



## *Ophiostomatoid* fungi associated with declined *Pinus pinaster* stands in Spain

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### Abstract

**Aim of study:** We studied the presence of fungi and distribution patterns in relation to the health status of declining *Pinus pinaster* trees.

**Area of study:** Trees in two declining stands in Central Spain were allotted to three declining classes.

**Material and Methods:** Trees in two declining stands in Central Spain were allotted to three declining classes (healthy, declining and recently dead) and 3 trees of each class were felled in each stand. Wood slides (phloem and xylem) were taken at six positions along the trees and samples collected from fungal identification.

**Main results:** A total of 21 fungal taxa were isolated and identified; eleven of these species belonged to the Ophiostomatoid group. *Ophiostoma minus* was the most frequently isolated fungus and was identified in 22% of the samples, mainly associated to dead and diseased trees.

**Research highlights:** Together these results suggest a putative association of *O. minor* with the decline in this area, and thus we suggest paying more attention to this fungus as a potential agent of decline in *P. pinaster* stands.

**Key words:** Ophiostomatoid fungi; forest pathology; bluestain fungi; multivariate analyses.

**Abbreviations:** UTM: Universal Transverse Mercator coordinate system; MEA: Malt Extract Agar; DNA: Deoxyribonucleic acid; ITS: Internal Transcribed Spacer; BLAST: Basic Local Alignment Search Tool; CCA: Canonical Correspondence Analyses; PWN: Pine Wood Nematode.

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### Introduction

Maritime pine (*Pinus pinaster* Aiton) is a western Mediterranean and north-African typical species that stretches down to the Atlantic coast. Most extensive forests are located in Spain, France and Portugal. In Spain, *P. pinaster* is an important source of natural goods; it covers naturally the largest surface (600.000 ha) (Del Río *et al.*, 2004) and is the pine species more intensively used in reforestation (800.000 ha) for wood and resin production, with 270.000 ha managed for resin tapping in the old sixties (40.000 ton per year, Serrada, 2004).

During the last years a general decline has appeared in some Maritime pine stands located at the north and centre of Spain (Álvarez *et al.*, 2008a). Symptoms expressed by declining maritime pines include sparse tree crowns, with

unusual crown transparency, and short, yellow-green needles, and death (Fig. 1). Blue-stain was always visible in the wood since the first stages of the disease, suggesting damages caused by ophiostomatoid or other fungi as *Diplodia pinea*. However, no damages caused by insects are usually found in these *P. pinaster* stands. The symptoms spread following a gradient of mortality indicating damages caused by a biotic agent. At the final stages of the disease, symptoms appear with extraordinary virulence being visible a sudden and entire necrosis of the crown, and the dead of the tree in some weeks after the more evident symptoms appearance (Álvarez *et al.*, 2008b, 2009). The mortality rate was important reaching 60% of the trees in some heavily affected stands. Apparently, there is no relationship between the decline and resin tapping, as decline can be also found in non-tapped trees.

Ophiostomatoid fungi are commonly associated to blue-stain diseases in conifers (Solheim & Langstrom, 1991; Wingfield *et al.*, 1993; Hausner *et al.*, 2005). *Ceratocystis* spp., *Ophiostoma* spp. and *Ceratocystiopsis* spp. are the main species causing blue-stain in stems, or roots of conifers (Solheim *et al.*, 1993; Grylls & Seifert, 1999), appearing both in their teleomorphic or anamorphics states (*Graphium* spp., *Leptographium* spp., *Chalara* spp.; Jacobs & Wingfield, 2001). Thus, *Leptographium wagnerii* is causing black-stain in roots of conifers in North America (Otrosina *et al.*, 1999) and other species like *L. wingfieldii* has been commonly associated to a general decline of *P. sylvestris* in Poland (Jankowiak *et al.*, 2007). In addition, *O. minus* has been shown as one of the most virulent ophiostomatoid fungi on pines (Masuya *et al.*, 2003). This fungus was associated with an important decline of *Pinus sylvestris* in France (Piou & Lieutier, 1989) and recently related with important diseases like these caused by *Dendroctonus frontalis* (the Southern Pine Beetle) in North America (Six & Klepzig, 2004) or by the pine wood nematode pathogen *Bursaphelenchus xylophilus* (Maehara *et al.*, 2005).

Although pathogenic and saprophytic fungal taxa have been described associated with different conifers (Ganley & Newcombe, 2006; Hu *et al.*, 2007; Zamora *et al.*, 2008; Botella *et al.*, 2010) the ophiostomatoid fungi associated to *Pinus pinaster*, particularly in Spain, and its influence on the health status of the tree, are still barely known. The main aim of this study was to identify the ophiostomatoid fungi associated to *P. pinaster* trees showing decline symptoms.

## Materials and Methods

### Sampling

*Pinus pinaster* samples were collected from two plots located in two pine stands with decline symptoms in Castilla & León region (Spain). These plots were selected because of the severity of the damages. One is sited at Burgos province (La Horra, UTM coordinates 37T, X-722292, Y-331796, soil type Inceptisol, suborder Xerochrepts) and the other at Ávila province (San Esteban del Valle, UTM coordinates 29T, X-738530, Y-4729138, soil type Inceptisol, suborder Xerochrepts and Xerumbrepts). The Burgos stand is 820 m.a.s.l., with dried gypsum soils and 500 millimetres rain per year. The maximum annual temperature is 29 °C and the minimum -2 °C. The Avila stand is about 1250 m.a.s.l., with dried soils and 1600 mm rain per year. Maximum annual temperature is 30 °C and minimum 0 °C.



**Figure 1.** *Pinus pinaster* stand affected by decline in Burgos province.

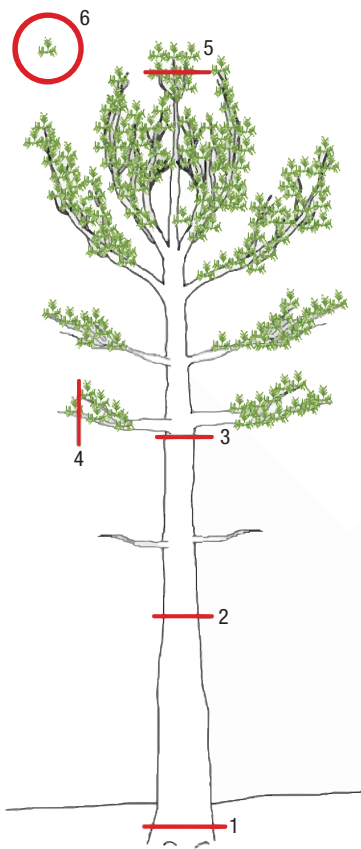
In October 2007, nine trees were randomly selected per stand according to their health status (i) three healthy, (ii) three with decline symptoms and (iii) three recently dead (Fig. 2). The trunk was cut with a chainsaw and samples were collected from six different levels (Fig. 3): (1) the collar, (2) the middle of the trunk, (3) the first alive branch, (4) a branch cross-section, (5) the terminal guide and (6) some needles from the top of the crown. Fungal identification was performed using phloem and xylem pieces from these slides. A total of 792 moist chambers were performed ([18 trees x 5 levels x 2 tissues (xylem and phloem) x 4 replicates each] + [18 trees x 1 level x 1 tissue (needles) x 4 replicates each]).

### Fungal isolation and identification

In order to identify the fungal taxa, xylem and phloem samples (1 x 2 cm<sup>2</sup>) as well as needles (2 cm in length) were sampled from each tree, and processed using moist chamber and culture media methodologies,



**Figure 2.** Health status of *Pinus pinaster* trees selected for the study: (i, left) three healthy, (ii, center) threes with different degrees of decline symptoms and (iii, right) three recently dead.



**Figure 3.** Different levels where samples were taken from *Pinus pinaster* trees, 1: collar, 2: middle of the trunk 3: fist alive branch, 4: branch cross section, 5: terminal guide, 6: needles.

as it has been outlined in previous fungal studies (Santamaría & Diez, 2005; Zamora *et al.*, 2008; Botella *et al.*, 2010; Botella & Diez, 2011). The moist chamber is based in finding fruiting bodies on plant tissues (xylem, phloem and needles) after incubation in Petri dishes at room temperature ( $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) (Zhou *et al.*, 2001) in diffused daylight containing wet paper. A total of 792 moist chambers were performed ([18 trees x 5 levels x 2 tissues (xylem and phloem) x 4 replicates each]+ [18 trees x 1 level x 1 tissue (needles) x 4 replicates each]).

The culture media method included growing mycelia on culture media. Tissue samples were cultured on MEA+tetracycline (33 g of malt extracts, 16 g of agar and 250 mg of tetracycline per litre of distilled water) and were processed into a laminar flow hood to avoid contaminations. Only the surface of needles was sterilized due to the fact that sodium hypochlorite used in sterilization can affect the growth of the ophiostomatoid fungi in the wood. Samples were washed in running tap water for one minute, soaked in 70% alcohol for two minutes, and soaked twice in 3% sodium hypochlorite solution, for two minutes each time. Finally, the samples were immersed twice in sterile distilled water, for two

minutes each time, to remove any possible remains of the hypochlorite. Petri dishes were stored for 7 days in the dark at  $25^{\circ}\text{C}$  and then carefully examined using binoculars. Fruiting bodies from the samples were identified with a microscope Nikon Eclipse E-400 model. A total of 792 Petri dishes were performed ([18 trees x 5 levels x 2 tissues (xylem and phloem) x 4 replicates each]+ [18 trees x 1 level x 1 tissue (needles) x 4 replicates each]).

Fungi in Petri dishes were identified according to morphological characteristics of spores and other reproductive structures, such as size, shape and colour. Different taxonomic keys were used for fungal identification (Barnett & Hunter, 1998; Hanlin, 1998; Goidanich, 1990; Lanier *et al.*, 1978; Sutton, 1980; Kiffer & Morelet, 1999). For those structures belonging to the ophiostomatoid fungi Wolfaardt *et al.*, (1992) and Grylls & Seifert (1999) keys were used. Some recalcitrant species like *Ophiostoma ips* (Rumb.) Nannf. were determined by DNA sequencing (primers ITS1 and ITS4) and BLAST match (Kuekam *et al.*, 2013).

### Statistical analysis

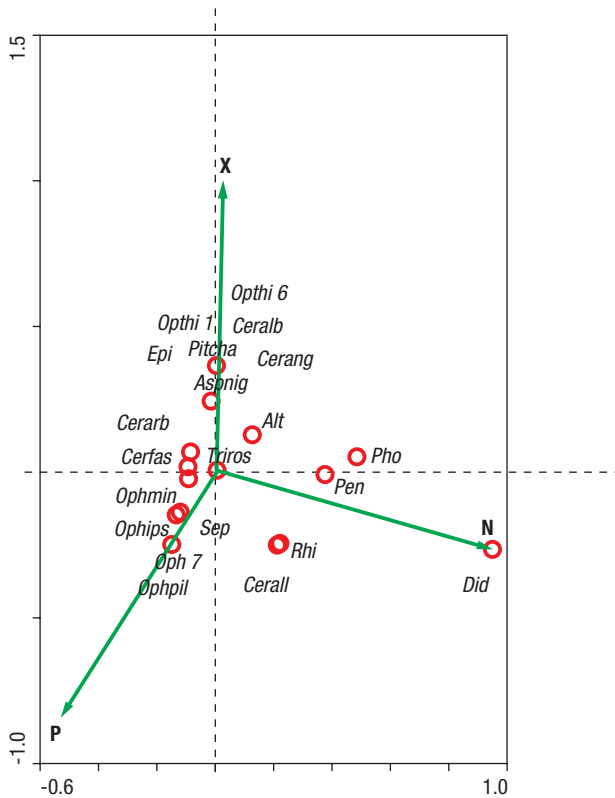
In order to assess the influence of the main explanatory variables on the fungal occurrence, a Canonical Correspondence Analysis (CCA) was carried out. The presence or absence of fungal species found for each sample was considered as the dependent variable. The independent variables considered were (i) stand (Burgos/Avila), (ii) tissue type (xylem/phloem/needles), (iii) level of sample in the tree (levels 1, 2, 3, 4, 5, 6) and (iiii) healthy status of the tree (healthy, diseased, dead). A forward selection procedure using the Monte Carlo's test was then applied to test the degree of significance, with 499 permutations for exploratory analysis and 999 for final results (Legendre & 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 & Smilauer, 2002).

### Results

The intensity of defoliation in the sampled stands ranged from 10 to 100% and the mortality associated reach the 60% of the trees at the Burgos stand, the more heavily affected by the decline.

A total of 21 fungal species were isolated and identified from the eighteen trees (Table 1). Eleven isolates belonged to the ophiostomatoid group being *Ophios-*

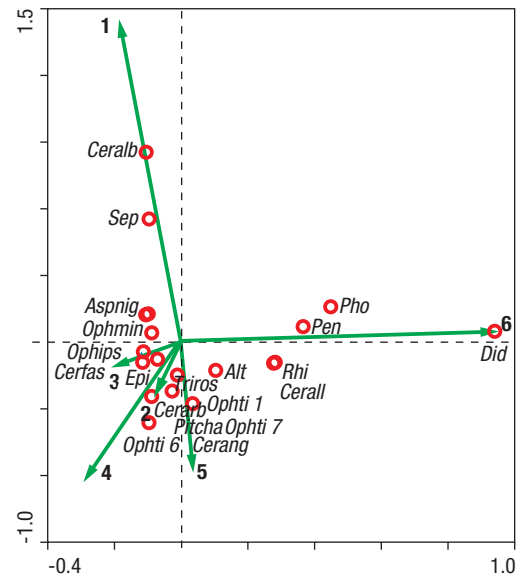




**Figure 4.** Correspondence analysis considering sampled tissues and identified species. X: xylem, P: phloem, N: needles. *Alt*: *Alternaria* sp., *Aspnig*: *Aspergillus niger*, *Ceralb*: *Ceratocystiopsis alba*, *Cerfas*: *Ceratocystiopsis fasciata*, *Cerall*: *Ceratocystis allantospora*, *Cerang*: *Ceratocystis angusticollis*, *Cerarb*: *Ceratocystis arborea*, *Did*: *Didymella* sp., *Epi*: *Epicoccum* sp., *Ophips*: *Ophiostoma ips*, *Ophmin*: *Ophiostoma minus*, *Ophil*: *Ophiostoma piliferum*, *Oph 1*: *Ophiostomatal with perithecia type 1*, *Ophi 6*: *Ophiostomatal with perithecia type 6*, *Ophi 7*: *Ophiostomatal with perithecia type 7*, *Pen*: *Penicillium* sp., *Pho*: *Phoma* sp., *Pitcha*: *Pithomyces chartarum*, *Rhi*: *Rhizopus* sp., *Sep*: *Septonema* sp., *Triros*: *Trichotecium roseum*.

*toma* and *Ceratocystis*, with three species each, the more represented genera. *Ceratocystiopsis* only appeared with two species. Three unidentified ophiostomatoids appeared as well with a low frequency in the *P. pinaster* samples. *Ophiostoma minus* (Hedgcock) was the most frequently isolated fungus, followed by *O. ips* (Rumbold). Some other taxa without any phytopathological importance were also identified (i.e. *Penicillium* sp. (Link), *Aspergillus niger* (Tiegh), *Rhizopus* sp. (Ehrenb)).

Canonical Correspondence Analysis (CCA) showed all the independent variables analyzed with significant values of probability: (i) stand (Burgos/Avila) ( $P = 0.002$ , data not shown), (ii) tissue type (xylem/phloem/needles) ( $P = 0.022$ , Figure 4), (iii) level of sample in the tree (levels 1, 2, 3, 4, 5, 6, Figure 5) ( $P = 0.044$ ) and (iiii) healthy status of the tree (healthy, diseased, dead) ( $P = 0.046$ , data not shown).



**Figure 5.** Correspondence analysis considering sampled tree levels (1-6) and identified species. *Alt*: *Alternaria* sp., *Aspnig*: *Aspergillus niger*, *Ceralb*: *Ceratocystiopsis alba*, *Cerfas*: *Ceratocystiopsis fasciata*, *Cerall*: *Ceratocystis allantospora*, *Cerang*: *Ceratocystis angusticollis*, *Cerarb*: *Ceratocystis arborea*, *Did*: *Didymella* sp., *Epi*: *Epicoccum* sp., *Ophips*: *Ophiostoma ips*, *Ophmin*: *Ophiostoma minus*, *Ophil*: *Ophiostoma piliferum*, *Oph 1*: *Ophiostomatal with perithecia type 1*, *Ophi 6*: *Ophiostomatal with perithecia type 6*, *Ophi 7*: *Ophiostomatal with perithecia type 7*, *Pen*: *Penicillium* sp., *Pho*: *Phoma* sp., *Pitcha*: *Pithomyces chartarum*, *Rhi*: *Rhizopus* sp., *Sep*: *Septonema* sp., *Triros*: *Trichotecium roseum*.

The relative frequency of taxa was higher in Avila (61.3%) than in Burgos (38.6%). *O. minus* was more abundant in Burgos (13.1%) than in Avila (8.9%), whereas *O. ips* and *C. fasciata* were more frequent in this second location with 11.3% and 8.9%, respectively (Table 1). Analyzing the vegetal tissues, *Ophiostoma minus* appeared three times more frequently in the phloem of Avila pine trees whereas in Burgos, this appearance was similar for both tissues (Table 2, Fig. 4). For *Ophiostoma ips*, we found the same pattern for both localities.

Almost all of the ophiostomatoid species appeared more frequently in the phloem (50.3%) than in the xylem (42.5%) (Fig. 4). The axis of this correspondence analysis explained the 38% of the data variability and the three types of tissue showed significant values of probability (P-needles = 0.032, P-phloem = 0.004 and P-xylem = 0.05). The more generalist species showed a clearer trend to appear on the needles such as *Penicillium* sp. or *Phoma* sp (Saccardo) whereas the ophiostomatoid species appeared exclusively in the vascular tissues. Thus, *O. minus* has a frequency of 13.7% in the phloem and 8.3% in the xylem, *O. ips*, 9.5% and 5.9% and *C. fasciata* with 7.1% and 5.3% respectively (Table 2).

**Table 1.** Absolute and relative prevalence of the fungal species . At each location, nine trees and six sampling position along each individual tree were studied.

SPECIES	STAND		TOTAL
	Burgos	Avila	
<i>Alternaria</i> sp. (Nees)	2 (1,19%)	4 (2,38%)	6 (3,57%)
<i>Aspergillus niger</i> (Tiegh)	1 (0,60%)	6 (3,57%)	7 (4,17%)
<i>Ceratocystiopsis alba</i> (DeVay <i>et al.</i> )	–	1 (0,60%)	1 (0,60%)
<i>Ceratocystiopsis fasciata</i> (Olchow. y J. Reid)	6 (3,57%)	15 (8,93%)	21 (12,50%)
<i>Ceratocystis allantospora</i> (Griffin )	–	3 (1,79%)	3 (1,79%)
<i>Ceratocystis angusticollis</i> (E.F. Wright y H.D. Griffin)	–	1 (0,60%)	1 (0,60%)
<i>Ceratocystis arborea</i> (Olchow. y J. Reid)	1 (0,60%)	1 (0,60%)	2 (1,19%)
<i>Didymella</i> sp. (Sacc. ex D. Sacc.)	1 (0,60%)	–	1 (0,60%)
<i>Epicoccum</i> sp. (Link)	1 (0,60%)	–	1 (0,60%)
<i>Ophiostoma ips</i> (Rumbold)	7 (4,17%)	19 (11,31%)	26 (15,48%)
<i>Ophiostoma minus</i> (Hedgcock)	22 (13,10%)	15 (8,93%)	37 (22,02%)
<i>Ophiostoma piliferum</i> (Fries)	1 (0,60%)	2 (1,19%)	3 (1,79%)
<i>Ophiostoma type 1</i>	–	1 (0,60%)	1 (0,60%)
<i>Ophiostoma type 6</i>	2 (1,19%)	–	2 (1,19%)
<i>Ophiostoma type 7</i>	1 (0,60%)	–	1 (0,60%)
<i>Penicillium</i> sp. (Link)	3 (1,79%)	9 (5,36%)	12 (7,14%)
<i>Phoma</i> sp. (Saccardo)	1 (0,60%)	3 (1,79%)	4 (2,38%)
<i>Pithomyces chartarum</i> (Berk and Curt.)	–	1 (0,60%)	1 (0,60%)
<i>Rhizopus</i> sp. (Ehrenb)	–	3 (1,79%)	3 (1,79%)
<i>Septonema</i> sp. (Corda)	9 (5,36%)	2 (1,19%)	11 (6,55%)
<i>Trichotecium roseum</i> (Pers.)	7 (4,17%)	17 (10,12%)	24 (14,29%)
<b>TOTAL</b>	<b>65 (38,69%)</b>	<b>103 (61,31%)</b>	<b>168 (100,00%)</b>

**Table 2.** Prevalence of fungi (%) per location and vegetal tissues.

SPECIES	Percentage of prevalence of fungi per stand					
	Burgos			Avila		
	Phloem	Xylem	Needles	Phloem	Xylem	Needles
<i>Alternaria</i> sp. (Nees)	–	2.86	0.95	1.49	1.49	–
<i>Aspergillus niger</i> (Tiegh)	0.95	4.76	–	–	1.49	–
<i>Ceratocystiopsis alba</i> (DeVay <i>et al.</i> )	–	0.95	–	–	–	–
<i>Ceratocystiopsis fasciata</i> (Olchow. y J. Reid)	8.57	5.71	–	4.48	4.48	–
<i>Ceratocystis allantospora</i> (Griffin )	1.90	–	0.95	–	–	–
<i>Ceratocystis angusticollis</i> (E.F. Wright y H.D. Griffin)	–	0.95	–	–	–	–
<i>Ceratocystis arborea</i> (Olchow. y J. Reid)	–	0.95	–	1.49	–	–
<i>Didymella</i> sp. (Sacc. ex D. Sacc.)	–	–	–	–	–	1.49
<i>Epicoccum</i> sp. (Link)	–	–	–	–	1.49	–
<i>Mucor</i> (P. Micheli ex L.)	0.95	–	–	–	–	–
<i>Ophiostoma ips</i> (Rumbold)	11.43	6.67	–	5.97	4.48	–
<i>Ophiostoma minus</i> (Hedgcock)	6.67	7.62	–	23.88	8.96	–
<i>Ophiostoma piliferum</i> (Fries)	1.90	–	–	1.49	–	–
<i>Ophiostoma type 1</i>	–	0.95	–	–	–	–
<i>Ophiostoma type 6</i>	–	–	–	–	2.99	–
<i>Ophiostoma type 7</i>	–	–	–	1.49	–	–
<i>Penicillium</i> sp. (Link)	1.90	3.81	2.86	–	1.49	2.99
<i>Phoma</i> sp. (Saccardo)	–	1.90	0.95	–	–	1.49
<i>Pithomyces chartarum</i> (Berk and Curt.)	–	0.95	–	–	–	–
<i>Rhizopus</i> sp. (Ehrenb)	1.90	–	0.95	–	–	–
<i>Septonema</i> sp. (Corda)	–	1.90	–	13.43	–	–
<i>Trichoderma</i> (Pers.)	–	–	0.95	2.99	–	–
<i>Trichotecium roseum</i> (Pers.)	7.62	7.62	0.95	4.48	4.48	1.49
<b>TOTAL</b>	<b>43.79</b>	<b>47.60</b>	<b>8.56</b>	<b>61.1</b>	<b>31.35</b>	<b>7.46</b>

The correspondence analysis shows ophiostomatoid species (excepting *Ceratocystis alba*) in other tissues that collar (heights 1) and needles (heights 6) (Figure 5). However, the generalist species showed a clear affinity for these tree positions, the closest to the ground and needles and clearly separated from the others. In this figure, variables explained the 4.1% of the total variability of the data and only the sampling positions 1 and 6 showed significant statistical differences (P-values of 0.018 and 0.033, respectively). The two more abundant phytopathogenic fungi, *Ophiostoma minus* and *O. ips*, appeared distributed in a fairly homogeneous pattern. The height 5 (terminal guide) hosted almost all the species of fungi identified, contributing almost with a quarter of the total variability (24.4%).

*O. minus* was mainly found in dead and diseased trees while *O. ips* appeared almost twice in healthy trees than in died ones (Table 3). *Ceratocystiopsis fasciata* (Olchow. and Reid) had also a remarkable representation (12.5%) and mainly in dead trees (8.9%). Other ophiostomatoid fungi were found with lower representation (between 0.6% and 1.7%) like *Ceratocystis allantospora* (Griffin), *Ceratocystis angusticollis* (Wright and Griffin), *Ceratocystis arborea* (Olchow

and Reid), *Ceratocystiopsis alba* (DeVay *et al.*), *Ophiostoma piliferum* (Fries) and the three unidentified specimens: *Ophiostoma* type 1, *Ophiostoma* type 6 and *Ophiostoma* type 7. Fungal species explained 37.4% of the affinity between plots. *Ophiostoma minus* and *Ophiostoma ips* had the highest representation in the sampling units as observed in the correspondence analysis graph. Most of the affinity between plots was explained by the presence of these two species. *Ceratocystiopsis fasciata* also highlights among the ophiostomatoid group.

## Discussion

In this study we identified eleven ophiostomatoid taxa associated to *Pinus pinaster* decline and, excluding typical laboratory contaminants (i.e. *T. roseum*), three of them were remarkable for their prevalence: *Ceratocystiopsis fasciata*, *Ophiostoma ips* and *O. minus*.

*Ceratocystiopsis fasciata* is a cryptic species not mentioned in any other publication, only one about its morphological description (Grylls & Seifert, 1999). In this study, it has emerged strongly related to the dead

**Table 3.** Absolute and relative prevalence of the fungal species related to the healthy state of the sampled trees.

Species	Healthy state			Total
	Healthy	Diseased	Dead	
<i>Alternaria</i> sp. (Nees)	4 (2,38%)	1 (0,60%)	1 (0,60%)	6 (3,57%)
<i>Aspergillus niger</i> (Tiegh)	4 (2,38%)	2 (1,19%)	1 (0,60%)	7 (4,17%)
<i>Ceratocystiopsis alba</i> (DeVay <i>et al.</i> )	–	1 (0,60%)	–	1 (0,60%)
<i>Ceratocystiopsis fasciata</i> (Olchow. y J. Reid)	7 (4,17%)	3 (1,79%)	11 (6,55%)	21 (12,50%)
<i>Ceratocystis allantospora</i> (Griffin)	2 (1,19%)	–	1 (0,60%)	3 (1,79%)
<i>Ceratocystis angusticollis</i> (E.F. Wright y H.D. Griffin)	–	–	1 (0,60%)	1 (0,60%)
<i>Ceratocystis arborea</i> (Olchow. y J. Reid)	1 (0,60%)	1 (0,60%)	–	2 (1,19%)
<i>Didymella</i> sp. (Sacc. ex D. Sacc.)	–	–	1 (0,60%)	1 (0,60%)
<i>Epicoccum</i> sp. (Link)	–	1 (0,60%)	–	1 (0,60%)
<i>Ophiostoma ips</i> (Rumbold)	15 (8,93%)	4 (2,38%)	7 (4,17%)	26 (15,48%)
<i>Ophiostoma minus</i> (Hedgcock)	8 (4,76%)	13 (7,74%)	16 (9,52%)	37 (22,02%)
<i>Ophiostoma piliferum</i> (Fries)	1 (0,60%)	1 (0,60%)	1 (0,60%)	3 (1,79%)
<i>Ophiostoma type 1</i>	–	–	1 (0,60%)	1 (0,60%)
<i>Ophiostoma type 6</i>	1 (0,60%)	–	1 (0,60%)	2 (1,19%)
<i>Ophiostoma type 7</i>	–	–	1 (0,60%)	1 (0,60%)
<i>Penicillium</i> sp. (Link)	5 (2,98%)	5 (2,98%)	2 (1,19%)	12 (7,14%)
<i>Phoma</i> sp. (Saccardo)	2 (1,19%)	–	2 (1,19%)	4 (2,38%)
<i>Pithomyces chartarum</i> (Berk and Curt.)	1 (0,60%)	–	–	1 (0,60%)
<i>Rhizopus</i> sp. (Ehrenb)	1 (0,60%)	–	2 (1,19%)	3 (1,79%)
<i>Septonema</i> sp. (Corda)	6 (3,57%)	4 (2,38%)	1 (0,60%)	11 (6,55%)
<i>Trichotecium roseum</i> (Pers.)	10 (5,95%)	7 (4,17%)	7 (4,17%)	24 (14,29%)
<b>TOTAL</b>	<b>68 (40,48%)</b>	<b>43 (25,60%)</b>	<b>57 (33,93%)</b>	<b>168 (100,00%)</b>

trees, but not particularly with the diseased trees suffering the decline. These results seem to indicate that *C. fasciata* is not the main fungus causing the decline, and their presence on the trees might be subsequent to the decline.

*Ophiostoma ips* is a pathogenic species and is listed as the most frequent of those registered associated to *Ips sexdentatus* (Zou *et al.*, 2001; Fernández *et al.*, 2004; Bueno *et al.*, 2010) and *Orthotomicus erosus* (Romón *et al.*, 2007). Although it can kill trees (Fernández *et al.*, 2004) *O. ips* could be not so virulent as *O. minus* (Popp *et al.*, 1995; Zhou *et al.*, 2002). In the present study it has emerged as the second more abundant (15.4% of the total), mainly associated to healthy trees.

Several reasons suggest that *O. minus* could be the more important ophiostomatoid related to *P. pinaster* decline in Burgos and Avila stands. : (1) it was the most prevalent, being isolated from the 22.0% of all identified trees, (2) it was the species more associated to diseased (7.7%) and dead trees (9.5%), (3) many references establish the high pathogenicity of this fungus (Langstrom *et al.*, 1993; Popp *et al.*, 1995; Jankowiak, 2006; Jankowiak & Rosa, 2007), even higher than *O. ips* in all the pathogenicity tests (Lieutier *et al.*, 1989; Popp *et al.*, 1995) and, (4) is a fungus associated in at least other two important pathologies around the world: the Southern Pine Beetle outbreak in Norteamérica (Six & Klepzig, 2004), and the Pine Wood Nematode disease (Togashi, 2004). This fungus is one of the most virulent Ophiostomatoids that have been ever described, since it has the capacity to kill trees when it was inoculated (Masuya *et al.*, 2003). Its strong presence in the samples and the detection on diseased and dying trees (Table 3) indicate that it could play an important role in the decay of the trees sampled. However, pathogenicity proofs should be performed to definitively establish its specific role in the decline.

However, we should take into account the possibility that the present state of the decline may be due to a complex relationship between fungal (*Ophiostoma minus*, *Heterobasidion annosum*, recently detected in some areas close to our sampling plots (Prieto-Recio *et al.*, 2012), or even other pathogens as *Phytophthora cinnamomi*...), soil, climatic and silvicultural factors. Anyway, further work is necessary to determine the pathogenicity of *O. minus* and other associated ophiostomatoid fungi on adult trees, with the aim to definitively establish the association of this fungus with the maritime pine decline.

*O. minus* have also been associated with a decline of *Pinus sylvestris* in France (Piou & Lieutier, 1989). The relationship between *O. minus* and the damage observed in this host suggested that this fungus had an

important role in the decline. On the other hand, Jankowiak *et al.* (2007), using two-year-old Scots pine seedlings, demonstrated that inoculation with *O. minus* produced significantly larger lesions on *Picea abies* than other ophiostomatoid fungi. . Comparing with other ophiostomatoid fungi, lesions induced by *O. minus* were significantly larger than lesions induced by *O. ips* (Popp *et al.*, 1995). These results suggest *O. minus* is a good candidate to be involved in the *P. pinaster* decline. However, pathogenicity proofs are needed to definitively confirm its importance on this decline.

*O. minus* have been associated with *Dendroctonus frontalis* in North America (Six & Klepzig, 2004). This bark beetle attacks and kills southern pines, introducing fungi into them. *Ophiostoma minus* may initially aid beetles in killing trees, but later this bluestain fungus becomes an antagonist, competing with larvae for host phloem (Scott *et al.*, 2008). Mite's abundance was strongly correlated with *O. minus* and was an important driving force in promoting bluestain prevalence within trees (Lombardero *et al.*, 2003). Spring abundances of mites and the prevalence of *O. minus* during *D. frontalis* infestation were strong predictors of beetle population decline (Hofstetter *et al.*, 2006a, 2006b). Further studies on the interactions of *O. minus* with other insect or fungi associated to *Pinus pinaster* decline should be performed to establish their influence on the disease.

*O. minus* have been also associated to Pine Wood Nematode (PWN) pathogen *B. xylophilus* (Warren *et al.*, 1995). This nematode produced a large population after eating this fungus, (Togashi, 2004). PWN was detected in Portugal three years ago, and recently in the Spanish border (Cáceres and Pontevedra provinces; DOE 2009, DOG 2012, respectively). The symptoms and the analysis performed by the CESANFOR Diagnostic Center (Castilla & León Government, Spain) discarded the presence of *Bursaphelenchus xylophilus* in the areas affected by *Pinus pinaster* decline. This fungus has been also associated to PWN vectors. Among ophiostomatoid fungi, *O. minus* and *O. piceae* were the most frequently isolated species from *M. galloprovincialis* adults (Jankowiak & Rossa, 2007). The interaction of *O. minus* with the PWN and its insect vector should be taken into account if the disease appears in these areas in the future.

## Conclusions

In our study of declining *P. pinaster* stands in Northern Spain we found a total of 21 fungal taxa isolated and identified; eleven of these species belonged to the



ophiostomatoid group. *Ophiostoma minus*, *O. ips* and *C. fasciata* were the most frequently isolated fungi, mainly associated to dead and diseased trees. Our results suggest paying more attention to *O. minus* as a potential agent of decline in *P. pinaster* stands. However, more studies are needed to establish the importance of abiotic (drought, resin tapping...) or biotic (*Heterobasidion annosum*, or *Phytophthora cinnamomi*...) in the *P. pinaster* decline.

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