Phytochemistry, 1970, Vol, 9. pp. 397 to 402. Pergamon Press. Printed in England.

SEASONAL VARIATION OF TERPENOID CONSTITUENTS IN NATURAL POPULATIONS OF *JUNIPERUS PINCHOTII* SUDW.

ROBERT P. ADAMS

Department of Botany and Plant Pathology, Colorado State University, Fort Collins, Colorado

(*Received* 28 *July* 1969)

Abstract—Foliage samples were taken from sixteen trees in eight natural populations of *Juniperus pinchotii* Sudw. in July and again in January to determine if seasonal variations occur in the volatile terpenoids and if the summer or winter collections would be more variable. Gas/liquid chromatographic analysis revealed that significant differences do occur from summer to winter in the relative composition of the terpenoids. The summer collections were more variable than the winter collections which indicates the desirability of winter sampling when practical.

INTRODUCTION

THERE has been considerable controversy centering about the influence of environmental factors upon the amounts of volatile terpenoid components in higher plants, particularly those associated with seasonal changes. Several workers have shown that variations in terpenes can and do occur in the aging process of specific organs.[1, 2] Much of this variation may be due to the losses resulting from differential volatilization in the aging process. Flück[3] suggests that the diurnal variations of the essential oil of *Salvia officinalis* may be due to both evaporation and resinification.

More recently Burbott and Loomis[4, 5] have shown that there is a rapid turnover of the monoterpenes in cuttings of peppermint, *Mentha piperita* L. (although one must admit that this represents an extreme condition for the unrooted shoot). Under more natural conditions, Sukhov[6] fed $^{14}CO_2$ to a whole pine tree and showed that the label reached a peak in the monoterpenes in 13 days and then declined. Whether these monoterpenes were catabolized or lost by volatilization is not known. Conversely, Hanover[7] has shown that terpenes of cortex oleoresin (in *Pinus* spp.) are under genetic control and that environmental influences, except as they affect aging, are relatively minor. In a classical study of the turpentine of the genus *Pinus*, Mirov[8] concluded that "... all evidence seems to indicate that turpentine composition varies little throughout the growing season ...".

Thus the chemosystematist may be faced with considerable and significant seasonal variation in the volatile constituents when working with distillates of fresh foliage from

[1] R. W. SCORA and J. E. NEWMAN, *Agr. Metereology* **4**, 11 (1967).
[2] T. A. GEISSMAN and R. MUKHERJEE, *J. Org. Chem.* **33**, 656 (1968).
[3] H. FLÜCK, in *Chemical Plant Taxonomy* (edited by T. SWAIN), Academic Press, London (1963).
[4] A. J. BURBOTT and W. D. LOOMIS, *Plant Physiol.* **42**, 20 (1967).
[5] A. J. BURBOTT and W. D. LOOMIS, *Plant Physiol.* **44**, 173 (1969).
[6] G. V. SUKHOV, in *Radioisotopes in Scientific Research* (edited by R. C. EXTERMANN), Vol. IV, Pergamon Press, Oxford (1958).
[7] J. W. HANOVER, *Phytochem.* **5**, 713 (1966).
[8] N. T. MIROV, Technical Bulletin 1239, United States Department of Agriculture, Washington, 25, D.C. (1961).

branches collected under natural conditions, as has been noted by von Rudloff[9] in his work on *Picea* species and by Scora and Torrisi[10] in their study of the essential oils of citrus plants.

The purpose of this paper is to examine in detail the extent of seasonal variation in the volatile terpenoids from the branches taken from natural populations of *Juniperus pinchotii*. Tree to tree variation and site differences will be considered in a more extensive study.[11]

The two principal questions considered in this study are: (1) Does the terpenoid composition of fresh foliage samples differ significantly from summer to winter? (2) Which collections are the least variable, summer or winter?

TABLE 1. SUMMER AND WINTER VALUES AND *t* TESTS FOR THOSE COMPOUNDS FOUND IN EIGHT POPULATIONS OF *J. pinchotii* WHICH SHOW SIGNIFICANT DIFFERENCES FROM SUMMER TO WINTER

Cpd. No.	Identity	Avg. value (per cent)		*t* Test‡
		Summer	Winter	
1	(Tricyclene)†	0·24	0·33	−2·545*
3	(Camphene)	0·32	0·43	−2·924*
5	Sabinene	16·63	22·94	−4·495**
8	α-Terpinene	1·60	1·16	+6·110**
11	γ-Terpinene	2·64	1·69	+9·677**
14	Terpinolene	0·98	0·74	+3·202**
15B	Unknown	0·00	0·01	−2·236*
18	Unknown	0·02	0·05	−5·000**
19	Unknown	0·04	0·06	−3·146**
23	Unknown alcohol	0·96	1·62	−8·352**
25	Citronellal	0·45	0·83	−3·341**
26	Camphor	29·55	36·51	−3·699**
27	(Linalool)	1·13	1·73	−7·584**
28	Unknown	0·69	0·62	+2·373*
32	4-Terpineol	9·11	5·21	+5·576**
33	(Trans-2-methyl-6-methylene-3,7-octadien-2-ol)	0·45	0·33	+4·964**
34	Unknown	0·26	0·13	+2·957**
36	Unknown	0·30	0·14	+2·732*
37A	Unknown	0·19	0·12	+3·654**
39	Borneol	2·03	1·13	+4·517**
41	(Methyl vinyl anisole)	0·68	0·33	+2·702*
44	Unknown	0·23	0·14	+3·125**
46	Citronellol	5·22	4·20	+3·968**
47	Unknown	0·14	0·08	+3·616**
51	(Geraniol)	0·06	0·08	−2·138*
54	Unknown	0·19	0·10	+2·785*
59A	Unknown	0·06	0·03	+3·318**
61	Unknown	0·54	0·28	+2·937*
62	Elemol	3·15	1·84	+3·411**
63	(Elemol acetate)	1·03	0·75	+2·740*
64	Unknown (C_{15})	2·00	1·48	+2·319*
66	(γ-Eudesmol)	1·74	0·68	+3·244**
69/70	(α- and β-Eudesmols)	2·28	0·56	+3·956**
74	(C_{15} ester)	0·38	0·24	+2·424*

† Compounds enclosed in parentheses are tentively identified based on comparisons of the retention times of terpenoids known to occur in species of *Juniperus*.

‡ * Significant (5 per cent confidence level); **, highly significant (1 per cent confidence level).

[9] E. VON RUDLOFF, *Can. J. Bot.* **45**, 891 (1967).
[10] R. W. SCORA and S. TORRISI, *Am. Soc. Hort. Sci.* **88**, 262 (1966).
[11] R. P. ADAMS, in press.

RESULTS

Table 1 summarizes the differences in the summer versus winter comparison of the terpenoids of *Juniperus pinchotii* using the method of paired observations. Paired observations are of great value in a study of this nature in that resampling the same tree (genotype) from one season to another allows one to discard tree to tree (genotypic) differences and site differences, since the variable under consideration is chiefly that of seasonal variation. It

TABLE 2. *F* VALUES FOR THOSE COMPOUNDS FOUND IN EIGHT POPULATIONS OF *J. pinchotii* WHOSE SUMMER AND WINTER VARIANCES ARE SIGNIFICANTLY DIFFERENT

Cpd. No.	Identity	$F =$ †	Variance greater in	
			Summer	Winter
2C	Unknown (trace component)	4·00*		+
7	Myrcene	5·33**	+	
8	α-Terpinene	3·59*	+	
11	γ-Terpinene	3·26*	+	
13	(Para cymene)	8·22**	+	
14	Terpinolene	3·13*	+	
18	Unknown	3·67*	+	
32	4-Terpinenol	12·04**	+	
33	(Trans-2-methyl-6-methylene-3,7-octadien-2-ol)	5·61**	+	
34	Unknown	6·44**	+	
36	Unknown	5·82**	+	
39	Borneol	3·85*	+	
42	(δ-Cadinene)	100·94**	+	
45	Unknown	6·40**	+	
47	Unknown	3·34*	+	
51	(Aromatic alcohol)	4·12**		+
54	(Alcohol)	3·69*	+	
60	Unknown	6·49**	+	
61	Unknown	4·05*	+	
62	Elemol	6·99**	+	
63	(Elemol acetate)	2·89*	+	
66	(γ-Eudesmol)	23·57**	+	
67	Unknown	3·39*	+	
68	Unknown	3·91*	+	
69/70	(α- and β-Eudesmols)	75·49**	+	
72	Unknown	56·66**	+	
73	Unknown	7·15**	+	
74	(C_{15} ester)	3·10*	+	
74A	Unknown	3·67*	+	
76	(Acetate II)	5·99**	+	
77	Unknown	6·70**	+	
78	Unknown	9·88**	+	
35A	Unknown	3·63*	+	

† $F_{0.05} = 2.86$*, $F_{0.01} = 4.07$,** df = 15, 15.

should be noted that in Table 1, even though the average values of the summer and winter samples are shown, calculations were based on the averages of the individual tree differences and *not* the differences between the summer and winter averages. The results summarized in Table 1 show that of the approximately seventy terpenoid components normally present in *J. pinchotii*, twelve are significantly (at the 5 per cent confidence level) and twenty-five are highly significantly (at the 1 per cent confidence level) different from summer to winter (or winter to summer).

The compounds are listed in the order that they are eluted from the gas chromatograph, which is dependent on their volatility and polarity, with the most volatile and nonpolar being eluted first. Thus it appears that the more volatile terpenoids decrease in the summer with the exceptions of α-terpinene, γ-terpinene, and terpinolene, which are all isomers.

From Table 2 one can see that there are thirty-three terpenoids whose variances are either significantly or highly significantly different from summer to winter. Of these, two terpenoids are more variable in the winter than summer at the indicated levels of significance, and thirty-one are more variable in the summer at the indicated levels of significance. This indicates that in *J. pinchotii* the variability in terpenoid composition is much greater in the summer than winter.

DISCUSSION

The loss of the more volatile terpenes of *Juniperus pinchotii* is not unexpected since some of the oil glands were ruptured on every tree examined. According to volatility one would also expect α-terpinene, γ-terpinene, and terpinolene to decrease in the summer since they are certainly more volatile than citronellal or camphor (which both decreased in the summer), but each shows a highly significant *increase*. This is also true in spite of the fact that the July oil samples were stored for 6 months at $-20°$ in tightly capped vials in a 40–60 per cent ether solution. One might lose some of the more volatile terpenes but it is not too likely in an ether solution of this concentration (cf. von Rudloff[9] for additional consideration of this aspect). Thus one must conclude that volatility alone is not responsible for the variation observed. Since the absolute quantity of each terpenoid was not determined, one cannot say if the actual amounts of α- and γ-terpinenes, and terpinolene are changing from summer to winter, or remain at the same absolute levels. Nevertheless, the discordant variation observed between the more volatile terpenes and the three above-mentioned terpenes suggests the following:

(1) Either α-terpinene, etc., are produced in the summer at a faster rate than they are being lost, or (2) the other more-volatile terpenes are being produced at a much lower rate than in the winter (unlikely), or (3) a combination of both (1) and (2).

A closer examination of Table 1 reveals that the shifts from citronellol (C—OH) to citronellal (C=O), and borneol (C—OH) to camphor (C=O) both occur from summer to winter, possibly favoring higher oxidation in the cooler temperatures. The author (unpublished) has found evidence for this phenomenon in growth-chamber studies of *Liquidambar styraciflua*, but definitive study of such activity has not yet been made.

The indication that summer collections were more variable than winter collections was not unexpected, since on *a priori* grounds one would expect the growth of new foliage, higher metabolic rates, etc. to increase the tree to tree variation in the summer; whereas in the winter, with reduced growth activity, the trees should reach a "steady state". Although it should be noted[12] that since the average July temperature varies from 78°F in the westernmost population (Eddy County, New Mexico) to 86°F in the easternmost population (Kimble County, Texas) and the average January temperature varies from 44°F in the westernmost population to 50°F in the easternmost population, this species does not have as well-defined dormant period as found in other species. Undoubtedly some photosynthesis and metabolism occurs in the winter. Nevertheless, these temperatures are sufficient to induce considerable evaporation in the summer and inhibit any new growth in the winter.

[12] *Climate and Man* (edited by GOVE HAMBRIDGE), United States Government Printing Office, Washington, D.C. (1941).

In addition to the above study, I have sampled four populations of *J. ashei* Buch. (eight trees) in a separate study[13] which yielded fewer differences but tended to confirm the existence of significant seasonal differences from summer to winter with increased variation in the terpenes from the summer collections.

Thus one can conclude (at least in *J. pinchotii* and *J. ashei* and perhaps other gymnosperms) that seasonal variation in the volatile terpenes from foliage samples is a factor to be reckoned with in chemosystematics and that winter collections are likely to be less variable than collections from trees during the summer when environmental factors are more variable.

EXPERIMENTAL

In July of 1967, eight populations of *Juniperus pinchotii* were sampled (Table 3). *Juniperus* species are evergreen with the leaves remaining functional for several years. In general, the current year's growth represents only a small portion of the total green foliage. Samples consisted of the fresh green foliage from four or five branches, 8–10 in. long, representing several years' growth. Samples were taken from high and low and on four sides of two trees from each of the eight aforementioned populations. These sixteen trees were tagged with permanent aluminum labels for future reference. The branchlets were sealed in plastic bags and frozen upon return. In January 1968, these sixteen trees were resampled as described above. The July and January samples were steam distilled within 1 week after having been frozen. Distillation was carried out using a modified Clevenger-type circulatory apparatus as described by von Rudloff.[9] The terpenoids (monoterpenes and sesquiterpenes) were collected in a layer of diethyl ether, concentrated, and stored in tightly capped vials at −20°.

TABLE 3. POPULATION LOCATIONS OF *J. pinchotii* (VOUCHERS DEPOSITED IN THE HERBARIUM, UNIVERSITY OF TEXAS AT AUSTIN)

Population no.	Location
16	Texas, McCulloch County
17	Texas, Coke County
18	Texas, Howard County
21	New Mexico, Eddy County
22	Texas, Culberson County
29	Texas, Terrell County
31	Texas, Val Verde County
43	Texas, Kimble County

The oil samples were run in 0·5 μl alioquots in January 1968 on a Varian gas chromatograph model 1520-C with a flame ionization detector (FID), with a 4 m × 3 mm stainless-steel column, vibrator-filled with 1 per cent diethylene glycol adipate (DEGA) and 4 per cent polyethylene glycol (PEG, 20 M) on 80/100 Gas Chrome Q, DMCS treated. A 5 per cent silicone column (SE-30), 1·5 m × 3 mm, on Gas Chrome Q, DMCS treated, was used in some of the purity checks of compounds. Quantification was by use of a Varian model 476 digital integrator with a relative accuracy of ± 0·3 per cent in duplicate runs for peaks greater than 3 per cent. On smaller peaks the relative error may increase upwards to 10 per cent.[4]

The chromatograph was temperature programmed as follows: Initial temp. 45°; linear increase for 52 min at 3°/min; isothermal at 211° for 4 min.

Column conditions were as follows: Injector temp. = 170°; detector temp. = 230°; carrier gas flow = 25 ml/min (helium); flame detector flow = 25 ml/min (hydrogen); air flow = 300 ml/min.

Individual components were isolated and collected using a Varian Autoprep model 700 and the 1520-C with a 3 m × 6 mm, 3% DEGA/12% PEG column and thermal conductivity detectors. The major terpenoids were identified by i.r. absorption spectra through comparison with the i.r. spectra of known compounds. Some of the smaller components were tentatively identified by comparisons with the retention times of terpenoids known to occur in other species of *Juniperus*.[14–16] Those tentatively identified are enclosed in parentheses in Tables 1 and 2.

[13] R. P. ADAMS, Ph.D. Thesis, University of Texas (1969).
[14] A. R. VINUTHA and E. VON RUDLOFF, *Can. J. Chem.* **46**, 3743 (1968).
[15] E. VON RUDLOFF, *Can. J. Chem.* **46**, 679 (1968).
[16] E. VON RUDLOFF and F. M. COUCHMAN, *Can. J. Chem.* **42**, 1890 (1964).

A computer program was written to compare the sample means using the paired observations (July and January) on each of the trees for each compound present to test the hypothesis that there were significant differences between summer and winter samples. The t test is 2-tailed, using $t_{0.05} = 2.131$,* and $t_{0.01} = 2.947$** for 15 degrees of freedom (df).[17] In addition the program tested the hypothesis that the summer and winter variances are statistically different by use of a 2-tailed F test of the larger variance divided by the smaller variance where $F_{0.05} = 2.86$,* and $F_{0.01} = 4.07$,** for 15 degrees of freedom in the denominator and numerator.[17]

Acknowledgements—This research was supported in part by NSF Traineeship grants for Environmental and Systematic study in the summers of 1967 and 1968, a NSF predoctoral fellowship for 1968–1969, and funds from NSF GB5448X and NIH GM-11111. The author wishes to thank Dr. Ernest von Rudloff of the Prairie Regional Laboratory, Saskaton, Saskatchewan, Canada, for his assistance and advice in the early stages of the gas chromatographic analyses as well as his aid in the identification of some of the terpenes. The author also thanks Drs. T. J. Mabry and B. L. Turner, Department of Botany, University of Texas at Austin, for their helpful criticisms during the preparation of this report.

[17] R. G. D. STEEL and J. H. TORRIE, *Principles and Procedures of Statistics*, McGraw-Hill, New York (1960).