USE OF MONOTERPENE COMPOSITION IN FOREST GENETICS RESEARCH WITH SLASH PINE

A. E. Squillace

Abstract.--The concentrations of 4 of the 5 major monoterpenes in cortical oleoresin of slash pine are controlled by single genes, with high being dominant or partially dominant over low amounts. Environmental effects are small. Large differences occur between trees, and examples of 15 of the 16 possible phenotypes have been found. Distinctive patterns of geographic variation occur for each of the 4 monoterpenes shown to be simply inherited, with clinal trends being a dominant feature over much of the species range. Such detailed knowledge of variation and inheritance permits use of monoterpenes as gene markers for studying genetic problems. Uses include identifying relatives and seed origin and determining the degree of selfing and of wild pollen contamination in seed orchards.

Additional keywords: *Pinus elliottii* Engelm., essential oils, turpentine.

Interest in monoterpene composition at our laboratory originated from our research to develop strains of slash pine (*Pinus elliottii* Engelm.) that would yield large amounts of oleoresin for gum naval stores. The monoterpenes occurring in this species vary greatly in value, and the original objective of this monoterpene research, beginning in 1961, was to increase yield of the most valuable component, (ß-pinene, (Squillace and Fisher 1966). This work was fruitful, but we also soon learned that monoterpene composition could help solve other tree breeding problems. Hence, monoterpene composition became a major part of our research program. In this paper I briefly summarize findings on the variation and inheritance of monoterpene composition in slash pine and give some examples of how we use this information in our genetics research.

INDIVIDUAL TREE VARIATION AND INHERITANCE

The oleoresin of slash pine consists of about 20 percent monoterpenes, the remainder being mainly resin acids. The monoterpene fraction of oleoresin from xylem tissue consists mostly of a-pinene, (ß-pinene, and ß-phellandrene. Frequently camphene, myrcene, a-phellandrene, and limonene occur as minor consitiuents, while traces of A-3-carene, and 1-terpinene occur occasionally. In oleoresin from cortical tissue of branch tips, the same constituents occur but the amounts of myrcene and/or limonene can be very high in some trees.

1/ Chief Plant Geneticist, Southeastern Forest Experiment Station, USDA Forest Service, Olustee, Florida 32072. The paper is largely based on a report presented at a Symposium sponsored by the Commission of the European Communities at Brussels, January 1977.

The oleoresin of stem xylem is of greatest commercial importance, but the oleoresin from cortical tissue has proved to be the most useful for genetics studies. The composition of monoterpenes in stem xylem oleoresin often varies with height in the tree and depends partly upon distance from the live crown (Roberts 1970 and Franklin 1976). This effect tends to complicate sampling procedures for stem xylem oleoresin. The oleoresin in cortical tissue, on the other hand, is relatively constant within the crown. This desirable feature, plus the fact that cortical oleoresin has 5 major constituents compared to 3 in xylem oleoresin, has lead us to favor cortical oleoresin for genetics studies. Monoterpene composition in oleoresin of needles tends to be similar to that of branch cortical tissue. However, we usually use the latter, because of its relative ease of collection--most trees readily exude a droplet of oleoresin sufficient for analysis when branch tips are excised. Occasionally it is necessary to concentrate samples (Goodwin 1977).

The relative amounts of most of the monoterpenes are usually either high or low. That is, frequency distributions for oleoresin from a large number of trees are usually bimodal (figures IA and B). One complication is that the location of modes can be affected by the presence or absence of other major constituents. For example, the mode for high -pinene is lower in trees containing high limonene (fig. 1A) than in trees containing low limonene (fig. 1B). After studying over 2000 trees, we developed the classification scheme given in table 1 for 4 of the major constituents. Since clear evidence of bimodality for a-pinene is lacking, this constituent is excluded from table 1.

Figure 1.--Frequency distributions for ß-pinene. (From Gansel and Squillace 1976).

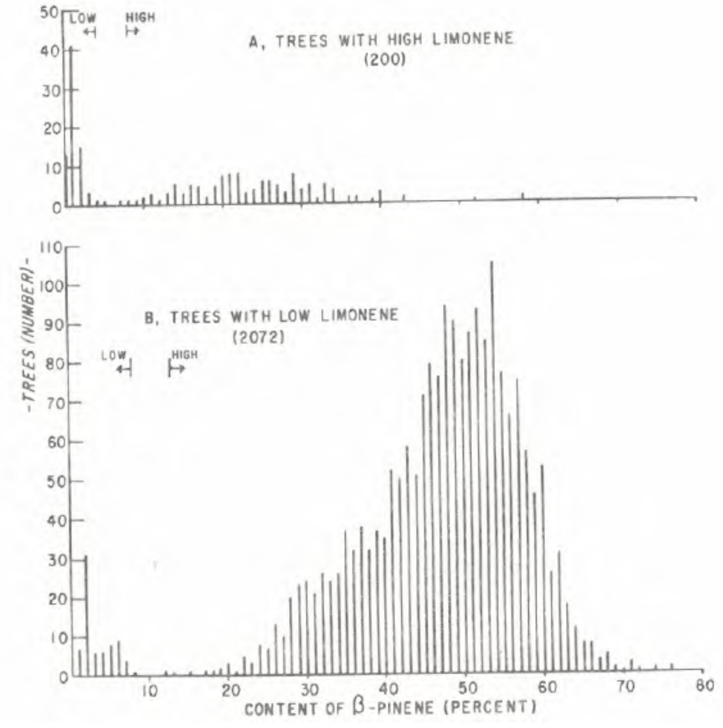
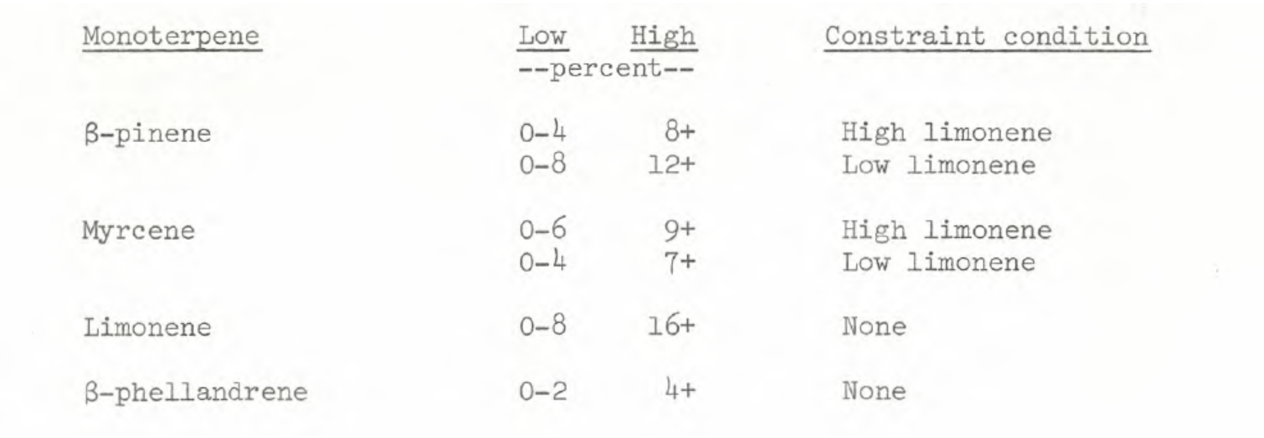


Table 1.--Criteria used for classifying trees as having high or low amounts of each monoterpene. (From Gansel and Squillace, 1976)



Monoterpene	Low	High	Constraint condition
	--percent--		
β-pinene	0-4	8+	High limonene
	0-8	12+	Low limonene
Myrcene	0-6	9+	High limonene
	0-4	7+	Low limonene
Limonene	0-8	16+	None
β-phellandrene	0-2	4+	None

Bimodality in the frequency distribution for a monoterpene suggests that the relative amount of it is controlled by a single gene with dominant gene action. Studies of parents and their self- and cross-pollinated offspring show that this is indeed the case for all constituents showing clear bimodality (table 2) (Squillace 1971, 1976a). Curiously, high was found to be dominant over low in all cases.

As the work progressed we were able to identify enough genotypes to study the degree of dominance expressed by monoterpenes. Preliminary indications are that dominance is partial rather than complete in most cases (table 3). In cases of incomplete dominance we may eventually be able to distinguish heterozygotes from homozygotes, enhancing the utility of monoterpenes as gene markers.

Environmental effects on monoterpene composition of cortical oleoresin of slash pine are small (Squillace and Fisher (1966) and Gansel and Squillace (1976)).

Since 4 of the major monoterpenes show bimodality, we can classify trees into 16 phenotypes as indicated in table 4. Note that many of the trees fall into a few of the phenotypic classes, but appreciable numbers occur in other classes. Only one class lacks representatives. Note especially that two trees occur which, lacking genes for high amounts of the monoterpenes known to be simply inherited, are almost entirely a-pinene. At the other extreme, some trees contain the high allele for all 4 monoterpenes.

GEOGRAPHIC VARIATION

In one of our studies, we sampled trees originating from all portions of the species range. Trees were classified as having either high or low amounts of each of the 4 monoterpenes shown to be simply inherited. Distinctive patterns occurred. For example no trees having high ß-pinene were

Table 2.--Segregation data for inheritance of four monoterpenes in branch cortical oleoresin of slash pine.

Type of mating	Families	Individuals			
		Observed		Expected	
		High	Low	High	Low
		Number			
				β-pinene	
BB x --	78	980	0	980.0	0.0
Bb x Bb	8	86	25	83.2	27.8
Bb x bb	2	9	10	9.5	9.5
bb x bb	0	---	--	-----	----
				Myrcene	
MM x --	2	32	0	32.0	0.0
Mm x Mm	17	151	44	146.2	48.8
Mm x mm	45	278	330	304.0	304.0
mm x mm	26	1	278	.0	279.0
				Limonene	
LL x ℓℓ	1	31	0	31.0	0.0
Lℓ x Lℓ	0	--	--	----	---
Lℓ x ℓℓ	2	14	19	16.5	16.5
L- x ℓℓ	1	2	0	$\geq$ 1.0	$\leq$ 1.0
Lℓ x wind	1	7	4	$\geq$ 5.5	$\leq$ 5.5
ℓℓ x ℓℓ	90	0	1052	.0	1052.0
				β-phellandrene	
PP x --	63	769	14	783.0	0.0
Pp x Pp	8	138	48	139.5	46.5
Pp x pp	17	43	49	46.0	46.0
pp x pp	2	1	50	.0	51.0

found in extreme south Florida (fig. 2). From this point the percentage of such trees increased rapidly to the north. A plateau was reached where all trees had high -pinene. Clinal patterns, with plateaus in some cases were also found for other monoterpenes (figs. 3 to 5).

UTILITY OF MONOTERPENE COMPOSITION

Identification of Seed Origin

The geographic patterns of variation in monoterpene composition offer

Table 3.--Degree of dominance for simply-inherited monoterpenes in slash pine.

Phenotypic group1/	Homozygous dominants		Heterozygotes		Homozygous recessives		Degree of dominance2/
	Basis, trees	Average content	Basis, trees	Average content	Basis, trees	Average content	
	No.	%	No.	%	No.	%	
				β-pinene			
BMℓP	156	47.0	12	44.3	60	3.1	0.88
BmLP	1	29.0	25	22.8	28	1.1	.56
BmℓP	257	51.0	8	39.2	9	5.7	.48
Wtd. Ave.3/							.63
				Myrcene			
BMℓP	2	34.5	228	21.7	1076	1.0	.24
				Limonene			
BMLP	1	37.0	11	38.4	548	.5	1.08
bmLP	1	75.0	5	70.0	9	.3	.87
bmLp	1	89.0	1	89.0	2	.0	1.00
Wtd. Ave.3/							1.01
				β-phellandrene			
BMℓP	168	18.6	61	11.5	83	.4	.22
BmℓP	183	16.4	24	11.3	269	.5	.36
bmLP	1	33.0	1	14.0	3	.0	-.15
Wtd. Ave.3/							.28

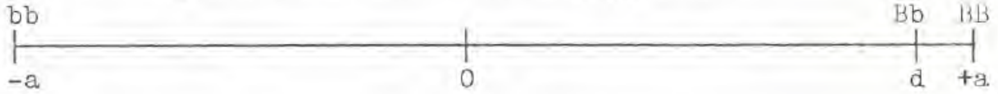
1/B,M,L, and P represent high amounts of β-pinene, myrcene, limonene, and β-phellandrene, respectively, while lower case letters represent low amounts.

2/Computed by methods outlined in Kempthorne (1957, p. 373). For example, the degree of dominance for β-pinene group BMℓP was computed as follows:

$$a = \frac{47.0 - 3.1}{2} = 22.0$$

$$d = 22.0 - (47.0 - 44.3) = 19.3$$

$$\frac{d}{a} = \text{degree of dominance} = \frac{19.3}{22.0} = .88$$



3/Weighted by number of homozygous dominants and heterozygotes involved.

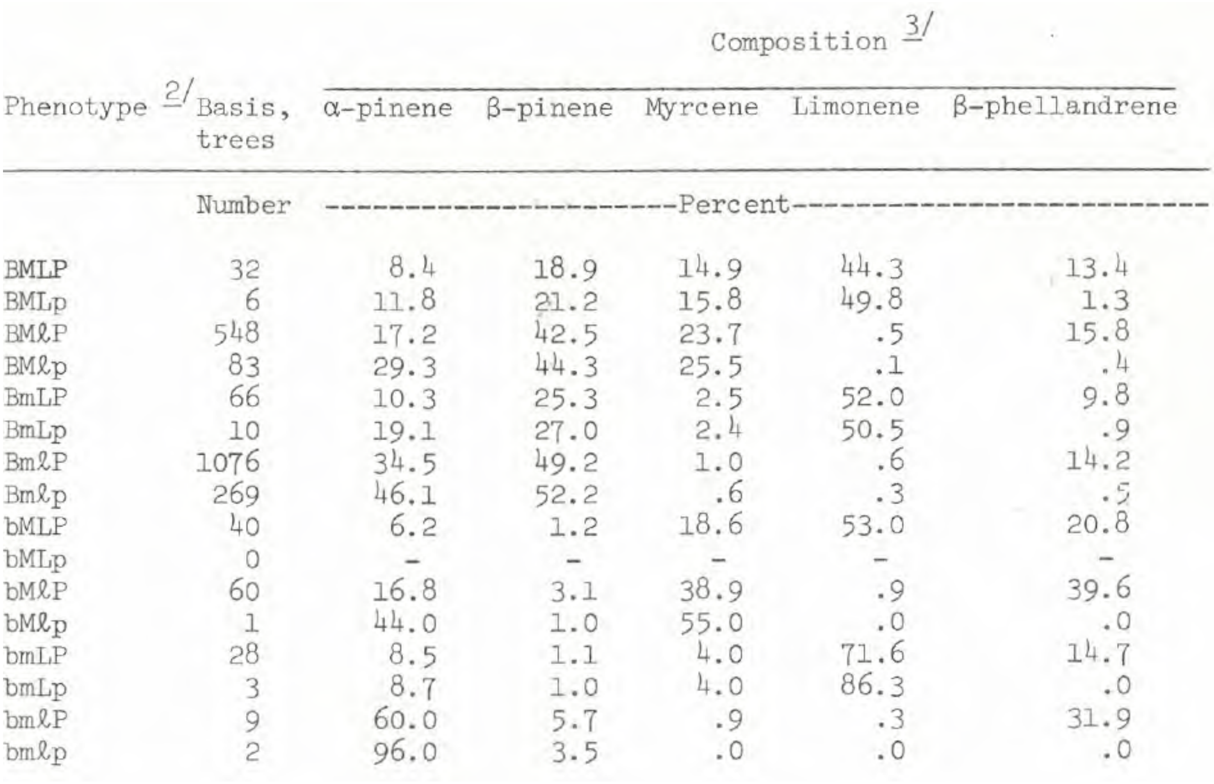
Figure 2.--Percent of trees having high ß-pinene (From Gansel and Squillace, 1976).

Figure 3.--Percent of trees having high myrcene (From Gansel and Squillace, 1976).

Figure 4.--Percent of trees having high limonene (From Gansel and Squillace, 1976).

Figure 5.--Percent of trees having high ß-phellandrene (From Gansel and Squillace, 1976).

Table 4.--Average monoterpene composition in branch cortical oleoresin of 15 slash pine phenotypes. [1]



Phenotype [2]	Basis, trees	Composition [3]				
		α-pinene	β-pinene	Myrcene	Limonene	β-phellandrene
	Number	Percent				
BMLP	32	8.4	18.9	14.9	44.3	13.4
BMLp	6	11.8	21.2	15.8	49.8	1.3
BMℓP	548	17.2	42.5	23.7	.5	15.8
BMℓp	83	29.3	44.3	25.5	.1	.4
BmLP	66	10.3	25.3	2.5	52.0	9.8
BmLp	10	19.1	27.0	2.4	50.5	.9
BmℓP	1076	34.5	49.2	1.0	.6	14.2
Bmℓp	269	46.1	52.2	.6	.3	.5
bMLP	40	6.2	1.2	18.6	53.0	20.8
bMLp	0	-	-	-	-	-
bMℓP	60	16.8	3.1	38.9	.9	39.6
bMℓp	1	44.0	1.0	55.0	.0	.0
bmLP	28	8.5	1.1	4.0	71.6	14.7
bmLp	3	8.7	1.0	4.0	86.3	.0
bmℓP	9	60.0	5.7	.9	.3	31.9
bmℓp	2	96.0	3.5	.0	.0	.0

[1] Sixteen phenotypes are theoretically possible, but no trees of the type bMLp occurred in the sample of 2233 trees.

[2] B, M, L and P represent high amounts of ß-pinene, myrcene, limonene, and ß-phellandrene, respectively, while lower case letters represent low amounts.

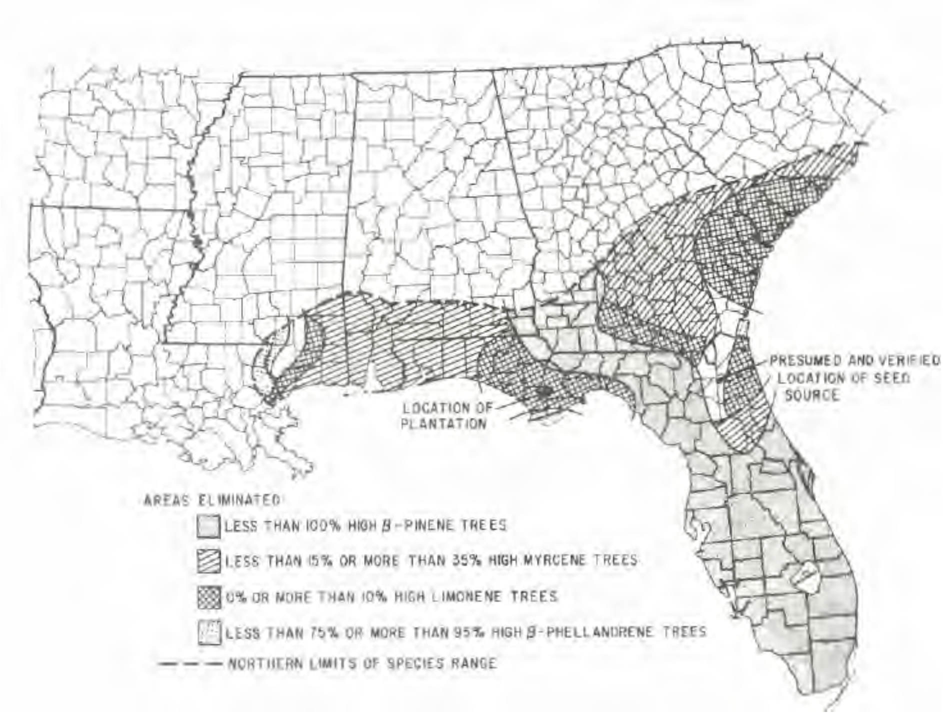
[3] Small amounts of camphene and/or a-phellandrene frequently occur.

possibilities for identifying the approximate geographic origin of seed used in plantations of unknown origin. We recently had an opportunity to test the procedure on 3 plantations that were planted in west Florida in about 1936. On the basis of uncertain records, the seed were believed to have come from several counties in northeast Florida. We sampled 30 trees in each plantation and concluded that the seed used for each were of roughly the same geographic origin (table 5). Hence, we used the averages to estimate their geographic origin. Taking each of the 4 chemicals successively, we eliminated areas where the seed could not likely have originated (fig. 6). All but two areas were eliminated, a small one in southeast Mississippi and a larger one in northeast Florida and southeast Georgia. Hence, the analysis suggested that the purported origin given in the plantation records, northeast Florida, was correct. Possibilities also exist for identifying seed orchard seed.

Table 5.--Percentages of trees having high amounts of 4 monoterpenes in 3 adjacent slash pine plantations.

Plantation	β-pinene	Myrcene	Limonene	β-phellandrene
A	100	23	3	87
B	100	13	3	87
C	100	37	7	80
Average	100	24	4	85

Figure 6.--Determination of probable origin of seed in a 40 year old slash pine plantation. (See table 3)



Identification of Relatives

Knowledge of the mode of inheritance of 4 major monoterpenes permits us to identify relatives in trees to about the same extent that relatives can be identified in human beings using blood types. Identification of ramets within clones is, of course, done rather easily. In several instances we have suspected that certain ramets had been mislabelled, on the basis of cone and seed characteristics, and then verified such suspicions by examining monoterpene composition. In some instances we were able to determine the proper clone. Identification of parents and progenies is, of course, less certain although in one instance we detected and corrected a serious labelling error in a progeny test.

Selling and Wild Pollen Contamination in Orchards

An unusual situation in one of our demonstration seed orchards permitted us to make rough estimates of both the extent of selfing and the degree of wild pollen contamination. The orchard covered 5 acres (2 hectares) and all slash pine trees within 400 feet (122 meters) had been removed. It contained 9 clones, which were all related as either half sibs or full sibs. We knew rtionoterpene genotypes of all the clones (table 6). One of the clones, No. 5, was thought to be suitable for estimating the degree of selling because 1/16 of its selfed progeny would be of the type bmlp, which could not be produced from matings among clones in the orchard. Hence, wind-pollinated seeds were collected from it and monoterpene composition was determined on seedlings grown from them.

Table 6.--The genotypes of nine clones in an experimental slash pine orchard being used to estimate selfing and wild pollen contamination.

Clone number	Genotype
1	BB Mm ℓℓ Pp
2	BB Mm ℓℓ Pp
3	BB mm ℓℓ Pp
4	BB Mm ℓℓ Pp
5	Bb mm ℓℓ Pp
6	BB Mm ℓℓ Pp
7	BB mm ℓℓ PP
8	Bb mm ℓℓ PP
9	BB Mm ℓℓ PP

Frequencies of the various phenotypes showed that 1.3 percent were of the type bmlp (table 7). Hence, we could guess that approximately 16 x 1.3 = 21 percent of the progeny of clone 5 were selfs. However, we also computed expected frequencies, first assuming that all matings were crosses among trees in the orchard, and then also by assuming that all progeny were sired by contaminate pollen. The latter were made on the basis of known gene frequencies determined for the region in which the orchard was located.

Comparison of observed and expected frequencies strongly suggested that considerable wild pollen contamination is occurring in this orchard. All seedlings containing high limonene plus those of the type bMl^{P} and bMlp are necessarily contaminants because they cannot be formed by any mating among orchard clones. Also, the correlation of observed frequencies with frequencies expected from orchard out-crosses is smaller than with frequencies expected from orchard contamination. Note also that bmlp trees can be produced by contamination as well as by selfing. Hence, a better estimate of the

Table 7.--Observed and expected phenotypic frequencies in 546 wind-pollinated progeny 1/ of clone 5 in an experimental slash pine orchard average.

Phenotype	Observed frequencies[1]	Expected frequencies if pollen is entirely: Self	Orchard outcross	Wild (contamination)
BMLP	0.000	0.0	0.0	0.002
BMLp	.000	.0	.0	.001
BMℓP	.106	.0	.253	.095
BMℓp	.017	.0	.065	.031
BmLP	.011	.0	.0	.013
BmLp	.002	.0	.0	.004
BmℓP	.578	.562	.569	.615
Bmℓp	.220	.188	.077	.203
bMLP	.000	.0	.0	.000
bMLp	.000	.0	.0	.000
bMℓP	.009	.0	.0	.004
bMℓp	.004	.0	.0	.001
bmLP	.000	.0	.0	.000
bmLp	.000	.0	.0	.000
bmℓP	.040	.188	.036	.023
bmℓp	.013	.062	.0	.008
Sums	1.000	1.000	1.000	1.000

[1] Averages of data from seed collections made in 1973 and 1974.

degree of selfing might be 16(1.3 - .8) = 8.4 percent and this agrees more closely with estimates of selfing that we have obtained in other orchards through use of chlorophyll-deficient seedlings as gene markers.

Thus, we believe that production of selfed seedlings in this orchard is low, but that contamination is high. Of course, these results apply only to one orchard, but they demonstrate the possible utility of monoterpene composition.

DISCUSSION

Several other uses of monoterpene composition in solving forestry problems have been suggested, as summarized by Squillace (1976b). For example, relationships have been shown between monoterpene composition and insect and disease resistance. Such correlations permit indirect genetic selection for these traits. Monoterpene composition is well suited for studies of relationships between species and for identification of hybrids. A recent paper (Squillace, et al. 1977) suggests gene flow from Caribbean pine (*P. caribaea* Mor.) into slash pine based on population analyses of monoterpene composition.

Utility of monoterpene composition will likely be enhanced by more modern gas chromatographs. A recent model, for example, provides for automatic injection of 35 samples, permitting around-the-clock analyses with a minimum of attendance. Thus, more intensive and extensive sampling will be feasible.

In short, monoterpene composition has proved to be very useful in many forest genetics studies and its use is likely to increase.

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