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VARIATION OF THE *PINUS PONDEROSA* NEEDLE OIL WITH SEASON AND NEEDLE AGE

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Abstract—Influence of season and needle age on the yield and composition of the *Pinus ponderosa* needle oil was investigated. Oil yields throughout the year averaged 0·13% on the basis of tissue green weight. The average composition was 11·9% α-pinene, 70·2% β-pinene, 8·0% 3-carene, 5·0% myrcene, 1·8% limonene, 2·2% β-phellandrene and 6·4% methyl chavicol (total monoterpenes = 100%). The amount of methyl chavicol and total monoterpenoids was highest in summer, lower in juvenile than in mature first-year needles, and decreasing thereafter with needle age. A significant increase in 3-carene and decrease in β-pinene was apparent in juvenile needles. The most important step towards decreasing the seasonal and age variability of needle samples in ecological or chemotaxonomic studies was found to involve exclusion of sample collections during a period between the initial appearance of young needles and the time that they reach maximum length.

INTRODUCTION

THE COMPOSITION and quantity of the volatile oil present in foliage is dependent upon plant genotypes as well as upon several nongenetic variables connected with the environment and the processes associated with plant development. Although several investigations are available on the seasonal changes in oil yield of *Pinus*,[1–9] the results are contradictory and the patterns vary from species to species. Diurnal variability of oil yield has been the subject of a single study; *Pinus sylvestris* foliage was used and rather low amplitudes of 5–9% reported.[10] Less information is available on the seasonal change in oil composition. Okay[11] reported little or no variation in four Turkish pine species which included *Pinus nigra* and *Pinus sylvestris*, while Stanković found an increase of the more volatile constituents in winter, as well as a maximum in β-pinene in fall, in *Pinus nigra* grown in Yugoslavia.[7] Juvonen, working with Finnish *P. sylvestris*, reported little seasonal variation in composition of the oil from mature needles, while oil from young, elongating needles varied greatly with α-pinene increasing from 7·4% in June to 36·4% in August. On the yearly changes in

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oil associated with needle age, Poltavtchenko *et al.*,[12] reported for the same pine the highest oil yields for the youngest needles (collected in winter); yields decreased with needle age but with no parallel differences in oil composition.

In connection with our current work on the ecological importance of the *Pinus ponderosa* leaf oil (correlation of the chemical parameters to the susceptibility of *P. ponderosa* to pathogens, insects and smog injury), it appeared necessary to determine the seasonal and year-to-year variability of the oil to standardize collection procedures and to evaluate results of other studies. The present paper represents results of the experimental efforts in this direction.

Pinus ponderosa Laws. twig-and-needle oil, obtained in 0·11% yield, has been previously investigated by Schorger, who found it to be composed of 2% (−)-α-pinene, 75% (−)-β-pinene, 6% (±)-limonene, 7% borneol, 2% bornyl acetate and 3% 'green oil'.[13]

RESULTS AND DISCUSSION

Qualitatively our results agreed with most of Schorger's data. Aside from finding several additional, quantitatively minor monoterpenoids, the only larger difference was in identification of methyl chavicol as the major constituent of the oil, occasionally amounting to 40% of the total. Higher-boiling constituents were minor in amount. Tree-to-tree variability was low as far as monoterpene hydrocarbons were concerned, but the variability of methyl chavicol appeared to be greater (Table 1).

TABLE 1. COMPOSITION OF *Pinus ponderosa* LEAF OIL

Tree No.	Oil yield %f.w.	Yield percent of total monoterpenes								
		α-Pinene	Camphene	β-Pinene	Δ^3-Carene	Myrcene	Limonene	β-Phellandrene	Methyl chavicol	Higher boiling unknowns
Five *Pinus ponderosa* trees used in this study*										
1	0 10	12 6	tr	80 7	3 5	2 1	0 5	0 6	3 1	0 2
2	0 06	12 4	tr	82 5	3 4	1 4	0 3	0 7	20 2	2 0
3	0 12	11 9	tr	83 2	1 5	1 5	1 2	1 2	55 0	2 6
4	0 09	11 6	tr	79 7	6 1	2 5	0 4	0 8	13 2	3 9
5	0 15	12 1	tr	81 5	1 0	3 7	1 0	1 7	2 7	4 4
Twenty-five *Pinus ponderosa* trees growing close by†										
Mean	0 13	11 9	—	70 2	8 0	5 0	1 8	2 2	6 4	
Standard deviation	0 05	1 5	—	6 9	2 8	3 7	1 1	1 5	5 8	Not determined
Coefficient of variation	0 35	0 12	—	0 09	0 35	0 74	0 61	0 68	0 90	

* Needles grown in 1968, collected 8 May 1969. Yield is expressed in per cent of fresh needle weight individual compounds (including methyl chavicol in this case) on the basis of monoterpene hydrocarbons set as 100%. Terpinolene was present in some samples, but never in large amounts; sabinene and α-phellandrene, as well as α- and γ-terpinenes were absent. Traces of an unknown compound, tentatively identified as *cis*-ocimene were present in some samples.

† Needles grown in 1968, samples in July 1969.

The general biosynthetic mechanisms leading to the constituents of *P. ponderosa* leaf oil can be considered as established. Thus it can be safely accepted that formation of monoterpenoids goes through mevalonate/neryl pyrophosphate path, followed by differentiation into individual compounds. However, methyl chavicol possessing a *n*-propyl side chain attached to a benzene nucleus para to a methoxyl, probably arises through the shikimic acid path. This has been demonstrated by radioactive labelling for anethole, the

[12] Y. A. POLTAVTCHENKO, T. N. TKATCH, V S. TKATCH and G. A. RUDAKOV, *Nauch. Dokl. Vysshei Shkoly, Biolog. Nauki*, p. 71 (1968).

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double bond isomer of methyl chavicol.[14,15] In view of the large difference between the biogenesis of methyl chavicol and that of monoterpenes, we decided to separate the analytical results by expressing the methyl chavicol and total monoterpene contents separately, the concentration of individual monoterpenes being expressed in per cent of their total. The variability studies were based on five separate sets of needle samples obtained from five *P. ponderosa* trees; sampling was performed each month from May to October and in December and March, keeping and analysing separately the needles grown in various years.

For examination of the differences between needles grown in various years, the analytical results were standardized separately for each tree and monthly collection by setting the values for the youngest mature needles (i.e. terminal needle whorls in March and May, and next-to-juvenile whorls for the other months) as = 1·0; in this way among tree variability was minimized. The mean values for the five trees examined for each month are given in Figs. 1–3. With mature needles the results indicate a highly significant decrease in methyl chavicol as needles become older (Fig. 1). The methyl chavicol content of the young, developing needles is, however, much lower than that of the neighboring mature needles. This difference is present even in October, well beyond the time needles reached their maximum extension (August). The results are qualitatively similar in the case of total terpene content of the needles, although differences are smaller and less well defined. As with methyl chavicol, the oldest and the developing needles have the lowest total terpene content (Fig. 2). However, the terpene level characteristic for the mature needles is reached somewhat earlier, probably before the maximal needle extension. Biosynthetically, methyl chavicol is more closely related to the formation of lignin than are terpenoids and the difference found

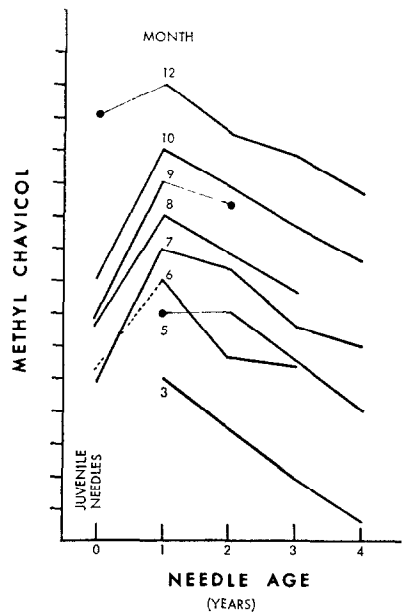

FIG. 1. YEARLY CHANGES IN METHYL CHAVICOL CONTENT RELATIVE TO DATA FOR YEAR-OLD NEEDLES TAKEN AS = 1·0, ORDINATE DIVISIONS = 0·1.
Heavy lines (▬▬) 0·01 level; broken lines (- - - -) 0·1 level; thin lines (————) not significant.

[14] K. KANEKO, *Chem. Pharm. Bull. Japan* **8**, 875 (1960).
[15] K. KANEKO, *Chem. Pharm. Bull. Japan* **9**, 108 (1961).

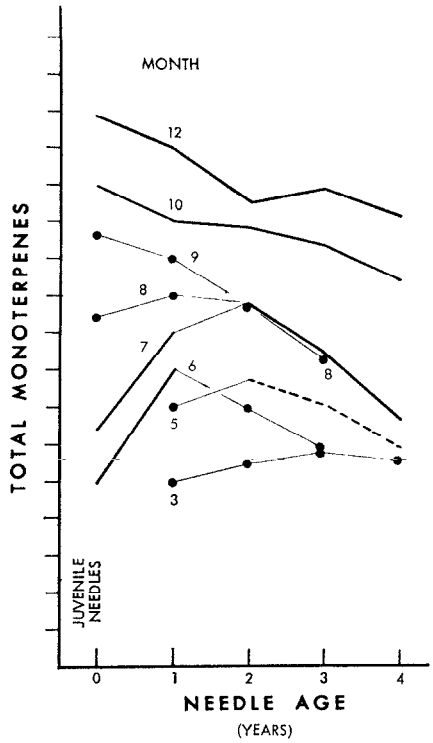

FIG. 2. YEARLY CHANGES IN TOTAL MONOTERPENE CONTENT RELATIVE TO DATA FOR 1-yr-old NEEDLES, TAKEN AS = 1·0, ORDINATE DIVISIONS = 0·1.

Significance as in Fig. 1.

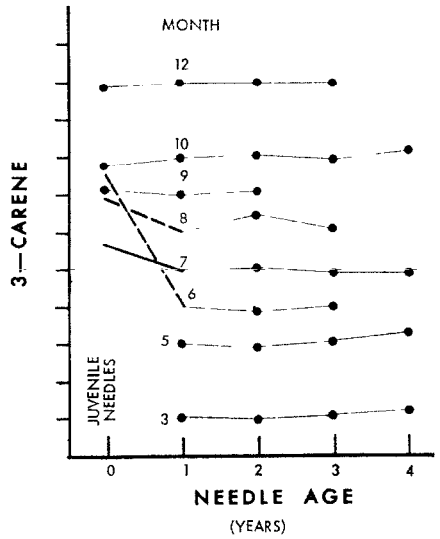

FIG. 3. YEARLY CHANGES IN 3-CARENE CONTENT OF MONOTERPENES RELATIVE TO DATA FOR 1-yr-old NEEDLES, TAKEN AS = 1·0, ORDINATE DIVISIONS = 1·0.

Significance as in Fig. 1, except for broken lines (0·05 level).

could be associated with the consumption of the common precursors needed for building the cell wall of the plant. At least some processes of growth[16b] (i.e., of lignin accumulation) and of photosynthesis[16c] are known to continue in pines well beyond the point of maximal needle extension.

Less regularity was observed in changes of the monoterpenoid composition. 3-Carene was significantly higher (Fig. 3) and β-pinene lower (0·83 in June and 0·91 in July) in the developing needles in summer months than at other times, while no regularity in changes was associated with α-pinene at all. The results also suggested a higher content of myrcene, limonene, β-phellandrene, and terpinolene in juvenile needles in summer although low percentages and corresponding analytical inaccuracy made results statistically significant only rarely at the 0·5% level (*t*-test). However, combining non-standardized results obtained in June, July and August produced significant differences between juvenile and the neighbouring mature needles for all four terpenes (Table 2), with practically no significant differences between samples derived from mature needles of different age collected in those or other months.

Changes in dispersion of values for all components of volatile oil were examined for

[16] P. J. KRAMER and TH. T. KOZLOWSKI, *Physiology of Trees*, (a) p. 344, (b) p. 25, 52, (c) p. 71, McGraw-Hill, New York (1960).

TABLE 2. CHANGES IN OIL CONTENT AND COMPOSITION IN NEEDLES OF VARIOUS AGE†

Compounds‡	Needle age (yr)	Mean content‡	Standard deviation	Coefficient of variation
Methyl chavicol	0	0·5***	0·7**	1·33*
	1	2·2	1·7	0·79
	2	2·1	2·1	1·00
	3	1·4	1·2	0·83
Total monoterpenes	0	7·8***	4·5	0·58**
	1	14·5	4·7	0·32
	2	13·7	4·8	0·35
	3	11·4	4·5	0·39
α-Pinene	0	12·2	1·6	0·13
	1	12·3	1·3	0·10
	2	12·8	2·3	0·17
	3	12·6	1·6	0·12
β-Pinene	0	69·4***	7·4**	0·11**
	1	76·9	4·2	0·055
	2	76·0	5·6	0·074
	3	76·8	3·9	0·050
3-Carene	0	11·0**	5·7**	0·52
	1	5·7	3·9	0·67
	2	6·1	3·6	0·58
	3	5·6	2·9	0·50
Myrcene	0	3·4*	1·3**	0·39
	1	2·3	1·1	0·47
	2	2·5	0·8	0·30
	3	2·8	0·6	0·23
Limonene	0	1·8*	0·8**	0·44*
	1	1·3	0·3	0·25
	2	1·2	0·4	0·31
	3	1·0	0·4	0·35
β-Phellandrene	0	2·0**	0·5	0·27**
	1	1·4	0·5	0·39
	2	1·3	0·6	0·47
	3	1·3	0·6	0·46
Terpinolene	0	1·1***	0·9**	0·81
	1	0·2	0·3	—
	2	0·1	0·2	—
	3	0·1	0·2	—

† Combined samples for the months of June, July and August. Significance level symbols for differences between the indicated and the value following: (*) < −0·05%, (**) < −0·01%, (***) < 0·001%. *t*-Test was used for means and *F*-test for standard deviation and coefficient of variation. Differences in means between the 1- and 3-yr-old needles were significant in case of total monoterpenes and limonene.

‡ Methyl chavicol and total monoterpenes in per cent of fresh needles weight times 100; monoterpenes in per cent of their total.

needles grown in various years by bulking June, July and August results again (Table 2). In most statistically significant cases larger standard deviations or coefficients of variation were associated with the developing needles; this can be rationalized in terms of the higher metabolic activity in juvenile foliage.

The month-to-month changes of the oil yield and composition were too variable to show any statistically reliable trends. For this reason, data for the two terminal mature needle whorls (June–October juvenile needle analyses were not considered) were bulked and then statistically analysed as four groups—winter (December and March), spring (May and June), summer (July and August) and fall (September and October) (Table 3). The differences between seasons with respect to both means and standard deviations, appear to be sizeable

TABLE 3. VARIATION OF VOLATILE OIL YIELD AND COMPOSITION WITH SEASON*

Compound	Season	Mean yield	Statistical significance of change in yield**	Standard deviation	Statistical significance of change in standard deviation†
Methyl chavicol					
	Winter	1·5	ns	1·0	ns
	Spring	0·9	0·001	1·1	0·02
	Summer	2·7	0·005	1·9	0·01
	Fall	1·5	ns	1·0	ns
Total monoterpenoids					
	Winter	10·5	ns	3·8	ns
	Spring	11·1	0·025	4·5	ns
	Summer	14·5	0·001	4·7	ns
	Fall	8·7	ns	4·1	ns
α-Pinene					
	Winter	11·8	ns	0·8	ns
	Spring	11·8	0·01	1·0	0·002
	Summer	12·9	ns	1·9	0·05
	Fall	12·4	ns	1·2	0 05
β-Pinene					
	Winter	78·5	0·005	3·0	ns
	Spring	81·4	0·001	3·1	0·05
	Summer	75·0	0·005	4·8	ns
	Fall	79·0	ns	3·5	ns
3-Carene					
	Winter	4·4	0·01	2·0	ns
	Spring	2·9	0·001	1·5	0·001
	Summer	6·9	0·001	3·7	0 02
	Fall	3·7	ns	2·1	ns
Myrcene					
	Winter	2·8	ns	0·7	ns
	Spring	2·5	ns	1·0	ns
	Summer	2·3	ns	0·7	ns
	Fall	2·4	ns	0·5	ns
Limonene					
	Winter	1·3	0·05	0·4	ns
	Spring	1·0	ns	0·5	ns
	Summer	1·2	0·025	0·4	ns
	Fall	1·6	0·05	0·4	ns
β-Phellandrene					
	Winter	1·8	0·001	0·4	ns
	Spring	1·1	ns	0·4	ns
	Summer	1·4	0 01	0·6	0·02
	Fall	1 9	ns	0·3	ns

* Significance tested using *t*-test for means and *F*-test for standard deviation; the values refer to differences between given and immediately following season.

† Figures indicate level of probability; ns—non-significant.

and associate themselves predominantly with the summer months. This suggests that increase in metabolic activity during needle growth affects mature as well as juvenile needles. Particularly noticeable in this respect were summer maxima involving methyl chavicol and total terpene yields and the minima for methyl chavicol in spring and total terpenes in fall. In terms of monoterpene composition a maximum in 3-carene in summer and a corresponding minimum in β-pinene were characteristic, and parallelled that found in juvenile needles (Table 2).

The total variance associated with changes in individual oil constituents due to season and needle age can be regarded as composed of two components—a systematic component, involving the same changes in foliage oil of the trees examined, and a random component. Both components can adversely affect any correlative studies based on these oils (e.g. correlations with susceptibility of *P. ponderosa* to fungal, beetle, or smog damage, genetic or chemotaxonomic studies) by obscuring any existing correlations through addition of extraneous variance (random changes) and by introducing non-existing correlations through addition of extraneous covariance (systematic changes). Thus, some standardization of sampling involving season and needle age is indicated. Evaluation of the efficiency of various sample-collection methods was performed by calculation of random and systematic variance components separately for various methods of sample collection. Table 4 shows the results with each variance component expressed as a per cent of the corresponding tree-to-tree variance (largely genetic). The highest systematic and random changes, approaching or even becoming higher than tree-to-tree variance, were associated with methyl chavicol

TABLE 4. EFFECTIVENESS OF FOLIAGE SAMPLING METHODS

Sample set	Methyl chavicol		Total monoterpenes		α-Pinene		β-Pinene		Δ^3-Carene	
	V_r*	V_s	V_r	V_s	V_r	V_s	V_r	V_s	V_r	V_s
All samples	214	104	53	25	64	65	38	24	85	77
All samples, juvenile needles not considered June–October	225	82	52	26	60	11	24	12	37	33
Two whorls following terminal needles considered only	248	120	49	25	57	15	19	13	41	35
Mature needles grown in the same year only	237	138	52	30	56	18	22	14	39	42
All samples with exception of June–July	133	17	40	8	34	1 5	17	6 5	27	7
All samples with exception of June–July–August	92	24	33	3	29	1 5	14	8 5	16	5 5
June–July–August samples only	277	261	74	58	112	17	60	22	160	86

* V_r, V_s—random and systematic variance components, expressed in per cent of tree-to-tree variance, V_g (methyl chavicol —0·49, total monoterpenes —17·56, α-pinene —2·25, β-pinene —47·6 and 3-carene —7·84), the latter calculated using Table 1 data for 25 trees.

and with June, July and August samples. Accordingly, the strongest suppression of either variance component was obtained by omitting these samples. Omitting juvenile needles usually improved results somewhat. Strangely enough, no improvement was obtained by further standardization of sampling in terms of needle age.

EXPERIMENTAL

Sample collection. Leaves were gathered from a pre-selected 5-tree plot of Ponderosa pine, 20–30 yr old, in the Blodgett University Forest (El Dorado Co., Central Sierra Nevada mountains, elevation 4200 ft) in 1969 about the middle of each month from branches matched as closely as possible. Juvenile needles were initiated in May; in July elongation was 65–85%; their maximum elongation was reached in August. Samples were put into sealed jars and placed in ice chests immediately after collection, then refrigerated at −10° within 4 hr.

Steam distillation of leaf volatiles. 100–200 g of frozen needles were cut into 2·5–5 cm segments, then pulverized to a coarse powder in a Waring Blender in the presence of dry ice. Crushed foliage was then steam-distilled for *ca.* 0·5 hr, using a circulating apparatus, the bulk of the oil coming over during the first 10–15 min. The oil was pipetted off the surface of the aqueous distillate collected in the receiver and placed into small vials containing a crystal of pyrogallol as antioxidant.

Oil analysis. Volatile oil components were analysed by gas chromatography, using a model 600C Aerograph GLC instrument with Varian Aerograph Model 470 digital integrator, and 0·4 λ samples of a *ca.* 1·5% solution of oil in CS_2. Monoterpenes—3 m × 3 mm o.d. column of 10% β,β′-oxydipropinitrile on

silylated Chromosorb P at 64° and 20 psi. Methyl chavicol and higher terpenoids—3 m × 3 mm column of 10% 20M Carbowax on silylated Chromosorb P at 140° and 25 psi.

Methyl chavicol was isolated using the Aerograph Autoprep A-700 instrument equipped with 7·5 m × 6 mm column containing 10% Carbowax 20M on silylated Chromosorb P. The IR spectra of the separated material and reference compound were identical.

Statistical calculations. The significance of differences in means was tested by *t*-test, while *F*-test was applied for the significance of differences in standard deviation or coefficient of variation. Systematic (V_s) and random (V_r) variance components were expressed in per cent of tree-to-tree variance (V_g), computed from Table 1 data for 25 trees (Table 4, footnote).

$$V_s = \frac{100\bar{r}^2 \sum_{i=1}^{i=n} V_i}{n.\ V_g};\quad V_r = \frac{100(1-\bar{r}^2) \sum_{i=1}^{i=n} V_i}{n.\ V_g};\quad \bar{r}^2 = \frac{\sum_{i=1}^{i=n} \sum_{j=1\ (j\neq i)}^{j=n} r^2_{i,j}}{n(n-1)}$$

i,j = running numbers assigned to individual trees analysed (here 1 to 5 each); V_i = variance of an essential oil component for a set of data obtained from tree 'i'; $r_{i,j}$ = correlation coefficient for two sets of data for the same component obtained from trees 'i' and 'j'; n = number of trees examined (here 5).

The significance of differences between needles grown in various years, as depicted in Figs. 1–3, was determined by *t*-test using sets of data corresponding to maximal and minimal end points of each line.

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