ONTOGENETIC VARIATION AND DIURNAL STUDY IN THE COMPOSITION OF ESSENTIAL OIL IN *ARTEMISIA DOUGLASIANA*

R.W. SCORA, J. KUMAMOTO,

Department of Botany and Plant Sciences, University of California, Riverside, CA 92521

P.F. HORNER,

Department of Biology, San Bernardino Valley College, San Bernardino, CA 92410

and J.L. HOLLENBERG

Department of Chemistry, Univerity of Redlands, Redlands, CA 92373

ABSTRACT.—The variation of 33 components in the essential oil from mature, adolescent, and juvenile leaves and terminal buds of *Artemisia douglasiana* Compositae, Anthemideae, a relictual species growing in its native California habitat, was compared by Bartlett's test for homogeneity of variance. A stepwise discriminant analysis selecting those components differentiating between life stages was followed by canonical analyses of these selected compounds differentiating all life stages from each other. Although no consistent diurnal pattern was observed, a diurnal study yielded primarily changes in the total volume of oil, while the composition of individual oil components remained relatively constant.

Artemisia douglasiana Bess. Compositae is an herbaceous perennial that grows in mesic coastal sagebrush and chapparal communities in California. It is a fairly abundant relictual species that thrives best in canyons where moisture is available throughout the year.

Numerous reports on leaf oils of other *Artemisia* species, as well as on those used in the flavor industry and for medicinal purposes, have been published. However, we have found only two reports on the constituents isolated from *A. douglasiana*.

In 1971, new guaianolide, sesquiterpene lactones, from the fall growth of *A. douglasiana* were reported (1). Leaf oils of *A. douglasiana* were included in an extensive review on the monoterpenes and systematics of the genus *Artemisia* (2). The authors stated that the North American species contained larger amounts of the irregular monoterpenes, artemisia ketone and artemisia alcohol. Previously, these had been found in *Artemisia japonica* (3) and in *Artemisia capillaris* (4).

Various constituents in the essential oils of *Artemisia dracunculus* were identified, and the seasonal changes of these, as well as the total yield of oil from various parts of the plant, were reported (5). When young leaves of *A. dracunculus* were compared with mature leaves, a higher yield of total oil in the younger leaves was observed (6). Older leaves had a higher content of methylchavicol. The authors reported further that the amount of oil rose from the beginning of the vegetative period to the beginning of bud development, then decreased somewhat, reaching its maximum during flowering time and then declining to leaf fall. Similar changes were observed for methylchavicol, while *trans*-ocimene and *cis*-α-ocimene decreased, and limonene remained moderately constant during the vegetative period. The maximum yield of essential leaf oils in *Artemisia abrotanum* was found in July (7). Three additional monoterpene compounds—an ester, an ether, and a lactone—were isolated from *Artemisia tridentata* in 1973 (8).

Volatile terpenes were proposed to be the allelopathic agents of herb exclusion in thickets of *Artemisia californica* (9), and the toxicity of its terpenes on the germination and length of radicles of *Madia sativa* seeds was studied (10). The necessity for clarifying the presence and nature of any diurnal cycling, when investigating leaf oils, has now been established in a variety of other genera.

The presence of diurnal fluctuations of individual terpene components in *Juniperus californica* was established, and it was suggested that changes in volume were due to volatilization from the leaf surface (11). Later, the volatilization of *Salvia mellifera* monoterpenes was found to be dependent primarily on the vapor pressure of the terpenes and the influence of the temperature, humidity of the air surrounding the leaf, and the surface area of oil present on the leaf (12). A study on *Juniperus scopulorum* showed that oxygenated terpenes and sesquiterpenes increased during the day, while sabinene increased during the early morning and then decreased until late evening (13). In a four-year investigation on the leaf oils of *Citrus sinensis*, a twofold diurnal change was observed, depending on the season of harvest. The amount of the main oil components varied with the total oils, so that the composition of oil showed only small variations (14).

A considerable variation in the volume of *Citrus aurantium* leaf oil was also observed. The minimum was at 0200 h, and a strong correlation existed where the amounts of individual terpenes paralleled the changes in volume of total oil. An alternative to the volatilization theory was proposed (15). In needles of *Pinus pinaster*, comparison of infrastructural and biochemical results showed that there are preferential sites of synthesis for monoterpene hydrocarbons (16). The following study has been undertaken in order to determine the terpene patterns characteristic in the steam-distilled oils of the different life stages of *A. douglasiana* and the nature of any diurnal changes. This information may be useful for taxonomic sampling procedures and in the direct harvest of oil from plants for flavoring, medicinal uses, or fuel and chemical needs.

RESULTS AND DISCUSSION

Totally furled terminal buds, maximum length 4-5 cm, yielded 0.117 ml of opaque to pale blue oil per 100 g fresh weight (FW). Unfurled juvenile leaves not exceeding 8 cm in length, 1.5 cm in width with nonserrated margins, yielded 0.197 ml of intensely deep blue oil per 100 g FW. Open, adolescent leaves exceeding 8 cm in length with nonserrated margins yielded 0.065 ml oil per 100 g FW. Serrated mature leaves yielded 0.064 ml oil per 100 g FW. The observation that the highest oil yield was obtained from the younger leaves is in agreement with the observation by Thieme and Nguyem (6) for *A. dracunulus*. While these authors reported a higher content of methylchavicol in mature leaves of *A. dracunculus*, we found camphene, ambrosial (17), camphor, and terpinene-4-ol to have the highest amount in the mature life stage of *A. douglasiana*. In the juvenile stage, it was sabinene, Hoerger's ether, and borneol, while β-thujone and compound 26 occurred in highest amounts in the terminal buds.

For the life stages, a one-way analysis of variance and Duncan's multiple range test were calculated for each peak (Table 1). Bartlett's test for homogeneity of variance was then calculated (Table 2) to compare the variation between the five samples at each life stage. The variances were not homogeneous for 11 of the peaks. The terminal bud group had higher variation than the mature leaf group for components 2, 10, 18, 26, 31, 32, and 33; whereas the mature group was higher for components 11, 12, 17, and 22 (for identification, see Table 1). Thus, the terminal bud group had more variation in 64% of the components, which showed significant differences in the variances. For all components, the terminal bud group had higher variance than the mature one for 58% of the components. β-Thujone was extremely variable, except in the mature leaf group. Although the yield of oil is the lowest for the mature leaf group, the overall homogeneity of the five samples was the best of the four life stages tested, and therefore the most suited for chemotaxonomic uses.

A stepwise discriminant analysis was used to select those components differentiating between life stages. The selected components were 6, 12, 26, 28, and 31. Each of

TABLE 1.   Life Stage in *Artemisia* Oils, Average Percent for Each Peak by Life Stage

	Peak	Terminal	Juvenile	Adolescent	Mature
1	αPinene	1.84 z[a]	2.38 z	2.67 z	1.61 z
2	α-Thujene	2.37 z	2.12 z	.37 z	1.44 z
3	Camphene	1.90 z	2.37 z	2.18 z	3.32 z
4	Unknown	.00 z	.01 z	.04 z	.07 z
5	β-Pinene	.80 z	1.04 z	1.31 z	1.06 z
6	Sabinene	9.15 y	11.37 y	10.46 y	2.99 z
7	Δ³-Carene	.91 y	.87 y	.40 yz	.16 z
8	β-Myrcene	.60 z	.74 z	.49 z	.45 z
9	Hoerger's ether	1.52 z	1.79 z	1.22 z	1.69 z
10	1,8-Cineole	4.35 z	7.14 z	9.18 z	8.61 z
11	γ-Terpinene	.81 z	.85 z	.82 z	1.24 z
12	p-Cymene	.20 z	.23 z	.68 y	.82 y
13	Ocimene	.41 z	,32 z	.31 z	.35 z
14	Artemisia ketone	3.35 z	2.34 z	1.86 z	2.72 z
15	Unknown	.23 z	.12 z	.12 z	.34 z
16	α-Thujone	.21 z	.22 z	.08 z	.13 z
17	Unknown	.05 z	.02 z	.04 z	.20 y
18	β-Thujone	14.83 z	12.68 z	13.88 z	1.69 z
19	Artemisia alcohol	2.28 y	1.59 yz	.40 z	.72 z
20	Unknown	.23 z	.37 z	.40 z	.72 z
21	Unknown	.00 z	.00 z	.00 z	.08 z
22	Ambrosial	1.31 z	7.93 yz	8.55 y	16.76 x
23	Camphor	16.40 y	8.66 z	9.46 z	22.37 y
24	Unknown	.38 z	.21 z	.36 z	.37 z
25	Caryophyllene	1.21 z	.69 z	.65 z	.84 z
26	Unknown	4.23 y	1.71 z	1.67 z	2.76 yz
27	Terpinen-4-ol	2.56 z	2.73 z	3.11 yz	4.97 y
28	Unknown	.66 z	.24 z	.70 z	1.45 y
29	Isoborneol	2.51 y	2.16 yz	1.69 yz	1.12 z
30	Borneol	13.45 z	18.79 z	16.07 z	13.52 z
31	α-Terpineol	7.11 z	5.34 z	6.87 z	3.71 z
32	Unknown	2.34 z	1.57 z	2.31 z	1.23 z
33	Unknown	1.82 z	1.36 z	1.64 z	.89 z

[a]Duncan's Multiple Range Test; life stages with any letter in common are not statistically different at the 5% level for that peak. Life stages with no letters in common are significantly different at the 5% level.

the five samples at each life stage was correctly assigned on the basis of the discriminant functions for these five components.

A canonical analysis was run for these five selected components. The first canonical variate separates the mature leaf stage from the younger stages. The second canonical variate separates the younger stages from each other (Figure 1).

Three clones were examined in a diurnal study. The composition of the oil from one of these clones, A, contained a significantly higher amount of artemisia ketones, $8.0 \pm 1.0\%$, than the other two at $0.08 \pm 0.09\%$ (B) and $0.6 \pm 0.2\%$ (C), respectively.

The two clones low in artemisia ketone had a minimum yield of oil of 0.56 ml/100 g dry weight (DW) (B) and 0.133 ml/100 g DW (C) at 1500 h, while the clone high in artemisia (A) had its maximum yield of 0.71 ml/100 g DW at 1500 . The minimum yield for this clone was 0.35 ml/100 g DW (A) at 1000 h, while the maximum for the other two was 0.87 ml/100 g DW (B) at 1500 h and 0.50 ml/100 g DW (C) at 1000 h.

As we have observed in other cases, the changes are primarily in the volume of oil and not in the composition of the individual constituents. The main constituent, camphor, had an average value of $22.0 \pm 4.0\%$ in A, $36.0 \pm 2.0\%$ in B, and $29.0 \pm 7.0\%$ in C. Sabinene had an average value of $4.0 \pm 0.6\%$ in A, $7.8 \pm 0.8\%$ in B, and $4.9 \pm 0.8\%$

TABLE 2.   Life Stages in *Artemisia* Oils, Variance of Five Replicates for Each Peak by Life Stage

Peak	Terminal	Juvenile	Adolescent	Mature	Significance[a]
1	.180	.517	2.524	.714	N.S.[b]
2	8.718	5.032	.103	2.293	H.S.[c]
3	1.000	.994	.456	1.669	N.S.
4	N.P.	trace	.006	0.22	H.S.
5	.103	.548	1.530	.640	N.S.
6	6.383	6.562	9.555	1.455	N.S.
7	.292	.154	.049	.040	N.S.
8	.094	.064	.031	.072	N.S.
9	.663	1.462	.623	1.667	N.S.
10	4.189	20.251	45.643	.890	H.S.
11	.025	.013	.228	.338	S.[d]
12	.004	.008	.189	.024	H.S.
13	0.25	.016	.021	.026	N.S.
14	25.800	9.630	10.360	10.800	N.S.
15	0.183	.040	0.68	.057	N.S.
16	.034	.028	.004	.009	N.S.
17	trace	trace	.001	.035	H.S.
18	276.300	251.000	209.400	.700	H.S.
19	1.819	1.800	.728	.437	N.S.
20	.171	.298	.704	.103	N.S.
21	N.P.[e]	N.P.[e]	trace	.025	H.S.
22	2.082	13.240	7.103	79.492	H.S.
23	20.060	8.390	12.110	53.030	N.S.
24	.012	.020	.012	.012	N.S.
25	.262	.281	.127	.405	N.S.
26	4.805	.058	.396	1.264	H.S.
27	1.124	.915	1.469	6.152	N.S.
28	.183	.014	.309	.135	N.S.
29	.832	.669	.565	.125	N.S.
30	31.410	67.240	69.880	21.410	N.S.
31	23.430	3.940	6.090	.550	S.
32	2.648	.298	.958	.105	S.
33	2.286	.573	.565	.030	H.S.

[a]Significance of Bartlett's test of homogeneity of variances.
[b]N.S. = not significant.
[c]H.S. = highly significant (significant at the 1% level).
[d]S = significant (significant at the 5% level).
[e]N.P. = peak not present.

in C. Camphene had an average value of $2.5\pm0.4\%$ in A, $5.0\pm0.8\%$ in B, and $4.2\pm0.9\%$ in C. β-Thujone had an average value of $1.2\pm0.2\%$ in A, $2.4\pm0.2\%$ in B, and $7.0\pm5.0\%$ in C.

It is interesting to note that the clone A, high in artemisia ketone, is low in camphor; and the other two, B and C, which are low in artemisia ketone, are higher in camphor. There does not seem to be any rationale to suggest that this sum should be conserved.

Although it was disappointing to find no consistent diurnal pattern in the yield of oil in the three different clones examined, the large difference between the minimum and maximum values obtained suggests a more active metabolic function for these terpenoids, which remains to be elucidated.

The continuous active synthesis of terpenoids was conclusively demonstrated by Banthorpe and Ekundayo (18) in their pulse labeling experiment in *Pinus palustris*. The maximum incorporation by 72 h, which was followed by a gradual decline and loss of label, suggests an active turnover in some unspecified metabolic process.
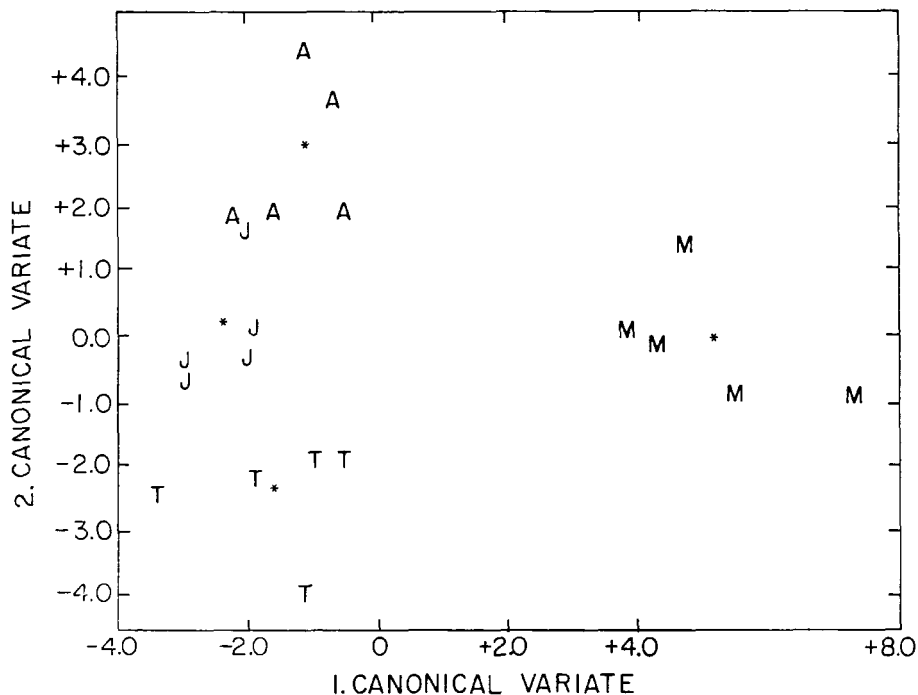
FIGURE 1.   Canonical variates for T=terminal bud, J=juvenile leaf, A=adolescent leaf, and M=mature leaf oil of *Artemisia douglasiana*.

EXPERIMENTAL

Healthy leaves were collected and separated into juvenile, adolescent, and mature foliage, and terminal buds.[1] Five samples, each consisting of 100-150 g of fresh leaf material, were collected for each life stage: three on March 8 and two on March 15.

For diurnal harvesting, a total of 100-150 g per sample of mature leaves from 20 plants of each of three clones was collected at 0500, 1000, 1300, 2000, and 0100 h on June 23, 1977. The clones were chosen from the beginning, the middle, and the end of a strip 14 m wide and 1000 m long, in Waterman Canyon, 1.5 km north of San Bernardino, CA, at about 300 m elevation, 30-35 mm precipitation per year. All leaf material was collected in plastic bags, frozen in the field with dry ice, and kept frozen until steam distilled in a Clevenger apparatus within seven days after harvest.

The crude oil was separated on a Varian 1520 gas chromatograph using two matched stainless steel columns 306 cm×0.64 cm o.d. packed with 20% LAC 2R446 on 60-80 mesh chromosorb W. The column temperature was nonlinearly programmed from 50-180° in a two-hour run. Injector and detector temperatures were 160° and 200°, respectively. The major peaks were trapped in capillary tubes, and ir spectra were obtained. Additional structural confirmation was obtained with a Finnigan 1015 S/L quadrupole mass spectrometer connected to a Varian Aerograph series 1400 gas chromatograph. The system was controlled with a pdp 8/m system/150 computer, and data were collected on magnetic tape. Mass spectral data were analyzed on an H.P. 3000 computer; mass spectra for reference compounds were obtained on the same instrument for direct comparison of fragmentation patterns.

LITERATURE CITED

1.   K.H. Lee, S. Matsueda, and T.A. Geissman, *Phytochemistry*, **10**, 405 (1971).
2.   R. Stangl and H. Greger, *Plant Syst. Evol.*, **136**, 125 (1980).
3.   K. Yano, *Flavour Ind.*, **1**, 328 (1970).
4.   M. Miyazawa and H. Kameoka, *Phytochemistry*, **16**, 1054 (1977).
5.   G.M. Nano, A. Martelli, and P. Sancin, *Riv. Ital. Essenze, Profumi, Piante Off., Aromi, Saponi, Cosmet. Aerosol*, **98**, 409 (1966).

[1]Voucher: P.F. Horner 31772 UCR.

6.   H. Thieme and T. Nguyen, *Pharmazie*, **27**, 324 (1972).
7.   A. Radu, M. Tamas, and E. Bancila, *Farmacia*, **21**, 417 (1973).
8.   E. Hoerger, *Proc. Montana Acad. Sci.*, **33**, 97 (1973).
9.   C.H. Muller, W.H. Muller, and B.L. Haines, *Science*, **143**, 471 (1964).
10. J.P. Halligan, *Ecology*, **56**, 999 (1975).
11. V.E. Tatro, R.W. Scora, F.C. Vasek, and J. Kumamoto, *Ann. J. Bot.*, **60**, 236 (1973).
12. W.A. Dement, B.J. Tyson, and H.A. Mooney, *Phytochemistry*, **14**, 2555 (1975).
13. R.P. Adams and A. Hagerman, *Am. J. Bot.*, **64**, 270 (1977).
14. J.A. Hopfinger, J. Kumamoto, and R.W. Scora, *Am. J. Bot.*, **66**, 111 (1979).
15. J.M. Ortiz, J. Kumamoto, and R.W. Scora, *Int. Flavours Food Add.*, **9**, 224 (1978).
16. C. Bernard-Dagan, J.P. Carde, and M. Gleizes, *Can. J. Bot.*, **57**, 255 (1979).
17. J. Kumamoto, R.W. Scora, and W.W. Payne, *J. Agric. Food Chem.*, **23**, 123 (1975).
18. D.V. Banthorpe and O. Ekundayo, *Phytochemistry*, **15**, 190 (1976).