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VOLATILE CONSTITUENTS OF NEEDLES OF FIVE *PINUS* SPECIES GROWN IN GREECE

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Key Word Index—*Pinus halepensis*; *P. brutia*; *P. nigra*; *P. pinea*; *P. canariensis*; terpenes; essential oil; volatile metabolites; chemotaxonomy.

Abstract—The chemical composition of the volatile metabolites from needles of *Pinus halepensis* Miller, *P. brutia* Tenore, *P. nigra* Arnold, *P. pinea* Linnaeus and *P. canariensis* Sweet and Sprengel, grown in natural habitats in Attiki, Greece, was analysed. The variability and chemotaxonomic importance of the terpenoid constituents are discussed.

INTRODUCTION

In the context of biosystematic investigations of herbivore–host plant chemical interactions [1, 2], we have initiated a study of the feeding preferences of the serious conifer lepidopteran pest *Thaumetopoea pityocampa* (Den & Schiff). This processionary moth is a monophagous herbivorous insect that feeds exclusively upon pine tree needles. Laboratory and field experiments have indicated that it is able to discriminate between pine species via chemical cues [3], as well as other means. From the total of 12 natural and introduced pine species found in various phytogeographic compartments and altitudes in Greek provenances, the more extensively grown species in natural stands are: *P. pinea*, once widely distributed throughout the Mediterranean region and now restricted to sandy coasts; *P. nigra*, extending from southern Europe northwards to Austria and the S. Carpathians; *P. canariensis*, which originated on the Canary islands and was planted extensively for timber in several Mediterranean countries where it escaped cultivation and became naturalized; *P. halepensis*, widely distributed throughout the Mediterranean region; and the oriental species *P. brutia* found from Asia Minor and Iran to areas of south western Europe and North Africa. Besides the morphologically distinct and taxonomically typified pine species, a large number of hybrids has evolved as a result of range expansion out of the refugia at the end of Pleistocene glaciations, a fact that provided opportunities for introgressive hybridization [4, 5]. The individual morpho-anatomical characteristics of these hybrids in several cases can be deceiving and can lead to wrong assignment to typified taxa. The high degree of genotypic variability observed in a number of *Pinus* species is reflected in the biochemical variability, which is usually studied at the levels of terpene composition and isozyme variation [6]. The volatile constituents, particularly monoterpenes, have been extensively studied, since it has been demonstrated that the monoterpene composition besides some non-genetic variabilities related to the environment [7], is dependent upon the plant's genotype and can be used for taxonomic purposes [8].

The chemical composition and the interspecific variation of various *Pinus* species volatiles have been the subject of numerous studies [9–13]. The bulk of the work though has been focused on North American and Western European species and only a limited number of chemically oriented reports dealt with southeastern Mediterranean pine species [10, 11, 14, 15]. Very little is known about the chemical composition of the volatile metabolites of pine species grown in Greece [10, 14, 15]. Some early studies dealt with the determination of physical constants and the qualitative analyses of turpentine and only the major volatile components were identified [16, 17]. Because of the ecological significance of the volatile secondary metabolites in the insect–host plant system, this study has focused on the chemical composition of the essential oil obtained from the needles of *P. halepensis*, *P. brutia*, *P. nigra*, *P. pinea* and *P. canariensis*.

RESULTS AND DISCUSSION

A total of 113 trees were selected for this study. They were chosen from natural stands occupied by more than one pine species so that the chemical differences would reflect mainly the genotypic and not the environmentally induced differences [18]. The leaves were selected as the chemical source because it is recognized that the basic tissue responsible for the production of terpenes is the needle resin canal epithelium [19], and so is expected to exhibit smaller tree-to-tree variation than other plant parts. Furthermore, the needle terpenoid profile is known to be directly related to the oviposition preference shown by *T. pityocampa* adults [20, 21]. The needles were collected from various parts of the crowns in order to

overcome the plant plasticity phenomena [22]. In order to remove the effect of seasonal variation, all of the samples were collected in July. This month is within the dormancy period of Mediterranean pines and is also the month during which the maximum oviposition activity of the herbivore occurs. Samples were stored at -70° until they were hydrodistilled. After removal of the brachyblast bases the needles were cut into pieces and hydrodistilled. Because the harsh conditions of steam distillation have been suspected as a source of artifacts, the head space volatiles of a random sample of trees were collected and analysed. As the head space analysis did not show any new peaks or loss of peaks observed in the foliage steam distillates, it was decided to proceed with the analysis of the essential oils by capillary GLC and GC-MS.

Fifty metabolites were detected, characterized and quantified on the basis of their retention data and mass spectra, as constituents of the essential oils. As expected the majority of the identified metabolites were found to be monoterpenes and sesquiterpenes. The taxonomic value of the terpenes is well documented and widely accepted, since they meet the basic requirements set by Harborne [23], i.e. structural variety, physiological stability, wide distribution in the plant families and ease of identification. Within the monoterpene domain were identified 14 hydrocarbons, five alcohols and seven ester or ether monoterpene derivatives. In addition to the 26 monoterpenes and monoterpene derivatives, a large number of less volatile metabolites, mainly sesquiterpenes and diterpenes, were detected and quantified (Table 1, nos 27–50). Sesquiterpenes have been commonly reported as 'unidentified' metabolites and only recent studies identify and report sesquiterpene constituents [24]. The highest levels of sesquiterpenes and diterpenes were found in the essential oils of *P. halepensis* and *P. canariensis* where they represent 48.33 and 60.34%, respectively, of the total essential oil. Caryophyllene, germacrene D and α-humulene were always the major constituents in the sesquiterpene fractions of all of the samples analysed in this study. Small amounts of the sesquiterpene alcohols elemol and guaiol were detected in the essential oils of *P. brutia* and *P. pinea*, respectively. The sesquiterpene alcohol farnesol and the corresponding acetate were detected in the *P. pinea* and *P. canariensis* distillates as minor metabolites, while the less common sesquiterpenes β-bourbonene and manoyl oxide, the latter identified previously in *P. strobus* L. extracts [25], were found in the essential oil of *P. nigra*. Strikingly, only *P. halepensis* samples contained large amounts of the diterpene macrocyclic cembrene hydrocarbons (cembrene-related unidentified compounds nos 40–44), cembrenes have been isolated in the past from other *Pinus* species and marine coelenterates [26, 27]. Noteworthy was the presence of significant amounts of (5,9α-,10β)-kaur-15-ene, the biosynthetic precursor of the tetracyclic plant hormones (giberellins) and also the immediate precursor of (5,9α-10β)-kaur-15-ene, bicyclic diterpene alcohol (11*E*,13*Z*)-labdadien-8-ol, in the essential oil of *P. pinea*.

Earlier studies on the monoterpene composition of *P. halepensis* populations showed the presence of high proportions of α-pinene in the extracts [10, 15], a finding that can be attributed to a number of factors, e.g. the sample collection procedures used (i.e. scraping or boring of the trunk), a tree response to various types of injury, as well as to the smaller number of monoterpenes identified, a fact that increased the relative percentages of the reported constituents. Our data on the *P. brutia* foliage chemical composition are comparable to those reported by Schiller and Grunwald [11] (high proportions of β-pinene and α-pinene) even though direct comparison with our data is not possible for all the above reasons.

In order to investigate the phenetic affinities of pine species, the composition data of all detected constituents were submitted to a minimum variance agglomerative centroid clustering algorithm [28]. Euclidean distance (Fig. 1) was used as a distance measure. Two groups G1 (*P. brutia*) and G2 (*P. pinea*, *P. nigra*, *P. canariensis* and *P. halepensis*), were initially recognized. The G1 group was characterized by high amounts of β-pinene, a relatively minor constituent in the G2 group. The large amounts of limonene in *P. pinea* oil were responsible for the discrimination of these populations within the G2 group. Within their cluster the populations of *P. canariensis* were discriminated on the basis of a large germacrene D contribution. In the *P. nigra* and *P. halepensis* populations cluster, the most significant discrimination factor was α-pinene; this terpenoid being present in large amounts only in the *P. nigra* oils.

Five chemotypes A–E corresponding to each of the five investigated pine species were recognized. According to the four terpenes with the highest discriminating weight, the chemotypes can be assigned as:

Chemotype A (*P. halepensis*) α-pinene > limonene > β-pinene > germacrene D

Chemotype B (*P. brutia*) β-pinene ≫ α-pinene > germacrene D > 3-carene

Chemotype C (*P. nigra*) α-pinene > germacrene D > limonene > β-pinene

Chemotype D (*P. pinea*) limonene ≫ germacrene D > α-pinene > β-pinene

Chemotype E (*P. canariensis*) germacrene D ≫ α-pinene > β-pinene > limonene.

Although the foliage chemical constituents of the five investigated pine species appear to be good separators and distinct chemotypes characteristic of the pine species are recognized, they fail to reproduce the evolutionary groups. The observed deviation of the hierarchical structure of the phenogram (Fig. 1) from the generally accepted morpho-geographical taxonomic scheme [29] is high. Similar failures to discriminate between major subgeneric evolutionary lines on the basis of leaf terpenoid metabolites have been reported for North American Haploxylon and Diploxylon pines [9]. It seems that high congruence between terpene-based and morphological classification can be achieved only at subspecific taxonomic levels and introgressions between closely related species e.g. *P. halepensis* and *P. brutia* [V. Roussis, P. V.

Table 1. Chemical composition of *Pinus* spp foliage essential oils

Compound	*P. halepensis*	*P. brutia*	*P. nigra*	*P. pinea*	*P. canariensis*	*F*-ratio/Prob.*
1 Thujene	0.16 (62.5)†	0.02 (350.0)	0.40 (55.0)	0.08 (25.0)	0.01 (100.0)	16.7***
2 α-Pinene	13.40 (47.4)	16.03 (30.9)	52.19 (12.4)	5.13 (26.7)	14.01 (66.9)	51.6***
3 Camphene	0.44 (68.1)	0.81 (38.3)	2.17 (22.6)	0.34 (85.3)	0.30 (103.3)	34.1***
4 Sabinene	1.27 (118.1)	0.88 (10.2)	0.80 (145.0)	0.35 (31.4)	0.02 (300.0)	3.7*
5 β-Pinene	1.13 (41.6)	45.66 (10.4)	2.03 (28.6)	2.65 (30.2)	1.86 (160.8)	391.1***
6 Mycrene	6.62 (131.1)	2.35 (32.8)	1.33 (35.3)	2.31 (16.5)	8.84 (82.7)	NS
7 α-Phellandrene	0.05 (60.0)	0.04 (85.7)	0.11 (45.5)	0.62 (38.7)	—	37.2***
8 3-Carene	6.87 (62.3)	0.50 (60.0)	0.40 (242.5)	—	0.01 (400.0)	18.6***
9 α-Terpinene	0.30 (70.0)	0.10 (100.0)	0.35 (168.6)	0.15 (73.3)	0.03 (66.7)	NS
10 Limonene	5.03 (54.7)	1.63 (20.9)	2.24 (38.8)	39.05 (17.8)	1.44 (109.7)	179.1***
11 β-Phellandrene	1.27 (110.2)	1.11 (35.1)	0.31 (48.4)	13.8 (17.8)	0.09 (100.0)	180.3***
12 Ocimene	1.77 (41.8)	1.85 (78.9)	1.20 (68.3)	1.59 (62.9)	0.91 (92.3)	NS
13 γ-Terpinene	0.42 (85.7)	0.26 (84.6)	0.23 (60.1)	0.22 (45.5)	0.11 (90.9)	NS
14 Terpinolene	3.07 (77.9)	1.32 (80.3)	1.06 (67.0)	0.47 (46.8)	0.18 (88.9)	6.9***
15 Linalool	0.78 (51.3)	0.66 (130.3)	0.14 (221.4)	0.12 (191.7)	0.05 (180.0)	3.4*
16 Fenchol	0.11 (127.3)	0.11 (100.0)	0.04 (200.0)	—	0.02 (300.0)	2.5*
17 Borneol	0.02 (100.0)	0.11 (63.6)	0.03 (200.0)	—	0.03 (300.0)	3.2*
18 4-Terpinenol	0.70 (128.6)	0.46 (119.6)	0.22 (122.7)	0.16 (75.0)	0.02 (200.0)	2.6*
19 α-Terpineol	0.54 (63.0)	1.20 (42.9)	1.00 (147.0)	0.91 (57.1)	0.75 (240.0)	5.7**
20 Linalyl acetate	—	0.24 (291.7)	0.02 (200.0)	—	—	NS
21 Methyl thymyl ether	0.10 (180.0)	—	—	0.66 (74.2)	—	11.8***
22 Fenchyl acetate	0.28 (85.7)	0.38 (86.8)	0.78 (85.9)	0.19 (31.6)	0.21 (95.2)	3.4*
23 Terpenyl acetate	0.01 (200.0)	0.81 (173.0)	0.37 (189.2)	—	0.27 (88.9)	NS
24 Citronellyl acetate	—	—	0.03 (233.3)	0.14 (107.1)	—	4.7**
25 Neryl acetate	0.36 (8.3)	0.06 (66.7)	0.12 (66.7)	0.09 (33.3)	0.22 (40.9)	20.1***
26 Geranyl acetate	0.19 (42.1)	0.33 (69.7)	0.07 (142.9)	0.08 (37.5)	0.44 (140.9)	NS
27 δ-Elemene	0.03 (133.3)	—	0.13 (76.9)	0.27 (44.4)	—	13.1***
28 Caryophyllene	19.05 (29.9)	4.85 (37.1)	5.67 (49.2)	2.21 (36.2)	0.05 (160.0)	11.9***
29 α-Humulene	3.36 (28.6)	0.92 (34.8)	0.97 (58.8)	0.41 (31.7)	1.47 (89.8)	8.7***
30 Calarene	0.39 (53.8)	0.06 (83.3)	0.09 (33.3)	0.15 (40.0)	0.26 (46.2)	9.9***
31 Germacrene D	0.50 (78.0)	7.63 (55.4)	14.27 (52.4)	4.23 (57.4)	50.55 (22.1)	72.9***
32 Aristolene	1.09 (43.1)	0.12 (133.3)	0.38 (128.9)	1.24 (36.3)	0.50 (68.0)	11.3***
33 Butanoic acid, 3-methyl, 2-phenyl ethyl ester	6.57 (21.0)	0.83 (91.6)	2.10 (168.6)	1.00 (44.0)	1.00 (65.0)	9.6***
34 α-Gurjunene	1.18 (85.6)	0.30 (80.0)	0.25 (72.0)	0.14 (57.1)	0.76 (52.6)	6.1**
35 α-Muurolene	0.53 (32.1)	0.12 (41.7)	0.45 (102.2)	0.28 (64.3)	0.91 (48.4)	8.4***
36 δ-Cadinene	0.55 (25.5)	0.35 (37.1)	1.09 (86.2)	0.35 (42.9)	3.26 (22.7)	45.5***
37 Elemol	0.36 (69.4)	0.31 (45.2)	0.06 (150.0)	0.08 (100.0)	0.29 (151.7)	NS
38 Guaiol	1.05 (4.8)	—	0.26 (150.0)	2.79 (28.7)	0.14 (321.4)	49.1***
39 Globulol	0.01 (200.0)	—	0.05 (260.0)	0.82 (29.3)	0.18 (177.8)	20.2***
40 Eudesmol	0.41 (26.8)	0.32 (90.6)	0.38 (78.9)	0.77 (32.5)	0.63 (92.1)	NS
41 Unidentified	—	0.08 (287.5)	—	—	—	NS
42 Unidentified	2.38 (52.5)	0.31 (106.5)	0.18 (161.1)	0.06 (116.7)	0.06 (216.7)	20.8***
43 Unidentified (*M*, 272)	0.98 (46.9)	0.12 (116.7)	0.05 (240.0)	—	0.01 (200.0)	26.8***
44 Unidentified (*M*, 272)	0.62 (46.8)	0.06 (116.7)	0.05 (240.0)	—	0.02 (250.0)	23.1***
45 Unidentified (*M*, 272)	0.13 (100.0)	0.02 (250.0)	—	—	—	5.8**
46 Uidentified *M*, 272)	0.86 (75.6)	0.28 (207.1)	0.22 (54.5)	0.30 (43.3)	0.18 (61.1)	2.5*
47 Cembrene	7.62 (62.2)	0.88 (125.0)	0.57 (243.9)	—	0.01 (400.0)	15.8***
48 Unidentified	0.36 (75.0)	0.09 (222.2)	0.01 (200.0)	—	0.03 (100.0)	5.5**
49 (5,9α-,10β)-Kaur-15-ene	—	—	—	4.62 (58.0)	—	21.6***
50 (11*E*,13*Z*)Labdadien-8-ol	0.30 (113.3)	—	—	0.87 (44.1)	0.03 (133.3)	23.8***

*F-ratios indicate how well the terpenes separate between pine species in a multiple comparison sense. They were derived by K-means clustering procedure. The number of formed clusters is equal to the number of pine species [30].

The asterisks denote probabilities associated with F-ratios (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, NS = not significant).

†Coefficients of variation are shown in parentheses.

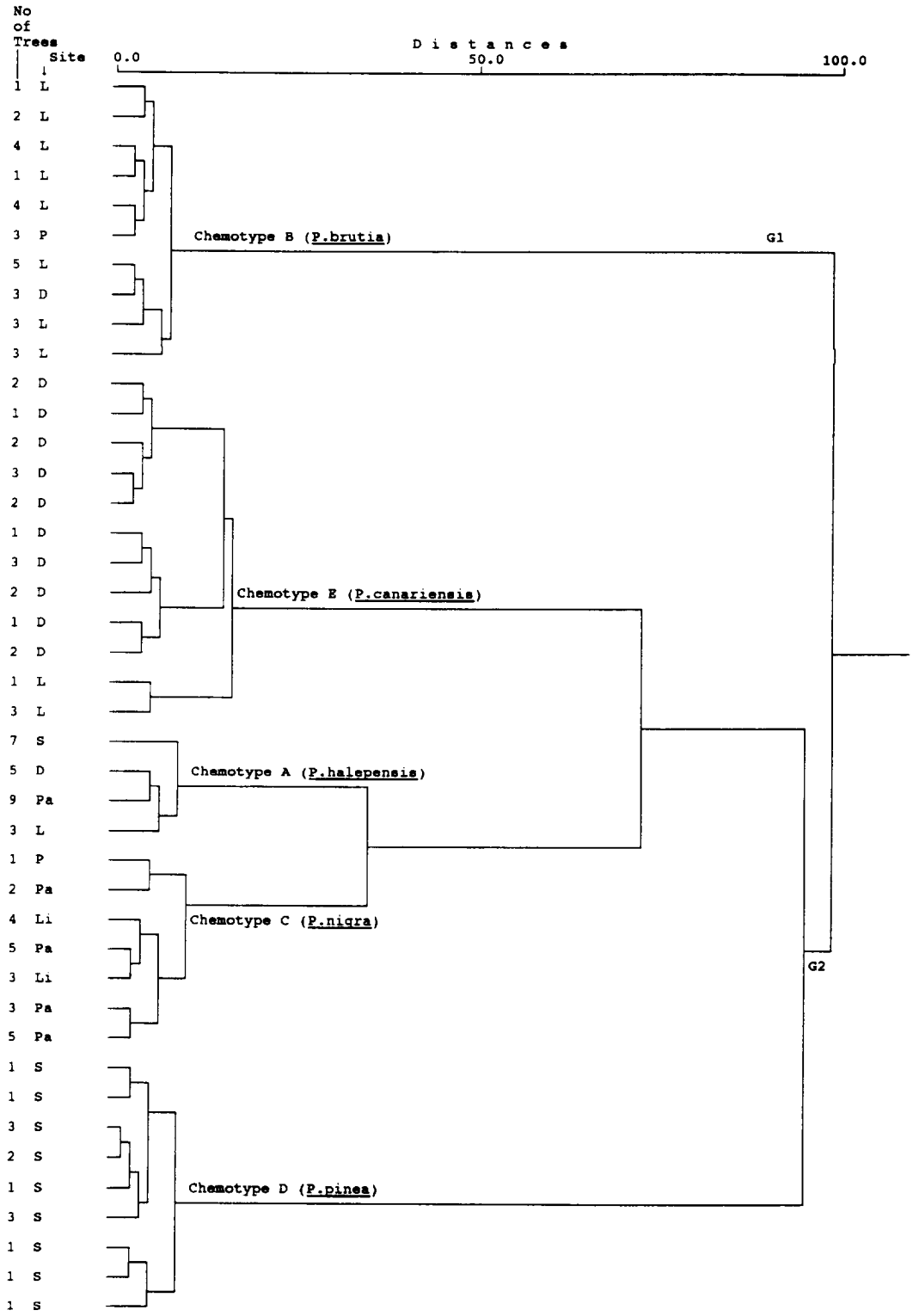

Fig. 1. Dendrogram showing the hierarchical classification of the five *Pinus* chemotypes in the terpene space (all constituents are incorporated). Site symbols: L = Loutropyrgos, Pa = Mt Parnis, S = Schinias, P = Kaissariani, D = Demokritos wood.

Petrakis, A. Ortiz and B. E. Mazomenos, unpublished results] or *P. contorta* Engelm. and *P. bankisiana* Lamb. [9]. In this study, β-pinene triggers distortions in the phenogram by separating *P. brutia* from the remaining species thus causing agglomeration of species that belong to different subsections.

EXPERIMENTAL

Sample collection. The study area selected was the provenance of Attiki in central Greece and the investigated trees were within a 30 km radius area, in natural forests. Based on their morphoanatomical characteristics, a total of 113 trees were chosen as representatives of the five pine species. Voucher specimens are deposited in the laboratory's herbarium at NCSR "Demokritos". Needles (50 g) were gathered in a random manner from various parts of the tree crowns to reduce plant plasticity effects. The needles of each tree were cut into small pieces (3–5 mm) and separately hydrodistilled.

Sample analysis. The needles were steam distilled for 2 hr in a modified apparatus with a water cooled oil receiver, to reduce hydrodistillation overheating artifacts. The essential oils were taken up in Et_2O and subsequently dried over $MgSO_4$. The GC conditions used were: DB-1 (30 m × 0.32 mm) or DB-5 (60 m × 0.25 mm) fused silica gel column; carrier gas He (2 ml min^{-1}); on column injector 200°; FID 250°; column temp. 50° for 5 min then 3° min^{-1} to 250°. Mass spectra were obtained from a GC-MS system operating in the EI mode (equipped with a 60 m × 0.25 mm DB-5 capillary column). The identification of the chemical constituents was based on comparisons of their R_ts and mass spectra with those obtained from authentic samples and/or the NIST/NBS and Wiley library spectra.

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