole and anisaldehyde. In all tables mean values are given with a 99% confidence interval.

Results and Discussion

The first series of determinations concerned methyl chavicol and (E)-anethole contents in the oils of 32 seedlings growing in the greenhouse in France, 27 of which were studied four times, at different periods of the year, while 5 others died before the end of the experiment and therefore have been studied only one to three times. Results show a clear partition of all plants among three very distinct groups, with respect to methyl chavicol and (E)-anethole percentages (Table I). They are: group E, whose oil consisted of almost pure methyl chavicol; group A, whose oil consists of almost pure (E)-anethole; and group AE, whose oil was a mixture of about 90% (E)-anethole and 10% methyl chavicol. Moreover, this partition is so clear that it is possible, for anybody familiar with the odors of methyl chavicol and (E)-anethole, to classify the plants into these three categories only by crushing and smelling their leaves. For each group, the oil composition was found to be stable through time (Table I).

A study on a larger sample (87 plants, including the 32 above) of MK's lineage confirmed the partition into the same three very homogeneous groups E, A and AE (Table II). Here anisaldehyde and (Z)-anethole were also analyzed. These compounds were present in very variable, although always small amounts, and it seemed impossible to distinguish any particular feature in their distribution.

The oil of the MK tree itself was also studied on 11 samples of leaves at different stages (young or mature) and in different light exposures (shade, i.e., at base of the tree crown, or full light, at top of the tree). Similarly, we studied eight leaf samples taken from the tree BI. Results show that both trees belong to group AE, and that the methylchavicol and (E)-anethole percentages in the oil are independent of age (Table III) and light exposure (Table IV). Finally, young trees 138 and 139 were analyzed only once, and proved to belong to groups E and A, respectively (Table V).

In spite of considerable variations in total oil content, no external cause could be involved to explain this distribution pattern and the remarkable stability of methyl chavicol and (E)-anethole ratios for each chemovariant. This clearly suggests a genetic origin, and the situation is as follows: MK, a tree belonging to chemovariant AE, bred a mixed lineage consisting of (schematically) 50% chemovariants AE, 25% E and 25% A (Table II). This strongly suggests Mendel's laws and the action of a biallelic gene with a partial dominance of one allel upon the other.

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Table I. Stability through time of methyl chavicol and (E)-anethole percentages in leaf oils of three distinct groups of seedlings born from the tree MK

	July 1990	September 1990	February 1991	May 1991
GroupA	n = 4	n = 4	n = 4	n = 4
(total oil in g/kg)	(28.45 ± 5.13)	(24.03 ± 7.87)	(4.78 ± 2.67)	(12.44 ± 2.45)
(E)-anethole%	99.47 ± 0.16	99.65 ± 0.31	99.9[a]	99.67 ± 0.29
Methyl chavicol %	0.53 ± 0.16	0.35 ± 0.31	0.1[a]	0.33 ± 0.29
GroupE	n = 8	n = 10	n = 8	n = 8
(total oil in g/kg)	(33.42 ± 7.71)	(29.36 ± 4.15)	(7.46 ± 3.01)	(21.14 ± 3.65)
(E)-anethole %	0.13 ± 0.10	0[b]	0[b]	0[b]
Methyl chavicol %	99.87 ± 0.10	100[b]	100[b]	100[b]
GroupAE	n = 16	n = 17	n = 16	n = 16
(total oil in g/kg)	(26.67 ± 4.34)	(24.95 ± 3.48)	(7.21 ± 2.10)	(13.79 ± 1.21)
(E)-anethole %	90.73 ± 0.86	88.04 ± 1.47	88.34 ± 0.73	89.54 ±1.00
Methyl chavicol %	9.27 ± 0.86	11.96 ± 1.47	11.66 ± 0.73	10.46 ± 1.00

[a] standard deviation = 0.20, theoretical confidence intervals include values < 0% or > 100%; [b] s. d. = 0

Table II. Characterization of three chemotypes in the lineage of tree MK (87 seedlings)

	Chemotype A	Chemotype E	Chemotype AE
Number of seedlings	n = 20	n = 24	n = 43
Distribution in sample	(23.0%)	(27.6%)	(49.4%)
Total oil (g/kg)	21.34 ± 3.15	28.53 ± 3.47	23.06 ± 2.62
(E)-anethole(%)	99.25 ± 0.36	0.04[a]	89.18 ± 0.76
Methyl chavicol (%)	0.43 ± 0.25	99.94 ± 0.1	10.21 ± 0.80
(Z)-anethole (%)	0.14 ± 0.10	0.02[b]	0.15 ± 0.08
Anisaldehyde (%)	0.18[c]	0[d]	0.46 ± 0.30

[a] standard deviation = 0.09, theoretical confidence interval includes values < 0%; [b] id., s. d. = 0.05; [c] id., s. d. = 0.43 ; (d) s. d. = 0

Table III. Comparative composition of the oils from young and mature leaves of trees MK and BI

Components	Young leaves (near apex, not fully expanded, light green) n = 9 (5 samples from MK + 4 from BI)		Mature leaves (base of twigs, fully expanded, dark green) n = 10 (6 samples from MK + 4 from BI)	
	%	Standard deviation	%	Standard deviation
(E)-Anethole	88.53 ± 1.17	1.37	89.69 ± 1.73	2.13
Methyl chavicol	11.17 ± 1.23	1.44	9.69 ± 1.75	2.15
(Z)-Anethole	0.09[a]	0.13	0.46 ± 0.22	0.27
Anisaldehyde	0.21 ± 0.18	0.21	0.16[a]	0.22

[a] theoretical confidence interval includes values < 0%

Table IV. Comparative composition of the oils from leaves of trees MK and BI in different light exposure

Components	Shade (bottom of the tree crown) n = 11 (7 samples from MK + 4 from BI)		Full light (top of the tree) n = 8 (4 samples from MK + 4 from BI)	
	%	Standard deviation	%	Standard deviation
(E)-Anethole	89.55 ± 1.55	1.99	88.58 ± 1.45	1.59
Methyl chavicol	10.13 ± 1.63	2.10	10.75 ± 1.64	1.80
(Z)-Anethole	0.15 ± 0.12	0.15	0.48 ± 0.29	0.32
Anisaldehyde	0.17 ± 0.16	0.21	0.20 ± 0.20	0.22

Table V. Composition of the oils from leaves of trees MK & BI and treelets 138 & 139

Plant characteristics	MK fruiting, 7 m high	BI flowering, 6 m high	138 treelet, 1.6 m high	139 treelet, 1.5 m high
Total oil (g/kg)	13.62 ± 5.86 (n = 11)	18.00 ± 2.31 (n = 8)	24.27 (n = 1)	21.41 (n = 1)
(E)-Anethole(%)	88.39 ± 1.59	90.18 ± 0.78	0	99.1
Methyl chavicol (%)	11.30 ±1.54	9.14 ± 0.95	100	0.6
(Z)-Anethole (%)	0.28 ± 0.21	0.40 ± 0.08	0	0
Anisaldehyde (%)	0.03[a]	0.29 ± 0.29	0	0.3
Chemotype	AE	AE	E	A

[a] s. d. = 0.09, theoretical confidence interval includes values < 0%

If it is the case, we should admit one of the following three hypotheses:

1) Isomers methyl chavicol and (E)-anethole have a common precursor, and each enzymatic reaction is coded by one of the two allels. If we call X the allel coding for (E)-anethole synthesis, and Y the allel coding for methyl chavicol synthesis, genotype XX would correspond to chemovariant A, genotype YY to chemovariant E and genotype XY to chemovariant AE.

2) Methyl chavicol is produced exclusively through transformation (isomerization) of (E)-anethole. Then, if Y is the recessive allel coding for the isomerase and X the dominant allel partially inhibiting this enzyme, we would have the following correlation between genotypes and chemovariants: XX for A, YY for E and XY for AE.

3) The reverse situation is also possible, methyl chavicol being the primary product and (E)-anethole the result of an isomerization. Then we would call X the dominant allel coding for this isomerase and Y the inhibiting allel.

A former study on *Pimpinella anisum* L. (Apiaceae) and *Ocimum basilicum* L. (Labiatae) indicates that "bifurcation of the pathway leading to allyl (e.g., methyl chavicol) and propenyl (e.g., anethole) isomers occurs likely at cinnamic acids level" (8), i.e., at p-methoxycinnamic acid for both anethole and methyl chavicol. While anethole derives directly from p-methoxycinnamic acid through reduction of the side-chain terminal carboxy group to methyl, the biosynthesis of methylchavicol involves a more complicated pathway : "loss of the carboxy group [of p-methoxycinnamic acid] and incorporation of an 'extra' carbon atom into the side-chain" (8). This point has been indirectly confirmed, through deuterium analyses, on *Foeniculum vulgare* Mill. (Apiaceae) and on *Illicium verum* Hook.f. (Illiciaceae) (9). In this scheme, the differences between the routes to anethole and methyl chavicol involve necessarily more than one gene. Actually, for those taxa whose oil contained more than traces of both anethole and methyl chavicol, such as *Ocimum basilicum* or *Foeniculum vulgare*, respective percentages of these

compounds vary significantly and continuously from one individual (or variety) to another. This is consistent with a multi-genetic difference between anethole and methyl chavicol biosyntheses, but seems to invalidate hypothesis 1), unless one imagines a different biosynthetic scheme in Rutaceae than in Apiaceae, Labiatae and Illiciaceae. In that view, it is noteworthy that high (E)-anethole and/or methyl chavicol contents in the oil from leaves have been measured in a few other taxa of the family Rutaceae: *Clausena anisata* (Willd.) Hook. f. ex Benth. (syn. *C. dunniana* H. Lév.) (10,11), *C. heptaphylla* (Roxb.) Wight et Arn. ex Steudel (1,12), *Vepris madagascarica* (Baill.) H. Perrier (13), *Melicope anisata* (H. Mann) T. G. Hartley et B. C. Stone and *M. christophersenii* (St. John) T. G. Hartley et B. C. Stone (formerly segregated, together with other Hawaiian *Melicope* spp., in the genus *Pelea* A. Gray) (14,15).

On the other hand, it has been noted that "an interconversion (allyl → propenyl) in vivo cannot be excluded in certain plants containing both isomers" (8). This would be in favor of hypothesis 3), but only if this isomerization is efficient enough to be almost complete, in chemotype A. There is now a great need for further studies on the biosynthetic pathways to methyl chavicol and (E)-anethole in *Clausena anisum-olens*, because of the potential economic importance of this plant as a natural source of (E)-anethole for food industry.

Acknowledgments

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