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Article

Differentiation of Five Pine Species Cultivated in Brazil Based on Chemometric Analysis of their Volatiles Identified by Gas Chromatography-Mass Spectrometry

Francisco A. Marques,*,[a] Gustavo Frensch,[a] Scheila R. M. Zaleski,[a] Noemi Nagata,[a] Beatriz H. L. N. Sales Maia,[a] Sonia M. N. Lazzari,[b] Cesar A. Lenz[c] and Arlene G. Corrêa[d]

[a]Departamento de Química, Universidade Federal do Paraná, CP 19081, 81531-990 Curitiba-PR, Brazil

[b]Departamento de Zoologia, Universidade Federal do Paraná, CP 19020, 81531-980 Curitiba-PR, Brazil

[c]Instituto de Tecnologia do Paraná, Cidade Industrial, 81350-010 Curitiba-PR, Brazil

[d]Departamento de Química, Universidade Federal de São Carlos, CP 676,13565-905 São Carlos-SP, Brazil

A composição dos voláteis emitidos por diferentes árvores é usada com sucesso como marcador em uma grande variedade de estudos, como a quimiotaxonomia. A caracterização dos voláteis emitidos por diferentes espécies de *Pinus* provou ser uma ferramenta importante para entender o processo de seleção de plantas hospedeiras por insetos herbívoros. Os voláteis dos galhos de cinco espécies de *Pinus* (*P. caribaea*, *P. elliottii*, *P. maximinoi*, *P. patula* e *P. taeda*) foram coletados por aeração e analisados por cromatografia gasosa com detector de ionização de chama (GC-FID), utilizando colunas de fase não-polar e quiral, e espectrometria de massas (GC-MS) com analisador de quadrupolo. A composição relativa foi usada para análises de componentes principais (PCA) e de agrupamento hierárquico (HCA) para a discriminação das cinco espécies.

The composition of the volatiles emitted by different trees has been successfully used as a marker in a wide variety of studies, such as chemotaxonomy. Characterization of the volatiles emitted by different species of *Pinus* has proven to be an important tool to understand the process of host-tree selection by herbivorous insects. The volatiles present in samples of the branches of five species of *Pinus* (*P. caribaea, P. elliottii*, *P. maximinoi*, *P. patula* and *P. taeda*) were collected by aeration, and the contents analyzed by gas chromatography using a flame ionization detector (GC-FID), applying non-polar and chiral column phases, and mass spectrometry (GC-MS) using a quadrupole mass analyzer. The relative composition of the different volatiles was used to perform a discriminant analysis among the five pine species, by means of cluster (HCA) and principal component (PCA) analyses.

Keywords: *Pinus*, volatiles, principal component analysis, hierarchical component analysis, chemotaxonomy

Introduction

The genus *Pinus* (Pinaceae) comprises 105 tree species, which are important and often dominant vegetation in large land areas of the Northern Hemisphere.[1] In addition to their wood and other products of high economic value, trees of *Pinus* influence ecosystems in various ways as they affect biochemical processes and hydrological regimes, and provide food and create habitat for animals. *Pinus* trees are cultivated in many countries, both inside and outside their natural habitats.[2]

In Brazil, pine trees, mainly *Pinus taeda* L. and *Pinus elliottii* Engelmann, have been cultivated on a commercial scale for over 30 years.[3] Currently there are about 2 million hectares of commercial pine plantations, in large continuous areas and generally in stands with a narrow genetic base,

*e-mail: tic@ufpr.br

mainly in the South and Southeast Regions.[4] The product of these plantations is mostly destined for the timber and cellulose industries.[5]

Since 2001, the banded pine weevil *Pissodes castaneus* (De Geer, 1775), which attacks preferentially *P. taeda*, has become an important pest of pine trees in Brazil. In 2002, 7.6% of the trees of a *Pinus taeda* plantation in Cambará do Sul City (Rio Grande do Sul State, Brazil) was attacked by *P. castaneus*; while in São Joaquim City (Santa Catarina State, Brazil), 16.53% of the trees was attacked.[4] A recent study found that *P. castaneus* adults are attracted to the volatiles emitted by *P. taeda*.[6]

Chemometric and bioinformatic methodologies are being extensively applied in taxonomic and metabolomic studies.[7-14] For instance, Scrivant *et al.*[15] succeeded in delimiting the species of *Bothriochloa*, by studying the essential oil composition by means of GC-MS (gas chromatography-mass spectrometry). Fischedick *et al.*[16] discriminated varieties of *Cannabis* by GC, using the quantitative data for monoterpenoids, sesquiterpenoids and cannabinoids. Similar studies involving *Pinus* were reported previously, for different species than those studied here, based on chemical analysis of the volatiles produced by different tissues including needles,[17-19] seeds,[20] cones,[21] xylem[22,23] and phloem.[24] The application of chemometry in studies in Brazil was recently reviewed by Bruns *et al.*[25]

In the present study, it was collected and characterized the volatiles emitted by branches of five pine species cultivated in Brazil, *P. elliottii*, *P. taeda*, *Pinus caribaea* Morelet, *Pinus maximinoi* H. E. Moore and *Pinus patula* Schiede ex Schlechtendal et Chamisso, by means of aeration, GC-FID (gas chromatography using a flame ionization detector), GC-MS and hierarchical cluster (HCA) and principal component (PCA) analyses, as potential methods to be used in the differentiation of these species.

Experimental

Standards

The (+)-α-pinene, (-)-α-pinene, (+)-β-pinene, (-)-β-pinene and the C8-C18 and C20 *n*-alkane standards were purchased from Aldrich (Deisenhofen, Germany).

Research materials

The volatiles were collected using aeration of branches from one-year-old trees of *P. caribaea*, *P. elliottii*, *P. maximinoi*, *P. patula* and *P. taeda* collected from stands located in Southern Brazil (Table 1).

Table 1. Sampling locations in Brazil

Species	Sampling site	Latitude	Longitude
P. caribaea	Colombo	25°33' S	49°15' W
P. elliottii	Colombo	25°33' S	49°15' W
P. maximinoi	Colombo	25°33' S	49°15' W
P. patula	Três Barras	26°11' S	50°30' W
P. taeda	Três Barras	26°11' S	50°30' W

Sample preparation

Branches from the five species of *Pinus* were aerated in a 1 L glass chamber. Aeration was carried out under controlled conditions at 23 ± 2 °C, relative humidity 70 ± 10% and a photoperiod of 12L:12D (12 h light:12 h darkness). Volatiles were trapped on a 0.4 cm long bed of Super Q resin (Alltech, Deerfield, Illinois, USA) held in place by glass-wool plugs in a glass tube (4 mm ID). Volatiles were collected for two days (flow 1.0 L min^{-1}), then eluted with hexane (3 × 0.5 mL). Extracts were concentrated as required (ca. 100 μL) under argon. All procedures were carried out in triplicate.

Analyses

GC-FID analysis

Extracts and *n*-alkane standards were analyzed by GC in the splitless mode with a Varian 3800 instrument equipped with a VA-5 capillary column (30 m × 0.25 mm × 0.25 μm), using helium as carrier gas, at flow of 1.02 mL min^{-1}. Oven temperature was initially held at 60 °C for 1 min and increased at a rate of 3 °C min^{-1} to 180 °C, and the final temperature was held for 25 min.

Enantiomeric analysis ((+)- and (-)-α-pinene and (+)- and (-)-β-pinene)

The enantiomeric monoterpenes were separated on a Varian 3800 instrument equipped with a Chirasil-Dex CB β-cyclodextrin (25 m × 0.25 mm × 0.25 μm) capillary column, using helium as carrier gas, at flow of 1.02 mL min^{-1}. Oven temperature was initially held at 60 °C for 1 min and increased at a rate of 3 °C min^{-1} to 180 °C, and the final temperature was held for 25 min.

The enantiomers were detected by co-injection of standards.

In order to determine the enantiomeric composition, the same analysis conditions were used for the extracts, (+)- and (-)-α-pinene and (+)- and (-)-β-pinene and *n*-alkane standards.

Identification of the terpenes

Extracts and *n*-alkane standards were also analyzed by coupled GC-MS with a Varian Saturn 2000 system equipped with a quadrupole detector using a CP-Sil 8 CB (Crossbond 5% phenyl/95% dimethylpolysiloxane) low-bleed column (30 m × 0.25 mm × 0.25 μm), using helium as carrier gas, at flow of 1.02 mL min. Oven temperature was initially held at 60 °C for 1 min and increased at a rate of 3 °C min^{-1} to 250 °C. The mass spectrometer operated in electron impact mode (70 eV) with a mass range set from 40 to 350 *m/z*. The interface and source temperatures were set at 250 °C.

Peak identification

The Kovats retention index (K.I.) was calculated for the compounds, and their mass spectra were compared with the literature.[26]

Principal component analysis and hierarchical clustering analysis

The PCA and HCA analyses were performed using the relative area percentages of the terpenes.[27,28] Both the racemic and the enantiomeric compositions were used as variables for the sample description. These two exploratory data analyses were applied to a data matrix of 15 × 18 (15 being the samples, 18 being the volatile compounds), by using PLS-Toolbox 3.0 (Eigenvector Research, Inc.) operating in MatLab 6.5 (Math Work Inc.).[29]

Results and Discussion

Identification of the volatiles

The chromatogram profiles of the volatiles produced by the five species of *Pinus* analyzed are exemplified in Figure 1. It was identified 14 monoterpenes and 4 sesquiterpenes in the volatiles of all five species. The enantiomeric compositions of α- and β-pinene for the species are shown in Table 2.

Principal component analysis

The PCA analysis was performed using the 18 terpenes (the two enantiomers of α- and β-pinene were considered as two separate components) identified as sample descriptors for all the *Pinus* species (Table 2).

The PCA analysis was carried out using autoscaled data. The first three principal components explained more than 82% of sample variability, with the first factor scoring 35.42%. The score and loading results, illustrated in Figures 2 and 3, respectively, show that the discrimination of *P. taeda* is strongly influenced by (+)-α-pinene (1), myrcene (7), methyl octanoate (13) and germacrene D (17). *P. elliottii* is discriminated based on the contents of camphene (3), (-)-β-pinene (6) and (*E*)-caryophyllene (15). The strong influence of (+)-α-pinene for *P. taeda* and (-)-β-pinene for *P. elliottii* was observed by Gomes da Silva *et al.*[17] in extracts from needles of *Pinus* from central Portugal.

P. patula is discriminated from the other species by the influence of *p*-mentha-2,4(8)-diene (12) and thymol methyl ether (14), and the larger amount of β-phellandrene (10). Meanwhile, (-)-α-pinene (2), (+)-β-pinene (5), δ-2-carene (8) and terpinolene (11) allowed the differentiation of *P. maximinoi* samples. Finally, *P. caribaea* is differentiated from the other species by the medium contents of the volatile compounds analyzed.

The volatiles *p*-mentha-2,4(8)-diene (12) and thymol methyl ether (14) were found only in *P. patula* samples, while methyl octanoate (13) was found only in *P. taeda*

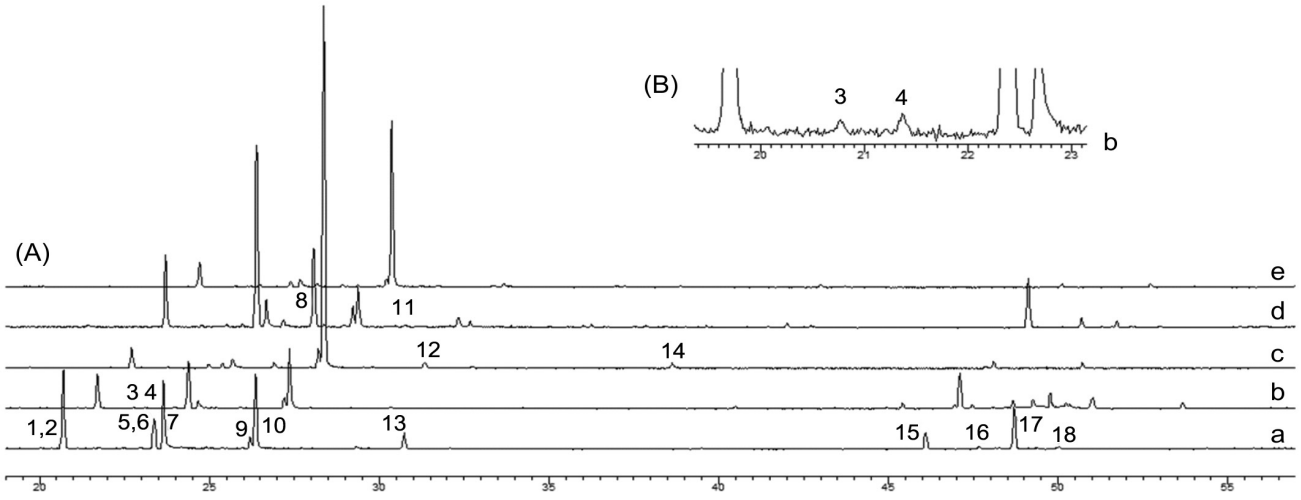

Figure 1. (A) Representative chromatograms of the volatiles emitted by *P. taeda* (a), *P. elliottii* (b), *P. patula* (c), *P. maximinoi* (d) and *P. caribaea* (e); (B) expansion of a region of the chromatogram of sample (b), showing good separation between compounds 3 and 4.

Table 2. Terpene compositions of five *Pinus* species

	Terpene	K.I.[a]	Relative mean area / %				
			P. taeda	*P. elliottii*	*P. patula*	*P. maximinoi*	*P. caribaea*
1	(+)-α-pinene	939	18.36 ± 1.5	9.52 ± 3.5	2.29 ± 3.1	5.15 ± 9.4	4.25 ± 4.4
2	(-)-α-pinene	939	4.37 ± 1.5	7.16 ± 3.5	2.27 ± 3.1	17.28 ± 9.4	5.70 ± 4.4
3	camphene	954	–	1.19 ± 0.1	–	0.35 ± 0.1	0.13 ± 0.1
4	sabinene	975	–	1.23 ± 0.1	0.92 ± 0.04	–	–
5	(+)-β-pinene	979	0.81 ± 1.6	0.47 ± 0.9	0.24 ± 0.2	10.06 ± 3.9	0.46 ± 0.7
6	(-)-β-pinene	979	9.56 ± 1.6	23.77 ± 0.9	0.88 ± 0.2	16.93 ± 3.9	1.16 ± 0.7
7	myrcene	991	16.14 ± 3.0	5.32 ± 0.3	2.39 ± 0.3	4.27 ± 1.5	3.92 ± 0.5
8	δ-2-carene	1002	–	–	–	18.54 ± 2.3	2.73 ± 1.6
9	limonene	1030	3.21 ± 0.3	–	4.45 ± 0.1	–	–
10	β-phellandrene	1032	17.08 ± 5.3	23.59 ± 1.0	80.66 ± 0.7	6.48 ± 1.5	69.21 ± 6.6
11	terpinolene	1089	–	0.49 ± 0.04	–	5.62 ± 2.9	–
12	*p*-mentha-2,4(8)-diene	1090	–	–	1.57 ± 0.2	–	–
13	methyl octanoate	1125	5.26 ± 0.5	–	–	–	–
14	thymol methyl ether	1237	–	–	1.49 ± 0.3	–	–
15	(*E*)-caryophyllene	1419	1.23 ± 0.3	12.80 ± 0.4	1.42 ± 0.1	7.11 ± 2.9	2.14 ± 0.6
16	α-humulene	1455	0.69 ± 0.1	–	–	–	1.05 ± 0.6
17	germacrene D	1485	15.91 ± 3.8	9.17 ± 2.0	1.31 ± 0.1	–	7.92 ± 6.6
18	α-muurolene	1501	1.11 ± 0.3	–	–	1.29 ± 0.5	–

[a]Kovats index.

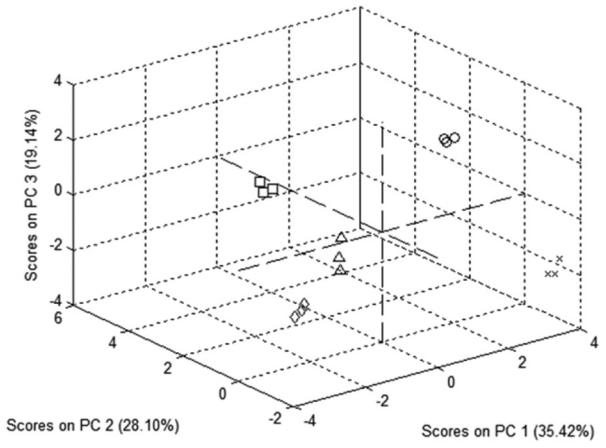

Figure 2. Score plot of PC analysis of *Pinus* species: (□) *P. taeda,* (○) *P. elliottii,* (◊) *P. patula,* (×) *P. maximinoi* and (△) *P. caribaea*.

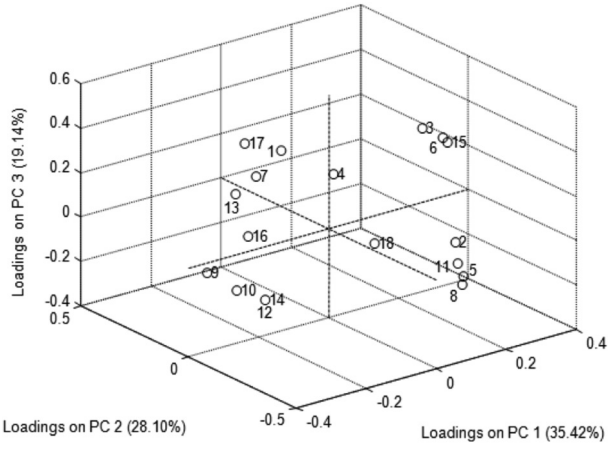

Figure 3. Loading plot of PC analysis of *Pinus* species (see Table 2).

samples (Table 2). If these compounds are in fact being produced only by these respective species, they could be used as chemical markers; however, the design of the present study does not permit this conclusion.

Hierarchical cluster analysis

The data matrix was autoscaled because of the different magnitudes of the relative compositions of the volatile compounds found. The Euclidean distance metric was used to measure the similarity, and k-nearest neighbor clustering was applied to obtain the dendrogram (Figure 4).

As seen in the dendrogram (Figure 4), *P. elliottii* was the most homogeneous group, while *P. maximinoi* showed greater heterogeneity than the other species studied. *P. caribaea*, *P. elliottii* and *P. taeda* were grouped together, while *P. patula* and *P. maximinoi* were isolated. This is consistent with the morphological classification[2]

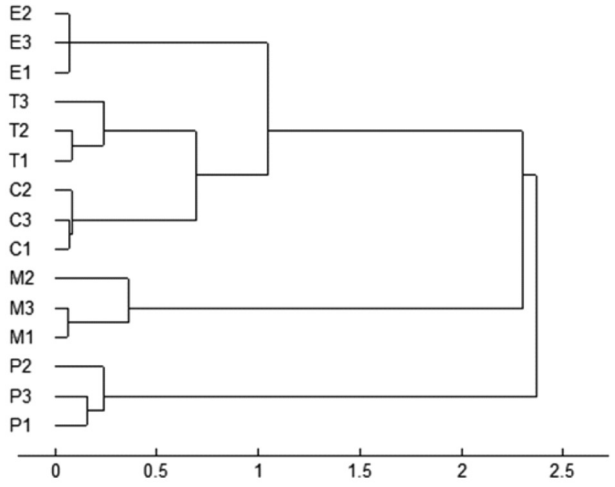

Figure 4. Dendrogram using the KNN (k-nearest neighbor clustering) method for hierarchical cluster analysis. *P. elliottii* (E), *P. taeda* (T), *P. caribaea* (C), *P. maximinoi* (M) and *P. patula* (P).

(Figure 5), in which *P. caribaea*, *P. elliottii* and *P. taeda* belong to the subsection *Australes*, while *P. patula* belongs to *Oocarpae,* and *P. maximinoi* to *Ponderosae*.

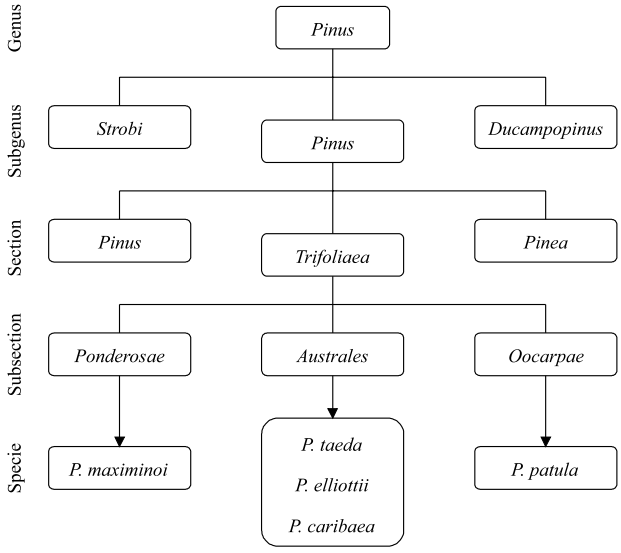

Figure 5. Morphological classification of the five species of *Pinus*.

Conclusions

Our results indicate that it is possible to discriminate *Pinus* species based on the terpenes emitted, using GC-FID and GC-MS followed by analysis of chemometrics. The discrimination among all five species using HCA reflected the morphological classification of this genus, and proved to be a powerful method to classify *Pinus* trees.

The preference of adults of *P. castaneus* to attack *P. taeda* and *P. elliottii* in Brazil makes it likely that this pest locates the host plants based on the specific volatiles that they emit. This study will be very helpful in further behavioral and electrophysiological studies to determine the role that each volatile plays in these insect-plant chemical-mediated communications.

Supplementary Information

The chromatograms in achiral and chiral columns, and the mass spectra of the identified compounds are available free of charge at http://jbcs.sbq.org.br as a PDF file.

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