Needle Terpenoid Composition of *Pinus halepensis* (Mill.) Trees Infested by the Scale Insect *Marchalina hellenica* (Genn.) in Greece

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Abstract

Needle terpenoid composition was determined by using GLC-MS in *Pinus halepensis* (Mill.) trees that were infested and not infested by the scale insect *Marchalina hellenica*. The study area was within the Forest National Park of the Cape Sounion, southern Attica region, Greece. A total of 43 compounds, 32 of which were identified, were detected, including monoterpenes, sesquiterpenes, and neutral diterpenes. The healthy trees showed higher mean concentration for the monoterpenes fraction as well as for the diterpene fraction than the mean concentration of infested trees; whereas, the concentration for the sesquiterpene fraction was more or less similar in infested and non-infested trees. The statistical analysis of terpene data showed the existence of quantitative differences between healthy and infested trees mainly for the components β-caryophyllene, neoabietal, α- humulene, cembrene, and neoabietol. A Ward cluster analysis based on selected major compounds classified all trees in two chemotypes, with the majority of healthy trees belonging to one chemotype and most of the infested trees belonging to the second.

Key words: *Pinus halepensis*, *Marchalina hellenica*, needle terpenoids

Introduction

Marchalina hellenica is a scale insect which in Greece infests mainly *Pinus halepensis* (Mill.) and *Pinus brutia* (Ten.) (Avtzis 1985). The insect attack results in the production of honeydew, which is used as a feeding substrate by honeybees and converted into honey (Erlinghagen 2001, Gounari 2006). During the mid-1990s, *M. hellenica* was artificially introduced into the pinewoods of the Attica region in Greece in order to increase the total honey yield. The artificial infestations resulted in an ecological imbalance in the ecosystems of regional Aleppo pine. Current field observations show the overpopulated occurrence of the insect in the pinewoods of the area (Gallis 2007). Terpene composition in conifers is usually strongly inherited, not greatly affected by environmental conditions, and offers a valuable tool to study several scientific problems such as: hybrid identification, introgression, tree resistance to insect attacks and diseases, and others (Hannover 1992, Squillace 1987). The objectives of this study are: a) to determine the qualitative and quantitative terpenoid composition in the needles of Aleppo pine trees that are infested and not infested by the scale insect *M. hellenica*, and b) to investigate if the needle terpenoid composition could be used to study the parasitism of the scale insect to Aleppo pine trees.

Materials and Methods

All trees investigated were sampled from two natural stands of Aleppo pine: the "Markati" and "Agia Triada" in the area of the National Park of Cape Sounion, southern Attica region, Greece. A total of

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22 trees, including 11 trees infested by *M. hellenica* and 11 healthy trees (not infested), were sampled in late March 2006. The sampling of the infested trees in the field depended on the macroscopic evaluation of the insect's attack on the tree as described in the literature (Erlinghagen 2001, Gounari 2006), including: white cottonish secretions, the degree of tree desiccation, and the existence and the extent of drained needles. The trees were of similar age (over 30 years old) in both stands, have been subjected to the same silvicultural treatments, and so far have been protected effectively from forest fires. As mentioned above, healthy and infested Aleppo pine trees occurred in the same area in both sampled stands in our study. Gounari (2006) states that it is normal that both infested and non-infested Aleppo pine trees occur in the same area. This differentiation is due to phenol-immunity phenomenon. The differentiation could also be permanent or temporary and periodic, with permanent immunity controlled genetically (Smirnoff and Valero 1975).

From each tree, 1-year-old needles were collected from the upper 1/3 part of the crown and from the same side to avoid any epigenetic variation in terpene composition due to tissue age, crown position, grown conditions, etc. After collection, the samples were put in plastic bags and stored in a refrigerator at -20 ^{o}C for about 2 months. The needle samples were transferred into a portable ultra low freezer with CO_2 (dry ice) and transported the same day by plane to Spain. Chemical analysis was conducted by the Department of Forestry Engineering, Escuela Tecnica Superior de Ingenieros (ETSI), Montes, Polytechnic University of Madrid, Spain.

The needles were cut into small pieces (2 to 4 mm). A known weight of needles (3 g approx.) was extracted with 5 ml petroleum ether/diethyl ether (1:1) for 24 hours at 4 ^{o}C. Isobuthylbencene (60 μg/ml), heptadecane (60 μg/ml), and heptadecanoic acid (60 μg/ml) were used as internal standards. One μl of the mixture was injected into a gas chromatograph. The samples were analyzed with an HP5890A gas chromatograph and connected to an HP 5971 mass detector (EI, 70 eV). The following temperature program was used: the oven temperature was initially at 60 ^{o}C, increasing at a rate of 4 ^{o}C/minutes to a final temperature of 270^{o} C, and was held for 10 min. The injector's temperature was set at 260 ^{o}C and the detector's temperature was set at 300 ^{o}C. A DB-5 capillary column, 30 m x 0.25 mm (0.25 μm film thickness), was used for component separation. Helium was used as a carrier gas with 15 psi column head pressure and a split ratio of 1:50. For quantitative measurements, by the internal standard method, additional injections of replicate samples were made using a flame ionization detector under the same working conditions. All compounds were identified on the basis of their retention time and their electron impact (EI) mass spectra by comparing them with those in the database and literature. Hewlett Packard Chemstation software was used for peaks integration. Components were quantified as a percentage contribution of each peak to the total terpenoids found in the chromatogram, i.e. total oleoresin basis. The concentration of each terpenoid fraction (monoterpenes, sesquiterpenes, and neutral diterpenes) was also calculated, using the internal standard method. For statistical analysis, a Statistical Packages for Social Sciences statistical package (SPSS/PC) was used. Thirteen components were selected due to their presence in amounts more than 2 percent in most samples. To separate the infested and healthy pine trees, a cluster analysis (Ward, squared Euclidian distance) on the basis of the percentage amounts of the selected components was used. A discriminate analysis was performed to see which components have the largest effect in classifying trees as healthy or infested. The percentages of the components were transformed into arcsine - square root functions (Kung 1988) before statistical analysis.

Results

A total of 43 compounds were detected in current year needles of Aleppo pine trees analyzed by Gas Liquid Chromatography Mass Spectrometry (GLC-MS), including monoterpenes, sesquiterpenes, and neutral diterpenes (table 1).

Table 1—Terpenoid composition (total oleoresin basis) in *Pinus halepensis* needles

		Components	Healthy Trees % mean	std	Wounded Trees % mean	std
1		α-thujene	0.13	0.19	0.19	0.15
2	●[a]	α-pinene	5.30	1.96	4.52	2.25
3		camphene	0.95	1.03	0.01	0.02
4	●	sabinene	2.41	4.19	2.01	1.04
5		β-pinene	0.78	0.69	0.47	0.26
6	●	myrcene	4.87	4.68	5.88	4.43
7		3-δ-carene	0.31	0.55	0.62	0.57
8		α-terpinene	0.20	0.23	0.10	0.22
9		limonene+phellandrene	0.51	0.79	0.31	0.21
10		trans-β-ocimene	0.15	0.21	0.17	0.18
11		γ-terpinene	0.13	0.19	0.14	0.14
12	●	terpinolene	2.47	2.82	2.03	0.97
13		terpine-4-ol	0.05	0.12	0.02	0.04
14		α-terpineol	0.01	0.04	0.03	0.10
15		bornyl acetate	0.02	0.05	0.11	0.20
16		α-copaene	0.24	0.12	0.22	0.22
17	●	β-caryophyllene	11.89	1.96	14.01	2.42
18	●	α-humulene *	2.03	0.32	2.39	0.41
19		germacrene-D	1.23	1.05	0.96	0.32
20	●	phenylethyl isovaleranate	3.99	1.33	3.31	1.59
21		α-Muurolene	0.31	0.20	0.19	0.14
22		δ- cadinene	0.39	0.25	0.49	0.19
23		β-cadinene	0.22	0.18	0.17	0.19
24		α-bisalolene	0.17	0.09	0.13	0.12
25		β-elemene	0.25	0.13	0.08	0.07
26		M+220	0.24	0.13	0.21	0.19
27		M+222	0.30	0.20	0.33	0.15
28		M+222	0.26	0.16	0.26	0.05
29	●	cembrene	33.03	11.27	24.69	9.22
30		M+286 (Diterpenic aldehyde)	1.09	1.21	0.90	0.35
31		dehydroabietal	1.23	0.92	1.38	0.48
32	●	methyl levopimarate	1.88	1.52	3.72	3.45
33	●	methyl dehydroabietate	1.81	1.31	2.28	0.90
34	●	neoabietal	1.43	1.05	2.77	1.82
35	●	methyl 8.13(15) abietadien 18 oate	2.57	1.77	2.78	1.36
36	●	neoabietol	2.10	1.42	3.79	2.31
37		M+332	0.92	0.64	1.71	1.19
38		M+ 314	1.91	1.07	1.66	1.35
39		M+330	1.35	0.69	1.60	1.24
40		M+332	0.83	0.49	1.53	1.03
41		M+330 ($C_{21}H_{30}O_3$)	1.12	1.16	1.81	1.00
42		M+316	0.74	0.55	1.43	0.83
43		M+406	0.62	0.63	1.16	0.58
		% monoterpenes total	19.68		19.41	
		% sesquiterpenes total	23.31		23.76	
		% diterpenes total	57.01		56.82	
	Mean concentration monoterpenes (mg g^{-1})		1.25		1.10	
	Mean concentration sesquiterpenes (mg g^{-1})		1.50		1.55	
	Mean concentration diterpenes (mg g^{-1})		1.27		0.89	

[a] Components selected for further evaluation.

The components α-pinene, sabinene, myrcene, and terpinolene were the major constituents in the monoterpenes fraction of all the samples analyzed. The sesquiterpene fraction is characterized by the high amounts of β-caryophyllene found to be in higher levels (14.01 percent) in infested trees compared with those (11.89 percent) in healthy trees. The macrocyclic diterpene cembrene is the major component among all the samples analyzed in our investigation. Cembrene is found with higher amounts (33.03 percent) in healthy trees than in infested trees (24.69 percent). The mean (mg g^{-1} dry weight of needles) concentration of monoterpene, sesquiterpene, and diterpene fractions for infested and healthy trees was also calculated too (table 1). The healthy trees showed higher mean concentration (1.25 mg g^{-1}) for the monoterpenes fraction in comparison to mean concentration (1.10 mg g^{-1}) of infested trees. The concentration of the sesquiterpene fraction was similar in infested (1.55 mg g^{-1}) and in healthy trees (1.50 mg g^{-1}). Concerning the diterpene fraction, the differences between healthy (1.27 mg g^{-1}) and infested (0.89 mg g^{-1}) trees are larger.

Compounds selected for statistical analysis were α-pinene, sabinene, myrcene, terpinolene, β-caryophyllene, α-humulene, phenylethyl isovaleranate, cembrene, methyl 8, 13 (15) abietadien 18 oate, neoabietol, methyl levopimarate, methyl dehydroabietate, and neoabietal. The last three compounds were present in amounts less than 2 percent in healthy trees, but were also included because they occurred in amounts more than 2 percent in infested trees. The dendrogram produced by hierarchical cluster analysis revealed the classification of all Aleppo pine trees in two major clusters (clusters A and B). Cluster A contains 12 trees in total (54.54 percent), while cluster B contains 10 pine trees in total (45.45 percent). The frequencies of clusters in each group of trees differ significantly. A total of 81.81 percent of the infested trees (nine trees) included in our analysis belong to cluster A, and 72.72 percent of the healthy trees (8 trees) belong to cluster B (table 2). The two clusters (A and B) differ in the amounts of the most components. The components: β-caryophyllene, α-humulene, neoabietal, cembrene, and neoabietol showed differences between clusters. Results of discriminate analysis showed that the above mentioned five compounds have the largest discriminate ability between healthy and wounded trees. The quantitative terpene composition of clusters expressed as percent contribution on the basis of the total area of 13 components is shown in table 2.

Table 2—Mean (percent) terpene composition of clusters, distribution, and percent frequency in healthy and wounded Aleppo pine trees

	Components	Cluster A (mean %)	Cluster B (mean %)
1	α-pinene	7,28	5,69
2	sabinene	2,89	3,08
3	myrcene	6,28	8,26
4	terpinolene	3,76	2,15
5	β-caryophyllene	19,86	14,36
6	α-humulene	3,40	2,44
7	phenylethyl isovaleranate	4,94	6,46
8	cembrene	29,35	48,51
9	methyl levopimarate	5,04	2,22
10	methyl dehydroabietate	3,61	1,70
11	neoabietal	4,14	1,37
12	methyl 8, 13 (15) abietadien 18 oate	3,74	3,43
13	neoabietol	5,67	1,99
	Total trees	12	10
	% total frequency	54,54	45,45
	Healthy trees	3	8
	% frequency of healthy trees	27,27	72,72
	Wounded trees	9	2
	% frequency of wounded trees	81,81	18,18

Discussion

The literature concerning the relation of terpenes in *P. halepensis* with an attack by *M. hellenica* are rather rare. Mitta et al. (2002) analyzed cortical oleoresin terpenes in Aleppo pine trees infested by the insect from Crete Island, Greece. The authors reported that sensitivity of the pine to attack by *M. hellenica* was significantly correlated with high levels of α- pinene and low levels of limonene and α - terpinyl acetate. In our study, statistically significant differences in quantitative terpene composition between infested and non-infested trees were found for β-caryophyllene, α-humulene, neoabietal, cembrene, and neoabietol. A Ward cluster analysis, based on relative (percent) quantity of 13 selected components, revealed that arrangement of all trees into two clusters occurred at different frequencies. The quantitative terpene composition of cluster A is characterized by high amounts of β - caryophyllene (19.86 percent) and the composition of cluster B by very high amounts (48.51 percent) of cembrene (table 2). With respect to the limited number of trees analyzed from our results, it could be suggested that the presence of cluster A indicates infested trees, while the presence of cluster B indicates healthy ones. To conclude, the needle terpenoid analysis by gas chromatography seems to be a valuable tool to help the scientists study the parasitism of *M. hellenica* to Aleppo pine. However, further research will be required, with analysis of a larger number of samples by GLC-MS, including trees from different seasons of the year as well from several locations around the area, to clarify the influence of *M. hellenica* on terpenoid content and composition of *P. halepensis* in order to study the interaction between insect and pine tree as well the parameters involved in this complicated phenomenon.

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