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Variation of endophytic cork oak-associated fungal communities in relation to plant health and water stress

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Summary

The main objective of this study was to obtain more comprehensive knowledge about the effect of water stress on endophytic fungal communities in asymptomatic and declining cork oak trees. Six asymptomatic and six declining cork oak trees were randomly selected in a natural cork oak forest located in Sardinia, Italy. In February 2003, the soil around three asymptomatic and three declining trees was covered with a circular plastic film to reduce rain water supply with the intention to induce water stress. The remaining six trees served as controls. Predawn xylematic water potential (PWP) was used as water status indicator and measured seasonally. Between July 2003 and June 2004, fungal endophytes were isolated every 2–4 months from twigs, branches and woody tissues. Significant differences in PWP between covered and control trees were detected mainly in autumn. Gas exchange was greatest in asymptomatic control plants. All tissues were colonized by endophytic fungi. Nineteen fungal species were isolated from 1620 plant fragments. *Biscogniauxia mediterranea* was the most frequently isolated fungus. Its isolation frequency was significantly higher in declining covered trees than in control trees ($p < 0.05$). Presence of this fungus in asymptomatic control trees was significantly higher in winter than in summer. Water stress seems to reduce species diversity of the endophytic mycobiota in cork oak and to promote proliferation of some potentially pathogenic endophytes.

1 Introduction

Cork oak (*Quercus suber* L.) is a sclerophyllous evergreen tree species widespread in the Western Mediterranean area, where it plays an important ecological and socio-economical role. The climate of cork oak growing areas is characterized by warm to hot dry summers, cool wet winters, and unpredictable occurrence of rainfall and extreme events (Oliveira and Penuelas 2004; Cowling et al. 2005). Summer drought, associated with high temperatures, low water availability in the soil and high values of vapour pressure deficit, are the main factors limiting the distribution and biomass production of plants living in these environments (Bradford and Hsiao 1982; Larcher 2000; Sánchez-Gómez et al. 2006).

Evergreen species growing in Mediterranean areas have developed several molecular mechanisms for cell survival during drought periods (Kozlowski and Pallardy 2002; Wang et al. 2004), as well as biochemical and physiological adaptations responsible for low-temperature responsive genes (Novillo et al. 2004; Suping et al. 2005). Thus, cell damage is prevented and protection of photosynthetic function provided by a series of adaptive and constitutive mechanisms that are key factors for the growth of evergreen species in the Mediterranean areas, especially in summer and winter (Oliveira and Penuelas 2004; Cavender-Bares 2007; Varone and Gratani 2007).

In particular, cork oak has developed various physiological strategies for resistance to environmental stresses (Tenhunen et al. 1984; Oliveira et al. 1992; Faria et al. 1996). A strong link between stomatal closure, changes in soil water status and changes along the hydraulic pathway allows this species to maintain leaf water potential above the xylem cavitation threshold during the summer drought, ensuring an optimal use of water and carbon resources during low soil water availability (Otieno et al. 2007). Despite these adaptive mechanisms to environmental stresses, an increase in oak decline events and tree mortality, due to the effect of several adverse abiotic and biotic factors and attack by various endophytic fungal pathogens, has been detected in many Mediterranean countries during past decades (Luque and Girbal 1989; David et al. 1992; Bakry and Abourouh 1995; Santos 1995; Gallego et al. 1999; Sánchez et al. 2003; Linaldeddu et al. 2007a). Environmental stresses such as low or high temperatures, drought, high irradiance, and lack of some soil nutrients may lead to physiological plant modifications and influence the plant susceptibility to fungal pathogens (Schoeneweiss 1975; Boyer 1995; Garrett et al. 2006). The endophytic microbial component of oak trees is reactive to host changes due to adverse environmental factors; in particular, several fungal endophytic pathogens appear to be involved in the rapid decline of stressed oak trees (Desprez-Loustau et al. 2006; Gonthier et al. 2006). Endophytic fungal communities include both mutualistic and pathogenic species (Carroll 1988; Redman et al. 2001; Mucciarelli et al. 2003; Ragazzi et al. 2003; Sieber 2007). Pathogenic fungi can persist in a latent phase in asymptomatic tissues for a certain period of time or until the onset of plant stress (Linaldeddu et al. 2005a; Slippers and Wingfield 2007).

The results of recent studies carried out in Southern Italy have highlighted *Biscogniauxia mediterranea* (De Not.) O. Kuntze, *Botryosphaeria corticola* Phillips, Alves *et* Luque, *Discula quercina* (Westend.) Arx and *Pleurophoma cava* (Schulzer) Boerema, Loer. & Hamers as the main endophytic species involved in the aetiology of oak decline (Anselmi et al. 2004). All of these

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species are able to latently colonize all canopy cork oak tissues, but also cause cankers, dieback and an imbalance of some metabolic processes including stomatal conductance and photosynthesis (Luque et al. 1999, 2000; Franceschini et al. 2005; Linaldeddu et al. 2009). Since only limited information about cork oak–endophytic pathogen relationships under drought conditions in the field is available, the aim of this study was to obtain more comprehensive knowledge about the temporal variation of endophytic fungi associated with cork oak trees in relation to plant health and water stress.

2 Materials and methods

2.1 Site description and experimental design

The study was carried out in a natural cork oak forest '*Foresta Demaniale Monte Pisanu*' located in Sardinia, Italy (40°26′N, 8°58′E, 820 m a.s.l.). The mean annual temperature is 11.4°C (min 5.9°C, max 16.5°C), and the mean annual rainfall is 610.2 mm (data from the period 1995–2004). The vegetation of the whole area is mainly composed of downy oak (*Quercus pubescens* Willd.), growing in some parts alongside cork oak and holm oak (*Quercus ilex* L.). Large forest areas are covered by cork oak without the other two species. Inside one area covered almost exclusively by cork oak, 12 mature cork oak trees (six asymptomatic and six declining) were randomly selected. To reduce rainfall water supply and to increase the level of water stress through the reduction in the soil water content, from February 2003 the soil around six of the selected cork oak trees (three asymptomatic and three declining) was covered under the crown projection by a circular plastic film structure (70 m^2), about 0.6 m above the soil (Fig. 1). Moreover, the tree trunk in correspondence with the roof was sealed by polyurethane glue, and a trench in correspondence with the roof border was made. The other selected trees (three asymptomatic and three declining) were used as a control. Weather data was provided by a meteorological station located near the experimental site by the Regional Agency for the Environmental Protection of Sardinia.

2.2 Ecophysiological measurements

During the period July 2003–June 2004, the predawn xylematic water potential (PWP) was measured seasonally. Three 3-year-old twigs from each of the 12 examined cork oak trees were enclosed in a plastic bag, cut and PWP was then immediately measured by an electronic pressure chamber (SKPM 1400; SKYE Instruments, Llandrindod Wells, UK). At the same time, one soil core of 5 cm diameter and 40 cm depth was sampled below each tree and the soil moisture content was measured by gravimetric method.

In June 2004, leaf gas exchange was measured as net photosynthesis (Pn) (μmol CO_2 m^{-2} s^{-1}), transpiration (E) (mmol H_2O m^{-2} s^{-1}) and stomatal conductance (g_s) (mmol H_2O m^{-2} s^{-1}) rates, using a semi-portable infrared gas analyzer (ADC model 2250; Analytical Development Company, Hoddesdon, UK) equipped with a Parkinson's leaf cuvette, on four mature leaves chosen *at random* from each of the 12 selected trees. Measurements were made at 11:00 am using artificial light with a photosynthetically active radiation (PAR) value near 1600 μmol m^{-2} s^{-1}. Air flux through the chamber was adjusted to 3.33 ml s^{-1}.

2.3 Isolation and identification of fungal taxa

Since July 2003 fungal endophytes were seasonally isolated from three 1-year-old twigs, three 3-year-old branches and three cylindrical wood cores (5 mm diameter) extracted with a Pressler incremented borer from the trunk of each of the 12 selected trees. All samples were first superficially sterilized through immersion for 15 min in 10% H_2O_2 and subsequently rinsed five times in sterile water. The surface sterilization was carried out in accordance with a method tested by Ragazzi et al. (2003), Franceschini et al. (2005) and Gonthier et al. (2006). Next, all samples were left to dry on sterile filter paper under aseptic conditions. Three fragments of bark of about 3–4 mm^2 were taken from each twig and branch, while three fragments of

Fig. 1. Cork oak tree with its root zone covered by a plastic roof for rainfall interception.

5–6 mm^3 were obtained from each wood core. All samples were placed in Petri dishes containing potato dextrose agar (PDA; Fluka, Sigma-Aldrich, Milano, Italy) amended with 0.06 g l^{-1} of streptomycin to limit the development of bacterial colonies. All inoculated dishes were transferred to a thermostat at 25°C and left in the dark for 5 days. Then the mycelium of each colony was again transferred to a Petri dish with PDA and incubated at 25°C until differentiation of the reproductive structures. To facilitate the formation of asexual or sexual reproductive structures necessary for taxonomic identification, fungal isolates were sub-cultured on PDA dishes with sterile cork oak twig fragments. Morphological and cultural features (conidial pigmentation, wall texture, number of septa, size and shape as well as mycelial growth rate) were used to identify fungal isolates using appropriate taxonomic keys (Sutton 1980; Pitt 1991; Ellis and Ellis 1997; Boerema et al. 2004; Phillips et al. 2005). Representative isolates of all endophytic species obtained in this study were stored in the culture collection of the Department of Plant Protection, University of Sassari.

2.4 Colonization rate and isolation frequency

For each plant the rate of endophytic colonization (CR) was calculated using the formula:

$$CR = N_c/N_t \times 100$$

where N_c represents the number of segments colonized by at least one fungal species, and N_t is the total number of segments examined.

The isolation frequency (IF) of a single endophyte *taxon* was defined by the following formula:

$$IF = N_i/N_t \times 100$$

where N_i and N_t are the number of segments colonized by the endophyte and the total number of segments examined, respectively.

2.5 Diversity of endophytic communities

The structure of the endophytic community was assessed by two indices:

Shannon's diversity index (H):

$$H = -\sum p_i \ln p_i$$

and Simpson's dominance index (D):

$$D = \sum p_i^2$$

where p_i is the relative abundance of $taxon_i$.

The dominance index ranges from 0 (all *taxa* are equally frequent) to 1 (the 'community' consists of only one *taxon*). Calculations were done using the freeware package past version 1.89 (http://folk.uio.no/ohammer/past) (Hammer et al. 2001).

2.6 Statistical analysis

All experimental data (CR, IF, predawn xylematic water potential and leaf gas exchange values) were subjected to analysis of variance (ANOVA). Means were compared by the least significant difference (LSD) test at p = 0.05, using xlstat software (Addinsoft, France). Percentage data were angular transformed before analysis.

3 Results

3.1 Meteorological data

The trend of air and dew temperature, and rainfall during the years 2003 and 2004 is shown in Fig. 2. In the year 2003, characterized throughout Europe by an exceptional heat wave, the mean annual temperature in the experimental site was 12.1°C (+0.7°C with respect to the climatic value); in addition, minimum and maximum annual values of air temperature recorded during 2003 were significantly higher than the climatic data (+0.3°C and +0.7°C, respectively). In 2004, mean annual values of air temperature were in accordance with the climatic values. The heat wave of 2003 caused a remarkable increase in the atmosphere evapotranspirative demand, also highlighted by the gap between air and dew temperatures. Annual rainfall values both in 2003 and 2004 were significantly higher than climatic data (+237.8 mm and +166.6 mm, respectively).

3.2 Predawn xylematic water potential

Reduction of rainfall water supply due to plastic covers caused a distinct decrease in the soil moisture content of covered trees (Fig. 3). The PWP values alternated between the covered and uncovered trees throughout the study. In July 2003, no significant differences in the PWP values between covered and uncovered asymptomatic trees were detected, whereas the PWP values were significantly lower in declining uncovered trees than in those which were covered (Table 1). In autumn (October and December 2003), the PWP values were lower in covered asymptomatic and declining trees than in uncovered ones. No significant differences in PWP values were detected between declining covered and uncovered trees, and between

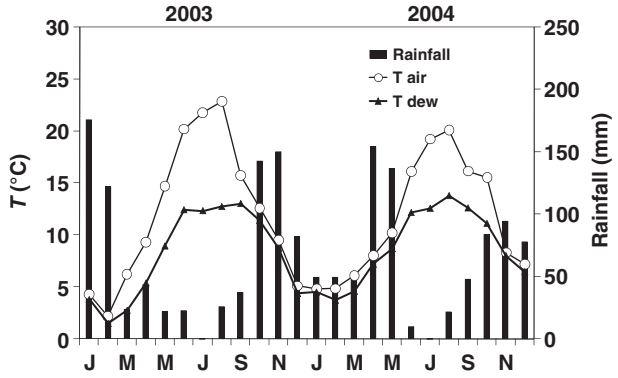

Fig. 2. Mean monthly air and dew temperatures, and monthly rainfall values recorded during the experimental period.

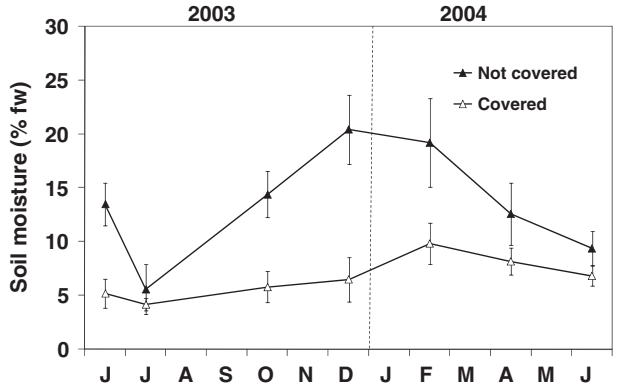

Fig. 3. Mean soil moisture values in covered and not covered (control) plots. Bars represent standard deviation.

Table 1. Mean predawn xylematic water potential (PWP) values (MPa) of examined cork oak trees measured during the experimental period.

	Period					
Treatment group	July 2003	October 2003	December 2003	February 2004	April 2004	June 2004
DC	−1.234a	−0.903c	−0.200b	−0.255b	−0.340a	−0.364a
HC	−1.319ab	−0.804bc	−0.270c	−0.143a	−0.410b	−0.525b
DNC	−1.505c	−0.700ab	−0.102a	−0.228b	−0.397ab	−0.449ab
HNC	−1.384bc	−0.673a	−0.121a	−0.212b	−0.397ab	−0.452ab

For each column values marked with different letters are significantly different according to LSD test ($p \leq 0.05$).
DC, declining covered; HC, asymptomatic covered; DNC, declining control; HNC, asymptomatic control.

asymptomatic covered and uncovered trees in the measurements taken in February, April and June 2004 except for the asymptomatic group in February 2004, when the PWP mean value of covered trees was significantly higher.

3.3 Gas exchange measurements

A summary of gas exchange measurements, made 16 months after starting the drought treatment, is shown in Table 2. The net photosynthetic values (Pn) ranged from 2.4 to 12.2 μmol CO_2 m^{-2} s^{-1}. The leaf transpiration rate (E) ranged from 1.2 to 4.2 mmol H_2O m^{-2} s^{-1}. Average net photosynthetic and transpiration rates were significantly higher in the non-covered asymptomatic control trees than in the other three treatments, indicating a better physiological status. With respect to stomatal conductance g_s, the highest mean values was observed in the asymptomatic control trees.

3.4 Endophytic infections

Fungal endophyte infections were detected in all examined cork oak tissues (Fig. 4). Significant differences in the colonization rate of cork oak trees were detected among the sampling periods and treatments (Table 3). In particular, the highest values of CR were observed between February and June 2004 in the declining covered trees.

Table 2. Mean (±SE) value of net photosynthesis (Pn), transpiration (E) and stomatal conductance (g_s) measured in cork oak trees.

Treatment group	Pn (μmol CO_2 m^{-2} s^{-1})	E (mmol H_2O m^{-2} s^{-1})	g_s (mmol H_2O m^{-2} s^{-1})
DC	8.2 ± 0.48a	2.4 ± 0.14a	107.1 ± 8.35ab
HC	7.8 ± 0.84a	2.1 ± 0.18a	89.5 ± 9.64a
DNC	8.3 ± 0.33a	2.3 ± 0.12a	130.9 ± 7.88bc
HNC	9.9 ± 0.26b	3.2 ± 0.17b	155.5 ± 13.59c

For each column values marked with different letters are significantly different according to LSD test ($p \leq 0.05$).
Data were recorded on June 8, 2004.
DC, declining covered; HC, asymptomatic covered; DNC, declining control; HNC, asymptomatic control.

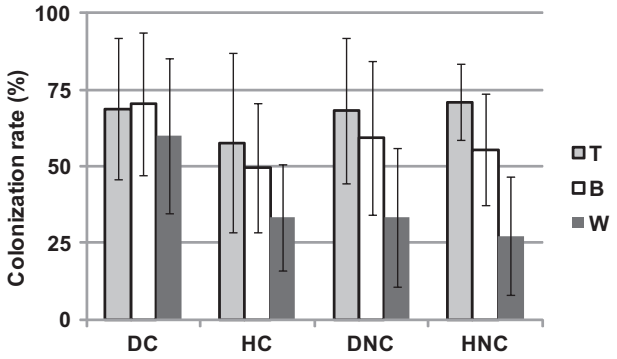

Fig. 4. Mean colonization rate of different cork oak tissues: twigs (T), branches (B) and trunk wood (W). DC, declining covered trees; HC, asymptomatic covered trees; DNC, declining control trees; HNC, asymptomatic control trees. Bars represent standard deviation.

Table 3. Endophytic colonization rate (CR), number of endophytic colonies, number of species isolated, Shannon diversity index and Simpson dominance index in the different sampling periods.

Period	Treatment group	CR (%)*	No. colonies	No. species	Shannon diversity index	Simpson dominance index
July 2003	DC	41.9b–d	36	7	1.34	0.41
	HC	33.3ab	28	11	1.73	0.31
	DNC	44.4b–e	38	9	1.66	0.28
	HNC	40.7a–d	36	13	2.06	0.20
October 2003	DC	49.4c–f	42	4	0.34	0.86
	HC	27.2a	24	5	0.87	0.58
	DNC	48.1cde	42	6	1.09	0.46
	HNC	41.9b–d	38	5	0.96	0.49
February 2004	DC	88.9i	78	7	0.75	0.68
	HC	62.9fg	57	11	1.70	0.31
	DNC	80.2hi	70	10	1.80	0.24
	HNC	72.8gh	63	13	2.16	0.17
April 2004	DC	72.8gh	62	9	0.97	0.61
	HC	58.0ef	51	15	2.16	0.20
	DNC	39.5abc	36	7	1.57	0.26
	HNC	48.1cde	43	12	2.03	0.19
June 2004	DC	79.0hi	66	5	0.60	0.73
	HC	53.1c–f	47	12	1.83	0.25
	DNC	55.6def	49	12	1.91	0.23
	HNC	53.1c–f	49	11	1.98	0.17

DC, declining covered trees; HC, asymptomatic covered trees; DNC, declining control trees; HNC, asymptomatic control trees; CR, colonization rate.
*Values in column marked with different letters are significantly different according to LSD test ($p \leq 0.05$).

Nineteen fungal endophytic species were isolated from the 1620 processed plant fragments; 18 were identified to species/genus level, one coelomycete could not be identified (Table 4).

Among the isolated fungi only *B. mediterranea* was detected in all examined trees and in each sampling period. The IF values of this xylariaceous fungus were always significantly higher in the declining covered trees than in other cork oak trees, with the exception of the first sampling (Table 4). The occurrence of this fungus in asymptomatic control trees was significantly higher

Table 4. Isolation frequency (%) of fungal endophytes from cork oak trees in the different sampling periods.

	Period																			
	July 2003				October 2003				February 2004				April 2004				June 2004			
Endophytes	DC	HC	DNC	HNC	DC	HC	DNC	HNC	DC	HC	DNC	HNC	DC	HC	DNC	HNC	DC	HC	DNC	HNC
Alternaria sp.	–	2.5	1.2	2.5	1.2	3.7	–	–	–	4.9	7.4	11.1	3.7	3.7	1.2	3.7	–	–	1.2	1.2
Aureobasidium pullulans	–	1.2	2.5	2.5	–	1.2	1.2	9.9	1.2	4.9	–	6.2	1.2	3.7	3.7	9.9	–	1.2	3.7	13.6
Biscogniauxia mediterranea	27.2bcd	18.5ab	22.2abc	17.3ab	48.1ef	22.2abc	33.3cd	24.7abc	79.0h	37.0de	37.0de	27.2bcd	59.3fg	25.9a–d	18.5ab	18.5ab	69.1gh	25.9a–d	25.9a–d	16.1a
Botryosphaeria corticola	3.7	–	–	–	–	–	–	–	6.2	–	–	–	–	–	–	–	1.2	–	1.2	–
Botrytis cinerea	1.2	–	–	1.2	–	–	–	–	1.2	1.2	3.7	2.5	–	1.2	4.9	1.2	–	1.2	–	–
Cladosporium sp.	–	1.2	2.5	1.2	1.2	1.2	4.9	9.9	–	3.7	4.9	3.7	1.2	3.7	–	6.2	–	1.2	–	1.2
*Coryneum depressum**	6.2	2.5	1.2	1.2	1.2	–	–	–	–	3.7	1.2	3.7	–	–	–	1.2	6.2	8.6	1.2	1.2
Cytospora sp.	–	–	–	–	–	–	–	–	4.9	7.4	13.6	3.7	1.2	1.2	–	–	3.7	1.2	3.7	–
Discula quercina	2.5	2.5	8.6	1.2	–	–	9.9	–	2.5	1.2	9.9	3.7	3.7	3.7	11.1	1.2	–	3.7	9.9	–
Dothiorella iberica	1.2	1.2	–	–	–	–	–	–	1.2	–	–	–	1.2	3.7	–	–	–	1.2	3.7	2.5
Epicoccum nigrum	–	1.2	–	4.9	–	–	–	–	–	3.7	4.9	6.2	–	3.7	3.7	3.7	–	–	3.7	3.7
Myrioconium sp.	–	–	–	–	–	–	–	1.2	–	1.2	–	–	–	–	–	1.2	–	–	–	1.2
Penicillium purpurogenum	–	–	–	1.2	–	–	–	–	–	1.2	2.5	3.7	–	3.7	–	–	–	1.2	1.2	–
*Pleurophoma cava**	–	–	1.2	–	–	1.2	–	–	–	–	–	1.2	1.2	3.7	–	–	–	1.2	–	–
Preussia aemulans	–	–	–	1.2	–	–	1.2	–	–	–	–	–	–	–	–	–	1.2	7.4	1.2	2.5
Sordaria fimicola	–	1.2	4.9	1.2	–	–	–	–	–	–	1.2	3.7	–	1.2	–	2.5	–	3.7	–	–
*Stereum gausapatum**	1.2	–	2.5	–	–	–	–	1.2	–	–	–	1.2	–	1.2	–	–	–	–	–	–
Trichoderma citrinoviride	–	1.2	–	6.2	–	–	–	–	–	–	–	–	3.7	1.2	1.2	2.5	–	–	3.7	11.1
Coelomycete unidentified	–	1.2	–	2.5	–	–	1.2	–	–	–	–	–	–	1.2	–	1.2	–	–	–	6.2

Biscogniauxia mediterranea values marked with different letters are significantly different according to LSD test ($p \leq 0.05$).
DC, declining covered; HC, asymptomatic covered; DNC, declining control; HNC, asymptomatic control.
*The identity was confirmed by the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

in winter (February 2004) than in summer (June 2004), showing clear seasonal patterns of endophyte infection. These results were similar to the findings obtained by Linaldeddu et al. (2005a) on the seasonal fluctuations of the colonization by *B. mediterranea* in a natural cork oak forest in Sardinia (Italy).

Remarkably, the occurrence of fungal species such as *B. corticola*, *Coryneum depressum* Kunze, *Cytospora* sp., *D. quercina*, *P. cava* and *Stereum gausapatum* (Fr. : Fr.), often reported as having weak pathogenic behaviour on *Quercus* spp. (Luisi et al. 1993; Muñoz et al. 1996; Franceschini et al. 2000; Luque et al. 2000), was detected (Table 4). However, all these species were isolated sporadically. *B. corticola*, the most virulent of these species (A. Franceschini and B. T. Linaldeddu, unpublished data), was detected mostly in February, and in the declining covered trees. *Coryneum depressum* was isolated more frequently from covered than from control trees. *Discula quercina*, the casual agent of oak anthracnose, canker and branch dieback on several oak species in Europe and northern America (Moricca and Ragazzi 2008), was isolated mainly from declining control trees.

In addition, several other fungal species were found which are well-known for their antagonistic behaviour against *B. mediterranea*, *B. corticola* and *D. quercina* (Linaldeddu et al. 2005b; Maddau et al. 2009), such as *Epicoccum nigrum* Link, *Penicillium purpurogenum* Stoll, *Preussia aemulans* (Rehm) Arx and *Trichoderma citrinoviride* Bissett. These species, despite having low values of IF, were mostly associated with asymptomatic control trees.

The ecological role of sporadically isolated endophytic fungi remains elusive; some species such as *Aureobasidium pullulans* (de Bary) G. Arnaud and *Sordaria fimicola* (Roberge ex Desm.) Ces. & De Not. are frequently reported as endophytes of forest trees (Pugh and Buckley 1971; Collado et al. 1999).

3.5 Endophytic community structure

The mean number of endophytic species isolated tended to be higher in asymptomatic than in declining cork oak trees, and to be higher in control than covered trees (Table 3). The Shannon diversity reflected the strong impact of the drought treatment on the microbial community diversity both in asymptomatic and declining cork oak trees (Table 3). Diversity indices were higher in asymptomatic control trees than in covered asymptomatic trees. During the experimental period the Shannon diversity index clearly decreased from 1.34 in July 2003 to 0.6 in June 2004 in the declining covered trees. Moreover, the fungal endophytic community associated with declining covered trees is characterized by a high dominance index, as a result of the high incidence of *B. mediterranea* infections.

4 Discussion

In this study the drought effects on the diversity of endophytic fungal communities associated with cork oak were analyzed.

The cover treatment notably reduced the soil moisture content, simulating drought conditions. Leaf gas exchange was affected by an artificially induced reduction of soil moisture by plastic roofs. In particular, the rates of net photosynthesis, transpiration and stomatal conductance, measured more than 1 year after the treatment was started, were higher in the control treatment. The reduction of gas exchange in cork oak trees during water stress periods has already been reported by several authors (Faria et al. 1996; García-Plazaola et al. 1997), as a consequence of reduced stomatal conductance and physiological changes related to the activity of the enzymes of the antioxidant system. These mechanisms allow cork oak to optimize the use of carbon and water resources during periods of limited water availability occurring in summer in the Mediterranean areas. The PWP values recorded throughout the whole experimental period confirm the efficiency of the plastic covers in reducing water availability; this was more evident after the summer of 2003, when the mean soil moisture difference between covered and control trees was higher than during the other periods. The lower water availability in covered trees seems to have induced a progressive qualitative and quantitative shift in the endophytic fungi associated with cork oak trees. The qualitative change in endophytic fungal community assemblages on oak trees during water stress periods has already been reported by Gonthier et al. (2006). The endophytic lifestyle has been described as a balanced antagonism between fungus and host plant (Schulz et al. 1999; Schulz and Boyle 2005). Thus, it is possible that water stress may have altered the plant defence of covered cork oak trees against some endophytic species, in particular *B. mediterranea*. The endophytic occurrence of this pathogen in declining covered trees was copious also in trunk wood tissues (data not shown). This result emphasizes that water stress induces *B. mediterranea* to switch from the latent to the pathogenic phase. The pathogenic behaviour of *B. mediterranea* on several Mediterranean oak species is frequently associated with severe water stress (Vannini et al. 1996, 2009; Luque et al. 2000; Capretti and Battisti 2007). The seasonal variation of *B. mediterranea* infections in cork oak trees recognized in this study concords with the data previously reported by Collado et al. (1999) on holm oak in Spain. Among the other opportunistic pathogens, *C. depressum* has been detected most frequently in declining covered trees suggesting a possible involvement of this fungus in cork oak decline. *Coryneum* spp. are often referred to as having weak pathogenic behaviour on severely water stressed hosts (Luque et al. 2000).

Water stress, besides favouring the endophytic infections of some opportunistic fungal pathogens, and causing a reduction in cork oak endophytic microbial biodiversity, seems to negatively influence the endophytic proliferation of some antagonistic fungal species. For example, *T. citrinoviride* was rarely detected in covered compared to control trees. This endophytic *Trichoderma* species is characterized by a strong antagonistic activity against the main pathogens involved in oak decline (Linaldeddu et al. 2005b, 2007b). Recently, the endophytic strain of *T. citrinoviride* from cork oak has been shown to produce a mixture of polypeptide antibiotics (peptaibols) in liquid culture. These antibiotics belong to the paracelsin family and showed a strong antifungal activity against seven dangerous forest tree pathogens (Maddau et al. 2009). Thus, the knowledge of plant-associated fungi and their antagonistic potential is essential not only to understand their ecological role, but also for future biotechnological applications in agriculture and forestry.

Finally, our results contribute to improving the knowledge of the cryptic presence of endophytic fungi in different tissues of cork oak trees, and demonstrate that the incidence of some fungal endophytes, mainly *B. mediterranea*, is linked to host phenology, and to changes in plant physiology caused by prolonged drought periods.

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