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Variations in Allelochemical Composition of Leachates of Different Organs and Maturity Stages of *Pinus halepensis*

Catherine Fernandez · Yogan Monnier · Elena Ormeño · Virginie Baldy · Stéphane Greff · Vanina Pasqualini · Jean-Philippe Mévy · Anne Bousquet-Mélou

Abstract We investigated changes in the occurrence of allelochemicals from leachates of different *Pinus halepensis* organs taking into account the stages of pine stand age (i.e., young <15-years-old, middle age±30-years, and old > 60-years-old). GC-MS analysis of aqueous extracts revealed approx. 59 components from needles and roots. The major constituents were divided into different phytochemical groups—phenolics (50%), fatty acids (44%), and terpenoids. Further analyses were carried out to characterize the distribution of allelochemicals in different organs and *P. halepensis* successional stages. Roots and needles had two distinct chemical profiles, while needle leachates were composed mainly of oxygenated terpenoids (e.g., α-eudesmol, α-cadinol, and α-terpineol). Roots mainly contained fatty acids. Needles from young pine stands had the highest content of monoterpenes, suggesting their role as potential allelochemicals that could help young pine stands to establish. Pooling the different functional chemical groups showed that needles and, to a lesser extent, old roots, had higher chemical diversity than the roots of young and medium-aged pines. The highest diversity in phenolic constituents and fatty acids was in young needles (D_{chem}=2.38). Finally, caffeic acid, a compound that has allelopathic properties was found in aqueous extracts at high concentrations in both young needles and old roots. The role of this compound in mediation of biological interactions in *P. halepensis* ecosystem functioning is discussed.

Keywords *Pinus halepensis* Miller · Allepo pine · Phenolic compounds · Terpenoids · Needles · Roots · Soil · Allelopathy

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Introduction

Plant secondary metabolites affects ecosystem processes and biodiversity. Release of secondary metabolites into the environment, an important driver of biotic interactions, occurs through litter decomposition, root exudates, vaporization into the air, and leaching from plant parts to the soil (Rice 1984). Production of secondary compounds is often associated with plant protection. Compounds in aboveground parts help to protect plants against microbes, herbivores (Vernenghi et al. 1986), and/or UV irradiation (Delfine et al. 2003). Root compounds may be produced in response to soil-borne pathogens (Valette et al. 1998).

Allelochemicals also may be involved with beneficial interactions (Dicke et al. 2003), such as attracting pollinators to flowers and leaves (Caissard et al. 2004) or signaling events in plant-plant, plant-microbe, or plant-nematode interactions (Hiltpold and Turlings 2008). Among vascular plants, almost all allelochemicals are secondary metabolites and have the potential to impact ecosystem structure and function. Allelopathic components

also play a role in regulating plant diversity (Chou 1999), establishment of invasive species (Bousquet-Mélou et al. 2005), and the dynamics in arid environments (Karageorgou et al. 2002).

In the Northern Mediterranean basin, *Pinus halepensis* Miller (Pinaceae, Pinales) is a pioneer and expansionist species that colonizes abandoned agricultural lands characterized by high biodiversity (Roche and Tatoni 1995). Owing to its richness of secondary metabolites (Macchioni et al. 2003), *P. halepensis* may play an important role in plant succession through several processes. For example, secondary compounds (terpenoids and/or phenolic compounds) can affect root symbionts and site quality, by interfering with decomposition, mineralization, and humification (Kainulainen and Holopainen 2002). They can also be involved with interspecific competition phenomena through allelopathic interactions (Rice 1984). Indeed, *P. halepensis* may inhibit seedling establishment of various species in pine stands, suggesting the allelopathic nature of litter, leaf leatchates, and/or root exudates (Fernandez et al. 2006, 2008; Navarro-Cano et al. 2009). Other conifers such as *Pinus sylvestris* L. (Bulut and Demir 2007), *P. densiflora* (Sieb. et Zucc.) (Kato-Noguchi et al. 2009), *Picea abies* (L.) Karst. (Pellissier 1994), or *Picea mariana* (Mill.) (Mallik and Newton 1988) also have allelopathic potential.

Allelopathic potential may be modified by several factors such as the age of the donor plant (Inderjit and Asakawa 2001). Therefore, to understand the role of *P. halepensis* in secondary succession, it is essential to evaluate whether allelochemicals vary in diversity, amount, or function in different plant organs (needles vs. roots), and at different successional stages. For this reason, we analyzed the composition of secondary metabolites in aqueous extracts of roots and needles of *P. halepensis* by searching for polar (fatty acids and phenolic compounds) and less polar compounds (terpenoids) known to be allelopathic (Rice 1984), and we determine whether the chemical diversity of aqueous extracts depends on changes in the age of *P. halepensis* stands.

Methods and Materials

Samples Sites In order to evaluate the variability and allelopathic potential of *P. halepensis* in relation to different stages of secondary succession, three age classes of *P. halepensis* were chosen: (i) Young *P. halepensis* (<15-years-old) called successional stage "Y"—that included meadows colonized by dispersed individuals; (ii) Medium-aged *P. halepensis* (±30-years-old) called successional stage "M"—monospecific or recently closed forest stands, without understory; and (iii) Old *P. halepensis* (>60-years-old) and called successional stage "O"—mature forest with well-developed understory.

Three replicates were collected from each successional stage. Sites were selected along the Southern hillside of the Luberon Mountains in the Natural Regional Park (South of France), on the basis of similar global index (climatic and topoedaphic conditions), by using a model developed by CEMAGREF (Ripert and Vennetier 2002). All sites featured deep agricultural soils (>1 m) of Rendoll in "Soil Taxonomy" (Soil Survey Staff 1999) with no slope and high fertility for *P. halepensis*.

Plant Material Collection and Aqueous Leachates Preparation Needles and roots were collected from five individuals at each site. Needles were harvested from the entire tree crown, and roots were sampled in close proximity to the pines (diam <1.5 m). Just after harvest, needle and root extracts were soaked in water (50 g fw of tissue in 250 ml of distilled water). Extracts were done at room temperature (18°–20°C) and kept in darkness for 24 h. Needle extracts simulated leaf leaching, while root extracts simulated root exudates.

Bioassays. The phytotoxicity of aqueous extracts was tested in previous studies with *Lactuca sativa* seeds, *Linum strictum* (allelopathy, Fernandez et al. 2006), and *Pinus halepensis* (autotoxicity, Fernandez et al. 2008). In these studies, phytotoxicity was analyzed in terms of germination rate and seedling growth (roots and hypocotyles) (Fernandez et al. 2006, 2008), but also in terms of sapling growth (Monnier et al. 2008).

Chemical Analyses

Instrumentation GC-MS analyses were performed on a Hewlett-Packard 6890 GC coupled with an HP5973N Mass Selective Detector. The GC was equipped with an HP-5MS capillary column (30 m×0.25 mm× 0.25 µm—J&W Scientific). Samples were injected with an ALS 7673 Automatic Injector in splitless mode (2 µl for 1 min) for polar compounds (phenolics and fatty acids), and in pulsed splitless mode (5 µl at 25 psi for 1 min) for less polar compounds (terpenoids). Purge flow was set to 50 ml/min after 1 min, except for qualitative studies of polar compounds, for which 30 ml/min were used. Helium (99.995%) was used as carrier gas. A constant flow of 1 ml/min was maintained throughout the runs. Three different oven temperatures were used for qualitative and quantitative studies of polar compounds, and one for less polar compounds. The first program began at 70°C, ramped to 270°C at 5°C/min, and remained at this temperature for 10 min. The second program began at 50°C, increased to 220°C at 5°C/min, and remained at this temperature for 6 min. The third program began at 50°C, increased to 160°C at 2°C/min, and remained at this temperature for 5 min. The

injector temperature (250°C) and MSD transfer line heater (280°C) were the same for all injections. The mass spectrometer parameters for EI mode were: ion source, 230°C; MS quadrupole, 150°C; electron energy, 70 eV; Electron Multiplier Energy 1100–1300 V. Data were acquired in scan mode from 40 to 500 amu for qualitative analyses and quantitative analyses of less polar compounds, and in SIM mode for quantitative analyses of polar compounds.

Chemicals HPLC grade methylene chloride, ethyl acetate, cyclohexane, and acetonitrile were obtained from SDS (Peypin, France). HPLC grade water was used for extractions and a Milli Q system was used for analytical procedures. N, O−bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was used as the derivatizing reagent for qualitative studies with polar compounds. Methylation reagent (methyle iodure, 99.5% purity), internal standard (3-chloroanisole, >97% purity or undecane, 99% purity), HPLC grade methanol, phenolics, fatty acids, phase transfer catalysts (tetrahexylammonium bromure—THAB, > 99% purity), and tri-n-butylmethylphosphonium polymer bound (1.4 mmol Cl^-/g resin—TBMP) were used for quantitative analyses. Analytical grade sodium chloride and potassium dihydrogenophosphate were provided by Prolabo (VWR, France).

Qualitative Analyses

Polar Compounds Qualitative studies of *P. halepensis* fatty (di)acids and phenolics of root and needle extracts were carried out with three randomly-sampled extracts (10%) mixed for each analysis.

The mixed solution was extracted×3 with ethyl acetate (25 ml). The resulting three fractions were pooled, concentrated to dryness, and suspended in methylene chloride (1 ml). The procedure was repeated twice, and the combined solutions were evaporated to dryness by using a stream of helium to remove residual water.

Acetonitrile (200 µl) and BSTFA+1%TMCS (200 µl) were added to the residue. The resulting solution was incubated for 1 h at 70°C, cooled, filtered, and analyzed by GC-MS to produce the trimethylsilyl derivatives.

Less Polar Compounds Aqueous extracts (10 ml of a 10% solution of each extract) were mixed with cyclohexane (1 ml) containing undecane (2 mg/ml) for 1 h. After phase separation, the organic layers were injected onto the GC-MS.

Compound identification was done by comparison of MS spectra to those of reference standards. Database searches in the HP mass spectral libraries were conducted for unidentified components. Retention indexes of compounds were determined relative to Wisconsin Diesel Range Hydrocarbons injection (Interchim, Montlucon, France) and confirmed by comparison with those expected in literature (Adams 1989).

Quantitative Analyses

Polar Compounds Quantitative analyses were performed on needle and root extracts of young, medium, and old pines using a method adapted from Fiamegos et al. (2004). An extraction-derivatization technique was used for phenolics via Phase Transfer Catalysis (PTC). The methylation and extraction methodology was improved to fit phenolics and fatty acids and diacids. The method was tested for a small number of compounds (2 or 3) from each chemical family: fatty acids (palmitic and stearic acids), fatty diacids (succinic and azelaic acids), simple phenols (catechol and pyrogallol), acetophenones (acetovanillone and acetosyringone), phenolic acids (4-hydroxybenzoic, protocatechuic, and gallic acid), and cinnamic acids (*p*-coumaric, caffeic, and sinapic acids). These compounds were selected based on their occurrence in *P. halepensis* (qualitative analysis) or ability to be methylated under these conditions. The quantified derivatives may include several allelochemicals because of the methylation process.

Stock solutions (1 mg/ml) of each compound were prepared by dissolving pure standards (25 mg) in deionized water (25 ml). Methanol/water (1:1 *v*/*v*) was used for less soluble compounds. THAB in dichloromethane (0.1 M; 217.3 mg in 5 ml) and the internal standard were dissolved in dichloromethane.

The procedure was carried out in 35 ml-Pyrex® tubes equipped with PTFE screw caps. Each individual extract (10 ml) was added with stirring to the following solution: KH_2PO_4 buffer (500 µl of a 1 M solution; pH 8.0), TBMP (50 mg), THAB in dichloromethane (100 µl of a 0.1 M solution), internal standard in dichloromethane (50 µl of a 100 µg/ml solution), dichloromethane (850 µl), and methyle iodure (100 µl). Tubes were sealed and heated (80°C) for 1 h to allow methylation. The solutions were cooled, saturated with NaCl, and vigorously shaken. After separation, the organic layer was removed, filtered through a filter syringe (0.45 µm), and analyzed by GC-MS.

The same procedure was used to methylate standards for calibration curves (constructed by taking the ratio of external variable standard to internal constant standard).

Less Polar Compounds An identical procedure was used for less polar compounds. Quantitation was relative to undecane.

Table 1 Mean concentration (± standard deviation) of compounds in root and needle extracts of *Pinus halepensis* (ng/ml)

RI	Common name	Roots			Needles					
		Young	Medium	Old	Young	Medium	Old	F	P	Sign
Monoterpenes										
970	α-Pinene*	2.5 (0.4) a	2.1 (0.3) a	2.3 (0.4) a	0.7 (0.1) b	0.7 (0.2) b	0.3 (0.1) b	12.630	0.001	***
982	Thuja-2,4(10)-diene**	5.2 (1.4) a	3.7 (0.5) a	3.1 (0.5) a	0.0 b	0.1 (0.1) b	Tr b	30.030	0.001	***
994	Sabinene**	0.4 (0.1) a	0.2 (0.1) a	0.1 (0.1) a	0.9 (0.1) b	1.1 (0.2) b	1.1 (0.1) b	19.780	0.001	***
996	β-Pinene*	0.1 (0.1) a	0.2 (0.1) a	0.1 (0.1) a	0.3 (0.1) a	0.1 (0.1) a	0.1 (0.1) a	0.730	0.605	ns
1007	β-Myrcene*	0.8 (0.2) a	0.4 (0.1) a	0.5 (0.1) a	1.1 (0.3) b	0.5 (0.1) a	0.5 (0.1) a	3.400	0.008	***
1026	α-Terpinene*	0.2 (0.1) a	Tr a	0.2 (0.1) a	0.8 (0.2) b	0.5 (0.1) b	0.5 (0.1) b	13.130	0.001	***
1033	p-Cymene*	0.6 (0.3) b	0.0 a	0.5 (0.1) b	0.1 (0.1) a	0.5 (0.2) b	0.5 (0.1) b	2.860	0.020	**
1064	γ-Terpinene*	0.2 (0.2) a	0.0 a	0.0 a	0.7 (0.3) b	0.4 (0.2) b	0.5 (0.2) b	3.140	0.001	***
1113	1,3,8-p-Menthatriene**	0.2 (0.2) a	0.0 a	0.0 a	0.0 a	0.2 (0.1) a	0.3 (0.2) a	1.950	0.095	ns
Oxygenated monoterpenes										
1071	trans-p-Menth-2-en-1-ol**	4.6 (1.1) a	3.7 (1.3) a	4.0 (1.4) a	3.9 (1.0) a	13.5 (1.7) b	12.4 (2.3) b	8.440	0.001	***
1076	cis-Linalool oxide**	0.7 (0.4) a	0.5 (0.3) a	0.3 (0.1) a	4.7 (0.6) bc	6.3 (0.8) c	3.0 (0.5) b	42.390	0.001	***
1091	Fenchone**	0.0 a	0.4 (0.2) b	1.1 (0.3) c	0.1 (0.1) a	0.0 a	0.0 a	8.870	0.001	***
1092	trans-Linalool oxide**	17.7 (13.3) b	1.6 (0.5) a	2.2 (0.6) a	8.8 (1.6) b	8.9 (0.9) b	6.2 (0.8) ab	7.760	0.001	***
1100	cis-p-Menth-2-en-1-ol**	4.6 (0.9) ab	2.2 (0.5) a	1.9 (0.4) a	7.5 (1.5) bc	12.6 (1.7) c	11.3 (1.8) c	14.740	0.001	***
1114	Fenchol, endo-**	0.2 (0.2) a	0.3 (0.1) a	1.1 (0.3) a	0.0 a	0.6 (0.6) a	Tr a	2.620	0.09	ns
1114	cis-Sabinene hydrate**	0.3 (0.1) a	0.2 (0.1) a	0.7 (0.2) a	2.4 (0.6) b	1.8 (0.2) b	1.9 (0.2) b	13.270	0.001	***
1136	trans-Pinocarveol**	10.3 (2.7) b	5.6 (1.1) ab	6.0 (0.9) b	5.7 (0.7) b	4.3 (0.6) a	3.7 (0.5) a	2.500	0.037	*
1138	cis-Verbenol*	4.5 (1.1) b	1.3 (0.4) a	4.4 (3.4) ab	0.6 (0.6) a	0.2 (0.1) a	0.2 (0.2) a	7.750	0.001	***
1141	Camphor*	20.4 (5.5) b	43.5 (9.5) c	50.1 (10.3) c	1.7 (0.2) a	3.6 (0.5) a	3.1 (0.5) a	7.750	0.001	***
1143	trans-Verbenol**	86.2 (28.4) b	45.7 (6.8) b	45.9 (6.6) b	5.8 (1.0) a	7.7 (2.5) a	5.0 (1.1) a	37.820	0.001	***
1163	Borneol*	44.2 (7.6) b	36.5 (6.3) b	46.5 (6.1) b	26.5 (2.5) ab	18.9 (3.2) a	27.4 (3.4) ab	5.570	0.001	***
1171	cis-3-Pinanone**	0.8 (0.2) bc	1.3 (0.3) c	1.7 (0.5) c	0.0 a	0.4 (0.2) ab	Tr a	13.070	0.001	***
1175	4-Terpineol*	14.1 (3.2) a	12.0 (3.8) a	25.1 (8.5) a	173.0 (40.3) b	88.0 (11.7) b	93.4 (11.4) b	25.970	0.001	***
1189	α-Terpineol*	7.2 (1.7) a	5.9 (1.0) a	14.2 (3.3) a	73.5 (7.9) bc	155.9 (7.8) c	117.1 (11.9) bc	91.76	0.001	***
1194	Myrtenol**	27.4 (4.9) b	23.1 (2.7) b	31.0 (4.5) c	2.3 (0.3) a	3.6 (0.4) a	2.4 (0.2) a	82.120	0.001	***
1203	Bornyl ou Isobornyl derivative (formate?)**	6.8 (1.9) ab	4.1 (0.7) a	4.0 (0.9) a	7.2 (0.9) ab	8.9 (1.0) b	5.7 (1.2) ab	4.020	0.001	*
1206	Verbenone*	43.2 (13.3) b	36.6 (4.3) b	36.7 (7.0) b	4.6 (0.6) a	9.3 (1.8) a	6.1 (1.0) a	23.770	0.001	***
1218	(E)-2-Caren-4-ol**	2.0 (0.7) bc	0.5 (0.3) a	0.9 (0.1) a	1.4 (0.2) ab	3.4 (0.4) c	2.0 (0.2) bc	10.420	0.001	***
1285	Bornyl acetate or Isobornyl acetate**	18.1 (2.7) c	13.5 (1.9) bc	11.1 (1.6) b	0.5 (0.4) a	0.0 a	0.0 a	28.990	0.001	***
Sesquiterpenes										
1415	β-Caryophyllene*	0.9 (0.2) bc	1.2 (0.3) c	0.4 (0.2) abc	0.6 (0.2) abc	0.3 (0.1) ab	0.0 a	5.320	0.001	***
1449	α-Caryophyllene*	0.1 (0.1) a	0.1 (0.1) a	Tr a	0.1 (0.1) a	0.1 (0.1) a	0.1 (0.1) a	1.160	0.338	ns
1498	α-Muurolene**	1.1 (0.6) ab	0.5 (0.5) a	3.1 (0.8) b	0.5 (0.5) a	0.8 (0.6) ab	0.5 (0.5) a	3.140	0.012	**
Oxygenated sesquiterpenes										
1548	Elemol**	0.0 a	0.0 a	0.0 a	2.6 (1.4) b	22.2 (2.7) b	17.8 (4.0) b	79.990	0.001	***
1577	Caryophyllene oxide*	8.5 (4.9) ab	2.3 (1.3) a	3.2 (0.8) ab	17.8 (9.4) b	18.2 (8.3) b	36.4 (26.3) b	4.640	0.009	**

Table 1 (continued)

RI	Common name	Roots			Needles					
		Young	Medium	Old	Young	Medium	Old	F	P	Sign
1595	Guaiol**	0.4 (0.3) a	14.7 (11.0) a	5.4 (3.5) a	11.8 (2.7) a	10.9 (2.4) a	7.1 (1.5) ba	1.06	0.397	ns
1644	β-Eudesmol**	1.5 (0.6) a	1.5 (0.5) a	2.0 (0.3) a	18.6 (3.1) c	8.5 (1.2) bc	8.3 (1.3) b	28.600	0.001	***
1651	α-Eudesmol**	0.4 (0.3) a	0.5 (0.5) a	0.0 a	8.4 (1.5) b	5.0 (0.7) b	4.6 (0.7) b	48.630	0.001	***
1651	α-Cadinol**	0.4 (0.2) a	0.5 (0.4) a	Tr a	9.0 (1.4) b	5.5 (0.8) b	5.2 (0.8) b	53.210	0.001	***
Fatty acids										
1041	Succinic Acid*	8161.1 (797.0) c	5654.5 (713.8) b	5229.9 (679.1) b	1466.6 (236.5) a	850.0 (34.7) a	985.3 (105.9) a	59.560	0.001	***
1557	Azelaic acid*	17.6 (4.3) a	11.9 (1.2) a	16.3 (2.1) a	51.5 (4.1) b	38.6 (2.4) b	47.5 (5.6) b	31.030	0.001	***
1935	Palmitic acid*	552.5 (73.6) d	336.3 (43.6) bcd	452.9 (46.4) cd	150.5 (26.4) abc	160.8 (20.6) ab	122.6 (19.0) a	9.760	0.001	***
2159	Stearic acid*	226.1 (24.0) bc	233.9 (33.1) bc	315.2 (32.0) c	140.9 (20.5) abc	102.7 (11.3) ab	85.3 (15.4) a	7.010	0.001	***
Phenolics										
1154	Catechol*	92.0 (83.3) b	5.5 (0.6) ab	5.6 (0.8) ab	2.8 (0.3) a	3.0 (0.2) a	3.6 (0.3) a	4.910	0.001	***
1325	Pyrogallol*	392.4 (37.4) c	388.5 (29.7) c	495.2 (79.0) c	156.9 (23.0) b	73.1 (8.7) a	85.9 (13.6) a	35.450	0.001	***
1384	4-Hydroxybenzoic acid*	120.9 (36.5) b	45.0 (9.6) a	55.9 (5.9) ab	340.4 (37.4) c	74.5 (8.7) b	86.9 (13.9) b	25.270	0.001	***
1602	Protocatechuic acid*	3127.1 (728.2) b	1985.7 (165.5) b	1905.1 (195.8) b	1994.3 (171.6) b	602.3 (76.0) a	851.2 (113.0) a	19.630	0.001	***
1731	Gallic acid*	1418.5 (385.8) b	1333.4 (193.7) b	1473.3 (203.6) b	840.7 (130.8) b	167.8 (29.1) a	198.5 (49.3) a	32.970	0.001	***
1576	Acetovanillone*	64.4 (9.4) a	43.3 (3.4) a	50.2 (3.8) a	216.4 (19.5) b	55.9 (4.1) a	65.5 (10.3) a	35.610	0.001	***
1689	Acetosyringone*	3.2 (1.6) b	1.2 (0.5) ab	1.4 (0.5) ab	4.7 (0.9) c	0.4 (0.1) a	0.5 (0.1) ab	10.570	0.001	***
1681	p-Coumaric acid*	905.3 (134.8) b	364.3 (85.6) a	323.0 (71.5) a	891.9 (93.7) b	236.1 (23.0) a	217.5 (26.9) a	19.340	0.001	***
1894	Caffeic acid*	366.7 (52.9) bc	292.3 (67.3) abc	801.2 (256.9) d	781.0 (110.3) d	166.9 (14.1) a	220.3 (35.5) ab	10.500	0.001	***
2035	Sinapic acid*	3.0 (0.6) a	4.5 (1.4) a	7.5 (1.0) b	7.5 (1.2) b	5.3 (0.8) a	6.3 (1.0) ab	3.650	0.005	**
Others										
987	Benzaldehyde*	0.2 (0.1) a	0.0 a	0.0 a	0.1 (0.1) a	0.1 (0.1) a	0.1 (0.1) a	1.350	0.253	ns
1021	o-Methylanisole**	0.6 (0.2) b	0.2 (0.1) ab	0.1 (0.1) a	0.1 (0.1) a	Tr a	Tr a	5.990	0.001	***
1114	Phenylethyl alcohol**	0.0 a	0.0 a	0.0 a	49.0 (9.3) c	22.0 (4.2) b	16.9 (4.4) b	74.930	0.001	***
1246	Ethyl phenylacetate**	0.0 a	0.0 a	0.0 a	4.3 (1.3) b	3.8 (0.6) b	2.2 (0.5) b	5.750	0.001	***
1408	Methyl eugenol**	0.3 (0.2) a	1.1 (0.4) ab	0.1 (0.1) a	1.3 (0.3) bc	3.4 (0.9) cd	5.2 (0.9) d	13.930	0.001	***
1492	Phenylethyl isovalerate**	5.7 (4.0) a	0.8 (0.7) a	1.0 (0.3) a	43.6 (10.9) b	51.5 (10.8) b	32.2 (6.1) b	61.200	0.001	***
1500	Methylisoeugenol**	0.0 ab	0.0 ab	0.0 a	1.6 (0.6) bc	5.0 (2.3) cd	9.7 (2.3) d	19.930	0.001	***
	Total fatty acids	8957.3 (853.0) b	6236.5 (705.4) b	5918.2 (697.6) b	1809.5 (272.4) a	1152.0 (45.1) a	1240.6 (126.2) a	60.13	0.001	***
	Total phenolics	6493.4 (1324.0)b	4463.7 (394.7) b	5118.4 (600.3) b	5236.5 (482.0) b	1385.2 (130.3) a	1736.1 (251.3) a	26.290	0.000	***
	Total monoterpenes	323.6 (69.3) ab	245.1 (31.3) a	295.4 (38.9) a	334.6 (53.4) b	351.9 (23.7) b	304.7 (23.2) a	10.01	0.265	ns
	Total sesquiterpenes	14.2 (6.7) a	22.0 (14.4) a	15.5 (3.7) a	75.4 (15.3) b	74.5 (12.8) b	82.9 (26.9) b	15.550	0.001	***

Tr traces, *RI* Retention index (retention index of fatty acids and phenolics are those of methyl derivatives because of the extraction/derivatization method employed)

*: compared to authentic standards; **: tentatively identified.

Statistical Analyses Variation in chemical composition by organ type and successional stage was analyzed by using a Canonical Analysis of Principal coordinates (CAP). This is a useful analysis of multivariate data by reference to prior hypotheses [here: no effect of pine compartment or age of pine (successional stage) on chemical composition (Anderson and Willis 2003)]. CAP was the most powerful test for compositional differences among assemblages. As factors appeared to be significant from CAP, they were analyzed by using a Principal Component Analysis (PCA). XL stat® (ver. 4.01) was used for this analysis. Finally, differences in the concentration of each compound by age and pine compartment were tested with the *Kruskall-Wallis test* followed by *post hoc NSK test.* Statgraphics® (version 2.1) was used for these statistical analyses. The chemodiversity index of each tree was calculated according to Iason et al., (2005): $D_{chem} = -\sum [c \log(c)]$ where c is the proportional concentration of each chemical compound.

Results

Chemical Composition Aqueous extracts from needles and roots from the different stands of *P. halepensis* showed a complex mixture of at least 59 identified compounds belonging to different functional phytochemical groups (Knudsen and Gershenzon 2006). Nine monoterpenes, 20 oxygenated monoterpenes, 3 sesquiterpenes, 6 oxygenated sesquiterpenes, 4 fatty acids, 10 phenolic compounds, and 7 others (Table 1) were found in mixtures and consisted predominantly of phenolics (50%), fatty acids (44%), monoterpenoids (5%), and sesquiterpenoids (1%).

Effect of Organ and Successional Stage on Leachates We observed a significant effect of organ type (Canonical Analysis of Principal coordinates, F=20.90; P<0.001) and successional stage (CAP, F=2.87, P<0.001) on chemical composition of leachates.

Figure 1 represents a two-dimensional mapping of the Principal Component Analysis. Axis 1 represents 25.7% of the information, and is characterized on the positive side by two oxygenated sesquiterpenes (α-eudesmol, α-cadinol) and α-terpineol (an oxygenated monoterpene). The negative side shows the concentration of total fatty acids, succinic acid, myrtenol (an oxygenated monoterpene), and a monoterpene derivative (bornyl or isobornyl acetate). Axis 2 represents 15.28% of the information, and is characterized on the positive side by oxygenated and non-oxygenated monoterpenes (e.g., *trans*-pinocarveol, ß-myrcene) and a phenolic compound (4-hydroxybenzoic acid). The hierarchical ascending classification distinguishes two main groups according to organ type. The 1st group is situated on the positive side of Axis 1 and includes all needles analyzed. Needles then were characterized by two oxygenated sesquiterpenes and three oxygenated monoterpenes (α-eudesmol, α-cadinol, and α-terpineol). The second group is located on the negative side of the Axis 1 and includes all the roots analyzed. Roots then were characterized by high concentrations of total fatty acids and other compounds (see above) such as succinic acid.

The "needles group" can be subdivided into 2 sub-groups: young needles (with some young roots) occurring on the positive side of Axis 2 had the highest monoterpene concentrations, while the older needles were located on the negative side of Axis 2.

The Chemodiversity Index (D_{chem}; Iason et al. 2005) was calculated with pooled data from all functional groups and shows that needles have a higher chemical diversity than young and medium-aged pine roots and, to a lesser extent old roots (Fig. 2). When the index is calculated for functional groups, needles have a higher diversity index for total sesquiterpenes (D_{chem} mean of 2.38 for needles and 1.19 for roots), while roots have a higher diversity index for monoterpenes (D_{chem} mean of 3.20 for roots and 2.40 for needles; Tukey tests and one-way Anova, P<0.05). For phenolics and fatty acids, young needles have the highest diversity (D_{chem}=2.38).

Discussion

Numerous compounds were observed in aqueous extracts of *Pinus halepensis*. Monoterpenoids (e.g., α-pinene; sabinene; β-pinene; β-myrcene; α-terpinene; *p*-cymene; γ-terpinene, fenchol, camphor, α-terpineol) and sesquiterpenoids (e.g., β-caryophyllene; α-muurolene; guaiol; α-eudesmol) were found in needles, litter, and needle emissions (Ormeño et al. 2007), as well as in *Pinus halepensis* essential oil (Macchioni et al. 2003). Moreover, the diversity index for monoterpenes in *Pinus halepensis* needles is high (1.7 to 3.1) compared to *Pinus ponderosa* or *Pinus sylvestris* needles (0.9 to 1.5 and 0.8 to 1.7, respectivly) (Iason et al. 2005; Thoss and Byers 2006). Several of these simple phenolics found in *P. halepensis* have been found previously (e.g., gallic acid, 4-hydroxybenzoic acid, vanillic acid, syringic acid, and *p*-coumaric acid) (Robles et al. 2003), as well as in other *Pinus* species (Alonso et al. 2002; Cannac et al. 2007). Fatty acids were recently found in the needles and litter of several conifers (Song and Cui 2003).

The influence of organ and successional stage on the chemical composition of leachates confirms the difference between root and needle leachates—an observation that

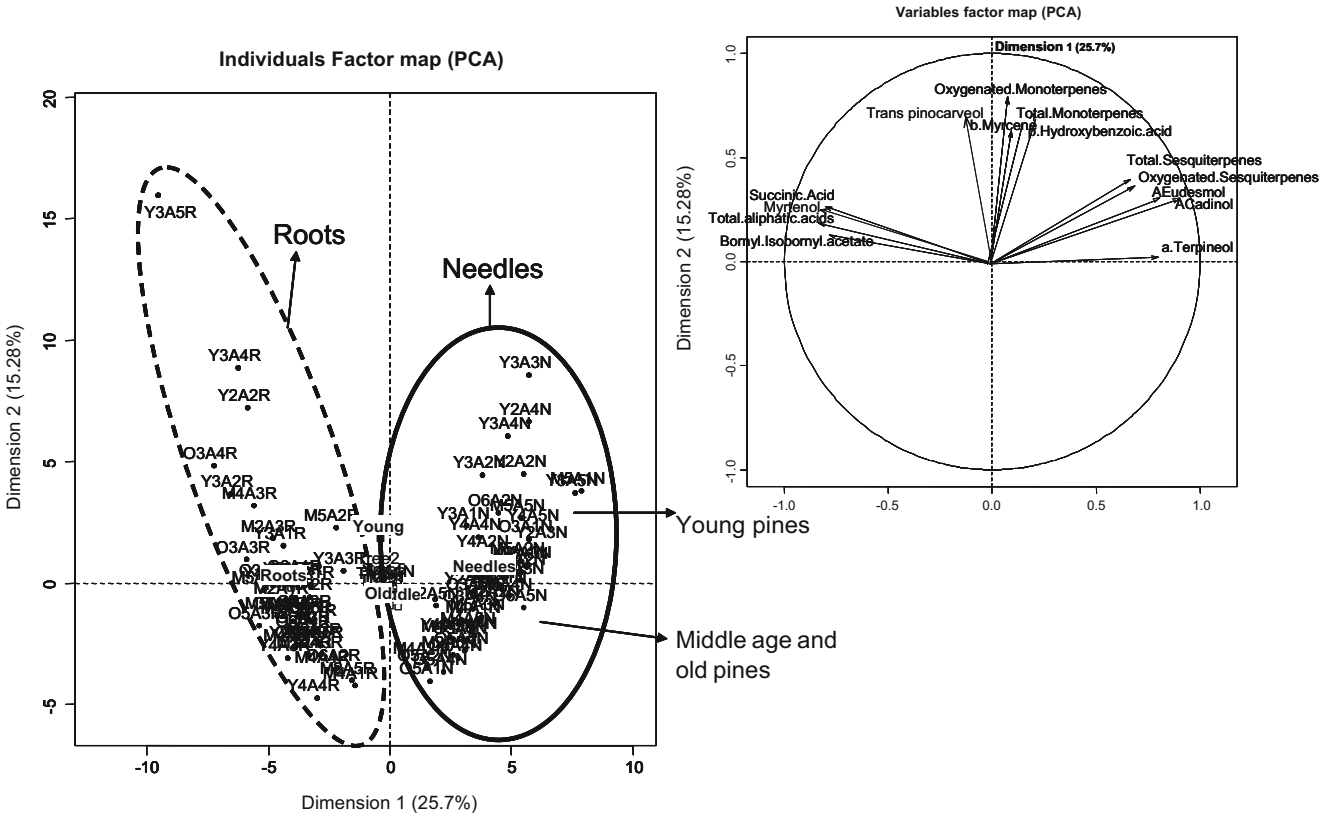

Fig. 1 Two-dimensional mapping of the Principal Component Analysis performed for chemical compounds from different age and organs of *Pinus halepensis* (Y: young <15 years; M: middle age±30 years; and O: old >60 years) and organ (last letter N: needles; R: roots)

parallels the work of Macchioni et al. (2003) with the essential oil of different *Pinus* organs. In general, the concentrations of allelochemicals in conifers decline with age (Liu and An 2003) as does composition and relative amounts of constituents (Julkunen-Tiitto et al. 1996). The chemical variability of *P. halepensis* organs and stage suggest that different organs (i.e., needles or roots) release different chemicals into the environment at different stages and times. Needles show greater variation with the age of the stand. Two sub-groups were identified that had differences between the dominant phytochemical groups in young vs. older pines stands. The highest monoterpene concentration occurred in younger pines needles (Fig. 1) and may be responsible for inhibiting seed germination (Vokou et al.

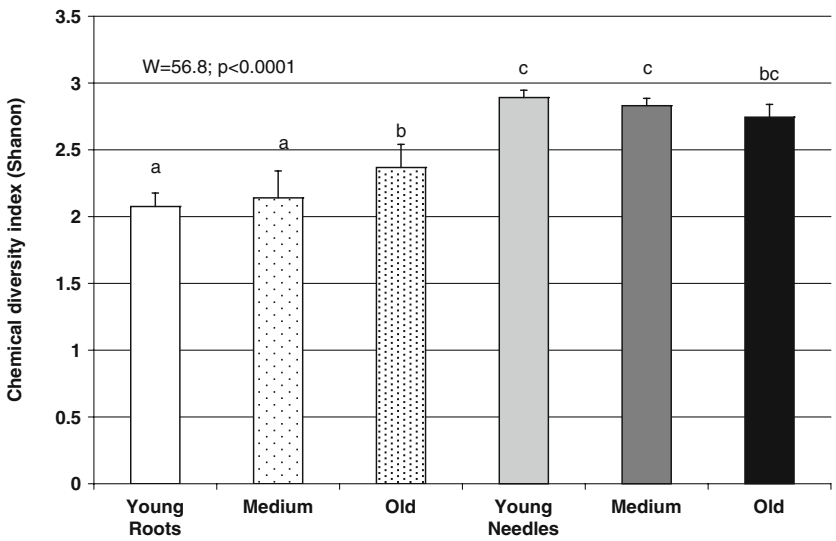

Fig. 2 Chemical diversity of *Pinus halepensis* leachates from different organs and age of calculated according to Iason et al., (2005) (Mean ± SD)

2003) and facilitating the establishment of young pine stands (Fernandez et al. 2006).

The capability of *P. halepensis* to synthesize a rich phenolic mixture during early stages of colonization may confer a competitive advantage in the competition among plants or pathogens. Moreover, the high diversity of compounds in older roots is not surprising given the abundance of competing microbes, insects, and roots of other plants in the same environment. Root monoterpene diversity also may impact tritrophic interactions in soil as these compounds are implicated in indirect defense mechanisms against root feeders. Several studies have now demonstrated that roots can recruit herbivore enemies by releasing chemical cues into soil (Hiltpold and Turlings 2008).

As mentioned previously, secondary metabolites may function in the defense of one plant against another via allelopathic processes. *P. halepensis* leachates, whether from needles or roots, are allelopathic toward some, but not all, plants. Biosensor plants—i.e., *Avena sativa, Lactuca sativa,* and *Lemna minor* (Nektarios et al. 2005)—are inhibited by *P. halepensis* leachates. The leachates also negatively affect wild plants, including: *Festuca arundinacea*, *Cynodon dactylon*, *Linum strictum*, and *Pinus halepensis* (Nektarios et al. 2005; Fernandez et al. 2006, 2008). These findings suggest that the release rate and level of allelochemicals are important factors in the environments in which they occur. Castaldi et al. (2009) showed that *Arbutus unedo* leaves and root extracts were detectable in soil extracts. Similarly, *P. halepensis* extracts may contain a chemical composition similar to those released in nature. Pine needles seem to have higher allelopathic activity in fresh tissue, moderate activity in senescing tissue, and low activity in decaying pine needles (Nektarios et al. 2005). In addition, *P. halepensis* needle litter also negatively affects herbaceous plants (Nektarios et al. 2005), but has no effect on other species—e.g., *Quercus ilex* (Broncano et al. 1998) or *Pistacia lentiscus* (Maestre et al. 2004).

Secondary coumpouds are recalcitrant to decomposition (Vitousek and Reiners 1991). As this constitutes one significant way for allelochemicals to enter the environment (Rice 1984), they can directly influence microbial activity (White 1994), and soil productivity (Bloom and Mallik 2004). *P. halepensis* forests are prone to accumulate relatively thick needle layers below their canopy (Garciaple et al. 1995) and would seem to have the potential to influence biotic interactions in litter (Inderjit and Nilsen 2003) and plant dynamics through allelopathic interactions and mechanical effects on seedling recruitment (Fernandez et al. 2008; Navarro-Cano et al. 2009).

Our previous data showed that young needles and old roots were responsible for most of the allelopathic and autotoxic interactions (Fernandez et al. 2006, 2008; Table 2). The present investigation gives more detail on the principal compounds in both young needles and old roots known to be allelopathic—i.e., sinapic and caffeic acids (phenolic acids) (Table 1) even if both are in low concentrations in *P. halepensis* leachates compared to other compounds (Table 1). Mixtures of phenolic acids and other organic compounds can cause inhibitory effects even though the concentration of individual compounds are well below inhibitory levels (Blum 1996). Caffeic acid (CA) had higher concentrations in both young needles and old roots.

Table 2 Sensitivity of several target species to the highest dose of *Pinus halepensis* extracts from roots and needles and stand age (Y: young < 15 years old, M: medium aged, O: old aged, >60 years old, S: senescent, D: decaying)

Target species	Target type	Roots/ Y	Roots/ M	Roots/ O	Needles/ Y	Needles/ M	Needles/ O	Needles/ S	Needles/ D	References
Lactuca sativa	herbaceous/ target reference	Ge 0 Gr 0	Ge 0 Gr −	Ge 0 Gr−−	Ge 0 Gr +	Ge 0 Gr ++	Ge 0 Gr ++			Fernandez et al. 2006
Linum strictum	herbaceous/ wild species	Ge−− Gr −	Ge − Gr−−	Ge−− Gr−−	Ge−− Gr−−−	Ge − Gr −	Ge−− Gr −			Fernandez et al. 2006
Festuca arundinacea	herbaceous/ wild species					Gr−−−		Gr−−	Gr −	Nektarios et al. 2005
Cynodon dactylon	herbaceous/ wild species					Gr−−−		Gr−−	Gr −	Nektarios et al. 2005
Avena sativa	herbaceous/ wild species					Gr−−−		Gr−−	Gr −	Nektarios et al. 2005
Pinus halepensis	tree/germination stage	Ge − Gr −		Ge−− Gr −	Ge−− Gr 0		Ge − Gr −			Fernandez et al. 2008
Pinus halepensis	tree/ sapling stage				Gr−−					Monnier et al. 2008
Quercus pubescens	tree/ sapling stage				Gr 0					Monnier et al. 2008

GE: GERMINATION; GR: GROWTH; 0: NO EFFECT;−: NEGATIVE EFFECT;+: POSITIVE EFFECT.

This acid is ubiquitous in plants. As with most cinnamic acids, caffeic acid is implicated in many biological interactions (Batish et al. 2008) including allelopathy (Rice 1984) and microbial interactions (Harrison et al. 2007). This compound induces stress in plants, alters physiological and resulting biochemical reactions, and detrimentally impacts plant growth. It is a potent root growth inhibitor (Gallet 1994; Barkosky et al. 2000), and disrupts plant-water relationships and photosynthesis (Barkosky et al. 2000). This phenolic acid changes protease, peroxidase, and polyphenol oxidase activities in root development (Batish et al. 2008) and either interfers with absorption of potassium and phosphorus (Glass 1974), or depolarizes cell membranes in roots (Glass and Dunlop 1974). Caffeic acid has many biological activities and may play a key role in giving *P. halepensis* a competitive advantage over other plants.

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