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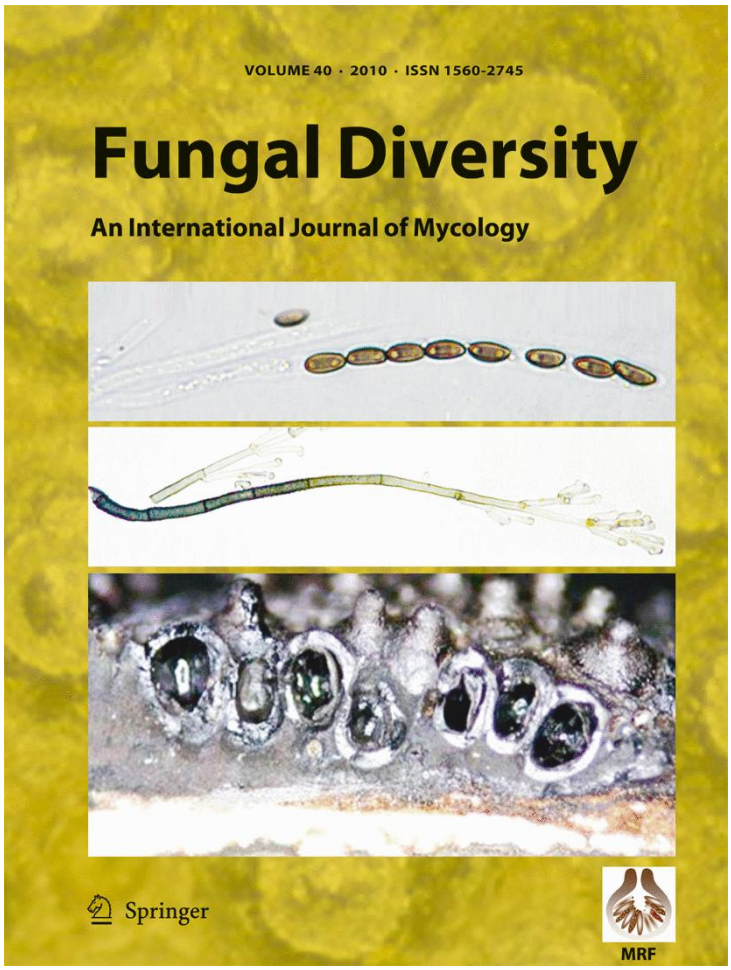

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Fungi associated with the decline of *Pinus halepensis* in Spain

L. Botella · O. Santamaría · J. J. Diez

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Abstract Fungal species richness and composition within needles and twigs in 55 stands of *Pinus halepensis*, spread out over the whole Iberian Peninsula, were determined. The aim was to evaluate the relationships of fungal communities with local environmental variables, in order to analyze the potential causes of the current decline of this pine species in Spain. A total of 35 fungal taxa were isolated from 1980 moist chambers analysed (990 per vegetal tissue). A taxon within the *Alternaria alternata* complex was most frequent, followed by *Leptostroma pinastri*, *Aspergillus niger*, *Diplodia pinea* and *Phomopsis* sp. At the tree level, tissue was a significant response variable and a higher species richness was found in needles as compared to twigs. On the other hand, the multivariate analysis showed the environmental variables 'age', 'shadow', 'elevation', 'mean temperature', 'illumination' and 'availability of water' significantly influenced fungal species composition. In particular, 'mean temperature', was an important variable implicated in the general weakening of this thermophilic pine species, and appeared to be inversely correlated with the occurrence of several conifer pathogens such as *Brunchorstia pinea, Cytospora* sp., *Diplodia pinea, Naemacyclus niveus*, *Pestalotiopsis stevensonii* and *Sclerophoma pythiophila*. This study shows a possible combined effect of abiotic and biotic stresses in causing the general decline of Allepo pine in Spain.

Keywords Abiotic stress · Allepo pine · Forest pathogens · Fungi · Global warming · Weakening

L. Botella (✉) · J. J. Diez
Departamento de Producción Vegetal y Recursos Forestales,
Universidad de Valladolid,
Avda Madrid 44,
34004 Palencia, Spain
e-mail: lbotella@pvs.uva.es

O. Santamaría
Departamento de Ingeniería del Medio Agronómico y Forestal,
Universidad de Extremadura,
Ctra. Cáceres s/n.,
06071 Badajoz, Spain

Introduction

Pinus halepensis, commonly named Allepo pine, is a species native to the Mediterranean region and is widespread over the western area, from Spain to Algeria. In Spain, virtually natural stands are distributed over the whole eastern coast, although due to its important ecological plasticity it has been intensively used for afforestation in north-western areas of the Iberian Peninsula, very often out of its natural habitat (Abelló 1998). In such areas, *P. halepensis* has been exposed to stressful environmental conditions which have resulted in loss of vigour and general weakness. Furthermore, in 1998, the Spanish Government, through its General Direction for the Nature Conservation (DGCN), also confirmed that many *P. halepensis* natural forests were loosing their vitality (SPCAN 1998). Since then, a generalized decline of *P. halepensis* forest in Spain has been recognised. Specific causes that explain this decline have not been determined to date. However, as with other tree species, a combination of abiotic and biotic factors is thought lead to the decline (Thomas et al. 2002; Arnold 2007). Unfavourable abiotic conditions determine the health of forests and result in trees being more susceptible to pathogens (Sieber 2007). *Pinus halepensis* forests, especially those located out of their natural range, grow in unfavourable conditions and many fungal species have been shown to enhance the weakening of the trees (Santamaria et al. 2008). *Gremmeniella abietina*

is an important aggressive pathogen, which was found to occur in afforested *P. halepensis* stands in north-western Spain in 1999 (Santamaria et al. 2003). *Sirococcus strobilinus* has also been described as an increasingly important pathogen responsible for shoot blight of *Pinus halepensis* (Muñoz López 1997). *Sclerophoma pythiophila* and *Cenangium ferruginosum* have also frequently been isolated from diseased conifers (Santamaria et al. 2007; Zamora et al. 2008) in spite of being considered as a secondary pathogen of other pine species (Brener et al. 1974; Phillips and Burdekin 1992). Although pathogenic and saprobic fungal taxa have been described associated with different conifers in several articles (Jurc et al. 1996; Müller and Hallaksela 1998, 2000; Hoff et al. 2004; Ranta and Saloniemi 2005; Wang et al. 2005; Ganley and Newcombe 2006; Hu et al. 2007) the mycota of *P. halepensis*, particularly in Spain, and its influence on their health status, is still barely known. Consequently, the main objectives of this study were to: (1) identify the composition of the fungi isolated from *P. halepensis* trees showing decline symptoms located in stands across the entire Spanish territory, and (2) try to establish distribution patterns of the frequency and fungal species richness in relation to the environmental variables at each stand.

Materials and methods

Sampling

During the spring-summer of 2006, 55 *Pinus halepensis* stands were sampled within its distribution area. They were randomly chosen from the European Network of Forest Damages (Level 1), Spain, and the coordinates were supplied by the General Direction for the Nature Conservation (DGCN) (Fig. 1). Likewise, the environmental variables of the stands used in the present study, 'slope', 'aspect', 'age', 'water' 'humus' and 'soil type' (whose values follow the described in the manuals of the Protective Service of Nocive Agents (SPCAN 1998) were provided by the DGCN; and 'pluviometry', 'mean temperature', 'solar radiation', 'elevation', 'illumination' and 'shadow' were taken from the Digital Climatic Atlas of the Iberian Peninsula (Ninyerola et al. 2005). The stands and their environmental variable values can be observed in Table 1.

Three trees showing decline symptoms were randomly selected from each stand and three branches were cut from each. A total of 165 trees were processed. Once collected,

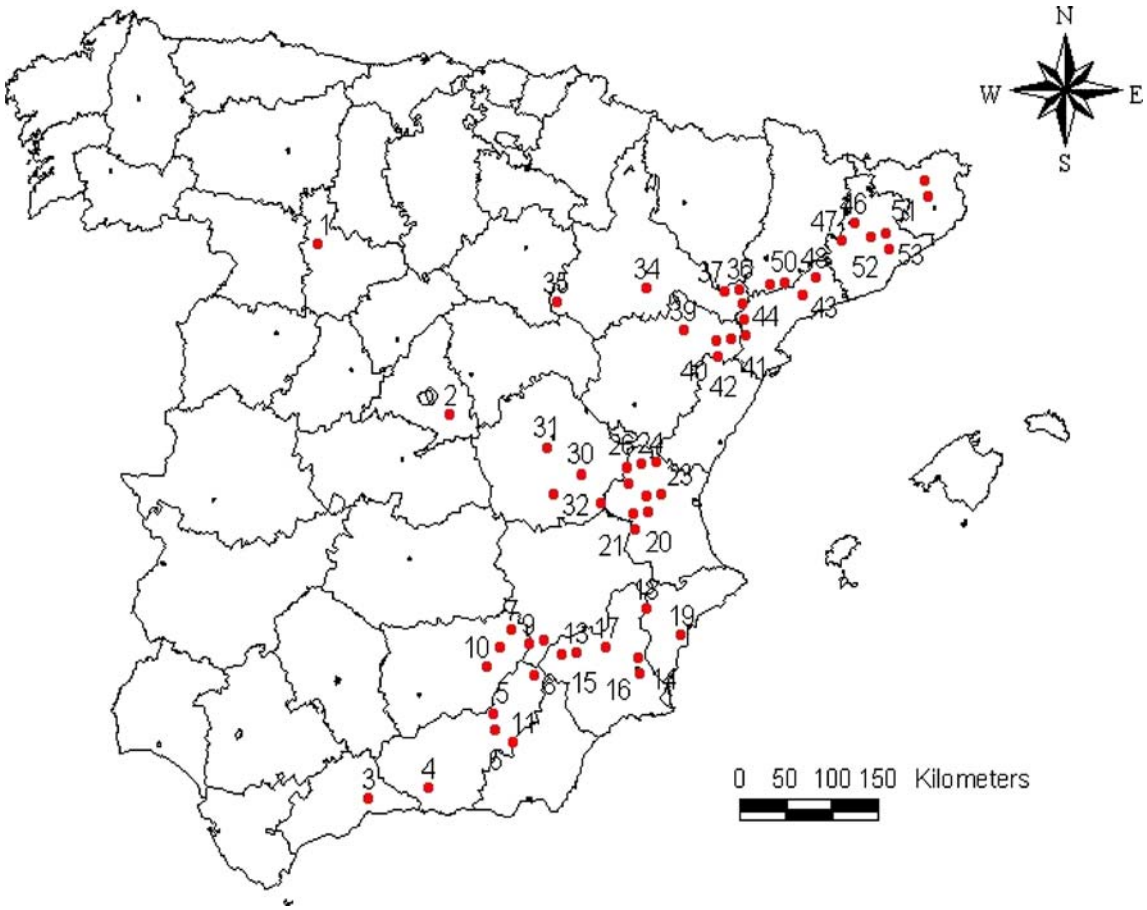

Fig. 1 Geographical distribution of the 55 stands sampled in this study

Table 1 Sampling locations and environmental variables

Stand code	Location	Province	UTM coordinates		[a]Slope	[a]Aspect	[a]Age	[a]Water	[a]Humus	[a]Ground	[b]Pluv (mm)	[c]Temp (ºC)	[d]Solar rad	[e]Eleva (m)	[f]Illum	Shadow (º)
			X	Y												
1-TOR	Tordehumos	Valladolid	321200,4915	4627200,704	35	1	1	1	3	149	447	11	2036	800	187	0
2-ARG	Arganda	Madrid	463900,3793	4457700,772	5	7	2	2	2	149	463	14	2033	701	187	1
3-COL	Colmenar	Málaga	376500,303	4077500,161	59	4	4	1	1	123	622	14	1979	699	189	6
4-ALB	Albuñuelas	Granada	440300,7262	4087200,816	13	3	8	1	2	116	644	14	1953	1046	196	4
5-ZUJ	Zújar	Granada	510400,6468	4161700,504	22	3	1	1	2	117	593	15	1789	800	197	4
6-BAZ	Baza	Granada	512800,7935	4146100,895	5	9	2	2	2	117	369	13	1950	1261	193	1
7-BEN	Benatae	Granada	530300,1867	4244500,413	43	1	1	2	2	149	793	14	1846	996	190	2
8-PUE	P. Don Fabrique	Granada	553600,5125	4200100,218	13	2	1	1	1	117	192	12	2140	1286	170	14
9-YES	Yeste	Granada	548700,5954	4231300,647	60	9	8	2	2	122	574	13	1840	899	177	9
10-MOG	Mogón	Granada	503100,3661	4208600,263	5	4	2	2	2	193	1070	14	1982	1076	193	6
11-AMA	Amarguillas	Granada	531200,9997	4132800,852	16	1	1	2	2	123	379	12	1859	1256	197	0
12-VVA	Vva. del Arzobispo	Granada	516700,6061	4226600,976	30	4	8	1	1	155	954	14	2316	897	149	30
13-MOR	Moratalla	Murcia	599200,7342	4222700,51	20	2	2	2	2	0	406	16	2004	564	188	0
14-ALM	Almudema	Murcia	668100,7494	4200900,895	50	8	2	2	2	0	326	17	2126	297	189	0
15-SAB	El Sabinar	Murcia	583200,6651	4220400,947	13	3	2	2	3	0	378	13	2122	1070	187	2
16-FOR	Fortuna	Murcia	665700,0495	4216500,384	30	2	1	1	1	117	322	18	2032	120	188	0
17-CIE	Cieza	Murcia	631300,6902	4227500,031	55	1	1	1	2	122	292	17	2075	349	186	1
18-VILL	Villena	Alicante	674600,1633	4265700,816	30	2	1	2	1	124	429	14	1927	908	190	0
19-ELC	Elche	Alicante	711500,7908	4239200,091	0	9	1	2	1	157	311	18	2050	68	186	1
20-COF	Cofrentes	Valencia	662700,0886	4343700,846	20	4	8	2	3	0	487	15	1836	575	196	1
21-POR	La Portera	Valencia	660300,8232	4359200,944	50	7	3	1	1	117	455	15	2005	603	187	3
22-BUÑ	Buñol	Valencia	676400,7438	4361600,174	25	6	3	2	3	122	515	15	1942	600	190	4
23-CHE	Cheste	Valencia	690100,7933	4379500,63	30	7	2	2	2	0	456	16	1979	479	191	0
24-AND	Andilla	Valencia	685300,1158	4410600,333	40	4	1	2	2	191	438	12	1959	1104	188	1
25-UTI	Utiel	Valencia	655500,9372	4390400,423	0	9	8	2	3	0	489	12	2055	1000	186	7
26-TUE	Tuéjar	Valencia	669200,4909	4408300,92	38	5	8	2	3	149	390	14	1875	806	188	2
27-REQ	Requena	Valencia	674000,577	4377100,713	20	3	8	2	3	0	455	15	2005	603	187	3
28-SIN	Sinarcas	Valencia	653100,0025	4405900,484	0	9	3	2	3	0	500	13	2082	888	186	1
29-NER	Nerpio	Albacete	564700,5223	4233700,915	30	2	8	1	1	117	326	14	1871	898	177	7
30-PAR	Paracuellos	Cuenca	604900,2654	4398900,887	0	9	8	2	1	149	462	12	2122	968	181	5
31-OLA	Villar de Olaya	Cuenca	567800,396	4425300,551	5	7	8	2	2	175	590	12	2080	1088	183	4
32-ALA	Alarcón	Cuenca	575100,1572	4378700,632	18	2	3	2	3	0	518	13	2031	803	187	1
33-MIN	Minglanilla	Cuenca	625700,2567	4370100,505	10	6	3	2	2	177	399	15	2045	600	187	2
34-FUE	Fuendetodos	Zaragoza	675100,0773	4583800,163	20	9	1	2	2	124	393	13	2050	587	187	0

Table 1 (continued)

Stand code	Location	Province	UTM coordinates		[a]Slope	[a]Aspect	[a]Age	[a]Water	[a]Humus	[a]Ground	[b]Pluv (mm)	[c]Temp (ºC)	[d]Solar rad	[e]Eleva (m)	[f]Illum	Shadow (º)
			X	Y												
35-ARI	Ariza	Zaragoza	578200,3207	4569700,503	4	9	1	2	2	149	412	13	2035	803	187	0
36-MEQ	Mequinenza	Zaragoza	774400,1369	4582300,652	20	2	8	1	1	175	401	15	2013	201	187	1
37-CAS	Caspe	Zaragoza	758200,5817	4579900,331	45	2	2	1	2	0	372	15	2033	197	187	1
38-NON	Nonaspe	Zaragoza	776800,7142	4566800,575	25	3	2	2	3	117	402	15	2025	198	187	2
39-ANR	Andorra	Teruel	714600,7448	4541900,1	50	7	8	1	1	117	465	13	2035	701	186	5
40-BEL	Belmonte de S. José	Teruel	749200,6988	4531100,954	5	1	8	2	1	117	506	13	2039	701	186	1
41-MAE	Maella	Zaragoza	765400,8934	4533400,863	0	9	3	2	3	0	555	14	2035	601	186	0
42-MON	Monroyo	Teruel	751600,8874	4515500,384	45	8	3	2	2	149	694	13	1969	806	191	0
43-MUS	La Mussara	Tarragona	841400,8231	4576100,758	10	9	3	2	2	122	646	12	1940	807	193	0
44-BAT	Batea	Tarragona	779100,1543	4551200,377	20	5	1	2	2	122	482	14	2012	441	189	1
45-HOR	Horta de Sant Joan	Tarragona	781500,3178	4535700,619	40	7	5	1	2	0	576	14	1786	453	185	6
46-NAV	Navás	Barceolna	896800,5554	4647400,023	20	6	2	2	3	116	633	13	2050	469	183	5
47-SUR	Súria	Barceolna	882900,5153	4629600,597	15	8	3	2	2	0	653	13	2043	411	184	6
48-SAR	Sarral	Barceolna	855200,5209	4593900,45	10	1	3	1	2	156	511	13	2118	505	185	3
49-GRA	La Granadella	Lleida	806700,3067	4586900,248	60	8	6	2	2	122	492	14	2021	397	186	4
50-CER	Cerviá de Garrigués	Lleida	822900,7352	4589200,825	50	3	2	2	2	122	494	13	2004	672	188	0
51-MAI	Maiá de Montcal	Girona	973100,7366	4690100,784	40	5	3	2	2	155	830	14	2135	216	185	2
52-MOC	Monistrol de Calders	Barcelona	915300,6057	4634300,733	30	1	3	2	1	149	69	13	1949	531	180	4
53-LLI	LliÇa D´Amunt	Barcelona	933800,6463	4621100,355	40	6	2	2	3	155	694	14	2066	205	184	4
54-BAN	Banyoles	Girona	975500,8188	4674600,709	20	5	4	2	1	127	847	13	2274	360	171	16
55-CEN	S. Martí de Centelles	Barcelona	931500,9993	4636600,046	30	5	2	2	2	127	801	11	1999	827	181	6

[a] Variables defined through the DGCN qualitative criteria (SPAN 1998). UTM coordinates. Universal Transverse Mercator, UTM, coordinates (in meters). [b] Pluviometry (mm). [c] Mean temperature (°C). [d] Solar radiation measure: 10 kJ/(m2*day*micrometre). [e] Elevation (m). [f] Illumination measure: (100+100*cos(incidence angle))

the samples were stored immediately at 4°C until further processing.

Fungal isolation

Six twig segments (0.5 cm diam., 0.5–1 cm long, including bark) and six needles were randomly selected from each tree, and processed according to the moist chamber method in order to identify the fungi present, as it has been outlined in previous fungal studies (Santamaria and Diez 2005; Zamora et al. 2008). The method consisted of finding fruiting bodies on plant tissues (twigs and needles) after incubation in Petri dishes at room temperature (22°C±2°C) in diffused daylight containing wet paper. The samples used in this method were not surface sterilised in order to find both endophytes and fungal epiphytes. The moist chambers were prepared into a laminar flow hood with the plant material stored in bags in the field and opened into the hood to avoid contaminations, as much as possible, since the samples were not surface sterilised. Cultures were identified according to morphological characteristics of spores and other reproductive structures, such as size, shape, colour. Different taxonomic keys were used for fungal identification. (Hanlin 1998; Goidanich 1990; Sutton 1980; Kiffer and Morelet 1999).

Statistical data analysis

Univariate statistic Two analyses were carried out; in the first case at tree level (Table 2), using species richness (SR) as a response variable, a two-way ANOVA was performed, with the stand (55 stands), sampling tissue (needles and twigs) and its interaction as explanatory variables. Fisher´s least significant difference (LSD) test was used for multiple comparison among treatments by means of the General Linear Model Procedure of SAS (Statistical Analysis Software v. 9.1.3) (Anonymous 1989) when significant differences were found in the ANOVA table. Transformation Ln (x+1) was carried out to stabilize the residual variance. In the second analysis at the stand level, the same procedure was used, with the SR also used as the response variable, but in this case all the edaphoclimatic variables were used as the explanatory ones.

Table 2 ANOVA table at tree level

Source	d.f.	F-value	*p*-value
Tissue	1	37.96	<0.001
Site	54	2.80	<0.001
Tissue*site	54	1.62	0.008

d.f. degrees of freedom

Multivariate analysis In order to assess in more detail the influence of the main explanatory variables on the fungal occurrence, but in this case using the 'isolated fungal species composition' as a response variable, a Canonical Correspondence Analysis (CCA) was carried out. A forward selection procedure using the Monte Carlo test was then applied to test the significance, with 499 permutations for exploratory analysis and 999 for final results (Legendre and Legendre 1998). The constrained ordination was performed by using default settings and untransformed species data by means of CANOCO for Windows version 4.5 (Ter Braak and Smilauer 2002).

Results

A total of 35 fungal species were isolated and identified from the 1980 moist chambers analysed (990 per vegetal tissue). The frequency of occurrence and the stands and tissue type where the fungi were collected from, can be seen in Table 3. *Alternaria alternata* species complex was the most frequently isolated fungus (it was identified in 70.9% of stands), followed by *Leptostroma pinastri* (56.4 %), *Aspergillus niger* (43.4 %) and *Diplodia pinea* and *Phomopsis* sp. (25.5%). Conversely, *Arthrinium caricicola*, *Chaetomiun atrobruneum*, *Gliocephalis* sp., *Preussia* sp., as well as *Brunchorstia pinea,* only occurred in one location. *Naemacyclus niveus* and *Diplodia pinea* were very common in the regions of Aragon and Catalonia. *Diatripella* sp. occurred in Murcia, Valencia and southern Albacete. *Dichomera* sp. was mainly found in the regions of Granada, Murcia and Jaén, albeit also appearing in a single location of Tarragona. The other taxa had a broad distribution.

When fungal species richness (SR), was calculated using the number of different fungal species, and was analysed at the tree level, the ANOVA showed 'tissue', 'stand' and their interaction were all significant variables (Table 2). In the case of the 'tissue' variable, the needles supported a greater species richness (SR=29±7.) than in twigs (SR=27±7). In order to study the variable 'stand' in more detail, the influence of the edaphoclimatic characteristics were evaluated (Table 1) on the species richness at a stand level. ANOVA showed only the variable "ground" (df=14; F-value=2.09; *p*-value=0.0679) and the variable "solar radiation" (df=2; F-value=2.88; *p*-value=0.0807) to have some influence on species richness.

The ANOVA analysis of edaphoclimatic variables was not conclusive, and therefore a multivariate analysis was carried out in order to analyze the influence of all the variables in more detail, but on the fungal species distribution. The exploratory analysis of the CCA test showed the 'illumination', 'shadow', 'age', 'mean temper-

Table 3 Fungi species, stands and tissue where they were recovered and repetitions

Fungi[a]	Abb.[b]	S[c]	T/N[d]	Stands where the fungus was isolated from, and frequency of isolation within each stand (numbers within parenthesis)
Alternaria alternata complex[e] (Fr.:Fr.)Keissl.	*Ala*	39	16/62	4-ALB (2), 5-ZUJ (4), 7-BEN (2), 8-PUE (2), 9-YES (3), 10-MOG (3), 11-AMA (2), 14-ALM (2) 15-SAB (3), 16-FOR (2), 20-COF (1), 21-POR (2), 22-BUÑ (1), 24-AND (2), 29-NER (2), 27-REQ (3), 28-SIN (1), 32-ALA (3), 33-MIN (2), 34-FUE (1), 35-ARI (1), 36-MEQ (2), 37-CAS (2), 38-NON (5), 39-ANR (2), 40-BEL (2), 41-MAE (2), 42-MON (1), 45-HOR (2), 43-MUS (4), 44-BAT (1), 46-NAV (1), 47-SUR (3), 48-SAR (3), 50-CER (1), 51-MAI (1), 52-MOC (1), 53-LLI (1), 55-CEN (3).
Arthrinium caricicola Kunze ex Fr.	*Arc*	*4*	0/4	7-BEN (1), 17-CIE (1), 23-CHE (1), 32-ALA (1).
Arthrobotrys dactyloides Drechsler	*Ard*	*1*	1/0	12-VVA (1).
Aspergillus niger[f] Van Tieghem.	*Asn*	*24*	15/21	4-ALB (2), 5-ZUJ (3), 6-BAZ (1), 8-PUE (1), 10-MOG (1), 17-CIE (1) , 21-POR (1), 22-BUÑ (1), 24-AND (3), 29-NER (1), 27-REQ (1), 30-PAR (1), 33-MIN (1), 34-FUE (3), 31-0LA (1), 40-BEL (3), 39-ANR (2), 36-MEQ (1), 43-MUS (1), 46-NAV (1), 49-GRA (2), 50-CER (1), 51-MAI (2), 54-BAN (1).
Brunchorstia pinea (Karst.) Höhn	*Brp*	*1*	1/0	1-TOR (1).
Chaetomiun globosum Kunze ex Fries	*Chg*	*1*	1/0	17-CIE (1).
Chaetomium cochliodes Palliser.	*Chc*	*2*	2/1	22-BUÑ (1), 25-UTI (1).
Chaetomium atrobruneum Ames.	*Cha*	*2*	2/0	2-ARG (1), 13-MOR (1).
Chaetomium fusiforme Chivers.	*Chf*	*8*	5/5	5-ZUJ (2), 7-BEN (1), 17-CIE (2),19-ELC (1), 22-BUÑ (1), 29-NER (1), 42-MON (1), 47-SUR (1).
Cladosporium herbarum (Pers.) Links. ex S. F. Gray	*Clh*	*11*	5/9	4-ALB (1), 13-MOR (1), 14-ALM (1), 18-VILL (1), 21-POR (1), 27-REQ (1), 43-MUS (2), 45-HOR (1), 53-LLI (1), 54-BAN (2), 55-CEN (2).
Camarosporium propinquum (Sacc.) Sacc.	*Cap*	*3*	1/2	32-ALA (1), 35-ARI (1), 45-HOR (1).
Cytospora sp. Ehrenb. ex Fr.	*Cy*	*8*	4/5	7-BEN (1), 10-MOG (1), 11-AMA (1), 28-SIN (1), 37-CAS (1), 42-MON (1), 47-SUR (2), 54-BAN (1).
Dichomera sp. Cooke.	*Di*	*6*	7/0	5-ZUJ (1), 10-MOG (1), 13-MOR (2), 26-TUE (1), 28-SIN (1), 45-HOR (1).
Diplodia pinea (Desm.) Kickx	*Dip*	*14*	27/11	1-TOR (1), 2-ARG (4), 10-MOG (1), 16-FOR (1), 19-ELC (2), 21-POR (2), 23-CHE (1), 25-UTI (1), 35-ARI (2), 46-NAV (2), 47-SUR (6), 52-MOC (8), 53-LLI (5), 55-CEN (2).
Diatripella sp. (Ces. & de Not) de Not.	*Di*	*3*	2/1	16-FOR (1), 22-BUÑ (1), 29-NER (1).
Epicoccum nigrum[f] Link.	*Epn*	*9*	5/5	2-ARG (1), 7-BEN (1), 10-MOG (2), 18-VILL (1), 19-ELC (1), 21-POR (1), 24-AND (1), 27-REQ (1), 46-NAV (1).
Gliocephalis sp.[e] Matr.	*Gl*	*1*	0/1	1-TOR (1).
Hendersoria acicula Münch et Tub.	*Hea*	*5*	5/1	1-TOR (1), 2-ARG (1), 3-COL (1), 4-ALB (1), 10-MOG (2).
Leptostroma pinastri (Desm.)	*Lep*	*32*	0/57	1-TOR (1), 2-ARG (2), 4-ALB (1) 5-ZUJ (2), 8-PUE (1), 9-YES (3), 10-MOG (3), 11-AMA (2), 13-MOR (2), 15-SAB (2), 18-VILL (1), 19-ELC (1), 20-COF (3), 22-BUÑ (2), 23-CHE (3), 29-NER (3), 25-UTI (2), 27-REQ (1), 30-PAR (1), 35-ARI (2), 32-ALA (2), 37-CAS (1), 39-ANR (2), 40-BEL (2), 41-MAE (1), 42-MON (2), 43-MUS (1), 44-BAT (2), 45-HOR (2), 50-CER (1), 52-MOC (2), 53-LLI (2).
Melampsora sp.[e]	*Me*	*2*	1/2	10-MOG(1), 19-ELC (2).
Naemacyclus niveus (Pers. ex Fr.) Fuck. ex Sacc.	*Nan*	*4*	0/5	32-ALA (2), 34-FUE (1), 44-BAT (1), 52-MOC (1).
Nectria coccinea (Pers.: Fr.) Fr.	*Nec*	*2*	3/0	40-BEL (2), 2-ARG (1).
Pestalotiopsis stevensonii Peck	*Pes*	*11*	7/11	1-TOR (2), 2-ARG (2), 12-VVA (1), 24-AND (1), 35-ARI (5), 36-MEQ (1), 37-CAS (2), 46-NAV (1), 51-MAI (1), 52-MOC (1), 55-CEN (1).
Penicillium sp.[f] *Link ex Fr.*	*Pe*	*12*	15/12	4-ALB (1), 6-BAZ (2), 11-AMA (3), 15-SAB (3), 20-COF (1), 22-BUÑ (2), 27-REQ (2), 36-MEQ(4), 37-CAS (1), 40-BEL (2), 48-SAR (1), 49-GRA (5).
Phoma sp. Sacc.	*Ph*	*10*	6/6	4-ALB (1), 10-MOG (1), 13-MOR (1), 15-SAB (1), 18-VILL (2), 20-COF (2), 45-HOR (1), 39-ANR (1), 40-BEL (1), 41-MAE (1).
Phomopsis sp. (Sacc.) Bubak.	*Pho*	*14*	9/8	7-BEN (1), 8-PUE (1), 10-MOG (1), 11-AMA (1), 14-ALM (3), 21-POR (1), 23-CHE (1), 24-AND (1), 26-TUE (1), 29-NER (1), 35-ARI (1), 42-MON (1), 46-NAV (1), 54-BAN (2).
Preussia sp. Fuckel	*Pr*	*1*	0/1	34-FUE (1).
Rhyzopus stolonifer[f] (Ehrenb. ex Fr.) Vuill.	*Rhs*	*6*	7/3	34-FUE (3), 39-ANR (2), 43-MUS (2), 44-BAT (1), 50-CER (1), 51-MAI (1).

Table 3 (continued)

Fungi[a]	Abb.[b]	S[c]	T/N[d]	Stands where the fungus was isolated from, and frequency of isolation within each stand (numbers within parenthesis)
Sclerophoma pithyophila (Corda.) Höhn	*Scp*	*11*	7/4	9-YES (1), 2-COL (1), 11-AMA (1), 22-BUÑ (1), 24-AND (1), 27-REQ (1), 28-SIN (1), 38-NON (1), 39-ANR (1), 40-BEL (1), 45-HOR (1).
Sordaria fimicola (Robergo ex Desmaz.)	*Sof*	*7*	6/1	4-ALB (1), 27-REQ (1), 29-NER (1), 37-CAS (1), 38-NON (1), 43-MUS (1), 49-GRA (1).
Spegazzinia sp.[e] Sacc.	*Sp*	*2*	0/2	5-ZUJ (1), 4-ALB (1).
Stachybotrys sp.[e] Cda	*St*	*10*	4/11	2-ARG (2), 6-BAZ (1), 7-BEN (2), 10-MOG (1), 12-VVA (1), 16-FOR (3), 17-CIE (1), 18-VILL (1), 19-ELC (1), 43-MUS (2).
Trichoderma viride Pers. ex S. F. Gray	*Trv*	*4*	0/4	1-TOR (1), 25-UTI (1), 31-OLA (1), 46-NAV (1).
Thyriopsis halepensis (Ck.) Thies & Syd.	*Thh*	*10*	0/14	13-MOR (1), 18-VILL (2), 16-FOR (2), 20-COF (1), 22-BUÑ (1), 23-CHE (1), 24-AND (2), 27-REQ (2), 39-ANR (1), 49-GRA (1).
Ulocladium sp. Preuss.	*Ul*	*6*	3/5	2-ARG (2), 4-ALB (1), 9-YES (2), 10-MOG (1), 16-FOR (1), 53-LLI (1).

[a] Fungal species nomenclature follows National Center of Biotechnology Information (www.NCBI.nlm.nih.gov). [b] Abbreviated name used in the graphs. [c] Number of stands were the fungi where found. [d] Number of occurrences of every fungus in Twigs (T) and Needles (N)

[e] Closest taxa reached according to the features analyzed. [f] Possible lab contaminants since samples were not surface sterilized

ature', 'elevation' and the 'availability of water' to be significant predictors for the fungal species composition. Thus another Monte Carlo analysis with 999 permutations was carried out with these six variables. The results of the model were: F-ratio=1.757 and *p*-value=0.0010. Eighenvalues (λ) for axis 1 was 0.275, axis 2 was λ=0.202, axis 3 was λ=0.158 and axis 4 was λ=0.124. Total λ sum was 4.99479.

The graphic representation of these results can be observed in Fig. 2. The variables "mean temperature", "availability of water" and "illumination" appear to have the greatest influence on the species distribution. Axis 1 seems to be closely related to the mean temperature, which greatly influence the occurrence of several fungi, either positive with *Arthrobotrys dactiloides*, *Melampsora* sp., *Chaetomium globossum*, *Chaetomiun fusiforme*, *Spegazzi-*

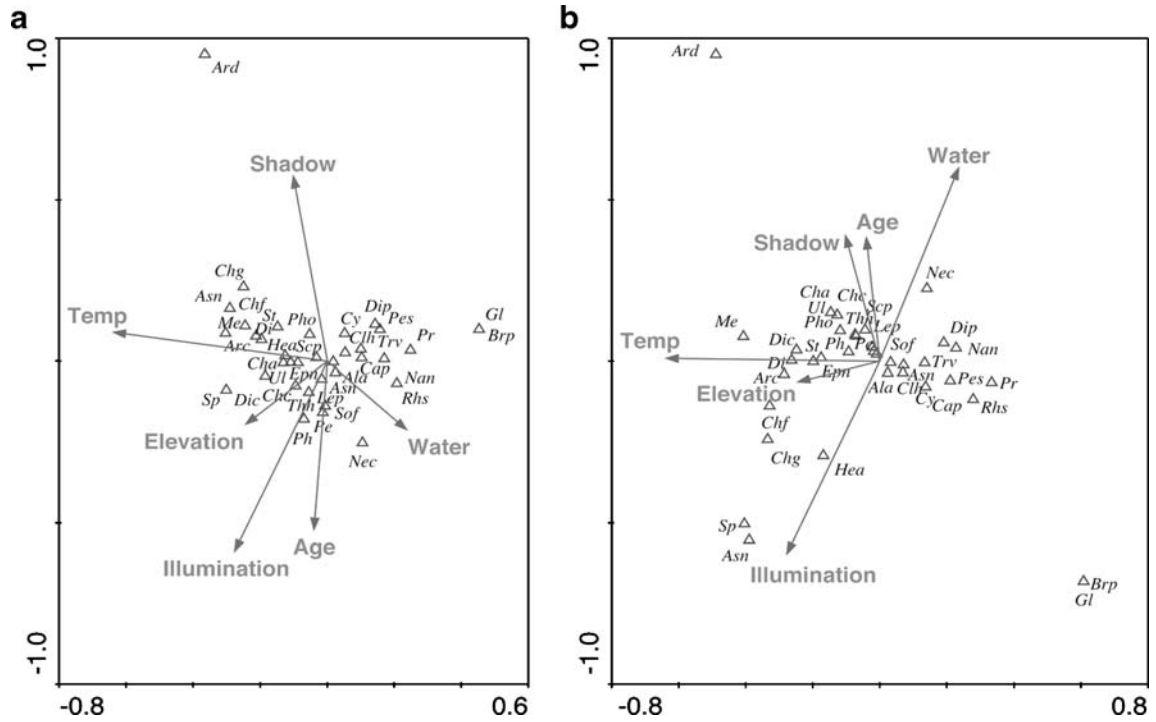

Fig. 2 **a** First and second axes, and **b** first and third axes for the Canonical Correlation Analysis (CCA) showing the environmental variables with a significant influence on the fungal species composition. The complete name for fungal species can be observed in Table 2. (Temp) Average temperature

nia sp., *Hendersoria acicola* and *Aspergillus niger*, or negative with *Gliocephalis* sp., *Brunchorstia pinea, Cytospora sp., Diplodia pinea, Naemacyclus niveus* and *Pestalotiopsis stevensonii*. In this sense, the variable 'illumination' was positively correlated with the 'mean temperature' variable and inversely correlated with the 'shadow' variable. Thus, *Spegazzinia* sp., *Aspergillus niger* and *Hendersoria acicola* were positively influenced by 'light exposure' and *A. dactyloides* was positively influenced by 'shadow'. In addition, *Spegazzinia* sp. was also much influenced by elevation. On the other hand, the older the stand, *Nectria coccinea*, *Phoma* sp., *Penicillium* sp., *Sordaria fimicola*, *Leptostroma pinastri* and *Thyriopsis halepensis* occurred more frequently. Finally, 'availability of water' positively determined the appearance of *Nectria coccinea*, *Diplodia pinea* and *Naemacyclus niveus*.

Discussion

In recent years there has been several publications that have tried to postulate the effects of the climate change on the health of trees (Thomas et al. 2002; Arnold 2007; Sieber 2007; La Porta et al. 2008; Gonthier et al. 2006; Giordano et al. 2009; Speer et al. 2009). This includes the effect of global warming on the appearance of new fungal diseases in forests where they had not previously caused health problems. The resulting abiotic conditions may cause physiological disturbances inside the trees that may favour pathogen spread and encourage disease development (Brandt et al. 2003; Desprez-Loustau et al. 2006; Gonthier et al. 2006; Giordano et al. 2009). In this sense, Mediterranean forests in Spain, particularly, those close to natural *Pinus halepensis* stands, are highly susceptible to abiotic stress, as desertification (Le Houerou 1992) or air pollution increases (Gimeno et al. 1995; Bussoti and Ferreti 1998). In the same way, forests of the northern Iberian Peninsula, which have been afforested with tree species out of their natural range, are also growing under abiotic threats such as the common occurrence of frosts, due to their thermophilic character (Thomsen 2009). Under this situation, many opportunistic fungal species might cause the generalized decline of the Allepo pine in Spain.

In the present study, species richness was significantly different among the sampled stands. This may indicate that different environmental conditions result in different fungal species richness and in different fungal species composition in the stands (Saikkonen 2007). 'Type of ground' and, especially 'solar radiation' appear to have some effect on fungal species richness. Parts of the tree with a longer exposure to the sun also have a lower humidity and consequently, less fungal mycota (Bahnweg et al. 2005). At the tree level, fungal species richness is clearly affected by the tissue type, being higher in needles than twigs. Tissue-pathogen specificity was also observed, with *Leptostroma pinastri*, *Thyriopsis halepensis* and *Naemaclyclus niveus* only occurring in needles. Needles probably represent the most biologically active part of the tree due to their permanent contact with the environment (Arnold 2007). Likewise, needles are more affected than twigs by stressful phenomena such as frosts (Calamassi et al. 2001).

Most of the fungi recorded in the present study have been also described previously for other conifers and broadleaf trees (Collado et al. 1996; Danti et al. 2002; Martín et al. 2004; Santamaria and Diez 2005; Zamora et al. 2008). Frequent species such as *Aspergillus niger, Penicillium* sp., *Trichoderma viridae* or *Arthrobotrys dactyloides* are recognised as saprobes. *Arthrobotrys dactyloides* has a phytopathological relevance since it has been described as a nematode-trapping fungus related to the biocontrol of root-knot nematodes such as *Meloidogyne javanica* (Stirling et al. 1998) and it is strongly limited by the mean temperature. *Alternaria alternata* and *Cladosporium herbarum*, which were the most frequently isolated taxa, are often recorded as endophytes (Guo et al. 2004; Ganley and Newcombe 2006; Promputtha et al. 2007; Huang et al. 2008). However, the most interesting findings, because of their potential implication in the *Pinus halepensis* decline, were those fungi described previously as needle and/or twig pathogens of conifers. *Brunchorstia pinea, Cytospora* sp., *Diplodia pinea* (syn. *Sphaeropsis sapinea* Dyko and Sutton), *Hendersoria acicola, Leptostroma pinastri*, *Naemaclyclus niveus*, *Pestalotiopsis stevensonii*, *Phoma* sp., *Phomopsis* sp., *Sclerophoma pythiophila* and *Thyriopsis halepensis* are pathogens that require further analyses in relation to their occurrence frequency and the environmental variables.

Leptostroma pinastri and *Thyriopsis halepensis*, which have been previously described as needle pathogens (Siebercanavesi et al. 1991; Phillips and Burdekin 1992) occurred in relation to the age of the stand. The older the stand, the more frequent was their presence. (Lehtijärvi and Barklund 2000; Arnold and Herre 2003). *Diplodia pinea*, *Naemaclyclus niveus*, *Pestalotiopsis stevensonii*, *Cytospora* sp., and to a greater extend, *Brunchorstia pinea* (anamorph of *Gremmeniella abietina*) were negatively associated with mean temperature. To explain this fact it is important to understand that temperature constitutes the limiting factor of natural Allepo pine distribution. Due to its early flowering it is very sensitive to late low temperatures, therefore, its natural habitat is generally distributed over temperate regions without frosts (average temperature of coldest month higher than −3°C) (Gil et al. 1996). In colder conditions, despite triggering off mechanisms of adaptation as delaying the formation of secondary needles (Climent et al. 2009), the tree may become weaker and, consequently,

be more susceptible to the infection of opportunistic pathogens. In particular, *Brunchorstia pinea* (Santamaria et al. 2003) is a psicrophylic fungus favouring colder climates (Thomsen 2009) as it happens in Tordehumos (1-TOR), Valladolid, location where although snow is rare the winters are cold enough (around 60 frosts per year) and where it was isolated for the first time. This confirmed its presence in this region.

Pestalotiopsis stevensonii and *Naemacyclus niveus,* which have been also considered secondary pathogens on pine needles (Lanier et al. 1978; Phillips and Burdekin 1992), occurred associated with *Diplodia pinea*, one of the most widespread pathogens of pines (Stanosz et al. 2007) and other conifers (Punithalingam and Waterson 1970; Farr et al. 1989). *Diplodia pinea* was considerably associated with areas with more availability of water, which is in contrast to that stated previously for *P. halepensis* (Paoletti et al. 2001) and other conifers (Bachi and Peterson 1985; Johnson et al. 1997; Stanosz et al. 2001). In such studies, susceptibility increased in water deficient conditions. However, according to the results obtained by Paoletti et al. (2001), water stress enhanced the damage caused by *D. pinea*, but it was not a necessary prerequisite for colonization. The taxonomy of *Diplodia* is presently undergoing revision (De Wet et al. 2003) and it will be interesting to establish if this belong in *Diplodia sensu stricto*.

Phomopsis sp. and *Sclerophoma pythiophila* were frequently identified in several stands spread over the whole peninsula (25.45% and 20%, respectively) and did not seem to respond to any variable strongly. This may signify that they occurred as ubiquitous species capable of adapting to different environmental conditions, as their wide distribution shows. Not only can they be found in Europe but also worldwide (Sutton and Waterston 1970). Specifically, *Sclerophoma pythiophila* has typically been isolated from Norway spruces and Scots pines (Rishbeth and Meredith 1957; Magan et al. 1995). In Spain, it has been detected in *Pinus halepensis* frequently associated to diseased twigs where *Gremmeniella abietina* also occurred (Santamaria et al. 2007, 2008). However, in the current study, *Sclerophoma pythiophila* did not appear in the same stand where *Gremmeniella abietina* was found. Further studies about the relation between edaphoclimatic factors and those fungal species may clarify the combined action of the environmental conditions and fungal pathogens upon the course of a forest disease.

In conclusion, this study identified 35 fungal species isolated from *P. halepensis* trees with symptoms of decline. Ten species have phytopathological importance. However, according to the generalized damages observed in the *P. halepensis* stands, their pathogenicity is not severe enough to be the only cause. Several edaphoclimatic variables such as, 'availability of water', 'shadow', 'light exposure', 'age', 'elevation' and 'mean temperature' seem to influence fungal species richness and composition in Allepo pine. In particular, 'low temperature', limits Allepo pine distribution. Therefore, a combination of stressful abiotic factors, which predispose the health of the trees and enhance the occurrence of several pathogenic fungi traditionally known as secondary pathogens, may explain the current situation of the *Pinus halepensis* decline in Spain.

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