ACTA BIOLOGICA CRACOVIENSIA Series Botanica 46: 95–100, 2004

FATTY ACID AND TOCOCHROMANOL PATTERNS OF TURKISH PINES

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Received July 10, 2003; revision accepted January 19, 2004

The plant family Pinaceae is known to produce a set of unusual fatty acids in the seed oils. In Turkey it has been less studied in respect to the fatty acid and tocochromanol (tocopherols and tocotrienols) composition of the seed oils, particularly in terms of chemotaxonomy. This study examined the fatty acid, tocopherol, tocotrienol and plastochromanol-8 content of *Pinus* L. taxa naturally growing in Turkey (*Pinus nigra* subsp. *pallasiana, P. nigra* subsp. *pallasiana* var. *pyramidata, P. halepensis, P. sylvestris, P. pinea, P. brutia, P. radiata, P . pinaster*). The fatty acids (oleic, linoleic, α-linolenic, Δ5-taxoleic, pinolenic and coniferonic acid) and tocochromanols are valuable components of *Pinus* seed oils. in the family Pinaceae. Unsaturated fatty acid comprises ~90% of the seed oil. While γ-tocopherol was found to be the largest component of all *Pinus* seed oil, β-tocotrienol was not found at all. Total tocopherols in the studied taxa was much higher than total tocotrienols. The distribution of fatty acid and tocochromanol patterns in the genus *Pinus* is discussed, and in particular the presence of Δ5-UPIFA acids such as taxoleic and pinolenic acids, as well as common and distinguishing features that may contribute to knowledge about the taxonomy and phylogeny of pines.

Key words: Pinaceae, *Pinus*, fatty acid, tocochromanol chemotaxonomy, phylogeny.

INTRODUCTION

Gymnosperms are generally divided into two main taxonomic groups: the coniferophytes and cycadophytes (Page, 1990). Many attempts have been made to gain insight into the phylogenetic interrelationships of conifers, because they are the most prominent components of the extant flora, with a very long history and a particularly rich fossil record, beginning in pre-Permian times (Rothwell, 1982). The Pinaceae family consists of 11 or 12 genera. *Pinus*, with over 100 extant species, is the largest genus of conifers and the most widespread genus of trees in the Northern Hemisphere (Price et al., 1998; Wolff et al., 2001).

The genus *Pinus* (Pinaceae) is represented by nine taxa in FLORA OF TURKEY (Davis, 1965; Güner et al., 2000). *Pinus sylvestris* L. is distributed throughout Europe, extending into Caucasia. It is a very complex species, split in different ways by various authors. *Pinus nigra* Arn. subsp. *pallasiana, P. brutia* Ten., *Pinus halepensis* Miller and *P. pinea* L. are other taxa naturally growing in Turkey (Davis, 1965). *Pinus nigra* Arnold. subsp. *pallasiana* (Lamb) Holmboe var. *pyramidata* (Acat.) Yaltirik (Ehrami black pine) has a very narrow distribution around Kütahya, the inner Anatolian region of Turkey. There are some morphological differences between these species in the cone and other characters (Davis, 1965). The morphological similarity between the Mediterranean *P. pinaster* and the New World hard pines has led to their classification in a single section (Van der Burgh, 1973).

Unusual fatty acids are often present in significant percentages in the seed fat, but are almost totally absent in leaves and other parts of the plant (Smith, 1970; Takagai and Itabashi, 1982). These interesting fatty acids and their sporadic occurrence

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PL ISSN 0001-5296

in seed lipids is genetically determined, and they are highly significant indicators of phylogenetic relationships (Aitzetmüller, 1995). The content of Δ5-unsaturated polymethylene-interrupted fatty acid (Δ5-UPIFA) and the fatty acid profiles of gymnosperm seeds are useful chemometric data for the taxonomy and phylogeny of that division, and they may also have some biomedical or nutritional applications (Wolff et al., 2000), like the unusual fatty acids found in some other groups (Compositae, *Anemone* spp., Boraginaceae, Cannabaceae) (Tsevegsüren and Aitzetmüller, 1993; Tsevegsüren et al., 1997; Bagci et al., 2003, 2004). Tocopherols, together with tocotrienols and plastoquinones, are known as tocochromanols (Seher and Ivanov, 1973; Krishna et al., 1997; Velasco et al., 2000). Some of them exhibit vitamin E activity.

In this study, fatty acid, tocopherol, tocotrienol and plastochromanol content was determined in *Pinus* species naturally growing in Turkey: *Pinus nigra* Arn. subsp. *pallasiana* Lamb Holmboe (Anatolian black pine), *Pinus nigra* subsp. *pallasiana* var. *pyramidata* (Ehrami black pine), *P. pinea*, *P. brutia*, *P. halepensis*, *P. radiata* Don., *P. pinaster* Ait. and *P. sylvestris*. This is the first study of the fatty acid and tocopherol composition of Turkish Pinaceae; it reveals a number of new sources of Δ5 (UPIFA) fatty acid.

MATERIALS AND METHODS

PLANT MATERIAL

Pinus seed samples were obtained in 1999–2001 from the following localities: *P. pinaster*, Yalova – Armutlu; *P. radiata*; Izmit – Kefken; *P. halepensis*; Izmir – Urla; *Pinus nigra* subsp. *pallasiana* var. *pyramidata*; Kütahya, Tavsanli – Ikizoluk; *Pinus sylvestris*, Kastamonu, Ilgaz – Gökdere; *P. brutia*, Adana, Kizyusuflu village; *P. nigra* subsp. *pallasiana*, Adana – Kozan; *Pinus pinea*, Mugla – Güvercinlik gulf.

OIL EXTRACTION AND PREPARATION OF FATTY ACID METHYL ESTERS (FAME)

Impurities were removed from the seeds and the cleaned seeds were powdered using a ball mill. Lipids were extracted with heptane in a straight-through extractor. The triglycerides were transesterified to methyl esters with potassium hydroxide in methanol according to DGF method 5509 (1989).

CAPILLARY GLC

Fatty acid methyl ester (FAME) composition was determined with two different gas chromatographs, Hewlett-Packard HP5890 (A) and HP6890 (B), each equipped with a fused silica WCOT capillary and FID:

(A) Silar 5 CP, 50 m × 0.25 mm ID, 0.24 μm film thickness, nitrogen as carrier gas, 1:50 split ratio, pressure 160 kPa, oven temp.: 5 min isothermal at 163°C, then 163 to 205°C at 1°C/min; Inj. = 230°C, Det. 260°C.

(B) DB-23, 60 m × 0.32 m (J&W), 0.25 μm film thickness, hydrogen as carrier gas, 1:50 split ratio, pressure 69 kPa, oven temp.: 1 min isothermal at 80°C, then 80 to 150°C at 25°C/min, then 150 to 240°C at 3°C/min, 5 min isothermal, PTV-Inj. 80 2°C, 12°C/s to 250°C, 5 min isothermal, Det. 250°C.

Data analysis was done with a D 2500 Chromato Integrator (Merck-Hitachi) and Chemstation integration software. Peaks were identified by comparing the relative retention times with those obtained from test mixtures of known composition on two different columns.

TOCOPHEROL ANALYSIS

Tocochromanols were determined by high-performance liquid chromatography (HPLC) according to the procedure of Balz et al. (1992). An aliquot of a solution of 50 mg oil in 1 ml heptane was injected in an HPLC system via a Rheodyne valve with a sample loop volume of 20 μl. Tocopherols were separated on LiChrospher 100 (Merck, Darmstadt, Germany), Diol phase, 5 μm particle size with an HPLC column 25 cm × 4.6 mm ID with an additional guard column 4 mm long and 4 mm ID, filled with LiChrospher Si 60, 5 μm particle size. The system was operated with an eluent of heptane/tert.-butyl methyl ether (96:4 v/v) and detection by a fluorescence detector F-1000 (Merck) at 295 nm excitation wavelength and 330 nm emission wavelength. A D-2500 Chromato Integrator was used for data acquisition and processing. Calibration was done using external standards with α, β, γ and δ-tocopherol (Calbiochem, Bad Soden, Germany). Tocotrienols are calculated with the same response factors as the corresponding tocopherols, and plastochromanol-8 was calculated with the same response factor as γ-tocopherol (Balz et al., 1992).

TABLE 1. Fatty acid composition in seeds of Turkish Pines. Data shown are peak area.

Species	Fatty acid components																				
	16:0	16:1 Δ7	17:0	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ5,9	18: 2 Δ9,12	18: 3 Δ5,9 12	18: 3 Δ9,12 15	18: 4 Δ5, 9, 12,15	20: 0	20: 1 Δ11	20: 2 Δ5,11	20: 3 Δ5,11, 14	22:0	22:1	24:0	Tot. SFA (%)	Tot. USFA (%)	Tot. Δ 5	Oil Cont. (wt%)
P. nigra subsp. *pallasiana*	4.2	0.2	0.0	1.9	17.8	0.5	3.2	47.1	17.9	0.5	0.1	0.3	1.0	0.3	3.8	0.1	0.1	0.0	6.5	92.4	25.3	28.3
P. nigra subsp. *pallasiana* var. *pyramidata*	4.7	0.2	0.0	2.0	16.5	0.5	3.4	46.7	18.6	0.4	0.0	0.3	1.1	0.4	3.4	0.1	0.1	0.0	7.1	91.1	25.5	36.6
P.halepensis	5.1	0.1	0.1	3.3	21.7	0.5	0.2	57.1	0.1	0.6	0.0	0.6	0.8	0.5	4.5	0.2	0.2	0.0	9.1	86.1	5.2	23.1
P. sylvestris	3.0	0.1	0.1	1.8	15.5	0.8	3.1	44.8	20.9	0.4	0.0	0.4	1.0	0.5	4.9	0.2	0.1	0.0	5.4	92.1	34.6	41.3
P. pinea	6.3	0.1	0.1	3.5	38.5	0.4	0.1	45.0	0.0	0.6	0.0	0.6	0.8	0.5	2.2	0.1	0.2	0.0	10.5	88.4	2.8	43.9
P. brutia	4.4	0.1	0.1	3.5	18.9	0.3	0.1	58.1	3.2	0.7	0.0	0.5	1.2	0.4	5.8	0.1	0.8	0.0	8.7	89.6	9.6	23.1
P. radiata	4.0	0.2	0.1	2.0	16.9	0.6	2.8	42.4	16.7	0.6	0.0	0.3	0.8	0.4	3.5	0.1	0.1	0.0	6.5	85.0	23.0	40.1
P. pinaster	4.1	0.1	0.1	2.7	17.8	0.4	0.1	52.9	8.1	1.0	0.1	0.4	1.1	1.0	7.2	0.1	0.1	0.0	7.4	89.8	16.0	28.6

SFA – Saturated Fatty acid; USFA – Unsaturated Fatty acid

RESULTS AND DISCUSSION

Fatty acid and tocochromanol composition in naturally growing Turkish pines were determined in this study. Fatty acid, tocochromanol content and the oil yield of taxa belonging to the genus *Pinus* are shown in Tables 1 and 2. Total lipid yield of the pines studied here ranged from 23.05% to 43.87%. *Pinus pinea* showed the highest oil content (Tab. 1). The seeds of this species are used in traditional home-made food and sweets in Turkey.

The seed oils of the eight studied *Pinus* species contained significant amounts of linoleic acid (18:2Δ9c,12c; between 58.1% and 42.4%), and also oleic acid (18:1Δ9c; between 15.5% and 38.5%), which is the next main fatty acid (Tab. 1). The contribution of saturated fatty acids (SFA), principally 16:0 and 18:0 acids, was very low (<10%). In Pinaceae, Wolff et al. (2001) found the most common saturated fatty acid to be linoleic acid (18:2), accompanied by fairly high levels of oleic acid (18:1) and very low levels of linolenic acid (18:3). The main FAs with straight chains have been found in some *Pinus* species (*P. caribaea* Morelet, *P. kesiya* Royle ex. Gordon, *P. dalatensis* Ferre, *P. krempfii* Lecomte and others) from Vietnam and Far Eastern regions, 18:2 (n-6) and 18:1 (n-9) (Imbs and Long, 1996).

Linoleic and linolenic acids are elongated to 11,14-20:2 and 11,14,17-20:3 acids, respectively. α-linolenic acid is a minor constituent of pine SFA, almost always less than 1%, but can reach 1.04% in *P. pinaster*; this is also reported in more of the *Pinus* species, but *P. merkusii* has a higher level, 2.7% in the seed oil (Wolff et al., 2000).

The amount of saturated fatty acid is between 5.4% and 10.5% of total seed oil. Total unsaturated fatty acid is between 85.0% and 92.5% of total seed oil. In a comparison with other genera of Pinaceae, Wolff et al. (2001) reported that total SFA in *Picea* A. Dietr. is ~5% of total FA, which is less than in most *Pinus* species (ś10%).

The Δ5-unsaturated polymethylene-interrupted fatty acid Δ5 UPIFA [Δ5,9-18:2 (taxoleic), Δ5,9,12-18:3 (pinolenic), Δ5,9,12,15-18:4 (coniferonic), Δ5,11-20:2, Δ5,11,14-20:3 (sciadonic) acids] was occasionally found in the studied *Pinus* taxa (Tab. 1). Pinolenic and coniferonic acids, which are the Δ5 desaturation products of linoleic and linolenic acids, were present at low levels or absent in the studied *Pinus* species. *P. sylvestris* and *P. radiata* had the highest levels of pinolenic acid. These differences between *Pinus* taxa may have chemotaxonomic importance in infrageneric classification of *Pinus*. Pinolenic acid was also found in the seeds of most Pinaceae; an interesting characteristic is its effect

TABLE 2. Tocochromanol composition of Turkish Pines. Data shown are peak area

Species	Tocochromanols								
	α-T	β-T	γ-T	δ-T	α-T3	β-T3	δ-T3	γ-T3	P-8
P. nigra subsp. *pallasiana*	31.9	0.0	66.2	0.6	0.7	0.0	0.4	0.0	0.2
P. nigra subsp. *pallasiana var. pyramidata*	21.5	0.0	77.0	0.4	0.0	0.0	0.3	0.4	0.8
P. halepensis	9.1	0.0	89.8	0.3	0.0	0.0	0.8	0.0	0.0
P. sylvestris	7.1	0.0	89.9	0.3	0.4	0.0	2.2	0.1	0.0
P. pinea	22.3	0.4	60.2	3.6	11.0	0.0	2.6	0.0	0.0
P. brutia	9.7	0.0	88.3	0.9	0.0	0.0	1.1	0.0	0.0
P. radiata	8.7	0.0	87.2	0.8	0.0	0.0	1.1	2.2	0.0
P. pinaster	9.2	0.0	89.4	0.4	0.0	0.0	1.4	0.0	0.0

on blood pressure (Sugano et al., 1994). Total Δ5 fatty acid concentrations varied greatly in the studied *Pinus* species, ranging from 2.8% to 34.6% (Tab. 1). Some *Pinus* species like *P. maximartinezii* (0.98%) and *P. nelsonii* (0.94%) have very low Δ5 USFA in the seed oils (Wolff et al., 1999).

The pinolenic acid concentration was very high in *Pinus sylvestris*; it was the fatty acid component with the second-highest level in the oil (Tab. 1). The seed FA composition of the *Pinus* species was generally uniform, but *P. sylvestris* had the highest level of unsaturated Δ5 fatty acids (Tab. 1). Taxoleic and pinolenic acids were the most variable components in the genus *Pinus*. Interspecific variation of pinolenic acid was higher than that of taxoleic acid. The lowest pinolenic and taxoleic acid levels were determined in *P. pinea* (0.1–3.1%) and *P. halepensis* (0.1–20.9%). Total Δ5-UPIFA fatty acids in *Pinus* seed oil was highest in *P. sylvestris* (34.6%), two *P. nigra* varieties (25.5%, 25.3%), *P. radiata* (23.0%) and *P. pinaster* (16.0%). The other studied taxa showed lower levels of this FA group (2.8–9.6%). Data on the presence and content of Δ5 olefinic acids can be of use as new supplementary chemometric markers for the taxonomy of Turkish pines and others such as reported by Wolff et al. (2001).

The distribution profiles of Δ5-UPIFA are similar in the majority of pines. In particular, C18 Δ5-UPIFA is present in considerably greater proportions than C20 Δ5-UPIFA. In the studied taxa, content of 5,9,12:18:3 acid was higher than that of 5,9-18:2 acid. This is reported in most *Pinus* species (Imbs and Long, 1996).

Δ5 (UPIFA) unsaturated fatty acid components constitute an important unsaturated fatty acid group containing most of the lipids of conifer seeds. These FA have been shown to be common constituents of seed oils from all coniferophyte families, and to be characteristic of some cycadophyte families (two subgroups of Gymnospermae) (Wolff et al., 1997, 1999). However, no Δ5 olefinic acids were reported in the seeds and/or leaves of a few cycadophytes such as *Welwitschia mirabilis*, *Gnetum scandens* and *Macrozamia communis* (Berry, 1980; Daulatabad et al., 1985; Mustafa et al., 1986); some of them were found to contain cyclopropene acids. 5,9 and 5,11 18:2 acids coexist in *Ephedra* spp. seed lipids, though in minor amounts. These are the same octadecadienoic acids that were identified in *Ginkgo biloba* seeds (Wolff et al., 1999).

Pine samples were selected and obtained from different regions of Turkey. The fatty acid composition of total seed lipids was similar in the studied *Pinus* species, in spite of the geographical differences. An assessment of geographical variations in the seed FA composition of *P. abies* showed that its intraspecific variation was of considerably smaller amplitude than within other *Picea* species (Wolff et al., 2001). This observation supports the use of seed FA composition as a chemotaxonomic marker, as it is practically independent of edaphic or climatic conditions.

The tocochromanol derivatives α-, β-, γ- and δ-tocopherols and tocotrienols and 8-plastochromanol were detected in *Pinus* seed oils. While γ-tocopherol was found in all studied *Pinus* taxa, β-tocotrienol (T3) was not found. γ-tocopherol was the tocopherol component with the highest concentration in all *Pinus* species (Tab. 2). α-tocopherol was the second-highest in all studied taxa, varying from 8.7% to to 31.9% in *P. radiata* and *P. nigra* subsp. *pallasiana*). The high concentrations of γ- and α-tocopherol and their distribution in the pines give some clues to the chemotaxonomic importance of these components in *Pinus* species. β-tocopherol was not found in the seed oil, except in *P. pinea* and *P. radiata* at low concentrations. δ-tocopherol was determined in all taxa at low levels (0.04–3.6%).

Pinus pinea showed the highest δ-tocopherol content (3.57%) (Tab. 2). We can say that tocopherol content was higher than tocotrienol content in the studied *Pinus* species. Plastochromanol-8 was not found in *Pinus* seed oil except in *Pinus nigra* varieties. High content of γ-tocopherol and α-tocopherol, and low content of δ- and β-tocopherols and total tocotrienols, may be characteristic in *Pinus*. Larger investigations of the genus are required. In Franzen et al. (1991) study, α-tocopherol was found in all living organs of 28 spruce species.

The ancestors of gymnosperms may be an extinct group of pteridiophytes or some still more remote ancestors. Interestingly, some extant species of the seed ferns can synthesize arachidonic (5,8,11,14-20:4) and eicosapentaenoic (5,8,11,14,17-20:5) acids in their green parts (Schlenk, 1965; Aitzetmüller et al., 2001; Aitzetmüller, unpublished data). This implies that they contain one Δ6 desaturase specific for polyunsaturated C18 acids, which is a feature common to more ancient groups of bryophytes such as mosses (Schlenk, 1965; Groenewald et al., 1997) and liverworts (Groenewald et al., 1997), many microalgae and algae, and some molds.

The present study examined the distribution of fatty acids and tocochromanols in *Pinus* species naturally growing in Turkey and compared them with some related species. Qualitatively, SFA from all pine species analyzed so far were identical, but there were some differences between tocochromanol patterns. Interspecific variation of fatty acid and tocochromanol in the studied *Pinus* species was not very high except in *Pinus pinea*. The chemotaxonomic importance of fatty acids and tocochromanols in *Pinus* and in the family Pinaceae was confirmed with regard to the studied taxa. We analyzed only a limited number of plant species for their seed lipid and tocochromanol composition. Such study has already proved its value in formulating phylogenetic relationships (Aitzetmüller et al., 1999). Evaluation of fatty acids and tocochromanols together as chemometric characters, or of only fatty acids in a wider range of species, is suggested as a powerful tool for characterizing the systematic positions and phylogenetic relationships among the genera of Pinaceae.

ACKNOWLEDGEMENTS

The first author is grateful to TUBITAK–DFG for awarding a fellowship and research grants during part of this investigation at the University of Muenster, Germany, and also to Dr. L. Bruehl and Professor Dr. K. Aitzetmüller for their invitation.

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