Fungi in needles and twigs of pine plantations from northern Spain

Zamora, P.[1*], Martínez-Ruiz, C.[2], and Diez, J.J.[3]

[1]Centro de Sanidad Forestal de Calabazanos. Polígono de Villamuriel, 34190, Villamuriel de Cerrato, Palencia, Spain
[2]Área de Ecología, E.T.S.II.AA. de Palencia, Universidad de Valladolid, Avda Madrid 44, 34071 Palencia, Spain
[3]Laboratorio de Entomología y Patología Forestal, Departamento de Producción Vegetal y Recursos Forestales, E.T.S.II.AA. de Palencia, Universidad de Valladolid, Avda Madrid 44, 34071 Palencia, Spain

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Fungal composition of pine needles and twigs from stands in Northern Spain has been studied. Also the influence of host, seasonal factors, sampling site, sampling tissue and isolation method have been evaluated. The material analysed consisted of dried up, green and fallen needles and twigs from four host species, Scots pine (*Pinus sylvestris*), Maritime pine (*P. pinaster*), Austrian pine (*P. nigra*) and Mountain pine (*P. uncinata*). These materials were cultivated in moist chamber plates and culture media with a surface sterilized material and not sterilized. A total of 45 species were isolated from 2592 plant fragments. There were no differences found in the fungal species richness and RIF (relative isolation frequencies) between sites and pines, but there were differences between seasons, isolation methods and tissues. Almost the whole of species isolated was recovered in autumn and nearly half of those exclusively at that season. Also the higher RIF value was reached in autumn. Differing tissues and isolation methods, the highest richness was reached from dried pine needles using moist chamber method, and the greatest number of RIF was reached from fallen and dried up needles using the moist chamber method. These results suggest that the season, isolation method and tissue health decisively affected the frequency and species distribution of fungi recovered on pines.

Key words: endophytes, isolation method, sampling site, season, tissues

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*Corresponding author: P. Zamora; e-mail:zambrapa@jcyl.es

Introduction

In Northern Spain many 40-50 years-old plantations of Scots pine (*Pinus sylvestris*), Maritime pine (*P. pinaster*), Austrian pine (*P. nigra*) and Mountain pine (*P. uncinata*) in monospecific or mixed stands can be found. These stands have a protective function against erosion and constitute a timber and mushroom source. They also are very interesting because of their landscape value and their role as a refuge for wildlife (Oria *et al*., 1996). The use of inappropriate cultivars of pine, together with the high tree density in the plantations and the species composition probably affect the micro-climatic conditions in the canopy (Müller and Hallaksela, 1998a) and that may thus promote the outcome of numerous pathologies limiting the pine stands growth and conditioning the survival of the trees.

The best known microbes living on trees are those causing perceptible diseases and damages (Müller and Hallaksela, 2000). The fungal species that affect the aerial part of the plant produce the most visible symptoms and signs. These fungal infections are accompanied by macroscopically visible changes as necrosis or hypertrophy, as well as by the presence of external fungal hyphae or fruiting bodies (Jurc and Jurc, 1995). The apparently harmless majority of fungi may, however, have a versatile impact on the function and fitness of individual trees and the forest ecosystem as a whole (Carroll, 1988; Müller and Hallaksela, 2000). The endophytic fungi are widespread but still poorly known because they go

unnoticed and in most cases are not pathogenic for their host (Stefani and Bérubé, 2006). But some of them are, however, opportunist and can cause disease after the host has been weakened by some other factor (Sieber, 2007). The presence of known pathogens among endophytes has prompted plant pathologists and epidemiologists to seriously reconsider the possibility that some plant pathogens are latent within their host tissues for a considerable period (Petrini, 1991; Slippers and Wingfield, 2007). Because fungi are mainly responsible for pine diseases (Butin, 1995; Hartman *et al*., 1995; Kovalski and Krygier, 1996) it will be necessary to identify the fungal species associated with different tissues in order to avoid this possible problem.

Several studies dealing with the microflora associated to needles of different conifers (Jurc and Jurc, 1995; Sesan and Taut, 1998; Martín *et al*., 2004; Ganley *et al*., 2004; Ganley and Newcombe, 2006; Stefani and Bérubé, 2006; Arnold *et al*., 2007; Hu *et al*., 2007; Wei *et al*., 2007) and broad leaves species (Bettucci and Saravay, 1993; Polishook *et al*., 1996; Collado *et al*., 1999; Collado *et al*., 2000; Arnold *et al*., 2003; Martín *et al*., 2004; Santamaría and Diez, 2005; Gonthier *et al*., 2006; Göre amd Bucak, 2007; Hyde *et al*., 2007) have been performed. Some works have been focused on the host specificity for the fungal colonization (Petrini and Fisher, 1990; Betucci *et al*., 1999; Collado *et al*., 2000; Martín *et al*., 2004; El-Morsy *et al*., 2006; Pinnoi *et al*., 2006; Pinruan *et al*., 2007). The variation in the number of endophytes from a particular tree species or collection sites in relation with seasonality has been also described (Collado *et al*., 1999; Suryanarayanan *et al*., 2002; Martín *et al*., 2004; 2006). Furthermore, it has been studied the influence of the fungal isolation methodology in the distribution of fungal communities from different hosts as *Eucaliptus globulus* (Betucci *et al*., 1999), *Pseudotsuga menziesii* and ponderos pine (Hoff *et al*., 2004), Scots pine (Ranta and Saloniemi, 2005), *Quercus ilex* and *Quercus faginea* (Collado *et al*., 1996; 1999; 2000), *Chamaecyparis thyoides* (Bills and Polishook, 1992), *Pinus monticola* (Ganley and Newcombe, 2006) and *Populus tremula* (Santamaría and Diez, 2005). In this way, fungal communities have been investigated using two different methods: growing mycelia from plant tissues planted on culture media (Carrol *et al*., 1977; Barklund and Rowe, 1983; Santamaría and Diez, 2005) and finding fruiting bodies on plant fragments (Solheim, 1989; Livsey and Barklund, 1992; Santamaría and Diez, 2005). Although endophytic fungi associated to different conifers have been described in several papers (Bills and Pollishook, 1992; Jurc and Jurc, 1995; Jurc *et al*., 1996; Müller and Hallaksela, 1998a; b; 2000; 2001; Hoff *et al*., 2004; Ganley *et al*., 2004; Ranta and Saloniemi, 2005; Ganley and Newcombe, 2006; Stegfani and Beruré, 2006; Mohali *et al*., 2007), the mycobiota of the pine species in Southern Europe and particularly in Spain is still largely unknown.

The aim of this work was: (i) to identify the composition of fungal assemblages of pine plantations in the northwest of Spain; (ii) to evaluate the incidence of the host and seasonal factors in the distribution of the fungal species and; (iii) to analyse the effects of sampling site, sampling tissue and isolation method on the frequency and richness of the fungi recovered from different pines.

Materials and methods

Sampling

Samples were collected at six sites in northern Spain (Fig. l), in areas where forest of various species of pines (*P. nigra*, *P. pinaster*, *P. sylvestris* and *P. uncinata*) are frequent. Localization, altitude, surveyed pines and some edaphic characteristics of sampling sites are given in Table 1. Each site was visited twice, in the spring and autumn of 2003, and samples were collected from a total of 72 trees (40-50 years old); three randomly chosen per species of *Pinus* presented in every surveyed site and season. From each tree, 2 to 3 year-old healthy and necrotic branches (living but with a portion of dead tissue), located 3-4 m above the ground, were collected from the periphery of the canopy, and fallen pine needles were also collected from the ground. The samples were brought to the laboratory, stored at 4°C and processed within 24 h.

Fig. 1. Geographical situation of the six surveyed sites: **(a)** in Spain: **(b)** in the province of Palencia.

Table 1. Sampling sites localization and characterization. Name, village the site is located in; A, altitude, in metres above sea level, of each sampling site; Soil type according to 'Mapa de Suelos de Castilla y León' (Forteza and Tejero 1987).

Site	Name	Surveyed species	A	Soil type
1	Valcobero	*Pinus uncinata* *Pinus sylvestris*	1480	Humic Ranker
2	Cristo Sierra	*Pinus uncinata* *Pinus sylvestris*	1630	Lithosol
3	Mantinos	*Pinus nigra* *Pinus pinaster* *Pinus sylvestris*	1180	Humic Cambisol
4	Villota del Páramo	*Pinus nigra* *Pinus pinaster*	1070	Humic Cambisol
5	Villaeles	*Pinus pinaster* *Pinus sylvestris*	930	Gleyic Cambisol
6	Villota del Duque	*Pinus nigra*	930	Humic Cambisol and Calcic Cambisol

Fungal isolation and identification.

From each tree, three two-centimetre pieces of dried up, green and fallen pine needles as well as twigs (a total of 2592 plant fragments) were processed by two different methods (Santamaría and Diez, 2005). The first consisted of finding fruiting-bodies on plant tissues (needles and twigs) after incubating them in Petri dishes with wet paper at room temperature (24 ± 2°C) in diffused daylight until fruit-body production. The samples used in this method were not surface-sterilized in order to find endophytes and fungal epiphytes. In twig samples, the bark was not removed, and hence the fungal species that were fruiting on the bark could be detected. The second method included growing mycelia on culture media with two approaches: in the first one, surface sterilization was performed by dipping in ethanol (70% v/v) before soaking in (2% w/v) sodium hypochlorite solution (5 min for needles and 10 min for twig samples) and washed three times in sterile distilled water, with the subsequent plating of the sterilized fragments on Petri dishes containing potato dextrose agar (PDA) medium; the plates were sealed with parafilm and incubated at room temperature in a dark place for a month; the outgrowing colonies were counted and transferred to fresh PDA and cultivated in pure culture at room temperature in diffused daylight until sporulation; cultures were identified according to morphological characteristics. The second approach was the same as the previous one but samples were not surface-sterilized before plating. One-third of the selected samples were used in each one of the three methods (one method + two approaches).

Statistical analyses

The effects of the environment (the six sites), pine species, season (spring, autumn),

sampling tissue (dried up, green and fallen pine needles, and twigs) and isolation method (MC, moist chamber; SPD, Petri dishes containing sterilized samples; UPD, Petri dishes containing non-sterilized samples) on the fungal species richness and on the relative isolation frequencies (RIF) were evaluated by analysis of variance. The RIF was calculated with the formula RIF = *nijk/Nijk* where *nijk* is the number of isolates recorded in the site *i*, tissue *j* and method *k*, and where *Nijk* is the number of samples examined in the site *i*, tissue *j* and method *k* (Santamaría and Diez, 2005). Assumptions of normality and equal variance for parametric testing had previously been checked for these data (Sokal and Rohlf, 1995). A Tukey-Kramer test for multiple comparisons was used by means of the general linear model procedure of SAS when significant differences were found in the analysis of variance.

To asses the influence of the main explanatory variables (season, method and tissue) on the isolated fungal-species composition 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 analyses and 999 for final results (Legendre and Legendre, 1998). The constrained ordination was performed using default settings and untransformed species data by means of CANOCO for Windows version 4.5 (Ter Braak and Šmiluaer, 2002).

Results

The fungal species recovered from the pines, as well as their relative frequencies of isolation, are shown in Table 2. A total of 45 species were isolated from 2592 plant fragments (one-third per method). The fungal species richness (estimated by the number of different species) not differed significantly between sites and pines, but differed between seasons, isolation methods and tissues (Table 3). Moreover, richness differences between methods depend on the season, and richness differences between tissues depend on the method (method x season and method x tissue interactions: $p < 0.01$ in both cases; Table 3). Almost the whole of species isolated were recovered in autumn (42 spp.; 93%), and nearly half of those (20 spp.; 48%) exclusively at that season. In spring, however, the number of fungal species isolated was lower (24 spp.; 53%) and just three of them recovered only at that time (*Cenangium ferruginosum*, *Chaetomium erectum* and *Sphaeropsis sapinea*). As a whole, the MC method provided the highest number of species (30 spp.; 67%), but only significantly different to the SPD approach in autumn (Tukey-Kramer tests, $p < 0.01$), when the (87%) of the fungal species were recovered by using both MC and UPD isolation methods. Differing tissues and isolation methods, the highest richness (42%) was reached from dried pine needles using the MC-method.

As for the fungal species richness, the RIF values were significantly different among seasons, isolation methods and tissues. Moreover, RIF differences among methods depend on the seasons and RIF differences between tissues depend on the method (method × season and method × tissue interactions: $p < 0.05$ and $p < 0.01$ respectively; Table 3). The higher RIF value was reached in autumn, when the Tukey-Kramer test revealed significant differences ($p < 0.01$) between the two approaches (sterilized and unsterilized before plating) within the culture-media method. The second approach (UPD) showed the highest RIF value that, was not different from this for MC ($p > 0.05$), as found out by the Tukey-Kramer tests. The fallen pine needles yielded the significantly greatest number of RIF (Tukey-Kramer tests, $p < 0.01$) that was similar to this reached in the dried pine needless and significantly different to those on green pine needles and twigs. But these differences only were significant when the MC-method was used, as found out by the Tukey-Kramer tests ($p < 0.05$).

Forward selection using CCA indicated that only isolation method and season, in this order, were significant explanatory variables for predicting the fungal species composition (Monte Carlo test: $F = 2.60$ and $F = 1.75$, respectively, $p < 0.01$, 499 permutations). So, the constrained model (CCA) examined fungi species response to the two significant variables defined by forward selection (Fig. 2). Eighenvalues (λ) for axes 1 and 2 were 0.358 and 0.233, respectively, and the model was significant according to the Monte Carlo test ($F = 1.458$, $p = 0.002$, 999 permutations). The first

Table 2. Fungal species distribution and isolation frequencies[1].

Fungi[3]	Code	SPRING												SP[2]	AUTUMN										AU[2]	TOTAL[2]
		MC				UPD				SPD					MC				UPD				SPD				
		d	g	f	t	d	g	f	t	d	g	f	t		d	g	f	t	d	g	f	t	d	g	f	t		
Alternaria alternata (Fr) Keissl.	Ala	4.76	-	11.1	7.7	36.4	6.7	23.5	8.3	23.1	9.1	16.7	6.7	14.5	7.5	9.1	4.9	5	24.4	16.1	14.3	14.7	8.7	-	-	11.8	11	12.5
Arthrinium caricicola Kunze&J.C. Schmidt.	Arc	-	-	-	-	-	-	-	-	-	-	-	6.7	0.5	-	-	-	-	-	-	-	2.9	-	-	-	-	3	0.4
Aspergillus sp.	As	-	-	-	7.7	4.5	6.7	5.9	-	-	-	11.1	13.3	4.3	-	-	-	-	2.2	3.2	9.5	2.9	4.3	7.7	-	-	2.2	3
Aspergillus sp. Type A	AsA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.9	4.3	7.7	9.5	5.9	1.9	1.2
Aspergillus sp. Type B	AsB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.3	7.7	-	-	0.6	0.4
Aureobasidium pullulans (De Bary) G. Arnaud	Aup	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.3	-	4.8	-	0.6	0.4
Botritys cinerea Pers.	Boc	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9.1	-	-	6.7	6.5	4.8	8.8	-	-	-	-	3.2	2
Cenangium ferruginosum Fr.	Cef	4.8	-	-	7.7	-	-	-	-	-	-	-	-	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4
Ceuthospora sp.	Ceu	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	-	12.2	-	-	-	-	-	8.7	-	-	-	2.5	1.6
Chaetomium cochliodes Palliser.	Chc	4.8	-	-	-	-	-	-	-	-	-	-	-	0.5	-	-	-	-	-	-	-	2.9	-	-	4.8	-	0.6	0.6
Chaetomium erectum Skolko&J.W. Goves	Che	-	-	-	7.7	-	-	-	-	-	-	-	-	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
Chaetomium fusiforme Chivers.	Chf	-	-	-	-	-	-	-	-	-	-	5.6	-	0.5	-	-	2.4	5	-	-	-	-	-	-	-	-	0.6	0.6
Cladosporium herbarum (Pers.) Links.	Clh	-	-	3.7	-	-	6.7	-	-	7.7	-	-	-	1.6	5	-	-	-	6.7	9.7	-	-	-	-	-	-	2.5	2.2
Cladosporium sp. Type A	ClA	9.5	-	7.4	-	22.7	13.3	-	16.7	23.1	18.2	11.1	6.7	11.3	7.5	27.3	2.4	-	22.2	25.8	9.5	2.9	21.7	-	9.5	-	11	11.3
Cladosporium sp. Type B	ClB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.9	-	7.7	-	-	0.6	0.4
Coniothirium fuckelii Sacc.	Cof	-	-	-	-	-	-	-	-	-	-	-	-	-	5	9.1	-	-	4.4	6.5	-	-	4.3	-	-	-	2.5	1.6
Cytospora sp.	Cy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35	-	-	-	-	-	-	-	-	2.2	1.4
Didymella sp.	Di	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	-	-	-	-	-	0.6	0.4
Dothistroma septosporum (Doro.) M. Morelet.	Dos	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.4	-	-	-	-	-	-	-	-	-	0.3	0.2

[1]The isolation frequencies for each species are the percentages with respect to the total number of isolates collected from each season (spring and autumn) each method (MC, moist chamber; UPD, culture media with non-sterilized samples; and SPD, culture media with sterilized samples) and each sampling tissue (dried up, d, green, g, and fallen, f, pine needles and twigs, t).

[2]The column labelled SP and AU refers to the percentages of isolates for each species with respect to the total number of isolates collected in spring and autumn, respectively. The column labelled TOTAL is the percentage of the total number of isolates from one species with respect to the total number of fungal isolates.

[3]Fungal species nomenclature follows National Center of Biotechnology Information (www.NCBI.nlm.nih.gov).

Table 2 (Continued). Fungal species distribution and isolation frequencies[1].

Fungi[3]	Code	SPRING												SP[2]	AUTUMN										AU[2]	TOTAL[2]
		MC				UPD				SPD					MC				UPD				SPD				
		d	g	f	t	d	g	f	t	d	g	f	t		d	g	f	t	d	g	f	t	d	g	f	t		
Epicoccum nigrum Link.	Epn	-	-	-	-	-	-	11.8	-	-	-	-	-	1.1	7.5	9.1	-	5	24.4	22.6	4.8	17.6	4.3	7.7	9.5	5.9	11	7.3
Fusarium poae (Peck)Wollenw.	Fup	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.8	-	-	-	-	-	0.3	0.2
Fusarium sp.	Fu	-	-	-	-	-	-	5.9	16.7	-	-	5.6	-	2.2	-	-	-	-	-	-	-	2.9	-	-	-	-	0.3	1
Geotrichum sp.	Ge	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.9	-	-	-	-	0.3	0.2
Harzia acremonioides (Hrz.) Costantin.	Haa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.9	-	-	4.8	-	0.6	0.4
Leptosphaeria coniothyrium (Fuckel)Sacc.	Lec	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	-	-	-	-	-	0.6	0.4
Leptostroma pinastri (Desm.)	Lep	23.8	-	22.2	-	-	-	-	-	-	-	-	-	5.9	17.5	-	24.4	-	-	-	-	-	-	-	-	-	5.4	5.5
Lophodermium pinastri (Schard.) Chevall	Lop	23.8	-	23.3	-	-	-	-	-	-	-	-	-	7.5	5	-	12.2	-	-	-	-	-	8.7	-	4.8	-	3.2	4.8
Mucor hiemalis Whemer	Muh	-	-	-	-	-	6.7	5.9	8.3	-	9.1	-	-	2.2	-	-	-	-	-	-	28.6	2.9	-	7.7	9.5	11.8	3.8	3.2
Naemacyclus niveus (Pers.) Fuck. ex Sacc.	Nan	19	-	11.1	-	-	-	-	-	-	-	-	-	3.8	20	-	24.4	-	-	-	-	-	-	-	-	-	5.7	5
Nigrospora oryzae (Berk et Broome) Petch	Nio	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2	-	-	2.9	-	-	-	-	0.6	0.4
Penicillium sp.	Pe	9.5	50	7.4	-	4.5	33.3	17.6	8.3	23.1	36.4	33.3	20	16.7	5	-	-	10	2.2	6.5	9.5	5.9	13	30.8	33.3	-	7.9	11.1
Pestalotiopsis funerea Desm. Steyaert	Pef	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9.1	-	-	-	-	-	-	-	-	-	-	0.3	0.2
Phoma sp.	Ph	-	-	-	23.1	-	-	-	-	-	-	-	-	1.6	2.5	18.2	4.9	5	2.2	-	-	-	4.3	-	-	-	2.5	2.2
Phomopsis sp.	Pho	-	-	-	-	-	-	-	-	7.7	-	-	-	0.5	-	-	-	5	-	-	-	-	-	7.7	-	-	0.6	0.6
Preussia sp.	Pr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.2	-	-	-	-	-	-	0.3	0.2
Rhizopus stolonifer (Ehrenb.) Vuill.	Rhs	-	-	-	-	-	-	5.9	-	-	18.2	-	-	1.6	-	-	-	-	-	-	4.8	-	-	-	4.8	5.9	0.9	1.2
Sclerophoma pithyophila (Corda) Höhn	Scp	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	-	-	-	-	-	-	-	-	-	-	-	0.3	0.2
Sirococcus strobilinus Preuss	Sis	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	-	-	-	-	-	-	-	-	-	-	-	0.3	0.2

[1]The isolation frequencies for each species are the percentages with respect to the total number of isolates collected from each season (spring and autumn) each method (MC, moist chamber; UPD, culture media with non-sterilized samples; and SPD, culture media with sterilized samples) and each sampling tissue (dried up, d, green, g, and fallen, f, pine needles and twigs, t).

[2]The column labelled SP and AU refers to the percentages of isolates for each species with respect to the total number of isolates collected in spring and autumn, respectively. The column labelled TOTAL is the percentage of the total number of isolates from one species with respect to the total number of fungal isolates.

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		MC				UPD				SPD					MC				UPD				SPD					
		d	g	f	t	d	g	f	t	d	g	f	t		d	g	f	t	d	g	f	t	d	g	f	t		
Sordaria fimicola (Roberge ex Desm.) Ces&De Not	Sof	-	-	-	-	9.1	-	5.9	8.3	7.7	-	11.1	13.3	4.8	-	-	-	-	-	-	-	-	-	-	-	5.9	0.3	2
Sphaeropsis sapinea (Fr.) Dyko.&Sutton	Sps	-	-	3.7	-	-	-	-	-	-	-	-	-	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
Stachylidium sp.	St	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	-	4.9	-	-	-	-	-	-	-	-	-	0.9	0.6
Trichoderma viride Pers.	Trv	-	-	-	46.2	-	-	-	25	-	-	-	26.7	7	2.5	-	-	10	-	-	9.5	17.6	4.3	-	4.8	52.9	6.9	6.9
Trichotecium roseum (pers) Link.	Trr	-	50	-	-	22.7	26.7	17.6	8.3	7.7	9.1	5.6	-	9.1	2.5	9.1	2.4	-	2.2	-	-	-	4.3	7.7	-	-	1.9	4.6
Verticillium albo-atrum Reinke&Berthold	Vea	-	-	-	-	-	-	-	-	-	-	-	6.7	0.5	-	-	-	-	-	-	-	2.9	-	7.7	-	-	0.6	0.6
Verticillium sp.	Ve	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	-	2.4	-	-	-	-	-	-	-	-	-	0.6	0.4

[1]The isolation frequencies for each species are the percentages with respect to the total number of isolates collected from each season (spring and autumn) each method (MC, moist chamber; UPD, culture media with non-sterilized samples; and SPD, culture media with sterilized samples) and each sampling tissue (dried up, d, green, g, and fallen, f, pine needles and twigs, t).

[2]The column labelled SP and AU refers to the percentages of isolates for each species with respect to the total number of isolates collected in spring and autumn, respectively. The column labelled TOTAL is the percentage of the total number of isolates from one species with respect to the total number of fungal isolates.

[3]Fungal species nomenclature follows National Center of Biotechnology Information (www.NCBI.nlm.nih.gov).

Table 3. ANOVA results for the fungal species richness and the relative isolation frequencies (RIF).

Source	d.f.	Richness			RIF		
		MS	*F*-value	*P*-value	MS	*F*-value	*P*-value
Site	5	0.43	0.25	0.939	0.23	0.22	0.953
Pinus spp.	3	0.70	0.41	0.744	0.25	0.22	0.885
Season	1	56.00	37.56	0.000	16.67	20.34	0.000
Method	2	12.02	7.48	0.001	5.01	6.11	0.001
Tissue	3	17.48	11.54	0.000	6.50	9.58	0.000
SxM	2	8.34	6.09	0.003	3.45	4.21	0.018
SxT	3	3.22	2.47	0.062	1.06	1.37	0.258
MxT	6	9.09	7.14	0.000	3.47	5.11	0.000

d.f.= degrees of freedom; MS= mean square; S= Sseason; M= Method; T= Tissue.

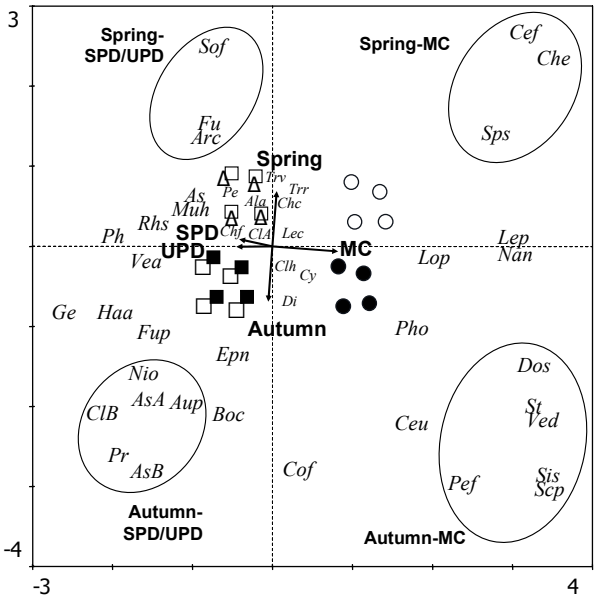

Fig. 2. CANODRAW CCA triplot: full species, samples and explanatory variables. Samples symbols: MC, moist chamber, ●○; Petri dishes containing sterilized samples; UPD, ■□; and Petri dishes containing non-sterilized samples, SPD, ▲Δ; (full and empty symbols refer to samples recovered in autumn or spring, respectively). Species abbreviations as Table 2 (first two letters of genus and first letter of species names).

axis is characterised by a gradient in the fungal species distribution strongly associated with the isolation method; from species exclusively isolated with the MC-method at the positive end (*Cef*, *Che*, *Sps*, *Lep*, *Nan*, *Dos*, *St*, *Ve*, *Sis*, *Scp*, *Pef*), to those exclusively isolated with one of the two approaches within the culture-media method at the negative end (*Sof*, *Fu*, *Arc*, *Rhs*, *Pho*, *Vea*, *Ge*, *Haa*, *Fup*, *Nio*, *ClB*, *AsA*, *Aup*, *Pr*, *AsB*). Axis two clearly shows a gradient associated with the season; from fungi species exclusively (*Cef*, *Che*, *Sps*) or mainly (*Sof*, *Fu*, *Arc*) isolated in spring, to those only isolated in autumn (*Nio*, *AsA*, *ClB*, *Pr*, *AsB*, *Aup*, *Boc*, *Cof*, *Ceu*, *Dos*, *St*, *Ve*, *Pef*, *Sis*, *Scp*).

Discussion

The number of taxa recorded in the present study was similar to those of previous surveys on fungal communities from other host, including conifers and broadleaf trees, under temperate climate (Bills and Polishook, 1992; Collado *et al*., 1996; 1999; 2000; Danti *et al*., 2002; Martín *et al*., 2004; Santamaría and Diez, 2005; Göre and Bucak, 2007). However, comparing with the richness of fungi in tropical plants like palms (Pinnoi *et al*., 2006; Pinruan *et al*., 2007) cocoa trees (Arnold *et al*., 2003) or *Heliconia mariae* (Bills and Polishook, 1994), the number of taxa found in our study was low. Tropical environments are known to differ from temperate habitats and they are characterized by warmer temperature, with little or no seasonality, and heavy precipitation during at least part of the year (Tsui *et al*., 1998). Endophyte infection is reduced in leaves of temperate trees when they are shielded from rain (Wilson, 1995; Suryanarayanan *et al*., 2002), this seems to be one reason of the lower number of taxa in temperate habitats in contrast with tropical ones where the leaves are densely colonized by endophytes during the wet season (Suryanarayanan, *et al*., 2002).

Considering the most abundant species as those with a RIF value higher than 3% (n = 11), we found that most of them are known as saprobes and ubiquitous (*Ala*, *As*, *ClA*, *Epn*, *Lep*, *Lop*, *Muh*, *Nan*, *Pe*, *Trv*, *Trr*). *Lophodermium pinastri* and its anamorphic

state (*Leptostroma pinastri*) where both found in dried up and fallen needles. The former is known to be an endophyte associated to some pine species, working as a primarily saprobe responsible for the initial decomposition of needles after abscission; hence its importance in forest resource cycling (Deckert and Peterson, 2000). Other species such as *Rhizopus stolonifer*, *Mucor hiemalis*, *Trichothecium roseum* and *Penicillium* spp. are pure saprobes and rarely occur as endophytes in healthy tissues, although they where isolated frequently from green, healthy and surface sterilized needles, probably as some sort of contamination. Among the twenty species exclusives from the autumn sampling, there are five relevant because of their pathogenity (*Boc*, *Dos*, *Pef*, *Scp*, *Sis*) (Butin and Peredo, 1986; Hartmann *et al*., 1995; Butin, 1995), although they were always isolated in very low frequency regardless of the isolation method used, and just from dry up or fallen needles. Only *Botrytis cinerea* and *Pestalotiopsis funerea* were also found in green needles, being present in healthy tissues. *Pestalotiopsis* is an anamorphic genus commonly present in tropical and subtropical species of plants (Tejesvi *et al*., 2007). The geographical distribution and ubiquitous occurrence of *Pestalotiopsis* suggest an important role for this genus in the temperate and tropical rainforest systems (Tejesvi *et al*., 2007). *Pestalotiopsis funerea* was first reported as an ecologically important endophyte from *Sequoia sempervirens* (Espinosa-García and Langenheim, 1990; Hu *et al*., 2007). But this widespread fungus also causes damping off, root rot of seedlings, needle and stem blight, twig dieback and stem cankers on many tree species (Bajo *et al*., 2008). There is known that some *Pestalotiopsis* species could have endophytic and pathogenic stages in their life cycle (Wei *et al*., 2007), and regardless the low frequency of isolation of *Pestalotiopsis funerea* it could get a problem whether trees are growing under bad conditions (Butin and Peredo, 1986). *Dothistroma septosporum* and *D. pini* are the two causal agents of *Dothistroma* needle blight of *Pinus* spp. in natural forests and plantations (Groenewald *et al*., 2007). *Dothistroma* needle blight is a mayor pest of pine plantations in the southern hemisphere, where both the host and the pathogen have been introduced, but in the northern temperate forest the damage level have historically been low (Woods *et al*., 2005). In the pine plantations studied, the incidence of *Dothistroma septosporum* was very low because it was isolated punctually in fallen needles. *Sirococcus strobilinus* causes shoot blight and seedling death of conifers (Smith, 1973; Shahin and Claflin, 1978; Sanderson and Worf, 1986; Rossman *et al*., 2008). It has many conifer hosts in the northern hemisphere, and in nature it is often encountered on cone scales, stems and needles, especially of *Picea* and *Pinus* spp. (Rossman *et al*., 2008). In southern Europe, *S. strobilinus* was found affecting Aleppo pines (*Pinus halepensis* Mill.) (Muñoz-López, 1997; Danti and Capretti, 1998; Konrad *et al*.,, 2007). The Spanish population of *S. strobilinus* seems to be a geographically isolated population and with host specificity because all the Spanish isolates found where collected from Aleppo pines (Konrad *et al*., 2007), but in this study *S. strobilinus* was isolated punctually from dried up needles of *P. sylvestris*. *Sclerophoma pytiophila* was also isolated punctually in dried up needles, and it has been shown to be generally a weak pathogen on several conifers (Brener *et al*., 1974); but in combination with other pathogenic species like *Gremmeniella abietina*, *S. pythiophila* seems to play a role in disease expression (Santamaría *et al*., 2006). From the three species isolated just in spring, *Cenangium ferruginosum* and *Sphaeropsis sapinea* are relevant because their pathogenicity, albeit they were in very low frequency in spring as for the pathogen species isolated exclusively in autumn. *Cenangium ferruginosum* is usually regarded as ubiquitous and opportunistic fungus which kills the bark and cambium of twigs and branches weakened by environment, other pests and pathogens, or natural senescence (Sinclair *et al*., 1987; Butin, 1995; Jurc and Jurc, 1997). However, Santamaría *et al*., (2006) demonstrated that *C. ferruginosum* is not able to damage healthy *Pinus halepensis* tissues. Incipient infections of *Cenangium* begin in summer and autumn and are held by host defences unless these are overcome (Sinclair *et al*., 1987; Jurc and Jurc, 1997). In

our study, the presence was so low probably because the infection was held by the host defences. The fungus *Sphaeropsis sapinea* is considered as an important opportunistic pathogen that intensely infects following unfavourable environmental conditions such as careless pruning, drought stress or hail, among others. The pines with a heavily *Sphaeropsis* infection can finally die (García and Diez, 2003) but in our study, this fungus was found in fallen needles and with very low frequency working most probably like a saprobe.

The multivariate analysis indicated that the most important variables for predicting the fungal species composition were the season and the isolation method. The highest richness and RIF values as a whole were found in autumn. Moreover, only in autumn differences in richness and RIF between isolation methods were statistically significant. The greater rainfall average in autumn than in spring (47.2 mm vs, 260.8 mm, respectively), in the different locations for our study in the sampling year, have probably promoted the fungal spores dispersion and the higher viability of the fungal propagules. Some authors have referred to the influence of the seasonal factors on the composition of fungal assemblages (Collado *et al*., 1999; Martín *et al*., 2004; 2006) and contrary to our results, they find out higher RIF and richness values in spring. However, in tropical forest where little or no seasonality is characteristic (Tsui *et al*., 1998) the rainfall is the major environmental factor determining endophyte infection (Wilson, 2000; Suryanarayanan *et al*., 2002).

On the other hand, although in the present study the moist-chamber method provided the highest number of species, 33% of them were exclusively isolated from cultures (UPD + SPD). The fungal species isolated with only one of the isolation methods were also different. Particularly, with the moist-chamber method, there were mostly isolated Coleomycetes (*Lep*, *Pef*, *Scp*, *Sis* and *Sps*) and with Petri dishes with PDA there were Hyphomycetes (*Fup*, *Fu*, *Ge*, *Haa* and *Nio*) and Zygomycetes (*Muh* and *Rhs*). Both methods led to isolate different species of Ascomycetes (*Cef*, *Che*, *Nan* with MC; *Lec*, *Pr* and *Sof* with UPD + SPD). The surface sterilization eliminates spores or mycelia fragments attached to the surface of the plant material (Petrini 1986; 1991) and this fact may explain the lowest richness and RIF values reached from the SPD approach. Hence, our results from the ANOVA test and the multivariate analysis suggested that the method used for isolation, determines the frequency and species distribution of fungi recovered from a certain host, as previously discussed by several authors (Bills and Polishook, 1992; 1994; Livsey, 1994; Jurc and Jurc, 1995; Collado *et al*., 1996; Polishook *et al*., 1996; Hyde *et al*., 2007). A combination of several isolation methods seems accordingly to be a good strategy for maximizing the diversity of species recovered in surveys on fungal communities. Furthermore, the use of the two different approaches (sterilized and unsterilized fragments) may detect the living condicions of the different species (Santamaría and Diez, 2005): whether it lives as an epiphyte or as an endophyte; whether the fungus inhabits only the surface of the tissue or grows within the tissue. In a previous study (Bissegger and Sieber, 1994), the effectiveness of surface-sterilizacion was also investigated, and an epiphytic mycobiota on unsterilized samples very similar to that recorded in the present study was found.

Also differences in richness and RIF of fungal species between tissues were found for the whole data and for every single isolation method. The highest values were recovered from necrotic tissues (dried needles and fallen needles) and significantly differed from those obtained from healthy tissues (green needles), according with other previous surveys (Petrini 1986; Hata and Futai 1995; Kowalski and Kher 1996; Bettucci and Alonso 1997; Bettucci *et al*., 1999; Frohlich *et al*., 2000). A great number of species was isolated from twigs but with quite low frequency and only the 13% where exclusive of them. In contrast to previous surveys (Petrini, 1986; Collado *et al*., 1996; Kowalski and Kehr, 1996; Sahashi *et al*., 1999; Barengo *et al*., 2000, Pinnoi *et al*., 2006), the low number of species recovered only from a single tissue in our study demonstrated a low degree of organ specificity of fungi. Nevertheless, some specificity was detected in relation to the healthiness of tissues as found by Kowalski and Krygier (1996), since considering together the green tissues (green

needles and twigs) and the necrotic ones (dry up and fallen needles) we obtained 27 and 22 % of specificity**,** respectively.

Among species of genus *Pinus*, no differences in fungal species richness and RIF were found. These results are consistent with those found in other studies in which no differences in fungal communities related to plant genus were found (Martín *et al*., 2004). There were also no differences in fungal species between sampling sites in contrast to other studies where the richness differences between sites could be explained by the environmental conditions (Collado *et al*., 1999; Suryanarayanan *et al*., 2002). The considerable effect that environmental conditions have on the frequency of fungi has been already stated with regard to diverse tree species (Helander *et al*., 1993; Elamo *et al*., 1999; Ragazzi *et al*., 2003). On the contrary, the difference in the frequency of endophytes within a single tree (Johnson and Whitney, 1989), even between trees within a site (Helander *et al*., 1993; Elamo *et al*., 1999), may be due to the influence of the genotype of the tree and the localized infection caused by any fungus (Elamo *et al*., 1999).

In conclusion, this study identifies the composition of main fungal assemblages of pine plantations in Northern Spain. The results also suggest that the method, season and tissue (healthy or necrotic) decisively affected the frequency and species distribution of fungi recovered on pines. The findings also indicate that there are not pathogenic fungi causing damages in these stands because most of them where recovered only in dead or dying tissues as in healthy or slightly damaged ones and in low frequency in both cases. Further investigations on the fungal species associated with the same hosts in other sites, during different seasons could yield more fungal taxa and therefore could further clarify the effect of the host on the fungal populations.

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