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Latent infection of *Biscogniauxia nummularia* in *Fagus sylvatica*: a possible bioindicator of beech health conditions

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***Biscogniauxia nummularia* is a xylariaceous fungus known as a common endophyte of European beech, living in plant tissues without development of symptoms, or even inducing strip-cankers and wood decay on trees stressed by drought. We studied the presence of the fungus in apparently healthy beech trees, growing in two different bioclimatic zones characterized by Continental and Mediterranean climates. Asymptomatic twigs were collected in each zone over the season and evaluated for the presence of *B. nummularia* infections using both cultural and qPCR methods. Results from qPCR indicated differences in the detection of *B. nummularia* among the seasons and between the study sites. In both sites the highest frequency of detection was in summer. *B. nummularia* was more frequently detected in the Mediterranean bioclimatic area, where drought is more common. These results suggest that *B. nummularia* may be a possible bioindicator of beech health stands.**

Keywords: *Fagus sylvatica*, Latent Pathogen, Real Time PCR, Xylariaceae

Introduction

European beech (*Fagus sylvatica* L.) is a late successional species widely distributed in European temperate forests. Beech forests are usually managed as high stands so that centenary trees are common (Dittmar et al. 2003).

Along the Italian peninsula, beech forests grow at elevations ranging from 300 to 2000 m a.s.l. They are distributed in two phytoclimatic regions: Northern or Continental, and Central-Southern or Peninsular (Piovesan et al. 2005). In the Alps and northern Apennines, the climate is continental and the sub-alliances of forest associations are *Abieti-Fagion, Eu-Fagion, Cephalanthero-Fagion* and *Luzulo-Fagion*. In central-southern Italy, the climate is Mediterranean and the associations are are *Aremonio-Fagion* and *Geranio versicoloris-Fagion* (Pignatti 1998, Piovesan et al. 2005, Di Pietro 2009). Beech forests located in the northern Apennines have greater affinity with the Alpine beech forests than with those in central Italy (Pignatti 1998, Piovesan et al. 2005). Because of its longevity, widespread distribution, and climate sensitivity, beech is a potential species for bio-monitoring programs on the status of European temperate forests (Biondi 1993, Piovesan & Adams 2001, Nielsen & Jørgensen 2003, Piovesan et al. 2005).

In a scenario of global climatic change, drought is expected to become one of the most limiting factors to beech forest sustainability, especially at lower latitudes (Weber et al. 2013). Monitoring of drought events and their impact on beech therefore are paramount needs in order to evaluate the latitudinal and altitudinal migration of the species and develop adaptation strategies. Immediate consequences of drought events on beech are extensive mortality of fine roots during peak drought (Leuschner et al. 2004), and an increase in host susceptibility to secondary pathogens (Schoeneweiss 1975, Desprez-Loustau et al. 2006). Several pathogens attack beech trees opportunistically after extended drought, such as the root rot fungi *Armillaria* spp. and occasionally *Heterobasidion annosum* (Wargo 1983, Capretti 1998, Popoola & Fox 2003, Capretti et al. 2007, Lakomy & Cieslak 2008). Other opportunistic pathogens that normally colonize symptomless tissue may have an indirect effect on the survival of stressed trees. Among these pathogens, *Biscogniauxia nummularia*, a Xylariaceous fungus, has been found in stressed beech trees (Granata & Whalley 1994, Paoletti et al. 1996, Capretti et al. 2003, Granata & Sidoti 2004). This fungus spends part of its life cycle as endophyte and may induce disease symptoms on its host under unfavorable environmental conditions. In prolonged summer drought, *B. nummularia* takes advantage of the altered host physiology, invades host tissue and causes elongated blackish bark lesions on trunk and branches, known as strip-cankers, and wood decay in mature trees (Hendry et al. 1998, Nugent et al. 2005). In the related species *B. mediterranea* (Vannini et al. 2009), outbreak of disease on oaks is likely to follow an increase in endophytic colonization. Therefore, monitoring of the endophytic stage of these fungi may provide an indication of drought-induced stress conditions of the host (Desprez-Loustau et al. 2006), *i.e.*, the water deficit that can predispose the plant to disease (Boyer 1995, Schoeneweiss 1975).

The detection of opportunistic pathogens within symptomless tissue is of primary importance to study disease progress and

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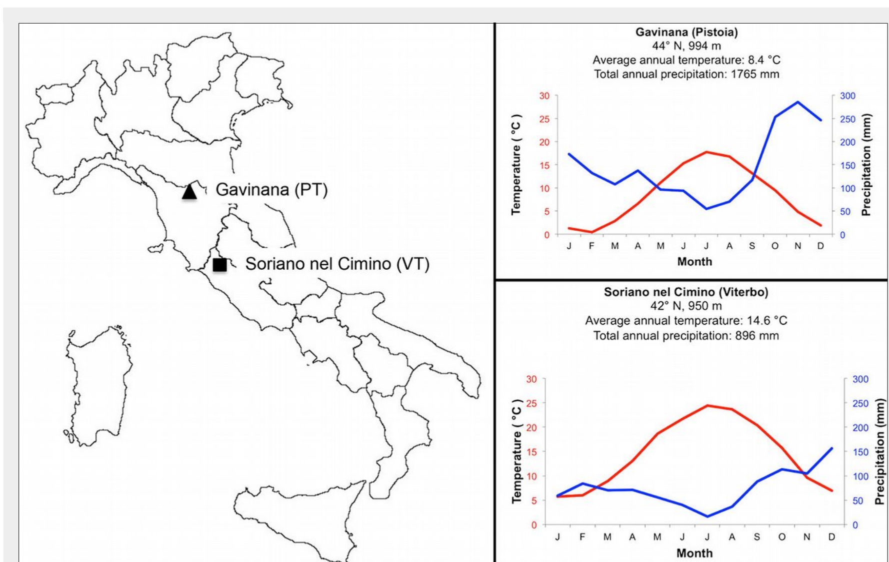

Fig. 1 - (Left panel): location of beech-sampling areas along the Apennines Mountains; (right panel): Walter-Lieth diagram of the two sampling sites in the period 2003-2008.

particularly the transition from latent to active tissue colonization. This process may also account for seasonal disease outbreaks caused by opportunistic pathogens, especially when these diseases are related to host decline.

In the past, the occurrence of endophytic microorganisms in healthy trees and the seasonality of occurrence in woody plants was detected by isolation from tissues on growth media in order to obtain axenic fungal cultures (Hallmann et al. 2006). However, these traditional isolation methods probably underestimate the frequency of a target microorganism (Catal et al. 2001).

Over recent years, rapid and sensitive molecular methods, such as real-time quantitative PCR (qPCR), have been developed to identify and quantify bacteria (Palacio-Bielsa et al. 2011) and fungi from woody plants, before symptoms occur in the host (Luchi et al. 2005, 2006). The effectiveness of this molecular method allows a target pathogen to be quantified directly in DNA extracted from plant tissue, reducing the time of diagnosis.

The aim of the work reported here was to investigate the potential of *B. nummularia* as bioindicator of beech health conditions in Italy, using a qPCR assay (Luchi et al. 2006) to assess the occurrence of this fungus in asymptomatic twigs.

Material and methods

Study area

The survey was carried out between 2007 and 2008 comparing beech samples from two distinct phytoclimatic regions: one forest in the Northern Apennines (Gavinana - Pistoia) and the second forest in the central Apennines (Soriano nel Cimino, Viterbo, Italy - Fig. 1). The geographic and climatic characteristics of the sites are given in Tab. 1. Climatic data were provided by the AgroMeteo (http://agrometeo.arsia.toscana.it) and the *Banca Dati Agrometerologica Nazionale* (http://cma.entecra.it) services for the Gavinana (Pistoia) and Soriano nel Cimino (Viterbo) site, respectively. The meteorological stations were at similar altitude and approximately 10 Km from the sampling sites. Sites were different in terms of climate conditions. In the period between 2003-2007 Gavinana had a shorter dry period (from May to September) in comparison with Soriano nel Cimino (from March to November - Fig. 1). During the sampling period (2007-2008), the annual precipitation was 1948 mm year^{-1} at Gavinana and 897 mm year^{-1} at Soriano nel Cimino. In the same period, highest values of precipitation generally occurred in November for Gavinana and October for Soriano nel Cimino. The average annual temperatures in 2007-2008 were 8.69 °C (Gavinana) and 14.8 °C (Soriano del Cimino).

For each sampling area a xerothermic index (X_i) was calculated using the following formula (eqn. 1):

$$X_i = \begin{cases} \sum(2TM - P) & \text{if } (2TM > P) \\ 0 & \text{if } (2TM \leq P) \end{cases}$$

where TM is the monthly mean of the maximum and minimum temperatures in °C and P is the monthly precipitation in mm (Grossmann et al. 2002). X_i was calculated on a historical data series from the last 10 years (1997-2007).

Previous studies showed the presence of *Phytophthora cactorum* and *B. nummularia* in the Soriano del Cimino forest (Vettraino et al. 2008, Ceccarelli 2011), while in Gavinana the stromata of *B. nummularia* were mainly associated with declining beech trees, especially on the edge of the forest (Ginanni 2007).

Latent infections of B. nummularia

For each area two slopes (N-S) were chosen in the same growing conditions and 10 asymptomatic beech trees of the same age (five on a northern and five on a southern facing slope) were arbitrarily selected inside the forest. Site topographies were gently sloping (5%). No symptomatic beech trees were present close to those sampled. Each tree was numbered and its position registered with GPS for the subsequent sampling. From each tree, three arbitrarily selected apical portions of current-year shoots were collected from the lower part of the crown, with a total of 30 twigs per stand. Collections were made in autumn (25 October 2007), winter (13 February 2008), spring (3 June 2008) and summer (1 September 2008). A total of 240 twigs were collected.

Twig segments (30 mm length, 5-6 mm diameter) were surface sterilized in the lab with 75% ethanol (1 min), 3% NaClO (3 min) and 75% ethanol (30 s) and rinsed three times with sterile water (Lodge et al. 1996). Each twig was split longitudinally into two parts containing both wood and bark tissue: one part was utilized for DNA extraction and the second for fungal culturing in agar media. The portion of twig used for fungal isolation was cut into 15 small fragments (2-3 mm each), placed on 1.5% PDA (potato dextrose agar - Difco, Milan, Italy) and incubated in darkness at 20°C for 10 days (Luchi et al. 2006).

The portions of twigs used for DNA extraction (100 mg fresh weight) were transferred to 2-ml microfuge tubes and ground with a Mixer Mill 300® (Qiagen, Valencia, CA, USA) for 2 min (20 Hz). DNA was extracted from all samples using the DNeasy® Plant Minikit (Qiagen), as described previously (Luchi et al. 2006). *B. nummularia* DNA was detected and quantified by real-time quantitative PCR (qPCR) using specific primers and the TaqMan™ probe (Luchi et al. 2006). Quantitation of *B. nummularia* DNA was expressed as pg per mg fresh

Tab. 1 - Characteristics of beech sampling forests in the Apennine Mountains (Italy).

Sampling site	Longitude N	Latitude E	Elevation (m a.s.l.)	Phytoclimatic unit	Xerothermic index (X_i)
Soriano nel Cimino (VT)	42°24′ 36.9″	12°12′ 08.9″	950	*Aquifolio-Fagion* Mediterranean climate	38.5
Gavinana (PT)	44°04′ 07.9″	10°48′ 41.7″	994	*Luzulo-Fagion* Sub-continental climate	0

twig weight (pg fungal DNA/mg fw).

Data analysis

Frequency of occurrence (OF) of *B. nummularia* was calculated with the formula (eqn. 2):

$$OF(\%)=\frac{N_i}{N_t}\cdot 100$$

where N_i is the number of twig segments in which *B. nummularia* was detected and N_t is the total number of segments tested.

Statistical analysis of data on the detection of *B. nummularia* among trees in each area, between sites, detection methods and seasons was calculated using the χ^2 test. The D'Agostino-Pearson K^2 test (D'Agostino & Pearson 1973) was applied to test for departure from normality of data ($\alpha = 0.05$). Data were also tested for homogeneity of variances using the Levene's test, and equality of variances using plots of residuals and the Shapiro-Wilks test. Comparisons of differences in the amount of *B. nummularia* were performed after log transformation. The results were analysed using one-way analysis of variance (ANOVA - $\alpha = 0.05$). Differences among means were tested by the *post-hoc* Tukey's test ($\alpha = 0.05$). An unpaired, two-tails test (*t*-test) was used to compare the sampling periods between sites. The temporal trend in *B. nummularia* abundance was analyzed by regression analysis using *B. nummularia* abundance (total pg fungal DNA) as dependent variable and the precipitation of the month before sampling (mm) as predictor. DNA data were log transformed to take into consideration the multiplicative effect of the independent variable (precipitation). All analyses were performed using the Graphpad Instat® software (San Diego, CA, USA).

Results

Detection of B. nummularia in symptomless beech trees

A total of 240 *F. sylvatica* twigs (120 for each forest) were processed to determine the presence of *B. nummularia*. The fungus was detected from samples using both cultural and molecular methods.

Frequency of occurrence of the fungus at each site was not significantly influenced by the tree aspect ($\chi^2_{[1]} = 2.09$; $P > 0.05$), thus data from both sites were pooled. No significant differences ($\chi^2_{[1]} = 0.02$; $P > 0.05$) were found in the annual frequency of occurrence of *B. nummularia* between the Soriano nel Cimino (OF = 25%) and Gavinana (OF = 23.3%) sites. No differences in isolation were observed between single trees. *B. nummularia* was significantly more frequently found using qPCR (72.5%) than the cultural method (24.1% - $\chi^2_{[1]} = 112.2$; $P < 0.001$). Overall frequency of detection using qPCR was greater for samples from Soriano nel Cimino (90.8%) than for those collected in Gavinana (54.1% - $\chi^2_{[1]} = 40$; $P < 0.001$). Due to the higher sensitivity of the molecular detection method, only data based on qPCR were used in further analyses.

Tab. 2 - Results of *Biscogniauxia nummularia* detection by qPCR from beech trees growing in two different forests in central Italy. Sampling periods: Autumn 2007, Winter 2008, Spring 2008 and Summer 2008.

Sampling period	Pres/Abs	qPCR detection of *B. nummularia* n. (%) of twigs inspected (out of 30 twigs)		Total	x^2	P
		Gavinana (PT)	Soriano nel Cimino (VT)			
Autumn	Presence	8 (26.7)	29 (96.7)	37	31.09	<0.001
	Absence	22 (73.3)	1 (3.3)	23		
	Total	30 (100)	30 (100)	60		
Winter	Presence	10 (33.3)	30 (100)	40	30.00	<0.001
	Absence	20 (66.6)	0	20		
	Total	30 (100)	30 (100)	60		
Spring	Presence	30 (100)	26 (86.7)	56	4.28	0.04
	Absence	0	4 (13.3)	4		
	Total	30 (100)	30 (100)	60		
Summer	Presence	17 (56.7)	24 (80.0)	41	1.4	>0.05
	Absence	13 (43.3)	6 (20.0)	19		
	Total	30 (100)	30 (100)	60		

Seasonality of B. nummularia occurrence

The percentage of qPCR-positive twig samples was significantly higher in the Soriano nel Cimino than in Gavinana site both in autumn ($\chi^2_{[1]} = 22.5$; $P < 0.001$) and winter ($\chi^2_{[1]} = 28.7$; $P < 0.001$). In contrast, spring detection of *B. nummularia* was higher in twigs collected at Gavinana (100%) than at Soriano nel Cimino (86.7% - $\chi^2_{[1]} = 4.28$; P=0.04 - Tab. 2). As for summer, no significant differences were observed between the two sampling sites ($\chi^2_{[1]} = 1.4$; P>0.05 - Tab. 2).

B. nummularia DNA in symptomless beech tissues was quantified by qPCR for each sample in both forests. Quantities ranged from 1.14E-02 to 2.26E+06 pg/mg of fresh weight beech tissue. Results from ANOVA showed significant differences in fungal DNA among sampling periods both in Soriano nel Cimino ($F_{[1, 119]} = 80.07$; $P < 0.001$) and Gavinana ($F_{[1, 119]} = 319.9$; $P < 0.001$). An increasing trend in the amount of fungal DNA was detected along the vegetative season, with a minimum in autumn (Soriano nel Cimino) or winter (Gavinana), and the maximum in summer at both sites (Fig. 2). The amount of *B. nummularia* DNA in healthy beech twigs was significantly higher in summer in Soriano nel Cimino (unpaired test, P=0.035) than in Gavinana (Fig. 2). No significant differences were detected for the other sampling periods (unpaired test, P > 0.05).

Regression analysis revealed that pathogen occurrence (in terms of DNA amount detected) was inversely proportional to

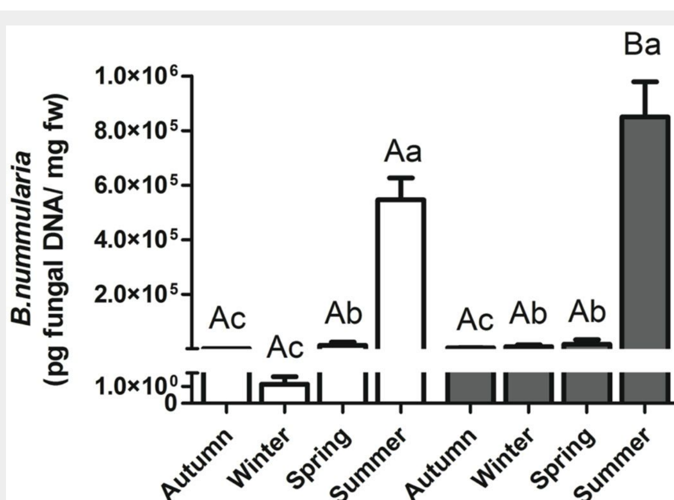

Fig. 2 - Quantification of *Biscogniauxia nummularia* DNA using qPCR in the northern (Gavinana, PT - white bars) and central Apennines (Soriano nel Cimino, VT - grey bars), in Autumn 2007, Winter 2008, Spring 2008 and Summer 2008. Different lowercase letters indicate significant differences among seasons (ANOVA, P < 0.05). Different capital letters indicate significant differences between two sites in the same season (Unpaired test, P < 0.05). Bars and whiskers represent the standard error and the mean value, respectively.

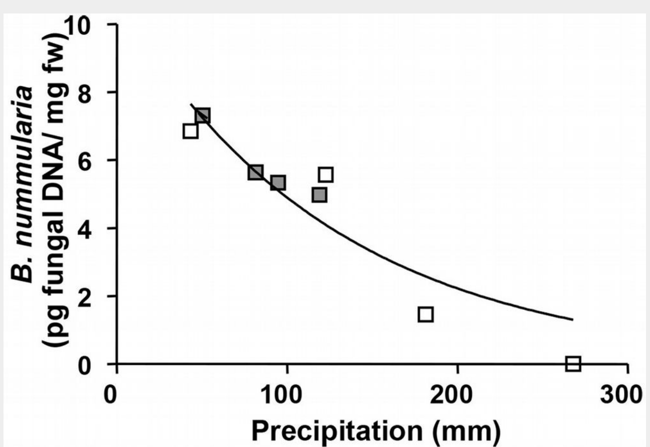

Fig. 3 - Amount of *Biscogniauxia nummularia* DNA detected in symptomless beech tissue by qPCR and inverse relationship with the precipitation (previous month of the sampling time) in Gavinana (white square) and Soriano nel Cimino sites (grey square).

the total precipitation of the month preceding the sampling ($P<0.05$, $R^2 = 0.86$ - Fig. 3).

Discussion

The results of this investigation confirmed that a specific qPCR assay could be used to detect *B. nummularia* DNA in the apical shoots of *Fagus sylvatica*, as already reported in a previous study (Luchi et al. 2006). The qPCR technique has been successfully applied to quantify the fungal pathogen in a range between 1.14E-02 to 2.26E+06 pg/mg of fresh weight. The sensitivity of this technique has significantly improved in the last few years. Indeed, qPCR is currently a reliable method to detect even small amounts of latent pathogens in the trees, before visible symptoms occur in the host (Luchi et al. 2005, Maresi et al. 2007).

Differential patterns of *B. nummularia* occurrence in symptomless beech trees in the two Apennines forests were likely due to their different climatic regimes, Mediterranean in Soriano nel Cimino (VT), and continental in Gavinana (PT). However, more pronounced differences in *B. nummularia* DNA amounts were observed in tissues at the end of the growing season in the site characterized by a warmer climate (X_i = 38.5), and with a longer arid period (Fig. 1). In this context, it can be hypothesized that water stress in beech stands may lead to an increase of the amounts of fungal inoculum within apparently healthy tissue, with possible changes in host susceptibility.

Interactions between water stress and the activity of forest pathogens have been recognized since long time (Paoletti et al. 2001, Moricca & Ragazzi 2008, Jactel et al. 2012). Both air moisture and soil drought have important roles in the epidemiology of tree diseases. Drought-induced tree diseases are often caused by secondary pathogens with endophytic abilities developing in secondary tissues (bark/wood) of stressed hosts (La Porta et al. 2008). Such fungi may establish interactions with the tree ranging from mutualism to antagonism depending on host physiology and environmental conditions. Nevertheless, water stress can trigger the disease development by endophytic fungi in asymptomatic hosts. In this sense, the presence of and fluctuations in populations of pathogenic endophytes can reflect physical changes in the environment (Desprez-Loustau et al. 2006).

In this study fluctuations in the amounts of *B. nummularia* were also observed in symptomless beech twigs, with significant differences occurring among sampling periods in both sites. The amount of fungal DNA was significantly higher during the vegetative season (summer) in both sampled forests. From an ecological point of view, *B. nummularia* behaves in a similar way to *B. mediterranea*, which proliferates in asymptomatic tissues of *Q. cerris* during dry growing seasons (Vannini et al. 2009).

The increase of quantities of *B. nummularia* within symptomless tissues may have a negative impact on beech health, possibly inducing the outbreak of disease, when climatic or host conditions change. Previous studies have also suggested that plant pathogenic fungi with endophytic behavior are sensitive to variations in host physiology driven by stress conditions (Leuschner et al. 2004, Desprez-Loustau et al. 2006). In conifers, outbreaks of *Diplodia sapinea* have been attributed to several stress factors and experimental work has underlined the interaction with water stress in the *Pinus - D. sapinea* pathosystem (Stanosz et al. 2001, Paoletti et al. 2001). In Fagaceae species of the Mediterranean area, *B. mediterranea* – the causal agent of charcoal disease of oak – can rapidly colonize the xylem and bark tissues of *Q. cerris* and *Quercus ilex* ssp. *ballota* subjected to water stress, inducing necrosis and canker formation and accelerating tree decline and death (Desprez-Loustau et al. 2006, Jurc & Ogris 2006, Capretti & Battisti 2007). Water stress favors the proliferation of the fungus in the endophytic phase; the pathogenic ability of *B. mediterranea* induces cankers and wood decay (Vannini & Scarascia Mugnozza 1991, Collado et al. 1999, Vannini et al. 2009). Similarly, Bassett & Fenn (1984) showed that asymptomatic seedlings of *Quercus alba* L. and *Q. velutina* Lam. naturally infected with *B. atropunctata* were rapidly colonized by the pathogen under water stress conditions.

The richness of fungal endophyte populations is strictly related to environmental parameters, such as temperature and rain (Hashizume et al. 2010, Zimmerman & Vitousek 2012). Recently, it was shown that the composition of leaf-associated fungi on *F. sylvatica* is correlated primarily with the annual mean temperature (Cordier et al. 2012, Coince et al. 2014). In the present work, the detection *B. nummularia* DNA in symptomless tissue of beech showed a significant decreasing trend as the mean precipitation of the month preceding the sampling increases (R^2= 0.86), *i.e.*, the amount of latent infection increased with decreasing precipitation. A relationship between environmental parameters and fungal behavior has been observed in several studies. Gange et al. (2011) showed that changing temperature and rainfall regimes cause different germination and growth rates of *Auricularia auricula-judae*. Straatsma et al. (2001) observed a correlation between the time of fruitbody appearance and temperature in a Swiss forest plot. In the *Diplodia*-Austrian pine pathosystem variations in the quantities of *D. sapinea* DNA in healthy pine shoots was positively correlated with the yearly amount of solar radiation received by trees (Maresi et al. 2007).

Variation in latent pathogens in apparently healthy plant tissues is important to consider when monitoring the health status of plants. Usually symptoms caused by biotic or abiotic factors represent successful bioindicators in assessing the health of forest trees (Schütt 1989). However, several studies have demonstrated the effectiveness of flora such as lichens as bioindicators of forest health (Loppi & Pirintsos 2003, Jeran et al. 2007, Mayer et al. 2009). Fungi colonizing plant tissues also could be considered as potential bioindicators of environmental changes. For example, Helander (1995) found a negative effect of air pollution on fungal endophytes in pine needles along a gradient from two factory complexes. Recently, Romeralo et al. (2012) showed that the frequency of fungi inside pine needles was negatively correlated with air quality. Furthermore, fungal endophytes were also used as bioindicators of tree vitality. Several authors reported a negative correlation between needle fungal endophytes and *P. abies* vitality (Barklund & Rowe 1983, Sieber 2007, Rajala et al. 2013). Some fungal species may change their behavior towards a plant host after the alteration of environmental conditions (Sieber 2007, Botella & Diez 2011).

The ability of latent pathogens to quickly adapt to new environmental conditions suggests that these organisms could be used as bioindicators of forest health (Vannini et al. 2009, Romeralo et al. 2012), in that: (i) their presence can be easily detected; (ii) they are sufficiently sensitive to provide an early warning of change; (iii)

they are widely applicable, and independent of sample size. Such characteristics match the criteria for their selection as good markers (Juutinen et al. 2006). In this study, *B. nummularia* was easily detected using a molecular method based on qPCR. In addition, the fungus occurred more frequent in the Mediterranean bioclimatic area, in which beech trees are more susceptible to drought stress, predisposing the host to fungal disease.

Conclusions

The identification of target species or functional groups for assessing the effects of climate on forests is particularly important in a scenario of global climatic change that affects the ecosystem functions and the survival of species (Staudinger et al. 2012). Italy and the Mediterranean basin in general are considered hot-spots for climatic change. Models for these areas predict extreme climatic events with prolonged drought periods and anomalous precipitation events that might seriously affect sustainability of several ecosystems (Coakley et al. 1999). Forests are particularly sensitive to climate change, because the long lifespan of trees does not allow for a rapid adaptation response (IPCC 2007).

Tools for assessing the general health status of individual trees or forest community, as well as for forecasting the outbreak of complex decline syndromes, may increase in importance in the next future. During recent years, climate change has altered host distributions, with shifts towards higher altitudes (IPCC 2007). This phenomenon has been observed in several tree species, including European beech that shifted upwards at the highest altitudes, displacing heath lands and grasslands and a few conifer species (Peñuelas et al. 2007). Such shifts could potentially favor the spread of pathogens into new bioclimatic zones causing additional damage. Considering that beech is a common forest species along the Italian peninsula and is particularly sensitive to climatic conditions, the interactions of beech with *B. nummularia* (or potentially other Xylariaceous endophytes) might be used as a tool in monitoring the health status of mountain forests. The seasonal variation in fungal quantities within healthy beech tissues suggest that *B. nummularia* should be considered a possible bioindicator of beech health condition.

Acknowledgments

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