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Composition and Genetics of Monoterpenes from Cortical Oleoresin of Norway Spruce and their Significance for Clone Identification

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Introduction

A considerable portion of the oleoresin found in conifers consists of the monoterpenes, commonly classified as secondary metabolites. Depending on their molecular stability, as well as their inter- and intraspeciifc variability, these compounds are frequently utilized for studies in the fields of taxonomy, population genetics, and genecology. In particular, the characteristics of qualitative and quantitative variation in monoterpene composition among individuals and populations enable an estimation of population heterogeneity, a determination of intraspecific variation patterns, and a specific characterization of individuals and populations, e.g. races, provenances (see for review LEVER and BURLEY 1974). The latter possibility has recently become particularly attractive in forestry research with respect to clonal and provenance identification, since, in many cases, morphological and physiological characters do not possess a sufficient specificity. In addition, the efficiency of such biochemical characters increases, if they can be found to be under strong genetic control, since then phenotypic differences directly indicate genetic variants. The findings, that several monoterpene components in pine species are controlled by relatively simple genetic systems (HANOVER 1966, 1971, SQUILLACE 1971, BARADAT et al. 1972, ROCKWOOD 1972, HILTUNEN et al. 1975), demonstrate the suitability of these metabolites for identification studies and furthermore indicate a possible application as so-called gene markers in population genetic investigations and tree breeding programs.

The monoterpene composition of Norway spruce (Picea abies) has been determined by v. SCHANTZ (v. SCHANTZ 1965, v. SCHANTZ and JUVONEN 1966) by means of GLC analyses of needle oils and wood extracts, and by SCHUCK and SCHÜTT (1975) through the study of volatile compounds from wood samples. In our investigations with Norway spruce reported in the present paper, cortical oleoresin collected from exuded drops was used for GLC analyses of monoterpene composition. A study of the intra- and interclonal variation should examine the possibility of clonal identification, and the data obtained from several full-sib families were scored with respect to inheritance patterns of individual monoterpene components. Furthermore, it was considered whether the monoterpene patterns are appropriate for constructing a mathematical method for clonal identification (differentiation).

Materials and Methods

a) Plant material

The 22 clones of Norway spruce (Picea abies (L.) KARST.) available for this study had three different origins: 9 of them were 10-year-old trees grown from cuttings of the Hessische Forstliche Versuchsanstalt, Hann. Münden; 9 were 6-year-old trees from cuttings of the Institut für Forstgenetik und Forstpflanzenzüchtung, Schmalenbeck; and 4 were 8-year-old clones originated from grafts of the Niedersächsische Forstliche Versuchsanstalt, Escherode.

For studies of the genetics of individual monoterpenes of cortical oleoresin, four full-sib families containing 16—22 progenies each have been analyzed. The following controlled crossings were available:

We 40—7	X We 40—7	selfing
We 47—1	X We 40—7	intraspecific crossing
We 48—5	X We 40—7	intraspecific crossing
P. abies var. acrocona	X We 40—7	intervarietal crossing

Five-year-old progenies are growing in Holzerode on the plantation of the Niedersächsische Forstliche Versuchsanstalt.

b) Collection *and* pretreatment of oleoresin samples

Oleoresin samples were collected for gas-liquid-chromatographic (GLC) analyses of monoterpene composition (MTC) in each plantation during one day in summer. The samples were sucked into micropipettes (5 μl) from cuts made in the cortex 1 cm below apical buds of the lateral branches of 2—6 trees from each clone. The micropipettes filled with fluid oleoresin were then closed into small ampoules for transport to the laboratory, where the resin was diluted in the same ampoules in 0.5 ml n-pentane (spectroscopic grade) and stored at —18° C. The samples treated in this manner could be used directly for GLC-analyses with a capillary system.

c) *GLC-analyses of monoterpenes*

GLC-analyses of monoterpenes were carried out with a Perkin-Elmer gas-chromatograph, model 900, equipped with a flame ionization detector (FID) and a 50 m long capillary column, type 23 G 3, (stationary phase: polypropylene glycol UCON LB-550-x). The velocity of the carrier gas (nitrogen) was 1 ml/minute. The temperature of the injection system was 180° C, of the column 100° C, and of the detector 200° C. 0.2 μl of the pentane-monoterpene dilution described above was injected in each analysis.

d) *Quantitative analyses of individual monoterpenes*

Quantitative analyses of individual monoterpene components were carried out with the help of a digital integrator (Perkin-Elmer, model D 26). According to the counts made by the integrator, relative amounts of individual monoterpenes were calculated using the following formula:

$$I_k = \frac{A_k}{(A_1 + A_2 \ldots + An)} \cdot 100,$$

where I_k is the relative peak area of the k-th monoterpene and A_k is the count for the k-th peak, with $k = 1,2,..n$ signifying the individual monoterpenes of the sample.

e) *Identification of individual monoterpenes*

The identification of individual monoterpenes found in oleoresin of Norway spruce was possible, both by means of comparing the relative retention times with those of pure control compounds*), and by adding the supposed component to the sample and confirming that the peak of the monoterpene in the sample was identical with that of the control compound. To avoid a wrong interpretation, the identification of each monoterpene component of oleoresin was confirmed by using columns with two different stationary phases (polypropylene glycol and apiezon).

Results

1. *Composition of cortical oleoresin of Norway spruce*

Eight principal monoterpenes could be identified in the cortical oleoresin of Norway spruce: α-pinene, camphene, β-pinene, myrcene, Δ-3-carene, limonene, β-phellandrene and terpinolene *(Fig. 1)*. In most of the investigated clones, the proportion of α- + β-pinene reached more than 50%, but clones with high amounts of Δ-3-carene, myrcene, and limonene + β-phellandrene were also found *(Table 1)*. Limonene and β-phellandrene have been combined in all analyses, because the successful separation of these compounds was possible only in a few clones which contained nearly equal amounts of both. An extremely high variation could be observed in Δ-3-carene contents, which could be grouped mainly into low and high values among clones investigated in this study; in fact, we obtained no intermediate values in Δ-3-carene contents in the cortical oleoresin of Norway spruce. Furthermore, the content of terpinolene seemed to be in strong positive correlation to that of Δ-3-carene, because the only clones in which we could obtain measurable amounts of terpinolene were those with a high content of Δ-3-carene. On the other hand, the combination of α- + β-pinene seemed to have a negative correlation to Δ-3-carene, because relative proportions of pinenes were low in clones in which the Δ-3-carene was high. Myrcene and the combination of limonene + β-phellandrene seemed also to correlate with the pinenes (clones 15 and 16 in *Table 1)*, but the nature of this correlation remains unclear in view of the plant material investigated in this study. We could obtain no correlation between Δ-3-

Table 1. — **Ranges of variation of the main monoterpenes found in cortex-oleoresin in a member of clones of Norway spruce.**

No.	Clone	α-pinene	Camphen	β-pinene	Myrcene	Δ-3-carene	Limonene β-phellandrene	Terpinolene
1	Ga 11	23.20-23.58	0.25-0.57	43.59-48.48	8.89-14.18	0- 2.21	16.97-18.07	0
2	Ga 22	26.79-27.83	0.66-0.73	54.07-55.23	2.81- 2.91	0.98- 2.40	12.00-13.49	0
3	Ga 23	7.63- 9.47	+	28.58-30.19	1.75- 2.32	48.97-50.87	4.58- 5.83	4.77-5.05
4	Ga 24	16.90-17.10	+	62.70-66.50	2.60- 3.80	5.80- 5.90	8.20-10.50	0
5	Ga 170	18.80-19.10	+	64.10-65.60	3.30- 4.10	+	11.50-13.50	0
6	Op 10	17.50-18.30	+	54.80-55.10	11.10-12.20	+	14.70-16.60	0
7	Op 11	3.72- 3.81	+	9.85-10.63	2.44- 2.52	61.42-62.57	14.36-15.41	5.83-7.43
8	Str 21	20.08-23.86	+-0.59	60.59-61.30	2.58- 3.46	1.63- 5.10	10.07-10.79	0
9	Wh 58/1	15.73-16.08	0.30-0.50	44.69-50.23	3.36- 4.50	10.43-11.24	19.95-22.99	0
10	Sch 6	18.34-21.44	0.24-0.85	52.53-53.91	9.96-11.40	+	12.56-18.11	0
11	Sch 15	15.97-19.68	+	42.43-50.61	12.16-22.05	7.35- 9.37	9.57-11.99	0
12	Sch 20	22.09-23.76	+	49.32-51.05	13.95-15.84	+	9.53-13.05	0
13	Sch 435	17.75-19.08	+-0.39	48.03-59.52	4.72- 7.29	5.44- 7.29	9.94-14.66	0
14	Sch 727	20.63-23.66	0.24-0.39	47.17-52.56	10.65-10.67	0.27-2.66	12.55-18.42	0
15	Sch 788	10.85-13.16	0.57-0.86	24.70-29.34	44.48-49.91	+	9.62-13.97	0
16	Sch 873	14.52-16.77	+-0.25	31.36-36.47	16.57-24.11	0-0.83	28.94-30.89	0
17	Sch 891	20.04-22.65	+-0.61	41.38-50.25	13.00-20.50	0.36-4.90	12.33-14.98	0
18	Sch 1208	19.67-21.41	0.24-0.69	59.97-64.88	5.03- 8.19	5.03-8.19	8.59- 9.41	0
19	We 40-7	19.58-24.56	+-1.39	50.83-57.27	8.76-12.72	0-1.00	10.99-13.72	0
20	We 47-1	16.23-16.37	+	40.55-41.92	13.66-19.45	0-2.75	21.10-28.64	0
21	We 48-5	17.01-19.73	+-1.31	45.25-51.06	21.12-26.94	+	7.97- 9.49	0
22	P.abies var.acrocona	4.76- 6.59	(+-0.14)	11.90-13.49	14.75-21.91	50.70-57.09	4.30- 7.41	3.26-3.79

+ = trace

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*) We are indebted to Fa. DRAGOCO, Holzminden, for supplying us with individual monoterpenes.

carene and myrcene, or between Δ-3-carene and limonene + βphellandrene.

With the sensitivity used in our analyses, measurable amounts of other monoterpenes ofund in Norway spruce by v. SCHANTZ and JUVONEN (1966) could not be recorded in our samples. For example, camphene could be found as traces or in small measurable amounts in all samples investigated, but its content did not reach more than 1.39%.

2. *Intra- and interclonal variation of monoterpene composition*

Six of the eight monoterpene components found in cortical oleoresin of Norway spruce showed a considerable

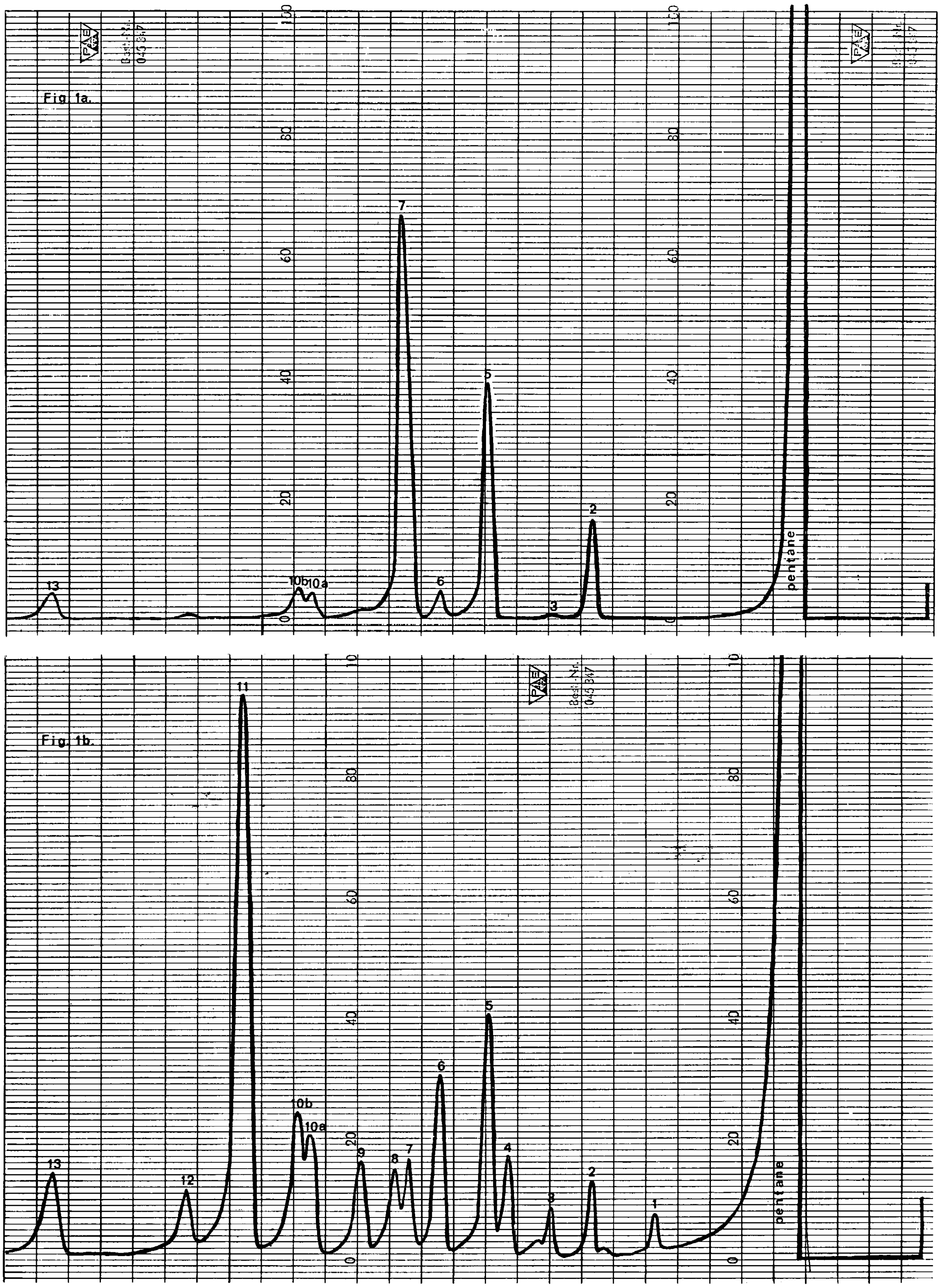

Fig. 1. — Gas-chromatographic separations of monoterpenes: *1a.* Monoterpenes from cortical oleoresin of Norway spruce. *1b.* Pure monoterpenes as control substances (1 = santene; 2 = α-pinene; 3 = camphene; 4 = sabinene; 5 = β-pinene; 6 = myrcene; 7 = Δ-3-carene; 8 = α-Phellandrene; 9 = α-terpinene; 10a = limonene; 10b = β-phellandrene; 11 = p-cymene; 12 = γ-terpinene; 13 = terpinolene).

variation among all trees investigated. As can easily be seen from the data in *Table 1*, the tree-to-tree (interclonal) variation is much greater than the intraclonal variation, which resulted from environmental effects on the MTC. Some monoterpene components vary between clones from 10% or less to 60% or more, indicating a very broad range of amounts. In particular, Δ-3-carene has been found to exhibit extreme differences between the minimal and maximal values (0—60%) of its proportion in the MTC, however, its intraclonal variation did not reach more than 7%. Although the differences can be easily observed for some monoterpene components, it is necessary for general purposes to precisely compare the amounts of intra- and interclonal variation.

Based on the data in *Table 1*, which represent the lowest and highest observed values (in percent) of all monoterpene components found within every clone, a comparison of the magnitudes of the two sources of variation appears to be meaningful under the following considerations. Since in this situation no reasonable assumption can be made about the distribution of the measured values, a method will be outlined that should allow for a suitable definition of interclonal variation as well as for a comparison with intraclonal variation. The basic conception of this treatment assumes that the variation interval (range) of each clone may be represented by a number chosen from the respective interval (*e.g.* arithmetic mean, median), and that the range of such numbers reflects the extent of interclonal variation. In order to reliably state whether the interclonal variation is greater than the intraclonal, it seems logical to compare the length of the largest intraclonal interval (d) with the length of the smallest interclonal variation interval (Δ), which arises from all possible combinations of numbers of the above-mentioned kind. Thus, if in any one case $\Delta > d$, in all other cases the estimated interclonal variation also has to be greater than the intraclonal variation. In the following, this approach is defined for an arbitrary monoterpene component.

Table 2. — Estimates of the interclonal variation (Δ), the intraclonal variation (d), and the relation of both for five monoterpene components.

Monoterpene component	Δ	d	Δ/d
α-pinene	22.98	4.98	4.61
β-pinene	53.47	11.49	4.65
myrcene	41.96	9.89	4.24
Δ-3-carene	60.59	6.39	9.48
limonene/phellandrene	23.11	7.54	3.07

$[\underline{a}_i, \bar{a}_i]$ shall be the variation interval of the i-th clone, whereby $\underline{a}_i$ and $\bar{a}_i$ represent the lowest and highest observed values, respectively. The length of the largest clonal interval (d) is given by

$$d := \max_i (\bar{a}_i - \underline{a}_i).$$

It is clear that the smallest interclonal range has lower and upper bounds $\max_i \underline{a}_i$ and $\min_i \bar{a}_i$, respectively, therefore

$$\Delta = \max_i \underline{a}_i - \min_i \bar{a}_i.$$

The two quantities, Δ and d, were calculated for just five monoterpene components, since terpinolene could only be found in three clones, and camphene appears only in very small amounts (*Table 1*). The resulting data given in *Table 2* show that for all other monoterpenes the estimate of the interclonal variation is far greater than the largest intraclonal interval.

This result, demonstrating a relatively high interclonal variation, provides a good possibility for characterizing and, subsequently, distinguishing different clones and populations by means of measuring MTCs. Additionally, the extremely low and high amounts found in a few cases among clones for one and the same monoterpene component indicate a relatively simple genetic control involving only one or a few genes.

3. *Inheritance pattern of several monoterpene components*

In addition to the great interclonal variation, a few monoterpene components are found to show a so-called bimodal distribution of their proportions among the 22 clones investigated, *i.e.* the proportions can be grouped into non-overlapping classes. In particular, the proportions of Δ-3-carene form two widely separated classes, a low class ranging from 0 to 11% and a high class ranging from 48 to 62% (*Table 1*). This phenomenon suggests Mendelian inheritance patterns, and, therefore, the segregation of these specific proportions of several monoterpene components was studied in four full-sib families. The parents involved in these controlled crossings include the clones We 40—7, We 47—1, We 48—5, and the spruce variety, acrocona (see *Table 1*), which exhibit specific proportions of the monoterpenes α-pinene, β-pinene, the combined components α- and β-pinene, and Δ-3-carene. The ranges of the "low" and "high" proportions found in the parents and progenies of all families are given in the following table.

	α-pinene	β-pinene	α- + β-pinene	Δ-3-carene
low range	3—12	10—28	13—39	0— 8
high range	16—29	41—68	48—93	43—71

The three parents We 40—7, We 47—1, and We 48—5 contained high proportions of the pinenes and low proportions of Δ-3-carene, respectively, whereas the fourth parent (var. *acrocona*) contained low proportions of the pinenes and high proportions of Δ-3-carene (see *Table 1*). The design and results of the family analyses are summarized in *Table 3*, whereby the phenotypes of the trees are only designated "high" or "low" according to the ranges in the table. The parent We 40—7 must be homozygous for genes controlling the proportions of the pinenes and Δ-3-carene, since no segregation could be found in the progenies after selfing. Accordingly, the parents We 47—1 and We 48—5 proved also to be homozygotes, because their crossings with We 40—7 yielded no segregating offspring (*Table 3*). Furthermore, these data allow the statement that these parent trees are homozygous for the same alleles, since all members of the families possess the same phenotypes.

Only the crossing We 40—7 × var. *acrocona* involving parents with opposite phenotypes resulted in progenies segregating for low and high proportions (*Table 3*). The values found in the distribution of α-pinene and Δ-3-carene phenotypes among the progenies suggested a 1 : 1 segregation ratio for low and high proportions. The deviation of the observed distribution from a hypothetical 1 : 1 ratio is statistically not significant ($\chi^2 = 1.5$, $P > 20\%$). Therefore, we may primarily conclude that the low and high proportions of α-pinene and Δ-3-carene are controlled by two alleles of single gene loci. However, the distribution of low and high β-pinene phenotypes deviates too much from a 1 : 1 segregation pattern ($\chi^2 = 4.17$, $P < 5\%$). Since this deviation resulted from a shift within the pinene pro-

Table 3. — Distribution of phenotypes with low or high monoterpene proportions in progenies of four full-sib families.

Crossings	Number of Progenies	α-Pinene		β-Pinene		α + β-Pinene		Δ-3-Carene	
		low	high	low	high	high	low	low	high
We 40—7 selfed	14		14		14		14	14	
We 40—7 × We 47—1	16		16		16		16	16	
We 40—7 × We 48—5	19		19		19		19	19	
We 40—7 × var. acrocona	24	15	9	17	7	15	9	9	15

portions, and since low and high proportions of α-pinene are nearly always correlated with low and high proportions of β-pinene, respectively, it seems to be reasonable to combine the two pinene components for further considerations. The resulting data show that the proportions of the combined pinenes could also be separated into a low and a high group, the phenotypic distribution of which would now be in accordance with an expected 1 : 1 ratio *(Table 3)*.

The data so far obtained by scoring the phenotypic distributions in four families lead to the conclusion that both the low and high proportions of Δ-3-carene and those of the combined pinenes are controlled by two alleles at a single gene locus. Since the parent acrocona must be heterozygous for both α- + β-pinene and Δ-3-carene proportions, the alleles for low pinene and high Δ-3-carene amounts are dominant over the alleles for high pinene and low Δ-3-carene amounts. After checking exactly the distribution of the pinene and Δ-3-carene proportions within each progeny, it was found that low amounts of the pinenes were always correlated with high amounts of Δ-3-carene and vice-versa. Therefore, it was postulated that both "quasi-qualitative" characters — low and high proportion of pinenes and low and high proportion of Δ-3-carene — are controlled by one and the same gene locus having one dominant allele causing low pinene and high Δ-3-carene amounts and one recessive allele causing high pinene and low Δ-3-carene amounts.

4. *Clone identification with the help of MTC*

The basic idea of clone identification can be characterized as follows: Given a fixed number of clones, a decision has to be made as to the affiliation of an individual under consideration to one of these clones, not excluding the possibility that it cannot be assigned to any of them. As a special case, this way of proceeding enables pairwise comparisons within the initially given set of clones, aiming at an investigation of their diversity and/or identifiability.

If we acept the conception that two individuals can be regarded as members of the same clone if and only if they possess identical genotypes (at all loci), unique assignment of individuals to clones in general requires knowledge of the total genotype, which in praxi is not obtainable. On the other hand, there of course exist special situations for which unique assignment can be performed, even if only a small number of gene loci are involved in the investigation of genotypes. In any case, two individuals differing at at least one gene locus can never belong to the same clone, so that a negative statement *(i.e.* an idividual does not belong to a clone) always is exact, a fact which suggests that the term 'clone differentiation' is in some situations more appropriate than 'clone identification'.

Since genotypes cannot be recognized immediately, but rather by their phenotypic expression which might be accessible to modifying environmental influences, it is common to differentiate between characters which do not and those which do depend on environmental conditions. In the first case, each genotype corresponds to a single phenotype; if this correspondence is detectable and one-to-one (*e.g.* one gene locus, no dominance) the character is called a gene marker. If the correspondence is not one-to-one, it is called an incomplete gene marker.

In the second case, where the characters (mostly metric) are dependent on environmental conditions, each genotype corresponds to a variety of phenotypes, whose ranges are theoretically determined by the set of environmental conditions that potentially affect the genotype. Clearly, two such genotypes can be properly distinguished only if their corresponding phenotypic ranges do not overlap. This idea shall serve as the foundation for further considerations, that is, the subject of clone identification (differentiation) shall be treated deterministically and not statistically as done within the theory of discrimination.

4.1 *Gene markers*

With the help of gene markers (complete or incomplete), unique assignment of an individual to a clone can be performed exclusively in those cases where this individual is known to belong to the set of clones and where its phenotype is present only once among the set of clones. This is genuine clone identification. For all other situations the only possible exact statement is, that an individual does not belong to a clone, and, consequently, these situations constitute examples of clone differentiation. The assumption that individuals must be known to belong to the set of clones under consideration in order to enable clone identification is inevitable, since a gene marker covers a more or less small section of the genome only and thus nothing is known about the genotypes at the remaining loci.

In particular, given an arbitrary number of clones, for which no information concerning possible common origins can be obtained, pairwise comparisons can only result in clone differentiation. On the other hand, indistinguishability between clones establishes an equivalence relation on the set of all clones, because two indistinguishable clones show the same phenotype. This in turn allows for a complete decomposition of this set into mutually disjoint subsets called equivalence classes, where the members of such a class are all indistinguishable and members of different classes always are distinguishable. This way of proceeding in a natural manner provides a means of reducing the total number of clones to a number of new units which can be effectively distinguished from each other with reference to the gene marker employed. Furthermore, the use of additional gene markers does not invalidate the already established equivalence class structure, but rather supplements it in the sense of a progressive refinement, which at the limit leads to classes that contain only members of the same clone. Clearly the number of classes associated with the gene markers cannot exceed the number of different phenotypes observable with these markers.

The experimental findings reported in this paper have been obtained under preconditions as are described in the previous paragraph in connection with pairwise comparisons of clones; in particular it was proved that the Δ-3-carene content constitutes an incomplete gene marker for a one-locus-two-allele model with dominance. Applying the above considerations to these results, the investigated 22 clones can be partitioned into two distinct classes, namely clones 3, 7, 22 with a high Δ-3-carene content and the remaining clones with a low Δ-3-carene content.

Consequently, it can be stated that two clones belonging to different classes are distinct in the sense that they do not result from vegetative propagation of the same tree.

4.2 *A method using the total MTC (interval-method)*

It was reasoned before that in some cases additional characters which are subject to the modifying influence of changing environments may be used for the differentiation of genotypes. For these cases, considerations are based on the phenotypic ranges of genotypes, where model assumptions concerning the constitution of the genotypes are not of primary interest as is the case with gene markers. The fact that MTC's investigated in this context show remarkable stability for a great variety of environments, that is, they have narrow phenotypic ranges of genotypes, increases the likelihood of finding clones whose phenotypic ranges do not overlap and thus are distinguishable.

For the following considerations, it is assumed that the experimentally realized ranges differ only negligibly from the theoretical ones. Furthermore, for each monoterpene component a phenotypic range of a clone is conceived as a closed interval on the real line. The left and right endpoints of such an interval corresponding to the k-th monoterpene component of a given clone are the lowest and highest values, respectively, of monoterpene content measured with the members of that clone and denoted by $\underline{a}_k$ and $\bar{a}_k$. Since the contents are given in the form of proportions, $0 \leqslant \underline{a}_k \leqslant \bar{a}_k \leqslant 1$ holds true, and the MTC of a tree can be represented by a stochastic vector, that is, a vector with non-negative components which sum up to 1.

The idea now is to construct a method of clone differentiation which is easy to handle and at the same time is based on experimental data which are quickly surveyable. For this purpose a clone A is assigned an n-dimensional interval (parallelotope) composed of the real closed intervals $[\![\underline{a}_k, \bar{a}_k]\!]$ defined above ($k = 1, \ldots, n$; n = number of monoterpene components); this n-dimensional interval shall be denoted $[\![\underline{a}, \bar{a}]\!]_n$. Note that $[\![\underline{a}, \bar{a}]\!]_n$ in general is not equal to, but rather contains, the phenotypic range of clone A, since not all points in $[\![\underline{a}, \bar{a}]\!]_n$ must be stochastic vectors. More precisely, the n-component stochastic vectors from an (n-1)-dimensional simplex S_{n-1} in the real n-dimensional euclidian space R^n, and the phenotypic range R_A of clone A simply is equal to the intersection of S_{n-1} and $[\![\underline{a}, \bar{a}]\!]_n$, i.e. $R_A = S_{n-1} \cap [\![\underline{a}, \bar{a}]\!]_n$. A geometric representation of these facts for two monoterpene components ($n = 2$) is given in *Figure 2*.

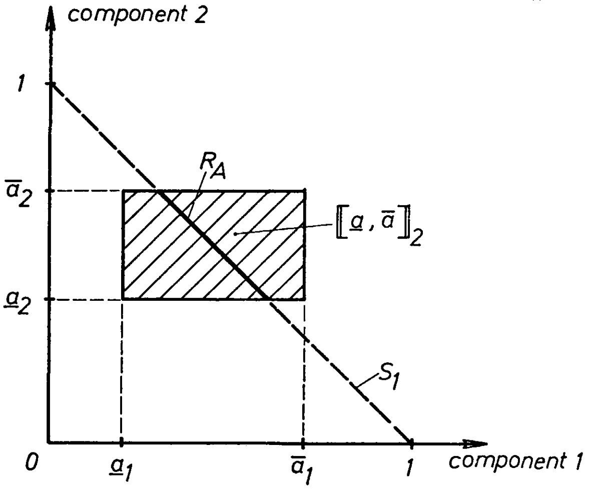

Fig. 2. — The phenotypic range R_A of a clone A with respect to an MTC consisting of two components. R_A is represented as the intersection of the one-dimensional simplex S_1 with the two-dimensional interval (parallelotope) $[\![\underline{a}, \bar{a}]\!]_2$ reflecting the variational ranges $[\![\underline{a}_i, \bar{a}_i]\!]$ $(i = 1{,}2)$ of each of the two components.

According to the previous statements, the genotypes of two clones A and B (and consequently those clones themselves) are distingusihable if the phenotypic ranges R_A and R_B do not overlap, i.e. the intersection $R_A \cap R_B$ is empty, which in particular holds true if for at least one component k the intervals $[\![\underline{a}_k, \bar{a}_k]\!]$ and $[\![\underline{b}_k, \bar{b}_k]\!]$ do not overlap. This condition is sufficient but not necessary for $R_A \cap R_B$ to be empty .In order to arrive at a necesary and sufficient condition, we call the intersection of $[\![\underline{a}, \bar{a}]\!]_n$ and $[\![\underline{b}, \bar{b}]\!]_n$ realizable if it is non-empty and contains at least one stochastic vector; furthermore we introduce the quantities

$\underline{ab}_k := \max\{\underline{a}_k, \underline{b}_k\}$ and $\overline{ab}_k := \min\{\bar{a}_k, \bar{b}_k\}$.

With this notation, $R_A \cap R_B$ is non-empty if and only if $[\![\underline{a}, \bar{a}]\!]_n \cap [\![\underline{b}, \bar{b}]\!]_n$ is realizable.

Now $[\![\underline{a}, \bar{a}]\!]_n \cap [\![\underline{b}, \bar{b}]\!]_n$ is non-empty if and only if

$$\underline{ab}_k \leqslant \overline{ab}_k \text{ for all } k = 1, \ldots, n \qquad \text{(a)}$$

and contains at least one stochastic vector if and only if

$$\sum_{k=1}^{n} \underline{ab}_k \leqslant 1 \text{ and } \sum_{k=1}^{n} \overline{ab}_k \geqslant 1. \qquad \text{(b)}$$

Consequently $R_A \cap R_B$ is empty, or equivalently, clones A and B are distinguishable if and only if at least one of the conditions (a) or (b) is not realized. Again for $n = 2$ the geometric significance of these considerations is represented in *Figure 3*.

Now that we have obtained a proper description of distinguishability, and thus of non-distinguishability of clones for the present situation, we might question whether the same line of reasoning traced out in section 4.1 con-

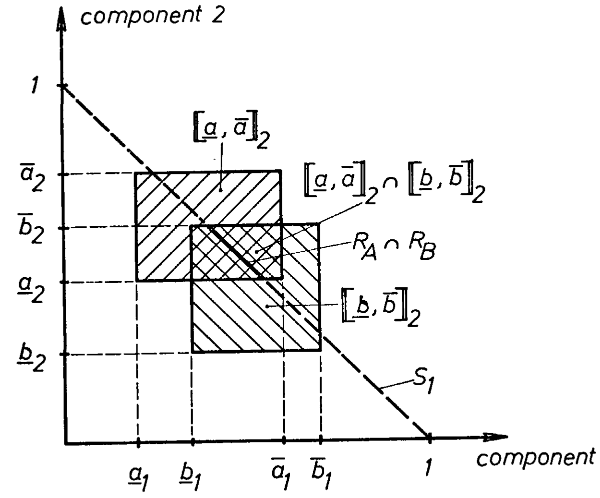

Fig. 3. — The intersection of the phenotypic ranges R_A, R_B of two clones A and B with respect to an MTC consisting of two components (compare with the legend of *Fig. 2*).

cerning the construction of equivalence classes apply to the total MTC. This is not the case, since the intersection of sets is not transitive. On the other hand, taking into account that the formation of equivalence classes for gene markers lead to the same essential result as the application of the present method, namely the specification of the set of clones which is distinguishable from a reference clone, the impossibility of constructing equivalence classes by no means indicates a disadvantage as compared to the method of gene markers.

Applying this method of clone differentiation to the set of data contained in Table 1, it turns out that each single clone is distinguishable from all of the respective remaining clones with the exception of clones 1, 10, 14 and 19, which form the non-distinguishable pairs (10; 19), (19; 14). and (14; 1). Clearly, these findings are compatible with those sated in section 4.1 of this chapter, particularly, since clones 1, 10, 14 and 19 all belong to the same equivalence class made up of trees with low Δ-3-carene content.

Discussion

The data of MTC found in our GLC-analyses of cortical oleoresin of Norway spruce appeared to be highly reproducible in samples stored for 6 months at —18⁰ C. The samples collected as described above were in all cases suitable for GLC-analyses with a capillary system; we could never obtain any contaminations of other substances in our samples (see *Fig. 1)*. Excluding limonene and β-phellandrene, all other monoterpenes found in our samples could easily be separated and identified. It is possible that with higher sensitivity of the gas-chromatograph or by using lower dilution of exuded oleoresin, more monoterpenes could be found, as described by v. Schantz (1965) and v. Schantz and Juvonen (1966) in their investigations of needle oils of several Picea species. However, the proportions of these probably remain low in all cases, as is the case with camphene in our study, so that they are without importance for many purposes, especially since possible environmental influences on these substances can hardly be controlled.

The MTC in cortical oleoresin of Norway spruce revealed some interesting features in our study: the amount of α-pinene in all investigated samples was about two- or three-fold lower than that of β-pinene, whereas v. Schantz and Juvonen (1966) have reported opposite ratios of these compounds in steamdistilled oils in several *Picea* species. However, the origin of the oleoresin in their investigation was more heterogeneous than in our study, in which the oleoresin was exuded directly from resin ducts of the cortex. The relative contents of limonene and camphene were also clearly lower in our study than in that of v. Schantz and Juvonen. On the other hand, we have obtained correlations between different monoterpene components in cortical oleoresin of Norway spruce similar to those observed by Juvonen and Hiltunen (1972 and 1974) in steamdistilled needle oils of *Pinus silvestris*, namely a strong positive correlation between Δ-3-carene and terpinolene and a negative correlation between the carene-group and the pinenes. Juvonen and Hiltunen (1972) likewise reported extremely high variations in Δ-3-carene contents in needle oils of pines grown in different parts of Finland.

Our data show that intraclonal variation representing the environmental modification of MTC's can be regarded as unessential for the main components, which indicates that nearly all phenotypic variation is genetically caused. This finding is in accordance with corresponding results obtained e.g. for slash pine (Squillace 1971) and Scotch pine (Hiltunen *et al.* 1975). Consequently, a strong genetic control of the amounts of individual monoterpene components is generally recognized, however, the identification of single genes causing the detectable content differences has been successful only in a few cases.

In accordance with the data of Squillace (1971), we found bimodal frequency distributions for several monoterpene components consisting of clusters of low and high proportions, respectively. Segregation analyses performed with four full-sib families demonstrated simple inheritance patterns for these low and high amounts occurring in the monoterpenes α-pinene, β-pinene and Δ-3-carene. This corresponds to the results of Squillace (1971), who identified single gene loci which control the content of β-pinene and myrcene. However, the phenomenon of the extreme differences between low and high proportions of Δ-3-carene seems to be widely distributed in conifers, since, in accordance with our results with Norway spruce, a monogenic control has also been found in *Pinus monticola* (Hanover 1966), *Pinus pinaster* (Baradat *et al.* 1972) and *Pinus silvestris* (Hiltunen *et al.* 1975).

Various data from clone investigations as well as from family analyses support our hypothesis that a dominant/recessive pair of alleles at one and the same gene locus causes either a low amount of Δ-3-carene and a high amount of the pinenes or vice-versa. Such a central regulation of the synthesis of these monoterpenes has already been postulated by Juvonen and Hiltunen (1972) for Scotch pine, using only factor and correlation analyses. Both investigations lead to the conclusion that a major gene locus primarily regulates the biosynthesis of two groups of monoterpene components from a common precursor (resulting from geranyl-P-P) in such way that a predominant synthesis of one group (Δ-3-carene, terpinolene) implies a minor synthesis of the other group (α-pinene, β-pinene, limonene, β-phellandrene) and vice-versa. However, the incorporation of myrcene in this biosynthetic system has so far not been possible with our results. Additionally, further factors — genetic as well as pure chemical ones — may modify this system by determining a further content distribution within each group and also probably causing the variations within the proportional ranges of the individual components.

The use of biochemical characters for the identification of Norway spruce clones has previously been proposed by Sauer, Kleinschmit and Lunderstädt (1973), but they could not find significant clonal differences in the respective patterns of these characters. For the comparison of the MTCs we used a simple interval-method, which allowed us to distinguish 18 out of the 22 clones investigated. The four clones which could not be distinguished originate from three different locations, for which the environmental factors are surely different, so that it might be suspected that these clones are genetically similar, particularly since those clones grown in the same place and coming from the same origin could easily be differentiated with our method. It is possible that the initial number of clearly distinguishable clones would decrease if additional clones were investigated. However, the inclusion of other secondary metabolites as characteristics, e.g. di- and sesquiterpenes and phenolics, should again increase the usefulness of the interval-method.

Summary

The monoterpene pattern in cortical oleoresin of Norway spruce *(Picea abies* (L.) Karst.) was determined by means of gas chromatographic analyses. The main components of this material were found to be α-pinene, β-pinene, myrcene, Δ-3-carene, limonene, and β-phellandrene, while camphene and terpinolene occurred only in traces or in relatively low concentrations. The investigation of 22 clones from different locations showed that for all monoterpene components, the intraclonal variation was far lower than the interclonal variation, which emphasizes the suitability of these metabolites for clone characterization.

Since several of the monoterpene components were shown to possess a bimodal variation pattern by means of classifying the 22 clones with respect to high or low content, the inheritance of low or high content for α-pinene, β-pinene, and Δ-3-carene was investigated with the help of full-sib families. The phenotypic distribution observed in the respective progenies allowed for the conclusion that low and high content of α-pinene, a- + β-pinene and Δ-3-carene is controlled by one dominant/recessive pair of alleles of the same gene locus. Based on these results and on further data from clonal analyses, we postulate that the identified gene locus regulates as a major gene the monoterpene biosynthesis from a common precursor alternatively in two directions. In one direction, the components Δ-3-carene and terpinolene are predominantly synthezised, while in the other, this is true for α-pinene, β-pinene, limonene, and β-phellandrene.

In order to meaningfully utilize the monoterpene composition for the purpose of clone characterization, some basic considerations about the possibilities of clone identification on the one hand and clone differentiation on the other hand have been performed. These considerations have been applied to gene markers as well as the total available monoterpene composition. Particularly, by utilizing the monoterpene composition, a method for clone differentiation was developed, which was built up on deterministic concepts and which combines the advantages of relatively high precision with simple computational application.

Key words: Monoterpene Composition, GLC-Analyses, Clone Identification, Gene Marker, Norway Spruce.

Zusammenfassung

Das Monoterpenmuster im Rindenharz der Fichte *(Picea abies)* wurde mit Hilfe von gaschromatographischen Analysen bestimmt. Die Hauptkomponenten in diesem Material waren α-Pinen, β-Pinen, Myrcen, Δ-3-Caren, Limonen und β-Phellandren, während Camphen und Terpinolen nur in Spuren oder sehr geringer Konzentration auftraten. Nach Untersuchung von 22 Klonen von verschiedenen Standorten zeigte es sich, daß bei allen Monoterpen-Komponenten die intraklonale Variation bei weitem geringer ist als die interklonale, was die Eignung dieser Stoffe zur Klon-Charakterisierung unterstreicht.

Nachdem einige Monoterpen-Komponenten ein bimodales Variationsmuster aufwiesen, wobei die untersuchten Klone in Klassen mit niedrigem oder hohem Gehalt eingestuft werden konnten, wurde bei 4 Vollgeschwisterfamilien die Vererbung des jeweils niedrigen und hohen Gehalts der Komponenten α-Pinen, β-Pinen und Δ-3-Caren untersucht. Die dabei beobachtete Verteilung der Phänotypen in den Nachkommen ließ den Schluß zu, daß niedriger und hoher Gehalt bei α-Pinen, a- + β-Pinen und Δ-3-Caren von jeweils einem dominant/rezessiven Allelpaar eines Genlocus kontrolliert werden. Aufgrund dieser Ergebnisse und weiterer Daten aus den Klonanalysen wird postuliert, daß der von uns identifizierte Genlocus als Majorgen die Monoterpen-Biosynthese aus einem gemeinsamen Prekursor alternativ in zwei Richtungen steuert, wobei einmal die Komponenten Δ-3-Caren und Terpinolen und zum anderen α-Pinen, β-Pinen, Limonen und β-Phellandren vornehmlich synthetisiert werden.

Um die Monoterpenzusammensetzung sinnvoll im Rahmen der Klon-Charakterisierung verwenden zu können, wurden einige grundlegende Betrachtungen über Möglichkeiten der Klonidentifizierung einerseits sowie der Klondifferenzierung andererseits angestellt. Diese Betrachtungen bezogen sich sowohl auf Genmarker als auch auf die gesamte verfügbare Monoterpenzusammensetzung. Insbesondere konnte unter Verwendung der Monoterpenzusammensetzung eine Methode der Klonidentifizierung entwickelt werden, die auf deterministischen Grundlagen aufbauend den Vorteil relativ hoher Genauigkeit und einfacher rechentechnischer Handhabung besitzt.

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