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Journal of Chromatography A, 1034 (2004) 183–189
JOURNAL OF CHROMATOGRAPHY A
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Characterisation of different clones of *Picea abies* (L.) Karst using head-space sampling of cortical tissues combined with enantioselective capillary gas chromatography for the separation of chiral and non-chiral monoterpenes

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Received 4 September 2003; received in revised form 2 February 2004; accepted 2 February 2004

Abstract

Head-space sampling (HS) has been combined with enantioselective gas chromatography (GC) for the analysis of chiral and non-chiral monoterpenes present in the cortical tissues of five different Norway spruce clones. (1*S*)-(−)-α-Pinene, (1*S*,5*S*)-(−)sabinene, (1*S*)-(−)-β-pinene, and (4*S*)-(−)limonene dominated over (1*R*)-(+)-α-pinene, (1*R*,5*R*)-(+)-sabinene, (1*R*)-(+)-β-pinene, and (4*R*)-(+)-limonene. Results showed a large variation in the enantiomeric composition of cortical tissues between different clones. The development of HS–GC greatly increased the speed of precise analyses of chiral monoterpenes in small samples and therefore offer excellent opportunities in studies on the ecophysiological and chemotaxomic roles of these chiral components.

Keywords: Enantiomer separation; Head-space analysis; *Picea abies* (L.) Karst; Cortical tissue; Monoterpenes; Pinene; Sabinene; Limonene

1. Introduction

In the past 10 years enormous strides have been made in understanding the functional role of plant monoterpenoids.

Although many monoterpenoids serve primarily as defences against insects and diseases, these compounds are implicated in multiple ecological roles at different levels of ecosystems [1].

Conifers are prolific producers of resin defense, which is a mixture of monoterpenoids, sesquiterpenoids and diterpenoids. These terpenoid mixture can be constitutive and synthesised de novo in response to attack by herbivores and microbes [2].

Previous studies showed relationships between variation in constitutive monoterpenes and resistance to diseases. For example, slash pine (*Pinus elliottii* Engelm. var. elliottii), loblolly pine (*Pinus taeda* L.) clones [3,4] and jack pine families (*Pinus banksiana* Lamb.) (Michelozzi et al. unpublished data) characterised by certain constitutive monoterpene profiles, tended to be less susceptible to fusiform rust caused by *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*. Other studies have shown that in general, the largest proportionate accumulation of the most toxic monoterpenes can occur in response to attacks by insects and fungi [5]. For example, an increase of δ-3-carene was reported in tissues of lodgepole pine trees infected by *Ceratocystis clavigera* [6].

In addition to their defensive role, monoterpenes are strongly inherited; therefore, analyses of monoterpene profiles offer excellent opportunities as biochemical markers in forest genetics to select chemotypes that are less susceptible to diseases and the attack by insects and animals [7–10].

The development of gas chromatographs equipped with electroantennographic detection (EAD) has clearly indicated that some insects can also distinguish the enantiomeric forms of mono- and sesquiterpenes [11]. For instance, both sexes of

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doi:10.1016/j.chroma.2004.02.001

Scolytus multistriatus positively respond to (−)-α-cubebene (3a*S*,3b*R*,4*S*,7*R*,7a*R*)-1H - cyclopental[1,3]cyclopropal[1,2] benzene,3a,3b,4,5,6,7-hexahydro-3,7-dimethyl-4-(1-methyl-ethyl) produced by *Ulmus* spp. ([12] and references therein). The capability to distinguish enantiomeric pairs is often associated with the fact that some insects use one chiral form of terpenoids present in the bark and phloem of the host tree to produce attracting or inhibitory pheromones. A good example of this type of behaviour is represented by the engraver *Ips typographus* (L.) [13], one of the most active pest in Europe. It has been shown that the "pioneer" male of this bark beetle uses (1*S*)-(−)-α-pinene present in *Picea abies* (L.) Karst (Norway spruce) to produce the pheromone (*R*)-*cis*-(−)-verbenol. This compound, together with "de novo" synthesised 2-methyl-3-buten-2ol, attracts other individuals of the same species to the tree for the purpose of mating and feeding [14]. The ability of the beetle to overcome the subcortical defenses is facilitated by mutualistic associations with phytopathogenic fungi that help kill the tree [15]. On the other hand, the survival of the tree subjected to the attack of bark beetle–fungal complexes is strictly related to variations in composition of constitutive and induced monoterpenes and their enantiomeric distribution.

Investigations on the ecophysiological role of enantiomeric plant terpenoids have been undertaken only recently thanks to the commercially availability of gas chromatography (GC) columns internally coated with cyclodextrin derivatives. Complete separation of some chiral monoterpene pairs in extracts of tree samples with solvent was reported using a two-dimensional approach by combination of conventional capillary columns with chiral columns [16]. Although very accurate, this method is more time consuming and expensive than the head-space sampling (HS)–GC method developed in this work.

It has been recently shown [17] that positive identification and accurate quantification of both enantiomeric and non-enantiomeric terpenoids released from plant leaves can be successfully achieved using a single column internally coated with β-cyclodextrin. The sampling method used in this study was, however, too complex and time consuming for detecting terpenoids sequestered in plant tissues as it involves the use of adsorption traps whose content must be analysed by thermal desorption. Head space was shown to be suitable for routine sampling monoterpenes in tree tissues as the sample preparation is simple and rapid. This technique was successfully used to derive monoterpene profiles of five Italian Norway spruce provenances using columns unable to separate enantiomeric pairs [18].

In this study, a gas chromatographic method for the detection of chiral monoterpenes in tree tissues sampled with the head-space technique has been developed and tested. The method has been used to differentiate five different Norway spruce clones on the basis of the chiral monoterpene content present in bark tissues. It was developed in the frame of a study aimed at finding the possible relationships existing between monoterpene profiles of *P. abies* (L.) Karst and the attack of *Heterobasidion annosum* (Fr.) Bref.

2. Experimental

2.1. Sampling and storage of cortical tissues of P. abies

Samples were collected from five different clones of *P. abies* growing at the University of Firenze's Agricultural and Forest Research Centre. 1-year-old cortical tissue samples were taken from 4 to 5 trees per each clone. Cortical samples were collected from the branch tips at the same elevational and horizontal position in the crown of each tree following the sampling procedure indicated by Squillace [19]. A 0.2 g of each cortical sample were ground in liquid nitrogen and placed in a glass vial (20 ml). Vials were tightly closed with Teflon septa and sealed with aluminum caps. Samples were stored at −20 °C until they were used for the analysis by headspace gas chromatography. Before the injection of gas samples into the column, the temperature of vials was risen and enough time was allowed to the sample to reach an equilibrium between the phases. The criteria followed in the selection of the most appropriate equilibration temperatures and times are discussed in Section 3. For peak identification by GC–MS, liquid extracts of cortical tissues were used. They were obtained by extracting 0.1 g shavings of spruce cortex in 3 ml of *n*-pentane. Prior to the injection, solid particles were separated from the liquid by centrifugation. With GC–MS, head-space samples of cortical tissues obtained by solid-phase microextraction (SPME) were also analysed. 0.1 g of cortical tissues were introduced in a sealed vial and emitted vapours adsorbed on fused silica fibres coated with 100 μm polydimethylsiloxane. Fibres used were supplied by Supelco (Bellefonte, PA, USA). They were exposed at 25 °C for 1 min to monoterpenes vapours.

2.2. GC–FID and GC–MS determinations

GC–FID analyses were performed using a Perkin-Elmer (Norwalk, USA) gas chromatograph (AutoSystem XL) equipped with a Perkin-Elmer TurboMatrix 40 automatic sampler for head-space analysis. Data collected were acquired and stored on the TotalChrom™ chromatography software. The separation of enantiomeric monoterpenes was performed on a Cyclodex-B capillary column 30 m-long and 0.25 mm-diameter supplied by J & W Scientific (CA, USA). Hydrogen was used as carrier gas to shorten the analysis time and reduce the maximum elution temperature. To combine short time with the best resolution, several tests were made by changing the initial temperature of the column and the temperature program because these two parameters are quite critical in determining the performances of β-cyclodextrin columns [17].

After these tests it was found that the best resolution for monoterpenes was achieved when the sample was injected

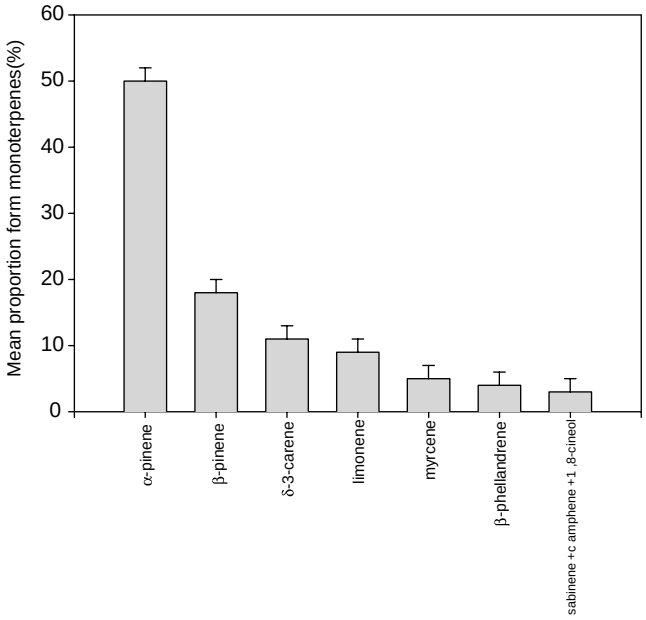

Fig. 1. Average percent composition of monoterpene compounds present in liquid extracts of cortical tissues from five different Norway spruce clones.

at 40 °C and the sequential elution obtained by applying a temperature gradient of 1.5 °C/min until a final temperature of 160 °C was reached. To better separate very volatile compounds (such as isoprene or acetone) from heavier isoprenoids, the initial temperature was maintained for 5 min. The pressure of the hydrogen as carrier gas was 0.68 atm. In these conditions all compounds present in the sample were eluted in less than 40 min.

First indications on the elution sequence of compounds present in cortical samples of Norway spruce were obtained by comparing the retention times of recorded peaks with those obtained by injecting pure enantiomeric monoterpenes supplied by Fluka (Buchs, Switzerland). The elution sequence was unambiguously confirmed by submitting to GC–MS analysis some of the tree samples analysed by GC–FID. In this case, analyses were performed on an Agilent Technology GC 6890 gas chromatograph (Cernusco sul Naviglio, Italy) coupled to a 5973 mass spectrometer from the same company. The MS was operated in electron impact mode (70 eV) and peaks were acquired by scanning positive ions in the mass range from 35 to 350 *m/z*. The final temperature of the column was increased to 200 °C because helium was used as carrier gas. Since the GC–MS system was not equipped with a head-space sampler, positive identification of monoterpenes was performed using extracts and head-space samples obtained by SPME. All these samples were analysed in the splitting mode using small aliquots (0.5 l) of liquid extracts. A 20:1 splitting ratio was used.

2.3. Statistical analyses

The amount of each monoterpene (in sufficient quantities to be considered in analysis of variance, ANOVA) was expressed as a percentage of total monoterpenes. Percentages of various components were transformed to arcsin-square root functions on the mean basis to fulfil the normality assumption. The transformed means were used for ANOVA and canonical discriminant analysis using the Systat statistical program (Systat Software Inc., Richmond, USA).

3. Results and discussion

3.1. Optimisation of the sampling and separation methods

Preliminary information on the percent composition of monoterpenes in the oleoresin of cortical tissues of Norway

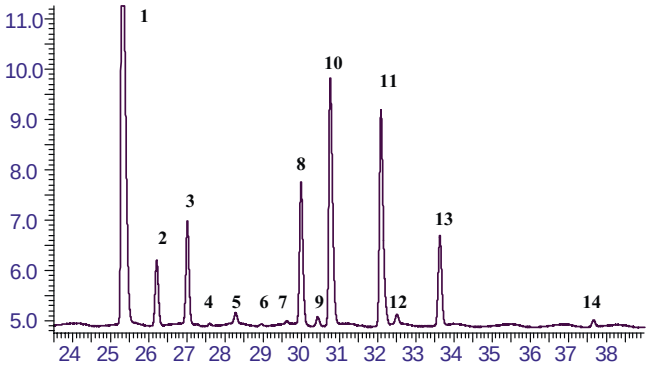

Fig. 2. Typical chromatogram showing the complete separation of chiral and non-chiral monoterpene compounds present in liquid extracts of cortical tissues collected from Norway spruce trees. **1** (1*S*)-(−)-α-pinene; **2** (1*R*)-(+)-α-pinene; **3** myrcene; **4** (1*R*,5*R*)-(+)-sabinene; **5** (1*R*)-(+)-camphene; **6** (1*S*,5*S*)-(−)-sabinene; **7** (1*S*)-(−)-camphene; **8** (1*S*)-(+)-δ-3-carene; **9** (1*R*)-(+)-β-pinene; **10** (1*S*)-(−)-β-pinene; **11** (4*S*)-(−)-limonene; **12** (4*R*)-(+)-limonene; **13** (4*R*)-(−)-β-phellandrene; and **14** 1,8-cineol.

Table 1
Mean monoterpene content (%) in cortical samples of Norway spruce detected at different equilibrium times

Equilibrium times (min)	(1*S*)-(−)-α-Pinene	(1*R*)-(+)-α-Pinene	Myrcene	(1*R*)-(+)-Camphene	(1*S*)-(+)-δ-3-Carene	(1*R*)-(+)-β-Pinene	(1*S*)-(−)-β-Pinene	(4*S*)-(−)-Limonene	(4*R*)-(+)-Limonene	(4*R*)-(−)-β-Phellandrene
10	20.8	2.8	1.6	0.6	1.1	1.2	57.1	1.9	0.4	4.8
20	21.1	2.9	1.6	0.6	1.1	1.2	57.7	2.0	0.4	4.9
30	21.1	2.8	1.6	0.6	1.0	1.2	57.6	2.2	0.5	5.0
40	21.0	2.8	1.5	0.6	1.0	1.5	57.6	2.1	0.4	4.9
50	21.2	2.9	1.5	0.6	1.0	1.4	57.2	2.1	0.4	4.9

Table 2
Mean proportion (%) for monoterpene content in five Norway spruce clones

	(1*S*)-(−)-α-Pinene	(1*R*)-(+)-α-Pinene	Myrcene	(1*R*,5*R*)-(+)-Sabinene	(1*R*)-(+)-Camphene	(1*S*,5*S*)-(−)-Sabinene	(1*S*)-(−)-Camphene	(1*S*)-δ-3-Carene	(1*R*)-(+)-β-Pinene	(1*S*)-(−)-β-Pinene	(4*S*)-(−)-Limonene	(4*r*)-(+)-Limonene	(4*R*)-(−)-β-Phellandrene	1,8-Cineol
Clone A	41.7	2.1	16.0	0.1	0.5	0.1	0.1	2.4	0.6	16.3	5.6	0.3	13.1	0.1
Clone B	29.7	7.3	8.1	0.1	0.7	0.1	0.2	0.7	0.7	34.2	9.2	0.6	6.8	0.6
Clone C	71.6	8.6	0.5	0.1	1.1	0.2	0.2	0.4	0.7	6.0	8.8	0.6	0.4	0.3
Clone D	16.8	0.5	0.8	0.1	0.8	0.0	0.1	52.8	0.4	20.6	4.1	0.3	1.7	0.2
Clone E	54.8	7.0	1.3	0.1	0.9	0.1	0.2	1.1	0.6	7.8	22.4	0.7	1.9	0.2

Table 3
Analysis of variance (ANOVA) for enantiomeric monoterpenes in cortical tissue of five clones of *P. abies*

Source of Variance	df	(1*S*)-(−)-α-Pinene	(1*R*)-(+)-α-Pinene	Myrcene	(1*R*,5*R*)-(+)-Sabinene	(1*R*)-(+)-Camphene	(1*S*,5*S*)-(−)-Sabinene	(1*S*)-(−)-Camphene	(1*S*)-(+)-δ-3-Carene	(1*R*)-(+)-β-Pinene	(1*S*)-(−)-β-Pinene	(4*S*)-(−)-Limonene	(4*R*)-(+)-Limonene	(4*R*)-(−)-β-Phellandrene	1,8-Cineol
		Mean square and significance													
Clone	4	752.1***	129.2***	217.1***	0.0	1.9***	1.5***	1.1	1390.6***	1.0	354.3***	125.5***	2.1***	164.7***	2.9
Error	15	8.5	3.1	8.4	0.4	0.4	0.2	1.3	3.8	0.4	5.8	5.0	0.3	6.7	1.2

*** Significant at $P \leq 0.001$.

spruce was needed to optimise the sampling and separation method of enantiomeric pairs. It was obtained by analysing liquid extracts of a mean sample of cortical tissues from all the different clones on a DB-5 column connected of a mass spectrometer. The average monoterpene composition of the five clones is reported in Fig. 1.

It shows that the number of monoterpenes present in the specimens investigated was quite limited and, in all cases, was dominated by α- and β-pinene. These two terpenoids accounted, by themselves, for ca. 67% of the total monoterpene content. About 20% of the total mixture was composed by δ-3-carene, limonene, myrcene and β-phellandrene. The remaining 3%, was composed by sabinene, camphene and 1,8-cineol present in variable amounts.

Among the nine monoterpenes identified, only two of them (namely myrcene and 1,8-cineol) did not have an asymmetric carbon in the molecule. An accurate quantification of 16 components was, thus, necessary to characterise the Norway spruce clones based on chiral and non-chiral components. By considering the large differences in concentration existing between the monoterpenes present in cortical tissues, a complete separation of all compounds was required to accurately evaluate the relative contents of components present at trace levels. Among them, the most critical to separate was (1*R*)-(−)-δ-3-carene because it is eluted between (+)- and (−)-β-pinene on β-cyclodextrin columns. To optimise the gas chromatographic conditions of the chiral column, a test mixture obtained by combining equal aliquots of all pentane extracts was used. This preliminary investigation suggested that our task was greatly facilitated by the lack of (1*R*)-(−)-δ-3-carene and (4*S*)-(+)-β-phellandrene in cortical tissues.

Fig. 2 shows the GC–FID profile of chiral and non-chiral monoterpene components that was obtained under optimum conditions. The maximum resolution was obtained by injecting the samples at 40 °C and by applying a moderate temperature gradient to the column (1.5 °C). The use of hydrogen as carrier gas allowed to reduce the final temperature of the column from 200 to 160 °C. The chemical nature of individual compounds was confirmed by GC–MS and by the injection of pure compounds. The observations made in cortical tissues confirmed the general predominance of the (−)-enantiomers in Norway spruce and in other conifers species of genus *Pinus* and *Abies* [20–22]. Once fixed the chromatographic conditions, experiments were performed to achieve a reliable and representative sampling of cortical tissues by headspace. Since the balanced-pressure technique is exploited by the TurboMatrix 40 apparatus used to collect and transfer the sample into the column, the pressurisation level of cortical samples was defined on the basis of the pressure of hydrogen necessary to achieve the best resolution with the β-cyclodextrin column used (0.68 atm.). The pressurisation time was set to 5 min based on the recommendation of the manufacturer who suggests values ranging from 3 to 5 min

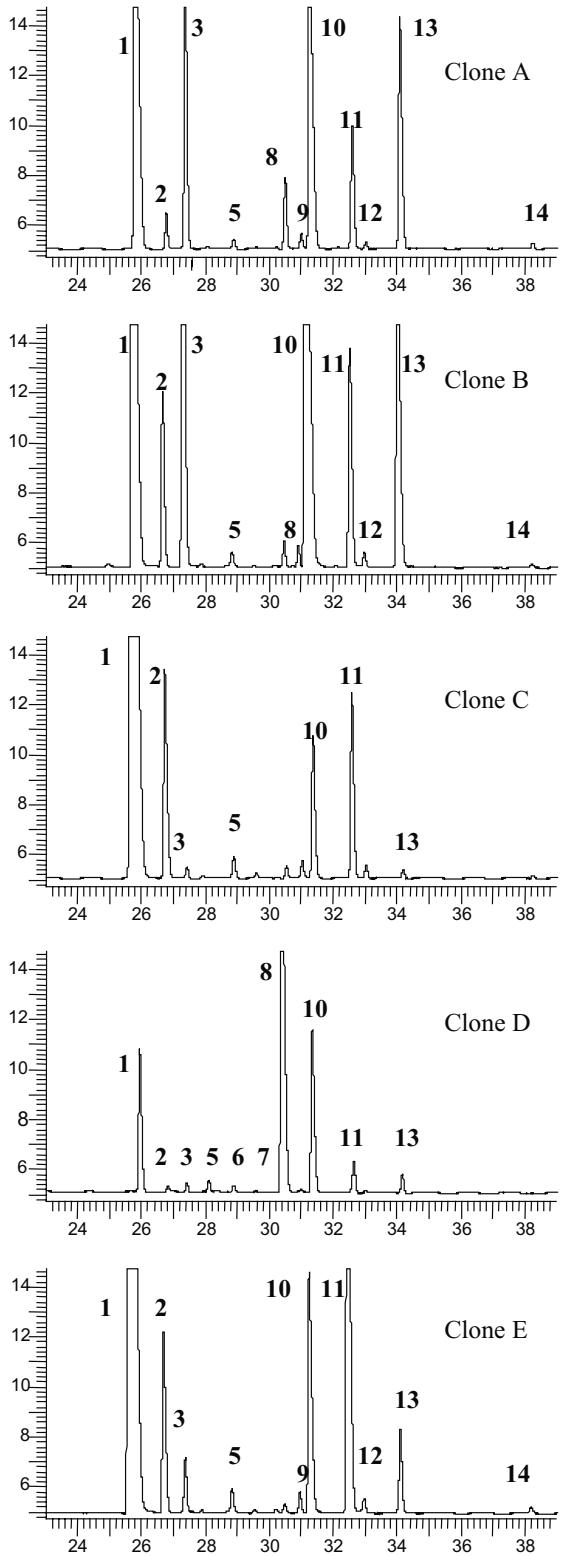

Fig. 3. GC profiles of chiral and non-chiral monoterpene components detected in the cortical samples of five different Norway Spruce clones analysed using head space in combination with chiral capillary chromatography. For peak assignment refer to the previous figure.

Table 4
Comparison of Italian clones of *P. abies* by means of the Duncan test

Clone	B	A	E	C
D	(1*S*)-(−)-α-Pinene (1*R*)-(+)-α-Pinene Myrcene (1*S*,5*S*)-(−)Sabinene (1*S*)-(+)-δ-3-Carene (1*S*)-(−)-β-Pinene (4*S*)-(−)-Limonene (4*R*)-(+)-Limonene	(1*S*)-(−)-α-Pinene Myrcene (1*S*,5*S*)-(−)Sabinene (1*S*)-(+)-δ-3-Carene (4*R*)-(−)-β-Phellandrene	(1*S*)-(−)-α-Pinene (1*R*)-(+)-α-Pinene (1*S*,5*S*)-(−)Sabinene (1*S*)-(+)-δ-3-Carene (1*S*)-(−)-β-Pinene (4*S*)-(−)-Limonene (4*R*)-(+)-Limonene	(1*S*)-(−)-α-Pinene (1*R*)-(+)-α-Pinene (1*S*,5*S*)-(−)Sabinene (1*S*)-(+)-δ-3-Carene (1*S*)-(−)-β-Pinene (4*S*)-(−)-Limonene (4*R*)-(+)-Limonene
C	(1*S*)-(−)-α-Pinene Myrcene (4*R*)-(−)-β-Phellandrene	(1*S*)-(−)-α-Pinene (1*R*)-(+)-α-Pinene Myrcene (1*R*)-(+)Camphene (1*S*)-(+)-δ-3-Carene (1*S*)-(−)-β-Pinene (4*R*)-(+)-Limonene (4*R*)-(−)-β-Phellandrene	(1*S*)-(−)-α-Pinene (4*S*)-(−)-Limonene	
E	(1*S*)-(−)-α-Pinene Myrcene (1*S*)-(−)-β-Pinene (4*S*)-(−)-Limonene (4*R*)-(−)-β-Phellandrene	(1*S*)-(−)-α-Pinene (1*R*)-(+)-α-Pinene Myrcene (1*S*)-(−)-β-Pinene (4*S*)-(−)-Limonene (4*R*)-(+)-Limonene (4*R*)-(−)-β-Phellandrene		
A	(1*R*)-(+)-α-Pinene (1*S*)-(−)-β-Pinene			

to get the most reproducible results with capillary columns. The injection time was set at 0.1 min to minimise peak broadening. Using these conditions, the influence of temperature and time on the partition equilibrium of chiral and non-chiral monoterpene between the solid and gas phase was investigated. The aim was to combine the highest sensitivity with the best resolving power. Based on previous studies [18,20], thermostatting temperatures ranging from 35 to 100 °C were investigated. These experiments were performed with rather long thermostatting times (30 min) to be sure that a stable equilibrium was achieved even at the lowest temperatures. No significant differences in monoterpene composition were detected between equilibration temperatures of 35, 50 and 80 °C; however 80 °C as thermostatting temperature was selected in order to detect trace concentrations of analytes. The samples should not be heated at 100 °C to avoid overloading of major components.

The effect of the equilibration time was then investigated to find out if shorter preparation times could have been used for routine analysis. Results reported in Table 1 show that 10 min were sufficient to cortical samples to reach an almost stable equilibrium between the solid and gas phase. Although such equilibration time was sufficient to provide reliable information on many monoterpene components, a value of 30 min was selected in order to get reproducible results.

4. Enantiomeric monoterpenes as biochemical markers for the characterisation of Norway spruce clones

Fig. 3 reports the typical profiles obtained by submitting to GC analysis head-space samples of cortical tissues collected from different clones of Norway spruce trees. The percent compositions obtained by averaging 4–5 samples collected from each one of the clones are reported in Table 2.

Table 5
Canonical variable for enantiomeric monoterpenes and cumulated percentages of discrimination on the two canonical axes

Terpenes	Canonical variates	
	Axis 1	Axis 2
(1*S*)-(−)-α-Pinene	1.319	0.913
(1*R*)-(+)-α-Pinene	−1.099	−1.208
Myrcene	−3.394	−1.898
(1*R*)-(+)-Camphene	−3.409	−1.346
(1*S*,5*S*)-(−)-Sabinene	−0.483	−0.148
(1*S*)-(+)-δ-3-Carene	−1.151	0.746
(1*S*)-(−)-β-Pinene	−2.258	−0.742
(4*S*)-(−)-Limonene	−0.232	−0.115
(4*R*)-(+)-limonene	3.128	1.172
(4*R*)-(−)-β-Phellandrene	0.985	0.648
Eigenvalues	437.771	109.480
% Cumulative	77.1	96.4

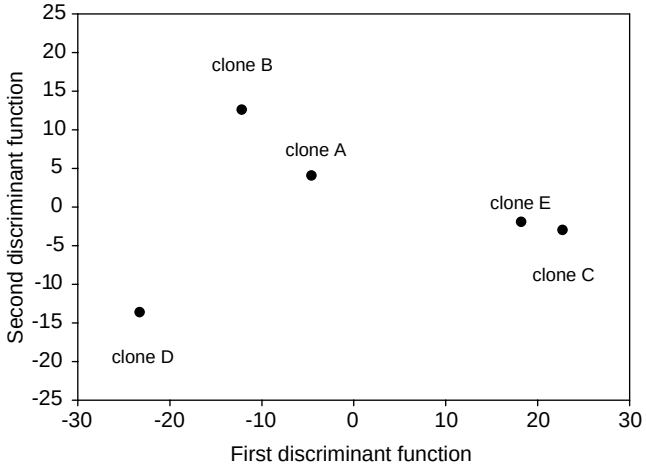

Fig. 4. Differentiation of the five Norway spruce clones by multivariate discriminant analysis using the percent composition of the 10 most significant monoterpenes determined by head-space gas chromatography of cortical tissues.

The results of the analysis of variance reported in Table 3 indicated that 10 of the 14 components separated by the column were highly significant to differentiate clones investigated. Table 4 lists monoterpenes that can be used to differentiate one clone from another according to the Duncan test at 5% level of significance. Cumulated percentages of discrimination calculated for the 10 significant enantiomeric monoterpenes on the two canonical axes are reported in Table 5. Data in the table indicate that the majority of total discrimination was concentrated on the first axis where (1*R*)-(+)-camphene, myrcene, (4*R*)-(+)-limonene and (1*S*)-(−)-β-pinene played the most important role. Myrcene, (1*R*)-(+)-camphene, (1*R*)-(+)-α-pinene and (4*R*)-(+)-limonene were fundamental for clone characterisation because of the values displayed in the second axis. Fig. 4 shows the position of the five clones on the plane of the first two canonical axes suggesting three groupings. Clone D results the most distinct one. Clone A and B appear relatively close, while trees C and E are very closely related especially on the second axis. The analysis of enantiomeric monoterpenes correctly classified all tress belonging to each clone.

5. Conclusions

These results clearly show that head-space analysis of cortical chiral monoterpenes provides a fast and reliable technique to distinguish Norway spruce clones. In addition to be useful in chemotaxomical studies, this method open new opportunities to study the ecophysiological role played by chiral monoterpenes produced and emitted by plants.

Acknowledgements

This work was supported by grants from the European Community Project QLRT-2000-00241 “Resistance of spruce to root and butt rot disease”. We thank Giuseppe Pieraccini and Gloriano Moneti (C.I.S.M.-University of Firenze) for technical advice and access the mass spectrometer facilities.

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