Phytochemistry, 1970, Vol. 9, pp. 2509 to 2515. Pergamon Press Printed in England.

OLEORESIN VARIABILITY IN *PINUS PONDEROSA*

EUGENE ZAVARIN* and FIELDS W. COBB, Jr.†

University of California Forest Products Laboratory, Richmond, California, U.S.A.

(*Received* 12 *January* 1970)

Abstract—Monoterpene composition of xylem oleoresin from 139 *Pinus ponderosa* trees was determined by GLC, together with related parameters of turpentine content of oleoresin, oleoresin exudation pressure, oleoresin yield, and oleoresin propensity to crystallize. Although noncompositional variables correlated poorly or not at all *inter se* or with turpentine composition, stronger quantitative relationships were found to exist between individual monoterpenes. The results are discussed from the viewpoint of their relevance to the susceptibility of *P. ponderosa* to bark beetle attack, as well as from the viewpoint of terpene biosynthesis.

INTRODUCTION

WHILE substantial progress has been made in recent years in regard to understanding the secondary (insect–insect) attraction of bark beetles due to the work of Silverstein, Wood, and others, on insect pheromones of *Ips confusus* and related species,[1–3] much less information is available on primary plant–insect interactions, responsible for initial host selection. Among many factors possible, xylem oleoresin has long been suspected of having some relation to the susceptibility of *Pinus* species to bark beetles and wood-inhabiting fungi. Recent studies by Chararas,[4] Smith,[5] and Cobb *et al.*[6] suggest that volatile terpenoids of the oleoresin are responsible for this effect. For studies of disease-induced susceptibility of *Pinus ponderosa* to attacks by species of *Dendroctonus*, it is important to obtain information on natural variability of the various parameters associated with the oleoresin of this pine.

The chemical composition of oleoresin of *P. ponderosa* has been examined on a number of occasions and results have been summarized by Mirov.[7] Mirov reported the composition of turpentine from oleoresin obtained by tapping trees growing near Placerville, California, in the Sierra Nevada, to be as follows: 1% (−)-α-pinene, 50% (−)-β-pinene, 30% 3-carene, 4–5% (−)-limonene, 3% myrcene, 3% cadinene and 1% of an unidentified sesquiterpene. Mirov also studied individual variability of turpentine composition in 27 trees; this, judged by the specific optical rotation ($[\alpha]^{25}_{5780} = -9{\cdot}1 - -39{\cdot}1$), appeared to be rather high.

Later, Smith[8] investigated by gas chromatographic methods the composition and individual variability of turpentines from the same pine, on the basis of sixty-four trees growing

* Forest Products Chemist, University of California Forest Products Laboratory, Richmond, California, U.S.A.

† Assistant Professor, Department of Plant Pathology, University of California, Berkeley, California, U.S.A.

[1] R. M. SILVERSTEIN, J. O. RODIN and D. L. WOOD, *J. Econ. Entomol.* **60**, 944 (1967).
[2] R. M. SILVERSTEIN, J. O. RODIN, D. L. WOOD and L. E. BROWNE, *Tetrahedron* **22**, 1929 (1966).
[3] D. L. WOOD, R. W. STARK, R. M. SILVERSTEIN and J. O. RODIN, *Nature* **215**, 206 (1967).
[4] C. CHARARAS, *Scolytides des Coniferes*, Editions P. Lechevalier, Paris (1962).
[5] R. H. SMITH, Resin quality as a factor in the resistance of pines to bark beetles, in *Breeding Pest Resistant Trees*, p. 189, Pergamon Press, Oxford (1966).
[6] F. W. COBB, M. KRSTIĆ, E. ZAVARIN and H. W. BARBER, JR., *Phytopathology* **58**, 1327 (1968).
[7] N. T. MIROV, *Composition of Gum Turpentines of Pines*, U.S. Dept. of Agr., Forest Service, Tech. Bull. No. 1239 (1961).
[8] R. H. SMITH, U.S. Forest Service Research Paper PSW-15 (1964); *Science* **143**, 1337 (1964).

in the central Sierra Nevada, and reported the following variability ranges: 4·4–7·9% α-pinene; 18·3–31·5% β-pinene; 24·6–46·8% 3-carene; 9·2–17·5% myrcene; 9·8–20·5% limonene; 1·6–2·4% β-phellandrene and 0·9–2·0% of an unknown.

From the above it is apparent that there is no information on variability of a number of important parameters. Although the effect of monoterpenes on bark beetles is not properly understood, it can be assumed that if there is a meaningful interaction it will take place between the beetles and the oleoresin or its vapor. Thus, it is the concentration of individual monoterpenes in oleoresin, rather than turpentine composition alone, which is of primary importance. This seems obvious in the case of direct beetle–oleoresin interaction, but holds also for the vapor phase as, according to the Raoult's law, at constant temperature the concentration of a monoterpene in oleoresin determines the concentration of the same terpene in the vapor phase. For establishment of meaningful correlations in studies on bark beetle toxicity of oleoresin, therefore, it is imperative either to have information on the turpentine content of the oleoresin or to use the oleoresin rather than the turpentine basis for expressing percentages of individual terpenes. Other important parameters which could relate to the susceptibility to bark beetles could also include oleoresin exudation pressure (OEP),[9–15] oleoresin productivity (yield)[16] as well as the propensity of oleoresin to crystallize.[17]

RESULTS AND DISCUSSION

This paper presents the results of studies on the individual variability of oleoresin from 139 trees of *Pinus ponderosa* from the central Sierra Nevada and covers the parameters of turpentine composition, turpentine content of the oleoresin, OEP, oleoresin yield, and oleoresin crystallizability. The trees exhibited a high degree of variability in each of the parameters investigated (Table 1). The goodness of fit into the normal Gaussian distribution was examined for all variables by the χ^2 method, and results are given in Fig. 1. Frequency distribution diagrams for α-pinene turpentine content, oleoresin exudation pressure, and oleoresin yield are given in Figs. 2 and 3. Of the noncompositional variables, yield and particularly OEP did not show normal distribution, while the percentage crystallization and turpentine content of oleoresin satisfactorily fitted the Gaussian distribution (Fig. 1). The OEP of most of the trees was high (90–155 ψ); the frequency of the trees with OEPs of 10–90 ψ was generally low, except for nine trees which had no measurable resin pressure. The strong deviation from normal distribution, with modes at 0, 130 and possibly at 40 ψ, was perhaps due to a relatively simple inheritance mechanism involving few genes only. Another reason could be the presence among the trees sampled of individuals infected by root disease fungi or some other disease organism resulting in a drop in OEP. However, no evidence of debilitating diseases was found upon examination of all trees included in the study. Yield, based on the quantity of resin collected during the first 24 hr, was highly variable, and the trees were more or less equally distributed throughout the range of yields (Fig. 2). This was not entirely unexpected because yield is influenced by several factors which are also

[9] F. W. COBB, JR., D. L. WOOD, R. W. STARK and P. R. MILLER, *Hilgardia* **39**, 127 (1968).
[10] F. W. COBB, Jr., D. L. WOOD, R. W. STARK and J. R. PARMETER, JR., *Hilgardia* **39**, 141 (1968).
[11] R. W. STARK, *Ann. Rev. Entomol.* **10**, 303 (1965).
[12] J. P. VITE, *Contrib. Boyce Thompson Inst.* **21**, 37 (1961); J. P. VITE and D. L. WOOD, *Contrib. Boyce Thompson Inst.* **21**, 67 (1961).
[13] J. A. RUDINSKY, *Can. Entomol.* **98**, 1339 (1966).
[14] J. A. RUDINSKY, *Proc. Int. Union Forest Res. Organ.*, 13th Congress, Vienna 1961, Teil 2, Band 1, 11 (1962).
[15] D. E. MATHRE, *Contrib. Boyce Thompson Inst.* **22**, 363 (1964).
[16] G. R. STRUBLE, U.S. Dept. Agr. Forest Serv. Res. Note PSW-60 (1965).
[17] F. S. SANTAMOUR, JR., U.S. Forest Service Note NE-39 p. 1 (1965).

variable—for example, yield would be expected to increase in proportion to oleoresin exudation pressure and to a decrease in viscosity of the oleoresin,[18] i.e. in proportion to the turpentine content of the oleoresin. The correlation analyses (Table 2) were in line with these assumptions, with the correlation coefficients, r, between yield and OEP and between yield

TABLE 1. VARIABILITY STATISTICS FOR TERPENE PERCENTAGES, OEP, CRYSTALLIZABILITY AND OLEORESIN YIELD

Variable	Units	Mean	Standard deviation	Maximum	Minimum
α-Pinene	% turpentine	7·35	2·55	20·0	3·5
	% oleoresin	1·80	0·65	4·4	0·65
β-Pinene	% turpentine	33·0	8·95	67·5	13·0
	% oleoresin	8·05	2·50	16·5	2·8
3-Carene	% turpentine	36·5	11·5	60·5	0·0
	% oleoresin	8·90	3·05	17·3	0·0
Myrcene	% turpentine	9·25	3·95	23·0	0·0
	% oleoresin	2·25	1·10	6·1	0·0
Limonene	% turpentine	13·05	7·25	34·0	0·0
	% oleoresin	3·15	1·85	11·9	0·0
Terpinolene	% turpentine	1·45	0·60	3·5	0·0
Total terpenes	% oleoresin	24·3	3·45	37·1	13·0
Yield	(ml/24 hr)	3·45	1·92	6·3	0·0
OEP	ψ	105·5	37·5	155·0	0·0
Crystallizability	(%/24 hr)	40·6	20·4	100·0	0·0

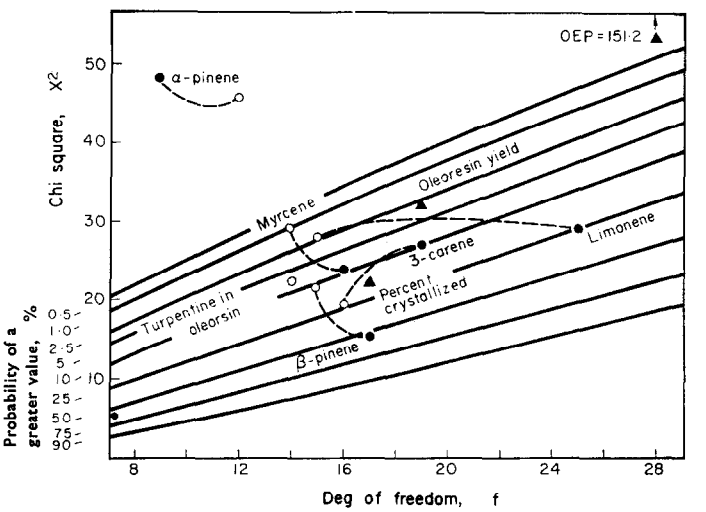


FIG. 1. GOODNESS OF FIT INTO NORMAL DISTRIBUTION OF PARAMETERS INVESTIGATED, USING χ^2 METHOD.
○—Designate values obtained using total oleoresin basis for expressing percentages.

and turpentine content both being positive and significant on the 1% and 5% levels, respectively. However, values were relatively small, $r = 0{\cdot}34$ and $r = 0{\cdot}21$, and together explained only about 16% of the total variability. Thus, unknown factors must predominate.

[18] P. F. BORDEAU and C. S. SCHOPMEYER, Oleoresin exudation pressure in slash pine, its measurement, hereditability, and relation to oleoresin yield, in *The Physiology of Forest Trees* (edited by KENNETH V. THIEMANN), p. 313, The Ronald Press, New York (1958).

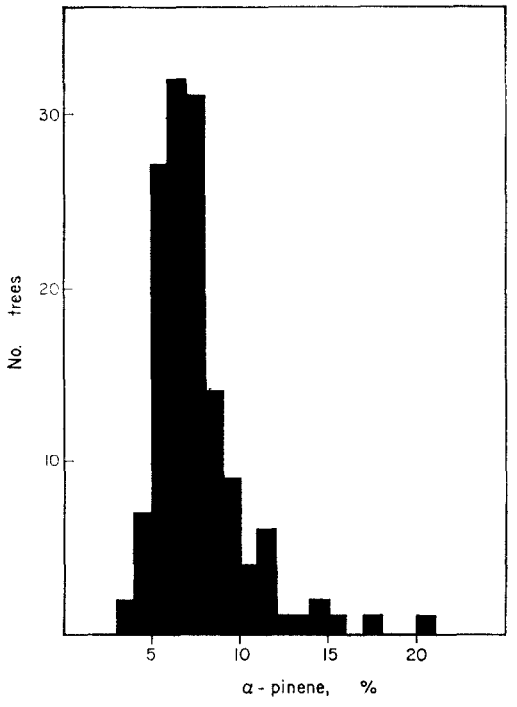


FIG. 2. FREQUENCY DISTRIBUTION DIAGRAM FOR α-PINENE.

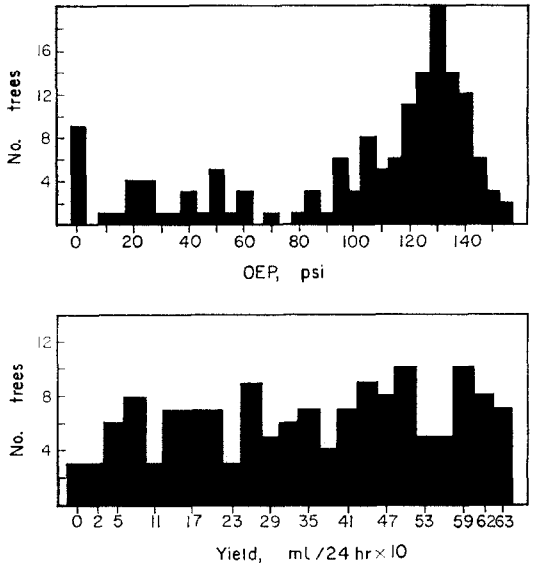


FIG. 3. FREQUENCY DISTRIBUTION DIAGRAMS FOR OEP AND OLEORESIN YIELD.

Composition of the turpentine agreed well with the previous reports (Table 1) except that β-phellandrene was present in traces only. Using GLC methods, Smith's unknown peak was found to be identical with terpinolene. The frequency distributions were close to Gaussian (Fig. 1) when percentages were expressed either on oleoresin or turpentine bases,

except for α-pinene distribution which was markedly skewed and suggested control by few genes only. Squillace and Fisher reported non-Gaussian distributions for several monoterpenes of *P. elliottii*,[19] and Hanover reported the same [20] for the cortical 3-carene of *P. monticola*. The results of Smith on xylem turpentines from *P. contorta*, *P. washoensis* and *P. coulteri*[21] suggest that the same is holding for several monoterpenes of these pines.

No significant correlations were obtained between turpentine content of the oleoresin, OEP, yield or crystallizability of the oleoresin and the monoterpene composition. The fact that comparatively large variations in monoterpene composition did not exert practically any influence on the changes (much less significant, too) of the total monoterpene content of oleoresin, suggests independent enzymatic control of the total material flow towards monoterpenes. However, many characteristic correlations were obtained between percentages of individual monoterpenes (Table 2).

TABLE 2. LINEAR REGRESSION STATISTICS FOR TERPENE PAIRS

Independent variable	Dependent variable	Intercept—(coef. A) (turpentine % basis)	Slope—(coef. B) (turpentine % basis)	Slope—(coef. B) (oleoresin % basis)	Correlation coef. R* (turpentine % basis	Correlation coef. R (oleoresin % basis	Standard error of estimate (turpentine % basis)
3-Carene	Limonene	21·4	−0·23	−0·19	−0·36	−0·31	6·8
3-Carene	Myrcene	12·6	−0·09	−0·018	−0·26	−0·052	3·8
3-Carene	β-Pinene	54·7	−0·59	−0·30	−0·79	−0·37	5·8
β-Pinene	3-Carene	68·8	−0·98	−0·45	−0·79	−0·37	7·5
Limonene	α-Pinene	8·5	−0·09	−0·064	−0·26	−0·19	2·5
3-Carene	α-Pinene	11·6	−0·12	−0·055	−0·53	−0·26	2·2
β-Pinene	α-Pinene	1·3	0·18	0·17	0·65	0·68	1·9
Limonene	Turpentine content of oleoresin	5·1	−0·059	—	−0·127	—	3·4
Myrcene		23·6	0·076	—	0·088	—	3·4
3-Carene		24·0	0·089	—	0·030	—	3·4
β-Pinene		23·7	0·019	—	0·050	—	3·4
α-Pinene		24·8	−0·065	—	−0·048	—	3·4

* $r_{5\%} = 0{\cdot}167$; $r_{1\%} = 0{\cdot}218$.

In one of our previous papers, we discussed the connection of correlations between percentages of terpenoids to biosynthesis.[22] The postulated rules required that strongly positively correlated compounds (percentages proportional) should be biosynthetically closer to each other than to compounds to which they are strongly negatively correlated. The rather high positive correlation coefficient, found between α- and β-pinenes (III and IV), together with low intercept, A, and high slope B (Table 1 and Fig. 4), suggests that these two terpenes should be biosynthetically closer to each other than to 3-carene (V) or limonene (VI). This is reasonable biosynthetically, since formation of both β-pinene (IV) and (−)-α-pinene (III) (the predominant enantiomer in *P. ponderosa*) should proceed through interaction of the positively charged C_8 with the double bond of the hypothetic 4-p-menthene-8 carbonium ion intermediate (II). The formation of 3-carene and limonene does not involve this double bond,[22] on the other hand.

[19] A. E. SQUILLACE and G. S. FISHER, *Proc. 2nd Genetics Workshop of the Society of Amer. Forest. and the 7th Lake States Forest Tree Improv. Conf.*, 1965, U.S. Forest Service Research Paper NC-6, pp. 53 (1966).
[20] J. W. HANOVER, *Forest Sci.* **12**, 447 (1966); *Heredity* **21**, 73 (1966).
[21] R. H. SMITH, *Forest Sci.* **13**, 246 (1967).
[22] E. ZAVARIN, *Phytochem.* **9**, 1049 (1970).

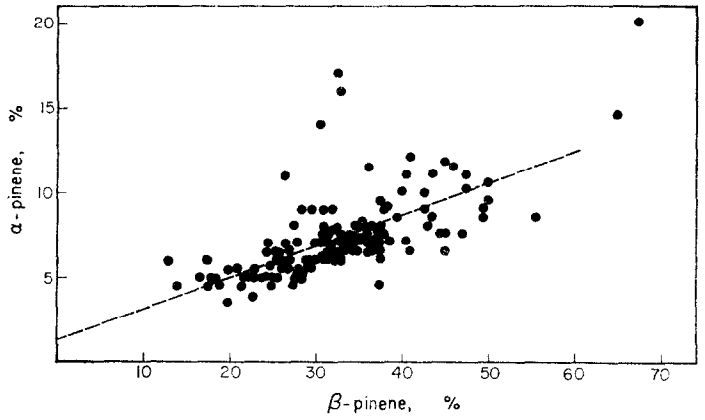
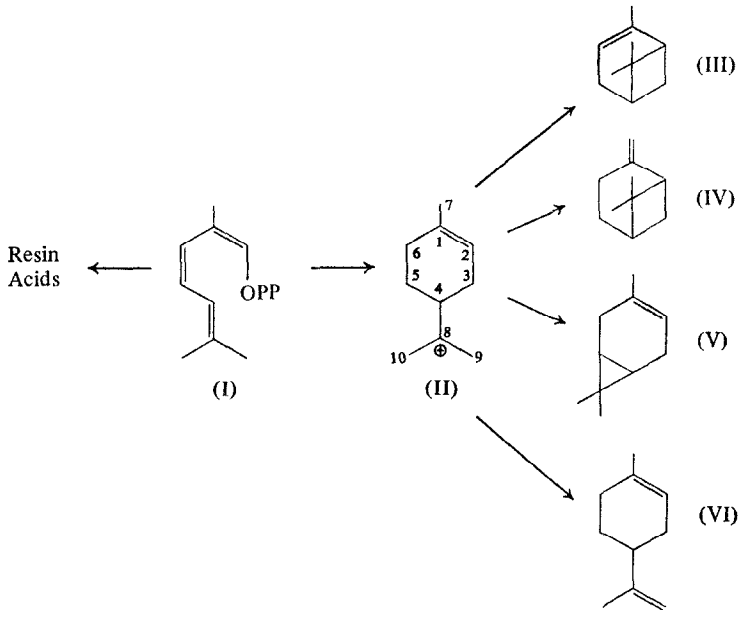


FIG. 4. % α-PINENE VS. % β-PINENE PLOT.

Myrcene did not correlate significantly with any of the terpenes, except for 3-carene, but even here the correlation coefficient was very small and explains less than 7% of the variability. This is reasonable because myrcene formation should not involve participation of 1-*p*-menthene-8-carbonium ion, while formation of cyclic monoterpenoids apparently goes through this intermediate, i.e. the two groups of terpenes are fairly well separated.[22]

Terpinolene correlated significantly and positively with 3-carene (V) ($r = +0{\cdot}27$); although the value of the correlation coefficient was low, this is most likely due to the low amounts of terpinolene present and the consequent analytical inaccuracy. The positive correlation between 3-carene and terpinolene has been noticed before,[22] and was explained by the closeness of biosynthetic paths, involving stabilization of 1-*p*-menthene-8-carbonium ion (II) by proton loss at C_5 vs. C_4.



The procedure of normalizing the analytical results to 100%, as is customary in terpene analysis, introduces some spuriousness in regression analyses which is at its maximum in two component systems with a mandatory $r = 1{\cdot}0$. The spurious part decreases, however, when we go on to multicomponent systems.[22] Spurious correlations can be detected by observing the changes in regression slopes and correlation coefficients for terpene pairs on changing from a turpentine to an oleoresin per cent basis. As can be seen from Table 2, no qualitative changes took place, although—and in contrast to our results with *Abies lasiocarpa* cortical oleoresin[23]—several negative slopes, B, and correlation coefficients, r, noticeably decreased. While this could indicate a certain amount of spuriousness in the results, it could also result from the introduction of an additional, independent variability when using the oleoresin per cent basis—i.e. variability in the total material flow (I → II) from the common precursor, neryl-pyrophosphate (I), to the monoterpenoids. As previously indicated, the latter seems to be independently controlled in *P. ponderosa* oleoresin.

CONCLUSION

Results indicate that if oleoresin is a factor in the resistance of trees to bark beetles or pathogenic fungi, studies to determine its effects under natural conditions should include measurements of as many variables as possible. The composition of turpentine may be one of the important factors, as reported by Smith,[5] but the quantity of turpentine within the oleoresin, the pressure with which it is forced into a wound, the amount of oleoresin exuding from a wound and the facility with which it can crystallize and be handled by the beetle[10] may be overriding factors. The relatively high variability and low correlations among these variables seem to minimize the chance that a single factor can be correlated well with resistance.

EXPERIMENTAL

One hundred and thirty nine *Pinus ponderosa* trees 40–50 years old were selected on the Blodgett Experimental Forest (El Dorado County, California) located in the Sierra Nevada mountains at about 4200–4400 ft elevation. All were presumably healthy, vigorously growing trees, 15–22 in. dia. 4·5 ft above the ground. Oleoresin was collected from each tree, and the OEP, percentage crystallization, and oleoresin yield were determined in July 1965.

OEP was determined by methods described earlier.[9] Standard hydrostatic pressure gauges were inserted into holes drilled into the xylem of the tree bole about 5 ft above the ground. Pressure readings were recorded at periodic intervals throughout the day beginning before sunrise. The highest reading obtained for each tree was used in the analyses reported in this paper. The methods for determining the amount of oleoresin crystallization and yield were also described in the earlier paper.[9] Briefly, the glass tubes were inserted into holes drilled into the xylem, and both the total amount of oleoresin which collected in the tubes and the percentage of resin which crystallized were determined at periodic intervals for up to 48 hr. The data reported here were those obtained 24 hr after the initial installation.

The GLC methods of analysis for turpentine content and composition have been described.[24] Calculations were made using the IBM 1620 as well as DC6400 computers of the University of California.

Acknowledgements—We are indebted to Professor Ronald W. Stark, for critical review of this paper, and to Mr. George Goldman, Dr. Thomas Reichert, and University of California Computer Center for writing the computer programs. Thanks are also due to Dr. Thomas Reichert and Mr. H. W. Barber for their help in analytical work. This study was financed in part by NSF Grant No. GB 5970, which is gratefully acknowledged.

[23] E. Zavarin, K. Snajberk, T. Reichert and E. Tsien, *Phytochem.* **9**, 377 (1970).
[24] P. R. Miller, F. W. Cobb, Jr. and E. Zavarin, *Hilgardia* **39**, 135 (1968).