CLONAL REPEATABILITY OF MONOTERPENE COMPOSITION OF CORTICAL OLEORESIN OF *PINUS RADIATA*

R.D.BURDON, R.E.GASKIN, C.B.LOW
and J.A.ZABKIEWICZ

New Zealand Forest Research Institute,
Private Bag 3020, Rotorua, New Zealand

(Received for publication 29 July 1992; revision 18 June 1993)

ABSTRACT

Forty-five clones of *Pinus radiata* D.Don were studied for repeatability (broad-sense heritability) of levels of individual monoterpenes in cortical oleoresin, using hedged cuttings that had come from 7- to 9-year-old ortets. In these cuttings the estimated clonal repeatabilities of levels of all monoterpenes were very high (generally >0.85). It appeared that clones could readily be distinguished in 99% of pairwise comparisons. For 22 of the clones 15-year-old ortets were sampled in the same month. Ramet-ortet repeatabilities were very high (>0.85) for three of the main monoterpene components (β-pinene, carene, and limonene), lower (c. 0.6) for a fourth (α-pinene), and lower still for some minor components, but higher repeatabilities were clearly attainable through straightforward refinements of the chemical analysis.

Keywords: monoterpenes; cortical oleoresin; broad-sense heritability; genetic fingerprint; clones; *Pinus radiata*.

INTRODUCTION

Monoterpene composition in the oleoresin of *Pinus radiata* varies widely among trees (Bannister *et al.* 1962; Burdon, Gaskin, Zabkiewicz & Low 1992) as it does in many conifers (Squillace 1976a, b). This, and the fact that chemical composition is a relatively direct expression of gene action, suggest that monoterpene analysis could provide a genetic fingerprint for a tree. A basic application would be to check the genetic identity of clonal material, which could be very useful for both tree breeding and seed orchard management. Speculative applications, based largely on the relatively high toxicity of some monoterpenes to insects (e.g., Smith 1972) and/or fungi (e.g., Chou & Zabkiewicz 1976), are uses as selection criteria for resistance to insect pests or fungal diseases, and strong genetic control of monoterpene composition is a prerequisite for any such applications.

The monoterpene composition in oleoresin from near the shoot tips of a tree is complex, with a number of monoterpenes occurring in significant but widely varying amounts. Provided the genetic control is strong, and the variation is largely independent among

monoterpenes, this multiplicity of major components affords good prospects for fingerprinting each of a large number of genotypes almost uniquely.

The monoterpene composition of oleoresin from the cortex of twigs in conifers has usually shown a good combination of variability and sampling stability, and cortical monoterpene composition has proved strongly heritable in various conifer species (Baradat *et al.* 1972, 1975; Squillace 1976a, b; Squillace *et al.* 1980). In *P. radiata*, seedling twins (pairs of seedlings emerging from single seeds) have shown either consistent differences or consistent similarities (Burdon & Zabkiewicz 1973) and populations have been shown to differ (Burdon, Zabkiewicz & Andrew 1992) in monoterpene composition. Nevertheless, the latter study showed some disconcerting non-genetic variation. This paper reports a study designed to quantify the repeatability of monoterpene composition in clonal material and to define better the range of conditions over which the genotypic differences are expressed consistently. It was begun on material chosen to study the genetic control of resistance to shoot dieback associated with infection by the fungus, *Diplodia pinea* (Desm.) Kickx, in connection with a hypothesis that resistance was closely related to monoterpene composition. Subsequent investigation (Burdon unpubl.) failed to confirm that hypothesis, possibly because only chemical moieties and not optical isomers were distinguished. For purposes of the study reported here, therefore, the clones were viewed as an essentially random sample of a typical New Zealand "land-race" population. The material sampled comprised both ramets (cuttings) of clones and ortets of a subset of the clones.

MATERIAL AND METHODS

Plant Material

The ortets were in unimproved stands in Tarawera Forest, on sites (altitudes 60–100 m) where infection by *Diplodia pinea* had caused severe shoot dieback. Most of the ortets were selected intensively for freedom from dieback and good tree form, and less intensively for dominant crown status, when they were 6–8 years from planting. One year later cuttings were taken from laterals in free-growing crown, and 45 clones were successfully propagated. The rooted cuttings were planted out in a single row-plot for each clone next to the FRI nursery at altitude 300 m but the same latitude as Tarawera Forest. They were kept hedged at about 1 m height.

Oleoresin Sampling

Twenty-two of the ortets that had been propagated at age 9 from planting were successfully sampled for oleoresin at age 14 from seed. Each oleoresin sample was taken on 17 April about 15 cm from the tip of a second-order lateral within the free-growing crown. A scalpel cut was made in the ridge of cortex subtending a cataphyll, and up to 5 µl of exuding resin collected in a capillary tube which was then sealed in a 1-ml vial.

Oleoresin was collected 12 days later from the cuttings, 5 years after they had been set. One oleoresin sample was taken from up to five cuttings each of all 45 clones, a total of 192 cuttings being successfully sampled. A sample was collected from each cutting as described for the ortets, except that it was taken from the zone of bare cataphylls on the topmost elongated shoot cycle of one of the vigorous upright shoots growing on top of the "hedge".

Samples were transferred promptly into refrigeration (–20°C) pending analysis by gas-liquid chromatography (GLC) which was done within 2 months.

Chemical Analysis

For analysis, diethyl ether (approx. 200 ml) was added to each sample and an aliquot (0.4 µl) was injected into a Pye 104 gas chromatograph. Conditions were: metal capillary FFAP SCOT column 50' × 0.02" i.d., oven temperature 82°C, helium gas flow 4 ml/min, nitrogen make-up gas flow 40 ml/min, automatic injection S4 unit, and peak area integration (HP3380A reporting integrator). The amount ("level") of each monoterpene was expressed as a percentage of the total monoterpene mixture based on individual peak areas. Identification of the individual monoterpenes was as described by Zabkiewicz & Allan (1975).

Statistical Analysis

For each monoterpene the data from the samples collected from the cuttings were subjected to unbalanced one-way analysis of variance. Clonal (ramet-ramet) repeatability was estimated for each monoterpene as $\hat{\sigma}_c^2 / (\hat{\sigma}_c^2 + \hat{\sigma}_w^2)$, where $\hat{\sigma}^2{}_c$ and $\hat{\sigma}^2{}_w$ are the estimates of between-clone and within-clone variance respectively, obtained from the analysis of variance.

Ramet-ortet clonal repeatability was estimated for each monoterpene as the unweighted regression of the mean for the ramets of a clone on the ortet value. As a cross-check it was also estimated by the correlation method of Franklin (1974), which has advantages if variances differ between the ramets and ortets.

All statistical analyses were made both on untransformed data and arcsin transformed values.

RESULTS

Thirteen monoterpenes were detected, and are listed in Table 1 in order of elution. Four of them, α-pinene, β-pinene, carene, and limonene, occurred frequently as major peaks, and were almost invariably detected. Sabinene and β-phellandrene sometimes occurred as major peaks but were often not detected. Myrcene and terpinolene were minor peaks that were almost always detected. Among the very minor peaks camphene and γ-terpinene were detected almost always, α-terpinene and p-cymene irregularly, and α-phellandrene rarely. Even among the major peaks levels varied very widely. Levels of most monoterpenes were generally similar between the ramets and the ortets (Table 1), the main exceptions being lower detected levels of β-pinene and higher detected levels of sabinene and β-phellandrene in the ramets.

The various estimates of clonal repeatabilities of monoterpene levels are shown in Table 2. Ramet-ramet estimates were consistently high and very highly significant ($p < 0.001$), and the lower values were associated either with detection of minor peaks for some ramets but not others, within particular clones, or failure of the integrator to resolve pairs of peaks for some ramets. Considerable between-clone heterogeneity of variances was evident (Bartlett tests), which occasions marginal reservations concerning the repeatability estimates.

Ramet-ortet estimates of repeatabilities were somewhat more variable. Some regression estimates marginally exceeded unity, but they involved monoterpenes that occurred at higher levels in the ramets than in the ortets, and the corresponding correlation estimates fell within bounds. By contrast, the correlation estimate for β-pinene, which occurred at higher

TABLE 1–Means and ranges for levels (untransformed percentages of individual monoterpenes) in ortets and their ramets

Monoterpene	Means		Lower/Upper bounds	
	Ortets	Ramets	Ortets	Ramets*
α-pinene	19.3	17.9	6.1 /47.3	6.1 /47.7 (66.1)
camphene	0.21	0.27	0 / 0.58	0 / 0.69
β-pinene	23.7	15.4	9.5 /71.4	4.2 /40.2 (2.4/51.4)
sabinene	4.3	8.5	0 /21.2	0 /26.6
carene	19.7	19.2	0.17/42.1	0.70/41.6
myrcene	3.3	4.4	0† / 7.7	0 /10.5
α-terpinene	0.17	0.18	0 / 1.7	0 / 0.50
limonene	22.1	24.5	0.73/38.7	0.93/44.3 (57.6)
β-phellandrene	1.82	3.8	0 /18.2	0 /23.0 (28.1)
γ-terpinene	0.37	0.34	0 / 0.81	0.08/ 0.59
p-cymene	0.97	1.36	0 / 0.05	0 / 0.36 (0.39)
terpinolene	3.97	5.10	0.95/11.8	0.91/11.6 (0.76/11.6)

* Clonal means. Figures in parentheses relate to wider bounds for all 45 clones and not just the 22 that were cross-referenced with their ortets.

† Peak evident on GLC trace but not resolved from carene by integrator.

TABLE 2–Estimates of clonal repeatability of levels (untransformed percentages) of individual monoterpenes

Monoterpene	Method		
	Ramet-ramet†	Ramet/Ortet	
		Regression ±s.e.	Correlation
α-pinene	0.93	0.58 ± 0.14	0.59***
camphene	0.93	0.83 ± 0.13	0.82***
β-pinene	0.85	0.59 ± 0.06	0.86***
sabinene	0.94	1.11 ± 0.08	0.73***
carene	0.82	0.84 ± 0.09	0.86***
myrcene	0.71	0.66 ± 0.16	0.58***
α-terpinene	0.74	0.12 ± 0.07	0.24 NS
limonene	0.94	1.07 ± 0.11	0.86***
β-phellandrene	0.98	1.06 ± 0.07	0.93***
γ-terpinene	0.64	0.26 ± 0.09	0.42***
p-cymene	0.74	0.47 ± 0.21	0.36*
terpinolene	0.94	0.95 ± 0.12	0.82***

NS $p > 0.05$
* $p < 0.05$
*** $p < 0.001$
† $p < 0.001$ throughout.

levels in the ortets, was higher than the regression estimate. The lower estimates tended to involve minor peaks; such peaks were often not detected in the ortet samples which in some cases were obtained in well below the ideal quantities. The one major component which gave relatively low ramet-ortet repeatability (<0.7) was α-pinene, for which three clones gave anomalously high levels in the ortet samples.

Use of arcsin transformation mitigated the non-normality of frequency distributions and heterogeneity of within-clone variances in some analyses, but in others it obviously inflated the errors arising when minor components were close to the limits of detection. In general the transformation had very little impact on repeatability estimates.

Interdependences among levels of different monoterpenes, other than those arising from the constraint of having to sum to 100%, were generally low. The one striking exception was a very close correlation (R = 0.96) for clonal (ramet) means between sabinene and terpinolene.

The low degree of interdependence among levels of different monoterpenes, plus the repeatabilities of the levels, led to clear differences among almost all clones. Reliable discrimination between clones could be made, based on levels of one or more monoterpenes, in 99% of pairwise comparisons, both for ramet means (no overlap between values for the ramets of different clones) and ortet values (with differences substantially exceeding variation among ramets within clones).

DISCUSSION

Overall, the results confirm the high degree of genetic control of monoterpene composition in *P. radiata*. If the clones correspond to a random population sample and the ramets are properly randomised the clonal repeatability amounts to broad-sense heritability (H^2). The non-randomness in the original choice of clones is unlikely to have been a significant factor. Failure to randomise the ramets would have tended to inflate the ramet-ramet repeatabilities, but not the ramet-ortet ones. Some of the latter repeatabilities were clearly depressed markedly by certain limitations of the GLC analyses; close inspection of GLC traces indicated some failures to detect minor peaks and to resolve sabinene from β-pinene and myrcene from carene, while some slope sensitivity errors may have depressed the observed repeatability of the level of α-pinene which follows soon after the solvent peak. There seems no doubt that the levels of almost all the monoterpenes are inherently very repeatable, but it is doubtful whether the minor peaks could add information that would warrant the extra trouble to determine them very accurately. Even so, it appears that use of monoterpenes as genetic fingerprints may require more precise chemical analysis than would be needed, say, for comparing populations.

Technical refinements of the GLC analysis could clearly eliminate much of the observed non-genetic variation by improving detection of minor components, and obtaining better resolution of peaks with similar elution times. Options include addition of a suitable compound to serve as an internal standard, using a different (less volatile) solvent, using better columns and integrators, or conducting slower analyses. Multivariate data analysis (cf. Blackith & Reyment 1971; Burdon, Zabkiewicz & Andrew 1992) may help cope with such factors as unavoidable slope sensitivity errors. An alternative to such refinements would be to analyse oleoresin samples from all genotypes in question using quick and cheap GLC analysis, and follow up equivocal results with other techniques e.g., isozymes and DNA studies, but high-quality GLC analyses have now become quite cheap (under NZ$100 per sample, except with a small batch of samples, R Franich, pers. comm.).

There is no information on the narrow-sense heritability (h^2) of cortical monoterpene levels in *P. radiata*, although some marked genetic dominance effects observed in some other conifers suggest that it could be appreciably lower than broad-sense heritability.

Any heritability or repeatability is specific not only to the trait concerned but also to the population, the particular environmental conditions, and the measure of the trait that is adopted. The ramet/ortet relationship represents a much more stringent criterion of repeatability than does the ramet-ramet relationship, since it embraces different sites, different types of shoot material, and presumably different "physiological ages" (maturation states). The site differences were admittedly not great, but the types of shoot material differed markedly in vigour which, along with the site differences, may have occasioned appreciable differences in shoot ripeness (lignification), while the physiological ages in the ortets and the ramets are likely to have been 11 and 14 years respectively. It should be noted that the calculated ramet/ortet repeatabilities implied an adjustment for the composite (fixed) main effect of classes of material, which embraced site, shoot type, and physiological age. Comparing monoterpene composition between ramets and ortets outside the common-garden context would thus entail appreciably lower repeatabilities (cf. Burdon, Zabkiewicz & Andrew 1992).

The observed discrimination rates between pairs of clones are potentially optimistic for two reasons. Firstly, they implicitly addressed a comparisonwise Type I error rate (although it is questionable whether it would be appropriate in this application to set a chosen Type I error rate to an "experimentwise" level, since identity is unlikely to come into question for more than the occasional clone). Secondly, the clonal values for the cuttings were means for several ramets, the repeatability of a clonal mean ($H^2_{\bar{c}}$) conforming to

$$H^2_{\bar{c}} = H^2/[H^2 - (1 - H^2)/n]$$

where n = effective number of ramets per clone (4.33 in this study), noting that H^2 was presumably theoretically subject to some upwards bias. Another possible concern is that the statistical properties of monoterpene data (with complex non-normality and undoubted relationships between variances and means) make it difficult to obtain rigorous and generalised statistical comparisons between individual clones, either monoterpene by monoterpene or using multivariate analysis. These considerations, however, seem unlikely to be crucial. The chemical analysis was manifestly capable of refinement, while the statistical niceties are likely to be decisive only in very occasional comparisons, particularly when other factors are potentially more critical. In another conifer, *Picea abies* L.Karsten, a high rate of clone-by-clone discrimination in a multi-component monoterpene mixture has also been observed (Esteban *et al.* 1976).

While the relative differences in average levels of some monoterpenes between the ortets and ramets were quite large (Table 1) they were very minor in relation to non-genetic differences that were observed in other circumstances (Burdon, Zabkiewicz & Andrew 1992).

The extremely close clonal association between levels of sabinene and terpinolene has been foreshadowed and discussed (Burdon, Gaskin, Zabkiewicz & Low 1992), and suggests that these compounds are involved in a common biosynthetic pathway in both *P. radiata* and *P. muricata* D.Don.

ACKNOWLEDGMENTS

Thanks are due to D.Briscoe for assistance with collecting the samples and to Dr K.G.Eldridge, Dr W.J.Libby, and Dr D.L.Rockwood for constructive comments on the draft. We also acknowledge receipt of Grant No.SR:14087 from the New Zealand Lottery Science Board to support publication of this series of papers.

REFERENCES

BANNISTER, M.H.; WILLIAMS, A.L.; McDONALD, I.R.C.; FORDE, M.B. 1962: Variation of turpentine composition in five population samples of *Pinus radiata*. *New Zealand Journal of Science 5*: 486–95.

BARADAT, P.; BERNARD-DAGAN, C.; PAULY, G.; ZIMMERMAN-FILLON, C. 1975: Les terpènes du pin maritime: Aspects biologiques et génétiques. III—Hérédité de la teneur en myrcène. *Annales des Sciences Forestières 32*: 29–54.

BARADAT, P.; BERNARD-DAGAN, C.; FILLON, C.; MARPEAU, A.; PAULY, G. 1972: Les terpènes du pin maritime: Aspects biologiques et génétiques. III—Hérédité de la teneur en monoterpènes. *Annales des Sciences Forestières 29*: 307–34.

BLACKITH, R.E.; REYMENT, R.A. 1971: "Multivariate Morphometrics". Academic Press, London.

BURDON, R.D.; ZABKIEWICZ, J.A. 1973: Identical and non-identical seedling twins in *Pinus radiata*. *Canadian Journal of Botany 51*: 2001–4.

BURDON, R.D.; ZABKIEWICZ, J.A.; ANDREW, I.A. 1992: Genetic survey of *Pinus radiata*. 8: Population differences in monoterpene composition of cortical oleoresin. *New Zealand Journal of Forestry Science 22*: 257–73.

BURDON, R.D.; GASKIN, R.E.; ZABKIEWICZ, J.A.; LOW, C.B. 1992: Genetic survey of *Pinus radiata*. 7: Variation and inheritance of pinene composition in wood oleoresin. *New Zealand Journal of Forestry Science 22*: 246–56.

CHOU, C.K.S.; ZABKIEWICZ, J.A. 1976: Toxicity of monoterpenes from *Pinus radiata* cortical oleoresin to *Diplodia pinea* spores. *European Journal of Forest Pathology 6*: 354–9.

ESTEBAN, I.; BERGMANN, F.; GREGORIUS, H.-R.; HUHTINEN, O. 1976: Composition and genetics of monoterpenes from cortical oleoresin and their significance for clone identification. *Silvae Genetica 25*: 59–66.

FRANKLIN, E.C. 1974: Adjustment of heritabilities estimated by correlation coefficients for typical forest tree breeding experiments. *Silvae Genetica 23*: 166–80.

SMITH, R.H. 1972: Xylem resin in the resistance of Pinaceae to bark beetles. *USDA Forest Service, Pacific Southwest Forest and Range Experiment Station, General Technical Report PSW-1.*

SQUILLACE, A.E. 1976a: Analysis of monoterpenes by gas-liquid chromatography. Pp. 120–57 *in* Miksche, J.P. (Ed.) "Modern Methods in Forest Genetics". Springer-Verlag, New York.

———1976b: Biochemical genetics and selection: Composition of volatile terpenes. Pp. 167–78 *in* Proceedings IUFRO Joint Meeting of Genetic Working Parties on Advanced-Generation Breeding, Bordeaux. INRA Laboratoire d'Amélioration des Conifères, Cestas, France.

SQUILLACE, A.E.; WELLS, O.O.; ROCKWOOD, D.L. 1980: Inheritance of monoterpene composition in cortical oleoresin of loblolly pine. *Silvae Genetica 29*: 141–51.

ZABKIEWICZ, J.A.; ALLAN, P.A. 1975: Monoterpenes of young cortical tissues of *Pinus radiata*. *Phytochemistry 14*: 211–2.