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Clone Certification by use of Cortical Monoterpenes as Biochemical Markers

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Summary

Monoterpenes can be useful biochemical markers for certifying clones in seed orchards. Cortical oleoresin from buds in the top branches of 81 clones in a research seed orchard were sampled for monoterpene composition to use in distinguishing between clones. Of 3240 possible paired combinations of clones, 2623, 2202, 880, and 302 combinations of clones differed in the composition of one, two, three, and four of the five major monoterpenes respectively. Only 19%, or 617, of the possible combinations of clones could not be separated at all. Beta-phellandrene separated the greatest number of combinations of clones and limonene, the least. Scions from a commercial seed orchard had been mislabeled in establishing a second and third seed orchard and errors were verified by using monoterpene analysis. It is recommended that trees in research seed orchards be subjected to monoterpene analysis to verify correct labeling so that errors are not perpetuated in breeding and genetic studiees or in commercial seed orchard establishment.

Key words: α-pinene, β-pinene, β-phellandrene, seed orchard, ramets, oleoresin, gum.

Zusammenfassung

Monoterpene können nützliche biochemische Marker zur Zertifizierung von Klonen in Samenplantagen sein. In einer Versuchssamenplantage mit 81 Klonen wurde Rindenbalsam von Terminalknospen auf den Gehalt an Monoterpenen sowie deren Eignung zur Klonunterscheidung untersucht. Von 3240 möglichen paarweisen Klonkombinationen unterschieden sich die Kombinationen 2623, 2202, 880 und 302 in 1, 2, 3 bzw. 4 der 5 Haupt-Monoterpene. Nur 19% oder 617 der möglichen Kombinationen von Klonen konnten überhaupt nicht getrennt werden. Anhand von Beta-Phellandren konnte die größte, von Limonen die geringste Anzahl der Klonkombinationen getrennt werden. Durch falsche Kennzeichnung von Pfropfreisern in einer kommerziellen Samenplantage waren in den nachgezogenen Plantagen Fehler entstanden, die durch die Monoterpen-Analyse aufgedeckt wurden. Es wird empfohlen, daß Bäume in Versuchssamenplantagen einer Monoterpen-Analyse unterzogen werden, um die korrekte Kennzeichnung sicherzustellen, so daß Fehler sich nicht bis in die Züchtung und in genetische Studien oder in eingerichtete kommerzielle Samenplantagen hinein fortsetzen.

Introduction

Scions may be accidentally mislabeled in breeding operations or in grafted seed orchards. Unless there are strong morphological differences in the vegetative structures of scions or in the morphology of cones and seeds, the errors can go unnoticed. And even if an error becomes apparent, the mislabeled material must be identified or discarded. A rapid and nondestructive means of clone certification is needed to avoid this problem.

Monoterpene composition of cortical or foliar oleoresin holds much promise for identifying conifer clones in breeding programs and seed orchards. It has been successfully used to identify cultivars of *Picea pungens* ENGELM. (ROTTINK and HANOVER, 1972) and *Juniperus horizontalis* MOENCH (FRETZ, 1977). Monoterpenes have also been used to separate clones of *Pseudotsuga menziesii* (MIRB.) FRANCO (RADWAN and ELLIS, 1975), *Pinus sylvestris* L. (THORIN and NOMMIK, 1974), and *Picea abies* (L.) KARST. (ESTEBAN *et al.*, 1976). Research on cortical monoterpenes has shown that the nongenetic within-tree variation in the monoterpene composition is much less than the variation among trees of a species (HANOVER, 1966). Thus, by identifying monoterpene phenotypes of trees one may be able to certify ramets from clones. HANOVER (1966) found that intraclass correlation coefficients for monoterpene composition in *Pinus monticola* DOUGL. ex D. DON were high. He suggested

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that a few samples from specific tissues in a tree should give a good measure of the monoterpene phenotype, which can be used as a fingerprint in identafying ramets. GANSEL and SQUILLACE (1976) studied geographic patterns in cortical monoterpene content in *Pinus elliottii* ENGELM. (slash pine) which have been used in determining the origin of slash pine plantations (SQUILLACE *et al.*, (1980). Inheritance of cortical monoterpenes in slash pine has been studied (SQUILLACE, 1971) but is not necessary for clone certification since ramets of a single clone propagated by grafting vary but little in cortical monoterpene composition (KOSSUTH and MUSE, 1986) even though within tree variation is very high (KOSSUTH and MUSE, 1985). Isozyme analysis can also be used for clone certification but monoterpene analysis is more convenient to use.

The goal of this study was to determine whether each ramet could be identified through a series of paired comparisons. The objectives were to: (1) determine if all the ramets in a seed orchard could be differentiated by monoterpene content, and (2) determine whether ramets in three commercial slash pine seed orchards, some with morphologically different cones, were indeed derived from the same or different ortets. The commercial seed orchards were Waterman orchard, ITT Rayonier, Inc., Yulee, Florida; Reidsville orchard, ITT Rayonier, Inc., Reidsville, Georgia; and Container Corporation of America orchard, Callahan, Florida.

Materials and Methods

In the summer of 1984, buds were sampled from four ramets from each of 81 clones of genetically improved high-gum-yielding slash pines in the USDA Forest Service research seed orchard, Olustee, Florida. Cortical oleoresin from the terminal bud of a shoot in the top of each tree was collected by severing the terminal centimeter of the bud and allowing the oleoresin to flow out. The oleoresin was then placed into small collection vials containing pentane (SQUILLACE, 1976). All samples were collected within a 2-week period. GLC analysis was conducted according to the method of KOSSUTH and MUNSON (1981) by using a 5840-A Hewlett Packard chromatograph with an automated sample injector and programmable integrator. The amount of each monoterpene was expressed as a percentage of the total monoterpenes since this method has been shown to have the least variation (POWELL and ADAMS, 1973).

The mean monoterpene composition for each clone was obtained by averaging the ramet values. There is no significant difference in monoterpene phenotypes among ramets of a clone (KOUSSUTH and MUSE, 1986) when the contents of five or more buds are averaged. Von RUDLOFF (1972) concluded that differences in monoterpene percent composition of 5 to 10% for major monoterpenes in leaves were outside of sampling and analytical error and would indicate different phenotypes. In this study ramets were considered to be from different clones if the relative amounts of one or more of the major monoterpenes differed by 10%. No attempt was made to separate clones based on a monoterpene that made up less than 10% of total monoterpenes for either clone.

In the Waterman seed orchard, some of the seven ramets of one clone appeared to have morphologically different cones. These ramets and the ramets established from these trees in the Reidsville seed orchard were sampled in October 1982. In December 1982, the same Waterman orchard ramets and the ramets from which they originated were sampled. The original ramets are in the Container Corporation orchard, which was established first. GLC analyses were conducted as described for ramets from the Olustee seed orchard.

Results

The major cortical oleoresin monoterpenes in all the slash pine seed orchards were α-pinene, β-pinene, β-phellandrene, limonene, and myrcene. A matrix analysis based on each monoterpene was conducted to separate clones in the Olustee seed orchard. Increasing the number of monoterpenes considered increased the number of clones that could be separated (*Table 1*). A total of 2623 combinations of clones of the possible 3240, or 81% could be separated by at least one of the five monoterpenes. A total of 2202 or 68% of the combinations of clones could be separated by two monoterpenes simultaneously. A total of 880, or 27% of the combinations of clones could be separated by any three monoterpenes simultaneously. A total of 302, or 9% of the combinations of clones could be separated by four monoterpenes simultaneously.

Beta-phellandrene, α-pinene, β-pinene, myrcene, and limonene alone separated 58, 52, 45, 25, and 5% of the clone pairs in the Olustee seed orchard, respectively. Beta-phellandrene could be used to distinguish between the largest number of combinations of clones, 1866, and the fewest number of combinations of clones were separated by limonene (*Table 2*). Whenever limonene could be used to sepa-

Table 1. — Increase in the number of combinations of clones that can be separated when increasing numbers of monoterpenes are considered. The number of combinations of clones that the last monoterpene alone separates is in parenthesis.

Monoterpene	Clone pairs separated
β-phellandrene (1866)	1866
+α-pinene (439)	2305
+α-pinene+β-pinene (255)	2560
+α-pinene+β-pinene+myrcene (63)	2623
+α-pinene+β-pinene+myrcene+limonene(0)	2623
α-pinene (1692)	1692
+β-phellandrene (613)	2305
+β-phellandrene+β-pinene (255)	2560
+β-phellandrene+β-pinene+myrcene (63)	2623
+β-phellandrene+β-pinene+myrcene+limonene (0)	2623
β-pinene (1467)	1467
+β-phellandrene (865)	2332
+β-phellandrene+α-pinene (228)	2560
+β-phellandrene+α-pinene+myrcene (63)	2623
+β-phellandrene+α-pinene+myrcene+limonene (0)	2623
Myrcene (824)	824
+β-phellandrene (1408)	2232
+β-phellandrene+α-pinene (291)	2523
+β-phellandrene+α-pinene+β-pinene (100)	2623
+β-phellandrene+α-pinene+β-pinene+limonene (0)	2623
Limonene (158)	158
+β-phellandrene (1773)	1931
+β-phellandrene+α-pinene (376)	2307
+β-phellandrene+α-pinene+β-pinene (253)	2560
+β-phellandrene+α-pinene+β-pinene+myrcene (63)	2623

Table 2. — Number of combinations of clones separated simultaneously by the base monoterpene and any 3 other monoterpenes (4 total); the base monoterpene and 2 other monoterpenes (3 total), exclusive of the 4 total; the base monoterpene and 1 other monoterpene (2 total), exclusive of the 4 and 3 total; and the base monoterpene alone exclusive of the 4, 3 and 2 totals [a]/.

No. monoterpenes simultaneously separating a clone pair	Base Monoterpene				
	β-phellandrene	α-pinene	β-pinene	Myrcene	Limonene
4	299	301	299	251	58
3 exclusive of 4	477	504	482	176	95
2 exclusive of 3 and 4	954	765	586	334	5
1 exclusive of 2,3 and 4	136	122	100	251	0
Total	1866	1692	1467	63	158
% of 3240 possible clone pairs.	58	52	45	25	5

[a]/ No combination of clones is counted more than once for each base monoterpene, i.e. clone pairs separated by 4 monoterpenes are not counted again in the number of clone pairs separated by 3, 2 or 1 monoterpenes for the base pair.

rate a combination of clones, there was always one other monoterpene or more that also separated that combination of clones. Those that could be separated by two or more monoterpenes are an expression of the restriction in percent analyses where the total is 100% (SQUILLACE, 1976). If one component increases, others necessarily decrease.

Of the 81 clones in the Olustee seed orchard, 13 contained one or two of four ramets that differed in monoterpene content by 10% or more. These were resampled in the manner suggested by KOSSUTH and MUSE (1986) by combining five buds before GLC analysis to obtain a more accurate phenotypic estimation of the monoterpene content. Eleven of the clones had only one outlying ramet. When these ramets were resampled by using five buds, eight turned out to be similar to the other three ramets of the same clone, and three were different. These three were removed from the seed orchard. Resampling the remaining 2 of the 13 clones that differed in monoterpene content showed that two ramets of clone 365 had 13 to 15% myrcene and two had 2% or less. All the clone bank ramets of clone 365 from which the seed orchard was established, had 13 to 15% myrcene indicating that the two ramets supposedly from clone 365 in the seed orchard with 2% or less myrcene were incorrectly labeled, and they were removed. Two ramets of clone 353 had 25% myrcene and two had less than 2%. Three of the five ramets from clone 353 in the clone bank had 2% or less myrcene and other two had 25%. Further study of the ramets used in establishing the clone bank is necessary to verify ramets of clone 353 in both the seed orchard and clone bank.

Monoterpene composition of the five ramets under study in the Container Corporation orchard were all similar, with myrcene content of 13.9 to 17.4% and β-phellandrene content of 9.0 to 15.6% (*Table 3*). Sampling of the seven ramets in October and December in the Waterman orchard gave the same monoterpene composition in both months. Three of the seven ramets (AU-10, BA-9, and W-2) were similar and differed from the other four in myrcene composition of 1—2% vs. 15—18%, and β-phellandrene of 40—47% vs. 17—20%. Four (N-11, P-6, P-9, and U-12) of the seven ramets were similar to those from the Container Corporation orchard and apparently are correctly labeled. The other three were were determined to be mislabeled.

Eleven scions from the Waterman orchard were grafted in establishing the Reidsville orchard. Seven of these ramets (B-10, D-32, I-17, L-40, M-3, V-12, and V-38) were similar in myrcene and β-pellandrene content to the four ramets in the Waterman orchard and to all of the ramets in the Container Corporation orchard. Three of the remaining four ramets (Q-18, U-1, and Z-48) were similar to the three mislabeled ramets in the Waterman orchard, and the fourth one (P-36) was different from all the ramets sampled in all three seed orchards. The fourth ramet had 33.0, 40.9, and 23.3% α-pinene, β-pinene, and β-phellandrene respectively, and also appeared to be mislabeled (*Table 3*).

Discussion

Clones can be verified by starch or polyacrylamide gel electrophoresis to separate isozymes, but the sample preparation, equipment and supplies required are much greater than those required for gas-liquid chromatography (GLC) of cortical monoterpenes. For monoterpene analysis in slash pine, oleoresin from apical bud cortical tissue is dissolved in pentane and injected automatically into gas chro-

Table 3. — Monoterpene composition (percent) of ramets in three commercial seed orchards that presumably originated from the same ortet. Container Corporation orchard established first; Waterman established from Container scions; Reidsville established from Waterman scions.

Orchard and ramet	Monoterpene				
	Myrcene	α-pinene	β-pinene	β-phell.	Limonene
Container					
A-11	15.0	20.9	49.9	12.9	1.3
BBB-6	13.9	20.3	54.8	9.0	1.4
DDD-1	17.4	21.0	47.3	13.0	1.3
R-11	17.0	18.5	47.3	15.6	1.3
Z-33	16.2	19.5	47.8	14.6	1.3
Waterman [a]/					
N-11	18.0(16.4)	16.7(21.4)	46.0(44.1)	17.3(16.9)	1.3(1.3)
P-6	16.7(15.0)	17.1(21.1)	47.5(50.1)	17.4(12.4)	1.3(1.4)
P-9	17.7(17.5)	20.2(19.4)	40.4(44.4)	19.6(16.6)	1.2(1.3)
U-12	18.2(17.0)	19.6(21.3)	42.5(44.6)	18.2(15.5)	1.2(1.4)
AU-10	1.7 (1.5)	16.6(17.4)	36.1(37.5)	43.5(40.2)	1.4(1.6)
BA-9	1.7 (1.5)	17.3(19.1)	31.7(36.2)	46.6(40.0)	1.7(1.5)
W-2	1.9 (1.8)	12.9(16.9)	38.5(38.2)	44.2(40.0)	1.5(1.7)
Reidsville					
B-10	14.9	20.3	46.2	16.7	1.2
D-32	13.2	20.3	51.0	13.5	1.2
I-17	16.0	19.7	46.9	15.3	1.2
L-40	14.1	20.3	46.2	16.7	1.2
M-3	15.8	16.6	45.1	20.0	1.2
V-12	15.9	19.0	47.0	17.2	1.0
V-38	13.6	20.0	51.7	12.7	1.3
P-36	1.5	33.0	40.9	23.3	1.4
Q-18	1.7	13.8	36.8	45.2	1.6
U-1	1.7	15.3	36.9	42.7	1.6
Z-48	1.7	16.9	35.5	43.4	1.6

[a]/ The first value is for an October sampling; values for a December sampling are in parenthesis.

matographs with integrators that give both quantitative and qualitative determinations of cortical monoterpenes. Since one is almost always able to separate clones by isozyme analysis or GLC of monoterpenes, both are useful techniques. However no studies with isozymes or monoterpenes have attempted to distinguish between all possible combinations of clones in a seed orchard. The use of isozymes should be considered when monoterpene analysis does not show ramets to be different. Because so may more loci can be studied using isozymes, the more lengthy procedure of isozyme analysis is justified to obtain more precision in making a determination. Hence, monoterpene analysis can be viewed as giving positive separation of clones but not necessarily positive similarity of the genetic make-up of ramets.

The large differences in monoterpene composition found among some ramets of the presumed same clone in the Olustee seed orchard clearly demonstrate that a few scions were mislabeled when establishing the seed orchard. In analyzing the clone bank ramets, it is apparent that some scions were also mislabeled when establishing the clone bank. Both of these plantings are used for advanced generation breeding and the establishment of high-gum-yielding seed orchards. The findings of this study indicate the necessity for roguing ramets of questionable identity in the seed orchard and returning to the original seed orchard or ortet to certify correct clone bank ramets. Within a year or two after establishment, seed orchards or clone banks that are to be used in tree breeding programs should be subjected to some kind of certification to avoid errors in the breeding program and in the establishment of commercial orchards. Errors at this stage are often perpetuated and magnified since all scions for a new planting may be taken from one incorrectly labeled ramet.

Myrcene and limonene are often minor monoterpenes (less than 6% of the total) (Gansel and Squillace, 1976) and are most useful in separating clones when one has a low content and the other a high content. The ramets in the commercial seed orchards fell into this category, and separations were straightforward. Increasing confidence in phenotype separations can be obtained with an increasing number of monoterpenes simultaneously separating the same ramet combinations. Furthermore, new research indicates one should sample five buds in determining a tree phenotype (Kossuth and Muse, 1986). A difference of 10% is a most conservative guide but is especially necessary when only one monoterpene is sufficiently different enough to obtain a separation.

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The Role of Reproductive Phenology upon the Mating System of a Douglas-fir Seed Orchard

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Abstract

The relations between reproductive phenology and the mating systems of a Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] seed orchard were studied using allozyme polymorphisms at six loci. Seeds were analysed from cones of 28, 84, and 35 trees, representing three non-overlapping early, intermediate, and late reproductive phenology classes, respectively. Significant differences ($P < 0.05$) were observed among the three maternal gene pools, but not among the three pollen pools Significant change of gene fixation was observed between the parental (negative F) and filial (positive F) generations for each of the three reproductive phenology classes, indicating the presence of some form of inbreeding. Estimates of outcrossing rates for the intermediate class were higher than those obtained for either the early or late classes, indicating maximum panmixia during the height of flowering.

Key words: Pseudotsuga menziesii, seed orchard, reproductive phenology, outcrossing.

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