Pergamon

S0031-9422(96)00119-7

Phytochemistry, Vol. 42, No. 5, pp. 1289–1297, 1996

0031-9422/96 $15.00 + 0.00

RELATIVE AMOUNTS AND ENANTIOMERIC COMPOSITIONS OF MONOTERPENE HYDROCARBONS IN XYLEM AND NEEDLES OF *PICEA ABIES*

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(*Received in revised form* 2 *January* 1996)

Key Word Index—*Picea abies*; Pinaceae; Norway spruce; plus-trees; needles; xylem; monoterpene hydrocarbons; enantiomers; two-dimensional gas chromatography; multivariate analysis.

Abstract—The relative amounts of 23 monoterpene hydrocarbons, including enantiomers, present in extracts of branch xylem and needles from 41 *Picea abies* plus-trees of widely different origins, were determined. A two-dimensional gas chromatographic system was used for the determination of the enantiomeric compositions of the seven major chiral monoterpenes. Data were evaluated by multivariate data analysis. Large variations were found in the enantiomeric compositions, as well as in the relative amounts of the monoterpenes, both within and among the trees. Trees from the same geographic area did not show a greater similarity in their composition of volatiles than trees from different geographic areas. In the xylem, (−)-β-pinene and (−)-β-phellandrene, and in the needle samples, (−)-α-pinene, (−)-limonene and (−)-camphene, dominated over their (+)-enantiomers. Among-tree variation in enantiomeric composition was higher in xylem samples than in needle samples, except for β-pinene and β-phellandrene.

INTRODUCTION

Knowledge of the variation of the monoterpenes including the enantiomers of the chiral monoterpenes among different trees and tissues could contribute to a better understanding of the complex host–insect relationships in forest ecosystems. Another application is to use the monoterpenes as genetic markers of individual clones, varieties or seed sources, thus enhancing the knowledge of the biodiversity among and within populations. Monoterpenes have been used in genetic studies of forest trees since the 1960s [1, 2]. A major field of research is on the genetic structure of forest-tree populations and the distribution of genetic variation among and within populations.

Earlier studies of Swedish populations of *Picea abies* (L.) Karst. by conventional non-chiral GC techniques revealed large variations in monoterpene compositions in needles both within and among populations [3]. Some monoterpenes (limonene, β-pinene and myrcene) showed a clinical variation with latitude, altitude and length of the growing season, indicating an adaptive value [3]. The relative amounts of the monoterpene hydrocarbons of *P. abies* of German origin were also found to be dependent on the plant genotype [4–6], whereas the variation of the monoterpene hydrocarbons within clones of the same species appeared to be small [7]. In head-space analyses of cortical monoterpenes in *P. abies* obtained from different provenances in Italy, some of the stands being regarded as genetically isolated [8], there were significant differences in the relative amounts of the monoterpenes sabinene, 3-carene, myrcene and *p*-cymene, among trees of different provenances.

The enantiomers of chiral monoterpene hydrocarbons were not separated in the above mentioned studies. However, the use of cyclodextrins as stationary phases for GC columns has facilitated such separations [9–11]. Differences in the ratios of (−)- to (+)-α-pinene in the phloem were found among individual *P. abies* trees [12]. The ratio of (−)-α-pinene to (+)-α-pinene was *ca* 10:1 in the needle oil of all *P. abies* trees from northern Italy [13]. Discriminant analyses of GC data of both chiral and achiral monoterpenes and sesquiterpenes from wood and foliage of 45 *Pinus radiata* trees [14] showed three groups corresponding to the three populations sampled. The enantiomers of α-pinene, camphene, β-pinene and limonene from the needles of the Macedonian *Pinus peuce* Griseb. have been separated by the use of a short non-chiral GC column, directly connected to a chiral main column [15]. The enantiomeric composition (see Experimental) of the monoterpenes in the needle oils of 10 individuals of

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each of *Abies sachalinensis* (Fr. Schm.) and *A. mayriana* Miy. et Kudo have also been determined [16]. The (−)-enantiomers of the monoterpenes appear to dominate over the corresponding (+)-enantiomers in both *P. peuce* and the two *Abies* species.

When using a two-dimensional GC technique with two GCs and chiral GC columns, based on cyclodextrins, it is possible to separate small quantities of monoterpene enantiomers found in the complex extracts of spruce needles and xylems [17]. The enantiomeric compositions of each of six major chiral monoterpene hydrocarbons were shown to be similar in the xylem of the trunk and in that of twig, but very different in the needles of *P. abies* [18]. In commercially available turpentine from thermo-mechanical pulping of *P. abies* [17, 19], the following enantiomeric ratios of the main compounds have been reported: α-pinene 63:37 (−)/(+), β-pinene 98:2 and limonene 83:17.

The present investigation is based on a larger number of trees than used in our previous studies [17, 18], its purpose being to increase our knowledge of the variation in the enantiomeric compositions of monoterpenes among and, especially, within trees. Results from analyses of the monoterpene fractions of branch xylem and needles of 41 selected plus-trees of *P. abies*, originating from different regions in Sweden and the European continent, are reported. The data obtained are presented as the variation among samples (Figs 1–3) and the variation among trees (Tables 1 and 2). The GC data obtained are evaluated by Principal Components Analysis (PCA) and the Projections to Latent Structures–Discriminant Analysis (PLS–DA) plots presented in Figs 4 and 5.

RESULTS

Relative amounts

One of our main interests concerns the inheritance of chiral monoterpene hydrocarbons in Norway spruce. For this reason, only the monoterpene hydrocarbons, which together constituted over 95% of the volatile fraction of the extracts were included in this study. The relative amounts of the monoterpenes in the xylem and in the needles varied greatly both within and among the trees (Table 1, Figs 1 and 2). The main constituents, in decreasing order, in the xylem were (−)-β-pinene, (−)-α-pinene and (+)-α-pinene, and in the needles, (−)-camphene, (−)-limonene, (−)-α-pinene and myrcene. The needle samples contained only minor amounts of (+)-α-pinene. The two xylem samples containing the smallest relative amounts of (−)-α-

Table 1. Relative amounts of 23 constituents of the monoterpene fraction including the enantiomers of the six main chiral monoterpene hydrocarbons in xylem and needles of *Picea abies* trees. Standard deviations are based on mean values from east and west branches of each monoterpene

	Needles				Xylem			
	Mean	SD	Highest	Lowest	Mean	SD	Highest	Lowest
Tricyclene	4.0	0.9	5.5	1.7	0.3	0.3	1.9	0.1
(−)-α-Pinene	18.2	2.8	22.2	11.7	27.5	1.0	53.0	13.6
(+)-α-Pinene	4.0	2.5	10.4	1.7	26.6	11.4	47.0	4.4
Unknown	0.2	0.4	1.7	<0.1	0.1	0.1	0.3	<0.1
(−)-Camphene	27.2	5.8	37.4	11.4	0.9	0.6	2.8	0.2
(+)-Camphene	3.6	0.9	5.3	1.5	0.2	0.1	0.4	0.1
(−)-β-Pinene	3.6	2.7	17.4	0.8	30.2	12.2	54.1	5.6
(+)-β-Pinene	0.3	0.1	0.5	0.2	0.8	0.1	1.3	0.5
(−)-Sabinene	0.1	<0.1	0.2	<0.1	0.2	0.3	1.7	<0.1
(+)-Sabinene	1.8	0.8	4.0	0.4	0.5	0.3	1.8	<0.1
2-Carene	<0.1	<0.1	0.3	<0.1	<0.1	<0.1	<0.1	<0.1
3-Carene	0.5	0.5	2.4	<0.1	2.5	6.0	29.1	0.1
Myrcene	9.4	4.3	21.8	3.2	1.8	1.8	11.5	0.2
α-Terpinene	0.1	<0.1	0.2	<0.1	<0.1	<0.1	0.1	<0.1
(−)-Limonene	21.4	7.3	41.1	10.6	2.1	2.9	12.5	0.3
(+)-Limonene	2.4	0.3	2.9	1.6	0.9	0.4	1.7	0.2
(−)-β-Phellandrene	0.9	0.5	2.5	0.4	4.3	2.4	14.4	0.6
(+)-β-Phellandrene	0.3	0.2	0.8	0.1	0.2	0.1	0.5	<0.1
Cis-β-Ocimene	0.1	<0.1	0.2	<0.1	<0.1	<0.1	<0.1	<0.1
γ-Terpinene	0.2	0.2	1.0	<0.1	0.1	0.1	0.4	<0.1
Trans-β-Ocimene	0.3	0.3	1.2	<0.1	<0.1	<0.1	<0.1	<0.1
p-Cymene	0.1	0.1	0.7	<0.1	0.1	0.1	0.3	<0.1
Terpinolene	0.5	0.1	0.8	0.1	0.4	0.4	2.6	<0.1
Others	<0.8	—	—	—	<0.3	—	—	—

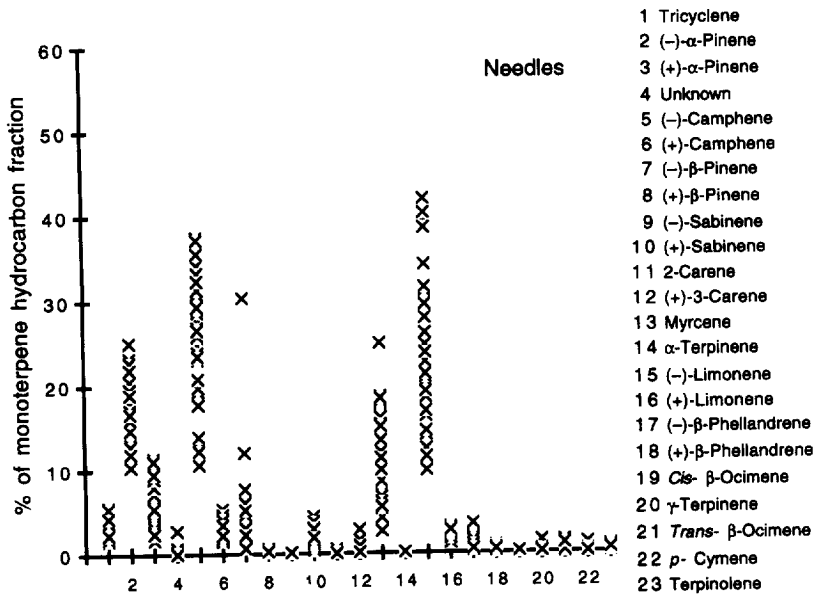

Fig. 1. Relative amounts of 23 monoterpenes (columns 1–23) including the enantiomers of the six main chiral monoterpenes present in one-year-old needles of 41 trees of *Picea abies*. Each sample is represented by one cross.

pinene, *ca* 13% (Fig. 2), had been taken from trees from northern Sweden and Germany, respectively.

Enantiomeric compositions

Large differences in enantiomeric compositions of the chiral monoterpenes were found both between needle and xylem tissues from one tree and among trees (Fig. 3, Table 2). The enantiomeric composition of 3-carene was determined in samples where it occurred in sufficient concentrations; nevertheless, only the (+)-enantiomer was detected. In needles, the mean value of the enantiomeric composition of each compound, except sabinene, exceeded 70%, whereas in xylem samples, the mean values of the enantiomeric compositions exceeded 70% only for β-pinene, β-phellandrene and camphene (Table 2).

In a few trees, the enantiomeric compositions of α-pinene, camphene, β-phellandrene and sabinene were almost the same both in needles and xylem, whereas in other individuals the enantiomeric compositions differed between the tissues by more than 60% for some monoterpenes (Fig. 3). The among-tree differences in enantiomeric composition of camphene, sabinene and limonene were low in needles but high in xylem. The opposite was true for β-phellandrene (Fig.

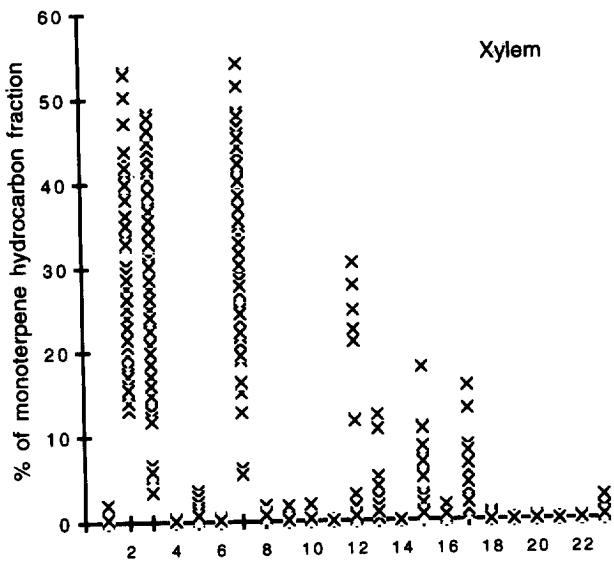

Fig. 2. Relative amounts of 23 monoterpenes, including the enantiomers of the six main chiral monoterpenes present in the branch xylem of 41 trees of *Picea abies*. Each sample is represented by one cross. Columns as Fig. 1.

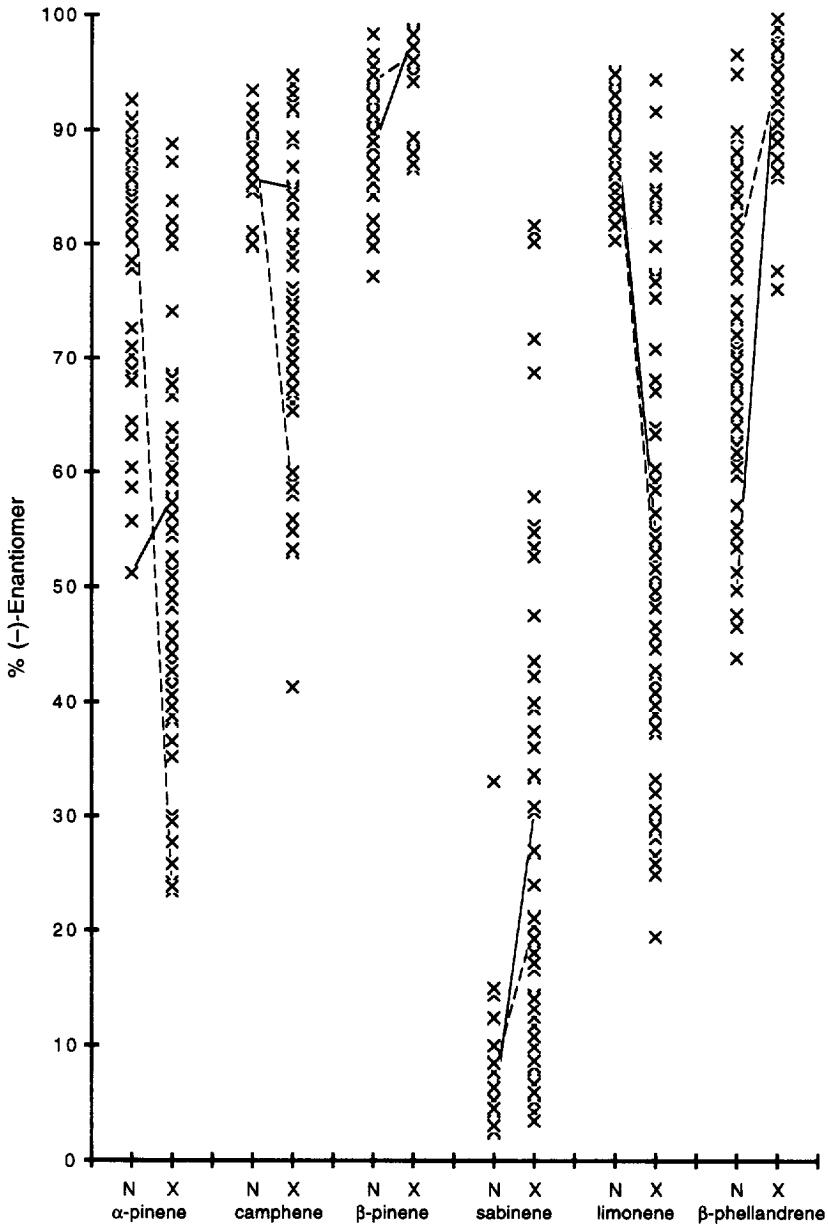

Fig. 3. Enantiomeric compositions of the six main chiral monoterpenes (% (−)-enantiomer of the sum of the (+)- and the (−)-enantiomers) in needle (N) and xylem (X) samples. The enantiomeric compositions of six chiral monoterpenes in the two samples that had the lowest values of (−)-α-pinene in the needle and the xylem, respectively, are marked by a full and a broken line. Each sample is represented by one cross.

Table 2. Enantiometric compositions of each of the six main chiral monoterpene hydrocarbons in xylem and needles of *Picea abies*. Standard deviations are based on mean values from east and west branches of each monoterpene

	% (−)-enantiomer							
	Needles				Xylem			
	Mean	SD	Highest	Lowest	Mean	SD	Highest	Lowest
α-Pinene	82.4	9.0	92.6	53.4	51.7	15.3	87.6	23.6
Camphene	88.0	2.7	93.5	79.4	75.0	11.6	94.0	49.6
β-Pinene	90.2	4.1	96.1	78.5	96.6	2.6	98.7	86.9
Sabinene	5.2	3.8	22.8	2.8	23.7	18.8	81.4	5.0
Limonene	89.1	3.1	95.1	81.7	55.9	20.3	89.7	22.7
β-Phellandrene	70.8	12.8	92.2	45.2	94.0	3.7	99.4	77.0

3, Table 2). Within-tree variation was studied by means of the difference in enantiomeric composition (1) between the samples of the east and west branches of the same tissue and (2) between tissues. The enantiomeric compositions in one tissue could not be predicted from the values found for the other tissue within the same tree (Fig. 3, in which two different lines connected the marks of the two samples containing the lowest values of (−)-α-pinene). In needles, the difference in enantiomeric composition between east and west branches within the same tree were *ca* ±20% units for β-phellandrene, ±10% units for β-pinene and ±5% units for α-pinene, camphene, sabinene and limonene. In xylem samples, the differences between the east and west branches within the same tree was slightly larger for most of the chiral monoterpenes, which indicated that the needle samples were more homogeneous than the xylem samples. The corresponding differences between needles and xylem samples were still larger, as shown in Fig. 5.

Multivariate data analysis

Both the Principal Components (PC)-plot based on data from GC analyses of needle samples (Fig. 4(A)) and the corresponding PC-plot of xylem data (Fig. 4(B)) showed the existence of a few outliers. The deviating needle and xylem samples did not originate from the same trees. The outlier-trees were all found to contain large relative amounts of (+)-3-carene in the xylem. No such group of high-3-carene trees could be traced among the needle samples. No tendency for a grouping of either the needle or the xylem samples of trees of the same origin was found. In some cases, the PC-plots also showed that there were a large difference in the composition of monoterpenes between samples from the east and the west branch of the same tree, i.e. the marks representing the samples were found far from each other in the plot (Fig. 4(A) and (B)).

The values of the relative amounts of the 23 monoterpenes present in the needle and xylem samples from the east and the west branches, respectively, were combined and subjected to PC Analysis (PCA). For both east and west branches, the PC-plots showed that needle and xylem samples formed two well-separated groups. However, one needle sample, the most extreme outlier in the PC-plot for needles (Fig. 4(A)), was more similar to the xylem samples than to the needle samples in its composition of monoterpenes. A Projections to Latent Structures–Discriminant Analysis (PLS-DA) (Fig. 5) including both needle and xylem samples from the east branches, confirmed that there was a difference in the relative amounts of monoterpene hydrocarbons. Two significant components were found. The same outlier among the needle samples was found as in the PC-plot for needles (Fig. 4(A)). The group of needle samples was resolved on the basis of a combination of tricyclene, (−)-camphene, (+)-camphene, (+)-sabinene, myrcene, (−)-limonene, (+)-limonene, *trans*-β-ocimene and *cis*-β-ocimene. The mean values of these constituents were found to be higher in the needles than in the xylem. Similarly, (+)-α-pinene, (−)-β-pinene, (+)-β-pinene and (−)-β-phellandrene were found to be of importance for the group of xylem samples.

Selectively normalized GC-data obtained from trees originating from the southern and the northern parts of Sweden, respectively, resulted in PLS-DA models that explained only a minor part of the variance in the data (branch xylem samples: 1 significant component, variance explained in X: 16%, variance explained in Y: 51%; needle samples: 1 significant component, variance explained in X: 9%, variance explained in Y: 56%).

PC-plots, based on non-chiral GC-data only, gave essentially the same information about outliers as did Fig. 4(A) and (B). A PCA including all of the 45 integrated peaks from the analyses of xylem samples resulted in a model having one significant component explaining 33% of the variance in the data. The information about outliers was found to be essentially the same as the one given by the plot in Fig. 4(B). A corresponding PCA based on GC-data from analyses of needle samples did not result in any significant component.

In a PLS model based only on the enantiomeric compositions, needle and xylem samples were separated from each other, although less distinctly so, compared to the model based on the relative amounts of the 23 monoterpenes (Fig. 5). The percentages of the (−)-enantiomers (the enantiomeric compositions) of sabinene, β-pinene and β-phellandrene were found to be of importance for grouping xylem samples, and the percentages of the (−)-enantiomers of α-pinene, camphene and limonene were important for needle samples.

DISCUSSION

The large among- and within-tree differences both in relative amounts and in enantiomeric compositions of monoterpene hydrocarbons, which were found in the spruce trees of this study, are probably of importance for host–insect interactions. Insects, attracted to or feeding on spruce trees, seem to be able to accept large variations in the composition of the volatiles of the host-tissue. The large among-tree variation observed for both tissues might obstruct mass attacks by forest insects.

The within-tree variation in enantiomeric compositions of monoterpene hydrocarbons might guide the insects to certain areas of the tree for aggregation and breeding. The enantiomeric compositions are most probably of greater importance for those forest insect taxa, which use certain constituents in the host-tree as precursors for their own pheromone production (e.g. *Ips typographus*, see below), than for insects such as *Tomicus piniperda* (L.) [25] and other bark beetles, that are attracted by the host odours. Electrophysiological investigations on *Dendoctonous valens* indicate that two sets of receptors, one responding to both (+)- and (−)-α-pinene and the other responding more strongly to

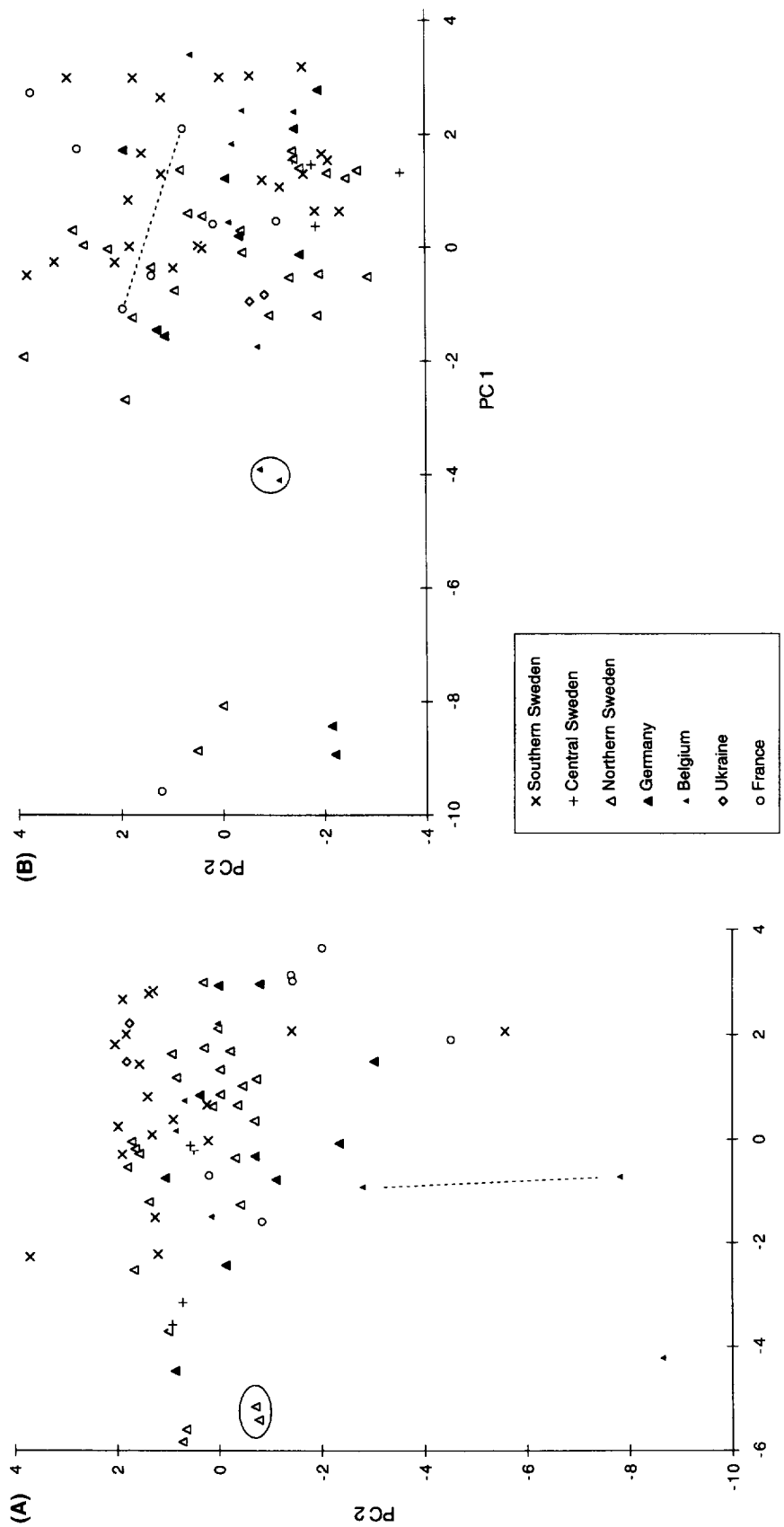

Fig. 4. Score plots from Principal Components Analysis based on selectively normalized data obtained by GC analysis of monoterpene hydrocarbons and monoterpene enantiomers. (A) Needles: the first and only significant component, as judged by cross-validation, explains 18% of the variance in the GC data. (B) Xylem: the first and only significant component, as judged by cross-validation, explains 28% of the variance in the GC data. Samples marked by a ring or a broken line are examples of the variation between east and west branches. The origin of the trees have the latitude (°N); altitude (m), for Sweden; 57°40′–66°04′; 55–550 m, Germany: 48°00′–52°00′; 260–850 m, Belgium: 50°00′; 550 m, Ukraine: 49°00′; 500 m.

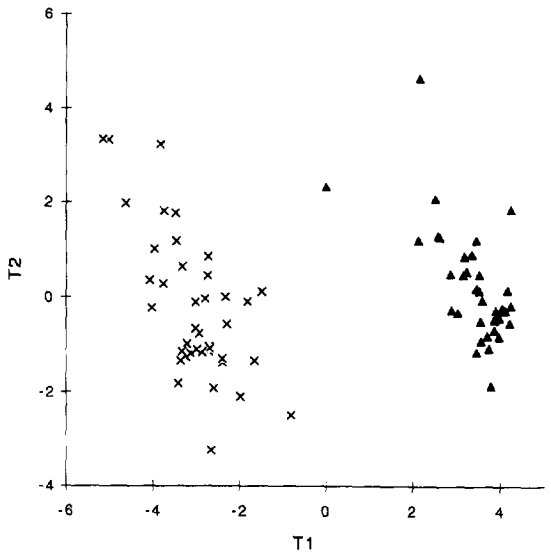

Fig. 5. PLS-DA based on the relative amounts (normalized to the total sum) of monoterpene hydrocarbons including enantiomers in the needle samples and branch xylem samples from east branches. The first and the second PLS-DA components (both significant as judged by cross-validation) explain, in total, 54% of the variance in GC data and 96% of the variance of the dummy variable *Y*. ×, Xylem samples; ▲, needle samples.

the (−)-enantiomer [26], are involved in the perception of volatiles. The behaviour tests of the beetles, however, reveal that (−)-α-pinene is less active than the (+)-enantiomer [27]. These test results may be biased by the difficulty in obtaining pure enantiomers for comparisons. The spruce bark beetle, *Ips typographus* (L.) needs (−)-α-pinene as a precursor for the *in vivo* synthesis of one of its aggregation pheromone components, (*S*)-*cis*-verbenol. The ratio between (−)-α-pinene and (+)-α-pinene in the phloem of *P. abies* is positively correlated to the presence of (*S*)-*cis*-verbenol in the hindgut of the bark beetles [28]. Thus, the variation in enantiomeric composition of α-pinene in the xylem and needles described in our study might influence the degree of aggregation of *I. typographus* on different trees.

Differences in the effects for the enantiomers of the chiral monoterpenes are less known in biological systems. (−)-α-Pinene was found to be more toxic to *Formica rufa* ants, *Dysdercus cingulatus* bugs and *Tenebrio molitor* beetles than (+)-α-pinene [29]. In *Monomorium pharaonis* ants, the LD_{50} of the (+)-enantiomer of α-pinene was significantly higher than that of the (−)-enantiomer [30].

In the present study, the differences in the composition of monoterpenes were greater between needle and xylem tissues than within each tissue (Fig. 5). The among-tree variations in needle samples were of the same magnitude as the within-tree variations, comparing needles from the east and west branches (Fig. 4(A)). The same relation was found for xylem samples (Figs 4 and 5). Despite this wide range in enantiomeric compositions of monoterpenes in the trees analysed (Fig. 3), it was possible to separate xylem and needle samples using only the enantiomeric compositions of six chiral monoterpenes in a PLS-DA. It would be of interest to determine whether the composition of these monoterpene enantiomers also provides sufficient information for distinguishing various *Picea* taxa from one another.

The number of investigated trees of each specific geographical area (Fig. 4) was too small for an evaluation of compositional differences between the origins. The PLS-DA on GC data of the two origins from northern and the two from southern Sweden showed, however, that only a small part of the variance in the data could be explained by compositional differences between these origins. The largest part of the remaining variance was probably due to variation within origin. A similar large variation within population was observed when applying univariate analysis to non-chiral GC data resulting from the analysis of needles from 15 Swedish populations of *P. abies* [3].

Due to the economically important biological connection between the spruce bark beetle (*I. typographus*) and its host, it would be of special interest to study the inheritance (controlled crosses, F1-generation, already exist) of the two enantiomers of α-pinene and include trees with extremely low amounts of the (−)-enantiomer in a tree-breeding programme. However, no trees in this study showed a lower relative amount of (−)-α-pinene than 13% in the branch xylem and an enantiomeric composition less than 23%, which most probably is a sufficient content to be used as a prepheromone by *I. typographus*. For cloning experiments, it would be desirable to have access to trees in which only the (+)-enantiomer of α-pinene is present and which are, thus, resistant to *I. typographus*. Great efforts are needed, if the purpose is to identify trees of natural origin that cannot interact in the pheromone system of *I. typographus*.

The plus-trees of Norway spruce included in our study were of widely different origins. They can be regarded as representatives of a large part of the variation in traits of economical importance present in *P. abies*. The large variations found within and between the two tissues in the trees, indicate the importance of specifying the tissue for genetic studies. Needles provides a homogeneous material, easy to collect and with a defined age.

EXPERIMENTAL

Plant material. The origins of the 41 plus-trees included in this study, all of which are growing in Sweden, are given in Fig. 4. Each tree was represented by one graft. Needle and xylem samples from all but three of the trees were collected in May 1992 in clone archives at Bogesund (latitude 59°25′) 25 km north-east of Stockholm, Sweden, at an outdoor temperature of 5–8°, before shoot elongation started. Samples from the three remaining trees were collected in seed orchards in

northern Sweden, at the same shoot developmental stage. Two branches from each graft, east- and west-oriented, were cut close to the main trunk at a height of 2 m and transported as such to the laboratory.

Sample preparation. Branches were kept outdoors at 5–8° for a maximum of 5 days before sample preparation. A few branches were stored at room temp. for 1 week, but this treatment did not give rise to outliers (i.e. samples having a composition of monoterpene hydrocarbons markedly deviating from those of other samples) in the PC-plots (see definition below). Twenty needles from the shoots of the previous year were taken from each branch and cut into 1-mm pieces. Xylem was taken from branches (one cylinder 1 mm × 20 mm) and cut into 1–2-mm pieces. All samples were extracted in 0.5 ml of hexane (Merck) for 24 hr and the solns filtered through 150–200 mg of silica gel, Matrex silica 90–130 mm, pore size 30 Å (AMICON™). The extracts were stored at −25° before GC analyses.

Analysis. Relative amounts of the constituents and enantiomeric compositions of the seven major chiral monoterpene hydrocarbons were determined by the use of a two-dimensional GC system [17]. In the first GC, a DB-WAX column (J&W Scientific; 30 m, 0.25 mm i.d., 0.25 μm film thickness) was used for the non-chiral separations of the monoterpene hydrocarbons with the following temp. progr.: 40° (2 min) followed by 50° min^{-1} up to 60° (1 min) followed by 4° min^{-1} up to 95°, thereafter a rapid increase to 180°. Terpinolene was eluted after 13 min. Small amounts of α-phellandrene, possibly present in the samples, were not separated from myrcene under the conditions used. In the second GC, a Cyclodex-B, permethyl-β-cyclodextrin/DB1701, fused silica capillary column (J&W Scientific; 30 m, 0.25 mm i.d., 0.25 μm film thickness) was used for the analysis of the chiral monoterpene hydrocarbons, except 3-carene. The temp. progr. was 55° (11 min) followed by 1° min^{-1} up to 75°. In samples containing sufficient amounts of 3-carene, its enantiomeric composition was determined using the second GC, but by means of a γ-cyclodextrin fused silica column (Lipodex E, dipentylbutyryl-γ-cyclodextrin, Macherey-Nagel; 25 m, 0.25 mm i.d.) at 30° isothermally. The inj. temp. was 180°. The detector temp. was 180° in the first GC and 150° in the second GC. For chiral analyses of 3-carene, the detector temp. was 120° in the second GC.

The constituents of the monoterpene frs were identified on a Finnigan 4500 MS instrument connected with a Varian 3400 GC using a DB-WAX fused silica capillary column (J&W Scientific; 30 m, 0.25 mm i.d., 0.25 μm film thickness) and a Finnigan SSQ 7000 instrument connected with a Varian 3400 GC using a DB-5 fused silica capillary column (J&W Scientific; 30 m, 0.25 mm i.d., 0.25 μm film thickness). The temp. prog. for both GCs was 40° for 4 min, followed by 4° min^{-1} up to 200°. Monoterpene enantiomers were identified by means of GC R_is, using naturally occurring or synthetic ref. compounds. Pure (+)-β-phellandrene was prepd as described earlier [20] and (−)-sabinene was collected from the flower fragrance of *Laserpitium latifolium* using an entrainment technique [21]. Data are presented in terms of *relative amounts* of the 23 monoterpene hydrocarbons identified, among which the enantiomers of the seven chiral monoterpene hydrocarbons are included as separate compounds (Table 1, Figs 1 and 2), as well as in terms of *enantiomeric compositions* (area of (−)-enantiomer peak divided by sum of areas of (−)- and (+)-enantiomer peaks, in %; Table 2, Figs 3 and 4) of the six major chiral monoterpene hydrocarbons.

Multivariate data analysis. GC data were evaluated by multivariate data analysis using the programme CODEX^R vers. 2.5 (Chemometric Optimization and Design for Experimenters, a product from SumIT System AB, Box 1936, S-171 19 Solna, Sweden) which was installed as an add-in module to Microsoft Excel^R. Data were subjected to both PCA and PLS-DA [22]. Raw data were normalized and logarithmic transformations made. Thus obtained, the data were then treated in accordance with the procedure used in an earlier investigation on *P. sylvestris* [23]. The PCA and PLS-DA score plots visualize the data structures. The closer two trees are found in a plot, the more similar are their monoterpene compositions. The corresponding loading plots (not shown here) give information about the importance of each constituent for making up the model. Two types of normalization of raw data were used for the PCAs performed on GC data obtained from needle samples or branch tissue samples separately. For the first type, the total integrated area was used as a normalizing factor, resulting in the relative amounts presented in Figs 1 and 2. For the second, selective type of normalization, used for the PCA presented in Fig. 4, the normalization factors consisted of the total integrated area minus the sum of the dominating peak-areas (in Fig. 4(A): (−)-α-pinene, (−)-camphene and (−)-limonene; in Fig. 4(B): (−)-α-pinene, (+)-α-pinene and (−)-β-pinene). In the latter way, one effect of closure, a negative correlation between large peaks, was reduced [24]. Those correlations between the variables (constituents) which were introduced by using the total sum as the normalization factor affected the PC models. For this reason, plots based on data normalized to the total sum were not used for further evaluation of the data when performing PCA or PLS-DA on branch xylem or needle data separately. Each variable was scaled to unit variance (autoscaling). The number of significant components was determined by cross-validation [22]. A component was judged to be significant, when CSV/SD was <0.95. In PLS-DA, a dummy Y variable, containing the numbers 1 and 0, respectively, for the two classes of samples, was used [22].

Acknowledgements—We gratefully acknowledge the helpful assistance of Mr Leiner Engsby in collecting the biological material. For valuable comments on an earlier version of this manuscript we thank Dr Gunhild Aulin-Erdtman. This work has been supported finan-

cially by The Carl Trygger Foundation, The Swedish Council for Forestry and Agricultural Research, and The Swedish Natural Science Research Council.

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