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Phytochemistry 66 (2005) 1729–1735
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Fatty acids from seeds of *Pinus pinea* L.: Composition and population profiling

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Received 18 March 2005; received in revised form 16 May 2005
Available online 5 July 2005

Abstract

Pinus pinea L. is widely disseminated all over the Mediterranean Basin. Qualitatively, *P. pinea* fatty acid seed composition is identical and typical of the genus *Pinus*. This composition is made of unsaturated oil with several unusual polymethylene-interrupted unsaturated fatty acids. Linoleic acid is the major fatty acid followed by oleic, palmitic and stearic acids. Quantitatively, for all Mediterranean populations, total amounts of fatty acids seem to be fairly constant and independent from their origin. When applying principal component analysis, it seems that there is not a distinct geographical variability. Tunisian populations appear to be integral part of the Mediterranean populations without any particular structuring. Taking into account this research and the data reported in the literature, we can confirm that *P. pinea* expresses no significant variability. This low genetic diversity revealed by fatty acid composition can be explained by anthropogenetic diffusion of genetically homogeneous reproductive material as early as the first explorations.

Keywords: *P. pinea* L.; Seed fatty acids; *cis*-5 olefinic acids

1. Introduction

Stone pine (*Pinus pinea* L.) is widely distributed all over the Mediterranean Basin. It was probably disseminated as early as the first human explorations (Fallour et al., 1997).

Pine populations in Tunisia are mostly artificial. They were introduced at the beginning of the 20th century without knowing the seed origin. They were reforested particularly to restore disturbed forest areas.

In Tunisia, *P. pinea* is the most planted species along the Mediterranean coast line for sand dune stabilization. It is also used for timber and resin production and its wood is well known to be stable even at high humidity. It can be used for construction purposes, furniture making and to a lesser extent for the pulp and paper industry. Resin from *P. pinea* is a complex mixture of many organic compounds tapped by wounding its bark. Apart from these industrial uses, *P. pinea* is much appreciated for its seed production widely used in food preparation and particularly in oriental pastry. The seed contains ca. 45% lipids and ca. 21% proteins on a dry weight basis (Nasri and Triki, 2004).

Very few works were carried out on *P. pinea* expressing their genetic variability. *P. pinea* is usually added to the list of the few conifer species with extremely low allozyme diversity (Fallour et al., 1997). Several seed components and particularly minor lipid compounds are often reliable biochemical indicators of the species (Heyrman et al., 1999; Mongrand et al., 1998, 2001;

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doi:10.1016/j.phytochem.2005.05.023

Ribeiro et al., 2002; Nasri et al., 2005) and the knowledge of these compounds usually indicates valuable information to differentiate among species, varieties and geographical origins (Armanino et al., 2002).

Among enzymes involved in lipid metabolism, acyl-ACP thioesterases, desaturases, hydrolases, transacyclases and elongases are strategic proteins in fatty acid biosynthesis. Fatty acids usually clearly reflect a close botanical relationship in the family of Pinaceae (Gunstone et al., 1995; Mongrand et al., 1998; Wolff et al., 2001; Armanino et al., 2002). Much information on chemotaxonomy of angiosperms has been obtained by studying the content of fatty acids of leaves from 468 angiosperm species (Mongrand et al., 1998). In this work, the authors have clearly put forward that fatty acids may be used as a powerful tool for chemotaxonomy purposes and could be used similarly for characterizing gymnosperms.

Gymnosperms contain the unusual delta-5 olefinic acids (Takagi and Itabashi, 1982; Wolff et al., 1999a,b, 2000, 2001; Nasri et al., 2005). These fatty acids could be a powerful tool to discriminate the Pinaceae from other gymnosperms families. They may be of physiological and biochemical relevance (Wolff and Bayard, 1995).

In the present study we have used principal component analysis on *P. pinea* seed fatty acids to establish correlations between and within some Mediterranean populations including some current Tunisian populations.

2. Results and discussion

Oil content of the fully ripen *P. pinea* seeds from Tunisian populations varied from 44.69% to 51.87% on a dry weight basis as seen in Tables 1 and 2. Results from Moroccan and European populations are shown in Table 3.

Tunisian *P. pinea* seeds are found to be rich in lipids, averaging 47.05% on a dry weight basis (Table 1). Qualitatively fatty acid composition is identical for all populations. Extracted oils from all populations are mainly unsaturated. Linoleic acid is the main fatty acid (48.52%) ranging from 437.3 mg g^{-1} of total fatty acids (TFA) in Bechateur population to 511.0 mg g^{-1} TFA in Hdada followed by the oleic acid (35.83%) ranging from 310.4 mg g^{-1} TFA in Bechateur to 380.7 mg g^{-1} TFA in Dar Chichou.

In addition to these usual fatty acids, seed oils are characterized by several unusual minor polymethylene-interrupted unsaturated fatty acids with *cis*-5 double bond (Table 2). Total amounts of these compounds vary from 15.8 mg g^{-1} TFA in Bechateur to 24.1 mg g^{-1} TFA in Oued El Bir. Acids 20:3 *cis*-5,11,14 (sciadonic) and 18:3 *cis*-5,9,12 (pinolenic) are found in larger quantities. For all of these, compositions seem to be fairly independent from origin. These results confirm previous work on Tunisian *P. pinea* populations (Nasri et al., 2005).

For Moroccan and European populations, the most important fatty acid is also the linoleic acid (<498.2 mg g^{-1} TFA) followed by the oleic (<431.1 mg g^{-1} TFA) Table 3. Identified saturated acids (always lower than 160.9 mg g^{-1} TFA) are: palmitic (<88.9 mg g^{-1} TFA), 17:0 (<1.5 mg g^{-1} TFA), stearic (<62.7 mg g^{-1} TFA), arachidic (<10.7 g g^{-1} TFA).

As seen in Table 3, the *cis*-5 olefinic acids are taxoleic acid 18:2 *cis*-5, 9 (<5.3 mg g^{-1} TFA), pinolenic (<3.7 mg g^{-1} TFA), 20:2 *cis*-5, 11 (<15.5 mg g^{-1} TFA) and sciadonic (<21.5 mg g^{-1} TFA). They are not significantly different from all other Mediterranean olefinic fatty acid compositions. As for *Pinus cembroides*, contents of these unusual fatty acids from *P. pinea* seeds

Table 1
Tunisian *Pinus pinea* seed fatty acid composition (mg g^{-1} total fatty acids)

Populations		BE	HD	ZO	DC	OB
Oil content (%)		51.87	48.64	45.1	44.97	44.69
FA composition		mg g^{-1} TFA	mg g^{-1} TFA	mg g^{-1} TFA	mg g^{-1} TFA	mg g^{-1} TFA
(FA1)	14:0	0.6	0.5	0.5	0.4	0.4
(FA2)	16:0	71.5	70.3	71.8	67.6	68.0
(FA3)	16:1	0.8	0.7	0,6	0.7	0.6
(FA4)	17:0	0.6	0.6	0.6	0.6	0.5
(FA5)	17:1	1.4	0.7	1.0	0.9	0.8
(FA6)	18:0	37.9	40.7	42.0	38.3	34.5
(FA7)	18:1 *cis*-9	310.4	376.3	350.4	380.7	377.0
(FA9)	18:2 *cis*-9,12	437.3	511.0	481.1	478.1	501.3
(FA11)	18:3 *cis*-9,12,15	4.1	5.5	4.9	4.9	6.1
(FA12)	20:0	7.5	6.7	7.4	6.9	6.3
(FA13)	20:1 *cis*-11	4.6	7.2	6.9	6.9	7.8
(FA15)	20:2 *cis*-11, 14	3.1	4.9	4.8	4.3	4.9
$\sum$ Saturated FA (mg g^{-1} TFA)		118.1	118.8	122.3	113.8	109.7

Table 2
Tunisian *Pinus pinea* seed *cis*-5 olefinic acid compositions (mg g^{-1} total fatty acids)

Population		BE	HD	ZO	DC	OB
Δ-5 fatty acid		mg g^{-1} TFA	mg g^{-1} TFA	mg g^{-1} TFA	mg g^{-1} TFA	mg g^{-1} TFA
(FA8)	18:2 *cis*-5,9	1.5	3.1	1.8	1.7	1.0
(FA10)	18:3 *cis*-5, 9,12	2.5	3.1	3.1	2.8	3.3
(FA14)	20:2 *cis*-5,11	1.4	0.9	1.5	1.1	1.5
(FA16)	20:3 *cis*-5, 11,14	10.4	13.6	12.1	14.6	18.3
Total (mg g^{-1} TFA)		15.8	20.7	18.5	20.2	24.1

Table 3
Mediterranean *Pinus pinea* seed fatty acid compositions (mg g^{-1} total fatty acids)

Fatty acids	M1	M2	F1	F2	I	G1	G2	E1	E2	T1	T2	T3	T4	T5	T6	T7	T8
14:0	0.5	0.5	0.6	0.4	0.5	0.4	0.5	0.1	0.5	0.5	0.5	1.6	0.5	0.4	0.5	0.6	0.6
16:0	76.9	68.3	75.0	64.9	67.0	66.6	66.7	88.9	68.6	69.6	66.8	74.7	76.3	65.2	76.0	70.2	69.0
16:1	0.7	tr	0.7	0.6	tr	0.6	0.7	0.7	1.4	0.7	1.1	0.7	0.9	0.4	0.9	0.9	0.7
17:0	0.6	tr	0.7	0.6	tr	0.4	0.5	0.7	0.6	0.4	0.5	1.5	0.4	0.9	0.5	tr	tr
17:1	0.6	4.0	0.7	1.4	1.1	1.2	1.2	2.3	0.7	1.2	0.7	4.5	0.8	1.2	1.1	1.2	1.4
18:0	44.8	43.1	48.3	45.5	40.7	41.8	43.3	62.7	45.4	39.6	37.7	42.8	38.0	57.9	43.6	41.9	40.0
18:1 *cis*-9	407.8	335.7	415.4	343.1	354.6	386.5	369.7	305.7	398.1	362.0	431.1	364.7	436.7	312.8	376.1	392.	335.4
18:2 *cis*-5,**9**	1.6	5.3	1.9	1.5	3.8	1.8	1.6	1.1	1.0	1.5	1.2	1.6	1.7	tr	1.5	2.5	2.0
18:2 *cis*-9,12	497.2	419.4	482.3	391.4	436.4	400.2	409.6	283.0	497.0	422.9	498.2	447.9	485.9	365.3	427.1	428.6	411.5
18:3 *cis*-5,9,12	3.3	tr	3.7	2.7	2.5	2.4	3.1	1.7	3.4	2.5	2.9	2.6	2.5	2.3	2.8	1.8	1.6
18:3 *cis*-9,12,15	6.1	5.5	6.6	5.3	4.6	4.3	5.4	3.3	7.7	4.7	4.8	4.9	4.7	4.2	4.5	3.3	3.7
20:0	7.7	7.5	6.6	6.2	6.0	7.0	6.4	8.5	6.6	6.5	6.7	10.7	6.3	6.8	6.8	7.5	6.7
20:1 *cis*-11	8.5	9.2	8.4	7.2	8.6	7.0	8.7	7.3	8.9	6.8	7.8	7.6	8.2	6.3	7.5	7.4	4.7
20:2 *cis*-5,11	1.4	15.5	3.0	1.8	tr	3.1	3.7	9.5	2.5	3.3	1.9	2.1	1.7	tr	2.4	1.8	2.6
20:2 *cis*-11,14	6.4	4.8	6.5	4.9	9.4	3.6	5.4	5.2	6.9	4.1	4.7	3.9	5.7	7.5	4.6	3.8	6.5
20:3 *cis*-5,11,14	17.9	10.8	21.5	15.3	14.3	13.1	18.1	8.1	19.6	13.2	15.3	13.7	13.8	11.6	14.3	13.5	10.0
$\sum$ FA *cis* − 5	24.2	31.6	30.1	21.3	20.6	20.4	26.5	20.4	26.5	20.5	21.3	20.0	19.7	13.9	21.0	19.6	16.2
$\sum$ saturated FA	130.5	119.4	131.2	117.6	114.2	116.2	117.4	160.9	121.7	116.6	112.2	130.3	121.5	131.2	127.4	120.2	116.3

are quite low (totaling less than 31.6 mg g^{-1} TFA). Wolff and Marpeau (1997) consider that these biochemical characteristics might be of possible acclimatation of *P. pinea* to particular environmental conditions. It may also be due to genetic control.

Fatty acid compositions of Tunisian populations are not significantly different from all other Mediterranean populations. For all of these, extracted oils are mainly unsaturated with linoleic as the major fatty acid. These unsaturated oils confer some important dietary properties to *P. pinea* seeds. In fact, it is recognized that unsaturated fatty acids can influence the physical properties of the membrane such as fluidity and permeability. Linoleic fatty acid is indispensable for the healthy growth of human skin (Bruckert, 2001). It can be transformed by the organism in a series of long fatty acid chains, precursors of eicosanoids, a family of compounds with 20 carbons including prostaglandins and leucotriens in particular. Prostaglandins have an important role at the vessel level and for blood coagulation. Another fatty acid found in large quantities in *P. pinea* seeds, the 18:1 *cis*-9 acid, is also very important in nervous cell construction. It can be changed by the organism into a set of compounds close to prostaglandins. Oleic fatty acid has a fundamental role in cardiovascular prevention.

2.1. Principal component analysis

A statistical analysis was done to differentiate populations within the Mediterranean *P. pinea* species by using 22 fatty acid compositions. These fatty acid compositions have been submitted to principal component analysis (PCA) using S.A.S statistics analysis. From this analysis, the following axes of inertia have been withheld as seen in Table 4.

These five axes of analysis were retained because they express 80.68% of the total variation. Axis 1 explains 28.27% of the total variation. This axis associates positively to 18:1 *cis*-9 (FA7), 18:2 *cis*-9,12 (FA9), 18:3 *cis*-5, 9,12 (FA10), 18:3 *cis*-9,12,15 (FA11) and 20:3 *cis*-5,11,14 (FA16). Along axis 2, the most discriminate variables explaining 18.69% of the variation are 17:0 (FA4), 17:1 (FA5), 20:0 (FA12). Axis 3 (14.52%) is characterized by 18:2 *cis*-5,9 (FA8), 20:1 *cis*-11 (FA13). Axis 4 (11.1%) is defined positively by 14:0 (FA1) and negatively by 18:0 (FA6). Finally, axis 5 that explain 8.07%

Table 4
Contribution of different characters to the definition of PCA axes on *Pinus pinea* fatty acid compositions

Components	PC 1	PC 2	PC 3	PC 4	PC 5
Inertia%	28.27	18.69	14.52	11.1	8.07
Cumulative%	28.27	46.97	61.5	72.61	80.68
Characters defining axes	+FA7				
	+FA9	+FA4			+FA2
	+FA10	+FA5	+FA8	−FA1	+FA3
	+FA11	+FA12	+FA13	+FA6	+FA14
	+FA16				+FA15

of the total variation is defined positively by 16:0 (FA2), 16:1 (FA3), 20:2 *cis*-5,11 (FA14) and negatively by 20:3 *cis*-5,11,14 (FA15).

Data projections on planes as defined by inertia axes of PCA (Figs. 1 and 2) do not show significant differences in Tunisian populations from the Mediterranean ones. The Tunisian populations appear to be an integral part of theses populations without any particular structuration. Even though the data on the types and distribution of fatty acids did not provide an unequivocal guide to the classification of plants (Mongrand et al., 1998), many correlations became apparent when applying PCA to the Mediterranean *P. pinea* populations. In fact, we can notice that the Tunisian Bechateur population (BE) is different from all other Tunisian populations. The same results have been observed by the morphological variability (Nasri et al., 2004). In this later study, we have suggested that the Tunisian Bechateur population had occupied most of the morphological parameters studied. It has been considered as a potential source of good seed production.

For all Mediterranean populations, *P. pinea* seed oils are rich in lipids >40% on a dry weight basis. In addition, when applying principal component analysis, all Mediterranean populations are not structured according to the geographical origin. We could not differentiate independent groups according to the origin. Total fatty acids amounts seem to be fairly constant and independent from their origin. This low genetic diversity reveled by fatty acid composition can be explained by the anthropogenetic diffusion of genetically homogeneous reproductive material as early as the first explorations. In fact it is recognized that many European populations of *P. pinea* could originate from one or a few of the Leb-

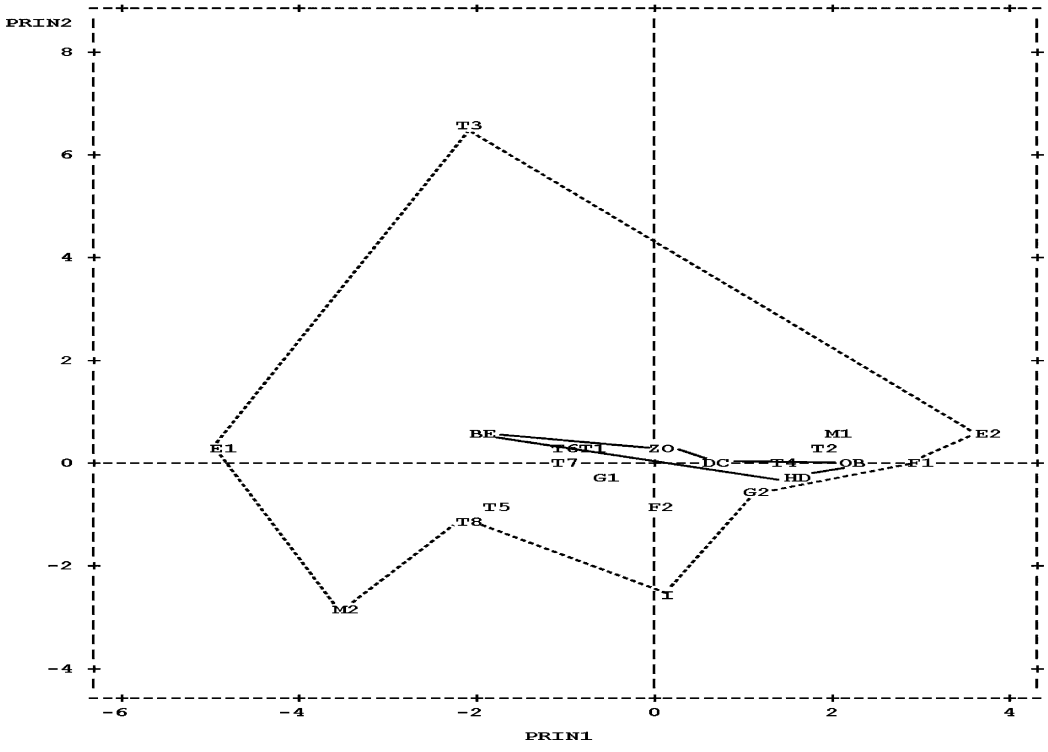

Fig. 1. Principal component analysis, dispersion of *Pinus pinea* populations in planes formed by axes PC1 and PC2 of the PCA. — Connect Tunisian populations, --- Connect Morocco and European populations.

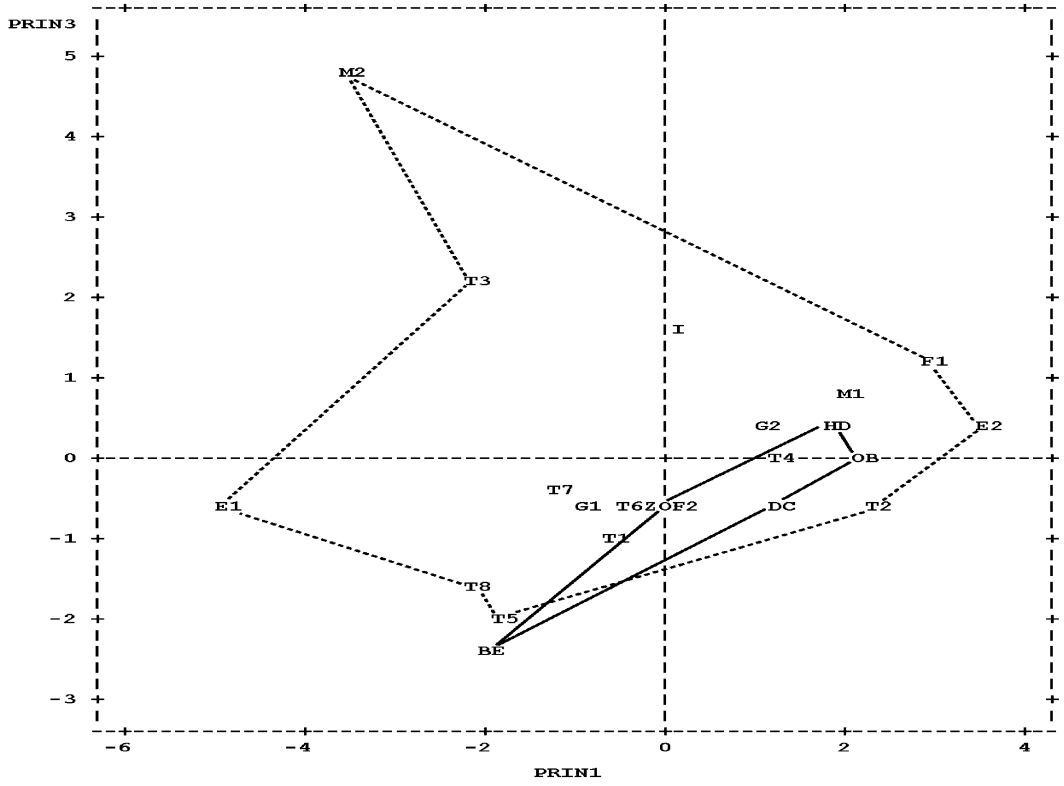

Fig. 2. Principal component analysis, dispersion of *Pinus pinea* populations in planes formed by axes PC1 and PC3 of the PCA. — Connect Tunisian populations, ---- Connect Morocco and European populations.

anese populations introduced by the Etruscans (Fallour et al., 1997). For Tunisian *P. pinea* populations, we are unaware the origin of seeds, no particular structuring with and within all other Mediterranean populations is noticed.

3. Conclusion

Concerning *P. pinea* fatty acid composition, results are in agreement with literature (Wolff and Bayard, 1995). Qualitatively and quantitatively, *P. pinea* fatty acid seed composition is identical and typical of the genus *Pinus*. This composition is made of unsaturated oil with several unusual polymethylene-interrupted unsaturated fatty acids with an ethylenic bond in the *cis*-5 position. Linoleic acid is the major fatty acid followed by oleic, palmitic and stearic acids. Quantitatively it does not show a meaningful variability. For all Mediterranean populations, total amounts of fatty acids seem to be fairly constant and independent from their origin. When applying principal component analysis to the fatty acid compositions considered as chemometric data (16 variables of 22 populations), it seems that there is not a distinct geographical variability. Data projections on planes defined by inertia axes of principal component analysis do not show an important differentiation of the Tunisian populations. They appear to be an integral part of these populations without any particular structuring. However, only the Bechateur population might have a different origin, the other populations show several morphological and biochemical features supporting their common origin. Taking into account this research and the data reported in literature (Fallour et al., 1997), we can confirm that *P. pinea* expresses no significant variability. This low genetic diversity revealed by fatty acid composition can be explained by anthropogenetic diffusion of genetically homogeneous reproductive material as early as the first explorations.

For this reason we are considering using other approaches such as unsaponifiable seeds lipid fraction, simple DNA sequence repeats (microsatellites) and pine needle terpene composition to better characterize genetic diversity of *P. pinea*.

4. Experimental

4.1. Seeds

Local sampling was performed on 5 Tunisian populations. Three to four cones per tree, on 15–25 trees per population were harvested in fall 2002: Bechateur

(BE), Hdada (HD), Zouaraa (ZO), Dar Chichou (DC) and Oued El Bir (OB).

Seeds from Morocco and European populations were obtained from INRA Avignon France: Turkey : Eceabat (T1), Izmir (T2), Mugla (T3), Serik (T4), Yalova (T5), Yatagan (T6), Kumluca (T7), and Antalya (T8), Spain: Meseta Castellana (E1), Cordillera Central (E2), France: Saintes-Maries (F1), Saint-Aygulf (F2), Morocco: Mezzine (M1), Cap Sartel (M2), Italy: Feniglia (I) and Greece: Greece (G1), Greece Agios (G2) Fig. 3.

4.2. Oil extraction

Oils from the seeds were extracted mainly according to Folch et al. (1957) modified by Bligh and Dyer (1959). Seeds (2.5 g) were fixed with 20 mL of boiling 1% NaCl (w/v) aqueous solution to denature the phospholipases. The aliquot was crushed in a mortar in the presence of 20 mL of methanol, and chloroform (20 mL) was then added. The total chloroform–methanol–NaCl (1%) homogenate was centrifuged at 3000*g* and the lower chloroformic phase containing the total lipids was kept. The solvents were removed in a rotary evaporator at 50 °C, and the total lipids were saved and stored at −20 °C (Vorbeck and Marinetti, 1965).

4.3. Fatty acid methyl ester preparation

Fatty acid methyl esters (FAMEs) were prepared according to Metcalfe et al. (1966) modified by Lechevallier (1966). An aliquot (0.2 mL) of total lipids was evaporated in a tube of methylation. Fatty acids were saponified with 4 mL of a methanolic sodium hydroxide solution (0.5 M) for 15 min in a boiling water bath at 65 °C. As for transmethylation, the mixture was homogenized with 3 mL of a methanolic solution of BF3 (20%, w/v) and the reaction was allowed to proceed for 5 min. FAMEs were extracted twice with 10 mL of petroleum ether, with 10 mL of water being added to the mixture.

4.4. Gas–liquid chromatography

FAMEs were analyzed by gas–liquid chromatography (GC) in a Hewlett–Packard HP-4890 D chromatograph equipped with a CP Wax 52 CB capillary column (1.2 μm film thickness; 50 m × 0.32 mm), operated isothermally at 200 °C with an inlet carrier gas (nitrogen) pressure of 100 kPa. The injector (split mode) was maintained at 230 °C and the flame-ionization detector (FID) at 250 °C. Unusual polymethylene-interrupted fatty acids were identified by comparison of their equivalent chain lengths (ECLs) with those calculated

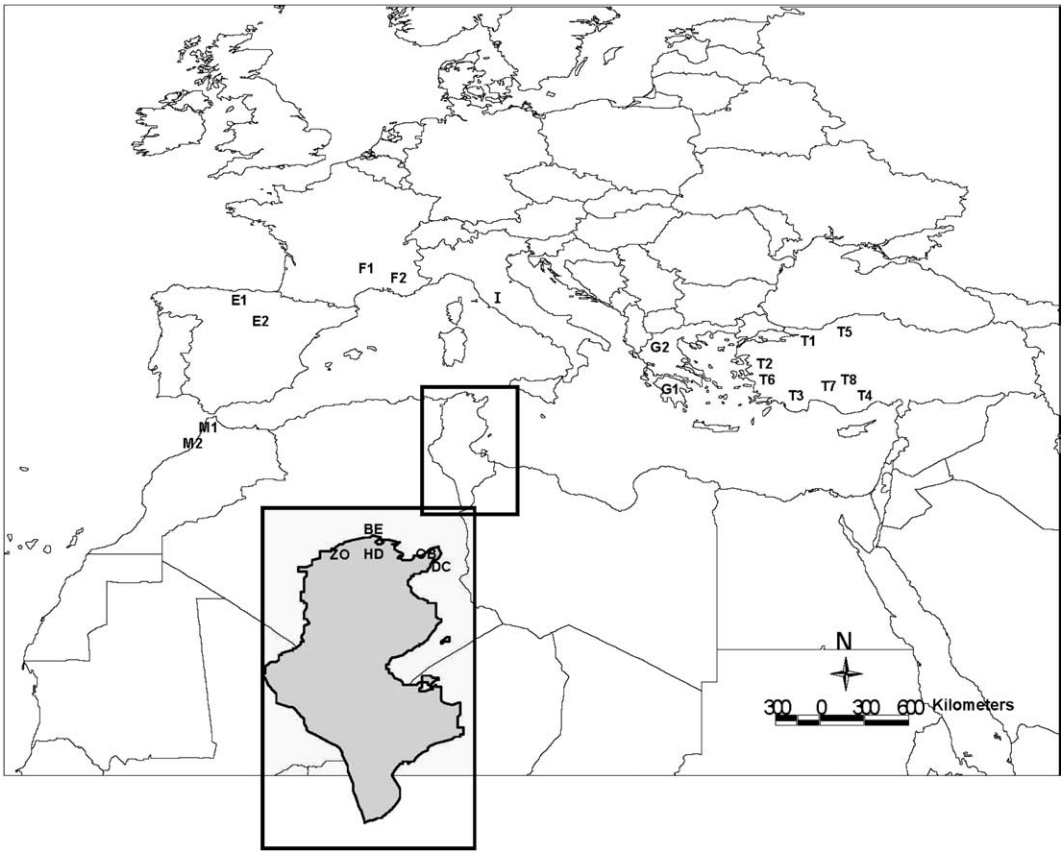

Fig. 3. Geographic location of the 22 *Pinus pinea* samples used for fatty acid population profiling.

with authentic related standards. ECLs were determined according to Wolff and Bayard (1995) with 16:0, 18:0, 18:1, 18:2, 18:3, 20:0, 22:0, 22:1 and 24:0 fatty acid methyl esters as reference components (Supelco, North Harrison, USA). FAMEs were analyzed by GC-FID under conditions similar to those above, and by gas–liquid chromatography-mass spectrometry with a HP chromatograph, model 5890 Series II, attached to an Agilent selective quadrupole mass detector under an ionization voltage of 70 eV at 250 °C, and connected to a computer with a HP ChemStation. The injector, in split mode, and the interface temperatures were maintained at 250 °C, and helium was used as carrier gas.

4.5. Chemometric method

To evaluate the information contained in experimental data, principal component analysis (PCA) was applied. PCA is a chemometric method to visualize information contained in experimental data and to find the true dimensions of a dataset (Miloun et al., 1992). The v-parameters (variables) measured for each sample describes each sample (object) in a v-dimensional space. PCA generates a set of new orthogonal variables (axes), linear combinations of the original ones, so that the maximal amount of variance contained in the dataset (information) is concentrated in the first principal components. The loadings are the coefficients of the original variables defining each principal component. The scores are the coordinates of the objects on the new axes (Armanino et al., 2002).

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