

## Monitoring endophyte populations in pine plantations and native oak forests in Northern Spain

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### Abstract

The replacement of native forest with plantations of other species may have important impacts on ecosystems. Some of these impacts have been widely studied, but very little is known about the effects on fungal communities and specifically endophytic fungi. In this study, endophyte assemblages in pine plantations (*Pinus sylvestris*, *P. nigra* and *P. pinaster*) and native oak forests (*Quercus pyrenaica*) in the north of the province of Palencia (Spain) were analyzed. For this purpose, samples of needles/leaves and twigs were collected from three trees in each of three plots sampled per host species. The samples were later processed in the laboratory to identify all of the endophytic species present. In addition, an exhaustive survey was carried out of the twelve sites to collect data on the environmental, crown condition, dendrometric and soil variables that may affect the distribution of the fungi. The endophyte assemblages isolated from *P. sylvestris* and *P. nigra* were closely related to each other, but were different from those isolated from *P. pinaster*. The endophytes isolated from *Q. pyrenaica* were less closely related to those from the other hosts, and therefore preservation of oak stands is important to prevent the loss of fungal diversity. Finally, the distribution of the endophyte communities was related to some of the environmental variables considered.

**Key words:** endophytic fungi; environmental variables; soil properties; crown condition; dendrometric variables.

### Resumen

#### Comunidades de hongos endófitos en plantaciones de pino y robledales del norte de España

La sustitución de los bosques autóctonos por plantaciones de otras especies puede tener importantes consecuencias sobre el ecosistema. Algunos de esos cambios ya han sido ampliamente estudiados, pero hasta el momento hay muy poca información acerca de lo que ocurre con las comunidades fúngicas y en especial con las de los endófitos que ahí viven. En este trabajo se analizaron las agrupaciones de endófitos existentes en las plantaciones de pino (*Pinus sylvestris*, *P. nigra* y *P. pinaster*) y bosques nativos de rebollo (*Quercus pyrenaica*) en el norte de la provincia de Palencia (España). Para ello se recogieron muestras de acículas/hojas y ramillos de cada una de las especies estudiadas que fueron procesadas en laboratorio para determinar qué especies de hongos endófitos portaban. Además se realizó una exhaustiva toma de datos en las doce parcelas estudiadas para analizar las variables ambientales, del estado fitosanitario de la copa, dendrométricas y edáficas implicadas en la distribución de los hongos. Las comunidades de endófitos encontradas en *P. sylvestris* y *P. nigra* resultaron ser similares entre sí, y al mismo tiempo diferentes de las de *P. pinaster*. Los endófitos aparecidos sobre *Q. pyrenaica* resultaron estar alejados de los del resto de los hospedantes, por lo que la conservación de los robledales es importante para evitar la pérdida de la diversidad fúngica. Finalmente se encontraron algunas variables ambientales relacionadas con la distribución de las comunidades de endófitos.

**Palabras clave:** hongos endófitos; variables ambientales; propiedades edáficas; estado de copa; variables dendrométricas.

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Received: 10-10-11. Accepted: 09-07-12.

## Introduction

In past centuries, the north of the province of Palencia (Castilla y León, Spain) was mainly covered by *Quercus pyrenaica* Willd. forests. However, in the 19th century many of these oak forests were cut down and replaced with crops and grazing land, to meet the demands of the growing population. Much later, in the 1960s, many pine plantations were established for productive and protective purposes. The most commonly used species were *Pinus sylvestris* L., *P. nigra* J. F. Arnold and *P. pinaster* Aiton, in monospecific or mixed stands. Monospecific plantations of *P. sylvestris* occupy 1,140,000 ha in Spain, those of *P. nigra* 870,000 ha and those of *P. pinaster* 1,800,000 ha (Serrada *et al.*, 2008). Most of these plantations coexist with native forests of *Q. pyrenaica*, which is still a very important tree species in Spain, occupying an area of more than 1,000,000 ha (Gil and Torre, 2007; Serrada *et al.*, 2008).

Researchers have made great efforts to assess the effects that replacing native vegetation with pine plantations have on macroscopic life forms (Proenca *et al.*, 2010; Galván and Benayas, 2011), but little attention has been paid to the potential effects on microbial diversity. Plants are known to harbour host-specific microorganisms (Petrini and Fisher, 1990; Ragazzi *et al.*, 2003; Martín-García *et al.*, 2011), and the replacement of native vegetation may cause changes in the microbial diversity, including that of endophytic fungi.

Many definitions of endophytes have been reported. Some researchers define endophytes as fungi that are able to infect their hosts without causing visible symptoms of disease (Petrini, 1991), while others consider the term endophyte to be synonymous with mutualism (Backman and Sikora, 2008). However, the distinction between pathogenic and endophytic organisms is not clear, and the same fungus or even the same isolate may behave as a saprophyte or pathogen, depending on the host vigour (Schulz *et al.*, 1999). In the present study, we considered endophytes as those fungi isolated from apparently healthy and asymptomatic tissues after surface sterilization.

In addition to the host, environmental conditions and stand characteristics are also important in determining the presence of endophytic communities (Petrini and Carroll, 1981; Helander *et al.*, 2006). Recently, Bottella *et al.* (2010) have demonstrated that several climatic variables appear to influence endophyte communities in Aleppo pine, and Martín-García *et al.* (2011) reported that nutrient status, forest health and

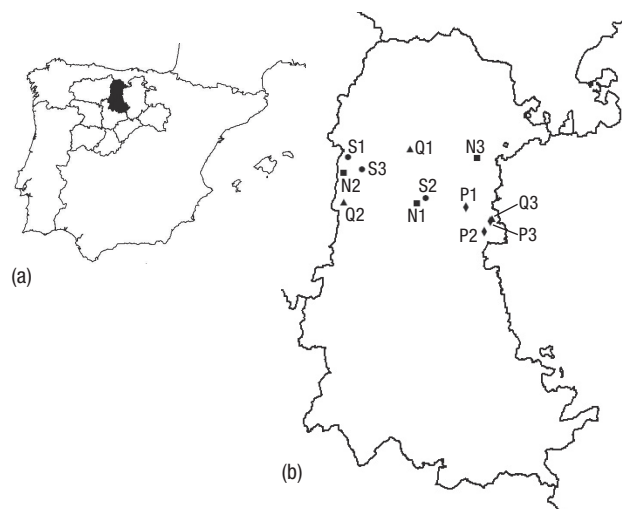
dendrometric variables appear to determine the types and distribution of endophyte communities in poplar plantations. However, so far no research has been carried out to determine the effects of the host or the overall stand characteristics. The main aim of this study was to describe and monitor endophyte populations in native and introduced forest species in Palencia (northern Spain).

## Material and methods

### Description of the study site and sampling procedure

The present study was carried out in Palencia (northern Spain) (Fig. 1), in areas where plantations of *P. sylvestris*, *P. nigra* and *P. pinaster* coexist with native forests of *Q. pyrenaica*. Three native oak stands and nine pine plantations (three stands of each pine species) were chosen for study. Three trees were chosen at random within each stand for sampling.

An exhaustive field survey was performed to obtain as much data as possible about environmental, phytosanitary, dendrometric and soil properties, with the aim of relating these variables to the distribution of the endophyte communities. The environmental variables studied were altitude, annual mean temperature, annual minimum mean temperature, annual maximum mean temperature, annual precipitation, spring precipitation, summer precipitation, autumn precipitation



**Figure 1.** Geographical location of the twelve sites surveyed; (a) in Spain; (b) in the province of Palencia. (N = *P. nigra*, P = *P. pinaster*, Q = *Q. pyrenaica* and S = *P. sylvestris*).

and winter precipitation. As regards forest health, crown transparency, discoloration and the percentage of dead crown were evaluated (Ferretti, 1997). Several dendrometric variables were measured at stand level (density, basal area and age) and at tree level (total height, crown base height, diameter at breast height [dbh], bark thickness and live crown ratio [calculated by dividing the length of the crown by the total tree height]).

Mineral soil samples were collected from the upper 30 cm soil layer in each subplot (four samples per plot). The samples were mixed together to make one composite sample per plot, and homogenized. The pH was determined potentiometrically with a pH meter, in a soil solution (1:2.5, soil:water). Organic matter was determined by the  $K_2Cr_2O_7$  method. Total N was determined by Kjeldahl digestion. Soil exchangeable cations ( $K^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$ ) were extracted with ammonium acetate and determined by atomic absorption/emission spectrometry.

Particle-size distribution was determined by the Bouyoucos method (hydrometer method), and the ISSS (International Society of Soil Science) classification was applied.

The Cationic Exchange Capacity (CEC) was determined by Bascomb's method (i.e. the exchange cations were displaced by Ba ions, which were then displaced by Mg ions and the remaining concentration of Mg was determined by titration against EDTA).

All of the variables analyzed and the respective values for each site sampled are shown in Table 1.

## Fungal isolation and identification

Healthy branches from the northern sector of the upper fourth of the canopy of each tree were collected in autumn. This type of branch was sampled because a preliminary study in two of these stands did not show any significant differences in endophyte communities according to sector (north cf. south) or height of canopy (upper and lower fourth). The branches were stored at 4°C and processed within 24 h.

The plant material (branches and needles/leaves formed during the ongoing season) was surface sterilized with ethanol (96% v/v, one minute), sodium hypochlorite (2% v/v, five minutes on needles/leaves and seven on twigs) and ethanol (70% v/v, one minute). Twelve pieces of needles/leaves (0.5 cm long/ 0.5 × 0.5 cm, respectively) and twelve twig segments (0.5 cm diam., 0.5-1 cm thick) from each tree were placed in Petri

dishes containing "potato dextrose agar" (PDA) medium. The plates were sealed with Parafilm® and incubated in the dark at room temperature for one month. The outgrowing colonies were transferred to a fresh PDA plate and grown in pure culture in diffused daylight until sporulation. Fungal isolates were observed under a stereomicroscope and identified according to morphological characteristics, by analysing the shape and colour of the colonies and the main characteristics of the fungal structures. Different taxonomic keys were used to identify the fungi.

## Statistical analysis

*Univariate statistics.* The effects of the host species (*P. sylvestris*, *P. nigra*, *P. pinaster* and *Q. pyrenaica*) and tissue sampled (needles/leaves and twigs) were evaluated by analysis of variance, by using relative isolation frequencies (RIF) and richness (number of fungal species per sample) as dependent variables. The RIF was calculated from the formula  $RIF = n_{ij}/N_{ij} \times 100$ , where  $n_{ij}$  is the number of isolates recorded in the host species  $i$  and tissue  $j$ , and where  $N_{ij}$  is the number of samples of species  $i$  and tissue  $j$  examined. Assumptions of normality and equal variance for parametric testing were confirmed. All of the statistical analyses were performed with Statistica 6.0 software for Windows (StatSoft Inc., Tulsa, Oklahoma, USA, 2004).

*Multivariate statistics.* Non-metric multidimensional scaling (NMDS) was performed to explore the relationships between the different types of pine and oak plantations (and associated dendrometric and forest health variables, soil properties and climatic characteristics) and the distribution of the endophyte communities isolated from needles/leaves only, twigs only and needles/leaves plus twigs. To our knowledge this is the first time this procedure has been used for this purpose.

NMDS is a nonparametric procedure, recommended for non-normal data because it uses ranked distances and does not depend on assumptions of linear relationships among variables (McCune and Grace, 2002). NMDS was conducted using Bray-Curtis as the distance metric, and the multivariate ordination was created using the metaMDS results. Environmental variables (dendrometric, forest health, soil properties and climatic characteristics) that were significantly correlated with one or more axes were overlaid on the ordination as vectors. NMDS was first performed at the stand level and then at the tree level.

**Table 1.** Summary of some selected environmental variables, dendrometric measures, crown condition variables and soil properties in the twelve stands under study. UTM Coordinates in European Datum 1950 (ED50) spindle 30. Climate data according to “Atlas Climático Digital de la Península Ibérica” (Ninyerola et al., 2005)

	<i>Pinus sylvestris</i>				<i>Pinus nigra</i>				<i>Pinus pinaster</i>				<i>Quercus pyrenaica</i>			
	S1	S2	S3	Mean ± S.D.	N1	N2	N3	Mean ± S.D.	P1	P2	P3	Mean ± S.D.	Q1	Q2	Q3	Mean ± S.D.
<b>Environmental variables</b>																
UTM X Coordinate	347,970	372,411	352,392	-	369,688	346,814	388,565	-	384,998	390,702	392,609	-	367,545	346,706	393,189	-
UTM Y Coordinate	4,728,484	4,715,563	4,724,462	-	4,713,892	4,723,449	4,728,054	-	4,712,559	4,704,958	4,708,014	-	4,730,747	4,714,199	4,708,947	-
Altitude (m)	1,097	1,077	943	1,039.0 ± 83.7	1,037	1,094	992	1,041.0 ± 51.1	910	938	893	913.7 ± 22.7	1,072	1,015	895	994.0 ± 90.4
Mean temp. (°C)	9	10	10	9.7 ± 0.6	10	10	11	10.3 ± 0.6	11	11	11	11.0 ± 0.0	10	10	11	10.3 ± 0.6
Annual min. mean temp. (°C)	3	4	4	3.7 ± 0.6	4	4	4	4 ± 0.0	5	4	5	4 ± 0.0	4	4	5	4.3 ± 0.6
Annual max. mean temp. (°C)	15	16	16	15.7 ± 0.6	17	16	17	16.7 ± 0.6	17	17	17	17.0 ± 0.0	16	16	17	16.3 ± 0.6
Annual precipitation (mm)	823	610	752	728.3 ± 108.5	610	756	616	654.0 ± 71.1	584	558	572	571.3 ± 13.0	736	650	577	654.3 ± 79.6
Spring precipitation (mm)	200.6	162.7	189.6	184.3 ± 19.5	159.1	183.3	169.1	170.5 ± 12.2	169.5	160.8	163.4	164.6 ± 4.5	187.9	176.5	166.8	177.1 ± 10.6
Summer precipitation (mm)	110.2	85.0	104.2	99.8 ± 13.2	80.8	104.2	93.1	92.7 ± 11.7	88.0	85.1	88.5	87.2 ± 1.8	100.2	96.4	88.8	95.1 ± 5.8
Autumn precipitation (mm)	280.5	193.9	251.8	242.1 ± 44.1	193.0	247.7	194.0	211.6 ± 31.3	179.5	174.5	178.8	177.6 ± 2.7	243.0	207.8	178.6	209.8 ± 32.3
Winter precipitation (mm)	219.4	168.4	205.2	197.7 ± 26.3	173.8	196.1	159.7	176.5 ± 18.4	143.2	135.6	139.3	139.4 ± 3.8	205.8	167.1	140.3	171.1 ± 32.9
<b>Dasometric measures</b>																
N (trees/ha)	733	1,100	678	837.0 ± 229.4	1833	689	1367	1,296.3 ± 575.3	478	667	689	611.3 ± 116.0	322	778	678	592.7 ± 239.7
G (m <sup>2</sup> /ha)	34	37.4	31.0	34.1 ± 3.2	40.0	21.0	40.8	33.9 ± 11.2	38.6	29.3	36.7	34.9 ± 4.9	13.6	24.0	2.0	13.2 ± 11.0
Age (years)	39	40	48	42.3 ± 4.9	29	36	39	34.7 ± 5.1	49	47	31	42.3 ± 9.9	103	62	19	61.3 ± 42.0
Total height (cm)	1,477.0	1,321.7	1,279.3	1,359.3 ± 104.1	814.3	1,140.3	937.0	963.9 ± 164.7	1,373.3	974.3	1,094.7	1,147.4 ± 204.7	1,132.0	1,175.7	446.7	918.1 ± 408.9
Crown base height (cm)	688.3	539.7	616.3	614.8 ± 74.4	267.0	473.0	280.0	340.0 ± 115.4	579.3	587.0	392.0	519.4 ± 110.4	549.0	593.3	25.7	389.3 ± 315.7
Diameter breast height (dbh)	24.1	21.6	20.2	21.9 ± 2.0	16.6	19.1	19.6	18.4 ± 1.6	30.3	27.1	25.6	27.7 ± 2.4	17.0	13.5	6.1	12.2 ± 5.6
Bark thickness (mm)	10.9	11.7	14.7	12.5 ± 2.0	12.0	10.8	20.0	14.2 ± 5.0	28.9	32.3	26.3	29.2 ± 3.0	11.5	8.0	4.8	8.1 ± 3.3
Live crown ratio	0.5	0.6	0.5	0.6 ± 0.0	0.7	0.6	0.7	0.7 ± 0.1	0.6	0.4	0.6	0.5 ± 0.1	0.5	0.5	0.9	0.7 ± 0.2
<b>Crown condition</b>																
Crown transparency (%)	20.0	18.3	8.3	15.6 ± 6.3	33.3	26.7	21.7	27.2 ± 5.9	13.3	20.0	16.7	16.7 ± 3.3	23.3	30.0	10.0	21.1 ± 10.2
Discoloration (0-4)	0.0	0.0	0.3	0.1 ± 0.2	0.7	3.0	0.7	1.4 ± 1.4	0.0	1.3	0.7	0.7 ± 0.7	2.3	2.0	2.3	2.2 ± 0.2
Dead crown (%)	11.7	11.7	13.3	12.2 ± 1.0	0.0	11.7	26.7	12.8 ± 13.4	8.3	5.0	5.0	6.1 ± 1.9	15.0	28.3	11.7	18.3 ± 8.8
<b>Soil properties</b>																
Bulk density (g cm <sup>-3</sup> )	1.1	1.1	1.1	1.1 ± 0.0	1.1	1.2	1.2	1.2 ± 0.1	1.1	2.0	1.1	1.4 ± 0.5	1.3	1.0	1.3	1.2 ± 0.2
Sand (%)	67.7	77.6	65.7	70.3 ± 6.4	74.7	79.6	72.7	75.7 ± 3.6	73.1	86.7	76.7	78.8 ± 7.1	68.7	57.6	69.6	65.3 ± 6.7
Silt (%)	15.9	14.0	21.9	17.3 ± 4.1	16.0	13.1	10.0	13.0 ± 3.0	11.3	5.6	15.6	10.8 ± 5.0	17.6	22.4	19.4	19.8 ± 2.4
Clay (%)	16.4	8.4	12.4	12.4 ± 4.0	9.3	7.3	17.3	11.3 ± 5.3	15.6	7.6	7.6	10.3 ± 4.6	13.6	20.0	11.0	14.9 ± 4.6
pH	4.4	4.3	5.3	4.7 ± 0.6	5.3	4.8	6.7	5.6 ± 1.0	5.5	5.7	5.8	5.7 ± 0.2	5.9	4.9	6.5	5.8 ± 0.8
CEC (cmol <sub>c</sub> kg <sup>-1</sup> )	20.9	18.4	20.9	20.1 ± 1.4	14.8	13.3	13.6	13.9 ± 0.8	16.4	11.7	23.4	17.2 ± 5.9	24.1	20.3	18.4	20.9 ± 2.9
Ca (g kg <sup>-1</sup> )	0.6	0.7	2.7	1.6 ± 1.2	1.8	0.5	5.5	2.6 ± 2.6	4.2	1.8	3.9	3.3 ± 1.3	0.6	1.1	2.4	1.4 ± 1.0
Mg (g kg <sup>-1</sup> )	0.2	0.1	0.7	0.3 ± 0.3	0.6	0.1	0.5	0.4 ± 0.3	1.1	1.0	2.6	1.5 ± 0.9	0.9	0.9	0.8	0.9 ± 0.1
K (ppm)	0.1	0.0	0.1	0.1 ± 0.1	0.1	0.0	0.1	0.07 ± 0.0	0.1	0.1	0.2	0.1 ± 0.1	0.2	0.1	0.2	0.2 ± 0.0
OM (%)	4.3	4.0	5.7	4.7 ± 0.9	8.6	3.7	2.7	5.0 ± 3.2	7.5	4.2	9.1	7.0 ± 2.5	4.6	5.3	2.7	4.2 ± 1.3
N (mg g <sup>-1</sup> )	1.0	0.9	1.2	1.0 ± 0.2	2.4	0.8	0.9	1.4 ± 0.9	2.4	1.1	2.5	2.0 ± 0.8	2.1	1.9	1.2	1.7 ± 0.5

The multiple response permutation procedure (MRPP) was used to test the null hypothesis, i.e. that there was no difference in the types of endophytes present in different habitats. The MRPP compares the observed intra-group average distance with the average distance that would have resulted from all the other possible combinations of the data under the null hypothesis. For this, MRPP calculates a variable A (chance-corrected within-group agreement) and a p-value to assess the significance of A. The MRPP is also a nonparametric procedure that does not depend on assumptions such as normally distributed data or homogeneous variances. The procedure was also performed using Bray-Curtis dissimilarity and running 2,000 permutations.

Non-Metric Multidimensional Scaling (NMDS) and Multiple Response Permutation Procedure (MRPP)

were carried out with the VEGAN package (Oksanen *et al.*, 2011) implemented in the R software environment (R-Development-Core-Team, 2008).

## Results

A total of 29 fungal species were isolated from 864 fragments from *P. sylvestris*, *P. nigra*, *P. pinaster* and *Q. pyrenaica*. The relative frequencies of isolation of these fungi are shown in Table 2. The most frequent endophyte was *Aureobasidium pullulans* Viala & Boyer, which appeared in 13.43% of the fragments analyzed. Some other fungi such as *Aspergillus niger* van Tieghem, *Penicillium* spp., *Cladosporium* spp., and *Alternaria alternata* complex. Ness ex Fr. also occurred

**Table 2.** The distribution of fungal species and their relative isolation frequencies (RIF). The column labelled “Total” refers to the percentage number of isolates for each fungal species with respect to the total number of the fragments cultured throughout the sampling

Fungal species	Code	<i>Pinus sylvestris</i>		<i>Pinus nigra</i>		<i>Pinus pinaster</i>		<i>Quercus pyrenaica</i>		Total
		Needles	Twigs	Needles	Twigs	Needles	Twigs	Leaves	Twigs	
		<i>Alternaria alternata</i> complex. Ness ex Fr.	Acom	7.41	3.70	–	22.22	–	7.41	
<i>Aspergillus niger</i> van Tieghem	Anig	3.70	7.41	3.70	7.41	22.22	14.81	7.41	3.70	8.80
<i>Aureobasidium pullulans</i> Viala & Boyer	Apul	7.41	37.04	–	51.85	–	3.70	–	7.41	13.43
<i>Botrytis</i> spp	Bspp	–	–	–	–	–	11.11	–	7.41	2.31
<i>Chaetomium cochliodes</i> Palliser	Ccoc	–	3.70	–	–	–	–	–	–	0.46
<i>Cladosporium herbarum</i> (Pers.) Link. ex S.F.Gray	Cher	–	–	–	–	7.41	–	–	–	0.93
<i>Cladosporium</i> spp	Cspp	–	18.52	11.11	11.11	–	7.41	–	–	6.02
<i>Cytospora leucosperma</i> (Pers.) Fr.	Cleu	–	–	–	–	–	–	–	3.70	0.46
<i>Cytospora</i> spp	Cysp	–	–	–	–	3.70	–	–	–	0.46
<i>Diplodia</i> spp	Dspp	–	3.70	–	–	–	–	–	–	0.46
<i>Epicoccum nigrum</i> Link.	Enig	3.70	–	–	3.70	3.70	3.70	–	–	1.85
<i>Nigrospora oryzae</i> (Berk. & Broome) Petch	Nory	–	–	–	–	–	–	–	7.41	0.93
<i>Penicillium</i> spp	Psp	14.81	11.11	22.22	14.81	–	–	–	3.70	8.33
<i>Pestalotiopsis funerea</i> (Desm.) Steyaert	Pfun	–	–	–	–	3.70	18.52	–	–	2.78
<i>Preussia</i> spp	Prsp	–	–	3.70	–	–	–	3.70	–	0.93
<i>Sordaria fimicola</i> (Roberge ex Desm.) Ces. & De Not.	Sfim	–	–	0.00	–	–	11.11	–	18.52	3.70
<i>Trichoderma viride</i> Pers. ex S.F.Gray	Tvir	–	–	3.70	7.41	7.41	3.70	14.81	3.70	5.09
<i>Trichothecium roseum</i> (Pers.) Link	Tros	–	–	–	–	–	–	–	14.81	1.85
<i>Deuteromycete 1</i>	Deu1	–	3.70	–	–	–	–	–	–	0.46
<i>Deuteromycete 2</i>	Deu2	–	22.22	–	–	–	–	–	–	2.78
<i>Deuteromycete 3</i>	Deu3	–	14.81	–	–	–	–	–	–	1.85
<i>Deuteromycete 4</i>	Deu4	–	–	–	–	–	–	3.70	–	0.46
<i>Deuteromycete 5</i>	Deu5	–	–	–	–	–	–	3.70	–	0.46
<i>Deuteromycete 6</i>	Deu6	–	–	–	–	22.22	29.63	–	–	6.48
<i>Deuteromycete 7</i>	Deu7	–	–	–	–	–	18.52	–	–	2.31
<i>Deuteromycete 8</i>	Deu8	–	–	–	–	–	7.41	–	–	0.93
<i>Deuteromycete 9</i>	Deu9	–	–	–	3.70	–	–	–	–	0.46
<i>Deuteromycete 10</i>	Deu10	–	–	–	11.11	–	–	–	–	1.39
<i>Deuteromycete 11</i>	Deu11	–	–	–	–	–	7.41	–	–	0.93
Species richness		2.67	4.67	1.67	5.00	2.67	5.67	3.00	3.00	3.54

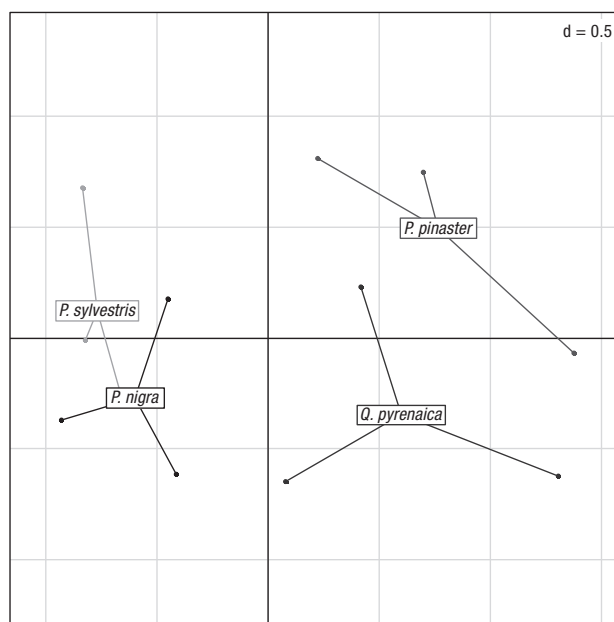
at high frequencies. On the other hand the frequencies of isolation of *Chaetomium cochliodes* Palliser, *Cytospora leucosperma* (Pers.) Fr., *Cytospora* spp., *Diplodia* spp. and some unidentified mitosporic fungi were lower. *A. niger* was the most ubiquitous fungus, and appeared in all hosts and tissues sampled. Similarly, some other endophytes such as *A. alternata* complex, and *A. pullulans* were isolated from all tree species studied. The fungal species *Cladosporium* spp. and *Epicoccum nigrum* Link. appeared in all three pine species, but were absent from oak.

The species richness of isolates obtained from pine plantations was not significantly different from that in oak forests ( $N = 12$ ,  $F = 0.713$ ,  $P = 0.558$ ), but did significantly differ between tissues (twigs > needles/leaves) ( $N = 12$ ,  $F = 12.500$ ,  $P = 0.003$ ). The same pattern was found for RIF values ( $N = 12$ ,  $F = 1.732$ ,  $P = 0.201$  and  $N = 12$ ,  $F = 18.572$ ,  $P < 0.001$ , respectively).

Multivariate analysis of the relative frequencies of endophytes isolated from needles/leaves only, twigs only or needles/leaves plus twigs revealed similar results, although the grouping of the stands according to the tree species was clearer for needles/leaves plus twigs. For this reason, the individual NMDS ordinations for leaves and twigs are not shown.

NMDS ordination and the MRPP clearly differentiated the host species ( $A = 0.14$ ,  $p = 0.005$ ). The ordination of the endophyte assemblages separated the *P. sylvestris* and *P. nigra* plantations from *P. pinaster* and *Q. pyrenaica* stands (Figure 2). Several endophytes such as *Cladosporium* spp., *Penicillium* spp., *A. alternata* complex, *A. pullulans* and Deuteromycete 2 were mainly associated with *P. sylvestris* and *P. nigra*, although they were not exclusive to these species. On the other hand, while *Sordaria fimicola* was mainly isolated from *Q. pyrenaica*, and to a lesser extent from *P. pinaster*, other endophytic fungi such as *Pestalotiopsis funerea* (Desm.) Steyaert and Deuteromycete 6 were almost exclusive to *P. pinaster* (Figure 3).

NMDS at stand level retained several significant environmental variables (altitude ( $p = 0.02$ ), winter precipitation ( $p = 0.02$ ), minimum mean temperature ( $p = 0.01$ ), and two soil exchangeable cations (Ca [ $p = 0.02$ ] and K [ $p < 0.01$ ])). The first ordination axis for endophyte assemblages, and to a lesser extent the second axis, appeared to indicate similar patterns for climatic and soil properties (Figure 3). Examination of the ordination shows several species associated with a mild winter and high concentrations of Ca and K, or low



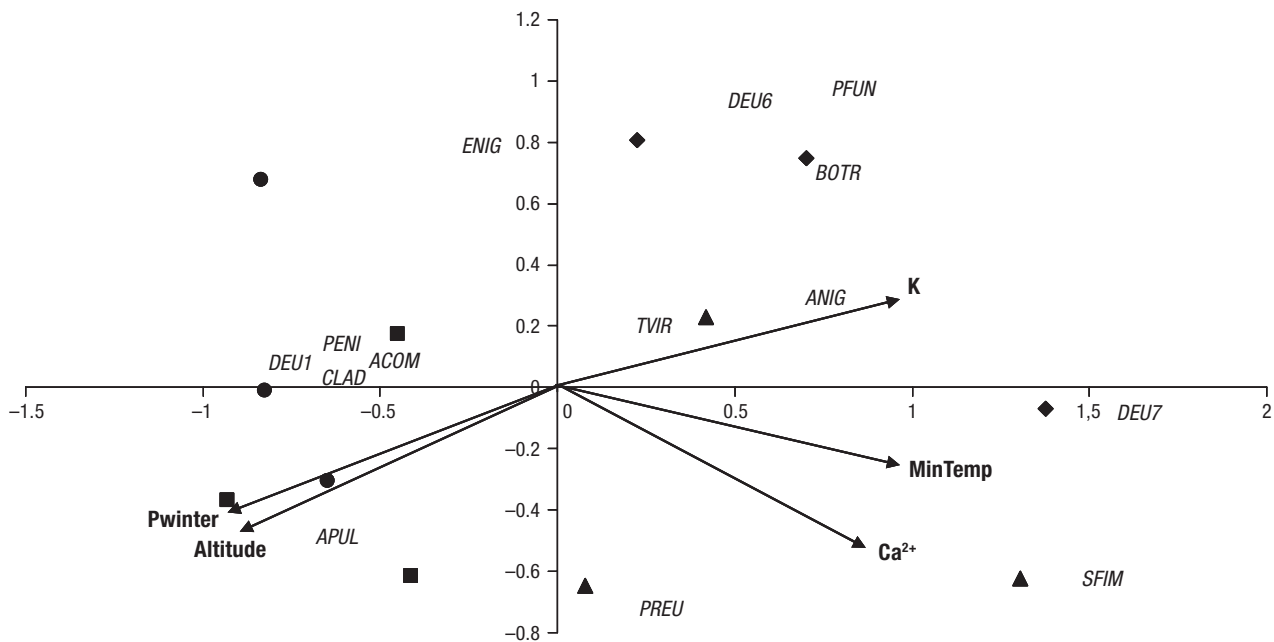
**Figure 2.** Non-metric multidimensional scaling (NMDS) at stand level. Dissimilarity distance = Bray-Curtis.

altitude and winter precipitation values (*S. fimicola* and Deuteromycete 7) or vice versa (*A. pullulans*, Deuteromycete 2, *Penicillium* sp. and *A. alternata* complex).

On the other hand, NMDS carried out at tree level retained two significant environmental variables, both of which are dendrometric variables (crown base height [ $p = 0.04$ ] and live crown ratio [ $p = 0.01$ ])). The ordination biplot appears to indicate the height of the lowest living branch and the ratio between this and total height as key factors for endophyte assemblages. In particular, several species appear to be associated with trees with high crown base height and low live crown ratio (*Cladosporium* spp., *A. alternata* complex and *Penicillium* spp.), and vice versa (*S. fimicola*) (Figure 4).

## Discussion

Similar numbers of taxa were recorded in the present study as in previous surveys on fungal communities from other hosts, including conifers and broadleaf trees under mild climate (Pettrini and Fisher, 1990; Collado *et al.*, 2000; Ragazzi *et al.*, 2003; Martín-Pinto *et al.*, 2004; Santamaría and Diez, 2005; Zamora *et al.*, 2008). The most abundant species observed in the present study (*A. pullulans*, *Penicillium* spp., *A. niger*, *Cladosporium* spp. and *A. alternata* complex) are ubiqu-



**Figure 3.** Multivariate analysis for fitting environmental vectors to NMDS (Non-metric multidimensional scaling) ordination plots. Symbols represent the sites (circle = *P. sylvestris*, square = *P. nigra*, rhombus = *P. pinaster*, triangle = *Q. pyrenaica*).

uitous taxa, which have often been isolated from very different host genera, such as *Quercus* (Fisher *et al.*, 1994; Collado *et al.*, 1999; Nicolotti *et al.*, 2003), *Pinus* (Martín-Pinto *et al.*, 2004; Zamora *et al.*, 2008; Botella *et al.*, 2010), *Populus* (Santamaría and Diez, 2005; Martín-García *et al.*, 2011; Martín-García *et al.*, 2012), *Eucalyptus* (Bettucci and Alonso, 1997), *Betula* (Green, 2004), *Picea* (Johnson and Whitney, 1992) and *Calocedrus* (Petrini and Carroll, 1981). Conversely, other species isolated are known secondary and opportunistic pathogens. This is the case with *Botrytis* spp. (Capieau *et al.*, 2004), *Cytospora* spp. (Rogers and Noskowiak, 1976), *Diplodia* spp. (Fabre *et al.*, 2011) and *Pestalotiopsis funerea* (Vujanovic *et al.*, 2000).

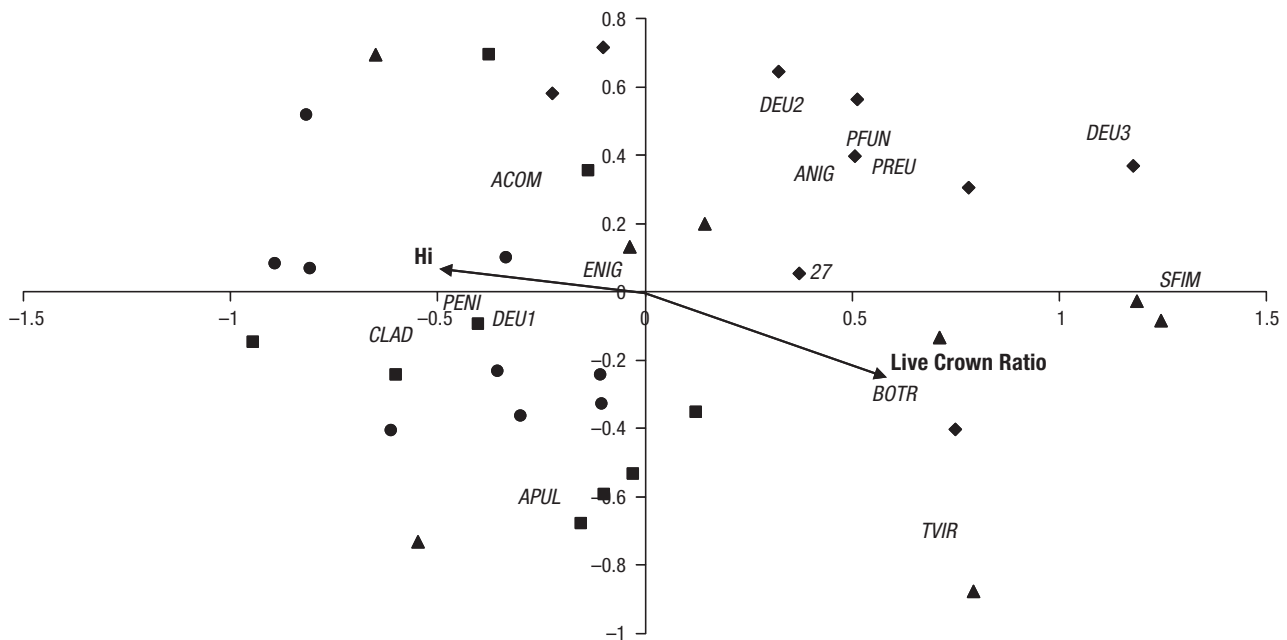
The present results show that species richness did not differ significantly between pine plantations and oak forests, although it was slightly higher in the pine stands. Arnold and Hoffman (2008) indicated that species richness may be higher in exotic species than in native species, so that it is perhaps not surprising that we did not find any differences between pines and oaks, despite the recent introduction of pines in the area.

Tissue clearly affected endophytic species richness in the tree species sampled, and more fungal taxa were found in hard tissues like twigs than in soft tissues such as leaves and needles. The needles of pines and leaves of oaks are both renewed periodically. When this hap-

pens, the fungi must colonize the new tissue, which may explain the lower number of isolates recorded in the needles/leaves than in the twigs. Similar findings have been reported for *Quercus ilex* L. (Collado *et al.*, 1999), *Q. faginea* Lam. (Collado *et al.*, 2000) and some *Pinus* spp. (Zamora *et al.*, 2008), but the opposite was found in *Cupressus arizonica* Greene (Arnold, 2007).

Analysis by NMDS and MRPP showed that endophyte assemblages differed according to host species, which is consistent with findings of other studies that compared hosts of different genera (Petrini and Fisher, 1990), the same genus (Collado *et al.*, 2000) and even a hybrid compared with its parent (Martín-García *et al.*, 2012).

Similar endophyte communities were found in *P. sylvestris* and *P. nigra* stands. These pine species occupy similar types of environments, and both grow in cold areas (Gil and Torre, 2007). The multivariate analysis revealed that the fungal assemblages found in *P. pinaster*, a western Mediterranean species, were different from those isolated from the other pine species. This appears to be consistent with the findings of Hata and Futai (1996), who reported that the taxonomic affinities of the host pines strongly affected the colonization patterns of the endophytes. The *Q. pyrenaica* mycoflora was very different from the mycoflora isolated from *P. sylvestris* and *P. nigra*, and only slightly related to that isolated from *P. pinaster*.



**Figure 4.** Multivariate analysis for fitting environmental vectors to NMDS ordination plots. Hi = crown base height. Live Crown Ratio was calculated by dividing the length of the crown by the total tree height. Symbols represent the trees (circle = *P. sylvestris*, square = *P. nigra*, rhombus = *P. pinaster*, triangle = *Q. pyrenaica*).

The NMDS revealed that the type and distribution of endophytic communities depend on edaphoclimatic variables as well as host specificity. The location of different host species is determined by edaphoclimatic factors, and therefore it is possible that the environmental variables may have been retained because of indirect correlations with the host. However, this is unlikely, since only exchangeable K was significantly related to the host species ( $N = 12$ ,  $F = 4.67$ ,  $P = 0.03$ ), and altitude, winter precipitation, minimum mean temperature and exchangeable cations Ca were not significantly related to host species ( $N = 12$ ,  $F = 2.33$ ,  $P = 0.15$ ;  $N = 12$ ,  $F = 3.27$ ,  $P = 0.08$ ;  $N = 12$ ,  $F = 2.22$ ,  $P = 0.16$ ;  $N = 12$ ,  $F = 2.96$ ,  $P = 0.10$ , respectively).

Altitude has previously been identified as a key factor in the pattern of colonization of endophytes in different host species (Sieber, 1989; Sieber *et al.*, 1999; Ragazzi *et al.*, 2003; Hashizume *et al.*, 2008; Botella *et al.*, 2010), although several authors have pointed out that the importance of altitude may actually be due to climatic conditions (Sieber, 1989; Hashizume *et al.*, 2008). The present findings are consistent with this hypothesis, since altitude was strongly correlated with both climatic variables retained in the NMDS analysis, winter precipitation and minimum mean temperature ( $r = 0.74$ ,  $p < 0.01$  and  $r = -0.78$ ,  $p < 0.01$ , respectively). On the other hand,

it is well known that the optimum growth rates of endophytes differ depending on the temperature (Hashizume *et al.*, 2008). Therefore, the minimum mean temperature may limit or favour the colonization and spread of endophytes according to their temperature requirements. Similarly, precipitation has traditionally been indicated as a key factor explaining the colonization pattern of endophytes (Kriel *et al.*, 2000). However no study has determined the period during which precipitation is essential. Arnold (2007) pointed out that endophytes move via contagious spread (horizontal transmission), and therefore precipitation during the sporulation season is expected to be the most influential variable. Nevertheless, the sporulation season varies according to the species, and e.g. Sieber *et al.* (1999) indicated favourable climatic conditions as a prerequisite for the establishment of endophytic thalli, but also pointed out that these conditions depend on the endophyte species and the sporulation season.

The concentrations of calcium and potassium in the soil were related to the type of fungal species isolated. Several studies have indicated that soil nutrient status has an important effect on endophytic communities (Sieber *et al.*, 1999; Kriel *et al.*, 2000; Martín-García *et al.*, 2011). However, so far no research has been carried out to determine the effects of soil properties on endophytes of pines and oaks.



Crown base height and live crown ratio explained the distribution of the fungi according to the NMDS analysis performed at tree level, whereas density was not related to the endophytic community, in contrast with other studies that indicated this variable as essential (Helander *et al.*, 1993; Kriel *et al.*, 2000). Live crown ratio appears to have a greater effect on endophyte assemblages than crown base height, according to the NMDS analysis. This may be because both crown base height and, indirectly, the distance from the lowest branch to sampled branch are included. Thus, taking into account that the fungus overwinters in fallen leaves and twigs from which spores are discharged into the canopy (Helander *et al.*, 2006), a lower crown base height should favour colonization by endophytes. The distance between the lowest branch (possible beginning of colonization) and the branch sampled may be decisive for the fungi colonization pattern.

## Conclusions

The endophyte assemblages identified in the pine plantations established in the 1960s were different from those isolated from native *Q. pyrenaica* forests. Therefore, taking into account the great importance of endophyte communities for the pharmaceutical and agrochemical industries (Schulz *et al.*, 2002) and the potential future benefits, oak stands should be preserved to maintain the existing biodiversity of endophytes.

## Acknowledgements

We thank Celia Herrero for the exhaustive data collection. Financial support for this study was provided by the European Union and the Regional Government of Castile and Leon, through the INTERREG IIIB Atlantic Area programme (FORSEE project).

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