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Research Papers

Species- and organ-specificity in endophytes colonizing healthy and declining Mediterranean oaks

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Summary. The specificity of fungal endophytes to trees has generally been investigated on allopatric populations. In the present study, specificity was investigated on *Quercus cerris* and *Quercus pubescens* growing in sympatry at the same site, to examine host-endophyte interactions without interference from habitat-specific selective pressure. In a two-year study, 4800 samples were obtained from photosynthetic and non-photosynthetic tissue (leaves and twigs) both of healthy and declining trees. Endophytes were isolated from surface-sterilized samples and identified by traditional or molecular methods. Twenty-two endophyte species in 19 genera were identified. Some taxa in the *Sordariomycetes*, *Dothideomycetes* and *Leotiomycetes* colonized both species, but quantitative differences were evident. Water shortages and extended droughts may have impacted more severely on *Q. cerris*, which exhibited a more diverse endophytic assemblage and greater infection levels than *Q. pubescens*. In both species, more isolates were recovered from twigs than from leaves, and more from declining than from healthy trees. Endophytes tended to be specific to each host, and to the organs of that host. Interaction between plant species and the environment and continued competitive interaction between endophyte species may have led to niche diversification, with selection favouring host-specific and organ-specific endophytes. This study advances understanding of the role of some pathogenic fungal endophytes in Mediterranean oak forests.

Key words: *Quercus cerris*, *Quercus pubescens*, fungal endophytes, oak decline.

Introduction

The Italian Peninsula, located centrally in the circum-Mediterranean eco-region, represents an important biodiversity hotspot zone, especially in its southern offshoots (Cowling *et al.*, 1996). During the last ice age the area was a glacial refuge for several oak species (Petit *et al.*, 2002). Schwarz (1964, 1993) listed 15 and 12 oak species as growing in Italy in his two editions of *Flora Europea* (the difference between publication dates was caused by taxonomic uncertainty regarding the ease with which oak species interbreed).

In Mediterranean oak forests a variety of biotic and abiotic factors induce ecosystem-level constraints that have far-reaching impacts on the survival and reproduction of living organisms. These forces exert strong selective pressures on the fungal endophytic communities, influencing the distribution of species as well as their commonness or rarity (Ragazzi *et al.*, 2012; U'ren *et al.*, 2012).

Since selective pressures vary significantly among ecosystems, the site where a tree grows is a major factor in determining the abundance, taxonomic composition and diversity of endophytic mycobiota, even within closely related tree species (Collado *et al.*, 1999). An aspect that has scarcely been investigated is whether closely related trees that grow in the same locality, under the same ecological conditions, also harbour similar assemblages of endophytic fungi, or whether such trees eventually develop their own specific endophyte communities. Identifying the endophytes that are dominant on a particular

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host tree can shed light on the factors (biotic and/or abiotic) that shape the plant-endophyte relationship, and clarify whether these endophytes affect the composition and dynamics of the tree population.

Turkey oak (*Quercus cerris* L.) and Pubescent oak (*Q. pubescens* Willd.) are ecologically and economically important Mediterranean oak species. The range of these trees partially overlaps but their habitat requirements are different. *Quercus pubescens* grows mainly on hilly areas from the sub-alpine belt down to the south, and including the Italian islands. This species marks the transition from the Mediterranean sclerophyllous oaks to the mesophilic broadleaves, and it thrives in warm areas with a sunny and dry climate. *Quercus cerris* has a more southerly range, frequently colonizing slopes from Tuscany southwards, but it is more mesophilic, preferring cooler sites and deeper soils (Bussotti and Bruschi, 2000).

Along the Italian Peninsula, some of the areas in which these oaks grow have intermediate characteristics, suitable for mesic-xeric forests. In these areas, the two oak species, both exhibiting a wide ecological amplitude, are found in mixed stands, together with other broadleaved tree species, the most common being *Acer campestre*, *A. monspessulanum*, *Carpinus betulus*, *Castanea sativa, Fraxinus excelsior*, *F. ornus* and *Q. ilex*.

Turkey oak has already been investigated in Italy for fungal endophytes as part of a broader study on oak decline (Ragazzi *et al*., 2004). Endophytes from both symptomatic and asymptomatic individuals of this species have been examined in various parts of the country, and their isolation frequencies have been determined in relation to the host phenology and to the tree organ affected (Ragazzi *et al*., 2001a). A distinctive species abundance and diversity was found for some endophyte fungi, depending on the geographic locality where the trees grew. Similar local variations have also been reported for endophytic assemblages on other tree species (Göre and Bucak, 2007; Hoffman and Arnold, 2008).

Most of the studies on endophyte occurrence, however, explored the endophytes colonizing a given plant taxon over an extensive geographical area, or focused on unrelated or only remotely related plant taxa. The habitat heterogeneity and lack of taxonomic relatedness of these plants often led to conflicting results. Some studies found strong evidence for host specificity (Petrini, 1996; Collado *et al*., 2000). Exclusive plant-fungus combinations were also reported, such as the singular case of *Abies alba* parasitized by *Viscum album*: although these plants grow in a very close union, yet each has a distinct endophytic assemblage (Petrini, 1996). Other studies reported a tendency to host-generalism in many endophytes, which colonize a number of related or unrelated hosts at different latitudes and over vast areas (Arnold, 2007). Examples are prominent members of the teleomorphic and anamorphic genera *Alternaria*, *Apiognomonia*, *Biscogniauxia*, *Botryosphaeria*, *Diplodia*, *Discula*, *Fusarium*, *Hypoxylon*, *Lasiodiplodia*, *Nectria*, *Phoma* and *Sphaeropsis*, which are important components of endophyte communities in various crop systems, from fruit orchards, to nurseries, tree plantations and forests all over the world.

The aim of the present study was to ascertain whether the endophytic fungi in the closely related Mediterranean oak species *Q. cerris* and *Q. pubescens* were host-specific. We investigated species composition and abundance of the dominant endophytes in a mixed stand of these trees, including healthy trees and trees with decline/dieback. To exclude any effects caused by variables other than the host itself (e.g. site, local climate, stand exposure, vegetation cover, anthropogenic disturbances) the endophytes studied came from oaks growing at the same site and under the same ecological conditions, in a mixed stand having an approximately equal proportion of each species. Endophytes were identified to determine: 1) whether each oak species, and different organs of each species (leaves or twigs), had distinct assemblages of endophytes; and 2) whether the impaired health conditions of declining trees affected the composition of the endophytic assemblages. We consider an endophyte a microorganism that lives asymptomatically inside a plant, without inducing any noticeable symptom at least for part of its life cycle (Moricca and Ragazzi, 2008).

Materials and methods

Study site

The study was conducted in the Alta Val di Cecina Forest, located in a hilly area (altitude 300–400 m a.s.l.) in the territory of Ulignano, Municipality of Volterra, Province of Pisa, Tuscany, Central Italy (UTM coordinates: X, 1655324.73; Y, 4813251.52), in a site previously utilised to investigate aspects of the epidemiology of the ascomycete *Apiognomonia quercina* (Ragazzi *et al*., 2007).

The soil characteristics varied, soils being composed of various types of clay intersecting each other, mostly clay and scaly marl with inserted layers of limestone (Geological Maps of Italy, I.G.M., scale 1:100,000). All these strata form poorly permeable soils of medium depth.

The forest is natural and has been managed in different ways. In the past it was used to pasture sheep and swine as well as to produce firewood. Currently it is situated within an area set aside for wildlife conservation. The compaction of the soil caused by the passage of domestic and wild animals is still creating problems to the roots of plants and favours erosion of the soil, revealing the naked rock underneath in various places.

The forest contains some woodlots that are drier than the rest. These woodlots face the south-west and have Turkey oak and Pubescent oak growing in them at fairly low density and showing evident signs of decline, which is mainly the result of summer droughts and a poor water supply. Many trees in these drier areas exhibited partial crown yellowing and withered branches, or they were dead though still standing. There were also some woodlots in cooler parts of the forest, with an east-north-easterly exposure, where mature and ageing trees grew in coppice; many of these trees were still vigorous and had full and green crowns.

As regards the stand structure in these woodlots, many of the oaks were in the dominant and co-dominant crown classes, while in the intermediate and lower crown classes there were a few dominated trees. The woodlots also contained trees with small diameters (up to 8–10 cm): *Sorbus domestica*, *S. terminalis*, *Fraxinus ornus,* and scattered individuals of *Quercus ilex* and *Pyrus pyraster.* The understory consisted of *Juniperus communis*, *Spartium junceum, Prunus spinosa, Crataegus monogyna, Ruscus aculeatus, Asparagus acutifolius* and *Erica arborea.*

The mean annual temperature of the forest is 12.9ºC and the mean annual precipitation 873 mm, according to data from the nearby weather-station at Volterra.

Sampling

Samples were taken from *Q. cerris* and *Q. pubescens* in June 2009 and June 2010, when the leaves of these oaks were fully expanded (both species are decidous, with more or less synchronous, late foliation in spring and late fall of leaves in autumn). The trees sampled were about 40–50 years old, grew in two even-aged woodlots and had not been harvested since at least the 1970s.

Two transects, each 500 m long and 10 m wide were laid out, one containing oaks that were mainly in good, though not necessarily very good condition, the other containing declining oaks. The transects had similar understory vegetation and similar soil types, with the two oak species growing intermixed. Declining oaks were scored on a 2-point disease scale in which 1 = slight decline (11–25% crown defoliation) and 2 = medium decline (26–60% crown defoliation), in accordance with international guidelines (Ferretti, 1994). This grading was made for the purpose of sampling plants showing approximately the same levels of decline in the two years. Every year, ten asymptomatic and ten declining oaks (five of each species) were sampled in the two transects. The trees sampled formed part of the main canopy (dominant crown class), had almost the same sized crowns, and had visually determined heights of 15–18 m and stem diameters of 18–22 cm at breast height (1.30 m above ground level).

The trees grew along parallel lines 10 m apart. From each tree, 20 current year twigs and 20 apical leaves, five from each cardinal point, were collected.

Isolation and identification of endophytes

Samples were processed within 72 h after collection. Each current-year twig was cut through at the base, mid-point and apex and from one of each of the cut surfaces one 5 mm diameter tissue segment was removed, giving 600 tissue segments for *Q. cerris* and 600 for *Q. pubescens* (300 segments from declining trees and 300 from asymptomatic trees for each species).

From each leaf, three fragments were cut, each approx. 5 mm^2 (one from the leaf apex, one from the peduncle, and one from the edge), giving 600 leaf samples per tree species.

Before the endophytes were isolated, samples were sterilized in 10% H_20_2 by dipping the twig samples for 15 min and leaf samples for 5 min, and washing five times in sterile, distilled water.

A control test was carried out on the leaf samples to ensure the effectiveness of the sterilization procedure: the lower surface of randomly chosen leaf fragments (approx. 5% of the total of leaves) was pressed

onto Petri dishes filled with potato dextrose agar (PDA), according to Schulz *et al*. (1998).

In each year of the experiment, 200 nine cm diam. Petri dishes per oak species were seeded with six samples each: fifty Petri dishes received twig samples and 50 dishes leaf samples of healthy oaks, while 50 Petri dishes received twig samples and 50 dishes leaf samples of declining oaks (total 1200 samples per oak species).

Each Petri dish contained 20 mL PDA, 0.06 g mL^{-1} streptomycin, 0.05 g L^{-1} ferric chloride and 0.02 g L^{-1} asparagin. The growth inhibitor Rose Bengal (BDH Laboratory Supplies, Poole, Dorset, UK) was added (30 mg L^{-1}) to the medium to prevent the development of fast growing fungi that may have masked slower growing endophytes that were present in the same tissue piece.

Petri dishes were incubated at 20°C in the dark for 7d, after which the colonies that had formed were transferred to 2% malt extract agar (MEA) and kept at 4°C. The colonies were grouped by their linear growth rates and culture characteristics (appearance, surface topography and texture, mycelium compactness, type of margin, growth of aerial mycelium, hyphal pigmentation and branching and growth rates). They were then identified by their microscopic features with the keys of Gams (1971), Carmichael *et al*. (1980), Sutton (1980) and Von Arx (1987).

A small group of non-sporulating isolates could not be identified by the above keys. To promote their sporulation, these taxa were grown on MEA under a range of temperatures (15, 18, 21 and 24°C).

Molecular identification of unresolved taxa

The identification on the basis of the morphological traits alone proved inconclusive or uncertain for a few taxa. These were represented by some commonly occurring isolates that we tentatively ascribed, by means of microscopic examination alone, to members of the genera *Acremonium*, *Diaporthe*, *Fusarium*, *Monochaetia* and *Phomopsis*. Representatives of these taxa were further examined by PCR amplification and sequencing of the rDNA ITS region.

Fresh cultures were obtained from hyphal tips taken under a dissecting microscope and incubated on MEA in the dark at 20°C. Genomic DNA was extracted from lyophilized mycelium ground under liquid N_2 to a fine powder as in Moricca *et al*. (2000). The rDNA ITS region was amplified using the general primers ITS1 and ITS4 (White *et al*., 1990). Amplification was carried out in 25 μL volumes containing: 0.2 μM of each primer; 2.5 μL of 10× Taq DNA polymerase buffer (10 mM Tris HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 0.1 mg gelatin); 200 μM each of dATP, dCTP, dGTP, and dTTP; 10 ng of template DNA, and 0.5 units of Taq DNA polymerase (Promega, Madison, WI). Incubation was performed in a GeneAmp PCR 9600 thermal cycler (Applied Biosystem Division, Foster City, CA) using the following cycle parameters: 94°C for 60 sec, 50°C for 60 sec, and an extension step at 72°C for 120 sec initially and increased by 1 sec per cycle, using the maximum ramp time between each temperature. The total number of cycles was 35, with an initial denaturation step of 2 min at 94°C and a final extension step of 8 min at 72°C. A negative control with all reagents except DNA was included in all reactions. Results of amplification were checked by electrophoresis on 1% agarose gel (Pharmacia), stained with ethidium bromide and visualized under a UV transilluminator.

The PCR-amplified ITS region was sequenced directly after isolation from low-melting agarose gel. Amplicons were purified with a Prep–A–Gene kit (BioRad, Richmond, CA), and sequenced using the USB Sequenase 2.0 sequencing kit (US Biochemical, Cleveland, OH). The two PCR primers ITS1 and ITS4 served as sequencing primers for both strands. Taxa were identified by comparison with sequences available in the GenBank database. Identification was assumed to be accomplished at the species level when the similarity between our sequences and those in the database were greater than 98%. When the match was between 95 and 98%, identification was considered to be accomplished at the generic level (Sánchez Márquez *et al*., 2008).

Frequency of isolation and relative frequency of endophytes

The isolation frequency (IF) of each endophyte was calculated using the formula $IF = Ni/Nt \times 100$, where Ni is the number of samples from which the fungus was isolated, and Nt the total number of samples. The relative frequency of endophytes, which is the number of colonies of those endophytes as a percentage of all colonies, was also calculated.

Data analysis

The significance of the data was determined with ANOVA, after the percent data were ARCSIN

transformed. The differences in endophyte isolation frequency were examined for significance using the Duncan's New Multiple Range Test. Spearman rank correlation coefficients (r_s) were used to compare the isolation frequency between the two years of the experiment since they gave the concordance as an ordinal scale.

Results

Isolation and identification of endophytes

The sterilization protocol was effective as only four fungal colonies, three *Penicillium* sp. and one *Aspergillus* sp., grew in total on the medium after 1 week of incubation from about 120 presses of leaf portions (3.3% contamination). Sterilization was thus effective in eliminating the normal phylloplane-inhabiting epiphytic fungi.

All of the 40 trees sampled in the two years harboured fungal endophytes. From a total of 4800 samples that were examined for endophyte colonization (2400 in 2009 and 2400 in 2010) 888 colonies were obtained, 737 (83%) from *Q. cerris* and 151 (17%) from *Q. pubescens* (Table 1). Of the 737 colonies from *Q. cerris*, 328 (44.5%) came from asymptomatic trees and 409 (55.5%) from declining trees, while of the 151 colonies from *Q. pubescens*, 67 (44.4%) were from asymptomatic trees and 84 (55.6%) from declining trees. Of the colonies from *Q. cerris*, 542 (73.5%) were from the twigs and 195 (26.5%) from the leaves, while of the colonies from *Q. pubescens*, 100 (66.2%) were from the twigs and 51 (33.8%) from the leaves.

The two oak species differed markedly in the percentage of colonization, or the percentage of samples having at least one endophyte colony. In *Q. cerris* samples, 3% of the leaves and 11% of the twigs were colonized in asymptomatic trees, and 32 of leaves and 74% of twigs were colonised in declining trees, while in samples of *Q. pubescens*, the figures were 1% in the twigs and 5% in the leaves of asymptomatic trees, and 9% in the leaves and 23% in the twigs of declining trees.

The endophytic mycobiota isolated from the oak species was entirely composed of Ascomycota and anamorphic fungi. Regarding *Gliomastix* as a synonym of *Acremonium* (Gams, 1971), a total of 22 species in 19 genera were identified (Table 2). Most of the endophytes were from the *Sordariomycetes*, with 14 species in 11 genera. The *Dothideomycetes* yielded seven genera, each with a single species. The *Leotiomycetes* were represented by a single species, *Colpoma quercinum*.

Table 1. Number of fungal colonies recovered from asymptomatic (A) and declining (D) individuals of *Quercus cerris* and *Q. pubescens*. Average of two years of sampling, 2009 and 2010.

Infected organ [a]	Parameter	*Q. cerris*		*Q. pubescens*	
		A	D	A	D
Leaves	Total No. of colonies	114	81	30	21
	Total No. of species	10	5	2	2
	No. of species per tree (avg.)	4	2	1	1
	No. of species per tree (range)	3–5	1–3	1	1
	Percentage of infection [a]	3	32	1	9
Twigs	Total No. of colonies	214	328	37	63
	Total No. of species	19	16	12	11
	No. of species per tree (avg.)	9	6	5	3
	No. of species per tree (range)	7–11	5–7	4–6	2–4
	Percentage of infection [a]	11	74	5	23

[a] A sample was taken to be infected if at least one endophyte colony was recovered from it.

Table 2. Fungal endophytes isolated from asymptomatic (A) and declining (D) individuals of *Quercus cerris* and *Q. pubescens* (L, leaves; T, twigs).

Endophyte	GenBank accession No.	*Q. cerris*				*Q. pubescens*			
		A		D		A		D	
		L	T	L	T	L	T	L	T
Gliomastix murorum	JX262799	+	+		+				
Acremonium sp.	JX262800	+	+			+	+		
Alternaria alternata	n.s.[a]	+	+	+	+	+	+	+	+
Apiognomonia quercina	n.s.	+		+	+			+	+
Aureobasidium pullulans	n.s.		+		+		+		+
Ceratocystis coerulescens	n.s.		+						
Cladosporium cladosporioides	n.s.	+	+						
Colpoma quercinum	n.s.		+		+				
Coryneum kunzei	n.s.		+						
Diplodia mutila	n.s.		+		+		+		+
Discula quercina	n.s.	+	+	+	+		+		+
Epicoccum nigrum	n.s.	+	+		+				
Fusarium solani f. sp. *eumartii*	JX262801						+		
Biscogniauxia mediterranea	n.s.		+	+	+		+		+
Monochaetia monochaeta	JX262802		+		+		+		+
Phoma cava	n.s.		+		+				
Phomopsis quercina	JX262803		+		+		+		+
Diaporthe eres	JX262804		+		+		+		+
Diaporthe sp.	JX262805		+		+				+
Trichoderma harzianum	n.s.	+	+		+		+		+
Trichoderma viride	n.s.	+	+		+		+		
Ulocladium chartarum	n.s.	+		+					
Sterile mycelia	--	+	+	+	+		+	+	+

[a] n.s. = not sequenced.

Asymptomatic *Q. cerris* trees yielded ten endophyte species from the leaves and 19 from the twigs, with eight species being common to both. Declining *Q. cerris* yielded five species from the leaves and 16 from the twigs, with an overlap of four species in common to both tissues.

Asymptomatic *Q. pubescens* trees harboured two species in the leaves and 12 in the twigs, with two species from the leaves also found in the twigs. Declining *Q. pubescens* trees yielded two species in the leaves and 11 in the twigs, with the two species from the leaves also occurring in the twigs.

Only one fungus, *Alternaria alternata,* was isolated from the leaves and the twigs of asymptomatic and declining individuals of both oak species.

The endophytes recovered from the twigs of

asymptomatic *Q. cerris*, already known from previous endophyte studies for being beneficial fungi or latent pathogens, had an IF varying from a minimum of 1.1% (*Phomopsis quercina*) to a maximum of 23.3% (*Gliomastix murorum*) (Table 3). In the twigs of declining *Q. cerris*, the IF varied from 2.4% (*Trichoderma harzianum*) to 25.1% (*Discula quercina*).

On the leaves of *Q. cerris,* the IF varied from 1% (*D. quercina*) to 23.1% (*Epicoccum nigrum*) on asymptomatic trees, and from 4.7% (*Biscogniauxia mediterranea*) to 33.6% (*Apiognomonia quercina*) on declining trees.

For *Q. pubescens* the IFs ranged as follows: on the twigs of asymptomatic trees, from 1.1% (*D. quercina*) to 10.9% (*A. alternata*); on the twigs of declining trees, from 6.4% (*D. quercina*) to 11.1% (*A. alternata*); on the leaves of asymptomatic trees, no isolations; on the leaves of declining trees, from 2.0% (*A. alternata*) to 4.2% (*A. quercina*).

The most common endophytes occurring in both tree species were, in decreasing order of isolation frequency: *Trichoderma harzianum*, *Apiognomonia quercina*, *Biscogniauxia mediterranea*, *Gliomastix murorum*, *Epicoccum nigrum*, *Discula quercina*, *Alternaria alternata*, *Trichoderma viride*, *Phomopsis quercina*, *Cladosporium cladosporioides*, *Colpoma quercinum* and *Diplodia mutila*.

The endophytes that according to the literature (Ragazzi *et al*., 2004; Moricca and Ragazzi, 2008) have been found to be highly virulent, i.e. *A. quercina* (including here the anamorph *D. quercina*), *D. mutila*, *B. mediterranea*, and *P. quercina*, had an IF of 67.1% from the twigs of declining *Q. cerris* and of 35.3% from declining *Q. pubescens*, *Q. cerris* and of 4.2% from declining *Q. pubescens*.

The figures were substantially lower from the twigs and the leaves of asymptomatic individuals of the two oaks, with an IF of 18.2% from the twigs of asymptomatic *Q. cerris* and of 3.2% from the twigs of asymptomatic *Q. pubescens*, and an IF of 3.8% from the leaves of asymptomatic *Q. cerris*, whereas no endophytes were recovered from the leaves of asymptomatic *Q. pubescens* trees.

Table 3. Isolation frequency (%) of endophytes, clearly recognized as beneficial or pathogenic fungi, from asymptomatic (A) and declining (D) individuals of *Quercus cerris* and *Q. pubescens*. Average data of the two years of the study, 2009 and 2010.

Endophyte	*Q. cerris*[a]				*Q. pubescens*[a]			
	Leaves		Twigs		Leaves		Twigs	
	A	D	A	D	A	D	A	D
Gliomastix murorum	18.1 a	-	23.3 a	4.3 a	-	-	-	-
Alternaria alternata	-	-	-	8.1 b	-	2.0 a	10.9 a	11.1 a
Apiognomonia quercina	2.8 b	33.6 a	-	-	-	4.2 a	-	7.7 a
Cladosporium cladosporioides	14.2 a	-	2.2 b	-	-	-	-	-
Colpoma quercinum	-	-	3.6 b	11.0 b	-	-	-	-
Discula quercina	1 b	-	2.6 b	25.1c	-	-	1.1 b	6.4 a
Diplodia mutila	-	-	1.3 b	11.3 b	-	-	-	-
Epicoccum nigrum	23.1c	-	18.2 a	3.1 a	-	-	-	-
Biscogniauxia mediterranea	-	4.7 b	13.2c	18.7d	-	-	-	10.3 a
Phomopsis quercina	-	-	1.1 b	12.0 b	-	-	2.1 b	10.9 a
Trichoderma harzianum	16.6 a	-	18.1 a	2.4 a	-	-	10.0 a	8.6 a
Trichoderma viride	13.6 a	-	13.1c	3.9 a	-	-	-	-

[a]Values in columns followed by the same letter do not differ significantly ($P \leq 0.05$, Duncan's New Multiple Range Test); isolation frequencies below the 1% treshold are not reported.

Table 4. Analysis of variance (1-way ANOVA) on isolation frequency (percent data arcsin transformed).

Variation	df	Deviance	Variance	F
Total	6	859.59	-	
Between oak species	1	436.68	436.68	250.96 *
Between asymptomatic and declining trees	1	393.66	393.66	226.24 *
Between sampling years	1	24.02	24.02	13.80 **
Error	3	5.23	1.74	

* Significant at $P \leq 0.01$.
** Not significant.

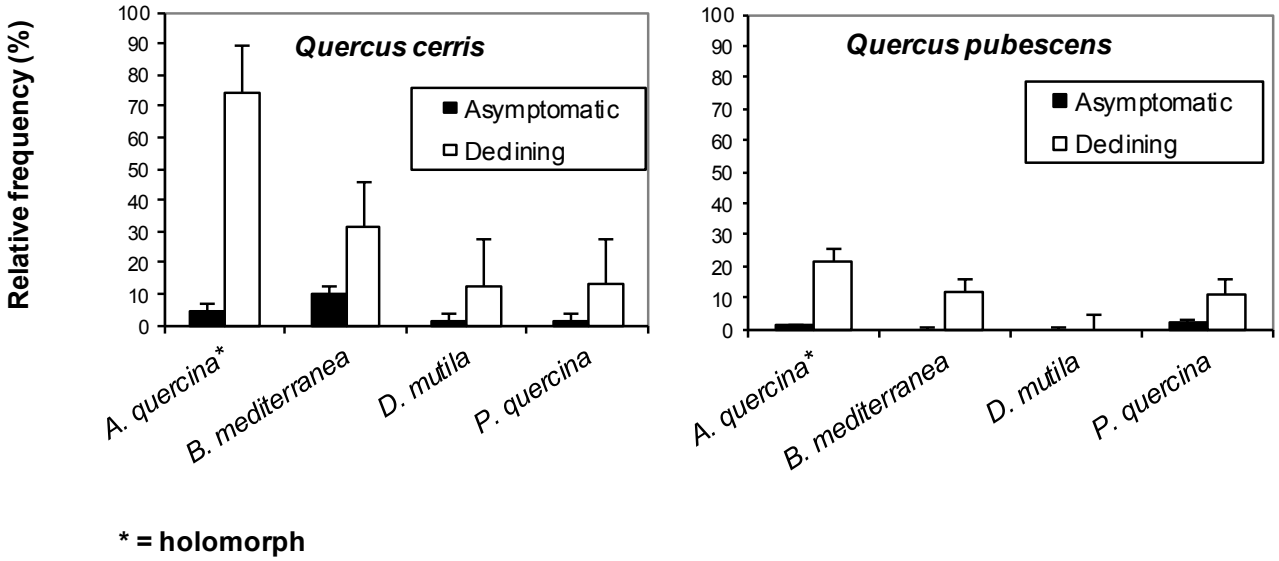

Figure 1. Relative frequency (%) of some pathogenic fungal endophytes recovered from asymptomatic and declining *Quercus cerris* and *Q. pubescens*. Average of two years of sampling twigs and leaves (2009 and 2010). Bars represent standard errors of the means.

Sterile mycelia were recorded in a number of samples, but their isolation frequency never exceeded 5%. These fungi constituted a miscellany of morphotypes which were grouped on the basis of their similar cultural characters in dark, hyaline or pink sterile mycelia. This heterogeneous assemblage of sterile mycelia was largely dominated by dark mycelia (80–85% of non sporulating morphotypes), followed by hyaline mycelia (15–18%), while pinkish mycelia occurred rarely (2% or less). Morphotypes remained sterile for several months, even after incubation at various temperatures (15, 18, 21 or 24 °C) on MEA at 20°C in the dark.

Sequence similarity comparisons (i.e. assessment of percentage nucleotide similarity) in the 5.8S gene and flanking ITS1 and ITS2 spacers, using the National Center for Biotechnology Information's GenBank nucleotide BLAST search, permitted unequivocal identification of five endophyte species, while two other taxa were identified at the genus level only (GenBank accession numbers are given in Table 2). Some isolates that were ascribed by their micromorphological characteristics to the genus *Acremonium* revealed 100% sequence identity with *Gliomastix murorum*. A subset of sequences did not match exactly those of known *Acremonium*/*Gliomastix* species, show-

ing greatest similarity to an *Acremonium* sp. originally isolated from the upper layer of forest soil. Similarly, sequences of isolates that were formerly assigned to the genus *Diaporthe* either shared 100% nucleotide identity with a *Diaporthe eres* strain (CBS No. 345.94) isolated from oak leaves or gave their best matches to *Diaporthe* sp. The rest of isolates exhibited a perfect (100%) match or had more than 98% similarity with reference sequences from the database, and could therefore be identified as *Fusarium solani* f. sp. *eumartii*, *Monochaetia monochaeta* and *Phomopsis quercina*.

Analysis of variance (Table 4) detected that there were highly significant differences in the IFs ($P \leq 0.01$) between oak species and between asymptomatic and declining trees. Between test years, on the other hand, the IFs did not differ significantly ($P \leq 0.01$).

The relative frequency (%) of the overt pathogenic endophytes *A. quercina*, *B. mediterranea*, *D. mutila* and *P. quercina* ranged from 12.6% (*D. mutila*) to 74.7% (*A. quercina*) on declining *Q. cerris*, and from 10.5% (*P. quercina*) to 21% (*A. quercina*) on declining *Q. pubescens* (Figure 1).

The occurrence of the pathogenic endophytes *A. quercina*, *C. quercinum*, *D. mutila*, *D. quercina*, *B. mediterranea* and *P. quercina* in the two oak species was similar in the years of sampling, 2009 and 2010; the isolation frequency in the first and second year was significantly and positively correlated based on the non-parametric (Spearman) correlation coefficient analysis (Table 5).

Table 5. Spearman correlation coefficients for the isolation frequency of some pathogenic fungal endophytes from the twigs of declining oaks (comparison of the data of 2009 with those of 2010).

Endophyte	Rs
Apiognomonia quercina	0.294 *
Colpoma quercinum	0.317 *
Diplodia mutila	0.656 ***
Discula quercina	0.703 ***
Biscogniauxia mediterranea	0.419 **
Phomopsis quercina	0.398 **

*, Significant at 0.05; **, significant at 0.01; ***, significant at 0.001.

Discussion

The numerous fungi isolated from the twigs and leaves of *Quercus cerris* and *Quercus pubescens* were sufficient to give a representative indication of the endophyte populations of these species.

The characterization of endophytic fungi by traditional techniques and keys, coupled with a DNA-based molecular approach, led to the identification of 22 endophyte species in 19 genera.

High agreement was found between traditional and molecular identification. Identification of unresolved endophytic strains by means of rDNA (ITS region) sequence comparison supported and extended the identity of taxa predicted by traditional methods. This approach confirmed the occurrence of two different taxa within the genus *Acremonium*; differentiated three distinct species within the *Diaporthe*/*Phomopsis* complex; and clarified the identity of the taxonomically controversial taxon *Fusarium solani* f. sp. *eumartii*, a member of the *Fusarium solani* species complex, formerly found in Italian oak forests and identified as *Fusarium eumartii* (Moricca and Ragazzi, 1991; Ragazzi *et al*., 2001b).

Studies sampling the same or related tree species elsewhere in Italy have reported very similar numbers of endophyte species and genera. In mixed stands of *Q. robur* and *Q. cerris* grown as coppices in the territory of Trino Vercellese (Piedmont), *Q. cerris* harboured 12 species in ten genera (Gennaro *et al*., 2002). A study of mixed stands on the north-eastern slopes of Mount Etna (Sicily) found that *Q. cerris* was host to 15 species in 14 genera, while *Q. pubescens* individuals growing in some woods yielded 15 species (Sidoti *et al*., 2002). The same number of species (15) was found in a *Q. pubescens* stand at Monte su Contru (Sardinia) (Franceschini *et al*., 2002).

The number of endophytes found in this study in Italy was also comparable with the number of endophytes reported by researchers elsewhere in the world for other oak species, namely for *Q. alba* and *Q. rubra* (Cohen, 2004), *Q. emoryi* (Faeth and Hammon, 1997) *Q. garryana* (Wilson and Carroll, 1994), *Q. ilex* (Fisher *et al*., 1994; Collado *et al*., 1996) and *Q. robur* (Pehl and Butin, 1994).

The most striking difference between the two oaks here examined was that more endophyte species were isolated from *Q. cerris* than from *Q. pubescens*, with some species, namely *Gliomastix murorum*, *Ceratocystis coerulescens*, *Cladosporium cladosporioides*, *Colpoma quercinum*, *Coryneum kunzei*, *Epicoccum*

nigrum, *Phoma cava* and *Ulocladium chartarum*, being found only in *Q. cerris*. Moreover, the isolation frequencies also revealed that some endophyte species were considerably more abundant in *Q. cerris* than in *Q. pubescens*.

A possible explanation for the differences in endophyte species composition and infection levels might be the environmental conditions that may have differentially affected the two oak species (Hughes, 2000). The ecology of *Q. cerris* and *Q. pubescens* is rather different: *Q. pubescens* is a heliophilous, thermophilic and xerophilous species which thrives in arid and stony soils, whereas *Q. cerris* is mesophilic, quite demanding in terms of soil moisture, and shunning heavy and shallow soils (Bussotti and Bruschi, 2000). The warmer climates that have occurred in the area examined during the last 2–3 decades, accompanied by prolonged droughts, often resulting in water shortages to the vegetation, may have impaired *Q. cerris* more severely than *Q. pubescens*, even in areas where the two species grow in close proximity, and where the same amount of water and nutrients is available to both. As a consequence, *Q. cerris* trees may have become more weakened and more prone to fungal infection and colonization than *Q. pubescens*. This also explains why the infection levels of *A. quercina, B. mediterranea, D. mutila*, and *P. quercina* were greater on the twigs of declining *Q. cerris*, the tree species that is more sensitive to changes in temperature and in the moisture balance. This confirms the fact that when some trees are subjected to physiological stress or to premature ageing, their defences become weakened or suppressed and they are no longer able to control the virulence of pathogenic endophytes (unbalanced antagonism) (Schulz and Boyle, 2005). This is especially true for opportunistic endophytes, i.e. those latent pathogens whose asymptomatic colonization as quiescent microthalli is only a transitory state, and they are always fully capable of inducing symptoms and causing disease as soon as the trees become weakened by some adverse factor (Sieber, 2007; Moricca and Ragazzi, 2008).

The tree organs of both oak species also differed in the number of endophytes they harboured. This finding was consistent with other studies on the *Fagaceae*. Differences in the IF of endophytes between tree organs have also been reported for other oak species: *Q. garryana* (Wilson and Carroll, 1994); *Q. faginea* subsp. *faginea* Lam. and *Q. ilex* subsp. *ballota* (Desf.) Samp. (Collado *et al*., 2000); *Q. petraea* (Mattus.) Liebl. (Halmschlager *et al*., 1993); *Q. robur* (Petrini and Fisher, 1990; Kehr and Wulf, 1993); and *Q. suber* L. (Franceschini *et al*., 2002). In *Fagus sylvatica,* too, the composition of the endophytic assemblage was reported to differ between organs in studies carried out in Switzerland (Sieber and Hugentobler, 1987), Germany (Pehl and Butin, 1994) and Italy (Danti and Sieber, 2004).

The study confirmed the known fact that some harmful endophytes colonized twigs or leaves to different extents, i.e. they exhibited organ preference. *Diplodia mutila* is common in twigs of various oak species and also sometimes in buds, but it is rare in leaves; *P. quercina* occurs in twigs, but only rarely in buds (Ragazzi *et al*., 2001a); as regards the holomorph *A. quercina*/*D. quercina*, the teleomorph is more common in leaves, while the anamorph occurs only in twigs and branches (Moricca and Ragazzi, 2008).

The present study also found that host- and organ-specific endophytes were mainly the dominant ones, i.e. those that had an IF of more than 10% in all the samples studied, and that had a greater relative IF on *Q. cerris* than on *Q. pubescens*. These were *B. mediterranea, D. mutila, P. quercina* and *Apiognomonia quercina*/*Discula quercina*.

When the IFs of the above endophytes were compared between years 2009 and 2010, they were significantly correlated (Spearman's correlation coefficient). The non-parametric statistic, Spearman rank correlation coefficient (rs) is an objective parameter for assessing the relationship between two variables, and it can be applied to the types of percentage data that are used to calculate relative abundance (i.e. % isolation). Application of this statistical test confirmed the prevalence of the endophytes *A. quercina*, *C. quercinum*, *D. mutila*, *D. quercina*, *B. mediterranea* and *P. quercina* on the same oak species in the two growing seasons. The interpretation obtained by Spearman rank correlation for the isolation frequencies of the dominant endophytes is in agreement with the research on endophytes of many woody plants of temperate regions. Studies have identified specific endophyte communities for several plant species, generally characterized by low and stable numbers of dominant fungal species (Petrini, 1996).

The specificity of endophytes to their host tree or trees has been previously investigated, with some studies examining the specificity of endophytes to trees in the genera *Quercus* and *Fagus* (Petrini and

Fisher, 1988, 1990; Kowalski and Kehr, 1992; Toti *et al*., 1992). The endophytes that dominate in a given plant genus or species are usually specialized or exclusive to that genus or species and, together with the other fungi on the plant, form a distinct assemblage, as was seen in the two oak species studied here, and as is found more generally also in other tree-endophyte associations (Toti *et al*., 1992; Cohen, 2004).

The specificity of endophytes to plants and plant organs is a widespread phenomenon (De Errasti *et al*., 2010). No microbe indifferently exploits all available resources (Sicard *et al*., 2007), most of them being restricted to hosts, populations, individuals, and even to certain organs of individuals. Specialization is a biotic mechanism that depends on a number of intrinsic and extrinsic factors, such as competition, parasitism, reinforcement of reproductive barriers, host resistance, and co-evolution of matching traits (Moricca and Ragazzi, 2008). This adaptive process leads to niche restriction, and may favour the durable coexistence of endophyte species in the same tree. The competitive interaction between the endophyte species to exploit a common resource (a host or a host organ) in time may have thus lead these invaders to become specialized to one oak species or to a particular tree-organ within each host.

In conclusion, if specialization may account for the dissimilarity in endophyte species occurrence between *Q. cerris* and *Q. pubescens*, the differential tolerance of the two oak species to adverse environmental conditions could explain the quantitative differences in endophyte colonization. This study provides evidence that the declining status of the trees favours the colonization of the host tissues by pathogenic endophytes.

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